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Communication

[*Comunicação*]

Real-time PCR for detection of *Brucella ovis* and *Histophilus somni* in ovine urine and semen

[*PCR em tempo real para detecção de* Brucella ovis *e* Histophilus somni *em urina e sêmen ovino*]

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Infectious ovine epididymitis affects rams worldwide, and it is considered to be one of the most important infectious diseases (Poester *et al*., 2013). *Brucella ovis* is one of the most important infectious agents associated with this disease, which may also be due to *Histophilus somni* infection, as well as other organisms (Díaz-Aparicio *et al*., 2009; Poester *et al.*, 2013; Moustacas *et al.*, 2014). *B. ovis* infection is associated with chronic epididymitis and infertility in rams and, occasionally, abortion in ewes and birth of weak lambs. *H. somni* is a component of the microbiota colonizing mucosal surfaces of cattle, goats, and sheep. However, *H. somni* may act as an opportunistic pathogen, and the infection may result in epididymitis as well as several other clinical manifestations such as vaginitis, placentitis, pneumonia, meningoencephalitis, mastitis, synovitis, septicemia, and other reproductive disorders (Díaz-Aparicio *et al*., 2009; Poester *et al.*, 2013; Moustacas *et al.*, 2014). The gold standard diagnosis of *B. ovis* infection is based on clinical examination, serology and semen bacteriology (Xavier *et al*, 2011; Poester *et al.*, 2013), while for *H. somni* infections, only clinical examination and bacteriology are routinely performed. Semen and urine are the samples of choice for diagnosis (Xavier *et al*., 2010; Costa *el al*., 2012; Moustacas *et al*., 2013). These techniques are labor intensive and slow, whereas molecular techniques have been increasingly used, including conventional PCR (Xavier *et al.*, 2010; Costa *et al.*, 2012), nested PCR (Costa *et al.*, 2013), and conventional multiplex PCR (Saunders *et al.*, 2007; Moustacas *et al.*, 2013). These methods are highly specific and faster than conventional techniques. Real-time PCR, also known as quantitative PCR, is another molecular tool that allows quantification of target DNA. Due to a shorter extension time, and no need of electrophoresis, real-time PCR is even less time consuming. Real-time PCR protocols have been developed for *Brucella* spp. detection, and also for differentiation among *Brucella* spp. species (Hinic *et al*, 2008), but none of these protocols are *B. ovis*-specific. Furthermore, there are no previous reports of primers and probes for amplification of *H. somni* DNA by real-time PCR. Thus, the goal of this study was to develop real-time PCR protocols for *B. ovis* and *H. somni* detection in urine and semen samples from rams.

B. ovis and *H. somni*-specific oligonucleotide primers and fluorescent dye-labeled probes were designed using Primer 3 software version 0.4.0. Primer pairs were designed to amplify approximately 100 base pairs (bp) of the target gene, with a melting point around 60°C. These parameters were verified by using the Oligo Analyzer 1.1.2 software. Specificity of the target sequences was evaluated by the Basic Local Alignment Search Tool from GenBank database in order to verify similarity with DNA sequences from other organisms. For amplification of *B. ovis* specific DNAa primer pair targeting a

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sequence within the *B. ovis*-specific genomic island was constructed (Tsolis *et al*., 2009). For *H. somni* DNA amplification, a primer pair and probe sequence were based on the genome sequence available on GenBank (accession number CP000947.1).

Probes were TaqMan MGB TM Probes (Applied Biosystems, USA) incorporating a FAM reporter for *B. ovis* and Cy5 for *H. somni* at the 5' end and a non-fluorescent quencher (BHQ, black hole quencher) at the 3' end. Sequences of primers and probes used for real-time PCR are detailed in Table 1.

H. somni was cultured on GC medium (base medium for chocolate agar) (Becton Dickinson USA), supplemented with 1% bovine hemoglobin and 0.5% of yeast extract (Becton Dickinson), incubated at 37°C for 48 h with an atmosphere of 5% CO_2. *B. ovis* was cultured on Thayer-Martin modified selective medium, composed by GC (DIFCO, USA), 1% of bovine hemoglobin (BBL, USA) and VCN inhibitor (BBL, USA), incubated at 37°C, in a 5% CO_2 atmosphere for 5 to 7 days. An isolated colony from each agent was suspended in 100µL of Tris-EDTA (Tris-HCl 1M, EDTA 0.5M) and then boiled during 10min for genomic DNA extraction. DNA extraction from biological samples was performed by the proteinase K and phenol/chlorophorm method as previously described (Matrone *et al*., 2009) using 500µL of fresh semen or 1 mL of thawed urine. All DNA samples were stored at -20°C until amplification.

