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RESEARCH ARTICLE

Detection of *Brucella* spp. in Vaginal Swab Samples of Aborting Cattle: Comparison of Immunoperoxidase to Bacteriological Culture Technique [1]

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Summary

Brucellosis is an important disease in terms of economic losses in cattle breeding. *Brucella* spp. associated abortion is common in Kars, Turkey. Determination of Brucellosis cases therefore poses great importance. In this study, an immunochemical staining technique was tested in detecting cattle with Brucellosis and compared to bacteriological culture technique. Post-abortion, total of 261 vaginal swab samples were collected from cows. *Brucella* spp. were isolated from 25 animals by cultural method. All *Brucella* isolates were identified as *Brucella abortus* field strain and biotyped as *B. abortus* biotype 3. Smears prepared from culture positive vaginal samples were stained by immunoperoxidase technique, and 22 out of 25 animals were detected as Brucella-positive. Sensitivity and specificity of the immunoperoxidase technique were calculated as 88% and 92%, respectively.

Keywords: *Brucella, Immunoperoxidase, Bacteriology, Cattle*

Abort Yapan Sığırların Vajinal Sıvap Örneklerinden *Brucella* spp. Tespit Edilmesi: İmmunoperoksidaz ve Bakteriyolojik Kültür Yönteminin Karşılaştırılması

Özet

Brusellozis sığır yetiştiriciliğinde ekonomik kayıplara neden olan önemli bir hastalıktır. Kars'ta *Brucella* spp. nedenli abortlar oldukça yaygındır. Bu nedenle Brusellozis olgularının tespiti büyük önem arz etmektedir. Bu çalışmada; Brusellozisin tespiti amacıyla immunokimyasal boyama yöntemi test edildi ve bakteriyolojik kültür yöntemi ile karşılaştırıldı. Abortu takiben toplam 261 vajinal svap örneği ineklerden toplandı. Kültür yöntemi ile 25 hayvanda *Brucella* spp. izole edildi. Tüm *Brucella* izolatları *Brucella abortus* saha suşu olarak identifiye edildi ve *B. abortus* biyotip 3 olarak tiplendirildi. Kültür pozitif vajinal örneklerden hazırlanan sürme preparatlar immunoperoksidaz yöntemi ile boyandı ve 25 hayvandan 22'si Brucella pozitif tespit edildi. İmmunoperoksidaz yönteminin duyarlılığı ve özgüllüğü sırasıyla %88 ve %92 olarak hesaplandı.

Anahtar sözcükler: *Brucella, İmmunoperoksidaz, Bakteriyoloji, Sığır*

INTRODUCTION

Brucellosis is an important and highly contagious zoonotic disease worldwide [1,2]. It causes prominent economic losses due to abortion, stillbirth, infertility, drop in milk and meat production and loss in breeding capacity in cattle. The etiologic agent of cattle brucellosis is *Brucella abortus*, which is the number one cause of bacteriological

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abortions, though *B. melitensis* and *B. suis* might also be responsible for infection [3-7]. The spread of the infection is mostly via aborted fetus, fetal membranes, lochia, urine, non-pasteurized milk and milk products [8]. *Brucella* spp. are Gram negative, aerobic, coccobacilli shaped, non-motile, non-spore forming, facultative intracellular pathogens with low metabolic activity and size of 0.5x0.7x1.5 µm [9].

Brucella spp. are as well the most important bacterial agent of abortion in cattle in Turkey [5,10]. Prevalence of Brucellosis in serological surveys was recorded as 1% in Kırıkkale [11], 15% in Kayseri [12], 20.9-21.7% in Van [13], 38.2-56.4% in Burdur [14], and 53.8% in Kars [15]. In Northeastern Turkey, *Brucella* spp. in aborted fetuses were determined as 42.25% and 74.4% in Erzurum [5,10] and 40%, 41.23%, and 59.7% in Kars [5,6,16] by bacteriological technique.

