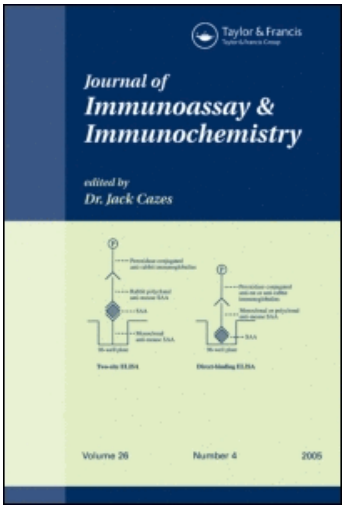

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Enzyme-Linked Immunosorbent Assay of Bovine Tuberculosis by Crude Mycobacterial Protein 70

Yun Sang Cho[a]; Suk Chan Jung[a]; Jong Man Kim[a]; Han Sang Yoo[b]
[a] Bacteriology and Parasitology Division, Animal Disease Research Department, National Veterinary Research and Quarantine Service, Mannan-gu, Anyang, Gyeonggi-do, Republic of Korea [b] Laboratory of Infectious Diseases, College of Veterinary Medicine and the School of Agricultural Biotechnology, Seoul National University, Seoul, Republic of Korea

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Yun Sang Cho, Suk Chan Jung, and Jong Man Kim
Bacteriology and Parasitology Division, Animal Disease Research Department, National Veterinary Research and Quarantine Service, Mannan-gu, Anyang, Gyeonggi-do, Republic of Korea

Han Sang Yoo
Laboratory of Infectious Diseases, College of Veterinary Medicine and the School of Agricultural Biotechnology, Seoul National University, Seoul, Republic of Korea

Abstract: MPB70 (mycobacterial protein of BCG 70) as T-cell stimulator has been tried with an intradermal skin test (IST) and enzyme-linked immunosorbent assay (ELISA) of bovine tuberculosis (BTB). In this study, crude mycobacterial protein 70 (CMP70) was prepared by anion exchange chromatography from the culture supernatant of *Mycobacterium bovis* AN5 and CMP70 ELISA was compared with purified protein derivative (PPD) ELISA. PPD and CMP70 ELISA have shown a positive reaction to the sera of *M. bovis* infected cattle and IST positive reactors. One of three IST negative cattle showed the nonspecific reaction in PPD ELISA, whereas all of the IST negative cattle ($n = 3$) were did not show the nonspecific reaction in CMP70 ELISA. When each ELISA was applied to sixty-two IST positive cattle, ELISA positive reactors were eighty four per cent to CMP70 antigen and fifty-two per cent to PPD. CMP70 has been shown to be more specific and sensitive than PPD in ELISA.

Keywords: Mycobacterial protein of BCG 70 (MPB70), Crude mycobacterial protein 70 (CMP70), Purified protein derivative (PPD), Intradermal skin test (IST), Bovine tuberculosis (BTB), Enzyme-linked immunosorbent assay (ELISA)

Address correspondence to Yun Sang Cho, Bacteriology and Parasitology Division, Animal Disease Research Department, National Veterinary Research and Quarantine Service, Mannan-gu, Anyang 480, Gyeonggi-do, Republic of Korea. E-mail: choys@nvrqs.go.kr

INTRODUCTION

Since Old Tuberculin (OT) was introduced by Robert Koch in 1891,[1] bovine tuberculosis (BTB) has been diagnosed by the difference of local reaction before and after tuberculin intradermal injection. Tuberculin has been developed for the improvement of sensitivity and specificity. Therefore, heat concentrated synthetic medium (HCSM) and purified protein derivative (PPD) tuberculin have been developed and, currently, PPD has been used for diagnosis of BTB. Immunologic diagnosis of bovine tuberculosis (BTB) can be classified as the methods using cell-mediated immunity (CMI) and humoral immunity (HI). The methods using CMI are intradermal skin test (IST), lymphocyte transformation test (LTT), and gamma-interferon (γ-IFN) assay.[2] The antigens of IST are old tuberculin (OT),[1] heat concentrated synthetic medium (HCSM) tuberculin,[3] and purified protein derivative (PPD) tuberculin.[4] In addition, the mycobacterial protein of BCG 70 (MPB70)[5] has been applied to the intradermal skin test.

