Evaluation of a MPB70-ELISA to differentiate *Mycobacterium bovis*- from *M. avium*-sensitized swine[1]

Carla D. Marassi[2], Flávia C.S. de Oliveira[3], Sonia R. Pinheiro[3], Sergio S. Azevedo[3], Francisco R.M. Soto[3], Walter Oelemann[4], Walter Lilenbaum[2]* and Silvio A. Vasconcellos[3]

ABSTRACT.- Marassi C.D., Oliveira F.C.S., Pinheiro S.R., Azevedo S.S., Soto F.R.M., Oelemann W., Lilenbaum W. & Vasconcellos S.A. 2014. **Evaluation of a MPB70-ELISA to differentiate *Mycobacterium bovis*- from *M. avium*-sensitized swine.** *Pesquisa Veterinária Brasileira 34(11):1069-1072.* Departamento de Microbiologia e Parasitologia, Universidade Federal Fluminense, Rua Professor Hernani Mello 101, Lab 309, Niterói, RJ 24210-130, Brazil. E-mail: mipwalt@vm.uff.br

Swine are susceptible to different mycobacteria species, being *Mycobacterium bovis* an agent of tuberculosis, with most significant zoonotic risks, while *M. avium* determines a granulomatous lymphadenitis with low zoonotic risk. Currently performed intradermal tests present some important limitations, such as the lack of ability to detect anergic animals or to differentiate among mycobacterial species. In order to improve the TB diagnosis, serological assays have been developed, with encouraging results. The purpose of this study was to evaluate the performance of a MPB70-ELISA in 82 piglets divided into four groups: sensitized by inactivated *M. bovis*, *M. avium*, inoculated with oil adjuvant, or with saline solution. The test was able to discriminate between an animal sensitized by *M. bovis* and animals of the three other groups, including *M. avium*-sensitized animals; for this reason, we suggest that MPB70-ELISA could be used as a complementary tool for discriminating the agent of the mycobacteriosis, and therefore to diagnose tuberculosis in a swine herd.

INDEX TERMS*:* Diagnosis, ELISA MPB70, swine, *Mycobacterium bovis*, *Mycobacterium avium.*

RESUMO.- [**Avaliação de um MPB70-ELISA para diferenciar entre suínos sensibilizados com *Mycobacterium bovis* ou *M. avium*.**] Suínos são suscetíveis a diferentes espécies de micobactérias, sendo *Mycobacterium bovis* agente de tuberculose (TB), com claro risco zoonótico, enquanto *M. avium* determina uma linfadenite granulomatosa (LG) de baixo risco zoonótico. Os testes intradérmicos atualmente realizados apresentam algumas limitações importantes, como a falta de habilidade em detectar animais anérgicos ou de diferenciar entre as espécies micobacterianas. Com o intuito de melhorar o diagnóstico de TB, testes sorológicos têm sido desenvolvidos, com resultados encorajadores. O objetivo do presente estudo foi avaliar um MPB70-ELISA em 82 leitões divididos em quatro grupos: sensibilizados por *M. bovis*, por *M. avium,* inoculados com óleo adjuvante ou com solução salina. O teste foi capaz de discriminar entre os animais sensibilizados com *M. bovis* dos demais três grupos, incluindo aqueles que foram sensibilizados com *M. avium;* desta forma, sugere-se que o MPB70-ELISA poderia ser utilizado como ferramenta complementar para discriminar o agente da micobacteriose, e portanto diagnosticar TB em um plantel de suínos.

TERMOS DE INDEXAÇÃO*:* Diagnóstico, ELISA MPB70, suínos, *Mycobacterium bovis*, *Mycobacterium avium.*

INTRODUCTION

Swine can be infected by several mycobacteria, causing variable degrees of disease. Among them, there are some agents with clear and significant zoonotic risk, such as

[1] Received on July 12, 2014.
Accepted for publication on October 2, 2014.

[2] Laboratório de Bacteriologia Veterinária, Departamento de Microbiologia e Parasitologia, Instituto Biomédico, Universidade Federal Fluminense (UFF), Lab 309, Rua Prof. Hernani Mello 101, Niterói, RJ 24210-130, Brazil. *Corresponding author: mipwalt@vm.uff.br

[3] Laboratório de Zoonoses Bacterianas, - Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária, Universidade de São Paulo (USP), Av. Prof. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil.

[4] Departamento de Imunologia, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro (UFRJ), Cidade Universitária, Rio de Janeiro, RJ 21941-902, Brazil.

mycobacteria from the tuberculosis complex, as *Mycobacterium bovis*, agent of bovine tuberculosis (TB) (Wisselink et al. 2010). Conversely, other mycobacteria, such as members of the *Mycobacterium avium* complex (MAC), lead to a granulomatous lymphadenitis (GL)in head and mesenteric lymph nodes that, despite its economic importance due to the condemnation of carcasses (Faldyna et al. 2012), present a lower public health risk, often restricted to immunocompromised people.

