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Research article

DEVELOPMENT OF ELISA MILK-BASED TEST FOR REFERENCE SERA PREPARATION AND FOR SEROLOGICAL DETECTION OF BOVINE BRUCELLOSIS

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The non-invasive nature and practical applicability of milk-based serological tests provide significant advantages over blood tests for the serological screening of brucellosis in dairy animals. Accordingly, several novel rapid chromatographic and filtration-based serological test formats have recently been applied and increasingly used in milk and milk serum (whey) for brucellosis detection. This study aimed to develop an ELISA test (wELISA) for the detection of anti-*Brucella* antibodies in milk and to establish whey collection for use in brucellosis diagnostics. For this purpose, wELISA was optimized and validated, and its performance characteristics were compared with those of the serum ELISA, which is recognized as an alternative in the reference test list for anti-*Brucella* antibody detection. Optimization was carried out based on the detection limit and serum dilution rates, using the percentage inhibition (PI) values in spiked samples and cut-off criteria. During the optimization process, the ELISA PI values of blood sera spiked into milk were compared with serum ELISA optical density (OD) values. Following optimization, all whey samples, including those from the control group were evaluated to determine the performance characteristics of wELISA. The whey samples used in the wELISA was prepared using the World Organization for Animal Health (WOAH, formerly OIE) reference sera and control sera. In conclusion, the lower detection limit of wELISA and its compatibility with the serum ELISA indicate that this test has potential for detecting anti-*Brucella* antibodies in milk and can be used for preparing reference collections for point-of-care tests in brucellosis diagnosis.

Keywords: bovine brucellosis, ELISA, point of care tests, standard milk whey

INTRODUCTION

Brucellosis diagnosis predominantly relies on serum and blood-based tests, rather than other biological materials, as recommended by the World Organization for Animal Health (WOAH) [1]. Given that abortion notification is mandatory in many countries, and that the proportion of the seropositive subclinically infected cows ranges between

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5-10 % in most countries, serological surveillance is essential in monitoring the disease status in livestock populations [2-4]. Accordingly, screening tests with higher sensitivity and specificity selected according to disease prevalence are widely employed [5,6]. Common screening methods include the Enzyme-Linked Immunosorbent Assay (ELISA), Rose Bengal Test (RBT) and Buffered Plate Agglutination Test (BPAT), which are generally preferred over less frequently used rapid tests [7]. For confirmation, tests such as Complement Fixation Test (CFT), Serum Agglutination Test (SAT), Competitive–ELISA (c-ELISA), Fluorescence Polarization Assay (FPA) and indirect ELISA (iELISA) have been frequently used [7-11]. However, the invasive nature of blood sampling for serological testing presents a challenge for large-scale screening on dairy farms. Therefore, highly accurate milk-based tests are often preferred over blood tests in these settings. Despite this preference, difficulties associated with preparation and standardization have limited the application and evaluation of milk-based brucellosis screening tests other than ELISA and Milk Ring Test (MRT) [12-17]. For these reasons, the purpose of this study was to prepare and standardize a reference anti-*Brucella* antibody-containing reference whey to serve as a control for rapid milk-based membrane assay and to evaluate the diagnostic performance of whey-based ELISA (wELISA) as an alternative to serum-based ELISA in brucellosis serology.

MATERIAL AND METHODS

Crude lipopolysaccharide

Crude lipopolysaccharide (LPS) antigen used in iELISA and wELISA was prepared from *Brucella abortus* S19 vaccine strain by the hot phenol method described by Caroff et al [18]. The presence of LPS was detected by SDS-PAGE and the reactivity to LPS was determined by Western Blot analysis using a mouse monoclonal anti-*Brucella* LPS antibody (IgG2a, clone 4B5A, IZS, Teramo, Italy).

Field and Reference sera

A total of 136 blood sera including 90 from brucellosis free dairy cattle obtained from Ministry of Agriculture and Forestry, Samsun Veterinary Control Institute (Turkey) and the remaining 46 from 2 groups of field sera obtained from the endemic region of Turkey where *B. abortus* was isolated. Nineteen out of 46 sera had been obtained from infected cows and the remaining 27 sera were from the confirmed serum collection tested with RBT, CFT and ELISA (Table 1). These field sera were evaluated based on the criteria recommended by WOAH [1]. The serum CFT titers ranged between 10 – 320 CFTU and RBT evaluated as +2 through +4 as positive and ELISA was evaluated as positive based on the cut-off value [19].

