

Monitoring of the immune response to *B. abortus* S19 conjunctival vaccine in cattle

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Summary: *Brucella abortus* S19 strain is one of the most preferred strains in vaccines against brucellosis in cattle. However, monitoring of the *B. abortus* S19 vaccine is difficult due to non availability of sustainable immunoreactive antigen and accurate test method. In this study, the humoral and the cellular immune response to S19 vaccine in one year old heifers and calves were monitored on post vaccination days (pvd) of 46, 85 and 169. Thus, the levels of Immunoglobulin (Ig)G and IgA isotypes against lipopolysaccharide (LPS) for humoral immunity and interferon gamma (IFN γ) against brucellergen for cellular immune response were investigated by home-made ELISAs. In this study, significant IgG positivity was observed on pvd 46 in calves (100%) and heifers (96.6%), but IgA positivity and IFN γ levels were not over 50%. Moreover, percentage of positive animals for IFN γ (13.3-43.3%) and IgA (0-44%) have shown no significance for monitoring the vaccine throughout the study. Therefore, IgG levels can be used to monitor the efficiency of *Brucella abortus* S19 conjunctival vaccine in cattle. On the other hand, novel antigen combinations along with brucellergen and LPS for monitoring the immunity would enhance the sensitivity of the test and could be recommended for future investigations.

Keywords: *B. abortus* S19 vaccine, conjunctival route, immunity.

Sığırlarda *Brucella abortus* S19 konjunktival aşısına karşı immün yanıtın izlenmesi

Özet: *Brucella abortus* S19 suşu sığırlarda brusellozise karşı en çok tercih edilen aşı suşlarından biridir. Bununla birlikte, *B. abortus* S19 aşısına bağlı immün yanıtın izlenmesi immünoreaktif bir antijeninin ve test yönteminin belirlenememesi nedeniyle zordur. Bu çalışma ile düve ve buzağılarda bu aşıya karşı gelişen humoral ve hücrel immün yanıtın aşılama sonrası örneklemenin yapıldığı 46, 85 ve 169. günlerde izlenmesi amaçlandı. Bu nedenle, humoral immünite için, lipopolisakkarit (LPS) antijenine karşı immünoglobulin izotip IgG ve IgA'nın ve hücrel immün yanıt için ise brusellerjen antijenine karşı hücrel immün yanıt göstergelerinden interferon gamma (IFN γ) home-made ELISA yöntemleri ile araştırıldı. Bu çalışmada, aşılama sonrası 46. günde buzağılarda (%100) ve düvelerde (%96.6) belirgin IgG pozitifliği gözlemlendi, fakat IgA pozitifliği ve IFN γ düzeyleri %50'nin üzerine çıkmadı. Ayrıca, IFN γ tespiti için %13.3-43.3 ve IgA tespiti için %0-44 aralığında belirlenen pozitif hayvan yüzde oranları aşı takibi için yetersiz olarak belirlendi. Bu nedenle, sığırlarda *B. abortus* S19 konjunktival aşısının etkinlik takibinde IgG düzeyleri kullanılabilir. Diğer taraftan, immüniteyi izlemek için brusellerjen ve LPS ile birlikte yeni antijen kombinasyonlarının kullanılması durumunda testlerin duyarlılığı artacak olup, gelecekteki araştırmalar için önerilmektedir.

Anahtar sözcükler: *B. abortus* S19 aşısı, immünite, konjunktival aşı.

