DOI: 10.1590/S0037-86822011000500011 Revista da Sociedade Brasileira de Medicina Tropical 44(5):587-590, set-out, 2011

Article/Artigo

Production of anti-*Cryptosporidium* polyclonal antibodies and standardization of direct immunofluorescence for detecting oocysts in water

Produção de anticorpos policlonais anti-*Cryptosporidium* e padronização da imunofluorescência direta para a detecção de oocistos na água

Silvia Cristina Osaki[1,2], Adriana Oliveira Costa[3], Ludmilla Della Coletta Troiano[1], Ernesto Renato Kruger[1,4], Juliana Tracz Pereira[5], Nelson Luis Mello Fernandes[1,2], Márcia Benedita de Oliveira Silva[6] and Vanete Thomaz Soccol[1]

ABSTRACT

Introduction: The production of anti-*Cryptosporidium* polyclonal antibodies and its use in direct immunofluorescence assays to determine the presence of *Cryptosporidium* in water are described in the present work. **Methods:** Two rabbits were immunized with soluble and particulate antigens from purified *Cryptosporidium* oocysts. The sera produced were prepared for immunoglobulin G extraction, which were then purified and conjugated with fluorescein isothiocyanate (FITC). Slides containing known amounts of oocysts were prepared to determine the sensitivity of the technique. To test the specificity, slides containing *Giardia duodenalis* cysts were prepared. **Results:** The conjugate was successfully used in water samples experimentally contaminated with *Cryptosporidium* oocysts, and it was possible to detect up to five oocysts/spot, corresponding to contamination of 250 oocysts/mL. **Conclusions:** The three immunizations performed in the rabbits were enough to produce antibodies against *Cryptosporidium*, the standard direct immunofluorescence assay permitted the detection of five oocysts in 20% of the samples, and no cross-reaction with *Giardia duodenalis* cysts occurred.

Keywords: Polyclonal antibodies. Direct immunofluorescence. Oocysts. Water.

RESUMO

Introdução: A produção de anticorpos policlonais anti-*Cryptosporidium* e sua utilização na imunofluorescência para determinar a presença de *Cryptosporidium* em água são descritas no presente trabalho. **Métodos:** Dois coelhos foram imunizados com antígeno solúvel e particulado provenientes de oocistos purificados de *Cryptosporidium.* O soro produzido foi preparado para a extração de imunoglobulinas G, que foram purificadas e conjugadas com isotiocianato de fluoresceína (FITC). Lâminas contendo quantidades conhecidas de oocistos foram preparadas para determinar a sensibilidade da técnica. Para testar a especificidade foram preparadas lâminas contendo cistos de *Giardia duodenalis.* **Resultados:** O conjugado foi usado com sucesso em amostras de água contaminadas experimentalmente com oocistos de *Cryptosporidium*, sendo capaz de detectar até cinco oocistos/*spots* que corresponde a uma contaminação de 250 oocistos/mL. **Conclusões:** As três imunizações realizadas nos coelhos foram suficientes para produção de anticorpos contra *Cryptosporidium*; a reação de imunofluorescência direta padronizada permitiu a detecção de cinco oocistos em 20% das amostras; não houve reação cruzada com cistos de *Giardia duodenalis.*

Palavras-chaves: Anticorpos policlonais. Imunofluorescência direta. Oocistos. Água.

1. Programa de Pós-Graduação em Processos Biotecnológicos e Biotecnologia, Divisão de Biotecnologia, Universidade Federal do Paraná, Curitiba, PR. 2. Disciplina de Ciências Agrárias/Epidemiologia, Universidade Federal do Paraná, Palotina, PR. 3. Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, MG. 4. Instituto Marcos Enrietti, Secretaria de Agricultura do Estado do Paraná, Curitiba, PR. 5. Departamento de Química, Instituto Federal do Paraná, Paranaguá, PR. 6. Disciplina de Parasitologia, Instituto de Ciências Biológicas e Naturais, Universidade Federal do Triângulo Mineiro, Uberaba, MG.

