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# Infectious epididymitis caused by *Brucella ovis* in Croatian sheep flocks

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**Abstract:** The distribution of infection by the species *Brucella ovis* in rams and abortion cases in sheep in Croatia was investigated in the period from 2011 to 2015. The investigation relied on serological testing in rams and sheep after abortion conducted using a complement fixation test and immunosorbent assay conducted with bacteriological testing of the testes in seropositive rams and aborted material. Seropositive reactions were confirmed in 1100 (6%) of rams samples and in 12 (2.3%) of ewe samples. Isolated strains were also confirmed on the molecular level. In 10 (45.5%) animals asymmetry of the scrotum and unilateral enlargement of the tail of the epididymis were confirmed. Pathomorphological examination revealed visible changes such as granulomas, fibrosis, and atrophy of the testes and epididymis in 12 (54.5%) ram testes. In bacteriological investigation of samples from 22 rams and fetuses following abortion in ewes, 13 (59.1%) and 2 (14.3%) isolations were performed, respectively. All 15 isolates were identified as *Brucella ovis* using a molecular method. The results show that infectious epididymitis caused by *Brucella ovis* is distributed throughout the study area in Croatia. The disease most often spreads through uncontrolled trade or contacts of sheep between flocks, which significantly hinders eradication measures.

**Key words:** *Brucella ovis*, ovine epididymitis, prevalence, multiplex PCR

## 1. Introduction

Ovine epididymitis is a chronic disease caused by the bacterium *Brucella ovis*, with characteristic changes in rams testes and epididymis and the sheep placenta. It causes significant economic losses in sheep flocks without disease control measures (1). Losses are seen as the reduced fertility of rams, abortion in ewes, early death of avital lambs, and the removal of affected animals from the flock and bans in trade. *B. ovis* is considered the most important infectious agent causing reproductive disorders in sheep worldwide (2–5). At the first appearance of the disease, the percentage of infected rams is very high, between 20% and 60%. It is possible to found 45%–75% infected flocks. In countries with advanced control programs, the incidence is significantly lower, though complete eradication is difficult to achieve (6,7).

Špičić et al. (8) first described infectious epididymitis in rams in Croatia. The disease was later proven to be present in most counties in the country (9). Infection with the species *B. ovis* has been confirmed in virtually all countries with prominent sheep production. It has been reported in the neighboring countries of Slovenia (10) and Serbia (5,11). It has also been confirmed in Romania (12), Austria (13), Italy (14), Switzerland (15), Spain (3), Russia (16), Ukraine (17), Australia (2,18), New Zealand (19,20), Canada (4,21), and the United States (22).

The objective of this study was to determine the prevalence of ovine epididymitis in different regions of Croatia. Serological testing was conducted over a 5-year period (2011 to 2015) annually in rams and in sheep after abortion, and material from rams and aborted fetuses was used to isolate and identify the causative agent using bacteriological and molecular methods.

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## 2. Materials and methods

During the 5-year study period (2011 to 2015), the blood serum of 18,324 rams originating from 20 counties and the city of Zagreb, and 521 ewes from flocks where abortions were reported, were tested serologically for *B. ovis* infection. Blood sample testing was conducted on 6028 samples from rams and 87 samples from ewes in 2011; 1477 ram samples and 67 ewe samples in 2012; 2312 ram samples and 87 ewe samples in 2013; 3871 ram samples and 153 ewe samples in 2014; and 4636 ram samples and 127 ewe samples in 2015. A total of 5 to 10 mL of blood was extracted from the jugular vein of each tested animal. Blood samples were centrifuged in the laboratory at 1500 rpm, and the serum samples were stored at –20 °C until testing.

### 2.1. Serological testing

To identify the antibodies for *B. ovis*, we used the indirect enzyme linked immunosorbent assay (iELISA) and the complement fixation test (CFT). We used the indirect immunoenzyme test CHEKIT – *Brucella ovis* (IDEXX, Germany). The procedure was performed according to the manufacturer's instructions, and the results were read on the Tecan Sunrise spectrophotometer at a wavelength of 450 nm. The CFT was performed on microtiter plates (micro method), according to World Organisation for Animal Health (OIE) recommendations (23). A positive result was considered as a quantity of antibodies of ≥50 ICFTU/mL serum. In the test, we used the R-LPS antigen *B. ovis* (VLA Waybridge, UK), an amboceptor (hemolysin) and 2% ram erythrocytes (CVI, Zagreb, Croatia).

