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Original Article

Comparison of Cattle Serum Antibody Responses to Five Different Mycobacterial Antigens in an ELISA System

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Abstract

The presence of common zoonosis diseases caused by *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM), such as Johne's and Crohn's diseases, poses a public health threat and economic losses to Iranian livestock. Therefore, the early detection of mycobacteria is of paramount importance. In this regard, enzyme-linked immunosorbent assay (ELISA) is a new, simple to use, rapid, and useful diagnostic tool. This study was performed to evaluate different crude antigens obtained from *Mycobacterium* species using an indirect ELISA test to identify the mycobacterial infection in infected livestock. Five different strains of Mycobacteria including *M. tuberculosis*, *M. phlei*, *M. bovis*, *M. avium subspecies paratuberculosis*, and *M. bovis* AN5 were cultured. The crude antigens in the samples were precipitated with trichloroacetic acid 4%. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis of crude antigens isolated from different *Mycobacterium* species was reported. The total level of protein was determined by the Lowry protein assay. After the crude antigen preparation, the ELISA test was performed and the results were compared with the purified protein derivative skin test. Data analysis was performed using SPSS software version 25. All five strains were detected in more than 92% of healthy animals. The highest sensitivity of ELISA tests was in *M. bovis* AN5 antigen which was greater than 83%. The highest diagnostic specificity and efficiency of assays were in *M. avium subspecies paratuberculosis* which was 95.83% and over 83%, respectively. Regarding the results, *M. avium subspecies paratuberculosis* and *M. bovis* AN5 antigens were promising candidates for the design of diagnostic ELISA due to their sensitivity, specificity, and efficiency.

Keywords: Mycobacteria, Nontuberculous mycobacteria, Purified protein derivative test, Enzyme-linked immunosorbent assay

1. Introduction

Worldwide, tuberculosis (TB), mainly caused by *Mycobacterium tuberculosis*, is one of the top 10 causes of death. According to the World Health Organization (WHO) in 2017, millions of people fall ill with TB every year. Globally, the best estimate is that 10.0 million people (range, 9.0–11.1 million) developed TB disease in 2017: 5.8 million men, 3.2 million women, and 1.0 million children.

TB is considered a significant health and economic threat, particularly in developing countries (1). In addition to mycobacterial pathogens, other species such as environmental, atypical, and nontuberculous mycobacteria (NTM) may cause disease both in

humans and livestock (2).

Mycobacterium avium subspecies paratuberculosis (MAP) is the etiologic agent of severe gastroenteritis in ruminants known as Johne's disease (3). Moreover, MAP has a zoonotic role in Crohn's disease among children and adolescents. This species is prevalent in domesticated animals worldwide and significantly affects the global economy (3).

Currently, there are several challenges in the diagnosis of disease using traditional tests (4). This might be due to the structural complexity of the bacterium and the presence of common antigens between pathogenic and non-pathogenic *Mycobacterium* species and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (5).

Mycobacterium species can be found using appropriate antigens for detection. In one study, mycobacterial antigens were classified into four groups by performing a gel double diffusion of bacterial antigens. The first group contained the antigens found in all mycobacteria. The second, third, and fourth groups comprised of antigens in slow- and fast-growers and species-specific antigens, respectively (6).

As a result, the identification and use of specific and immunogenic antigens play a critical role in the diagnosis of disease by the constitution of serologic methods (7). For instance, ELISA is a rapid, easy-to-perform, and user-friendly test for early detection of infection (5).

The purpose of this study was to investigate different *Mycobacterium* species, including pathogenic *Mycobacteria*, NTM, and *M. bovis* BCG with whole proteins. Therefore, the ELISA method tested the protein antigens of different mycobacterial strains and compared with the purified protein derivative (PPD) skin test.

