

ARTÍCULO ORIGINAL/ARTIGO ORIGINAL

# Serological evidence of *Cryptosporidium* infections in a group of pregnant women attended by the prenatal routine care at a public hospital in Sao Paulo (SP), Brazil

Evidência sorológica de infecção por *Cryptosporidium* entre gestantes atendidas pela rotina da assistência pré-natal em um hospital público de São Paulo (SP), Brasil

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## Abstract

This paper had as objective to evaluate the previous exposition to *Cryptosporidium*, and possible risk of infection, of a group of pregnant women attending a prenatal care program. Serum samples from 48 patients in the prenatal care, attended at the "Hospital do Servidor Público Estadual Francisco Morato de Oliveira", Sao Paulo (SP), Brazil, were submitted to ELISA for detection of anti-*Cryptosporidium* IgG antibodies by using a crude extract of disrupted oocysts as antigen. For the standardization step, positive and negative control sera were obtained after a preliminary screening for anti-*Cryptosporidium* antibodies carried out among laboratory workers. Serum samples from patients with other parasite infections were also evaluated for the presence of anti-*Cryptosporidium* antibodies, and the results compared to the ones obtained with the group of pregnant women. The high frequency of 54.2% observed for this group, suggesting previous exposition to the parasite in a great number of pregnant women submitted to the study, might indicate high risk of infection in the population, and a need for a further investigation related to the sanitary and environmental aspects that conditioned such high level of exposition.

**Key words:** *Cryptosporidium*, maternal exposure, enzyme-linked immunosorbent assay, IgG antibodies, risk factors, serology.

## Resumo

O trabalho teve como objetivo avaliar a exposição prévia ao *Cryptosporidium*, e possível risco de infecção, de mulheres grávidas atendidas em um programa pré-natal. Amostras séricas de 48 pacientes, atendidas pelo Programa de Assistência Pré-Natal do Hospital do Servidor Público Estadual "Francisco Morato de Oliveira", São Paulo (SP), foram submetidas ao teste ELISA para detecção de anticorpos IgG anti-*Cryptosporidium*, utilizando como antígeno extrato bruto de oocistos rompidos. Na etapa de padronização, soros controle positivo e negativo foram obtidos após triagem preliminar realizada entre funcionários de

um laboratório de parasitologia, para presença de anticorpos anti-*Cryptosporidium*. Amostras séricas de pacientes com outras infecções parasitárias foram também avaliadas quanto à presença de anticorpos anti-*Cryptosporidium*, e os resultados comparados com os encontrados no grupo de mulheres grávidas. A elevada frequência de 54,2% observada neste grupo, sugerindo prévia exposição ao parasito por um número relativamente grande de mulheres grávidas avaliadas no presente estudo, poderia indicar elevado risco de infecção na população e uma necessidade de novas investigações com relação a aspectos sanitários e ambientais que condicionaram este alto nível de exposição.

**Palavras-chave:** *Cryptosporidium*, exposição materna, ELISA, anticorpos IgG, fatores de risco, sorologia.

### Introduction

*Cryptosporidium* is a coccidian protozoan parasite that is widely spread in the environment and recognized as responsible for diarrhea disease in both, immunocompromised and immunocompetent individuals. In these healthy hosts the *Cryptosporidium* infection is normally self limited and often asymptomatic, but in the immunosuppressed individuals, such as those with acquired immunodeficiency syndrome (AIDS), cancer and congenital immunoglobulin deficiencies, or receiving immunosuppressive drugs, cryptosporidiosis can be severe, long-lasting, and sometimes fatal.<sup>(1)</sup>

The transmission of *Cryptosporidium* to a susceptible host is usually by fecal-oral route, through the ingestion of infective oocysts, eliminated in the environment by the infected hosts. The direct human-to-human transmission can occur among family members, sexual partners, children in day-care centers, hospitalized patients and staff of hospital. Zoonotic transmission is also possible, involving pets or farm animals, and by accidental infection of veterinary workers.<sup>(2)</sup>

