

Communication

[Comunicação]

**A comparison of two agar gel immunodiffusion methods and a complement fixation test for serologic diagnosis of *Brucella ovis* infection in experimentally infected rams**

[Comparação entre dois métodos de imunodifusão em gel de Agar e um método de fixação de complemento para o diagnóstico sorológico da infecção por *Brucella ovis* em carneiros experimentalmente infectados]

M.N. Xavier<sup>1</sup>, F.M. Sant'Anna<sup>1</sup>, T.M.A. Silva<sup>1</sup>, E.A. Costa<sup>1</sup>, V.S. Moustacas<sup>1</sup>, F.A. Merlo<sup>1</sup>,  
C.A. Carvalho Júnior<sup>1</sup>, M.G. Dasso<sup>2</sup>, L.A. Mathias<sup>3</sup>, A.M.G. Gouveia<sup>1</sup>,  
A.P. Lage<sup>1</sup>, R.L. Santos<sup>1\*</sup>

<sup>1</sup>Escola de Veterinária - UFMG  
Av. Antônio Carlos, 6627  
31270-901 - Belo Horizonte, MG

<sup>2</sup>Laboratório de Brucelose, Instituto de Pesquisas Veterinárias Desidério Finamor - IPVDF - Eldorado do Sul, RS

<sup>3</sup>Faculdade de Ciências Agrárias e Veterinária - Universidade Estadual Paulista - Jaboticabal, SP

Ovine brucellosis due to *Brucella ovis* infection is worldwide considered one of the most important infectious diseases of sheep in raising areas. The disease is caused by a Gram-negative bacteria of the genus *Brucella*, which are facultative intracellular coccobacilli that belong to the  $\alpha 2$ -Proteobacteriacea family (Xavier *et al.*, 2009). Clinical signs of the infection consist of chronic epididymitis and infertility in rams (Xavier *et al.*, 2009) and, occasionally, abortion in ewes and birth of weak lambs (Molello *et al.*, 1963). In Brazil, a seroprevalence of 13.4% (ranging from 6.9 to 50%) positive sheep flocks has been reported in the state of Rio Grande do Sul, whereas a seroprevalence of 5.57% has been reported in the Northeastern part of the country (reviewed by Xavier *et al.*, 2009).

Serologic diagnosis of *B. ovis* infection may be achieved through a wide range of tests, including complement fixation (CF) (Alton *et al.*, 1988), agar gel immunodiffusion (AGID) (Myers and Siniuk, 1970), and the enzyme immunoassays (ELISA) (Gall *et al.*, 2003) are the most commonly used for routine diagnosis of the disease. Importantly, CF has been applied as the standard serological test in *B. ovis* eradication programs worldwide. However, CF is also

considered a laborious technique, which is time consuming and requires well trained personnel (Alton *et al.*, 1988). Conversely, AGID is considered highly sensitive, and has a simple and low cost execution. Consequently, AGID has been used in replacement of CF for the diagnosis of *B. ovis* infection in several countries, including Brazil (Manual..., 2009). The aim of this work was to evaluate three serological tests using commercially available antigens for the diagnosis of *B. ovis* infection in experimentally infected rams.

A total of 181 ovine serum samples were used in this study, being 117 from experimentally infected rams. The remaining 64 samples included nine samples obtained from the experimentally infected rams prior to inoculation, and the other 55 serum samples were obtained from sheep of different ages belonging to a *B. ovis*-free flock, which had no history of epididymitis or infertility, and was located in Belo Horizonte, Brazil. Nine 1 to 3-year-old cross-breed rams were experimentally infected as previously described (Xavier *et al.*, 2010). Blood samples were collected from the jugular vein, serum was separated, and stored at -20°C. This experiment was approved by the Universidade Federal de Minas Gerais Committee for Experimental Animal Use (CETEA, protocol 02/2007).

