

Comparative antibody response of five recombinant antigens in related to bacterial shedding levels and development of serological diagnosis based on 35 kDa antigen for *Mycobacterium avium* subsp. *paratuberculosis*

Sung Jae Shin^{1,2}, Han Sang Yoo², Sean P. McDonough¹, Yung-Fu Chang^{1*}

¹College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

²Department of Infectious Diseases, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea

Eighty-five complex (85A, 85B and 85C), 35-kDa and superoxide dismutase (SOD) were cloned, expressed and purified as antigens in an enzyme-linked immunosorbent assay (ELISA) to compare the serological reactivity of cows with different shedding levels of *Mycobacterium avium* subsp. *paratuberculosis* (MPT). Antibody responses to all recombinant antigens positively increased depending on shedding levels. In particular, antibody responses to the 35 kDa were higher than those to the others in all shedder groups. Also, the mean of O. D. values among Ag 85 complex, 85B showed slightly higher response than others with high sensitivity and specificity in all shedder groups. In receiver operating characteristic (ROC) curve analysis, the result of 35 kDa ELISA yielded an area under the curve value of 0.945 (95% confidence interval = 0.895 – 0.996), which indicated that this 35 kDa is more accurate indicator of MPT infection than other antigens. At the cut-off point recommended by the ROC curve analysis, the sensitivity and specificity of 35 kDa ELISA were higher than those of other antigens with 93.3% and 86.4%, respectively. Finally, a commercially available ELISA kit was used to clarify 200 positive and 200 negative sera. We then re-tested these serum samples with our ELISA test using the 35-kDa antigens. 35 kDa ELISA and commercial kit showed almost similar results in ROC curve analysis even though two of positive sera in commercial kit were negative in 35 kDa ELISA. The sera, which showed difference in the comparison with commercial ELISA kit, they also did not react with 35 kDa in Western blot. These results suggest that a 35-kDa based ELISA can be useful for detecting MPT infection.

Key words: *Mycobacterium avium* subsp. *paratuberculosis*, 85 A, 85 B, 85 C, 35-kDa, Superoxide dismutase, Sensitivity, Specificity, ELISA, ROC curve analysis

Introduction

Johne's disease is a chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MPT). Disease occurs worldwide and affects cattle, sheep, goats, deer and members of the camelid family [11,15]. Johne's disease is of tremendous economic importance to the worldwide dairy industry, causing major losses due to reduced production and early culling of animals with estimates of 20% of U.S. dairy herds affected and costs of \$220 million per year to dairy industry [17,23]. In addition to direct economic losses, premature culling of infected animals reduces the herd manager's ability to cull for other reasons such as low productivity or other health problems and can result in the loss of valuable genetic potential [20]. This organism has been suggested to cause Crohn's disease in people [6,11]. However, this issue is highly controversial and others report that this bacterium is not present in specimens obtained from Crohn's patients [4]. Further studies are needed to resolve the question of the zoonotic potential of MPT [22].

At present, no specific therapy or vaccination program effectively prevents Johne's disease [11]. Although good management practices can lead to a reduced incidence, eradication is dependent on early detection and culling of infected animals [5,15]. Unfortunately, eradication and control programs to limit the impact of this disease are hampered by the lack of simple and specific diagnostic tests that can detect the disease in subclinically infected (infected but symptom-free) animals [5].

Isolation of MPT by fecal culture is the definitive test for diagnosis of Johne's disease. Fecal culture techniques are currently the most sensitive and specific ante-mortem test for MPT infection in cattle [25]. However, culture techniques using solid media require 6 to 12 weeks to produce a result and performance varies because of lack of standardization of culture procedures [13,19].

Recently, gene probes and PCR assays for the detection of

*Corresponding author

Phone: 607-253-3675; Fax: 607-253-3943

E-mail: yc42@cornell.edu

MPT in feces have been developed. However, these nucleic acid-based techniques require specialized equipment, are expensive, and are less sensitive than conventional fecal culture, especially in low shedding animals [8]. In order to overcome these limitations, research has been directed towards the development of new serologic tests with improved sensitivity for the identification of paratuberculosis [12,16,18,19]. Currently available serological tests for Johne's disease are the complement fixation test (CFT), agar gel immunodiffusion (AGID) and various forms of enzyme-linked immunosorbent assays (ELISA) [12,14,19,26]. However, since seroconversion occurs relatively late during the course of the disease, the utility of these tests is limited [12,18,19]. The specificity of ELISA tests for sheep and cattle is improved by removing cross-reacting antibodies by absorbing sera with *Mycobacterium phlei*. This increases the specificity of serological testing to 98.8% and 99.8% in sheep and cattle respectively. However, the sensitivity for detection of fecal shedders under field conditions is reportedly only 57%, depending on the disease status of the animals tested [3,19,26].