Contaminated water, including drinking and recreational water, might represent the major source of infections for humans. *Cryptosporidium* oocysts may remain viable in water for over four to five months, being very resistant to the most common disinfectants and difficult to be destroyed by conventional chlorination treatment. Consequently, cryptosporidiosis has been recognized as an important waterborne disease, with several outbreaks reported.<sup>(2,3)</sup>

Diagnosis of human cryptosporidiosis is based on clinical signs and symptoms, parasitological, immunological and molecular assays, with development of a great variety of tests. The most usual laboratory

diagnosis relies on microscopic examination and morphological identification of parasite oocysts in the stool. As an alternative diagnostic method, monoclonal and polyclonal antibodies have been used for detection of *Cryptosporidium* excretory-secretory antigens in stool, with excellent performance.<sup>(1,2)</sup>

Specific anti-*Cryptosporidium* IgG, IgM and IgA antibodies have been detected in sera of immunocompetent and immunocompromised patients by different serological methods, such as indirect immunofluorescence test, enzyme-linked immunosorbent assay (ELISA) and Western blot. Using these methods it is possible to assess the immune response during the infection and realize epidemiological studies, using the frequency data of circulating antibodies in the population as an indicator of exposure to the parasite.<sup>(4-9)</sup>

The aim of the present study was to evaluate the previous exposition to this protozoan parasite, and possible risk of infection, in a group of pregnant women attending a prenatal care program, through detection of anti-*Cryptosporidium* IgG antibodies, and to compare the data with other different groups of individuals.

### Material and methods

#### Sera

Serum samples from 48 pregnant women, attending the Prenatal Care Program (PCP) of the *Hospital do Servidor Público Estadual Francisco Morato de Oliveira*, Sao Paulo, SP, Brazil, were kindly donated for this study. Additionally, banked specimens (n = 72) with seropositivity to another parasite infections, such as toxoplasmosis (TOX), Chagas' disease (CHA), leishmaniasis (LEI), schistosomiasis (SCH), and cysticercosis (CYS), were also included in this study, for detection of anti-*Cryptosporidium* antibodies. All samples were examined without any personal identification.

#### Recovery of *Cryptosporidium* oocysts for antigen preparation

A calf, five days old, was infected with  $5 \times 10^6$  oocysts purified from stool of naturally infected calves. The diarrhea started six days after infection and the feces were collected daily, mixed with a 2.5% potassium dichromate solution and stored at 4°C. After washing five times in an equal volume of PBS (0.01 M phosphate-buffered saline, pH 7.2), oocysts were concentrated and purified from stool samples by flotation on sucrose gradient, with density of 1.2 g/ml, according to Sheather's technique, with some modifications.<sup>(10)</sup> Approximately 2 ml of oocysts suspension was added to 10 ml of sucrose solution, homogenized and centrifuged for 10 min at

400 x g in a 50 ml centrifuge tube. The surface of the supernatant was carefully removed with a Pasteur pipette and transferred to a clean tube. Oocysts were suspended in PBS and washed by centrifugation at 450 x g for 10 min until removing all the sucrose. To remove bacteria and fungi, sodium hypochlorite solution (5%) were added to oocysts solution, homogenized and centrifuged at 4.000 x g for 2 min in 1.5 ml centrifuge tubes. After five washings, the number of oocysts was estimated in a Neubauer camera and results expressed as oocysts/ml.

#### **Antigen preparation**

Oocyst suspension containing about  $2.5 \times 10^6$  oocysts/ml was submitted to seven cycles of freeze/thaw steps and then sonicated (20 cycles of 15 seconds at 60 KHz each), according to Moss and Lammie,<sup>(11)</sup> with modifications. This suspension was centrifuged at 4.000 x g for 30 min at 4°C and the supernatant stored at -20°C, for further using in ELISA assays. The amount of protein in the soluble antigen was determined by Lowry<sup>(12)</sup> method.

