Comparison of recombinant MPB70 and SahH and a native 20-kDa protein for the detection of bovine tuberculosis by ELISA

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Research article

Keywords: Mycobacterium bovis, enzyme-linked immunosorbent assay, MPB70, SahH, purified protein derivative

Posted Date: February 21st, 2020

DOI: https://doi.org/10.21203/rs.2.24167/v1

Abstract

Background Bovine tuberculosis (bTB) is a zoonosis mainly caused by *Mycobacterium bovis* . Test-and-cull protocols and gross pathological examinations of abattoir animals as well as milk pasteurisation have been implemented for preventing the spread of tuberculosis from animals to humans worldwide, including in the Republic of Korea. Despite the importance of precise and rapid diagnostic tests, conventional methods, including intradermal skin tests and γ-interferon assays, are limited by the high rate of false negative results for cattle in the late infectious stage as well as laborious and time-consuming procedures. Therefore, antibody detection methods, such as enzyme-linked immunosorbent assay (ELISA), are urgently needed to supplement established approaches and to expand the diagnostic window. In this study, we developed a bTB ELISA by evaluating candidate recombinant and native proteins and various assay parameters.

Results We produced recombinant MPB70 and SahH (rM70S) and a native 20-kDa protein (20K). The 20K ELISA showed 94.4% sensitivity and 98.2% specificity and had an optimal sample-to-positive (S/P) ratio cut-off of 0.531. rM70S ELISA showed 94.4% sensitivity and 97.3% specificity with an S/N ratio cutoff of 1.696.

Conclusion The assays showed the same sensitivity but the specificity was higher for 20K ELISA than for rM70S ELISA. Both assays had acceptable diagnostic efficiency and are expected to be useful for bTB diagnosis in combination with established methods for herd screening and for expanding the diagnostic window.

Background

Bovine tuberculosis (bTB) is caused by Mycobacterium bovis, which can infect both humans and other taxa, including cattle [1, 2]. In South Korea, the frequencies of bTB in cattle were 0.08% (2,898 heads) at the individual level and 0.41% (427 farms) at the farm level in 2018 (https://www.kahis.go.kr/home/lkntscrinfo/selectLkntsOccrrnc.do,http://library.mafra.go.kr/skyblueimage/28195.pdf). Infected cattle are subjected to test-and-cull and compensation. Accordingly, the economic losses due to bTB in South Korea are high, in addition to the labour-intensive process of bTB diagnosis by national control agencies [3–5]. Furthermore, the potential spread from M. bovis-infected cattle to owners and workers is an important issue[6–10], even though a human M. bovis infection has not been reported to date in South Korea.

Currently, test-and-destroy and abattoir surveillance as well as milk pasteurisation are used for preventing the transmission of animal tuberculosis from animals to humans in South Korea [4, 5, 11]. bTB has been detected by cell-mediated immunity-based diagnosis (CEMID), including intradermal skin tests (IST) and γ-interferon assays, based on the cell-mediated immune reaction to M. bovis [2, 12, 13]. However, CEMID has a bTB diagnostic window with a high false-negative rate and is laborious, requiring two farm visits for injection and interpretation, retesting at bTB-positive farms, and evaluations of non-reactors by IST [14–16]. To complement the CEMID, methods for the humoral immunity-based diagnosis (HUMID) of bTB are urgently needed.

In this study, we produced recombinant MPB70 and SahH (rM70S) and compared it with a native 20-kDa protein and a purified protein derivative (PPD) to establish a serological bTB assay with high sensitivity and specificity. To the best of our knowledge, this is the first evaluation of the combination of MPB70 and SahH antigens for bTB ELISA.

