

Study on differentiation of pathogen-nonpathogen Mycobacterial infections using ESAT6-CFP10 in ELISA system

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Abstract

Pathogenic mycobacteria, including the causative agents of tuberculosis, are responsible for considerable morbidity and mortality worldwide. The availability of a laboratory method that can distinguish between two groups of pathogens; *Mycobacterium tuberculosis* complex (MTC or MTBC), nontuberculous mycobacteria (NTM) and those who have only had a history of dealing with non-pathogenic or recently vaccinated individuals is of great importance. In this regard, different strains of mycobacteria were cultured in the Dorset-Henley liquid medium and the inactivation of the cultures is carried out by the heat. The bacteria were separated from the liquid medium containing secreted proteins with EKS filters. Low molecular weight proteins in *Mycobacterium tuberculosis* precipitated with ammonium sulfate were purified by Sephadex-G50 gel chromatography and crude antigens in other mycobacteria precipitated with TCA. Protein concentrations determined with lowry protein assay, antigens coated on the ELISA plate and the results were analyzed with SPSS software and investigated. In this study, all antigens had more than 92% detection ability in healthy livestock in the ELISA method. The highest specificity was related to ESAT-6/CFP10 and *M. avium* subsp. *Paratuberculosis* antigens were 83.66% and 95.83% respectively and the highest efficiency of diagnostic tests were over 83% concerning these two antigens. It concluded that the two antigens ESAT-6/CFP10 and *M. avium* subsp. *Paratuberculosis* are suitable candidates for the design of the diagnostic ELISA system due to their sensitivity, specificity and efficiency and also reliable detection of healthy livestock from sensitized livestock.

Keywords: Mycobacteria, ELISA, Tuberculosis, livestock

INTRODUCTION

Mycobacterium is a genus of Actinobacteria, given its own family, the Mycobacteriaceae. Mycobacteria can be classified into several major groups for diagnosis and treatment: *Mycobacterium tuberculosis* complex, which can cause tuberculosis and nontuberculous mycobacteria (NTM) are all the other mycobacteria, which can cause pulmonary disease resembling tuberculosis, lymphadenitis, and skin disease or disseminated disease ^[1, 2].

Tuberculosis (TB) remains a global health problem, affecting millions of people annually ^[3]. The main cause of TB is *Mycobacterium tuberculosis* (MTB), a small, nonmotile, aerobic bacillus ^[4].

Traditional culture tests are prone to a high rate of false positives due to cross-reactivity with nontuberculous mycobacteria although wider use of liquid cultures are recommended by the World Health Organization (WHO) in countries with high tuberculosis (TB) burden. Thus, it is important to rapidly identify MTB in positive liquid cultures to confirm TB infection ^[5, 6].

Sensitive and rapid TB diagnostics play an important role in detecting and preventing the disease. Various culture filtrate protein fractions have been proposed for the detection of the disease, including MPT64, ESAT-6, CFP-10, Ag85 complex ^[7, 8]. ESAT6/CFP10 are early-secreted proteins with molecular weights of 6 and 10 kDa, respectively. In culture, they are some of the most abundant MTB antigens. They are not found in *Mycobacterium bovis* BCG or most

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How to cite this article: Bahmanjeh, A., Ataei Kachooei, S., Faezi Ghasemi, M., Mosavari, N., Hassanzadeh, S. M. Study on Differentiation of Pathogen-Nonpathogen Mycobacterial Infections Using ESAT6-CFP10 in ELISA System. Arch Pharma Pract 2020;11(2):28-36.

Mycobacterium other than tuberculosis (MOTT) but are present in *Mycobacterium kansasii*, *Mycobacterium szulgai*, *Mycobacterium marinum*, and *Mycobacterium riyadhense* [9].

MPT64 is a secreted, immunogenic protein of MTB. It is highly specific for the *Mycobacterium tuberculosis* Complex (MTBC), and most importantly; it is not found in MOTT [10].

The Mycobacterium Ag85 complex is involved in biological processes including immunogenic responses and cell wall biosynthesis. For the intracellular survival of MTB within the macrophage, Ag85 expression is essential [11].

ESAT6/CFP10 are also being studied as alternatives to PPD (purified protein derivative). They have shown high sensitivity in the diagnosis of active and new TB infections. Furthermore, no false positives in BCG-vaccinated people have been observed. It has been found that TB proteins actively secreted in culture (Mentioned above) is stronger than that resulting from the standard PPD [12].

