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Performance of the Dot-blot test method for detecting antibodies to *Sarcocystis* spp. in cattle[1]

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ABSTRACT.- Ferreira, M.S.T., Fernandes F.D., Alves, M.E.M, Braunig P., Sangioni, L.A. & Vogel F.S.F. 2020. **Performance of the Dot-blot test method for detecting antibodies to *Sarcocystis* spp. in cattle.** *Pesquisa Veterinária Brasileira 40(5):385-388*. Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Avenida Roraima 1000, Santa Maria, RS 97105-900, Brazil. E-mail: fagnermedvet@gmail.com

Serological techniques can detect antibodies against *Sarcocystis* spp., *Neospora caninum* and *Toxoplasma gondii* antigens in single or mixed infections. Immunofluorescent antibody tests (IFAT) is considered the gold standard technique for Sarcocystosis diagnostic in cattle serum and a positive IFAT result reflects *Sarcocystis* spp. infection. Therefore, the aims of the present study were to compare IFAT and Dot-blot for sarcocystosis diagnostic in experimentally infected mice and to investigate serological cross-reactions with *N. caninum* and *T. gondii* in these methods. Mice (*Mus musculus*) were inoculated intraperitoneally with bradizoites of *Sarcocystis* spp. or tachyzoites of *N. caninum* or *T. gondii.* Serum samples were obtained and analyzed by IFAT and Dot-blot for the three protozoa. Serum from *N. caninum* and *T. gondii* experimentally infected mice were tested by IFAT and reacted only to *N. caninum* or *T. gondii* antigens, respectively. Specific antibodies against *Sarcocystis* spp. were present in all animals experimentally infected with this protozoan, with IFAT titers from 10 to 800. Serum samples from mice experimentally infected with *Sarcocystis* spp., *N. caninum* and *T. gondii* and tested by Dot-blot demonstrated no cross reaction between protozoa. A Dot-blot using *Sarcocystis* spp. antigen appears to be a good alternative to IFAT in the serological diagnosis of Sarcocystosis.

INDEX TERMS: Dot-blot test, antibodies, *Sarcocystis* spp., cattle, serological diagnosis, IFAT, protozoan.

RESUMO.- [**Desempenho do teste Dot-blot para detecção de anticorpos para *Sarcocystis* spp. em bovinos.**] As técnicas sorológicas podem detectar anticorpos contra os antígenos de *Sarcocystis* spp., *Neospora caninum* e *Toxoplasma gondii* em infecções únicas ou mistas. O teste de anticorpos imunofluorescentes (IFAT) é considerado a técnica padrão-ouro para o diagnóstico de sarcocistose no soro de bovinos e um resultado positivo de IFAT reflete *Sarcocystis* spp. infecção. Portanto, os objetivos do presente estudo foram comparar IFAT e Dot-blot para diagnóstico de sarcocistose em camundongos infectados experimentalmente e investigar reações cruzadas sorológicas com *N. caninum* e *T. gondii* nesses métodos. Os camundongos (*Mus musculus*) foram inoculados intraperitonealmente com bradizoítos de *Sarcocystis* spp. ou taquizoítos de *N. caninum* ou *T. gondii*. As amostras de soro foram obtidas e analisadas por IFAT e Dot-blot para os três protozoários. O soro de *N. caninum* e *T. gondii* infectados experimentalmente foram testados por IFAT e reagiram apenas aos antígenos de *N. caninum* ou *T. gondii*, respectivamente. Anticorpos específicos contra *Sarcocystis* spp. estavam presentes em todos os animais experimentalmente infectados com este protozoário, com títulos de IFAT de 10 a 800. Amostras de soro de camundongos infectados experimentalmente com *Sarcocystis* spp., *N. caninum* e *T. gondii* e testadas por Dot-blot não demonstraram reação cruzada entre protozoários. Um Dot-blot usando *Sarcocystis* spp. O antígeno parece ser uma boa alternativa ao IFAT no diagnóstico sorológico da sarcocistose.

TERMOS DE INDEXAÇÃO: Dot-blot, anticorpos, *Sarcocystis* spp., bovinos, diagnóstico sorológico, IFAT, protozoário.

