



## Diagnostic and Vaccine Chapter

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### ARTICLE INFO

### ABSTRACT

The first report in this chapter describes the development of a killed composite vaccine. This killed vaccine is non-infectious to humans, other animals, and the environment. The vaccine has low reactivity, is non-abortive, and does not induce pathomorphological alterations to the organs of vaccinated animals. The second report of this chapter describes the diagnostic value of a competitive enzyme-linked immunosorbent assay for detecting *Brucella*-specific antibodies and its ability to discriminate vaccinated cattle from infected cattle. The results indicated that the competitive enzyme-linked immunosorbent assay is more sensitive than traditional tests for detecting antibodies to *Brucella abortus* in naturally and experimentally infected cattle.

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### 1. Introduction

To control the spread of a zoonotic disease, it is important to be able to assess the contamination of animal products and the environment, and the health of animals and humans. Brucellosis has been eradicated in the United States (U.S.) through the use of vaccines to immunize animals, and cattle herds in the U.S. are now brucellosis-free in all 50 states. The incidence of human brucellosis, on the other hand, is approximately 100–200 cases per year due to contact with immigrants from endemic areas, contact with wild animals, etc. The eradication of brucellosis takes time; in the US, the brucellosis eradication program, which includes the use of vaccines and the implementation of good agricultural health policies, was initiated over 70 years ago. Much of the world, however, continues to have endemic brucellosis in both animal and human populations. There are approximately 500,000 new cases of human brucellosis each year worldwide [1].

Eradication of brucellosis requires efficacious vaccines and appropriate food safety practices. Through well-directed and well-managed animal health programs, use of the existing vaccines would substantially reduce the incidence of brucellosis worldwide. Effective vaccines that are multivalent and produce a good immunological response with limited seroprevalence in the host are in high demand. During this symposium, several papers were presented on the development of vaccines for the control of brucellosis or on programs within individual countries based on existing vaccines to control the disease. The first report presented in this

chapter is a representative study of vaccine development from Kazakhstan, performed by Kokanov from the Branch of the National Biotechnology Center of the Republic of Kazakhstan in Stepnogorsk, together with Ten, Abutalip, Mustafin, and Daugalieva from the Joint-Stock Company KazAgroInnovatsiya, Almaty, Kazakhstan, in which the authors describe the development of a killed composite vaccine. The advantages of the vaccine is that it has low reactivity, is non-abortive, and does not cause pathomorphological alterations to the organs of the vaccinated animals.

In addition, although there are several diagnostic tests for brucellosis, there is a need to develop a more sensitive and specific diagnostic test to confirm the presence of *Brucella* and to identify the species without sacrificing the host. Some of the current tests are used mainly for rapid screening of multiple samples, especially liquid samples, e.g., milk. These tests usually rely on manual observation by the analyst and the endpoint of the tests are colorimetric or precipitates. Although these tests achieve their purpose in allowing an analyst in the field or laboratory to quickly assess whether the milk comes from a cow with brucellosis, they cannot be used to confirm the presence of the bacteria or distinguish other properties of the infection, e.g., bacterial species, vaccine, etc. Current diagnostic tests also fail to distinguish a vaccinated animal from a naturally infected animal. This is important because several of the commonly used vaccines for brucellosis produce a high titer of seroprevalence for long periods of time in the host post-vaccination.

Brucellosis is a chronic disease and the phenotypic and symptomatic expression are not always obvious to the observer, therefore confirmatory tests have relied solely on culturing of the *brucella* organism from the sample or host. These tests not only take days to complete, but also have high false negative rates due

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to the fact that the bacteria are not always present in the blood or in all organ tissues. Thus, there is strong demand for a diagnostic test that can confirm the presence of *Brucella* and identify the species without destroying the host. To this end, the second report in the chapter by Verkhovskiy, Kalnov, and Sklyarov details the development of a competitive enzyme-linked immunosorbent assay (ELISA) for diagnosing bovine brucellosis.

During this symposium, several papers addressing research efforts to develop and test new vaccines and diagnostic tests were presented. Two of these presentations are summarized in this chapter as Report I and Report II. Other papers addressing these topics in more detail are included in this issue as separate papers.

