

Development of Indirect ELISA (IELISA) for Diagnosis of Bovine Brucellosis, Comparison of Three Different Labeled Detection Reagents

Abstract

The aim of this study was to develop Indirect Enzyme-Linked Immunosorbent (IELISA) assay for diagnosis of bovine brucellosis and to compare the performance characteristics of developed IELISA by different kinds of enzyme labeled conjugate. Three IELISA tests were developed using *Brucella abortus* smooth lipopolysaccharide (sLPS) as coating antigen with three different enzyme labeled conjugates: the mouse monoclonal (mAb) anti-bovine IgG1, the polyclonal anti-bovine antibody and the recombinant A/G protein, all conjugated to Horseradish peroxidase enzyme were used for this study. More than 180 bovine serum samples which tested by conventional serological tests like Rose Bengal Test (RBT) and 2-Mercapto-Ethanol (2-ME) were used for assessment of performance characteristics of our developed IELISA. Cut-off value for three different enzyme labeled conjugates were OD 0.23 as calculated, the specificities and sensitivities of our three developed IELISA were 100%, 100% when mouse monoclonal anti-bovine IgG1 enzyme conjugate was used, 98.9, 100% once polyclonal anti-bovine labeled conjugate was used, and eventually 100%, 98.8% in those with recombinant A/G protein conjugate.

Keywords: Indirect ELISA (IELISA); Bovine Brucellosis; Protein A/G conjugate; ELISA validation

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Abbreviations: IELISA: Indirect Enzyme-Linked Immunosorbent; RBT: Rose Bengal Test; RPAT: Rapid Plate Agglutination Test; SAT: Standard Agglutination Test; CFT: Complement Fixation Test; FPA: Fluorescence Polarisation Assay; TMB: Tetra-Methyl Benzidine; HRP: Horseradish Peroxidase; OD: Optical Density

Introduction

Brucellosis is caused by several species of the genus *Brucella*, mainly *Brucella abortus*, *B. melitensis* and *B. suis*. Infection with *Brucella* in cattle is usually caused by *B. abortus*, less frequently by *B. melitensis*, and occasionally by *B. suis*. *Brucella melitensis* is the main causative agent of infection with *Brucella* in sheep and goats [1].

Brucellosis is still considered as one of the most important zoonotic diseases in the world which is globally spread [2]. Brucellosis is not eradicated in many parts of the world especially in Asia, Africa, central and south America and some parts of Europe [3]. The golden standard for diagnosis of brucellosis is isolation and identification of causative bacterium, but it required high security laboratory (biological containment level 3 facilities), highly skilled personnel, an extended turn-around time for results and it is considered a hazardous procedure [4]. Therefore, Diagnosis of brucellosis are mainly perform by conventional serological screening tests like Rapid Plate Agglutination Test (RPAT), Rose Bengal Test (RBT), Standard Agglutination Test (SAT), 2-Mercapto Ethanol Test (2ME) and Complement Fixation Test (CFT) as the brucellosis diagnostic tests [4-10]. Other tests like ELISA and Fluorescence Polarisation Assay (FPA) tests with high sensitivity

and specificity are normally used as complementary tests [4-6]. Meanwhile, SAT cannot be recommended as a reliable diagnostic test due to the high possibility of false positive reactions [4].

The bovine immune system reaction against the disease started with initial and quick response of IgM isotype. The production of IgM followed quickly by IgG1, IgG2 and IgA antibodies. Last two proceeding antibody classes exist in low and unstable amount, therefore, the most important antibody produced against *Brucella* in bovine is IgG1 [4]. ELISA method poses a great opportunity of identification of all of the four antibody classes [11].

In this study, we explained utilization of three different enzyme labeled reagents including monoclonal anti bovine IgG1-HRP conjugate (IELISA m), polyclonal anti-bovine HRP conjugate (IELISA p) and protein A/G-HRP conjugate (IELISA protein A/G) and then comparing them in terms of sensitivity and specificities. To validate developed IELISA, their results were compared with positive and negative known samples, already or simultaneously tested by direct agglutination tests like SAT, RBT and 2ME tests. Among conjugates utilized in our IELISA, Protein A/G has special interest; this protein conjugate has four specific sites for Fc part of immunoglobulin G in many species. Two of them are related to the protein A (*Staphylococcus aureus* spp. Cowan) which has 42 KDa molecular weight and the remaining two sites pertaining to protein G (*Streptococcus* spp. group G) which has 30 KDa MW. The recombinant protein A/G is able to detect both bovine IgG1 and IgG2 subclasses as well as different animal species immunoglobulin isotypes. The enzyme labeled conjugate of this protein is used to identify the immunoglobulin G class against the brucellosis in both human and livestock [8]. We will also explained

about high sensitivity of our developed IELISA and problem that we were faced in development process of our IELISA and that was positive reaction in samples with low insignificant antibody titer tested by SAT or in other word false positive reactions.

