International Journal of Advanced Biotechnology and Research (IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol-7, Issue-1, 2016, pp1-9
http://www.bipublication.com

Research Article

Development and optimization a high sensitive and specific ELISA system for rapid detection of paratuberculosis in cattle

Keshavarz Rouhollah*, Mirjalili Ali, Mosavari Nader, Tadayon Keyvan,

Ghaemmaghami Shamseddin, Fardid Farzaneh and Dashti pour shojaat

Agricultural Research and Education Organization,
Razi vaccine & Serum Research Institute, Karaj, Iran.

*Corresponding author: Email: piroozkeshavarz@gmail.com

[Received-21/12/2015, Accepted-29/12/2015, Published-05/01/2016]

ABSTRACT:

John's disease is a chronic gastroenteritis of cattle which caused by *Mycobacterium avium* subsp. *Paratuberculosis* and has a wide spread all over the world. This bacterium can be found in feces and milk and transmits the disease easily. Therefore, rapid detection of infection is highly important. There are different ways for detection of paratuberculosis that ELISA has the highest importance between conventional methods because of its convenience and high accuracy. The aim of this study is development and optimization an ELISA system for rapid detection of paratuberculosis in cattle. For this purpose, secreted antigens of *Mycobacterium avium* subsp. *Paratuberculosis* by trichloroacetic acid (TCA) method were precipitated and the best concentration of antigen and antibody were determined by checkerboard and the system's cut-off was determined with RUC curve; The specificity and sensitivity of developed ELISA system were evaluated for 1000 serum samples.

In this study, the best concentration of antigen and antibody were 1.1 µg/l and 0.01 respectively and based on RUC curve results the amount of cut-off was evaluated 0.2 (95% CI, 82.94 to 99.91). Finally, specificity and sensitivity of system were determined 99% and 86% respectively that this system was more sensitive in comparison with IDEXX ELISA kit. These results showed that secreted antigens of *Mycobacterium avium* subsp. *Paratuberculosis* can be the best choice for developing new ELISA systems because the most present kits are using cellular antigens.

Key Words: ELISA-paratuberculosis - checker board- IS*900* PCR- ROC

[I] INTRODUCTION

Nowadays Paratuberculosis (PTB, Johne's disease) is highly regarded between many pervasive chronic bacterial diseases of ruminants in agriculturally developed countries (2,8,17). PTB is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) which grows gradually and is a mycobactin-dependent organism. Clinical signs of PTB include diarrhea, weight loss, decreasing of milk production and death due to dehydration of ruminants. The economic loss caused by this infectious disease in America is estimated 200 million dollars with prevalence of 20% per year and at least 100 million dollars per year (6) in Iran with prevalence of at least 15%.

Since there is no effective treatment for Johne's Disease and many of infected animals can remain asymptomatic for years while they transmit the pathogen via fecal contamination and milk, disease control is first priority which requires infected herds identification, separation and elimination of the shedder animals in the herd.

Conventional methods to identify paratuberculosis ranged from simple direct smear exam, cultivation and isolation of fecal bacteria to molecular and serological methods. Fecal direct smear is the first choice for the clinical cases of disease. The sensitivity of this method is very low and distinguishing between pathogenic and nonpathogenic (saprophytic) Mycobacterium is very difficult. Cultivation and isolation of fecal bacteria are used for diagnosis of PTB according to the gold standard and has the ability of detecting infection while the amount of bacteria in the fecal is higher than 100 CFU/g. Prolong incubation time (5 to 16 weeks), lack of reproducibility and unability to distinguish between transmitted and colonized Mycobacterium paratuberculosis in the intestine are disadvantages of this method.

