



Comparing recombinant MPB70/SahH and native 20-kDa protein for detecting bovine tuberculosis using ELISA

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ABSTRACT. Bovine tuberculosis (bTB) is a zoonosis caused by *Mycobacterium bovis*. Test-and-cull protocols and gross pathological examinations of abattoir animals as well as milk pasteurization have been implemented to prevent the spread of tuberculosis from animals to humans worldwide. 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 and due to laborious and time-consuming procedures. Therefore, antibody detection methods such as enzyme-linked immunosorbent assay (ELISA) are urgently needed to supplement the established approaches and expand the diagnostic window. This study was conducted to develop a bTB ELISA by evaluating recombinant and native proteins and various assay parameters. We produced recombinant MPB70 and SahH (M70S) and a native 20-kDa protein (20K) and optimized the ELISA protocol. The 20K ELISA showed 94.4% sensitivity and 98.2% specificity with an optimal sample-to-positive ratio cut-off of 0.531. The sensitivity and specificity of M70S ELISA were 94.4% and 97.3%, respectively, with an optimal sample-to-negative ratio cut-off of 1.696. Both assays showed acceptable diagnostic efficiency and could be used for bTB diagnosis in combination with established methods for herd screening and to expand the diagnostic window.

KEY WORDS: *Mycobacterium bovis*, receiver operating curve, sensitivity, serological diagnosis, specificity

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Bovine tuberculosis (bTB) is caused by *Mycobacterium bovis*, which infects cattle and other mammals, including humans [14, 16]. In South Korea, the prevalence of bTB in cattle was 0.08% (2,898 heads) at the individual level and 0.41% (427 farms) at the farm level in 2018. Infected cattle are subjected to test-and-cull and compensation. Therefore, economic losses due to bTB in South Korea are high in addition to the labor-intensive process of bTB diagnosis [7, 17, 18]. Furthermore, the potential spread from *M. bovis*-infected cattle to people coming in contact is an important issue [4, 9, 21, 26].

Currently, test-and-cull and abattoir surveillance as well as milk pasteurization are used to prevent the transmission of tuberculosis from animals to humans in South Korea [5, 7, 17]. bTB has been detected through cell-mediated immunity-based diagnosis (CEMID) such as intradermal skin tests (IST) and γ -interferon assays [2, 14, 30]. However, CEMID has a bTB diagnostic window with a high false-negative rate and is laborious, requiring two farm visits for injection and interpretation [1, 8, 37]. To complement CEMID, methods for humoral immunity-based diagnosis of bTB are urgently needed for the rapid screening of bTB suspicious herds and/or individuals in a more convenient manner compared to diagnostic methods based on CEMID.

Here, we produced recombinant MPB70 and SahH (M70S) and compared it with a native 20-kDa protein and purified protein derivative (PPD) to establish a serological bTB assay. To the best of our knowledge, this is the first study to combine the use of MPB70 and SahH antigens in a bTB enzyme-linked immunosorbent assay (ELISA).

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MATERIALS AND METHODS

Ethics approval

The Animal and Plant Quarantine Agency (APQA) is a national central research institute in the Republic of Korea that has been researching and developing diagnostics, treatment, and prevention technologies for animal diseases since 1911. The Institutional Animal Care and Use Committee (IACUC) of APQA for animal testing was first organized and operated in 2008. This study was conducted prior to the organization of IAUCC in APQA, and the regulations on animal welfare were conducted in accordance with general regulations. Blood was collected at the time of bTB testing, and bTB-positive sample collection was performed at the time of slaughtering in accordance with the national animal disease prevention policy.

Purified protein derivative

Mycobacterium bovis AN5 was cultured on Sauton broth to harvest PPD according to the national standard protocol in Korea [5]. Briefly, after culture for 8 weeks at 37°C, the sample was heated at 100°C for 3 hr and the supernatant was harvested through ultracentrifugation to remove bacteria. The culture supernatant was precipitated with 40% trichloroacetic acid. The precipitate was washed continuously with 1% trichloroacetic acid, acetone, and ethyl ether. The washed pellet was dried at 37°C overnight. The protein concentration was determined using a BCA Protein Assay Kit (Pierce, Waltham, MA, USA). The PPD was aliquoted at 1 mg per vial, dried, and stored at -20°C until use.

Purification of 20-kDa antigen

The 20-kDa (20K) protein was purified from the culture supernatant of *M. bovis* AN5 using a MonoQ HR 5/5 column with an NaCl gradient on an AKTA explorer (GE Healthcare, Little Chalfont, UK) [7]. The culture supernatant was loaded onto a MonoQ HR 5/5 column and fractionated over a linear gradient of 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 determined at an optical density (OD) of 280 nm, salt concentration, and fraction count were recorded. Fractions with absorbance values >1.0 at 280 nm were further analyzed using the BCA protein assay (Pierce), SDS-PAGE, and western blotting. Fractions corresponding to 20 kDa were collected, mixed, and stored at -20°C until use.

Recombinant MPB70 and SahH

The plasmid pGS (Bionote Co., Hwaseong, Republic of Korea), *E. coli* Top10F', and *E. coli* BL21 competent cells were prepared and stocked. The primers were designed and synthesized according to the gene sequences encoding MPB70 and SahH of *M. bovis* strain AF2122/97 (GenBank accession number NC002945) (Table 1). Recombinant MPB70 and SahH proteins were expressed in *E. coli* BL21. Genomic DNA from *M. bovis* AN5 was isolated using the GuSCN/silica method. The MPB70 and SahH DNA fragments were amplified from *M. bovis* AN5 genomic DNA by using the polymerase chain reaction using *Taq* DNA polymerase (Bioneer, Daejeon, Republic of Korea). PCR conditions for MPB70 were as follows: initial denaturation at 95°C for 12 min; followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, and extension at 72°C for 2 min; and a final extension at 72°C for 10 min. The PCR conditions for SahH were: an initial denaturation at 94°C for 5 min; 28 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min; and 1 cycle at 94°C for 1 min, 60°C for 1 min, and 72°C for 10 min. The amplified 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 using colony polymerase chain reaction and sequenced using vector primers. Each MPB70 and SahH plasmid was transformed into *E. coli* BL21. The transformed *E. coli* cells were inoculated in Luria-Bertani broth and cultivated overnight at 37°C in a shaker. When the A600 (optical density at 600 nm) value reached 1.0, isopropyl-β-D-thiogalactoside was added to a final concentration of 2.5 mM. The mixture was further grown for 5 hr and the cells were harvested through centrifugation at 5,000 ×g for 15 min at 4°C. The protein was purified on a DEAE-Sepharose gel after harvesting the inclusion bodies through ultrasonication. Each fraction was examined using SDS-PAGE and western blotting.