Diagnosis of swine TB relies on intradermal tests, but there is a lack of definite patterns of interpretation, either for the single intradermal test (SIT) that uses *M. bovis* purified protein derivative (bovPPD) or for the comparative intradermal test (CIT), that uses bovPPD and also *M. avium* PPD (avPPD). Although theoretically those tests should be able to distinguish TB (*M. bovis*) from GL (*M. avium)*, those agents share a number of antigens that makes that differentiation difficult and often not reliable by current skin tests. Besides that, intradermal tests are based on the detection of the cell-mediated immune response (CMI) triggered by the mycobacterial infection. However, as the infection progresses, antibodies became more easily detectable (Walravens et al. 2002), and, depending on the employed capture antigen (as MPB70), specific antibodies can be detected even at the early stages of the disease (Wiker 2011). MPB70 is one of the most studied antigens used in ELISAs for TB diagnosis in ruminants (Marassi et al. 2011). It has been employed as a complementary tool, mainly providing the detection of anergic animals that fail to be detected by intradermal tests (Marassi et al. 2009, Waters et al. 2011, Zarden et al. 2013).

Few studies addressed the diagnosis of mycobacterial infections in swine. Although serological assays have been developed with promising results (Boadella et al. 2011, Wisselink et al. 2010), as well as an Interferon-gamma release assay (Faldyna et al. 2012), they are mainly focused on the diagnosis of lymphadenitis, which is determined by MAC. To our knowledge, there are no studies addressing the diagnosis of *M. bovis* infections in swine and/or designed to discriminate between infections determined by *M. bovis* or MAC.

Therefore, the purpose of this study was to evaluate the performance of a MPB70-ELISA in swine experimentally sensitized by *M. bovis*-or *M. avium*-oil suspensions and its discriminatory capability.

MATERIALS AND METHODS

Animals. This research was protocoled as number 1696/2009, certified by the Bioethical Commission of the School of Veterinary Medicine and Animal Science of University of São Paulo. A commercial herd certified as free of tuberculosis located in São Paulo, Brazil, for at least five years was studied. Until 21 days-old, animals were kept in individual boxes with controlled temperature and milking. When piglets were 30 days-old (Day 0), after a negative CIT, 82 animals were randomly chosen to be studied. All of them presented physiological parameters of normality.

Experimental groups. After a 60-day interval period, the 82 animals (90 days old/Day 60) were divided into four groups for sensitization. Subsequently, 21 piglets were intramuscularly inoculated (IM) with an oil suspension of inactivated *Mycobacterium avium* D4 strain (Group A); 21 piglets were inoculated with an oil suspension of inactivated *Mycobacterium bovis* AN5 strain (Group B); 20 piglets were inoculated with oil adjuvant (Group C), and 20 piglets were inoculated with saline solution (Group D). After a 30-day interval (Day 90), all animals were tested by CIT. All the inoculations and tests were performed according to Brazilian official recommendations (Brasil 2011).

Sera samples. Blood samples for ELISA were collected from all 82 animals in three different moments: at the first CIT conducted before sensitization (Day 0); at the moment of sensitization (Day 60) and 30 days after sensitization (Day 90). Samples were always collected a few moments before injection of antigens for CIT.

MPB70-ELISA. ELISAs were conducted according to the protocol previously validated for bovines (Marassi et al. 2011). Briefly, MPB70 was diluted to a concentration of 0.5µg/mL in a 0.05M carbonate buffer, (pH 9.8) and used to coat a 96 well plate (Nunc Maxisorb – Sigma-Aldrich) overnight at 8°C. After three washes, the plate was blocked with a solution composed of 5% skim milk diluted in PBST buffer (0.05% Tween20, NaCl 0.2M, pH 7.2) and incubated for 2hs/37ºC. the plate was washed three times and swine sera were diluted 1/100 in PBST buffer with 1% of skim milk and distributed in duplicate. Sera dilutions were incubated at 37°C under continuous agitation for 60 minutes. After five washes, a secondary antibody composed by a peroxidase conjugated anti-swine IgG (Sigma-Aldrich) was used at 1/40,000 and incubated for one hour at room temperature under continuous agitation. The reaction was revealed with TMB (Sigma-Aldrich) (1mg/mL) and stopped with 1N HCl (Merck) after 30 min. in the dark. Optical density (OD) readings were taken using a spectrophotometer (model 680 BioRad) at a wavelength of 450 nm.

Statistics. Comparison between tests and differences among samples were analyzed by *t*-Test and Friedman analysis performed by MedCalc® statistics program (version 12.4).