The diagnosis of BTB by IST has been improved in its specificity. But, the IST has some disadvantages, such as the difficulty of intradermal injection, the inconsistency of the diagnostic efficiency according to the injection volume, and time needed for the final interpretation. Therefore, trials have been conducted for development of laboratory diagnostic methods to overcome the disadvantages of IST. Thus, LTT and γ-IFN assay using CMI has been developed. The complement fixation test (CFT) and ELISA, using HI, has been also tried. PPD, MPB70, and lipoarabinomannan (LAM) had been used as the antigens for BTB ELISA.[6–8] In addition, lipopolysaccharide (LPS) was applied to ELISA[9] and recombinant MPB70 was also used as ELISA antigen.[10]

IST has been utilized worldwide with the national eradication program of BTB. However, it has some disadvantages, such as low specificity and sensitivity; it is laborious and time-consuming. An ELISA has been developed to diagnose BTB efficiently and economically as the ancillary test for BTB. Thus, ELISA has been attempted to be used as the screening method of the exposure to *M. bovis* of cattle in the diagnosis of BTB, and the sensitivity and specificity of ELISA has been improved through research for the purified antigen. In this study, crude mycobacterial protein 70 (CMP70) has been prepared, which contained MPB70 (22 kDa) identified as specific antigen of BTB.[11] Also, ELISA using PPD and CMP70 were applied to cattle and compared with IST.

EXPERIMENTAL

Bacterial Strains and Challenge

Mycobacterium bovis AN5 (ATCC 35726), *M. phlei* (ATCC 11758), and *M. avium* P18 (ATCC 12227) were used for the production of PPD and

CMP70 and the challenge to cattle. Each strain was cultured on Sauton broth (asparagine 4.8 g, citric acid 2.4 g, $MgSO_4$ 0.6 g, K_2HPO_4 0.6 g, ferric ammonium citrate 0.06 g, glycerine 72 mL, $ZnSO_4$ 0.0096 g, $CuSO_4$ 0.0012 g, ammonia water 2.7 mL, D.W. 1,200 mL, pH 7.0–7.2) for six weeks and then was harvested by centrifugation. Each harvested strain was adjusted with sterilized saline to 10^7 CFU/mL and 10 mL was challenged intravenously to cattle. All cattle were bled and control serum was prepared 3 weeks later after being challenged.

Preparation of PPD

After *M. bovis* AN5 was cultured on Sauton broth for six weeks, the cultures were sterilized at 100°C for 3 h. The culture filtrate was precipitated by adding 40 per cent trichloroacetic acid (TCA), of which the volume was one tenth of the total culture filtrate, and then reacting at 5°C for 3 h. After these precipitates were centrifuged at 6,000 rpm for 30 min, the resultants were washed twice with one per cent TCA, acetone, and ethyl ether, sequentially. The washed precipitates then were dried at 37°C overnight. The dried powder was used as PPD tuberculin and stored at 4°C.

Preparation of CMP70

CMP70 antigen was prepared by anion exchange chromatography. The broth culture was autoclaved at 100°C for 3 h and then the supernatant was filtered. The proteins in the filtered solution were precipitated by 70% ammonium sulfate and then dialyzed. After dialyzing, the resultant was loaded on a DEAE-Sepharose CL-6B column and fractionated with a molar gradient of NaCl from 0 to 500 mM. The fractions containing MPB70 protein after anion exchange chromatography were used as CMP70 antigen for ELISA. MPB70 was further purified by QAE Sepharose A-50 gel filtration and MPB70 in CMP70 was confirmed as 22 kDa by SDS-PAGE.

ELISA

Positive control sera were prepared from cattle infected with *M. bovis* AN5 ($n = 2$), and also other control sera from cattle infected with *M. phlei* ($n = 1$) and *M. avium* P18 ($n = 1$), respectively, were prepared. We used fetal bovine serum (FBS) as negative control sera, and the sera of intradermal skin test negative cattle ($n = 20$) from Livestock Association in Republic of Korea as negative sera. Also, test sera for ELISA were prepared from sera of 62 cattle which were the positive reactors in IST.

