Use of embryonated egg membranes in the standardization of immunohistochemistry protocols for *Toxoplasma gondii**

Alexandre Dias Munhoz[1], Márcio de Barros Bandarra[2], Rosemeri de Oliveira Vasconcelos[2], Tiago Wilson Patriarca Mineo[3] and Rosangela Zacarias Machado[2+]

ABSTRACT. Munhoz A.D., Bandarra M.B., Vasconcelos R.O., Mineo T.W.P. & Machado R.Z. **Use of embryonated egg membranes in the standardization of immunohistochemistry protocols for *Toxoplasma gondii*.** [Utilização de membranas de ovos embrionados na padronização de protocolos de imuno-histoquímica de *Toxoplasma gondii*.] *Revista Brasileira de Medicina Veterinária 36(4):401-404, 2014.* Departamento de Patologia Animal, Universidade Estadual Julio Mesquita Filho, Unesp/Jaboticabal, Rod. Paulo Castellane s/n, Jaboticabal, São Paulo, SP 14884-900, Brazil. E-mail: zacarias@fcav.unesp.br

The chorioallantoic membranes of embryonated eggs can be used in the standardization of immunohistochemistry (IHC) protocols to detect *Toxoplasma gondii* antigens. On the 10th day of embryogenesis, four groups received, respectively, 1×10^4, 1×10^5, 5×10^5 and 1×10^6 *T. gondii* tachyzoites (RH strain)/ 100 µL, and a control group received PBS (pH 7.2). Three forms of heat induced antigen retrieval were employed (double boiler, electric cooker and Pascal pressure chamber) in the IHC. A diffuse distribution of the parasite was observed in the IHC of all infected groups. The results of the various antigen retrievals were similar for all slides, with no differences demonstrated as to the retrieval forms applied. The use of membranes as positive controls in IHC reactions for *T. gondii* provides parasite-rich material that is easy to prepare and shows quick results, making it a useful tool in the standardization of protocols for this purpose.

KEY WORDS. Diagnosis, toxoplasmosis, chorioallantoic membrane.

RESUMO. O uso de membranas corioalantóides de ovos embrionados pode ser utilizado na padronização de protocolos de Imunohistoquímica (IHQ) na detecção de antígenos de *Toxoplasma gondii*. No 10º dia de embriogênese quatro grupos receberam, respectivamente 1×10^4, 1×10^5, 5×10^5 e 1×10^6 taquizoítos de *T. gondii* (cepa RH)/ 100 µL e um grupo controle recebeu PBS (pH 7,2). Utilizaram-se três formas de recuperação antigênica pelo calor (banho maria, panela elétrica de cozimento e câmara de Pressão Pascal) na IHQ. Na IHQ de todos os grupos infectados observou-se uma distribuição difusa do parasito. O resultado das diferentes recuperações antigênicas foi similar em todas as lâminas, não demonstrando diferenças quanto às formas de recuperação utilizadas. A utilização de membranas como controle positivo em reações de IHQ para *T. gondii*, em qualquer uma das concentrações de parasito utilizados neste trabalho, fornece um material rico em parasito, de fácil preparo e rápido resultado, tornando-se uma ferramenta útil na padronização de protocolos para este fim.

PALAVRAS-CHAVE. Diagnóstico, toxoplasmose, membrana corioalantóide.

INTRODUCTION

Toxoplasma gondii is a coccidium with cosmopolitan distribution, capable of infecting a large variety of

Rev. Bras. Med. Vet., 36(4):401-404, out/dez 2014

401

animals (McColgan et al. 1988). A definitive toxoplasmosis diagnosis usually relies on several procedures as serology, identification of compatible microscopic lesions in tissues, positive PCR and direct immunodetection by immunohistochemistry (IHC) (Buxton 1998, Pescador et al. 2007, Dagleish et al. 2010).

The IHC technique to detect *T. gondii* bradyzoites in tissues is highly specific (Hamir & Dubey 2001, Dubey et al. 2003, Thomas et al. 2007), with low (Pescador et al. 2007) or high sensitivity (Thomas et al. 2007), depending on the parasite load present in the tissue. It has the advantage of being sensitive, in relation to parasite isolation indegenerated tissues (Buxton 1998), and rarely presents cross-responses with other related protozoa (Granstrom et al. 1991, Dubey et al. 1998). Variations in the results obtained by the application of this technique has been reported in several laboratories, which usually occurs due to operator experience, application of different processing protocols and tissue fixation time, as occurs in IHC for *Neospora caninum* (Van Maanen et al. 2004, Furuta et al. 2007, Mineo et al. 2009).