### 2.2. Clinical and pathomorphological examinations

Biological material was taken with the owner's consent from serologically positive rams for pathomorphological and bacteriological tests. The testes, epididymis, and lymph nodes (*lnn. inguinalis*, *lnn. ilici mediales*, and *lnn. lumbales aortici*) were sampled after castration (11 rams) and slaughter (11 rams). These animals originated from the following 4 counties: Zagreb, Primorje-Gorski Kotar, Bjelovar-Bilogora, and Sisak-Moslavina. The organs and stomach contents of 8 aborted fetuses and 6 placentas taken at the time of ewe abortion were also tested for *B. ovis* infection.

### 2.3. Bacteriological isolation

Several grams of material (testes, lymph nodes, placenta, and fetal organs and 1 mL of stomach content of aborted fetuses) were processed, and approximately 1 mL of homogenate was inoculated on selective agars, i.e. blood agar (blood agar base, Cat. No. 110328, Merck KGaA, Darmstadt, Germany), *Brucella* agar (*Brucella* medium base, Oxoid CM0169, Oxoid Ltd., Basingstoke, United Kingdom), and modified semiselective agar according to Thayer-Martin with added VCN Selective Supplement SR0101E (Oxoid Ltd.) (24,25). Petri dishes with inoculated materials were incubated at 37 °C in the presence of 10% $CO_2$, and colony growth was observed at daily intervals.

### 2.4. Classical identification

#### 2.4.1. Morphological characteristics

Isolates were identified on the basis of colony morphology, growth in the presence of 10% $CO_2$, production of $H_2S$, growth on media with the addition of 20 μg/mL thionine and basic fuchsine, and agglutination of antiserums (24,26).

#### 2.4.2. Molecular identification

Fifteen isolates were examined using the polymerase chain reaction (PCR) test. A loopful of bacterial culture was mixed in 100 μL of distilled water (UltraPure DNase⁄RNase-Free Distilled Water, Invitrogen, Paisley, UK), boiled at 95 °C for 20 min, and centrifuged at 14,000 × *g* for 1 min. The supernatant was used in the PCR reaction. The controls used in molecular investigations were standard *Brucella* strains: *B. abortus* 544, *B. suis* 1330, *B. melitensis* 16M, and *B. ovis* 63/290. PCR based on replication of the part of the genome that codes for the synthesis of the protein BCSP-31, characteristic for the genus *Brucella*, was used in order to identify the genus *Brucella*. The expected product size was approximately 440 bp (27). A multiplex PCR (Bruce-ladder) was used to identify the *Brucella* species (28). The expected sizes of the PCR products were 1072, 794, 587, 450, and 152 bp for *B. ovis*; 1682, 794, 587, 450, and 152 bp for *B. abortus*; 1682, 1072, 794, 587, 450, and 152 bp for *B. melitensis*; and 1682, 1072, 794, 587, 450, 272, and 152 bp for *B. suis*. The products were analyzed using a QIAxcel capillary electrophoresis system (QIAGEN, Hilden, Germany).

### 2.5. Statistical analysis

Numerical data were processed using the Stata 13.1 statistical package (Stata Corp., USA). Univariate analysis was performed using chi-square or Fisher exact tests in order to compare frequencies of seropositive animals between counties, groups of counties, and years of the research. The geographic position of a county of animal origin was the criterion for groupings of counties. Group 2 consisted of southern counties attached to the coastline, while group 1 contains all other counties. A logistic regression model was used to analyze the association of the *Brucella* test results with the year of observation and geographic location.

