First evidence of Brucella ovis *infection in rams in the Pirot Municipality, Serbia*

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Brucella ovis,
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Rams,
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Summary
This paper describes a research on *Brucella ovis* infection in rams in the Pirot Municipality of South Serbia. A positive result with indirect immunoenzyme test (i-ELISA) was confirmed in 67 (29.8%) and suspicious in 31 (13.8%) out of 225 tested rams. Complement fixation test (CFT) was used as a confirmation test on 67 ELISA positive sera and gave positive reaction in 41 (61.2%) ram serum samples. Rams originated from 113 flocks with 4751 sheep, from 28 villages in the Pirot Municipality of southern Serbia. Clinical examination was performed on epididymis and testes of 12 rams from 7 seropositive flocks by inspection and palpation. The examination showed scrotum asymmetry and unilateral increase of the epididymistail in 5 (41.7%) out of 12 seropositive rams. Pathomorphological examination of testes and epididymis confirmed pathological changes in 7 (58.3%) of the 12 examined rams. One-sided epididymitis with pronounced hypertrophy of the epididymitis was also confirmed. Twelve rams were tested for the presence of bacteria, i.e. 21 epididymis, testes and lymph nodes samples. We isolated 20 *Brucella* strains from 11 (91.7%) of the 12 examined animals. All isolates were identified with bacteriological and molecular techniques as *B. ovis*. This is the first evidence of ovine epididymitis (*B. ovis*) in Republic of Serbia.

Infezione da Brucella ovis *in arieti del distretto di Pirot in Serbia meridionale*

Parole chiave
Ariete,
Brucella ovis,
Brucellosi,
Epididimite,
Serbia

Riassunto
In questo lavoro sono riportati i risultati dell'indagine sulla diffusione dell'infezione da *Brucella ovis* in arieti del distretto di Pirot in Serbia meridionale. I campioni di siero prelevati da 225 arieti, appartenenti a 113 greggi (4751 pecore) distribuite nel territorio di 28 villaggi, sono stati analizzati utilizzando il test ELISA indiretto per identificare la presenza di anticorpi *Brucella ovis*. Su 225 campioni, 67 (29,8%) sono risultati positivi e 31 (13,8 %) sospetti. I 67 campioni positivi sono stati testati di nuovo utilizzando il test di fissazione del complemento, di questi 41 campioni (61,2%) sono risultati positivi. L'esame clinico ha permesso di rilevare in 5 (41,7%) dei 12 arieti sieropositivi l'asimmetria dello scroto e l'aumento unilaterale della coda dell'epididimo. Le alterazioni patologiche a carico di testicoli ed epididimi, tipiche dell'infezione, sono state confermate all'esame macroscopico in 7 (58,3%) dei 12 arieti sieropositivi. In questi 7 campioni è stata rilevata epididimite unilaterale con marcata ipertrofia della coda, del corpo e della testa dell'epididimo. L'analisi batteriologica è stata condotta su 21 campioni di tessuto dell'epididimo, dei testicoli e dei linfonodi dei 12 arieti. Dai campioni di 11 (91,7%) arieti sono stati identificati, con metodi batteriologici e molecolari, 20 isolati appartenenti alla specie *Brucella ovis*. Lo studio è la prima segnalazione della presenza dell'infezione da *Brucella ovis* negli arieti del distretto di Pirot, in Serbia.

Introduction

Brucella ovis infection is considered the most significant infective cause of reproductive disorders in sheep worldwide (Afzal and Kimberling 1986, Bulgin and Anderson 1983, Burgess 1982, Kalinovski *et al.* 1995). It was first reported in sheep in 1953 in Australia and New Zealand (Bulgin and Anderson 1983), currently it is present in South and North America, Australia, New Zealand, South Africa and Southern Europe (OIE Terrestrial Manual 2009, Bagley *et al.* 1985). In France, the number of infected flocks has increased after *Brucella melitensis* Rev-1 vaccination although it helped stoping *B. melitensis* infection in 2008 (Serpe *et al.* 1999). The disease has so far been confirmed in other countries, i.e. in Romania (Denes and Glavitz 1994), Croatia (Corbel *et al.* 1983, Sancho *et al.* 1985) and Slovenia (Kirćanski 2009).

Brucella ovis is the causative agent of ovine epididymitis, a contagious infectious disease of rams and ewes. Chain-like mode of spreading, chronic course and poorly pronounced clinical symptoms, which largely hinder its timely discovery, control and eradication, characterise the disease. Scrotum asymmetry, epididymitis and orchitis in rams, miscarriages in ewes, stillbirths and disvital lambs, increased perinatal mortality or fewer births in comparison to previous years are symptoms of a possible *B. ovis* infection. The conclusive diagnosis can only be made using laboratory tests. The causative agent can also be isolated from seronegative and clinically normal rams (Alton *et al.* 1988, Blasco *et al.* 1983, Bulgin 1990).

In recent decades in the Republic of Serbia and Macedonia, *B. ovis* seropositive animals have been confirmed, though the causative agent was usually never isolated and identified (Krt 1992). In Serbian municipalities of Presevo and Bujanovac, indirect enzyme-linked immunosorbent assay (i-ELISA) on samples from 2,273 rams and ewes showed a *B. ovis* infection seroprevalence of 4.53% (29). ELISA, complement fixation (CFT) and agar gel immunodiffusion tests (AGID) on 200 tested sheep showed a *B. ovis* infection prevalence of 7.5% (Kimberling and Schweitzer 1989).

