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Communication

[*Comunicação*]

Adaptation and evaluation of polymerase chain reaction for *Brucella ovis* detection in semen, urine and organs of rams experimentally infected

[*Adaptação e avaliação da reação em cadeia da polimerase para detecção de Brucella ovis em sêmen, urina e órgãos de carneiros infectados experimentalmente*]

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Brucella ovis is the main cause of a clinical or subclinical disorder characterized by epididymitis and subsequent decrease in fertility in rams (Manterola *et al*., 2003). Diagnosis of *B. ovis* infection is reached by a combination of clinical examination, isolation of the bacterium and detection of anti-*B. ovis* antibodies in blood serum (Webb *et al.*, 1980; Xavier *et al.*, 2011). Nowadays, more specific techniques such as Polymerase Chain Reaction (PCR) are used in *Brucella* spp. detection (Cortez *et al.*, 2001; Manterola *et al.*, 2003; Keid, 2004). The objective of the present study was the adaptation and evaluation of PCR for *B. ovis* in semen, urine and organs samples collected from experimentally inoculated animals. Results were compared with those of microbiological culture, and the applicability of the technique in routine diagnosis was analyzed.

A lyophilized *B. ovis* REO 198 strain was obtained at the Centro de Pesquisa Veterinária Desidério Finamor – Eldorado do Sul/RS. The Institutional Ethics and Animal Welfare Commission of the FMVZ/UNESP/Campus de Botucatu approved this study (ethics committee protocol n# 69/2008). Thirty-one rams, from one to two-year-old, were used. A suspension containing $2x10^9$CFU/mL *B. ovis* was administered, 2mL by intrapreputial route and 50μL by intraconjunctival route, simultaneously. After inoculation, blood for serology and urine and semen for bacterial culture and PCR were collected every week. Culture of the samples was carried out on the same day of collection (Brown *et al.*, 1971). A pool of weekly samples of urine and semen of each animal was used in PCR totalizing one monthly sample of semen and urine of each animal.

Animals were euthanized each 15 days until the 4^{th} month and monthly subsequently till one year post challenge, and organs (urinary bladder, lungs, spleen, liver, ampoule, bulbourethral gland, prostate, lymph nodes, epididymis and testicles) were collected. Pools of each organ of every two animals euthanized in each moment were used. Protocols followed by different authors (Cortez *et al.*, 2001; Manterola *et al.*, 2003) were used in the preparation of the samples and in the extraction procedure. PCR sensitivity for semen, urine and organ samples was analyzed by means of a concentrated *B. ovis* suspension at 10^{-1}, according to McFarland scale. Semen, urine and organ samples were contaminated in order to evaluate the detection threshold. To achieve this aim, 1μg of genomic DNA of each sample was added to 1μL of each bacterial dilution.

Contaminated samples were submitted to the following amplification protocol. Semen, urine (approximately 500μL) and organs samples (approximately 200μL) were thawed and added

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to 500μL Tris-EDTA (TE) buffer, pH 8.0 (10mM Tris-HCL pH 8.0; 1mM EDTA pH 8.0) for semen and urine and 800μL Tris-EDTA (TE) for organs samples. Semen and urine were incubated at 80ºC for 10min. After that, they were centrifuged at 13,000xg for 15min. The supernatant was discarded and the sample was washed for 2-3 times, until the supernatant was clear. Extremely creamy semen samples were submitted to up to four washings. After being washed, the precipitate for organs samples was resuspended in 350μL Tris-EDTA (TE) buffer, pH 8.0, made up of 10mM Tris-HCl pH 8.0, 25mM EDTA, pH 8.0, 100mM NaCl.

Organs samples were incubated at 80ºC for 10min and added to solution made up of SDS 1% and 12μL proteinase K (20mg/mL). It was then incubated at 37ºC for 24h. The aqueous phase containing nucleic acid was extracted using the phenol/chloroform/isoamyl alcohol method (Cortez *et al.,* 2001). The final pellet was resuspended in 60μL TE, pH 8.0, and incubated at 56ºC for 30 min.

The amplification procedure was the same for semen, urine and organs samples. Primers were ISP1 and ISP2, designed for the *Brucella* nucleotide sequence IS 6501 (ISP1 F: 5´- GGTTGTTAAAGGAGAACAGC – 3´ and ISP2 R: 5´- GACGATAGCGTTTCAACTTG – 3´) (Manterola *et al.,* 2003). PCR reaction mix was based on Keid *et al*. (2007), in a final volume of 25μL. The amplification procedure was carried out as described elsewhere (Manterola *et al.,* 2003). The analysis of the amplified product was carried out by 2% agarose gel electrophoresis (w/v). For the statistical analysis, samples were classified as positive or negative in PCR and microbiological culture. This was done considering the frequency of occurrence of results for PCR and bacterial culture in the monthly samples. Percentage of association between test results was done using Wilcoxon nonparametric test.