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INTRODUCTION

Sarcocystis spp., *Neospora caninum* and *Toxoplasma gondii* are coccidians belonging to Sarcocystidae family. They have worldwide distribution and may cause infections in ruminants inducing productive and economic losses (Tenter 1995, Dubey 2003, 2009). Although the prevalence of *Sarcocystis* spp. infection in cattle herds is approximately 90% to 100% in many countries, Sarcocystosis in cattle are frequently asymptomatic making difficult the diagnosis (Moré et al. 2011, Ruas et al. 2001, Akhlaghi et al. 2016).

Diagnosis of acute Sarcocystosis is difficult because, the clinical signs are uncommon, unspecific and the parasitemia is frequently low and consequently undetectable (Ndiritu et al. 1996, Dubey et al. 2016). Diagnosis of chronic Sarcocystosis is usually made by conventional laboratory methods as microscopy, immunoassays and tissue digestion (Fayer & Dubey 1986, Gajadhar et al. 1987, Cawthorn & Speer 1990). Several serological methods as immunofluorescent antibody tests (IFAT), enzyme linked immunosorbent assays (ELISA), and immunoblots (as Western blot and Dot-blot) have been developed for diagnosis the bovine Sarcocystosis (Moré et al. 2008). Serological techniques detect antibodies against the protozoans *Sarcocystis* spp., *N. caninum* and *T. gondii* antigens in single or mixed infections (Uggla et al. 1987, Dubey et al. 1996). However a specific and sensitive serological test which detects acute and chronic infection and suitable for screening a large number of animals is lacking for *Sarcocystis* spp.

The aims of the present study were a) to compare two serological tests (IFAT and Dot-blot) for Sarcocystosis diagnostic, and b) to check the cross-reactions of *N. caninum* and *T. gondii* using IFAT and Dot-blot in serum of experimentally infected mice.

MATERIALS AND METHODS

Animal inoculation and serum samples. Seven mice (*Mus musculus*), male, approximately 6 weeks of age with initial weight of 30g, without previous contact with parasites were used to obtain hyperimmune serum against *Sarcocystis* spp., *Neospora caninum* and *Toxoplasma gondii*. Two animals were inoculated intraperitoneally with $2x10^5$ bradyzoites of *Sarcocystis* spp. obtained from cysts of a naturally infected bovine heart and purified by previously described by García-Lunar et al. (2015). Similarly, two animals were inoculated intraperitoneally with $2x10^5$ tachyzoites of *N. caninum* and two animals with *T. gondii*. Parasites were obtained from culture in VERO cells as described in the item 2.2. After 20 days of infection, blood was collected from cardiac puncture and serum was obtained. One naive animal was used as a negative experimental control. Samples were identified and stored at -20°C until processing.

Antigens preparation of sorological tests. Tachyzoites from the NC-1 strain of *N. caninum* (Dubey et al. 1988) and RH-strain of *T. gondii* (Sabin, 1941) were cultured in VERO cells (African green monkey kidney cells) in RPMI 1640 culture medium (Invitrogen, Brazil), supplemented with 10% fetal bovine serum (Nutricell, Brazil) with 5% CO_2 at 37°C. Tachyzoites from *N. caninum* were obtained after cellular suspension and disruption, and solution was decanted during 30 minutes, at 4°C, in sterile tube to diminish cell debris. Tachyzoites from *T. gondii* were obtained from frozen aliquots and supplemented with RPMI and fetal serum. Supernatant was recovered from each suspension and centrifuged at 1.500 x g for 10 minutes. Tachyzoites were counted using Neubauer's chamber and re-diluted with RPMI to a final concentration of $2x10^5$ tachyzoites/mL. The pellets were stored at −80°C until use for total protein extraction or IFAT (Pinheiro et al. 2005).

Sarcocystis spp. bradyzoites and/or merozoites were obtained from microscopic analysis of myocardium samples from naturally infected cattle and then purified as described by García-Lunar et al. (2015) and then used as antigen (Moré et al. 2011). Briefly, 100g of minced myocardium were mixed with 400ml of digestion solution (2.5% pepsin, 1% HCl) and were placed in a magnetic stirrer for 20 minutes at 37°C. The homogenate was filtered using 300µm gauze into a 50ml tubes and centrifuged at 500 x g for 5 minutes. The supernatant was discarded and the pellet was washed in 30ml of PBS, 13.5ml of isotonic Percoll® (GE Healthcare) and 1.5ml saline solution (1.5M NaCl) and then centrifuged (4.000 x g for 10 min) (Pertoft et al. 1980). The supernatants and the upper layer above the pellet were discarded and the pellet was washed three times with PBS to a final concentration of $2x10^5$ bradyzoites/mL. Pellets with bradyzoites were frozen at -80°C until use for total protein extraction or for their use in IFAT (Moré et al. 2008, Fernandez-García et al. 2009).