Table 1. Categorization of control sera with known brucellosis status using serological tests.

Evaluation of blood sera						
Brucellosis status	***RBT*[1]**		***CFT*[2]**		***iELISA*[3]**	
(no. of samples)	+	−	+	−	+	−
Aborted or/culture + *(n=46)*	40	6	43	3	46	0
Healthy cattle *(n=90)*	0	90	0	90	0	90
Kappa value	0.891		0.947		1.000	

Abbreviations: [1] and [2] evaluation was performed according to the recommendations by WOAH (2022). **CFT** titers ranged between 10 to 320 CFTU, **RBT** ranging from +2 through +4 evaluated as positive and **ELISA** was performed according to Genç et al. (2010)

A WOAH reference serum containing 1000 Complement Fixation Test Units (CFTU) of anti-*Brucella* antibody (obtained from Veterinary Control Institute of Samsun, Turkey) was used for optimization and performance evaluation of the ELISA tests. *Brucella* strong positive, weak positive and negative reference sera obtained from VLA (Weybridge, UK) were also tested for the reactivity to LPS by mouse monoclonal anti-*Brucella* LPS antibody (IgG2a, clone 4B5A, IZS, Teramo, Italy).

Milk samples

Ten milk samples obtained from a certified *Brucella*-negative herd, tested for the presence of anti-*Brucella* antibody by Milk Ring Test (MRT Antigen, Pendik Veterinary Control Institute, Turkey), and ELISA were used [19].

Spiked samples

A total of 20 control blood sera containing 10 out of 27 positive sera from endemic region of Kars and 10 out of 90 negative sera from non-endemic region of Samsun were tested to detect optimal test dilution based on the percent inhibition and cut-off evaluation.

Whey preparation

The whey from 10 milk samples spiked with control and reference sera were prepared according to Genç *et al.*, [20] with some modifications. Briefly, an equal quantity of reference blood sera was transferred into the anti-*Brucella* antibody negative milk and each sample was incubated with rennin (Turkish rennet, Yayla) at 37 °C for 30 minutes. After centrifugation at 10.000xg for 10 minutes, the wheys were applied in wELISA testing.

Whey Enzyme-Linked Immunosorbent Assay (wELISA)

In the study, milk spiked with 10 positive and 10 negative control sera were used for optimization of the wELISA. Additionally, a total of 136 blood sera and WOAH reference sera spiked into 10 milk samples were used for evaluation of diagnostic parameters related to blood sera and whey samples. The wELISA was carried out following the methods described by [3,20] with some modifications. Briefly, microplates were coated with B. *abortus* crude LPS antigen in carbonate buffer (0.1 M, pH 9.6) and incubated overnight at +4 °C. The micro wells were then saturated with blocking solution. After incubation and the washing step, spiked-milk wheys and also the reference and control blood sera were incubated to evaluate the inhibition effect and determine the appropriate dilutions. Then, the reaction was developed by addition of the conjugate (alkaline phosphatase conjugated sheep anti-bovine IgG, Novus Biologicals, NB776) and pNPP (p-Nitrophenyl Phosphate, Sigma, N2765, USA) substrate respectively, and after stopping the reaction, the absorbance was read at 405 nm in ELISA reader (Multiskan EX, Fisher Scientific, Shanghai, China). The assays were carried out in duplicate and the cut-off points were determined by Area Under Curve (AUC) values. It was used for comparison of diagnostic parameters of both tests by Receiver Operating Curve (ROC) analysis and Youden index score [21].

Determination of the detection limit

The detection limit of ELISAs was determined by diluting the WOAH reference serum twofold from 8 to 0.125 CFTU.

Statistical analysis

Detection of the diagnostic parameters and determination of optimal working serum dilution by percent inhibition rate and cut-off evaluation by ROC analysis were performed by MedCalc version 22.014. And categorization of control sera relative to RBT, CFT and ELISA was performed by kappa statistics with SPSS version 17.0.

