Unit of Foodborne and Neglected Parasitic Diseases
Istituto Superiore di Sanità

Identification of *Giardia duodenalis* cysts at the Assemblage level by PCR/RFLP

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1. Aim and field of application

To determine the identity of cysts of the protozoan *Giardia duodenalis* at the assemblage level by a PCR/RFLP analysis. This method can be applied to faecal material of human and animal origin that have been tested positive for the presence of *Giardia* cysts.

2. Principle of the method

The PCR (polymerase chain reation) 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. Nested-PCR is a modification of the PCR technique that allows higher sensibility by performing two consecutive PCR reactions. In the first reaction an external oligonucleotide pair is used, while in the second reaction an internal oligonucleotide pair is used, both targeting the same DNA fragment.

It is possible to combine the "standard PCR" with the "Restriction Length Fragment Polymorphism" (RLFP), i.e., the analysis of DNA restriction fragments. The RFLP technique allows distinguishing PCR fragments of similar length by enzymatic digestion with one or more endonucleases, which are enzymes that cut DNA by recognition of short and specific oligonucleotide sequences. Therefore, it is possible to amplify the same portion of DNA from different species and then to distinguish them based on the number and size of the restriction pattern.

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 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/RFLP have allowed the identification at the Assemblage level of *G. duodenalis* cysts present in human and animal faecal samples. Among these methods, those based on the amplification of the beta-giardin gene (coding for a structural protein) has been widely used. The two consecutive PCR amplifications, obtained with specific oligonucleotides, and the subsequent digestion of the PCR fragment with the restriction endonuclease HaeIII allow the identification of each *G. duodenalis* assemblage based on the restriction pattern (Sulaiman et al. 2003; Lalle et al., 2005).

The size of the fragment obtained with the two consecutive PCR amplification, targeting the beta-giardin gene, are 723 and 511 base pairs (bp), respectively.

The size of the fragments produced by HaeIII digestion of the nested beta-giardin PCR fragment (511 bp) for each *G. duodenalis* Assemblage are shown in Table A.

Table A - Size (in base pairs) of the beta-giardin fragments after HaeIII endonuclease digestion expected for each G. duodenalis Assemblage.

Assemblage	Digestion fragments
A	201, 150, 110, 50
B	150, 117, 110, 84, 26, 24
C	194, 150, 102, 50, 15
D	200, 194, 117
E	186, 150, 110, 26, 24, 15
F	186, 150, 110, 50, 15
G	194, 165, 102, 50

Using the PCR/RFLP technique, it is possible to distinguish *G. duodenalis* Assemblage A, B, C. D, E, F and G, based on the number and size of the fragments obtained by digestion of the 511bp fragment of the beta-giardin gene with the HaeIII enzyme.

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4. Definitions

Beta-Giardin, the sequence encoding for a structural protein of the *G. duodenalis* cytoskeleton.

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

SetA, mix of 2 oligonucleotide pairs amplifying a 723 bp fragment of the beta-giardin gene from all *G. duodenalis* Assemblages.

SetB, mix of 2 oligonucleotide pairs amplifying a 511 bp fragment of the beta-giardin gene from all *G. duodenalis* Assemblages.

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

Positive control for the amplification, purified genomic DNA from faeces containing cysts of *G. duodenalis*; the control is used in the amplification session to verify the efficacy of the PCR.

Negative control for the amplification, reagent grade water; the control is used in the amplification session to verify the absence of contamination in the PCR reaction.

Restriction Enzyme. Restriction enzymes are enzymes of bacterial origin able to cut DNA at specific sites, allowing DNA fragmentation in a reproducible and specific manner. The recognition sites are short (4-8 bp) DNA sequences specific for each restriction enzyme. Enzyme concentration is expressed as "enzymatic units" (U). In particular, 1U corresponds to the amount of enzyme required to completely digest 1 µg of DNA in 1 hour at the proper temperature.