SDS-PAGE and western blotting

PPD, MPB70, SahH, and the 20-kDa protein were analyzed using SDS-PAGE and western blotting, as previously described [7]. Equal volumes of serum samples ($n=3$) from a bTB-free farm (confirmed by annual ISTs and no previous report of bTB) were mixed and used as the negative control. Serum from cattle immunized with *M. bovis* AN5 ($n=1$) was used as a positive control.

Table 1. Primer sequences of MPB70 and SahH

Target gene	Primer (F/R)	Oligonucleotides primer sequence	PCR product size (bp)
MPB70	Forward (<i>Bam</i> HI)	5' <u>GCGGATCC</u> GGCATGAAGGTA3' ^{a)}	600
	Reverse (<i>Sal</i> I)	5' <u>CCGTCGACT</u> <i>TAA</i> ACGCCGAGGCATTAGCAC3' ^{b)}	
SahH	Forward (<i>Bam</i> HI)	5' <u>GGATCC</u> ATGACCGGAAATTGGTC3' ^{a)}	1,488
	Reverse (<i>Sal</i> I)	5' <u>GTCGACT</u> <i>TAGT</i> AGCGGTAGTGGTC3' ^{b)}	

a) *Bam*HI recognition sequence is underlined. b) *Sal*I recognition sequence is underlined and stop codon is in bold and italics.

Ten milliliters of heat-inactivated *M. bovis* AN5 (5×10^9 cells/injection) with incomplete Freund's adjuvant was inoculated subcutaneously 4 times on day 14, day 28, and day 56 after the first inoculation. The antibody titer was checked using ELISA with *M. bovis* AN5 sonicated antigen and the positive serum was bled at 31 days after the last 4th injection. The proteins were separated using SDS-PAGE and then analyzed using western blotting [7].

ELISA

A total of 18 serum samples from *M. bovis*-positive cattle from 8 farms (confirmed by ISTs and *M. bovis* isolation) and 975 serum samples from *M. bovis*-negative cattle from 14 farms (confirmed by ISTs and absence of clinical signs of infection) in South Korea were tested using PPD, M70S, and 20K ELISA. Blood samples were collected from the jugular or tail vein, followed by serum separation and inactivation at 56°C for 30 min. All sera were stored at -20°C until use. Indirect bTB ELISA was performed to detect *M. bovis* antibody, as previously described [6, 7]. The optimal concentration for PPD, M70S, and 20K proteins was 1 µg/ml. The sera were diluted to 1:200 with PBS prior to use. One hundred microliters of rabbit anti-bovine IgG-conjugated horseradish peroxidase diluted 1:2,000 with Tween-PBS were added to each well. The substrate solution was prepared just before use; 100 µl was added to each well, and the plates were incubated for 30 min at room temperature ($22 \pm 2^\circ\text{C}$). The reaction was stopped using 1.0 M H₂O₂ and the OD at 405 nm was calculated [6, 7]. ELISA results were analyzed according to three criteria: the OD value, sample/negative (S/N) ratio, and sample/positive (S/P) ratio. The diagnostic efficiency was compared based on sensitivity and specificity. Positive and negative control sera were used in each ELISA for validation [6, 13, 16, 32]. The optimal method for effectively diagnosing bovine tuberculosis was based on the area under the receiver operator characteristic (ROC) curve (AUC) values determined, and the sensitivity and specificity of each parameter were compared.

Statistical analysis

ELISA results were analyzed using one-way analysis of variance (ANOVA) with Bonferroni *post hoc*. All data are presented as mean \pm standard deviation (error bars). Statistical analysis was performed using Microsoft Excel 2010. All statistical values were considered significant at $P \leq 0.05$. ROC analyses were performed using GraphPad Prism.

RESULTS

Protein profile and antigenicity

The major protein component of PPD was a 20-kDa protein on SDS-PAGE. The 20-kDa protein of PPD is also a major immunoreactive protein against *M. bovis*-positive serum. The 20-kDa antigen was purified using anion exchange chromatography [7] and evaluated using western blotting (Fig. 1A). M70S was produced and evaluated with respect to size and antigenicity against *M. bovis*-positive serum. The sizes of MPB70 and SahH were 37 kDa and 75 kDa, respectively, according to western blotting against *M. bovis*-positive serum (Fig. 1B).