RESULTS

Spiked-whey samples were prepared by adding blood sera, as detailed in Table 1, and indirect ELISA previously described by Genç *et al*., [20] was optimized for testing whey samples. Cut-off values for both serum and whey samples were established using 10 negative and 10 positive serum samples. Optimal serum dilution was assessed through serial dilutions of milk and serum ranging from 1:2 to 1:256. The percent inhibition (PI) of spiked samples was evaluated based on the determined cut-off value. In the whey ELISA, the lowest statistical variation was observed at 1:2 dilution. Therefore, this dilution was selected as the minimum dilution for antibody concentration testing and was used for all serum testing throughout the study (Table 2). The cut-off values for serum and whey samples are shown in Table 2.

Table 2. Detection of optimal dilution of spiked samples based on percent inhibition and cut-off value

ELISA with			
Serum dilution (n=number)	**Serum (ODtest/ODneg)**	**Spiked milk (ODtest/ODneg)**	**Percent Inhibition (%) of spiked milk**
1:2 (n=10)	5.15-5.87 (5.51)	4.67-5.12 (4.90)	11.07
1:4 (n=10)	5.01-5.23 (5.12)	4.15-4.34 (4.25)	16.99
1:8 (n=10)	4.70- 5.41 (5.06)	3.90-4.14 (4.02)	20.55
1:16 (n=10)	4.63-5.06 (4.85)	3.78-4.06 (3.92)	19.17
1:32 (n=10)	4.46- 5.14 (4.80)	3.55-3.85 (3.70)	22.92
1:64 (n=10)	3.67-4.41 (4.04)	2.92-3.40 (3.16)	21.78
1:128 (n=10)	2.58-2.93 (2.76)	2.16-2.45 (2.31)	16.30
1:256 (n=10)	1.67-1.89 (1.78)	1.37-1.54 (1.46)	17.98
	$\overline{X}$ ± SD	$\overline{X}$ ± SD	
Negatives (n=10) 0.423 ± 0.06	0.423 ± 0.06	0.462 ± 0.06	
Cut-off (n=20)	0.603	0.642	

Abbreviations: $\overline{X}$: arithmetic mean, **SD**: standard deviation, **cut-off**: ($\overline{X}$)+2SD

From the standpoint of analytical evaluation, the detection limit (analytical sensitivity) of the reference serum in whey was established at 0.5 CFTU and 1 CFTU (Table 3).

Diagnostic performances were determined according to the ELISA results. The sensitivity and specificity of serum and wELISA were 100 %, 100 % and 92.9 %, 100 % respectively with a confidence limit of 95 %. Among the diagnostic parameters, there was no significance ($p<0.001$) (Table 4).

Table 3. Detection limit of serum and spiked samples based on the criteria below and cut-off value.

Reference serum unit (CFTU)	Blood Sera	Spiked Milk Sera
8	p	p
4	p	p
2	p	wp
1	wp	wp
0.500	wp	negative
0.250	negative	negative
0.125	negative	negative
	(X) ± SD	(X) ± SD
Negative serum	0.439 ± 0.02	0.464 ± 0.024

Abbreviations: CFTU: Complement Fixation Test Unit, results were categorized based on test/negative OD ratio; **p**: positive (>2.0), **wp**: weak positive (1.5-1.9), **n**: negative (under the ratio of weak positive <1.5), **cut-off value**: over the mean OD ratio of negative serum

Table 4. Evaluation of diagnostic parameters of serum-ELISA and whey-ELISA using control blood sera and whey

Diagnostic parameters	Serum	Whey
Sensitivity	100 (97.2–100)	92.9 (80.5–98.5)
Specificity	100 (97.2–100)	100 (96–100)
AUC	1000 (0.93–0.99)	0.971 (0.93–0.99)
Youden index (J)	1.00	0.93
Significance level P	<0.001	<0.001
Cut-off (n=90)	>0.360	>0.340

Abbreviations: AUC: Area Under Curve, **Youden index (J):** $J = \max_c \{Se(c) + Sp(c) - 1\}$, **c:** optimal cut-point

In the study, the consistency of control sera was tested by using RBT, CFT and ELISA tests. ELISA was found to be perfect in agreement (kappa: 1.0) compared to RBT and CFT (Table 1). The evaluation of these serum and whey samples was carried out by using this ELISA protocol based on cut-off and AUC values, respectively (Fig 1A and Fig 1B).