## 3. Results

### 3.1. Serological results

During the study period from 2011 to 2015, a total of 18,324 serum samples were examined from breeding rams, and positive reactions were found in 1100 (6%) of animals (Figure 1; Table 1). From 521 ewe blood samples, positive reactions were found in 12 (2.3%) of the samples. Observed

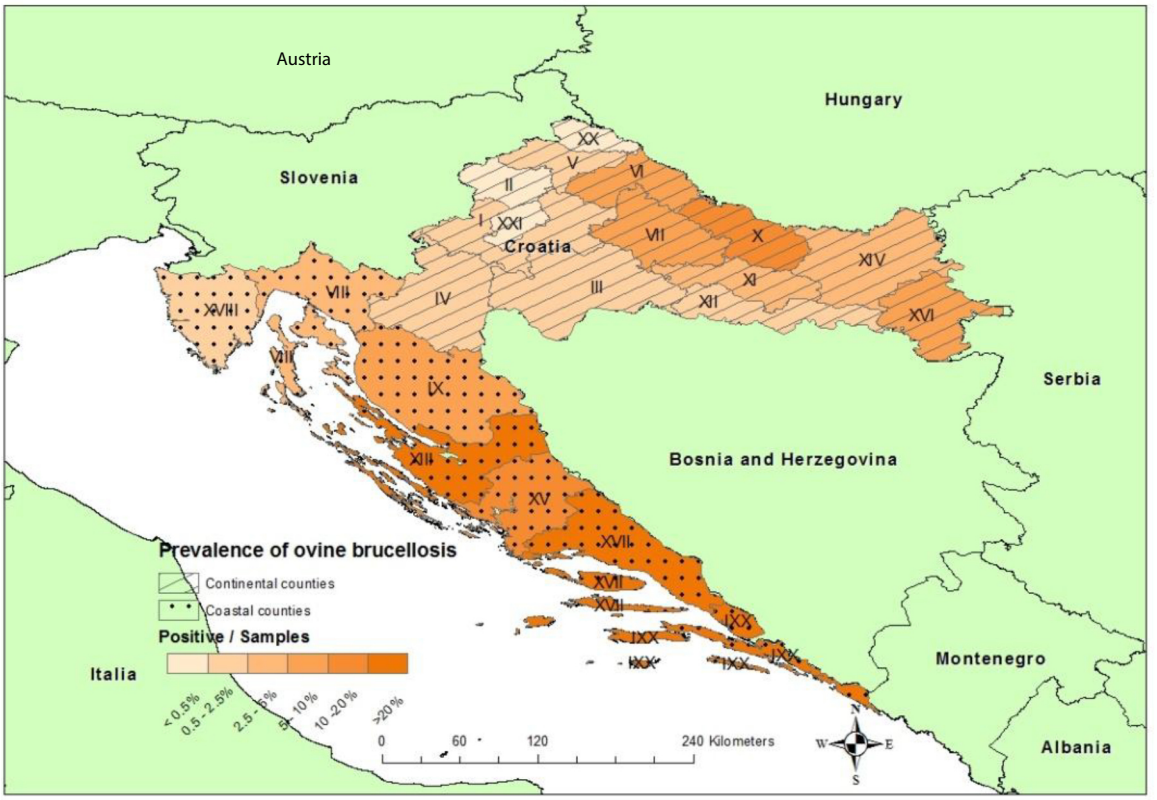


**Figure 1.** Distribution of ovine epididymitis in Croatia.

**Table 1.** Seroprevalence of ovine epididymitis in rams during 2011–2015 by counties.

| County | Negative | Positive | (%) | Total |
|---|---|---|---|---|
| I. Zagreb | 1545 | 36 | (2.3%) | 1581 |
| II. Krapina-Zagorje | 525 | 1 | (0.2%) | 526 |
| III. Sisak-Moslavina | 3856 | 32 | (0.8%) | 3888 |
| IV. Karlovac | 2478 | 41 | (1.6%) | 2519 |
| V. Varaždin | 279 | 2 | (0.7%) | 281 |
| VI. Koprivnica-Križevci | 418 | 42 | (9.1%) | 460 |
| VII. Bjelovar-Bilogora | 1823 | 134 | (6.5%) | 1957 |
| VIII. Primorje-Gorski Kotar | 261 | 9 | (3.3%) | 270 |
| IX. Lika-Senj | 662 | 56 | (7.8%) | 718 |
| X. Virovitica-Podravina | 867 | 12 | (12.%) | 992 |
| XI. Požega-Slavonija | 826 | 43 | (4.9%) | 869 |
| XII. Brod-Posavina | 187 | 2 | (1.1%) | 189 |
| XIII. Zadar | 314 | 136 | (30.3%) | 450 |
| XIV. Osijek-Baranja | 1055 | 39 | (3.6%) | 1094 |
| XV. Šibenik-Knin | 1069 | 223 | (17.2%) | 1292 |
| XVI. Vukovar-Srijem | 102 | 6 | (5.6%) | 108 |
| XVII. Split-Dalmatia | 312 | 154 | (33%) | 466 |
| XVIII. Istria | 388 | 3 | (0.8%) | 391 |
| XIX. Dubrovnik-Neretva | 21 | 15 | (41.7%) | 36 |
| XX. Međimurje | 17 | 0 | (0%) | 17 |
| XXI. City of Zagreb | 219 | 1 | (0.5%) | 220 |
| Total | 17,224 | 1100 | (6%) | 18,324 |

differences of ovine epididymitis occurrence between years are statistically significant (P < 0.0001, Table 2) and observed differences of ovine epididymitis frequency between groups of counties are statistically significant (P < 0.0001, Table 3). Animals that originated from southern counties (group 2) had 10.46 times higher odds of having ovine epididymitis compared to the animals from group 1 (P < 001). The odds ratio of seroprevalence in rams was higher by 1.52 times each consecutive year (P < 0.001, Table 4).