Introduction

Brucellosis is a bacterial zoonosis of worldwide importance that causes devastating losses to the livestock industry including livestock holders (12). Many countries managed to control bovine brucellosis by implementing the test and slaughter policy, practicing sanitary conditions and vaccination (6). *B. abortus* S19 used as vaccine is a stable smooth attenuated organism with high immunogenicity and antigenicity. Therefore, S19 vaccine has been used to prevent brucellosis for more than seven decades (1, 9, 19). Although generation and persistence of antibody response depends on age, dose, and route of vaccination (6), the conjunctival vaccination overcomes

disadvantages like abortion and persistent antibody titers (6, 18). Studies in mice have shown that S19 and RB51 induce a strong Th1 cell-mediated immune response producing IFN γ and IL-2 in immunized animals (2, 9). A useful method to reveal the presence of a cell-mediated immune response against *B. abortus* is production of gamma interferon following lymphocyte stimulation with the specific antigen. Previous studies demonstrated that *Brucella* spp. are able to elicit a cellular response through the production of IFN γ by the stimulated T lymphocytes both in mice (20) and cattle infected with *B. abortus* (23). Therefore, in vitro IFN γ or other cytokine detection methods that depend on the use of specific antigens are

preferred to the complicating tests that require radioactivity and also to in vivo counterparts. Brucellin and other recombinant proteins (BP26, heat shock proteins and others) are selected to determine the best proteins for induction of in vivo and in vitro stimulation (7, 10, 23). Outer Membrane Proteins (OMPs) are the choice of immunogen for IFN γ production. They were shown to be more antigenic than that of total live bacterial cell (5) and *B. abortus* and *B. melitensis* strains those have common antigens on cellular immune responses (3).

The purposes of this study were to investigate the humoral and cellular immune response triggered by conjunctival *B. abortus* S19 vaccine and to determine the period of immune responses in calves and heifers using home-made ELISAs, based on major antigens.

Materials and Methods

Sera: A panel of *Brucella abortus* positive and negative blood sera were obtained from the collection of Genç et al. (13) and sandwich ELISA serum references were from Genç et al. (14).

Antigens: Brucellergen (Brucellergen OCB) antigen was used for stimulation of blood cells in vitro and LPS antigen was used for the detection of anti-*Brucella*IgG and IgA isotype antibodies.

Secondary Antibodies: Anti-bovine IgG (Novus, NB776) and IgA (Biorad, AAI20AB) and streptavidin conjugates were used in iELISA, sandwich ELISA and competitive ELISAs (cELISAs). Anti-mouse IgG (Sigma A-2429) and anti-rabbit IgG (Sigma, A3687) conjugates were also used in competitive ELISA.

Substrates: pNPP (para-nitro-phenyl-phosphate) (Amresco, 0617) and streptavidin alkaline phosphatase (Code 3310-8, Mabtech ab) were used for the detection of both direct-indirect and cELISAs.

Animals and samples: Thirty Holstein calves, 3-5 months age (Group 1), and 30 Holstein heifers (1 year old, Group 2) were provided from a dairy cattle farm located in Tokat Province, Turkey. Calves and heifers were vaccinated with S19 vaccine by conjunctival route and then heifers were boosted. Blood samples were taken twice from each animal before vaccination and at pvd of 46, 85 and 169. Blood samples were taken into two sets of tubes with anticoagulant for plasma and without anticoagulant for serum samples.

All procedures that were done for obtaining animal sera were authorized by the scientific and animal experiments committee of 19 Mayıs University.

Vaccination: The freeze-dried S19 vaccine (Brupen A, Istanbul, Turkey) was manufactured at Pendik Veterinary Control and Research Institute, Turkey. The vaccine was used to vaccinate 3-5 months old calves and 1 year old heifers. Heifers were then boosted when they

were 15-17 months old with the same dose of the vaccine. The booster dose of S19 vaccine comprising of 5×10^9 CFU per 0.05 ml was administered on the conjunctiva as prescribed in the OIE manual (16).

Preparation of plasma: Plasma separation and blood cell induction were performed as follows (11, 23). In the first step, blood samples in heparin containing tubes were transferred to cell culture plates (TPP, 92024) and induced with PBS, Concanavalin A (ConA, Sigma C5275) and brucellergen. Blood induced with the brucellergen and the controls were incubated at 37°C, 5% CO $_2$ incubator (Nuair DH Autoflow) for 18 hours to induce lymphocytes to produce and release IFN γ . After 18 hours of incubation, each blood sample was centrifugated at 1000 g for 10 minutes to separate plasma. Plasma samples were freeze-dried until used. In the second step, the levels of IFN γ in each blood aliquot were determined using the sandwich ELISA (14).