## 2. Report I: evaluation of a killed composite vaccine

### 2.1. Introduction

Kokanov, Ten, Abutalip, Mustafin, and Daugalieva pointed out the need for a killed vaccine to support the biosafety of prepared animal biologicals. Although there are a number of efficacious vaccines that control brucellosis in many animal species, these live vaccine strains can migrate into nonimmune hosts and have a prolonged persistence in the environment. Therefore, the team at the Kazakh Veterinary Research Institute developed a killed composite vaccine. This killed vaccine is non-infectious to humans, other animals, and the environment. In addition, this vaccine has low reactivity, is non-abortive, and does not induce pathomorphological alterations to the organs of vaccinated animals. The sterility, safety, reactivity, and antigenicity of the vaccine have also been confirmed.

### 2.2. Results/discussion

#### 2.2.1. Sterility testing

Using sterile technique, a 1-ml aliquot of the vaccine was poured into two test tubes containing meat peptone agar. Meat peptone broth was added to one of the tubes and the tubes were incubated for 10–15 days. No bacterial growth was observed at the end of the incubation period, confirming the sterility of the vaccine and supporting the conclusion that reversion, migration, or persistence of this vaccine strain was impossible.

#### 2.2.2. Safety testing in mice, ewes, cows, and camels

The safety of this vaccine was demonstrated in albino mice. Testing of the vaccine in ewes, cows, and camels showed no vaccine-related abortive properties.

#### 2.2.3. Reactogenicity testing in rabbits, cattle, camels, sheep, and dogs

In vaccinated rabbits, the body temperature rose by a mean of 0.25 °C for 1–2 days after the vaccination. Redness and slight swelling were noted at the vaccine injection site, but disappeared

within 5–7 days. Studies conducted in cattle, camels, sheep, and dogs produced similar results. In cattle and camels, 1 month after the vaccination the mean swelling at the injection site was 2.5 cm in diameter.

#### 2.2.4. Antigenic testing in sheep, cattle, and camels

The titers of complement-fixing antibodies in sheep, cattle, and camels were reduced at months 3, 4, and 5, respectively, and the levels of agglutinating antibodies also decreased at months 3.5, 4, and 5, respectively. These data are very encouraging given the fact that testing of sheep, cattle, and camels is typically performed more than 6 months after vaccination.

Titers of the complement-fixing antibodies in the blood serum of sheep and goats post-vaccination are presented in Table 1. In this test the composite vaccine titers were compared to those of a live vaccine, *Brucella melitensis* Rev-1. The composite vaccine titers were negative by day 150 post-vaccination as compared to the live vaccine in which the titers were still above 80 at the same time point. A similar test showing the titers of complement-fixing antibodies was performed in vaccinated cattle. The results were similar to those of the tests run on sheep and goats (Table 2).

The immunogenic properties of the composite vaccine were compared to two other live vaccines, *Brucella abortus* strain 82 and *B. melitensis* Rev-1 (Table 3). In this test, organs of the vaccinated animals were tested for live brucella 12 months post-vaccination. The immunity produced by the composite vaccine was as effective as that produced by the two live vaccines.

The composite vaccine was then field-tested in several provinces of the Republic of Kazakhstan. The results of these field trials indicated that brucellosis elimination occurred 2–3 times faster using the composite vaccine compared to the live vaccines, *B. abortus* strain 82 and *B. melitensis* Rev-1.

## 3. Report II: evaluation of a C-ELISA for diagnosing bovine brucellosis

### 3.1. Introduction

To control the spread of a zoonotic disease, it is important to be able to assess the contamination of animal products, man, and the environment. Culture of the disease organism is confirmatory and the most reliable method of identifying a causative agent, but it is sometimes not possible, or is too time consuming or costly. Therefore, other diagnostic tests have been developed to provide reliable data to determine the infection status of the host. Many serological tests are available for brucellosis. Here, we provide a report by Verkhovskiy, Kalnov, and Sklyarov at the Research Institute of Diagnostics and Prevention of Human and Animal Diseases in Moscow, Russia, on the development of a competitive ELISA (C-ELISA) to determine the infection status in cattle.

Detection of *Brucella* antibodies is a useful method of diagnosing brucellosis. This method is still used for monitoring animals

**Table 1**  
Average titers for complement-fixing antibodies in the blood serum of sheep and goats.

Days after vaccination	Animal groups					
	Composite vaccine			Live vaccine from strain <i>B. melitensis</i> Rev-1		
	Number of reacting animals	%	Average titer	Number of reacting animals	%	Average titer
30	8	100	87.5	8	100	63.3
60	6	75	80.0	8	100	100
90	5	52.5	5.2	7	87.5	87.5
120	3	37.5	20.0	7	87.5	100
150	–	–	–	7	87.5	82.5

