

Development of Qualitative and Quantitative ELISA Models for Bovine Brucellosis Diagnosis ^[1]

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[1] This work was kindly supported by the Scientific Research Projects Commission of Ondokuz Mayıs University (Project no: VET-005)

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Makale Kodu (Article Code): KVFD-2009-692

Summary

In this study, a qualitative and a quantitative ELISA models (iELISA and qELISA, respectively) have been developed for screening and quantifying IgG isotype anti-Brucella abortus LPS antibody. For developing high sensitive and specific ELISA models, the nature and concentration of blocking-diluting reagents have been found very critical since milk components such as casein and particularly β -lactoglobulin, but not α -lactalbumin, strongly inhibited the anti-LPS antibody detection by an unknown mechanism. The iELISA as well as qELISA models have been developed and validated by OIE reference sera and well-known field sera evaluated with RBT, CFT and cELISA. The results demonstrated that both ELISA models, iELISA in large-scale screening and qELISA in standard determination of anti-LPS antibody concentration, developed in this study can be included as valuable and effective immunodiagnostic tools in brucellosis monitoring-eradication and vaccination surveillance programs in endemic countries.

Keywords: Bovine brucellosis, ELISA, quantitative ELISA, LPS, IgG antibody

Sığır Brusellozis Tanısı için Kalitatif ve Kantitatif ELISA Modellerinin Geliştirilmesi

Özet

Bu çalışmada, IgG izotipi anti-Brucella abortus LPS antikorlarının taranması ve nicelendirilmesi için kalitatif ve kantitatif ELISA modelleri (iELISA ve qELISA) geliştirildi. Yüksek düzey duyarlılık ve özgünlüğe sahip ELISA modellerinin geliştirilmesinde doyum-dilüsyon reaktiflerinin tabiatı ve konsantrasyonları çok kritik bulunmuştur. Bu çerçevede, süt bileşenlerinden α -laktalbuminin inhibitör etkisi bulunmadığı ve tanımlanmamış bir mekanizmadan dolayı kazein ve özellikle β -laktoglobulinin anti-LPS antikor tespitini güçlü olarak inhibe ettiği belirlenmiştir. iELISA ve qELISA modelleri OIE referans serumlarının yanı sıra RBT, CFT ve kompetitif ELISA ile değerlendirilerek belirlenmiş saha serumları yardımıyla geliştirilerek valide edilmiştir. Elde edilen sonuçlar, bu çalışmada geliştirilen her iki ELISA modelinden iELISA'nın geniş ölçek taramalara ve qELISA'nın ise anti-LPS antikor konsantrasyonunun standart bir şekilde belirlenmesine yönelik olarak, endemik ülkelerde brusellozis tarama-eradikasyon ve aşı izleme programlarında etkin ve geçerli immunodiagnostik araçlar olarak kullanılabileceğini göstermektedir.

Anahtar sözcükler: Sığır brusellozisi, ELISA, kantitatif ELISA, LPS, IgG antikor

INTRODUCTION

Brucellosis, an important zoonotic disease, is characterized by abortions and reproductive failures in animals. Brucellosis may be diagnosed by bacteriological and serological methods and DNA-based techniques ¹⁻⁴.

Serological methods include Rose Bengal Test (RBT), Buffered Plate Agglutination Test (BPAT), Complement Fixation Test (CFT), indirect and competitive enzyme linked immunosorbent assays (iELISA and cELISA), immuno-

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blotting techniques and Fluorescence Polarization Assay (FPA) ⁴. The RBT and BPAT as well as ELISAs and FPA are considered as suitable screening tests ⁵.

Numerous ELISA models have been developed for bovine brucellosis screening ⁴. These tests principally detect bovine antibody directed to the lipopolysaccharide (LPS), the immunodominant antigen of *Brucella abortus* ^{4,6}. Indirect and in a lesser extent competitive ELISA models, are mainly used to screen the presence of the Brucella specific antibody in both blood serum and milk ⁷. Indirect ELISA models, at least as sensitive and specific as cELISA ^{8,9} are considered as especially valuable for the detection of latent carriers ⁶.

