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Short communication

Development and evaluation of a species-specific PCR assay for the detection of *Brucella ovis* infection in rams

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ABSTRACT

Brucella ovis infection is a major cause of epididymitis and infertility in rams, resulting in reproductive failure and significant economic losses worldwide. The goal of this study was to develop a PCR test targeting specific *B. ovis* genomic sequences. Specific primer pairs were designed targeting 12 of those ORFs. Samples of blood, serum, semen, urine, and preputial wash were collected from experimentally infected rams ($n = 9$) every other week up to 180 days post infection (dpi), when tissue samples were obtained. Blood, serum, semen, urine, and preputial wash samples were obtained, in weekly intervals for 1 month, from eight rams belonging to a *B. ovis*-free flock. Semen samples were also obtained from rams belonging to naturally infected flocks ($n = 40$). The limit of detection of this PCR protocol was 100, 10, and 1 CFU/mL for semen, urine and prepucial wash samples, respectively. Sensitivity and specificity values obtained with this PCR method were similar to that of bacteriology when evaluating biological samples. Agreement between PCR and bacteriology results was greater than 90%. These results clearly indicate that this species-specific PCR method is highly efficient for the diagnosis of *B. ovis* infection in semen, urine, preputial wash and tissue samples from infected rams.

1. Introduction

Ovine brucellosis due to *Brucella ovis* infection is considered one of the most important infectious diseases of sheep worldwide (Burgess, 1982). The disease is caused by a Gram-negative coccobacilli of the genus *Brucella* (α2-Proteobacteriacea family) (Garrity, 2001). Clinical signs of infection consist of chronic epididymitis and infertility in rams (Biberstein et al., 1964; Searson, 1987) and, occasionally, abortion in ewes and birth of weak lambs (Molello et al., 1963; Osbourn and Kennedy, 1966). Unlike most *Brucella* spp., *B. ovis* does not cause disease in humans (Blasco, 1990). Importantly, sheep are considered preferential hosts not only for *B. ovis*, but also for *Brucella melitensis*, which is the most pathogenic *Brucella* species for humans (Blasco, 1990). Therefore, the differential diagnosis between *B. ovis* and *B. melitensis* infection in sheep has significant public-health implications.

Diagnosis of *B. ovis* infection is based on clinical examination, serological tests, and bacteriology of semen samples (Webb et al., 1980; Burgess, 1982). Molecular

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diagnosis based on the amplification of *Brucella* spp. DNA in semen samples has been applied to diagnosis of *B. ovis* infections (Manterola et al., 2003; Saunders et al., 2007), but these tests have target sequences that are preserved in all classical *Brucella* species. Therefore, there are no species-specific PCR assays currently available for direct diagnosis of *B. ovis* infection.

The genome of *B. ovis* (strain ATCC25840) has been completely sequenced, resulting in the identification of a *B. ovis*-specific island in the chromosome II (Tsolis et al., 2009). Considering the potential of these ORFs as amplification-targets in the development of a specific PCR assay for the diagnosis of *B. ovis* infection, as well as the lack of a rapid and specific method for definitive identification of this agent in biological samples, the present study aimed to develop and evaluate a species-specific PCR-based assay for the diagnosis of *B. ovis* infection in semen, urine, preputial wash and tissues samples from rams.

2. Materials and methods

2.1. Primers specificity and analytical sensitivity

To determine the specificity of primers previously described by Tsolis et al. (2009), strains of different bacteria species that can potentially cause epididymitis in rams were used, including *Actinobacillus seminis* (ATCC15768), *Histophilus somni* (3384Y and D0614057), *Corynebacterium pseudotuberculosis* (D0507204 and D0503218), *Arcanobacterium pyogenes* (D0602705 and D06022438), *Mannheimia haemolytica* (D0614057) and *Staphylococcus aureus* (ATCC 12600), as well as species phylogenetically related to *B. ovis*, including *Ochrobactrum intermedium* (LM3301) and *Ochrobactrum anthropi*. All bacteria were grown in tryptic soy agar (DIFCO, USA) plates with 5% ovine blood for 2–3 days at 37 °C. Genomic DNA was extracted from pure cultures as previously described (Romero et al., 1995), followed by PCR reaction as described below.