Although current serological tests are useful in detecting cattle with clinical paratuberculosis, the application of this procedure in identifying cattle in early stages of infection or in subclinical stages has proven to be of limited value. Also, the low sensitivity of the commercially available ELISAs might, at least in part, be due to the heterologous nature of the antigen they are apparently based on (derived from MTB strain 18, which was recognized to be *M. avium* subsp. *avium*) [13]. Development of sensitive serologic tests for the rapid identification of infected animals requires identification of protein antigens or epitopes specific for MPT [16]. Several specific antigens of MPT have been reported. Among these antigens, the 85 A, B and C complex, 35-kDa (p35) and superoxide dismutase (SOD) elicit strong T- and/or B- cell immune responses in MPT infection [2,9,15].

In this study, these 5 recombinant proteins were purified and used as ELISA antigens to evaluate the sensitivity and specificity of the test with sera from cows with different levels of bacterial shedding using receiver operating characteristic (ROC) curve analysis (Analyse-it Software, www.analyse-it.com) and, then ELISA based on 35 kDa antigen was compared with a commercial kit.

Material and Methods

Antigen preparation

Bacterial strains and plasmid

E. coli Top10 was used for Zero-Blunt and TA vector cloning (Invitrogen, USA). *E. coli* DH5 α was used as a host for plasmids pBSK (Bluescript k+) (Stratagene, USA) and pET22B (Novagen, USA). *E. coli* BL21 (DE3) pLysS strain and *E. coli* BL21 (DE3) (Novagen, USA) served as hosts

for the expression of the 85 complex, 35kDa and *sod* genes as previously described [9]. All *E. coli* strains were cultured in LB medium with appropriate antibiotics. MPT field isolate (A198638) was cultured at 37°C in Middle brook 7H9 medium supplemented with OADC, Mycobactin J and Tween 80 as previously described [9,25]. This strain (IS900 positive and mycobactin dependent) was isolated from a cow with Johne's disease [9].

Cloning of 85A, 85B, 85C, 35-kDa protein and Sod genes

Genomic DNA of MPT strain A198638 was isolated and used as a template in PCR as previously described [9,24]. The primers specific for 85A, 85B, 85C, 35-kDa and *sod* genes were prepared as previously described and reported to Genbank as AF280067, AF219121, AF280068, AF333435 and AF333434, respectively. PCR was performed as previously described [9]. The amplified PCR products were cloned and the presence of appropriate insert was confirmed by restriction enzyme analysis.

Expression and purification of 85A, 85B, 85C, 35kDa and SOD proteins

The specific primers for expression of 85 complex (85A, 85B and 85C) were prepared as previously described [10]. The specific primers for 35 kDa, forward 5'CAT ATG ACG TCG GCT CAA AAT G3' and reverse 5'GAA TTC TCA CTT GTA CTC ATG GAA CTG3'. The primers for SOD, forward 5'CAT ATG GCT GAA TAC ACC CTG CCC GA3' and reverse 5'CTC GAG TCA GCC GAA GAT CAG GCC TT3'. PCR was performed as previously described [9]. The PCR products of each gene were cut with *Nde*I and *Eco*RI or *Xho*I and inserted into pET22B digested with the same enzymes. The recombinant plasmids were transformed into *E. coli* BL21 for protein overexpression. Cultures were grown at 37°C until OD₆₀₀ = 0.6 and then induced with different concentrations (0 mM to 5 mM) of isopropyl- β -D-thiogalactopyranoside (IPTG). The overexpressed proteins formed inclusion bodies that were partially purified as described previously [9]. An aliquot of the cell pellet of overexpressing bacteria was denatured in sample buffer and subjected to 12% SDS-PAGE. All proteins were further purified by preparative continuous elution using a polyacrylamide tube gel apparatus (Bio-Rad, USA) following the manufacturer's instructions [9]. Purified proteins were subjected to N-terminal amino acid sequence to prove that these proteins were the desired gene products.