Bacteriological and serological means are widely used in determination of Brucellosis. However, bacteriological culture technique, which is accepted as the unequivocal method in detection of *Brucella* spp., takes long time, and serological tests give cross reactions and false readings due to vaccinated animals [17,18].

The purpose of this study is to apply immunoperoxidase staining technique, and to compare the results of it to that of bacteriological culture technique in diagnosis of Brucellosis in smears prepared from vaginal swab samples collected post-abortion in cattle. Therefore, the current investigation is aimed to rapid and reliable detection of Brucellosis, which is the major cause of abortion in Kars province, Turkey.

MATERIAL and METHODS

Vaginal Swab Samples

This research (Study No: 06/29) was approved by the animal local ethics committee, Kafkas University. The study materials of vaginal swab samples were collected on February to May 2008 and March 2009 to March 2010 in Kars province from those aborting cows that were known to be non-vaccinated against *Brucella*. For bacterial isolation total of 261 vaginal swab samples were cultured. Total of 75 smear samples of which 25 from Brucella culture positive vaginal swab samples, 25 from randomly chosen Brucella culture negative swab samples and 25 from Brucella culture were used for immunoperoxidase staining. Sample collection sites and number of Brucella positive and negative vaginal swab samples were listed in *Table 1*.

Bacterial Isolation and Identification

Vaginal swab samples were collected and then inoculated onto 7% defibrinated sheep blood agar (Oxoid, CM0271) and onto Brucella Medium Base (Oxoid, CM0169) containing Brucella Selective Supplement (Oxoid, SR083). The inoculated plates were incubated at 37°C in aerobic and microaerobic environment for 4 to 7 days. Following incubation, *Brucella* spp. suspected colonies were characterized by colony morphology, Gram staining, oxidase, catalase and urease activities. Agglutination test using A and M monospecific antisera, H_2S activity, stain susceptibility tests and Tbilisi phage typing were also performed for identification and biotyping. Identification of isolates as field or vaccine strains were made based on growing in the presence of penicillin and l-erythritol [19]. As control strains, *B. abortus* biotype 1, *B. abortus* biotype 2, *B. abortus* S19 and *B. melitensis* biotype 1, *B. melitensis* biotype 2 obtain from Pendik Veterinary Control and Research Institute were used.

Immunoperoxidase Technique

A total of 75 smear samples prepared from 25 Brucella culture-positive and 25 Brucella culture-negative vaginal swab samples and 25 Brucella culture medium were used in immunoperoxidase technique. All smear samples were stained by avidin-biotin immunoperoxidase staining method as follow; smears were fixed in alcohol and then treated with 3% H_2O_2 for 30 min to block endogenous peroxidase activity. Antigen retrieval was succeeded by application of 1% Trypsin solution at 37°C for 25 min. Following three times washes in 0.1 M Phosphate Buffered Saline (PBS), pH 7.4, non-specific staining was blocked by 10% non-immune goat serum for 30 min. Then, smears were incubated for 30 min with rabbit anti-Brucella polyclonal antibody (Becton Dickinson, Cat No: 240934) diluted 1:50 with PBS. Biotinylated secondary antibody and Streptavidin peroxidase complex (Zymed Histostain-Plus Bulk Kit, Cat No: 85-9043) were consecutively applied for 30 min each, and three times PBS washes were performed between the applications. Antibody binding was visualized by color development using 3,3 diaminobenzidine/H_2O_2. Finally, the sections were counterstained with Harris hematoxylin, rinsed with tap water, immune-mounted and observed under a light microscope. Negative controls of the staining were done by replacing primer antibody with PBS. As positive control, smears prepared from *B. abortus* standard strain were used.

Quantitative Assessment of Immunoperoxidase Technique Findings

A semi-quantitative grading scale was applied. Smears stained with immunoperoxidase technique were observed in 5 randomly chosen fields under 2 x 100 objective and the numbers of stained bacteria were counted and the means were calculated. The results were scored as follow; (-) No bacteria, (+) 1-10 bacteria, (++) 11-25 bacteria, (+++) >25 bacteria.