To assess analytical sensitivity of the assay, samples of semen, blood, urine and preputial wash from *B. ovis*-free rams were obtained and spiked with tenfold serial dilutions of *B. ovis* strain ATCC25840 for final concentrations ranging from 10^6 to 10^0 CFU/mL. Samples were then processed for DNA extraction and PCR.

2.2. Experimental animals and sample collection

Nine cross-breed rams, 1–3-year-old were used for experimental infections. Prior to inoculation, samples of serum, blood, semen, urine, and preputial wash were collected to confirm that the rams were free of *B. ovis* infection. Rams were inoculated intraconjunctivally and intrapreputially with a total of 3.6×10^9 CFU/ram of *B. ovis* (strain ATCC25840). After inoculation, blood, serum, semen, urine, and preputial wash samples were collected in 15 days intervals for 180 days ($n = 117$ for each biological sample) for serology, bacteriology and PCR. Rams were conditioned to ejaculate into an artificial vagina for semen sampling. Urine sampling was performed by blocking breathing for 30 s. Preputial wash was performed by introduction of 10 mL of a sterile PBS into the preputial cavity, followed by mucosal massage for 1 min and recovery of the liquid into a sterile 15 mL tube (adapted from Clark and Dufty, 1978). All sampling was performed using sterile equipment and precautions to prevent cross-contamination. This experiment was approved by the Universidade Federal de Minas Gerais Ethics Committee in Animal Experimentation (CETEA, protocol: 02/2007).

In order to determine tissue distribution of *B. ovis*, infected rams were sedated with xilazine (Copazine, Schering-Plough Coopers, Brazil), euthanatized by eletroconcussion followed by necropsy at 180 days post infection. Fragments of tail, body and head of both epididymis, both testes, ampulla of the ductus deferens, both seminal vesicles, both bulbo-urethral glands, prepuce, glans penis, inguinal lymph node, iliac lymph node, spleen, liver, kidney and bladder were collected. Samples were placed in a 50 mL Falcon tube containing 2 mL of sterile PBS solution for bacteriology or stored at −80 °C until DNA extraction.

As negative controls, eight Santa Inês mature rams, with negative serology for *B. ovis* by immunodiffusion in agar gel (ID) from a *Brucella*-free flock without history of epididymitis or infertility were submitted to blood, semen, urine and preputial wash sampling as described above, in weekly intervals during 4 weeks ($n = 32$ for each biological sample).

Lyophilized semen samples from 40 rams belonging to naturally infected flocks with different *B. ovis* bacteriological and serological status as determined by indirect ELISA (Nielsen et al., 2007), were obtained from the Instituto Nacional de Tecnologia Agropecuária (INTA), Bariloche. These semen samples were resuspended in 1 mL of sterile PBS solution prior to DNA extraction.

2.3. Serology

Serum samples from experimentally infected rams and from the negative control flock were tested by immunodiffusion in agar gel (ID) test, performed as previously described (Marín et al., 1989). The antigen used in ID tests was made from soluble extract of heat-inactivated *B. ovis* strain REO198 (Instituto de Pesquisas Desidério Finamor, Rio Grande do Sul, Brazil).

2.4. Bacteriology

B. ovis isolation was performed by plating 100 μL of semen, blood, urine or preputial wash onto selective Thayer–Martin modified media (Brown et al., 1971; Alton et al., 1988). Tissues samples were macerated in sterile PBS with a homogenizer (Hamilton Beach, USA) and then plated. Plates were incubated at 37 °C in 5% CO_2 for 5–7 days. Suspected colonies were plated on GC media (DIFCO, USA) with 1% bovine hemoglobin (BBL, USA) and incubated at 37 °C in 5% CO_2 for 5–7 days. Colonies were confirmed by the specific *B. ovis* PCR assay described in this study.