Refolding of proteins and determination of protein concentration

Refolding of proteins was performed using a Refolding kit (Novagen) following the manufacturer's instructions. The concentration of purified recombinant protein was determined using a BCA protein assay kit (Bio-Rad, USA).

Dialysis of proteins and removal of endotoxins

The recombinant proteins were dialyzed against PBS at 4°C for 3 days with three changes of PBS using SPECTRAPOR membrane (Spectrum, USA). After dialysis, the protein solution was centrifuged at 5,000 g for 10 min. The supernatant was collected and endotoxins were removed using Detoxi-Gel Affinity Pak Columns (Pierce, USA)

Polyacrylamide gel electrophoresis and Western blot

SDS-PAGE and Western blot analysis were performed as previously described [9]. Monoclonal antibody specific to 85-complex antigen of *M. tuberculosis* was used (obtained from Colorado State University, Clone# CS90). Also, serum from MPT infected cows and non-infected cows, as determined by IS900 PCR and fecal culture, were used as primary antibodies for identification of 35-kDa and SOD proteins. Goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Cappel, USA) and anti-bovine immunoglobulin G conjugated to alkaline phosphatase (Sigma, USA) were used as secondary reagents.

Serological tests

A total of 82 sera from asymptomatic cows were divided into four groups based on shedding level as shown in Table 1. Fecal culture and IS900 PCR tests for MPT infection were performed to determine positive and negative samples [8,24].

Checkerboard titration was used to determine the optimum concentration of protein (2.5, 5 or 10 µg/ml) and serum dilution for use in an indirect ELISA. Flat-bottom 96-well plates (Maxisorp, Denmark) were coated with 100 µl of each antigen in carbonate-bicarbonate buffer (14.2 mM Na₂CO₃, 34.9 mM NaHCO₃, 3.1 mM NaN₃, pH 9.5) at 4°C overnight, followed by washing three times with PBS containing 0.05% Tween 20 (PBST, washing buffer) using microwell plate washer Bio-Tek ELx405 (BioTEK Instruments, USA). Uncoated sites in the wells were blocked with 5% skim milk in PBST at 37°C for 1 h. One hundred microliter of sera (1 : 100 diluted) from each group were added to plate and incubated at 37°C for 1 hr. The plate was washed twice with PBST and 100 µl of optimally diluted (1 : 25,000) HRP conjugated anti-bovine IgG (Sigma, USA) was added to all the wells and incubated at 37°C for 1 h. The plates were again washed three times in PBST. Next, 100 µl of 2-2'-Azino-Bis-Thiazoline-6-Sufonic acid (Sigma, USA) was added to each well and the plates were incubated for 30 minutes at 37°C in the dark. Stop solution (1M HCl, 50 µl) was added and the plates were read 3 times at 405 nm at 2-minute intervals in a Bio-Tek 312e ELISA reader (BioTEK Instruments, USA). Positive and negative sera along with antigen and antibody controls were included in each plate.

Table 1. Grouping with respect to shedding level and IS900 PCR test

Infected level	Number of cows	*Fecal culture (CFU/g feces)
Highly infected	22	> 300
Moderately infected	15	31-300
Low infected	23	1-30
Negative control	22	0

*Based on the criteria adopted by New York State Diagnostic Laboratory for Johne's test, Cornell University.

Comparison of an ELISA using the 35 kDa protein antigen and a CSL commercial kit

Two hundred positive and negative bovine sera for MPT infection, as determined by the Johne's Absorbed EIA kit (CSL Veterinary, Australia) were used in an ELISA assay using the 35 kDa protein. Wells were coated with 1 µg of 35-kDa protein and alkaline phosphatase conjugated rabbit anti-bovine IgG (Sigma, USA) was used as the secondary reagent. Absorbance was read twice at 405 nm using a Bio-Tek 312e ELISA reader (BioTEK Instruments, USA).

Evaluation of tests and statistical analysis

Wilcoxon signed-ranks test was used for analysis of the mean of O. D. values between individual antigens in same shedding levels using SAS version 8.0 Software. Differences were considered to be significant if probability values of $P < 0.05$ were obtained.

In addition, ROC curve analysis (Analyse-it Software, www.analyse-it.com) was performed on the ELISA results of individual antigens to determine the optimal cut-off point (at which the serum of the sensitivity and specificity values is maximal) [21] for distinguishing between positive and negative result. The ROC curve (a plot of the true positive rate (sensitivity) against the false positive rate (1-specificity) that is obtained at each cut-off point) was constructed and the area under the curve (AUC) value was calculated as a measure of the accuracy of the test. Also, ELISA was compared with fecal culture used as indicator in this study by kappa statistic.

