

PCR amplification of triosephosphate isomerase gene of *Giardia lamblia* in formalin-fixed feces

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ABSTRACT. *Giardia lamblia* (syn *G. intestinalis*, *G. duodenalis*) is the intestinal protozoan producing non-bacterial diarrhea most prevalent in the world. PCR genotype classification of *Giardia* in feces depends on the quality and quantity of purified DNA and the removal of a great number of inhibitors. The aim of this study was to adapt a PCR protocol to the amplification of the triosephosphate isomerase (*tpi*) gene of *Giardia lamblia* in formalin-fixed feces. The *tpi* gene of *G. lamblia* was amplified in 28 of the 34 analyzed samples (82.35%) and the B genotype was obtained in all cases. Two major modifications were implemented to improve the performance of PCR from formulated fecal matter. One of these improvements was the use of polyvinylpyrrolidone (PVP) and the other was the addition of bovine serum albumin (BSA). The PCR protocol used in this study showed an amplification percentage exceeding the values reported by other authors with high sensibility and specificity.

Key words: *Giardia lamblia*, PCR, genotype, formalin fixed feces.

RESUMEN. *Giardia lamblia* (sin. *G. intestinalis*, *G. duodenalis*) es el protozoo intestinal productor de diarrea no bacteriana diagnosticado con más frecuencia en todo el mundo. La genotipificación de *Giardia* por PCR a partir de materia fecal depende de la calidad y cantidad del ADN purificado y de la eliminación de una gran variedad de inhibidores. El objetivo de este trabajo fue adaptar un protocolo de PCR para la amplificación del gen *tpi* de *Giardia lamblia* en materia fecal formolada. El gen *tpi* de *G. lamblia* amplificó en 28 de las 34 muestras analizadas (82.35%) y en todos los casos se obtuvo el genotipo B. Dos modificaciones se implementaron para mejorar el rendimiento de la PCR a partir de materia fecal formolada. Una fue el uso de polivinilpirrolidona (PVP) y la otra fue el agregado de albúmina sérica bovina (BSA). El protocolo de PCR utilizado en este trabajo presentó un porcentaje de amplificación que superó los valores reportados por otros autores, con una alta sensibilidad y especificidad.

Palabras clave: *Giardia lamblia*, PCR, genotipo, heces formoladas.

INTRODUCTION

Giardia lamblia (syn *G. intestinalis*, *G. duodenalis*) is one of the most common intestinal parasites in the world. It primarily affects children in both developed and developing countries.¹⁰ The prevalence varies between 2% and 5% in industrialized countries and may exceed 30% in developing countries.¹¹ In 1988, the World Health Organization (WHO) estimated that around 280 million people are annually infected with *Giardia* spp in Asia, Africa and Latin America.²⁰

The present taxonomy of *Giardia* is based on morphological characteristics but does not reflect genetic and phenotypic heterogeneity.¹ DNA analysis and isoenzyme profiles indicate that *Giardia lamblia* is a complex species and exhibits a high genetic diversity.^{15,26} Two evolution-marking genes are known: slow (ribosomal RNA and alpha elongation factor), used to distinguish the main genotypes, and fast (triosephosphate isomerase, glutamate dehydrogenase, and beta giardin), to identify different genotypes and sub-genotypes. *Giardia* isolations identified as human pathogens are classified into two major groups named genotype A and genotype B; *Polish* and *Belgian* or Groups 1/2 and Group 3, respectively.¹⁷

The PCR-RFLP analysis of the triosephosphate isomerase (*tpi*) gene permits to distinguish between genotypes A (I and II) and B of *Giardia*.^{2,5}

Genotype classification of *Giardia* by PCR from feces depends on the quality and quantity of purified DNA and removal of a great variety of inhibitors such as biliary salts, bilirubin, urobilinogen, hemoglobin, pollen, cellulose, complex polysaccharides, and phenolic compounds which may inhibit the activity of polymerases even in low concentrations.^{4,18,25,29} In Argentina, it is frequent that the collection of feces for parasitologic analysis be done seri-

Abbreviations: PCR, polymerase chain reaction; *tpi*, triosephosphate isomerase; PVP, Polyvinylpyrrolidone.

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ally with preservatives such as PAF (phenol-alcohol-formalin), SAF (sodium acetate in formalin) or 10% formalin. Therefore, the presence of formalin may inhibit polymerases and react with DNA, thus making the subsequent amplification difficult and resulting in false negatives.^{8,9,16,28}

The objective of this study was to adapt a PCR protocol for the amplification of the *tpi* gene of *Giardia lamblia* in formalin-fixed feces.