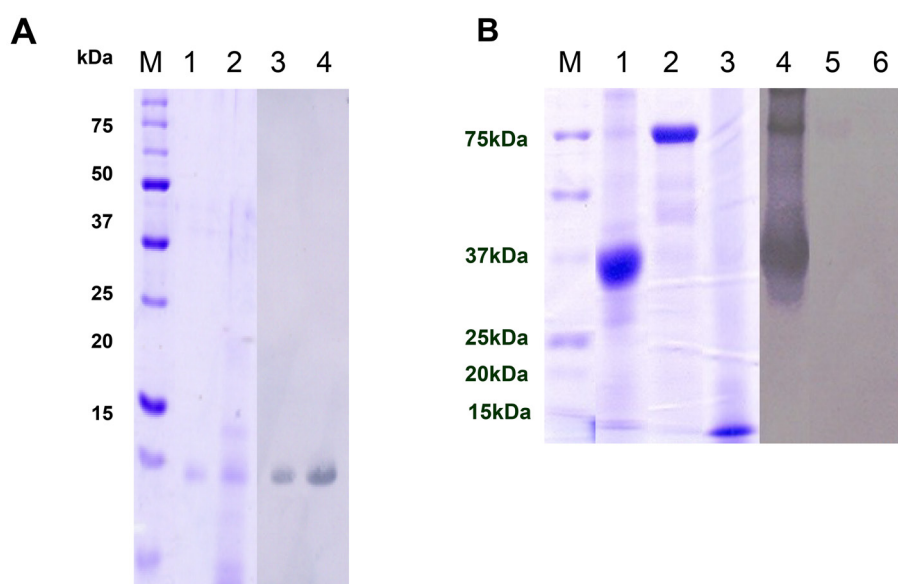


Fig. 1. SDS-PAGE analysis and western blotting of native 20 kDa antigen (A), and recombinant MBP70 and SahH antigen (B). (A) M, molecular weight marker. Lane 1 to 2, SDS-PAGE of native 20 kDa antigen. Lane 3 to 4, western blotting of native 20 kDa antigen with serum from cattle infected with *Mycobacterium bovis* AN5. (B) M, molecular weight marker. Lane 1 to 3, SDS-PAGE of recombinant MBP70 and SahH, and purified protein derivative (PPD). Lane 4 to 6, western blotting of recombinant MBP70 and SahH, and purified protein derivative (PPD) with serum from cattle immunized with *M. bovis* AN5.

ELISA for purified protein derivative

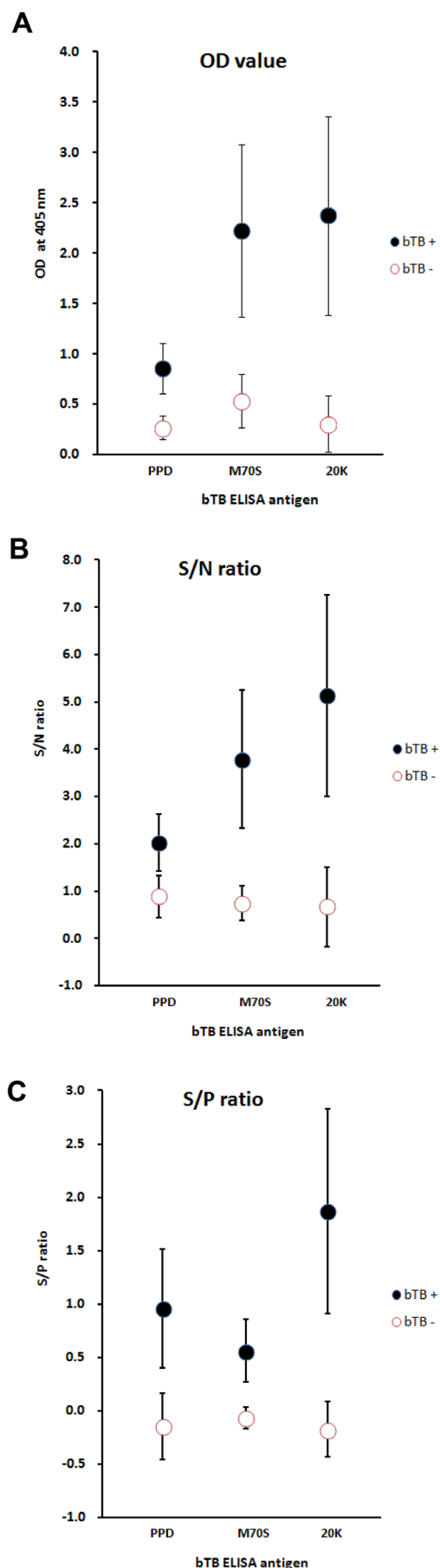
We evaluated the sensitivity and specificity of three antigens through ELISA using 18 bTB-positive serum samples and 975 bTB-negative serum samples. The 18 bTB-positive serum samples were confirmed as *M. bovis* infections by *M. bovis* isolation. The average OD value of *M. bovis*-positive serum samples ($n=18$) using PPD was 0.860, whereas that of *M. bovis*-negative serum samples ($n=975$) using PPD was 0.273 (Fig. 2A). The results of PPD ELISA by OD values for bTB-positive and -negative sera were significantly different from those of M70S and 20K ELISA ($P<0.001$). Compared to M70S and 20K ELISA, the difference in PPD ELISA reactivity between positive and negative sera was significantly lower when the ELISA results were analyzed using the average OD value (Fig. 2A). The average S/N ratio of *M. bovis*-positive serum samples ($n=18$) using PPD was 2.026, whereas that of *M. bovis*-negative serum samples ($n=975$) using PPD was 0.886 (Fig. 2B). The results of PPD ELISA by S/N ratio in bTB-positive and -negative sera were also significantly different from those of M70S and 20K ELISA ($P<0.001$), and the difference in the S/N ratio between positive and negative serum samples was significantly narrow for PPD among the three antigens (Fig. 2B). The ROC curve for PPD ELISA based on the S/N ratio also showed the lowest diagnostic efficiency (Table 2 and Fig. 3B). The average S/P ratio for *M. bovis*-positive serum samples ($n=18$) using PPD was 0.958, whereas that of *M. bovis*-negative serum samples ($n=975$) using PPD was -0.146 (Fig. 2C). The results of PPD ELISA by S/P ratio in bTB-positive and -negative sera were significantly different from those of M70S and 20K ELISA ($P<0.05$). Unlike the comparisons of OD value and S/N ratio, the difference in the S/P ratio between positive and negative serum samples for PPD was intermediate (Fig. 2C). The ROC curve for the PPD ELISA based on the S/P ratio showed the lowest diagnostic efficiency (Fig. 3C). The optimal cut-off values for the OD value, S/N ratio, and S/P ratio in PPD ELISA were 0.618, 1.289, and 0.271, respectively, based on the highest AUC value (Table 2 and Fig. 3). The OD value, among the three analytic criteria assessed, was the most appropriate 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 2).