Results

Protein profile and antigenicity

The major protein component of PPD was a 20-kDa protein, but the overall protein pattern was complex (Fig. 1). The 20-kDa protein of PPD is a major immunoreactive protein against M. bovis-positive serum. Compared with culture filtrate proteins (CFP), PPD had no proteins of larger than 20 kDa. However, the immunoreactive protein of CFP was only 20 kDa, similar to PPD. Other protein fractions from M. bovis were also analysed by SDS-PAGE and western blotting, including insoluble proteins (INS) and soluble proteins (SOL). The patterns of INS proteins were all diffuse, and the major SOL proteins were 20 and 40 kDa. The pattern of INS proteins in PPD was very different from that of CFP, while the pattern of SOL proteins was similar to that of CFP. Of INS. A 40-kDa protein showed the highest immunoreactivity by western blotting with M. bovis-positive serum. However, immunoreactive proteins were not detected in SOL. We also analysed M. avium Johnin, M. avium PPD, and M. phlei PPD. No protein bands were detected for M. avium Johnin PPD, and the major protein bands of M. phlei PPD were 22 kDa and 40 kDa. The three sources from M. avium and M. phlei did not show immunoreactive proteins against M. bovis-positive serum.

We targeted a 20-kDa major immunoreactive B cell antigen, MPB70. The 20-kDa antigen was purified at two laboratories, the Animal and Plant Quarantine Agency (APQA) and ChoongAng Vaccine Laboratory (CVL), by anion exchange chromatography [5]. We evaluated the size and protein profile by SDS-PAGE and antigenicity by western blotting (Fig. 1B). Non-reactive proteins with molecular weights exceeding 20 kDa were removed by purification.

rM70S was produced and evaluated with respect to size and antigenicity against M. bovis-positive serum (Fig. 1C). The sizes of MPB70 and SahH were 37 kDa and 75 kDa, respectively. The immunoreaction of the recombinant rMPB70 was higher than those of other recombinant proteins, such as ESAT6, HspX, PhoS, MPB64, Antigen 65, and SahH. The 37-kDa MPB64 and MPB70, 30-kDa Antigen 65, 75-kDa SahH, and 20-kDa PPD were clearly immunoreactive against M. bovis-positive serum.

ELISA for the purified protein derivative

We evaluated 18 bTB-positive serum samples and 975 bTB-negative serum samples. The bTB-positive serum samples were confirmed for M. bovis infections by microbiological isolation, microscopic diagnosis, histopathological findings, and genetic diagnosis. We analysed the results of ELISA using the PPD antigen based on the sample/positive (S/P) ratio, sample/negative (S/N) ratio, and optical density (OD). The average OD value for M. bovis-positive serum samples (n = 18) was 0.860, while that of M. bovis-negative serum samples (n = 975) was 0.273 (Fig. 2). Compared with other ELISA antigens, such as M70S and 20K, the reactivity of PPD ELISA differed little between positive and negative sera. The differentiation efficiency of PPD based on the OD value was the lowest among the three ELISA antigens. However, PPD ELISA exhibited an equivalent efficiency to those of 20K and M70S ELISA based on OD values and receiver operating characteristic (ROC) curves (Fig. 3). The average S/N ratio of M. bovis-positive serum samples (n = 18) was 2.026, while that of M. bovis-negative serum samples (n = 975) was 0.886. Similar to the comparison of OD values, the difference in the S/N ratio between positive and negative serum samples was the narrowest for PPD among the three ELISA antigens. The differentiation efficiency of PPD by the S/N ratio was the lowest among the three ELISA antigens. The ROC curve for PPD ELISA based on the S/N ratio showed the lowest diagnostic efficiency (Fig. 3). The average S/P ratio for M. bovis-positive serum samples (n = 18) was 0.958, while that of M. bovis-negative serum samples (n = 975) was − 0.146. Unlike the comparisons of OD values and S/N ratios, the difference in the S/P ratio between positive and negative serum samples for PPD was intermediate among the three ELISA antigens. The differentiation efficiency of PPD based on the S/P ratio was intermediate. The ROC curve for PPD ELISA based on the S/P ratio showed the lowest diagnostic efficiency (Fig. 3). Optimal cutoffs for the OD value, S/N ratio, and S/P ratio were 0.618, 1.289, and 0.271, respectively (Table 2). The OD value was the most appropriate criteria for PPD ELISA in terms of sensitivity (77.8%), specificity (98.8%), positive predictive value (PPV) (53.8%), and negative predictive value (NPV) (99.6%).

Table 1
Primer sequences of MPB70 and SahH

Target gene	Primer (F/R)	Oligonucleotides primer sequence	PCR product size (bp)
MPB70	Forward (BamHI)	5'GCGGATCCGGCATGAAGGTA3'[a]	600
	Reverse (SalI)	5'CCGTCGACTTAAACGCCGGAGGCATTAGCAC3'[b]	
SahH	Forward (BamHI)	5'GGATCCATGACCGGAAATTTGGTC3'[a]	1,488
	Reverse (SalI)	5'GTCGACTTAGTAGCGGTAGTGGTC3'[b]	

[a] BamHI recognition sequence was underlined.