Molecular techniques such as PCR-IS*900* are highly interested due to the specificity and high speed of the test; But the high cost, the need for specific laboratory facilities and lack of distinguishing between transmitted and colonized *Mycobacterium paratuberculosis* have limited use of the technique. Between the serum assays, ELISA technique has been widely used in diagnostic laboratories around the world because of no need to special laboratory facilities, high speed and low cost. Since ELISA kits for diagnosis of paratuberculosis, have high specificity (90% to 99%) but low sensitivity (13.5 to 42 percent), designing ELISAs with high sensitivity (while maintaining high specificity) is strongly considered and this improvement is highly dependent on the selection of antigen. Therefore, one of the main challenges in developing an effective ELISA is detection of proper antigen to recognize all stages of infection, especially early stage and subclinical stage. Different types of ELISAs are designed based on various types of antigens and depending on the stage of the infection, the sensitivity is evaluated(7,16). Recently, the secreted proteins of *Mycobacterium paratuberculosis* have been identified as important antigens with high sensitivity for detection of bovine paratuberculosis.

It is indicated that antibodies have stronger reactions with culture filtrate (CF) antigens compared to cellular extraction antigens and they have the ability to detect paratuberculosis in the early stages of infection. Moreover, different methods are used for the concentration of secreted proteins. But, no report is published yet on the use of trichloroacetic acid protein precipitation (TCA) method. Thus, the aim of this study was development of an ELISA by using secreted proteins of *Mycobacterium avium* subspecies *paratuberculosis* condensed with TCA and to increase specificity of test by reducing non-specific antibodies using *Mycobacterium phlei* (MP) antigens.

[II] MATERIALS AND METHODS

The 4-phase and 3-years study (2010-2012) was performed in Razi Vaccine and Serum Research Institute, Karaj, IR Iran.

Phase 1: Preparation of secreted proteins

1-1: Culturing standard strain 316F and extraction of DNA:

Standard strain 316F was cultivated in Herrold's egg yolk agar supplemented with mycobactin J. It was incubated at 37 ° C for 2 to 3 months to complete its growth. DNA was extracted using the Vansooligen2002 extraction method and aliquot into appropriate concentration and held in the refrigerator at 4 ˚C as working solution and the remained DNA was transferred to freezer at temperature of -20˚C as long as tests were running.

2-1: PCR-IS *900* identification test to confirm strain:

The presence of Specific Genetic Marker IS *900* was evaluated in the target bacteria genome. Application of PCR-IS *900* in amplification and observation of a 560-bp fragment indicates the identity of the strain under investigation as MAP.

3-1: Extraction of secreted antigens:

The bacteria were mass cultured in Dorset-Henley synthetic liquid medium. After incubation about two months at 37°C, the bacteria were removed from the culture medium (By centrifugation at 10,000×g for 30 min), the supernatant was filtered through 0.2-µm-pore-size (Nalge Nunc International, Rochester, NY) and to concentrate Culture Filtrate (CF) trichloroacetic acid protein precipitation (TCA) method was used with 40% TCA. Adding TCA to CF with 1:9 ratios led to dissolution of CF completely and created a solution composed of TCA with final concentration of 4%. In the next step the solution was incubated overnight at room temperature. By using aspiration and after elapsing the mentioned time, the most of supernatant was removed and the remaining supernatant with the precipitation re suspended and then divided into centrifuge bottles. The bottles were centrifuged (2500g for 15 min). The obtained precipitations were washed using TCA 1% and NaCl 10% two times and one time respectively are preferred. The precipitation obtained now was washed and dissolved in solvent buffer (34 mM Na2HPO4, 86 mM NaCl) and pH was set around 7.0-7.1 with 10 N NaOH. To determine the level of protein micro-Kjeldahl method was used

Phase 2: Preparation of soluble antigens of Mycobacterium phlei

In order to removing non-specific antibodies in the bovine serum, soluble antigens of Mycobacterium Phlei (ATCC 11758) were used. Mass culture of Mycobacterium phlei has taken place in Dorset Henley medium and after incubation for 1 month, bacteria were washed 3 times with saline buffer and then centrifuged (3000 g for 15 min). Created pellet bacteria are weighted and 1ml PBS (10mM PH: 7.2, volume weight 1mg / ml) was added and homogenization (Fisher Scientific™ Laboratory Homogenizer, Model 125) process was carried out for 20 min during 20 times, each time 30 seconds with the maximum power in the ice. The suspension created was centrifuged (5000g for 15 minutes). After determining the protein of supernatant with Lowry method, 4% BSA was added to the supernatant and it was aliquot into 200 macro liter and stored at -20 C.

