EVALUATION OF ANTI-*Toxoplasma gondii* ANTIBODY DETECTION BY MODIFIED AGGLUTINATION TEST IN TISSUE OF EXPERIMENTALLY INFECTED MICE

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ABSTRACT

Toxoplasma gondii infection affects homoeothermic animals around the world, and many infections, in animals and man, can result in abortion, congenital disorders, ocular or neuromuscular disease, with serious impact in animal and public health. A variety of antibody detection methods have been proposed since initial description of Sabin-Feldman dye test, usually using blood sera as sample for detection. Tissues and fetal liquids can be used for antibody detection too, but few research compare the performance of this detection in pairwise sera, and the evaluation of autolysis effect was not been verified. This work shows the results of sensibility, specificity, and the efficiency of direct agglutination method for anti-*T. gondii* antibody detection in fresh or autolysed samples of liver, spleen, and muscle, obtained of chronically infected mice.

Keywords: *Toxoplasma*; modified agglutination method; tissues; ROC curves.

AVALIAÇÃO DA DETECÇÃO DE ANTICORPOS ANTI-*Toxoplasma Gondii* PELO MÉTODO DE AGLUTINAÇÃO DIRETA EM TECIDOS DE CAMUNDONGOS EXPERIMENTALMENTE INFECTADOS

RESUMO

A infecção pelo *Toxoplasma gondii* afeta animais homeotérmicos em todo o mundo, e muitas infecções, nos animais e no homem, podem resultar em abortamentos, alterações congênitas e doença ocular ou neuromuscular, com sérios impactos em saúde animal e saúde pública. Diversos testes de detecção de anticorpos têm sido propostos desde a descrição inicial do teste de Sabin-Feldman, usualmente utilizando o soro sanguíneo como amostra para a detecção. Tecidos e líquidos fetais também podem ser utilizados para a pesquisa de anticorpos, entretanto poucos trabalhos comparam o desempenho desta detecção em amostras pareadas com soro sanguíneo, e a avaliação do efeito da autólise não foi verificada. Este trabalho apresenta os resultados de sensibilidade, especificidade e a eficiência do método de aglutinação direta na detecção de anticorpos anti-*T. gondii* em amostras de fígado, baço e musculatura, recém coletadas, ou autolisadas, oriundas de camundongos cronicamente infectados.

Palavras-chave: *Toxoplasma*; método de aglutinação direta; tecidos; curvas ROC.

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EVALUACIÓN DE LA DETECCIÓN DE ANTICUERPOS ANTI-*Toxoplasma Gondii* POR EL MÉTODO DE AGLUTINACIÓN DIRECTA EN TEJIDOS DE RATONES INFECTADOS EXPERIMENTALMENTE

RESUMEN

Infección por *Toxoplasma gondii* afecta a los animales homeotérmicos en todo el mundo, y muchas infecciones en animales y humanos puede resultar en abortamientos, enfermedad congénita y neuromusculares o de los ojos, con graves impactos en la salud pública y sanidad animal. Pruebas de detección de anticuerpos se han propuesto desde la descripción inicial de la prueba de Sabin-Feldman, generalmente con el suero de la sangre como muestra para la detección. Líquidos y los tejidos fetales pueden utilizarse también para el anticuerpo de detección, sin embargo, pocos estudios comparan el performance de esta detección en muestras pareadas con suero de la sangre, y no se comprobó la evaluación de los efectos de autólisis. Este trabajo presenta los resultados de sensibilidad, especificidad y eficiencia del método de aglutinación directa en la detección de anticuerpos contra *T. gondii* en muestras de hígado, bazo y los músculos, recién recogidos, o autolisadas, de ratones crónicamente infectados.

Palabras-clave: *Toxoplasma*; aglutinación directa; tejidos; curvas ROC.

INTRODUCTION

Toxoplasmosis is a worldwide zoonosis, caused by intracellular parasite *Toxoplasma gondii*. The frequency in which infection with this parasite occurs in regions of the planet is variable and linked to factors such as cultural patterns of population, food habits, age, rural or urban origin, among others[1].

T. gondii can be isolated from tissues by inoculation of laboratory animals, mostly mice, and cells in tissue culture. However, the isolation can be difficult, depending on the conditions of the tissue and the amount of sample, in addition, especially when the use of animals, consume a varied time of 20 to 60 days for a final diagnosis, and even require successive inoculations of the material. Tissues can be examined for the presence of *T. gondii* by cytological and histopathological techniques, however, depending on the stage of infection, the parasite can become difficult, due to the irregular distribution of tissue cysts in the organs and even in a particular organ[2].