### 3.2. Clinical and pathomorphological results

Inspection and palpation of the epididymis and testes was performed on 22 seropositive rams, and in 10 (45.5%) animals asymmetry of the scrotum and unilateral enlargement of the epididymis tail were found.

### 3.3. Pathomorphological results

Macroscopic examination of the epididymis and testes in 22 rams showed pathological changes (mostly granulomas, fibrosis, and atrophy), suggesting infection by *B. ovis*. Positive results were found in 12 (54.5%) of the animals.

### 3.4. Bacteriological results

A total of 13 (59.1%) isolates were isolated from ram testes and 2 (14.3%) isolates from the aborted fetuses and placenta.

### 3.5. Molecular identification

In the PCR test (*BCSP-31* gene), all 15 isolates were identified as belonging to the genus *Brucella* spp. All 15 *Brucella* isolates were further identified as belonging to the species *B. ovis* using the Bruce-ladder method (Figure 2).

**Table 2.** Results of serological testing of rams in the period from 2011 to 2015.

| Year | Number of ram blood samples | Number of positive samples | % of positive samples |
|---|---|---|---|
| 2011 | 6028 | 276 | 4.25 |
| 2012 | 1477 | 71 | 4.81 |
| 2013 | 2312 | 122 | 5.28 |
| 2014 | 3871 | 307 | 7.93 |
| 2015 | 4636 | 324 | 6.99 |
| Total | 18,324 | 1100 | 6.0 |

Observed differences of ovine epididymitis frequency between years are statistically significant (P < 0.0001) (Figure 1).

**Table 3.** Seroprevalence of ovine epididymitis in rams by groups of counties.

| Group of counties | Negative | Positive | Total |
|---|---|---|---|
| 1* | 14,197 | 504 | 14,721 |
| 2* | 3027 | 596 | 3623 |
| Total | 17,244 | 1100 | 18,324 |

*Group 2 consists of southern counties attached to the coastline; group 1 contains all other counties.

**Table 4.** Association of seroprevalence in rams in space and time.

| Factor | OR | SE (OR) | P | 95% CI |
|---|---|---|---|---|
| Location | 10.46 | 0.77 | 0.000 | 9.04–12.11 |
| Year | 1.52 | 0.03 | 0.000 | 1.45–1.59 |

OR - Odds ratio; SE (OR) - standard error of odds ratio; P - P-value; 95% CI - 95% confidence interval.