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

5. Devices/instruments

Bench top centrifuge for 1.5 mL tubes, minimum 10,000 g

Freezer ≤-15°C

Bench shaking homogenizer for tubes (e.g. Fast Prep instrument)

Thermomixer with vibration, temperature range 25÷100°C

PCR thermocycler

Refrigerator, temperature range 1÷8°C

Horizontal electrophoretic apparatus

Digital imaging system

Adjustable volume pipettes, volume range: 1-1000µL

Reagent grade water system production

Vortex

Analytical balance, readability 0.1g

UV transilluminator

Orbital shaker

Qiaxcel, vertical capillary electrophoresis system

6. Reagents and chemicals

6.1 Reagents and chemicals extraction protocol A

Lysis vial. Screw top tube containing a mixture of ceramic and silica particles (e.g. Lysis Matrix E tube, FastDNA Spin kit for Soil, MP Biochemicals). Commercially available.

Restoring buffer. Sodium phosphate buffer (e.g. Sodium Phosphate Buffer, FastDNA Spin kit for Soil, MP Biochemicals). Commercially available. Store according to manufacturer's recommendations.

Homogenization buffer. Commercial solution (e.g. MT buffer, FastDNA Spin kit for Soil, MP Biochemicals). Store at room temperature.

Lysis buffer. Commercial solution (e.g. PPS buffer, FastDNA Spin kit for Soil, MP Biochemicals). Store at room temperature.

Silica resin. Commercial solution (e.g. Binding Matrix, FastDNA Spin kit for Soil, MP Biochemicals). Store at room temperature.

Collection Column. Commercially available (e.g. SPIN filter, FastDNA Spin kit for Soil, MP Biochemicals).

Washing buffer. Commercially available (e.g. SEWS-N, FastDNA Spin kit for Soil, MP Biochemicals). To prepare according to manufacturer's instruction. Store at room temperature.

Collection tube. Commercially available 2 mL tube (e.g. Catch tube, FastDNA Spin kit for Soil, MP Biochemicals).

Elution buffer. Commercial solution (e.g. DES, FastDNA Spin kit for Soil, MP Biochemicals). Store according to manufacturer's recommendations.

6.2 Reagents and chemicals extraction protocol B

InhibitEX Buffer. Commercially available reagent: QIAamp Fast DNA Stool, QIAGEN. Store according to manufacturer's instructions.

Proteinase K. Commercially available reagent: QIAamp Fast DNA Stool, QIAGEN. Store according to manufacturer's instructions.

Lysis buffer. Commercially available solution: QIAamp Fast DNA Stool and identified by the manufacturer as 'AL' solution**.** Store according to manufacturer's instructions.

Absolute ethanol. Commercially available reagent. Store according to manufacturer's instructions.

Recovery column. Commercially available material: QIAamp Fast DNA Stool, QIAGEN, and identified by the manufacturer as QIAmp Mini Spin Columns.

Collection tube. Commercially available material: QIAamp Fast DNA Stool, QIAGEN, and identified by the manufacturer as Collection tubes (2 ml).

Wash buffers. Commercially available solutions: QIAamp Fast DNA Stool, QIAGEN.

Prepare according to the manufacturer's specifications, labeling this solution with the abbreviations 'AW1' and 'AW2'. Store according to the manufacturer's specifications.

Elution buffer. Commercially available solution: QIAamp Fast DNA Stool, QIAGEN, and labeled by the manufacturer as Buffer AE. Store according to the manufacturer's specifications.

6.3 Reagents and chemicals for the following steps

PCR master mix. Commercial solution to perform DNA amplification reactions. Store according to the manufacturer's recommendations.

Oligonucleotides (primers). Commercial preparation (Table B), the lyophilized products is reconstituted, with reagent 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 for up to 10 years.

SetA. The oligonucleotide mixture used for the PCR; the mixture is obtained by combining an equal volume of the two oligonucleotides BGFor71 and BGRev794 diluted in reagent grade water or MilliQ at 20 pmol/µL. The final concentration corresponds to 10 pmol/µL. 100µL aliquots are prepared and stored frozen up to 10 years.

