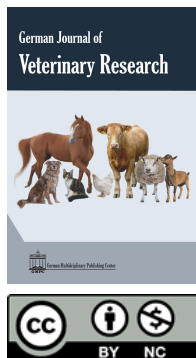Research Article

Development of antigen-capture ELISA using monoclonal antibodies for the detection of brucellae in milk

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Abstract

In this study, a *Brucella* antigen-capture ELISA (Ag-cELISA) prototype was developed. To study the validity of the developed Ag-cELISA, milk samples collected from *Brucella*-positive goats (n=120) and cattle (n= 64), as well as from unknown *Brucella*-status cattle (n=105) and sheep (n=65) herds were tested by Ag-cELISA, I-ELISA, and culture method. All *Brucella*-positive samples were confirmed using PCR. It was found that the developed Ag-cELISA could detect 50-100 bacteria per well (equivalent to 10^3 to 2×10^3 cells per mL) as the lowest limit of detection (LOD) and was therefore considered moderately sensitive to detect brucellae in milk. In an infected goat herd, out of 120 milk samples, 41, 32, and 17 were positive by Ag-cELISA, I-ELISA, and culture, respectively. Ag-cELISA detected 15 positive cases out of 17 culture-positive milk samples. Two culture-positive milk samples were not detected in Ag-cELISA. The relative sensitivity and specificity between Ag-cELISA and I-ELISA were 78% and 100%, respectively. In an infected cow herd, out of 64 milk samples, 32, 23, and 11 were found positive by Ag-cELISA, I-ELISA, and culture, respectively. Ten out of 11 culturally positive milk samples were found positive by Ag-cELISA. The relative sensitivity and specificity between the Ag-cELISA and I-ELISA were 71.9% and 100%, respectively. From randomly collected 105 cow and 110 sheep milk samples from herds of unknown *Brucella*-infection status, three (2.85%) and five (4.5%) samples were found positive using Ag-cELISA, respectively. These results showed that Ag-cELISA could detect brucellae in milk more practically and safely than bacterial culture. On the other hand, this information reaffirms that milk can be an important source of brucellosis and creates a public health risk in humans; therefore, increased public awareness is of utmost importance.

Keywords: Antigen-capture ELISA, Brucellosis, Milk, Diagnosis

Introduction

Brucellosis is one of the most common zoonotic diseases with important veterinary and public health concerns and economic impacts. Brucellosis is endemic in many countries worldwide (Gwida et al., 2010; Dean et al., 2012). The control and eradication of brucellosis is primarily based on bacteriological, serological, and molecular diagnosis of the disease. However, the isolation of *Brucella* spp. from cattle with positive serology is the only definitive diagnosis of the disease. Sera from different stages of infection may not give positive results, and there is currently no single serological test able to detect all stages of infection.

The most common way of brucellosis transmission to humans is through consuming raw milk and unpasteurized dairy products (Tekin et al., 2012). In dairy animals, *Brucella* spp. replicate in the mammary gland and supra-mammary lymph nodes, and these animals continually excrete the pathogen into milk throughout their lives. Rapid and accurate bacteriological, serological, and molecular diagnosis of the disease is crucial for epidemiologic surveillance, investigation of outbreaks, and follow-up of a control program (Corbel, 1997; Pappas and Memish, 2007).

The gold standard for diagnosis of brucellosis is the culturing of bacteria from clinical specimens. However, it could be a lengthy process and pose a health risk for the laboratory workers who conduct the test. and the success of culturing is very low even in experienced laboratories (Güllüce et al., 2003; Ilhan et al., 2008). Besides, there are relatively low numbers of *Brucella* in milk and milk products as bacterial shedding is intermittent (WOAH-OIE, 2019). To isolate bacteria from milk specimens, it might take 6 weeks before claiming

the specimen is negative for brucellosis (WOAH-OIE, 2019). Therefore, we aimed to develop an antigen-capture ELISA (Ag-cELISA) to detect *Brucella* (alive or dead) in milk samples. This would be safer to handle specimens and save enormous time compared to the classical bacteriological culture method. The method is also much simpler and less expensive to perform than PCR, which also requires less laboratory clean room infrastructure and fewer contamination control issues.