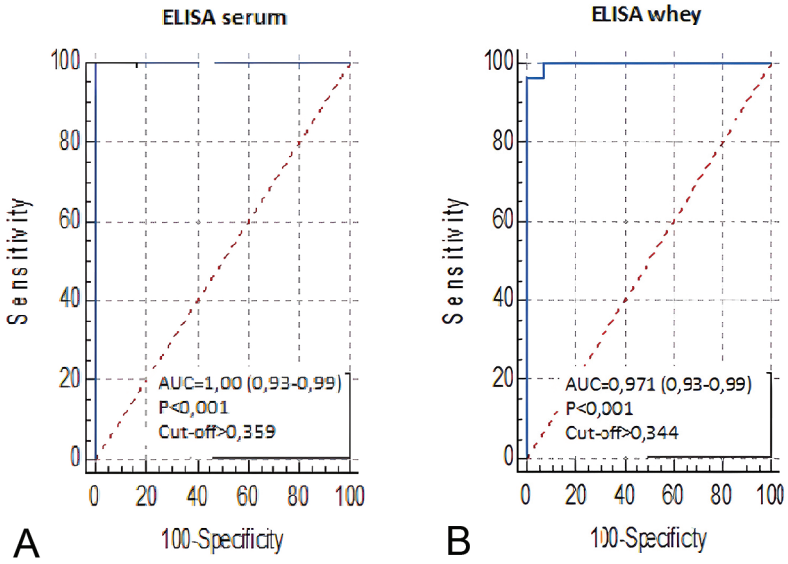

Figure 1A. Cut-off and AUC values of ELISA with control blood sera.
Figure 1B. Cut-off and AUC values of ELISA with whey.

DISCUSSION

In recent years, a significant number of investigations have focused on the applicability of alternative methods for detecting brucellosis in various biological materials [22-24]. Milk has become a preferred sample for anti-*Brucella* antibody detection due to its ease of non-invasive collection and sufficiently detectable antibody levels. Consequently, ELISA and Milk Ring Test (MRT) have been adapted for screening purposes using milk samples [8,20,22]. Due to its higher diagnostic performance, ELISA is considered the best alternative until the validation of other methods is established [22,25]. Studies have shown that iELISA has the highest sensitivity and specificity [26], whereas lower values have been obtained with the common blood-based tests. A similar study by Genç *et al.* [20] reported high diagnostic sensitivity (DSe) (98.3 %) and specificity (DSp) (100 %) for blood serum-ELISA [27-29]. For accurate diagnosis, rapid and reliable field diagnostics are essential [6,27]. This and other related studies on milk and blood sera indicate that iELISA is the test of choice for detection of anti-*Brucella* antibodies [12,16,17,20, 24,25].

However, disadvantages such as the need for optimization with different chelating agents for milk-based ELISA tests, [25], and the low sensitivity, specificity and laboratory-restricted applicability of MRT led to the search for alternative applications. Besides sensitivity and specificity, diagnostic speed is also crucial, since brucellosis is a contagious and zoonotic disease. In recent years, the development of rapid field tests with high diagnostic performance for brucellosis diagnosis has gained momentum [30,31]. These tests, initially capable of detecting antibodies in blood serum, have been later on adapted to test various materials such as milk by using filtration techniques [30]. Nevertheless, it was hypothesized that developing a methodology that would evaluate the presence of anti-*Brucella* antibodies in milk could be an alternative to filtration applications for rapid tests and would offer a significant advantage for monitoring *Brucella* antibodies in milk. Therefore, iELISA was selected to test whey, due to its higher sensitivity, specificity and the accuracy of its results and also its potential for semi-quantitative evaluation. Subsequently, the reference control whey was prepared by diluting the milk spiked with blood in ratios ranging from 1:2 to 1:256, aiming to determine the optimal serum dilution based on the inhibitory effect of milk on spiked samples and cut-off correlation. The lowest coefficient of variation (10.28 %) was detected at 1:2 dilution in wELISA, and this dilution was selected as the lowest dilution for testing antibody concentration (Table 2). Additionally, the low detection limit of iELISA using both blood and whey spiked with milk made this test a good choice for detecting 0.5 IU and 1.0 IU of anti-*Brucella* antibody, respectively (Table 3). In this study, the ELISA protocol was modified using whey instead of milk, and its diagnostic performance is shown in Table 4, Figure 1A and 1B. These results, based on cut-off values, J score, and sensitivity and specificity values, indicate that whey instead of milk can be applied for rapid test formats [27,29].