20-kDa ELISA

The average OD value for *M. bovis*-positive serum samples ($n=18$) with 20K was 2.376, whereas that of *M. bovis*-negative serum samples ($n=975$) at 20K was 0.310 (Fig. 2A). The results of 20K ELISA by OD values to bTB-positive serum samples were not significantly different from those of M70S ELISA ($P=0.623$), while the results of 20K ELISA for bTB-negative serum samples were significantly different from those of M70S ELISA ($P<0.001$). Compared to PPD and M70S ELISA, the reactivity of the 20K ELISA exhibited the greatest difference between the positive and negative sera (Fig. 2A). The ROC curve constructed for 20K ELISA based on OD values showed the highest diagnostic efficiency, such that the AUC of the 20K ELISA was 0.991 (Table 2 and Fig. 3A). The average S/N ratio for *M. bovis*-positive serum samples ($n=18$) at 20K was 5.138, whereas that of *M. bovis*-negative serum samples ($n=975$) at 20K was 0.671 (Fig. 2B). The results of 20K ELISA by S/N ratio to bTB-positive sera were significantly different from those of M70S ELISA ($P=0.033$), while the results of 20K ELISA for bTB-negative sera were not significantly different from those of M70S ELISA ($P=0.219$). The reactivity of the 20K ELISA based on the S/N ratio exhibited the greatest difference between positive and negative sera among the three antigens (Fig. 2B). The ROC curve of the 20K ELISA based on the S/N values showed intermediate diagnostic efficiency, such that the AUC of the 20K ELISA was 0.991 (Table 2 and Fig. 3B). The average S/P ratio for *M. bovis*-positive serum samples ($n=18$) at 20K was 1.869, whereas that of *M. bovis*-negative serum samples ($n=975$) at 20K was -0.174 (Fig. 2C). The results of 20K ELISA by S/P ratio in bTB-positive and -negative sera were significantly different from those of M70S ELISA ($P<0.001$). The reactivity of the 20K ELISA based on the difference in the S/P ratio between positive and negative sera was the highest among the three antigens (Fig. 2C). The ROC curve for 20K ELISA based on S/P values exhibited the highest diagnostic efficiency, such that the AUC of the 20K ELISA was 0.994 (Table 2 and Fig. 3C). The optimal cut-off values for the OD value, S/N ratio, and S/P ratio were 1.149, 2.139, and 0.531, respectively, based on the highest AUC value (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%) (Table 2).

MPB70/ SahH ELISA

The average OD value for *M. bovis*-positive serum ($n=18$) with M70S was 2.224, whereas that of *M. bovis*-negative serum ($n=184$) with M70S was 0.533 (Fig. 2A). Compared with the PPD and 20K ELISA analyses employing the average OD value, the difference in M70S ELISA reactivity between positive and negative sera was similar but slightly less than that in 20K ELISA reactivity and greater than that in PPD ELISA reactivity (Fig. 2A). The ROC curve for M70S ELISA based on the OD values showed a diagnostic efficiency similar to that of the 20K ELISA, with an AUC of M70S ELISA of 0.990 (Table 2 and Fig. 3A). The average S/N ratio of *M. bovis*-positive serum samples ($n=18$) with M70S was 3.788, whereas that of *M. bovis*-negative serum samples ($n=184$) with M70S was 0.748 (Fig. 2B). The reactivity of M70S ELISA based on the difference in S/N ratios between positive and negative serum samples was intermediate among the three antigens (Fig. 2B). Interestingly, the ROC curve for M70S ELISA based on the S/N ratio showed the highest diagnostic efficiency, with an AUC of M70S ELISA of 0.994 (Table 2 and Fig. 3B). The average S/P ratio for *M. bovis*-positive serum samples ($n=18$) with M70S was 0.561, whereas that of *M. bovis*-negative serum samples ($n=184$) with M70S was -0.068 (Fig. 2C). The difference in M70S ELISA reactivity based on the S/P ratio between positive and negative serum samples was the narrowest among the three antigens (Fig. 2C). The ROC curve for M70S ELISA based on the S/P ratio showed intermediate diagnostic efficiency, such that the AUC of M70S ELISA was 0.992 (Table 2 and Fig. 3C). The optimal cut-off values for the OD value, S/N ratio, and S/P ratio were 1.320, 1.696, and 0.143, respectively, based on the highest AUC value (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%) (Table 2).



DISCUSSION

We compared the sensitivity and specificity of three bTB ELISA antigens: PPD, M70S, and 20K. In particular, we used PPD as a crude protein antigen mixture, 20K as a purified protein antigen, and M70S as a recombinant protein antigen combination. M70S and 20K were more sensitive and specific than PPD in bTB ELISA. M70S comprised of a new combination of antigens, MPB70 and SahH. Interestingly, M70S ELISA showed sensitivity and specificity equivalent to those of the 20K ELISA. We evaluated three antigen ELISAs using three criteria: OD values, S/N ratio, and S/P ratio (Table 2). The criteria for OD values showed the highest specificity, but the lowest sensitivity. The S/N and S/P ratios showed the highest sensitivity. The specificity of the S/N ratio was the lowest, and that of the S/P ratio was moderate.

ELISAs using various recombinant proteins (MPB70, MPB83, and ESAT6) as well as native antigens (P22, 20 kDa, CMP, and PPD) have been developed as a supplementary strategy for bTB serological diagnosis [6, 7, 14, 16, 30]. Initially, native proteins such as MPB70, MPB64, MPB59, P32, P70, P65, and CMP70 were evaluated as bTB ELISA antigens [6, 11, 12, 15, 38]. Purified proteins such as CMP, 20kDa, and MPB70 exhibit higher sensitivity and specificity compared to crude proteins such as PPD. In this study, the purified 20-kDa antigen also showed higher sensitivity and specificity than those of PPD. Immunospecific-recombinant proteins of *M. bovis*, such as MPB70, MPB83, ESAT6, CFP10, Mb0143, PE5, PE13, TB10.4, TB15.3, Rv3615c, Rv3020c, and ESAT6/MPB70/MPB83 have been previously used as bTB ELISA antigens [13, 29, 30, 32, 34, 35]. Here, we mixed MPB70 with SahH as a novel recombinant antigen combination. SahH was detected as a high molecular weight immunoreactive protein group through 2D-gel electrophoresis and immunoblot analyses. SahH encodes *S*-adenosylhomocysteinase, which catalyzes the reversible hydrolysis of *S*-adenosylhomocysteine to homocysteine and adenosine and is involved in mycobacterial stress responses [28, 33]. SahH enhances bacterial attachment to IL-8 and promotes its entry into neutrophils [10]. However, the B cell antigenic characteristics of SahH are unclear. In this study, the antigenicity of recombinant SahH was confirmed with a size of 75 kDa determined using western blotting.

