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Evaluation of the indirect fluorescent antibody test and modified agglutination test for detection of antibodies against *Toxoplasma gondii* in experimentally infected pigs

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ABSTRACT

The study determined the sensitivity and specificity of the indirect fluorescent antibody test (IFAT) and modified agglutination test (MAT) for anti-*Toxoplasma gondii* antibody detection by analyzing sera from 46 experimentally infected pigs. Values for sensitivity were 95.7% (confidence interval 95%: 84.0-99.2%) and for specificity 97.8% confidence interval 95%: 87.0-99.9%) in both tests. There was an optimum agreement of results between IFAT and MAT evidenced by a Kappa test of 0.86. These results validate these tests for the detection of *T. gondii* infection in pigs. IFAT and MAT despite methodologies with different characteristics and readings have similar accuracy in pig serum samples.

Index terms: *Toxoplasma gondii*, swine, antibodies, serology, indirect fluorescent antibody test (IFAT), modified agglutination test (MAT).

INTRODUCTION

Toxoplasmosis is a zoonotic disease of cosmopolitan distribution caused by *Toxoplasma gondii* (Nicolle & Manceaux 1909), an intestinal coccidian whose definitive hosts are felines, especially the domestic cat (Navarro et al. 1992). The social and economic impact of toxoplasmosis on public health is demonstrated by the loss of the quality of life caused by blindness, neurological disorders and death by abortion or encephalitis (Roberts & Mcleod 1999, Goméz-Mariz et al. 2000).

The world-wide prevalence of antibodies against *T. gondii* in swine varies from 1 to 90% (Souza 1995). In the USA, Gamble et al. (1999) estimated that pig toxoplasmosis varies between 3 and 20%, depending on the region studied and animal age. In North Paraná herds, Brazil, Garcia et al. (1999) detected 24% of seropositive pigs. Many of the variations in the antibody rates against *T. gondii* result from management conditions and type of breeding, as well as from the different serological tests used (Vidotto 1992).

According to Dubey (1986) swine is a major source of toxoplasmosis infection for humans in the USA. The detection of *T. gondii* cysts in swine is very difficult, as tissue cysts vary in size from 60 to 100 mm (Kreier 1993) and are unpredictable in localization. Additionally, bioassay requires inoculation of the digested or crushed tissue, and involves much time to obtain results. Serological tests are quicker and more practical, associating the detection of antibodies against *T. gondii* with the presence of tissue cysts (Hirvelã-Koski 1990). Therefore, serology is proper to determine the prevalence of the agent in swine herds (Aranda et al. 2000).

Indirect fluorescent antibody test (IFAT) and modified agglutination test (MAT) are two tests of proven accuracy to detect anti-*T. gondii* antibodies in animal and human sera. The present study aimed to determine the specificity, sensitivity and Kappa agreement for IFAT and MAT for detection of IgG against *T. gondii* in sera of experimentally infected pigs. Additionally, the complexity of the techniques tested was assessed, considering the antigen cost, time spent to perform the tests and difficulties in reading the results.

MATERIALS AND METHODS

Origin of sera

Negative sera, by IFAT, from 46 mixed breed (Large White x Landrace) male pigs and boars, from commercial farms with intensive sanitary control and without clinical signs or history of reproductive problems in the herds, previously utilized in two experiments (Garcia 2002, Freire et al. 2003), were used. The pigs were kept in quarantine for adaptation to the experimental facilities, and blood collection was repeated immediately before carrying out the experimental inoculation with *Toxoplasma gondii.* Sera obtained from these blood samples (pre-inoculation) were kept at -18°C and used in this study as negative samples. After that, twenty-eight pigs were orally infected with 5.0 x 10^4 oocysts of *T. gondii* (10 animals received the VEG^6 strain and 18 animals the AS-28 strain). The 18 remaining pigs were inoculated intramuscularly with 1.0 x10^7 tachyzoites (RH strain). Blood samples were collected again 22 days after infection with *T. gondii* and the sera obtained were used as positive samples.

Serological examination

IFAT. The test was carried out as described by Camargo (1973). The antigen was prepared with inactivated *T. gondii* RH strain tachyzoites in formaldehyde solution at 1% for 30 minutes at 37°C. After washing in physiological saline solution and centrifuging, the tachyzoites were diluted and fixed on glass. Sera samples of 100 ml were diluted in phosphate buffered saline (PBS 0.015M, pH 7.2) and tested in two-fold dilutions, from 1:16 until the greatest dilution considered positive (1:1024). Ten microliters of diluted sera were pipetted in each delimited circle on the slides previously adsorbed with *T. gondii* antigen. The slides were incubated at 37°C for 35 minutes in a wet chamber. After three washes with PBS the slides were dried and incubated with 10 ml of a rabbit anti-pig IgG fluorescein conjugate, diluted 1:4000 in PBS, during 35 minutes at 37°C. At the end of incubation, the slides were washed again to remove the remaining free conjugate, then covered with buffered glycerin (pH 8.0) and slide cover and read in an epifluorescent microscope. The reagent sera with reciprocal of the title ^{3}64 were considered positive (Vidotto et al. 1990).