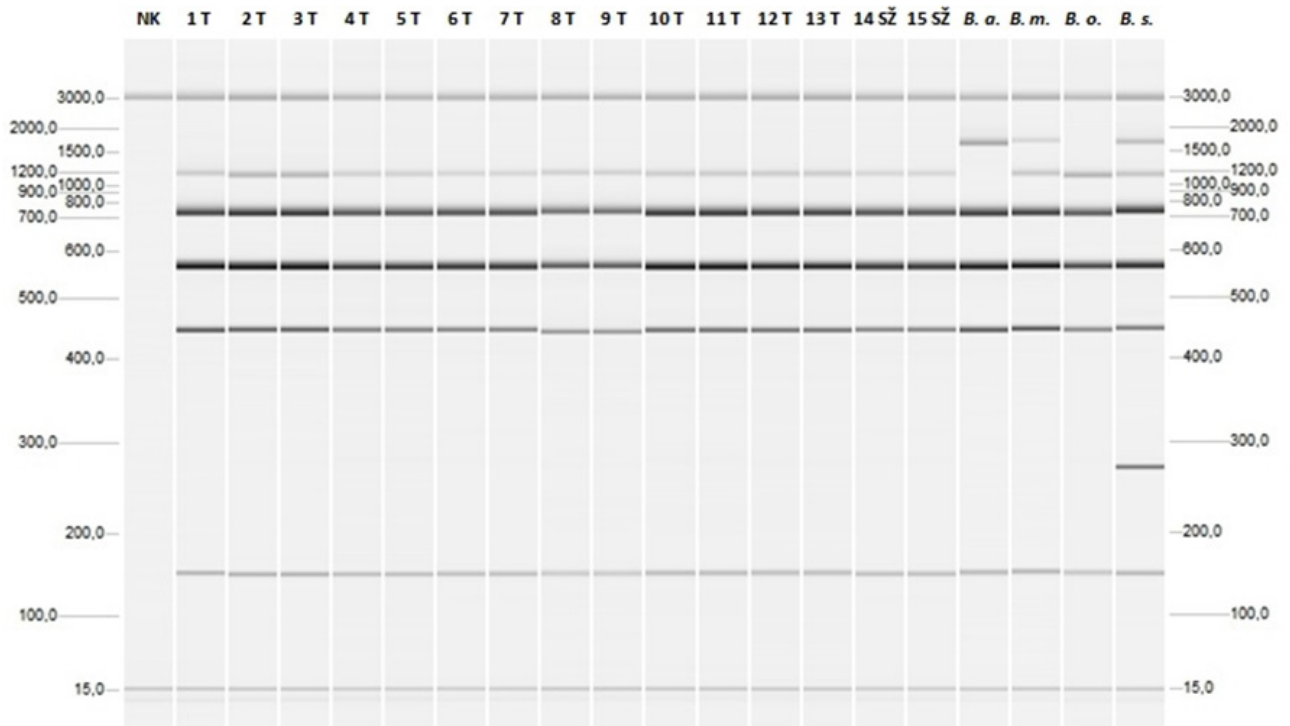


**Figure 2.** Identification of *B. ovis* strains by multiplex PCR (Bruce-ladder).
NK - Negative control; 1 T – 13 T - *B. ovis* strains isolated from the testicles of rams; SR 14 and 15 - *B. ovis* strains isolated from the stomach contents of aborted fetuses of sheep; Positive control - B. a. - *B. abortus* 544; B. m. - *B. melitensis* 16M; B. o. - *B. ovis* 63/290; B. s. - *B. suis* 1330; designations on the left- and right-hand columns represent the values of markers indicating the size of the product of multiplying the base pairs: 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000, and 3000 bp.

