Identification of the assemblages A and B of *Giardia duodenalis* by multiplex PCR

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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 fecal material of human and animal origin known to be positive for the presence of *Giardia* cysts and/or trophozoites. Among *G. duodenalis* assemblages, only assemblage A and B may infect humans, so this method permit the diagnosis of human giardiasis and the identification of potential zoonotic risk in animal samples.

2. Principle of the method

The polymerase chain rection (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. In the multiplex PCR, two or more oligonucleotide pairs are used at the same time to generate amplicons different in size among assemblages. The different size of the amplicons is originated by variation in the same gene.

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 divide 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 ingestion of cysts that undergoes excystation into trophozoites in the proximal small intestine after the exposure to the acidic environment of the stomach. Nine species have been described based on the host specificity, the morphology and the phenotype: Giardia agilis in amphibians, G. varani in lizards, G. muris and G. microti in rodents, G. cricetidarum in cricetids, G. peramelis in quenda, G. ardeae and G. psittaci in birds, and G. duodenalis (syn. lamblia and 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 based on genetic analysis. Only Assemblages A and B have been isolated from humans and a wide range of mammals, whereas the other Assemblages (C-G) have distinct host specificities 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.

Ryan, UM, Feng, Y, Fayer, R, Xiao, L. (2021) Taxonomy and molecular epidemiology of *Cryptosporidium* and *Giardia* - a 50 year perspective (1971-2021). Int J Parasitol. 51(13-14):1099-1119

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.

Wielinga, C, Williams, A, Monis, P, Thompson, RCA. (2023) Proposed taxonomic revision of *Giardia duodenalis*. Infect Genet Evol. 111:105430

ISO/FDI 20837:2006(E). Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Requirements for sample preparation for qualitative detection

ISO/FDI 20838:2006(E). Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Requirements for amplification and detection for qualitative methods

ISO 22174: 2005. Microbiology of food and animal feeding stuffs. Polymerase chain reaction (PCR) for the detection of food-borne pathogens. General requirements and definitions

Qiagen: QIAamp Fast DNA Stool Handbook.

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 fecal sample containing cysts of *G. duodenalis,* Assemblage A or B analysed in the same working session of test samples, to verify the efficacy of the DNA extraction session.

Positive control for the amplification, purified genomic DNAs of *G. duodenalis* Assemblage A and 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 absence of contamination in the PCR 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 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 Reagent grade water system production

5.10 Vortex

5.11 Analytical balance, readability 0.1g

5.12 UV transilluminator

5.13 Orbital shaker

5.14 QIAxcel, vertical electrophoresis apparatus

6. Reagents and chemicals

6.1 InhibitEX Buffer. Commercial solution: QIAamp Fast DNA Stool, QIAGEN. Store according to manufacturer's recommendations.

6.2 Proteinase K. Commercial solution. Store according to manufacturer's recommendations.

6.3 Lysis buffer. Commercial solution: QIAamp Fast DNA Stool, QIAGEN, identified as "AL" buffer. Store according to manufacturer's recommendations.

6.4 Ethanol (96–100%). Commercially available solution.

6.5 Binding Column. Commercially available product: QIAamp Fast DNA Stool, QIAGEN, identified as "QIAamp Mini Spin Columns".

6.6 Collection tube. Commercially available product: QIAamp Fast DNA Stool, QIAGEN, identified as "Collection tube" (2 mL).

6.7 Washing buffers. Commercial solutions: QIAamp Fast DNA Stool, QIAGEN, to be prepared according to manufacturer's recommendations and identified as "AW1" and"AW2". Store according to manufacturer's recommendations.

6.8 Eluting buffer. Commercial solution: QIAamp Fast DNA Stool, QIAGEN, identified as "AE" buffer. Store according to manufacturer's recommendations.

6.9 PCR master mix. Commercially available solution suitable for PCR amplification (i.e., Qiagen HotStarTaq Master Mix Kit).. Store according to the manufacturer's recommendations.

6.10 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 20 years; the reconstituted product can be stored frozen up to 10 years.

6.11 Set11. The oligonucleotide mixture (6.11) 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), starting from a 10 pmol/µL concentration of each oligonucleotide. The final concentration corresponds to 10 pmol of each oligonucleotide in the amplification mix. 100µL aliquots are prepared and stored frozen up to 10 years.

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

6.12 Loading buffer. Commercial product allowing DNA molecule visualization during electrophoresis, it may be included in the PCR Master Mix solution (point 6.9). Store according to the manufacturer's

recommendations.

6.13 Agarose. Commercial product suitable for performing DNA molecule electrophoresis. Store according to the manufacturer's recommendations.

6.14 TAE solution 50x. Commercially available solution (2M Tris-acetate, 50mM EDTA, pH 8.2–8.4 at 25°C). Store at room temperature for up to 24 months.

6.15 TAE solution 1x. 1000 mL preparation: take 20 mL of the 50x solution and bring to 1000 mL with water. To prepare immediately before use.