Previously, the sensitivity and specificity of bTB ELISA using recombinant proteins were reported as 63.0–83.2% and 75.5–98.0%, respectively [13, 29, 30, 34]. MPB70 and MPB83 are the most important recombinant antigens in ELISA [1, 3, 19, 22]. Commercially available ELISA kits using MPB70 and MPB83 showed 50% sensitivity and 97.5% specificity [3]. In this study, upon comparison of the MPB70 and SahH antigen combinations with a commercial kit based on a previous report [3], a similar specificity and a higher sensitivity of 94.4% were obtained (Table 2). Theoretically, the size and pI of MPB70 are 22 kDa and 4.1, respectively, which are similar to those of MPB83; however, the

Fig. 2. Comparison of purified protein derivative (PPD), M70S, and 20K ELISA for bovine tuberculosis (bTB)-positive and negative sera analyzed based on three criteria—optical density (OD) value (A), S/N ratio (B), and S/P ratio (C). bTB+ represents the sera of *M. bovis*-infected cattle ($n=18$), and bTB- represents the sera of *M. bovis*-negative cattle in PPD ELISA ($n=975$), M70S ELISA ($n=184$), and 20K ELISA ($n=975$). bTB, bovine tuberculosis. M70S, recombinant MPB70 and SahH protein combination. 20K, native purified 20-kDa protein from *Mycobacterium bovis* culture filtrate proteins. S/N ratio, ratio of sample OD/negative control OD. S/P ratio, ratio of sample OD/positive control OD.

Table 2. Sensitivity, specificity, positive predictive value, negative predictive values at optimal cutoff of bTB ELISA antigens

Antigen	Criteria	Cutoff ^{a)}	Sensitivity ^{b)}	Specificity ^{c)}	PPV ^{d)}	NPV ^{e)}	Accuracy ^{f)}	AUC ^{g)} (95% CI ^{h)})
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	1.32	77.8	99.5	93.3	97.9	97.5	0.990 (0.979–1.000)
	S/N	1.696	94.4	97.3	77.3	99.4	97	0.994 (0.986–0.998)
	S/P	0.143	94.4	96.2	70.8	99.4	96	0.992 (0.967–0.999)

a) The optimal cutoff value was determined at the highest area under curve (AUC) value in the receiver operating curves (ROC) curve. b) Sensitivity= $[(\text{true pos.})/(\text{true pos.} + \text{false neg.})] \times 100$. c) Specificity= $[(\text{true neg.})/(\text{true neg.} + \text{false pos.})] \times 100$. d) PPV (positive predictive value)= $[(\text{true pos.})/(\text{true pos.} + \text{false pos.})] \times 100$. e) NPV (negative predictive value)= $[(\text{true neg.})/(\text{false neg.} + \text{true neg.})] \times 100$. f) Accuracy= $[(\text{true pos.} + \text{true neg.})/(\text{true pos.} + \text{false pos.} + \text{false neg.} + \text{true neg.})] \times 100$. g) AUC: ROC area under the curve. h) CI: confidence interval.

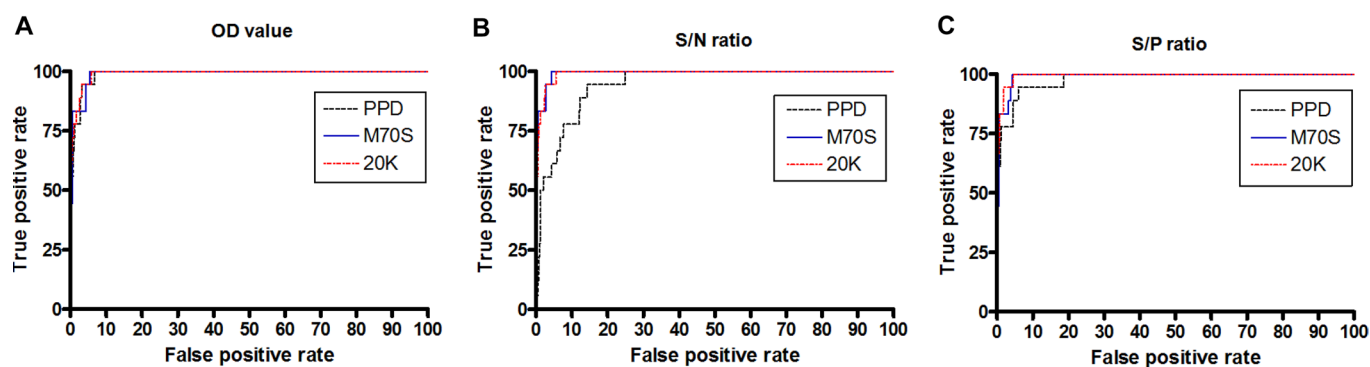


Fig. 3. Comparison of receiver operating curves (ROC) curve of purified protein derivative (PPD), M70S, and 20K ELISA. Criteria of cut-off were optical density (OD) value (A), S/N ratio (B), and S/P ratio (C). Bovine tuberculosis (bTB)+ represents the sera of *Mycobacterium bovis*-infected cattle ($n=18$), and bTB- represents the sera of *M. bovis*-negative cattle in PPD ELISA ($n=975$), M70S ELISA ($n=184$), and 20K ELISA ($n=975$). M70S, recombinant MPB70 and SahH protein combination. 20K, native purified 20-kDa protein from *M. bovis* culture filtrate proteins.

theoretical size and pI of SahH are 60 kDa and 4.7, respectively. Since MPB70 and MPB83 have the similar protein sizes and pI values, and the immune response to these two proteins in cattle infected with *M. bovis* is the most potent, they are thought to have similar and overlapping immune reactions. However, since SahH has a significantly different protein size and pI value compared to these two widely used proteins, bTB-positive cattle, which showed no antibody response to MPB70 or MPB83, exhibited antibody-positive reactions to SahH. Therefore, the combination of MPB70 and SahH used in this study is considered to be more sensitive than the previously used single antigens and the MPB70/MPB83 combination. Thus, the MPB70 and SahH antigen combinations can increase sensitivity without inhibiting specificity.

