A modified method for detecting *Cryptosporidium* oocysts using DNA templates extracted from environmental samples

Método modificado para detecção de oocistos de *Cryptosporidium* utilizando DNA extraído de amostras ambientais

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RESUMO
O protozoário parasito *Cryptosporidium* tem emergido como um dos mais importantes contaminantes da água, causando surtos de diarreia de veiculação hídrica em todo mundo. Para avaliar o significado, para a saúde pública, da presença desse agente patogênico em amostras ambientais, vários métodos têm sido desenvolvidos para isolar e detectar oocistos de *Cryptosporidium*. No presente estudo foi padronizado um método confiável e reprodutível para detectar e identificar oocistos de *Cryptosporidium* em amostras de água no Estado de São Paulo, Brasil, como o primeiro passo para futuros estudos de genotipagem. Amostras de água foram concentradas por filtração, submetidas a ultrasom em solução de Tween 80 a 0.1%; o sedimento obtido foi transferido para microtubos contendo 1,0 ml de água destilada e conservado a -20ºC. O DNA foi extraído com adição de 1% de PVP no tampão de lise; a extração foi realizada em tubo Phase Lock Gel Heavy®. Houve amplificação do fragmento esperado de 214 bp em cinco das 11 amostras de água analisadas. Os resultados deste estudo demonstraram a utilidade de aplicação do teste padronizado em estudos epidemiológicos e em programas de vigilância, em virtude da técnica ter apresentado sensibilidade para incrementar significativamente a quantidade de produto amplificado.
Palavras-chave. *Cryptosporidium*, reação em cadeia da polimerase, poluição da água, saúde pública.

ABSTRACT
The protozoan parasite *Cryptosporidium* has emerged as one of the most important water contaminants, causing outbreaks of waterborne diarrhea worldwide. In order to assess the importance for public health of this pathogen's presence in environmental samples, several methods have been developed to isolate and detect *Cryptosporidium* oocysts. In the present study, a reliable and reproducible method has been standardized for detecting and identifying *Cryptosporidium* oocysts in water samples in the State of São Paulo, Brazil as the first step for future genotyping studies. Water samples were concentrated by filtration, and then subjected to ultrasound in Tween 80 0.1%, the obtained sediment was transferred into micro tubes containing 1.0 ml of distilled water and stored at -20ºC. DNA was extracted with the addition of 1% PVP in lysis buffer, the organic extraction was performed in Phase Lock Gel Heavy®. There was a 214 bp amplification on the expected fragment in five out of the 11 water samples analyzed. The results of this study demonstrated the application usefulness of the standardized test in epidemiological studies and surveillance programs because the technology allowed to increase significantly the amount of amplified product.
Key words. *Cryptosporidium*, polymerase chain reaction, water pollution, public health.

The coccidian protozoan *Cryptosporidium* is responsible for many outbreaks of human disease worldwide. Epidemiological studies have indicated that the main routes of transmission for human cryptosporidiosis are human-animal, person-to-person spread and the waterborne infection. In São Paulo State, Brazil, studies have described the presence of *Cryptosporidium* oocysts in water sources[1,2], sewage[3-5], mineral water[6] and outbreaks of cryptosporidiosis in day-care-centers[7,8], but no waterborne infection had been reported. The oocyst is the environmentally stable stage and is resistant to inactivation by commonly used drinking water disinfectants.

Currently, there are several methods in the literature for detection, identification and enumeration of *Cryptosporidium* oocysts in water and fecal samples. Therefore, the data obtained using the conventional immunofluorescent assay (IFA) for environmental samples and optical microscopy for clinical samples, do not provide information on the *Cryptosporidium* species, genotypes and specific hosts. Nevertheless, in Brazil, as far as we know, to date, there is only one study of molecular method applied to detect *Cryptosporidium* oocysts in fecal samples adapted to our conditions[9].

The present study was designed to achieve a methodology to extract genomic DNA and detect *Cryptosporidium* oocysts from water samples.

Raw water samples were harvested from the same site in Ribeirão da Fazenda River which belongs to Ribeirão da Fazenda Basin, at the North coast of the city of São Sebastião, São Paulo State, Brazil.

The samples were collected in sterilized plastic containers and treated as described by Franco et al 2001[1] with modifications. Briefly, 10L of water were passed through membrane filters (145 mm diameter, 0.45 µm, Millipore®, Brazil), which were transferred to a plastic bag containing 200 ml of the water sample and 300 ul of 0.1% Tween 80 solution, and submitted to ultrasonic irradiation (Thornton T50) for 15 minutes. Afterwards, the content was transferred to a 50 ml centrifuge tube and centrifuged at 5.000 rpm for 15 minutes at 4°C. Pellets were transferred to a unique 2.0 ml microcentrifuge tube with 1.0 ml of distilled water and stored at –20ºC.