Phase 3: Collecting serum samples:

Serum samples were obtained at different phases of the study from healthy, naturally infected or experimentally infected cattle with *M. avium* subsp. *paratuberculosis*. Finally, they were separately aliquot and stored at -20 C in the freezer.

3-1: *M. avium* subsp. *paratuberculosis*-positive serum: It was obtained from pooling 10 Holstein-Friesian cows that were positive in ELISA absorbance test (IDEXX, Westbrook, ME) and confirmed by fecal culture. They were used in the first and second Checker Board.

3-2: *M. avium* subsp. *paratuberculosis*-negative serum: It was obtained by mixing 30 Holstein-Friesian cows which were evaluated negative in non-absorbed ELISA tests (ID Vet-France), fecal and PCR culture for three consecutive years and had no clinical signs of disease and they were used in first Checker Board. Moreover, 950 samples of *M. avium* subsp. *paratuberculosis*-negative serums with condition stages of 1-3 and 50 samples of *M. avium* subsp. *paratuberculosis*-positive serum with condition stages of 2-3 were separately prepared and stored for determining the sensitivity, specificity and maintained in a freezer at -20 C.

3-3: *Mycobacterium phlei* positive Serum:

Hence 2 Holstein bulls aged 1 to 6 years which had the lowest antibody titers against MP, were sensitized by using somatic antigen MP; based on

OIE guidelines, MP antigens were mixed with adjuvant and then injected intramuscularly , 1 ml solution, 3 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. This serum was used as a negative control in the second Checker Board. We had assumed that *Mycobacterium Phlei* antigens with the ability of removing *Mycobacterium phlei* positive serum antibodies, are able to remove antibody against *Mycobacterium Phlei* in bovine serum.

Phase 4: Optimizing the best concentration of MAP antigens and antibody dilution

1-4: First Checkerboard: In purpose to obtaining the best concentration of MAP antigen and the best dilution of antibody, positive and negative serums in 1-3 and 2-3 stages were used.

By adding CF antigens of *Mycobacterium avium* ssp *paratuberculosis* (Concentration range 0.1-20 micrograms per liter), to the bicarbonate coating buffer 0.1 M (pH: 9.6), serial dilution was prepared and added 100 □l in each well of ELISA microtiter plate (PolySorp Nunc-Immuno 96-microwell plate) from column 1 to 11 and the last column was considered as negative control (coating buffer without antigen). microtiter plate was incubated for 18 h at 4 ° C. At the end of this time, the wells were washed three times with 10 mM PBS solution and then each well was filled with 150 □l solution of 2.5% casein (Maravel - UK) as a blocker and incubated for one hour at room temperature. obtained dilutions of 1/50 and 1/100 negative and positive control that were prepared in 1-2-3 stages by using 10 mM PBS/Tween 20 with BSA 1%, were added to each well with 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 [pH 7.2] containing 0.05% Tween 20), optimized dilution of 1/5000 was prepared in 10mM PBS(pH 7.2) from HRP cojugate (Mixture of monoclonal mouse anti bovine and polyclonal goat anti bovine Ig G with 1:1 ratios) (AbD Serotec-UK) and was added 100 ml to each wells and the plate was incubated again for 30 min at room temperature. Subsequently, after washing wells (according to previous stage), 100 microliters of tetra-methyl benzidine (TMB) substrate was added to wells and the plate maintained for 15 minutes in darkness at room temperature. Finally 100 µl of a stop solution (HCL 1 N) was added and the plate was read at wavelength of 450 nm by an ELISA reader (Bio Rad-Model 620). The considered antigen and antibody dilution levels after optimizing test conditions were evaluated by different Checker Boards by indirect ELISA method in duplicate. Blocker buffers used in the optimization process include the Bovine Serum Albumin solution 2%, casein solution 2.5% and fat-free milk powder 2% dissolved in PBS. In fact, the optimization of test conditions was possible in order to obtain the most favorable outcome for the evaluation of serum samples, minimizing non-specific reactions, saving material consumption and also obtaining the best results in the shortest time.

