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Communication

[*Comunicação*]

Diagnosis of *Brucella ovis* infection by serology and PCR in urine samples from naturally infected rams in the State of Piauí

[*Diagnóstico da infecção por Brucella ovis por sorologia e reação em cadeia pela polimerase (PCR) em amostras de urina de carneiros naturalmente infectados no Estado do Piauí*]

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Ovine brucellosis caused by *Brucella ovis* is a disease with worldwide distribution. *B. ovis* is a facultative intracellular Gram-negative coccobacillus belonging to the genus *Brucella* (Xavier *et al*., 2009). The infection in sheep may be often asymptomatic, but it is usually characterized by epididymitis and orchitis in rams, and occasionally abortion in ewes, and neonatal mortality. Therefore, *B. ovis* infection ultimately leads to infertility, early culling, and consequently economic losses (Burgess, 1982). Prevalence of infection is highly variable among different regions in the world, ranging from 2.4 to 46.7% of infected animals and 2.1 to 67% of soropositive flocks (Robles *et al*., 1993, Sergeant, 1994). In Brazil, prevalence data is fragmented, with reports of 13.7% in the State of Rio Grande do Sul (Magalhães Neto and Gil-Turnes, 1996), 8.6% in Paraíba (Clementino *et al*., 2007), 17.5% in Pernambuco (Coleto *et al*., 2003), 12% in São Paulo (Nozaki *et al*., 2004), and 11.3% in Rio Grande do Norte (Azevedo *et al*., 2004). There are no previous reports of *B. ovis* infection in the State of Piauí.

Traditionally, diagnosis of *B. ovis* infection is based on clinical examination, serological tests, and bacteriology of semen samples (Burgess, 1982). Several serologic methods are employed to detect antibodies against *B. ovis*, including agar gel immunodiffusion (AGID), complement fixation (CF), and enzyme linked immunosorbent assay (ELISA) (Marín *et al*., 1989). However, serological diagnosis is not entirely satisfactory since it commonly provides highly variable results, with high frequency of false-negative (Nozaki *et al*., 2004). Although bacteriology is considered the gold standard for diagnosis, it is laborious, time consuming, and its results may be compromised by environmental contamination (Manterola *et al*., 2003). Molecular techniques based on amplification of *Brucella* spp. genomic DNA have been applied to the diagnosis of *B. ovis* infections (Manterola *et al*., 2003; Saunders *et al*., 2007). Our group has recently developed a species-specific PCR assay for detection of *B. ovis* genomic DNA in biological samples, including semen, preputial wash, and urine (Xavier *et al*., 2010). This study aimed to evaluate the applicability of a species-specific PCR method under field conditions, using urine samples to detect *B. ovis* by PCR and to compare to serology (AGID).

Serum and urine samples were collected from 90 rams belonging to 31 herds located in the State of Piauí. Blood samples were collected with vacuntainer tubes without EDTA (BD - Becton, Dickinson Co., USA) from the jugular vein, and then centrifuged. Serum samples were aliquoted in cryogenic tubes. Urine samples were collected by blocking breathing for 30 seconds and aliquoted in cryogenic tubes. Serum and urine were stored in liquid nitrogen.

Serum samples were tested by the AGID method as previously described by Marín *et al*. (1989). The used antigen was produced from soluble extract of heat-inactivated strain of *B. ovis* strain REO 198 by Instituto de Pesquisas Desidério Finamor, Rio Grande do Sul, Brazil.

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DNA extraction from urine samples was performed with 1mL of urine according to the protocol previously described by Xavier *et al*. (2010). Two pairs of primers of a specific genetic island of *B. ovis*, determining the specific amplification of *B. ovis,* were used in this study (Tsolis *et al*., 2009). The primers were designed to target a specific *B. ovis* open reading frame, namely AO503 (Tsolis *et al*., 2009): 5'-GCCTACGCTGAAACTTGCTTTTG-3' and 5'-ATCCCCCCATCACCATAACCGAAG-3'. PCR reaction was performed using 23µL of commercial PCR Supermix (Invitrogen, Brazil), 0.5µL of each primer at 25µM, 0.25µL of Taq polymerase (Invitrogen, Brazil), and 3µL of template DNA (100-500ng of DNA per reaction). Cycling parameters consisted of denaturation at 95°C for 5 minutes, 35 cycles of denaturation (95°C for 1 minute), annealing (55°C for 1 minute), extension (72°C for 1 minute) and a final extension at 72°C for 5 minutes. PCR products were resolved by 1% agarose gel eletrophoresis. Specific PCR products had 228 base pairs.

