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A Systematic Detection for Brucellosis at Chronic Stage of Infection in Semen of Sheep and Saanen Goats

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ABSTRACT

The study was conducted in a herd (n: 244) in which goats (n: 206) and sheep (n:38) had a history of brucellosis in Bursa which is located in Northwestern of Turkey between the years 2012-2014. For the detection of *Brucella* spp. and the other zoonotic bacterial agents, semen samples were taken from Saanen goats (n: 35) and rams (n: 8). Samples were tested by routine diagnostic procedures and PCR. The serum samples of male animals were also tested for Brucellosis by C-ELISA and I-ELISA. The culture results represented *Trueperella pyogenes* (n:2), *Pasteurella pneumotopica* (n: 5), *Esherichia coli* (n: 3), *Aeromonas salmonicida subs.* Salmonicida (1), *Brevundimonas vesicularis* (n: 2) and *Mycoplasma bovigenitalium* (n: 1) and *Mycoplasma arginini* (n: 1) from semen samples. Rams had no symptoms due to epididymitis or epididymoorchitis in clinical examination, but two bucks showed orchitis and they were serologically positive for brucellosis. Also, one seronegative buck showed epididymitis in a flock. There were no statistically significant differences between the serologically positive and negative animals in an examination of semen samples in terms of their volume, concentration, mass activity, motility and defectivity rate for acrosome. Although 20 of the serum samples were negative for anti-*Brucella* antibody, 23 of them were serologically positive for brucellosis. As a result of this study, *Brucellae* were not detected by bacteriologically and molecularly while there were some positive serum samples for brucellosis. This could be attributed that these samples might have been collected from chronically infected animals in which animals generally do not shed the organisms. Therefore, it was thought that sampling with regular intervals might help for the definitive incidence of brucellosis.

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Koyun ve Saanen keçilere ait semenlerden sistematik deteksiyon ile kronik Bruselozisin tanısı

MAKALE BİLGİSİ

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ÖZ

Çalışma, 2012-2014 yılları arasında Türkiye'nin kuzeybatısında yer alan Bursa ilinde brucellosis geçmişli keçi (n: 206) ve koyunlara (n: 38) ait bir sürüden (n: 244) örneklemeler suretiyle yapıldı. Saanen keçilerinden (n: 35) ve koçlardan (n: 8) alınan semen örnekleri *Brucella* spp. ve diğer zoonotik bakteriyel ajanların tespiti amacıyla çalışıldı. Örnekler, rutin tanısal prosedürler ve PCR ile test edildi. Erkek hayvanlardan alınan serum örnekleri ayrıca C-ELISA ve I-ELISA ile Bruselloz yönünden test edildi. Kültür sonuçları; Semen örneklerinden *Trueperella pyogenes* (n: 2), *Pasteurella pneumotopica* (n: 5), *Esherichia coli* (n: 3), Aeromonas *salmonicida* sub. Salmonicida (n:1), *Brevundimonas vesicularis* (n: 2) ve *Mycoplasma bovigenitalium* (n: 1) ve *Mycoplasma arginini* (n: 1) izole edildi. Klinik muayenede ise koçlarda epididimitis veya epididimoorşit kaynaklı semptom olmamasına karşın serolojik olarak bruselloz pozitif olan iki adet koç orşitis idi. Ayrıca bir seronegatif koç epididimitis semptomu gösterdi. Semen örneklerinin muayenesinde serolojik olarak pozitif ve negatif hayvanlar arasında akrozomun hacmi, konsantrasyonu, kütle aktivitesi, motilitesi ve defektivite oranı açısından istatistiksel olarak anlamlı bir fark bulunmadı. Serum örneklerinden 20 adedi anti-Brucella antikoru için negatif olmasına rağmen, 23 adedi bruselloz için serolojik olarak pozitifti. Selektif besiyerinde yapılan katı ve sıvı kültürler ile semen örneklerinden yapılan tür spesifik PCR testi sonucunda örneklerden Brucella saptanmadı. Bu durum, numunelerin; hayvanların genellikle organizmaları saçmadığı kronik enfekte hayvanlardan toplanmış olabileceğini göstermektedir. Periyodik örneklemenin brusellozun kesin tanısında ve insidensinin saptanmasında yardımcı olabileceği düşünülmektedir.

