

COMPARISON OF EFFICIENCY OF VARIOUS DNA EXTRACTION METHODS FROM CYSTS OF *GIARDIA INTESTINALIS* MEASURED BY PCR AND TAQMAN REAL TIME PCR

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Summary: COMPARISON OF EFFICIENCY OF VARIOUS DNA EXTRACTION METHODS FROM *GIARDIA INTESTINALIS* CYSTS MEASURED BY PCR AND TAQMAN REAL TIME PCR

The aim of the presented study was to work out an effective method of extraction of DNA from *Giardia intestinalis* cysts as well as a sensitive and specific method for detection of DNA of this protozoan using a polymerase chain reaction (PCR). Twelve protocols for DNA extraction have been compared. Purification and extraction of DNA were preceded by additional actions in order to destroy the cysts' wall. The highest effectiveness of DNA extraction was obtained in case of alternating application of freezing the samples in liquid nitrogen and their incubation in water bath in the temperature of 100 °C, and then the extraction with the QIAamp DNA Tissue Mini Kit (QIAGEN) – T kit – with an all night long incubation with proteinase K in 56 °C. Effectiveness of DNA extraction with the use of each kit after extraction with each treatment was measured by nested PCR product of β -giardin gene fragment and C_T values of real time PCR of the SSU rRNA gene of *G. intestinalis*. The detection limit, defined as the lowest number detected in 100 % cases, was 100 cysts per 200 μ l when effectiveness was evaluated with nested PCR and 50 oocysts with real time PCR after extraction DNA with T kit. Results of our comparative studies have shown that all stages preceding the molecular detection of *G. intestinalis* DNA are equally important, and materially influence on the final effect and this version of method seems to be very useful for the sensitive detection of DNA of *G. intestinalis*.

Keywords: DNA extraction, *Giardia intestinalis*, PCR, real time PCR.

Résumé : ÉTUDE COMPARATIVE DE L'EFFICACITÉ DE DIFFÉRENTES MÉTHODES D'EXTRACTION D'ADN DE KYSTES DE *GIARDIA INTESTINALIS* PAR PCR ET PCR EN TEMPS RÉEL (TAQMAN).

Le but de la présente étude est de proposer une méthode d'extraction d'ADN efficace à partir des kystes de *Giardia intestinalis* et une méthode de détection, à la fois sensible et spécifique, de l'ADN de ce protozoaire par PCR. Douze protocoles d'extraction d'ADN ont été comparés. La purification et l'extraction de l'ADN ont été précédées d'étapes visant à détruire la paroi kystique. L'efficacité optimale a été obtenue en alternant la congélation des échantillons dans l'azote liquide et leur incubation dans de l'eau portée à 100 °C, puis par extraction avec le QIAamp DNA Tissue Mini Kit (QIAGEN) – T kit – avec incubation la nuit entière en présence de protéinase K à 56 °C. L'efficacité d'extraction obtenue avec chaque kit, selon chaque traitement, a été estimée par observation de l'amplicon du gène de la β -giardine obtenu par PCR nichée et d'après les valeurs de C_T obtenues par PCR en temps réel avec des amorces amplifiant le gène SSU rARNr de *G. intestinalis*. La limite de détection, définie d'après le plus faible pourcentage de cas détectés était de 100 kystes pour 200 microlitres. Les résultats de nos études comparatives montrent l'égle importance de toutes les étapes précédant la détection moléculaire de l'ADN de *G. intestinalis* et leur influence sur la détection finale, et que cette version méthodologique semble très utile pour une détection sensible de l'ADN de *G. intestinalis*.

Mots clés : extraction d'ADN, *Giardia intestinalis*, PCR, PCR en temps réel.

INTRODUCTION

Giardia intestinalis, also called *Giardia lamblia* or *Giardia duodenalis*, is a protozoan that inhabits in the upper small intestine of humans and several other vertebrates (Graczyk *et al.*, 1998, 2008; Majewska & Kasprzak, 2000; Adam, 2001; Sprong *et al.*, 2009). This parasite infects thousands of people all over the world and is the cause of giardiasis - acute and chronic manifestations including diarrhea and malabsorption (Adam, 2001; van der Giessen *et al.*, 2006).

The formation of the cyst allows the parasite to survive outside the host and to resist the adverse conditions (Graczyk *et al.*, 2008; Majewska *et al.*, 2009). *Giardia* infections are initiated by ingestion of cysts from contaminated food or drinking and recreation water, either by direct fecal-oral contact (Lujan *et al.*, 1997; Graczyk *et al.*, 2008; Monis *et al.*, 2009).