2-4: Second Checker Board: determining the best (lowest) concentration of MP soluble antigens for absorption of non-specific antibodies in Bovine serum by using positive and negative serums obtained from stages 1-3 and 3-3.

For this purpose, based on the results obtained from stage 2-3 and after determining the best concentration of CF antigens and appropriate dilution of antibody, two plates containing MAP secreted antigens were prepared and MP soluble antigens (in a not-coated ELISA Plate) with different concentrations of 0.5 to 100 micrograms with 250 ml diluted positive and negative serum obtained from the previous stages Serial Dilution was prepared and incubated at room temperature for 30 minutes. After the initial incubation, 100 ml was added to main plate and the process was done

like the first Checker Board (test was conducted on duplicate plates). The lowest concentration of MP antigens that created the highest ratio S/N (Signal/Noise) was established as the best concentration. Signal indicates the optical density (OD) of seropositive and noise determines the seronegative OD.

In this stage, 4 types of positive and negative controls were used. Positive control 1 includes positive control resulting from stage 1-3 diluted with serum dilution of absorption commercial kit based on instructions of the kit and used after initial incubation and removal of non-specific antibodies. Positive control 2, includes seropositive resulting from stage 1-3 used after incubation with different concentrations of MP antigens. The serums resulting from stage 2-3 were used as negative control. Furthermore, positive and negative serums of commercial kit which diluted with our serum diluent (with different concentrations of MP antigens) made positive and negative controls of number 3. Finally, serum diluent of commercial kit with its own positive and negative controls made positive and negative controls of number 4.

3-4: Cut-off calculation of designed ELISA system:

In this study we try to utilize Receiver Operating Characteristics (ROC) curve which is a reliable method to evaluate the performance of a new diagnostic test. To determine cutoff value, 170 serum samples were used in which 29 samples were confirmed by positive stool culture and the remaining 141 were negative by stool culture. Samples were tested simultaneously by both IDEXX ELISA kit and our indigenous homemade ELISA. An IDEXX ELISA result wasreported as S/P format, based on manufacturer instruction for use and our ELISA resultwas based on test OD value.

At each assigned cutoff value; sensitivityand specificitywith their confidence interval, werecalculated for both methods. All analysis and graphs are drawn by Sigmaplotfor windows version 12.5 and results are shown in following tables and graphs(3,7).

Phase 5: The sensitivity and specificity of designed ELISA compared with fecal culture results:

1000 cows from different animal herds proven to be infected and free by paratuberculosis were selected and fecal (in three times weekly) and serum samples were taken and *Sensitivity and Specificity* was determined *by* formula "Sensitivity= true positives/(true positive + false negative)" and "Specificity=true negatives/(true negative + false positives)" based on new S/P.

[III] RESULTS

Strain MAP confirmation: In this experiment, the presence of specific Genetic Marker IS*900* was targeted in the genome of the bacteria. By implementation of the optimized IS*900* PCR protocol on the extracted MAP strain 316F genomic DNA, a fragment in size of 560 bp was amplified and the identity of this strain was confirmed as MAP.

The results of the protein analysis: According to Lowry's technique used in this study, the protein content of CF and MP were 1.1 and 2.2 mg per liter, respectively.

