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Research Article

Seroprevalence of Brucellosis and Leptospirosis in Aborted Dairy Cows

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Abstract: This study was designed to determine the seroprevalence of brucellosis and leptospirosis in aborted dairy cattle. For this purpose, serum samples were collected from 163 aborted dairy cattle that had no history of vaccination against either brucellosis or leptospirosis. The antibodies against *B. abortus* were detected in these serum samples as 68.1%, 65.6%, 58.9% and 55.2%, respectively by the Competitive Enzyme-Linked Immunosorbent Assay (C-ELISA), Complement Fixation Test (CFT), Rosebengal Plate Test (RBPT) and Serum Agglutination Test (SAT). In the study, the serum samples were also collected from 15 healthy dairy cattle which had been vaccinated with reduced dose of *B. abortus* S-19 vaccine six months before the collection of the serum samples. These serum samples were used to compare the differences in the antibody titers between the vaccinated and naturally infected dairy cows by using C-ELISA and CFT. It was found that the animals vaccinated with this vaccine should be revaccinated before six months.

Serum samples of aborted cows were then used to detect dominant *Leptospira serovars* by ELISA and Microscopic Agglutination Test (MAT). MAT was used as a standard test to establish the sensitivity and specificity of ELISA for leptospirosis. The sensitivity and specificity of ELISA were 92.6% and 92%, respectively, when *L. hardjo* antigen was used for both tests. It is found that the total of 66 (40.5%) of sera were positive for both *L. grippotyphosa* and *L. hardjo* antigens and 46 (28.2%) sera were positive for antibodies against *L. hardjo* and 39 (23.9%) of these sera were positive for antibodies against *L. grippotyphosa* by MAT. None of the sera was positive for other *Leptospira serovars* tested.

Key Words: Brucellosis, leptospirosis, serology, abortion, cattle.

Abort Yapmış İneklerde Brusellozis ve Leptospirozis Seroprevalansının Belirlenmesi

Özet: Bu araştırma abort yapmış süt sığırlarında brusellozis ve leptospirozis'in seroprevalansının belirlenmesi amacıyla planlandı. Bu amaçla hem brusellozis hem de leptospirozis'e karşı aşılanmamış 163 abort yapmış sığır kan serum örnekleri toplandı. Aynı serum örneklerinin Competetive Enzyme-Linked Immunosorbent Assay (C-ELISA), Complement Fixation Test (CFT), Rosebengal Plate Test (RBPT) ve Serum Agglutination Test (SAT) ile değerlendirilmesinde *B. abortus*'a karşı sırasıyla % 68,1, % 65,6, % 58,9 ve % 55,2 oranında pozitiflik belirlendi. Araştırmada *B. abortus* S-19'un ergin dozu ile aşılanmış 15 sığırdan 6 ay sonra alınan kan serum örnekleri CFT ve C-ELISA kullanılarak aşılı ve doğal infekte sığırlar arasında antikor titrelerini karşılaştırmak amacıyla kullanıldı ve 6 ay önce aşılanan hayvanların 6 aydan önce yeniden aşılanması gerektiği sonucuna varıldı.

Dominant *leptospira* serovarlarını belirlemek amacıyla abort yapmış sığır kan serum örnekleri mikroskobik aglütinasyon test (MAT) ve ELISA teknikleri kullanılarak değerlendirildi. Leptospirozis için ELISA'nın sensitivite ve spesifitesini belirlemek amacıyla mikroskobik aglütinasyon test standart test olarak kabul edildi. Her iki testde *L. hardjo* antijeni kullanıldığında ELISA'nın sensitivitesi % 92,6, spesifitesi ise % 92 olarak belirlendi. Toplam 66 (% 40,5) serumda hem *L. grippotyphosa* hem de *L. hardjo*'ya karşı pozitiflik tespit edilirken, bu serumların 46 (% 28,2)'sında *L. hardjo*'ya, 39 (% 23,9)'unda *L. grippotyphosa*'ya karşı MAT ile pozitiflik tespit edildi. Serumların hiçbirinde diğer *leptospira* serovarlarına karşı herhangi bir pozitiflik tespit edilmedi.

Anahtar Sözcükler: Brusellozis, leptospirozis, seroloji, abort, sığır.

Introduction

Brucellosis caused by members of the genus Brucella is an important bacterial disease of domestic animals and humans (1-4). Brucella genus consists of six *Brucella* species. *Brucella abortus* is the most common causative agent of brucellosis in cattle, while the infection with

Brucella melitensis and *Brucella suis* is rarely seen in cattle (5-7). *B. abortus* in cattle localizes in genital organs such as testes, uterus and udder. It causes abortion, mastitis, orchitis, arthritis and infertility in cattle (7,8).