The variability of the test procedure, reagents and results described in the literature does not allow comparing different studies ^{6,7,9}. In this respect, the purpose of this study was to develop qualitative and quantitative ELISA models for bovine brucellosis screening and quantifying anti-Brucella antibody, respectively.

MATERIAL and METHODS

Brucella antigen

Smooth lipopolysaccharide (LPS) from *B. abortus* 2308 strain kindly provided by Dr. I. Moriyon (University of Navarra, Spain) was used as antigen to detect anti-Brucella antibody. Detailed description of the LPS preparation was published elsewhere ¹⁰.

Reference and Field sera

Strong and weak reference positive sera were kindly provided by Veterinary Laboratory Agency (Weybridge, UK), Brucella reference laboratory of OIE (World Organization for Animal Health). Reference negative sera were from Institut Pourquier (France) and Svanovir (Sweden). Fetal bovine sera of Brucella-free herds were also used as negative control (Biochrom, S 0415). From a total of 597 field sera collected from different regions of Turkey, 3 main groups were constituted as follows: i) Sera from High Prevalence Region (HPR, from Kars and Ardahan, with high rate abortion), ii) Sera from Low Prevalence Region (LPR, including 2 localities of Samsun, Havza and Çarşamba districts, with abortion) and iii) Vaccinated cattle (V, from different regions).

i. Sera from High Prevalence Region (HPR): HPR group constituted of 125 sera previously evaluated with RBT, CFT and cELISA (Svanovir, Sweden) have been supplied from the collection of Dr. Genç ¹¹. One hundred twenty five sera evaluated with RBT, 67 sera tested with cELISA and 95 sera screened with CFT compared with our qualitative ELISA (iELISA) were used for screening assay. To

determine anti-LPS antibody concentration with our quantitative ELISA (qELISA) model, 67 positive serum samples from the study of Dr. Genç et al. ¹¹ and additional 84 positive samples out of 159 sera tested only with RBT from the same region (HPR) were used in this study.

ii. Sera from Low Prevalence Region (LPR): LPR group of 52 positive samples among 265 bovine sera evaluated with RBT and ELISA, were included in the evaluation of qELISA for quantifying anti-LPS antibody.

iii. Sera from vaccinated cattle (V): V group containing 48 sera from at least twice vaccinated cattle were collected from low rate prevalence regions. The CFT positive sera of this group were evaluated with qELISA for quantifying IgG isotype anti-LPS antibody.

ELISA Procedures and Establishment of Bovine IgG Standard Calibrator

After optimization, the procedure of the qualitative ELISA model was established as follows. Microwell plates (Poly Sorp; Nunc) were coated with 100 µl of LPS antigen solution (5 µg/ml) by overnight incubation at 4°C. The plates were washed twice with PBS containing 0.05% Tween 20 (PBST) and blocked for 2 hours at 37°C with 1% and 5% of skim milk or cold water fish gelatin (Sigma G7765) (PBST/M or PBST/FG). After washing, 100 µl of sera diluted 1:200 were added and incubated for 1 hour at 37°C. The plates were washed and 1:30 000 dilution of AP/conjugated anti-bovine IgG (Sigma A0705) was added and incubated for 1 hour at 37°C. The plates were washed and 100 µl of pNPP (Sigma N9389) was added and incubated at 37°C. The optical density (OD) was measured at 405 nm in ELISA reader.

For quantitative ELISA, a bovine IgG standard calibrator has been established and included at least in triplicate into each assay. The procedure of qELISA was identical to iELISA, except the results were expressed as microgram per milliliter of the sera by linear regression calculation in respect to the IgG standard calibrator. The concentration of anti-LPS antibody equal or superior to 50 ng/ml of bovine IgG was considered as positive. In order to establish IgG standard calibrator, purified bovine IgG (Sigma I5506) solutions at different concentrations have been coated in micro-wells and tested in the same manner as described for qualitative ELISA. After the determination of the lower and upper detection limits of qELISA, the bovine IgG solutions giving the most significant correlation coefficient were determined as IgG standard calibrator.