Checker Board results:

The results of the first Checker-Board showed that the best selected concentrations of CF antigen after optimization of test conditions (to raise the ratio (S / N)) was 2 micrograms per milliliter; and preferred dilution of positive and negative control serum was 1/100.

Moreover, the casein solution 2.5% was selected between 3 different blocking buffers under study to create the highest S/N ratio(13).Meanwhile, two other blockers(BSA, fat- free milk) had the S/N ratio 10 and 9 respectively.

In reviewing second Checker-Board, the most appropriate concentration of MP antigens to absorb non-specific antibodies resulting from

checking plate ELISA coated with 2 micro-grams per ml of avium subsp. paratuberculosis culture filtrate (CF) antigens and serum controls PC3 and NC3 was 50 macro grams in milliliters (S/N = 13).

Determining new Cutoff for ELISA system designed for ELISA absorption system:

At each assigned cutoff value; sensitivityand specificitywith their confidence interval, werecalculated for both methods.

All analysis and graphs are drawn by Sigmaplotfor windows version 12.5 and results are shown in following tables and graphs.

Table 1: . Test S/P value of IDEXX ELISA kit with their related sensitivity and specificities

Cutoff > S/P	Sensitivity %	95% CI	Specificity %	95% CI
0.185	82.76	64.23% to 94.15%	61.7	53.15% to 69.76%
0.195	75.86	56.46% to 89.70%	69.5	61.20% to 76.97%
0.205	68.97	49.17% to 84.72%	77.3	69.50% to 83.93%
0.215	62.07	42.26% to 79.31%	78.01	70.27% to 84.55%

Following graph also displayed relationships between sensitivity, specificity and best cutoff value for IDEXX ELISA kit

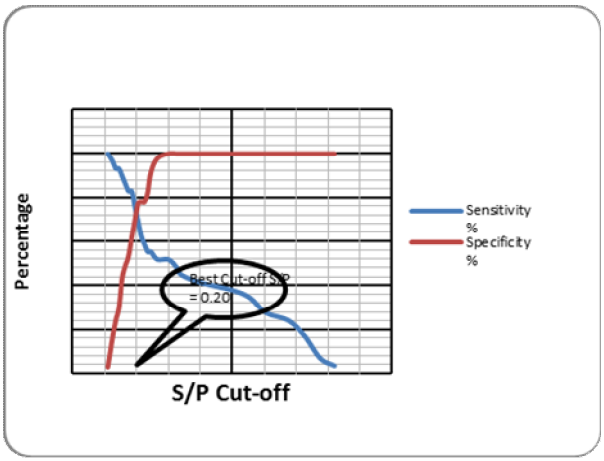

Fig 1: Cut- off Determination: Sensitivity versus specificity of IDEXX ELISA

Table 2:. Test OD value of our homemade ELISA kit with their related sensitivity and specificities

Cutoff >	Sensitivity %	95% CI	Specificity %	95% CI	LR+	LR-
0.205	96.55	82.24% to 99.91%	68.79	60.45% to 76.33%	3.0936	0.05
0.215	96.55	82.24% to 99.91%	75.89	67.97% to 82.69%	4.0046	0.046
0.225	93.1	77.23% to 99.15%	75.89	67.97% to 82.69%	3.8615	0.091
0.235	89.66	72.65% to 97.81%	78.72	71.04% to 85.16%	4.2133	0.131
0.245	86.21	68.34% to 96.11%	83.69	76.54% to 89.37%	5.2857	0.165

And following graph also displayed relationships between sensitivity, specificity and best cutoff value for our homemade ELISA.