**Table 2**  
Average titers for complement-fixing antibodies in the blood serum of cattle.

Days after vaccination	Animal groups					
	Composite vaccine			Live vaccine from strain <i>B. melitensis</i> Rev-1		
	Number of reacting animals	%	Average titer	Number of reacting animals	%	Average titer
30	10	100	70.5	10	100	170.5
60	9	90	76.0	9	90	70.5
90	7	70	66.0	9	90	80.0
120	5	50	44.0	9	90	95
150	–	–	–	9	90	95

certified to be officially free of brucellosis. The most commonly used tests include buffered *Brucella* antigen agglutination test, complement fixation test (CFT), and ELISA performed using serial or parallel testing strategies [2–4]. Smooth lipopolysaccharide (S-LPS), an immunodominant component of *Brucella*, and LPS antibodies are involved in all traditional tests used for brucellosis diagnosis [5,6]. The results obtained when only one serological test is used are not always easy to interpret and a combination of several tests is often required to ensure an accurate diagnosis of brucellosis [2,7,8]. Indirect ELISA (I-ELISA) and C-ELISA for the detection of antibodies to S-LPS of *Brucella* have several advantages, i.e., higher sensitivity and specificity, better standardization, robust and specific applications, objective results, adaptability to automated procedures, and commercially available kits. At present, the C-ELISA kit developed by some manufacturers is a multi-species assay that can be used to discriminate between animals vaccinated with *B. abortus* strain 19 and naturally infected animals.

In accordance with the regulations of Russian medical practice, the ELISA for brucellosis is used to diagnose all known forms of the disease and for epidemiological observations and selection of humans for appropriate vaccination (with attenuated vaccine from *B. abortus* strain 19-BA). Also, ELISA is used to detect *Brucella* antigens in the environment and to identify bacterial isolates from cell cultures and other biological specimens. ELISA for brucellosis diagnosis is not used in veterinary practice in Russia, which is why none of the commercially available kits have been evaluated in Russia.

The purpose of this study was to evaluate the performances of C-ELISA kit for the diagnosis of bovine brucellosis in comparison to the conventional serological tests routinely used in Russia. The ability of the C-ELISA kit to discriminate vaccinated from infected cattle was also investigated.

## 3.2. Materials and methods

### 3.2.1. Animals and sera

**3.2.1.1. Bovine sera from a brucellosis-free herd.** Serum samples were prepared from blood samples taken from 198 animals on farms located in the Moscow region that have been officially free from brucellosis for at least 10 years.

**3.2.1.2. Bovine sera from *B. abortus*-infected herds.** Serum samples were prepared from blood samples taken from 461 animals of naturally infected or suspected brucellosis-infected herds located in the Krasnodar and Rostov regions (southern Russia).

**3.2.1.3. Bovine sera from experimentally vaccinated and non-infected animals.** Serum samples were prepared from blood samples taken from 377 animals of brucellosis-free vaccinated herds located in the Krasnodar, Rostov, and Altay regions (eastern Russia). All animals were immunized subcutaneously with commercial vaccines with  $100 \times 10^9$  cells of *B. abortus* strain 82 or  $100 \times 10^9$  cells of *B. abortus* strain 75/79-AB. Both strains used in the vaccines are live attenuated and weak agglutinogenic strains. Blood samples were collected within 7 months after vaccination.

**3.2.1.4. Sera from experimentally vaccinated and experimentally infected animals.** Eighteen animals from a brucellosis-free herd in the Altay region were vaccinated with  $100 \times 10^9$  cells of *B. abortus* strain 75/79-AB. After 10 months, all animals were injected with  $15 \times 10^6$  cells of virulent *B. abortus* strain 54M. Blood samples were collected just before and 45 days after the challenge.

### 3.2.2. Serology

**3.2.2.1. Traditional tests.** Classical serological tests, i.e., the Rose Bengal test, and CFT, were performed and interpreted as previously described [9,10]. Three additional methods that are used in Russia, the serum agglutination test, agar gel immunodiffusion test, and an indirect hemagglutination test were performed in accordance with established protocols.