2.5. Amplification of B. ovis DNA by PCR

DNA extraction was performed with 500 μL of semen or blood fresh samples, 1000 μL of thawed urine or preputial

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wash samples, and approximately 500 μL of macerated thawed tissue samples, as previously described (Matrone et al., 2009). The present study used the 12 primer pairs targeting a *B. ovis*-specific genomic island as previously described (Tsolis et al., 2009) for assessing specificity of amplification. For amplification of *B. ovis* genomic DNA in biological samples, primer pairs targeting the ORFs AO503 (F: 5′-GCCTACGCTGAAACTTGCTTTTG-3′ and R: 5′-ATCCCCCCATCACCATAACCGAAG-3′) and AO512 (F: 5′-TTCAGGCGACTGCTAATGGCAC-3′ and R: 5′-AAACCGA-TACCTCATCCCCGAG-3′) were used. PCR reactions were performed using 23 μL of a commercial PCR mix (PCR Supermix, Invitrogen), 0.5 μL of a 25 μM solution of each primer, 0.25 μL of Taq Polymerase (Invitrogen, Brazil), and 1–3 μL of template DNA (100–500 ng of DNA per reaction). Cycling parameters were denaturation at 95 °C for 5 min; 35 cycles of denaturation (95 °C for 1 min), annealing (55 °C for 1 min), and extension (72 °C for 1 min); and a final extension at 72 °C for 5 min. PCR products were resolved by 1% agarose gel electrophoresis. Reactions were considered positive when they yielded products of 228 and 135 bp for primers targeting AO503 and A0512, respectively.

2.6. Statistical analysis

Frequencies of *B. ovis* detection by PCR and bacteriology were compared by Fisher's exact test using the GraphPad InStat software, version 3.05 (GraphPad InStat software, Inc., USA). PCR test sensitivity and specificity were assessed according to Henken et al. (1997). Proportion of agreement between diagnosis methods was assessed by Kappa test (Smith, 2005), with Minitab 15 software (Global Tech, Brazil).

3. Results

3.1. Specificity and analytical sensitivity of PCR primers

Tsolis et al. (2009) demonstrated that all 12 primer pairs used in the present study did not amplify specific target sequences in all other classical species of *Brucella*. Moreover, all 12 target sequences were conserved in 18 different *B. ovis* field strains. In the present study, we demonstrated that all 12 target sequences were also absent in other bacteria species that can potentially cause epididymitis in rams including *A. seminis*, *H. somni*, *C. pseudotuberculosis*, *A. pyogenes*, *Chlamydophila abortus*, *M. haemolytica* and *S. aureus*, as well as species phylogenetically related to *B. ovis*, including *O. intermedium* and *O. anthropi* (data not shown).

The specific PCR assay had a detection limit of 10^2 CFU of *B. ovis*/mL in spiked semen and blood samples, with a higher sensitivity in urine (10 CFU/mL) (Supplementary Fig. 1) and preputial wash (1 CFU/mL). Uninfected samples were negative by culture and PCR.

3.2. Detection of B. ovis in semen, urine and preputial wash samples by specific PCR

The experimental challenge was enough to result in infection in all rams, since *B. ovis* antibodies were detected by ID in at least one time point during experimental infection in all rams (data not shown). *B. ovis* detection by PCR and bacteriology started at 45 days post infection (dpi) for semen (Fig. 1 and Supplementary Table 1) and preputial wash samples (Fig. 1 and Supplementary Table 2), and remained intermittent during the course of the experiment. The same pattern was observed in urine samples. However, in this case, the detection began at 30 dpi (Fig. 1 and Supplementary Table 3). Detection of *B. ovis* in individual biological samples of experimentally infected rams by PCR and bacteriology was similar during the course of infection (Fig. 1). Moreover, when results from semen, urine and preputial wash samples were combined, the percentage of positive rams by PCR and bacteriology was higher, reaching 77.8% (7/9) of positive animals at 135 dpi (Fig. 1). Importantly, *B. ovis* was not detected by PCR or bacteriology in blood samples from infected rams at any time point during the course of infection.

