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Original Scientific Article

EPIZOOTIOLOGICAL, CLINICAL AND PATHOLOGICAL CHARACTERISTICS OF SHEEP FLOCKS INFECTED WITH *BRUCELLA OVIS* IN THE REPUBLIC OF SERBIA

Miloš Petrović[1], Silvio Špičić[2], Aleksandar Potkonjak[3], Branislav Lako[3], Miloš Kostov[4], Željko Cvetnić[2]

[1]Veterinary Specialist Institute Niš, Serbia; [2]Croatian Veterinary Institute Zagreb, Croatia; [3]Faculty of Agriculture, University of Novi Sad, Department for Veterinary Medicine, Novi Sad, Serbia; [4]Military Hospital, Pathology Department, Niš, Serbia

*Corresponding author, E-mail: cvetnic@veinst.hr

Summary: This paper describes a study on *Brucella ovis* infection of sheep flocks in southern Serbia. Using serological testing, positive reactions were confirmed in 67 (29.8%) and suspicion in 31 (13.8%) out of the total 225 tested sheep sera samples. Rams originated from 113 flocks with 4751 sheep from 28 settlements in the Pirot region of southern Serbia. Pathological changes indicative of *Brucella ovis* infection were confirmed by macroscopic examination in the testes of 7 (58.3%) out of 12 examined rams. In 7 (58.7%) animals, unilateral epididymitis with pronounced hypertrophy of the tail, body and head of epididymis was confirmed. Using bacteriological and molecular techniques, the presence of *B. ovis* was confirmed in the samples (testes, epididymis, lymph nodes) in 11 (91.7%) out of 12 rams. The clinical manifestation of ovine epididymitis arose following the procurement of rams and mating season. The disease was more pronounced in flocks in which it occurred for the first time. On the basis of the results presented here, it can be concluded that *B. ovis* infection causes substantial economic losses in sheep production, and is manifested in reduced conception (24.85%), miscarriages (5.38%), reduced number of lambs (0.74 lambs per ewe) and increased perinatal mortality (7.78%).

Key words: *Brucella ovis*; epizootiology; clinical changes; pathological changes; Serbia

Introduction

Brucella (B.) ovis causes chronic disease in sheep which manifests in characteristic testes and epididymis changes in rams and placentitis in ewes. It is estimated that this disease causes substantial economic losses in flocks where there is no disease control (7). Losses are seen in reduced fertility of rams, miscarriages in ewes, early deaths of avital lambs, removal of infected animals from the flock and bans on trade. *B. ovis* is considered to be the most important causative agent of infectious reproductive disorders in sheep worldwide (1, 11, 12, 28).

The main symptoms of the disease in rams are epididymitis and, less commonly, orchitis. Infected rams have lower sperm quality and reduced fertility. Infection can result in sterility. Changes are usually unilateral, rarely bilateral. Pathological changes are most often found in the tail, less often in the body and the head of the epididymis. The percentage of infected rams in the country is very high, and ranges from 20–60%, and infected flocks from 45–75%. In countries with advanced control programmes, the prevalence is significantly lower,

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though complete eradication is difficult to achieve. It strongly depends on situation in country/region (6, 7, 8, 11, 25, 27, 39).

Infection with *Brucella ovis* has been confirmed in virtually all countries with relevant sheep production (5, 9, 15, 33). It has been proven in neighbouring countries, i.e. Romania (21) and Croatia (18, 42), and has been described in Slovenia (30), Austria (40), Italy (23), Switzerland (26), Spain (35) and Ukraine (19).

The objective of this study was to confirm the epizootiological, clinical and pathomorphological characteristics of *Brucella ovis* infection in sheep flocks in a sheep-raising region in Serbia.

Material and methods

Epizootiological survey and clinical examination

On the basis of results of serological testing (indirect enzyme linked immunosorbent assay, ELISA; and complement fixation test, CFT), an epizootiological survey and clinical examination of seropositive rams were conducted. Serological testing was conducted on 225 ram sera samples originating from 113 flocks with 4751 sheep, from 28 settlements in the Pirot region of southern Serbia. The epizootiological survey and clinical examination of seropositive rams was conducted in 7 (30.4%) out of 23 seropositive flocks of sheep, in 6 (42.9%) out of 14 settlements in which seropositive animals were confirmed. The surveyed flocks were labelled with codes S36, S46, S57, S58, S63, S64 and S74.