[b] SalI recognition sequence was underlined and stop codon was in bold and italics

Table 2
Sensitivity, specificity, positive predictive value, negative predictive value at optimal cutoff according to bTB ELISA antigens

Antigen	Criteria	Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC (95% CI)
PPD	OD	0.618	77.8	98.8	53.8	99.6	98.4	0.989 (0.981–0.998)
	S/N	1.289	94.4	85.7	10.9	99.9	85.9	0.946 (0.914–0.977)
	S/P	0.271	94.4	94.1	22.7	99.9	94.1	0.979 (0.958-1.000)
20K	OD	1.149	83.3	97.8	41.7	99.7	97.6	0.991 (0.983–0.998)
	S/N	2.139	94.4	97.4	40.5	99.9	97.4	0.991 (0.984–0.998)
	S/P	0.531	94.4	98.2	48.6	99.9	98.1	0.994 (0.988-1.000)
M70S	OD	1.320	77.8	99.5	93.3	97.9	97.5	0.990 (0.979–1.001)
	S/N	1.696	94.4	97.3	77.3	99.4	97.0	0.994 (0.986–0.998)
	S/P	0.143	94.4	96.2	70.8	99.4	96.0	0.992 (0.967–0.999)

20-kDa ELISA

The average OD value for M. bovis-positive serum samples (n = 18) was 2.376, while that of M. bovis-negative serum samples (n = 975) was 0.310 (Fig. 2). Compared with other ELISA antigens, such as PPD and rM70S, the reactivity of 20K ELISA exhibited the greatest difference between positive and negative sera. The differentiation efficiency of 20K based on the OD value was the highest among three ELISA antigens. The ROC curve for 20K ELISA based on OD values showed the highest diagnostic efficiency (Fig. 3). The average S/N ratio for M. bovis-positive serum samples (n = 18) was 5.138, while that of M. bovis-negative serum samples (n = 975) was 0.671. Similar to the comparison of OD values, the reactivity of 20K ELISA based on the S/N ratio exhibited the greatest difference between positive and negative sera among the three ELISA antigens. The differentiation efficiency of 20K by S/N ratio was the highest among three ELISA antigens. The ROC curve of 20K ELISA based on S/N values showed the highest diagnostic efficiency (Fig. 3). The average S/P ratio for M. bovis-positive serum samples (n = 18) was 1.869, while that of M. bovis-negative serum samples (n = 975) was − 0.174. Similar to the comparisons of OD values and S/N ratio, the

reactivity of 20K ELISA based on the difference in the S/P ratio between positive and negative sera was higher among the three antigens. The differentiation efficiency of 20K by the S/P ratio was the highest among the antigens. The ROC curve for 20K ELISA based on S/P values exhibited the highest diagnostic efficiency (Fig. 3). Optimal cutoffs for the OD value, S/N ratio, and S/P ratio were 1.149, 2.139, and 0.531, respectively (Table 2). The S/P ratio was the most appropriate criterion for 20K ELISA in terms of sensitivity (94.4%), specificity (98.2%), PPV (48.6%), and NPV (99.9%).