Materials and methods

Milk samples

All procedures for working with infected and suspicious milk samples were carried out in safety conditions by working under ClasII plus cabinet and wearing protective clothing, including goggles. Aerosolization was avoided by working extremely cautiously. In ELISAs studies, milk cream was removed, including spiked samples, because the cream layer tends to bind antibodies unspecifically. Milk samples were collected from infected, unknown, and healthy animals. Herds, where *B. abortus* was previously isolated and identified were considered infected herds (Alton et al., 1988). Free herds were chosen based on the information received from official historical records of these farms. Herds with no information about the presence of brucellosis were referred to as unknown herds. From an infected goat flock and an infected cattle herd, 120 and 64 milk samples were collected, respectively. From different villages, 110 sheep and 105 cattle milk samples were collected randomly from herds of unknown *Brucella*-infection statuses.

Test strain

B. abortus S19 vaccine strain was used to spike milk samples. This strain has been stored in the culture stock in the Microbiology Laboratory of the Faculty of Veterinary Medicine, Harran University.

Preliminary tests for Ag capture ELISA

To detect the limit of detection (LOD), negative cattle milk samples were spiked with live *B. abortus* S19 vaccine strain rather than reference or field strains for safety reasons. In the test design, each well of the top left and right quadrants of the plates contained 10^5 colony-forming units (CFU) and 10^3 CFU per mL of milk, respectively. Milk spiked with 10^4 CFU per mL and milk containing no *Brucella* were added to the down left and right quadrant's wells, respectively. All wells contained 100 μL of respective milk samples.

Monoclonal antibodies (Mabs)

Monoclonal antibodies (Mabs) BrF11 (anti-A>M OPS) and BM40 (anti-M OPS) were kindly supplied by Dr. John McGiven (APHA, Weybridge, England).

Analysis of milk samples using Ag-cELISA

The ELISA was developed and optimized by testing various reagent types and concentrations against each other and selecting the combination that most effectively differentiated between spiked milk samples with and without *Brucella* cells. Monoclonal antibodies (MAbs) BrF11 (anti-A>M OPS) and BM40 (anti-M OPS) were used, mixed together in equal amounts, for both capture and detection of *Brucella* cells. The MAbs were labeled with biotin to facilitate subsequent binding to streptavidin-HRP conjugates. Biotinylation to amine groups in the MAbs was done using EZ-Link Sulfo-NHS-Biotin (ThermoFisher Scientific, #A39256, USA). Free biotin was used separated from biotinylated MAbs using desalting columns (ZebaTM Spin Desalting Columns, 7 K MWCO, 2 mL, #9889, ThermoFisher Scientific, USA).

The capture MAbs concentrations tested were 10, 5, 2.5, and 1.25 μL. The detector MAbs concentrations tested were 10, 5, 2.5, 1.25, 0.625, and 0 μL. Two types of Streptavidin-HRP conjugates were evaluated, each at 0.1 and 0.5 μL, standard streptavidin-HPP (ThermoFisher Scientific, #21140, USA) and streptavidin poly-HRP (Streptavidin poly-HRP, Thermo Fischer Scientific, #21140, USA). Each combination was applied to the same milk samples spiked with 0, 10^3, 10^4, and 10^5 CFU per mL of *B. abortus* S19. The final selection was made based on more differences seen between samples with and without brucellae.

ELISA plates were coated with capture MAbs in 0.05 M carbonate buffer, pH 9.6 at 4°C for 18 hours. Plates were blocked with 5% non-fat dry milk in phosphate buffer saline (BLOTTO) for two hours. Plates were washed three times with PBS containing 0.05% tween 20 (PBS/T). After a washing step, 100 μL reference and test milk samples diluted 1:2 in PBS/T were added into each well of the plates. Plates were incubated at room temperature for 1 hour. Then washed, and 100 μL of biotinylated detector MAbs were added to the wells. Plates were incubated at room temperature for 1 hour, then washed, and 100 μL of Streptavidin-HRP conjugate diluted in BLOTTO was added per well.

Two different streptavidin (Streptavidin poly-HRP, Pierce, Thermo Fischer cat number 21140, USA) and Streptavidin Protein HRP, Pierce, Thermo Fischer cat. Num 21126, USA) was used in the test design for comparison. Plates were incubated at 37°C at dark for 1 hour. Finally, after washing steps, 100 μL of the chromogenic substrate (10 mg o-Phenylenediamine tablet, (P-8287, Sigma, St. Louis, MO, USA) in 25 mL of 0.05 M phosphate-citrate buffer pH 5.5, with 10 μL 30% H2O2) were added to all the wells. The reaction was stopped with 2 N H2SO4. The plates were shaken on an orbital shaker for 15 min before reading at OD_{450} nm in a microplate reader (VERSAmax 3.13/B2573). Any OD value bigger than the mean of the negative milk samples plus 3 standard deviations was accepted as the cut-off value. Therefore, LOD was roughly estimated based on positive OD values.