The objective of this study was to test flocks in Pirot Municipality of the Serbian Republic in order to confirm the presence of ovine epididymitis. Infection in rams was confirmed by serological testing, possible pathomorphological changes on testes, and isolation and identification of the causative agent using bacteriological and molecular methods.

Materials and methods

Serological tests

Serum samples

Pirot municipality is situated in Southern Serbia, between 22°36' East longitude and 43°09' North latitude, covers an area of 1,232 km^2 and altitude between 368 and 2,169 meters above sea level. According to 2011 data, the municipality consists of 71 villages with 1,564 flocks, 15,566 sheep and 500 rams. In 28 villages, the owners of flocks, with mostly intensive farming, agreed to participate in the investigation. A village represents the smallest territory unit with its own authority. Semi-extensive and extensive sheep flock management with joint pasturing characterise Pirot. Previous investigations on sheep brucellosis (*B. melitensis*) in this region always gave negative results. In order to get an insight into presence of *B. ovis* infection, from 2010 to 2011 we tested 225 ram sera samples. The rams originated from 113 flocks, with a total of 4,751 sheep, and they were of the Pramenka breed. Five to 10 ml of blood were collected from the rams' jugular vein. Blood samples were then centrifuged in the laboratory at 1,500 rpm and the obtained sera stored at -20°C until analysis.

Serological testing

In Veterinary Specialists Institute Nis (Serbia) ELISA (CHEKIT – *Brucella ovis*, IDEXX, Bern, Switzerland) kit was used to prove the presence of *B. ovis* antibodies. A complement fixation test (CFT) was used as a confirmation test. The ELISA test was performed according to manufacturer's instructions and the results were read on Tecan Sunrise (Tecan Austria Gesellschaft M.B.H., Salzburg, Austria) spectrophotometer at 450 nm and interpreted according to manufacturer's instructions. Only positive results were retested with a more specific test, CFT, in order to exclude possible cross-reactions with antibodies to other environmentally present pathogens in the tested area. Complement fixation test (CFT) was conducted in Croatian Veterinary Institute Zagreb (Croatia) on microtitration plates (micromethod) according to OIE recommendations (Schopf and Khaschabi 1997). The rough-LPS *B. ovis* antigen (Veterinary Laboratory Agency, VLA, Waybridge, UK), CFT amboceptor and CFT complement (Simens, Marburg, Germany) and 2% sheep erythrocytes (Croatian Veterinary Institute, Zagreb, Croatia) were used in the test according to manufacturer's instructions. Titre of more than

50 ICFTU/ml was considered as positive (Dobrean *et al*. 2002, Schopf and Khaschabi 1997).

Rams which resulted positive to ELISA and CFT were selected for castration and further gross pathology examination and bacteriological testing were performed. Clinical examination, castration or culling were conducted in only 6 of the seropositive 14 villages *i.e.* 7 out of 23 seropositive sheep flocks because some owners did not agree to further testing (Figure 1, Table I).

Clinical examination, pathomorphological and bacteriological testing

Samples

At the time of examination, the number of seropositive rams was reduced due to death or elimination from the flock. The majority of rams' owners eliminated animals with swollen testes from

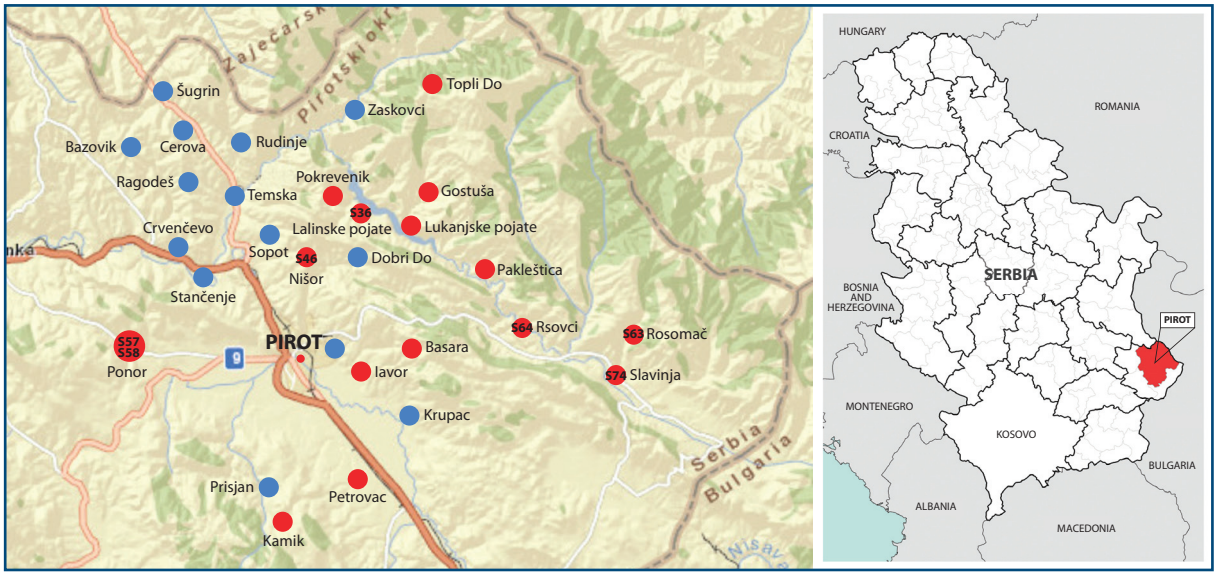

Figure 1. *Pirot Municipality, Republic of Serbia.* The villages included in this study between 2010 and 2011, the blue dot indicates the villages where serologic tests have been conducted, the red dot indicate the villages where seropositive flocks have been reported: S 36, S 46, S 57, S 58, S 63, S 64 and S 74- seropositive flocks with carried clinical, pathomorfological and bacteriological testing.