Traditionally, the laboratory diagnosis of intestinal protozoan infections relies on the detection of trophozoites and cysts by microscopic stool examination. However, the majority of diagnostic methods used in the clinical practice have a limited application regarding the detection of protozoans in water samples (Randi *et al.*, 2003; Guy *et al.*, 2003). The most important restriction is much lower concentration of cysts in water in comparison with material collected from patients. In order to improve the monitoring of oocysts of *Cryptosporidium* in water,

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the United States Environmental Protection Agency (USEPA) introduced the 1622 (USEPA 2001a) Method and then 1623 (USEPA 2001b), used to concentrate and detect the oocysts of *Cryptosporidium* and cysts of *Giardia* in water samples. Both methods are used to determine the presence and concentration of the resistant forms in water, and they consist of: filtration, concentration of (oo)cysts, immunomagnetic separation, fluorescent antibody and counter staining, differential interference contrast, microscopic detection and enumeration. These methods do not allow on the identification of *Cryptosporidium* or *Giardia* species, as well as the origin of species, or the pathogenic potential of oocysts and cysts. Such data can be obtained with the use of one of the molecular methods based on the nucleic acids isolated from the resistant forms of these parasites, which were obtained with the discussed methods. Because samples of water have only a few *Giardia* cysts in the presence of many other microorganisms, the recovery of *Giardia* DNA during DNA extraction is very important (Guy *et al.*, 2003; Skotarczak, 2009). Numerous direct DNA extraction methods have been tested in the preparation of DNA from *G. intestinalis* cysts, we tried sonication, freezing in liquid

nitrogen and boiling in water bath preceding the usage of three commercial kits for the extraction of DNA from *G. intestinalis* cysts (twelve DNA extraction protocols). The effectiveness of DNA extraction was measured with semi-nested PCR signal and C_T values of TaqMan real time PCR targeting a specific region of rDNA of *G. intestinalis*.

MATERIALS AND METHODS

Purified bovine derived preparations of *Giardia intestinalis* cysts were obtained from Bulk Stock Live, BTF Biomérieux, Australia. Samples containing 7.5×10^3 cysts of *G. intestinalis* with PBS in volume of 1 ml were used for genome DNA extraction. Twelve DNA extraction protocols were investigated successively (Table I). In the protocols 1-3 the DNA extraction was carried out with the use of three kits without any preceding actions. In the protocols 4-12 the extraction with three kits was preceded by different combinations of freeze-thaw or sonication rounds. The effectiveness of DNA extraction was evaluated with semi-nested PCR and TaqMan real time PCR after

Protocol 1	No treatment - QIAamp DNA Stool Kit (QIAGEN) – St kit; A. incubation with proteinase 70°C/10 min B. incubation with proteinase 56°C/night
Protocol 2	No treatment - QIAamp DNA Tissue Mini Kit (QIAGEN) – T kit ; A. incubation with proteinase 56°C/3 h B. incubation with proteinase 56°C/night
Protocol 3	No treatment - FastDNA SPIN Kit for Soil (MP Biomedicals) – So kit; modified protocol, extraction carried out without FastPrep Instrument, incubation with proteinase 56°C/night
Protocol 4	Boiling (100°C/5 min) and freezing (-70°C/5 min) repeated 5× - QIAamp DNA Stool Kit (QIAGEN) – St kit; A. incubation with proteinase 70°C/10 min B. incubation with proteinase 56°C/night
Protocol 5	Boiling (100 °C/5 min) and freezing (-70°C/5 min) repeated 5× - QIAamp DNA Tissue Mini Kit (QIAGEN) – T kit; A. incubation with proteinase 56°C/3 h B. incubation with proteinase 56°C/night
Protocol 6	Boiling (100°C/5 min) and freezing (-70°C/5 min) repeated 5× - FastDNA SPIN Kit for Soil (MP Biomedicals) – So kit; modified protocol, extraction carried out without FastPrep Instrument, incubation with proteinase 56°C/night
Protocol 7	Boiling (100°C/2 min) and freezing in liquid nitrogen (2 min) 3× - QIAamp DNA Stool Kit (QIAGEN) – St kit; A. incubation with proteinase 70°C/10 min B. incubation with proteinase 56°C/night
Protocol 8	Boiling (100°C/2 min) and freezing in liquid nitrogen (2 min) 3× - QIAamp DNA Tissue Mini Kit (QIAGEN) – T kit; A. incubation with proteinase 56°C/3 h B. incubation with proteinase 56°C/night
Protocol 9	Boiling (100°C/2 min) and freezing in liquid nitrogen (2 min) 3× - FastDNA SPIN Kit for Soil (MP Biomedicals) – So kit; modified protocol, extraction carried out without FastPrep Instrument, incubation with proteinase 56°C/night
Protocol 10	Sonication (3×2 min) - QIAamp DNA Stool Kit (QIAGEN) – St kit; A. incubation with proteinase 70°C/10 min B. incubation with proteinase 56°C/night
Protocol 11	Sonication (3×2 min) - QIAamp DNA Tissue Mini Kit (QIAGEN) – T kit; A. incubation with proteinase 56°C/3 h B. incubation with proteinase 56°C/night
Protocol 12	Sonication (3×2 min) - FastDNA SPIN Kit for Soil (MP Biomedicals) – So kit; modified protocol, extraction carried out without FastPrep Instrument, incubation with proteinase 56°C/night