Considering individual samples, the specific PCR assay was able to detect *B. ovis* DNA in 17.9% (21/117); 19.7% (23/117) and 22.2% (26/117) in semen, urine and preputial wash samples, respectively (Table 1). Conversely, bacteriology resulted in 16.2% (19/117); 18.8% (22/117) and 16.2% (19/117) positivity in semen, urine and preputial wash samples, respectively. No significant differences ($P > 0.05$) were observed between bacteriology and PCR, or among the different biological samples.

Forty lyophilized semen samples from rams with variable serological test results (indirect ELISA) belonging to flocks naturally infected with *B. ovis* were evaluated (Supplementary Table 4). From those samples, 80% (32/40) belonged to rams serologically positive for *B. ovis*. Importantly, only 23 out of these 40 samples were processed for isolation of *B. ovis*, with 47.8% (11/23) of positivity by bacterial isolation. Considering the animals with serological diagnosis, PCR was able to detect *B. ovis* DNA in 70% (28/40) of semen samples. Yet, considering samples with diagnosis based on bacteriology, the PCR assay detected *B. ovis* in 69.6% (16/23) of the cases. No significant differences ($P > 0.05$) were observed between tests in lyophilized semen samples from naturally infected rams.

Considering that all rams exposed to experimental challenge became infected since all of them had positive serological results at some time point during the course of experimental infection, the PCR assay sensitivity was 19.4, 21.3, 24.1 and 20.4% for semen, urine, preputial wash, and all samples, respectively. In that case, the bacteriology sensitivity was 17.6, 20.3, 17.6 and 17.6% for semen, urine, preputial wash, and all samples, respectively. As there was no *B. ovis* detection by PCR nor bacteriology in all 32 semen, 32 urine and 32 preputial wash samples from negative control rams, the specificity of both methods was 100%. When PCR assay relative sensitivity was calculated considering the bacterial isolation as gold standard for the diagnosis of *B. ovis* infection, thus considering all samples with positive bacteriology as true positive samples, the estimated sensitivity of the PCR method increased markedly. In this scenario, the sensitivity values were 89.5, 82, 100 and 90.3% for semen, urine, preputial wash, and all samples, respectively. However, as the PCR

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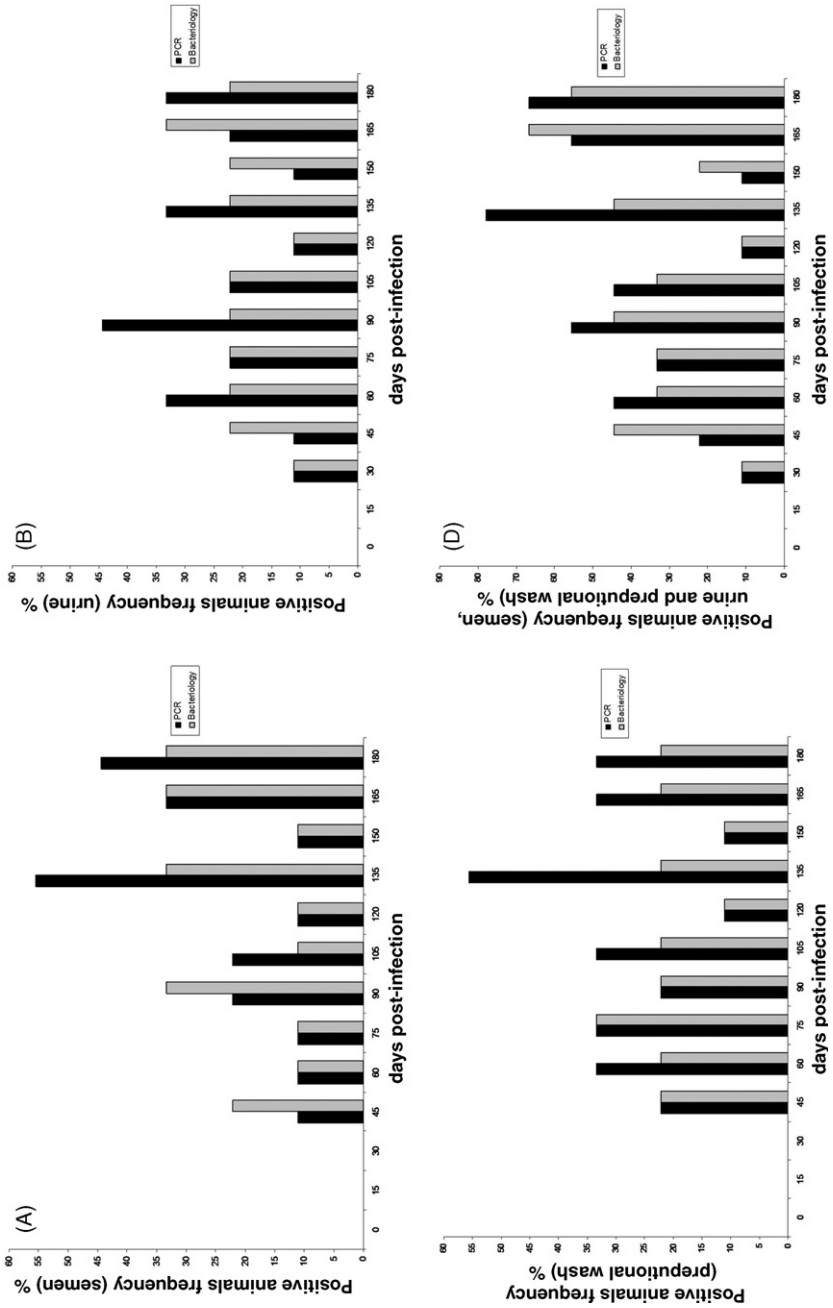