2. Materials and Methods

2.1. Bacterial Strains

In this study, five strains of *Mycobacteria* including *Mycobacterium Tuberculosis* (ATCC:35808), *phlei* (ATCC:11758), *bovis* (ATCC:1173-P2 BCG), *bovis* AN5 (ATCC:35726), and MAP (ATCC:316F) were provided by the Department of tuberculosis of Razi Vaccine and Serum Research Institute to investigate. The presence of all these strains has already been confirmed by the polymerase chain reaction test.

The bacteria were cultured on a Lowenstein-Jensen medium and incubated at the temperature of 37˚C for two months. The samples were transported to a biphasic potato-Dorset-Henley liquid medium for 60 days at the temperature of 37˚C for bacterial adaption. For expression of bacterial antigens, the samples were transferred to Henley-Dorset liquid medium for 60 days at the temperature of 37˚C. To inactivate bacteria, a Fernbach culture flask containing media and bacteria was placed at the temperature of 68˚C for 60 minutes. The proteins in the culture were filtered using 0.22 µm Eks filters and precipitated by adding trichloroacetic acid (TCA).

2.2. TCA Precipitation

Precipitation of tuberculin was performed by adding TCA 40% with the ratio of 9:1 for 24 hours. This solution was allowed to stand overnight at room temperature without stirring. The next day, most of the supernatant was removed by aspiration and precipitated residue was used to resuspend and transfer to centrifuge bottles. The bottles were centrifuged at 2500 rounds per minute for 10 min to remove residual supernatant. The precipitate was washed once with TCA 1% then with NaCl 10%.

At last, the extracted tuberculin was diluted with 3.5 mM $Na_2HPO_4.7H_2O$, 10% Glycerol, 5 mM Phenol, 8.5 mM NaCl, and D.W up to one liter. The pH was adjusted to 6.9 ± 0.1 with 10 N NaOH. This method of precipitation was used for five strains of *M. tuberculosis*, *M. phlei*, *M. bovis* AN5, *M. bovis* BCG, *M. avium subspecies paratuberculosis*.

2.3. Determination of Protein Amount by Lowry Method

The extracted protein was measured by Lowry assay, which is more sensitive, rapid, and accurate in

comparison to other methods (8). This stage was performed on bacteria mass, which was inactivated by water steam (68°C) for one hour. In this process, bovine serum albumin (BSA) was used as a protein concentration standard. The samples' absorption was read at 750 nm by a spectrophotometer.

2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

After gel (12%) staining and visualization with Coomassie Blue dye, the Laemmli system was used as an SDS-PAGE method for separating proteins under denaturing conditions (9).

2.5. Study Population

Serum samples were collected from 54 livestock and obtained at different phases of the study from healthy, naturally infected, or experimentally sensitized cattle with different antigens of *Mycobacterium tuberculosis, phlei*, *bovis* BCG, *bovis* AN5, and MAP crude antigens. In this study, a total of 12 Holstein bulls aged 1 to 6 years with the lowest antibody titers against mycobacterial antigens were included. They had negative mycobacterial culture test results for two months before and after sensitization. The samples were sensitized by crude antigens of mycobacteria based on World Organization for Animal Health guidelines.

The negative control group included five samples with negative culture test results and no clinical symptoms. Moreover, the positive control group contained five samples whose fecal and ELISA test results confirmed the presence of infection. Sensitization was performed with crude antigens in the Deputy of tuberculin and mallein production department, Razi Vaccine and Serum Research Institute, University of Tehran, Tehran, Iran.

2.6. Tuberculin Skin Test

All the study population was evaluated by the tuberculin skin test provided by the Deputy of tuberculin and mallein production department, Razi Vaccine and Serum Research Institute. Before injection, skin thickness was measured using a caliper. The skin test reaction was read after 72 hours, and the induration was measured and recorded. The cattle were classified as PPD-positive if the skin-fold thickness at the injection site was more than 4 mm, negative if it was less than 2 mm, and suspicious if it was between 2 and 4 mm.