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Introduction

Brucellosis is manifested by reproductive failure, which includes abortion, the birth of unthrifty lamp and kids, and retained placentae in female animals. The disease is important for animals and humans because of the zoonotic potential of the disease. In Europe, animal brucellosis is still seen in Mediterranean and Balkan countries (Portugal, Spain, Italy, Malta, Bosnia-Herzegovina, Croatia, Greece, Cyprus, Turkey, Macedonia, and Serbia). Outside of this area, countries in Africa (Algeria), Asia (Syria, Iran) and Latin America (particularly Brazil and Mexico) are endemic countries for brucellosis in farm animals. Brucellosis is also a notifiable disease in Turkey, USA and Canada (Bauerfeind et al., 2016).

Brucella ovis causes clinical or subclinical disease of chronic evolution, characterized by testicular changes derived from the inflammatory process in the epididymis with seminal vesiculitis and ampulitis, followed by orchitis with subsequent loss of fertility in rams (Ridler and West, 2011, Carrera-Chávez, 2016). Lesions in Brucella-infected males are largely confined to the genital organs including testicles, seminal vesicles and epididymes (Morgan and MacKinnon, 1979). Brucellae are commonly present in the seminal fluid, accompanied by specific antibodies and may be transmitted to susceptible females through intra-uterine insemination (Crawford et al., 1990). It is noteworthy to mention that the artificial insemination is one of the main routes for infections of female animals. Although the semen from Brucella-infected bulls contains from 100 to 50,000 organisms/ml, infected bulls rarely transmit the disease through natural insemination (Morgan and MacKinnon, 1979; Adel et al., 2001).

For large scale diagnosis, the use of serological tests is recommended, plate agglutination tests with antigens prepared by smooth and rough strains, enzyme-linked immunosorbent assay and complement fixation tests, agar gel diffusion test are used mostly (Ridler and West, 2011). Infected rams may have intermittent excretion or no excretion of bacteria, which makes bacteriological analysis not practical or reliable. The clinical diagnosis by scrotal palpation is valid only if the animal has developed lesions and should be considered that epididymitis may be caused by other bacteria; moreover, infected rams without apparent clinical signs, may excrete the bacteria (Ficapal et al., 1998; Mejid et al., 2010, Carrera-Chávez et al., 2016). Rams/bucks and teaser animals should comply with the requirements before entring into an artificial insemination center and isolation at the pre-entry isolation facility where the country or zone of origin is not free from the diseases. OIE refers Brucellosis, Ovine epididymitis, Contagious agalactia, Peste des petits ruminants, Contagious caprine pleuropneumonia, Paratuberculosis, Scrapie, Maedi-Visna, Caprine arthritis/encephalitis, Bluetongue and Tuberculosis for ram and bucs (2019 © OIE - Terrestrial Animal Health Code - 10/08/2018).

Molecular testing of reproductive fluids (uterine/vagina discharge, semen) and urine from bovines is a useful tool for diagnosis of brucella but there is a little literature in this area for goat and sheep. Commercial ELISA kits are available for the diagnosis of Brucellosis. The routine diagnosis of brucellosis by detecting Brucella-DNA by PCR has been previously investigated (Fekete et al., 1990; Adel et al., 2001). Although authors have evaluated the relationship between palpable lesions of epididymitis and semen quality in rams (Carvalho et al., 2012), according to the literature review, sperm quality in rams serologically positive to *Brucella* spp. naturally infected has been evaluated before by Carrera-Chávez et al. (2016). Therefore, this study aimed to evaluate sperm quality in naturally brucella-infected rams and goats which are also serologically positive for brucellosis and to isolate and identify *Brucella* spp. and the other bacteria in semen and tissues by bacteriological and molecular methods.