Address to: Dra. Vanete Thomaz Soccol. PPGBIOTEC/Div. Biotecnol/UFPR. Rua Cel. Francisco H. dos Santos 100/2º andar, Centro Politécnico, Prédio de Administração, 81531-990 Curitiba, PR, Brasil. Phone: 55 41 33613272
e-mail: vanete.soccol@pq.cnpq.br

Received in 22/02/2011
Accepted in 09/06/2011

INTRODUCTION

The direct immunofluorescence assay (DIF) for detecting *Cryptosporidium* was developed in the 1980s and showed advantages in relation to the technique used up until that point, which involved floating with a zinc sulfate solution and Lugol staining[1]. When compared with acid fuchsin staining, the immunofluorescence assay technique shows greater sensitivity and specificity[2].

One of the problems in using DIF in water samples is the presence of a large number of organic and inorganic substances capable of binding to antibodies and producing a false-positive result[3]. This problem can be minimized by establishing a purifying stage; the purpose of which is to eliminate the particles that can bind to marked antibodies producing fluorescence that could confuse the less-experienced technician.

This technique and others that use immunofluorescent methods are not able to identify the *Cryptosporidium* species, the genotype and specific hosts[4], but are still very important in epidemiological studies and in the evaluation of the water following treatment. Additionally, methods 1,622 and 1,623 of the American Environment Protection Agency[5,6], developed for monitoring *Cryptosporidium* in water, require a final stage that involves fluorescent monoclonal antibodies capable of staining the membrane of *Cryptosporidium* oocysts.

Considering the high cost for performing methods 1,622 or 1,623, the proposal of the present work was to produce polyclonal antibodies that can be used in immunofluorescence assays in water samples from impounding or treated water, aimed at determining contamination levels by *Cryptosporidium* oocysts.

METHODS

Antigenic extract preparation

Cryptosporidium parvum oocysts were purified from calf feces by sucrose gradient as previously described[7]. A total of 10^8 oocysts diluted in 10mL of

phosphate buffered saline (PBS), pH 7.2, were disrupted by sonication at 60 Hz (five cycles/min in an ice bath followed by a 1-min rest). The material was observed under phase contrast microscopy to ensure the efficacy of sonication to rupture the membrane. The extract was centrifuged for 30 min at 9,000 x *g* at 4ºC, and the soluble antigen (supernatant) and particulate antigen (sediment) were separated into two aliquots. The protein concentration was determined by the Bradford method[8] using 1mg/mL bovine serum albumin (BSA) as standard. Absorbance readings were performed using a 595-nm filter.

Immunization

Two male New Zealand rabbits, approximately 7 months old, were used for immunization after they were submitted to an adaptation period of 30 days.

Three subcutaneous immunizations were administered in the scapular area of each rabbit, alternating between the right and left sides. The first dosage of the antigenic extracts contained 1.25mg and 0.85mg of the soluble and particulate antigen, respectively, and complete Freund's adjuvant (Sigma Chemical Company). The second immunization was performed after 15 days, using the antigenic extract in the same concentration described before and incomplete Freund's adjuvant (Sigma Chemical Company). The third immunization was repeated 15 days after the application of the second dosage, using identical concentrations of antigenic extract and adjuvant. Blood collections were performed on days 0, 15, 30, 45, 60, 90, and 120 to determine the antibody titers. The production blood collections were performed on days 45, 60, and 75, and the sera were stored at -20ºC until use.

