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DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR THE DETECTION OF *BRUCELLA OVIS* DNA IN CLINICAL SAMPLES

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Summary. The etiological agent of infectious ovine epididymitis is *Brucella ovis* and for its direct indication in clinical samples several PCR protocols are proposed. This study describes a design and selection of the oligonucleotides for real-time PCR targeting conservative BOV_A0504 gene. The specificity of a real-time PCR was validated using 25 *B. ovis* field isolates and 14 microorganisms of closely related species. The detection limit of *B. ovis* in bacterial culture was determined as 3.5×10^1 CFU/mL with Ct value of 37.8. There are no detectable fluorescence signals in the clinical samples from intact animals, whereas bacteriologically confirmed material such as urine and testicle tissue samples were positive. It confirms that the assay is highly specific for detection of *B. ovis* DNA. Thus, the proposed real-time PCR assay enables fast detection and quantification of *B. ovis* in clinical material, which can be used as additional test for estimation of the health status of a sheep herd

Keywords: specific primers, infectious ovine epididymitis, sheep

Introduction. *Brucella* (*B.*) *ovis* is associated with the infectious ovine epididymitis (IOE) that is one of the most important infectious diseases in sheep causing significant economic losses worldwide (OIE, 2018).

This infection is characterized by epididymitis and orchitis in rams, abortion in ewes as well as birth of weak lambs.

The diagnosis is based on the gold standard methods such as serology (CFT, ELISA and AGID) and pathogen isolation (Alton et al., 1988).

As an additional and express test several molecular techniques were elaborated, including conventional PCR (Bricker, 2002; Manterola et al., 2003; Xavier et al., 2010), nested PCR (Costa et al., 2013), and multiplex PCR (Saunders et al., 2007; López-Goñi et al., 2008; Moustacas et al., 2013). These assays are highly specific and faster than serological and bacteriological methods. Moreover PCR could help to confirm *B. ovis* circulating in some clinical cases or during bacterial culture characterization. Real-time PCR is an effective molecular tool allowing quantification of target DNA without electrophoresis that is less time-consuming.

For the differentiation among *Brucella* spp. species a real-time PCR assay have been developed (Hinić et al., 2008) as well as a protocol for simultaneous *B. ovis* and *H. somni* indication in urine and sperm samples (Moustacas et al., 2015), but there is a lack of *B. ovis*-specific protocol.

Thus, **the aim of this study** was to design specific primers and probe as well as develop real-time PCR protocol for *B. ovis* DNA detection in clinical samples.

Materials and methods. Oligonucleotide sequences for the real-time PCR primers and probe were designed using AmplifX v. 2.0.7 (https://inp.univ-amu.fr/en/amplifx-manage-test-and-design-your-primers-for-pcr) and BioEdit v. 7.0.0 (Rozen and Skaletsky, 2000) based on sequence data available from GenBank (https://www.ncbi.nlm.nih.gov/genbank). These sequences were compared using the basic local alignment search tool BLAST (https://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1990). Primers and the FAM-labeled probe were commercially synthetized.

To assess analytical specificity of real-time PCR a panel of DNA samples was prepared, which included 25 field isolates of *B. ovis*, reference strain *B. ovis* 63/290, that was kindly provided by Dr. Claire Ponsart (ANSES, France), other species of the genus *Brucella*, *Yersinia enterocolitica*, *Campylobacter fetus* subsp. *fetus*, *Corynebacterium pseudotuberculosis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. Enteritidis (all strains were obtained from the National Collection of Microorganisms of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine).

For DNA extraction 3–5 colonies of each microorganism were transferred into 200 µL of sterile saline buffer and boiled at 95°C for 10 min. The cell lysate was centrifuged at 17,380 g for 1 min. The 5 µL of supernatant was used for amplification.

Clinical materials (urine, vaginal swabs, testicles and lymph nodes) were sampled from infected and intact animals in two farms in Kharkiv Region. DNA extraction was done using innuPREP DNA/RNA Mini Kit (Analytic, Jena, Germany) following the manufacturer's instruction.