Pathomorphological and bacteriological testing

Biological materials were sampled from seropositive rams for pathomorphological and bacteriological testing. The testes and epididymis were sampled after the castration of three rams, and the testes, epididymis and lymph nodes (*lnn. inguinalis, lnn. ilicimediales* and *lnn. lumbalesaortici*) were sampled following the slaughter of nine rams. The observed macroscopic changes are described below in detail.

During the pathoanatomical examination, several tissue sections were taken from the macroscopically altered parts of the organs, and from the direct surroundings of the seemingly unaltered tissue. The size of tissue sections was typically 1.5 cm x 1 cm x 0.5 cm.

Tissue sections were fixed in 10% buffered neutral formalin, and prepared according to standard procedures (37). Tissue preparations were stained using the standard hematoxylin and eosin (HE) method and were microscopically analysed on a *Leica DM 1000* microscope with a digital *Leica EC3* camera (32). In the staining process, hematoxylin (*Mayer's hematoxylin, Bio Optica*, Italy) and eosine (1% aqueous solution, BioGnost, Croatia) solutions were used.

Bacteriological processing and identification of isolates

Bacteriological testing was conducted on 21 samples of epididymis, testes and lymph node tissues originating from 12 rams from 7 flocks, from 6 settlements. Several grams of delivered materials (testes and lymph node tissue) were examined and about 1 ml of homogenate was inoculated onto a selective nutritional substrate, i.e. blood agar, Brucella agar and modified semi-selective nutritional substrate according to Thayer-Martin. Plates with inoculated material were incubated at 37°C in the presence of 10% CO_2, and colony growth was observed in daily intervals. Isolates were identified on the basis of colony morphology, growth in the presence of an atmosphere with 5–10% CO_2, production of H_2S, growth on substrates with the addition of 20μg/ml thionine and basic fucsin and agglutination of antiserum R (4, 17, 34). In order to prove that the isolates belong to the genus *Brucella,* a PCR method based on the replication of the part of the genome that codes the synthesis of protein BCSP-31 characteristic for genus *Brucella* was used. The expected size of the replication product is approximately 440 bp (41). For identification of *Brucella* species, a multiplex PCR (Bruce-ladder, Ingenasa, Spain) method was used (22).

Results

Results of the serological testing

Using indirect enzyme immunoassay testing, a positive reaction was found in 67 (29.8%) and suspicion in 31 (13.8%) out of 225 examined ram

serum samples. Seropositive rams originated from 16 settlements (57.1% of the investigated settlements in the Pirot region) and 34 flocks (30.1% of investigated flocks in the Pirot region). The complement fixation test confirmed a positive reaction in 41 (18.2%) out of 225 examined rams. Seropositive rams originated from 14 settlements (50% of investigated settlements in the Pirot region) and 23 flocks (20.4% of investigated flocks in the Pirot region).

Results of the epizootiological survey

The results of the epizootiological survey indicate that the clinical manifestations of epididymitis in sheep in all flocks appeared after introduction of new rams or ewes and during the mating season. Manifestation of the disease was more pronounced in flocks in which the disease appeared for the first time. Based on results of the epizootiological survey in this study, it can be concluded that infection with *B. ovis* causes substantial economic losses in sheep production, seen in consequential reduction of conception (24.85%), miscarriages (5.38%), reduced number of lambs (0.74 lambs per ewe) and perinatal mortality (7.78%) (Table 1).