IFAT. Slide preparation and IFAT for *N. caninum* and *T. gondii* were performed as described by Dubey et al. (1988). Bradyzoites of *Sarcocystis* spp. were fixed in slides as described by García-Lunar et al. (2015).

In order to investigate antibodies against *N. caninum* and *T. gondii*, serum samples were used at the dilution of 1/64 (Devens et al. 2014) in phosphate-buffered saline solution (PBS; phosphate 0.1M, NaCl 0.33M, pH 7.2). Serum samples were analyzed for antibodies against *Sarcocystis* spp. in serial dilutions starting at 1/10, dilution in PBS (Tenter 1988). A commercial goat anti-mouse IgG fluorescein isothiocyanate conjugate (Sigma Bio Sciences, St Louis, USA) was used as secondary antibody. Mouse positive and negative sera controls were included on each slide to each parasite. After incubations, slides were observed at 400x magnification under fluorescent microscope (Leica CTR 4000/EBQ 100, Leica Microsystems, Germany) and complete fluorescence of tachyzoites and bradyzoiytes was considered positive. IFAT were used as a gold standard serologic test, since it presents good sensitivity and specificity to diagnose *Sarcocystis* spp., *N. caninum* and *T. gondii* infections (Moré et al. 2008).

Total protein extraction. Total protein extraction was performed using the Radio-Immunoprecipitation Assay Buffer (RIPA Buffer - Sigma Bio Sciences, St Louis, USA) buffer following the manufacturer's recommendations. RIPA Buffer were added (300µL) were added to the pellets containing *N. caninum*, *T. gondii* tachyzoites, *Sarcocystis* spp. bradyzoites, VERO cells and bovine myocardium. The lysate was centrifuged at 8.000 x g for 10 minutes at 4°C to sediment the cellular debris. The supernatant containing the protein solution was carefully transferred to a microtube where 3µl of protease inhibitor cocktail (Sigma Bio Sciences, St Louis, USA) was added and stored at -20°C until the time of use.

Dot-blot. Nitrocellulose membrane with 0.45µm porosity was used and approximately 12µg/mL of each parasites purified antigenic protein was distributed. After drying for 20 minutes at room temperature, membranes were washed three times for five minutes under shaking with PBS-T wash solution (0.05% Tween 20 and PBS) and then blocked with blocking solution (PBS-T with 5% non-fat dry milk) for 16 hours at 4°C. Mice serum samples and negative control serum, were diluted (1/50) in PBS-T and then distributed on the membrane and incubated at 37°C for one hour under slight shaking as previously described (Tenter 1988, Pinheiro et al. 2005). After

incubation, the membrane was washed three times for five minutes and then incubated with horseradish peroxidase (HRP)-conjugated produced in goat anti-mouse immunoglobulin G (1/2000, Sigma Bio Sciences, St Louis, USA) under shaking for 1 hour at 37°C. The enzymatic reaction was revealed with a developing solution (9mL of 50mM Tris-HCl, 1mL of 0.3% Nickel Sulphate, 30µL of Hydrogen Peroxide, 0.006g of DAB) (Sigma Bio Sciences, St Louis, USA) for 20 minutes at room temperature (Pinheiro et al. 2005).

RESULTS AND DISCUSSION

Neospora caninum or *Toxoplasma gondii* experimentally infected mice tested by IFAT for the 3 protozoans (*N. caninum*, *T. gondii* and *Sarcocystis* spp.) reacted only to *N. caninum* or *T. gondii* antibodies, respectively. Specific antibodies against *Sarcocystis* spp. were found in two animals experimentally infected only by this protozoan (one animal showed titer of 50 and other animal presented titer of 800 at IFAT). As previously reported, IFAT is considered the gold standard technique for Sarcocystosis serologic diagnostic in cattle and a positive IFAT result reflects *Sarcocystis* spp. infection (Garcia et al. 2008, Moré et al. 2008). Therefore, in the present study was established that IFAT would be the standard technique to be compared with Dot-blot test. Moré et al. (2008) have described IFAT at 1:25 dilution to be a suitable method for diagnosis of Sarcocystosis in cattle.