Given the limitations of the direct detection of the parasite, the most frequent alternative for the detection of infection is the detection of serum antibodies. The first antibody detection test was the Sabin-Feldman dye tetst[3], that was followed by other methods, such as immunofluorescent antibody test – IFAT, and modified agglutination test – MAT[2]. Classically these tests use blood serum as sample for the detection of antibodies. However in some situations, obtaining blood serum can be difficult, and the detection of antibodies and other serum markers may be unable[4]. Tissue samples from slaughterhouses, or from animals found dead, as well as aborted fetuses fluids can be an alternative for the presence of antibodies.

In Japan, Hagiwara, Katsube[5] detected significant correlation (r=0.82) in titles of antibody determined by reaction of Sabin-Feldman, with 190 pig serum samples examined paired with suspensions of musculature.

Arthur, Blewett[6] examined the fetal fluids of 171 aborted lambs for detection of antibodies against *T. gondii* by IFAT for IgG antibodies. The degree of deterioration of the samples evaluated did not influence the results for this method, obtaining 28 positive samples with dilutions higher than two. According to the authors, the detection of fetal specific

antibodies to *T. gondii* by IFAT provides a way of rapid and reliable diagnosis of infection with this parasite.

Thoracic fetal fluids of 738 aborted pigs in Argentina were examined for the detection of antibodies to *T. gondii* by IFAT and MAT. Of these, fifteen animals had antibodies to *T. gondii* in the first method, and ten were *T. gondii*-positive for the second[7].

The prevalence of *T. gondii* infection was investigated by analysis of 807 suspension of muscle suspension samples collected from ten pig slaughterhouses for Lundén et al.[8], which used for the enzyme-linked immunosorbent assay (ELISA) and found 42 (5.2) positive samples.

Despite being used as samples for the presence of antibodies, tissues and fetal fluids from infected animals have not been systematically evaluated for their performance in the detection of antibodies against *T. gondii* by MAT, comparing with standardized tests, such as the detection of antibodies in blood serum or tissue cysts in the tissues. This study aims to compare the detection of antibodies against *T. gondii* by MAT in samples of liver, spleen, and muscles of mice chronically infected, with the detection of antibodies in serum samples of the same animals as well as verify the influence of autolysis in the performance of the detection of antibodies in tissues.

MATERIAL AND METHODS

For the assessment of MAT in the detection of antibodies against *T. gondii* in tissues of chronically infected mice, two experiments were conducted. The first with tissues collected from animals after euthanasia, while the second with tissues collected from animals at 24, 48, 72 and 96 hours after euthanasia, to evaluate the sensitivity and specificity of the test in autolysed tissues.

We used mice infected and uninfected, kept at Preventive Veterinary Medicine and Public Health Laboratory at the Paranaense University (LMVP/UNIPAR).

All experimental protocols were analyzed and authorized by Institutional Committee on Ethics in Research Use of Animals before it`s research beginning (Protocol #14707/2009).

Experiment I

Animals

Were examined mice chronically infected with *T. gondii*, used for maintenance of two strains of the parasite, known as ME49 and UMU01. Blood was collected to obtain serum and the organs, for preparation of suspensions. As negative control group mice were obtained from Experimental Animal Facilities at UNIPAR/Campus Cruzeiro.

Experimental Groups

Mice chronically infected with the parasite were anesthetized in chamber saturated with isoflurane vapor, blood collected and then suffered euthanasia by anesthesia excess for collection of organs. Similarly, *T. gondii* negative animals have blood and tissue samples collected, constituting the negative control group.

Sample collection

Animals' blood was collected by the retro-orbital sinus puncture with glass capillary and transferred to plastic microtubes, and then identified by a protocol number. After collection, blood samples were centrifuged at 1650 g for 15 minutes to promote the serum separation, and aliquots of 0.5 ml of serum were stored at -20ºC until the examination for antibodies.

Mice suffered euthanasia by anesthesia excess. Animals were fixed in Styrofoam plate, for incision of the abdomen and abdominal cavity exposure to collection of liver and spleen, and dissected to collect muscles, especially of the thoracic and pelvic limbs. These samples were pounded into Ceramic mortar, added an equal volume of phosphate buffered saline solution (PBS), pH 7.2, for suspension of the tissue, homogenized and centrifuged at 1650 g for 15 minutes, and aliquots of 0.5 mL of the supernatant was stored in plastic microtubes properly identified and frozen at -20ºC until the examination for antibodies.

