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Acta Veterinaria-Beograd 2024, 74 (1), 133-144
UDK: 636.32/.38.09:616.993-07(497.11)
DOI: 10.2478/acve-2024-0010

*Research article*

# RELIABILITY OF MOLECULAR TESTS IN DIAGNOSING OVINE BRUCELLOSIS CAUSED BY *BRUCELLA OVIS*

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(Received 01 February, Accepted 04 March 2024)

*Brucella ovis* infects sheep and causes a clinical or subclinical disease characterized by genital lesions and reduced fertility in rams, placentitis and abortions in ewes, and increased perinatal mortality in lambs. As part of this study, analyses were conducted on a sheep farm near Belgrade (Serbia). Of the serological tests, indirect ELISA was performed. A total of 94 blood sera were analyzed, 33 from rams and 61 from ewes. The results showed 23 (69.7%) positive findings in rams and 2 (3.3%) positive findings in ewes, with an overall prevalence of 25.4% bounded by a 95% confidence interval. Bruce-ladder multiplex PCR and Real time PCR were performed on 19 serologically positive rams and two serologically positive ewes. The results indicate a slightly higher sensitivity of Real time PCR compared to conventional PCR in diagnosing *B. ovis* from the reproductive tissues of rams. However, the differences in sensitivity between different nucleic acid extraction protocols were not significant. Most rams showed a positive PCR result in only one sample of reproductive tissue, suggesting the recommendation to take multiple samples from each animal. Further research is needed to bring the sensitivity of molecular tests in diagnosing ram epididymitis closer to the sensitivity of serological tests.

**Keywords:** *Brucella ovis*, ELISA, molecular diagnostics, sheep

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## INTRODUCTION

*Brucella ovis* is a Gram-negative bacterium with a naturally rough cell wall type and is a causative agent of a disease known as ovine epididymitis [1]. When it comes to domestic animals, only sheep with genital lesions and reproductive disorders are affected. In rams, the dominant clinical finding is epididymitis (more often unilateral than bilateral), orchitis and infertility [2]. In ewes, placentitis, abortion, and avital stillborn lambs [3].

The disease is mainly transferred by mating, the sheep are passive carriers of the disease, while rams develop a subacute or chronic infection with intermittent excretion of *B. ovis* in the semen, genital secretions and urine for at least 2 to 4 years [3].

*B. ovis* infection does not lead to the appearance of pathognomonic clinical symptoms, it is often of subclinical course and can circulate in the herd for a long time without noticeable symptoms. Furthermore, more than half of the cases of *B. ovis* disease do not lead to clinically manifest epididymitis, which complicates the clinical suspicion of this disease [4]. The diagnosis of brucellosis caused by the *Brucella ovis* species is established in laboratories. [5,6]. Antibody detection tests such as ELISA, AGID and CFT, bacterial isolation from semen, urine or genitals, and molecular tests (PCR) that detect pathogen DNA can be used [7]. To make a diagnosis with greater certainty, periodic re-sampling is recommended as the dissemination of *B. ovis* is intermittent. Although ELISA is more sensitive than CFT, CFT is more commonly used due to the lack of an internationally recognized standardized ELISA test [3].

This research aimed to examine the sensitivity of classical and real-time PCR in the detection of sheep infection with *B.ovis* in comparison to a widely used serological test with high sensitivity and specificity. Available literature indicates the lack of sensitivity of molecular tests compared to serological tests that detect the presence of specific antibodies to *B.ovis* [8-10].

## MATERIAL AND METHODS

### Sample collection

The research was conducted on a sheep farm near Belgrade. The farm is of a non-commercial type, the rams are kept separately from the ewes with the exception of a few rams in the breeding season. On the farm, visible palpable changes in the testicles were noticed in most of the rams. The material was collected from a total of 94 animals, 33 males and 61 females of the Ile de France sheep breed. For serological tests, blood samples from animals were obtained by jugular vein *puncture.* Respecting the principle of asepsis, blood was taken in sterile glass tubes in an amount of about 6 ml. After spontaneous coagulation, the blood was centrifuged at 2000xg for 15 minutes. Blood sera were then separated into Eppendorf microtubes and stored at -20 °C until further processing. Preputial and vaginal swabs were taken from serum

ELISA-positive animals and soaked in phosphate-buffered saline PBS (Thermo Fisher Scientific, USA). Samples were frozen and stored in the freezer at -20 ºC until use.