Materials and Methods

Sample Processing

This study was performed between the years 2012-2014 from a herd in Bursa which is located in Northwestern of Turkey. The herd included 206 goats and 38 sheep, totally 244 and had a history of brucellosis infection. For the detection of *Brucella* spp. and the other zoonotic bacterial agents, vaginal swabs and milk samples were taken from female goat (n:56) and ewe(n:5). Semen samples (n: 42) were collected from Saanen goats (n:34) and ram (n:8). The serum samples of sheep (n: 38) and goats (n:206) were also tested for Brucellosis by C-ELISA and I-ELISA. For this purpose, the team from Department of Artificial Insemination received separate ejaculation samples from, Saanen (n: 34), Ivesi (n: 3) and Kıvırcık (n: 5) for their spermatological examination.

Clinical Examination and Semen Collection

A total of 43 semen samples from adult rams (n:8) and goats(n:35) were collected for semen examinations. Animals were 3 to 4 years old and were, maintained in a breeder herd in Turkey. Each animal was examined by genital palpation to detect macroscopic testicular alterations. Clinical examination was performed by manual palpation of both testicles and epididymis simultaneously. Comparing to each other, symmetry, size, shape and consistency of both testicles were assessed and recorded. The epididymides were palpated, starting from the head, continuing to the body and the tail. Semen was collected by electrostimulation with an electroejaculator standardized for small ruminants, produced by MINITUBE-Germany (Ustuner et al., 2016). To collect semen, ram and goats were restrained physically and lubricated probe was inserted into the rectum with downward pressure on the front of the probe so the electrodes remained nearby the upper portion of the ampullary region. The electrical stimulation was applied for 4-8 s. While the electro stimulation was stopped briefly (3-4s), further massage was applied with the probe. This cycle was repeated until 1.0-2 ml semen was collected (usually 3-4 electro stimulations). Collected semen was placed in a warm water bath (30°C). Macroscopic analysis of semen characteristics, including volume, color, and viscosity was done clinically. Then, semen was evaluated immediately for consistency, wave motion (0-5 scale), percentage of motile spermatozoa (%) and defected acrosome rate (%) (Ustuner et al., 2016).

Semen Evaluation

All semen parameters were assessed by the same person on each occasion throughout the study. Sperm counts were performed with the hemocytometer method. Sperm motility was assessed subjectively using a phase-contrast microscope (Olympus BX 51) (400x) with a warm slide (38°C). Acrosomal and other morphological integrity were evaluated by Giemsa staining technique (Ustuner et al., 2016).

Culture

Each of semen samples (n:43) were investigated for the diagnosis of *Mycoplasma* spp., *Ureaplasma* spp., *E.coli, Klebsiella* spp, *Acinetobacter* spp., *Trueperella pyogenes, Brucella melitensis, B.ovis, Listeria monocytogenes,* and *Campylobacter* spp. by routine bacteriological procedures. For this purpose, a loop of all freshly collected semen samples were inoculated on to Columbia agar (COS43041; bioMérieux) with 7 percent defibrinated sheep blood, MacConkey's agar (CM115; Oxoid) and Levine eosin methylene blue agar (CM0069B; Oxoid), XLT-4 Agar Base (BD, Difco®- 223420) supplemented with XLT-4 Supplement (BD Difco™-100 m) for the detection of aerobic bacteria. The plates were incubated for 24 hours at 37°C. Listeria Selective Agar (Oxoid®-CM0856B) containing Listeria Selective Supplement Oxoid®-SR0141E) were used for the detection of *Listeria* species from semen samples. Semen samples were diluted in Mycoplasma Broth Base (CM0403; Oxoid) containing Mycoplasma Supplement G (SR0059C; Oxoid) to 10^{-3} and inoculated to Mycoplasma Agar Base (CM0401B; Oxoid) plates containing Mycoplasma Supplement G (SR0059C; Oxoid) for isolation of *Mycoplasma* spp. All samples were also streaked onto *Brucella* Selective Medium Base Oxoid®-CM0169 containing Brucella Selective Supplement (Oxoid® SR0083) and Butzler SelectiveMedium (Colombia Blood Agar Base) (Oxoid®-CM0331B) containing Butzler- Campylobacter Selective Supplement (Oxoid®- SR-0085E) for the isolation of *Mycoplasma, Brucella and Camphylobacter* species, respectively. All plates were incubated for seven days at 37°C in a humidified atmosphere with 5-10% CO_2. Mycoplasma broth media and agar plates were examined daily for signs of mycoplasma growth. Mycoplasma-like colonies and typical 'fried egg' colonies were examined under 35X magnification of stereomicroscope. Mycoplasma-like growth was tested to digitonin sensitivity for Acholeplasmas and urease activity for Ureaplasmas using standard methods (Poveda et al., 1998).

