First evidence of *Brucella ovis* infection in Republic of Croatia

Die ersten Nachweise von *Brucella ovis* Infektion in Kroatien

Summary:
We researched the spread of *Brucella ovis* (*B. ovis*) infection in sheep during 2002 and 2003 in Croatia. A total of 30,635 sheep blood samples were examined using the enzyme-linked immunosorbent assay (ELISA). In 2002, 1014 out of 14,404 examined sheep blood samples (7%) from six counties gave positive reactions while 2060 (14.3%) were found suspicious. In 2003, 638 out of 16,221 examined sheep blood samples in nine counties (3.9%) tested positive while 1083 (6.7%) were suspicious. In rams and sheep that were serologically positive specific pathological changes were found in 68 (43.6%) out of 156 examined rams and in 5 (3.8%) out of 133 examined sheep. *B. ovis* was isolated from ram tissues from three counties and identified with classical microbiological procedures and the polymerase chain reaction (PCR). This research proves that *Brucella ovis* is present in sheep flocks in Croatia which is also the first proof of its existence in the country.

Keywords: *Brucella ovis*, Croatia, immunosorbent assay, PCR

Zusammenfassung:
Die pathologische und kulturelle mikrobiologische Untersuchung von seropositiven Böcken und Schafen ergaben bei 68 (43,8 %) von 156 untersuchten Böcken und 5 (3,8 %) von 133 untersuchten Schafen spezifische Befunde. *B. ovis* wurde aus den Organen von Böcken aus 3 Provinzen isoliert und die Isolate wurden mit kulturellen mikrobiologischen Methoden und Polymerase-Kettenreaktion (PCR) identifiziert. Bei diesen Untersuchungen konnten *Brucella ovis*-Infektionen und die Infektions-Epididymitis der Schafe in Kroatien erstmals nachgewiesen und eine relativ weite Verbreitung gezeigt werden.

Schlüsselwörter: *Brucella ovis*, Kroatien, Enzymimmunoassay (ELISA), Polymerase-Kettenreaktion (PCR)
**Introduction**

Brucellosis is a globally widespread infectious disease caused by some bacterial species of the genus *Brucella*. There are four species known to cause human disease: *B. melitensis*, *B. abortus*, *B. suis* (biovar 1 and 3) and *B. canis*. Each of these has a specific animal reservoir. Up to this research only confined sheep and goat infections with *B. melitensis* were determined: in Istra in 1990 and in Dalmacija in 2004. Also, in 2004 four seropositive humans were identified, all owners of infected sheep and goats. Positive reactions in pigs were detected in 5 counties from 2002–2004 (Pozega-Slavonija, Vukovar-Srijem, Sisak-Moslavina, Osijek-Baranja i Krapina-Zagorje), all in small rural herds except one larger farm. No humans that came in contact with infected pigs were reported ill. Whole-herd “stamping out” method was used to restrain these infections with *B. melitensis* in sheep and goats, and *B. suis* in pigs.

*Brucella ovis* infection in rams and sheep causes either clinical or subclinical disease and it is not pathogenic for humans. The disease in rams is characterized by pathological changes in epididymes with consequently lower fertility. Clinically visible signs of infection in females are less common. More often the infection of females is characterized by lower fertility rate, abortions, lower vitality and the perinatal mortality of lambs. Sheep and ewes are considered as an important link in the natural spreading of the infection among rams. According to simulation models *B. ovis* infection causes significant economic losses in flocks with no control measures although there is no exact confirmation (Blasco and Marin, 1990; Krt, 1992).

At the moment of first evidence in a country prevalence among rams is usually high and varies between 20 and 60% while percent of infected flocks lies between 45 and 75%. Implementation of control programmes reduces prevalence although eradication is hardly achievable (Blasco et al., 1983; Sancho et al., 1985).

So far *B. ovis* infection has been registered in almost all countries with sheep farming (Blasco and Marin, 1990; OIE, 2004).