For the purpose of molecular testing, testis and epididymis tissue samples were taken from 19 serum ELISA-positive rams. After induction into general anesthesia, orchidectomy was performed. Two pooled tissue samples of the testis and epididymis were obtained from each ram. Reproductive organs were treated in the Stomacher machine after being previously chopped with sterile scissors and tweezers. The organs were homogenized in a Bag Mixer 400 P (Interscience, France), magnitude 8 beats/s. Organ dilutions with saline in a ratio of 1:2 were used. Samples were homogenized in bags with porosity filters < 250 μm (Bag Filter P), with a volume of 400 ml (Interscience, France). The material homogenized was packed in Eppendorf microtubes and stored at -20 ºC until use. To conduct this study, authorization was obtained from the Ethical Committee for the Protection and Well-being of Laboratory Animals at the Faculty of Veterinary Medicine, University of Belgrade (Approval No. 01-522, dated May 29, 2023).

## Serological testing

Serums were tested for the presence of specific antibodies against Brucella ovis by indirect ELISA commercial kit Ingezim Brucella ovis (Ingenaza, Spain). The test was performed according to the manufacturer`s recommendations. A total of 94 serum samples were examined by an Ingezim Brucella ovis indirect ELISA test, 33 from male and 61 from female animals. According to the manufacturer, diagnostic sensitivity and specificity values of the used ELISA kit were 99% and 98%, respectively.

## Molecular methods

Bruce-ladder multiplex PCR and Real time PCR tests were performed on testicular and epididymal samples of 19 adult rams that were positive for the presence of antibodies against B. ovis. Both tests were also performed on preputial swab samples of the 19 serologically positive rams, and on vaginal swab samples of two serologically positive ewes, and whole blood samples of all serologically positive animals. Two different commercial nucleic acid extraction kits were used in the study, ThermoScientific "DNA extraction kit" (Thermo Fisher Scientific, USA) and QIAlamp Cador Pathogen "DNA extraction" mini kit (Qiagen, GERMANY). Currently, there is no available literature specifically addressing the comparative use of different extraction protocols for the genomic confirmation of *B. ovis*.

Samples of extracted DNA from homogenized organs were tested with the Bruce-ladder multiplex PCR method described by Garcia-Yoldi et al., [11]. After preliminary testing, the protocol was modified by adding 10 μl of extracted DNA to the PCR mixture instead of 1 μl of DNA, and the amount of total PCR mixture was 50 μl. Primers were used at a final concentration in the PCR mixture of 0.25 pmol/μl. The final concentration of dNTP (Thermo Scientific, USA) was 0.4 mM. Taq polymerase

(Thermo Scientific, USA) was added in the amount of 0.5 µl. The program on which the PCR reaction was performed included: initial denaturation, at 95 °C, for 7 minutes, followed by 25 cycles of template denaturation at 95 °C, for 35 seconds, annealing at 64 °C, for 45 seconds, and primer extension at 72 °C, 180 seconds, and then final extension at 72 °C, for 6 minutes. Visualization of obtained PCR products was performed using the method of horizontal electrophoreses in agarose gel with a concentration of 1 % (Serva, Germany) with the addition of GreenSafe Premium dye (NZYTech, Portugal) in the final concentration of 1%.The DNA of previously isolated and confirmed *Brucella melitensis and Brucella ovis Reo 198* (CO2-independent strain) was used as a positive control, and the Mass ruler DNA 100 bp ladder (Thermo Scientific, USA) as a DNA marker. The appearance of five (absence of 1682 bp and 272 bp) bands of the following lengths was considered positive: 1071, 794, 587, 450, and 152 bp.

Real time PCR was completed using a commercial kit, Luna® Universal qPCR Master Mix (NEB, USA). The target sequence was IS711 with primers: forward GCTTGAAGCTTGCGGACAGT, reverse GGCCTACCGCTGCGGAAT, probes FAM-AAGCCAACACCCGGCCATTATGGT-TAMRA. The reaction mix was composed of 2.5 µl template DNA, 5.5 µl Master Mix, 0.44 µl of each primer (10 µM), 0.22 µl of probe (10 µM) previously published by Hinić et al., [12], and 3.4 µl Rnase-free water. Amplifications of the IS711 genome region were accomplished using Quant Studio 3, Applied Biosystems (USA) and temperature profile as follows: initial denaturation at 95 °C for 1 min, 40 cycles of denaturation at 95 °C for 15 s, and 60 °C for 30 s. The samples with a Ct value lower than 35 were considered positive.