PCR primers ISP1 and ISP2 amplified DNA in samples of semen, urine and organs in dilutions up to 10^{-4}, except for lymph node and spleen samples, which only showed positive results up to dilution 10^{-3}. As for the 236 semen samples tested, PCR showed higher sensitivity than culture, detecting 51 positive samples whereas culture detected only 19 samples. In urine samples, sensitivity of the techniques was similar. PCR of the organs showed higher sensitivity than bacterial culture. Statistical analysis showed that tests were independent, and that there was no agreement between microbiological culture and PCR (Table 1). The Table 2 represents the agreement percentage, between PCR and bacterial culture. However, no statistical significant difference was observed between semen and urine samples when the percentage of agreement between the tests was evaluated.

Table 1. Result of microbiological culture and PCR for *B. ovis* in rams experimentally inoculated with *B. ovis*, according to clinical material

	Semen	Urine	Organs
Culture	19/236 (8.0%)	24/236 (10.1%)	7/209 (3.3%)
PCR	51/236 (21.6%)	30/236 (12.7%)	45/209 (21.5%)

Proportion: number of positives/number of samples

Table 2. Descriptive measures of the agreement percentage of PCR and microbiological culture for semen and urine of rams experimentally infected with *B. ovis*

Descriptive measure	Type of sample		P value
	Semen	Urine	
Minimum value	33.3%	33.3%	
1º Quartile	61.5%	62.5%	
Med	72.7%	75%	
3º Quartile	83.7%	100%	P>0.05
Maximum value	100%	100%	
Mean±standard deviation	73±17.9%	76.1±21%	

Detection threshold in lymph nodes and spleen reached only dilution 10^{-3}. Some authors reported the presence of inhibiting substances, such as hemoglobin, influencing the reaction (Morata *et al.,* 1998) that could explain the lower detection threshold in these samples compared to the other organs suspensions. Studies on the detection threshold of PCR for *B. ovis* in organs, semen and urine of sheep were not found in the literature analyzed, except for the report by Saunders *et al.* (2007) who used multiplex PCR, observing sensitivity equal to 25CFU.

Analytical sensitivity observed in the other organs, semen and urine samples were equal to $3x10^2$CFU/mL, similar to the results by Amim *et al.* (2001). Sensitivity of $1.0x10^0$CFU/mL was observed in pools of semen samples obtained from dogs naturally infected by *B. canis* (Keid, 2007). In the present study, positive results were observed in 8.0% of the semen samples submitted to isolation and in 21.6% of the samples submitted to PCR. For urine samples, 10.1% were positive in isolation and 12.7% in PCR.

The low percentage of rams showing positive bacteriological results in the present study was similar to the findings of other researchers, who reported that the percentage is always lower than the number of clinically affected or serologically positive rams (Ficapal *et al.,* 1998). The low percentage of isolation was justified by the low sensitivity of modified Thayer-Martin agar for semen culture (Manterola *et al.,* 2003). The results of microbiological culture of infected animals in the present study were lower compared with those of a published report (Biberstein *et al.,* 1963). This difference may be explained by the culture medium used in the trial. The highest percentage of isolation was observed in genital organs (testicles, seminal vesicle, epididymis and bulbourethral gland), demonstrating once more the preference of the bacterium for the reproductive tract. PCR used in semen samples showed higher sensitivity than bacterial culture.

The negative results observed in some samples may be explained by the intermittent shedding of the bacterium in the semen (Paolicchi *et al.,* 2000). Urine culture showed positive frequencies, with some samples negative in PCR, what may be explained by the excess of DNA in the samples tested, a potential PCR inhibitor. PCR sensitivity in semen samples (21.6%) was higher when compared with bacterial isolation (8.0%), as reported by other authors (Hamdy and Amim, 2002). Different results were reported by some authors using the same primer pairs, with PCR sensitivity equal to 51.9% (Manterola *et al.,* 2003). PCR sensitivity in urine samples (12.7%) was almost similar to isolation (10.1%), whereas PCR sensitivity in organs was equal to 21.5%. Results found in the present study were similar to those reported by other authors (Cortez *et al.,* 2001). In the present study, PCR detected a greater number of positive samples than microbiological culture, showing that this adapted technique may be a diagnostic alternative in the confirmation of infection, due to prompt diagnosis. Isolation is too time-consuming, and not practical to be used in routine testing of asymptomatic animals.

Keywords: ram, semen, urine, brucellosis, culture

RESUMO

O objetivo do estudo foi adaptar e avaliar a PCR para detecção de Brucella *ovis e comparar os resultados com aqueles obtidos por cultivo microbiológico do sêmen, urina e dos órgãos de carneiros infectados experimentalmente. Dos 31 animais infectados experimentalmente, amostras de PCR do sêmen apresentaram maior sensibilidade (21,6%) do que o cultivo (8,0%). Em amostras de urina, a sensibilidade das técnicas foi semelhante (10,1% para a cultivo e 12,7% para PCR). PCR detectou a presença do agente em 21,5% das amostras testadas, enquanto os órgãos de cultivo detectaram em apenas 3,3% das amostras. PCR detectou um maior número de amostras positivas do que o cultivo microbiológico.*

Palavras-chave: brucelose, cultivo, carneiro, sêmen, urina

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