MAT. The test was performed as described by Dubey & Desmonts (1987). *T. gondii* RH strain tachyzoites fixed in formaldehyde solution antigen was used. Briefly, sera were tested on 96-well plates with a u-shaped bottom (25 ml of *mix* antigen plus 25 ml of serum), based on two-fold serial dilutions, from 1:25 until the greatest dilution considered positive (1:200). The plates were sealed with plastic film and kept overnight in a chamber at 37°C. An agglutination reaction (mesh or net) identified the positive sera and a well-defined blue point identified the negative sera. Serum with titer ^{3}25 was considered positive (Dubey 1997). The MAT was kindly supplied by Dr. J.P. Dubey of the United States Department of Agriculture, Beltsville, Maryland, USA.

Serological test record card

Each test was accompanied by a record card, where the time spent analyzing the 46 samples, complexity of the technique, and the difficulty in reading the results and expenses with the products used were recorded, taking into consideration that the antigen used in MAT is not produced in Brazil. The purchase cost of the epifluorescence microscope used in the IFAT reading was not considered.

Statistical analysis

The results were tabulated and the sensitivity, specificity (Gart & Buck, 1966) and Kappa agreement (Landis & Koch, 1977) was calculated. The Epi Info 6.04 program was used for the statistical analyses (Dean et al. 1992).

RESULTS AND DISCUSSION

As seen in Table 1, there were found identical values with the same number of positive and negative animals by both tests, with similar values of sensitivity and specificity. The values of 95.7% for sensitivity and 97.8% for specificity in both tests showed excellent agreement. Of the 46 positive sera, two were considere negative by IFAT (false negative, Swine 8 and 45) and two by MAT (Swine 11 and 20). Among the negative sera one was identified as positive by IFAT (false positive, Swine 17) and one by the MAT (Swine 31). Using a confidence interval of 95%, sensitivity was 84.0-99.2% and specificity was 87.0-99.9%.

Table1. Reactivity showed by the pig sera: experimentally inoculated (infected) and pre-inoculated (not infected) by indirect fluorescent antibody test (IFAT) and modified agglutination test (MAT)

Tests		Infected pigs	Not infected	Total
IFAT	+	44	01	45
	-	02	45	47
	Total	46	46	92
MAT	+	44	01	45
	-	02	45	47
	Total	46	46	92

Sensitivity = positives / total infected x 100 = 95.7 % (IC 95%: 84.0-99.2).
Specificity = negatives / total not infected x 100 = 97.8 % (IC 95%: 87.0-99.9).
Predictive positive value = positives / total positives x 100 = 97.8 % (IC 95%: 87.0-99.9).
Predictive negative value = negatives / total negatives x 100 =95.7 % (IC 95%: 84.0-99.2).
Accuracy = 96.7% .
Kappa = 0.93.

The concordance of the results showed by Kappa = 0.86 (Table 2) is considered optimum according to Landis & Kock (1977). Both tests were able to detect IgG antibodies against *T. gondii* in pigs during this period of infection.

Table2. Agreement between indirect fluorescent antibody test (IFAT) and modified agglutination test (MAT) analyzing pig sera: experimentally inoculated (infected) and pre-inoculated (not infected)

	IFAT+	IFAT-	Total
MAT+	42	03	45
MAT-	03	44	47
Total	45	47	92

Sensitivity = positives / total infected x 100 = 93.3% (IC 95%: 80.7 - 98.3).
Specificity = negatives / total not infected x 100 = 93.6% (IC 95%: 81.4 – 98.3).
Predictive positive value = positives / total positives x 100 = 93.3% (IC 95%: 80.7 - 98.3).
Predictive negative value = negatives / total negatives x 100 = 93.6% (IC 95%: 81.4 – 98.3).
Accuracy = 93.4 %.
Kappa = 0.86.

The excellent agreement of these tests observed in this study is enforced by the agreement of them with the Die Test (DT), considered by Dubey (1997) as one of the most specific serological tests for detection of anti-*T. gondii* antibodies in swine. Camargo (1964) compared the IFAT and DT and reported total concordance for positivity or negativity by both tests. Similarly, MAT was as suitable as DT for IgG detection in human beings (Desmonts & Remington 1980).

