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TECHNICAL REPORT

PROTOCOL FOR DNA EXTRACTION OF *Cryptosporidium* spp. OOCYSTS IN FECAL SAMPLES

Elenice M.N. GONÇALVES(1), Ronalda S. ARAÚJO(2), Magali ORBAN(1), Glavur R. MATTÉ(2), Maria Helena MATTÉ(2) & Carlos E.P. CORBETT(3)

SUMMARY

Molecular characterization of *Cryptosporidium* spp. oocysts in clinical samples is useful for public health since it allows the study of sources of contamination as well as the transmission in different geographical regions. Although widely used in developed countries, in Brazil it is restricted to academic studies, mostly using commercial kits for the extraction of genomic DNA, or in collaboration with external reference centers, rendering the method expensive and limited. The study proposes the application of the modifications recently introduced in the method improving feasibility with lower cost. This method was efficient for clinical samples preserved at -20 °C for up to six years and the low number of oocysts may be overcomed by repetitions of extraction.

KEYWORDS: *Cryptosporidium* spp.; Diagnosis; Molecular techniques.

The genus *Cryptosporidium* has several species and genotypes that infect different hosts. At present the species recognized by many researchers as causing human cryptosporidiosis are: *C. parvum* (*C. parvum* bovine genotype or genotype 2 or genotype C or type II) and *C. hominis (C. parvum* human genotype or genotype 1 or genotype H or type I), responsible for most of the human infections; *C. felis, C. meleagridis* and *C. canis* (C. type dog) reported in immunocompetent patients; and *C. muris, C. baileyi, C. felis, C. meleagridis, C. canis, C. suis* (C. type pig) related to immunosuppressed patients[4,5,23,27,29,30].

Cryptosporidiosis represents a great public health issue, because many cases are asymptomatic, there is no effective clinical treatment and it may be fatal in immunocompromised individuals[11,17,27]. Epidemiologic studies on human cryptosporidiosis are made difficult by the different transmission pathways and by the limitation in identifying species using conventional methods, such as staining and immunological tests[5,11]. Progress in molecular techniques produced a great impact on the knowledge of taxonomy, biology, pathogenesis, epidemiology and transmission of the agent and also on evaluation of cryptosporidiosis treatment, as well as helping epidemiologists regarding control and determination of infection sources[4,5,14,17,30] . Thus, among the molecular methods developed for the detection and differentiation of different *Cryptosporidium* species and genotypes, PCR is the mostly used for both clinical and environmental samples[8,11,28], but its sensitivity is affected by the presence of inhibitors existing in fecal samples which may be minimized with changes in the extraction protocol[6,8,10,15,16,22,26]. In Brazil, most laboratories do not identify *Cryptosporidium* spp. oocysts routinerely[7,15] and there is no standardization of molecular techniques for genotypic characterization in isolated samples of fecal material. This suggests that information about circulating species and genotypes are scarce in our country[1,2,3,6,12,13,14,15]. Preservation of fecal samples for molecular analysis may be performed in 2.5% potassium dichromate at 4 °C[10,12,14,18], in absolute ethanol at room temperature[19,22], in 10% formalin[18,31], under refrigeration at 4 °C[23], at -20 °C[21,28] or at -80 °C[24] or else, after a drying process with silica[21]. There are reports on the inhibitory effects of potassium dichromate and formalin on PCR that may lead to a false-negative result of this reaction[18,19,25]. Another factor that may contribute to non-amplification of the fragment of interest, mainly in *Cryptosporidium* study, may be inadequate DNA extraction from fecal samples[31].