Brucellosis is still endemic in many regions of the world, although it has been successfully eradicated in some countries (3). The infection threatens both domesticated animal and human health and causes considerable economic losses (5,9,10). It is especially widespread in South Europe, North Africa and the Middle East (11). Brucellosis is also the most important infection of cattle causing abortion in Turkey (9,10,12).

The definitive diagnosis of brucellosis is made by the methods of culture and isolation procedures (13). Besides, it is not practicable when large number of animals are involved (14,15). Isolation of Brucella species depend on the concentrations of bacteria in the collected specimens at the time of collection. Even, it may be in low concentration for its isolation. Therefore, Polymerase Chain Reaction (PCR) are preferred to cultural methods for the diagnosis of brucellosis (16). On the other hand, several conventional serological methods have been used for serodiagnosis of brucellosis in domesticated animals and humans (17,18). These serological tests are including SAT (19,20), RBPT (19), CFT (19,21), ELISA (22-24) and Fluorescence Polarization Assay (FPA) (25,26). However, presence of cross-reactions especially with Salmonella, Yersinia spp. and *E. coli* creates problems for serodiagnosis of Brucellosis (16). On the other hand, agglutination tests were found to be sensitive but non-specific, whereas indirect ELISA was developed and used as the most specific serological test for brucellosis. However, it is very difficult to distinguish cows vaccinated with *B. abortus* S-19 vaccine from the cows naturally infected with *B. abortus* in endemic areas. Therefore, C-ELISA has been developed to distinguish naturally infected animals with *B. abortus* from the vaccinated animals or from animals infected with cross-reacting Gram-negative bacteria (22-24,27).

Brucellosis and leptospirosis widely occur in Kars district and causes considerable economic losses due to abortion, death of cows, reduction in the production of animal products and increase in farming expenses including treatment and labor. It is also well known that animal infected with either Brucella spp. or Leptospira spp. contaminate the environment and play an important role in the spread of these infections to other susceptible animals. Moreover, these infections are zoonotic and populations such as veterinary surgeons, shepherds, farm workers and abattoir workers are also at risk of these infections. Therefore, it is essential to conduct serological tests to detect carriers and keep the flocks free of the infections.

The purpose of this study was to investigate the seroprevalence of brucellosis and leptospirosis in aborted dairy cows raised in Kars and Ardahan district of Turkey. It was also aimed to evaluate C-ELISA to distinguish naturally infected aborted cows from the cows vaccinated with *B. abortus* strain 19. In addition, we aimed to detect dominant Leptospira serovars of aborted cows and to evaluate sensitivity and specificity of the ELISA for the serodiagnosis of Leptospira spp.

Materials and Methods

Materials

A total of 178 blood sera collected from 16 settlements of Kars and 4 settlements of Ardahan provinces were used to detect antibodies specific to *B. abortus* and Leptospira serovars. 163 sera were collected from aborted dairy cows which had no history of vaccination against either brucellosis or leptospirosis, while remaining 15 sera were collected from healthy cows vaccinated with a reduced dose of *B. abortus* strain 19 vaccine.

Antigens and control sera

In the study, RBPT, SAT and CFT antigens used for brucellosis were kindly provided by Pendik Central Veterinary Control and Research Institute, while standard *L. hardjo*, *L. grippotyphosa*, *L. pomona*, *L. ichterohaemorrhagica* and *L. hebdomadis* serovars were supplied from Etlik Central Veterinary Control and Research Institute. Negative sera used in CFT, RBPT and SAT were obtained from Kafkas University, Veterinary Faculty, Department of Microbiology reference sera collections. For leptospirosis, the sera found positive for MAT in a titer of 1:800 or over were used as positive control and the sera found negative were used as negative control for ELISA.

Method

Serological test for Brucella

RBPT, SAT, CFT and C-ELISA were used to detect antibodies against *B. abortus*. RBPT and SAT were

applied as described by Alton et al. (19). CFT was performed by the method of Alton et al. (19) at Pendik Central Veterinary Control and Research Institute. A commercially available C-ELISA kit (Svanovir™) was used to detect specific antibodies for *B. abortus* according to the manufacturer's instructions (28).