Materials and Methods

Samples

One hundred and eighty bovine sera were randomly collected from Tehran, Alborz dairy farms of Iran. Samples were collected from vaccinated and non-vaccinated animals and they were verified by screening tests like direct agglutination such as RBPT followed by SAT and 2ME tests. Those samples with positive reaction by RBPT were tested by SAT and 2 ME which determine acute or chronic status of infection. Based on those serological tests 85 positive and 95 negative samples were used for our study.

IELISA antigen and buffers

Antigen component of IELISA which was used in this study was the smooth lipopolysaccharide (sLPS) of *Brucella abortus* S1119-3, (Peace River™ Company) which was diluted in carbonate buffer (0.05 M, pH: 9.6) as coating buffer. The blocking buffer was Phosphate Buffer Saline (PBS, 0.8g of NaCl, 0.02g of KCl, 0.144g of Na₂HPO₄, 0.024g of KH₂PO₄, 0.01 M, pH 7.2) containing 1% of Bovine Serum Albumin (BSA, fraction V Merck). To overcome the problem observed by low titer sera (1:40) tested by SAT, serum diluents buffer (PBS + 0.05% Tween 20) prepared by adding 1% casein, 30 mM EDTA and different concentrations of urea ranging from 0.06 M - 2 M concentration. Sample diluent comprising urea can be used to increase the stringency of binding and removing reactivity from low affinity antibodies which may leads to false positive assay results or insignificant OD in our developed IELISA.

The washing buffer was prepared by PBS +0.05% Tween 20. The conjugate dilution buffer was prepared by PBS 0.01M pH 7.2, EDTA 30mM, 1% BSA, 0.05% Tween 20, 0.2% casein, 0.3% Kathon™ CG and 0.2% sucrose. Sugar was used to stabilize our secondary conjugate.

Development of IELISA

Polystyrene microtiter plate was coated by 1µg/ml concentration of sLPS of *Brucella* antigen (Peace River™ Company) diluted in carbonate buffer (0.05 M, pH: 9.6). To coat the antigen, 100 µl diluted sLPS (1µg/ml obtained by checkerboard) was added into ELISA microtiter wells (Pishtaz Teb diagnostics of IRAN) and were incubated at 4°C overnight. Then, microtiter wells were washed three times by PBS containing 0.05% Tween-20 buffer (300 µl/ well). To reduce nonspecific binding of antigen, microtiter plates were blocked using 100 µl of blocking buffer (PBS +1% BSA) per well and plates were incubated at room temperature for an hour.

Blocking solution was discarded and wells were sharply strike onto absorbent papers to pouring out remains of blocking solution and finally plates leave to dry at room temperature and kept at 4°C until use. To run the test, Initially 100 µl of diluted (1:50 in PBST + 1%casein sample dilution buffer + 30 mM EDTA) positive and negative serum samples was added into each well of formerly coated microplate. Further development optimization of our IELISA, showed addition of 2 M urea to serum dilution buffer was improved our IELISA results. Diluted serums were incubated

for 30 minutes at room temperature and then washed four times with washing solution (300 µl/ well). Then, 100 µl of the each of horseradish peroxidase (HRP) labeled conjugate was diluted in appropriate conjugate dilution buffer were added in separate microtiter strips. In this study, three kinds of HRP labeled conjugate reagents were used; i.e. polyclonal anti-bovine (1:3000 dilution), mouse monoclonal anti-bovine IgG1 (1:4000 dilution) and recombinant protein A/G (1:2500 dilution) all purchased from Peace River™ Company, were added into separate strips and incubation takes place for 30 minutes at room temperature followed by four times wash step (300 µl /well). Finally, 100 µl of ready to use substrate reagent of Tetra-Methyl Benzidine (TMB) provided by Pishtaz Teb Diagnostics was added into wells and microplates incubated for 15 minutes at room temperature in darkness. The reaction was stopped by adding 100 µl HCl 1M (Merck) and optical density (OD) of the samples were read at 450 nm wavelength with the 630 nm reference filter using Hitech ELISA reader.

Validation of IELISA

Cut off was determined by testing 50 negative samples. The cut off was determined by formula of mean of negative sera OD plus 2 × Standard Deviation (SD) of negative sera OD.

Precision study was performed by intra-assay or within run as well as inter-assay or between run/day test on three levels of negative, low and high positive serum. To assess intra-assay of our IELISA, 20 repeats of each of three level samples were run simultaneously in one test and for inter-assay test the three level samples were tested in five consecutive days, one run per day, each with four replicates.