In a previous study, the sensitivity and specificity of sonicated supernatant cultures in ELISA were 47.5% and 94.4%, respectively [27]. Interestingly, ELISA using M70S and 20K antigens showed a significant improvement in sensitivity [27]. Moreover, the sensitivity and specificity of M70S and 20K ELISA were superior to those of culture filtrates and the lipoarabinomannan ELISA [31]. Identification of the 20-kDa antigen confirmed that it was composed of 22 proteins [7]. MPB70 and MPB83 were included along with the other 20 proteins such as MPT64, CFP21, GroEL1, LppX, CFP10, MPT32, and EsxN [7]. Due to the composition of these immunogenic proteins in 20-kDa antigens, the sensitivity and specificity of 20K ELISA were improved over those of ELISA employing the crude antigen PPD [7]. Furthermore, the 20K ELISA was more reactive in the positive and false-positive reactors than the MPB70-only ELISA in a previous study [7]. Moreover, the 20K ELISA showed a higher specificity than the MPB70-only ELISA, with a lower antibody titer to the IST-negative cattle in a previous study [7]. In a previous study, the sensitivity of ELISA using the MPB83-only antigen was 37.5% and specificity was 89% [25]. Thus, the 20-kDa ELISA was confirmed to have superior sensitivity and specificity compared to the MPB70-only and MPB83-only ELISA [7]. The recombinant protein combination of MPB70 and SahH ELISA showed equivalent sensitivity and specificity to the 20K ELISA (Table 2). Thus, the MPB70 and SahH ELISA showed improved sensitivity and specificity compared to the MPB70-only and MPB83-only ELISA.

Antibody measurements for individual antigens are known to differ significantly between individuals [24]. Therefore, the antigen combination is an important determinant of sensitivity and specificity in ELISA. ELISA using three recombinant protein antigens, MPB70, MPB83, and ESAT6, showed 69.5% sensitivity and 96% specificity in a previous study [20]. The ELISA sensitivities

of PC (MPB83, MPB70, Rv2650c, Mb1498, SerS), DID38 (MPB70, MPB83), and DID65 (MPB70, PstS1, CFP10) were 90.5%, 85.1%, and 81.1%, respectively, and the specificities were 98.1%, 99.1%, and 98.2%, respectively, in a previous study [23]. In this study, for ELISA using the antigen combination 22-kDa MPB70 and 60-kDa SahH, the sensitivity was 94.4% and the specificity was 97.3% based on the S/N ratio criteria (Table 2). The sensitivity of M70S ELISA was higher than that of the ELISA employing the recombinant antigen combinations reported previously, and the specificity was similar. Thus, M70S is an appropriate antigen combination. In the future, a recombinant protein combination composed of proteins with confirmed B cell antigenicity is expected to show further improved sensitivity.

In conclusion, M70S and 20K ELISA will be useful for individual testing as well as herd screening [14, 16, 29]. M70S ELISA showed sensitivity and specificity equivalent to those of native 20K ELISA. M70S is more convenient for commercialization with respect to mass production, standardization, and known concentrations of antigen components compared to native 20K [6, 34]. In slaughterhouses, randomly selected serum samples can be evaluated, followed by tracking of antibody-positive reactors [13, 30, 36]. For controlling bTB, ELISA can decrease labor and time requirements. Furthermore, humoral immunity-based diagnosis can account for the gap in the diagnostic window of CEMID, such as false-negatives of late infection. The M70S and 20K ELISA proposed in this study can be useful as a supplementary serological method for diagnosing bovine tuberculosis.