---

Recebido em 27 de fevereiro de 2011

Aceito em 21 de março de 2011

\*Autor para correspondência (*corresponding author*)

E-mail: rsantos@vet.ufmg.br

AGID was performed using two commercially available antigens: (i) an antigen manufactured by the Instituto de Tecnologia do Paraná (TECPAR, Brazil), containing *B. ovis* (strain REO 198) lipopolysaccharide and cellular wall protein extracts; and (ii) an antigen manufactured by the Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF, Brazil), containing soluble extracts from log phase heat-inactivated *B. ovis* strain REO 198. AGID test was performed as previously described (Marín *et al.*, 1989). Briefly, 4.6mL of 1.1% agarose gel (Invitrogen, Brazil) diluted in 0.1M borate buffer pH 8.6 was placed onto a clean microscope slide. A central well of 5.3mm and six outer wells of 4.0mm in diameter were cut, with distance between the central well and outer wells of 3.0mm. Two diametrically opposed wells were filled with 30µL positive control serum provided by each kit and the other four wells with 30µL of test serum. The central well was filled with 30µL of antigen (either TECPAR or IPVDF). The slides were incubated for 72h at 4°C in a humid atmosphere. Reaction was visualized with indirect light. Positive sera developed sharp precipitation lines. Any sera giving a line of identity with the positive control lines were considered positive.

CF was performed in microtitre plates as described by Alton *et al.* (1988). Complement, antigen, and hemolysin dilutions were determined by titration (Alton *et al.*, 1988). IPVDF antigen was used in a dilution of 1:60. The test sera and positive or negative controls were first diluted at 1:2 with Triethanolamine buffer (TBS; 0.05% gelatin, 0.15M CaCl<sub>2</sub>, 0.5M MgCl<sub>2</sub>, 0.12M NaCl, 0.28% triethanolamine, and 0.017 M HCl) in glass tubes and inactivated at 60°C for 30min. Sera were distributed in the microplates in double dilutions from 1:2 to 1:128 in Triethanolamine buffer, with a final volume of 25µL in each well. After dilution, 25µL of antigen and 25µL of preserved complement diluted to contain 2.5 minimal hemolytic doses were added to each well, with 25µL of TBS replacing the antigen in serum control. The microplates were incubated at 37°C for 30 minutes. Then, an aliquot of 25µL of a 2% suspension of sheep red blood cells sensitized with hemolysin, was added to each well and again incubated at 37°C for 30min.

After centrifugation at 1,500xg for 20min, results were visually read at each dilution as 0 (complete hemolysis), 1 (75% hemolysis), 2 (50% hemolysis), 3 (25% hemolysis), or 4 (no hemolysis). The titre was the reciprocal of the highest dilution of serum in which there was less than 75% hemolysis. Serum samples that presented titre  $\geq 4$  were considered to be positive for *B. ovis* infection (Alton *et al.*, 1988). Geometric means of CF titers considering all experimentally infected rams at a given time point during infection were calculated.

Frequencies of positive sera samples by the three serological methods were compared by Fisher's exact test using the GraphPad InStat software, version 3.05 (GraphPad InStat software, Inc., USA) with a 95% confidence level. The sensitivity and specificity were assessed according to Henken *et al.* (1997), with GraphPad software, version 3.05 (InStat Inc., USA) with 95% confidence level. Proportion of agreement between diagnosis methods was assessed by Kappa test, with Minitab 15 software (Global Tech, Brazil) with 95% confidence level.

The three methods evaluated in this study detected antibodies against *B. ovis* in 55.6% (5/9), 77.8% (7/9), and 77.8% (7/9) of experimentally infected rams as early as 15 days post-infection (dpi), for AGID-TECPAR, AGID-IPVDF, and CF, respectively (Figure 1). The highest percentages of positive rams were observed from 30 to 60 dpi for all serological tests, with 100% (9/9) of positivity by CF from 30 to 45 dpi, whereas both AGID tests detected antibodies against *B. ovis* in up to 88.9% (8/9) of the rams at the same time points (Figure 1).