Antibody detection test

Detection of anti-*T. gondii* antibodies in serum samples and organ suspensions was made by the modified agglutination test (MAT) using antigen fixed by formalin-MAT-AF[9], prepared in LMVP/UNIPAR. Dilution of samples was performed initially to 1: 2 in SSTF, followed by serial dilutions at the base two to determination of antibody titer in samples.

Experiment II

Experiment II followed the same protocol for collection and preparation of samples and for the detection of antibodies, however, were comprised four groups of samples from five mice chronically infected and four groups of three uninfected mice samples, totaling 32 animals evaluated. Serum samples were collected with the animals anesthetized, but samples of tissues (spleen, liver, and muscles) only at 24, 48, 72 and 96 hours after euthanasia. During these periods, the carcasses of the animals were maintained at room temperature (22 ± 5ºC), in boxes protected from contact with flies.

Data Analysis

For the experiment I, the association between the results of detection of antibodies in serum and tissue suspension was verified by McNemar's test, kappa association index calculation, and the sensitivity, specificity, false-positive rate, false-negative rate, and agreement of the tests[10]. In addition, it was determined the curve ROC (Receiver Operating Characteristic) and the area of the curve for diagnostic quality comparison of antibody detection in each one of the tissues examined, taking as the gold standard the detection of antibodies in serum[11, 12]. For the experiment II, was calculated the sensitivity and specificity for each assessed tissue, regardless of time of autolysis, and tissues compared with each other by determining the ROC curve and the associated area. P values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Among the mice chronically infected and uninfected, evaluated in experiment I, were examined seventy samples of serum, liver and spleen, and 69 samples of musculature, corresponding to experiment I. The associations of the results of MAT between serum, liver, spleen, and muscle are presented in table 1.

From samples compared to the gold standard for MAT, the liver showed thirty true positive results, 32 true negatives results and four both false positives and false negatives. Of the sixty-nine muscle samples, 25 have true positive results, 36 true negatives, eight false positives and no false negative. In spleen samples was obtained thirty-one true positives, 34 true negatives, three false positives and two false negatives. Between samples evaluated, the muscle got less sensitivity (75.8 ± 7.4), with 24.4 chance to have a false positive result.

However, presented 100 of specificity, the biggest result for detection of true negatives, with efficiency of 88.4 ± 3.8 (table 2).

Table 1. Association of direct agglutination method results (MAD) *Toxoplasma gondii* antibody detection in serum, liver, spleen and muscle of mice chronically infected or not.

		Liver[1]		**Spleen[2]**		**Muscle[3]**	
		Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Serum	Positive	30	4	31	3	25	8
	Negative	4	32	2	34	0	36
Total		34	36	33	37	25	44

Statistics: [1]McNemar χ^2 test χ^2=0.13 (p=0.7237); [2]χ^2=6.13 (p=0.0133); [3]χ^2=0.00 (p=1.0000).

Table 2. Validation statistics (E-estimate ± SD – standard deviation estimates; 95-95 confidence interval) of the results of *Toxoplasma gondii* antibodies detection by direct agglutination method on suspension of liver, spleen and muscle of mice chronically infected.

Statistics	**Liver**		**Spleen**		**Muscle**	
	E ± sd	**95%CI**	**E ± sd**	**95%CI**	**E ± sd**	**95%CI**
Sensibility	88.2±5.5	72.6-96.7	91.2±4.9	76.3-98.1	75.8±7.5	57.7-88.9
Especificity	88.9±5.2	73.9-96.9	94.4±3.8	81.3-99.3	100.0±0.0	90.3-ND
Eficiency	88.6±3.8	78.7-94.9	92.9±3.1	84.1-97.6	88.4±3.8	78.4-94.9
False positive	11.1±5.2	3.1-26.1	5.6±3.8	0.7-18.7	0.0±0.0	ND-9.7
False negative	11.8±5.5	3.3-27.4	8.8±4.9	1.9-23.7	24.2±7.5	11.1-42.3
Kappa	77.1±7.6	62.2-92.0	85.7±6.2	73.6-97.8	76.5±7.6	61.7- 91.4

95%CI = 95% confidence interval; ND = not definied.

When checked the ROC curves and the associated areas, however, there was no difference in efficiency between tissues (table 3, Figure 1) on the detection of antibodies against *T. gondii* (p>0.05).

Table 3. Differences in the area on the ROC curve (AUC Δ), confidence interval 95 (95) and value of P to the Toxoplasma gondii antibodies detection by direct agglutination method in suspensions of samples of liver, spleen and muscle of mice chronically infected.