Table I. – DNA extraction protocols.

extraction with each preisolation treatment. For the determination of the detection limit samples containing 7.5×10^3 cysts of *G. intestinalis* were diluted in distilled water to 1×10^3 , 5×10^2 , 1×10^2 , 5×10^1 and 1×10^1 cysts per 200 μ l. These dilutions are approximate, because the cysts were not counted.

SEMI-NESTED PCR AMPLIFICATION

A highly polymorphic region of the β -giardin gene was amplified by semi-nested PCR protocol (Caccio *et al.*, 2002). The method involves the amplification of an approximately 753 bp-long primary product followed by a secondary reaction of an internal fragment with a length of approximately 384 bp. For the I. PCR step, a PCR product was amplified by using primers G7 5'-AAGCCCAGACCTCACCCGAGTGC-3' and G759 5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3' (Caccio *et al.*, 2002). For the II PCR step, a PCR product was amplified by using primers G376 5'-CATAACGACGCATCGCGGCTCTCAGGAA-3' and G7595'-GAGGCCGCCCTGGATCTTCGAGACGAC-3' (Caccio *et al.* 2002). Each PCR mixture (total volume, 10 μ l) contained 1 μ l of Sigma (USA) 10 \times PCR buffer, 30 mM MgCl₂, 0.3 nM of each deoxynucleotide triphosphate, 5 pM of each primer, 0.5 U of *Taq* polymerase Sigma (USA), and 1 μ l of DNA template. The reactions were performed in a DNA thermal cycler (Biometra, Germany and MJ Research, USA). Thermal-time profiles in first and second PCR were the same as described by Castro-Hermida *et al.* (2008). Negative control reaction mixtures contained sterile distilled water in place of template DNA. PCR products were visualized by 1.5 % agarose gel electrophoresis.

TAQMAN REAL TIME PCR

A region of the small subunit rRNA gene of *G. intestinalis* was used as a target sequence for real time PCR. Primers Gd-80F and Gd-127R amplifying a 62-bp fragment (Haque *et al.*, 2007) were used. The PCR samples (volume 10 μ l) contained 14 pM of each primers, 8 pM of the probe Gd-FT (Genomed, Poland), 0.5 nM of each dNTP (Novazym, Poland), 15 mM MgCl₂, 1.5 U Taq DNA Polymerase (Sigma, USA) and 2 μ l of DNA template. Reactions were performed in Rotor Gene 6000 (Corbett, Australia). Oligonucleotides sequences and thermal profile were performed as described previously (Haque *et al.*, 2007) with modification of cycles' number (65 cycles). The threshold cycle (C_T) was calculated for individual samples by the internal software of the cycler using the manual threshold setting at a fluorescence value of $10^{-1.75}$. Appropriate negative controls were included in each PCR run. All analysis were carried out in four replicates. TaqMan real time PCR reaction was developed to show reproducible PCR efficiency values with regard to all samples preceded by different protocols.

RESULTS

Comparison of efficiency of various *G. intestinalis* undiluted cyst treatments prior to DNA extraction with the use of twelve protocols, measured with semi-nested PCR signal and C_T values of real time PCR have shown that the best results were obtained after extraction of DNA with T kit, preceded with triple liquid nitrogen/water bath in 100 °C for 2 minutes and with proteinase K digestion all night long (protocol 8 B, Table II).