Table I. *Bacteriological and clinical findings in seropositive rams in the Pirot Municipality, Republic of Serbia, 2010-2011.*

Village name	Flock ID	Ram ID	Ram age in years	Serological testing		Clinical examination		Bacteriology testing			
				ELISA	CFT	Unilateral epididymitis	Bilateral epididymitis	Sample/ID epididymis and testes	*Brucella ovis*	Sample/ID lymph node	*Brucella ovis*
Velika Lukanja	S 36	6150254*	2	+	+	0	0	ET 8	+	-	-
Nišor	S 46	6629594**	3	+	+	0	0	ET 11	+	LČ 11	+
		4337349**	3	+	+	1	0	ET 12	+	LČ 12	+
Ponor	S 57	4412845**	2,5	+	+	0	0	ET 4	+	LČ 4	+
	S 58	4412829**	3,5	+	+	1	0	ET 2	+	LČ 2	+
		2452058**	4,5	+	+	1	0	ET 6	+	LČ 6	+
Rosomač	S 63	1448438**	2,5	+	+	1	0	ET 3	+	LČ 3	+
		9413681**	2,5	+	+	0	0	ET 10	+	LČ 10	+
Rsovci	S 64	6414294*	5	+	+	1	0	ET 9	+	-	-
Slavinja	S 74	9332931**	2,5	+	-	0	0	ET 7	+	LČ 7	+
		5412026*	2,5	+	+	0	0	ET 1	Not carried due contamination		
		5625496**	2,5	+	+	0	0	ET 5	+	LČ 5	+

*Castrated rams; **Culled rams.

the flock by slaughter as a routine procedure over the year. The clinical examination was performed on epididymis and testes of 12 rams from 7 seropositive flocks in 6 villages by inspection and palpation. Pathomorphological testing of testes and epididymis and sampling for bacteriological testing were carried out after castration of 3 rams out of 3 flocks. In cases where owners allowed ram slaughter, testes, epididymis and lymph nodes (*ln. inguinalis, ln. ilicimedialesand ln. lumbalesaortici*) were sampled from 9 rams out of 6 flocks (Table I).

Bacteriological testing

Bacteriological testing was conducted in Croatian Veterinary Institute Zagreb (Croatia) on 21 epididymis, testes and lymph nodes samples from 12 rams originating from 7 flocks in 6 villages (Table I). Around 25-50 grams of material (testes and lymph nodes) were processed, and approximately 1 ml of homogenate inoculated on each plate with blood agar, *Brucella* agar and modified semi-selective agar according to Thayer-Martin (Alton *et al.* 1988)[1]. Plates were then incubated at 37°C in the presence of 10% CO_2. Colony growth was observed on daily basis over 10 days.

Identification of isolates

Morphological characteristics

Isolates were identified on the basis of colony morphology, growth in the presence of 5-10% CO_2, production of H_2S, growth in presence of 20 µg/ml thionine and basic fucsin, and agglutination with antisera A, M and R (Alton *et al.* 1988, Clapp *et al.* 1962, Schopf and Khaschabi 1997).

[1] Lamb Epididymitis. http://www.optimalag.com/cleonscorner/Article 002. aspx.

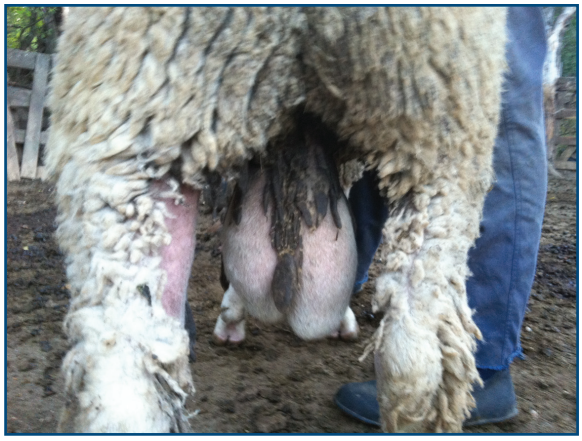

Figure 2. *Asymmetry of the scrotum in rams infected with the species* B. ovis*, in the Pirot Municipality, Republic of Serbia, 2010-2011.*

Molecular identification

Twenty isolates originating from 11 rams were examined using the polymerase chain reaction (PCR) test. Loop full of bacterial culture was mixed in 100 µl of distilled water (UltraPure™ DNase/RNase-Free Distilled Water, Invitrogen, Paisley, Scotland, UK), boiled at 95°C for 20 minutes, and centrifuged at 14,000 g for 1 minute. The supernatant was used in the PCR reaction. The controls used in molecular investigations were standard *Brucella* strains: *Brucella abortus* 544, *Brucella suis* 1330, *B. melitensis* 16M, *B. ovis* 63/290. We used a PCR based on replication of the part of the genome that codes the synthesis of the protein BCSP-31, characteristic for the genus *Brucella* in order to identify isolates as *Brucellae*. The expected replication product size was approximately 440 bp (Praud *et al.* 2012). A multiplex PCR (Bruce-ladder) was used to identify the *Brucella* species (Garcia-Yoldi *et al.* 2006). The expected size of the PCR products