SetB. The oligonucleotide mixture used for the PCR; the mixture is obtained by combining an equal volume of the two oligonucleotides BGintFor and BGintRev diluted in reagent grade water or MilliQ at 20 pmol/µL. The final concentration corresponds to 10 pmol/µL. 100µL aliquots are prepared and stored frozen up to 10 years.

Table B – Oligonucleotides present in the setA and setB, their codes and amplified nucleotide sequences.

Oligonucleotide sequence	Code	Amplified sequence
5'-CCCGACGACCTCACCCGCAGTCG-3' 5'-GCCGCCCTGGATCTTCGAGACGA-3'	BGFor71 BGRev794	Beta-giardin (external primers)
5'-GAACGAACGAGATCGAGGTCCG-3' 5'-CTCGACGAGCTTCGTGTT-3'	BGinfFor BGintRev	Beta-giardin (internal primers)

Loading buffer. Commercial product allowing electrophoresis of DNA molecules. Do not add if already present in the PCR master master mix. Store according to the manufacturer's recommendations.

Agarose and high-resolution agarose. Commercial products suitable for performing gel electrophoresis. The high-resolution agarose is suitable for the analysis of small DNA fragments (25-700 bp), improving their electrophoretic separation. Store at room temperature for up to 24 months.

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

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.

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.

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

L100. Commercial product containing DNA molecular weight markers, multiple of 100 bp within the 100-1500 bp range. Store refrigerated according to the manufacturer's recommendations.

Reagent grade water, Milli-Q.

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

Reference DNA, purified genomic DNA from feces containing cysts of *G. duodenalis* or DNA extracted from in vitro cultured trophozoites of *G. duodenalis* WBC6 clone (Assemblage A). Store frozen for up to 10 years.

Restriction enzyme *HaeIII*. Commercial product suitable for DNA enzymatic digestion. Store refrigerated according to manufacturer's recommendations. The oligonucleotide sequence recognized by *HaeIII* is reported in Table C.

Table C - Oligonucleotide sequence recognized by HaeIII restriction enzyme.

Restriction Enzyme	Target sequence
HaeIII	**5'…GG▼CC…3'** **3'…CC▲GG…5'**

Restriction enzyme buffers. Commercial products with defined pH and saline concentration suitable for DNA enzymatic digestion. The buffers are supplied together with the corresponding restriction enzyme. Store refrigerated according to manufacturer's recommendations.

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.

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

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

7. Procedure

7.1 Sample preparation

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

7.2 Method

Perform DNA extraction with one of the two following alternative protocols, reported as **Protocol A** and **Protocol B**

7.2.1 DNA extraction from faecal samples to be tested (Protocol A)

If not otherwise stated, the procedure is carried out at room temperature.

Each working session requires that an aliquot of the positive control for the DNA extraction is submitted to the DNA extraction procedure, and identified as "positive control for the extraction".

NB: the reference fecal material, preserved in 50% ethanol, requires the removal of the ethanol with a centrifuge at 5000 rpm for 5 minutes and the resuspension of the pellet with 400 µL of reagent grade water, then proceed as indicated in point "a".

a) Transfer 450 µL of faecal sample into a lysis vial.

b) Add 978 µL of restoring buffer and 122 µL of homogenization buffer.