The average serological titers obtained by the CF method also reached the highest values from 30 to 60 dpi, but positive titers ( $\geq 4$ ) were maintained only until 75 dpi (Figure 2). Frequency of positive rams as assessed by both AGID methods oscillated from 60 dpi until the end of the study period (Figure 1), with a tendency to a decreasing positive frequency over the time course of infection. Conversely, the frequency of detection of serological positive rams by the CF method showed a tendency of constant drop throughout the course of the experimental infection (Figure 1). Indeed, the average serological titers obtained by CF followed this same pattern (Figure 2).

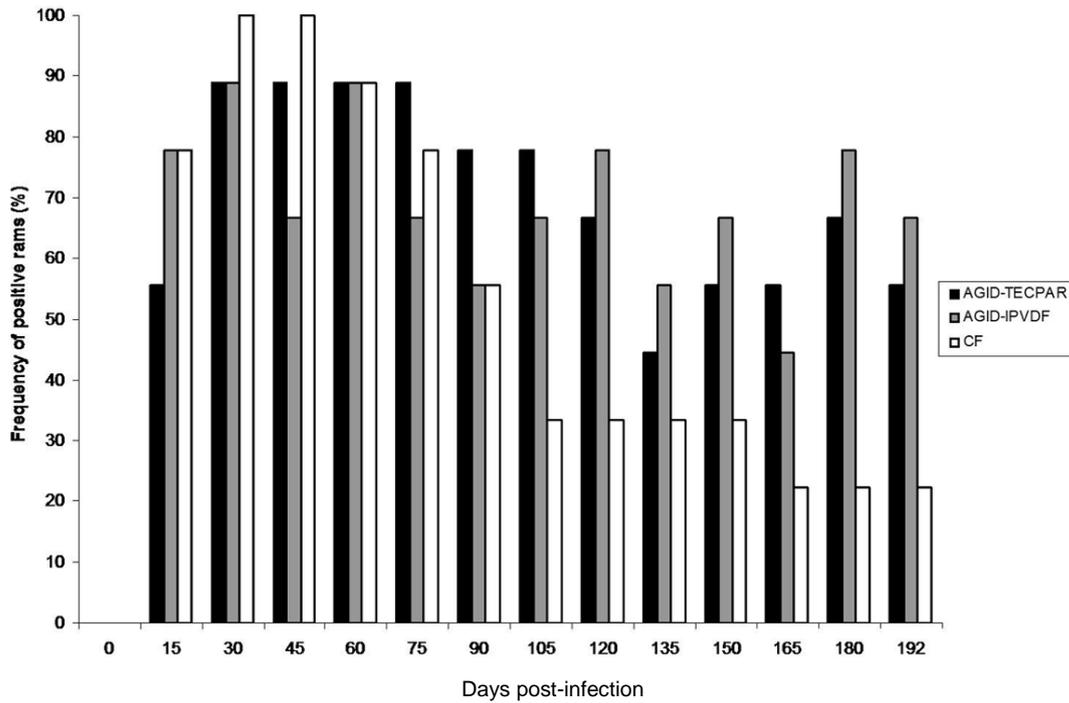


Figure 1. Frequency (%) of *Brucella ovis* serological positive rams by agar gel immunodiffusion (AGID) with the TECPAR antigen (AGID-TECPAR) or the Desidério Finamor antigen (AGID-IPVDF), and by complement fixation (CF) throughout the course of experimental infection (up to 192 days post infection).