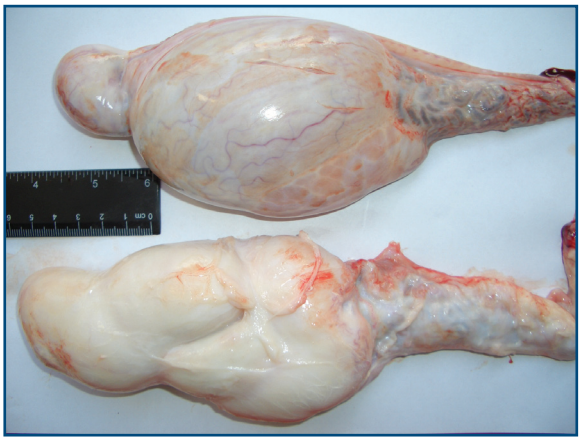

Figure 3. *Testes with membranes removed; testicle with normal structure (above) and an atrophic testicle and enlarged epididymis, with nodular changes, an altered anatomical structure, displaying ribbon-like growths (below) of rams from the Pirot Municipality, Republic of Serbia, 2010-2011.*

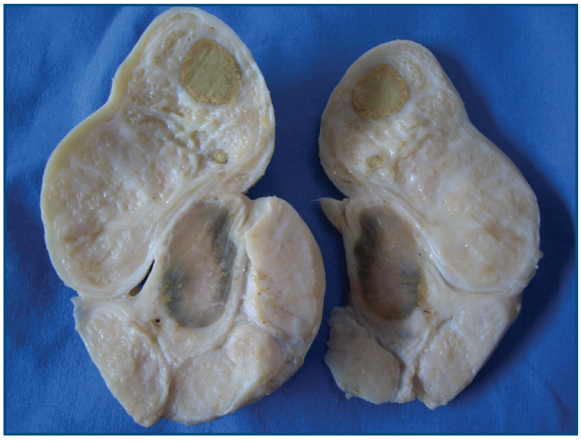

Figure 4. *Chronic inflammation caused by the species* B. ovis *is characterised by granulomatomic region in the epididymis of rams from the Pirot Municipality, Republic of Serbia, 2010-2011.*

were 1072, 794, 587, 450 and 152 base pairs (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* (Farina *et al.* 1995). The replication products were analysed using a QIAxcel capillary electrophoresis system (Qiagen, Hilden, Germany).

Results

Results of the serological testing

The indirect ELISA confirmed a positive reaction in 67 (29.8%), and a suspicious reaction in 31 (13.8%) out of 225 tested ram serum samples. The seropositive rams originated from 16 out of 28 villages and 34 out of 113 flocks.

The complement fixation test confirmed a positive reaction in 41 out of 67 examined rams. Seropositive rams originated from 14 out of 16 tested villages and from 23 flocks out of 34 tested flocks in the same region.

Results of the clinical examinations

The clinical examination, by adspection and palpation of epididymis and testes of 12 rams from 7 seropositive flocks in 6 villages confirmed scrotum asymmetry (Figure 2) and an unilateral increase in epididymis tail in 5 (41.7%) seropositive rams. Sensitivity and pain in epididymis was confirmed in 4 (80%) of 5 rams exhibiting changes. This manifested in ram's resistance and pulling away during palpation. The region temperature was not established. Testes were mobile in scrotum.

Results of pathomorphological testing

Macroscopic examination of epididymis and testes was performed after castration or culling. Pathological changes indicating infection were confirmed in 7 (58.3%) of the 12 examined ram testes. Various degrees of damage, characteristic for acute and chronic stages of this disease, were established. In the acute case, changes were of necrotic character, while in the chronic case, granulomas, fibrosis and testes and epididymis atrophy were observed.

A characteristic macroscopic finding was unilateral epididymitis in 7 (58.3%) of 12 examined rams (Figures 3 and 4). All 7 rams with enlarged epididymis had marked hypertrophy of the tail, 4 (33.3%) had hypertrophy of the body and 2 (16.7%) had hypertrophy of the head of epididymis. In 2 (16.7%) rams, spermatoceles were found in the epididymis