Since semen samples may contain relatively few brucellae, *Brucella spp.* detection procedure was based on two-stage culturing i.e., selective enrichment in liquid culture medium and inoculation on to three different selective solid media, namely Farrel's, modified Thayer-Martin and Cita. For selective enrichment tryptic soy broth (BBL) containing 5-10% fatal calf serum amphotericin B (1 mg / ml), and vancomycin (20 mg / ml) was used. Semen samples (0.1ml) were inoculated into each of this broth and incubated at 37°C in air supplemented with 5-10% CO_2, for six weeks, with weekly passages on to solid selective media. Farrel's medium was prepared by adding 5-10% serum and antibiotic supplement into the basic medium (Oxoid SR0083A). Modified Thayer-Martin's (GC Medium Base, 38 g/litter; BIOLIFE Laboratories, Milan, Italy) was prepared by adding hemoglobin (10 g/litter, Difco), colistin methanesulphonate (7.5 mg/litter), vancomycin (3 mg/litter), nitrofurantoin (10 mg/litter), nystatin (100,000 International Units [IU]/litter =17.7 mg) and amphotericin B (2.5 mg/litter) (Sigma) to the basic media. Cita medium included basic medium, 5-10% fatal calf serum, colistin methanesulphonate (7.5 mg/litter), by adding vancomycin (20 mg/litter), nitrofurantoin (10 mg/litter), nystatin (100,000 International Units [IU]/litter = 17.7 mg) and amphotericin B (4 mg/litter) (Sigma)

Competitive ELISA (C- ELISA)

Svanovir® (Svanova) *Brucella* diagnostic C-ELISA kit was used according to the manufacturer instructions. for screening *Brucella* lipopolysaccharide (LPS) antigen of smooth *Brucella* strains. Positive, negative, weak positive and conjugate controls (20 µl of sample dilution buffer) and serum samples were prediluted 1/10 in tubes by adding 20 µl of respective control or sample into 180 µl of Sample Dilution Buffer. Prediluted controls and samples in the volume of 50µl were placed into appropriate wells, respectively Mouse monoclonal antibody (mAb) specific of an epitope on the O-polysaccharide of S-LPS antigen of *Brucella abortus*, of were added 50µl in each well. The plates were incubated with the test serum and mAbs by shaking for 5 minutes and then 30 min. without shaking in room temperature. All plates were rinsed four times and then 100 µl of Substrate Solution containing goat anti-mouse IgG antibody conjugated with horse-radish peroxidase was added into wells and the plates were incubated 30 min for a binding reaction.

Indirect ELISA (I –ELISA)

Chekit® (Idexx) Brucellosis serum antibody Test kit was used according to the manufacturer instructions for Indirect- ELISA. The volume of 90 µl of washing solution was plated each well and 10 µl of undiluted serum sample and controls were added into appropriate wells comprising up to final dilution at 1:10. After the incubation for 60 min at 37°C, all wells were dispended with 300 µl of washing solution three times. Check it –Brucellosis Anti Ruminant Ig-PO conjugate was added then and the plate was incubated for 60 minutes at 37°C.