#### **Standardization of ELISA for detection of anti-*Cryptosporidium* antibodies**

ELISA was standardized according to Ungar et al.,<sup>(13)</sup> with some modifications. During the standardization step, stool and blood venous samples were collected from clinically normal individuals (n = 20), among the workers of the Laboratory of Parasitology Staff (LPS) at *Instituto Adolfo Lutz*, Sao Paulo (SP), Brazil, after signing the informed consent statement. All the blood samples from LPS group were submitted to an ELISA carried out on microtiter plates sensitized with an arbitrary concentration of *C. parvum* antigen preparation, for detection of anti-*Cryptosporidium* IgG antibodies. Three samples that showed the higher optical density (OD) readings, in this preliminary ELISA, were selected as positive controls and used for the standardization of the ELISA; eight serum samples that provide OD readings  $\leq 0.30$  were selected as negative controls. One positive and one negative serum samples were used in the checkerboard titrations for determination of the optimal concentrations of reagents. After standardization, the ELISA was carried out as follows: wells on flat polystyrene microtiter plates (*Alamar Techno Científica LTDA.*) were coated with 50 µl of *C. parvum* antigen preparation, containing about 60 µg/ml of proteins, in order to have 3 µg of protein per well. Coated plates were blocked with 100 µl of PBS containing 1% skimmed milk (PBS-M) for 50 min at 37°C. Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T). Test sera diluted 1:100 in PBS containing 0.05% of Tween-20 and 1% skimmed milk (PBS-T-M) were incubated

at 37°C for 50 min. After three washes, peroxidase-conjugate anti-human IgG (Sigma Chemical Company, St. Louis, MO, USA) diluted to 1:10.000 was added and incubated for 50 min at 37°C, followed for another series of three washes. A chromogenic mixture of H<sub>2</sub>O<sub>2</sub> and o-phenylenediamine (OPD) was added and the plates incubated for 15 min in the dark. After addition of 50 µl of stop solution (H<sub>2</sub>SO<sub>4</sub> 1N), OD was measured at 492 nm, in a microplate reader (Labsystems Multiskan MS). The test samples were assayed in duplicate, and for each reaction day, serial dilution of a positive control serum were added, and the cutoff value (0.300) was determined by calculating the arithmetic mean of the OD readings of eight negative control sera diluted to 1:100, plus two standard deviations.

#### **Absorption of anti-*Cryptosporidium* antibodies**

A *Cryptosporidium* antigen containing 590 µg/ml of protein was used as absorbent. To define the best condition for absorption, different dilutions of the absorbent (1:25, 1:50, 1:100, 1:200, 1:400 and 1:800) and different periods of incubation (1, 2, 3, 4 and 18 hours), at 37°C, were tested. The positive control serum was diluted to 1:100 to establish the conditions of absorption. ELISA was done before and after the absorption for some selected positive sera presenting the highest OD readings from different groups.

#### **ELISA for detection of anti-*Toxoplasma* antibodies**

Detection of IgG antibodies to *Toxoplasma gondii* was done for some serum samples by ELISA, according to technique routinely used in the Parasitology Service of *Instituto Adolfo Lutz*.

#### **Statistical analysis**

Data were analyzed by using Epi-Info version 6.04 (World Health Organization). A confidence interval of 95% was considered to calculate the positivity rates for each analyzed sera group.

#### **Ethical considerations**

The study is in compliance with the norms of Resolution n. 196, of 10/10/1996, of the National Health Committee and was approved by the Research Ethics Committee of the *Faculdade de Ciências Farmacêuticas da Universidade de São Paulo*, Sao Paulo, SP, Brazil.

#### **Results**

Table 1 shows the positivity rates for anti-*Cryptosporidium* IgG antibodies obtained among the pregnant women of PCP group, and the results compared to other studied groups. The frequency of 54.2%, obtained in the PCP group, was higher than the frequency of 41.7% observed for the total of the other parasite seropositive groups. With relation to

**Table 1. Positivity rates for anti-*Cryptosporidium* IgG antibodies by ELISA for different groups of human sera. Sao Paulo (SP), Brazil**

Group	Tested samples	Positive	
		N°	% (CI)
Workers of the Laboratory of Parasitology Staff (LPS)	20	6	30.0% (12.8 – 54.3)
Pregnant Care Program (PCP)	48	26	54.2% (39.3 – 68.4)
Other parasite infection groups			
Chagas' disease patients (CHA)	21	14	66.7% (43,1 – 84,5)
Cysticercosis patients (CYS)	16	7	43.7% (20,7 – 69,4)
Leishmaniasis patients (LEI)	10	4	40,0% (13,7 – 72,6)
Schistosomiasis patients (SCH)	10	2	20,0% (3,5 – 55,8)
Toxoplasmosis patients (TOX)	15	3	20,0% (5,3 – 48,6)
<b>Total</b>	<b>72</b>	<b>30</b>	<b>41,7% (30,3 – 53,9)</b>