Paired samples	**ΔAUC**	**95%IC**	**P-Value**
Liver-Spleen	0.033	-0.062 – 0.128	0.500
Liver-Muscle	0.002	-0.102 – 0.107	0.962
Spleen-Muscle	0.030	-0.043 – 0.103	0.417

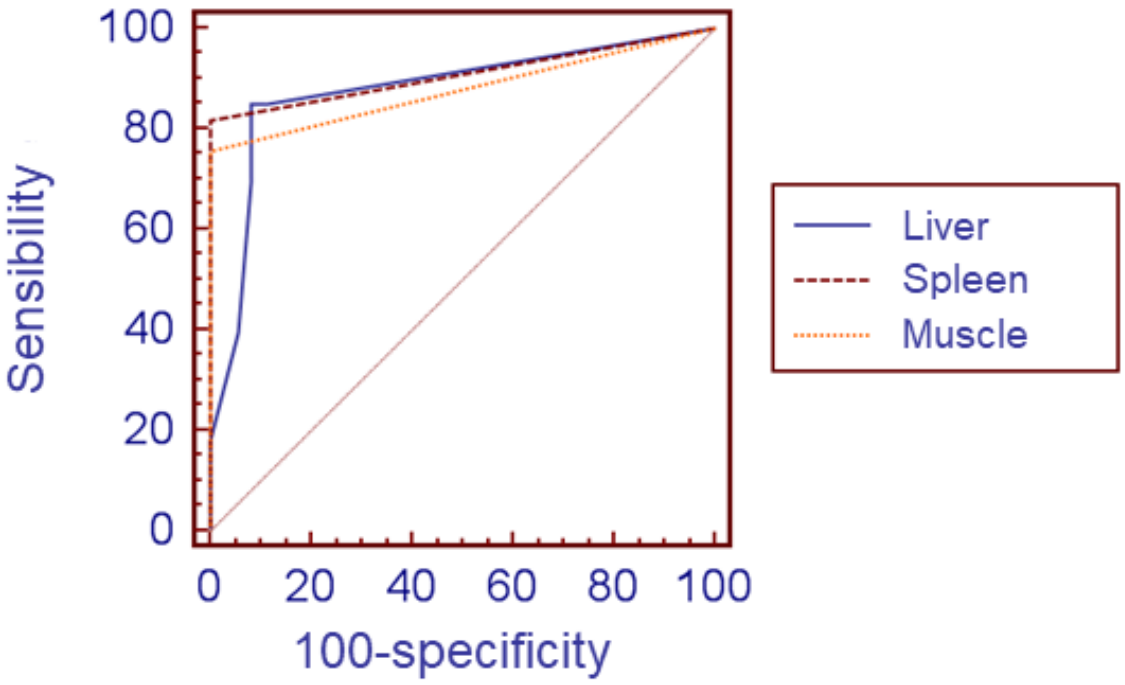

Figure 1. ROC curve for the results of *Toxoplasma gondii* antibodies in suspensions of liver, spleen and muscle of chronically infected mice.

As in other works by detecting antibodies in suspensions of musculature, the sensitivity of MAD was smaller. Lundén et al.[8] found 94 sensitivity and specificity of 92 when compared ELISA test results in muscle suspension with the reaction of Sabin-Feldman in the serum of naturally infected animals. Hill et al.[13], also comparing the ELISA in suspensions of experimentally infected animals' muscles, found sensitivity of 76.9, while the MAD had a sensitivity of 80.6 and ELISA, 100 sensitivity. On the other hand, despite the lowest sensitivity, specificity was 100 in this work, and Hill et al.[13] found a negative predictive value of 100 to the ELISA in suspensions of musculature.

The experiment II sought to verify the influence of autolysis in the performance of the detection of antibodies in samples of tissues of mice chronically infected. There were no differences, regardless of performance rate, for MAT results in tissue at different sampling times (24, 48, 72 or 96 hours; $p>0.05$), and with this, to the final analysis, the results, independent of the time of collection, were considered together, to each of the tissues.

Table 4 presents the parameters of sensitivity and specificity of antibody detection by MAD, for each of the tissues, when the validation criterion were the titers 0, 2 and 20. As can be seen, regardless of the titer there was decrease of expressive parameters. The least affected was the muscle, as in sensitivity and in specificity, and when considered larger than titer 2, was the best performing tissue (Figure 2).

Table 5 shows that, even with the differences between sensitivity and specificity, these were not sufficient to indicate significant differences between tissues in detecting antibodies in autolyzed samples.

Table 4. Sensitivity (SE) and specificity (ES) – of estimate and 95-95 confidence interval-the results of *Toxoplasma gondii* antibodies detection by direct agglutination method on suspension of liver, spleen and muscle of mice chronically infected in samples collected between 24 to 96 hours after euthanasia.