Serological test for Leptospira

a. MAT

Standard *leptospira* strains for MAT were grown in EMJH (Ellinghausen McCullough Johnson Harris) media at 30 ºC for 4-10 days at Etlik Central Veterinary Control and Research Institute. After incubation, cultures were suspended to give a final concentration $2x10^8$ bacteria/ml. Serial dilutions of test serum were prepared ranging from 1:50 to 1:6400 and an equal volume of diluted test sera and antigen suspension was added to each well. Following incubation at 28-30 ºC for 2 hours, the plates were then examined with a dark-field microscope for the presence and degree of agglutination and lysis (29).

b. ELISA

Preparation of sonicated antigens for ELISA

Leptospira antigen for ELISA was prepared as described by Berchovich et al. (30). Briefly, preparations of *L. hardjo* and *L. grippotyphosa* were grown on EMJH. Strains were incubated at 30 ºC for 14 days. After incubation cultures were centrifuged at 8000 rpm for 20 minutes. Sediment was suspended with 0.01 M PBS at a ratio of 1:10 and washed twice. After washing, cultures were sonicated (Bandelin, Sonopuls HD2070) for 3 minutes. And its protein contents was determined according to the method of Lawry (31) using a commercially available kit (BCA-1, Sigma, Germany). Protein content of *L. hardjo* antigen suspension was 40 µg/ml and it was used for ELISA. But, the protein content of *L. grippotyphosa* antigen suspension was found to be very low and it was not used as an antigen for ELISA.

ELISA

The ELISA for the detection of *L. hardjo* specific antibodies was performed as described by Theirmann and Garrett (32). ELISA plates were coated with *L. hardjo* antigen by incubating at 4 ºC overnight. Optimal antigen and serum dilution was determined by checker-board method using positive and negative control sera as 1:100 and 1:100, respectively.

c. Statistical analysis

In the study Win Episcope 2.0 program (33) was used to evaluate sensitivity, specificity, proportion of agreement and confidence intervals of ELISA for the detection of Leptospirosis.

Results

A- In non-vaccinated cows

a-RBPT, SAT, CFT and C-ELISA results

In non-vaccinated dairy cows, 96 (58.9%), 90 (55.2%), 107 (65.6%) and 111 (68.1%) of 163 sera were found to be positive for antibodies specific to *B. abortus* by RBPT, SAT, CFT and C-ELISA respectively (Table 1). In the study, while only 10 of the 111 sera was found weak positive by C-ELISA, the remaining 101 sera was found positive according to the manufacturer's instructions. Weak positive results were evaluated as positive in infected animals by C-ELISA.

b-MAT and ELISA results

Serum samples were positive for *L. grippotyphosa* and *L. hardjo*; none of them were positive for other serovars tested. *L. grippotyphosa* and *L. hardjo* antigens were used in MAT, whereas only *L. hardjo* antigen was used for ELISA, therefore antibodies against *L. grippotyphosa* was not detected in ELISA.

In the study, a total of 63 (38.6%) sera were positive to ELISA and 46 (28.2%) sera were positive for MAT, when *L. hardjo* antigen was used. A total of 66 sera (40.5%) were positive to leptospira serovars, including 27 (16.6%) against *L. hardjo*, 20 (12.3%) against *L. grippotyphosa* and 19 (11.7%) against both of them as measured by MAT (Table 2).

B- In vaccinated cows

In the study, one serum sample was found to be positive by four serological tests. But 13 samples were found to give some degree of agglutination (+ to +++) by RBPT. 1:20++/1:80+++ and 1:5++/1:10+ titer was detected by SAT and CFT, respectively. There was no antibody detected in any of the 4 sera by CFT or C-ELISA (Table 3).

There was no antibodies against any Leptospira spp. in serum samples collected from vaccinated cows (Table 3).

Table 1. SAT, CFT, RBPT and C-ELISA results of sera of cows against brucellosis.

Assay	number of samples positive (%)	number of samples negative (%)	number of samples
SAT	90 (55.2%)	73 (44.8%)	163
CFT	107 (65.6%)	56 (34.4%)	163
C-ELISA	111 (68.1%)	52 (31.9%)	163
RBPT	96 (58.9%)	67 (41.1%)	163

Table 2. MAT results of the sera of aborted 163 cows.

Serotypes	number of samples positive (%)	number of samples negative (%)	number of samples
L. hardjo	27 (16.6%)	136 (83.4%)	163
L. grippotyphosa	20 (12.3%)	143 (87.7%)	163
L. hardjo and *L. grippotyphosa*	19 (11.7%)	144 (88.4%)	163
Total	66 (40.5%)	97 (59.5%)	163

Table 3. RBPT, SAT, CFT, C-ELISA and MAT results of sera of cows vaccinated with reduced dose of *B. abortus* S-19 vaccine.