CI = Confidence Interval with 95% of confidence.

each parasite seropositive groups, the frequency of the pregnant women group was lower than the rate observed for the group of Chagas' disease patients (66.7%) and higher than the positivity rates obtained for other groups: cysticercosis, leishmaniasis, schistosomiasis and toxoplasmosis patients (43.7%, 40.0%, 20.0% and 20.0%).

Anti-*Toxoplasma* antibodies were detected in 14.6% of the serum samples from the pregnant women group, and positive results for both, anti-*Cryptosporidium* and anti-*Toxoplasma* antibodies were observed in only one serum of this group. For the LPS group, anti-*Toxoplasma* antibodies were detected in 15% of the samples, and no simultaneous positive result for anti-*Cryptosporidium* and anti-*Toxoplasma* antibodies was observed for any member of the group.

The best conditions for absorption of the anti-*Cryptosporidium* antibodies were defined as antigen dilution of 1:25 and incubation of 4 hours, at 37°C, resulting in an optical density (OD) reading of the positive control serum lower than the cut off value.

The results of the experiments related to the absorption of anti-*Cryptosporidium* antibodies in some arbitrary selected serum samples were summarized on Table 2. Serum samples previously defined as positive for anti-*Cryptosporidium* antibodies and submitted to ELISA after incubation with the *Cryptosporidium* antigen showed OD readings lower than the cut off value.

## Discussion

The *Cryptosporidium* infection shows higher prevalence in developing countries than in industrialized countries. Positivity rates of 1% and 4% reported in Canada and Australia could be considered

**Table 2. Optical Density (OD) readings for some serum samples of different groups, submitted to ELISA for detection of anti-*Cryptosporidium* antibodies, before and after absorption with *Cryptosporidium* antigen. Sao Paulo (SP), Brazil**

Group	Serum	OD reading		Percentage of absorption
		Before absorption	After absorption	
PCP	1	0.275	0.152	55,2%
	2	0.680	0.254	32,9%
	3	0.523	0.268	51,2%
	4	0.738	0.287	38,9%
LPS	1	0.590	0.230	39,0%
	2	0.853	0.280	33,6%
	3	0.453	0.230	50,7%
	4	0.570	0.248	43,5%
CHA	1	0.909	0.367	41,3%
	2	0.446	0.232	52,0%
	3	0.393	0.160	40,7%
	4	0.567	0.234	41,2%
CYS	1	0.288	0.201	69,7%
	2	0.279	0.209	74,4%
	3	0.345	0.205	59,4%
	4	0.560	0.233	43,7%
SCH	1	0.257	0.190	73,9%
	2	0.363	0.155	42,6%
TOX	1	0.342	0.135	39,4%
	2	0.533	0.171	32,0%
	3	0.284	0.176	61,9%

PCP = Pregnant Care Program; LPS = Laboratory of Parasitology staff; CHA = Chagas' disease patients; CYS = Cysticercosis patients; SCH = Schistosomiasis patients; TOX = Toxoplasmosis patients. Cut off = 0.300

low when compared to 7.9% and 11.1%, observed among children in Liberia or Ruanda.<sup>(14,15)</sup>

A study realized in six communities, in British Columbia, Canada, evaluating sera from 4.097 pregnant women by ELISA, for detection of antibodies to the 27 kDa *Cryptosporidium parvum* sporozoites surface antigen, showed high seropositivity rates (77% to 92%) in the communities, with or without occurrence of waterborne outbreak.<sup>(9)</sup> Another study in Canada evaluated the seroprevalence for *Cryptosporidium* among residents of three communities, using different water sources. Sera from 1.944 women between 15 to 40 years old were tested for anti-*Cryptosporidium* IgG antibodies, by immunoblot, and the results showed that the higher seropositivity (52.5%) was observed for the community where an outbreak have been detected.<sup>(16)</sup>

