Identification of the assemblages A and B of *Giardia duodenalis*

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1. Aim and field of application

To determine the identity of cysts of the protozoan *Giardia duodenalis* belonging to the two assemblages A and B infecting humans, by a PCR analysis. This method can be applied to faecal material of human and animal origin known to be positive for the presence of *Giardia* cysts.

2. Principle of the method

The PCR is a molecular biology technique that allows for the amplification of specific nucleic acid fragments, of which the initial and terminal nucleotide sequences are known (oligonucleotide pair). If a species (or genotype) has its own characteristic DNA portion, due to its composition and/or dimension, it is possible to choose an oligonucleotide pair allowing for its amplification. The PCR amplification is characterized by a high sensitivity and specificity.

The protozoan parasites of the genus *Giardia* infect the upper part of the small intestine of vertebrates, including humans. The parasite's life cycle consists of a vegetative stage, the trophozoite, a teardrop-shaped binucleated cell, which divides by binary fission and colonizes the host intestine, and the tetranucleated cyst, the infective and resistant stage, which is able to survive outside of the host. Infection is acquired by cysts ingestion that undergoes excystation into trophozoites in the proximal small intestine after the exposure to the acidic environment of the stomach. Six species have been described based on the host specificity, the morphology and the phenotype: *Giardia agilis* in amphibians, *G. muris* and *G. microti* in rodents, *G. ardeae* and *G. psittaci* in birds, and *G. duodenalis* (syn. *G. lamblia* and *G. intestinalis*) in mammals. *Giardia duodenalis* is the causative agent of giardiasis, and it is the only species infecting both humans and other mammals, including livestock and companion animals. Seven morphologically indistinguishable Assemblages of *Giardia duodenalis* (referred to as Assemblages A to G) have been described, which can be identified based on the genetic analysis. Only Assemblages A and B have been isolated from humans and a wide panel of mammals, whereas the other Assemblages (C-G) have host specificity and are not infectious for humans (Monis et al., 1999; Monis et al., 2003; Sulaiman et al., 2003).

Molecular methods based on PCR have allowed the identification at the Assemblage level *G. duodenalis* cysts present in human and animal faecal samples. The method is based on the amplification of a portion of the genetic locus 4E1-HP that produces two PCR products of different size depending on the assemblage present in the sample (Vanni et al., 2012).

The expected sizes of the fragments produced by PCR are shown in Table A.

Table A - Size (in base pairs) of the 4E1-HP PCR fragments expected for the Assemblages A and B of G. duodenalis.

Assemblage A	Assemblage B
165	**272**

3. References

Monis, P.T., Andrews, R.H., Mayrhofer, G., Ey, P.L. (1999) Molecular systematics of the parasitic protozoan *Giardia intestinalis*. Mol Biol Evol. 16, pp. 1135-1144.

Monis, P.T., Andrews, R.H., Mayrhofer, G., Ey, P.L. (2003) Genetic diversity within the morphological species *Giardia intestinalis* and its relationship to host origin. Infect Genet Evol. 3, pp. 29-38.

Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H., Trout, J.M., Schantz, P.M., Das, P., Lal, A.A., Xiao, L. (2003) Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. Emerg Infect Dis. 9, pp. 1444-1452.

Vanni I, Caccio` SM, van Lith L, Lebbad M, Svard SG, et al. (2012) Detection of *Giardia duodenalis* Assemblages A and B in Human Feces by Simple, Assemblage-Specific PCR Assays. PLoS Negl Trop Dis 6(8): e1776.

Qiagen: QIAamp DNA Stool Handbook, Second edition April 2010.

4. Definitions

4E1-HP, genetic locus corresponding to a coding sequence for a hypothetical protein with a high variability between the assemblage A and assemblage B of *G. duodenalis.*

Oligonucleotide, short sequence (15/30 nucleotide bases) used to amplify a specific DNA fragment.