MATERIAL AND METHODS

Fecal Samples and PCR

Thirty four samples of human feces positive for *G. lamblia* detected by optical microscopy and belonging to residents of a rural community in General Mansilla, province of Buenos Aires, Argentina, were selected. Each individual's feces were collected in a container with 10% formalin for 5 days and stored at room temperature until processed. Cysts were concentrated in sucrose gradient,²¹ counted in Neubauer chamber and kept at 4°C. The cyst rupture was done in 3 cooling/heating cycles (-80°C, 30 minutes/+80°C, 30 minutes) and 15 minutes at 100°C. Later, they were incubated for 24 hours at 60°C with lysis buffer E (Tris-HCl 100 mM, EDTA 100 mM, SDS 2%, NaCl 0.2 M, Mercaptoethanol 1 mM, proteinase K 1 mg/ml) in equal parts.^{7,23} The samples were kept at -20°C. DNA was purified by QIAamp DNA Stool minikit (Qiagen®) according to the manufacturer's protocol and preserved at -20°C.

A heminested PCR was done to amplify the *tpi* gene of *G. lamblia*, using the primers designed by Amar et al.⁵ (Table 1). After optimization of PCR with primers set for MgCl₂ concentration and annealing temperature the following conditions were found to be optimal for both PCRs.

First step: PCR mixtures (30 µl) contained buffer PCR 1X, MgCl₂ 3 mM, dNTPs 0.25 mM, primers (TPIA-FI, TPIA-R, TPIB-FI y TPIB-R) 0.25 µM each, bovine serum albumin (BSA) 0.1 µg/µl, Taq DNA polymerase 0.1 U/µl and 3 µl DNA purified. All reactions involved an initial denaturation step at 94°C/4 minutes, 30 cycles of 94°C/30 seconds, 52°C/30 seconds, 72°C/1 minute, and a final extension of 72°C/10 minutes.

Second step: PCR A and B mixtures (30 µl) contained PCR 1X, MgCl₂ 1.5 mM, dNTPs 0.25 mM, primers 1 µM each, BSA 0.1 µg/µl, Taq DNA polymerase 0.05 U/µl and 2 µl amplicons (10⁻³). This assay used TPIA-FII and TPIA-R primers for the amplification of genotype A (PCRII A), and TPIB-FII and TPIB-R primers for genotype B (PCRII B). All reactions involved an initial denaturation step at 94°C/4 minutes and 33 cycles of 94°C/30 seconds, 54°C/30 seconds, 72°C/1 minute with a final extension of 72°C/10 minutes. Positive DNA control genotypes A and B (1 ng/µl) courtesy of Dr. Van Keulen, Cleveland University, and bidistillate water as negative control were used in each PCR round.

PCRII products were analyzed by electrophoresis in 1.5% agarose gel. (Labnet Transilluminator®, TM-26). Results were analyzed by the Mann-Whitney Test.

DNA Purification with PVP

The DNA of the samples positive for *Giardia* spp by optical microscopy and not amplified was treated with PVP according to the methodology by Lawson et al.¹⁶ Briefly, 50 µl DNA were incubated with 150 µl PVT-TE (10% PVP in buffer TE) at room temperature for 10 minutes. DNA was precipitated with 100 µl ammonium acetate 2 M and 600 µl isopropanol for 30 minutes at -20°C. The obtained DNA was kept at -20°C so as to perform the PCR again.

Table 1. Primers sequences and amplicons of PCR.⁵

	Genotype A	Genotype B
First step	TPIA-FI: CGAGACAAGTGTGAGATG	TPIB-FI: GTTGCTCCCTCCTTTGTGC
	TPIA-R: GGTCAAGAGCTTACAACACG	TPIB-R: CTCTGCTCATTGGTCTCGC
Products	576 bp	208 bp
Second step	TPIA-FII: CCAAGAAGGCTAAGCGTGC	TPIB-FII: GCACAGAACGTGTATCTGG
	TPIA-R: GGTCAAGAGCTTACAACACG	TPIB-R: CTCTGCTCATTGGTCTCGC
Products	476 bp	140 bp

Specificity analysis

DNA from 3 human fecal samples negative for *Giardia* spp, containing *Blastocystis hominis*, *Chilomastix mesnili* and *Entamoeba coli* was purified. PCR protocol was followed as previously described.