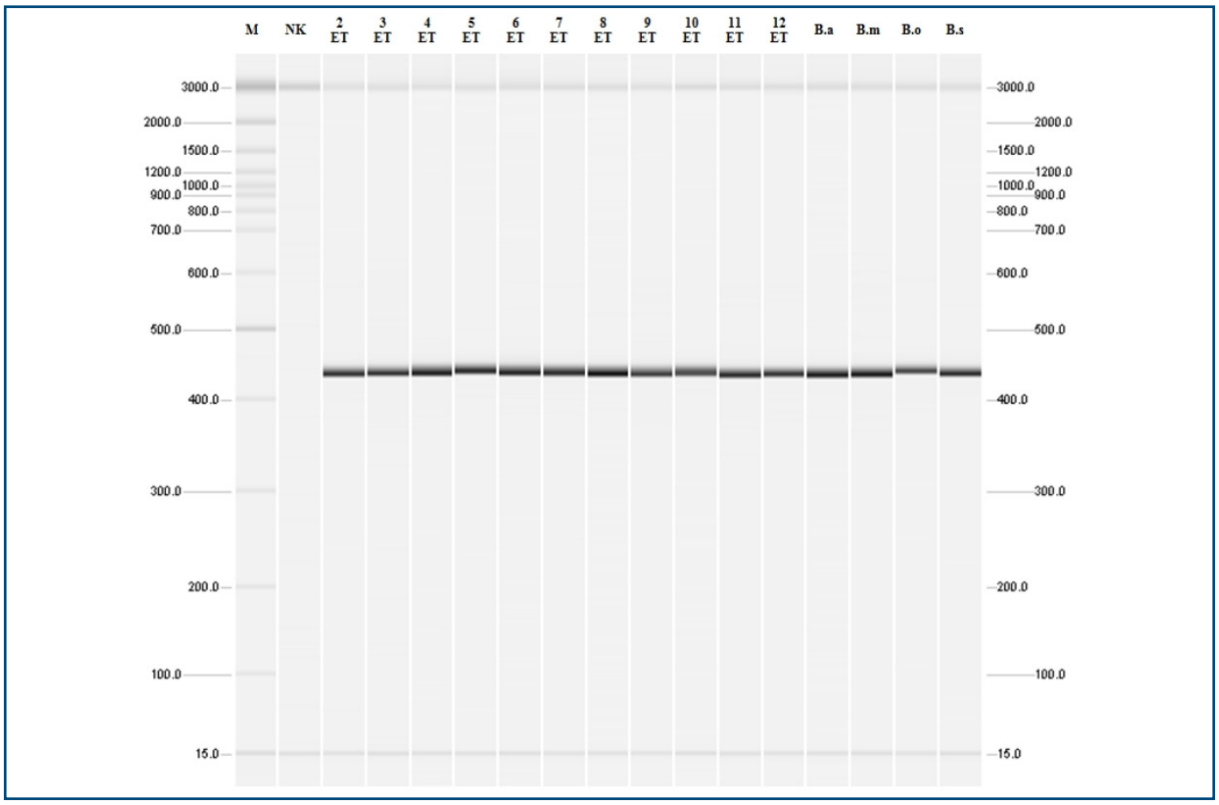

Figure 5. *Molecular identification* Brucella *genus for isolates from the epididymis and testicle tissues from rams in the Pirot Municipality, Republic of Serbia, 2010-2011.*

M = marker with replication product, sizes 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000 and 3000 base pairs; NK = negative control; ET2 - ET12 = Isolates of *Brucella* from tissue of the epididymis and testes of rams; B.a = *B. abortus* 544; B.m = *B. melitensis* 16M; B.o = *B. ovis* 63/290; B.s = *B. suis* 1330.

