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Investigation of *Brucella* agents in soil-fertilizer mixtures and animal feed samples from cattle farms after extinguished brucellosis outbreaks

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ABSTRACT

Although brucellosis is under control in developed countries, it still threatens the health of animals and humans in the endemic regions. In these endemic regions although the outbreaks in some cattle farms have been officially extinguished, the farmers have wondered whether the bacteria are still present in some farm materials after extinguishment processes. The situation must be clarified so that farm production activities can continue, and new animals can be procured in time. With this study, it was aimed to investigate the presence of *Brucella* bacteria in critical materials that may effectively transmit the disease after extinguishment procedures. For this reason, soil-fertilizer mixtures and animal feed were selected and investigated in three cattle farms where the disease was officially extinguished after the brucellosis outbreaks. The samples were collected approximately twenty days after completing extinction procedures for brucellosis. The soil-fertilizer mixtures and animal feed samples were collected in 68 and 55 pieces, respectively. The classic culture methods for the bacterial diagnosis of brucellosis were used with some modifications. After the growth of bacterial colonies on selective media, they were diagnosed by genus and species-specific PCRs. Five positive results were obtained by genus-specific PCR, but only one could be confirmed with species-specific multiplex PCR. For the remaining four, it was thought that they could belong to some soil bacteria genetically close to the *Brucella* genus. As a result, the brucellosis extinguishment procedures implemented could be considered adequate, and the farms were safe regarding the contamination. Although the official rules for disease extinction are fully implemented after outbreaks, similar studies are needed on more farms for more robust evaluations.

Söndürülen bruselloz salgınlarından sonra sığır çiftliklerinden alınan toprak-gübre karışımları ve hayvan yemi örneklerinde Brucella etkenlerinin araştırılması

ÖZET

Bruselloz gelişmiş ülkelerde kontrol altına alınmış olsa da endemik bölgelerde hayvan ve insan sağlığını olumsuz etkilemektedir. Bu bölgelerdeki sığır çiftliklerinde çıkan hastalıklar resmi olarak söndürülmüş olsa da, çiftçiler söndürme işlemlerinden sonra çiftliklerinde bulunan bazı materyallerde bakterilerin mevcut olup olmadığını merak etmektedir. Çiftlik üretim faaliyetlerinin devam edebilmesi ve yeni hayvanların zamanında tedarik edilebilmesi için bu hassas durumun açığa kavuşturulması bir ihtiyaç haline gelmiştir. Bu çalışmada, söndürme işlemlerinden sonra hastalığı etkili bir şekilde bulaştırabilecek kritik materyallerde *Brucella* bakterilerinin varlığının araştırılması amaçlamıştır. Bu sebeple, bruselloz salgınlarından sonra hastalığın resmi olarak sonlandırıldığı üç sığır çiftliğinde toprak-gübre karışımları ve hayvan yemleri seçildi ve incelendi. Örnekler, bruselloz için söndürme prosedürleri tamamlandıktan yaklaşık yirmişer gün sonra, toprak-gübre karışımlarından 68, hayvan yemlerinden 55 adet toplandı. Brusella bakterilerinin teşhisi için klasik kültür yöntemleri bazı modifikasyonlarla kullanıldı. Bakteri kolonileri seçici besiyerlerinde üretildikten sonra, Brusella bakterileri için cins ve tür spesifik PCR testleriyle araştırıldı. Cins-spesifik PCR ile 5 pozitiflik elde edildi. Bunlardan sadece bir tanesi tür-spesifik multipleks PCR ile doğrulanabildi. Kalan dördünün, genetik olarak yakın bazı toprak bakterilerine ait olabilecekleri düşünüldü. Sonuç olarak, bruselloz söndürme prosedürlerinin uygun olduğu ve çiftliklerin bulaş açısından güvenli sayılabileceği kanaatine varıldı. Hastalık çıkışlarından sonra hastalığın yok edilmesine yönelik resmi kurallar tam olarak uygulansa da, daha güvenilir değerlendirmeler için daha fazla çiftlikte benzer çalışmalara ihtiyaç duyulmaktadır.

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1. Introduction

Brucellosis is the general name for animal and human infections (1). The disease is caused by different bacterial species affiliated with the genus *Brucella* (2), mainly *B. melitensis*, *B. abortus*, and *B. suis* (1). Cattle are usually infected by *B. abortus*, less often by *B. melitensis*, and sometimes by *B. suis.* In animals, the disease is identified by abortion and infertility (3). There are limited symptoms to suspect the presence of the disease; however, many diagnostic tests can be used to make a diagnosis. Conventional culture can be used as a gold standard method. With this, molecular and serological methods are the most common diagnostic methods for brucellosis (1).