IFN γ Sandwich ELISA: Sandwich ELISA modified by Genç et al. (14) was used for evaluation of the plasma samples.

Home-made ELISAs: *B. abortus* LPS antigen was prepared from *B. abortus* S19 strain by hot phenol-water method as previously described by Caroff et al. (4). A total of 240 sera were screened by ELISAs according to the procedure for anti-*Brucella*IgG detection by Genç et al. (13) and a modified procedure was developed in this study for anti-*Brucella*IgA detection. The cut-off values of IgG and IgA ELISAs based on LPS were determined and the performance of the tests were evaluated according to sensitivity and specificity. Specificity of both tests was found 93.3%, while sensitivity of the tests were 96.7% and 93.3% for IgG and IgA, respectively.

Competitive ELISA: In order to eliminate cross-reactivity to *Y. enterocolitica* O:9 and *E. coli* O157:H7, positive samples by IgG and IgA pertaining to the prevaccination period of group-1 was tested by cELISA using *Brucella*LPS. Accordingly, the protocol was applied as outlined in OIE (16).

Data Analysis: For sandwich ELISA, index value was calculated as mean value of brucellergen induced bIFN γ divided by mean value of PBS stimulated bIFN γ at the same dilution. In this study, results were interpreted according to SI score (1,15, 17) and were evaluated as positive when SI was found ≥ 2.5 . For competitive ELISA, percent inhibition of the sera between the range of 0.3-0.7 OD was accepted as *Brucella* positive. Chi-square test was used for comparison of IFN γ , IgG and IgA results and P value was calculated by using the SPSS 23.0 program package (SPSS Inc, Chicago, IL, USA). In all the statistical analyses made in the study, p values under 0.05 were considered to be statistically significant.

Results

Home-made IgG and IgA ELISA: Maximum IgG proportions were obtained on 46 pvd in both calves (100%) and boosted-heifers (96.6%). The percentage of positive animals for IgG were decreased in following periods and it did not reach to 90%, which is an acceptable diagnostic level. These results with IgG shown that only 46 pvd can be valuable for detecting the immune responses in cattle vaccinated with S19 vaccine. Sufficient IgG levels were not detected later the time points.

Competitive ELISA: Seven sera belonging to the prevaccination period in group 1 were found reactive for IgG and 5 sera were positive for IgA, for that reason, these samples were then checked for any cross reactivity with *Y. enterocolitica* O:9 and *E. coli* O157:H7 by cELISA. In the test, 5 sera positive by both IgG and IgA ELISA were found reactive to *Y. enterocolitica* O:9 and only 2 IgG

positive sera were reactive to *E. coli* O157:H7 and they were excluded from the study.

Cellular immunity: The level of IFN γ was measured and evaluated as positive according to Stimulation Index (SI) $\geq 2.5-9$. Percentage of positivity for IFN- γ were shown as mean and mean of the standard deviations of the SI was demanstrated in Table 2. A total of nine blood samples, of seven reactive to LPS and 2 haemolysed, from group-I were tested with *Y. enterocolitica* O:9 and *E. coli* O:157:H7 and excluded from the study. As the level of both IgA and IFN γ responses were lower than IgG and the level of immunity was prevailed under 45% (Table 1). Although, percentages of positivity to IFN γ was between 13.3 and 43.3%, SI was between 3.47 ± 0.30 and 7.5 ± 1.01 (Table 2). This table shows that cell-mediated immunity is moderate but the proportion is low for estimating cellular immunity at S19 vaccinated cows.

Table 1. Level of anti-IgG and anti-IgA in sera of heifers boosted after primary vaccination with *B. abortus* S19 conjunctival vaccine based on various sampling times.

Tablo 1. Çeşitli örnekleme zamanlarına dayalı konjunktival *B. abortus* S19 aşısı ve ardından rapel doz uygulanan buzağı ve düvelerin kan serumlarında anti-IgG ve anti-IgA seviyeleri.