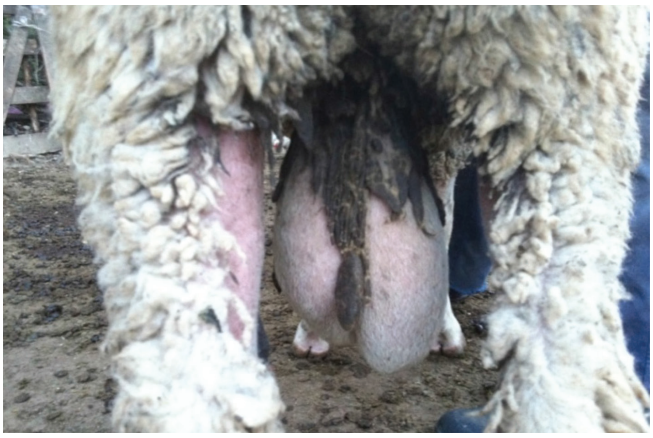

Figure 1: Scrotum asymmetry

Results of clinical examination

Clinical examination by adspection and palpation of epididymis and testes was conducted on 12 rams from 7 seropositive flocks, and in 5 (41.7%) seropositive rams, asymmetry of scrotum and unilateral enlargement of the epididymis tail were established. The enlargement of the epididymis was up to the size of chicken's egg. Increased sensitivity and pain during palpation was exhibited by 80% of rams with changes to epididymis (Table 2 and Figure 1).

Table 1: Manifestation of sheep epididymitis in seropositive flocks based on the epizootiological survey

Flock code	No. of rams	No. of ewes	Duration of disease (years)	Asymmetry of testes			Sheep		Lambs	
				Enlargement of epididymis and testes			Did not conceive	Miscarried	Births per ewe	Perinatal mortality
				Unilateral	Bilateral	Total				
S 36	3	55	4	(0/3)	(0/3)	(0/3)	(5/55)	(0/50)	(52/55)	(0/52)
				0%	0%	0%	9.09%	0%	0.95	0%
S 46	4	87	1	(1/4)	(0/4)	(1/4)	(34/87)	(3/53)	(55/87)	(16/55)
				25%	0%	25%	39.08%	5.66%	0.63	29.09%
S 57	1	7	1	(0/1)	(0/1)	(0/1)	(1/7)	(0/6)	(6/7)	(0/6)
				0%	0%	0%	14.28%	0%	0.86	0%
S 58	2	47	1	(2/2)	(0/2)	(2/2)	(14/47)	(0/33)	(34/47)	(6/34)
				100%	0%	100%	29.79%	0%	0.72	17.65%
S 63	11	310	4	(3/11)	(1/11)	(4/11)	(34/310)	(16/276)	(271/310)	(15/271)
				27.27%	9.09%	36.36%	10.96%	5.80%	0.87	5.54%
S 64	1	11	3	(1/1)	(0/1)	(1/1)	(2/11)	(0/9)	(9/11)	(0/9)
				100%	0%	100%	18.18%	0%	0.82	0%
S 74	8	175	1	(1/8)	(0/8)	(1/8)	(82/175)	(9/93)	(87/175)	(3/87)
				12.50%	0%	12.50%	46.86%	9.68%	0.5	3.45%
TOTAL:	30	692		(8/30)	(1/30)	(9/30)	(172/692)	(28/520)	(514/692)	(40/514)
				26.67%	3.33%	30%	24.85%	5.38%	0.74	7.78%

Table 2: Results of the clinical examination of epididymis and testes of seropositive rams by adspection and palpation

Settlement	Flock code	Ram ID	Ram age	Serological results		Asymmetry of scrotum Enlargement of epididymis		
				ELISA	RVK	Unilateral	Bilateral	Total
Lukanjske pojate	S 36	6150254	2	+	+	0	0	(0/1) 0%
Nišor	S 46	6629594	3	+	+	0	0	(1/2) 50%
		4337349	3	+	+	1	0	
Ponor	S 57	4412845	2.5	+	+	0	0	(0/1) 0%
	S 58	4412829	3.5	+	+	1	0	(2/2) 100%
		2452058	4.5	+	+	1	0	
Rosomač	S 63	1448438	2.5	+	+	1	0	(1/2) 50%
		9413681	2.5	+	+	0	0	
Rsovci	S 64	6414294	5	+	+	1	0	(1/1) 100%
Slavinja	S 74	9332931	2.5	+	-	0	0	(0/3) 0%
		5412026	2.5	+	+	0	0	
		5625496	2.5	+	+	0	0	
Total:				12	11	(5/12) 41.67%	(0/12) 0%	(5/12) 41.67%