Statistical Analysis

SPSS 13.0 programme was used for statistical analysis

for the determination of sensitivity, specificity, positive and negative predictive values, probability at 95% confidence level, coefficient correlation and linear regression.

RESULTS

In preliminary assays, weak (OIE-WP) and strong positive (OIE-SP) reference sera obtained from OIE as well as strong positive field sera were significantly inhibited in the detection of anti-LPS antibody by blocking-diluting reagents 5% (89.8%±3.8 of inhibition) and 1% (76%±10.1) of skim milk (M) and in a lesser extent 5% (30.8%±4.4) of fish gelatin solutions (FG) when compared to 1% FG solution giving optimal antibody detection. Since the inhibitory effect with 5% and 1% M solutions was very high, two different concentrations of three major milk whey proteins designated as C1, C2 for casein (Sigma C6780), α -LA1, α -LA2 for alpha-lactalbumin (Sigma L6010), and β -LG1, β -LG2 for beta-lactoglobulin (Sigma L2506) were used to determine their inhibitory effect on anti-LPS antibody detection in comparison with 1% FG solution (Table 1). The results clearly demonstrated that α -lactalbumin solutions did not have any inhibitory effect while particularly β -lactoglobulin and casein exerted significant inhibitory effect on the detection of anti-LPS antibody.

Table 1. Inhibitory effect of milk components in comparison with 1% of fish gelatin

Tablo 1. Süt bileşenlerinin inhibitor etkisinin %1 balık jelatini ile karşılaştırılması

Serum properties ^(a)	Mean of inhibition percentage with ^(b)						Mean of OD405 (\pm SD) with 1% FG ^(c)
	C1	C2	α LA1	α LA2	β LG1	β LG2	
OIE (reference weak +)	33	56	NI(d)	NI	61	85	1.486±0.087
OIE (reference strong +)	20	31	NI	NI	36	71	2.403±0.115
15 (strong +)	12	16	NI	NI	18	65	2.421±0.110
21 (strong +)	33	30	NI	NI	29	76	2.250±0.121
55 (strong +)	16	23	NI	NI	21	63	2.414±0.163
Total Inhibition % (\pm SD)	23±10	31±15	NI	NI	33±17	72±9	NI

^(a) Properties are detailed in Material and Methods. The strong positive sera 15, 21 and 55 were supplied from the collection of Dr. Genç ²¹. All sera at 1:200 dilution tested in triplicate in three experiments. ^(b) Blocking-diluting solutions prepared with 2.16 mg/ml of casein (C1), 10.8 mg/ml of casein (C2), 0.27 mg/ml of α -lactalbumin (α -LA1), 1.35 mg/ml of α -lactalbumin (α -LA2), 0.54 mg/ml of β -lactoglobulin (β -LG1) and 2.7 mg/ml of β -lactoglobulin (β -LG2) correspond to 1% and 5% of skim milk content, respectively. ^(c) Mean and SD of OD405 values obtained with all sera diluted at 1:200 in 1%FG. Mean and SD of OD405 values of the negative sera diluted at 1:200 in 1%FG, have been at 0.340±0.170 (n=67). Negative control sera from Institut Pourquier and Svanovir, fetal bovine sera (Biochrom) and the sera from the collection of Dr. Genç ²¹. ^(d) NI= Non-inhibition

The results given in Table 2 show that the percentage of total negative and positive sera evaluated with iELISA was identical to that of RBT and only slightly, but not significantly different to those observed with cELISA or CFT (P>0.05). No statistical difference between cELISA and iELISA was observed at the level of the negative predictive values (94.23% versus 93.55%) and in the relative sensitivity (96.05% versus 95.31%). On the

contrary, the relative specificity (94.75%) and the positive predictive value (96.06%) of iELISA in respect to those of cELISA (84.48% and 89.02%, respectively) showed that iELISA was more specific than cELISA and enabled to detect higher number of the positive samples. However, when the significance of relative correlation of the iELISA and cELISA results were analyzed in respect to those of RBT and CFT, statistically significant correlation observed for iELISA ($X^2=177.19$) was clearly higher than that of cELISA ($X^2=89.85$).