### Statistical analyses

The statistical analyses were done using the statistical software package Microsoft Office Excel 2016 (XLSTA for Excell). The generated results were evaluated by the battery of statistical tests, i.e., the chi-square test ($\chi 2$ –test) and Fisher's exact test, as well as the association coefficient Cohen's kappa. For the ELISA test, the values of the association coefficients as well as the predictive values of the test, likelihood ratios, the rate of false positive and false negative results, for the specificity and sensitivity of the test given by the test manufacturer were calculated.

## RESULTS

### Pathomorphological changes

Clinical examination showed unilateral enlargement of the tail of the epididymis in 63.16% (12/19) rams, enlargement of the head of the epididymis in 31.56% (6/19), while pain was noted in 21.05% (4/19) rams, the testicles and epididymis were mobile in the scrotum in 57.89% (11/19) of rams, while a firmer consistency of testicles and

epididymis was noted in 47.37% (9/19) of rams. Histopathological changes in the testicles and epididymis were found in all examined animals (19/19).

## Serological testing

Out of the 33 ram serum samples, 23 were positive (69.7%), as well as two (3.3%) out of the 61 ewes serum samples. The overall apparent seroprevalence of *Brucella* infection was 26.6% bounded by a 95% confidence interval (CI) of 17.664% and 35.528%, and the true seroprevalence was 25.4% bounded by 95 CI of 16.6% and 34.2%, respectively (Table 1).

**Table 1.** Properties of ELISA test

| | **Value** | **Lower bound (95%)** | **Upper bound (95%)** |
|---|---|---|---|
| Correct classification | 98.25% | 95.605% | 100.00% |
| Misclassification | 1.75% | 0.00% | 4.39% |
| Sensitivity | 99.00% | 96.989% | 1.00% |
| Specificity | 98.00% | 95.170% | 1.00% |
| True + | 23 | | |
| **False +** | 1 | | |
| True - | 69 | | |
| **False -** | 1 | | |
| Infected | 23 | | |
| False positive rate | 2.00% | 0.00% | 4.83% |
| False negative rate | 1.00% | 0.00% | 48.87% |
| Apparent prevalence | 26.60% | 17.66% | 35.53% |
| True prevalence | 25.36% | 16.56% | 34.15% |
| PPV (Positive Predictive Value) | 95.83% | 91.79% | 100.00% |
| NPV (Negative Predictive Value) | 98.57% | 96.17% | 100.00% |
| LR+ (Positive likelihood ratio) | 49.50 | 4.63 | 528.96 |
| LR- (Negative likelihood ratio) | 0.01 | -0.01 | 0.03 |
| Relative risk | 67.08 | 61.43 | 73.26 |
| Odds ratio | 1587.00 | 95.38 | 26,405.94 |

## Molecular methods

From 38 samples of homogenized testicles and epididymis Bruce-ladder multiplex PCR method on extracts performed with QIAamp Cador Pathogen mini kit and Thermo Scientific kit had a total of six (15.79%) and four (10.53%) positive results, respectively. On the same samples, Real time PCR had a total of 11 (28.95%) and 10 (26.32 %) positive results (Figure 1A). If we look at the number of rams that had a positive result on at least one of the paired samples, then classic PCR had a sensitivity

of 31.6% (6/19) and 21.1% (4/19), respectively, while Real time PCR had a sensitivity of 52.6% (10 /19) and 47.4% (9/19) (Figure 1B).