Titering sera samples

The immunoenzymatic assay (enzyme-linked immunosorbent assay, ELISA) was used to determine the kinetics of anti-*Cryptosporidium* antibody production. The protocol adopted for the experiment followed that proposed by Frost et al.[9]

Briefly, flat-bottom plates (Immulon 2, Dynatech Laboratories, Inc.) were adsorbed with soluble antigen or particulate antigen at four different concentrations (250, 500, 1,000, and 1,500ng) diluted in a carbonate buffer (0.05 M, pH 9.6). The rabbit sera were diluted 1:100, 1:200, 1:400, 1:800, and 1:1600 in incubation buffer (PBS; 0.25% casein; 0.05% Tween 20). The rabbit anti-IgG immunoglobulin conjugated to the enzyme peroxidase (Sigma Chemical Company, A-0545) diluted at 1:2500 and 1:5000 was added, and the enzymatic activity was revealed using orthophenylenediamine solution (OPD) diluted in substrate buffer (4mg of OPD+10.5mL of citrate buffer pH 5.0+4µL of H_2O_2). The reaction was interrupted using 50L of 5% H_2SO_4. The negative control of the reaction was performed using the serum collected from the same rabbit on day 0 of the experiment, prior to inoculation with the antigenic extract. The serum collected on day 45 of the experiment was used as a positive control, in accordance with the proposal of Fernandes et al.[10]

The absorbance readings of the ELISA test were performed in the microplate reader (BIORAD Model 550) using a 492nm wavelength filter.

Glycoprotein extraction

The extraction of glycoproteins was performed according to method proposed by Penha[11]. A pool of sera collected on days 30, 45, and 60 was centrifuged at 3,700 x *g* for 5 min at 4ºC. The supernatant was harvested, and 5% dextran was added in the proportion of 1.25mL of dextran to 25mL of serum, drop by drop, under stirring at room temperature. The mixture was maintained for 30 min at room temperature, stirring every 10 min. An 11.1% $CaCl_2$ solution was added, drop by drop, under agitation in an ice bath, up to a final concentration of 1%. The mixture was allowed to stand for 30 min in the ice bath and was then centrifuged at 14,400 x *g* for 15 min. The supernatant was separated, and the same volume of PBS pH 7.2 was added. Twice the volume of saturated ammonium sulphate (pH 7.6) was added, and the mixture was left under slow magnetic stirring for 1h at room temperature. Following precipitation of immunoglobulins G, the sample was centrifuged for 30 min at 14,800 x *g* at 4ºC.

Dialysis was performed using a membrane (Sigma, Dialysis Sacks) that permits a 12kDa exclusion limit for protein retention and ammonium sulfate release. The process was performed for 3 days in a flask with two liters of PBS pH 7.2, with changes every 12h. The immunoglobulins were centrifuged at 3,700 x *g* for 15min at 4ºC to remove impurities. The dialyzed material was centrifuged at 1,330 x *g* for 15min at 4ºC to separate the immunoglobulins. Proteins were quantified in a Metrolab 1,700 Spectrophotometer, with UV rays, at 260nm and 280nm.

Fluorescein isothiocyanate conjugation

The fluorescein isothiocyanate (FITC) was dissolved in carbonate/bicarbonate buffer and added, drop by drop, to the immunoglobulin suspension in the proportion of 1.6µg of FITC to 1mg of protein, at room temperature under slow magnetic stirring. The mixture was maintained under slow magnetic stirring for 2h, taking care to avoid foaming.

The sample was then submitted to molecular exclusion chromatography in a Sephadex G 25 column (Sigma Aldrich). After hydrating the Sephadex with ultrapure water, it was washed five times with borate saline and transferred to a gel filtration column. The volume of the column was estimated respecting the proportion of 20mL of gel for 1mL of conjugate. The initial fraction (conjugate) was separated and dialyzed in borate saline for 48h, centrifuged at 1,330 x *g* for 10min at 4ºC, aliquoted and stored at -20ºC.