A quantitative ELISA model, based on the qualitative iELISA and a pre-established bovine IgG standard calibrator was developed for quantifying anti-LPS antibody. The lower and upper detection limits of qELISA were 25 and 800 ng/ml of IgG, respectively. Six different concentrations of bovine IgG (25, 50, 100, 200, 400 and 800 ng/ml) giving a significant correlation coefficient ($R^2 > 0.972$, n=24) were defined as IgG standard calibrator and thus, included at least in triplicate into each quantitative assay. In comparison with this standard calibrator, the concentration of anti-LPS antibody of OIE weak and strong positive reference sera was determined as 15-20 μ g/ml and 70-90 μ g/ml, respectively. Based on these results, 3 categories of anti-LPS antibody positivity were defined as "Weak Positive" (WP, 10-30 μ g/ml), "Positive" (P, 30-70 μ g/ml) and "Strong Positive" (SP, >70 μ g/ml) (Table 3).

Three groups of field sera (designated as HPR, LPR and V) described in detail in material and methods were used to evaluate and validate the qELISA model. The LPS antibody concentration of these sera was individually determined and the results were given in Table 3. With qELISA model, high percentage of SP anti-LPS antibody (>70 μ g/ml) was detected for the cattle of V (77.1%), LPR (63.4%) and HPR groups (55.7%). Sera from HPR and V

groups contain significantly lower percentage of WP anti-LPS antibody (10-30µg/ml) when compared to LPR group. The percentage of P anti-LPS antibody of HPR (37%) was significantly higher than those of V (8.3%) and LPR groups (13.5%) ($P < 0.05$).

Table 2. Comparison of the iELISA results with RBT, cELISA and CFT

Table 2. iELISA sonuçlarının RBT, cELISA ve CFT ile karşılaştırılması

Sera ^(b)	No. and (%) of samples	No and (%) of the sera evaluated by iELISA ^(a)	
		Negative	Positive
RBT Negative	52 (41.6)	49 (94.2)	3 (5.8)
RBT Positive	73 (58.4)	3 (4.1)	70 (95.9)
cELISA Negative	26 (38.8)	25 (96.1)	1 (3.9)
cELISA Positive	41 (61.2)	2 (4.9)	39 (95.1)
CFT Negative	40 (42.1)	38 (95.0)	2 (5.0)
CFT Positive	55 (57.9)	3 (5.5)	52 (94.5)

^(a) Number and percentage of negativity and positivity of the sera evaluated by qualitative iELISA in comparison with those detected by RBT, cELISA and CFT

^(b) Sera evaluated by RBT, cELISA and CFT were supplied from the collection of Dr. Genç¹¹. Statistical analysis demonstrate that iELISA is at least as sensitive and specific as cELISA and there is a high significance of correlation between iELISA and CFT and RBT ($\chi^2=177.19$, $P < 0.001$)

Table 3. Anti-LPS antibody concentration levels of positive bovine sera

Table 3. Pozitif sığır serumlarının anti-LPS antikor konsantrasyon düzeyleri

Sera groups and properties ^(b)	No and (%) of LPS antibody positivity ^(a)			
	WP	P	SP	N
HPR (CFT/RBT, RBT positive)	11 (7.3)	56 (37.0)	84 (55.7)	151
LPR (ELISA positive)	12 (23.1)	7 (13.5)	33 (63.4)	52
V (CFT positive)	7 (14.6)	4 (8.3)	37 (77.1)	48
Total sample number	30	67	154	251

^(a) Three categories of anti-LPS antibody positivity were constituted on the basis of OIE weak and strong positive reference sera, which contain 15-20 and 70-90 µg/ml of IgG isotype antibody, respectively. The categories WP (weak positive), P (positive) and SP (strong positive) contain 10-30, 30-70 and >70 µg/ml of IgG isotype anti-LPS antibody

^(b) Serum groups and their properties are detailed in Material and Methods **HPR**: high prevalence region, **LPR**: low prevalence region, **V**: vaccinated, **N**: total number of samples