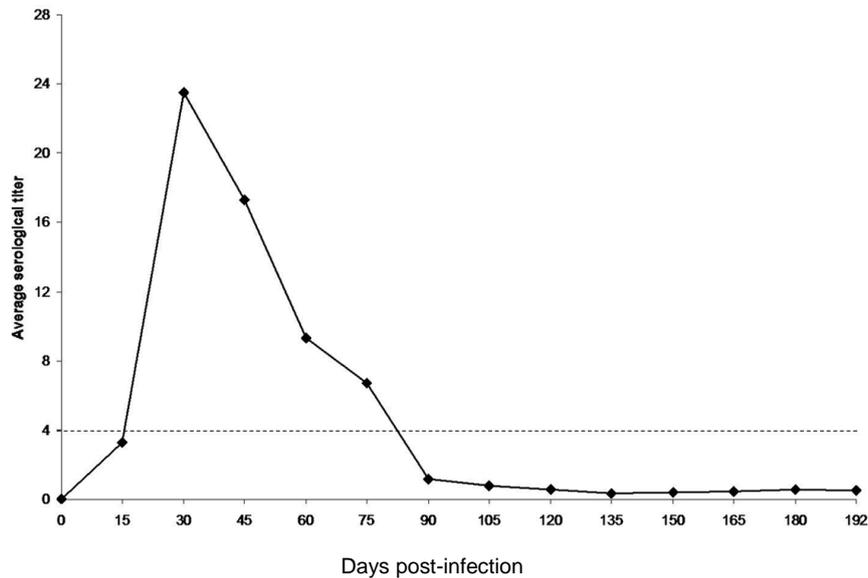


Figure 2. Average serological titers of antibodies against *Brucella ovis* as assessed by complement fixation (CF) in sera from experimentally infected rams throughout 192 days of experimental infection. The dotted line represents the cutting point of positivity (titer ≥ 4).

*A comparison of two agar gel...*

All 117 sera samples obtained from experimentally infected rams were considered as truly positive samples, since all nine rams were subjected to a *B. ovis* high challenge. Conversely, the remaining 64 sera samples were considered as truly negative samples, since they were obtained from rams during the pre-inoculation period (n=9) or from a *B. ovis*-free flock (n=55). The overall percentages of positive samples as assessed by AGID were 45.3% (82/181) and 44.7% (81/181) when considering AGID-TECPAR and AGID-IPVDF, respectively, which were similar (P>0.05). However, the overall frequency of positivity by CF was 33.7% (61/181), which was statistically lower than the percentage obtained by both AGID tests (P>0.05).

Sensitivity results are shown in Table 1. In order to achieve a more precise evaluation of sensitivity, serum samples from experimentally infected rams were grouped into monthly periods post-infection, from one to six months, with a n=18 for each period, with exception of six months post-infection (mpi), with n=27. The AGID-TECPAR method had the highest sensitivity value of 88.8% at 2mpi, followed by statistically similar sensitivity values until 4mpi. However, in the last 2mpi (i.e. 5<sup>th</sup> and 6<sup>th</sup> mpi) the sensitivity of this method decreased

significantly (P<0.05), reaching values as low as 50%. Conversely, AGID-IPVDF maintained statistically similar sensitivity values throughout the time course of experimental infection (P>0.05).

CF had the highest sensitivity values during the first 2mpi, reaching 88.8% and 94.4% at the 1<sup>st</sup> and 2<sup>nd</sup> months, respectively. However, CF sensitivity values decreased significantly beginning at the 4<sup>th</sup> mpi (P<0.05), reaching values as low as 22.2% in the last mpi. When comparing all three serological tests, statistically significant differences were observed beginning at the 4<sup>th</sup> mpi, when CF had lower sensitivity (P>0.05) when compared to AGID. Both AGID methods had statistically similar sensitivity throughout the course of experimental infection. Indeed, the same pattern was observed when sensitivity results considering all six months of infection were evaluated together, but FC had a significantly lower sensitivity (P>0.05). In this case, sensitivity values were of 70.1%, 69.2%, and 53.8% for AGID-TECPAR, AGID-IPVDF, and CF methods, respectively. All 64 samples considered to be truly negative for *B. ovis* infection had negative results in all three serological tests evaluated (data not shown). Therefore, both AGID methods as well as the CF test had 100% specificity.