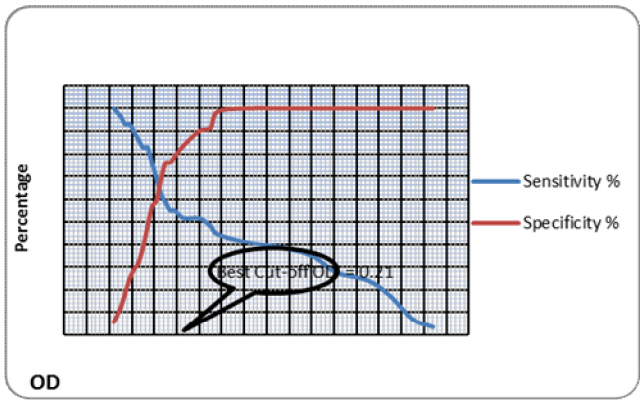

Fig 2: Cut off determination: Sensitivity versus specificity of Homemade ELISA

Based on above data the ROC curves for both methods are shown in fig (3).

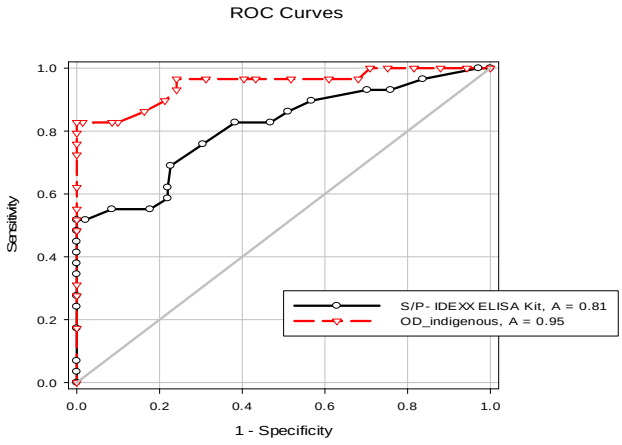

Fig 3: Graph ROC for both methods

Determining sensitivity and specificity of ELISA absorption system designed to compare with fecal culture results: Sensitivity and specificity of our studied test according to new cut off were determined 86% and 99% respectively; meanwhile, sensitivity and specificity of IDEXX kit according to manufacturer user manual is evaluated 70% and 99% respectively.

[IV] DISCUSSION

Near 60 years have passed from the diagnosis of paratuberculosis in Iran, but information available about the epidemiology of this disease is limited, due to limitation of diagnostic techniques for cultivation and isolation of bacteria from feces in herd. Problems associated with the cultivation and isolation of bacteria on the one hand and the high cost of imported ELISA kits on the other hand causes no specific program to evaluate the epidemiology and consequently controlling the disease in this country. Designing in house ELISA systems for this purpose has always been interested by research centers in various countries and in this regard(9), the most important principle is to extract proper antigens derived from proper strain of MAP. Standard strain 316F was used in this study because it did not need mycobactin-J for growth to be commercially viable and have more growth rate than strains which are related to mycobactin while other reported studies using strains JTC303; TEPS; ATCC19698 which are mycobactin-J dependent(10,11,12). In this study, comparing these strains with the others because the lack of access to other strains was not possible; however, insignificant difference in obtained CF and CE antigens had been reported by changing strain. The first report about the use of *M. avium* subsp. *paratuberculosis*-secreted antigens, that shows these antigens with higher sensitivity for diagnosis of early and subclinical infections in ELISA, dates back to 2008(15). Sensitivity and specificity in this study have been reported as 74% and 99 %respectively. However, in our study, the sensitivity and specificity were 86% and 99%, respectively; meanwhile sensitivity of commercial kits was determined maximum 50% in this study. This increased sensitivity can depend on three factors:

First, stage of infection in the herds of our study; Second, Sequestering obtained antigens and finally, the use of conjugated mixture