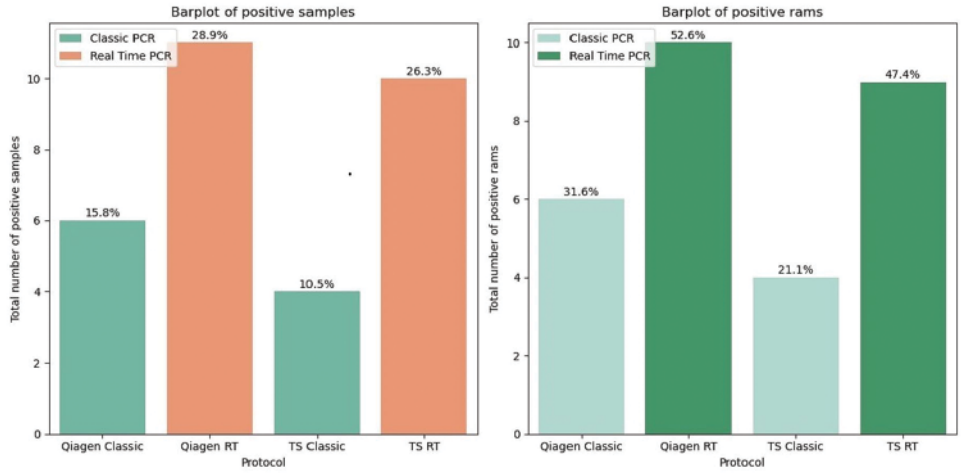


**Figure 1. A)** Total number and percentage of positive results obtained by different PCR protocols on testicular and epididymal tissue; **B)** Total number and percentage of positive rams on different PCR protocols. A ram was considered positive if any of the two pooled testicular and epididymal tissue samples were positive.

Classical PCR had a good concordance of 94.74% and a substantial Cohen's kappa value of 0.771 (95% CI 0.614 – 0.929) on the samples of both extraction protocols. On the other hand, Real time PCR protocols have a significantly worse concordance of 71.05% and a Cohen's kappa value of 0.277 (95% CI 0.093 – 0.461).

Observing all four PCR protocols separately, only on two occasions one ram was positive for both reproductive tissue samples (Table 2).

Considering both DNA extraction kits used 31.57% (6/19) rams had positive Bruce ladder multiplex PCR results and 73.68% (14/19) had positive Real time PCR results. On the chi-square test ($\chi 2$ –test) and Fisher's exact test for a significance level of 0.05, these differences were statistically significant.

Whole blood and preputial/vaginal swabs did not have positive results on any of the four PCR protocols.

**Table 2**. Comparison of results of four PCR tests for *Brucella ovis* on reproductive tissues

| Ram | Qiagen classic PCR | Thermo Scientific classic PCR | Qiagen Real time PCR | Thermo Scientific Real time PCR |
|---|---|---|---|---|
| 1 | - | - | - | ++ |
| 2 | + | + | + | + |
| 3 | - | - | - | - |
| 4 | + | + | + | + |
| 5 | - | - | + | - |
| 6 | - | - | - | - |
| 7 | - | - | + | - |
| 8 | - | - | + | - |
| 9 | - | - | - | - |
| 10 | - | - | - | - |
| 11 | - | - | - | - |
| 12 | + | + | ++ | + |
| 13 | + | + | + | + |
| 14 | - | - | + | - |
| 15 | - | - | + | - |
| 16 | + | - | - | + |
| 17 | - | - | - | + |
| 18 | + | - | + | + |
| 19 | - | - | - | + |

- =Negative, + =Positive with one sample, ++ =Positive with two samples

## DISCUSSION

On the territory of the Republic of Serbia, ram epididymitis was initially confirmed, and *B. ovis* was first isolated in the vicinity of Pirot [13]. Serological methods have proven to be very reliable in laboratory diagnosis and eradication of the disease, and the most commonly used methods are ELISA, CFT and somewhat less frequently, AGID [3,4]. In one study, compared to the CFT test, the indirect ELISA (Chekit *B. ovis*, Idexx, France) exhibited a sensitivity of 91.7% and a specificity of 95.2% [14]. In another study, also compared to the CFT test, the indirect ELISA (modified according to the method reported by Vigliocco et al. [15]) demonstrated a sensitivity of 96.3% and a specificity of 99.6% [16]. The high percentage of infected rams, coupled with the observation that the vast majority of them had no contact with sheep, highlights a significant role of inter-ram interactions in the transmission of *B. ovis* in this study. Rams are more susceptible to infection than ewes. Ewes rarely become actively infected and transmit the disease during abortion. Because only a few infected ewes abort or have dead or weak lambs, ewes appear to be relatively resistant to infection

and usually act as mechanical vectors [17,18]. Regarding low seroprevalence in sheep (3.3%), similar results were obtained by Galluzzo et al. (3.5%) [10].