Aliquots of fecal samples selected from the diagnostic routine of the Parasitology Service of Division of Central Laboratory, "Hospital das Clínicas", University of São Paulo Medical School, during the period of February 2000 to June 2006, were distributed in 1.5 mL microtubes and preserved in 10% buffered formalin at the 1:3 proportion, under refrigeration (4 °C) and "in natura" frozen at -20 °C. *Cryptosprodium* spp. oocysts were detected by the modified Kinyoun method[15], in smears prepared with 10 µL of fecal sample "in natura" or, in a small proportion and when formed fecal samples, in buffered formalin, on 76 x 26 mm glass slides for microcopy, fixed with methanol PA. When detected, they were measured with the help of an ocular micrometer during reading under a light microscope, 100x objective, and for each slide the mean was calculated. The amount of oocysts was estimated as 1+ (1-10 oocysts/smear); 2+ (10 ≤ 50 oocysts/smear);

(1) Serviço de Parasitologia da Divisão de Laboratório Central do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo/LIM 03, São Paulo, SP, Brasil.
(2) Laboratório de Saúde Pública da Faculdade de Saúde Pública da Universidade de São Paulo, São Paulo, SP, Brasil.
(3) Laboratório de Patologia das Doenças Infecciosas da Faculdade de Medicina da Universidade de São Paulo/LIM 50, São Paulo, SP, Brasil.