2.7. Checkerboard Titration Experiment to Optimize ELISA Parameters

To get the best results from your ELISA assay, the dilution factors of the sample and the detection antibodies must be optimized. If your sample or antibodies are too concentrated, you risk saturating the assay. If they are not concentrated enough, your signal will be weak and difficult to detect. For strong, quantifiable signals, use a checkerboard titration to test for the optimal concentration of sample and detection antibodies.

Dilution levels were evaluated by separate checkerboards with an indirect ELISA method to optimize test parameters, antibodies, and antigens (Kashyap, Rajan, et al. 2007). Serial dilution was performed by adding different mycobacteria antigens to discrete plates with the concentrations of 1-20 µg/mL to 0.1 M bicarbonate coating buffer, pH 9.6. Thereafter, 100µl of the solution was poured into the wells of ELISA microtiter *Nunc-Immuno™ 96* well MicroWell™ *PolySorp*® plates from column A to G. The last column was considered as a negative control containing coating buffer without antigen.

The microtiter plate was incubated for 16 to 24 hours at a temperature of 4°C. Then, the wells were washed three times with 300 µl of 10 mM phosphate-buffered saline (PBS) solution. At this point, each well was filled with 150µl of PBS Tween-20 containing 2.5% casein (Maravel, Ashford, Kent, United Kingdom) as a blocker and placed for one hour at room temperature.

The obtained dilutions of 1/50 V/V and 1/100 V/V negative and positive control sera provided by 10 mM PBS Tween-20 containing 1% BSA were added to each well with the volume of 100 µl and incubated for 30 minutes at room temperature (20 to 25°C). After five

times of washing with 300 µL of 1X Phosphate-Buffered Saline, 0.1% Tween® 20 Detergent, and 10 mM PBS containing 0.1% Tween-20, the optimized dilution of 1/5000 was prepared in 10mM PBS, pH 7.2, from anti-bovine anti-IgG Horseradish Peroxidase, conjugated antibody (AbD Serotec-UK).

Thereafter, 100 µl of the solution was added to each well for 30 minutes at room temperature. Consequently, after washing the wells, according to the previous stage, 100 µl of tetra-methyl benzidine substrate was poured into the wells, and the plate was maintained for 15 minutes in darkness at room temperature. Finally, 100 µl of a stop solution, H_2SO_4 0.1M was added and the plate was read at a wavelength of 450 nm by an ELISA reader (Bio Rad-Model 620).

2.8. Indirect ELISA Protocol

According to the checkerboard, the best serum and antigen dilutions were identified, and indirect ELISA was performed with 54 samples. The appropriate concentrations of different mycobacterial antigens in the separate plate were mixed to 0.1 M bicarbonate coating buffer, pH 9.6. The other procedures were executed under the checkerboard and appropriate serum dilution of 1/50.

2.9. Data Analysis

Data analysis was performed using SPSS software version 25. The cutoff value for the ELISA assay was calculated using the receiver operating characteristic (ROC) curve based on 54 sera with 38 and 16 negative and positive PPD skin tests, respectively, as a standard test, and the sensitivity and specificity were determined. In all the measurements, P-value less than 0.05 was considered statistically significant.

3. Results

3.1. TCA Precipitation and Determining Protein Concentration with Lowry Protein Assay

The TCA precipitation of proteins is often used to concentrate protein samples or remove contaminants, involving salts and detergents, before downstream applications such as SDS-PAGE or 2D-gels. According to the absorbance readings by Lowry protein assay, the standard curve in Excel software was plotted and the protein concentration was calculated using the obtained formula. BSA or standard solutions were used to draw the standard curve. The concentration of *M. bovis* BCG, *M. phlei*, MAP, *M. tuberculosis*, and *M. bovis* AN5 proteins precipitation TCA was estimated by Lowry protein assay as 1.5 mg/ml, 5.65 mg/ml, 1.8 mg/ml, 1.5 mg/ml, and 1.4 mg/ml, respectively.