TCA precipitation was used in this experiment because proteins found in CF are precipitated and it is important for increasing the sensitivity of the test. Concerns about the possibility of cross-reactions and reduction of specificity due to antigenic diversity in this way of deposition were not observed (results not shown) However, the use of TCA for precipitation of secreted proteins is reported for the first time. The third factor to increasing sensitivity is to use conjugated pool so that 6 serums of *Mycobacterium avium* subsp. *paratuberculosis* in infected cattle were evaluated negative when the conjugated mouse monoclonal anti-bovine was used alone in ELISA while the use of conjugated polyclonal goat anti-bovine IgG was clearly positive. On the other hand, 8 serums of infected cattle on the contrary presented better results with conjugated mouse monoclonal anti- bovine while they were almost negative in our conjugated polyclonal (results not shown). This indicates the positive effect of using two conjugate mixtures. Similar results indicate that combination of two conjugated monoclonal and polyclonal had increased ELISA sensitivity with 10.4% that had been reported previously, with the difference that conjugated polyclonal related to sheep anti bovine was in versus goat anti bovine Ig G (our selected HRP conjugate)(14).

The purpose of using 4 positive and negative controls were performing the cross test in this study that were used for evaluation of extracted MP antigen; As MP antigens had the ability of absorption of non-specific antibodies in the cattle serums, there were not any significant effects on the positive control OD that was shown in

previous studies. Furthermore, 50 µg of MP antigen had the ability of absorption of *Mycobacterium Phlei* antibodies; it had the ability of absorption non-specific antibodies in negative control of commercial absorbance IDEXX kit too.

To improve results, different blocker buffers were used. The buffer blocker casein was selected because of creating higher S/N against S/N of other blockers. Also, reducing the incubation time from one hour to 30 minutes and the incubation temperature of 37 ° C to room temperature did not show significant difference in results(results not shown) that similar results were previously reported.

[V] CONCLUSION

In this experiment and Based on our results, developed homemade ELISA is completely comparable to IDEXX ELISA and even in terms of sensitivity and specificity by ROC curve analysis it is showed our homemade ELISA has more improvement insensitivity and specificity results. At cut off point S/P =0.2, IDEXX kit has close to 70% sensitivity and 77% specificity but our homemade system at cut off point OD = 0.21, has more than 95% sensitivity and 75% specificity(for 120 serum samples).This difference also displayed in Area Under Curve (AUC) of ROC curve. Our home made ELISA has higher AUC of 0.95 versus 0.81 of IDEXX ELISA kit. Therefore, with comparing specificity of both systems, our homemade ELISA has higher sensitivity which leads to higher chance of detecting infected animals. Moreover, it is using simple test OD results for test status interpretation which is a lab user friendly aspect.

ACKNOWLEDGEMENT:

The authors would like to thanks from manager of Razi Vaccine and Serum Research Institute, Dr. Kohram and Tuberculin department of Razi Institute and also it's branch in Ahwaz province. Special thanks to Dr. Denico in Arak province.

REFERENCES:

1- Akos S, Csaba N, AKos L, Zolta N , Lilia T, Judit F (2000) Comparison of Recoveries of *Mycobacterium tuberculosis* Using the Automated BACTEC MGIT 960 System, the BACTEC 460 TB System, and Lo¨wenstein-Jensen Medium. JOURNAL OF CLINICAL MICROBIOLOG. p. 2395–2397

2- Ayele W.Y., Machackova M., Pavlik I. (2001): The transmission and impact of paratuberculosis infection in domestic and wild ruminants. Veterinarni Medicina, 46, 205–224. http://www.vri.cz/docs/vetmed/46-8-205

3- Collins M. T (2002) Interpretation of a Commercial Bovine Paratuberculosis Enzyme-Linked Immunosorbent Assay by Using Likelihood Ratios. CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Nov,9(6), p. 1367–1371

4- Coad M, Clifford D. J, Vordermeier H. M, Whelan A. O(2013) The consequences of vaccination with the Johne's disease vaccine. Gudair on diagnosis of bovine tuberculosis.Veterinary Record