Analysis of milk samples using I-ELISA for antibody detection

To detect anti-*Brucella* antibodies in milk, smooth lipopolysaccharide (sLPS) antigen extracted from *B. abortus* S99 using the hot phenol technique described (WOAH-OIE, 2019). Ninety-six-well polystyrene microplates (Nunc 69620, USA) were coated with 100 μL sLPS antigen at a dilution of 1:6000 in 0.05 M carbonate buffer, pH 9.6 for 18–24 hrs at 4°C. All the wells were rinsed three times with a washing solution (0.1 mM disodium hydrogen orthophosphate, 0.01% Tween 20, [PBS/T]). The control and test milk samples were added to each of the wells directly without diluting as 100 μL, and plates were incubated at room temperature for 1 hour on a rotary shaker. After five washes in PBS/T, a predetermined dilution of 100 μL protein A/G horseradish peroxidase conjugate (ThermoFisher Scientific, #32490, USA) in PBS/T was applied to all wells.

The plates were then incubated for 1 hour at room temperature (RT) on a rotary shaker. Finally, after five washes in PBS/T, 100 μL of the chromogenic substrate (10 mg o-Phenylenediamine tablet, (P-8287, Sigma, St. Louis, MO, USA) in 25 mL of 0.05 M phosphate-citrate buffer pH 5.5, with 10 μL 30% H_2O_2) per well were added. The plates were shaken continuously on an orbital shaker for 15 min prior to reading at OD_{450} nm in a microplate reader (VERSAmax 3.13/B2573). The cut-off level was determined by adding three standard deviations to the mean of the OD of negative controls. Any samples with ODs greater or equal to this value were considered positive. Sensitivity and specificity were calculated using real negative and positive test sera.

Culture studies in milk samples

Milk samples found positive by sandwich ELISA and milk antibody ELISA were cultured for the presence of brucellae. Randomly taken milk samples were cultured if they were positive by Ag-cELISA. Milk samples were centrifuged, and a deposit and cream layer of milk samples were directly streaked on Farrel's and Modified Thayer-Martin media. Also, they were inoculated into tryptic soy broth tubes containing 20 μg vancomycin and 1 μg amphotericin B, and all inoculated tubes were incubated at 37°C in air + 5-10 % v/v CO_2 for up to six weeks with weekly subculturing on solid selective media while streaked plates were incubated at same conditions up to one week. Samples were considered negative when no *Brucella* spp. were isolated after six weeks of incubation.

The culture was identified in three steps. The first step included checking for colony morphology by stereomicroscope and agglutination with neutral acriflavine (0.1%, w/v) (Sigma-A8126, Taufkirchen, Germany). In second step included species and biotype determination; the following tests were performed; Serum and CO_2 requirement for growth, the production of H_2S (Lead acetate paper, Fluka 37104), oxidase and urease production, and lysis with Tbilisi phage (Tbø) at routine test dilution (RTD) and $10^4\times$ RTD and R/C

phage at RTD, growth in media containing thionine (Sigma-T3387, Taufkirchen, Germany) (20 μmL) and basic fuchsine (Merck-115937, Taufkirchen, Germany) (20 μmL), agglutination with A and M monospecific antisera, and R antiserum (Alton et al., 1988).

Molecular confirmation of *Brucella* spp.

All isolated *Brucella* species were tested by multiplex PCR (Bruce-ladder) according to the method described by Mayer-Scholl et al. (2010). For bacterial DNA extraction, a loopful of bacterial culture was suspended in 200 μL PCR-grade water. The resulting mixture was boiled at 99°C for 10 min and centrifuged at 12000 $\times$g for 20 s. The resulting supernatants were used as the DNA template for Bruce-ladder. The assay was carried out in a 25 μL reaction mixture containing 2$\times$ QiagenMultiplex Master Mix (Qiagen, Hilden, Germany), 0.2 μM of each primer in a cocktail of nine primer sets, and 1μL template DNA. Amplifications were initiated by denaturing the sample for 15 min at 95°C followed by template denaturation at 94°C for 30 s, primer annealing at 58°C for 90 s, and primer extension at 72°C for 180 s for a total of 30 cycles. After the last cycle, amplicons were incubated for an additional 10 min at 72°C. Amplification products were separated on 1.5% agarose gels.