The protein concentrations of PPD and CMP70 antigen were calculated with the BCA protein assay kit (Pharmacia). Each antigen for ELISA was resuspended with coating buffer (Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, NaN_3 0.2 g, D.W. 1,000 mL, pH 9.6) at the optimal concentration and coated onto a 96-well ELISA plate at 2–8°C, overnight. The solutions for ELISA were stored in a refrigerator, for example, Tween-PBS (NaCl 8.5 g, KH_2PO_4 0.22 g, Na_2HPO_4 1.15 g, KCl 0.2 g, Tween20 0.5 mL, D.W. 1000 mL, pH 7.4), citrate buffer (citric acid solution (1.92 g/100 mL) 24.3 mL, Na_2HPO_4 solution (2.84 g/100 mL) 25.7 mL, D.W. 50 mL, pH 5.0), stock solution of 2,2-azino-di(3-ethylbenzothiazoline-6-sulphonate) (ABTS) (ABTS 0.54 g, D.W. 25 mL) (Sigma), 0.5 M H_2O_2, and equilibrated at room temperature (RT) for 2–3 h before ELISA. The sera were diluted to 1:200 with PBS (NaCl 8.5 g, KH_2PO_4 0.22 g, Na_2HPO_4 1.15 g, KCl 0.2 g, D.W. 1000 mL, pH 7.4). Tween-PBS with 1% gelatin was used as the blocking solution. The blocking solution was injected (150 μL) into each well of the ELISA plates and incubated at 37°C for 1 h. Two repetitions of the diluted sera (100 μL) were injected into each well as the same as positive and negative control sera to each plate. After washing the plates, 100 μL of rabbit (or sheep) anti-bovine IgG-conjugated horseradish peroxidase (Sigma), appropriately diluted with Tween-PBS, was injected into each well and then the injected plates were shaken for 1 min. The injected plates were then incubated for 30 min at RT. Substrate solution, which was composed of 0.5 M H_2O_2 0.1 mL, stock ABTS 0.25 mL, and citrate buffer 24.65 mL, should be made fresh just before its injection. Substrate solution was injected, 100 μL, then the injected plates were incubated for 30 min at RT. The reactions in the ELISA plates were stopped by injection of 50 μL 1.0 M H_2O_2 and the optical density of each well was determined within 10 min at 405 nm. The OD of negative control serum should be below 0.2; the criterion of positivity and negativity of test sera might be above 2 and below 1 as S/N (sample OD/negative OD) ratio, respectively.

RESULTS AND DISCUSSION

The culture supernatant was precipitated by ammonium sulfate and dialyzed, and was then fractionated by anion exchange chromatography (Fig. 1). The second peak of the chromatogram (Fig. 1A) was CMP70, which contained MPB70, a 22 kDa protein (Fig. 1B). Further purification of MPB70 was done by QAE-Sephadex A-50 column chromatography. Its chromatogram showed three peaks (Fig. 2A) and the second peak of QAE-Sephadex A-50 column chromatography contained MPB70 (Fig. 2B). The fraction of CMP70 was confirmed by SDS-PAGE (Fig. 3).

The optimal antigen concentrations of CMP70 and PPD were 2 and 10 μg/mL, respectively, when the reactivity of each antigen to the positive and negative sera was compared with the diluted antigen (Figs. 4 and 5).