Many endemic countries use different fighting strategies to control and eradicate brucellosis (4, 5). These strategies are formed according to the conditions of countries, such as geographic borders, disease prevalence, the budget for the control of disease, infrastructure of farms, organizations of veterinary services, feasible diagnostic tests, etc. (6). In Türkiye, the "test and slaughtered" strategy with vaccination has been conducted with various projects since the 1980s. According to the valid regulation when the study was carried out in Türkiye (Circular No: 2019-6), in case of any abortion in any herd, the culture method was used to determine *Brucella* bacteria in the herd. For bacterial cultures, some organs and abomasal fluid of aborted fetuses, samples of vaginal discharges, placenta, and milk were critical materials for isolations. In those times, if any *Brucella* agent was determined with conventional methods, all animals in the same herd were tested serologically (Rose Bengal rapid agglutination test and Complement fixation test), and seropositive animals were slaughtered in the contracted slaughterhouses. This practice was continued until negative results were determined twice in the same herd at two-month intervals. After taking two consecutive negative test results for each animal, disinfection was made, and the disease was considered extinguished within the enterprises.

Some researchers reported that *Brucella* bacteria could also be shed in urine and feces except in aborted fetuses, birthing and aborting fluids, placenta, semen, and milk (7). After shedding, the bacteria can be transmitted to animals or humans through digestion, inhalation, conjunctiva, and even intact skin (1, 8, 9). This uncontrolled shedding and transmission have led many researchers to investigate the persistence of *Brucella* bacteria in soil and other materials. It was reported that the bacteria can survive up to 250 days in soil (7, 10), although contamination varies according to many factors. Temperature, direct sunlight, and soil moisture could play a prominent role in the persistence of the bacterium in soil (11). All these cause question marks in the farmers' minds and lead to uneasiness about whether the danger of contamination is still continuous after the official disease extinction processes in the farms.

This study aimed to investigate the presence of *Brucella* bacteria in the soil-fertilizer mixture and animal feed at the cattle farms where the disease had been officially extinguished and evaluate these materials for the risk of contamination by *Brucella* agents.

2. Material and Methods

Samples

The study was conducted in three cattle farms where authorized units carried out official eradication procedures for brucellosis in 2020. The extinction procedures were continuous for each farm for approximately eight months. The morbidity rates of the disease in these farms were 65%, 60%, and 42%, respectively. Almost twenty days following the official extinction date for each farm, samples were chosen from the feed stored in the farms' areas and from risky regions in terms of contamination, such as birthing paddocks and walking areas. Pieces of 32, 21, and 15 soil-fertilizer mixtures and 20, 22, and 14 feed samples were taken from the first, second, and third farms, respectively. Samples for the soil-fertilizer mixture were collected from the topsoil surface at about 0 to 5 cm depth. Approximately 400-500 g samples were taken using a sterile spatula and gloves. After being placed into sterile containers, all samples were brought to the laboratory under cold chain conditions as soon as possible.

Standard strains

Standard strains were obtained from the strain collection of Harran University, Faculty of Veterinary Medicine, Department of Microbiology. *B. abortus* Tulya (ATCC 23450), the reference strain of *B. abortus* biotype 3, was used in the tests as the reference strain.

Bacterial isolation

All tests were carried out in the Laboratory of the Department of Microbiology in the Faculty of Veterinary Medicine of Harran University. *Brucella* selective broth and agar (Farrell's media) were prepared using the base media tryptic soy broth and agar (Oxoid, England) with adding a commercial *Brucella* selective supplement (Oxoid, England). Farrell's media were used for conventional bacterial isolation (1, 9, 12).

Each sample was mixed thoroughly before inoculations. Twenty-five g of each sample was inoculated into selective broth at a ratio of 1/10. Inoculated media were incubated at 37 °C in microaerobic conditions (Panasonic, MCO-18AC-PE, Japan) for six weeks. During the incubation period, cultures were refreshed weekly with the additional medium at a ratio of 1/5. Then, 200 µl from these media was passed onto selective agars every two weeks. These agars were incubated under the same conditions with broth media for five days. After five days, DNA extractions were performed from the colonies for the molecular diagnosis of *Brucella* bacteria. These PCRs were repeated every two weeks up to 6 weeks.