Sensitivity and specificity of the immunoperoxidase technique were determined based on the formula described by Moore et al.[20].

RESULTS

Results of the bacterial isolation were summarized in *Table 1*. In cultural method, *Brucella* spp. were isolated in 25 (9.57%) out of 261 vaginal swab samples. *Brucella* spp. isolation from the vaginal swab samples were succeeded by both Brucella selective agar and Sheep blood agar. The most successful bacterial isolation was accomplished in those samples that were collected 3-30 days post-abortion, and less were from the samples collected longer than 91 days post-abortion. Suspected colonies were characterized as *Brucella* spp. by microscopic morphology, positive oxidase, catalase and urease activities. All *Brucella* isolates were identified as *B. abortus* by their CO_2 requirement at initial growth, positive H_2S activity, and Tbilisi phage susceptibility. All *B. abortus* strains were biotyped as *B. abortus* biotype 3 by positive reaction with antiserum A and negative reaction with antiserum M via slide agglutination test and growth in the presence of basic Fuchsin and Thionin.

The results of the immunoperoxidase technique were detailed in *Table 2*. All of the smears prepared from Brucella culture media gave positive results by immunoperoxidase. Out of 25 Brucella culture positive vaginal

Table 1. *Number of vaginal swab samples, locations and number of Brucella isolation*

Tablo 1. *Vajinal sıvap örnek sayısı, örnek odakları ve Brucella izolasyon sayısı*

Location	Number of Samples Collected	Number of *Brucella* Isolation
Digor	2	0
Kağızman	2	1
Kars/Merkez	197	14
Selim	55	10
Susuz	5	0
Total	261	25

Table 2. *Data about abortion time, sample collection time post-abortion, and results of immunoperoxidase technique with bacterial intensities in smears prepared from Brucella culture positive vaginal samples*

Tablo 2. *Abort zamanı, abortu takiben örnek toplamaya kadar geçen süre ve Brucella kültür pozitif vajinal örneklerden hazırlanan sürme preparatlarda bakteri yoğunluğu ile immunoperoksidaz yönteminin sonuçları*

Case No	Estimated Abortion Time (m) in Gestation	Time past Post-abortion until Collection of Samples	Immunoperoxidase Technique	Bacterial Intensity in Smear
1	5-6	1 m	+	+++
2	8-9	1 m	+	+++
3	8-9	40 d	+	+++
4	5-6	10 d	+	+++
5	6-7	1 m	+	+++
6	7-8	1 m	+	+++
7	7-8	10 d	+	+++
8	7-8	3 m	+	+
9	6-7	2 m	+	++
10	7-8	2 m	-	-
11	8-9	20 d	+	+++
12	ND	2 m	+	+++
13	ND	2 m	+	+++
14	ND	10 d	+	+++
15	ND	1 m	+	+++
16	8-9	1 m	+	+++
17	8-9	20 d	+	+++
18	5-6	10 d	+	+++
19	8-9	4 m	-	-
20	5-6	3 m	+	++
21	6-7	12 m	+	+++
22	6-7	4 d	+	+++
23	Stillbirth	2 d	+	+++
24	7-8	40 d	-	-
25	Stillbirth	40 d	+	++

ND: Not determined, ***d:*** day, ***m:*** month

swab samples, 22 showed positive immunoreactivity for *Brucella* *(Fig. 1a,b,c)*. These positive stained bacteria were observed as single or mostly in aggregates. In smears prepared from Brucella culture negative samples, 2 false positive immunoreactivities were detected. Brown stained coccobacilli shaped bacteria in smears prepared from the culture media were observed mostly in large groups and less as single or groups of 3 to 5 *(Fig. 2)*. All of the smears prepared from Brucella culture positive vaginal samples showed positive immunostaining. No immunostained bacteria were observed in negative controls.