## 4. Discussion

Ovine epididymitis has been reported in all countries with a sheep-breeding tradition. The causative agent can also be isolated from seronegative and clinically healthy rams (29). The first report of the disease in Croatia was in 2002, and positive reactions were confirmed in 7% of investigated flocks (8). A later study described the incidence of 2% serologically positive sheep in 10 Croatian counties (9). The aim of this study was to determine the recent prevalence of ovine epididymitis in different counties of Croatia. This study confirmed a positive reaction in 1100 (6%) of 18,324 ram blood samples and 12 (2.3%) of ewe blood samples from 20 Croatian counties. In a southern region of Serbia, positive reactions were found in 29.8% of studied rams (5). A similar investigation in France found that about half of rams were effectively seropositive by iELISA (53.7%) and by CFT (37.2%) (7). The same authors also confirmed an association between *B. ovis* shedding in semen and seropositivity (CFT and iELISA) (7). Another investigation conducted in Brazil using the AGIT test on 705 flocks found 20 (2.5%) positive flocks (30). In the western United States, the adjusted seroprevalence using iELISA was detected as *B. ovis* antibodies among tested rams at a rate of 10.0% (31). The high seroprevalence of infection in rams is still the consequence of the lack of implementation of control programs over the years (32,33). Epizootiological data indicate that the disease has spread through flocks via sexual contact during natural mating. The odds ratio for having ovine epididymitis is higher in southern counties compared to northern counties (OR = 10.46, $P < 0.0001$). The level of implementation of any disease control in southern counties is generally considered as lower than in the rest of Croatia due to several reasons, mainly demographics and postwar status. In sheep breeding, rams are often introduced without any controls and are commonly traded between flocks, which furthers the spread of the disease. Furthermore, the possibility of uncontrolled exchange of breeding animals with owners from the neighboring countries cannot be overlooked. The rate of infection has been steadily rising over the years (OR = 1.52, $P < 0.0001$), which also supports the statement regarding the lack of effective control.

In the present study, visible pathological changes were proven in 54.5% of ram testes in previously seropositive animals. Owners most often observe asymmetry of the scrotum or enlarged epididymis and testes in one-third of rams. Other studies also found characteristic changes like unilateral epididymitis with hypertrophy of the tail,

body, or head of the epididymis (28). In investigation conducted in Ukraine on seropositive rams found chronic pathological changes in 60% of examined rams (17). A conducted experimental infection of 9 rams with *B. ovis* found that 6 (66.7%) animals developed clinical changes in the epididymis tail, and 5 of those 6 had unilateral changes (83.3%) (34). Of 233 rams seropositive for *B. ovis*, 125 (53.6%) were subclinical cases, a finding that supports the importance of this test in rams (31). Similar results were found in study conducted in Spain, where some kinds of alterations in ram testes were found in 60.6% animals. All animals were previously seronegative using the immunogel diffusion technique (35).

It has been proven that sperm of infected rams without pathological changes may also shed *B. ovis* (36). In this study, *B. ovis* was isolated in 59.1% of samples, which is a significant link between the visible pathological changes, which were found in 54.5% of examined testes samples. In a French study, *B. ovis* was isolated from 89 (44.95%) of 198 rams and the authors found a significant association ($P < 0.05$) between the results of bacteriological testing and clinical examinations (7). The isolates in the present study were identified as belonging to the species *B. ovis*, with an identical PCR profile in relation to the reference strain *B. ovis* 63/290, which confirms the high specificity of the multiplex PCR Bruce-ladder method.

The obtained results show that ovine epididymitis caused by *B. ovis* is widely distributed in Croatia. The disease primarily spreads through the uncontrolled trade of rams between flocks, which significantly hinders disease control. There are a number of approaches to controlling infection of rams by *B. ovis* applied in different countries and regions, depending on the economic capabilities. Eradication of the disease (testing and slaughter) is the most desirable means where this is logistically and financially feasible (20,37). It was stated that this approach includes a combination of serological (iELISA and CFT) and auxiliary (clinical examination) tests and bacteriological testing of semen (20). It is recommended that this control measure be applied before every mating season and prior to the introduction of new rams into a flock to ensure that they are disease-free. In flocks with a high incidence of the disease, this strategy could be financially unsustainable. There are good indications in the development of a new vaccine against *B. ovis* that has thus far been tested on mice (38). However, to date, only the vaccine *B. melitensis* Rev 1 has proven to be effective in the control of infections with the species *B. ovis* (1,7).