Sample no.	RBPT	SAT	CFT	C-ELISA	MAT
1	-	1/20 ++	-	-	-
2	+	1/20 ++	-	-	-
3	+	1/40 +	1/10 +	(+)	-
4	+	1/80 +++	1/10 ++	+	-
5	+	1/40 +	1/10 +	(+)	-
6	+	1/40 +	1/5 ++	(+)	-
7	+	1/20 ++	-	-	-
8	+	1/40 ++	1/10 +	(+)	-
9	+	1/20 ++	1/10 +	(+)	-
10	+	1/20++++	1/10 +	(+)	-
11	+	1/20++++	1/5 ++	(+)	-
12	+	1/40 ++	1/10 +	(+)	-
13	+	1/20++	1/10 +	(+)	-
14	-	1/20++	-	-	-
15	+	1/40++	1/10 +	(+)	-

(+): weak positive, +: positive - : negative
+(25%), ++(50%), +++(75%), ++++(100%) agglutination

Sensitivity and specificity

In antibody detected sera by MAT against only *L. hardjo* antigen, the sensitivity and specificity of ELISA were found 92.6% and 92% respectively. Proportion of agreement was 0.920 and confidence intervals (kappa) was found 0.745. Moreover, in seropositivity detected sera against *L. grippotyphosa* and *L. hardjo* serovars, sensitivity and specificity of the ELISA were found 84.2% and 92.4, respectively. Proportion of agreement observed 0.914 and confidence intervals (kappa) was 0.647.

Discussion

Brucellosis has been eradicated in Norway, Sweden, Finland, Lithuania, New Zealand, Australia and Japan to

reduce its effect on economy and international trade of animal products (5). Nevertheless, brucellosis continues to constitute one of the major problems in developing countries (11,34).

The isolation of *Brucella* spp. is the only certain method for the diagnosis of brucellosis; however, for a variety of reasons diagnosis is often made on the basis of serological tests. Isolation techniques and PCR are used for direct diagnose of the agent (16,19). Several serological techniques such as RBPT, SAT (19,20), CFT (19,21) and ELISA (22,35) have been used for diagnosis of brucellosis.

Although serological evaluations of brucellosis constitute majority of the studies in the vicinity (35-37) isolation studies (38,39) are also performed.

In the study, a total of 163 aborted cow sera obtained from 19 settlements were found positive for *B. abortus* as measured by C-ELISA, CFT, RBPT and SAT as 68.1%, 65.6%, 58.9% and 55.2%, respectively.

These findings and reported studies indicate that the prevalence of brucellosis may differ from region to region and even in settlements in the same region. Factors such as climate, geographical area, density of animal kept in the area may act for this disparity and may explain the variety of the results observed by the researchers.

It is known that RBPT is widely used for the diagnosis of brucellosis in only non-vaccinated animals. If antibody titers of unvaccinated cattle over 100 IU per ml of blood sera, it is accepted as infected by National Brucellosis Control Program of Ministry of Agriculture since 1990. Therefore, titers are 1:40 ++ and over by SAT, 1:10++ and over by CFT are accepted as positive or infected (40).

In this study, the lowest seropositivity was found as 55.2% by SAT. However, it was found to be positive at rates of 58.9%, 65.6% and 68.1% by RBPT, CFT and C-ELISA, respectively. As IgM is more efficient than IgG1 and IgG2 in SAT, the test is more efficient for the detection of acute brucellosis than chronic form (19,21). In the study, we found 58.9% and 55.2% sera positive by RBPT and SAT, respectively. In the present study, the ratio of seropositivity for RBPT was found higher than SAT, which shows IgG are the predominant antibodies in the sera examined.

Eradication of brucellosis in the region is practiced by adult (reduced) and young dose of *B. abortus* S-19 vaccine. In this study, we used animals vaccinated with adult dose of *B. abortus* S-19 to evaluate the importance of C-ELISA and to detect antibody titers of the animals 6 months later after this vaccination.

If adult dose (reduced dose) of vaccine is used, no agglutination test is preferred (40). Because agglutinating antibodies produced during initial stages of infection may not last long. Hence, these may not be detectable in later stages for a long period (41). Serological responses in animals vaccinated with reduced dose of *B. abortus* S 19 last at least 6 months (40). In this study, 13 sera from 15 cows vaccinated with reduced dose of *B. abortus* S 19 were found positive by RBPT. When the same sera were examined by SAT, titers ranged between 1:20 and 1:40. In the study, only one sera was found positive (strongly positive) by C-ELISA and gave a titer of 1:80+++ and 1:10++ by SAT and CFT, respectively. *B. abortus* was also isolated from vaginal swabs of this cow indicating the efficacy of C-ELISA to differentiate infected cow from vaccinated animals. But it is not possible to distinguish vaccinated animal from infected animal by CFT.