For detection and control of bovine tuberculosis (TB), tuberculin test is the most common method used. Due to having low specificity and sensitivity of this tuberculin test, it is better to perform another test in parallel for the investigation of the disease. Recently, due to having the ability of being performed in intermediate-level laboratories with relatively simple equipment, antigen or antibody detection tests such as enzyme immunoassays have largely replaced culture for the diagnosis of many infectious diseases. The ELISA test can be an appropriate test for performing in parallel with the tuberculin test for accurate detection due to bein user-friendly. Several antigens have been evaluated for their serodiagnostic potential in detecting circulating antibodies by enzyme-linked immunosorbent assay (ELISA).

In this study, it was attempted to used ELISA tests based on in cattle for differentiation of the pathogen and non-pathogen mycobacterial infections with proteins smaller than 10 kDa (specially ESAT-6/CFP-10) for the detection of TB and some other antigenic preparations seral antibodies.

MATERIALS AND METHODS

Bacterial species. Four strains of Mycobacterium including *Mycobacterium tuberculosis* (ATCC: 35808), *Mycobacterium phlei* (ATCC: 11758), *Mycobacterium bovis* (ATCC: BCG Pasteur 1173P2), and *Mycobacterium avium* subsp. *Paratuberculosis* (ATCC: 316F) were used in this study. All the strains were supplied by the Tuberculin and Mallein Production & Research Department at Razi Vaccine and Serum Research Institute and identifications were confirmed by specific PCR methods previously.

Bacterial culture. The bacteria were subcultured on the Dorset-Henley liquid medium, for 60 days at 37°C to produce a seed culture. After 60 days, Fernbach flask containing medium and bacterium within 60 minutes in the temperature

68° were used for inactivation of live bacteria. The cells were removed from the culture medium by 0.22 µm filters (EKS) filter.

TCA precipitation. The proteins in the culture filtrate of *Mycobacterium tuberculosis* were mixed thoroughly while one part TCA was added to nine parts culture filtrate resulting in a solution with a final concentration of 4% TCA, described as [13]. This method of precipitation was used for the treatment of 4 strains including *Mycobacterium tuberculosis*, *Mycobacterium phlei*, *Mycobacterium bovis* BCG, and *Mycobacterium avium* subsp. *Paratuberculosis*.

Ammonium sulfate precipitation. The proteins in the culture filtrate were precipitated by adding a concentration gradient of ammonium sulfate. The amount of ammonium sulfate that should be added to the solution can be determined by using an online calculator. 561 g ammonium sulfate (<http://www.encorbio.com/protocols/AM-SO4.htm>) instead of per liter was added from the liquid medium to prepare the solution and then the prepared solution was mixed for 16 h at for 4° C. Then this solution was allowed to stand overnight at room temperature without stirring. The solution was centrifuged in 3,000 rpm for 15 min and its supernatant was discarded, then phosphate buffer, pH:6.8, was added to the residue precipitation. Dialysis against deionized water was used for 72 h to omit the remaining salts in the protein solution, to reassure the exit of salt from the protein solution by conductometer, the conductivity of above solution was studied. After the dialysis process, the contents of the dialysis bag were concentrated with polyethylene glycol, Sigma-Aldrich [14].

Chromatography. Gel chromatography using Sephadex-G50, in a column with a dimension of 150×2 cm, was used for protein fractionation. Phosphate buffer (50 mM, pH 6.8) was added to the concentrated protein solution. Impurities of the protein solution were removed by centrifugation (15 min at 4°C at 10,000 rpm). The supernatant was applied to a gel chromatography column equilibrated previously with 50 mM phosphate buffer (pH 6.8). The column was eluted by 50 mM phosphate buffer (pH 6.8). After chromatography steps for fractionating the obtained proteins, the absorption of protein solutions was read by spectrophotometer in the wavelength 280 nm and its chromatograph was drawn [15].

Protein determination. The amount of yielded proteins in solutions was measured by the Lowry method [16]. In this process, Bovine Serum Albumin (BSA) was used as a standard. The absorbance of samples was read at 750 nm by a spectrophotometer. For protein measurement, Lowry protocol was used as described [16].

SDS-PAGE. Protein electrophoresis was carried out as described by Laemmli [17], using 12% acrylamide/bisacrylamide gels. The proteins were diluted in sample loading buffer, heated to 95°C for 10 min in loading

buffer and loaded onto the gel. Silver nitrate and Coomassie Blue R-250 staining were used to detect proteins after electrophoretic separation on polyacrylamide gels [18, 19].