3.2. SDS-PAGE of Different Mycobacterial Crude Antigens

The separation was carried out in SDS-PAGE, based on the molecular weight of the compounds. The crude antigens were loaded on a 12% gel with a 10 to 180 kDa molecular weight marker. After deposition of the samples and staining with the Coomassie Blue method, the presence of proteins with different molecular weights was determined. As a result, different proteins were detected with molecular weights of 20 to 180 kDa, the highest protein accumulation in molecular weights was above 50 kDa. The number of bands observed per *Mycobacterium* was about 10 bands. The SDS-PAGE proteins of these *Mycobacteria* are demonstrated in figure 1.

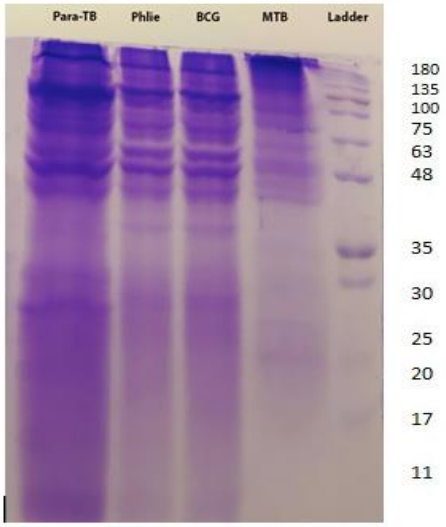

Figure 1. SDS-PAGE* gel staining with Coomassie Blue in different mycobacterial crude antigens
SDS-PAGE of TCA** precipitated proteins, right to left, 10 to 180 kDa protein ladder, *M. tuberculosis*, *M. bovis* BCG, *M. phlei*, *M. avium* subsp. *Paratuberculosis*, respectively. The number of bands observed in each *Mycobacterium* preparation was about 10 bands. The highest protein accumulation in molecular weights was above 50 kDa.
* sulfate-polyacrylamide gel electrophoresis
****** trichloroacetic acid

3.3. Tuberculin Skin Test Results in Livestock

All 54 samples were examined by the PPD skin test, in which 38 livestock had negative skin test results, four of them had positive skin test results due to illness, and 12 samples had positive skin test results due to sensitization by *Mycobacterium tuberculosis*, *phlei*, *bovis* BCG, *bovis* AN5, and MAP antigens. Therefore, approximately 30% of PPD skin tested were positive and 70% were negative (Figure 2; Table 1).

3.4. Checkerboard Results in the ELISA Method

The best concentration of antigen, according to the results of the checkerboard was about *M. bovis* BCG (7.5 µg/ml), *M. phlei* (15 µg/ml), MAP (0.9 µg/ml), *M. tuberculosis* (7.5 µg/ml), and *M. bovis* AN5 (0.7 µg/ml). The adequate dilution used for 54 sera and positive and negative control was 1/50. The amount of optimum density of five antigens is shown in table 2.

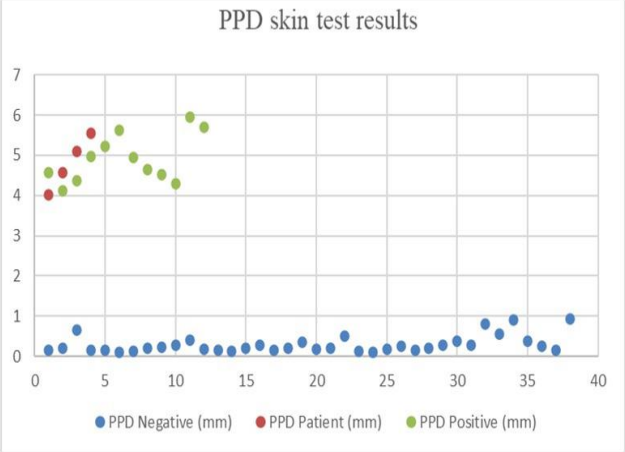

Figure 2. Tuberculin skin test results in livestock
54 livestock were examined by the PPD* skin test. There were 38 livestock with negative skin test results, four samples with positive skin test results due to illness, and 12 livestock positive skin test results due to sensitization by a specific antigen.
Negative results are shown in the diagram with blue color, sick livestock are shown with red color and positive due to sensitization in green color.
***** Purified protein derivative