Results

SDA-PAGE and Western blot analysis

Monoclonal antibody specific to 85-complex of *M. tuberculosis* showed strong reaction to recombinant 85A, 85B and 85C of MPT in Western blot as previously described (data not shown). Also, overexpressed 35-kDa and SOD (22-kDa) proteins in *E. coli* BL21 (DE3) pLysS cells were purified by preparative continuous elution using a polyacrylamide tube gel apparatus and also reacted strongly against sera from MPT infected cattle. In contrast, no reaction was observed with sera

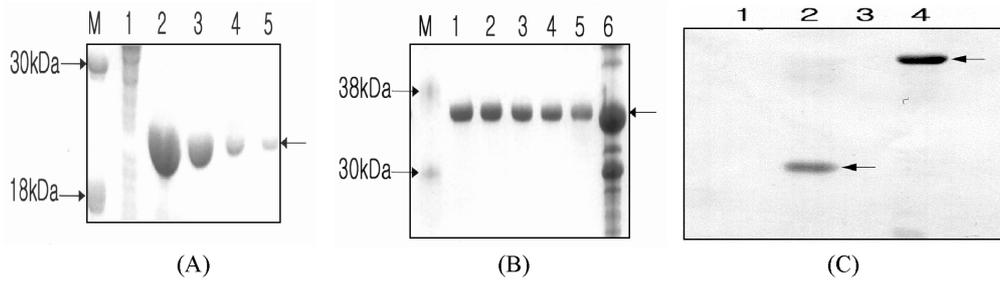


Fig. 1. Continuous purification of overexpressed SOD and 35-kDa proteins using 12% a Polyacrylamide gel with 491 Prep Cell (A and B) and Western blot analysis of Sod and 35kDa purified proteins against serum from MPT infected cows and non-infected cows determined by IS900 PCR, fecal culture and CSL ELISA test (C). (A) SDS-PAGE: M, molecular mass markers, Lane 1, Un-induced cell lysates, Lane 2 to 5, purified Sod protein (B) SDS-PAGE: M, molecular mass makers, Lane 2 to 5, purified 35-kDa protein fraction s, Lane 6, Induced cell lysates (C) Lane 1: Sod with negative serum, Lane 2: Sod with positive serum, Lane 3: 35-kDa with negative serum and Lane 4: 35-kDa with positive serum.

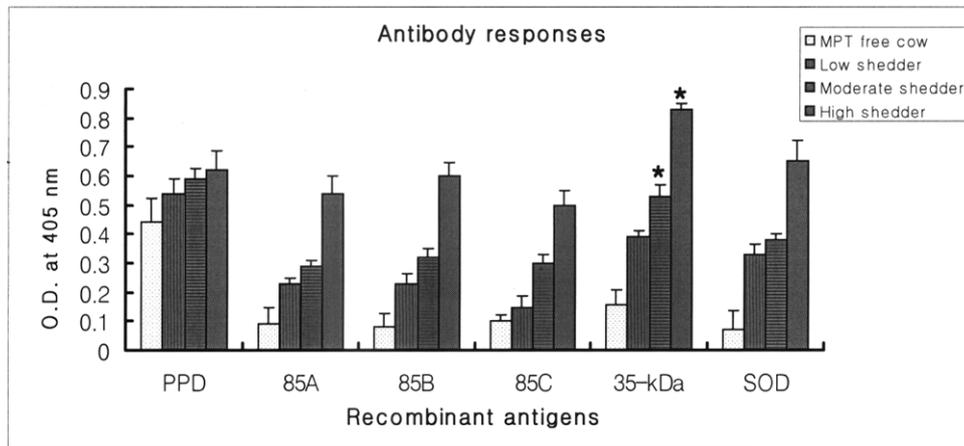


Fig. 2. IgG seroreactivity to recombinant proteins in sera for cattle infected with MPT. The data representd mean of O. D. at 405 nm with S.D. *indicated the significant differences between individual antigens in the same shedding levels ($P < 0.05$, Wilcoxon signed-ranks test).

from MPT-free cattle (determined by fecal culture and IS900 PCR test) in Western blot (Fig. 1).