Study populations. Serum samples obtained at different phases of the study from healthy, naturally and infected or experimentally infected cattle with a different antigen of *M.tuberculosis*, *M.phlei*, *M.bovis*, *M.avium* subsp. *paratuberculosis* crude antigens. Serum samples were collected from 54 livestock, 37 cows that three continual tuberculin tests were negative and 17 serum samples were positive (in the tuberculin test). Eight of the seventeen sera were sensitized for having positive sera of different strains of mycobacteria at the Tuberculin and Mallein Production & Research Department in Razi Vaccine and Serum Research Institute with killed mycobacteria (crude antigens). Hence 8 Holstein bulls aged 1 to 6 years which had the lowest antibody titers against mycobacterial antigens, were sensitized by using crude antigens of mycobacteria; based on OIE guidelines, crude antigens were mixed with adjuvant and then injected intramuscularly, 1 ml solution, three times with two weeks interval (first injection with Freund's complete adjuvant and next injections with incomplete Freund's adjuvant) and each time before each injection, serum was taken from the cow to investigate antibody titer. All eight livestock were negative for mycobacterial culture before and after two months of sensitization. The other nine positive sera were positive by both the tuberculin test and culture from lymph nodes (or tissues) of tuberculin positive cows.

Tuberculin Skin test. Each dose (0.1 ml) contains 3,250 International Units (IU) Purified Protein Derivatives of *Mycobacterium bovis* AN5 (PPD). It was provided by the Tuberculin and Mallein Production & Research Department in Razi Vaccine and Serum Research Institute. Before injection, skin thickness was measured using a caliper. 72 hours after injection, the skin of the area was examined, and if any bumps and stiffness were observed at the injection site, its thickness was measured with a caliper and recorded. The thickness is greater than 4 mm in cattle with tuberculosis, between 2 and 4 mm in suspicion, and less than 2 mm in non-infected cattle.

Optimizing the best concentration of mycobacteria, antigens and antibody dilution with Checkerboard and Indirect ELISA test protocol. Due to the optimization of test conditions, separately the checkerboard evaluated antibodies and antigens dilution levels with indirect ELISA method. By adding antigens of different mycobacteria in a separate plate, with concentration range between 1-20 µg/mL, to the bicarbonate coating buffer 0.1 M, pH: 9.6, serial dilution was prepared and 100µl was added in each well of ELISA microtiter plate, PolySorp Nunc-Immuno 96-microwell plate, from column A to G and the last column H, was considered as negative control, coating buffer without antigen. According to the checkerboard, the best serum and antigen dilutions were identified. The microtiter plate was incubated for 16 to 24 hours at 4°C. Then, the wells were

washed three times with 300 µl of 10 mM PBS solution and then each well was filled with 150µl of PBS Tween20 and solution of 2.5% casein (Maravel -UK) as a blocker and incubated for one hour at room temperature. The obtained dilutions of 1/50 and 1/100 negative and positive control serum that were prepared by using 10 mM PBS/Tween 20 with BSA 1%, was added to each well with the volume of 100 µl and were incubated for 30 minutes at room temperature, 20 to 25°C. After 5 times washing with 300 µL of PBST, 10 mM PBS, containing 0.1% Tween 20, optimized dilution of 1/5000 was prepared in 10mM PBS, pH 7.2, from HRP conjugate, Anti-Bovine Anti-IgG Horseradish Peroxidase Conjugated Antibody, AbD Serotec-UK, and 100 µl was added to each well and the plate was incubated again for 30 min at room temperature. Subsequently, after washing wells, according to the previous stage, 100 microliters of 3,3',5,5'-Tetramethylbenzidine or TMB substrate were added to wells and the plate maintained for 15 minutes in darkness at room temperature. Finally, 100 µl of a stop solution, H2SO4 0.1M, was added and the plate was read at a wavelength of 450 nm by an ELISA reader, Bio Rad-Model 620. Blocker buffers used in the optimization process include the PBS Tween20 and solution of 2.5% casein (Maravel -UK).

Sensitivity and specificity of the ELISA test. Sensitivity and Specificity were determined by formula "Sensitivity= true positives/ (true positive + false negative)" and "Specificity= true negatives/ (true negative + false positives)" based on new S/P.

Data Analysis. The cutoff value for the ELISA assay was calculated using the ROC curve based on 54 serums with 37 negative PPD skin tests and 17 positive PPD skin as a standard test. Statistical analyses were performed with the SPSS software version (25.0.0.0).

RESULTS

Ammonium sulfate precipitation. Since it is possible to separate proteins with molecular weights below 30 kDa by ammonium sulfate precipitation, for isolation of ESAT-6/CFP-10 at a molecular weight of 14 kDa from the *M. tuberculosis* crude antigens, ammonium sulfate precipitation was used. After dialysis, the conductometer showed a number below 1000 rpm, indicating salt was removed from the solution.

Chromatography for protein determination. To isolate different fractions of the proteins present in the crude antigens of *M.tuberculosis*, gel filtration was used. After separation of fractions and drawing a chromatograph, three peaks were observed (Fig. 1) and studying the protein concentration in each fractions with Lowry protein assay determined that out of, 8.65 mg/ml protein in fraction 1, 3.56 mg/ml protein in fraction 2, 0.6 mg/ml protein in fraction 3 proteins were the proteins from high to low molecular weight. (Fig. 1).