Set11, mix of 4 oligonucleotides amplifying a fragment of the 4E1-HP locus from A and B assemblages of *G. duodenalis*.

Positive control for the DNA extraction, aliquots of faeces containing cysts of *G. duodenalis* Assemblages analysed in the same working session of test samples, to verify the efficacy of the DNA extraction session.

Reference faecal DNA, purified genomic DNA from faeces containing cysts of *G. duodenalis* Assemblage A.

Positive control for the amplification, purified genomic DNAs of *G. duodenalis* Assemblage A and Assemblage B; these controls are used in the amplification session to verify the efficacy of the PCR.

Negative control for the amplification, reagent grade water; this control is used in the amplification session to verify the efficacy of the PCR.

PCR, Polymerase Chain Reaction.

The definitions and terminology used in the UNI EN ISO 22174 standard are applied in the present protocol.

5. Devices/instruments

5.1 Bench top refrigerated centrifuge for 1.5 mL tubes, minimum 20,000xg

5.2 Freezer ≤-15°C

5.3 Thermomixer with vibration, temperature range 25÷100°C

5.4 PCR thermocycler

5.5 Refrigerator, temperature range +1 - +8 °C

5.6 Horizontal electrophoretic apparatus

5.7 Digital imaging system

5.8 Adjustable volume pipettes, volume range: 1-10µL, 2-20µL, 20-100µL, 50-200µL, 200-1000µL

5.9 Analytical grade water system production, resistivity ≥ 18 Mohm /cm

5.14 Vortex

5.10 Analytical balance, readability 0.1g

5.11 UV transilluminator

5.12 Orbital shaker

6. Reagents and chemicals

6.1 Lysis buffer. Commercial solution: QIAamp DNA Stool Handbook, QIAGEN, identified as "ASL" buffer. Store at room temperature.

6.2 InhibitEX Tablet. Commercial solution: QIAamp DNA Stool Handbook, QIAGEN, identified as "ASL" buffer. Store at room temperature.

6.3 Proteinase K. Commercial solution: QIAamp DNA Stool Handbook, QIAGEN, identified as "ASL" buffer. Store according to manufacturer's instruction.

6.4 Tampone di lisi. **Lysis buffer.** Commercial solution: QIAamp DNA Stool Handbook, QIAGEN, identified as "AL" buffer. Store at room temperature.

6.5 Ethanol (96–100%). Commercial solution.

6.6 Binding Column. Commercial solution: QIAamp DNA Stool Handbook, QIAGEN, identified as QIAmp Mini Spin Columns.

6.7 Collection tube. Commercial solution: QIAamp DNA Stool Handbook, QIAGEN, identified as Collection tube (2 mL).

6.8 Washing buffers. Commercial solutions: QIAamp DNA Stool Handbook, QIAGEN, to be prepared according to manufacturer's instruction and identified as 'AW1'e'AW2'. Store at room temperature.

6.9 Eluting buffer. Commercial solution: QIAamp DNA Stool Handbook, QIAGEN, identified as "AE" buffer. Store at room temperature.

6.10 PCR master mix. 2x commercial solution, Promega, codes: M7501, M7502, M7505 (composition: dATP 400 µM, dCTP 400 µM, dGTP 400 µM, dTTP 400 µM, MgCl2 3mM, Taq DNA polymerase 50 U/mL), other commercial PCR master mixes should be considered suitable for PCR amplification (i.e., Qiagen HotStarTaq Master Mix Kit). Store according to the manufacturer's recommendations.

6.11 Set11. The oligonucleotide mixture (6.12) used for the PCR; the mixture is obtained combining an equal volume of the 4 oligonucleotides 4E1-HP A For, 4E1-HP A rev, 4E1-HP B For and 4E1-HP B Rev (Table B). The final concentration corresponds to 10 pmol/µL each; 100µL aliquots are prepared and stored frozen up to 24 months.