In conclusion, surveillance of ovine epididymitis at the national herd level could be conducted with serological testing of rams and cases of abortions in sheep. First evidence of disease should be confirmed by bacteriological or molecular investigation. Further national programs of control and eradication should be applied at the herd level without exceptions.

## References


1. Blasco JM. *Brucella ovis*. In: Nielsen K, Duncan JR, editors. Animal Brucellosis. Boca Raton, FL, USA: CRC Press; 1990. pp. 351-378.
2. Burgess GCW. Ovine contagious epididymitis: a review. Vet Microbiol 1982; 7: 551-575.doi:10.1016/0378-1135(82)90049-9doi:10.1016/0378-1135(82)90049-9.
3. Ficipal A, Jordana J, Blasco JM, Moriyon I. Diagnosis and epidemiology of *Brucellaovis* infection in rams. Small Rum Res 1998; 29: 13-19.
4. Arsenault J, Girard C, Dubreuil P, Belanger D. Lack of evidence of *Brucellaovis* infection in rams in Quebec. Can Vet J 2004; 45: 312-314.
5. Petrović M, Špičić S, Potkonjak A, Lako B, Kostov M, Cvetnić Ž. First evidence of *Brucella ovis* infection in ram sin the Pirot Municipality, Serbia. Vet Ital 2014; 50: 259-268.
6. Blasco JM, Buen L, Estrada J, Garcia J, Llena J, Ortilles A. Alteraciones testiculares v brucelosis en moruecos de la region Aragonesa. Noticias Neosan 1983; 211: 147 (in Spanish).
7. Picard-Hagen N, Berthelot X, Champion JL, Eon L, Lyazrhi F, Marois M, Peglion M, Schuster A, Trouche C, Garin-Bastuji B. Contagious epididymitis due to *Brucella ovis*: relationship between sexual function, serology and bacterial shedding in semen. BMC Vet Res 2015; 125: 2-7.
8. Špičić S, Marjanović S, Zdelar-Tuk M, Cvetnić Z. First evidence of *Brucella ovis* infection in Republic of Croatia. Dtsch Tierär Wochen 2009; 116: 209-213.
9. Špičić S, Marjanović S, Zdelar-Tuk M, Cvetnić Z. Serological, bacteriological and molecular diagnosis of brucellosis in domestic animals in Croatia. Cro Med J 2010; 51: 320-326.
10. Krt B. Evaluation of similar serological methods for the diagnosis of ovine brucellosis – infection with *Brucellaovis*. MSc, University of Ljubljana, Ljubljana, Slovenia, 1992.
11. Petrović M, Špičić S, Potkonjak A, Lako B, Kostov M, Cvetnić Ž. Epizootiological, clinical and pathological characteristics of sheep flocks infected with *Brucella ovis* in the Republic of Serbia. Slov Vet Res 2013; 3: 117-124.
12. Dobrean V, Opris A, Daraban S. An epidemiological and surveillance overview of brucellosis in Romania. Vet Microbiol 2002; 90: 157-163.

13. Schöpf K, Khaschabi D. Experiences in the eradication of *Brucella ovis* infections in sheep in Tyrol. Tierarztl Prax Ausg G Grosstiere Nutztiere 1997; 5: 413-416.

14. Farina R, Cerri D, Andreani G, Renzoni P, Gaudachini F, Lombardi G. Prima segnalazione sulla presenza di *Brucella ovis* in Italia. Sel Vet 1995; 36: 285-291 (in Italian).

15. Hold F, Zerobin K. *Brucella ovis* infection in rams of the "white Alp" breed. Schweiz Arch Tierheil 1993; 135: 44-50.

16. Kalinovski AI, Repina LP, Innokenteeva TI. Brucellosis in Siberia and the Far East. Med Parazitol (Moskva) 1995; 4: 42-45.

17. Denes B, Glavitz R. Bacteriologically confirmed cases of ovine epididymo-orchitis caused by *Brucella ovis* in Sub-Carpathia. Acta Vet Hung 1994; 42: 25-33.