SDS-PAGE of fraction-2 antigen. It was observed that fraction 2 (F2) has a two-band containing the proteins with low molecular weight 10 and 14.4 kD (Fig. 2), using molecular weight assay by SDS-PAGE and staining with silver nitrate. The molecular weights of protein from *M. tuberculosis* ranged from 6 to 180 kDa at this stage. The ESAT6/CFP10 protein complex is one of the most important *M. tuberculosis* secreted proteins with molecular weight 14 kD (Fig. 2). All processes were performed on fraction 2.

TCA precipitation for protein determination. The TCA precipitated the method with different concentrations of proteins of both high molecular weight and low molecular weight (total proteins). The concentration of *M. bovis* BCG, *M. phlei*, *M. avium* subsp. *Paratuberculosis* and *M. tuberculosis* proteins precipitation TCA were estimated by Lowry protein assay at 1.5 mg/ml, 5.65 mg/ml, 1.8 mg/ml and 1.5 mg/ml, respectively.

SDS-PAGE of different mycobacterial crude antigens. Crude antigens isolated by TCA were loaded onto a 12% separator gel with 10 to 180 kDa Protein Markers to evaluate the weights and the presence of proteins in different mycobacteria. After deposition of the samples and staining with Coomassie Blue method, the presence of proteins with different molecular weights was determined. SDS-PAGE proteins of *M. tuberculosis*, *M. avium* subsp. *Paratuberculosis*, *M. phlei*, *M. bovis* BCG are shown in (Fig. 3). As a result, the protein accumulation occurs at different molecular weights with 35 to 180 kDa, the number of bands observed in each Mycobacterium preparation is about 15 to 17 bands.

Checkerboard results in the ELISA method. The results of the Checkerboard showed that the best-selected concentrations of *M. bovis* BCG, *M. phlei*, *M. avium* subsp. *Paratuberculosis* and *M. tuberculosis*, ESAT6/CFP10 antigens after optimization of test conditions to increase a ratio (S/N) were 7.5 µg/ml, 15 µg/ml, 0.9 µg/ml, 7.5 µg/ml, and 7.5 µg/ml, respectively; and the appropriate dilution used for 54 sera, positive and negative control was 1/50.

Determining Cutoff for ELISA system with ROC curve. ROC curve is a graph or graphical schema that uses the true positive rate (sensitivity) on the x-axis and the false positive rate (specificity-1) on the Y-axis at different thresholds (different cutoff points) is created. All analyses and graphs are drawn with SPSS software version (25.0.0.0) and based on the above data the ROC curves for the method are shown in (Fig. 4). The areas under the curves with 95% CIs and with cut off value were calculated in (Table 1). Accordingly, the optimal cutoff values of the optical densities of antibody responses to the ESAT-6/CFP10, *M. tuberculosis*, *M. phlei*, *M. avium* subsp. *Paratuberculosis*, *M. bovis* BCG antigens for diagnosing livestock contaminated

with these mycobacteria were 0.921, 0.452, 0.257, 0.436 and 0.923, respectively (Table 1).

Determining the sensitivity and specificity of the ELISA absorption system to compare with skin test results. Statistical indices of sensitivity, specificity, positive and negative predictive value were calculated for data analysis. Test sensitivity for ESAT-6/CFP10, *M. tuberculosis*, *M. avium* subsp. *Paratuberculosis*, *M. phlei*, *M. bovis* BCG antigens is 60%, 83.33%, 33.33%, 66.66%, 66.66% respectively, having specificity, 88.63%, 72.91%, 95.83%, 70.83%, 68.75%, respectively. Efficiency of test is 83.33%, 74.07%, 88.88%, 70.37%, and 68.51%, respectively (Table 2).

Agreement of the tests based on Kappa coefficient calculation

Based on the PPD skin test as the standard, other tests were compared with this test. In ESAT6/CFP10 antigen, *M. tuberculosis*, *M. avium* subsp. *Paratuberculosis*, *M. phlei* and *M. bovis* BCG with PPD skin test, Kappa agreement coefficient is 0.468 (P<0.01), 0.3 (P<0.01), 0.341 (P<0.05), 0.2 (P<0.05) and 0.182 (P<0.05), respectively.

DISCUSSION

Tuberculosis remains a global health problem. In 2017, there were an estimated about 11 million new cases and 1.4 million deaths due to TB, worldwide [20]. The prompt diagnosis of mycobacteria especially MTB is a crucial problem [21]. Although the golden standard for diagnosing active TB remains the culture of the causative organism, but takes a long time and is not always successful. The serological method is an effective diagnostic tool of TB in vitro, which would be an attractive progress as immunoassays are simple, rapid, inexpensive and may offer the possibility to detect cases missed by standard sputum smear microscopy [21].