Table 3: Percent of established pathoanatomical changes in seropositive rams

Flock code	Ram ID	Hypertrophy of the epididymis				Spermato-celes	Atrophy of the testes	Granulomas in epididymis and testes	Total rams with confirmed changes
		total	tail	body	head				
S 36	615024	0	0	0	0	0	0	0	(0/1) 0%
S 46	662954	0	0	0	0	0	0	0	(1/2) 50%
	433739	1	1	1	0	0	0	0	
S 57	441285	0	0	0	0	0	0	0	(0/1) 0%
S 58	441289	1	1	0	0	0	0	0	(2/2) 100%
	245208	1	1	1	1	1	1	1	
S 63	144848	1	1	1	1	1	0	1	(1/2) 50%
	941361	0	0	0	0	0	0	0	
S 64	641424	1	1	1	0	0	1	1	(1/1) 100%
S 74	933291	0	0	0	0	0	0	0	(2/3) 66,67%
	541206	1	1	0	0	0	0	0	
	562546	1	1	0	0	0	0	0	
Total:		(7/12) 58,33%	(7/12) 58,33%	(4/12) 33.33%	(2/12) 16.67%	(2/12) 16.67%	(2/12) 16.67%	(3/12) 25%	(7/12) 58,33%

Results of pathomorphological testing

The macroscopic examination of epididymis and testes of 12 rams confirmed changes indicating *B. ovis* infection in 7 (58.33%) rams. The examination revealed lesions of varying degrees characteristics for acute and chronic phases of the disease. In acute phase, changes were necrotic, while in chronic phase granulomas, fibrosis and atrophy of testes and epididymis were observed (Table 3, Figures 2 and 3).

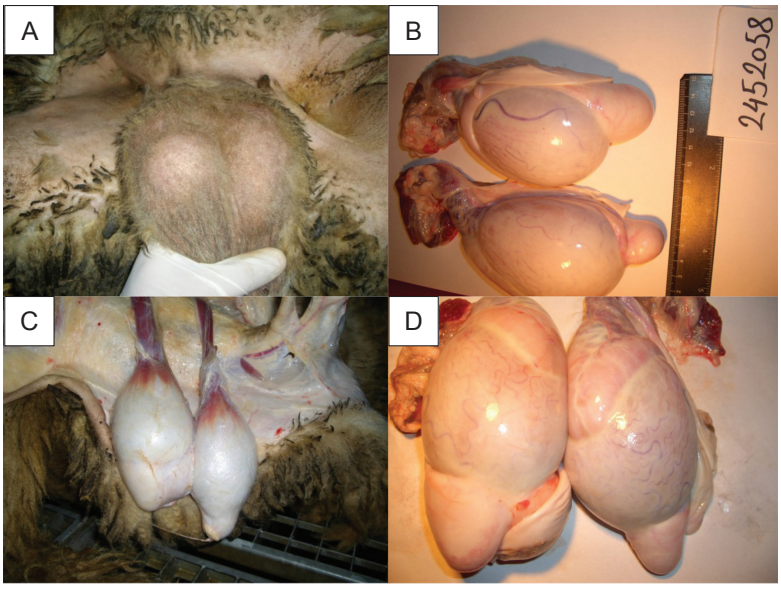

Figure 2: A) Increased epididymis tail. B) The unilateral enlargement of the whole epididymis, partly with lagging, accrued *tunica vaginalis*. C) "*In situ*" position of both testicles: abnormal, enlarged right testis and epididymis tail with spermatocele. D) Spermatocele of the epididymis tail, attached to the tunic vaginitis, which does not separate easily.