Molecular identification from bacteria

The genomic DNAs were isolated from the cultures using boiling (13). The isolated DNAs were stored at -20ºC until analyzed by genus and species-specific PCRs.

The method of Queipo-Ortuno et al. (14) was used with slight modifications for the genus-level PCRs. B4 and B5 primers (Sentebiolab, Türkiye) were preferred from the gene region encoding *Brucella* cell surface salt extractable protein 31 (Bcsp31) (15). Briefly, five µl of 10 X PCR solution containing a mixture of 100 mM Tris-HCl (pH:8.4, 500 mM KCl, and 15 mM $MgCl_2$,) 38.25 µl of PCR water, 1µl of primer mix stock (Final concentration 0.2 µM), dNTP mix stock 0.5 µl (Final concentration 0.2 mM), 0.25 µl of enzyme (5 IU/µl) (Final concentration 1.25 IU). Finally, five µl of DNA template extracted directly from the cultures was added to the tubes to complete the PCR mixture. The PCR tubes were placed, and the amplification process was completed in the thermal cycler by applying a total of 35 cycles, following pre-denaturation at 93 ºC for 5 minutes, one minute at 90 ºC (denaturation), 30 seconds at 60 ºC (annealing), one minute at 72 ºC (extension) and 7 minutes at 72 ºC (last extension) at the end of the cycles.

The species-specific multiplex PCR was carried out using the method of Mayer-Scholl et al. (16). In the PCRs, nine pair primers (Sentebiolab, Türkiye) were used (16, 17) with a commercial ready-to-use master mix kit (Qiagen, Germany).

In all tests, the genomic DNA of *B. abortus* bt 3 and the PCR-grade water were used as positive and negative (NTC) controls respectively.

The obtained amplicons were electrophoresed into 1.5% agarose gel at 100 volts for 45 minutes. The band formations were investigated after visualizing with the UV illuminator (Vilber-Lourmat, France).

3. Results

After the incubation periods of the samples, none of the cultures were pure, although the selective media had been used. So, mixed cultures, including various bacterial colonies, were used for molecular analyses.

Five positive results were determined at the end of the first two weeks (Table 1, Figure 1).

Table 1: The samples and results of the molecular tests
Tablo 1: *Örnekler ve moleküler test sonuçları*

Farms	Samples	Number of Samples	Results of genus-specific PCR		Results of Species-specific PCR	
			Positive	Negative	Positive	Negative
1. Farm	Soil-fertilizer mixture	32	3	29	1	31
	Animal feed	20	0	20	0	20
2. Farm	Soil-fertilizer mixture	24	1	23	0	24
	Animal feed	22	0	22	0	22
3. Farm	Soil-fertilizer mixture	15	1	14	0	15
	Animal feed	14	0	14	0	14

Among the five positive samples for genus-specific PCR, only one *B. abortus* field strain was identified by multiplex PCR from the DNAs of the soil-fertilizer mixture sample taken from the grazing area of the first farm. The other four samples were found to be negative by species-specific PCR. (Table 1, Figure 2).

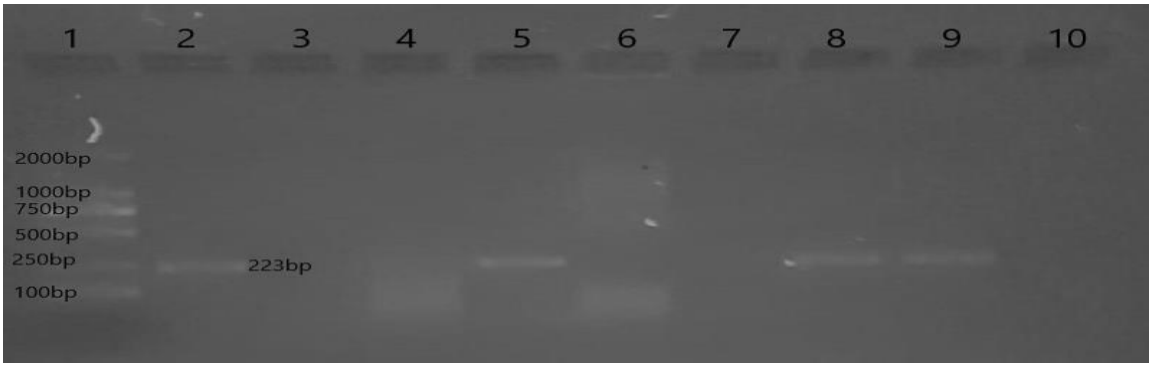

Figure 1: Agarose gel view for the genus-specific PCR
Şekil 1: *Cins spesifik PCR için agaroz jel görüntüsü*
Line 1: Marker 2000bp, Line 2, 5, 8, 9: Some positive results; Line 3, 4, 6, 7, 10: Some negative results.