The use of embryonated eggs for the production of *T. gondii* antigens was described by Warren & Russ (1948), which demonstrated simplicity in inoculation and egg maintenance, low cost, and presents a naturally sterile culture medium (Wunderlin et al. 1997).

The objective of this work is to use chorioallantoic membranes from embryonated eggs experimentally infected with *T. gondii* for the standardization of immunohistochemistry protocols for the detection of parasitic antigens.

MATERIALS AND METHODS

Parasites and preparation of inoculum

Toxoplasma gondii strain RH tachyzoites were used from the peritoneal wash of previously infected mice. Following euthanasia of the animals, 5mL of sterile PBS (pH 7.2) were inoculated in the abdominal cavity and the retrieved wash was centrifuged at 250Xg during 10 minutes. The pellet was resuspended in sterile PBS (pH 7.2) and centrifuged again at 250Xg during 10 minutes for three consecutive times. In the end, the pellet was resuspended in PBS containing antibiotics/antimycotics [penicillin (1000U), streptomycin (100µm) and amphotericin B (25µm) per mL (Gibco™, USA). The tachyzoites were counted in a Neubauer chamber and distributed among four suspensions containing $1x10^4$, $1x10^5$, $5x10^5$ and $1x10^6$ parasites/ 100µL.

Inoculation of embryonated eggs

Thirty pathogen-free embryonated chicken eggs (SPF) on their 1st day of embryogenesis were used in the experiments (Hy-Line do Brasil Ltda). Eggs were incubated at 37.7°C±2, with 55%±5 humidity. From the 7th day onwards, the eggs were taken to the ovoscope daily for diagnosis of embryo viability.

Egg inoculation followed previously described technique (Furuta et al. 2007). Briefly, eggs with 10 days of incubation were randomly distributed into five groups of six eggs each, named $1x10^4$, $1x10^5$, $5x10^5$, $1x10^6$ and negative controls.

The eggs were perforated with the help of a needle (25x7mm) introduced in a rubber stopper, allowing exposure of the tip only. Following the perforation, 100 microliters of the group's corresponding suspension was inoculated into the egg's allantoic cavity. An identical volume of fluid was administered to the control group eggs, which received only sterile PBS (pH 7.2) with antibiotic/antimycotic solution. After inoculation, the eggs were examined twice a day. Embryos that died up to 72 hours following inoculation were eliminated from the experimental groups.

Histopathology and Immunohistochemistry

Fragments from the chorioallantoic membranes of embryos were collected and kept in a 10% formaldehyde buffered solution for 24 hours. After fixation, the membranes were transferred to 70% ethanol and embedded in paraffin blocks.

Tissue sections with 3-5mm were dewaxed and hydrated prior to antigen retrieval. Three types of antigen retrieval methods were tested using heat: double boiler at 95°C for 30 minutes; electric cooker (Stteamer®) for 40 minutes; and Pascall™ (mod.S2800) pressure chamber according to the manufacturer's recommendation (Dakocytomation, Denmark). The three types of retrieval used a pre-heated citrate buffer solution (pH 6.0). Endogenous peroxidase was blocked with a hydrogen peroxide solution at 8% in methanol for 20 minutes in a dark environment. Non-specific binding sites within tissues were blocked (Protein Block, DakoCytomation, Denmark) and was followed by the addition of anti-*T. gondii* mouse polyclonal at 1:16,000 dilution, in a humid chamber, overnight at 4°C. A commercial biotinylated multi-species anti-IgG solution was used as secondary antibody, followed by an incubation with streptavidin-HRPO (LSAB, DakoCytomation, Denmark). Reaction was revealed by diaminobenzidine (DAB) tetrahydrochloride chromogen (DakoCytomation, Denmark), and counter-stained with Harris hematoxylin, before dehydration and mounting with Entelan (Merck, Germany).

In order to assure specific reactions, negative control membranes were incubated with anti-*T. gondii* mouse polyclonal serum, used in the present study, and the membranes infected with *T. gondii* were incubated with anti-*Neospora caninum* polyclonal serum from a cow with a history of abortions due to neosporosis. All described steps were followed by a cycle of washes in distilled water and Tris-HCl (pH 7.2).

