Experimental Parasitology 157 (2015) 84–87

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Research brief

Multilocus amplification of genomic DNA from single cysts of *Giardia duodenalis* separated using micromanipulation technique

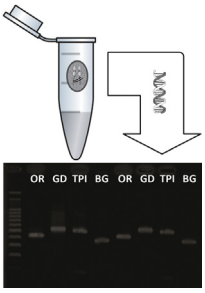
Juliana Martins Aguiar [a], Sheila Oliveira da Silva [a], Valdir Antônio dos Santos [b], Giselle Ayres Razera Rossa [a], Sueli Akemi Taniwaki [a], Fabio Gregori [a], Lara Borges Keid [c], Rodrigo Martins Soares [a, *]

[a] *Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil*
[b] *Departamento de Farmácia e Laboratório Clínico do Hospital Universitário, Universidade de São Paulo, São Paulo, SP, Brazil*
[c] *Faculdade de Zootecnia e Engenharia de Alimentos, Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, São Paulo, SP, Brazil*

HIGHLIGHTS

- Micromanipulation for separation of single cysts of *G. duodenalis*.
- New set of primers for the detection of *G. duodenalis* assemblages A and B.
- Simultaneous detection of four genes of single cysts of *G. duodenalis*.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 April 2015
Received in revised form
20 June 2015
Accepted 8 July 2015
Available online 11 July 2015

Keywords:
Giardia duodenalis
Cysts
Multilocus PCR
Micromanipulation

ABSTRACT

Giardia duodenalis is divided into at least eight groups, named assemblages A to H. Assemblages A and B are the only ones able to infect humans and other mammals. The species status for these assemblies is a moot point, but has not gained general acceptance because sexual activity in Giardia is not completely understood. Heterozygosity in *G. duodenalis* can be detected through simultaneous identification of multiple loci in single cysts or trophozoites. In this paper, we describe a technique that enables simultaneous detection of fragments from four genes from single cysts of *G. duodenalis* recovered from stool samples. Each cyst from a fecal sample of human origin was separated, the DNA was extracted and amplified by means of multiplex PCR directed to four genes and the multiplex PCR product was further re-amplified using four single PCR (one for each gene). The following loci were detected: beta giardin (bg), GLORF-C4 (orfC4), triose phosphate isomerase (tpi) and glutamate dehydrogenase (gdh). This procedure should make it possible to investigate multiple genes from a single cyst of *G. duodenalis* assemblage A or B.

© 2015 Elsevier Inc. All rights reserved.

Giardia duodenalis (synonyms: *Giardia intestinalis* and *Giardia lamblia*) is an enteric parasite that affects a large variety of domestic and wild animals and also humans (Ryan, Caccio, 2013). *G. duodenalis* has been described as a complex species in which the isolates

* Corresponding author. Av. Prof. Dr. Orlando Marques de Paiva, 87, 05508-270, São Paulo, SP, Brazil.
E-mail address: rosoares@usp.br (R.M. Soares).

http://dx.doi.org/10.1016/j.exppara.2015.07.004
0014-4894/© 2015 Elsevier Inc. All rights reserved.

present few morphological variations but can be differentiated into at least eight genetic groupings called assemblages (A to H) (Caccio, Ryan, 2008). Assemblages A and B are the only ones capable of infecting not only humans but also other mammals, and are therefore considered potentially zoonotic (Lalle et al., 2005; Sprong et al., 2009). The other assemblages seem to be confined to specific hosts. Several genetic markers have been used to differentiate the assemblages of *G. duodenalis* (Ryan, Caccio, 2013), and the commonest of these comprise investigations of molecular diversity: small subunit ribosomal RNA; beta-giardin (bg); glutamate dehydrogenase (gdh); elongation factor 1-α; triose phosphate isomerase (tpi); GLORF-C4 (orfC4); spacer region of ribosomal RNA; ferredoxin; histone H2B; histone H4; and ribosomal protein L7a (Monis et al., 1999; Caccio et al., 2002; Lasek-Nesselquist et al., 2009; Almeida et al., 2010).

In the present study, we describe a protocol that enables molecular identification of *G. duodenalis* cysts individually. Each cyst in a fecal sample is separated out and its DNA is extracted and then amplified by means of multiplex PCR directed towards four genes. Following this, the product from the multiplex PCR is re-amplified using four different PCRs (one for each gene), in nested PCR format. This technique makes it possible to study the presence of gene sequences originating from a single cyst and thus to ascertain whether this cyst has different copies from a single allele, with the possibility of elucidating questions relating to allele sequence heterozygosity and gene recombination.

Two feces samples of human origin that were positive for *G. duodenalis*, from individuals without any clinical symptoms of the infection, were used. The Ethics Committee of the University Hospital of the University of São Paulo had given approval for usage of the clinical samples. Each fecal sample was subjected to the technique of centrifugation-floatation in sucrose (modified Sheather technique) (Sheather, 1923) described elsewhere (Souza et al., 2007).

The cysts were recovered on a sterile Petri dish, after washing the slide and cover slip that had been used in observing the cysts, using 1.5 mL of TE buffer (10 mM Tris-HCL pH 8.0; 1 mM EDTA pH 8.0). The product thus obtained from washing was transferred from the dish to a microtube of capacity 1.5 mL and this was then centrifuged at 12,000 g for 10 min. The supernatant was discarded, 1 mL of the same buffer was added and centrifugation was performed again under the same conditions as before. The supernatant was again discarded and the sediment containing the *G. duodenalis* cysts was immediately subjected to a micromanipulation technique.