Sensitivity and specificity of developed IELISA were determined by comparison of our IELISA with serologically known positive and negative bovine sera.

Results and Discussion

Our developed IELISA was reacted with low titer serums (≤ 1:40 by SAT) and it was showed considerable OD of 0.3-0.4 which leads to high cut off value for ELISA or potential of creation of false positive reaction. Therefore, we tried to reduce OD of this group of samples without significant reduction on positive OD samples by using 2 M urea in our serum dilution buffer (Table 1).

The IELISA with high sensitivity can lead to false positive responses in such cases but the urea minimizes this interferences.

The cut-off value of developed IELISA was determined using formula mentioned in our materials and methods section (Table 2) and it was OD of 0.23 which mean those results higher than this OD value will be considered as positive.

Test precision including intra and inter assay tests was performed at three sample levels on negative, low and high positive serums. The results of intra and inter-assay, were shown in Table 3. Coefficient of variation was calculated by dividing standard deviation to mean value in each serum levels.

To estimate sensitivity and specificity and detection efficiency (EF) of our developed IELISA, 85 serologically

positive and 95 serologically negative bovine sera were used. Results were showed in Table 4.

Table 1: Effect of different urea concentration on low serum titer samples in IELISA optimization process.

Urea Concentration (Mole/L)	0.062	0.125	0.25	0.5	1	2	Control
High Positive Sera	2.81	2.81	2.8	2.8	2.79	2.78	2.85
Low positive Sera	0.9	0.88	0.87	0.85	0.84	0.82	0.93
Negative (Low Serum Titer $\leq 1:40$ by SAT)	0.39	0.32	0.3	0.25	0.2	0.14	0.4
Negative Sera	0.14	0.13	0.1	0.08	0.05	0.04	0.16

Table 2: Cut off Determination using both serologically low titer and negative bovine sera.

Negative sera ODs									
SAT Low titer ($\leq 1:40$) samples (Consider as Negative in Our Study)					Negative Samples				
0.24	0.19	0.138	0.14	0.15	0.07	0.03	0.024	0.022	0.05
0.15	0.175	0.185	0.18	0.13	0.09	0.065	0.021	0.04	0.029
0.194	0.129	0.161	0.147	0.21	0.128	0.1	0.046	0.035	0.11
0.15	0.167	0.198	0.141	0.168	0.086	0.04	0.07	0.12	0.098
0.14	0.135	0.17	0.13	0.187	0.059	0.042	0.125	0.025	0.05

Total Number	50
Mean	0.11
Standard Deviation (SD)	0.06
Estimated cut-off (Mean negative OD + 2SD)	0.23

Based on our results, the best test specificity was displayed in those tests using either protein A/G-HRP or Anti-bovine IgG1-HRP as labeled reagent. The diagnostic specificity obtained for each of the developed IELISA using polyclonal conjugated antibody, monoclonal conjugated antibody and the protein A/G enzyme conjugate (protein A/G-HRP) were 98% - 100% and 100% respectively (Table 4).

IELISA is a sensitive and specific test for bovine brucellosis diagnosis. Different antigens including sLPS of Brucella bacteria are normally used in various IELISA development studies [2,5,6,8,9,12-15]. The test protocol also varied between different studies [5,15,16].

The sensitivity of some serologic tests such as RBT, SAT, CFT, CELISA (Competitive ELISA), FPA and IELISA were also evaluated [5,6,9]. It has been shown the highest sensitivity was related to IELISA [7]. The result of this study was also displayed high sensitivity of IELISA.

There are several studies which compare IELISA with SAT, RBT and 2ME. In such studies, the sLPS *Brucella abortus* S1119-3 was

used [2,5,14,15,17]. The sensitivity and specificity of this antigen were higher than other antigenic indices like Outer Membrane Protein (OMP₂₈) or native haptan (NH) [1,12].

Another important consideration for IELISA test results is vaccination of animal. The *Brucella abortus* S19 and RB51 vaccine are used in the brucellosis control and campaign program. Animal vaccination by S19 strain provokes humoral immune response with consequent antibody rising which leads to false positive reactions in serological test. But those animals vaccinated by RB51 (including our sera samples) provoke cell mediated immunity against sLPS of Brucella bacteria without false positive results of vaccination in serological tests [1,3,4].

During IELISA optimization process we also assessed different urea concentration in the serum diluting buffer. Buffers containing 2 M of Urea concentrations reduce nonspecific binding of low titer negative sera ($\leq 1:40$ titer by SAT test) (Table 1). Other studies has been shown that urea treatment cause dissociation of low affinity antibody from immune complex and just high affinity antibodies remain in reaction [18-20].

Table 3: Intra and inter-assay (Between days) test used to showed test imprecision, OD results in three levels of negative, low positive and high positive samples. Coefficient of variation (CV%) is representative of our IELISA low imprecision.