PCR for *Toxoplasma gondii* in chorioallantoic membranes

Membrane fragments were kept at -70°C for extraction of DNA and performance of PCR. The DNA was

402

Rev. Bras. Med. Vet., 36(4):401-404, out/dez 2014

extracted using the DNeasy (Qiagen™) *kit* according to the manufacturer's recommendations. The PCR to *B1* gene was conducted using primers (Sense: 5'-GGAAC-TGCATCCGTTCATGAG-3'; Anti-sense: 5'-TCTTTAA-AGCGTTCGTGGTC-3'), with amplification products of 193 base pairs (Burg et al. 1989). The amplification reactions were carried out with a final volume of 25μL:5 μL of each DNA sample, 1.0μM of each initiator oligonucleotide, 2.5mM of MgCl2, 100 mM of dNTPs and Taq polymerase mixture (Invitrogen 0.625U/reaction), as well as ultrapure distilled water to complete the volume. The amplifications were processed in a PTC-200 thermocycler (MJ Research) with 35 cycles programmed for denaturation (93°C/1 minute), annealing (55°C/1 minute), and extension (72°C/2 minutes), followed by a final, 5-minute extension.

RESULTS

All embryos died between the 5th and 7th day, and no relation was observed between the infecting dose and the day of death. There were no embryo deaths in the control group.

The necropsy revealed necrotic foci, thickening of CA membrane, and bleeding (Figure 1A). It was observed a positive correlation between the infecting dose and observed clinical findings. Histopathological examination evidenced congestion, necrosis and fibrosis in all membranes of the infected groups, as well as a diffuse distribution of the parasite, also observed in IHC (Figure 2). The tested antigen retrieval protocols yielded similar results, with no interference and/or enhancement in immunostaining. Amplification of the parasite DNA by PCR was possible in all membranes of the infected groups (Figure 3). There were no signs of tissue damage observed in the negative control membranes, which were negative in the PCR and had no anti-*T. gondii* immunostaining in IHC. Additio-
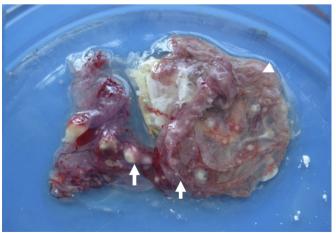

Figure 1. Focal bleeding, foci of necrosis (←) and thickening (◄) of the chorioallantoic membrane in embryonated egg infected with 5x10⁵ *Toxoplasma gondii* tachyzoites.
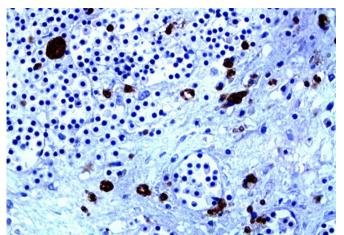

Figure 2. Diffuse positive immunoreactivity in immunohistochemistry, in chorioallantoic membrane of embryonated egg infected with 1x10⁶ *Toxoplasma gondii* tachyzoites (400x). Mouse primary antibody, LSAB and DAB. Obj.40X.

nally, no cross-reactivity was observed with *T. gondii* infected membranes and incubated with anti-*N. caninum* serum in the IHC.

DISCUSSION

The use of membranes as positive control in IHC reactions for *T. gondii* provides parasite-rich material that is easy to prepare and shows quick results. As observed in this experiment, 15 to 17 days is the period necessary to obtain an infected membrane that can be separated into various sections, providing a large volume of material to be embedded. This allows the standardization of various forms of antigen retrieval (as carried out in this work), of types and concentrations of primary antibodies, and of secondary complexes such as PAP; ABC (Uggla et al. 1987); LSAB (Furuta et al. 2007); EnVision™ (Thomas et al. 2007) and Advanced™.

Eggs containing embryos that died within 72 hours of inoculation should be discarded, since we and others have observed that the lethality originated from the inoculation procedures and not from the parasitism itself (Furuta et al. 2007). Loss of embryonated eggs due to inoculation protocols came to 20%. During ovoscopy, the observation of little embryo motility, reduced thickness and number of vessels, as well as the presence of spots resembling blood clots in the membrane are indicative of the start of fetal death. The 10th day of embryogenesis is the best moment for experimental inoculation because it presents an embryo that is stronger to infection and at the same time it is easy to locate favorable spots for the procedure (Furuta et al. 2007).

Depending on the time of infection and the concentration of inoculated parasites, it is possible to

observe histopathological alterations and moderate to severe IHC markings, making it possible to use them in a semi-quantitative IHC and to produce a gold standard (Van Maanen et al. 2004), since the high parasite load associated with the absence of concomitant infectious agents assures the tissue has high sensitivity and specificity in relation to other materials.

At last, the use of membranes in the production of these antigens can minimize the use of animals for this purpose (Eckert 1997), since the experimental use of infected mice that may or may not receive regular doses of sulpha is one of the ways used to produce positive control samples for IHC.