5- Facciuolo A, Kelton D F, Mutharia L M. (2013) Novel Secreted Antigens of *Mycobacterium paratuberculosis* as Serodiagnostic Biomarkers for Johne's Disease in Cattle., Clinical and Vaccine Immunology. Volume 20 Number 12 p. 1783–1791

6- Hasonova L, Pavlik I (*2006)* Economic impact of paratuberculosis in dairy cattle herds. a review. *Veterinarni Medicina, 51, (5): 193–211*

7- Hirpa E, Ameni G, Lawrence J, Tafess K, Worku A, Sori T , Zewdie O (2014) Performance Evaluation of Mycobacterium bovis Antibody Test for the Diagnosis of

Bovine Tuberculosis in Ethiopia., Academic Journal of Animal Diseases. 3(3): 33-38,

8- Hruska K. (2004): Research on paratuberculosis: Analysis of publications 1994–2004. Veterinarni Medicina, 49, 271–282. http://www.vri.cz/docs/vetmed/49-8-271

9- Köhler H, Burkert B, Pavlik I, Diller R, Geue L, Conraths FJ, Martin G. (2008) Evaluation of five ELISA test kits for the measurement of antibodies against Mycobacterium avium subspecies paratuberculosis in bovine serum. Berl Munch Tierarztl Wochenschr,;121(5-6):203-10

10- Marassi C, Fonseca L , Ristow P, Ferreira R, Walter Lilenbaum, Oelemann (2005) IMPROVEMENT OF AN IN-HOUSE ELISA FOR BOVINE PARATUBERCULOSIS SEROLOGY IN BRAZIL. Brazilian Journal of Microbiology 36:118-122

11- Nielsen S, Jorgensen J, Ahrens P, Feld N(1992) Diagnostic Accuracy of a Mycobacterium phlei-Absorbed Serum Enzyme-Linked Immunosorbent Assay for Diagnosis of Bovine Paratuberculosis in Dairy Cows. JOURNAL OF CLINICAL MICROBIOLOGY, , p. 613-618

12- Nielsen S S, Houe H, Thamsborg S M, Bitsch V (2001) Comparison of two enzyme-linked immunosorbent assays for serologic diagnosis of paratuberculosis (Johne's disease) in cattle using different subspecies strains of Mycobacterium avium.., J Vet Diagn Invest. Mar;13(2):164-6.

13- Shin S, Cho D, Collins M T *(2008)* Diagnosis of Bovine Paratuberculosis by a Novel Enzyme-Linked Immunosorbent Assay Based on Early Secreted Antigens of *Mycobacterium avium* subsp. *Paratuberculosis*. *Clin Vaccine Immunol ,vol. 15 no. 8 1277-1281*

14- Singh A. V, Singh S. V, Verma D. K, Yadav R, Singh P. K, Sohal J. S(2011) Evaluation of "Indigenous Absorbed ELISA Kit" for the Estimation of Seroprevalence of *Mycobacterium avium* Subspecies *paratuberculosis* Antibodies in Human Beings in North India., ISRN Vet Sci. 2011;: 636038

15- Shin S, Cho D, Collins M T (2008) Diagnosis of Bovine Paratuberculosis by a Novel Enzyme-Linked Immunosorbent Assay Based on Early Secreted Antigens of *Mycobacterium avium* subsp. *Paratuberculosis*. CLINICAL AND VACCINE IMMUNOLOGY, Aug. p. 1277–1281

16- Rajib D, Vivek K , Vijay K (2011) Conventional vs. Recombinant Antigen Based Detection of Mycobacterim avium subspecies paratuberculosis Infection in Animals**.** Vol. 6 No. 1, Article 75.

17- Vecerek V., Kozak A., Malena M., Tremlova B., Chloupek P. (2003): Veterinary meat inspection of bovine carcasses in the Czech Republic during the period of 1995–2002. Veterinarni Medicina, 48, 183–189. http://www.vri.cz/docs/vetmed/48-7-183