6.16 DNA intercalating agent. Commercial product able to fit in the DNA double helix, used to view the amplification product on agarose gel. Store according to the manufacturer's recommendations.

6.17 L50, ladder 50. Commercial product containing markers for DNA molecular weight multiple of 50 bp, within the 50-500 bp range, can be used. Store refrigerated according to manufacturer's recommendations.

6.18 Reagent grade water, Milli-Q.

6.19 Reference fecal sample, fecal sample containing cysts of *G. duodenalis* analysed in the same working session of test samples, to verify the efficacy of the DNA extraction. Store frozen for up to 10 years.

6.20 Reference DNAs, purified genomic DNAs from fecal sample containing cysts of *G. duodenalis* or cultured trophozoites, assemblages A and B. Store frozen for up to 10 years.

6.21 QIAxcel high resolution kit: commercial products from Qiagen. Include separation cartridge and buffers for sample preparation and gel running. To use with QIAxcel. Store each component as indicated by the manufacturer.

6.22 Alignment marker: commercial products from Qiagen. To use with QIAxcel. Store according to manufacturer's instructions.

6.23 DNA size marker: commercial products from Qiagen. To use with QIAxcel. Store according to manufacturer's instructions.

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 (6.20) will be submitted to the DNA extraction procedure and identified as "positive control for the extraction".

Note: before use, reference fecal material, preserved in ethanol 50%, is subjected to a single wash with reagent grade water (6.18) to eliminate ethanol, through 5 minutes of centrifugation at 5000 rpm. The deriving pellet is resuspended with 200 µL reagent grade water (6.18), the protocol starts from point "b"

a) Transfer 200 µL of faecal sample in 2 mL vials, previously marked with a code.
b) Add 1mL of InhibitEX buffer (6.1) and vortex 1 minute to homogenate the sample.
c) Incubate 10 minutes at 95 °C in a thermomixer under shaking at 1,400 rpm r.
d) Centrifuge vials 1 minute at 12,000 x g .
e) Put 25 µL of proteinase K (6.2) in a 1.5 mL tube.
f) Transfer 600 µL of supernatant (point "d") in the tube containing the proteinase K (point "e")
g) Add 600 µL of AL lysis buffer (6.3) and vortex briefly.
h) Incubate 10 minutes at 70 °C in the thermomixer .
i) Add 600 µL of absolute ethanol (6.4) and vortex briefly.

j) For each sample, set a binding column (6.5) in a collection tube (6.6).

k) Transfer 600 µL of the lysate (point"i") in the binding column (6.5) and centrifuge 1 minute at 12,000xg.

l) Discard the collection tube (6.6) and transfer the binding column (6.5) in a new collection tube (6.6).

m) Repeat from point "k" to "l" twice.

n) Add 500 µL of AW1 wash buffer (6.7) to the binding column (6.5) and centrifuge 1 minute at 12,000xg.

o) Discard the collection tube (6.6) and transfer the binding column (6.5) in a new collection tube (6.6).

p) Add 500 µL of AW2 wash buffer (6.7) to the binding column (6.5) and centrifuge 3 minutes at 12,000xg.

q) Transfer the binding column (6.5) in a new 1.5 mL vial.

r) Add 100 µL of ATE elution buffer (6.8) to the binding column (6.5) and incubate for 1-2 min.

s) Centrifuge 1 minute at 12,000xg, discard the binding column (6.5), store the 1.5 mL vials with the eluted DNA.

t) The obtained DNA will be defined 'DNA/fecal sample' and store frozen for up to 10 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 (6.20) as positive controls and water (6.18) 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 (6.9), Set11 (6.11) and positive amplification controls (6.21).

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

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

Table D – single sample amplification mix: components and volumes

2x PCR MasterMix (6.9)	25 µL
H_2O (6.18)	16 µL
Set11 (6.11)	4 µL
Total	45 µL

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

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 tube, mix by vortexing and centrifuge at maximum speed for few seconds.

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.

NB: In the case of "hot start" Taq polymerase (e.g. HotStarTaq DNA Polymerase, Qiagen), follow the manufacturer's recommendation to activate the enzyme (e.g. 90° C 10 minutes)

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

pre-denaturation length may vary according to the Master Mix manufacturer's recommendations

i) At the end of the amplification phase, centrifuge the tubes at maximum speed for ew seconds.

j) Keep tubes on ice or refrigerated until starting electrophoresis.