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REFERENCES

1. Amadori, M., Lyashchenko, K. P., Gennaro, M. L., Pollock, J. M. and Zerbini, I. 2002. Use of recombinant proteins in antibody tests for bovine tuberculosis. *Vet. Microbiol.* **85**: 379–389. [Medline] [CrossRef]
2. Bezos, J., Casal, C., Romero, B., Schroeder, B., Hardegger, R., Raeber, A. J., López, L., Rueda, P. and Domínguez, L. 2014. Current ante-mortem techniques for diagnosis of bovine tuberculosis. *Res. Vet. Sci.* **97** Suppl: S44–S52. [Medline] [CrossRef]
3. Buddle, B. M., Wilson, T., Luo, D., Voges, H., Linscott, R., Martel, E., Lawrence, J. C. and Neill, M. A. 2013. Evaluation of a commercial enzyme-linked immunosorbent assay for the diagnosis of bovine tuberculosis from milk samples from dairy cows. *Clin. Vaccine Immunol.* **20**: 1812–1816. [Medline] [CrossRef]
4. Cadmus, S., Akinseye, V. O. and van Soolingen, D. 2019. *Mycobacterium bovis* in humans and *M. tuberculosis* in animals in Nigeria: an overview from 1975–2014. *Int. J. Tuberc. Lung Dis.* **23**: 1162–1170. [Medline] [CrossRef]
5. Cho, Y. S., Jang, Y. B., Lee, S. E., Cho, J. Y., Ahn, J. M., Hwang, I., Heo, E., Nam, H. M., Cho, D., Her, M., Jean, Y. H., Jung, S. C., Kim, J. M., Lee, H. S., Lee, K. and Belisle, J. T. 2015. Short communication: Proteomic characterization of tuberculin purified protein derivative from *Mycobacterium bovis*. *Res. Vet. Sci.* **101**: 117–119. [Medline] [CrossRef]
6. Cho, Y. S., Jung, S. C., Kim, J. M. and Yoo, H. S. 2007. Enzyme-linked immunosorbent assay of bovine tuberculosis by crude mycobacterial protein 70. *J. Immunoassay Immunochem.* **28**: 409–418. [Medline] [CrossRef]
7. Cho, Y. S., Lee, S. E., Ko, Y. J., Cho, D., Lee, H. S., Hwang, I., Nam, H., Heo, E., Kim, J. M. and Jung, S. 2009. Definition of purified enzyme-linked immunosorbent assay antigens from the culture filtrate protein of *Mycobacterium bovis* by proteomic analysis. *J. Immunoassay Immunochem.* **30**: 291–304. [Medline] [CrossRef]
8. de la Rúa-Domenech, R., Goodchild, A. T., Vordermeier, H. M., Hewinson, R. G., Christiansen, K. H. and Clifton-Hadley, R. S. 2006. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. *Res. Vet. Sci.* **81**: 190–210. [Medline] [CrossRef]
9. Diallo, M., Diarra, B., Sanogo, M., Togo, A. C., Somboro, A. M., Diallo, M. H., Traoré, B., Maiga, M., Koné, Y., Tounkara, K., Sarro, Y. D., Baya, B., Goita, D., Kassambara, H., Dembélé, B. P., Siddiqui, S., Murphy, R. L., Dao, S., Diallo, S., Tounkara, A. and Niang, M. 2016. Molecular identification of *Mycobacterium bovis* from cattle and human host in Mali: expanded genetic diversity. *BMC Vet. Res.* **12**: 145. [Medline] [CrossRef]
10. Dziadek, B., Brzostek, A., Grzybowski, M., Fol, M., Krupa, A., Kryczka, J., Plocinski, P., Kurdowska, A. and Dziadek, J. 2016. *Mycobacterium tuberculosis* AtsG (Rv0296c), GlnU (Rv1018c) and SahH (Rv3248c) proteins function as the human IL-8-binding effectors and contribute to pathogen entry into human neutrophils. *PLoS One* **11**: e0148030. [Medline] [CrossRef]
11. Fifis, T., Costopoulos, C., Corner, L. A. and Wood, P. R. 1992. Serological reactivity to *Mycobacterium bovis* protein antigens in cattle. *Vet. Microbiol.* **30**: 343–354. [Medline] [CrossRef]
12. Fifis, T., Plackett, P., Corner, L. A. and Wood, P. R. 1989. Purification of a major *Mycobacterium bovis* antigen for the diagnosis of bovine tuberculosis. *Scand. J. Immunol.* **29**: 91–101. [Medline] [CrossRef]
13. Fontana, S., Pacciarini, M., Boifava, M., Pellesi, R., Casto, B., Gastaldelli, M., Koehler, H., Pozzato, N., Casalnuovo, F. and Boniotti, M. B. 2018. Development and evaluation of two multi-antigen serological assays for the diagnosis of bovine tuberculosis in cattle. *J. Microbiol. Methods* **153**: 118–126. [Medline] [CrossRef]
14. Garbaccio, S. G., Garro, C. J., Delgado, F., Tejada, G. A., Eirin, M. E., Huertas, P. S., Leon, E. A. and Zumárraga, M. J. 2019. Enzyme-linked immunosorbent assay as complement of intradermal skin test for the detection of *mycobacterium bovis* infection in cattle. *Tuberculosis (Edinb.)* **117**: 56–61. [Medline] [CrossRef]
15. Harboe, M., Wiker, H. G., Duncan, J. R., Garcia, M. M., Dukes, T. W., Brooks, B. W., Turcotte, C. and Nagai, S. 1990. Protein G-based enzyme-linked immunosorbent assay for anti-MPB70 antibodies in bovine tuberculosis. *J. Clin. Microbiol.* **28**: 913–921. [Medline] [CrossRef]
16. Infantes-Lorenzo, J. A., Moreno, I., Roy, A., Riscalde, M. A., Balseiro, A., de Juan, L., Romero, B., Bezos, J., Puentes, E., Åkerstedt, J., Tessema, G. T., Gortázar, C., Domínguez, L. and Domínguez, M. 2019. Specificity of serological test for detection of tuberculosis in cattle, goats, sheep and pigs under different epidemiological situations. *BMC Vet. Res.* **15**: 70. [Medline] [CrossRef]
17. Jeon, B. Y., Kim, S. C., Je, S., Kwak, J., Cho, J. E., Woo, J. T., Seo, S., Shim, H. S., Park, B. O., Lee, S. S. and Cho, S. N. 2010. Evaluation of enzyme-linked immunosorbent assay using milk samples as a potential screening test of bovine tuberculosis of dairy cows in Korea. *Res. Vet. Sci.* **88**: 390–393. [Medline] [CrossRef]