DISCUSSION

In this study, a qualitative and a quantitative ELISA models have been developed for screening and quantifying anti-LPS antibody, respectively. Both indirect ELISA models detect IgG isotype antibody directed to LPS of *Brucella abortus*. The use of *B. abortus* smooth LPS in both ELISA models was supported by the fact that rough LPS- and perosamine polysaccharide-based indirect ELISA and cELISA models did not outperform smooth LPS-based

indirect ELISA models^{9,10,12}. Smooth LPS-based tests appear as the most sensitive for bovine brucellosis screening though the false-positivity due to *Yersinia enterocolitica* O:9 infection was detected in certain circumstances^{13,14}. The false-positivity has been overcome by using a highly specific skin test with *Brucella abortus*-soluble protein (brucellin)^{14,15}. Besides, the immunoassays with the mixture of *Brucella* proteins were found highly specific and higher sensitive than the skin test^{2,9}.

The higher specificity and positive predictive value of the iELISA model in respect to cELISA and no statistical difference at the level of the negative predictive values and the sensitivity between iELISA and cELISA indicate the validity of iELISA model developed in this study. However, the fact that high statistically significant correlation observed between iELISA and, RBT and CFT demonstrate that iELISA can be considered as valuable as cELISA, CFT and RBT for brucellosis screening. The results reported by other authors with indirect ELISA support our iELISA model^{6,8-10} but, indirect ELISA models often qualitative does not allow standardizing the results for large-scale brucellosis screening. For this reason, a quantitative qELISA model was developed to establish a standard criterion for the determination of IgG isotype LPS antibody concentration. It has been made possible by establishing a bovine IgG standard calibrator and by determining anti-LPS anti-body concentration of OIE weak and strong positive reference sera. Based on these results, 3 categories of anti-LPS antibody positivity were defined as weak positive (10-30 µg/ml), positive (30-70 µg/ml) and strong positive (>70 µg/ml) and qELISA model was evaluated with 3 groups of field sera (Table 3). High percentage of strong positivity was detected from the cattle of at least twice vaccinated group (V) was detected. In addition, significantly low percentage of weak positivity as well as higher percentage of positivity (30-70 µg/ml) from high prevalence region in comparison with LPR group have been detected with qELISA model (Table 3).

The optimal condition of our ELISA models concerning the serum dilutions and the blocking-diluting reagent was substantially different from those described in most studies^{6,7,9}. The nature and concentration of blocking-diluting reagent have been found as a critical parameter in the detection of bovine anti-LPS antibody, which was efficiently inhibited by skim milk at 5% and 1% dilutions and in a lesser extent fish gelatin at 5% dilution. The experiments conducted with the major milk whey proteins clearly demonstrated that casein and particularly β-lacto-globulin, but not α-lactalbumin, strongly inhibited the binding of anti-LPS antibody. The mechanism of this inhibition exerted by β-lactoglobulin and casein, alone or together, is not known whether this effect is due to

the displacement of LPS from micro-well surface or the high affinity binding of these components to the immunodominant epitopes of LPS making it inaccessible for anti-LPS antibody.

In fact, it is known that some milk constituents such as lactadherin, lactoferrin and β -lactoglobulin, act as inhibitor by hindering the attachment of microorganisms to mammalian cells or their extract¹⁶⁻¹⁸. However, the binding affinity of an 11-amino-acid amphipathic peptide derived from lactoferrin to bacterial LPS has been demonstrated by polymyxin displacement¹⁹. Therefore, the results of this study allow suggesting that β -lactoglobulin alone or together with casein may inhibit the antibody binding to LPS via quenching the immunodominant epitopes. To explain the actual mechanism of this unknown effect, the study has been recently undertaken in our laboratory.

In conclusion, the results obtained with qELISA indicate that the determination of anti-LPS antibody concentration would be a standardized indicator for bovine brucellosis screening and vaccination surveillance. Taken together, the ELISA models developed in this study can be included as valuable diagnostic tools in brucellosis monitoring-eradication and vaccination programs in endemic countries.

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