DNA extraction was performed according to Carvalho-Almeida et al 2005[9] with minor modifications. In brief, 200 µl of the filtrated sediment were mixed to 500 µl of lysis buffer [50 µl of100 mM Tris – HCl (pH = 8.0) + 50 µl of 50 mM EDTA (pH = 8.0) + 50 µl of SDS 10% + 3.5 µl de 70 mM β-mercaptoetanol + 1 µl of polyvinilpyrrolidone (PVP) 1% + 345.5 µl de Milli-Q water]. The second incubation time was reduced to two hours. DNA was extracted with 500 µl phenol-chloroform-isoamyl (25:24:1) using tubes Phase Lock Gel Heavy (Eppendorf®, Germany) following the manufacturer's instructions. The resulting DNA was eluted in 50 µl of TE buffer and stored at 4ºC until used for amplification.

PCR assays were performed targeting a polymorphous region of the 18S rRNA gene of *Cryptosporidium* using primers previously described by Coupe et al 2005[10]. Primary PCR amplifies a fragment of 1035 bp, and was performed with forward primer SCL1 (5´-CTGGTTGATCCTGCCAGTAG-3`) and reverse primer CPB-DIAGR (5`-TAAGGTGCTGAAGGAGTAAGG-3`) in five replicas for each sample. PCR reaction had a final volume of 25 µl in each tube containing 5.0 µl of DNA, 14.2 µl MilliQ water, 2.5 µl of 1X PCR reaction buffer, 1 µl of PVP 1%, 1.25 µl $MgCl_2$ 50 mM, 0.2 µl of 25 mM dNTP, 0.3 µl of each primer and 1.25 U of TaqDNA polymerase (Taq Gen Biosystem). The PCR program included 5 min of initial denaturation at 94ºC followed by 39 cycles of denaturation at 94ºC for 30 s, annealing at 60ºC for 45 s and extension at 72ºC for 90 s and a final extension at 72ºC for 5 min. To clean the products of PCR amplification, all five replicas of each sample were transferred to one fresh tube and purification was performed using the Perfectprep® PCR Cleanup Kit (Eppendorf, Germany) according to manufacturer´s instructions. The second round PCR amplifies a 214-bp fragment using the forward primer SCL2 (5`-CAGTTATAGTTTACTTGATAATC-3`) and the reverse primer SCR2 (5`-CAATACCCTACCGTCTAAAG-3`). To perform the second round of PCR, again five replicas of each purified amplicon were made, in a final volume of 25 µl using 5.0 µl of the product of the first round amplification. The conditions were the same as those for the first round PCR, except that no PVP was added and annealing was at 58ºC temperature, extension lasted 30 seconds and final extension lasted 10 minutes. To achieve optimal detection limits the products of nested-PCR were purified wth Perfectprep® PCR Cleanup Kit (Eppendorf, Germany) and analyzed by electrophoresis in 2.0% agarose gel stained with ethidium bromide.

In this study, the procedure could amplify the expected fragment of 214 bp from five out of eleven water samples. This result is similar to one study in which authors found six positive out of 15 water samples examined after incubating the DNA in 4% PVP solution and performing

two step of column purification[11]. To the best of our knowledge, it is the first report of *Cryptosporidium* oocysts detected by PCR from river water in Brazil.

Failure to obtain positive PCR results from several types of water samples containing *Cryptosporidium* oocysts has also been reported by others. One of the main challenges for PCR assays of coccidian parasites is to provide sufficient DNA free from PCR inhibitors. To overcome this problem we added, 1% PVP to the PCR reaction mixture to each replica tube containing the DNA template, and cleaned the product of the first round PCR in a spin column (Perfectprep® PCR Cleanup Kit Eppendorf, Germany). From this purified PCR product, we used 5 µl as the template for the second round PCR, made five replicas and again, performed a new purification before running the electrophoresis.

The epidemiology of *Cryptosporidium* is complex, with hundreds hosts and 15 recognized species, being *C. hominis* and *C. parvum* the cause of the majority of human disease. Besides these two main species, another species have been detected in human stools and can be found in the aquatic environment: *C. meleagridis*, *C. felis*, *C. muris* and *C. canis*[12].

Results of this study suggest that the modifications made here in the DNA extraction protocol and the concentration of the amplicon would improve the sensitivity and specificity for detecting C*ryptosporidium* spp. oocysts in water samples, using nested-PCR. This molecular tool can be very useful in assessing the human-infective potential of *Cryptosporidium* oocysts in water and the source of *Cryptosporidium* contamination. Despite the fact that this procedure is time consuming to be employed for diagnostic purposes, it may be valuable in directing operational management and surveillance in potential outbreaks situation. This procedure can be useful in epidemiological studies and surveillance programs since it significantly increased the amount of amplicon obtained, even with very low counts of oocysts in the water sample.

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