Fig. 1. Frequency (%) of *Brucella ovis* detection by bacteriology or PCR in biological samples (*y* axis) from rams throughout the course of infection (up to 180 days post infection). (A) Semen samples; (B) urine samples; (C) preputial wash samples; (D) all samples (semen, urine, and preputial wash) combined.

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Table 1
Frequency (%) of *Brucella ovis* detection by species-specific PCR and bacteriology of semen, urine, preputial wash, and tissue samples from experimentally infected rams, during 180 days of infection, and frequency (%) of agreement and kappa test value.

Sample	Method		Agreement (%)[a]	Kappa[a]
	PCR (%)	Bacteriology (%)		
Semen[b]	17.9 (21/117)	16.2 (19/117)	93.60 (88.85–96.76)	0.796
Urine[b]	19.7 (23/117)	18.8 (22/117)	94.59 (89.63–97.64)	0.786
Preputial wash[b]	22.2 (26/117)	16.2 (19/117)	95.30 (90.56–98.09)	0.817
Tissue	28.6 (54/189)	20.1 (38/189)	90.48 (85.37–94.26)	0.741
Total	23.0 (124/540)	18.1 (98/540)	93.31 (91.13–95.10)	0.781

There was no significant difference between methods or biological samples by Fisher's exact test ($P < 0.05$).

[a] Values in brackets represent confidence intervals at a confidence level of 95%. Considering semen samples from experimental infection ($n = 117$), natural infection ($n = 23$) and negative controls ($n = 32$). Considering urine and preputial wash samples from experimental infection ($n = 117$) and negative controls ($n = 32$).

[b] These are cumulative results from samples collected every other week throughout the course of infection (up to 180 days post infection).