c) Homogenize the sample in a bench shaking homogenizer, for 40 seconds at speed 6.
d) Centrifuge vials for 10 minutes at 12,000 g.
e) Transfer the liquid phase into a 2 mL vial and add 250 µL of lysis buffer, mix by inverting the vial 10 times.
f) Centrifuge the vials for 5 minutes at 12,000 g.
g) In the meanwhile, vortex the bottle containing the silica resin for 30 seconds.
h) Transfer the liquid phase, obtained after centrifugation (point "f"), in a new 5 mL or 15 mL vial, add 1 mL of silica resin and mix in the orbital shaker or invert the vial for 2 minutes.
i) Leave the vial in a vertical position for 3 minutes to let the silica resin sediment.
j) Gently remove 500 µL of supernatant without disturbing the resin.
k) Using a micropipette, suspend the silica resin with the remaining supernatant.
l) Transfer 600 µL of the mix in the collection column set on a collection tube.
m) Centrifuge the collection column and vial for 1 minute at 12,000 g.
n) Empty the collection vial.
o) Repeat from point "l" to "n" until all the mix is transferred to collection column.
p) Add 500 µL of washing buffer to the collection column and gently suspend the silica resin with a micropipette.
q) Centrifuge for 1 minute at 12,000 g.
r) Empty the collection vial.
s) Centrifuge for 2 minutes at 12,000 g.
t) Transfer the collection column in a new vial.
u) Dry the silica resin for 5 minutes.
v) Gently suspend the silica resin in 100 µL of elution buffer with a micropipette.
w) Incubate for 5 minutes at 55 °C (±3 °C) in a thermomixer.
x) Centrifuge for 1 minute at 12,000 g, discard the collection column, and keep the vial containing the DNA.
y) The obtained DNA is defined 'DNA/fecal sample' and store frozen for up to 10 years.

*7.2.2 DNA extraction from faecal samples to be tested (**Protocol B**)*

If not otherwise stated, the procedure is carried out at room temperature.

Each working session requires that an aliquot of the positive control for the DNA extraction is submitted to the DNA extraction procedure, and identified as "positive control for the extraction".

NB: the reference fecal material, preserved in 50% ethanol, requires the removal of the ethanol with a centrifuge at 5000 rpm for 5 minutes and the resuspension of the pellet with 400 µL of reagent grade water, then proceed as indicated in point "a".

a) Transfer 200 µL of fecal sample into 2 mL vial previously marked with the appropriate identification code.
b) Add 1 mL of InhibitEX buffer and vortex for 1 minute to homogenize the sample.
c) Incubate for 10 minutes at 95°C in a thermoblock. During the incubation, leave to shake at approximately 1400 oscillations per minute.
d) Centrifuge for 1 minute at 12000 rpm
e) Place 25 µL of proteinase K into a 2 mL vial
f) Transfer 600 µL of the supernatant (point "d") into the vial containing proteinase K (point "e").
g) Add 600 µL of lysis buffer, buffer AL and vortex for 15 seconds.
h) Incubate at 70°C for 10 minutes in a thermoblock.
i) Add 600 µL of absolute ethanol and vortex briefly.
j) For each sample, place a recovery column in a collection tube.
k) Transfer 600 µL of lysate (point "i") to the recovery column and centrifuge for 1 minute at 12,000 rpm.
l) Discard the collection tube and place the recovery column in a new collection tube.

m) Repeat steps “k” to “l” two more times until all the lysate at point “k” is used up.

n) Add 500 µL of AW1 wash buffer to the recovery column and centrifuge for 1 minute at 12,000 rpm.

o) Discard the collection tube and place the recovery column in a clean collection tube.

p) Add 500 µL of AW2 wash buffer to the recovery column and centrifuge for 3 minutes at 12,000 rpm.

q) Centrifuge the tube with the recovery column again at 12,000 rpm for 3 minutes, without adding any more liquid.

r) Transfer the recovery column into a new 1.5 mL tube.

s) Add 100 µL of ATE elution buffer to the recovery column and incubate for 1-2 minutes.

t) Centrifuge for 1 minute at 12,000 rpm, discard the recovery column, keeping the tube containing the extracted DNA.

u) The DNA thus prepared is defined as ‘DNA/fecal sample’ and stored in a freezer. Under these conditions it can be stored for up to 10 years.

7.2.3 PCR amplification

Unless otherwise stated, store tubes on ice; use tips with barrier and wear disposable gloves.