MPB70 and SahH ELISA

Average OD value for M. bovis-positive serum (n = 18) was 2.224, while that of M. bovis-negative serum (n = 975) was 0.533 (Fig. 2). Compared with the other ELISA antigens, such as PPD and 20K, the difference in reactivity of rM70S ELISA between positive and negative sera was similar but slightly less than that of 20K ELISA and greater than that of PPD ELISA. In M. bovis-positive cattle, the difference in OD values between rM70S and 20K was not significant (p = 0.623). The differentiation efficiency of M70S based on OD values was similar to that 20K ELISA in bTB-positive cattle. The ROC curve for rM70S ELISA based on OD values showed similar diagnostic efficiency to that of 20K ELISA (Fig. 3). The average S/N ratio of M. bovis-positive serum samples (n = 18) was 3.788, while that of M. bovis-negative serum samples (n = 975) was 0.748. Similar to the comparison of OD values, the reactivity of rM70S ELISA based on the difference in S/N ratios between positive and negative serum samples was intermediate among the three ELISA antigens. In M. bovis-negative cattle, the difference in the S/N ratio between rM70S and 20K was not significant (p = 0.230). The differentiation efficiency of 20K based on the S/N ratio was the highest among the three ELISA antigens. The ROC curve for M70S ELISA based on the S/N ratio showed a similar diagnostic efficiency to that of 20K ELISA (Fig. 3). The average S/P ratio for M. bovis-positive serum samples (n = 18) was 0.561, while that of M. bovis-negative serum samples (n = 975) was − 0.068. Similar to the comparisons of OD values and S/N ratios, the difference in reactivity of rM70S ELISA based on the S/P ratio between positive and negative serum samples was the narrowest among the three ELISA antigens. The differentiation efficiency of rM70S based on the S/P ratio was the lowest among antigens. The ROC curve for rM70S ELISA based on the S/P ratio showed the highest diagnostic efficiency, similar to that of 20K ELISA (Fig. 3). Optimal cutoffs for the OD value, S/N ratio, and S/P ratio were 1.320, 1.696, and 0.143, respectively (Table 2). The S/N ratio was the most appropriate criterion in M70S ELISA in terms of sensitivity (94.4%), specificity (97.3%), PPV (77.3%), and NPV (99.4%).

Discussion

We compared three bTB ELISA antigens confirmed as highly reactive to M. bovis-specific antibodies. In particular, we used PPD as a crude protein antigen mixture, 20K as a purified protein antigen, and rM70S as a recombinant protein antigen. rM70S and 20K were more sensitive and specific in bTB ELISA than PPD. Interestingly, rM70S, which showed high sensitivity and specificity, was composed of a new combination of antigens for bTB serological diagnosis, MPB70 and SahH.

ELISAs have been developed for the serological diagnosis of bTB as a supplementary strategy using various recombinant proteins (MPB70, MPB83, and ESAT6) as well as native antigens (P22, 20 kDa, CMP, and PPD) [1, 2, 5, 12, 17]. Initially, crude and purified native proteins were evaluated as candidate bTB ELISA antigens, such as MPB70, MPB64, MPB59, P32, P70, P65, and CMP70 [17–21]. Purified native antigens, such as CMP, 20 kDa, and MPB70, exhibit higher sensitivity and specificity than those of crude native antigens (PPD). In this study, the native 20-kDa antigen showed higher sensitivity and specificity than those of PPD. Immunospecific-recombinant proteins of M. bovis have been applied as bTB ELISA antigens, such as MPB70, MPB83, ESAT6, CFP10, Mb0143, PE5, PE13, TB10.4, TB15.3, Rv3615c, Rv3020c, and ESAT6/MPB70/MPB83 (fusion protein) [12, 22–26]. In this study, we newly mixed MPB70 with SahH as a recombinant antigen. It was detected as an immunoreactive protein group by 2D-gel and immunoblot analyses. SahH encodes S-adenosylhomocysteinase, an enzyme that catalyses the reversible hydrolysis

of S-adenosylhomocysteine to homocysteine and adenosine and is involved in the mycobacterial stress response [27, 28]. SahH enhances attachment to IL-8 and promotes entry into neutrophils [29]. However, the serological characteristics of SahH have not been reported to date. In this study, the antigenicity of recombinant SahH was confirmed with a size of 75 kDa determined by western blotting (Fig. 1c). Reported values for the sensitivity and specificity of recombinant proteins are 63.0–83.2% and 75.5–98.0%, respectively [12, 22, 25, 26]. In this study, rM70S showed higher or equivalent sensitivity and specificity than those in previous studies. The antigen combination is an important determinant of the sensitivity and specificity of ELISA. In this study, rM70S exhibited comparable sensitivity and specificity to those of the native 20 kDa ELISA antigen. Therefore, rM70S is an appropriate antigen combination with easy and simple production and standardisation.