6.12 Oligonucleotides. Commercial preparation (Table B); the lyophilized products is reconstituted with analytic grade water, according to the manufacturer's recommendations, at a concentration of 100 pmol/µL; the lyophilized product can be stored frozen for up to 5 years; the reconstituted product can be stored frozen up to 24 months.

Table B – Oligonucleotides present in the set11 (6.11), their codes and amplified nucleotide sequences.

Oligonucleotide sequence	Code	Amplified sequence
5'-AAAGAGATAGTTCGCGATGTC-3' 5'-ATTAACAAACAGGGAGACGTATG-3' 5'-GAAGTCATCTCTGGGGCAAG-3' 5'-GAAGTCTAGATAAACGTGTCGG-3'	4E1-HP A For 4E1-HP A Rev 4E1-HP B For 4E1-HP B Rev	4E1-HP

6.13 Loading buffer 6x. Commercial product allowing DNA molecule electrophoresis to be performed. Store according to the manufacturer's recommendations.

6.14 Agarose. Commercial products suitable for performing DNA molecule electrophoresis. Store at room temperature for up to 24 months.

6.15 TAE solution 50x. Commercial product (2M Tris-acetate, 50mM EDTA, pH 8.2–8.4 at 25°C). Store

according to the manufacturer's recommendations.

6.16 TAE solution 1x. 1000 mL preparation: take 20 mL of the 50x solution and bring to 1000 mL with water. Store at room temperature for up to 1 month.

6.17 Ethidium bromide solution. Commercial product 10 mg/mL. For the working condition, dilute 1:100,000; for 100 mL solution, add 1.0 µL. Store according to the manufacturer's recommendations.

NOTE: Ethidium bromide is potentially mutagenous, carcinogenic and teratogenic; wear disposable gloves and handle the solution containing this substance very carefully.

6.18 L50. Commercial product containing markers for DNA molecular weight multiple of 50 bp. All commercial products containing molecules of multiples of 50 bp within the 50-500 bp range can be used. Store refrigerated according to manufacturer's recommendations.

6.19 Milli-Q grade water. Resistivity ≥ 18 Mohm/cm.

6.20 Reference faecal sample for the DNA extraction, aliquots of faeces containing cysts of *G. duodenalis* Assemblage A analysed in the same working session of test samples, to verify the efficacy of the DNA extraction session. Store in refrigerator (5.5) for up to **2 years** or frozen (5.2) for up to **5 years**.

6.21 Reference DNAs, purified genomic DNAs from cultured trophozoites of *G. duodenalis* WB strain (Assemblage A) and GS strain (Assemblage B). Store frozen for up to **5 years**.

6.22 Reference DNA (*Trichinella spiralis*) for the inhibition control: genomic DNA (1 ng/µl) purified from reference larvae provided by *Trichinella* Reference Laboratory (TRL). The *Trichinella* DNA is prepared pooling reference larvae following the procedures described in Sambrook, J., Fritsch, E.F., and Maniatis, T., in Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3 (1989). Store frozen for up to **5 years**

6.23 SetB. Olinucleotide mix (6.12) used for the multiplex-PCR. The mix is obtained combining an identical volume of each of the 10 oligonucleotides (6.12). The final concentration corresponds to 10 pmoles/µL for each primer. Aliquots of 100 µL are prepared and stored -20° C up to 24 months.

Table C. Sequences of the SetB oligonucleotides (6.23), their codes and amplified nucleotide sequences.