The DNA concentration was estimated using DeNovix DS spectrophotometer and converted to copy

number based on an average genome size of 3.28 Mb estimated from completed *B. ovis* ATCC 25840 genome available in GenBank (accession number CP000708 and CP000709). The standard DNA copies were prepared based on the size of the genome. Sensitivity of the method was proved by testing of *B. ovis* DNA in dilution from 10^7 to 10^1 to determine the detection limit of bacterial copy number. The coefficient of determination (R^2) and the slope value (s) of the regression curve were calculated.

PCR was performed in a 25 μL reaction volume using 2xABsolute Blue QPCR Mix, low ROX in plates MicroAmp® and 7500 Real-time PCRSystem (Applied Biosystems, USA) due to the optimal reaction conditions such as incubation times and temperatures, concentrations of the primers and dNTPs.

The detection of a fluorescent signal was done by the FAM channel (492–516 nm) and data was registered during the extension step. The results were interpreted by the presence (or absence) of the intersection of the fluorescence curve with the threshold level (Ct) set at the appropriate level. The sample was considered positive if for this sample the Ct value ≤ 38 on the FAM channel; if the value of Ct was absent (the fluorescence curve does not cross the threshold line) or greater than 38 the sample was considered negative or questionable respectively. Agreement between culture and real-time PCR was assessed by Kappa test.

Results and discussion. To develop a PCR assay for the indication of *B. ovis* genetic material, available complete genomes and different genes (IS711, 16S and 23S rRNA, Ykwd, HSP70, HSP40, CSP, Omp2, Omp31, ABC transporter and others) of *B. ovis* strains were analyzed and specific primers and probe were selected.

To select promising DNA targets, the publication regarding the comparison of complete genomes of the pathogens was analyzed (Rajashekara et al., 2004).

For the differentiation of *B. ovis* loci which are partially or completely absent at representatives of this kind can be used, namely: BMEI0899-0907, BMEI0993-1012, BMEII0129, BMEII0185-0226, BMEII0405, BMEII0708, BMEII0811-0815, BMEII0875-0878. The genes BMEI0994 and BMEII0812 were promising for the further study.

The first locus is located in the region of BMEI0993-1012, the genes of which are involved in the genetic markers of the Bruce-Ladder system (BMEI0998-0997), as *B. ovis* characterization (López-Goñi et al., 2008).

The second locus is specific according to the results of analysis *in silico*. Therefore, in order to develop a test system for the indication of *B. ovis* genetic material by real-time PCR, we additionally searched for conservative regions of pathogen's genes to construct specific primers and probes. To do this, we studied openly published complete genomes and genes using GenBank nucleic acid sequence databases and bioinformatics using BioEdit v. 7.0.0 software. In general, 71 genes of different strains of *B. ovis* were analyzed.

It was found that the considered sequences are not suitable for the primers construction, because the divergence within these genes of different *Brucella* species is only 3%. Therefore, the complete genome of the reference strain *B. ovis* ATCC 25840 was further analyzed.

Based on the results of this work, genes encoding hypothetical conserved proteins BOV_0019, BOV_0245, BOV_0650, BOV_0651, BOV_0950, BOV_1227, BOV_12284, BOVA were selected. Among the selected sequences, the genes BOV_A0504 and BOV_A0509 were promising for the construction of primers, and the last one is close to the gene BPI_II597 of *B. pinnipedialis* B2/94.

For the further primers construction for PCR detection of *B. ovis* DNA, the BOV_A0504 gene was selected. Using the program AmpliFx by iterations determined the sequences of primers based on their thermodynamic characteristics — melting point and enthalpy (Table 1).