At each working session, use positive and negative amplification control. Use reference fecal DNA as positive control and water as negative control.

The following procedure uses a 2x concentrated PCR master mix, in case of different concentration adjust the protocol according to the supplier.

a) Thaw DNA/fecal sample, 2x PCR MasterMix, SetA, and the positive amplification control, reference fecal DNA.

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

c) Prepare an adequate volume of the amplification mix. Calculate the volume based on a single sample amplification mix (Table D) and of the total number of samples added of two reactions (one for the positive amplification control and one for the negative control).

Table D – single sample amplification mix: components and volumes

2x PCR MasterMix	25 µL
H_2O	19 µL
SetA	1 µL
Total	45 µL

d) Vortex the amplification mix and centrifuge at maximum speed for few seconds.

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

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

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

h) Start the amplification cycle (Table E) on the thermocycler device; wait until the temperature reaches 95°C, then pause the instrument and insert the tubes in the device.

Table E – amplification cycles

Pre-denaturation [#]	3 min/94°C
Amplification	30 s/94°C 30 s/55°C 60 s/72°C
Number of cycles	35
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 reaction, centrifuge the tubes at maximum speed for few seconds.

l) Keep tubes on ice or refrigerate until the Nested PCR is performed.

7.2.4 Nested PCR

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

b) Prepare an adequate volume of the amplification mix. Evaluate the volume based on a single sample amplification mix (Table F) and of the total number of samples plus one (negative control).

Table F – single sample amplification mix: components and volumes

2x PCR MasterMix	25 µL
H_2O	19 µL
SetB	1 µL
Total	45 µL

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

d) Transfer 45 µL of the amplification mix to each PCR tube (point "a").

e) Add 5 µL of the previously obtained PCR product to each tube (7.2.3 point "l").

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

g) Start the amplification cycle (Table E) on the thermocycler device; wait until the temperature reaches 94°C, then pause the instrument and insert the tubes into the device.

Table E – amplification cycles

Pre-denaturation [#]	3 min/94°C
Amplification	30 s/94°C 30 s/53°C 60 s/72°C
Number of cycles	35
Final extension	7 min/72°C

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

h) At the end of the amplification reaction (Nested PCR), centrifuge the tubes at maximum speed for few seconds.

i) Keep tubes on ice or refrigerated until electrophoresis is performed.

7.2.5 Visualization of the results

The analysis will be primarily conducted by capillary gel electrophoresis. In case the number of samples to be analysed is less than eight, or the capillary electrophoresis apparatus is out of order, the analysis can be done by conventional agarose gel electrophoresis.

a) Switch on the Qiaxcel instrument 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"; 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;

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;

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". 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 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;

l) print the results to archive;

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

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

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

ii) Add 2 gr agarose in 100 mL TAE 1x in a glass beaker.

iii) Gently resuspend the powder by rotation.

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

v) Restore the volume lost by boiling with water.

vi) Allow the agarose solution to cool.

vii) Before it solidifies, add DNA intercalating agent according to the manufacturer's instructions.

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

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

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

xi) Cover the gel with TAE 1x buffer and gently pull out the comb.

xii) Add, in each tube containing the amplification product, the loading buffer, according to the manufacturer's specifications, if not present in the PCR master mix used.

xiii) Mix the samples by vortexing and centrifuge the tubes at maximum speed for a few seconds.

xiv) Load 10 µL of the amplification product (point 7.2.4 "i") in each well, respecting the progressive numbering of the tubes (point 7.2.4 "a").

xv) The first and the last wells are loaded with 15 µL of the L100 solution.

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

xvii) 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.

xviii) After 30 min, switch off the power supply, place the gel under UV illumination and check the band separation. The electrophoresis run is adequate if all the bands of molecular weight marker ranging from 250 and 2000 bp are easily distinguishable. If the separation is incomplete, continue the run.