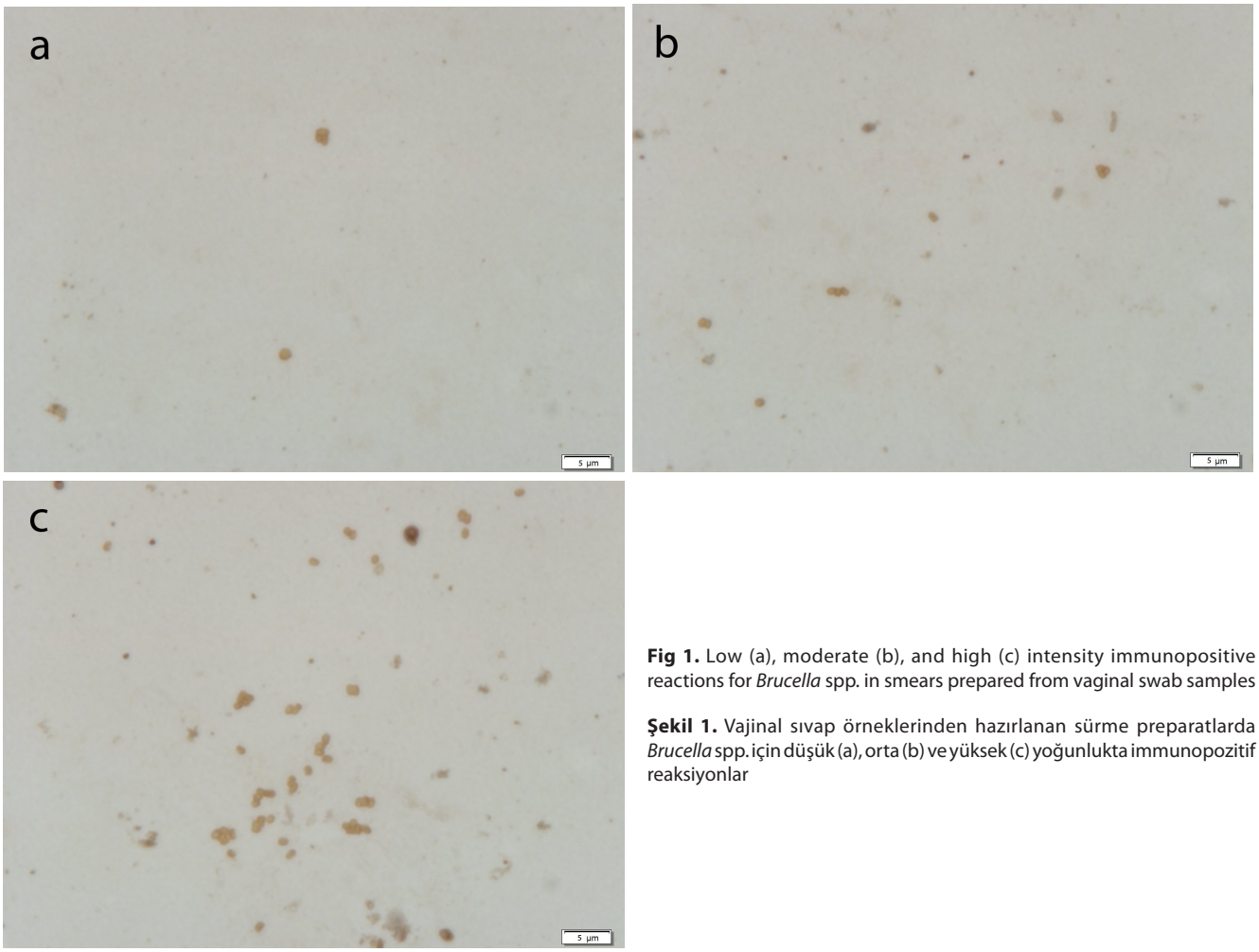

Fig 1. Low (a), moderate (b), and high (c) intensity immunopositive reactions for *Brucella* spp. in smears prepared from vaginal swab samples