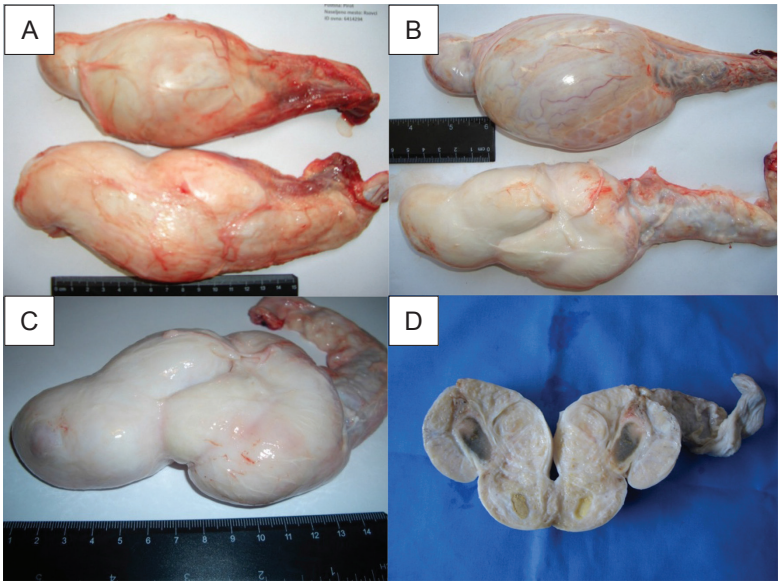

Figure 3: A) Both testes with accompanying envelopes. B) The testes without sheath. Upper testicle has distinctive appendages; lower atrophic testis with enlarged epididymis presents nodular changes, atypical anatomy, and is sporadically covered with stripe adhesions. C) Right testis with nodular changes, epididymis tail with cystic, slightly wave like wall. D) Sagittal section of the right testis and epididymis. Enlarged, deformed atrophic testis with gnarls and epididymis with granulomatous areas.

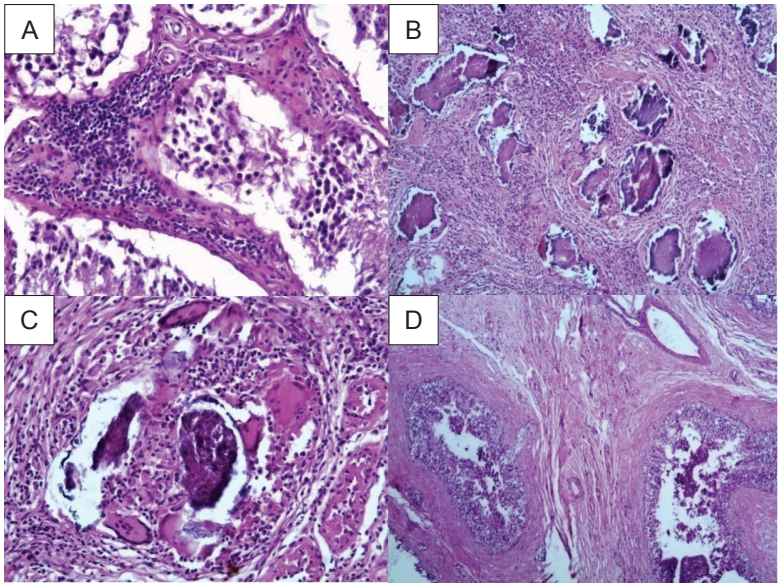

Figure 4: A) Pronounced interstitial mononuclear inflammatory infiltrate and seminiferous tubules with reduced sperm production and desquamated germinative epithelium (H & E, x200). B) The testicular atrophy with numerous microcalcifications, indicated by diffuse interstitial fibrosis and accumulation of inflammatory cells (H & E, x100). C) Spermatic granuloma with central accumulation of spermatozoa, surrounded by chronic inflammatory mononuclear and multinucleated giant cells in the testis (H & E, x200). D) Papillary hyperplasia with necrosis of epithelial cells, and interstitial fibrosis (H & E, x100)

Microscopic examination of epididymis confirmed vacuolar degeneration, papillary hyperplasia and necrosis of duct epithelial cells, and oedema and fibrosis of the interstitial area. In individual ducts, the formation of microabscesses and intraluminal necrotic tissue detritus were observed. In the interstitial area, mixed cell inflammatory infiltrate composed of plasma cells, lymphocytes and histiocytes was observed. Testes significantly reduced production of spermatozoids, with desquamated germinative epithelium in places. Individual preparations showed spermatic granulomas with a central accumulation of spermatozoids surrounded by chronic mononuclear inflammatory and multinucleated giant cells (Figure 4).