However, there has been no record of sheep and ram infections caused by *B. ovis* in Croatia. Routine serology for brucellosis (*B. melitensis*, *B. abortus* and *B. suis*) is based on use of *Brucella abortus* strain 99 or strain 1119-3 and *B. ovis* specific antigen obtained from *B. ovis* in the ELISA for brucellosis and *B. ovis* infection was never diagnosed *B. ovis* prior to this investigation.

The aim of this study was to conduct a serological control of animals at risk, remove positive reactors and vary the presence of the disease via bacteriological and fast molecular methods.

**Material and Methods**

**Serology**

*Serum samples*

Systematic ram sera testing for *B. ovis* infection began in 2002 based on the instruction from Ministry of Agriculture and Forestry of Republic of Croatia. Relatively big differences in the number of tested sera in various counties are a result of different representation of sheep farming as well as the way these sheep are kept. Breedings are mostly extremely extensive in their nature and therefore almost impossible to control and apply measures for eradicating this disease. In every county several veterinary organisations are active and they are authorised by the Department of Veterinary Medicine of Republic of Croatia to implement these measures. In the period in question these veterinary organisation provided us only with ram sera. In case of a positive serological reaction to *Brucella ovis* the entire flock had to be tested. We examined ram and sheep serum samples from different counties of Croatia. In 2002, we examined 14 404 and in 2003, 16 221 samples. Totally, 30 625 samples from 18 counties of Croatia were examined.

*Serological test*

Detection of antibodies against *B. ovis* was performed using ELISA test (CHEKIT-Brucella ovis enzyme immunoassay kit, Bommeli Diagnostic, Switzerland) according to producer’s recommendations.

**Pathologic and bacteriologic examination**

**Pathology**

Pathology changes were observed at abattoir in the sexual organs of 156 rams and 133 sheep that had reacted positive to *B. ovis* in serology. Tissue samples were submitted to the laboratory for further analysis.

Tissue samples. After a ram positive serological reaction to *B. ovis* infection was identified, ram and sheep culling was recommended to provide sampling for bacteriological examination. Also, large numbers of seropositive rams were castrated. Female sheep were excluded from the herd by the hand of the owner. In these cases no post mortem investigation was possible. Tissue samples from 156 rams and 133 sheep were examined. All these samples originated from three counties in which we observed the highest seroprevalence. Examined samples included: testicles and accessory sexual glands of rams, uterus and mammary glands of sheep as well as lymph nodes (sub-mammary, iliaci, scapulares, submandibulares and retropharyngeales).

Examined animals originated from County of Karlovac (10 rams, 12 sheep), County of Virovitica-Podravina (98 rams, 90 sheep) and County of Osijek-Baranja (48 rams, 31 sheep).

**Bacteriology**

Several grams of tissue (testis, uterus or lymph node) were homogenized, 1 ml of homogenate was inoculated on blood agar, *Brucella* agar and Thayer-Martin agar (Alton et al., 1988, Marin et al., 1996). Inoculated plates were checked daily. Grown colonies were identified based on morphology (small, translucent, convex and rough (R)), growth in atmosphere with 10% CO₂ H₂ production, growth on media supplemented with 20 μg/ml of thionin and basic fuchsin, and the ability to agglutinate with antisierum R (Corbel et al. 1983; Alton et al. 1988). Samples from Karlovac were incubated in a thermostat equipped with CO₂ generator (Haereus Instruments, Germany) while samples from County of Virovitica-Podravina and County of Osijek-Baranja were incubated in hermetically closed jars with added CO₂ (GENbox-bioMérieux, France). Lower isolation rate using jars was probably due to the inability to maintain appropriate CO₂ concentra-
Molecular identification of isolates

*B. ovis* was isolated from 5 rams from County of Karlovac, 11 rams from County of Virovitica-Podravina and 8 rams from County of Osijek-Baranja. All 24 isolates were analysed with polymerase chain reaction. We used the following standard strains: *B. abortus* strain 544, *B. suis* strain 1330, *B. melitensis* 16M, *B. ovis* 63/290 and *B. ovis* REO 198 for comparison.