PCR

A target sequence of a gene encoding 31 kDa protein (BCSP31) which is common in all *Brucella spp.* was amplified by PCR. F B4 (5 'CTC GGT TGC TGG TAT CAA CAA 3') and B5, R (5 'CGC GCT GGT TCA CTT CTG TGC 3') (Sigma-Aldrich) were used as primers. Amplification protocol was performed described by Baily et al. with minor modifications (1992). Bacterial DNA was obtained by boiling method from culture medium containing semen samples. The reaction volume was 50 ul including 10 mMTris-HCl (pH8.4), 50 mMKCl, 1 mM magnesium chloride, 200 mMdNTP (Fermentas), 1.25 U of Taq polymerase (Roche). Five microliters of the PCR amplicon were loaded into a 1.5 % (wt/vol) agarose gel (SeaKem –Lonza) in 1X TAE buffer using EzVision One loading dye (Amresco) and run concurrently. DNA Ladder (100 bp) (SM0241, 50 µg Thermo Scientific) and the PCR products obtained from the positive and negative control were included in each run. No bands at 223 bp were visualized in an UV-Tech Fire Reader **(**Progen Scientific**,** Merton, London, SW19 3UU).

Statistical Analysis

All data were analysed using the SPSS statistical package (SPSS 23.0 for Windows; SPSS, Chicago, IL, USA). Means of obtained semen parameters were analysed using the Mann Whitney U test. Data were considered statistically significant when $P \leq 0.05$.

Results and Discussion

Clinical and Semen Laboratory Examinations

There were no symptoms in rams at clinical examination, however, two serologically positive rams showed orchitis and one ram showed epididymitis, which was negative serologically for brucellosis. The volume and concentration of semen, mass activity, percentages of motility and defective acrosome rates in serologically positive and negative small ruminants are presented in Table 1.

There were no significant differences between serologically positive and negative animals for volume, concentration, mass activity, motility and defective acrosome rates ($P>0.05$). Although there were no differences in spermatological parameters between serologically positive and negative animals, motility rate was 20% less in two animals with orchitis and serologically positive.

Bacteriological Results

As a result of bacteriological culture *Trueperella pyogenes* (n:2), *Pasteurella pneumotopica* (n: 5), *Esherichia coli* (n: 3), *Aeromonas salmonicida subs.* Salmonicida (1), *Brevundimonas vesicularis* (n:2), *Mycoplasma bovigenitalium* (n:1) and *Mycoplasma arginini* (n:1) were isolated from semen samples of ram and goats. *Listeria* spp., *Campylobacte*r spp., *Corynebacter* spp., *Aeromonas* spp., *Staphylococcus* spp., *Streptococcus* spp., *Klebsiella* spp. *Ureaplasma* spp. and *Salmonella* sp. were not isolated from any of the specimens. *Brucella* spp. were not isolated from the semen samples.

Molecular Results

No specific band for genus-specific PCR was observed in PCR.

Serological results

Competitive ELISA and Indirect ELISA results were positive for 8 serum samples taken from ram and goats. Weak positivity was seen in 17 samples and 18 were negative for both C-ELISA and I-ELISA. The results were shown in Table 2.

Discussion

In this study, semen samples which were collected from the serologically positive herd were subjected to examination for spermatologic characteristics and diagnosis by culture method, and PCR assay for the detection of *Brucella spp.* DNA. *Brucellae* did not grow culturally from the semen of rams and goats. Even serologically positive animals and weak positives were negative for *Brucellae* by culture method and PCR. That may be attributed to the sampling time. Samples collected at chronic stages of infection might contain few organisms or not at all. It was previously reported that during the chronic stages of the disease when the foci of infection become walled off, infected animals stop excreting the organism (Morgan and MacKinnon, 1979). On the other hand, Corbel (1985), declared that some animals may show false seropositive results due to infection by other Gram-negative microorganisms. *Brucella* spp. were not isolated from the semen samples either directly plated from the solid selective medium or, liquid cultures weekly sub cultured. The negative or false-negative bacteriological results may also be attributed to massive contamination of semen samples and antibiotics given to animals by the owner, (Blasco, 1992). Alton et al. (1988) and Adel et al (2001) declared that a wide variety of culture methods used in different laboratories resulted in considerable differences in performance, variable success and some degree of difficulty. Khamesipour et al. (2013) randomly collected 84 bull's and ram's testis, 45 cattle semen and 315 cattle and sheep blood specimens from slaughterhouses and Artificial Insemination Centre in various parts of Iran. PCR detection for *Brucella* was directly studied from blood, semen and testis samples. PCR results showed that 14 out of 45 cattle semen samples (31.11%), and 63 out of 180 cattle blood samples (35%), and 42 out of 135 sheep's blood samples (31.11%), were positive for *Brucella* spp. Their study indicated that the testis samples in 15.21% of bulls and the testis samples in 10.52% of rams served as a reservoir of disease in Iran and these samples might have been a potential risk for spread of Brucella both in animal and in human. In our study, the testicular tissues were not used as a sample, only semen samples were taken from each ram separately. Despite specimens and serum samples were serologically titrated after vaccination or infections; *Brucella* infection was not spread out through the semen in this study.