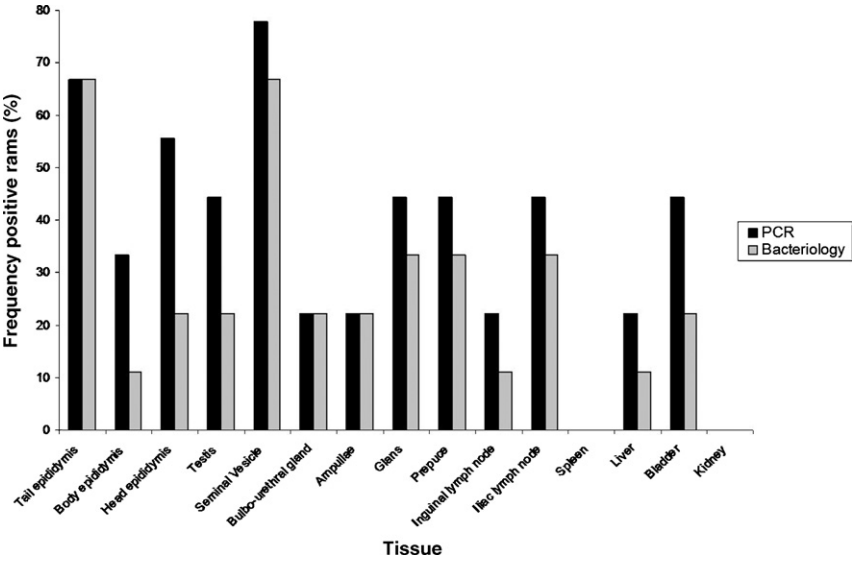

Fig. 2. Frequency (%) of *Brucella ovis* detection by bacteriology and PCR in tissue samples from rams at 180 days post experimental infection.

method was capable of detecting a higher number of positive samples when compared to bacteriology (Table 1), the specificity values decreased to 97.8, 98.8, 92.1 and 96.2% for semen, urine, preputial wash, and all samples, respectively.

Notably, the percentage of agreement between bacteriology and PCR was considerably high for all biological samples from experimentally infected rams, with values of 93.6, 94.59, 95.3 and 90.48% for semen, urine, preputial wash, and all samples, respectively (Table 1). Furthermore, kappa statistics values over 0.78 for all biological samples confirmed a high agreement between these methods (Table 1).

3.3. Detection of *B. ovis* in tissue samples by specific PCR

Evaluation of tissue samples from experimentally infected rams demonstrated that 77.8% (7/9) of them had evidence of *B. ovis* infection either by bacteriology or by the specific PCR method at 180 dpi (Supplementary Table 5). Considering PCR and bacteriology results, *B. ovis* was mostly identified in genital organs, particularly in the seminal vesicle and tail of the epididymis (Fig. 2). Importantly, *B. ovis* was also often detected in the external genitalia, including the prepuce and glans penis as well as in the iliac lymph nodes and urinary bladder. However, the organism was seldom or not at all detected in systemic sites of infection, such as the liver and spleen (Fig. 2).

As observed with the other biological samples, the specific PCR assay detected a similar number of positive tissue samples when compared to bacteriology (Table 1). While 20.1% (38/189) of samples were positive by bacteriology, 28.6% (54/189) were positive for *B. ovis* by PCR, with no significant difference between these methods. Furthermore, the agreement between these techniques in tissue samples was 90.48% with a kappa value of 0.741 (Table 1).

4. Discussion

Bacterial isolation is still considered the gold standard for the definitive diagnosis of *B. ovis* infection (Alton et al., 1988). Nevertheless, bacteriological identification of the organism may take up to 2 weeks, it is a complex procedure

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requiring optimal laboratory conditions and trained personnel, and the organisms must remain viable in the samples until reaching the laboratory (Bricker, 2002). Therefore, PCR-based assays are considered a rapid and sensitive alternative to overcome the limitations of bacterial isolation (Bricker, 2002), especially if the PCR method is direct and identifies the agent at the species level. The PCR assay developed in this study proved to be highly specific for detecting *B. ovis*, since amplification of the target sequences was absent in all classical species of *Brucella* (Tsolis et al., 2009) as well as in other bacteria that may potentially cause epididymitis in rams. Importantly, previously described PCR assays for diagnosis of *B. ovis* infection are limited to bacterial identification at the genus level, i.e. these methods detect *Brucella* spp. (Manterola et al., 2003; Saunders et al., 2007). The fact that the method described here could easily distinguish *B. ovis* from *B. melitensis* infection in rams is of great importance for controlling and monitoring the risk for human brucellosis in countries where small ruminants represent the most important source of *B. melitensis* infection for humans. Moreover, surveillance in *B. melitensis*—free areas that have *B. ovis* infection of sheep may be considerably improved by a rapid and efficient diagnosis method.