In this study, the results of the SDS-PAGE method of precipitation with TCA have almost the same band pattern because of the isolated protein complex in mycobacteria having the same protein profile. Ultimately, the SDS-PAGE method revealed that the ammonium sulfate precipitate was better than the precipitate with TCA because nonspecific bands were rarely observed. Three fractions obtained by chromatography, fraction-2 of *M. tuberculosis* antigen were more suitable than other fractions because they had appropriate molecular weight and acceptable ELISA results, and fraction-2 of *M. tuberculosis* antigen was used in the ELISA test.

There is a Kappa coefficient between ESAT6/CFP10 antigen with PPD skin test 0.468 and P> 0.05, this test can be a good alternative to the PPD test. Notable P> 0.05 indicates that the results are not random.

ESAT6/CFP10 antigens with a sensitivity of 60% and a specificity of 88.63% indicate the ability of the test to

acceptably differentiate non-diseased livestock. These antigens are a suitable candidate for the design of the diagnostic ELISA system with a test efficiency of 88.33%. The negative predictive value of this experiment was 90.69%, indicating a good ability to identify healthy livestock and positive predictive value was 54.54%.

Given the false-negative results of the ELISA test, there may be a recent infection with *M. tuberculosis* that has been treated or despite *M. tuberculosis* in livestock, the PPD skin test is unable to detect active disease states or memory antibodies in livestock serum. It was also shown by our previous study in 2014 that performing the ELISA test based on proteins lower than 10 kDa M.W. like ESAT-6 antigen isolated from sonicated bacteria was precipitated with ammonium sulfate, that can be a suitable way for screening besides the tuberculin test for accurate detection [22].

In NTM mycobacteria like *M. avium* subsp. *Paratuberculosis* antigens, there is a kappa coefficient between *M. avium* subsp. *Paratuberculosis* with PPD skin test 0.341 and $P > 0.05$, so the agreement between the two tests is low and this test cannot replace PPD skin test. With a sensitivity of 33.33% and a specificity of 95.83%, this test is capable of detecting healthy livestock; the test efficiency is 88.88%. Given the above feature, it is possible to design an ELISA system for the diagnosis of *M. avium* subsp. *Paratuberculosis*. Negative predictive 92%, indicates a good ability to detect healthy livestock and positive predictive value of 50%, indicates good testability in the detection of *M. avium* subsp. *Paratuberculosis* cases. In one of our previous studies in 2016, specificity and sensitivity of the system were determined by 99% and 86%, respectively. These results showed that secreted antigens of *Mycobacterium avium* subsp. *Paratuberculosis* can be one of the top choices for developing new ELISA systems because the most present kits are cellular antigens. Compared to the method performed, to remove non-specific antibodies in the bovine serum, soluble antigens of *M. phlei* were used [23].

In *M. phlei* antigens, there is a kappa coefficient between *M. phlei* with PPD skin test 0.2% and $P > 0.05$, so the agreement between the two tests is not suitable and this test cannot replace PPD skin test. This test with 66.66% sensitivity, 70.83% specificity and 70.37% efficiency can detect healthy livestock but the ability of the test to correctly detect sick livestock is mediocre. There is a low possibility with this method to design an ELISA system for detection of *M. phlei*. The negative predictive value was 94.44% which indicates a good ability to detect healthy livestock and 22% positive predictive value indicating very low ability in detecting livestock infected with *M. phlei*, because of the high antigenic crosses observed in the diagnosis of *M. phlei* in this study.

In *M. bovis* BCG antigens, there is a Kappa coefficient between *M. bovis* BCG with PPD skin test 0.182 and $P > 0.05$ between *M. bovis* BCG with PPD skin test, so the agreement between two tests is not appropriate and this test cannot

replace PPD. This test, with a sensitivity of 66.66% and a specificity of 68.75% has an average ability to detect healthy and infected livestock. The test efficiency is 68.51%. It is impossible with this system to design an ELISA system for the detection of *M. bovis* BCG. Negative predictive value 94.28, indicates a good ability to detect healthy livestock and positive predictive value 21.05% indicating the very low ability of testing due to high cross-antigenic is detectable in *M. bovis* BCG cases. As a result, cattle recent immunization with the BCG vaccine, cannot be detected by ELISA system. High antigenic crosses were present in the detection of BCG and *M. phlei* -infected sera in this research.