Molecular methods, although generally very sensitive and specific, showed a large lack of sensitivity in this study. Of course, there is a probability that some of the 19 ELISA positive results were false positives. By analyzing the characteristics of the ELISA test, we determined that it is possible to expect a rate of false positive results of 2% (95% CI 0.00 – 4.83%). A study conducted in the US state of Wyoming in 2019 found that 29% (5/17) seropositive rams have negative PCR, cultural and histopathological results [8]. The presence of *B. ovis* in different tissues of the reproductive tract is different and is most often detected in the tissues of the epididymis (especially the tail), seminal vesicles and ampullas, while in the testicular tissue the percentage of positive PCR findings in serologically positive animals varies from 0-5% [8]. Therefore, some authors recommend that several different tissues should be taken in duplicate or even triplicate to perform molecular tests [8]. Bearing in mind that in our research we had only two pooled samples of testicular and epididymal tissue per ram, the obtained results were expected. When looking at the sensitivity of the classic PCR tests in proving the presence of the *B. ovis* genome from the reproductive tissue, it ranges from 10-80% [9] and 0-45% [8] depending on the type of tissue.

Looking at each PCR protocol, only two rams had positive results on both pooled reproductive tissue samples. This result could perhaps be explained by the well-known fact that epididymitis in rams is often unilateral. Even when the changes are bilateral, there is a possibility that there are no bacteria or a small number of bacteria can be found in the tissue sample, which is below the detection limit of the molecular test. The insufficient yield of nucleic acids after extraction as well as the poor efficiency of amplification on the thermal cycler could explain the lack of sensitivity of molecular tests. Also, any inhibitor in DNA samples from any sources may limit the use of these methods [19]. The results of different PCR protocols in this study showed a significant degree of disagreement even though the same homogenized pooled testis and epididymis samples were used in all of them. A greater number of positive samples with the Real time PCR protocol indicates their greater sensitivity, although this study is too small to confirm this. Based on the power analysis, the ideal sample size should be 220 samples to obtain a more confident conclusion. It is interesting that both Real time PCR protocols, although with a small difference in the total number of positive results, showed a significant level of disagreement. Both protocols used the same samples, the same PCR mix and had identical conditions in the thermal cycler, the only difference was that the template was obtained by different extraction protocols. Although we cannot rule out the possibility of sample contamination during manipulation, there is a possibility that the reason for this phenomenon is the variable yield of *B. ovis* nucleic acid during the extraction process. The results obtained by several authors suggest that different nucleic acid extraction protocols differ in nucleic acid recovery, reproducibility and linearity [20,21].

When comparing classic and Real-time PCR protocols, most samples positive in classic PCR also showed positivity in Real time PCR, except for one discrepancy in ram number 16 (Table 2). Conversely, 12 samples (six in each extraction protocol) were negative in classic PCR but positive in Real time PCR. Despite using the same extracts, indicating the presence of *B. ovis* DNA, Bruce-ladder multiplex PCR protocols failed to detect it. This implies lower sensitivity in Bruce-ladder multiplex PCR, likely due to low target DNA concentrations in the template. Notably, classic and Real time PCR target different segments of the *B. ovis* genome, leading to varying yields of DNA. Abdel-Hamid et al. confirmed the higher diagnostic sensitivity of Real time PCR compared to classic PCR in the diagnosis of brucellosis in cattle [22].

Some research suggests that preputial/vaginal swabs may be a valid sample for detection of *B. ovis* DNA with Real time PCR [9,10]. Our results show that a preputial/vaginal swab can in no way be a valid sample for the diagnosis of ovine epididymis. It has been proven that *B. ovis* is occasionally excreted in the semen and urine [9], and it is likely that preputial/ vaginal mucosa, although the primary site of entry of bacteria into the body, is not the site of retention. Such low sensitivity of PCR tests in this study may be due to the inappropriate or ineffective DNA extraction protocol we chose, low brucellosis concentration or their complete absence in the samples [23,24]. False-negative results can also occur for several reasons such as the presence of EDTA, RNAase, DNAase, heme, heparin, phenol, polyamine, plant polysaccharides, urine, calcium alginate and perhaps some other reagents [19,25].