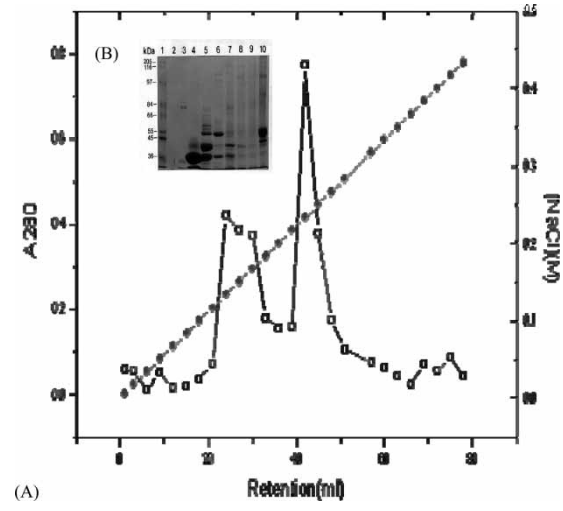

Figure 1. Chromatogram (A) and SDS-PAGE analysis (B) of crude *M. bovis* secretory protein by DEAE Sepharose CL-6B column chromatography. (A) Absorbance at 280 nm (.) of each fraction with mole gradient of NaCl (ø). (B) Lane 1, high molecular weight marker; lane 2, fraction (F)18; lane 3, F21; lane 4, F24; lane 5, F27; lane 6, F30; lane 7, F33; lane 8, F36; lane 9, F36; lane 9, F42.

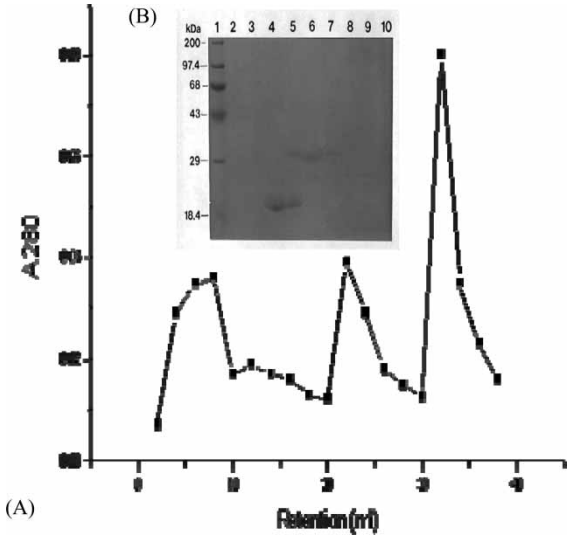

Figure 2. Chromatogram (A) and SDS-PAGE analysis of CMP70 by QAE-Sephadex A-50 column chromatography for identification of MPB70. (A) Absorbance at 280 nm of each fraction (■). (B) Lane 1, molecular weight marker; lane 2, F22; lane 3, F24; lane 4, F26; lane 5, F28; lane 6, F30; lane 7, F32; lane 8, F34; lane 9, F36; lane 10, F38.

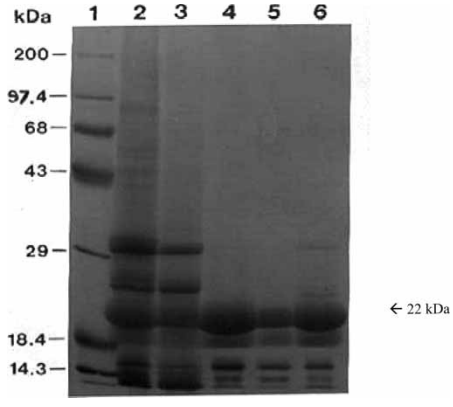

Figure 3. SDS-PAGE analysis of crude and purified *M. bovis* secretory protein. Lane 1, molecular weight marker; lane 2, culture supernatant after ammonium sulfate precipitation and dialysis; lane 3, fractions containing 22 kDa protein after DEAE-Sepharose CL-6B column chromatography; lane 4, 5, and 6, fractions containing 22 kDa protein after QAE-Sephadex A-50 column chromatography.

In PPD ELISA, the specific reaction to two *M. bovis* AN5 infected cattle and two IST positive cattle has been shown, while *M. avium* P18 infected cattle, *M. phlei* infected cattle, and two IST negative cattle reacted as low as the negative control. But, one IST negative cattle showed a nonspecific reaction in PPD ELISA (Fig. 4). In CMP70 ELISA, two *M. bovis* infected cattle and