REFERENCES

Burg J.L., Grover C.M., Pouletty P., Boothroyd J.C. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J. Clin. Microbiol.*, 27:1787-1792, 1989.

Buxton D. Protozoan infections (*Toxoplasma gondii, Neospora caninum* and *Sarcocystis* spp.) in sheep and goats: recent advances. *Vet. Res.*, 29:289-310, 1998.

Dagleish M.P., Benavides J. & Chianini F. Immunohistochemical diagnosis of infectious diseases of sheep. *Small Rum. Res.*, 92:19-35, 2010.

Dubey J.P., Abbitt B., Topper M.J. & Edwards J.F. Hydrocephalus associated with *Neospora caninum* infection in an aborted bovine fetus. *J. Comp. Path.*, 118:169-173, 1998.

Dubey J.P., Zarnke R., Thomas N.J., Wong S.K., Van Bonn W., Briggs M., Davis J.W., Ewing R., Menseh M., Kwok O.C.H., Romandi S. & Thulliez P. *Toxoplasma gondii, Neospora caninum, Sarcocystis neurona,* and *Sarcocystis canis*-like infections in marine mammals. *Vet. Parasitol.*, 116:275-296, 2003.

Eckert J. Alternatives to animal experimentation in parasitology. *Vet. Parasitol.*, 71:99-120, 1997.

Furuta P.I., Mineo T.W.P., Carrasco A.O.T., Godoy G.S., Pinto A.A. & Machado R.Z. *Neospora caninum* infections in birds: experimental infection in chicken and embryonated eggs. *Parasitology*, 134:1931-1939, 2007.

Granstrom D.E., Ralph C.G., Tuttle P.A., Williams N.M., Poonacha K.B., Petrites-Murphy M.B., Tramontin R.R., Swerczek T.W., Hong C.B., Rezabek G.B., Lyons E.T. & Drudge J.H. Immunohistochemical diagnosis of protozoan parasites in lesions of equine protozoal myeloencephalitis. *J. Vet. Diagn. Invest.*, 3:75-77, 1991.

Hamir A.N. & Dubey J.P. Myocarditis and encephalitis associated with *Sarcocystis neurona* infection in raccoons (*Procyon lotor*). *Vet. Parasitol.*, 95:335-340, 2001.

Mineo T.W.P., Carrasco A.O.T., Marciano J.A., Werther K., Pinto A.A. & Machado R.Z. Pigeons (*Columba livia*) are a suitable experimental model for *Neospora caninum* infection in birds. *Vet. Parasitol.*, 159:149-153, 2009.

McColgan C., Buxton D. & Blewett D.A. Titration of *Toxoplasma gondii* oocysts in non-pregnant sheep and the effect of subsequent challenge during pregnancy. *Vet. Rec.*, 123:467-470, 1988.

Pescador C.A., Oliveira E.C., Pedroso P.M.O., Bandarra P.M., Okuda L.H., Corbellini L.G. & Driemeier D. Perdas reprodutivas associadas com infecção por *Toxoplasma gondii* em caprinos no sul do Brasil. *Pesq. Vet. Bras.*, 27:167-171, 2007.

Thomas N.J., Dubey J.P., Lindsay D.S., Cole R.A. & Meteyer C.U. Protozoal Meningoencephalitis in Sea Otters (*Enhydra lutris*): a Histopathological and Immunohistochemical Study of Naturally Occurring Cases. *J. Comp. Path.*, 137:102-121, 2007.

Uggla A., Sjölaiid L. & Dubey J.P. Immunohistochemical demonstration of toxoplasmosis in fetuses and fetal membranes of sheep. *Am. J. Vet. Res.*, 48:348-351, 1987.

Van Maanen C., Wouda W., Schares G., von Blumröder D., Conraths F.J., Norton R., Williams D.J.L., Esteban-Redondo I., Innes E.A., Mattson J.G., Björkman C., Fernández-García A., Ortega-Mora L.M., Müller N., Sager H. & Hemphill A. An interlaboratory comparison of immunohistochemistry and PCR methods for detection of *Neospora caninum* in bovine foetal tissues. *Vet. Parasitol.*, 126:351-364, 2004.

Warren J. & Russ S.B. Cultivation of toxoplasma in embryonated egg; an antigen derived from chorioallantoic membrane. *Proc. Soc. Exp. Biol. Med.*, 67:85-89, 1948.

Wunderlin E., Wild P. & Eckert J. Comparative reproduction of *Cryptosporidium baileyi* in embryonated eggs and in chickens. *Parasitol. Res.*, 83:712-715, 1997.