Results of bacteriological testing

Bacteriological testing was conducted on 21 epididymis, testes and lymph node tissue samples originating from 12 rams. A total of 20 isolates were obtained from the material of 11 (91.7%) rams.

Results of molecular testing

All 20 isolates and the standard referential strains of *B. abortus 544, B. melitensis 16M, B. ovis 63/290* and *B. suis 1330* were identified as *Brucella* using PCR. They were identified on the basis of the replication product of around 440 base pairs.

Species identification was done using multiplex PCR method (*Bruce-ladder, Ingenasa, Spain*). All 20 brucella isolates were identified as *Brucella ovis*. The PCR profile of isolates overlapped with the profile of referential strain *B. ovis 63/290*. The expected sizes of PCR products for *Brucella ovis* were 1071, 794, 587, 450 and 152 base pairs.

Discussion

In recent decades, serologically positive animals on *B. ovis* infection have been confirmed in the Republic of Serbia, but the causative agent has never been isolated and identified (29, 31, 36).

In this study, indirect enzyme immunosorbent assay tests confirmed positive reactions in 67 (29.8%) and suspicion in 31 (13.8%) of the total 225 tested ram blood sera samples. Seropositive rams originated from 16 settlements (57.1% investigated settlements in the Pirot region) and 34 flocks (30.1% of investigated flocks in the Pirot region).

Complement fixation test confirmed a positive reaction in 41 (18.2%) of the 225 investigated rams. Seropositive rams originated from 14 settlements (50% of investigated settlements in the Pirot region) and 23 flocks (20.4% of the investigated settlements in the Pirot region).

All seropositive flocks showed clinical manifestations of sheep epididymitis that appeared following the purchase of infected rams, less commonly ewes, and during the mating season. In flocks where the disease occurred for the first time after mating, flock owners reported a high incidence of the disease, and in flocks where sheep epididymitis was present for years the symptoms intensity was lower. Literature states that *B. ovis* infections in sheep caused lambing reduction by 30% in newly infected flocks, and 15–20% in flocks where the disease is endemic (9). Following infection, the number of live births can be reduced by a quarter, 16% of lambs die within the first 6 weeks of life, and 20% of lambs remain infertile (30). The results of the epizootiological survey presented here closely corroborate these reports.

Earlier studies showed that about 50% of *B. ovis* infected rams exhibit epididymitis, though clinical symptoms often withdraw (8). After test inoculation of nine rams intraprepucially and conjunctively with *B. ovis,* the following was seen: eight of nine rams were serologically positive (AGID and CFT). Thirty days after infection, six (66.7%) out of nine rams developed clinical changes in epididymis tail (increased volume and change in consistency) which regressed in 50% of those rams. Five (83.3%) out of six rams had unilateral changes, while four (66.6%) showed sensitivity to palpation (13). Numerous pathogens other then *B. ovis* are often responsible for macroscopic testicular alterations in rams. This restricts the significance of clinical examination of epididymis and testes of rams in diagnosis of sheep epididymitis (7, 8, 43, 44). Various bacterial species *(Actinobacillus seminis, Histophilus somni, Salmonella enterica subsp. diarizonae, Brucella melitensis, Escherichia coli)* can also cause clinical epididymitis (3, 14, 16, 20, 24). Also, it has been shown that in many cases of ram epididymitis, sterile granulomas are caused by trauma (44).