Table 1. PPD skin test results in millimeters after 72 hours in 54 livestock

No.	PPD results (mm)	No.	PPD results (mm)
1	0.146	28	0.211
2	0.207	29	0.268
3	0.659	30	0.385
4	0.163	31	0.289
5	0.155	32	0.795
6	0.101	33	0.541
7	0.124	34	4.01
8	0.199	35	0.91
9	0.223	36	4.56
10	0.268	37	0.369
11	0.404	38	0.258
12	0.177	39	0.159
13	0.161	40	5.1
14	0.136	41	0.941
15	0.213	42	5.56
16	0.271	43	4.56
17	0.155	44	4.126
18	0.192	45	4.364
19	0.351	46	4.981
20	0.185	47	5.215
21	0.211	48	5.621
22	0.491	49	4.951
23	0.138	50	4.654
24	0.111	51	4.521
25	0.171	52	4.294
26	0.241	53	5.952
27	0.141	54	5.698

72 hours after injection, the skin of the area was examined for induration at the injection site and its thickness was measured with a caliper.
The thickness in livestock with tuberculosis was considered more than 4 mm, in suspected cattle between 2 to 4 mm, and in non-infected livestock less than 2 mm.

Table 2. The optical density of different mycobacterial strains

No.	MTB*	*M.bovis* AN5	M.Para TB	*M.Phlei*	*M.bovis* BCG
1	0.033	0.063	0.066	0.155	0.275
2	0.033	0.073	0.341	0.114	0.248
3	0.04	0.117	0.143	0.09	0.149
4	0.382	0.104	0.104	0.233	1.004
5	0.355	0.086	0.187	0.237	0.462
6	0.391	0.101	0.302	0.281	0.598
7	0.37	0.108	0.163	0.196	0.77
8	0.509	0.104	0.161	0.213	0.894
9	0.131	0.076	0.084	0.192	0.13
10	0.232	0.091	0.097	0.132	0.476
11	0.153	0.073	0.411	0.098	0.315
12	0.262	0.255	0.277	0.196	0.345
13	0.174	0.09	0.115	0.143	0.251
14	0.252	0.074	0.078	0.155	0.369
15	0.275	0.11	0.124	0.19	0.728
16	0.101	0.063	0.061	0.103	0.247
17	0.239	0.081	0.302	0.142	0.355
18	0.408	0.15	0.179	0.277	1.091
19	0.137	0.066	0.078	0.105	0.161
20	0.203	0.072	0.227	0.118	0.2
21	0.312	0.108	0.508	0.23	0.643
22	0.373	0.1	0.187	0.228	1.016
23	0.297	0.086	0.101	0.136	0.321
24	0.283	0.085	0.233	0.137	0.639
25	0.807	0.118	0.143	0.279	1.241
26	0.195	0.093	0.136	0.12	0.173
27	0.448	0.248	0.086	0.137	0.19
28	0.371	0.205	0.279	0.16	0.154
29	0.356	0.067	0.128	0.184	0.564
30	0.292	0.156	0.172	0.088	0.165
31	0.162	0.063	0.069	0.106	0.229
32	0.796	0.084	0.09	0.365	1.549
33	0.083	0.096	0.097	0.114	0.196
34	0.341	0.212	0.226	0.277	0.351
35	0.222	0.082	0.089	0.113	0.355
36	0.501	0.095	0.106	0.315	0.953
37	0.213	0.074	0.085	0.116	0.38
38	0.129	0.064	0.068	0.094	0.14
39	0.104	0.089	0.103	0.11	0.146
40	0.457	0.12	0.142	0.203	0.587
41	0.124	0.088	0.172	0.1	0.282
42	0.625	0.113	0.136	0.191	1.484
43	1.185	0.374	0.37	0.559	2.121
44	1.155	0.349	0.362	0.55	2.2
45	1.38	0.35	0.359	0.542	2.135
46	1.344	0.346	0.256	0.548	2.14
47	0.881	0.252	0.295	0.49	1.55
48	0.897	0.242	0.291	0.509	1.652
49	1.142	0.402	0.423	0.843	2.43
50	1.151	0.407	0.47	0.851	2.46
51	0.897	0.264	0.296	0.331	1.635
52	0.905	0.274	0.321	0.341	1.64
53	1.325	0.412	0.45	0.353	1.885
54	1.214	0.427	0.484	0.348	1.994