Tissue	Parameter	Criteria (antibody titer)					
		>0		>2		>20	
		%	95%CI	%	95%CI	%	95%CI
Liver	SE	90.0	68.3-98.8	25.00	8.7-49.1	5.00	0.1-24.9
	ES	66.7	34.9-90.1	91.67	61.5-99.8	100.00	73.5-100.0
Spleen	SE	100.0	83.2-100.0	50.00	27.2-72.8	25.00	8.7-49.1
	ES	41.7	15.2-72.3	83.33	51.6-97.9	100.00	73.5-100.0
Muscle	SE	90.0	68.3-98.8	70.00	45.7-88.1	45.00	23.1-68.5
	ES	75.0	42.8-94.5	91.67	61.5-99.8	100.00	73.5-100.0

95%CI = 95% confidence interval.

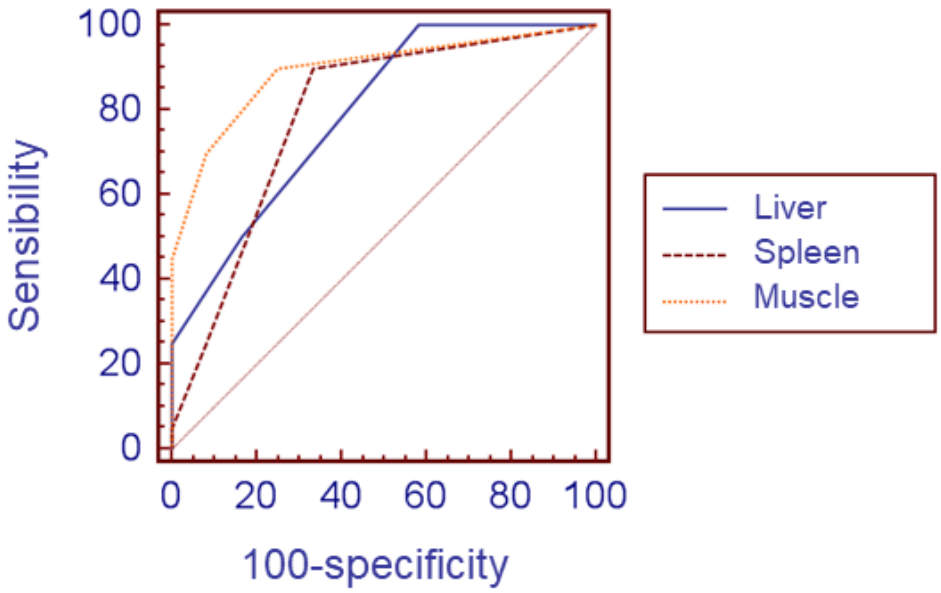

Figure 2. ROC curve for the results of *Toxoplasma gondii* antibodies in suspensions of liver, spleen and muscle of mice chronically infected in samples collected between 24 to 96 hours after euthanasia.

Table 5. Differences in the area on the ROC curve (AUC Δ), confidence interval 95 (95) and value of P to the *Toxoplasma gondii* antibodies detection by direct agglutination method in suspensions of samples of liver, spleen and muscle of mice chronically infected in samples collected between 24 to 96 hours after euthanasia.

Paired samples	ΔAUC	95%CI	P-Value
Liver–Spleen	0.002	-0.243 – 0.248	0.987
Liver–Muscle	0.102	-0.065 – 0.269	0.231
Spleen–Muscle	0.104	-0.088 – 0.296	0.287

The literature does not present results of detection of antibody in autolyzed tissue samples. However, a source for the presence of antibodies, potentially changed by cadaverous phenomena are fetal fluid samples taken in cases of abortion. Arthur, Blewett[6] reported that

only antibody above 256 could be considered sensitive and specific for thoracic fluid specimens obtained from aborted lambs; smaller than titer eight were not associated with histopathological lesions of fetal cotyledons compatible with toxoplasmosis. Venturini et al.[7] found a difference in detection of antibodies in fetal fluids from pigs when using the IFAT and MAT, the first being most sensitive. In this case the authors report the contamination of samples as a factor for decreased performance of the agglutination method.

Despite the loss of sensitivity, especially when evaluated in samples of liver and spleen, the detection of antibodies against *T. gondii* in tissue samples may be indicated when the blood serum is not available. Fall of independent performance of agglutination test in autolyzed samples, the advantage of the method in dispense species-specific reagents can justify its use especially when wild animals are involved, due to greater difficulty in obtaining species-specific reagents required by techniques such as IFAT and ELISA.

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