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

7.2.6 Interpretation of the Nested PCR results

The amplification test is considered valid if:

i) the amplification of the positive control shows an amplification product of 511bp;

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

iii) the positive control of the extraction product shows an amplification product of 511bp.

The data analysis shall consider only the bands that satisfy the following requirements:

- The band size is larger than 50 bp;
- The band is comprised between the two Alignment marker bands;
- The intensity of the emission peak is greater than the threshold value of 5%.

In case of overlapping 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" 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 (511bp) revealed by the electrophoresis is evaluated by their comparison

with the reference molecular weight L100 and with the positive control of extraction and amplification. The visual evaluation is considered sufficient and adequate.

If an unexpected band is present, the enzymatic digestion is not performed, the result of the test is expressed as “undeterminable assemblage”.

If the sample shows no amplification, reference DNA of *G. duodenalis* is added to the DNA/fecal sample to be tested and amplified according to paragraph 7.2.7 in order to exclude the presence of PCR inhibitors. If the expected 511 bp fragment is not amplified, the result of the test is expressed as “undeterminable assemblage”.

7.2.7 Test for the presence of inhibitors by PCR

If nor otherwise stated, keep tubes on ice or in a refrigerated box, use tips with barrier and wear disposable gloves.
At each working session, use reference DNA as positive control.
The following procedure uses a PCR master mix at a 2X concentration, to be modified in case of different concentration.

a) Thaw DNA/fecal sample (7.2.1 point “y”, 7.2.2 point “u”), 2x PCR MasterMix, Set A and the positive amplification control, reference DNA.
b) Mark with a progressive number an adequate number of 0.2 mL PCR tubes.
c) Prepare an adequate volume of the amplification mix. Calculate the volume based on a single sample amplification mix (tabella H) and of the total number of samples added of two reactions (one for the positive amplification control and one for the negative control).

Table H. single sample amplification mix: components and volumes

2x PCR MasterMix	25 µL
H_2O	14 µL
Set A	1 µL
Reference DNA	5 µL
Totale	45 µL

d) Vortex the amplification, mix and centrifuge at maximum speed for few seconds.
e) Transfer 45 µL of the amplification mix to each PCR tube (7.2.7 point “c”).
f) Add in each tube 5 µL of DNA/fecal sample (7.2.1 punto “y”, 7.2.2 point “u”) to analyze, in the positive control add 5 µL of reagent grade water.
g) Close the tubes, mix by vortexing and centrifuge at maximum speed for few seconds.
h) Start the amplification cycle in the thermocycler according to table E, paragraph 7.2.3. Wait until the temperature reaches 95°C, then pause the instrument and insert the tubes in the device, exit from the pause mode.
i) At the end of the amplification reaction, centrifuge the tubes at maximum speed for few seconds.
j) Keep tubes on ice or refrigerated until the Nested PCR is performed, according to paragraph 7.2.4.

7.2.8 Visualization of the results

To visualize the results, follow the procedure described at point 7.2.5.

7.2.9 Interpretation of the PCR amplification results to verify the presence of inhibitors

To interpret the results, follow the procedures described in 7.2.6.

The amplification test is considered valid if:
i. the positive control shows an amplification product of 511bp;
ii. the amplification of the negative control does not show any amplification product or, eventually, only bands related to unused oligonucleotides and/or primer dimer.

If the expected 511bp fragment is amplified, the presence of inhibitors will be excluded. The sample is considered as “negative” for the presence of *Giardia*, the assemblage of the sample is not identified, and the result of the test is expressed as “undeterminable assemblage”.

7.2.10 Enzymatic DNA digestion with endonuclease

Unless otherwise stated, store tubes on ice; use tips with barrier and wear disposable gloves.
At each working session, digestion reactions with the *HaeIII* enzyme are performed. Moreover, a positive control, represented by the amplification product of the reference fecal DNA, is processed alongside to verify the proper execution of the digestion reaction.