Brucella spp. detection procedure was implemented by using Farrel's, modified Thayer-Martin and Cita selective solid media. *Brucella ovis* was not detected in any of semen samples neither by culture nor by PCR. Therefore, it is essential that the diagnosis of *B. ovis* be carried out by serological diagnosis, and be part of the evaluation of fertility in rams, since other bacteria can cause epididymitis or there may be infected animals without apparent clinical signs and relative difficulty in isolating causative agent in chronically infected animals (Ficapal et al., 1998; Mejid et al., 2010). Chronically infected animals and latent carriers may have subfertility or maintain normal fertility, increasing the risk of infection in the flock (Xavier et al., 2009). The culture results of the semen samples in this study showed a variety of the organisms excreted by semen such as; *Trueperella pyogenes* (n:2), *Pasteurella pneumotopica* (n:5), *Esherichia coli* (n:3), *Aeromonas salmonicida subs. Salmonicida* (1), *Brevundimonas vesicularis* (n:2) and Mycoplasma *vesicularis* (n:2), *Mycoplasma bovigenitalium* (n:1) and *Mycoplasma arginini* (n:1). Low sperm motility (60%) and the semen contamination by Mycoplasmas and Urea plasmas were observed in reproductive animals (Eaglesome et al., 1992; Fish et al., 1985, Catania et al., 2014). *Mycoplasma bovigenitalium*, previously called *Mycoplasma* ovine/caprine serogroup 11 (Corrales et al., 2007; Nicholas et al., 2002), was originally isolated from infertile ewes having been introduced by infertile rams that showed abnormal sperm morphology and motility (Lambert, 1987). It has been associated with vulvovaginitis, epididymitis, orchitis, and infertility, resulting in economic losses. *Mycoplasma bovigenitalium* was introduced by infertile rams showing abnormal sperm morphology and motility (Nicholas et al.,1999).

Table 1 The effect of *Brucella spp.* on sperm parameters (means±SEM).

Group	n	Volume (ml)	Mass Activity (+, +++)	Motility (%)	Concentration (x109 spermatozoa/mL)	Defective Acrosome (%)
Brucella (-)	17	2.07±0.28	2.71±0.28	58.24±4.39	2.91±0.32	15.19±1.68
Brucella (+)	25	1.75±0.24	2.76±0.21	55.00±4.08	2.40±0.22	19.25±3.95

There was no significant difference (P>0.05).