Conclusions

Even though PPD showed the lowest specificity and sensitivity among the three ELISA antigens evaluated in this study, these values were acceptable for herd screening. PPD has been used as an IST reagent and can easily be applied to bTB ELISA. Overall, native 20K ELISA exhibited higher sensitivity and specificity than those of rM70S. However, rM70S is more convenient for commercialisation with respect to mass production, standardisation, and known concentrations [17, 25].

bTB ELISA using purified and recombinant antigens will be useful for individual tests as well as herd screening [1, 2, 26]. In slaughterhouses, randomly selected serum samples can be evaluated, followed by the tracking of antibody-positive reactors [12, 22, 30]. For the control of bTB, ELISA will decrease labour and time requirements. Furthermore, HUMID can account for the gap in the diagnostic window of CEMID. Therefore, the combination of HUMID and CEMID provides a comprehensive diagnostic system for bTB.

Methods

Purified protein derivative

Mycobacterium bovis AN5 was cultured on Sauton broth to harvest purified protein derivative (PPD), which was produced following the national standard protocol [11]. Briefly, after culture for 8 weeks at 37 °C, the sample was heated at 100 °C for 3 h and the supernatant was harvested by ultracentrifugation to remove bacteria. The culture supernatant was precipitated by 40% trichloroacetic acid (TCA). The precipitate was washed with 1% TCA, acetone, and ethyl ether. The washed pellet was dried at 37 °C. The protein concentration was determined using a BCA Protein Assay Kit (Pierce, Waltham, MA, USA). PPD was aliquoted at 1 mg/vial, dried, and stored at -20 °C.

Purification of the 20-kDa antigen

The 20-kDa protein was purified from the culture supernatant of M. bovis AN5 after killing by heating using a MonoQ HR 5/5 column with an NaCl gradient with AKTA explorer (GE Healthcare, Chicago, IL, USA) for anion exchange chromatography [5]. The culture supernatant was loaded onto the MonoQ HR 5/5 column and fractionated by 1.0 mL/min of a linear gradient from 0 to 2.0 M NaCl using buffer A (30 mM Tris-HCl with 2% butanol, pH 8.8) and buffer B (buffer A with 2.0 M NaCl) at a flow rate of 1.0 mL/min. The protein concentration, as determined by the optical density (OD) at 280 nm, salt concentration, and fraction count were recorded. Fractions with absorbance values of > 1.0 at 280 nm were further analysed by the BCA protein assay (Pierce), SDS-PAGE, and western blotting. The fractions corresponding to 20 kDa were collected, mixed, and stored at -20 °C.

Recombinant MPB70 and SahH

The plasmid pGS (Bionote Co., Hwaseong, Republic of Korea), Escherichia coli Top10F', and E. coli BL21 competent cells were prepared and stocked. The primers were designed according to the sequences of genes encoding MPB70 and SahH of the M. bovis AF2122/97 strain (GenBank accession number NC002945) and synthesised. Recombinant MPB70 and SahH proteins were expressed in E. coli BL21. Genomic DNA from M. bovis AN5 was isolated by the GuSCN/silica method. The MPB70 and SahH DNA fragments were amplified from AN5 genomic DNA by polymerase chain reaction (PCR). The amplified DNA fragments were purified using the Geneclean Kit (Bio 101, Inc., La Jolla, CA, USA), digested with the appropriate restriction enzyme, cloned into the pGS vector, and transformed into the E. coli strain Top10F'. The positive clones were screened by colony PCR and sequenced using vector primers. Each MPB70 and SahH plasmid was transformed into E. coli BL21. Transformed E. coli cells were inoculated in LB and cultivated overnight at 37 °C in a shaker. When the A600 value reached 1.0, isopropyl-β-d-thiogalactoside was added to a final concentration of 2.5 mM. The mixture was grown for another 5 h. The cells were harvested by centrifugation at 5,000 × g for 15 min at 4 °C. The protein was purified on a DEAE-Sepharose gel after harvesting inclusion bodies by ultrasonication. Each fraction was examined by SDS-PAGE and western blotting.