Sensitivity analysis

Serial decimal dilutions of genotype-B reference DNA (initial concentration 100 ng/μl) were carried out as well as a PCR round with the protocol described above.

Table 2. Number of cysts/mm³ of *Giardia* in formalin-fixed feces obtained after purification with sucrose, treatment of DNA with PVP and genotype obtained with PCR. (-) without treatment, (+) with treatment, ND not detected.

Code of feces sample	Number of cysts/mm ³	PVP	Genotype
1	11	(-)	B
2	485	(-)	B
4	27	(+)	ND
6	140	(-)	B
7	75	(+)	B
8	13	(-)	B
9B	80	(-)	B
20	30	(-)	B
21	25	(-)	B
22	90	(-)	B
23A	190	(-)	B
23B	80	(-)	B
26B	90	(+)	B
28	80	(+)	ND
32	7	(+)	B
34	6	(-)	B
39	6	(-)	B
37A	865	(-)	B
37B	75	(+)	ND
40	565	(-)	B
44	75	(-)	B
45	42	(-)	B
46	460	(+)	ND
48	28	(-)	B
49	30	(-)	B
50	175	(-)	B
51	230	(-)	B
53	75	(-)	B
54	90	(+)	B
56	11	(+)	ND
57	450	(-)	B
58	20	(-)	B
60	200	(-)	B
61	33	(+)	ND

Amplification of reference DNA in human feces

Four decimal dilutions of genotype-A and genotype-B reference DNA (initial concentration 100 ng/μl) were performed. Each dilution was mixed in equal parts with DNA from a human fecal sample positive for *G. lamblia* by optical microscopy and negative for PCR (PCR negative feces). The eight samples of both DNAs were used to amplification with the same protocol. Three controls were used: a) reference DNA, b) DNA from PCR negative feces and c) bidistillate water.

Amplification of reference DNA in human feces

DNA from 3 human fecal samples PCR positive (codes: 1, 23A and 57) were each one mixed with DNA from PCR negative feces and used to amplification with the same protocol.

Reproducibility

The amplification of a DNA sample of human feces positive for *Giardia* by PCR was done seven-fold. DNA extraction and PCR protocol were done as previously described.

RESULTS

Amplification

The *tpi* gene of *G. lamblia* was amplified in 28 of the 34 samples analyzed (82.35%). The samples analyzed turned out to be genotype B and an expected product (140 bp) was observed in all of them (Fig. 1). The number of

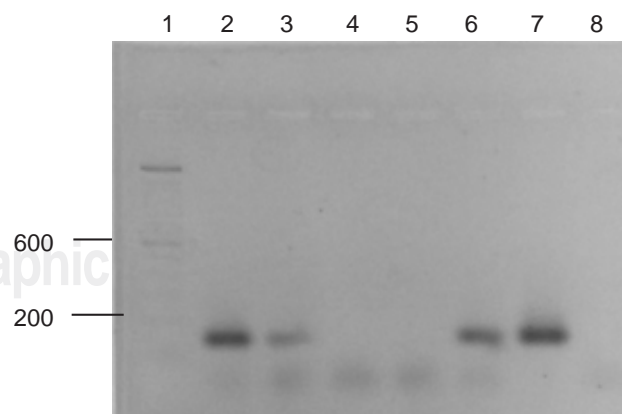


Figure 1. Agarose gel stained with ethidium bromide corresponding to PCR// B: Line 1 Molecular weight marker (100 bp), 2-6 DNA from human feces 2, 34, 28, 26B (without PVP), 26B (post-PVP), 7 positive control, 8 negative control.

cysts/mm³ of *G. lamblia* concentrated by the sucrose technique in human feces samples ranged from 6 to 865 (Table 2). They showed quite an asymmetric distribution, with a median of 54 in the samples that did not amplify and 77.5 in those that did. When the influence of the number of cysts in the sample amplification was evaluated, no significant differences were observed between the number of cysts/mm³ and the result of the amplification (Mann-Whitney Test, $p=0.619$). The genotype-A reference DNA could only be amplified with "A" primers (TPIA FI, FII and R) and not with "B" primers (TPIB FI, FII and R). Likewise, genotype-B reference DNA was only amplified with "B" primers and not with "A" primers.