Oocyst detection by direct immunofluorescence

To determine the sensitivity of the direct immunofluorescence (DIF) using the polyclonal antibodies, the slides were adsorbed with 1,000, 500, 100, 50, 10, 5, and 1 oocyst/spot, in quintuplicate, diluted in PBS, pH 7.2. As each spot was filled with 20µL, these amounts are equivalent to 50,000 to 50 oocysts/mL. Slides containing 1,000, 500, 100, and 10 of *Giardia duodenalis* cysts were prepared, also in quintuplicate, aimed at evaluating the specificity of the technique. Following the addition of oocysts, the slides were left standing at room temperature for 15 min for the oocysts to sediment and then incubated at 37ºC for 1h, or until completely dry. Subsequently, the slides were placed in light-proof containers and frozen at -20ºC until use.

Immediately before use, the slides were thawed at 37ºC for 10 to 15 min and submitted to three washes with PBS pH 7.2. The conjugate diluted in Evans's blue was added to the slides pure or in the following dilutions: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128. The slides were incubated in a moist chamber for 30 min in the dark. After performing three washes with PBS, pH 7.2, the slides were incubated at 37ºC for 10 min to dry and were examined under an Olympus BX4TF fluorescence microscope.

Ethical considerations

All procedures related to managing the animals and material collection were approved by the Ethics Committee on Animal Experimentation of the Federal University of Parana (CEEA/UFPR), protocol no. 23075.029322/2007-47, certificate 250.

RESULTS

The amount of protein obtained from 10^8 oocysts was 2.5mg/mL and 1.7mg/mL for the soluble and particulate antigens, respectively. The production kinetics of anti-*Cryptosporidium*-specific antibodies was tested by the ELISA technique, in which 500ng of protein/mL as antigen, 1:100 dilution of the serum, and 1:2,500 dilution of the conjugate were used. The cutoff point was determined by estimating the average absorbance of the negative controls plus two standard deviations, corresponding to 0.100. Thus, samples with absorbance equal to or higher than 0.100 were considered positive. The rabbit sera collected during the experiment were titered, and all showed levels of absorbance higher than 0.100, verifying the production of anti-*Cryptosporidium* antibodies (**Figure 1**).

The purified and FITC-conjugated anti-*Cryptosporidium* polyclonal antibodies tested in DIF assays detected oocysts in all replicates that contained 1,000 to 100 parasites (**Figure 2**). Two out of the five replicates containing 50 to 10 oocysts were positive, only one replicate with five oocysts was positive, and no sample containing only one oocyst was positive (**Table 1**).

No cross-reaction with *Giardia duodenalis* cysts was observed in any of the slide spots.

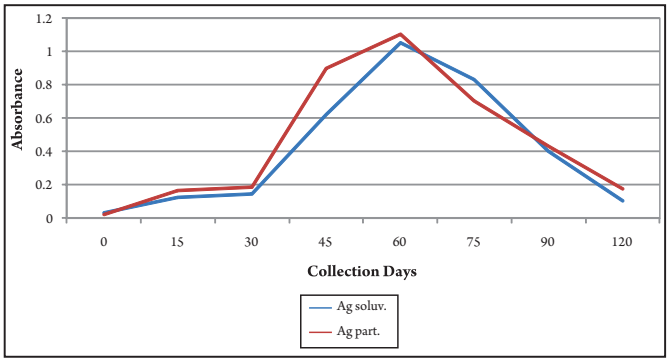

FIGURE 1 - Kinetics of antibodies production in rabbits immunized with antigenic extract of *Cryptosporidium parvum* oocysts, evaluated by ELISA performed with soluble and particulated antigen.

TABLE 1 - Direct immunofluorescence assays using policlonal antibodies in samples containing 1,000 to 1 oocyst/spot of *Cryptosporidium*.