In *M. tuberculosis* antigens, there is a Kappa coefficient between *M. tuberculosis* with PPD skin test 0.30 and $P > 0.05$ between *M. tuberculosis* with PPD skin test, so the agreement between the two tests is low and this test cannot replace PPD skin test. The sensitivity of the ELISA test is 83.33% specificity of the test is 72.91% and the efficiency of the test is 74.04%. This antigen is a suitable candidate for the design of the diagnostic ELISA system. The negative predictive value of this test was 97.22% indicating a good ability to detect healthy livestock. The positive predictive value of 27.77 indicates a low test ability to detect cases of *M. tuberculosis* and the presence of the antigenic cross. The report about the use of *M. tuberculosis*-secreted antigens, that shows these antigens have a sensitivity of 67% and a specificity of 79% for patients with *M. tuberculosis* with ELISA, dates back to 1984 [24]. Higher sensitivity was observed in the present study compared with the Zeiss *et al.* study.

It is concluded from the present study that the two antigens ESAT-6/CFP10 and *M. avium* subsp. *Paratuberculosis* crude antigens are proper candidates for the design of diagnostic ELISA due to their sensitivity, specificity, and efficiency. The highest sensitivity of ELISA assays was in *M. tuberculosis* antigen which was 83.33%. The highest specificity was related to ESAT-6/CFP10 and *M. avium* subsp. *Paratuberculosis* antigens which were 83.66% and 95.83%, respectively and the highest efficiency of diagnostic tests were over 83% concerning these two antigens. The actual negative results in ESAT-6/CFP10 antigen and *M. avium* subsp. *Paratuberculosis* antigens had more than 90% detection rate in healthy livestock. *M. avium* subsp. *Paratuberculosis* was well diagnosed in two crude antigen-sensitive livestock of *M. avium* subsp. *Paratuberculosis*. All antigens studied had more than 90% detection ability in healthy livestock.

This study showed that precipitating *M. tuberculosis* antigens with ammonium sulfate salts compared with TCA precipitating, had higher specificity and positive predictive value. The many different protein fractions present in the whole-protein antigen extract may explain its greater reactivity. It is recommended that one or more specific or recombinant antigens be used to obtain better results in the design diagnostic ELISA system or to remove non-specific

antibodies in the bovine serum, soluble antigens of *M. phlie* can be used [23].

More studies are required in the format of field trials as a proof for ELISA systems developed by the present study.

ACKNOWLEDGMENTS

We are thankful to all personnel of Razi Vaccine and Serum Research Institute especially Tuberculin and Mallein Production & Research Department, Vaccine Production Unit, Research & Production Complex, and also Arna Gene Avis Company that provided support.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

Conceptualization and methodology: Nader Mosavari and Saeed Ataei Kachooei

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Table 1. The cutoff value was calculated for different mycobacteria

Test Result Variable(s) OD	cut off value	Area	Std. Error ^a	Asymptotic Sig. ^b	Asymptotic 95% Confidence Interval(CIs)	
					Upper Bound	Lower Bound
ESAT-6/CFP10	0.921	0.751	0.075	0.014	0.605	0.897
<i>M. tuberculosis</i>	0.452	0.724	0.068	0.076	0.590	0.858
<i>M. phlei</i>	0.257	0.773	0.084	0.031	0.608	0.937
<i>M. avium</i> subsp. <i>Paratuberculosis</i>	0.436	0.616	0.117	0.356	0.387	0.846
<i>M. bovis</i> BCG	0.923	0.677	0.081	0.160	0.517	0.837

The test result variable(s): ESAT-6-OD, *M. tuberculosis*, *M. avium* subsp. *Paratuberculosis*, *M. phlei*, *M. bovis* BCG has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Table 2. Determining of sensitivity, specificity, and efficiency of different Mycobacterium

sensitivity and specificity	ESAT-6/CFP10 Antigen	<i>M. tuberculosis</i>	<i>M. avium</i> subsp. <i>Paratuberculosis</i>	<i>M. phlei</i>	<i>M. bovis</i> BCG
Sensitivity (%)	60	83.33	33.33	66.66	66.66
Specificity (%)	88.63	72.91	95.83	70.83	68.75
Positive predictive value (%)	54.54	27.77	50	22.22	21.05
Negative predictive value (%)	90.69	97.22	92	94.44	94.28
Efficiency (%)	83.33	74.07	88.88	70.37	68.51

Determination of sensitivity and specificity, positive and negative predictive value, and the efficiency of the studied livestock based on true positive and true negative and false-positive and false-negative results.