The clinical examination of 12 seropositive rams by epididymis and testes adspection and

palpation showed an increased percentage of rams with changes to epididymis and testes in comparison to epizootiological data. Asymmetry of scrotum and unilateral enlargement of epididymis tail was confirmed in 5 (41.7%) rams. Pathomorphological examination of epididymis and testes of 12 rams confirmed characteristic macroscopic findings of unilateral epididymitis with pronounced hypertrophy of epididymis tail in 7 (58.3%) rams, changes in epididymis body were found in 4 (33.3%) rams and in epididymis head in 2 (16.7%) rams. Microscopic examination of epididymis confirmed vacuolar degeneration, papillary hyperplasia and necrosis of duct epithelial cells, oedema and fibrosis of interstitial areas. In individual tubules, formation of microabscesses and intraluminal necrotic tissue detritus was observed. The interstitial area contains mixed cell inflammatory infiltrate composed of plasma cells, lymphocytes and histiocytes. Individual testes showed pronounced atrophy and fibrosis of sperm canals, presence of numerous microcalcifications with surrounding giant cell reactions and marked interstitial fibrosis with diffuse accumulation of chronic inflammatory cells.

The present study corresponds to research of other authors. Study conducted on 267 serologically positive rams, palpation confirmed enlarged testes in 125 (46.8%) rams. Following pathohistological examination, the number of animals exhibiting enlarged testes and epididymis was increased (7). Pathoanatomical changes were found in 68 (43.6%) out of 156 rams. An enlarged epididymis was confirmed in 38 (24.4%) rams, testicular atrophy in 14 (8.9%) and granulomas in testes and epididymis tissue in 16 (10.3%) tested rams. The pathohistological examination of epididymis in rams infected with *B. ovis* revealed interstitial oedema, fibrosis and perivascular infiltrates of lymphocytes and plasma cells. The same author confirmed epithelial hyperplasia with intraepithelial cysts containing neutrophils mixed with mononuclear cells (42). Granulomas surrounded by lymphocytes, epithelial and giant cells were a common find. A proliferation of intertubular connective tissue, small extratubular spermatozoic granulomas, necrosis and calcification were observed in individual preparations (38).

Epizootiological analysis of obtained results, clinical and pathomorphological examination in the present study indicate a higher percentage of rams with changes to epididymis and testes in relation to results of cited authors. This is a direct consequence of the lack of diagnostic testing for sheep epididymitis, long-term presence of the infection and lack of disease eradication programmes.

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EPIZOOTIOLOŠKE, KLINIČNE IN PATOLOŠKE ZNAČILNOSTI ČRED OVC, OKUŽENIH Z *BRUCELLO OVIS* V REPUBLIKI SRBIJI

M. Petrović, S. Špičić, A. Potkonjak, B. Lako, M. Kostov, Ž. Cvetnić

Povzetek: Članek opisuje raziskavo okužbe čred ovc z *Brucello ovis* na jugu Srbije. Pri serološkem testiranju so bile potrjene pozitivne reakcije v 67 (29,8 odstotka) in sumljive reakcije v 31 (13,8 odstotka) od skupno 225 testiranih seroloških vzorcih ovc. Ovni so izvirali iz 113 čred z 4751 ovcami iz 28 naselij v pokrajini Pirot v južni Srbiji. Patološke spremembe, ki kažejo na okužbo z *Brucello ovis*, so potrdili z makroskopskim pregledom mod pri 7 (58,3 odstotka) od 12 preučevanih ovnov. Pri 7 živalih (58,7 odstotka) so potrdili enostransko vnetje nadmodka z izrazito hipertrofijo repa, telesa in glave nadmodka. Z uporabo bakterioloških in molekularnih metod so potrdili prisotnost *B. ovis* v vzorcih (moda, nadmodek, bezgavke) pri 11 od 12 ovnov (91,7 odstotka). Klinična izraženost vnetja nadmodka ovnov je nastala po nabavi ovnov in po parjenju. Bolezen je bila izrazitejša v čredah, v katerih je prišlo prvič do pojava bolezni. Na podlagi predstavljenih rezultatov je mogoče sklepati, da okužba z *B. ovis* povzroča velike gospodarske izgube v proizvodnji ovc, ki se kaže v zmanjšani zmožnosti obrejitve (24,85 odstotka), s splavi (5,38 odstotka), z zmanjšanim številom jagnjet (0,74 jagnjeta na ovco) in povečano umrljivostjo jagnjet ob rojstvu (7,78 odstotka).

Ključne besede: *Brucella ovis*; epizootiologija; klinične spremembe; patološke spremembe; Srbija