None of the whole blood samples tested positive for *B. ovis* DNA. Similar results were obtained by Xavier et al. [9]. After getting infected through preputial, vaginal, oronasal or conjunctive mucosa, *Brucella ovis* through afferent lymphatic vessels and regional lymph nodes enter the bloodstream spreading throughout the entire body [26]. They are then localized in the epididymis, seminal vesicles, bulbourethral glands, ampoules and bladder. *B. ovis* remains in the bloodstream for a short period, thus the blood is not a good sample to prove the presence of *B. ovis* DNA. However, the possibility of loss *of B. ovis* nucleic acid during the process of its extraction from the blood cannot be ruled out. In humans, washing the blood a few times with water or lysis buffer until all the hemoglobin disappears before DNA extraction increases the PCR sensitivity substantially [27]. A PCR method that incorporates this washing procedure, a higher number of PCR cycles (40 cycles instead of 35), and primers for the gene encoding the *Brucella* cell surface salt-extractable (BCSP) 31-kDa protein can detect 700 CFU/ mL of peripheral blood [28].

In conclusion, we can state that Real time PCR tests showed a slightly higher sensitivity compared to classic PCR tests, while there were no significant differences in sensitivity between the different extraction protocols. The lower sensitivity of classical PCR protocols is probably due to their inability to detect low concentrations of target DNA in the template. Insufficient agreement with Real time PCR tests possibly indicates the need to find more efficient extraction protocols that would be able to give maximum nucleic acid yields from samples in which a small number of *B. ovis* is present. The

fact that the largest number of rams was positive only on one of the two aggregate samples of reproductive tissues examined indicates the need to take a larger number of samples from one animal. Further research is necessary to bring the sensitivity of molecular tests in the diagnosis of ovine epididymitis closer to the sensitivity of serological tests.

**Acknowledgements**

The study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract No 451-03-47/2023-01/200143).

**Authors' contributions**

MZ contributed in material sampling, laboratory work and wrote the manuscript. SR and DM conceived the study, participated in material sampling, its design and coordination, and interpretation of results. SS performed the statistical analysis and interpreted the data. NS participated in material sampling and laboratory work. VM participated in laboratory work and interpretation of molecular test results. MA participated in material sampling. OV performed the statistical analysis of the serological tests and edited the manuscript. All authors read and approved the final manuscript.

**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## POUZDANOST MOLEKULARNIH TESTOVA U DIJAGNOSTICI BRUCELOZE OVACA IZAZVANE VRSTOM *BRUCELLA OVIS*

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*Brucella ovis* inficira ovce i uzrokuje kliničku ili subkliničku bolest koja se karakteriše genitalnim lezijama i smanjenom plodnošću kod ovnova, placentitisom i pobačajima kod ovaca te povećanom neonatalnom smrtnošću kod jaganjaca. U okviru ovog istraživanja, sprovedene su analize na farmi ovaca u blizini Beograda (Srbija). Od seroloških testova, rađena je indirektna ELISA. Ukupno je analizirano 94 krvna seruma, 33 od ovnova i 61 od ovaca. Rezultati su pokazali 23 (69,7%) pozitivna nalaza kod ovnova i 2 (3,3%) pozitivna nalaza kod ovaca, s ukupnom prevalencijom od 25,4% uz 95% interval pouzdanosti. *Bruce-ladder multiplex* PCR i *Real time* PCR su rađeni kod 19 serološki pozitivnih ovnova i dve serološki pozitivne ovce. Rezultati pokazuju da je *Real time* PCR pokazao nešto veću osetljivost u poređenju s konvencionalnim PCR-om u dijagnozi *B. ovis* iz reproduktivnih tkiva ovnova. Ipak, razlike u osetljivosti između različitih protokola ekstrakcije nukleinske kiseline nisu bile značajne. Većina ovnova je imala pozitivan PCR rezultat samo kod jednog zbirnog uzorka reproduktivnog tkiva, što ukazuje na preporuku uzimanja više uzoraka od svake životinje. Potrebna su dalja istraživanja kako bi se osetljivost molekularnih testova u dijagnozi epididimitisa ovnova približila osetljivosti seroloških testova.