**3.2.2.2. C-ELISA.** The “*Brucella*-Serotest” kit was manufactured to detect antibodies to *B. abortus* and *B. melitensis* in both domestic and wildlife species. The kit procedure is based on C-ELISA using *B. abortus* S-LPS as the antigen and a monoclonal antibody (mAb) specific for an epitope on the o-polysaccharide part of S-LPS. Briefly, serum samples diluted 1:1 in Tris-buffered saline containing 0.1% Tween 20 were added to the plates and incubated together with the mAb for 30 min at room temperature. Negative control serum, positive control serum, and dilution buffer were added to each plate as

**Table 3**  
Comparative characteristics of immunogenic properties of brucellosis vaccines.

Animal species	Vaccine	No. tested		Positive cultures	Immunity	
		Animals	Organs		Abs. number of animals	%
Sheep	Composite vaccine	10	110	3	7	70
	Live vaccine from strain <i>B. melitensis</i> Rev-1	10	62	6	7	70
Cattle	Composite vaccine	10	120	5	7	70
	Live vaccine from strain <i>B. abortus</i> 82	10	120	9	7	70
Camels	Composite vaccine	5	60	3	6	60
	Live vaccine from strain <i>B. abortus</i> 82	5	60	5	6	60

**Table 4**  
Specificity of the Brucellosis C-ELISA “Brucella-Serotest” (N = 377).

Status of group	Region/number of animals	Number of animals per group classified negative by different tests				
		SAT	RBT	CFT	AGID	C-ELISA
Brucellosis-free	Moscow/198	198	198	198	198	198
Vaccinated by <i>B. abortus</i> strain 82	Krasnodar/81	81	81	81	81	81
Vaccinated by <i>B. abortus</i> strain 82	Rostov/55	55	55	55	55	55
Vaccinated by <i>B. abortus</i> strain 82	Rostov/8	8	8	8	8	6
Vaccinated by <i>B. abortus</i> strain 75/79-AB	Altay/35	35	35	35	35	34

SAT, serum agglutination test; RBT, Rose Bengal test; CFT, complement fixation test; AGID, agar gel immunodiffusion; C-ELISA, competitive enzyme-linked immunosorbent assay.

a control. Positive control serum comprised a pool of sera collected from confirmed *B. abortus*-infected cattle. Antibody binding was detected using a peroxidase conjugate (specific for mouse IgG). The conjugate was incubated for 30 min at room temperature. Tetramethylbenzidine in substrate buffer containing H<sub>2</sub>O<sub>2</sub> was used to visualize the peroxidase activity. Optical density of the controls and samples was measured at 450 nm in a microplate photometer (Multiscan EX; Thermo Fisher Scientific). Results are expressed as percent inhibition values for samples in comparison with the control serum. A positive sample was defined as having a 30% reduction of the negative control serum value.

### 3.2.3. Bacteriology

Blood cultures and pertinent organs from the *B. abortus*-infected animals were subjected to bacteriological examination in accordance to standard protocols [9].

## 3.3. Results

### 3.3.1. Evaluation of C-ELISA specificity

Specificity of the C-ELISA was evaluated based on results obtained with 198 serum samples obtained from cattle in the officially brucellosis-free region and with 179 serum samples obtained from brucellosis-free cattle vaccinated with different strains of *B. abortus* (Table 4).

The specificity of the C-ELISA was 100% when sera from brucellosis-free herds were tested and the test correctly identified all 198 serum samples as negative. The remaining 179 serum samples were obtained from vaccinated cattle and 176 of these samples were negative and 3 serum samples were classified as weakly positive. Thus, the C-ELISA specificity for sera from vaccinated cattle was 98.4%.

**Table 5**  
Comparison testing of cattle for brucellosis.

Bovine sera <sup>a</sup>	SAT (IU/ml)	C-ELISA		I-ELISA <sup>b</sup> (antibody titer)
		% Inhibition	Interpretation of the results	
Brucellosis-negative	– <sup>c</sup>	17.4	–	–
	–	13.8	–	–
	–	14.4	–	–
	–	18.4	–	–
Brucellosis-positive or brucellosis-suspicious	–	72.7	+	1:800
	200	75.9	+	1:1600
	400	88.7	+	>1:3200
	–	73.1	+	n/t <sup>d</sup>
	–	77.0	+	1:1600
	400	78.6	+	1:800
100	81.2	+	1:100	

<sup>a</sup> Status of bovine brucellosis infection was confirmed based on isolation and identification of *B. abortus* by bacteriological testing

<sup>b</sup> SVANOVIR® Brucella-Ab I-ELISA kit (SVANOVA, Sweden) was used in this experiment.

<sup>c</sup> –, Negative; +, positive.

<sup>d</sup> n/t, Not tested; SAT, serum agglutination test; C-ELISA, competitive enzyme-linked immunosorbent assay; I-ELISA, indirect enzyme-linked immunosorbent assay.