A survey carried out in the city of Campinas (SP), Brazil, showed that water samples collected from the

Atibaia River were all positive for *Cryptosporidium* oocysts and *Giardia* cysts.<sup>(17)</sup> In an impoverished semi-urban community in Fortaleza (CE), Brazil, a seroprevalence for anti-*Cryptosporidium* IgG antibodies of 75% was obtained among 40 children under 4 years old.<sup>(18,19)</sup> But the real frequency among immunocompetent individuals is not known, since the majority of the researches about prevalence of cryptosporidiosis are related to immunocompromised patients, mainly those with human immunodeficiency virus (HIV). Also, it was not found in the literature, studies related to the seroprevalence of cryptosporidiosis amongst pregnant women in Brazil.

A study with 512 samples, including 39 pairs of maternal-cord sera, done to investigate age-specific seroprevalence in a suburban population of Sao Paulo (SP), Brazil, revealed low seropositivity in infants, and quick increasing to nearly 60% by 5 years, and 80% by the age of 10 years.<sup>(9)</sup> Another study in Sao Paulo, by using an indirect immunofluorescence technique, verified 54% of seropositivity for IgG antibodies among 72 patients with *Cryptosporidium* oocysts in stool examination, and only 9% of seropositivity among 101 patients with negative results for *Cryptosporidium* oocysts in fecal examination.<sup>(9)</sup>

For this study, the ELISA for detection of anti-*Cryptosporidium* antibodies had to be standardized. Positive and negative control sera were selected among the workers of the laboratory of parasitology, where the study was carried out. Even though all fecal samples were negative for the presence of *Cryptosporidium* oocysts in these individuals, the presence of anti-*Cryptosporidium* antibodies in six of the 20 laboratory workers submitted to ELISA (30%) could be explained for the previous exposition to this parasite, on account of labor activities, or even at home or other common situation, once the high possibility of risk exposition to *Cryptosporidium* infection in the population, as already been demonstrated by other researchers.<sup>(2,8,17-19)</sup>

The ELISA results in the present work showed a high positivity index (54.2%) for anti-*Cryptosporidium* antibodies among the pregnant women involved in this study. The presence of these antibodies does not indicate active infection, but suggest previous contact with this parasite.

Higher positivity rates for anti-*Cryptosporidium* antibodies were observed among the patients with Chagas' disease (66.7%). Possibility of cross-reaction was discharged by treatment of the sera with *Cryptosporidium* antigen for removal of the anti-*Cryptosporidium* antibodies, without interference on the reactivity against *Trypanosoma cruzi*. In the CHA group, some serum samples submitted to immuno-

fluorescence antibody (IFA) test for detection of anti-*Trypanosoma cruzi* antibodies after incubation with the *Cryptosporidium* antigen, demonstrated decrease in the values of OD readings for anti-*Cryptosporidium* antibodies without alteration in the positivity of the IFA test for Chagas' disease.

Considering the high seroprevalence indices for toxoplasmosis, commonly obtained in the communities,<sup>(20)</sup> and the phylogenetic proximity between the genera *Cryptosporidium* and *Toxoplasma*, both coccidian parasites with similar biological cycles, a study was done to verify occurrence of cross-reactions between these two species. The frequency of anti-*Toxoplasma* antibodies among the pregnant women enrolled in this study was relatively low (14.6%), if compared to the frequency of anti-*Cryptosporidium* antibodies (52.0%), and only one patient presented antibodies to both parasites. These results suggest possible absence of cross-reactivity between the two parasites. The specificity of the anti-*Cryptosporidium* antibodies detected in the ELISA was also demonstrated by testing some positive sera before and after incubation with the specific antigen, for absorption of anti-*Cryptosporidium* antibodies.

The ELISA assay, as carried out in this study, showed to be a potentially useful tool for assessing possible risk of exposition to *Cryptosporidium* infection in population or community studies. Our results suggest high risk of exposition to this parasite among the pregnant women enrolled in this study, pointing out that special attention should be given to education in health, alerting about *Cryptosporidium* sp. as an important pathogen of water transmission, responsible for diarrhea in small children, aged people and immunocompromised individuals.

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