Şekil 1. Vajinal sıvap örneklerinden hazırlanan sürme preparatlarda *Brucella* spp. için düşük (a), orta (b) ve yüksek (c) yoğunlukta immunopozitif reaksiyonlar

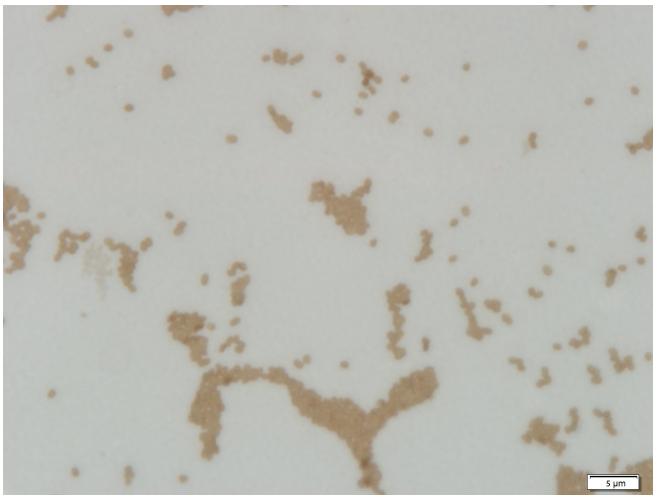

Fig 2. Positive immunoreactivity in a smear prepared from *Brucella* positive bacteriologic culture

Şekil 2. *Brucella* pozitif bakteriyolojik kültürden hazırlanan sürme bir preparatta pozitif immunoreaktivite

Out of 25 Brucella culture positive samples, 21 were determined to have abortion within the first two months. These animals, with only one exception, also showed great bacterial intensity in vaginal samples. Two samples collected from animals 3 months post-abortion showed positive immunostaining with low to moderate bacterial intensity. Total of 3 samples, two collected from animals aborted within the two months and one collected at 4 months post-abortion, did not show positive immunoreactivity.

Compared to the bacteriological culture technique, immunoperoxidase staining showed 12% false negative and 8% false positive immunoreactivity. According to these results, sensitivity and specificity of the technique were calculated as 88% and 92%, respectively.

DISCUSSION

Kars province is an important location for cattle breeding in Turkey. As in most other cities in Turkey, Brucellosis is an endemic disease in Kars [21,22]. Abortion in cattle in this location is the most devastating breeding problem in terms of economic losses. *Brucella* spp. are long known to be the most causative bacterial agents of abortion in cattle. Bacterial isolation of *Brucella* spp. in aborted fetuses at high levels in previous surveys indicates that the agent is the most important abortion cause in this city [5,6,16]. Besides abortion especially in the last trimesters of gestation, *Brucella* spp. might cause stillbirth and birth with low survival chance in cattle [2]. In accordance with the literature, animals surveyed in this study mostly had abortion in the last part of gestation and few had stillbirth.

Spread of *Brucella* agents in cattle mostly occurs through aborted fetus, fetal membranes, urine, uteral and vaginal secretions [8,23]. It has been reported that the agent could be shed in vaginal excretions for 6 weeks post-abortion [19]. *Brucella* agents could also be present in the uterus for 200 days post-abortion [24]. In the present study, the highest bacterial isolation was detected in vaginal swab samples of animals that had abortion within 2 months. Similarly, smears prepared from these samples showed the highest bacterial presence. We have shown that the bacteria can be isolated from the vaginal swabs up to 4 months post-abortion, however no immunopositive reactivity were able to be detected in smears at that time. Based on the results, up to 3 months, *Brucella* agents were detectable in smears by immunoperoxidase technique. These findings prove that the vaginal secretions at least 3 to 4 months pose big risk for the spread of disease. In the current investigation, isolation of bacteria from vaginal swab sample in an animal that had abortion 1 year ago was thought to be due to presence of metritis in the animal. False positive immunoreactivity in two samples that were negative by bacteriological mean was related to possible contamination of the samples.