Genomic DNA isolation

All 24 isolates as well as standard cultures were suspended in 50 µl of Q water (Sigma, Germany). Suspensions were heated during 15 minutes on 99°C in a thermoblock and occasionally shaken. Tubes were then centrifuged at 14 000 g during one minute. We used 2 or 5 µl of supernatant liquid for subsequent analysis.

To confirm that isolated strains belong to genus *Brucella* we amplified a genomic structure responsible for synthesis of the BCSP-31 protein. This protein is a membrane antigen specific for all members of genus *Brucella*. We used primers BRU-UP (GGG CAA GGT GGA AGA TTT) and BRU-LOW (CGG CAA GGTTGCTGTTT) and the size of the amplification product was about 440 base pairs (bp) (Serpe et al., 1999). The DNA amplifications were carried out in 50 µl reaction volume each containing 46µl of Hot Star Taq MasterMix and water (Qiagen, Germany), 1 µl (100 mM) of each of the primers (Invitrogen, Scotland) and 2 µl of genomic DNA. The program for amplification was as follows: polymerase activation (95°C/15 min), followed by 35 cycles of denaturation (95°C/1 min), annealing (58°C/1 min) and final extension step (72°C/5 min) (GeneAmp® PCR System, Applied Biosystems, USA).

To confirm that isolated strains belong to species *B. ovis* we focused on a nucleotide sequence within the IS711 insertion sequence specific for *B. ovis* (AMOS PCR, Bricker and Halling, 1994). We used 5 primers (Invitrogen, Scotland) specific for differentiation of *B. abortus* biovar 1 and 2, *B. melitensis* biovars 1, 2 and 3, *B. suis* biovar 1 and *B. ovis*. Reaction mixture consisted of 5 µl genomic DNA, 0.2 µM each of the 4 primers (*B. abortus* specific primer-GAC GAA CGG AAT TTT TCC AAT CCC, *B. melitensis* specific primer-AAA TCG CCT TGC TGG TCT GAC, *B. ovis* specific primer- CGG GTT CTG GCA CCA TCG TC, *B. suis* specific primer-GCG CCG GTT TTG TCT GAA GGT TCC GGG), 0.8 µM IS711 of a specific primer (TGC CGA TCA CTT AAG GGC CTT CAT), 250 mM of each nucleotide (GeneAmp® dNTP Blend, Applied Biosystems, USA) and 0.25 U Taq polymerase (AmpliTaq Gold® DNA Polymerase, Applied Biosystems, USA). Final volume of the reaction mixture was 50 µl. The samples were cycled (1 min at 94°C, 1 min at 57°C and 1 min at 72°C) 35 times with final extension step of 72°C (GeneAmp® PCR System, Applied Biosystems, USA). The expected amplification product size for *B. abortus* biovars 1 and 2 is ~498 bp, for *B. melitensis* biovars 1, 2 and 3 is ~731 bp, for *B. suis* biovar 1 is ~285 bp and for *B. ovis* is ~976 bp.

Amplification products were separated in 2% agarose gel and stained by ethidium bromide. The visualisation was done by using the UV transiluminator and the camera BioCapt Document System (Vilbert Lourmat – France).

Results

Serological results

A total of 1014 samples out of 14 404 examined sheep and rams from 6 countries in 2002 (7%) reacted positively and 2060 (14.3%) reacted suspiciously. In 2003, out of 16221 examined sheep and ram serum samples from 9 counties, 638 gave positive (3.9%) and 1083 (6.7%) gave suspicious results (Tab. 1).