18. Hopkinson WI, Lloyd J, Micke BM. *Brucella ovis* in Merino rams in western Australia. Aust Vet J 1979; 55: 200-201.

19. Sergeant ES. Seroprevalence of *Brucella ovis* infection in commercial ram flock in the Tamwort area. N Z Vet J 1994; 42: 97-100.

20. Ridler AL, Smith SL, West DM. Seroconversion and semen shedding in rams experimentally infected with *Brucella ovis*. NZ Vet J 2014; 62: 47-50.

21. Niilo L, MacDonald DW, Godkin GF, Stone MW. Ovine brucellosis in Alberta. Can Vet J 1986; 27: 245-249.

22. Bagley CV, Paskett ME, Matthews NJ, Stequist NJ. Prevalence and causes of ram epididymitis in Utah. J Am Vet Med Assoc 1985; 186: 798-801.

23. OIE-World Organisation for Animal Health. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris, France: OIE; 2014.

24. Alton GG, Jones LM, Angus RD, Verger JM. Techniques for the Brucellosis Laboratory. 1st ed. Paris, France: INRA; 1988.

25. Marin CM, Alabart JL, Blasco JM. Effect of antibiotics contained in two *Brucella* selective media on growth of *B. abortus*, *B. melitensis* and *B. ovis*. J Clin Microbiol 1996; 34: 426-428.

26. Corbel MJ, Gill KPW, Thomas EL. Methods for the Identification of *Brucella*. New Haw, UK: MAFF; 1983.

27. Serpe L, Gallo P, Finandza N, Scaramuzzo A, Fenizia D. Single-step method for rapid detection of *Brucella* spp. in soft cheese by gene-specific polymerase chain reaction. J Dairy Res 1999; 66: 313-317.

28. Garcia-Yoldi D, Marin CM, De Miguel PM, Munoz PM, Vizmanos JL, Lopez-Goni I. Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. Clin Chem 2006; 52: 779-781.

29. Olsen SC, Palmer MV. Advancement of knowledge of *Brucella* over the past 50 years. Vet Pathol 2016; 51: 1076-1089.

30. Machado G, Santos DV, Kohek I, Stein MC, Hein HE, Poeta AS, Vidor ACM, Corbellini LG. Seroprevalence of *Brucella ovis* in rams and associated flock level risk factors in the state of Rio Grande do Sul, Brazil. Prev Vet Med 2015; 12: 1183-1187.

31. Van Metre DC, Rao S, Kimberling CV, Morley PS. Factors associated with failure in breeding soundness examination of Western USA rams. Prev Vet Med 2012; 105: 118-126.

32. Bulgin MS, Anderson BC. Association of sexual experience with isolation of various bacteria in cases of ovine epididymitis. J Am Vet Med Assoc 1983; 182: 372-374.

33. Blasco JM, Marin CM. Brucelosis ovina: Etiologia, diagnostico bacteriologico. Ovis 1990; 8: 15-22 (in Spanish).

34. Carvalho Júnior CA, Moustacas VS, Xavier MN, Costa EA, Costa LF, Silva TMA, Paixão TA, Borges AM, Gouveia AMG, Santos RL. Andrological, pathologic, morphometric, and ultrasonographic findings in rams experimentally infected with *Brucella ovis*. Small Rum Res 2011; 102: 213-222.

35. Mozo R, Galeote AI, Alabart JL, Fantova E, Folch J. Evaluating the reproductive ability of breeding rams in North-Eastern Spain using clinical examination of the body and external genitalia. BMC Vet Res 2015; 11: 289.

36. Swift BLF, Craddock H, Hancock A. Ram epididymitis: a clinical report. Theriogenology 1982; 17: 343-347.doi:10.1016/0093-691X(82)90094-2doi:10.1016/0093-691X(82)90094-2.

37. Ridler AL, West M. Control of *Brucella ovis* infection in sheep. Vet Clin N Am Food A 2011; 27: 61-66.

38. Sancho P, Tejedor C, Sidhu-Munoz RS, Fernandez-Lago L, Viscaino N. Evaluation in mice of *Brucella ovis* attenuated mutants for use as live vaccines against *B. ovis* infection. Vet Res 2014; 45: 61.