This table shows the amount of optical density of different mycobacterial strains based on the appropriate dilution of antigen and antibody determined by the checkerboard.
**Mycobacterium tuberculosis*

3.5. Determining Cutoff for ELISA System with ROC Curve

Based on the results of optical absorption in indirect ELISA and PPD skin test, the ROC curve was plotted by SPSS software. Considering the best sensitivity and specificity, the optimum cutoff point in the graph was determined, which was 0.452, 0.436, 0.257, 0.111, and 0.923 for *M. tuberculosis*, MAP, *M. phlie*, *M. bovis* AN5, and *M. bovis* BCG, respectively (Figure 3).

3.6. Determining the Sensitivity and Specificity of the ELISA Test and Agreement of the Tests Based on Kappa Coefficient Calculation

Statistical indices of sensitivity, specificity, and positive and negative predictive values were used for data analysis. The sensitivity of the test for *M. tuberculosis*, MAP, *M. bovis* AN5, *M. phlie*, *M. bovis* BCG was 83.33, 33.33, 87.5, 66.66, and 66.66, respectively. The Specificity of the test for these species was 72.91, 95.83, 76.08, 70.83, and 68.75, respectively. In addition, the efficiency was measured as 74.07, 88.88, 77.77, 70.37, and 68.51, respectively (Table 3; Figure 3).

Based on the comparison of PPD skin test and ELISA results, in all crude antigens Kappa agreement coefficient was significant (P<0.05).