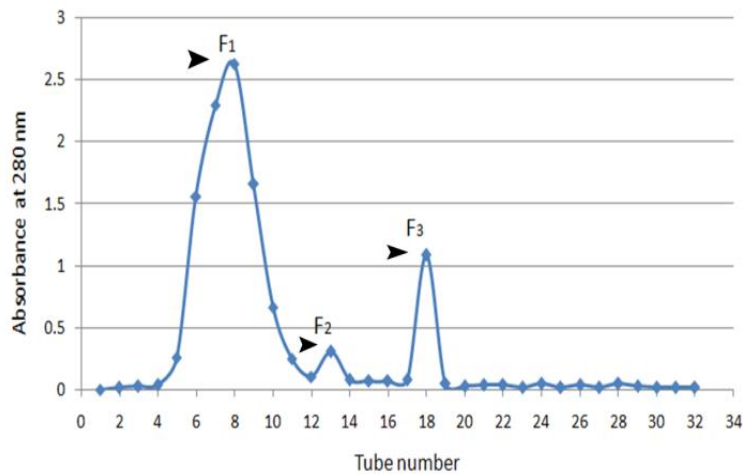


Figure 1. Gel filtration chromatography on crude *Mycobacterium tuberculosis* antigens Sephadex G-50 chromatography of *M.tuberculosis*; Three peaks (F1, F2, and F3) of optical density obtained by chromatography were observed. The first peak consisted of high molecular weight proteins and the second with a medium molecular weight and third peaks contained low molecular weight proteins.

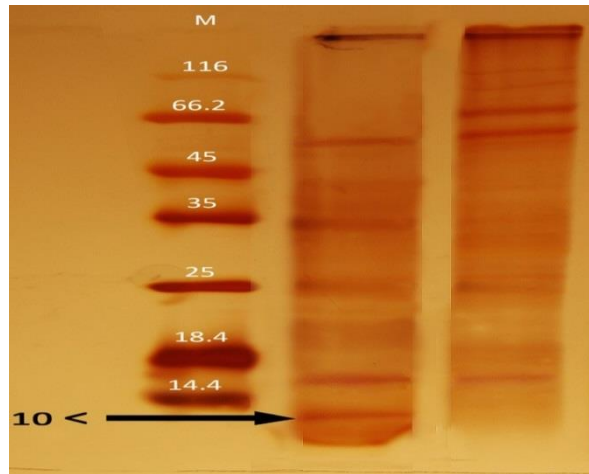


Figure 2. Staining of SDS-PAGE gel with silver nitrate in low molecular weight proteins from *M. tuberculosis* and the effect of heat and centrifugation on proteins SDS-PAGE of *M. tuberculosis* proteins. Two bands are seen in the region of 10 and 14.4 kDa molecular weight, which is in association with the ESAT-6/CFP10.M: Protein marker with molecular weights of 10 to 180 kDa.

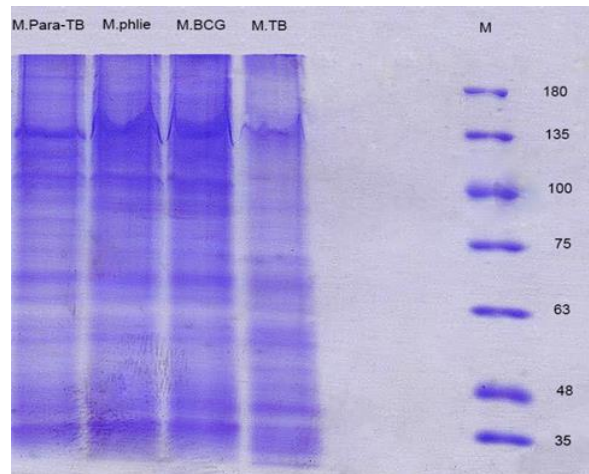
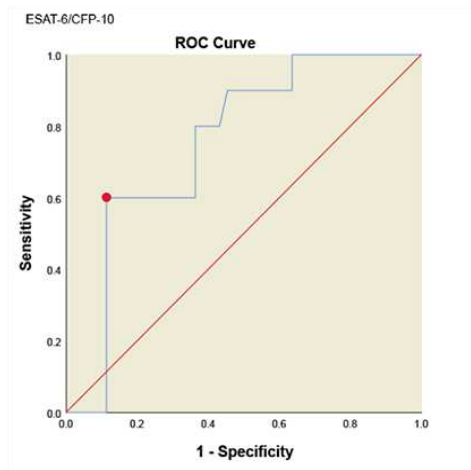
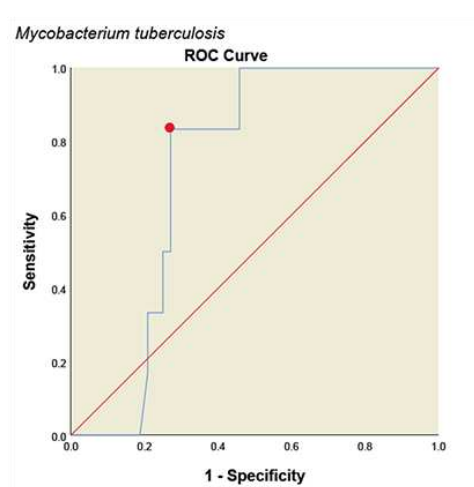