The procedure uses a restriction enzyme at the initial concentration of 10 U/µl and 10x concentrated restriction enzyme buffer. In case of different concentrations, adjust the protocol according to the manufacturer's recommendations.

a) Thaw PCR products, restriction enzymes and 10X restriction enzyme buffers.

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

c) Prepare an adequate volume of the enzymatic digestion mix for each restriction enzyme. Calculate the volume based on a single sample enzymatic digestion mix (Table H) and of the total number of samples plus the positive control.

Table H – Enzymatic digestion mix for a single sample: components and volumes

10x buffer	2 µL
Restriction enzyme	1 µL (10U)
PCR product (7.2.4 point "i")	10 µL
H_2O	7 µL
Total	20 µL

d) Mix each enzymatic digestion mix by vortexing and centrifuge at maximum speed for few seconds.

e) Transfer 10 µL of the cumulative digestion mix (point "c") to each 0.2 mL tube.

f) To each tube, add 10 µL of the nested PCR product (7.2.4 point "i") to be tested.

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

h) Incubate the tubes in a thermomixerat 37°C for 3h without shaking.

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

l) Keep tubes on ice or refrigerated until the electrophoresis is performed.

7.2.11 Visualization of the results

The visualization of the result is carried out as described in paragraph 7.2.5.

7.2.12 Interpretation of the results of enzymatic digestion

Interpretation of the result of enzymatic digestion is as described in paragraph 7.2.6.

In case of Assemblage D, it is acceptable that the 194 bp and 200 bp bands will appear as a single band of higher intensity.

In the case of assembly B, the failure to resolve the two 26 and 24 bp bands which may appear as a single band is acceptable.

The size of the restriction fragments, revealed by the electrophoresis, is evaluated by comparison with the reference molecular weight DNA size marker or L50 and with the positive control of digestion. As reported in Table A, the differences in size of the restriction fragments are evident, so the visual evaluation is considered sufficient and adequate.
The enzymatic digestion test is valid if the digestion of the positive control shows a pattern in accordance with Table A.

After the enzymatic digestion of the amplified fragments, the identification at the assemblage level is made by comparing the size of the bands produced by the sample(s) with those shown in Table A.
In case the sample shows unexpected bands, the identification at the assemblage level is not possible and the result of the test is expressed as "undeterminable assemblage".

8. Results

The results are expressed as follows, whenever only some bands are identified, underlined bands are essential to define the assemblies:

If the digestion profile with HaeIII comprises bands of 201, 150, 110, 50 bp, then the sample is identified as *G. duodenalis* assemblage A.

If the digestion profile with HaeIII comprises bands of 150, 117, 110, 84, 26, 24 bp, then the sample is identified as *G. duodenalis* assemblage B.

If the digestion profile with HaeIII comprises bands of 194, 150, 102, 50, 15 bp, then the sample is identified as *G. duodenalis* assemblage C.

If the digestion profile with HaeIII comprises bands of 200, 194, 117 bp, then the sample is identified as *G. duodenalis* assemblage D.

If the digestion profile with HaeIII comprises bands of 186, 150, 110, 26, 24, 15 bp, then the sample is identified as *G. duodenalis* assemblage E.

If the digestion profile with HaeIII comprises bands of 186, 150, 110, 50, 15 bp, then the sample is identified as *G. duodenalis* assemblage F.

If the digestion profile with HaeIII comprises bands of 194, 165, 102, 50 bp, then the sample is identified as *G. duodenalis* assemblage G.

In case the digestion test is valid but the sample shows a profile of bands not present in Table A, the identification at the assemblage level is not possible and the result of the test is expressed as “undeterminable assemblage”.

9. Characteristics of the method

This method has been characterised in terms of repeatability and specificity. The results of the validation protocol are used to confirm that the method is suitable for the expected aim and are reported in the validation report, which can be sent upon request.

10. Safety measures

This method has to be carried out only by authorized personnel. The operator should wear individual protection devices during the test performance. For general safety measures, refer to the guidelines of CDC.