Real-time PCR was set up in a final volume of 25µL, with 12,5µL of commercial supermix (Platinum Quantitative PCR SuperMix-UDG, Invitrogen, USA), 1µL of a solution of 10 mM of each primer, 0.5µL of a solution of 5mM of the TaqMan probe, 1µL of ROX (passive reference fluorochrome), 3µL of DNA template and 6µL of nuclease-free water. As a positive control, 1µL of DNA from pure cultures of *B. ovis* (ATCC 25840) and *H. somni* (3384Y) was used. Sterile nuclease-free water was used as a negative control. Reactions were performed at 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s, using a 7500 Fast Real-Time PCR System (Applied Biosystems, USA).

Sensitivity of the real-time PCR was assessed by performing reactions in triplicates with 0, 0.2, 2, 20, and 200 ng of genomic DNA from pure cultures of *B. ovis* (ATCC 25840), and *H. somni* (3384Y). To assess the specificity of real-time PCR, genomic DNA templates from bacterial species that can potentially cause epididymitis in rams were used, including *B. ovis* (ATCC 25840), *H. somni* (3384Y), *Actinobacillus seminis* (ATCC 15768), *Staphylococcus aureus* (ATCC 12600), *Manheimia haemolitica* (D0614057), *Corynebacterium pseudotuberculosis* (D0507204), and *Trueperella* (*Arcanobacterium*) *pyogenes* (D0602705) as well as an organism phylogenetically related to *B. ovis*, i.e. *Ochrobactrum anthropi* (ATCC 49188).

The real-time PCR methods developed in this study were validated by using semen and urine, the biological samples of choice for diagnosis of ovine infectious epididymitis (Xavier *et al*., 2010; Moustacas *et al*. 2013). Thus, 21 semen and 15 urine samples which were positive for *B. ovis* by conventional PCR from experimentally infected rams (Xavier *et al*., 2010), and 30 semen samples and 15 urine samples positive for *H. somni* by conventional PCR from experimentally infected rams (Moustacas *et al*., 2013) were used in this study. Negative controls included semen (n = 20) and urine (n = 20) samples from *B. ovis*- and *H. somni*-free rams, based on serology, semen bacteriology and semen conventional PCR for *B. ovis* detection, and bacteriology and conventional PCR for *H. somni* detection (Moustacas *et al*., 2013). Animal experiments were approved by the Universidade Federal de Minas Gerais Ethics Committee in Animal Experimentation (protocols CETEA 02/2007, 285/2008, and 2/2010). PCR reactions were performed using 200-500 ng of DNA from biological samples per reaction. Frequency of positive samples through real-time PCR was compared to frequency obtained by conventional PCR by Fisher's exact test using GraphPad Instat software version 3.10. Differences were considered significant when $P<0.05$. Agreement between conventional and real time PCR was assessed by Kappa statistics.

Several authors have evaluated real-time PCR assays for detection of *Brucella* spp. (Hinic *et al*., 2008). However, this is the first report of a *B. ovis*-specific real-time PCR. The species-specific diagnosis of *B. ovis* infection in rams is

extremely important for differentiation of other *Brucella* species with high zoonotic potential (i.e. *Brucella melitenis*), which can also infect sheep. Therefore, the differential diagnosis between *B. ovis* and *B. melitensis* infections in sheep has significant public-health implications. Targeting the *B. ovis*-specific genomic island (Tsolis *et al*., 2009), non specific amplifications are efficiently avoided and primers and probe designed in this study had high specificity. Too date, there are no reports of real-time PCR for *H. somni*-DNA amplification. Thus, this is the first report of *H. somni* real-time PCR protocol and its applicability in biological samples.

According to *in vitro* assays, the real-time PCR for both agents was highly sensitive since DNA amplification was successful at all concentrations tested, including the minimum concentration evaluated in this study (0.2ng DNA/reaction) as well as all other concentrations (2, 20, and 200ng/reaction). Figure 1 demonstrates representative amplification curves. Analytical specificity was evaluated with DNA samples from other agents. *B. ovis*-specific real time PCR amplified only *B. ovis* template DNA and yielded negative results with template DNA from all other organisms included in this experiment. Similarly, *H somni*-specific real time PCR amplified only *H somni* template DNA.