SDS-PAGE and western blotting

PPD, MPB70, SahH, and 20-kDa proteins were analysed by SDS-PAGE and western blotting [5]. Equal volumes of serum samples (n = 3) from a bTB-free farm (confirmed by annual ISTs and the lack of clinical signs of infection) were mixed and used as a negative control. Mycobacterium bovis AN5-positive serum (n = 1) was used as a positive control. Each serum sample was diluted as 1:500 for western blotting. The protein (10 μg per lane) was separated by SDS-PAGE and transferred onto a nitrocellulose (NC) membrane soaked in 600 mL of transfer buffer (15.6 mM Tris and 120 mM glycine) plus 150 mL methanol at 100 V and 400 mA for 1 h [5]. The membranes were then incubated in M. bovis-infected cattle serum diluted 1:500 in 0.01 M PBS at 37 °C for 1 h, followed by anti-bovine IgG horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) diluted 1:1,000 in 0.01 M PBS at 37 °C for 1 h. After washing with 0.01 M PBS (pH 7.4), the membranes were incubated in 3,3'-diaminobenzidine (Sigma, St. Louis, MO, USA) for 5 min at room temperature (22 ± 2 °C).

ELISA

Eighteen serum samples from M. bovis–positive cattle at 8 farms (confirmed by ISTs and the isolation of M. bovis) and 975 serum samples from M. bovis-free cattle at 14 farms (confirmed by ISTs and the lack of clinical signs of infection) were subjected to testing by PPD, MPB70/SahH, and 20-kDa ELISA. Diagnostic efficiency was compared based on sensitivities and specificities. Positive control and negative control sera were used for each ELISA for validation. ELISA results were analysed according to three criteria, the OD value, S/N ratio, and S/P ratio [1, 17, 22, 23]. The optimal method for effectively diagnosing bovine tuberculosis was determined by comparisons of the sensitivity and specificity of each parameter.

Statistical analysis

ELISA results were analysed by Student's t-tests. All data are presented as means ± standard deviations (error bars). All statistical values were considered significant at $P \leq 0.05$.

Abbreviations

bTB: Bovine tuberculosis; PPD: Purified protein derivative; ELISA: Enzyme-linked immunosorbent assay; IST: Intradermal skin test; CEMID: Cell-mediated immunity-based diagnosis; HUMID: Humoral immunity-based diagnosis; CFP: Culture filtrate proteins; INS: Insoluble proteins; SOL: Soluble proteins; S/P: Sample/positive; S/N:

Sample/negative; PPV: Positive predictive value; NPV: Negative predictive value; ROC: Receiver operating characteristic; PCR: polymerase chain reaction

Declarations

Ethics approval and consent to participate

This study was approved by the Animal and Plant Quarantine Agency's Institutional Animal Care and Use Committee, Republic of Korea. Verbal informed consent for the study was obtained from all owners prior to sample collection from the cattle.

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was funded by the Animal and Plant Quarantine Agency (APQA). The funders had no role in the design of the study, data collection, analyses, interpretation of data, or preparation of the manuscript.

Authors' contributions

YSC conceived of the study. YSC, SEL, J-TW, JO, HWC, JHK, J-TK, GH, and CP performed the experiments. SJ and JMK helped with data analysis. YSC, SJ and JMK interpreted the results and YSC wrote the article. All authors read and approved the final manuscript.

Acknowledgments

The authors would like to thank Editage (www.editage.co.kr) for English language editing.