Figure 3. SDS- PAGE gel staining with coomassie blue in different mycobacterial crude antigens SDS-PAGE of TCA precipitated Proteins, right to left, 10 to 180 kDa protein ladder, *M. tuberculosis* (M.TB) , *M. bovis* BCG (M.BCG), *M. phlei*, *M. avium* subsp. *Paratuberculosis* (M.Para-TB), respectively. The number of bands observed in each Mycobacterium preparation is about 15-17 bands. The protein accumulation occurs at different molecular weights.



(a)



(b)

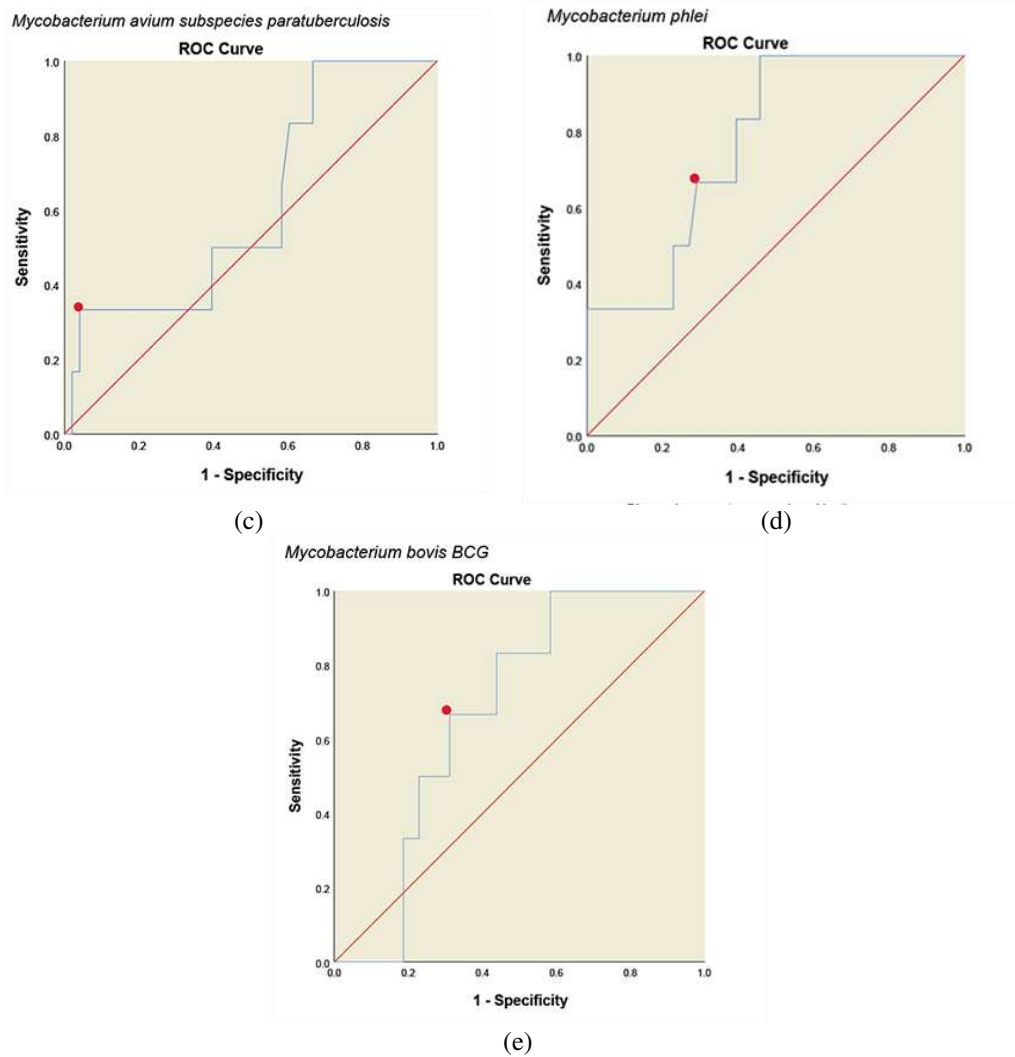


Figure 4: ROC diagram plotted for different Mycobacterium antigens

ROC diagram plotted for different mycobacterial antigens. a: ESAT6/CFP10 antigen, b: *M. tuberculosis*, c: *M. avium* subsp. *Paratuberculosis*, d: *M. phlei*, e: *M. bovis BCG*. The PPD skin test was considered as the standard. The red dot on each graph represents the cut point desired based on the sensitivity and specificity.