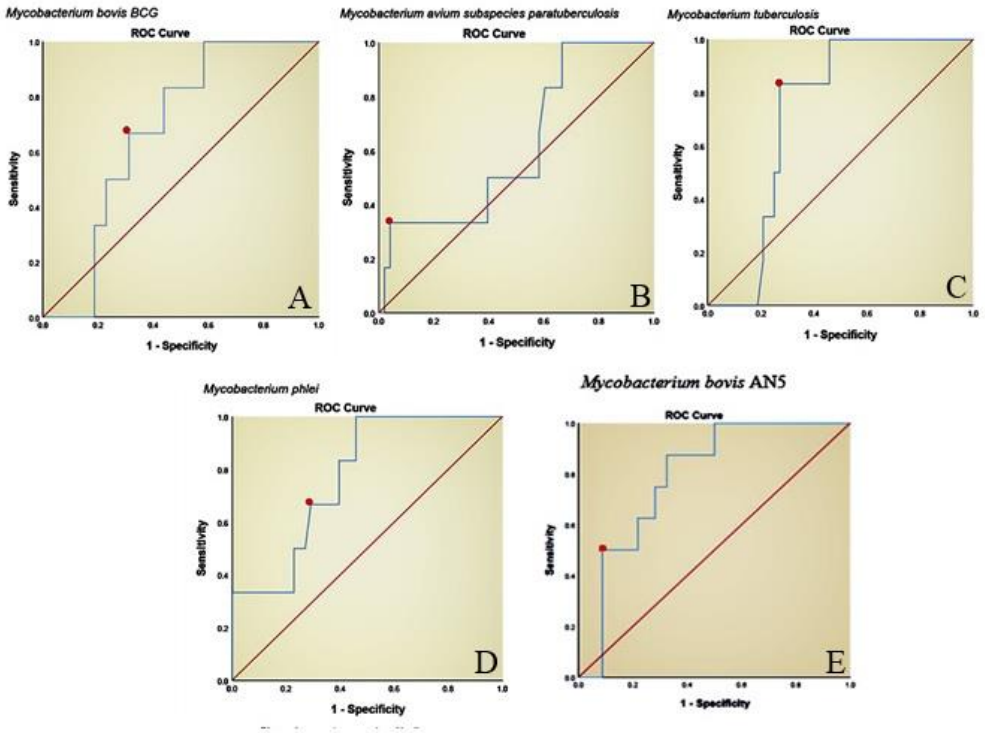

Figure 3. ROC* diagram plotted for different Mycobacterium antigens
This figure shows the ROC diagram plotted for different mycobacterial antigens. **A:** *M. bovis BCG*, **B:** *M. avium* subsp. *Paratuberculosis*, **C:** *M. tuberculosis*, **D:** *M. phlei*, **E:** *M. bovis* AN5. The PPD skin test was considered the standard. The red dot on each graph represents the cutoff point desired based on sensitivity and specificity.
* receiver operating characteristic

Table 3. Determining of sensitivity, specificity, and efficiency of different *Mycobacteria*

Determination of sensitivity and specificity	*M. tuberculosis*	*M. avium subsp. Paratuberculosis*	*M. phlei*	*M. bovis*BCG	*M. bovis*AN5
True Positive (TP)	5	2	4	4	7
True Negative (TN)	35	46	34	33	35
False positives (FP)	13	2	14	15	11
False Negative (FN)	1	4	2	2	1
Sensitivity (%)	83.33	33.33	66.66	66.66	87.5
Specificity (%)	72.91	95.83	70.83	68.75	76.08
Positive predictive value (%)	27.77	50	22.22	21.05	38.88
Negative predictive value (%)	97.22	92	94.44	94.28	97.22
Efficiency (%)	74.07	88.88	70.37	68.51	77.77

This table shows the calculation of sensitivity and specificity, positive and negative predictive values, and the efficiency of the studied livestock based on true positive and true negative and false-positive and false-negative results.

4. Discussion

TB, the second deadliest infectious disease worldwide is caused by bacteria called *Mycobacterium tuberculosis* complex (10). NTM is an infectious disease like TB, but it does not cause TB. This complex can cause a wide range of infections, the most often is pulmonary infection (65–90 %) (11).

Mycobacterium tuberculosis BCG is the only vaccine widely used in tuberculosis and most countries, including Iran, have used it. Although the BCG vaccine can prevent the spread of tuberculosis, it may not provide complete immunity to people with tuberculosis (12).

PPD test which is used for immunologic diagnosis of *Mycobacterium tuberculosis* infection has many constraints, such as being confounded by bacillus Calmette-Guerin (BCG) vaccination or exposure to NTM (13). Therefore, a system design is needed to identify and differentiate mycobacteria.

In recent years, culture tests were mainly replaced with antigen or antibody detection tests such as enzyme immunoassays for the diagnosis of tuberculosis. These methods can be performed in intermediate-level laboratories with relatively simple equipment (14).

In this study, the crude antigens were prepared after precipitation with 4% TCA mycobacteria to have crude proteins, followed by Lowry protein assays to confirm the presence and concentration of proteins. At last, the ELISA plate was coated and adjacent to the target sera and the obtained data were analyzed with SPSS software version 25.

According to the results, the sensitivity, specificity, and efficiency of this method for MAP were 33.33%, 95.83%, and 88.88%, respectively. Therefore, this test was capable of designing a diagnostic ELISA system for MAP. A positive predictive value of 50% indicated suitable testability in the detection of MAP cases. On the other hand, a negative predictive value of 92% indicated a proper ability to detect healthy livestock.