Table 1. Sensitivity of agar gel immunodiffusion (AGID) with the TECPAR antigen (AGID-TECPAR) or the Desidério Finamor antigen (AGID-IPVDF) and complement fixation (CF) for detection of antibodies against *Brucella ovis* in ovine serum samples throughout six months of experimental infection

Method	Months post-infection						Total
	1	2	3	4	5	6	
AGID-TECPAR	72.2Aab	88.8Aa	83.3Aab	72.2Aab	50.0Ab	59.3Ab	70.1Aab
AGID-IPVDF	83.3Aa	77.7Aa	61.1Aa	72.2Aa	61.1Aa	62.9Aa	69.2Aa
CF	88.8Aa	94.4Aa	66.6Aa	33.3Bbc	33.3Abc	22.2Bc	53.8Bb

Values followed by distinct lower case letters in the same row differ statistically by Fisher's Exact Test (P<0.05). Values followed by distinct upper case letters in the same column differ statistically by Fisher's Exact Test (P<0.05).

Agreement between AGID methods was 86.74%, with a kappa statistics value of 0.734, confirming a high agreement between the two tests. Conversely, agreement between CF and both AGID methods was lower, with values of 54.14% and 54.7% when CF was compared to the AGID-TECPAR and AGID-IPVDF, respectively. Such lower agreement was confirmed by the kappa statistics values of 0.201

and 0.215 between the CF and AGID-TECPAR or AGID-IPVDF, respectively.

Diagnosis of *B. ovis* infection is based on the association of clinical examination to detect lesions, serological tests, and bacteriology of semen samples from infected rams (Webb *et al.*, 1980; Burgess, 1982). This combined approach is recommended since no single procedure is entirely satisfactory for identification of all

infected animals in a flock (Lawrence, 1961). The most common clinical sign of *B. ovis* infection is chronic epididymitis in mature rams, which may be absent in most infected animals (Burguess, 1982; Xavier *et al.*, 2009). The occurrence of these asymptomatic infections limits the efficiency of clinical diagnosis. Moreover, shedding of *B. ovis* in the semen is known to be intermittent and it is usually detectable by bacteriology or polymerase chain reaction (PCR) only after 45 to 60 dpi (Burguess, 1982; Xavier *et al.*, 2010). Therefore, the efficacy of direct methods during the early stages of infection is also considerably limited. The present study demonstrates that antibodies against *B. ovis* in experimentally infected animals are highly detectable by AGID and CF as early as 15 to 60 dpi. Consequently, serological methods may be the best alternative for detection of infected animals during the early stages of infection.

It was demonstrated an oscillation in the frequency of rams serologically positive for *B. ovis* by both AGID methods after 75 dpi as well as a constant decrease in the number of positive rams and antibody titers by CF. Additionally, AGID-TECPAR and CF had a statistically significant decrease in sensitivity mainly after the third month of experimental infection. Some

experimentally infected rams are able to overcome the infection, which results in absence of detectable antibody titers or bacterial isolation after the acute phase of infection (Burguess, 1982).

Sensitivity of AGID and CF described in this study was lower than those previously described in the literature, which reported sensitivity values over 90% for these tests (Marín *et al.*, 1989). However, in these previous studies ovine serum samples from naturally infected rams were used, in which case the stage of infection as well as the infectious challenge could not be determined.

In conclusion, both AGID antigens were equally sensitive and specific for the serological diagnosis of *B. ovis* infection in rams, whereas CF resulted in lower sensitivity after the acute phase of infection. Considering that AGID and CF were less sensitive after 90 dpi, it is recommended that serological diagnosis should be associated to other available diagnostic tests, including bacteriology and PCR for improving the efficiency of the diagnosis of *B. ovis* infection in rams.

Keywords: ram, *Brucella ovis*, serology, complement fixation, immunodiffusion

## RESUMO

*A infecção por Brucella ovis é considerada uma das principais causas de epididimite e infertilidade em carneiros, resultando em falhas reprodutivas e perdas econômicas significativas em rebanhos ovinos ao redor do mundo. O estudo teve o objetivo de avaliar três testes sorológicos disponíveis para o diagnóstico da brucelose ovina por B. ovis, utilizando 181 soros ovinos. Amostras de soro provenientes de carneiros experimentalmente infectados foram coletadas ao longo de 192 dias pós-infecção (n=117) e durante o período pré-infecção (n=9). Adicionalmente, amostras de soro foram obtidas de ovinos provenientes de um rebanho livre para B. ovis (n=55). As técnicas de imunodifusão em gel de agar (IDGA), utilizando dois antígenos disponíveis comercialmente, e de fixação de complemento foram comparadas (FC). Foram obtidos resultados de sensibilidade e especificidade semelhantes para ambos os métodos de IDGA e ainda, a técnica de IDGA foi mais eficiente do que a da FC para o diagnóstico sorológico da infecção por B. ovis.*