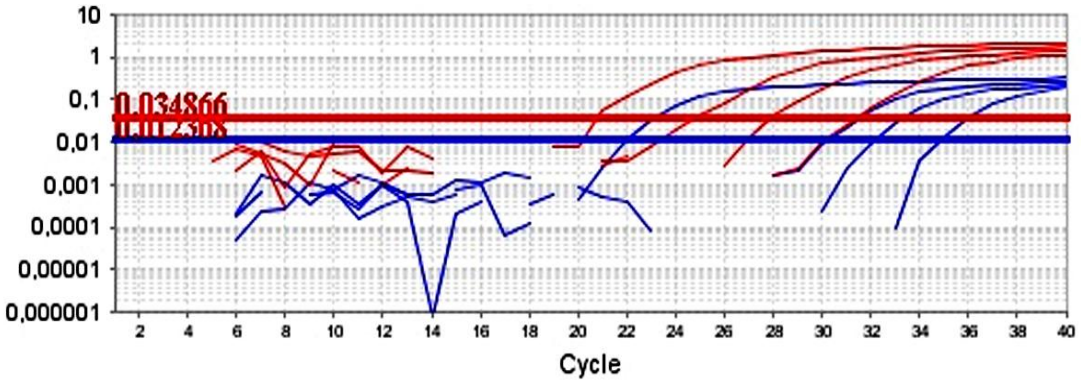

Figure 1. Representative amplification plot with amplification curves of *Brucella ovis* (red lines) and *Histophilus somni* (blue lines).

Table 1. Primers and fluorescent probes sequences designed for *Brucella ovis* and *Histophilus somni* detection by real-time PCR

PCR Target	Primers (5' - 3')		Fluorescent probes (5'Fluorophore - 3'Quencher)	PCR products size (bp)
	Forward	Reverse		
B. ovis	GGCCAACACGTACTGGAGAT	ACCGTTTCGGACGTGTCTAC	6-FAM/ CCTTCCAAAACGACATCCAT /BHQ1	104
H. somni	GATCAGAGCCATTGGCAAC	TGTATTTGCGCATCGGATAA	CY5/GGCAAGATTTCCAACAACCA /BHQ2	104

6-FAM = Carboxifluorescein; BHQ1 = Black Hole Quencher 1; CY5 = Cyanine 5; BHQ2: Black Hole Quencher 2

The applicability of this technique in biological samples was also confirmed since there was no loss of efficacy (P>0.05) when compared to conventional PCR with semen and urine samples from experimentally infected rams (Table 2). Once again the assay's specificity was confirmed due the absence of fluorescence signals in all 40 samples of semen and urine from the negative control (Table 2).

In conclusion, the real-time PCR assays described in this study are sensitive and highly specific. Therefore, these assays have a potential to be used as additional tools for a faster diagnosis of ovine infectious epididymitis associated with *B. ovis* or *H. somni* infections, using semen or urine samples.

Keywords: infectious epididymitis, ovine, diagnosis, PCR

Table 2. *Brucella ovis* and *Histophilus somni* detection by conventional and real-time PCR in semen and urine samples from experimentally infected rams, and agreement between these techniques

	Semen				Urine					
	Infected rams		Negative control		Infected rams		Negative control			
	conventional PCR	real-time PCR	conventional PCR	real-time PCR	conventional PCR	real-time PCR	conventional PCR	real-time PCR	Kappa	Agreement (%)
B. ovis	21/21 (100%)	16/21 (76.2%)	0.0% (0/20)	0.0% (0/20)	15/15 (100%)	70.6% (12/15)	0.0% (0/20)	0.0% (0/20)	0.787	89.5
H. somni	30/30 (100%)	21/30 (70.0%)	0.0% (0/20)	0.0% (0/20)	15/15 (100%)	86.7% (13/15)	0.0% (0/20)	0.0% (0/20)	0.744	87.1

RESUMO

A epididimite infecciosa ovina é uma das principais enfermidades reprodutivas de carneiros. O presente estudo teve por objetivo desenvolver protocolos de PCR em tempo real para B. ovis *e* H. somni *e avaliar sua aplicabilidade em amostras de sêmen e urina de carneiros. Delinearam-se primers e sondas espécie-específicos para cada agente. As sondas foram delineadas com o sistema TaqMan incorporando um marcador FAM para* B. ovis *e Cy5 para* H. somni *na extremidade 5' e um quencher na extremidade 3'. A PCR em tempo real para* B. ovis *e* H. somni *foi altamente sensível, uma vez que a amplificação de DNA ocorreu com até 0,2ng de DNA/reação. A especificidade dos iniciadores e sondas foi avaliada com amostras de DNA de outros agentes causadores de epididimite ovina e nenhuma amplificação inespecífica foi observada. A aplicabilidade da técnica em amostras biológicas também foi confirmada, pois não houve perda de eficácia (P>0,05) quando comparada à PCR convencional com amostras de sêmen e urina de carneiros experimentalmente infectados.*

Palavras-chave: epididimite infecciosa, ovinos, diagnóstico, PCR

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