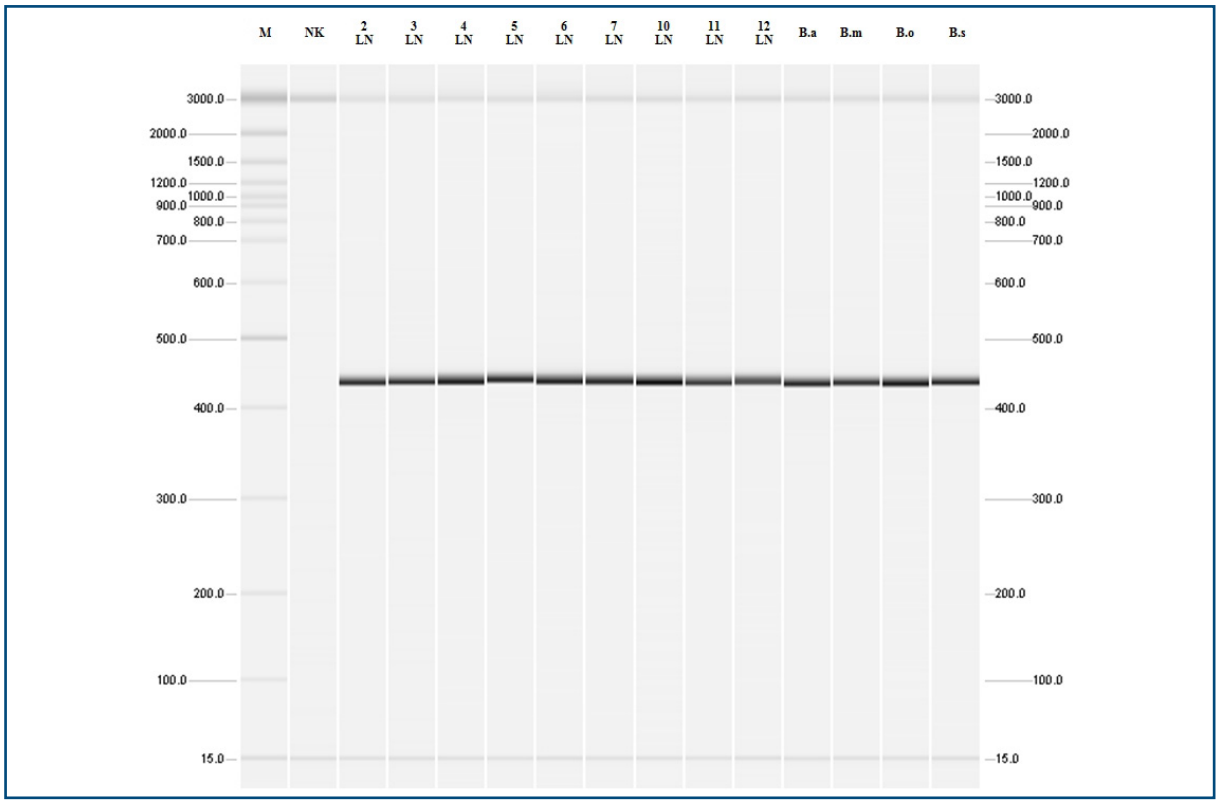

Figure 6. *Molecular identification* Brucella *genus for isolates from the lymph nodes from rams in the Pirot Municipality, Republic of Serbia, 2010-2011.*
M = markers with replication products of sizes 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000 and 3000 base pairs; NK = negative control; Lč2 - Lč7 and Lč10 - Lč12 = Isolates of *Brucella* from the ram lymph nodes; B.a = *B. abortus* 544; B.m = *B. melitensis* 16M; B.o = *B. ovis* 63/290, B.s = *B. suis* 1330.

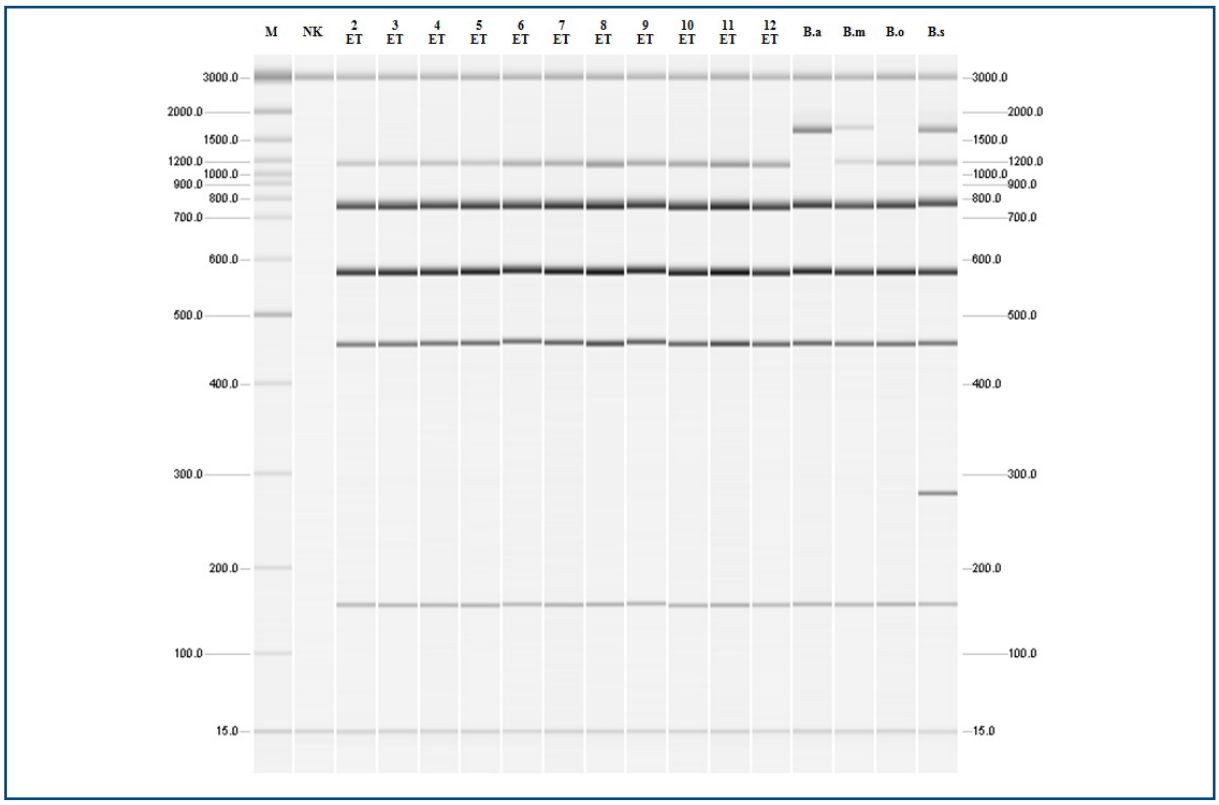

Figure 7. *Molecular typing of* Brucella *isolates from epididymis and testicle tissues of rams in the Pirot Municipality, Republic of Serbia, 2010-2011 tested by multiplex PCR (Bruce-ladder).*
M = markers with replication products with sizes 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000 and 3000 base pairs; NK = negative control; ET2 - ET12- *Brucella* isolates from epididymis and testicle tissues; B.a = *B. abortus* 544; B.m = *B. melitensis* 16M; B.o = *B. ovis* 63/290; B.s = *B. suis* 1330.