PVP treatment

Of the 34 analyzed samples, 24 amplified with the PCR protocol without pre-treatment. Ten samples received the treatment with PVP and only 4 of them could be amplified. The 4 samples analyzed post-PVP turned out to be genotype B (Fig. 1).

Specificity assay

No amplification product was obtained after PCR with the DNA from human feces with *B. hominis*, *C. mesnili* and *E. coli*.

Sensitivity assay

The PCR assay showed high sensitivity since it amplified A and B reference DNA from *Giardia* up to 1 pg/ μ l.

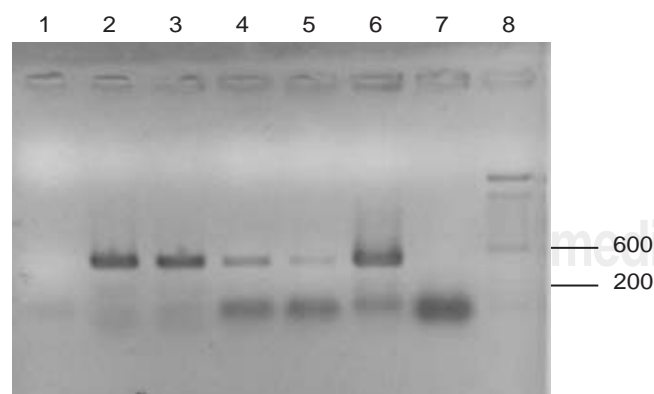


Figure 2. Amplification of genotype A reference DNA in PCR negative feces: Line 1 PCR negative feces DNA, 2-5 PCR negative feces DNA with genotype A DNA in different concentrations (2 1 ng/ μ l, 3 0.1 ng/ μ l, 4 10 pg/ μ l, 5 1 pg/ μ l), 6 positive control, 7 negative control, 8 molecular weight marker.

Amplification of reference DNA

Both genotypes reference DNA (A or B) mixed with DNA from PCR negative feces amplified in all the assayed dilutions. No amplification product was observed when DNA from PCR negative feces or bidistillate water were used (Fig. 2 and 3).

Amplification of reference DNA in human feces

Amplification products (genotype B) were obtained after each PCR.

Reproducibility

The PCR technique performed showed a reproducibility of 85.71%.

DISCUSSION

Use of PCR for microorganism detection in biologic samples is conditioned, partly, by the presence of substances inhibiting PCR or reducing amplification efficiency. PCR inhibitors may act in 3 levels: interference in the cellular lysis, degradation or uptake of nucleic acids, or inactivation of thermostable polymerases.³ The fecal samples for molecular method diagnosis are generally collected without preservatives since these reduce the sensitivity of the techniques due to DNA degradation. Therefore, the use of fresh feces would prevent the false negative results.^{9,28} In our laboratory, the samples intended for copro-parasitologic study were serially collected in a container for 5 days, so the use of formalin as preservative was necessary. In this study, the inhibiting substances present in the feces were absorbed in the Inhibitex (Qiagen®) matrix and

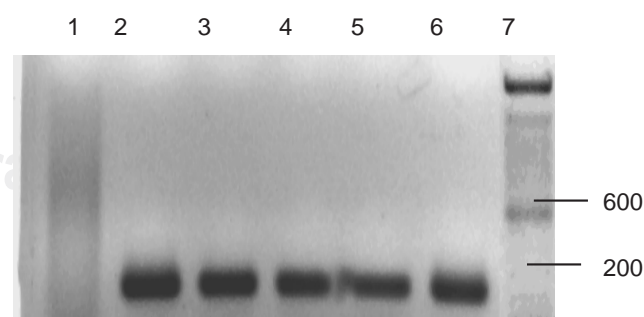


Figure 3. Amplification of genotype B reference DNA in PCR negative feces: Line 1 PCR negative feces DNA, 2-5 PCR negative feces DNA genotype B DNA in different concentrations (2 1 ng/ μ l, 3 0.1 ng/ μ l, 4 10 pg/ μ l, 5 1 pg/ μ l), 6 positive control, 7 molecular weight marker.

removed in the extraction process. Nevertheless, 10 samples did not amplify. Several authors suggest ensuing purification treatments to remove inhibitors.^{6,14,16}

Two major modifications were implemented in order to improve the performance of PCR from human feces samples. One of them was the use of PVP, which permitted the amplification in 40% (4/10) of the treated samples. The mechanism by which PVP removes PCR inhibitors from DNA is not precisely known, but it is suggested that PVP absorbs phenolic compounds which covalently bind to DNA.¹⁶ The other was to add bovine serum albumin (BSA), which increased the reaction efficiency in formalin fecal samples, improved the specificity of PCR and the fidelity of DNA synthesis.^{4,9,19} In spite of these modifications, 6 (17.65%) of the 34 samples analyzed could not be amplified.