Table 2 Semen culture and ELISA results of ram and goats used in this study

List	Animal	Breed	C-ELISA	I-ELISA	Semen Culture
1	Goat	Saanen	+?	?	
2	Goat	Saanen	?	_	*Brevundimonas vesicularis*
3	Goat	Saanen	_		*Aeromonas salmonicida subs. Salmonicida*
4	Goat	Saanen	-	-	
5	Goat	Saanen	-	-	*Mycoplasma arginini, Trueperella pyogenes*
6	Goat	Saanen	_	_	
7	Goat	Saanen	?	_	
8	Goat	Saanen	?	?	
9	Goat	Saanen	+?	?	*E.coli 1*
10	Goat	Saanen	?	_	*Mycoplasma agalactiae*
11	Goat	Saanen	?	_	
12	Goat	Saanen	-	_	*Brevundimonas vesicularis*
13	Goat	Saanen	-	?	
14	Ram	İvesi	?	_	*Mycoplasma agalactiae*
15	Goat	Saanen	?	?	*Mycoplasma agalactiae*
16	Goat	Saanen	?	_	
17	Goat	Saanen			
18	Goat	Saanen	_	_	*E.coli 1, Pasteurella spp.*
19	Goat	Saanen	+	+	
20	Goat	Saanen		?	
21	Goat	Saanen	+?	-	
22	Goat	Saanen	?	?	
23	Goat	Saanen	_	-	
24	Goat	Saanen		-	
25	Goat	Saanen	+	+	*Pasteurella pneumotropica*
26	Goat	Saanen	?	?	
27	Goat	Saanen	?	_	
28	Goat	Saanen	+?	_	*Mycoplasma bovigenitalium*
29	Goat	Saanen	+	?	
30	Goat	Saanen	_	_	
31	Goat	Saanen	_	_	
32	Goat	Saanen	_	_	
33	Goat	Saanen	?	_	*Pasteurella pneumotropica*
34	Ram	Kıvırcık	+?		
35	Ram	İvesi	?	?	
36	Ram	İvesi	_	?	
37	Ram	Kıvırcık	_	_	
38	Ram	Kıvırcık	_	_	*Trueperella pyogenes, E.coli*
39	Goat	Seannen	_	_	
40	Goat	Seannen	-	-	
41	Ram	Kıvırcık			*E.coli 1*
42	Ram	Kıvırcık			*Pasteurella pneumotropica*
43	Goat	Saanen	?	_	

Brevundimonas is one of few bacteria showing high survival rates under simulated Martian conditions (Dartnell et al., 2010). The *Brevundimonas* species are ubiquitous in the environment but are rarely isolated from clinical samples. In this study, two of Seannen goats carried *Brevundimonas vesicularis* without any symptoms, also they were serologically negative for *Brucella*. In this study, *Aeromonas salmonicida subs. salmonicida* was also identified from a goats' ejaculate for the first time. In a study, *Aeromonas hydrophila* was isolated from cases of bovine seminal vesiculitis in south Brazil (Moro et al., 1999). Seminal vesiculitis in teaser animals associated with *Actinomyces pyogenes, Escherichia coli, Brucella abortus, Mycoplasma bovigenitalium, Haemophilus somnus,* and *Pseudomonas aeruginosa* have been described before (Ball et al., 1968, Quinn et al., 2003) Bacterial organisms can migrate up the urethra of teaser animals to the internal reproductive organs and urethra (ascending infection) is a

possible route of an infection. Also, the homosexual activity of young goats and rams confined together is high, and if there were an infectious agent within a group of bucks, adequate opportunity for preputial and penile contamination could exist (Ball et al., 1968; Moro et al., 1999). A theory concerning the pathogenesis of seminal vesiculitis syndrome associated with the neurophysiologic events that could result in reflux of spermatozoa and urine into vesicular glands in teaser animals. Semen or urine could cause chemical irritation and loss of glandular integrity, resulting in further colonization by microorganisms (Linhart et al., 1988).

In this study, the serological results by Competitive ELISA and Indirect ELISA showed positivity for twenty-five [n:25 (59.52%)] serum samples. Seventeen [n:17 (40.48%)] were negative for both C-ELISA and I-ELISA, which are the serological tests employed in this study are proved to be highly sensitive according to Alton et al., (1988). However, in our study, we used serological tests that only detect smooth lipopolysaccharide which is absent in *B.ovis*. Therefore, in our study, all serologically positive animals were assumed to be exposed to smooth Brucellae. On the other hand, it has been reported that male sheep and goats in an infected herd were not infrequently become infected with *B.melitensis* themselves. In our study, orchitis was diagnosed in two bucks in the serologically positive group. These findings agree with Andreani et al. (1967), who observed that rams were positive serologically, yet none developed any symptoms, and all were negative pathologically and bacteriologically when sacrificed 7 or 8 months after exposure to infection source. Additionally, seven (n:7) out of twenty-six (n:26) *B. ovis* strains (26.9%) were cultured from the epididymites of rams which did not show palpable epididymal and/or testicular lesions (Dénes and Glávits, 1994). The reason for the absence of testicular inflammation in all serologically positive cases may be associated with the chronicity of the infection. In a study done by Van Metre et al. (2012) indicated that 53.6% of seropositive rams to *B. ovis* had a normal fertility evaluation, without abnormalities detected on physical examination or semen. In this study, similar results were observed in terms of semen volume, concentration, mass activity, motility and defective acrosome rates in serologically positive and negative groups (P>0.05). However, sperm motility and other spermatologic characteristics were negatively affected by testicular or epididymal lesions regardless of serological reactions to smooth Brucella spp.

