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

Željko CVETNIĆ1, Maja ZDELAR-TUK1, Sanja DUVNJAK1, Miroslav BENIĆ2, Željko MIHALJEVIĆ3, Boris HABRUN4, Irena REIL1, Marija CVETNIĆ5, Silvio ŠPIČIĆ1,*

1Laboratory for Bacterial Zoonoses and Molecular Diagnostics of Bacterial Diseases, Department of Bacteriology and Parasitology, Croatian Veterinary Institute, Zagreb, Croatia
2Laboratory for Mastitis and Raw Milk, Department of Bacteriology and Parasitology, Croatian Veterinary Institute, Zagreb, Croatia
3Department of Pathological Morphology, Croatian Veterinary Institute, Zagreb, Croatia
4Laboratory for General Bacteriology and Mycology, Department of Bacteriology and Parasitology, Croatian Veterinary Institute, Zagreb, Croatia
5Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

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 avitai 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.
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 \textit{Brucella} 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 \textit{B. ovis}, we used the indirect enzyme linked immunosorbent assay (iELISA) and the complement fixation test (CFT). We used the indirect immunoenzyme test CHEKIT – \textit{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 \textit{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 (\textit{lnn. inguinalis, lnn. iliici 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 \textit{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), \textit{Brucella} agar (\textit{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\textsubscript{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\textsubscript{2}, production of H\textsubscript{2}S, growth on media with the addition of 20 µg/mL thionine and basic fuchsine, and agglutination of antiseraums (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 $\times$ g for 1 min. The supernatant was used in the PCR reaction. The controls used in molecular investigations were standard \textit{Brucella} strains: \textit{B. abortus} 544, \textit{B. suis} 1330, \textit{B. melitensis} 16M, and \textit{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 \textit{Brucella}, was used in order to identify the genus \textit{Brucella}. The expected product size was approximately 440 bp (27). A multiplex PCR (Bruce-ladder) was used to identify the \textit{Brucella} species (28). The expected sizes of the PCR products were 1072, 794, 587, 450, and 152 bp for \textit{B. ovis}; 1682, 794, 587, 450, and 152 bp for \textit{B. abortus}; 1682, 1072, 794, 587, 450, and 152 bp for \textit{B. melitensis}; and 1682, 1072, 794, 587, 450, 272, and 152 bp for \textit{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 \textit{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.

<table>
<thead>
<tr>
<th>County</th>
<th>Negative</th>
<th>Positive</th>
<th>(%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Zagreb</td>
<td>1545</td>
<td>36</td>
<td>(2.3%)</td>
<td>1581</td>
</tr>
<tr>
<td>II. Krapina-Zagorje</td>
<td>525</td>
<td>1</td>
<td>(0.2%)</td>
<td>526</td>
</tr>
<tr>
<td>III. Sisak-Moslavina</td>
<td>3856</td>
<td>32</td>
<td>(0.8%)</td>
<td>3888</td>
</tr>
<tr>
<td>IV. Karlovac</td>
<td>2478</td>
<td>41</td>
<td>(1.6%)</td>
<td>2519</td>
</tr>
<tr>
<td>V. Varaždin</td>
<td>279</td>
<td>2</td>
<td>(0.7%)</td>
<td>281</td>
</tr>
<tr>
<td>VI. Koprivnica-Križevci</td>
<td>418</td>
<td>42</td>
<td>(9.1%)</td>
<td>460</td>
</tr>
<tr>
<td>VII. Bjelovar-Bilogora</td>
<td>1823</td>
<td>134</td>
<td>(6.5%)</td>
<td>1957</td>
</tr>
<tr>
<td>VIII. Primorje-Gorski Kotar</td>
<td>261</td>
<td>9</td>
<td>(3.3%)</td>
<td>270</td>
</tr>
<tr>
<td>IX. Lika-Senj</td>
<td>662</td>
<td>56</td>
<td>(7.8%)</td>
<td>718</td>
</tr>
<tr>
<td>X. Virovitica- Podravina</td>
<td>867</td>
<td>12</td>
<td>(12.%)</td>
<td>992</td>
</tr>
<tr>
<td>XI. Požega-Slavenija</td>
<td>826</td>
<td>43</td>
<td>(4.9%)</td>
<td>869</td>
</tr>
<tr>
<td>XII. Brod-Posavina</td>
<td>187</td>
<td>2</td>
<td>(1.1%)</td>
<td>189</td>
</tr>
<tr>
<td>XIII. Zadar</td>
<td>314</td>
<td>136</td>
<td>(30.3%)</td>
<td>450</td>
</tr>
<tr>
<td>XIV. Osijek-Baranja</td>
<td>1055</td>
<td>39</td>
<td>(3.6%)</td>
<td>1094</td>
</tr>
<tr>
<td>XV. Šibenik-Knin</td>
<td>1069</td>
<td>223</td>
<td>(17.2%)</td>
<td>1292</td>
</tr>
<tr>
<td>XVI. Vukovar-Srijem</td>
<td>102</td>
<td>6</td>
<td>(5.6%)</td>
<td>108</td>
</tr>
<tr>
<td>XVII. Split-Dalmatia</td>
<td>312</td>
<td>154</td>
<td>(33%)</td>
<td>466</td>
</tr>
<tr>
<td>XVIII. Istria</td>
<td>388</td>
<td>3</td>
<td>(0.8%)</td>
<td>391</td>
</tr>
<tr>
<td>XIX. Dubrovnik-Neretva</td>
<td>21</td>
<td>15</td>
<td>(41.7%)</td>
<td>36</td>
</tr>
<tr>
<td>XX. Međimurje</td>
<td>17</td>
<td>0</td>
<td>(0%)</td>
<td>17</td>
</tr>
<tr>
<td>XXI. City of Zagreb</td>
<td>219</td>
<td>1</td>
<td>(0.5%)</td>
<td>220</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>17,224</td>
<td>1100</td>
<td>(6%)</td>
<td>18,324</td>
</tr>
</tbody>
</table>
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 < 0.001). 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).

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

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of ram blood samples</th>
<th>Number of positive samples</th>
<th>% of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>6028</td>
<td>276</td>
<td>4.25</td>
</tr>
<tr>
<td>2012</td>
<td>1477</td>
<td>71</td>
<td>4.81</td>
</tr>
<tr>
<td>2013</td>
<td>2312</td>
<td>122</td>
<td>5.28</td>
</tr>
<tr>
<td>2014</td>
<td>3871</td>
<td>307</td>
<td>7.93</td>
</tr>
<tr>
<td>2015</td>
<td>4636</td>
<td>324</td>
<td>6.99</td>
</tr>
<tr>
<td>Total</td>
<td>18,324</td>
<td>1100</td>
<td>6.0</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Group of counties</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>14,197</td>
<td>504</td>
<td>14,721</td>
</tr>
<tr>
<td>2*</td>
<td>3027</td>
<td>596</td>
<td>3623</td>
</tr>
<tr>
<td>Total</td>
<td>17,244</td>
<td>1100</td>
<td>18,324</td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th>Factor</th>
<th>OR</th>
<th>SE (OR)</th>
<th>P</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>10.46</td>
<td>0.77</td>
<td>0.000</td>
<td>9.04–12.11</td>
</tr>
<tr>
<td>Year</td>
<td>1.52</td>
<td>0.03</td>
<td>0.000</td>
<td>1.45–1.59</td>
</tr>
</tbody>
</table>

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

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.
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* and clinical examinations (17). 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