Oligonucleotide sequence	code	amplified sequence
5'-GTT.CCA.TGT.GAA.CAG.CAG.T-3'	cp1.F	ESV
5'-CGA.AAA.CAT.ACG.ACA.ACT.GC-3'	cp1.R	
5'-GCT.ACA.TCC.TTT.TGA.TCT.GTT-3'	cp2.F	ITS1
5'-AGA.CAC.AAT.ATC.AAC.CAC.AGT.ACA-3'	cp2.R	
5'-GCG.GAA.GGA.TCA.TTA.TCG.TGT.A-3'	cp3.F	ITS1
5'-TGG.ATT.ACA.AAG.AAA.ACC.ATC.ACT-3'	cp3.R	
5'-GTG.AGC.GTA.ATA.AAG.GTG.CAG-3'	cp4.F	ITS2
5'-TTC.ATC.ACA.CAT.CTT.CCA.CTA-3'	cp4.R	
5'-CAA.TTG.AAA.ACC.GCT.TAG.CGT.GTT.T-3'	cp5.F	ITS2
5'-TGA.TCT.GAG.GTC.GAC.ATT.TCC-3'	cp5.R	

7. Procedure

7.1 Sample preparation

Faecal samples, known to be positive for the presence of *Giardia* cysts, are inspected to verify the preservation conditions. Vials must be intact without any sign of material leakage. If the conditions are not suitable the test is not performed.

7.2 Method

7.2.1 DNA extraction from faecal sample to be tested

If not otherwise specified, the procedure is carried out at room temperature. Each working session requires that an aliquot of the positive control for the DNA extraction (6.21) will be submitted to the DNA extraction procedure and identified as “positive control for the extraction”.

a) Transfer 1 mL of each faecal sample containing 50% ethanol in 1.5 ml vials.

b) Centrifuge (5.1) vials at 8,000 x g for 5 min.

c) Discard the supernatant and add a volume of H_2O equivalent to the starting volume of the sample.

d) Centrifuge (5.1) vials at 8,000 x g for 5 min.

e) Repeat washing as in ‘c’.

f) Transfer 200 µL of faecal sample in 2 ml vials.

g) Add 1.4 mL of ASL lysis buffer (6.1) and vortex to homogenate the sample for the DNA extraction (6.21) from the positive control, add the ASL lysis buffer (6.1) directly to the sample, then vortex to homogenate the sample.

h) Incubate at 95 °C in the thermomixer (5.3) at 1,400 rpm for 10 min.

i) Centrifuge (5.1) vials at 12,000 x g for 1 min.

j) Collect supernatant and transfer in new 2 ml vials.

k) Add one tablet of InhibitEX (6.2) and vortex for 1 min.

l) Incubate for 1 min.

m) Centrifuge (5.1) vials at 12,000 x g for 3 min.

n) Add 25 µL of Proteinase K (6.3) in a new 2 mL vial.

o) Collect supernatant (m) and add to the 2 mL vial (j)

p) Add 600 µL of the AL lysis buffer (6.4) in the same vial.

q) Incubate at 70 °C in the thermomixer (5.3) for 10 min.

r) Add 600 µL of ethanol (6.5) and vortex briefly.

s) For each sample, put one binding column (6.6) in a collection tube (6.7).

t) Transfer 600 µL of the lysate (q) in a binding column (6.6) and centrifuge (5.1) at 12,000 x g for 1 min.

u) Discard the collection tube (6.7) and transfer the binding column (6.6) in a new collection tube (6.7).

v) Repeat from ‘s’ to ‘t’ for additional two times.

z) Add 500 µL of the AW1 wash buffer (6.8) to the binding column (6.6) and centrifuge (5.1) at 12,000 x g for 1 min.

a1) Discard the collection tube (6.7) and transfer the binding column (6.6) in a new collection tube (6.7).

b1) Add 500 µL of the AW2 wash buffer (6.8) to the binding column (6.6) and centrifuge (5.1) at 12,000 x g for 3 min.

c1) Transfer the binding column (6.6) in a new 1.5 mL vial.

d1) Add 200 µL of elution buffer (6.9) to the binding column (6.6) and incubate for 1-2 min.

e1) Centrifuge (5.1) at 12,000 x g for 1 min., discard the binding column (6.6), store the 1.5 mL vials with the eluted DNA.

f1) The obtained DNA will be defined ‘DNA/faecal sample’ and store frozen (5.2) for up to **5 years**.