Abortion in cattle especially in the last trimesters of gestation in endemic locations could be greatly related to *Brucella* spp., however final diagnosis is still required. Diagnosis of Brucellosis is generally based on direct or indirect laboratory techniques [19,25]. Direct diagnosis of the agent could be made by microscopic investigation of stained smears by modified Ziehl-Neelsen and Köster methods. However, bacterial morphology of *Brucella* spp. in these techniques might be confused with *Chlamydophila abortus* and *Coxiella burnetii* [25,26].

Bacterial isolation and identification is the golden standard in determining *Brucella* spp. Although bacteriological technique is quite a specific method its sensitivity might vary greatly depending on the agent's survival within the sample, type of sample, and the number of samples tested. The technique also has some other drawbacks such as, it is time consuming; might result with no bacterial isolation from samples with low bacteria and in chronic disease cases; requires a special laboratory environment; and poses risk for zoonotic spread to laboratory personal [17,19].

Polymerase chain reaction (PCR) has become a commonly used method for identification of a variety of agents including *Brucella* spp.[27]. PCR has high sensitivity; however it might have a great variation in specificity. Although the technique could detect those agents that are difficult to determine with conventional techniques, it takes time and money. Requirements for a special laboratory equipment and skilled personal are some other disadvantages of PCR technique. In addition, false positive and false negative results might be seen in varying degrees due to need for standardization [28]. Although commercial kits are greatly standardized for *Brucella* spp., they are quite expensive and, therefore they could be used in limited samples. Moreover, since PCR technique also detects DNA segments from death bacteria the results might not be useful in post-treatment studies.

Indirect detection of *Brucella* spp. could be made by serological techniques. Agglutination test, complement fixation test and ELISA are often the methods used for this purpose [29,30]. These methods are mostly useful in vast surveillance or in eradication studies. However, low sensitivity and specificity due to vaccinations, previous infections and cross reactions with other Gram negative bacteria might hamper its use [18].

Immunoperoxidase technique has become a widely used technique in many areas, including cancer and bacterial disease research. Due to problems mentioned above in direct and indirect detection methods value of this technique in use increases. Especially in fixed tissue samples immunoperoxidase renders good results. The technique could detect not only live microorganisms but also bacterial parts and microorganisms with broken wall. Immunoperoxidase technique has shown to give better results compared to direct or acid-fast staining methods in determining many bacteria in samples with low presence [31]. Although histochemical detection of *Brucella* spp. in tissue sections were reported to be difficult and nonspecific [32], sensitivity and specificity of immunoperoxidase in determining *B. abortus* in lochia of infected cows were reported as 82% and 94%, respectively [33]. In the last investigation 36% false positive and 2.3% false negative reactions were recorded. In the current investigation, immunoperoxidase staining on smears prepared from vaginal swab samples gave satisfactory results. The sensitivity and specificity of the technique in this study were found as 88% and 92% with 12% false negative and 8% false positive, respectively, which are similar to the previous research.

In conclusion, we have shown that immunoperoxidase technique could be used in search of *Brucella* spp. in vaginal swab samples. Smears prepared from vaginal swabs that would be collected from animals post-abortion could be quickly stained and give result in the same day. Best results might be obtained in samples collected 2 months post-abortion. The results of this investigation have shown that bacterial isolation and identification method is still the golden standard, however, where it is not possible immunoperoxidase technique might be used with high confidence. The technique might also find usage in other sample types such as fetal membranes and fetus itself. Based on the encouraging results, smears prepared from abomasal content and/or mucosa of the aborted fetus could be tested for detecting *Brucella* spp. in future studies.

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