Results

Ag-cELISA

As a newly developed test to use antigen capture sandwich ELISA for testing milk samples, preliminary tests were applied, and each parameter in the test was evaluated accordingly. According to the test design, 5 μmL mix MAbs for capture and 2.5 μmL for mix MAbs detector antibody and standard streptavidin HRP (0.1 or 0.5 μmL) created a more reproducible difference between samples with and without brucellae. Also, the background was light when we used Streptavidin-HRP compared to Streptavidin-Poly HRP. This demonstrated that the Ag-cELISA had a lower detection limit at 10^3 CFUs per mL.

Serological analysis of samples from infected herds

In the current study, 120 milk samples from an infected goats herd revealed positive reactions in 41, 32, and 17 samples using Ag-cELISA, I-ELISA, and culture methods, antigenically, serologically, and bacteriologically, respectively. The relative sensitivity and specificity between the Ag-cELISA and I-ELISA were 78% and 100%, respectively (Table 1). Of 17 culture-positive milk samples, 15 were positive by Ag-cELISA, and two samples were not detected in Ag-cELISA. All culture-positive samples from goats were confirmed as *B. melitensis* biotype 1 (n=17) by PCR. All isolated *Brucella* spp. gave typical *B. melitensis* band profiles in Bruce ladder PCR consistent with culture results.

In 64 milk samples collected from an infected cattle herd, 32, 23, and 11 were found positive using Ag-cELISA, I-ELISA, and culture methods, respectively. From 11 culturally positive milk samples, 10 samples

Table 1: Relative sensitivity and specificity between Ag-cELISA and I-ELISA in the analysis of goat milk collected from infected herds.

Ag-cELISA	I-ELISA		Total
	Positive	Negative	
Positive	23[a]	0[b]	23[a+b]
Negative	9[c]	79[d]	88[c+d]
Total	41[a+c]	79[b+d]	120[a+b+c+d]

Relative sensitivity= a/a + c= 32/32+9= 78%.
Relative specificity= d/b + d= 79/0+79= 100%.
Ag-cELISA= antigen capture ELISA.
I-ELISA= indirect ELISA.

Table 2: Relative sensitivity and specificity between Ag-cELISA and I-ELISA in the analysis of cow milk collected from infected herds.

Ag-cELISA	I-ELISA		Total
	Positive	Negative	
Positive	23[a]	0[b]	23[a+b]
Negative	9[c]	32[d]	41[c+d]
Total	32[a+c]	32[b+d]	64[a+b+c+d]

Relative sensitivity= a/a + c= 32/32+9= 78%.
Relative specificity= d/b + d= 79/0+79= 100%.
Ag-cELISA= antigen capture ELISA.
I-ELISA= indirect ELISA.

were found positive by Ag-cELISA. The relative sensitivity and specificity between the Ag-cELISA and I-ELISA were 71.9% and 100%, respectively (Table 2). All culture-positive samples from goats were confirmed as *B. abortus* biotype 3 (n=11) by PCR. All isolated *Brucella* spp. gave typical *B. abortus* band profiles in Bruce ladder PCR consistent with culture results.

Analysis of random samples using Ag-cELISA
From randomly collected 105 cow and 110 sheep milk samples from herds of unknown infection status, three (2.85%) and five (4.5%) were found positive by Ag-cELISA, respectively (Table 3).

Discussion
Isolation of *Brucella* from milk is a time-consuming process and required at least six weeks of incubation period before considering the specimen as negative (WOAH-OIE, 2019). Molecular tracing of *Brucella* isolates in milk may improve milk-borne pathogens surveillance in Turkey. Several studies reported the presence of *Brucella* spp. in milk samples using classical culture tools, molecular techniques, and serology (Terzi Gülel, 2006; Abdelkareem et al., 2011; Arasoğlu et al., 2013; Altun et al., 2017; Babaoglu et al., 2018; Seçil et al., 2018; Taşkin Kafa and Sümer, 2020), however, only one article diagnose brucellosis antigenically was found in Turkey (Güllüce et al., 2003). Results show wide variability from 1% to 81.7% based on region and source of samples. Therefore, this picture tells us that milk and milk products might represent public threats to consumers. So we thought there is a need to develop a test to detect *Brucella* spp. live or dead in milk samples quickly and safely.