7.2.3 Result display

a) Switch on the Qiaxcel instrument (5.14) and the relative Qiaxcel ScreenGel management software on the PC;

b) access the "Process Profile" panel; indicate under "Cartridge Type" the option "DNA HighRes"; indicate the desired profile and Experiment Directory;

c) move the tube tray to the "Access Position" by selecting the "Load Position" item from the "Status Information" panel;

d) insert in MARKER1 position the 12 tubes containing at least 10 µL of the chosen "Alignment Marker" (6.22); then return the tray to the initial position by selecting the "Park Position" from the "Status Information" panel;

e) starting from the "A" row, position the samples to analyze (minimum volume 10 µL) in rows of 12 tubes. If the samples to be analyzed are not enough to complete the row of 12, add the required number of tubes containing the QX DNA dilution buffer (minimum volume 10 µL) supplied with the QIAxcel DNA High Resolution kit (6.21);

f) for each analysis session (which may include up to a maximum of 8 runs of 12 samples), include a tube containing the DNA size marker (6.23);

g) in "Run Parameters" set the option 0M500 under "Method"; select the runs on the virtual plate in the "Sample Row Selection" side panel;

h) in "Sample Selection" set the run parameters as follows: "Plate ID": PCR + data "Alignment Marker" (6.27). In "Sample Information" enter the names of the samples in the corresponding boxes;

i) in "Run Check" check that all the selected rows are occupied by tubes containing PCR products to be analyzed or QX DNA dilution buffer (6.26) and that the alignment Marker has been loaded, then select the appropriate boxes; finally select "Run";

j) visualize the results by selecting the "Absolute migration time" mode from the "Image options" menù and process the data with the "Start analysis" command;

k) scroll through the electropherogram of each sample to check peaks above the highest band of the alignment marker (6.22);

l) print the results to archive;

m) at the end of the run, close the program and turn off the instrument.

If the instrument 5.6 is out of service for an extended period proceed with agarose gel following the protocol below:

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

b) Add 2 gr agarose (6.13) in 100 mL TAE 1x (6.15) 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 DNA intercalating agent (6.16) according to the manufacturer's instructions

h) Shake gently to dissolve uniformly the DNA intercalating agent and pour the agarose in the gel tray previously prepared (point "a").

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

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

k) Cover the gel with TAE 1x buffer (6.15) and gently pull out the comb.

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

m) The first and last wells are loaded with 15 µL of the L50 solution (6.17).

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

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

p) After 30 minutes, switch off the power supply, place the gel under UV illumination and check the band separation. The electrophoresis run is adequate if it is possible to distinguish easily all bands of molecular weight marker ranging from 100 and 500 bp. If the separation is incomplete, continue the run.

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

7.2.4 Result Interpretation

The amplification test is considered valid if:

i) the amplification of the positive controls shows a product in accordance with table A;

ii) the amplification of the negative control does not show any product or, eventually, only bands related to unused oligonucleotides and/or primer dimer;

iii) the extraction positive control shows an amplification product according to Table A.

The data analysis shall consider only those bands satisfying the following requirements:

1) Band size bigger than 50 bp;
2) Comprised between the two Alignment marker bands (6.24);
3) Intensity of the emission peak greater than a threshold value of 5%.

In case of overlapped peaks, only the peak showing the higher intensity is considered; if peak values are comparable, the result is rejected.

The size of the amplification bands revealed by the electrophoresis is evaluated by:

i) visual comparison with the "DNA size marker" (6.25) and with the positive extraction and amplification controls on the virtual gel;

ii) comparison between the band size calculated by the software and the expected band size.

The size of the amplification bands (see Table A) revealed by the electrophoresis is evaluated by their comparison with the reference molecular weight L50 (6.17) and with the positive controls of extraction and amplification. The visual evaluation is considered sufficient and adequate.

7.2.5 Test for the presence of inhibitors by PCR

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. Mix reference DNA (6.2) to DNA/fecal sample (7.2.1 point "t") and amplify following the next steps to exclude the presence of inhibitors within the DNA/fecal sample.

In each working session, a reference DNA (6.21), positive control, is foreseen to verify the efficacy of the amplification reaction.
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/faecal samples, 2x PCR MasterMix (6.9), Set11 (6.11) and amplification positive control (reference DNA, 6.20).

b) Mark with a progressive number an adequate number of 0.2 mL 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 added of two reactions (one for the positive amplification control and one for the negative control).

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

2x PCR MasterMix (6.9)	25 µl
H_2O (6.18)	14 µl
Set11 (6.11)	1 µl
Reference DNA (6.20)	5 µL
Total	45 µl

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

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

f) To each tube, add 5 µL of the DNA/fecal samples to be tested (7.2.1 point “t”).

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

h) Start the amplification cycle on the thermocycler device, according to Table E, paragraph 7.2.2. 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.

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

l) Leave the tubes on ice or in a refrigerator 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 specific fragment of 165 o 272 bp, according to the reference DNA used, is visualized, the presence of inhibitors of the PCR reaction can be excluded and the sample will be confirmed as “negative”; the result of the test is expressed as “undeterminable assemblage”.

Differently, if any specific fragment of 165 o 272 bp, according to the reference DNA used, is visualized, the sample is reprocessed starting from the extraction. If any amplification is still available, the result of the test is expressed as “undeterminable assemblage”.

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”, the result of the test is expressed as “undeterminable assemblage”.

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.