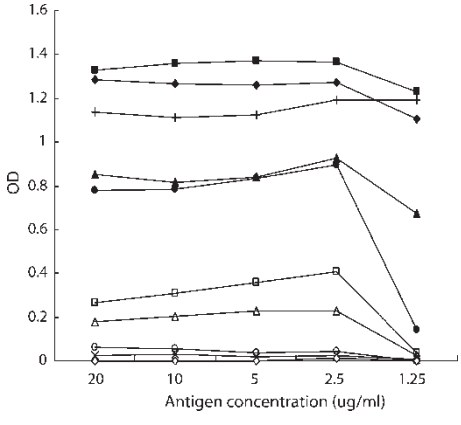

Figure 4. PPD ELISA for diagnosis of BTB. The optimal concentration of PPD was 10.0 μg/ml. □, *M. bovis* infected cattle 1; ■, *M. bovis* infected cattle 2; ▲, Skin positive reactor 1; +, Skin positive reactor 2; ○, *M. avium* P18 infected cattle; X, *M. phlei* infected cattle; ●, Tuberculosis negative cattle 1; □, Tuberculosis negative cattle 2; □, Tuberculosis negative cattle 3; □, Fetal bovine serum.

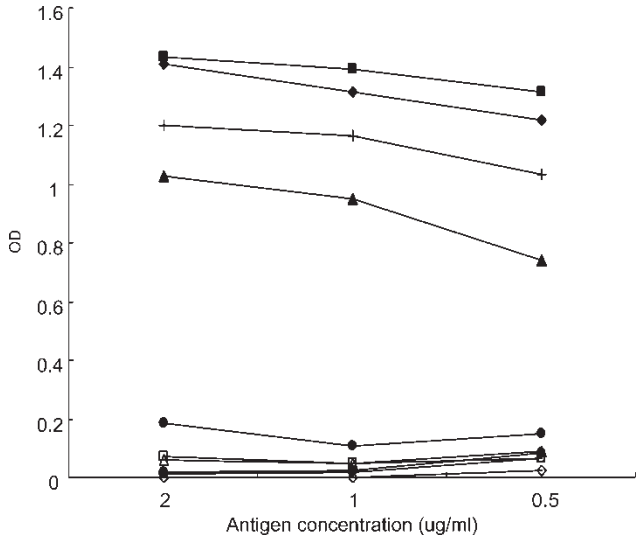

Figure 5. CMP70 ELISA for diagnosis of BTB. The optimal concentration of CMP70 was 2.0 μg/ml. □, *M. bovis* infected cattle 1; ■, *M. bovis* infected cattle 2; ▲, Skin positive reactor 1; +, Skin positive reactor 2; ○, *M. avium* P18 infected cattle; X, *M. phlei* infected cattle; ●, Tuberculosis negative cattle 1; □, Tuberculosis negative cattle 2; □, Tuberculosis negative cattle 3; □, Fetal bovine serum.

two IST positive cattle reacted specifically, and *M. avium* infected cattle, *M. phlei* infected cattle, and three IST negative cattle reacted as low as the negative control (Fig. 5). IST positive cattle ($n = 62$) and negative cattle ($n = 20$) were diagnosed by ELISA, using both antigens (Table 1). In PPD ELISA, the positive cattle ($n = 32$) were 52 per cent of the IST positive cattle, while it was 84 per cent ($n = 52$) in CMP70 ELISA.

CMP70 ELISA showed 82 per cent positives of IST positive reactors in this study and CMP70 showed higher sensitivity than PPD. In addition to the difficulty of the antigen mining, due to the lipid of *M. bovis* and the individual difference of the host immune reaction to *M. bovis*, there is another obstacle for improving the sensitivity of BTB ELISA. The dominant immune reaction of BTB is CMI rather than HI because BTB is an intracellular microorganism, and the HI reaction is too slow to detect by the conventional serological assay, such as ELISA. Additionally, the initial stage of the infection was related to CMI more than HI, while chronic or latent stage of infection was related to HI more than CMI. The infectivity of BTB has been increased according to the infectious stage because of its excretion. Therefore, the control of BTB is more important at late infectious stages, rather than at the initial infectious stage, to prevent highly infectious cattle from contacting other animals. This is the reason why the specific and sensitive BTB ELISA should have been developed.