Sampling time (post vac day)	IgG, GI test results			Sampling time (post vac day)	IgG, GII test results		
	neg	pos	% pos		neg	pos	% pos
0	23	0	0 ^a	0 ^b	14	16	53.3
46	0	23	100	46	01	29	96.6
85	18	05	21.8	85	15	15	50
169	13	10	43.5	169	09	21	70

Sampling time (post vac day)	IgA, GI test results			Sampling time (post vac day)	IgA, GII test results		
	neg	pos	% pos		neg	pos	% pos
0	25	0	0 ^c	0 ^d	26	04	13.3
46	19	6	24	46	27	03	10
85	17	8	32	85	30	0	0
169	11	14	44	169	20	10	33.3

IgG samples; in Group-I (GI) from calves vaccinated with conjunctival route of *B. abortus* S19 vaccine at 3-5 months of age, in Grup-II (GII) from heifers boosted conjunctivally with the same vaccine after 1 year postvaccination.

^a: As 7 sera were found reactive with competitive ELISA, they were not included in the study.

^{b,d}: Sampling time in group II denotes the beginning of post vaccination time after boosting in heifers previously vaccinated at calthood period.

^c: As 5 samples were found reactive with competitive ELISA, these sera were not included in the study.

Table 2. Results of bovine IFN γ -ELISA according to Stimulation Index.

Tablo 2. Stimülasyon İndeksine Göre Sığır IFN γ -ELISA Sonuçları.

Groups	Sampling time (day)	Negative	Positive(%)	SI (AM \pm SD)
Group-I	0	21	0 (0) ^a	-
	46	18	3 (14.3)	4.6 \pm 0.60
	85	21	0 (0)	-
	169	18	3 (14.3)	3.47 \pm 0.30
Group-II	0	26	4 (13.3)	4.65 \pm 0.44
	46	17	13(43.3)	5.34 \pm 1.33
	85	26	4 (13.3)	7.5 \pm 1.01
	169	25	5 (16.6)	6.5 \pm 0.48

bIFN γ results; in Group-I, calves conjunctivally vaccinated at 3-5 months age, in Group-II, heifers boosted conjunctivally with *B. abortus* S19 vaccine after 1 year postvaccination.

^a: Nine samples were excluded from the study due to reactivity to LPS specific antibodies.

Discussion and Conclusion

The availability of diagnostic tests capable of detecting vaccinated animals is essential for the determination of immunogenicity of the vaccine. Due to this issue and also to increase the performance of the tests to detect the immune response for longer periods or at defined time limits, different native and recombinant antigens have been preferred (8, 22). Data on the cellular immunity triggered after infection with *Brucella* spp, a cornerstone in the protection, is more limited, particularly regarding the vaccine. Having some practical advantages and easy evaluation of the test results and higher sensitivity, IFN γ test is preferred to in vivo Delayed Type Hypersensitivity (DTH) test. Brucellin and other specific proteins from rough strains such as *B. abortus* RB51 and *B. melitensis* B115 are selected and used as stimulation antigens in in-vitro tests (8,21, 23). In this study, commercially available brucellergen as the most immunogenic antigen was used as the induction antigen because *B. abortus* and *B. melitensis* have both remarkably similar antigenic moiety which is different from R strains that are devoid of Smooth LPS (S-LPS) (1) and OMPs which are constituents of brucellergen (5,22). In this study, IFN γ response was evaluated in blood of calves and heifers evoked with brucellergen after vaccination with the conjunctival *B. abortus* S19 vaccine in terms of the cellular immunity. Humoral immunity to *Brucella* antigens associated with this vaccine have not been cited extensively, but in this study, it was planned to show the level of antibody responses and if any, targeted to find out which immunoglobulin is acting in immunity. As there is no strict consensus on the detection time, types of test and the antigens, different suggestions are made for monitoring the immune response. In a study, IFN γ response by Perez-Sancho et al. (17) was between 10-50% against *B. melitensis* Rev-1 vaccination during 60 to 180 days postvaccination in sheep. The immune response was followed for 42 days after challenging through one year. IFN γ response was detected as 40-70% at the last day of the study, which is similar to the first sampling time of the boosted group. This result is higher than that of this study particularly in calfhood vaccinated group (0-14.3%) but approximate in heifer vaccinated group (13.3-40.3%) (Table1) and therefore, it can be concluded that some fluctuations can occur one month after the vaccination. Nevertheless, the fact that this method can detect infection very early in time and in high proportion, it is suggestive that this test can significantly contribute to the existing eradication program for bovine brucellosis (23). However, in this study it wasn't possible to estimate the results for the first 46 days. The results in our study were obtained at different time points of 46, 85 and 169 days postvaccination. The highest response in IFN- γ has been