Serological evaluation of 5 recombinant antigens

Antibody responses to all recombinant antigens positively increased depending on shedding levels. In particular, the mean of O.D. values of 35 kDa was higher than those of other antigens in moderate and high shedder groups ($P < 0.01$, Wilcoxon signed-ranks test). Among Ag 85 complex, 85B showed slightly higher response than others with high sensitivity and specificity in all shedder groups but no statistical differences were observed according to individual antigens with same shedding levels except 35 kDa ($P > 0.05$, Wilcoxon signed-ranks test) (Fig. 2).

Table 2 summarized cut-off point, sensitivity and specificity of all recombinant antigens analyzed by the ROC curve. The result of 35 kDa ELISA yielded an area under the curve value of 0.945 (95% confidence interval = 0.895 – 0.996) (Fig. 3), which indicated that this 35 kDa is more

accurate indicator of MPT infection than other antigens. At the cut-off point recommended by the ROC curve analysis, the sensitivity and specificity of 35 kDa ELISA were higher than those of other antigens as 93.3% and 86.4%, respectively (Table 2). Also, 35 kDa ELISA could detect 100% of MPT-infected cattle, which had shedding levels over than moderate shedding level (Table 3).

Comparison of 35-kDa and a commercial ELISA kit for herd screening

An ELISA test was developed using the 35-kDa and was compared with the CLS Commercial kit that has been used for herd screening in the field. Only 2 positive sera in CLS were negative in the 35-kDa ELISA.

All sera tested except 2 positive sera in CLS were matched with 35-kDa ELISA (Table 4). The sera were negative in 35-kDa ELISA also showed non-reactivity with the antigen by Western blot.

Table 2. Comparison of cut-off point, sensitivity and specificity of individual recombinant antigen in ELISA assay

Antigens	Cut-off point of O.D.	Sensitivity (%)	Specificity (%)
85A	0.17	66.7	81.8
85B	0.174	71.7	86.4
85C	0.152	68.3	81.8
35 kDa	0.21	93.3	86.4
SOD	0.16	80.8	90.9

Table 3. Comparison of identical ratio between fecal culture and ELISA based on 35 kDa antigen

35 kDa ELISA	Subjects		Infected ^a		Non-infected ^b	
	No. of tested	22 (H)	15 (M)	23 (L)	22 (N)	
35 kDa	No. of positive in ELISA (%)	22 (100%)	15 (100%)	19 (82.3%)	3 (13.6%)	

^aInfected animals based on the combined results of fecal culture and IS900 PCR

^bNon-infected cows based on fecal culture and IS900 from healthy flock

(H = High shedder, M = moderate shedder, L = Low shedder, and N = Non-infected cattle)

Table 4. Comparison of between 35-kDa and commercial kit

		CSL kit	
		positive	negative
35-kDa	positive	198	0
	negative	2*	200

*These two sera did not show any specific reactivity with 35 kDa antigen in Western blot.

Discussion

Control of paratuberculosis in diary herds requires preventing transmission of MPT to calves by culling cows that are shedding organisms in their feces (MPT shedders). However, there are no simple and accurate diagnostic methods for herd screening to detect MPT shedders. [5,11]. Thus, research has focused on improved serological methods to demonstrate antibodies against MPT [10,12,16, 18,26]. Compared to fecal culture and PCR tests, serologic tests are inexpensive, rapid, and easy to perform. Currently, complement fixation test (CFT) and an absorbed enzyme-linked immunosorbent assay (ELISA) are the serologic tests used most frequently. Western blot analysis has also been used for the identification of MPT infection. However, it is too labor extensive to perform western blot analysis especially if there are too many serum samples [5,11].

The specificity of Johne’s ELISA testing has been increased into the range of 95% to 100% by the introduction of absorbed ELISAs, which reduce cross-reactivity and use species-specific recombinant proteins [3,18,26]. However, sensitivity is still low and limited by variations in the stage of infection. In the attempt to increase sensitivity, several modifications have been tried including using a combination of antigens or a single recombinant antigen [10,16].