7.2.2 PCR amplification

Unless otherwise clearly stated, store tubes in ice; use tips with aerosol filter and wear disposable gloves. At each working session, use DNA from Reference faecal sample, two positives and one negative amplification controls. Use reference DNAs (6.21) as positive controls and water (6.19) as negative control. The following procedure uses a 2x concentrated PCR master mix, in case of a different concentration, adjust the protocol according to the supplier.

a) Thaw DNA/faecal sample, 2x PCR MasterMix (6.10), Set11 (6.11), positive amplification controls, and reference faecal DNA (6.20).

b) Mark with a progressive number an adequate number of 0.2 µL PCR tubes.

c) Prepare a adequate cumulative volume of the amplification mix. Evaluate the volume on the basis of a single sample amplification mix (Table D) and of the total number of samples plus four (one for the reference faecal DNA (6.20), two for the positive amplification controls (6.21), and 1 for the negative control).

Table D – single sample amplification mix: components and volumes

2x PCR MasterMix (6.10)	25 µL
H_2O	16 µL
Set11 (6.11)	4 µL
Total	45 µL

d) Mix the amplification mix by vortexing and centrifuge (5.1) at maximum speed for a few sec.

e) Transfer 45 µL of the cumulative amplification mix to each PCR tube (point “b”).

f) Add 5 µL of the DNA/faecal sample to be tested to each tube.

g) Close the tubes, mix by vortexing (5.14) and centrifuge (5.1) at maximum speed for a few sec.

h) Start the amplifying cycle (Table E) on the thermocycler device; wait for the temperature to reach 94° C and insert the tubes in the thermoblock by pausing the instrument.

Table E – amplification cycles

Pre-denaturation	5 min/94 °C
Amplification	30 s/94 °C 30 s/56 °C 30 s/72 °C
Number of cycles	40
Final extension	7 min/72 °C

i) At the end of the amplification phase, centrifuge (5.1) the tubes at maximum speed for a few sec.

l) Keep tubes in ice or refrigerated (5.5) until starting electrophoresis.

7.2.3 Agarose gel electrophoresis

a) Assemble the electrophoresis apparatus (5.6) according to the manufacturer’s recommendations. For the gel preparation, use a comb suited for the number of samples.

b) Add 2 gr agarose (6.14) in 100 mL TAE 1x (6.16) in a glass beaker.

c) Gently resuspend the powder by rotation.

d) Boil the agarose suspension for 30 sec. If the solution is not homogeneous, continue to boil for another 30 sec.

e) Restore with water the volume lost by boiling.

f) Allow the agarose solution to cool.

g) Before it solidifies, add 1.0 µL of ethidium bromide solution (6.17).

h) Shake gently to dissolve uniformly the ethidium bromide and pour the agarose in the gel tray previously prepared (a).

i) Wait for the gel to solidify, which requires at least 30 min.

j) Place the tray with the gel in the electrophoresis apparatus.

k) Cover the gel with TAE 1x buffer (6.16) and gently pull out the comb.

l) Load in each well 10 µL of the amplification product (point 7.2.2 "n"), respecting the progressive numbering of the tubes (point 7.2.2 "b").

m) The first and last wells are loaded with 15 µL of the L50 solution (6.18).

n) Connect the electrophoresis apparatus with the power supply (5.6) and set 10 v/cm of gel.

o) Run the gel for about 30 min or until the fastest dye, contained in the loading buffer, reaches a distance of 1 cm from the gel border.

p) After 30 min, switch off the power supply, place the gel under UV illumination (5.12) and check the band separation. The electrophoresis run is adequate if it is possible to easily distinguish all bands of molecular weight marker ranging from 250 and 1500 bp. If the separation is incomplete, continue the run.

q) At the end of the run, transfer the gel to the imaging system (5.7) and print the result.