recorded on 46 pvd in adult vaccinated group (43.3%), the responses on other periods in the group were lower than 20%. The fluctuations in test sensitivity limit the possibility of detecting animals vaccinated with S19 and do not preclude the use of the test as a monitoring method to identify vaccinated herds with S19 vaccine. Discrepancies on the results with the other authors may come from differences of the sampling times and differences in the antigen content of brucellergen. To explain this situation, study with the S19 vaccine should be monitored from the beginning of the postvaccination to thereafter 45 days. Cha et al. (5) showed that peak response was detected during first 5 weeks. Dorneles et al. (9), observed significant IFN γ response on day 28, that were decreased after a year. IFN γ has been presented to be detected at a very early time during the infection and determined in a high proportion in infected animals (7), however in this study IFN γ was detected very low. Moreover, these results are not compatible with that of Weynants et al. (23) for the periods of 80 days based on the brucellergen induction.

Another approach monitoring the immune response is based on the serology. For this purpose, different serological tests and antigens such as SLPS, (Rough LPS (RLPS), O polysaccharide (OPS), native hapten polysaccharide, OMP, cytosolic proteins, BP26 have been proposed for the diagnosis of brucellosis in vaccinated and infected animals to increase the specificity of the test especially in ELISA with different monoclonal Abs (15). In this study, immunoglobulin responses to major *Brucella* specific LPS antigen were evaluated by ELISAs based on the serum samples throughout the study. Although IgA response didn't give any conclusive data for diagnostic perspective, the result was meaningful for IgG. The maximum IgG positivity was detected 100% in calves and 96.6% in adults. According to the IgG results, antibody responses can be detectable in vaccinated animals during that period. However, these results could not be interpreted in terms of IgA isotype. In a similar way, Chand et al. (6), monitored the antibody response to conjunctival *B. abortus* S19 vaccine for 3 months. In their study, sera obtained in 3 weeks intervals for 4 periods were evaluated by Rose Bengal Test (RBT) and maximum positivity was determined at 3 weeks postvaccination period as 79.2%. In 6 weeks, it was reduced to 29.2% and after 9 weeks it was 4.2% and 12 weeks later no antibody was detected. In another study, Perez-Sancho et al. (17) detected IgG response in *B. melitensis* Rev-1 vaccinated sheep at 60 to 180 days postvaccination period. In the study, RBT and Complement Fixation Test (CFT) results were at the range of 20-60%. In the same study one year after challenging, immune response was followed for 42 days. Humoral immunity was over 90% at this time. It

could be concluded that as both studies had the results with the same percentages (6, 17), there can be some fluctuations after the period of one month. Based on the time and the immune response, IgG results could be detectable for up to 46 pvd, but IgA level was lower than expected for screening of the immune status of vaccinated calves. Humoral immunity was detected in calves as 100% by CFT in another study (19), which supports the results of 6 weeks period.

These overall results show that it is not possible to monitor the vaccine status according to the post vaccination times with high detection rate. It would be better to monitor the immunity from pvd 7 to 180 and accumulating datas for detecting different antibody classes and cellular immunity markers based on different antigens either recombinant or native. Besides, our results should be evaluated to produce a standart protocol for vaccine monitoring.

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