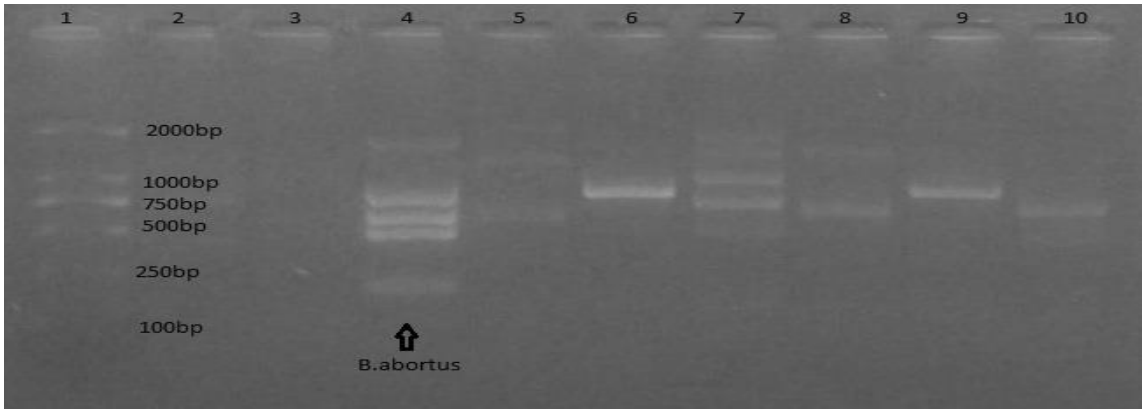

Figure 2: Agarose gel view for the species-specific PCR
Şekil 2: *Tür spesifik PCR için agaroz jel görüntüsü*
Line 1: Marker 2000bp, Line 2, 3, 8, 10: Some negative results, Line 4: Positive results (*B.abortus*-Band sizes: 1682, 794, 587, 450, and 152 bp), Line 5, 6, 7, 9: Genus-specific PCR positive but species-specific PCR negative results.

4. Discussion and Conclusion

Brucella agents can be shed in many different ways according to the clinical symptoms of diseased and porter animals during their lifetime. The most common way of spreading the agents of brucellosis is via aborted fetuses and their membranes. The others are vaginal discharges, semen, milk, hygroma, or arthritis fluids (1). Although many researchers and institutions of international health organizations consider the excretion of *Brucella* agents in feces possible, the transmission of the disease in this way is generally evaluated as insignificant in the epidemiology of the disease. On the other hand, studies show that brucellosis-bearing animals can also spread the agent through feces. Morales-Estradaa et al. (7) isolated *B. abortus*, *B. melitensis*, and *B. suis* species, a total of ten pieces from cattle and goat feces. They reported that contaminated feces is a risk factor in endemic areas. In this study, soil-fertilizer mixtures were chosen as the research subject because they carry a high risk of contamination. Both feces and secretions-excretions of animals, as well as abortion or postpartum shedding, were considered to be high risk for transmission.

Because the spread of *Brucella* agents in the environment by a latent carrier animal might last a lifetime, the possibility of transmission of these bacteria through soil has been a matter of curiosity, and many studies have been conducted on this topic. In the studies conducted, it has been reported from the past that *Brucella* species scattered with feces or by other means can remain in soil and dust for several weeks (18-21). Leski et al. (22) took soil samples from 19 locations in Kuwait and Iraq and analyzed samples molecularly. The researchers reported that bacteria from the *Brucella* genus were detected in 8 locations. Aune et al. (23) reported in their study conducted in the Greater Yellowstone Area that the persistence of *Brucella* bacteria in soil lasted up to 43 days in the places where contamination occurred through bison births or their waste, and they reported that the persistence of bacteria in soil was directly related to exposures of temperature and sunlight. Ahmed et al. (24) reported collecting 1280 soil samples from 256 villages in 9 regions of the Punjab province of Pakistan and investigating the DNA of *Brucella* bacteria. They detected positive results in 27 samples from the DNAs obtained using a soil DNA extraction kit. As a result, researchers investigated not only soil but also potential infectious materials such as fomites (25) and water (19), and they concluded that these materials could be a risk factor for transmission. In this study, animal feedstuffs were not taken off the farms' area, so they were considered a potential risk source for new animals. So, in addition to the soil-fertilizer mixture, contamination of stored feeds by various means was also considered possible, and these feeds were included as research material in the study. Essential carriers of the agent of brucellosis, such as soil and animal feeds, have the potential to harbor, preserve, and transmit the agent under appropriate conditions; they were made the subject of this research in the farms where the disease emerged.