Sera of animals experimentally infected with *Neospora caninum*, including pigs, did not react with *T. gondii* when analyzed by MAT at 1:15 dilution (Dubey et al. 1996). Dubey (1997) confirmed the specificity of MAT for *T. gondii* antibody detection in pig serum and did not find any cross-reaction with *Sarcocystis miescheriana*, virus, helminths, and gnotobiotic pigs and fetal sera from laboratory-bred sows.

The cost of purchasing the antigen and supplies used in each serological test was calculated at US$ 2.64 for IFAT and US$ 6.4 for MAT. The time taken to carry out the 46 tests was 3.5h for IFAT and 3.0h for MAT. However, it should be considered that with MAT could be tested a greater number of sera at the time. An advantage is that IFAT can be read shortly after the end of performing the technique (Vidotto et al. 1990). The interpretation of IFAT is more subjective, anddepends on the number of tachyzoites with total peripheral fluorescence per field and on the number of fields observed.

Although it has a longer incubation time (overnight) the reading of the MAT results is simple, because a microscope is not needed, making it more practical. MAT reading allows a greater number of sera to be analyzed in a certain period of time compared to IFAT. The major limitation of MAT observed in this study was the purchase of the

antigen, because it is still not produced in Brazil and imported from Canada. The antigen used in IFAT can be purchased from national suppliers or can easily be produced in cell culture or with the continual maintenance of live *T. gondii* in mouse.

Although the IFAT antigen is cheaper compared to that used in MAT, the need to use an anti-IgG conjugate for each animal species, necessary for IFAT, constitutes a considerable disadvantage for this technique. Differently, MAT can be used to analyze various animal species, as it does not require the presence of a specific conjugate (Oksanen et al. 1998). Waltman et al. (1984) compared ELISA with other tests for serological diagnostic in swine and reported some advantages of ELISA over IFAT, such as a greater number of sera, which can be analyzed at the same time, and the automated technique; but IFAT results were more in agreement with DT, used as standard test in the experiment. ELISA showed 72.9% sensitivity and 85.9% specificity, but less accuracy when compared with MAT (Dubey et al. 1995).

In conclusion, the excellent accuracy and agreement between the IFAT and MAT results obtained in the present study validate these tests for anti-T. gondii antibody detection in swine sera.

REFERENCES

Aranda F.S., Galisteo Jr A.J., Hiramoto R.M., Cardoso R.P., Meireles L.R., Miguel O. & Andrade Jr H.F. 2000. The prevalence and avidity of *Toxoplasma gondii* IgG antibodies in pigs from Brazil and Peru. Vet. Parasitol. 91:23-32. [Links]

Camargo M.E. 1973. Introdução às técnicas de imunofluorescência. Revta Bras. Patol. Clinica 10:143-171. [Links]

Camargo M.E. 1964. Improved technique of indirect immunofluorescence for serological diagnosis of toxoplasmosis. Revta Inst. Med. Trop., São Paulo, 6:117-118. [Links]

Dean A.G., Dean I.A. & Burton A.H. 1992. EPI INFO version 6.04: a world processing, data base and statistic program for epidemiology on microcomputers. Center for Disease Control, Atlanta, Georgia, USA. 302p. Disponível em http//:www.cdc.gov [Links]

Desmonts G. & Remington J.S. 1980. Direct agglutination test for diagnosis of Toxoplasma infection: method for increasing sensitivity and specificity. J. Clin. Microbiol. 11:562-568. [Links]

Dubey J. P. 1986. Toxoplasmosis. J. Am. Vet. Assoc.189:166-170. [Links]

Dubey J.P. & Desmonts G. 1987. Serological responses of equids fed with *Toxoplasma gondii* oocysts. Equine Vet. J. 48:1239-1243. [Links]

Dubey J.P., Thulliez P., Weigel R.M., Andrews C.D., Lind P. & Powell E.C. 1995. Sensitivity and specificity of various serologic tests for detection of *Toxoplasma gondii* infection in naturally infected sows. Am. J. Vet Res. 56:1030-1036. [Links]

Dubey J.P., Lindsay D.S., Adams D.S., Gay J.M., Baszler T.V., Blagburn B.L. & Thulliez P. 1996. Serologic responses of cattle and others animals infected with *Neospora caninum.* Am. J. Vet. Res. 57:329-336. [Links]

Dubey J.P., Andrews C.D., Thulliez P., Lind P. & Kwok O.C.H. 1997. Long-term humoral antibody responses by various serologic tests in pigs orally inoculated with oocysts of four strains of *Toxoplasma gondii*. Vet. Parasitol. 68:41-50. [Links]