In our study, we developed capture ELISA which can detect 50-100 bacteria per well (equivalent to 10^3 to 2×10^3 cells per mL) as the lowest limit of detection (LOD). In a similar study, Zhang et al. (2016) found the detection limit of *E. coli* (EHEC 0157:H7) was 1×10^3 CFU/mL. In another study, the LOD was found equal to 0.9×10^3 cells/mL for *Bacillus cereus* in food (Zhu et al., 2016). These findings were consistent with our results, which might tell us that Ag-Capture ELISA is a quite sensitive test to detect *Brucella* spp. in milk samples. The reason that we used two different MAbs BrF11 (anti-A>M OPS) and BM40 (anti-M

OPS) to develop a more sensitive test since heterogenous *Brucella* infections in both cattle, as well as sheep and goats, might happen. Our results showed that the highest positivity was found by milk (antibody) ELISA followed by antigen capture ELISA and culture in both goats and cattle from infected herds. The reason that antibody ELISA detected more positive samples than others could be explained by false positive results because of cross-reactions with the LPS layer of some bacteria e.g., *Yersinia enterocolitica* O:9, *F. tularensis*, *E. hermanii*, etc. *Yersinia enterocolitica* O:9 is known to be widespread in dairy herds worldwide (Muñoz et al., 2005; Wareth et al., 2014).

Ag-cELISA did not detect ten serologically positive milk samples in infected goat herds and 9 in cattle herds. This was considered normal because some infected animals secrete bacteria intermittently or might be chronically infected (WOAH-OIE, 2019). On the other hand, Ag-cELISA detected four serologically negative goats and three serologically negative cattle, which is also considered normal because animals could be in the initial stage of infection before antibodies are induced. Ag-cELISA detected 25 out of 28 culture-positive animals in both animal groups. This was surprising since culture yielded positive results and Ag-cELISA should have also been positive. This finding highlighted the ability of the Ag-cELISA to detect a low number of bacteria in mılk samples. It was considered that Ag-cELISA could detect bacteria in culture-positive cows relatively safely. Besides, the Ag-cELISA does appear to have the potential to help quantify the amount of *Brucella* in a sample, whereas culture routinely does not. Since Ag-cELISA can detect dead cells that may have died within milk samples before they were cultured, this test could be evaluated to be more sensitive than culture as our results support this assumption.

In the current study, 2.85% of randomly collected cow milk from the vicinities Urfa City and 4.5% of the randomly collected sheep milk were found to be positive for Ag-cELISA, although no *Brucella* was isolated from positive Ag-cELISA milk samples. The latter made us think that bacteria might be dead due to their intermittent secretion in the infected animals. Although the number of samples is not large enough to comment precisely, we still had a rough estimate of

Table 3: Number and percentages of randomly collected milk samples tested positive using Ag-cELISA.

Source of milk	Number of milk	Ag-cELISA positive (%)
Cow milk	105	3 (2.85 %)
Sheep milk	110	5 (4.5%)
Total samples	215	8 (3.7%)

seropositivity rates of milk containing brucellae. The generated epidemiological data will be useful in the future.

To detect the specificity of the test, milk samples that had been collected from 100 animals from farms assumed to be free from brucellosis were tested by Ag-cELISA, and no positive results were determined. This showed that the test was quite specific. However, more investigations with a large number of samples are required. Our test design aimed to compare two different streptavidin, namely Streptavidin poly-HRP and Streptavidin- HRP. We recommend using Streptavidin HRP, although poly HRP offers more sensitivity. However, poly–HRP increased the level of signal-to-noise ratio compared to streptavidin-HRP. Poly HRP is recommended by the manufacturer when only a minimal amount of specific antigens and antibodies were available. Possibly this was not the case when we conducted our test.

Transmission of *Brucella* from milk and milk products can be occurred due to the consumption of raw milk and the poor preparation process of milk products. This situation is an important concern for people consuming these products and living in rural areas of endemic countries (Chen et al., 2014). Our results show that brucellae in milk may constitute a public hazard, and this information should be shared with regional dairy associations. It was thought that the Ministry of Agriculture and Forestry launching a risk awareness program for both farmers and end users should be necessary.

In conclusion, antigen capture ELISA based on the biotin-streptavidin system was found to be sensitive enough to detect 50 bacteria per well. As an outcome, the newly developed test managed to detect 50 bacteria per well and is considered a safe, practical, quick test that could be advised to be used in milk screening together with serology.

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