Blood serum and milk serum (whey) samples have been used in immunochromatographic lateral flow tests [30-32] and immunofiltration based rapid tests for brucellosis detection [20,33]. While the WOAH recommends strong positive (sp), weak positive (wp) and negative sera as references for blood serum based tests, similar reference materials may also be required for milk-based testing, especially for membrane-based tests [30-32]. The ability to detect anti-*Brucella* antibodies at 0.5 CFTU in serum ELISA and at 1 CFTU in whey, and the capability to prepare these sera directly or at desired concentrations, provides a significant advantage for rapid milk tests.

In conclusion, testing can effectively be performed using milk serum for antibody monitoring. Since whole milk may cause membrane blockage in rapid tests such as lateral flow and immunofiltration, preferring whey instead of milk can resolve this issue. Furthermore, as some antibodies may bind to milk proteins and reduce the antibody concentration in the sample, whey prepared according to the methods described in the study can be utilized in test controls and optimization studies.

CONCLUSIONS

Production of standardized reference anti-*Brucella* sera and whey derived from these sera is feasible. In this study, milk wheys were prepared from the reference and well-characterized reference and control serum samples and their performance was compared in accordance with the original sera. Statistically, no significant difference was observed between the results of serum ELISA and wELISA when using whey derived from milk samples spiked with blood sera. This suggests that these experimentally prepared whey samples are suitable for brucellosis screening. Although field validation is pending, the high diagnostic performance of wELISA which was compatible with that of serum ELISA, establishes wELISA as an alternative for testing milk samples. Therefore, its use could be recommended for brucellosis monitoring. Furthermore, the approach of using whey samples prepared by spiking with reference blood sera offers a valuable method for preparing the standardized controls essential for the development and standardization of rapid test formats.

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Authors' contributions

EG, carried out the ELISA studies and participated in all studies and drafted the manuscript. ÖBY carried out the immunoassays and participated in the design of the study and helped to draft the manuscript. OG conceived of the study, and participated in its design and coordination and helped to draft the manuscript and performed the statistical analysis. All authors read and approved the final manuscript.

Ethical approval

This study was approved by the Animal Ethics Committee of 19 Mayıs University for Animal Experiments (E-68489742-604.02.03-255675) and it was conformed to ethical standards and the journal's policies.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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RAZVOJ ELISA TESTA NA BAZI MLEKA ZA PRIPREMU REFERENTNIH SERUMA I ZA SEROLOŠKO DETEKCIJU BRUCELOZE KOD GOVEDA

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Neinvazivna priroda i praktična primenljivost seroloških testova na bazi mleka pružaju značajne prednosti u odnosu na testove krvi za serološki skrining bruceloze kod mlečnih životinja. Shodno tome, nekoliko novih brzih hromatografskih i filtracionih seroloških testova je nedavno primenjeno i sve više se koristi u mleku i mlečnom serumu (surutci) za otkrivanje bruceloze. Cilj ove studije bio je razvoj ELISA testa (wELISA) za otkrivanje antitela protiv bruceloze u mleku i način sakupljanja surutke za upotrebu u dijagnostici bruceloze. U tu svrhu, wELISA je optimizovana i validirana, a njene karakteristike učinka su upoređene sa karakteristikama serumskog ELISA testa, koji je prepoznat kao alternativa na referentnoj listi testova za otkrivanje antitela protiv bruceloze. Optimizacija je sprovedena na osnovu granice detekcije i brzine razblaživanja seruma, koristeći procentualne vrednosti inhibicije (PI) u uzorcima sa primesama i kriterijume graničnih vrednosti. Tokom procesa optimizacije, vrednosti ELISA PI seruma u krvi dodatih u mleko upoređene su sa vrednostima optičke gustine (OD) seruma u ELISA testu. Nakon optimizacije, svi uzorci surutke, uključujući i one iz kontrolne grupe, procenjeni su kako bi se odredile karakteristike učinka wELISA testa. Uzorci surutke korišćeni u wELISA testu pripremljeni su korišćenjem referentnih seruma i kontrolnih seruma Svetske organizacije za zdravlje životinja (WOAH, ranije OIE). Zaključno, donja granica detekcije wELISA testa i njegova kompatibilnost sa serumskim ELISA testom ukazuju na to da ovaj test ima potencijal za detekciju antitela protiv brucele u mleku i da se može koristiti za pripremu referentnih kolekcija za testove na terenu u cilju dijagnoze bruceloze.