In a study conducted in 2016 to develop and optimize an ELISA system for rapid detection of paratuberculosis in cattle, the secreted antigens of MAP was precipitated by the TCA method. The ELISA method was performed on the crude antigen. The Specificity and sensitivity of the system were determined as 99% and 86%, respectively. The results of the mentioned study showed that this system was more sensitive in comparison with the IDEXX ELISA kit (15).

Regarding the results, the sensitivity, specificity, and efficiency of the test for *M. phlei* antigens were 66.66%, 70.83%, and 70.37%, respectively. In this study, there was high antigenic cross-reaction considering the detection of *M. phlei*. The positive and negative predictive values of 22% and 94.44%

indicated inappropriate and proper ability in detecting *M. phlei* infected and healthy livestock, respectively. Therefore, it was not possible to design an ELISA system for the detection of *M. phlei*. In a study performed in 1984, the preabsorption of tested sera with *M. phlei* was performed to avoid false-positive reaction on detection of MAP with ELISA method in bovine serum (16).

In *M. bovis BCG* antigens, with the test sensitivity of 66.66%, a specificity of 68.75%, and efficiency of 68.51%, it had an average ability to detect healthy and infected livestock. It is impossible with this system to design an ELISA system for the detection of *M. bovis* BCG or vaccinated cattle. A positive predictive value of 21.05% indicated the inconvenient ability of the test due to high antigenic cross-reactions. The negative predictive value was 94.28%, which indicated an acceptable ability to detect healthy livestock. In one study carried out in 1994 (17) on the crude antigen of *M. bovis* BCG, SDS-PAGE analysis showed 10 major and 10 minor structural polypeptides, and all regions were found with SDS-PAGE had cross-reaction with various mycobacteria.

In *M. tuberculosis* antigens, the sensitivity of the ELISA test was 83.33%, the specificity of the test was 72.91%, and the test efficiency was 74.07%. The negative predictive value of this test was 97.22% indicating a satisfactory ability to detect healthy livestock. The positive predictive value of 27.77% demonstrated a low ability to detect cases of *M. tuberculosis* and the presence of the antigenic cross-reactions. This antigen was a suitable candidate for the design of the diagnostic ELISA system if the precipitation of protein or fractionation with chromatography was performed. In one study conducted in 1983 (18), by measuring the IgG antibody to PPD antigen using the ELISA technique, patients with active pulmonary TB could be distinguished from patients with other pulmonary diseases.

In *M. bovis* AN5 crude antigen, with a sensitivity of 87.5%, a specificity of 76.08%, and 77.77% test efficiency, the test was capable of detecting healthy and infected livestock. This antigen was a suitable candidate for the design of the ELISA system. The negative predictive value in this test was 97.22% indicating an appropriate ability to detect healthy animals, while the positive predictive value of 38.88% showed that the ability to detect infected samples was moderate. Regarding the results of a study performed in 2009 (19), crude protein fractions were used for the identification of new *M. bovis* antigens. A proteomic approach allows the identification of new antigens for the detection of bovine TB. Even if the sensitivity and specificity of the tests based on these antigens were not optimal, they may form part of a combination or cocktail for the diagnosis of bovine TB.

In this study, all antigens studied had more than 92% detection ability in healthy animals. The highest sensitivity in ELISA tests was related to the crude antigen of *M. bovis* AN5, which was more than 87.5%. The highest diagnostic specificity was for MAP crude antigen (95.83%). In addition, the highest efficiency of diagnostic tests with more than 88.88% was about this antigen. In conclusion, MAP and *M. bovis* AN5 antigens were suitable candidates for the design of diagnostic ELISA due to their sensitivity, specificity, and efficiency.

Authors' Contribution

All authors had an equal role in the design, work, statistical analysis, and manuscript writing.

Ethics

All animal injections and bleeding activities were fully compliant with general animal ethics. Moreover, animal housing was according to standard operating procedures of the Razi Institute, which were mainly based on normal farming methods.

Conflict of Interest

The authors declare that they have no conflict of interest.

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