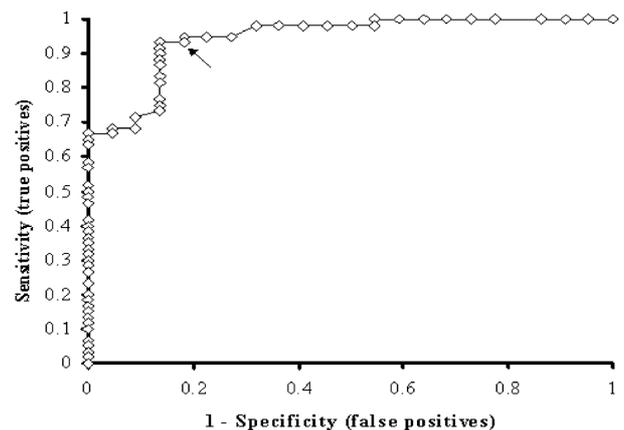


Fig. 3. The representative of ROC curve obtained from the analysis of ELISA based on 35 kDa and fecal culture that were obtained for the sera from 60 cattle infected with MPT and 22 of MPT-free cattle. The false positive rate (1-specificity (x-axis)) is plotted against the true positive rate (sensitivity (y-axis)) for each cut-off point applied. An optimal cut-off point (0.21) is indicated (arrow). AUC = 0.945, 95% confidence interval = 0.895 – 0.996).

Initial screening in this study was performed using crude antigens such as purified protein derivate (PPD) but animals with different levels of shedding could not be distinguished. However, OD values were statistically significantly higher for serum samples from cattle shedding high numbers of MPT when the 35kDa protein was used as compared to the other recombinant antigens tested. The ELISA data of all recombinant antigens were subject to ROC curve analysis [21], which estimates the sensitivity and specificity of a test at every possible cut-off point and provides a measure of test accuracy.

The results obtained in our study with sera in related to shedding level confirmed by fecal culture and IS900 PCR

test that 35 kDa protein showed most sensitive against MPT infections compared to the other recombinant antigens in the ROC curve analysis. The sensitivity and specificity was 93.3% and 86.4%, respectively. Also, O.D. values of 35 kDa in moderate shedders were as high those of other antigens in high shedder and 35 kDa was much better to detect positive sera even in low shedders.

Importantly, O.D. values to 35-kDa in moderate shedders were as high as those in high shedder to the other antigens. Thus, 35-kDa was superior to the other antigens testes for detecting positive sera even in low shedders.

In a previous study, 35-kDa was recognized by sera from all 16-reference animals with advanced Johne's disease (clinical stage) and 15 of 20 (75%) reference cattle with early infection (subclinical stage) [10]. In addition, 35-kDa did not react with sera from 15 MPT-free control cows. Use of recombinant 35-kDa antigen and/or a monoclonal antibody against an epitope on 35-kDa have recently been described for detection of MPT infection [2,10].

In this study, 85B and 85A had slightly higher seroreactivity than 85C, consistent with a previous study performed in tuberculosis patients. However there was no significant difference between 85A and 85B. Thus, the low sensitivity of these recombinant antigens were not effective for detection of MPT infection [9]. Previous serological studies showed the presence of antibodies to SOD in all tuberculosis patients tested and in 84% of leprosy patients [15]. Even though we found in this study that SOD was more sensitive than 85 complex, it is not recommended for field use to screen herds because the sensitivity and negative predictive values are low compared to 35-kDa

We tried to combine the 35-kDa and SOD antigens to detect MPT infection. The results were unsatisfactory compare to a single antigen used due to an increased signal to noise ratio (data not shown). An ELISA based on the 35 kDa compared favorably with the CLS commercial kit, as only two positive CSL sera were negative in our ELISA assay. The cut-off between positive and negative samples in commercially available kits is extremely narrow which makes these kits prone to subjective error [1]. In contrast, O.D. values between positive and negative sera exceeded 0.25 in our 35-kDa-based ELISA. Also, in kappa statistic compared with fecal culture, 35 kDa ELISA (0.51) were relatively higher than a commercial kit (0.276) [7].

These results suggested that ELISA based on 35 kDa antigen could be used as a sensitive tool for the serodiagnosis of MPT infection. The technical simplicity, speed and low cost of this serological assay, makes it very attractive for in conjunction with a test that measures the CMI response, such as the lymphocyte stimulation test, the gamma interferon test or the skin test, or, possibly even as a stand-alone screening test.

Although the results are promising, other proteins, which have not been studied so far in the antibody-based assays in

MPT infection and more serum samples, are necessary to be further studied.

Acknowledgments

This work was supported by the New York State Science and Technology Foundation. Sung Jae Shin was supported by overseas visiting fellowship provided by the Brain Korea 21 project.

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