Shedding of *B. ovis* in the semen of infected rams is considered the most important source of infection (Burgess, 1982). Consequently, semen samples have been used as the specimen of choice for *B. ovis* detection (Burgess, 1982; Manterola et al., 2003; Saunders et al., 2007), although *B. ovis* excretion in ram semen is known to be intermittent, which limits the efficiency of diagnostic methods (Burgess, 1982; Manterola et al., 2003; Saunders et al., 2007). Shedding of *B. ovis* in the urine has been previously described (Burgess, 1982; Cerri et al., 2002), but this specimen as well as preputial wash is not usually considered for diagnostic purposes. This study demonstrated that the frequency of *B. ovis* detection either by bacteriology or PCR was statistically similar when comparing samples of semen, urine or preputial wash. Additionally, the frequency of positive rams was higher when more than one biological sample was considered for diagnosis. Therefore, urine and preputial wash samples should be considered as suitable specimens for direct diagnosis of *B. ovis* infection. *B. ovis* was not detected in blood samples either by bacteriology or PCR during the entire experimental period. Therefore blood samples should not be used for diagnostic purposes.

The species-specific PCR assay developed in this study demonstrated a remarkably good sensitivity when compared to bacterial isolation, the gold standard method for direct diagnosis of *B. ovis* infection. Additionally, the values of sensitivity obtained in the present study were similar to previously described non-specific PCR assays (Manterola et al., 2003). Furthermore, PCR and bacterial isolation had an agreement of more than 90% for all biological samples, with kappa test values of more than 0.79, clearly indicating a strong agreement between these two methods. Therefore, the PCR method developed in this study may be used as an alternative to bacteriology for the direct diagnosis *B. ovis* in biological samples. The agreement and kappa test values between bacteriology and PCR were slightly lower when semen samples from naturally infected rams were evaluated since PCR detected a higher number of positive samples when compared to bacteriology. Although limitations due to the selective medium used for *B. ovis* isolation were not detected during the experimental procedure, such limitations have been described, including inhibition of growth of some *B. ovis* strains as well as overgrowth of contaminants (Manterola et al., 2003).

PCR sensitivity in this study was measured considering all experimentally infected rams as truly positive subjects since all of them were challenged and developed serological responses. This approach may result in an apparent low sensitivity, which is related to some aspects of the biology of *B. ovis* infection, i.e. the fact that shedding of *B. ovis* usually begins after 45 dpi (Burgess, 1982; Blasco, 1990), and a considerable number of serologically positive rams may never excrete *B. ovis* (Burgess, 1982; Plant et al., 1986). Additionally, in the present study, 22.2% (2/9) of experimentally infected rams had no evidence of *B. ovis* infection in tissue samples by PCR or bacteriology after 180 dpi. This result corroborates other studies, which demonstrated that some experimentally infected and serologically positive rams may overcome the infection after its acute phase (Biberstein et al., 1964; Webb et al., 1980; Burgess, 1982).

B. ovis was mainly detected by PCR or bacteriology in sexual organs and regional lymph nodes, and only rarely it was detected at systemic sites of infection. These findings reinforce *B. ovis* tropism for the male genital system (Biberstein et al., 1964; Burgess, 1982; Plant et al., 1986), after a transient and acute bacteremic phase of the disease (Biberstein et al., 1964). Importantly, *B. ovis* was frequently detected in bladder of experimentally rams. This apparent urinary tract colonization by the agent correlates with the high frequency of PCR positive urine samples.

In conclusion, the species-specific PCR assay for the diagnosis of *B. ovis* infection developed in this study demonstrated to be highly specific and sensitive when compared to bacteriology in samples of semen, urine, and preputial wash from infected rams. Therefore, this specific PCR assay is suitable for routine diagnosis of this disease, allowing a concurrent differential diagnosis with *B. melitensis*, and it can be used as a practical alternative for bacterial isolation.

Conflict of interest

The method described in this study has been included in a patent application submitted to the Instituto Nacional de Propriedade Industrial (INPI, Brazil).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2010.02.037.

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