RESULTS

Serum samples taken at day 0 presented low ODs in the ELISA, confirming that all animals were negative. The average values of the four experimental groups were 0.05, with no difference among the four tested groups ($P>0.05$). Similar results were obtained for samples collected at the moment of the sensitization of the animals (Day 60).

Nevertheless, for samples taken at Day 90, important differences could be observed among the four groups. Average ODs values were 0.05 for group A, 0.12 for group B, and 0.02 for groups C and D. In Group A, 18/21 presented ODs values ≤0.10 while for Group B the contrary was observed and 11/21 swine presented ODs ≥0.10. Difference between those two groups was statistically significant ($P<0.05$), as well as between group B and the controls (Groups C and D), which were not different among themselves ($P>0.05$). Moreover, average ODs of groups A, C and D at Day 90 did not significantly differ from those of Day 0 or Day 60, while for Group B average OD at Day 90 was significantly different ($P<0.05$) of that on Day 0 and Day 60. Results are depicted in Figure 1.

DISCUSSION

As expected, since we have studied a TB-free herd, average OD values were low for all tested animals, with no signi-

ficant difference among the four groups, both at the first testing moment (Day 0) and at the sensitization moment (Day 60). Those data reduce the possibility of a bias and indicate that the alterations that were later observed were most probably due to the injection of the mycobacterial oil suspensions.

In contrast, at Day 90, i.e. 30 days after sensitization, a significant increase on average ODs was observed only for animals of Group B (inoculated with inactivated *Mycobacterium bovis*), and 11/21 of that group presented high ODs (≥0.10). Importantly, at that moment average ODs values were unaltered for the animals of Groups A, C and D (Fig.1).

When compared to studies conducted in ruminants naturally infected with *M. bovis* (Waters et al. 2011), all ODs were relatively low. Although unusual, our findings were not totally unexpected. ELISA was performed on non-infected animals from a free herd, which had most probably no previous contact with either virulent or environmental mycobacteria. Additionally, it is well-known that ELISAs performed with a single purified protein lead to lower ODs (Marassi et al. 2011) and animals were sensitized only once, which reduces the intensity of the reaction (Wisselink et al. 2010). Additionally, it has been reported that age can influence ELISA results in swine (Garcia-Bocanegra et al. 2012) and in the present study we have only tested young animals.

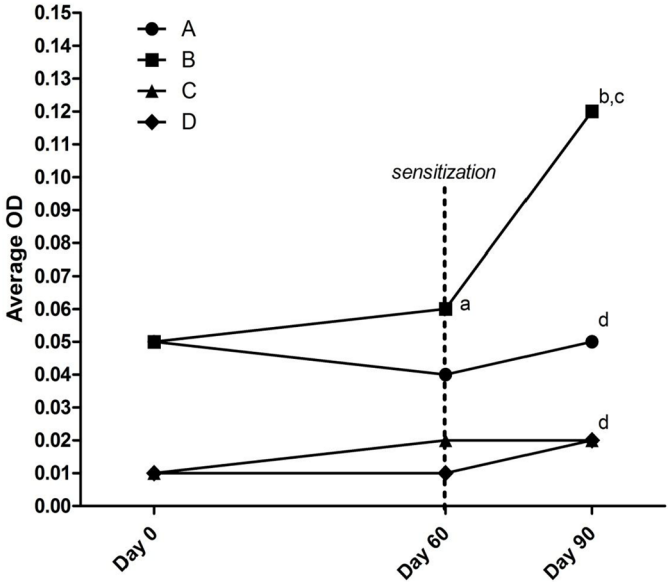

Fig.1. Optical Densities (ODs) average values of MPB70-ELISA tested in four groups of swine in different moments after experimental sensitization. Group A = swine sensitized by *Mycobacterium avium;* Group B = swine sensitized by *Mycobacterium bovis*; Group C = swine with an oil adjuvant inoculation; Group D = swine inoculated with saline. Day 0 = samples obtained at the moment of the first screening by intradermal tuberculinization test; Day 60 = samples obtained at the moment of the sensitization with one of the inoculums; Day 90 = samples obtained at the moment of the intradermal tuberculinization test; a-b = significant difference between Day 60 and Day 90 for Group B; c-d = significant difference between Group B and Groups A, C and D at Day 90.

Importantly, our results could clearly demonstrate the capability of MPB70-ELISA for detecting *M. bovis*-sensitized animals with no cross-reaction with *Mycobacterium avium*--sensitized animals. This can be useful for epidemiological understanding and, consequently, for control programs. Although humoral response in swine is not often investigated, recently a rapid serological test using bovine PPD and a dual path platform for bovine TB diagnosis was reported for *M. bovis*-infected wild boars (Boadella et al. 2011), with encouraging results. This study demonstrated that swine also present a detectable humoral response after mycobacterial infection. Therefore, ELISA can be employed for that species, with similar advantages that have been observed in cattle and other species, such as the possibility of testing samples as a batch, possibility of retesting inconclusive results and no need of a second visit to the herd (Marassi et al. 2011).