Pathology findings

Pathological changes specific for infection caused by *B. ovis* were observed in 68 (43.6%) out of 156 examined seropositive rams. Unilateral atrophy of testicles was found in 14 (8.9%) rams, unilateral epididymis enlargement in 38 (24.4%) rams and abscesses and granulomas inside epididymes and testicles in 16 (10.3%) rams (Fig. 1 and 2). Abscesses were not found in any other organs. Purulent content in uterus (purulent endometritis) which could be related to *B. ovis* infection was observed in 5 (3.8%) out of 133 examined seropositive sheep.

Bacteriological examination

A total of 289 post mortem samples were bacteriologically examined (156 rams and 133 sheep). Colonies became visible after 4 days of incubation. All isolated strains, grown in 10% CO₂ atmosphere, on media supplemented by thionate, did not produce H2S and with no agglutination using A and M standard antisera. *B. ovis* was isolated from 5 rams from County of Karlovac, 11 rams from County of Virovitica-Podravina and 8 rams from County of Osijek-Baranja (Tab. 2).

Results of molecular identification

All 24 isolates were identified as *B. ovis* based on classical microbiological identification system as well as using polymerase chain reaction. PCR confirmed that all isolates were members of genus *Brucella* based on amplification product ~440 bp in size. All isolates were then identified as *B. ovis* based on AMOS PCR results (Fig. 3).

Discussion

Brucellosis caused by *Brucella ovis* is a chronic disease. Affected animals have lower reproductive performance due to pathological changes in genital organs. Economical losses due to *Brucella ovis* infection are result of lower fertility rates of rams, abortions and higher mortality rate of newborn lambs. Although natural mating is the main way of spreading, infection passes from ram to ram by direct contact (Clapp et al. 1962).

*B. ovis* infection is widespread around the world. It was first described in New Zealand in 1952 and Australia in 1953. Behrens and Loeliger (1959) reported about epididymitis due to *B. ovis* in Greg horned heath sheep. Hold and Zerobin (1993) reported on typical pathology findings as well as isolation of *B. ovis* from rams in Switzerland. Schopf and Khaschabi (1997) found 10% positive rams by immunoenzym method during the 1990–1992 period in Austrian province of Tyrol. Cerri et al. (2002) reported the first isolation of *B. ovis* from testicles of two rams in Italy in 1994. Denes and Glavitz (1994) described findings of *B. ovis* in Ukraine. Robles et al. (1998) investigated the infection in Argentina finding prevalences between
and 6.3% during a 3 year period. Bagley et al. (1985) found epididymitis in rams from Utah while Bulgin (1990) isolated *B. ovis* in 6 out of 9 seronegative rams in Idaho. In 2002, we found 1014 (7%) positive and 2060 (14.3%) suspicious animals out of 14 404 examined from 15 counties. In 2003, these percentages were lower: 3.9% positive and 6.7% suspicious animals out of 16 221 from 17 counties. Robles et al. (1998) found the prevalence of *B. ovis* infection in Argentina between 2.1 and 6.3% during the three years of investigation. Schopf and Khaschabi (1997) reported on 10% seropositive rams during 1990/1992 while in 1995 the same authors found 3.5% positive rams in Tyrol.

In our abattoir survey we examined 156 rams from 3 counties for presence of pathological changes. We found changes that could be the result of *B. ovis* infection in Argentina between 2.1 and 6.3% during the 3 years of investigation. Schopf and Khaschabi (1997) reported on 10% seropositive rams during 1990/1992 while in 1995 the same authors found 3.5% positive rams in Tyrol.