Intra-assay test				Inter-assay (Between-day) test				
Repeat	Negative Serum	Low Positive sample	High Positive sample	Day	Replicate	Negative Serum	Low Positive Serum	High Positive Serum
1	0.05	0.7	1.55	Day 1	1	0.05	0.54	1.25
2	0.052	0.74	1.572		2	0.05	0.55	1.25
3	0.054	0.72	1.543		3	0.054	0.48	1.28
4	0.053	0.76	1.542		4	0.055	0.50	1.22
5	0.052	0.736	1.533	Day 2	5	0.048	0.54	1.3
6	0.04	0.74	1.5		6	0.06	0.50	1.3
7	0.054	0.735	1.568		7	0.05	0.50	1.36
8	0.055	0.73	1.56		8	0.05	0.51	1.29
9	0.053	0.687	1.556	Day 3	9	0.05	0.52	1.22
10	0.052	0.677	1.585		10	0.05	0.54	1.35
11	0.054	0.71	1.55		11	0.05	0.50	1.27
12	0.049	0.694	1.53		12	0.04	0.54	1.34
13	0.04	0.713	1.65	Day 4	13	0.06	0.58	1.32
14	0.05	0.697	1.487		14	0.054	0.63	1.26
15	0.05	0.712	1.55		15	0.05	0.48	1.27
16	0.044	0.73	1.53		16	0.06	0.50	1.36
17	0.045	0.72	1.62	Day 5	17	0.04	0.60	1.26
18	0.052	0.69	1.493		18	0.05	0.52	1.34
19	0.049	0.714	1.534		19	0.06	0.53	1.28
20	0.047	0.75	1.55		20	0.05	0.48	1.25
Mean	0.05	0.72	1.55	Mean		0.05	0.53	1.29
Standard Deviation	0.00	0.02	0.04	Standard Deviation		0.006	0.040	0.044
Coefficient of Variation (CV %)	9%	3%	2%	Coefficient of Variation (CV %)		11%	8%	3%

In this study, different kinds of conjugates used in IELISA test and their performance characteristics are shown in Table 4. The sensitivity of our designed IELISA in those using Anti-bovine IgG1-HRP conjugate was 100% which is better than other similar studies [5]. Our specificity result also displayed higher than other studies [6]. Our results also displayed use of labeled recombinant protein A/G in IELISA, leads to specificity and sensitivity of 100% and 98.8% respectively which is conformed to other studies using the same labeled reagent [18]. Our results also showed use of labeled anti-bovine IgG1 monoclonal antibody as secondary conjugate has greater sensitivity and specificity. Other studies also

revealed another advantage of exploiting of labeled anti-bovine IgG1 which is reduction in cross reaction results between sLPS of *Brucella abortus* and *Yersinia enterocolitica* [5] which is a common problem in SAT. They also introduced a competitive ELISA with sensitivity and specificity of 98.2% and 97.5% respectively [5]. Studies carried out by Nielsen et al using both labeled Protein A/G and monoclonal anti-bovine IgG1-HRP showed 100% sensitivity when labeled protein A/G protein is used [4]. Our developed IELISA can be used in field study with more number of sera which need further investigation.

Table 4: Sensitivity and specificity of our three kind developed IELISA.

IELISA ¹ types	IELISA P ²		IELISA M ³		IELISA Protein A/G ⁴	
	Positive	Negative	Positive	Negative	Positive	Negative
Optimum dilution	1/3000		1/4000		1/2500	
Sample type	Positive	Negative	Positive	Negative	Positive	Negative
Serologically regarded as positive	85	-	85	-	84	1
Serologically regarded as Negative	1	94	-	95	-	95
Specificity (%)	98.9		100		100	
Sensitivity (%)	100		100		98.8	
PPV ⁵ (%)	98.7		100		100	
NPV ⁶ (%)	100		100		97.9	
EF ⁷ (%)	99.4		100		98.8	

¹: Indirect Enzyme-Linked Immunosorbent Assay ²: Polyclonal anti-bovine antibody HRP conjugate, ³: Mouse monoclonal (mAb) anti-bovine IgG₁ HRP conjugate, ⁴: Protein A/G HRP conjugate, ⁵: Positive Predictive Value, ⁶: Negative Predictive Value, ⁷: Efficiency

Conclusion

Bovine brucellosis still remains as one of important animal health issue in different countries and subsequent human involvement leads the disease as one of immense zoonotic disease. IELISA can be used as one of good alternative diagnostic tools for diagnosis of bovine brucellosis. Although simple, cheap tests like SAT, RBT, etc. are used widely but development of IELISA using labeled anti-bovine IgG1 as conjugate reagent has great advantage of sensitivity, specificity with rapid results.

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