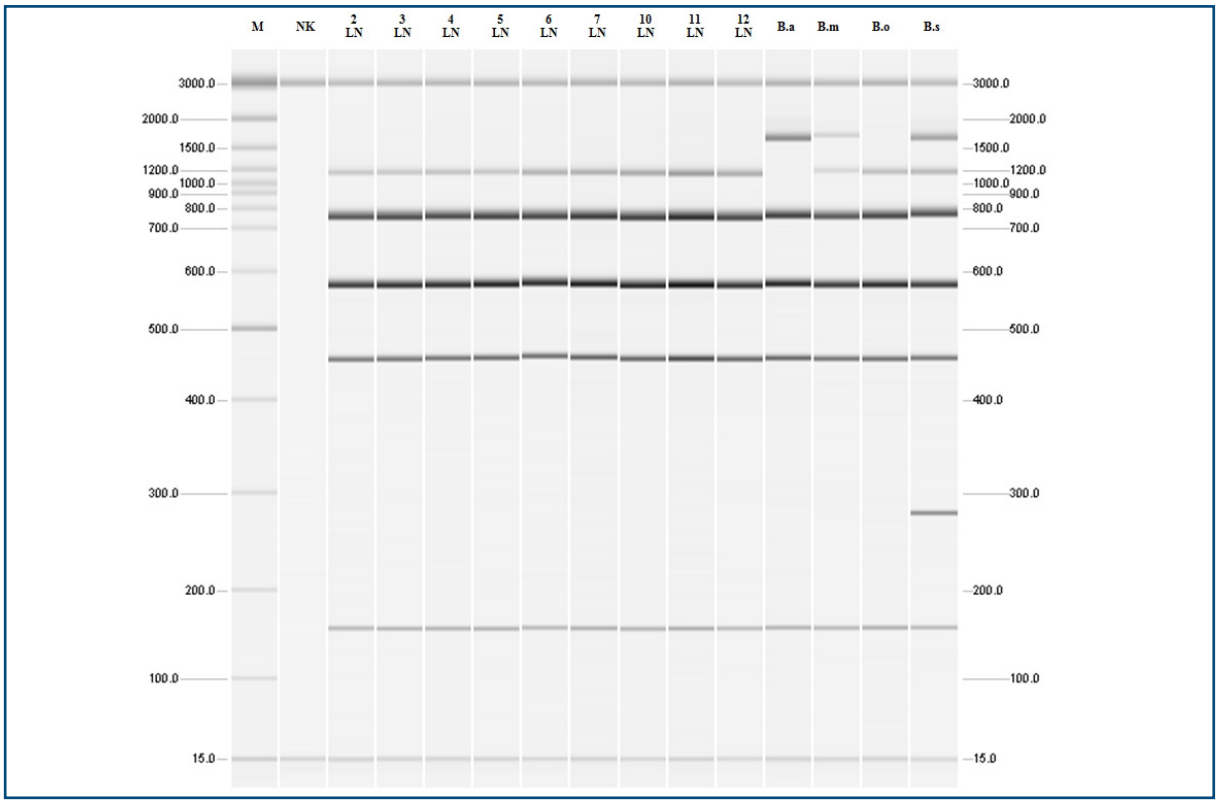

Figure 8. *Molecular typing of* Brucella *isolates from lymph nodes tissue of rams in the Pirot Municipality, Republic of Serbia, 2010-2011tested by multiplex PCR (Bruce-ladder).*

M = markers with replication products with sizes 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000 and 3000 base pairs; NK = negative control; Lč2-Lč7 and Lč10-Lč12 = *Brucella* isolates from lymph node tissue; B.a = *B. abortus* 544; B.m = *B. melitensis* 16M; B.o = *B. ovis* 63/290; B.s = *B. suis*.

tail. Changes in the size of epididymis head ranged from 5 to 12 mm and epididymis body and tail from 2 to 6 mm. The size of spermatoceles was up to 32 mm. Examination of lymph nodes established no macroscopic changes.

Results of bacteriological testing

Bacteriological testing was conducted on 21 samples of epididymis, testes and lymph nodes from 12 rams. We were not able to conduct the testing on 1 sample because of too much contamination. We identified a total of 20 isolates from 11 (91.7%) out of 12 tested rams: 11 isolates were selected from epididymis and testicle tissue, and 9 isolates from lymph node tissue.

Colonies were visible on selective agars incubated in atmosphere with addition of 10% CO_2 at 37°C on the third and fourth day. All isolated colonies were rough (R growth phase), round, convex with straight and full edges. The microscopic smears of 24h old cultures were Gram stained. Bacteria were Gram negative. The agglutination test with monospecific antisera A, M and R gave a visible agglutination with R monospecific antiserum in all isolates. Testing with monospecific antisera A and M resulted in no agglutination.

Results of the molecular testing

Twenty isolates and standard referential strains *B. abortus* 544, *B. melitensis* 16M, *B. ovis* 63/290 and *B. suis* 1330 were identified as *Brucellae* according to the presence of 440 bp long replication product (Figures 5 and 6).

Brucella isolates typing was conducted using a multiplex PCR method called Bruce-ladder. All 20 *Brucellae* isolates were identified as *Brucella ovis*. The PCR profile of the isolates corresponds to the profile of the standard referential *B. ovis* 63/290 strain: 1071, 794, 587, 450 and 152 base pairs (Figures 7 and 8).

Discussion

Ovine epididymitis is characterised by a chain-like way of spreading, chronic course and poorly pronounced clinical symptoms, which largely hinder its timely discovery, control and eradication. Final diagnosis can only be made by laboratory tests, and the causative agent can also be isolated from seronegative and clinically normal rams (Alton *et al.* 1988, Blasco *et al.* 1983, Bulgin 1990).

Sheep farming is a significant branch of livestock production in Serbia. According to the data,

1,460,295 sheep populated Serbia in 2011. In recent decades, *B. ovis* seropositive animals have been confirmed in Serbia, though the causative agent was not isolated and identified (Krt 1992). In Serbian municipalities of Presevo and Bujanovac, indirect immunoenzyme testing of sera from 2,273 rams and ewes identified a seroprevalence of *B. ovis* infection of 4.53% (Marín *et al.* 1989). The ELISA, CFT and AGID tests performed on samples from 200 sheep showed a prevalence of *B. ovis* infection of 7.5% (Kimberling and Schweitzer 1989). In neighboring Croatia, in 2008, the disease was present in 12 out of 20 counties (Špičić *et al.* 2010). However, regardless of the results of performed serological investigations, ovine epididymitis control is still not officially conducted in Serbia.