Number of oocyts	Positive samples/replicates
1,000	5/5
500	5/5
100	5/5
50	2/5
10	2/5
5	1/5
1	0/10

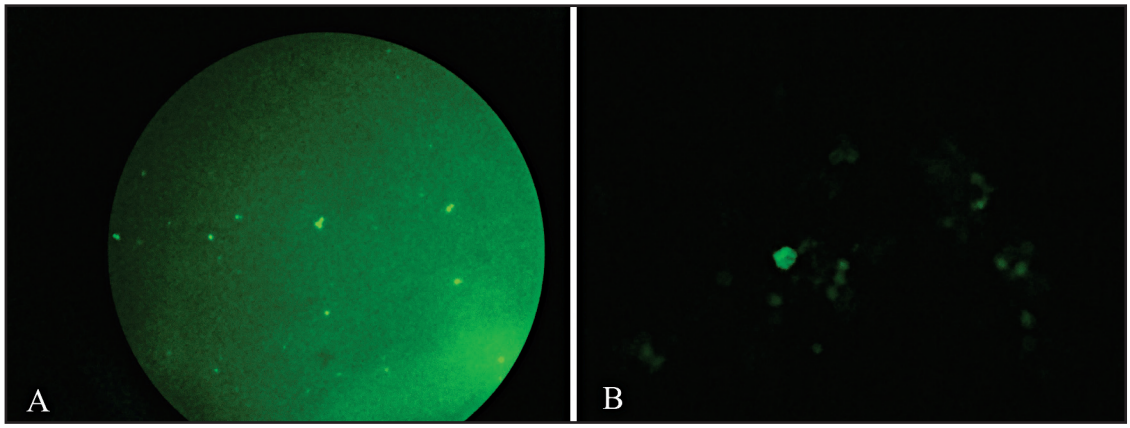

FIGURE 2 - *Cryptosporidium* oocysts detected by direct immunofluorescence assay using polyclonal antibodies produced by rabbit immunization. A. 200X, B. 400X.

DISCUSSION

Identification of *Cryptosporidium* in water is impaired by the low number of oocysts and by adverse environmental factors that may alter their morphology[1]. Thus, techniques that can concentrate and correctly identify the parasites are required. Methods 1,622 and 1,623 of the American Environment Protection Agency[5,6] are considered a reference for detecting *Cryptosporidium* in water. Among the several steps required to perform these methods, the final morphological identification involves the use of differential interference contrast microscopy, 4'-6-diamino-2-phenylindole (DAPI) staining, and immunofluorescence using a commercial kit. In Brazil and other developing countries, poly- or monoclonal antibodies against *Cryptosporidium* are not produced by national companies. Thus, the importation of immunofluorescence kits is costly for sanitation companies to monitor the parasites in water. Considering these aspects, the present work standardized a direct immunofluorescence reaction using anti-*Cryptosporidium* polyclonal antibodies produced by immunization in rabbits. These animals were chosen because they present greater volume compared with other animals and, consequently, produce a larger quantity of blood.

The anti-*Cryptosporidium* antibodies were successfully produced and used in DIF assays, which revealed sufficient sensitivity to detect 5 oocyst/spot or 250 oocysts/mL.

Cross-reactions can occur in direct immunofluorescence tests performed in water samples, as the presence of a large number of fluorescent particles may cause difficulties during the microscopic confirmation of suspect oocysts[3, 12, 13]. In the present work, the reactivity of the anti-*Cryptosporidium* antibodies with *Giardia duodenalis* cysts was tested, and no cross-reaction was observed. Despite the large morphological difference between oocysts and cysts, the intent of the specificity test was to precisely observe the presence of possible components present in the cysts that might be recognized by the anti-*Cryptosporidium* antibodies.

Although some unspecific reactions do occur, the DIF is a good alternative for detecting environmental contamination by *Cryptosporidium* oocysts[1]. Moreover, the technique can be associated with DAPI staining and differential contrast microscopy, which would improve the chances of correct identification.

Eventually, the development of DIF that uses monoclonal antibodies could make it possible to indicate hosts as the source of contamination by identifying individual *Cryptosporidium* species, as some of these present antigenic differences[14]. Monoclonal antibody production involves more laborious procedures and is the next step to be developed by our group. As screening methodologies with affordable costs are demanded by sanitation companies to maintain the optimum prices of the services provided, in the present work, we believe the standardization of a DIF with polyclonal antibodies for immediate use was the best initial choice. This should be considered as the first initiative to produce inputs for immunodetection of *Cryptosporidium* at a national level, and the results indicated that it can be used for screening of environmental samples.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

Fundação Nacional de Saúde (Ministério da Saúde - Brasil); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brasil).