Table 1 — Selection of the primer sequences using the AmpliFx soft

Name	Sequence	Start position	Direction	Length, number of nucleotides	Temperature, °C
Primer Set 1: 100 bp					
query_L1	AGAAGCTGTCGCCATAAGGT	1470	Forward	20	56.12
query_R1	TTGTCTGCAGTTCTGTTGGC	1550	Reverse	20	56.06
query_P1	CCCGAACGCGGTTCCTCCGC	1511	Forward	20	65.55
Primer Set 2: 100 bp					
query_L2	ACGAGTCTATCGTCGGACAC	1322	Forward	20	56.13
query_R2	CAAAGGCTATTCACCGCACA	1402	Reverse	20	55.93
query_P2	CGCAAGACCAAGCACCGGCT	1354	Forward	20	63.11
Primer Set 3: 105 bp					
query_L3	ATCAATCGGCTGCTTTAGCG	1084	Forward	20	56.18
query_R3	ATTAAGGGCGCCGAGGAATA	1169	Reverse	20	55.96
query_P3	CGGAAATTTCCTTAGCCTTACCCTGCG	1140	Forward	27	62.07

Thus, the third set of the sequences was selected, which flanks a site of 105 bp. These sequences were also analyzed using the BLAST algorithm to confirm their specificity *in silico*. Synthesized primers were used for *in vitro* amplification and the reaction was optimized regarding cycling parameters: 94°C for 2 min followed by 40 cycles of 94°C, 15 s and 60°C, 30 s.

To obtain a standard curve serial 10-fold dilutions of *B. ovis* DNA and corresponding Ct values were used (Fig. 1).

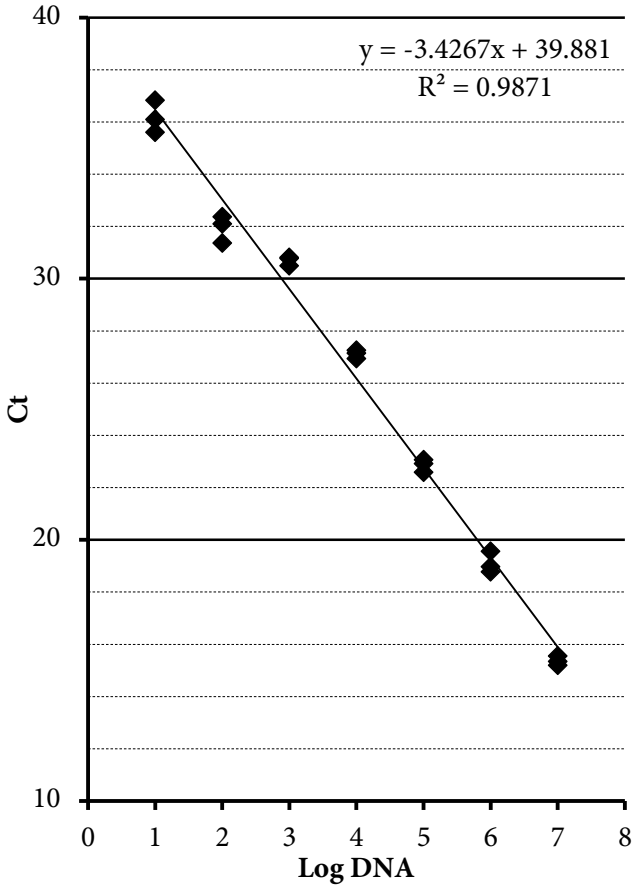

Figure 1. Standard curve showing the relationship between Ct values and standard dilution series of DNA *B. ovis* 65/65939 (Slope value = –3.427; R^2 = 0.987; Amplification factor = 1.96; qPCR Efficiency = 95.8%)

The obtained data showed that linearity was maintained (slope = –3.427 and coefficient of determination $R^2 > 0.98$). There is linear correlation between template concentrations ranged from 10^1 to 10^7 copies of DNA. The detection limit of *B. ovis* in bacterial culture was determined as 3.5×10^1 CFU/mL with Ct value of 37.8.

To assess an analytical specificity of the test a panel of DNA samples, isolated from 25 *B. ovis* strains and other bacteria, was studied. The results are showing in Table 2.

None of the bacterial species except *B. ovis* gave positive Ct value.

For the estimation of the diagnostic specificity a panel of DNA extracted from different clinical samples was tested (Table 3).