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Figures

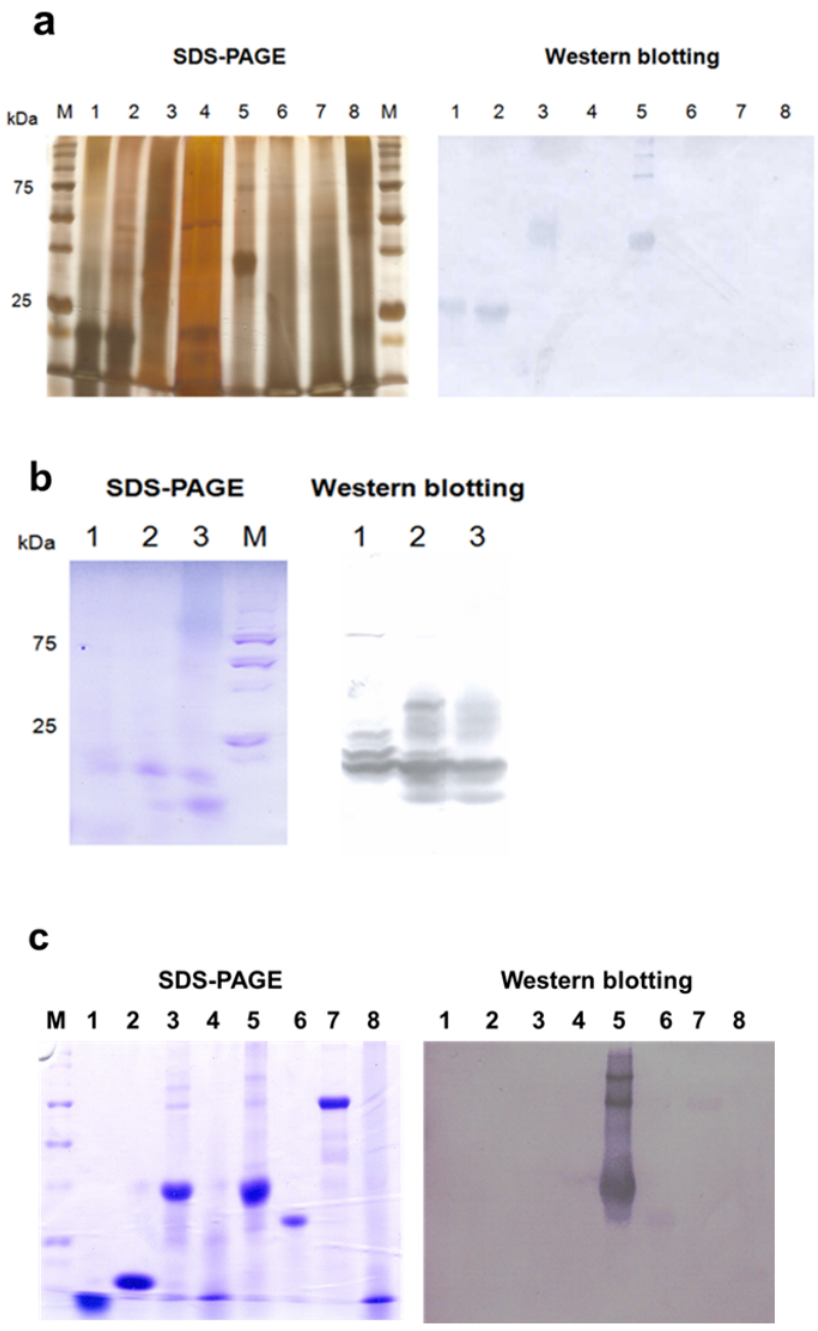

Figure 1

SDS-PAGE and Western blotting of PPD, 20kDa, MPB70, and SahH proteins. In (a), PPDs, CFP, INS, SOL, and MPB70 were produced from M. bovis. Johnin and PPDs were produced from M. avium, and PPD from M. phlei. M. protein marker, lane 1. M. bovis PPD, lane 2. M. bovis CFP, lane 3. M. bovis INS, lane 4. M. bovis SOL, lane 5. Recombinant MPB70, lane 6. M. avium Johnin, lane 7. M. avium PPD, lane 8. M. phlei PPD. In (b), native 20 kDa was analyzed by SDS-PAGE and Western blotting. Lane 1. APQA 20 kDa, lane 2. CVL 20 kDa, lane 3. M. bovis PPD, M. protein marker. In (c), With recombinant MPB70, ESAT6, HspX, PhoS, MPB64, Antigen 85, SahH, and native PPD from M. bovis were analyzed. M. protein marker, lane 1. ESAT6, lane 2. HspX, lane 3. PhoS, lane 4. MPB64, lane 5. MPB70, lane 6. Antigen 85, lane 7. SahH, lane 8. M. bovis PPD.