*Palavras-chave: ovino, Brucella ovis, sorologia, fixação de complemento, imunodifusão*

## ACKNOWLEDGEMENTS

We thank Adriana Amantino M. Martins for technical assistance. MNX, TMAS, EAC, VSM, APL, and RLS are recipients of fellowships from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). RLS is a fellow of the

John Simon Guggenheim Memorial Foundation. Work in RLS lab is supported by CNPq and FAPEMIG (Fundação de Amparo a Pesquisa do Estado de Minas Gerais).

## REFERENCES

- ALTON, G.G.; JONES, L.M.; ANGUS, R.D. *et al.* *Techniques for the Brucellosis Laboratory*. France: INRA, 1988. 192 p.
- BURGUESS, G.W. Ovine contagious epididymitis: a review. *Vet. Microbiol.*, v.7, p.551-575, 1982.
- GALL, D.; NIELSEN, K.; VIGLIOCCO, A. *et al.* Evaluation of an indirect-linked immunoassay for presumptive serodiagnosis of *Brucella ovis* in sheep. *Small Ruminant Res.*, v.48, p.173-179, 2003.
- HENKEN, A.M.; GRAAT, E.A.M.; CASAL, J. Measurement of disease frequency. In: NOORDHUIZEN, J.P.T.M.; FRANKENA, K.; VAN DER HOOFD, C.M. *et al.* (Eds.). *Application of Quantitative Methods in Veterinary Epidemiology*. The Netherlands: Wageningen Pers, 1997. p.63-98.
- LAWRENCE, W.E. Ovine brucellosis: a review of the disease in sheep manifested by epididymitis and abortion. *Br. Vet. J.*, v.117, p.435-446, 1961.
- MANUAL of diagnostic tests and vaccines for terrestrial animals. Paris: OIE, 2009. 1200p. Disponível em: <[http://www.oie.int/eng/normes/mmanual/A\\_suumry.htm](http://www.oie.int/eng/normes/mmanual/A_suumry.htm)>. Acessado em: 15 dez. 2009.
- MARÍN, C.M.; JIMENES DE BAGUES, M.P.; BLASCO, J.M. *et al.* Comparison of three serological tests for *Brucella ovis* infection of rams using different antigenic extracts. *Vet. Rec.*, v.125, p.504-508, 1989.
- MOLELLO, J.A.; JENSEN, R.; FLINT, J.C. *et al.* Placental pathology. I. Placental lesions of sheep experimentally infected with *Brucella ovis*. *Am. J. Vet. Res.*, v.24, p.897-904, 1963.
- MYERS, D.M.; SINIUK, A. Preliminary report on the development of a diffusion-in-gel method for the diagnosis of ram epididymitis. *Appl. Microbiol.*, v.19, p.335-337, 1970.
- WEBB, R.F.; QUINN, C.A.; COCKRAM, F.A. *et al.* Evaluation of procedures for the diagnosis of *Brucella ovis* infection in rams. *Aust. Vet. J.*, v.56, p. 172-175, 1980.
- XAVIER, M.N.; COSTA, E.A.; PAIXÃO, T.A. *et al.* The genus *Brucella* and clinical manifestations of brucellosis. *Cienc. Rural* v.39, p.2252-2260, 2009.
- XAVIER, M.N.; SILVA, T.M.A.; COSTA, E.A. *et al.* Development and evaluation of a species-specific PCR assay for detection of *Brucella ovis* infection in rams. *Vet. Microbiol.*, v.145, p.158-164, 2010.