Frequencies of positivity by AGID and PCR were compared by the Fisher's exact test using GraphPad Instat software, version 3.05 (GraphPad Instat, Inc., U.S.A.). Agreement between these two methods was evaluated by the Kappa test using Minitab 15 software (Global Tech, Brazil).

Eighteen out of 90 urine samples analyzed (20%) were positive by PCR, while 16 (17.8%) serum samples were positive by AGID. Four out of 31 farms assessed had positive rams by PCR, AGID, or both tests corresponding to 12.9% of positive herds. There was no statistical difference between methods considering the frequency of positive rams ($P>0.05$). However, kappa statistics indicated a low concordance between these methods ($k=0.02$). In spite of this low level of agreement between serology and PCR, combination of these methods resulted in a significantly increase frequency of positive diagnosis when compared to any of the methods individually ($P<0.05$), resulting in identification of 34.4% of positive rams.

These results clearly indicates that neither PCR nor serology are completely reliable diagnostic methods for individual rams since serologically negative rams can excrete the organism whereas serologically positive rams may not excrete the organism. Previous studies from our group have demonstrated that this species-specific PCR method has levels of sensitivity comparable or superior to bacterial isolation (Xavier *et al*., 2010). Furthermore, there is no good agreement between serology and PCR results even during the course of experimental infections (Xavier *et al*., 2010). The high occurrence of asymptomatic infections by *B. ovis* makes it very difficult to establish a clinical diagnosis. According to Burgess (1982), shedding of *B. ovis* in the semen is considered the main source of infection in the herd. Therefore, semen is considered the sample of choice for detection of *B. ovis* (Manterola *et al*., 2003, Saunders *et al*., 2007). However, *B. ovis* elimination in semen is intermittent, which can impair diagnostic sensitivity (Manterola *et al*., 2003, Saunders *et al*., 2007). In addition, considering that semen collection under field conditions is quite laborious, urine samples have been tested and proved to result in similar levels of sensitivity as compared to semen samples from experimentally infected rams (Xavier *et al*., 2010).

It is noteworthy that sheep is also susceptible to *B. melitensis* infection, and that in sharp contrast to *B. ovis* (that does not infect humans), *B. melitensis* is the species within the *Brucella* genus with the highest zoonotic potential (Xavier *et al*., 2009). Therefore, the species-specific PCR method employed in this study is highly desirable since it allows a differential diagnosis in areas where *B. melitensis* is enzootic, favoring epidemiological investigations and implementation of an appropriate control program.

In conclusion, in spite of a low agreement between serology and PCR, the species-specific PCR method employed in this study demonstrated to be a suitable complementary diagnostic method for identification of *B. ovis* infected rams. Furthermore, this method allows identification of serologically negative rams shedding the organism in the environment. These results support the notion that PCR of urine samples can be considered an important tool for the diagnosis of natural infections by *B. ovis*.

Keywords: *Brucella ovis*, sheep, diagnosis, PCR, AGID

RESUMO

A brucelose ovina causada pela Brucella ovis é uma doença reprodutiva de carneiros caracterizada por epididimite, orquite, com consequente diminuição da fertilidade e prejuízos econômicos significativos. O presente trabalho teve por objetivo avaliar a aplicabilidade da técnica de PCR como um método de diagnóstico em campo, comparado-a com a técnica de IDGA. Foram coletadas amostras de urina e soro de 90 carneiros oriundos de 31 rebanhos localizados no Estado do Piauí. Quatro das 31 (12,9%) propriedades avaliadas apresentaram animais positivos. Dezoito (20%) amostras de urina foram positivas pela PCR, enquanto o método de IDGA identificou 16 (17,8%) carneiros soropositivos. Embora os métodos tenham apresentado concordância baixa na estatística Kappa (k=0,04), não foi observada diferença estatística entre as técnicas (P>0,05) pelo teste exato de Fisher. A combinação dos dois testes aumentou significativamente a detecção de animais positivos para 34,4% (P <0,05), sugerindo que a associação de métodos de diagnóstico como a técnica de PCR em amostras de urina e sorologia por IDGA e a avaliação clínica dos animais é necessária para um diagnóstico eficiente na infecção por B. ovis.

Palavras-chave: Brucella ovis, *ovinos, diagnóstico, PCR, IDGA*

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