18. Jeon, H. S., Shin, A. R., Son, Y. J., Kim, J. M., Jang, Y., Kim, S., Lee, K. I., Choi, C. H., Park, J. K. and Kim, H. J. 2015. An evaluation of the use of immunoglobulin A antibody response against mycobacterial antigens for the diagnosis of *Mycobacterium bovis* infection in cattle. *J. Vet. Diagn. Invest.* **27**: 344–351. [[Medline](#)] [[CrossRef](#)]
19. Lightbody, K. A., Skuce, R. A., Neill, S. D. and Pollock, J. M. 1998. Mycobacterial antigen-specific antibody responses in bovine tuberculosis: an ELISA with potential to confirm disease status. *Vet. Rec.* **142**: 295–300. [[Medline](#)] [[CrossRef](#)]
20. Liu, S., Guo, S., Wang, C., Shao, M., Zhang, X., Guo, Y. and Gong, Q. 2007. A novel fusion protein-based indirect enzyme-linked immunosorbent assay for the detection of bovine tuberculosis. *Tuberculosis (Edinb.)* **87**: 212–217. [[Medline](#)] [[CrossRef](#)]
21. Luciano, S. A. and Roess, A. 2020. Human zoonotic tuberculosis and livestock exposure in low- and middle-income countries: A systematic review identifying challenges in laboratory diagnosis. *Zoonoses Public Health* **67**: 97–111. [[Medline](#)] [[CrossRef](#)]
22. Lyashchenko, K., Whelan, A. O., Greenwald, R., Pollock, J. M., Andersen, P., Hewinson, R. G. and Vordermeier, H. M. 2004. Association of tuberculin-boosted antibody responses with pathology and cell-mediated immunity in cattle vaccinated with *Mycobacterium bovis* BCG and infected with *M. bovis*. *Infect. Immun.* **72**: 2462–2467. [[Medline](#)] [[CrossRef](#)]
23. Lyashchenko, K. P., Grandison, A., Keskinen, K., Sikar-Gang, A., Lambotte, P., Esfandiari, J., Ireton, G. C., Vallur, A., Reed, S. G., Jones, G., Vordermeier, H. M., Stabel, J. R., Thacker, T. C., Palmer, M. V. and Waters, W. R. 2017. Identification of novel antigens recognized by serum antibodies in bovine tuberculosis. *Clin. Vaccine Immunol.* **24**: 24. [[Medline](#)] [[CrossRef](#)]
24. Lyashchenko, K. P., Pollock, J. M., Colangeli, R. and Gennaro, M. L. 1998. Diversity of antigen recognition by serum antibodies in experimental bovine tuberculosis. *Infect. Immun.* **66**: 5344–5349. [[Medline](#)] [[CrossRef](#)]
25. McNair, J., Corbett, D. M., Girvin, R. M., Mackie, D. P. and Pollock, J. M. 2001. Characterization of the early antibody response in bovine tuberculosis: MPB83 is an early target with diagnostic potential. *Scand. J. Immunol.* **53**: 365–371. [[Medline](#)] [[CrossRef](#)]
26. Robinson, P. A. 2019. Farmer and veterinarian attitudes towards the risk of zoonotic *Mycobacterium bovis* infection in Northern Ireland. *Vet. Rec.* **185**: 344. [[Medline](#)] [[CrossRef](#)]
27. Silva, E. 2001. Evaluation of an enzyme-linked immunosorbent assay in the diagnosis of bovine tuberculosis. *Vet. Microbiol.* **78**: 111–117. [[Medline](#)] [[CrossRef](#)]
28. Singhal, A., Arora, G., Sajid, A., Maji, A., Bhat, A., Virmani, R., Upadhyay, S., Nandicoori, V. K., Sengupta, S. and Singh, Y. 2013. Regulation of homocysteine metabolism by *Mycobacterium tuberculosis* S-adenosylhomocysteine hydrolase. *Sci. Rep.* **3**: 2264. [[Medline](#)] [[CrossRef](#)]
29. Souza, I. I., Melo, E. S., Ramos, C. A., Farias, T. A., Osório, A. L., Jorge, K. S., Vidal, C. E., Silva, A. S., Silva, M. R., Pellegrin, A. O. and Araújo, F. R. 2012. Screening of recombinant proteins as antigens in indirect ELISA for diagnosis of bovine tuberculosis. *Springerplus* **1**: 77. [[Medline](#)] [[CrossRef](#)]
30. Souza, I. I. F., Rodrigues, R. A., Gonçalves Jorge, K. S., Silva, M. R., Lilenbaum, W., Vidal, C. E. S., Etges, R. N., Kostovic, M. and Araújo, F. R. 2019. ELISA using a recombinant chimera of ESAT-6/MPB70/MPB83 for *Mycobacterium bovis* diagnosis in naturally infected cattle. *J. Vet. Med. Sci.* **81**: 9–14. [[Medline](#)] [[CrossRef](#)]
31. Sugden, E. A., Stilwell, K., Rohonczy, E. B. and Martineau, P. 1997. Competitive and indirect enzyme-linked immunosorbent assays for *Mycobacterium bovis* infections based on MPB70 and lipoarabinomannan antigens. *Can. J. Vet. Res.* **61**: 8–14. [[Medline](#)]
32. Trost, B., Stuber, T., Surujballi, O., Nelson, J., Robbe-Austerman, S., Smith, N. H., Desautels, L., Tikoo, S. K. and Griebel, P. 2016. Investigation of the cause of geographic disparities in IDEXX ELISA sensitivity in serum samples from *Mycobacterium bovis*-infected cattle. *Sci. Rep.* **6**: 22763. [[Medline](#)] [[CrossRef](#)]
33. Viswanathan, G., Joshi, S. V., Sridhar, A., Dutta, S. and Raghunand, T. R. 2015. Identifying novel mycobacterial stress associated genes using a random mutagenesis screen in *Mycobacterium smegmatis*. *Gene* **574**: 20–27. [[Medline](#)] [[CrossRef](#)]
34. Waters, W. R., Buddle, B. M., Vordermeier, H. M., Gormley, E., Palmer, M. V., Thacker, T. C., Bannantine, J. P., Stabel, J. R., Linscott, R., Martel, E., Milian, F., Foshaug, W. and Lawrence, J. C. 2011. Development and evaluation of an enzyme-linked immunosorbent assay for use in the detection of bovine tuberculosis in cattle. *Clin. Vaccine Immunol.* **18**: 1882–1888. [[Medline](#)] [[CrossRef](#)]
35. Waters, W. R., Palmer, M. V., Stafne, M. R., Bass, K. E., Maggioli, M. F., Thacker, T. C., Linscott, R., Lawrence, J. C., Nelson, J. T., Esfandiari, J., Greenwald, R. and Lyashchenko, K. P. 2015. Effects of serial skin testing with purified protein derivative on the level and quality of antibodies to complex and defined antigens in *Mycobacterium bovis*-infected cattle. *Clin. Vaccine Immunol.* **22**: 641–649. [[Medline](#)] [[CrossRef](#)]
36. Welby, S., Govaerts, M., Vanholme, L., Hooyberghs, J., Mennens, K., Maes, L. and Van Der Stede, Y. 2012. Bovine tuberculosis surveillance alternatives in Belgium. *Prev. Vet. Med.* **106**: 152–161. [[Medline](#)] [[CrossRef](#)]
37. Whelan, C., Shuralev, E., Kwok, H. F., Kenny, K., Duignan, A., Good, M., Davis, W. C. and Clarke, J. 2011. Use of a multiplex enzyme-linked immunosorbent assay to detect a subpopulation of *Mycobacterium bovis*-infected animals deemed negative or inconclusive by the single intradermal comparative tuberculin skin test. *J. Vet. Diagn. Invest.* **23**: 499–503. [[Medline](#)] [[CrossRef](#)]
38. Wood, P. R., Corner, L. A., Rothel, J. S., Ripper, J. L., Fifis, T., McCormick, B. S., Francis, B., Melville, L., Small, K., de Witte, K., Tolson, J., Ryan, T. J., de Lisle, G. W., Cox, J. C. and Jones, S. L. 1992. A field evaluation of serological and cellular diagnostic tests for bovine tuberculosis. *Vet. Microbiol.* **31**: 71–79. [[Medline](#)] [[CrossRef](#)]