REFERENCES

1. Cartusio-Neto R, Franco RMB. Ocorrência de oocistos de *Cryptosporidium* spp. e cistos de *Giardia* spp. em diferentes pontos do processo de tratamento de água, em Campinas, São Paulo, Brasil. Rev Hig Alim 2004; 18:52-59.
2. Xiao L, Cama V. Foodborne Parasites. Ortega YR, editor. *Cryptosporidium* and Cryptosporidiosis. Food Microbiol and Food Safety, Springer, New York, US; 2006. p. 57-108.
3. Rodgers MR, Flanigan DJ, Jakubowski W. Identification of algae which interfere with the detection of *Giardia* cysts and *Cryptosporidium* oocysts and a method for alleviating this interference. Appl Environ Microbiol 1995; 61:3759-3763.
4. Hashimoto A, Sugimoto H, Morita S, Hirata T. Genotyping of single *Cryptosporidium* oocysts in sewage by semi-nested PCR and direct sequencing. Water Res 2006; 40:2527-2532.
5. United States Environmental Protection Agency. Method 1,622 - *Cryptosporidium* in water by filtration/IMS/FA. 821-R-97-023U.S. Washington (DC): Environmental Protection Agency Publication. U.S. Environmental Protection Agency; 1997.
6. United States Environmental Protection Agency. Method 1,623 - *Cryptosporidium and Giardia* in water by filtration/IMS/FA. U.S. 821/R-99-006. Washington (D.C): Environmental Protection Agency Publication. U.S. Environmental Protection Agency; 1999.
7. Silva MBO. Infecção natural e experimental de *Cryptosporidium* em caprinos: avaliação clínica, parasitológica e da resposta humoral. [Tese]. [Belo Horizonte]: Universidade Federal de Minas Gerais; 2000. 187 p.
8. Bradford MM. A rapid and sensitive method for the quantitation of protein utilizing the protein-dye binding. Anal Biochem 1976; 72:248-254.
9. Frost FJ, Cruz de La AA, Moss DM, Curry M, Calderon RL. Comparisons of ELISA and Western blot assays for detection of *Cryptosporidium* antibody. Epidemiol Infect 1998; 121:205-211.
10. Fernandes NLM, Thomaz-Soccol V, Pinto SB, Minozzo JC, Oliveira CAL. Resposta imune-humoral e celular em bovinos da raça Nelore imunizados com extrato de larvas (L2 e L3) de *Dermatobia hominis* (Linnaeus Jr, 1781). Cienc Rural 2007; 37:789-795.
11. Penha TR. Produção e caracterização de anticorpos monoclonais anti-fragmento Fc de IgG de bovino. [Dissertação]. [Curitiba]: Universidade Federal do Paraná; 2007. 98 p.
12. Bull S, Chalmers R, Sturdee AP, Curry A, Kennaugh J. Cross-reaction of an anti-*Cryptosporidium* monoclonal antibody with sporocysts of *Monocystis* species. Vet Parasitol 1998; 77:195-197.
13. Quintero-Betancourt W, Peele ER, Rose JB. *Cryptosporidium parvum* and *Cyclospora cayetanensis*: a review of laboratory methods for detection of these waterborne parasites. J Microbiol Methods 2002; 49:209-224.
14. Sturbaum GD, Schaefer DA, Jost BH, Sterling CR, Riggs MW. Antigenic differences within the *Cryptosporidium hominis* and *Cryptosporidium parvum* surface proteins P23 and GP900 defined by monoclonal antibody reactivity. Mol Biochem Parasitol 2008; 159:138-141.