7.2.4 Interpretation of the PCR amplification results on agarose gel electrophoresis
The size of the amplification bands (see Table A) revealed by the electrophoresis is evaluated by their comparison with the reference molecular weight L50 (6.18) and with the positive controls of extraction and amplification. The visual evaluation is considered sufficient and adequate. The PCR will be considered valuable if:

i) positive controls of amplification and extraction show amplification products in agreement with those showed in table A;
ii) negative control of amplification does not show any amplification product.

7.2.5 Test for the presence of inhibitors by PCR for Trichinella spiralis
If nor otherwise stated, keep tubes on ice, use tips with barrier and wear disposable gloves. At each working session, use a positive and a negative amplification control. Use reference DNA (6.22) as positive control and water (6.19) as negative control.
The following procedure uses a PCR master mix at a 2X concentration. If the concentration is different, modify the procedure following the manufacturer's recommendations.

a) Thaw: DNA/fecal samples, 2x PCR MasterMix (6.10), SetB (6.23), amplification positive control (reference DNA, 6.25).

b) Mark with a progressive number an adequate number of 0.2 µL PCR tubes.

c) Prepare an adequate cumulative volume of amplification mix. Calculate the volume on the basis of a single sample amplification mix (Table F) and of the total number of samples plus two (1 for the positive amplification control and 1 for the negative control).

Table F – Amplification mix for a single sample: components and volumes

2x PCR MasterMix (6.10)	25 µl
H_2O	9 µl
SetB (6.23)	1 µl
Total	35 µl

d) Mix the amplification mix by vortexing and centrifuge (5.1) at maximum speed for a few seconds.

e) Transfer 35 µL of the cumulative amplification mix to each PCR tube (point "b").

f) Add 10 µL of reference DNA (6.22) and 5 µL of the DNA/fecal samples to be tested to each tube.

g) Close the tubes, mix by vortexing and centrifuge (5.1) at maximum speed for a few seconds.

h) Start the amplification cycle (Table G) on the thermocycler device (5.4); wait until the temperature reaches 95°C and insert the tubes in the thermoblock by pausing the instrument. Close the lid and restart the cycle.

Table I – Amplification cycle

Pre-denaturation	4 min/95 °C
Amplification	10 s/95 °C 30 s/55 °C 30 s/72 °C
Number of cycles	35
Final extension	3 min/72 °C

i) At the end of the amplification phase, centrifuge (5.1) the tubes at maximum speed for a few seconds.

l) Add 10 µL of loading buffer 6x (6.13) if not present in the PCR master mix.

m) Mix by vortexing and centrifuge (5.1) the tubes at maximum speed for a few seconds.

n) Leave the tubes on ice or in a refrigerator (5.5) before the electrophoresis.

7.2.6 Results

To visualize the results, follow the procedure described at point 7.2.3.

7.2.7 *Interpretation of the PCR results on agarose gel*

If the 173 bp specific fragment of *T. spiralis* is visualized, the presence of inhibitors of the PCR reaction can be excluded and the sample will be confirmed as "negative"; differently if the 173 bp specific fragment amplification product is not observed, a new DNA extraction will be performed from the sample. If the repeated extraction and a new amplification for 4E1-HP do not yield any of the expected products (table A), the result of the assay will be *undetermined*.

8. Results

The result will be expressed as follows:

a) If the band is estimated of 165 bp, the sample will be considered positive for *G. duodenalis* assemblage A;

b) If the band is estimated of 272 bp, the sample will be considered positive for *G. duodenalis* assemblage B;

c) If two bands are observed, one estimated of 165 bp and one estimated of 272 bp, the sample will be considered positive for both *G. duodenalis* assemblage A and B (mixed infection);

d) If the test is valid but a sample shows one or more bands not present in Table A, the species identification will be classified as "negative".

9. Characteristics of the method

This method has been characterised in terms of specificity, sensitivity and repeatability. The results of the validation process confirmed that the method is suitable for the specified aim.

10. Safety measures

This method has to be carried out only by authorized personnel. The operator should wear individual protection devices while performing the test.