Researchers were used isolation methods with molecular methods to identify *Brucella* agents at the genus and/or species level. Garrido-Haro et al. (26) performed bacterial isolation from samples obtained from cattle populations in Ecuador in the last 3 years. They confirmed the diagnosis by using genus-specific and species-specific PCRs. Mazwi et al. (27) performed bacterial isolation from animals at a slaughterhouse in South Africa and confirmed these isolates by PCR. In a study conducted in Northeastern Ethiopia Tekle et al. (28) isolated *Brucella* bacteria from eight out of 64 samples. After using species-specific PCR, they reported that four goat isolates were *B. melitensis*. Similar to previous studies, classical isolation methods were used with together a molecular diagnostic method, PCR, in this study.

The genus *Brucella* phylogenetically belongs to the family Rhizobiaceae in the α-2 subgroup of the class Proteobacteria. The genera of *Rhizobium* and *Ochrobactrum* are also in the same family (29). Researchers reported that these species are abundant in the soil and that soil bacteria are the closest relatives of *Brucella* agents. (30). Researchers reported that members of the *Ochrobactrum* genus are the closest phylogenetic relatives of *Brucella* agents and that the 16S rRNA gene shows more than 97% similarity to the *Brucella* genome sequence. Species such as *O. antrophi* and *O. intermedium* are closer to *Brucella* species than other species in the genus. This striking similarity requires attention to identify *Brucella* agents and diagnose infection correctly (31). Moreover, some researchers have reclassified the genus *Ochrobactrum* and included its species in the genus *Brucella* based on extensive genome comparisons using advanced molecular technology (32, 33).

PCR methods targeting the highly conserved 16S rRNA, the gene region encoding the Bcsp31 protein, and other genetically conserved regions have been developed to differentiate the genus *Brucella* (15, 34, 35). However, it should be noted that these regions may cross-react with species genetically close to the *Brucella* genus, such as *Ochrobactrum anthropi* and *Ochrobactrum intermedium* (34, 36, 37). For the results that were positive according to the genus level PCR but could not be confirmed by species-specific PCR, it was thought that these positive PCR results may be caused by saprophytic soil bacteria that are genetically close to the *Brucella* genus. On the other hand, this result may be due to the ability of the PCRs with different minimum detection limits to detect different amounts of specific DNA (38). Other bacteria can grow with *Brucella* agents, and they can limit the growth of the specific agents. So, the amount of specific DNA obtained may have been below the detection limit for species-specific PCR.

According to the results of this study, all positive samples were found in areas with soil-fertilizer mixtures. No positivity was detected in sampling from feed storage areas. These results were considered reasonable regarding the possibility of contamination of the materials. In addition, the preference of the culture method in the analyses was critical in obtaining viable microorganisms. Molecular methods cannot give us information about viability.

One of the most critical limitations of this study is that similar samples could not be taken from the farms where the study was conducted during the active disease period. Unfortunately, the farm owners requested analyses after the disease was extinguished, so comparisons of samples before and after the disease was extinguished could not be made.

It is also crucial for farm workers to follow biosafety protocols to prevent human infections, especially in case of outbreaks, since *Brucella* species are zoonotic pathogens.

With this study, the idea was obtained, especially in these or similar farms, how long the areas where new animals will be bought could be safe and whether the disease control methods applied in the farms could be effective after achieving disease extinction. The results of this study suggest that in-house risky areas can be considered epidemiologically safe for the spread of brucellosis on farms where the official rules for disease extinction are fully implemented after outbreaks. However, carrying out similar studies on more farms will make the evaluations on this subject even more reliable.

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Conflict of Interest

The author declared that there is no conflict of interest

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Ethical Statement

The ethical declaration has been obtained from the authors that the data, information and documents presented in this article have been obtained within the framework of academic and ethical rules, and that all information, documents, evaluations and results are presented in accordance with the rules of scientific ethics and ethics.

According to the Regulation on the Working Procedures and Principles of Animal Experimentation Ethics Committees (Article 2 - paragraph 2), this study does not require ethics committee approval. Because the study involves "non-experimental clinical veterinary medicine practices".

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