Due to more simple execution, we started this study testing animals by ELISA (Dobrean *et al.* 2002, Serpe *et al.* 1999). The i-ELISA kit used proved slightly more sensitive but less specific than the CFT (Serpe *et al.* 1999). In countries where prevalence of the disease is high (10% or greater), this test would be very good and inexpensive in comparison to AGID and CFT (Dobrean *et al.* 2002, Gall *et al.* 2003). However, prevalence of *B. ovis* infection in Pirot was unknown but considered very high so we also used CFT to increase the specificity of the analysis (Serpe *et al.* 1999).

The present study used indirect ELISA testing to confirm a positive reaction in 67 (29.8%) and suspicious in 31 (13.8%) of the 225 tested ram serum samples. The seropositive rams originated from 16 villages (57.1% of the surveyed villages in the Pirot municipality) and 34 flocks (30.1% of surveyed flocks in the Pirot municipality).

The complement fixation test confirmed a positive reaction in 41 out of 67 tested rams. Seropositive rams originated from 14 villages (50% of the surveyed villages in the Pirot municipality) and 23 flocks (20.4% of the surveyed flocks in the Pirot municipality). Although the research was not conducted on all flocks in the Pirot Municipality *i.e.* Serbia, identified prevalence is comparable to those of neighbouring countries. In Republic of Croatia and Bosnia and Herzegovina, studies showed that 21.7% of flocks were infected with *B. ovis* (Corbel *et al.* 1983). In southern France, in areas where the disease is endemic, in 2008, the prevalence was 22% (Serpe *et al.* 1999). The infection seroprevalence in rams and sheep in Croatia in 2002 was 7% and 3.9% in 2003 (Sancho *et al.* 1985). After introduction of disease eradication program, based on castration of seropositive rams, in 2008, seroprevalence was 2% (Špičić *et al.* 2010).

At the time of examination, the number of seropositive rams was reduced due to death or elimination from the flock. The majority of owners eliminated rams with enlarged testes by slaughter. Owners used their own rams for breeding and before mating season noticed scrotum asymmetry and enlargement of epididymis and testes. According to owners observations, about 30% of rams had scrotum asymmetry with unilateral enlargement and after mating about 25% of ewes were not fertilized. Miscarriages were seen in 5% of ewes, with the flock maximum being around 10%. The average birthing was 0.74 lambs per ewe (from 0.5 to 0.95), with 7.78% of lambs (maximum 29.09%) dying within the first month. It was proven that newly *B. ovis* infected flocks experienced 30% reduced lambing, opposed to 15-20% in flocks were the disease was endemic (Bulgin 1990). It was established that after *B. ovis* infection, number of live births can be reduced by 25%; 16% of lambs die within 6 weeks and 20% of ewes remain infertile (Kirćanski 2009).

Sheep owners are aware of these problems and remove potentially infected rams with changes in scrotum from flocks themselves. Our clinical research conducted on 12 seropositive rams found scrotum asymmetry and unilateral increase of epididymis tail in 5 (41.7%) rams.

The clinical detection of the disease is difficult because other bacteria, such as *Actinobacillusseminis, Histophilusovis, Haemophilus* spp., *Corynebacterium pseudotuberculosis ovis*, *Chlamydophila abortus* or *B. melitensis* cause similar symptoms and more than 50% of *B. ovis* infected animals do not show any palpable epididymitis lesions (OIE Terrestrial Manual 2009). Further pathomorphological testing found unilateral epididymitis in 7 (58.3%) out of 12 tested rams. All 7 rams showing enlarged epididymis had pronounced hypertrophy of the tail, 4 (33.3%) had hypertrophy of the body and 2 (16.7%) of the head of epididymis. Spermatoceles in epididymis tail were confirmed in 2 (16.7%) rams. The analysis of lymph nodes did not identify any macroscopic changes.

Based on results of bacteriological and molecular testing of material from rams, all the isolates were identified as *Brucellae*, *i.e. B. ovis*. A total of 20 *B. ovis* isolates were bacteriologically isolated from 11 (91.7%) of the 12 seropositive rams. Eleven isolates were selected from epididymis and testicle tissue, and 9 isolates from lymph node tissue. The material belonging to 1 ram was inappropriate for bacteriological testing. All isolates in the present study were identified as *B. ovis* with the identical PCR profile as the referential strain *B. ovis* 63/290.

On a sample of the 12 seropositive rams, we found a different diagnostic value of clinical examination (5, 41.7%), histopathological changes (7, 58.3%) and bacteriological examination (11, 91.7%). To prevent damage caused by the disease it is not enough just to clinically study rams in flocks. According to our findings, any eradication program

of this economically important disease in the region or across the country should be preceded by serologically testing of all rams in order to identify the extent of disease spread. Cost-benefit analysis should follow after which the most appropriate program should be chosen (Carpenter *et al.* 1987). In order to eradicate the disease it is necessary to conduct controls on both male and female animals (Serpe *et al.* 1999, Špičić *et al.* 2009).

This study is the first evidence of the presence of *B. ovis* infection in sheep in Pirot municipality and in Serbia.

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