The failures in the amplification would derive from the low quality DNA of the samples, either due to their degrading in time or because of chemical modifications caused by several substances, formalin among them.^{9,28,29} Also, these failures could be the result of the fact that the proportion of DNA in the feces samples was not enough to counteract the effect of the inhibitors that would have co-purified with the nucleic acids. Regarding this, Ghosh et al.¹³ were able to amplify an intergenic region of the ribosomal RNA gene of *Giardia* from only 10 trophozoites in culture, but they needed 2000 when mixing them with human feces.

When the relationship between the number of cysts/mm³ in the processed samples and the PCR amplification was evaluated, the median of the group of amplified samples was observed to be different from the group that did not amplify, but the difference was not significant. These results differ from those found by Amar et al.,⁵ who obtained differences between the percentage of positive PCRs and the number of cysts/mm³.

Our amplification percentage (82.35%) exceeded the values reported by several authors. El-Shazly et al.¹² reported an amplification percentage of 73.3% when working with fresh feces. Troll et al.²⁸ amplified the 16rRNA gene of *Entamoeba histolytica* in 19 feces samples with SAF and reported an amplification percentage of 36.8% (7/19). Traub et al.²⁷ analyzed 29 feces samples preserved in either potassium dichromate or formalin and obtained amplification of the *Giardia tpi* gene in 55% (16/29) of the human feces and 35% in canine feces. On the other hand, Amar et al.,⁵ working with fresh feces, reported the following amplification percentages for the *tpi* gene: 94% in sporadic giardiasis, and 88% in a giardiasis outbreak in a neonatology service. However, when the same group of researchers analyzed cysts taken from stained preparations, they obtained 59% of amplification due, according to the authors themselves, to the low number of cysts present in those preparations.

The primers used in this reaction were highly specific for genotypes A and B of *G. lamblia*. Besides, no amplification was observed with DNA from samples containing *Blastocystis hominis*, *Chilomastix mesnili* and *Entamoeba coli*.

The analytical sensitivity published for each specific primer of *G. lamblia* has a range of 1 to 10 cysts per reaction mix.^{13,24} From the amplification of the glutamate dehydrogenase gene (*gdh*), Read et al.²² obtained a sensitivity of 2 pg/μl. By using a region of the ribosomal RNA gene, Ghosh et al.¹³ reported a sensitivity of 2.5 pg/μl. Like other authors, our PCR assay had high sensitivity (1 pg/μl).

When the reference DNA was added to the PCR negative feces, an amplification of both genotypes in all the assayed dilutions was obtained. This could be due to the fact that the added DNA had better quality and purity, or to the dilution of the inhibitors present in the feces. According to Al-Soud and Rådström,⁴ adding better-quality DNA, or a higher quantity of it, would permit to reduce the effect of the inhibitors interfering with the template DNA or reversibly attaching to the polymerases action site. It is agree with the results obtained when DNA from PCR positive samples was added to PCR negative samples.

The reproducibility achieved by our work group exceeded the one reported by Amar et al.,^{5,6} who obtained 77% reproducibility with DNA from fresh feces. The sub-optimal reproducibility in this study could have been caused by the PCR inhibitors in the feces co-purified with DNA, by the existence of DNA of intestinal flora, by a low quantity of template DNA in the samples, or by the degradation of DNA during storage.⁶ New assays of DNA extraction are being explored to improve reproducibility.

In this study, a PCR protocol was adapted for its application in formalin-fixed feces. The modifications concerning this technique were the extraction of DNA with a commercial method to achieve higher pureness of DNA, ensuing purification with PVP for the samples that did not amplify, and the addition of BSA as reaction facilitator to the mixture.

The non-amplification might be due to the lower quality of template DNA, to the PCR inhibitors, or to the presence of local genotypic variants of the *tpi* gene not detected with the primers used. However, the PCR used in this study showed an amplification percentage exceeding the values reported by other authors. Besides, this PCR assay had high sensitivity and specificity. Therefore, the PCR amplifying the *tpi* gene of *Giardia lamblia* in formalin-fixed feces would be of great application in our environment.

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