Swine may be infected by either *M. bovis* or *M. avium*, determining different diseases (TB or GL, respectively) with different zoonotic and Public Health impacts. Thus, cross-reactions may confuse the serological diagnosis and consequently reduce the reliability of serological tests in that species when non-specific antigens are employed. MPB70 is a secreted protein known as species-specific for *M. bovis* (Wiker et al. 2011), and ELISAs employing that protein as antigens have been used with encouraging results for detecting TB in different species, as bovines (Waters et al. 2011), goats (Marassi et al. 2009) and buffalos (Zarden et al. 2013). Therefore, the discrimination between those two agents is mandatory, and we are not aware of any other study that has addressed that issue before.

CONCLUSION

The present study demonstrated that MPB70-ELISA not only detected sensitized animals but could specifically differentiate between the *Mycobacterium bovis*- and *M. avium*-sensitized animals.

We propose that MPB70-ELISA could be used as a complementary tool for identifying the agent of the mycobacteriosis in a swine herd.

REFERENCES

Boadella M., Lyashchenko K., Greenwald R., Esfandiari J., Jaroso R., Carta T., Garrido J.M., Vicente J., de la Fuente J. & Gortázar C. 2011. Serologic tests for detecting antibodies against *Mycobacterium bovis* and *Mycobacterium avium* subsp. *paratuberculosis* in Eurasian wild boar (*Sus scrofa scrofa*). J. Vet. Diagn. Invest. 23:77-83.

Brasil 2001. Regulamento Técnico do Programa Nacional de Combate e Erradicação da Brucelose e Tuberculose. Ministério da Agricultura, Pecuária e Abastecimento, Brasília, DF. Disponível em <HTTP://www.agricultura.gov.br/das/dda/in10.htm> Accessed on July 6, 2014.

Faldyna M., Göpfert E., Kudlackova H., Stepanova H., Kaevska M., Slana I. & Pavlik I. 2012. Usability of a gamma interferon release assay in the diagnosis of naturally infected pigs with *Mycobacterium avium* subspecies *hominissuis.* J. Vet. Diagn. Invest. 24:376-378.

García-Bocanegra I., Pérez de Val B., Arenas-Montes A., Paniagua J., Boadella M., Gortázar C. & Arenas A. 2012. Seroprevalence and risk factors associated to *Mycobacterium bovis* in wild artiodactyl species from southern Spain. 2006-2010. Plos One 7:e34908.

Marassi C., Almeida C., Pinheiro S., Vasconcellos S. & Lilenbaum W. 2009. The use of MPB70-ELISA for the diagnosis of caprine tuberculosis in Brazil. Vet. Res. Commun. 33:937-943.

Marassi C.D., Medeiros L., McNair J. & Lilenbaum W. 2011. Use of recombinant proteins MPB70 or MPB83 as capture antigens in ELISAs to confirm bovine tuberculosis infections in Brazil. Acta Trop. 118:101-104.

Walravens K., Marché S., Rosseels V., Wellemans V., Boelaert F., Huygen K. & Godfroid J. 2002. IFN-gamma diagnostic tests in the context of bovine mycobacterial infections in Bellgium. Vet. Immunopathol. 87:410-406.

Waters W.R., Buddle B.M., Vordermeier H.M., Gormley E., Palmer M.V., Thacker T.C., Bannantine J.P., Stabel J.R., Linscott R., Martel E., Milian F., Foshaug W. & Lawrence J.C. 2011. Development and evaluation of an enzyme-linked immunosorbent assay for use in the detection of bovine tuberculosis in cattle. Clin. Vac. Immunol. 18:1882-1888.

Wiker H.G. 2011. MPB70 and MPB83: major antigens of *Mycobacterium bovis*. Scand. J. Immunol. 69:492-499.

Wisselink H.J., Van Solt-Smits C.B., Oorburg D., Van Soolingen D., Overduin P., Maneschijn-Bonsing J., Stockhofe-Zurwieden N., Buys-Bergen H., Engel B., Urlings B.A. & Thole J.E. 2010. Serodiagnosis of *Mycobacterium avium* infections in pigs. Vet. Microbiol. 142:401-407.

Zarden C.F.O., Marassi C.D., Oeleman W. & Lilienbaum W. 2013. A complementary diagnosis of naturally occurring tuberculosis in water buffaloes (*Bubalus bubalis*) in Rio de Janeiro using a MPB70-ELISA. Brazil. Trop. Anim. Health Prod. 45:1203-1206.