This micromanipulation was performed with the aid of an inverted Nikon Eclipse TS100 microscope, sterile Petri dishes and a device constructed in the laboratory for this study, composed of the following materials: microhematocrit capillary tube with the tips tapered by means of heat treatment (molded in the same way as a Pasteur pipette), filter for syringe with pore size of 0.22 μm, tubing for serum and tip of 20 μL with filter. Aspiration was done with the help of a P20 micropipette (P20 Pipetman®, Gilson Inc, WI, USA).

Approximately 20 μL of each sediment containing the parasite cysts recovered as described above were diluted in a drop of 20 μL of TE placed on a sterile Petri dish. This procedure was repeated successively in serial dilutions while maintaining the same proportions (volume/volume), until a drop of 20 μL was obtained at a concentration that would result in microscope fields with very low cyst density (one cyst for every five microscope fields examined). The observations were made using an inverted microscope at a magnification of 200×. Each cyst was aspirated and transferred to a microtube for PCR with a capacity of 200 μL that already contained 5 μL of TE. Each cyst was aspirated with a volume of approximately 1 μL of TE. Thus, each 200 μL tube that was used for cyst recovery contained a final volume of 6 μL of TE.

DNA extraction was performed in the same microtubes in which the *G. duodenalis* cysts had been isolated. A further 5 μL of TE were added to the 6 μL of TE suspension containing the individualized cyst, and the suspension was then frozen at a temperature of −20 °C for 24 h. This was then defrosted at room temperature and 1 μL of proteinase K (20 mg/ml) was added. The suspension was subjected to two incubation periods: firstly for 12 h at 37 °C and secondly for 15 min at 95 °C.

Four pairs of primers were used simultaneously for amplification of fragments of the genes gdh, tpi, orfC4 and bg (Table 1). The primers were designed on sites that are common to sequences of *G. duodenalis* assemblages A and B that are available in GenBank. Primers to orfC4 and tpi are at the same binding sites as primers described elsewhere (Yong et al., 2002; Sulaiman et al., 2003). Primers to orfC4 are based on consensus sequences to AF293413, XM001704865, AJ291756, and M90390.

The following reagent mix was added to each microtube from which the cysts had been extracted (containing 11 μL of TE and digested cyst): 23.2μL of ultrapure water; 1 μL of dNTP (10 mM of each nucleotide); 5 μL of 10× PCR buffer (Platinum® Taq Polymerase, Invitrogen); 1 μL of each primer (sense and antisense) (10 pmol/μL); 1.5 μL of $MgCl_2$ (50 mM); and 0.3 μL Taq DNA Polymerase (5 U/μL) (Platinum® Taq Polymerase, Invitrogen).

Following this, four nested PCRs were performed using samples of 5 μL of the product obtained from multiplex PCR, which had previously been treated using enzymes of the ExoSAP-IT kit (USB® ExoSAP-IT® PCR product cleanup), in accordance with the manufacturer's recommendations. This was done in order to be able to eliminate residues of unincorporated nucleotides, primers and other molecules from single-strand nucleic acids.

Each nested PCR was performed in a different microtube. The primers used in the nested PCRs were same as those used in the multiplex PCR. The following reagent mix was added to each microtube: 29.2μL of ultrapure water; 1 μL of dNTP (10 mM of each nucleotide); 5 μL of 10× PCR buffer (Platinum® Taq Polymerase, Invitrogen); 1 μL of each primer (sense and antisense) (10 pmol/μL); 1.5 μL of $MgCl_2$ (50 mM) and 0.3 μL of Taq DNA polymerase (5 U/μL) (Platinum® Taq Polymerase, Invitrogen).

The thermocycling conditions for all the reactions were: initial denaturing at 95 °C for 2 min, followed by 35 cycles of denaturing at 95 °C for 30 s, hybridization at a temperature of 60 °C for 20 s and extension at 72 °C for 20 s; and a final extension at 72 °C for 7 min. The cycle was performed in Veriti™ Thermal Cycler equipment (Applied Biosystems).

The amplified products were viewed by means of the technique of electrophoresis on 1.5% agarose gel stained using a 0.5 μg/mL solution of ethidium bromide as described (Sambrook and Russel, 2001).

Table 1
Primers used for amplifying *Giardia duodenalis* cysts that have been individualized using the micromanipulation method.

Name[a]	Length[b]	Sequence[c]
TPI16F22	520	CCCTTCATCGG**I**GGTAACTTCA
TPI515R21		ACGCCCGTGCC**R**AT**R**GACCACA
ORFIV231F20	433	GCTCATC**W**TCGTCCTCTAGC
ORFIV644R20		GCATACGA**Y**GC**R**ACGACCTT
GDH418F21	550	GTCATGCGCTTCTGCCAGTCC
GDH947R21		CGCA**R**GGCATGATGCAATCCA
BG268F22	383	ATCAAGGAGGAGATCGACACCA
BG633R18		ATCTCCGAGGCGACGTTC

[a] Names of primers. F and R between numbers refers to Forward and Reverse, respectively.
[b] Expected lengths of base pairs in the fragments amplified.
[c] Primer sequences. The positions identified with the letters W, R and Y constitute degenerated positions, following the IUPAC code. The letter I corresponds to inosine. BG: beta giardin. ORFIV: GLORF-C4. TPI: triose phosphate isomerase. GDH: glutamate dehydrogenase.

Download English Version:

https://daneshyari.com/en/article/6290813

Download Persian Version:

https://daneshyari.com/article/6290813

Daneshyari.com