Table 2 — The specificity of the *B. ovis* real-time PCR assay by testing of DNA samples obtained from different bacteria

No	Strain	Ct, Fam (Mean ± SD; n = 3)	Result
1	*B. ovis* 67/Б	21.6 ± 0.34	+
2	*B. ovis* 76/982	26.1 ± 0.21	+
3	*B. ovis* 83/7315	24.3 ± 0.65	+
4	*B. ovis* 103/33479	25.1 ± 0.58	+
5	*B. ovis* 156/7808	22.1 ± 0.22	+
6	*B. ovis* 159/8406	22.0 ± 0.93	+
7	*B. ovis* 162/08337	23.0 ± 0.32	+
8	*B. ovis* 166/13575	24.0 ± 0.52	+
9	*B. ovis* 168/1807	21.7 ± 0.18	+
10	*B. ovis* 175/1257	24.3 ± 0.8	+
11	*B. ovis* 179/00441	23.6 ± 0.24	+
12	*B. ovis* 182/Taison	23.1 ± 0.15	+
13	*B. ovis* 183/13206	21.3 ± 0.41	+
14	*B. ovis* 186/21116	23.8 ± 0.24	+
15	*B. ovis* 64/64	26.2 ± 0.84	+
16	*B. ovis* 74/139	20.0 ± 0.03	+
17	*B. ovis* 154/8206	19.0 ± 0.08	+
18	*B. ovis* 155/8162	18.7 ± 0.15	+
19	*B. ovis* 158/4151	22.4 ± 0.2	+
20	*B. ovis* 178/00440	21.0 ± 0.03	+
21	*B. ovis* 181/6967	22.0 ± 0.49	+
22	*B. ovis* 184/03785	23.4 ± 0.03	+
23	*B. ovis* 185/03377	20.4 ± 0.7	+
24	*B. ovis* -/644	20.8 ± 0.91	+
25	*B. ovis* 65/c	19.4 ± 0.59	+
26	*B. abortus* 544	0.00	–
27	*B. abortus* 88/7-26	0.00	–
28	*B. suis* 58/1330	0.00	–
29	*B. melitensis* Rev-1	0.00	–
30	*B. canis* M20	0.00	–
31	*Y. enterocolitica* O:9	0.00	–
32	*Y. enterocolitica* O:3	0.00	–
33	*C. fetus* subsp. *fetus*	0.00	–
34	*L. monocytogenes*	0.00	–
35	*S. enterica* subsp. Enteritidis	0.00	–
36	*E. coli*	0.00	–
37	*S. aureus*	0.00	–
38	*C. pseudotuberculosis*	0.00	–
39	*P. aeruginosa*	0.00	–

Table 3 — Diagnostic specificity of the *B. ovis* real-time PCR assay by testing of DNA samples obtained from different clinical material

Sample	Ct, Fam (Mean ± SD)
Urine from infected rams (n = 5)	32.4 ± 0.82
Testicle tissues and lymph nodes from infected rams (n = 6)	30.64 ± 1.19
Samples from intact sheep (n = 42)	0.00

There are no detectable fluorescence signals in the clinical samples from intact animals, whereas bacteriologically confirmed material such as urine and testicle tissue samples were positive. It confirms that the assay is highly specific for detection of *B. ovis* DNA. Additionally, two samples of the testicle tissues were positive in real-time PCR, but the pathogen was not isolated (Kappa = 0.896).

High specificity and short period of testing are the most important advantages of PCR. It is an effective additional tool for routine laboratory diagnostic for the *B. ovis* detection in clinical samples. However, due to the OIE recommendations indirect diagnosis based on serological tests is preferred for herd surveillance (OIE, 2018).

The real-time PCR is recommended as additional method for early diagnostics and prevention of IOE during herd completing and could be widely used in the practice of state veterinary laboratories. Real-time PCR for detection of *B. ovis* DNA in clinical samples is a fast and reliable alternative for culturing.

Conclusion. A rapid, specific and sensitive quantitative PCR assay for the detection of *B. ovis* was developed. Its application makes possible to confirm the diagnosis in short time, to improve the efficiency of rehabilitation of sheep in problematic positive herds. PCR analysis is recommended as additional test for early diagnosis and prevention of IOE during herd recruitment and can be widely used in the practice of state veterinary laboratories.

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