Dubey J.P. 1997. Validation of the specificity of the modified agglutination test for toxoplasmosis in pigs. Vet. Parasitol. 71:307-310. [Links]

Freire R.L., Navarro I.T., Bracarense A.P.F.R.L. & Gennari, S.M. 2003. Vaccination of pigs with Toxoplasma gondii antigens incorporated in_immunostimulating complexes (iscoms). Arq. Bras. Med. Vet. Zoot. 55: 388-396. [Links]

Gamble H.R., Brady R.C. & Dubey J.P. 1999. Prevalence of *Toxoplasma gondii* infection in domestic pigs in the New England states. Vet. Parasitol. 82:129-136. [Links]

Garcia J.L., Navarro I.T., Ogawa L. & Oliveira R.C. 1999. Soroprevalência do *T. gondii*, em suínos, bovinos, ovinos e eqüinos e sua correlação com humanos, felinos e caninos, oriundos de propriedades rurais do norte do Paraná-Brasil. Ciência Rural, Santa Maria, 29:91-97. [Links]

Garcia J.L. 2003. Avaliação da Resposta Imune em suínos, com vacina de fração antigênica (roptrias e grânulos densos) de *Toxoplasma gondii* incorporados a complexos imunoestimulantes (iscoms). Tese (Doutorado), VPS, FMVZ-USP, São Paulo. [Links]

Gart J.J.& Buck A.A. 1966. Comparison of a screening test and a reference test in epidemiologic studies. Am. J. Epidemiol. 83:593-602. [Links]

Gomez-Marin J.E., Montoya-De-Londono M.T., Castano-Osorio J.C., Heine F.A, Duque A.M., Chemla C., Aubert D., Bonhomme A. & Pinon J.M. 2000. Frequency of specific anti-

Toxoplasma gondii IgM, IgA and IgE in Colombian patients with acute and chronic ocular Toxoplasmosis. Mem. Inst. Oswaldo Cruz 95:89-94. [Links]

Hirvelã-Koski V. 1990. Evaluation of ELISA for the detection of Toxoplasma antibodies in swine sera. Acta Vet. Scand. 31:413-422. [Links]

Kreier J. P. 1993. Parasitic Protozoa. 2nd Edition. Academic Press, San Diego, p.5-57. [Links]

Landis J.R. & Kock G.G. 1977. The measurement of observer agreement for categorical data. Biometrics 33:159-174. [Links]

Navarro I.T., Vidotto O., Giraldi N. & Freire, R. L. 1992. *Toxoplasma gondii*. Isolamento a partir de carne e cérebro de suínos comercializados na região de Londrina, PR. Semina 13:15-18. [Links]

Nicolle C. & Manceaux L. 1909. Sur un protozoaire nouveau du gondii. Compte Rendu H. Acad. Sci. 148:369-372. [Links]

Oksanen A., Tryland M., Johnsen K. & Dubey J.P. 1998. Serosurvey of *Toxoplasma gondii* in North atlantic marine mammals by the use of agglutination test employing whole taquizoites and dithiothreitol. Comp.Immunol. Microbiol.& Infect. Dis. 21:107-114. [Links]

Roberts F. & Mcleod R. 1999. Pathogenesis of toxoplasmic retinochoroiditis. Parasitol. Today 15:51-57. [Links]

Souza W.J.S. 1995. Epidemiologia da toxoplasmose. Avaliação sorológica em suínos e trabalhadores de abatedouros da mesma região do Grande Rio de Janeiro. Tese Doutorado), UFRRJ, Rio de Janeiro.102 p. [Links]

Waltman W.D., Dreesen D.W., Prickett M.D., Blue J.L. & Oliver D.G. 1984. Enzime-linked immunossorbent assay for the detection of toxoplasmosis in swine: Interpreting assay results and comparing with other serologic tests. Am. J. Vet. Res. 45:1719-1725. [Links]

Work K., Eriksen L., Fennestad K.L., Moller T. & Siim J.C. 1970. Experimental toxoplasmosis in pregnant sows. Acta. Path. Microbiol. Scand. 78:129-139. [Links]

Vidotto O., Costa A.J. & Balarin M.R.S. 1987. Toxoplasmose experimental em porcas gestantes. I. Observações clínicas e hematológicas. Arq. Bras. Med. Vet. Zoot. 39:623-639. [Links]

Vidotto O., Navarro I.T., Giraldi N., Freire R.L. & Mitsuka R. 1990. Estudos epidemiológicos da toxoplasmose em suínos da região de Londrina, PR. Semina 11:53-59. [Links]

Vidotto O. 1992. Toxoplasmose: epidemiologia e importância da doença na saúde animal. Semina 13:69-75. [Links]

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