Serum samples from mice experimentally infected with *Sarcocystis* spp. and tested by Dot-blot demonstrated the presence of antibodies against this protozoan (at 1:50 dilution) but no cross-reactions were observed in the same sample and same dilution against *N. caninum* or *T. gondii*. Only antibodies against *N. caninum* and no serological cross-reactions with *T. gondii* and *Sarcocystis* spp. were detected in serum samples from mice inoculated with *N. caninum*. As well as, no cross-reactions against *N. caninum* and *Sarcocystis* spp. was observed in serum samples from mice infected by *T. gondii*, showing only specific antibodies against *T. gondii*. These results showed the Dot-blot sensitivity for coccidian immunological diagnostic in mice and suggest this method as a potential serological test for bovine *Sarcocystis* spp. detection.

Although there are no studies that indicate the best experimental model for infection with *Sarcocystis* species that infect cattle, in this study was possible to produce antibodies against *Sarcocystis* spp. in *Mus musculus* mice as demonstrated by the two serological diagnostic tests performed. Animals inoculated with *Sarcocystis* spp. revealed antibodies against this protozoan at Dot-blot technique and showed a high titer of antibodies at IFAT (1:800). Dot-blot proves to be a sensitive test for IgG antibodies against *Sarcocystis* spp. in this study and similar result was observed by Tenter (1988) in mice infected with *Sarcocystis* and in agreement with results described by Ndiritu et al. (1996) in experimentally infected bovine. Tenter (1988) compared Dot-blot with the conventional diagnostic methods ELISA and IFAT for serological detection of *Sarcocystis muris* in immunized and experimentally infected rodents and demonstrated that Dot-blot was sensitive for detection of IgG in immunized mice showing sensitivity equivalent to ELISA and IFAT using serum from infected animals.

The results of the present study suggest that Dot-blot using *Sarcocystis* spp. antigens present good sensitivity and specificity compared to IFAT and this method should be evaluated using bovine serum samples from naturally infected animals as a possible screening test to detect antibodies against *Sarcocystis* spp. that could be applied into the field and consequently Dot-blot may be used as an alternative to IFAT, the gold standard technique to bovine Sarcocystosis.

IFAT is a technique that requires expensive equipment as fluorescence microscope is laborious, and difficult to interpret requiring trained technicians (Pappas 1988, Saville et al. 2004). Therefore, diverse serological diagnostic methods have been developed, to make the diagnosis faster and more precise (Moré et al. 2008, Dubey et al. 2015). In addition, the clinical signs of bovine Sarcocystosis when present are non-specific and not always causing visible macroscopic lesions during meat inspection, therefore these serological methods help in infection diagnosis in order to establish disease control measures (Blagojevick & Antic 2014). Serological tests also allow the diagnosis of *N. caninum* and *T. gondii,* in individual or mixed infections (Dubey et al. 1996, Moré et al. 2008). Therefore, Dot-blot may be considered as an alternative specific and sensitive method which makes possible to evaluate a large number of samples into the field in order to facilitate the diagnosis of *Sarcocystis* infection in cattle herds (Holmdahl et al. 1993, Ndiritu et al. 1996, Guclu et al. 2004). Further studies should be employed allowing the standardization of this method for diagnostic of *Sarcocystis* spp. in bovine serum samples from naturally infected animals.

CONCLUSION

Dot-blot showed same specificity and sensibility as IFAT for immunological diagnostic of *Sarcocystis* spp. in experimentally infected mice and this immunoblot test did not demonstrate serological cross-reactions with *Neospora caninum* and *Toxoplasma gondii*.

Compliance with ethical standards.- All experimental practices involving animals were approved by the Ethics Committee for Animal Experimentation at "Universidade Federal de Santa Maria" (UFSM) (Protocol number 87352511).

Conflict of interest statement.- The authors declare that they have no conflict of interest.

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