Table 1. PPD and CMP70 ELISA to IST positive reactors

	S/N ratio			
Antigen[a]	X[b] < 1	$1 < X < 2$	$X > 2$	Total (%)
PPD	4 (6)	26 (42)	32 (52)	62 (100)
CMP70	0 (0)	10 (16)	52 (84)	62 (100)

[a]The concentrations of PPD and CMP70 were 10.0 and 2.0 μg/ml, respectively.

[b]Number (percentage) of cattle corresponding to S/N ratio.

Note: S/N ratio of intradermal test negative cattle ($n = 20$) was 1.02 ± 0.15.

Various antigens from *M. bovis* have been developed for BTB ELISA. PPD, MPB70, and lipoarabinomanan (LAM) were applied to the cattle sera whether the cattle had the specific antibody to react the specific antigen of BTB. MPB70 has been known as a T cell antigen, and it also reacted as a B cell antigen. Therefore, it has been investigated in ELISA.[6,12–14] At first, MPB70 had been isolated and identified by Nagai (1990);[11] it was a mycobacterial protein of BCG with 70 per cent electrophoretic mobility. Then, MPB70 was investigated for ELISA and showed low sensitivity and high specificity.[6] The low sensitivity of MPB70 ELISA is due to the individual difference of the immune reaction. With TB, it has been known that the individual difference of the immune reaction has been very common. Therefore, the ideal antigen for ELISA would be the specific B cell antigen pool that should be mixed with each B cell antigen. In addition, MPB70 had been recombinant and then applied to ELISA.[10] The sensitivity and specificity of recombinant MPB70 ELISA was not sufficient to diagnose BTB in the field. It showed low sensitivity as the previous ELISA result, which was the critical defect for using as the screening test of BTB. Their combination would be useful for the serological diagnosis of bovine tuberculosis. In this study, CMP70 showed higher sensitivity than PPD. CMP70 has been mixed with unidentified mycobacterial antigen complex rather than only MPB70; the novel B cell antigens in CMP70 should be identified for the antigen cocktail for BTB ELISA by state-of-the-art proteomics tools, such as two-dimensional electrophoresis and mass spectrometry. Furthermore, various antigens of BTB which were 25 kDa antigens,[12] MPB70,[6] MPB64,[13] MPB59,[13] and MPB83[14] could be used as the antigens of the serological test.

CMP70 ELISA specificity was increased, compared with PPD ELISA. But, pure MPB70 showed lower reactivity than CMP70 in previous reports.[6,10] This may result from non-reaction to MPB70 in BTB positive cattle. Therefore, CMP70 ELISA will be useful for the control of BTB, used as the screening test for BTB. Consequently, other specific diagnosis of BTB, such as IST,

culture and acid-fast staining from specimen of related clinical signs, and PCR using early culture soup for early detection of BTB, could be applied to the ELISA positive after ELISA positive reactions were traced. CMP70 ELISA could be applied to detect the cattle exposed to *M. bovis*. Therefore, it could be applied as the screening and ancillary test to BTB in the field where the national outbreak of BTB was negligible.

CONCLUSIONS

CMP70, which contained MPB70 as a potent B cell antigen of *M. bovis*, was fractionated by DEAE-Sepharose CL-6B column chromatography from a culture filtrate protein of *M. bovis* AN5. CMP70 was shown to have higher specificity than PPD in ELISA for the serological diagnosis of BTB. CMP70 ELISA for BTB diagnosis would be useful for the screening test of BTB.

ABBREVIATIONS

BTB, bovine tuberculosis; CMI, cell-mediated immunity; LTT, lymphocyte transformation test; γ-IFN, gamma-interferon; CFT, complement fixation test; HI, humoral immunity; IST, intradermal skin test; OT, old tuberculin; HCSM, heat concentrated synthetic medium; PPD, purified protein derivative; MPB70, mycobacterial protein of BCG 70; LAM, lipoarabinomannan; LPS, lipopolysaccharide; CMP70, crude mycobacterial protein 70; ABTS, 2,2-azino-di(3-ethylbenzothiazoline-6-sulphonate); RT, room temperature; PCR, polymerase chain reaction.

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