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### Table 1: Results of serological examination with immunosorbent assay during 2002 and 2003

<table>
<thead>
<tr>
<th>County of</th>
<th>Examined blood samples from sheep and rams in 2002 and 2003</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No. of examined</td>
</tr>
<tr>
<td></td>
<td>Year 2002</td>
</tr>
<tr>
<td>Zagreb</td>
<td>9</td>
</tr>
<tr>
<td>Krapina-Zagorje</td>
<td>1</td>
</tr>
<tr>
<td>Sisak-Moslavina</td>
<td>35</td>
</tr>
<tr>
<td>Karlovac</td>
<td>1288</td>
</tr>
<tr>
<td>Varazdin</td>
<td>1</td>
</tr>
<tr>
<td>Koprivnica-Križevci</td>
<td>522</td>
</tr>
<tr>
<td>Primorje-Gorski kotar</td>
<td>32</td>
</tr>
<tr>
<td>Bjelovar-Bilogora</td>
<td>48</td>
</tr>
<tr>
<td>Liška-Senj</td>
<td>0</td>
</tr>
<tr>
<td>Virovitzka-Podravina</td>
<td>10 234</td>
</tr>
<tr>
<td>Pozega-Slavenija</td>
<td>117</td>
</tr>
<tr>
<td>Brod-Posavina</td>
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</tr>
<tr>
<td>Zadar</td>
<td>1</td>
</tr>
<tr>
<td>Osijek-Baranja</td>
<td>1946</td>
</tr>
<tr>
<td>Sibenik-Knin</td>
<td>0</td>
</tr>
<tr>
<td>Vukovar-Srijem</td>
<td>158</td>
</tr>
<tr>
<td>Split-Dalmacija</td>
<td>10</td>
</tr>
<tr>
<td>Medimurje</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>14 404</td>
</tr>
</tbody>
</table>

### Table 2: Results of bacteriological examination for *B. ovis*

<table>
<thead>
<tr>
<th>County of</th>
<th>No of examined samples*</th>
<th>No of bacteriological positive samples</th>
<th>% positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karlovac</td>
<td>22</td>
<td>5</td>
<td>22.7</td>
</tr>
<tr>
<td>Virovitzka-Podravina</td>
<td>188</td>
<td>11</td>
<td>5.9</td>
</tr>
<tr>
<td>Osijek-Baranja</td>
<td>79</td>
<td>8</td>
<td>10.1</td>
</tr>
<tr>
<td>Total</td>
<td>289</td>
<td>24</td>
<td>8.3</td>
</tr>
</tbody>
</table>

*a sample represents one animal*
These results have given us the insight into \( B. \text{ovis} \) infection status of sheep flocks in Croatia. Obviously the infection is present in counties in which sheep breeding represents important part of economy. Flocks suffering from higher rates of perinatal lamb mortality and abortions should always be under suspicion. According to flock owner’s statements, mortality rate in infected flocks, included in our study, varied from insignificant (County of Virovitica-Podravina) to extremely high (County of Karlovac). Unfortunately, we were not able to carry out thorough analysis of economic losses in flocks due to \( B. \text{ovis} \) infection. In cooperation with a veterinary organisation in County of Karlovac in one herd with 225 sheep and ewes, only 70 lambs were present in lambing season and shortly after lambing further 40 lambs died. Other possible reasons for reduced fertility and increased mortality of lambs after lambing like malnutrition, management and other infectious diseases could not be excluded. Investigation of rams in this herd revealed 70% of the rams seropositive to \( B. \text{ovis} \).

Control programmes are directed towards finding and eliminating of the infected animals, especially rams infected due to the transient nature of infection in females. Eradication is possible in closed and controlled flocks by testing and elimination scheme. Furthermore, separation of rams according to their age to the extent of no physical contact is an effective measure in \( B. \text{ovis} \) control. This is the first confirmation of \( B. \text{ovis} \) infection in sheep flocks in Croatia.

References

Bricker BJ, Halling SM (1994): Differentiation of \( B. \text{abortus} \) bv. 1, 2, and 4, \( B. \text{melitensis} \), \( B. \text{ovis} \), and \( B. \text{suis} \) bv. 1 by PCR. J Clin Microbiol 32: 2660–2666.


Address for correspondence:
Silvio Špičić, MSc, DVM
Laboratory for Bacterial Zoonosis and Molecular Bacteriology
Croatian Veterinary Institute Savska cesta 143
10 000 Zagreb
Croatia
yspicic@veinst.hr