and 3+ (> 50 oocysts/smear). For all fecal samples positive for *Cryptosporidium* spp. during reading under a light microscope, aliquots "in natura" (-20 °C) were defrosted to room temperature and diluted in deionized water, at a 1:1 proportion, in 2 mL tubes, to a final volume of 200 µL; to this fecal sample 500 µL lysis buffer [50 µ; Tris - HCl 100 mM (pH = 8.0); 50 µL EDTA 50 mM (pH = 8.0); 50 µL 10% SDS; 3.5 µL 70 mM β-mercaptoethanol; 1 µL 1% polyvinylpyrrolidone (PVP); 345.5 µL ultra-pure water (Milli-Q)] were added and incubated for 90 minutes in a 65 °C water bath. After incubation 20 µL proteinase K (20 mg/mL) were added to each tube, being again incubated for a period of 120 minutes at the same temperature. The samples were incubated in an ice bath for 15 minutes. DNA was extracted with 500 µL phenol-chloroform-isoamyl alcohol (24:25:1), the tubes were homogenized and the mixture was transferred to Phase Lock Gel light (Eppendorf®, Germany) tubes which were centrifuged at 12,000 rpm for five minutes at 4 °C. The aqueous phase, present at the surface, was transferred to a new microtube, 500 µL chloroform were added, homogenized and centrifuged at 12,000 rpm for 15 minutes at 4 °C. The supernatant was transferred to another microtube and precipitated with 500 µL ice-cold isopropyl alcohol and maintained in an ice bath for 10 minutes, centrifuged at 12,000 rpm, for three minutes at 4 °C. The supernatant was carefully discarded and the precipitated DNA washed with 500 µL 70% ethyl alcohol and centrifuged at 12,000 rpm, for three minutes at 4 °C. The supernatant was discarded and the DNA dried under vacuum, eluted with 50 µL TE buffer and maintained at 4 °C until utilization for amplification of the fragment of interest, according to the protocol described by COUPE *et al*[9]. In this protocol, lysis of the oocyst wall and DNA extraction was performed in fecal samples stored at -20 °C which suffered two subsequent incubations: one for rupture and the other for digestion of the oocyst cell wall. First, they were incubated with the lysis solution (Tris-HCl + EDTA + 10% SDS + β-mercaptoethanol). EDTA starts cell wall rupture, Tris-HCl equilibrates the pH (= 8.0) assuring lysis, SDS acts on the membrane lipid layer, and β-mercaptoethanol delays DNA oxidation facilitating its recovery. Incubation was performed with proteinase K, promoting cell wall digestion. For DNA extraction phenol-chloroform-isoamyl alchol (24:25:1) was used. Phenol-chloroform denaturizes proteins that form an interface layer between the aqueous and organic phases, the DNA being retained in the aqueous phase. Isoamyl alcohol removes organic residues and precipitates DNA in the aqueous solution. The step of removal of the aqueous phase was a critical point during the test since the interface layer could not be transferred due to the fact that, when in contact with alcohol, a permanent DNA precipitate was formed which made its posterior amplification impossible. This problem was eliminated by the introduction of the Phase Lock Gel light tube (Eppendorf®, Germany) in the separation step of the aqueous and organic phases. Introduction of PVP that assures efficient removal of inhibitors that may co precipitate with DNA during extraction after treatment with a lysis solution, improved the final amplification. Among the factors which may result in non-amplification by PCR are: the fecal sample containing variable concentrations of inhibitory substances such as biliary salts, proteins, polysaccharide complexes, bilirubins and phenolic compounds, depending on diet, intestinal flora and health conditions of the host, or, also, presence of a small number of oocysts, not uniformly distributed in the fecal sample, or not present in the aliquot separated for extraction, implying absence of target DNA, and also inadequate preservation or degradation of the DNA during storage. The used fecal samples were stored at -20 °C eliminating possible inhibitory effects by potassium dichromate and formalin on PCR[18,19,23], and the intrinsic inhibitors were minimized using PVP. Although the literature reports an inverse relationship between time of storage of the fecal sample and quality of extracted DNA with subsequent loss of sensitivity of PCR[26], preliminary results (data not shown) demonstrated that no statistically significant correlation was obtained ($p > 0.05$) for year of collection, result that corroborates to those of NSUBUGA *et al.*[22] who also did not observe interference on their results on analyzing samples conserved for 33 months. A study performed by LIMOR *et al.*[20], with samples preserved with dichromate for one year, did not reveal interference of the preserver on the PCR reaction in real time. In the study performed by HIGGINS *et al.*[16] was observed decrease in sensitivity of the PCR reaction in real time for samples preserved for three months in formalin. Most samples examined in this study for morphologic detection presented a small amount of oocysts (maximum of 10 oocysts/10 µL fecal sample) and additional tests were performed in five replica for DNA extraction and the final products were mixed and then subjected to PCR reaction, which increased concentration of material and guaranteed product amplification (data not shown). This strategy was used by XIAO *et al.*[28] increasing PCR efficacy from 62% to 86% in environmental samples with a low number of present oocysts, increasing also the possibility of detection of multiple genotypes in surface waters. We believe that the low number of *Cryptosporidium* oocysts detected on microscopy influences the result, probably due to the heterogeneous distribution in the sample or even by preserving aliquots without the target DNA. Discrepant results between microscopy and PCR were reported by MAGI *et al.*[21] who detected in their samples 20% positivity by PCR, due to the presence of few oocysts distributed in a non-homogeneous form in the analyzed fecal material, similarly to PENG *et al.*[24] with 35.3% positivity suggesting long storage time in formalin of the seventeen fecal samples studied. The general data obtained by PCR (37.23%) in this study resulted extremely positive when compared with those derived from former partnerships performed with protocols sedimented for PCR, in addition to the use of commercial kits for DNA extraction and which resulted in 49.2% and 20.9% amplification of samples[15]. The technique here used demonstrates its utilization in this context, once being associated with RFLP using only two restriction enzymes, is able to distinguish *C. hominis* and *C. parvum* (data not shown), responsible for most human infections from other related species, despite failures observed in samples with a low number of oocysts during microscopic screening, which may be overcomed with repetition of extractions.

RESUMO

Protocolo para extração de DNA de oocistos de *Cryptosporidium* spp. em amostras fecais

A caracterização molecular de oocistos de *Cryptosporidium* spp. em amostras clínicas é útil à saúde pública, pois permite estudo das fontes de contaminação e a transmissão em determinadas regiões geográficas. Apesar de largamente utilizada em países desenvolvidos, no Brasil está restrita aos estudos acadêmicos, na maioria utilizando kits comerciais para extração do DNA genômico, ou em colaborações com centros de referência externos, o que torna o método caro e limitado. Este estudo propõe a introdução de modificações nos métodos existentes para melhorar a viabilidade e baixar custos. O método proposto foi eficiente em amostras clínicas preservadas a -20 °C por

até seis anos e o baixo número de oocistos pode ser contornado por replicadas extrações de DNA.

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