In this study, we compared antibody titers against *B. abortus* between infected and vaccinated cows by C-ELISA. 91 (82%) of the 111 sera examined by C-ELISA was found positive; likewise 10 (9.1%) of the sera was found weak positive. In the study, all the sera (positive and weak positive) were accepted as positive because the vaccine has not been implemented.

In vaccinated animals, when CFT was accepted as standard test, sensitivity and specificity of C-ELISA was 100%. However, all the titers detected by CFT was accepted as low vaccine titer. In the study, in vaccinated group, 4 animals were found negative by CFT and C-ELISA and detecting of very low level of antibody by CFT from remaining 10 sera indicating decrease antibody titers to undetectable levels during 6 months. This supports the regulations of National Brucellosis Control Program. (40) Therefore, it indicates that vaccination of adult animals with reduced dose of *B. abortus* S 19 vaccine should be repeated before 6 months. The study shows that instead of CFT and RBPT, C-ELISA should be practiced regularly where vaccinated and infected animals kept together.

Most prevalent leptospira serovars of cattle and sheep were *L. grippotyphosa* and *L. hardjo* in our country (42-

46), which is consistent with the results reported in foreign countries (47,48).

In this study, a total of 66 (40.5%) sera were found to be positive for only *L. grippotyphosa* and *L. hardjo* serovars as measured by MAT. 27 (16.6%), 20 (12.3%) and 19 (11.7%) of these sera were positive for *L. hardjo* and *L. grippotyphosa* and for both serovars, respectively.

In the study, a total of 63 (38.6%) sera were positive to ELISA and 46 (28.2%) sera were positive for MAT, when *L. hardjo* antigen was used. The sensitivity and specificity of ELISA were found 92.6% and 92% respectively. Sera that were found positive against both *L. grippotyphosa* and *L. hardjo* by MAT were evaluated with coating plates using only *L. hardjo* antigen by ELISA. Sensitivity and specificity of the ELISA were found 84.2% and 92.4%, respectively. In the study, while specificity of the ELISA remaining the same level, whereas sensitivity of the ELISA was found lower when both *L. grippotyphosa* and *L. hardjo* antigens were used. in MAT

In this study, we found antibody titers of *L. grippotyphosa* higher than that of *L. hardjo* by MAT (not illustrated). When *L. grippotyphosa* antigen was not used in ELISA, we could not detect antibodies against *L. grippotyphosa*. Therefore, sensitivity of the ELISA was declined from 92.6% to 84.2%. As a result of this study, both *L. grippotyphosa* and *L. hardjo* antigens should be used for increasing the sensitivity of the ELISA.

The result of the study indicated that *L. hardjo* and *L. grippotyphosa* were the dominant leptospira serovars and they play an important role as causative agent of abortion in the cattle of the vicinity. Dominant Leptospira serovars were detected as *L. grippotyphosa* and *L. hardjo* by Çetinkaya et al. (44) by MAT from healthy animals. In the same area 16% and 23.3% of the examined sera were found positive against *L. hardjo* and *L. grippotyphosa* by MAT and ELISA, respectively in Elazığ district by Ertaş et al (45). It was detected that 33.63% and 36.26% of sera was positive in Kars district using MAT and ELISA by Şahin et al. (46) respectively. In this study, 69 (42.3%) sera were found positive by ELISA and 66 (40.5%) sera by MAT when only *L. hardjo* antigen was used. Our results are higher than the results of researchers (44-46), which suggests that the places where the samples were collected can be focus of leptospiras and it may be cause of abortion in this district. We concluded the leptospira vaccine prepared for the region must include *L. hardjo* and *L. grippotyphosa* serovars.

Brucellosis and leptospirosis occur in a high prevalence in the aborted dairy cattle of Kars district of Turkey. Vaccination of adult animals with reduced dose of *B. abortus* S 19 vaccine should be repeated at least after 6 months to obtain adequate protection for brucellosis. The *leptospira* vaccine including *L. hardjo* and *L. grippotyphosa* serovars must be applied to this region regularly. C-ELISA should be practiced where vaccinated and infected animals kept together. Precautions should be applied to the region to reduce the spread of these infections and to eliminate risk of humans having these highly zoonotic infections. The elimination of these infections will also increase the quality and the safety of the animal products for the region.

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