### 3.3.2. Evaluation of C-ELISA sensitivity

The sensitivity of the C-ELISA was compared with that of the traditional serological tests, which were performed in parallel. Preliminary results indicate that the C-ELISA was highly sensitive and could be used to process a large number of samples. Typical results are shown in Table 5.

The comparative efficacy of the traditional serological tests and C-ELISA for the diagnosis of bovine brucellosis was assessed in two regional veterinary laboratories located in southern Russia. Sera from 461 cattle of *B. abortus*-infected or brucellosis-suspected herds were tested and 140 bovine serum samples obtained from four herds were selected for additional study. Eighteen vaccinated and *Brucella*-challenged cattle were also examined. The comparative data (Table 6) confirm the advantages of the C-ELISA based on *B. abortus* S-LPS antigen and mAb to the o-polysaccharide part of the S-LPS. Several commercially available test systems are commonly used for detecting antibodies against *B. abortus* and *B. melitensis* in the blood serum and milk of cattle [4,11–13].

The C-ELISA results compared to the results of each conventional test are shown in Table 4. All samples that were positive in one of the conventional assays were also positive in the C-ELISA. No serum from any of the animals produced negative results in C-ELISA and positive results in any of the conventional tests.

Based on the C-ELISA results shown in Tables 4 and 6, this assay can distinguish between *Brucella*-infected animals, and *B. abortus* strain 82 and *B. abortus* strain 75/79-AB vaccinated cattle.

## 3.4. Discussion

Brucellosis remains a serious epidemiological problem in the Russian Federation. In 2008, more than 7000 cattle from more than 100 herds were naturally infected by *B. abortus*. The brucellosis eradication strategy includes combining vaccination and diagno-

**Table 6**  
Comparison of the efficacy of the Brucellosis C-ELISA and conventional serological tests (N = 158).

Status of group	Region/number of animals	Number of positive test results				
		SAT	RBT	CFT	AGID	C-ELISA <sup>a</sup>
<i>B. abortus</i> -infected	Krasnodar/6	3	3	3	3	5
<i>B. abortus</i> -infected	Krasnodar/6	4	6	5	2	6
<i>B. abortus</i> -infected	Rostov/78	13 (3) <sup>b</sup>	13(3)	13 (3)	n/t <sup>c</sup>	28
<i>B. abortus</i> -infected	Rostov/50	0 (10)	0 (10)	0 (10)	n/t	6
Vaccinated by <i>B. abortus</i> strain 75/79-AB and experimentally infected	Altay/18	13	12	n/t	4	18

<sup>a</sup> These cattle were selectively examined by the bacteriological method to confirm the positive C-ELISA results for bovine brucellosis.

<sup>b</sup> (·) Indicates the number of questionable results.

<sup>c</sup> n/t, Not tested; SAT, serum agglutination test; RBT, Rose Bengal test; CFT, complement fixation test; AGID, agar gel immunodiffusion; C-ELISA, competitive enzyme-linked immunosorbent assay.

sis. The current vaccines for bovine brucellosis available in the Russian Federation are *B. abortus* strain 82 and *B. abortus* strain 75/79-AB. In accordance with the epidemiology of each region and the vaccination program, in 2008 more than 2 million injections were performed using the *B. abortus* 82 vaccine and 191 thousand injections were performed using the *B. abortus* 75/79-AB vaccine. Therefore, new specific methods are necessary for assessing the current epidemic in herds and for subsequent immunoprophylactic measures. None of the conventional assays currently used in Russia, however, are capable of distinguishing vaccinated animals from naturally infected animals. Therefore, the objective of this study was to assess the ability of C-ELISA to detect *Brucella*-specific antibodies and to discriminate between vaccinated cattle and infected cattle. For this purpose, serum samples (N = 1054) obtained from cattle with a different brucellosis status were tested by five serological methods and C-ELISA. Our results clearly demonstrated that the C-ELISA we developed is more sensitive than conventional tests for detecting antibodies to *B. abortus* in naturally and experimentally infected cattle. This finding confirms and supports previous reports that C-ELISA is a reasonable alternative verification assay for the serological diagnosis of brucellosis [11,14,15].

Monitoring herds for the presence of infection is crucial to decrease the risk to transmitting the disease to humans and to decrease the economic impact due to widespread brucellosis infection in animals. Our results prove the potential of C-ELISA as a complementary assay for the control of brucellosis and the eradication program in Russia where *B. abortus* vaccination is mandatory.

#### Conflict of Interest Statement

All authors declare they have no conflict of interest

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