Generally, in Brucella infections, epididymal ducts show epithelial hyperplasia with interepithelial cysts containing neutrophils and cellular debris. Testicular atrophy derives regressive process in the testicular epithelium and cessation of spermatogenesis. Infected rams show poor semen quality and lowered fertility. Both the total concentration and the proportion of normal living spermatozoa are spectacularly reduced in rams with testicular alterations Even in the absence of palpable epididymitis, infected animals may have poor semen quality. (Blasco, 1990; Cameron and Lourman, 1976). In the present study, the infection with *Brucella* spp. occurred naturally; considering this, the percentage of animals serologically positive that presented testicular lesion was lower (4.76%) than (30.56%) decelerated by Carrera-Chávez et al. (2016).

In this study, the distribution of *Brucella*-DNA in semen was determined by separating naturally infected semen samples into seminal plasma which were then analysed by the PCR assay. All semen samples derived from different rams and goats were fractionated. The template DNA from each seminal fraction derived from equivalent amount of semen was analysed by PCR using specific primers for Brucella genus detection (Bricker and Halling, 1994). None of the amplicons yielded 223 bp fragment in PCR. A protocol was adopted to purify *Brucella*-DNA from bovine and ovine semen samples by Von Beroldingen et al. (1990) who concluded that direct PCR detection of *B. melitensis* DNA in semen was impractical due to the presence of inhibitory components in semen (Von Beroldingen et al., 1990). Preliminary experiments regarding the isolation methods suggested that most *Brucellae* might be present in the non-sperm cell fraction. This finding may be supported by the conclusions previously reported by Adel et al. (2001). The inhibition might have been observed in whole semen samples was primarily due to the high DNA concentrations. A similar finding was previously reported by Von Beroldingen et al. (1990) and Adel et al. (2001). It is important to prevent transmission of brucellosis by artificial insemination, only Brucella-free semen collected from *Brucella* free donors should be used (Adel et al., 2001). The first PCR assay for detection of Brucellae in semen samples of goats and rams was reported by Adel et al. (2001). They concluded that the PCR assay was a good supplementary test for detection of Brucella organisms in the semen of infected animals. They also recommended the use of the PCR, as a routine assay, for the detection of Brucella-DNA in seminal fluid and non-sperm cell fractions rather than on whole semen samples. They recommended the PCR assay could be of value in examining semen samples obtained from donor animals of the unknown history of the disease or suspicious animals; showing week serological results. Considering the data of our work, although serological tests were defined as weak positive or positive for smooth *Brucellae*, they were all negative in culture and PCR from semen samples. This contradiction suggests that the scattering of *Brucella* with semen may be intermittent depending on the pathogenesis of the disease. In sheep and goat males, the infection can be located in testis, epididymis, seminal vesicle and ampulla of deferent ducts, producing inflammation of genital organs. In the acute phase, it can be detected orchitis with inflammation of tunica vaginalis, and the scrotal sac can be distended by either hemorrhagic or fibrino-purulent exudate. In a chronic stage, hygromas and joints' inflammation can be observed in male goats. The main output of the disease in males is semen of bad quality and a consequent fertility loss (Megid et al., 2010; Enright, 1993; Leon, 1994; Jubb, 1993). This study commentated that *Brucella* might not be detected via spread out through the sperms, which may be attributed to the stage of sampling or few organisms in semen that might not allow isolation of the organism. Asymptomatic carriage of *Brucella spp.* in rams and goats should be detected in herds and artificial insemination centres every 6 months. The surveillance protocols should be applied to reduce the risk of transmission of Brucella infection to prevent the rams and goats. To achieve these goals, serological tests that also detect rough *Brucella* species

including B.ovis need to be developed and used routinely as a part of the serologic diagnosis of brucellosis in general for a more accurate evaluation of the disease in sheep and goats.

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