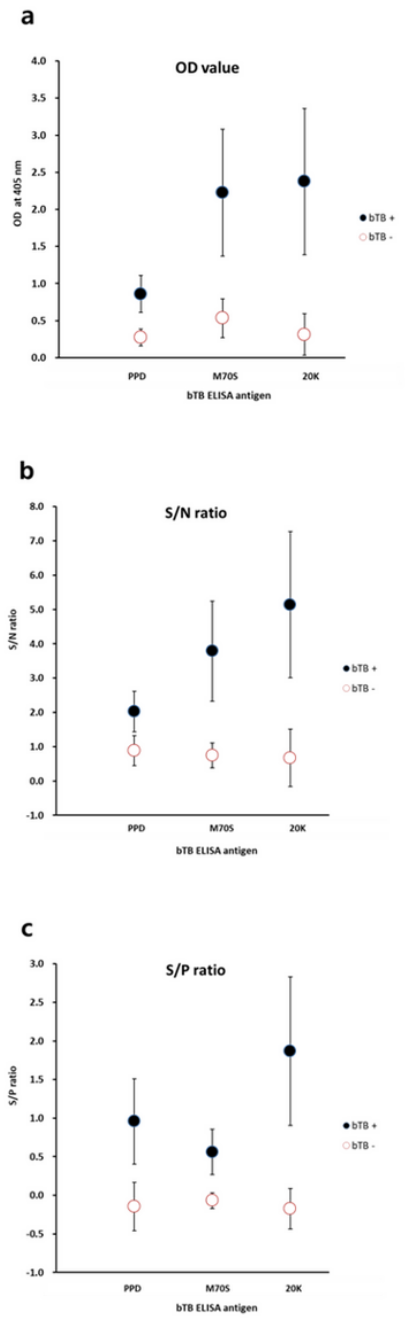

Figure 2

Comparison of OD value (a), S/N ratio (b), and S/P ratio (c) of PPD, M70S, and 20K ELISA. bTB+ was M. bovis-infected cattle's sera (n = 18), and bTB- M.bovis-negative cattle's sera in PPD ELISA (n = 975), M70S ELISA (n = 174), and 20K ELISA (n = 954).

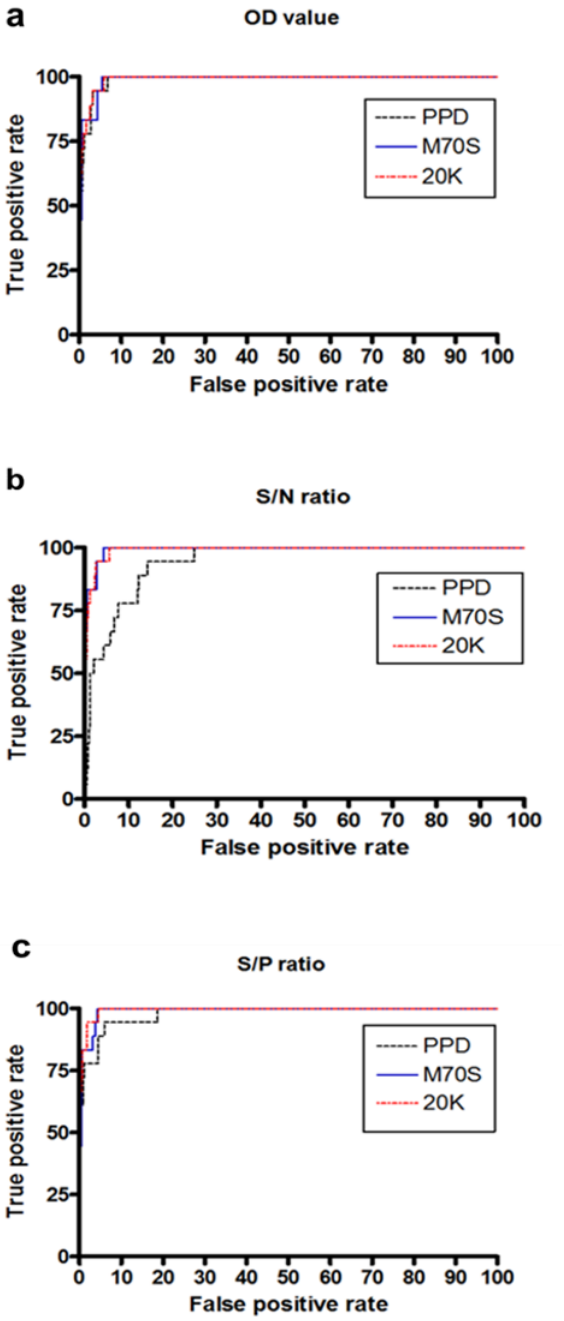

Figure 3

Comparison of ROC curve of PPD, M70S, and 20K ELISA. Criteria of cutoff were OD value (a), S/N ratio (b), and S/P ratio (c).