The assessment of sensitivity was determined using the analysis of five different diluted suspensions (Table III) of *G. intestinalis* cysts in four replicates each. The detection limit, defined as the lowest number detected in 100 % cases, was 100 cysts per 200 μ l when effectiveness was evaluated with PCR and 50 cysts with real time PCR after extraction of DNA with T kit. Whereas the detection limit after DNA extraction with two other kits was significantly higher (100 – for St kit and 1000 for So kit measured by real-time PCR) (Table III; Figs 1, 2).

DNA extraction protocols	Results of semi-nested PCR ¹	Results of Taqman RealTime PCR (threshold cycle - C_T)
Protocol 1	A	–
	B	–
Protocol 2	A	–
	B	–
Protocol 3	–	–
Protocol 4	A	–
	B	–
Protocol 5	A	–
	B	–
Protocol 6	–	–
Protocol 7	A	52.8 \pm 0.56
	B	15.2 \pm 0.82
Protocol 8	A	48.4 \pm 0.25
	B	13.5 \pm 0.78
Protocol 9	–	49.7 \pm 0.48
Protocol 10	A	–
	B	21.8 \pm 0.42
Protocol 11	A	53.8 \pm 0.66
	B	18.9 \pm 0.73
Protocol 12	–	54.8 \pm 0.35

+ + + + very strong intensity of the DNA band as determined by ethidium bromide stained-agarose gel electrophoresis; + + + strong intensity of the DNA band; + + medium intensity of the DNA band; +, weak intensity of the DNA band; – no DNA band visualized on ethidium bromide stained-agarose gel electrophoresis.

Table II. – Comparative efficiency of various *G. intestinalis* cysts undiluted treatments prior to DNA extraction with the use of twelve protocols, measured with PCR signal of product of the β -giardin gene and Threshold cycle (C_T) of real time PCR (18S rRNA gene).

DNA extraction protocols	Results of semi-nested PCR ¹					Results of TaqMan real time PCR (threshold cycle - C _T)				
	Number of cysts					Number of cysts				
	1×10 ³	5×10 ²	1×10 ²	5×10 ¹	1×10 ¹	1×10 ³	5×10 ²	1×10 ²	5×10 ¹	1×10 ¹
7 B	++	+	–	–	–	26.2 ± 0.18	28.4 ± 0.52	33.7 ± 0.48	41.4 ± 0.16	-
8 B	+++	+++	+++	++	–	21.5 ± 0.93	25.9 ± 0.38	28.9 ± 0.41	33.8 ± 0.53	48.2 ± 0.82
9	–	–	–	–	–	43.8 ± 0.29	49.5 ± 0.38			

+++ strong intensity of the DNA band; ++ medium intensity of the DNA band; + weak intensity of the DNA band; – no DNA band visualized on ethidium bromide stained-agarose gel electrophoresis.

Table III. – The detection level of *G. intestinalis* cysts in water suspensions defined by semi-nested PCR signal and threshold cycle (C_T) of real time PCR with the use of three protocols (7 B, 8 B, 9). Data represent mean +/- standard deviation for positive samples.



Fig. 1. – Products of amplification of gene fragment encoding β -giardin of *G. intestinalis* obtained from DNA isolated with protocol 8 B (3x boiling (100 °C/2 min) and freezing in liquid nitrogen (2 min), QIAamp DNA Tissue Mini Kit, QIAGEN, incubation with proteinase 56 °C/night)

M – marker of molecular masses; paths 1-4 – amplicons obtained from DNA isolate received from 10 cysts; paths 5-8 – amplicons obtained from DNA isolate received from 50 cysts; paths 9-12 – amplicons obtained from DNA isolate received from 100 cysts; paths 13-16 – amplicons obtained from DNA isolate received from 500 cysts; paths 17-20 – amplicons obtained from DNA isolate received from 1,000 cysts; paths 21, 22 and 23 – amplicons obtained from positive controls; path 24 – negative control



Fig. 2. – Products of amplification of gene fragment encoding β -giardin of *G. intestinalis* obtained from DNA isolated with protocol 7 B (3x boiling (100 °C/2 min) and freezing in liquid nitrogen (2 min), QIAamp DNA Stool Kit, QIAGEN, incubation with proteinase 56 °C/night)

M – marker of molecular masses; paths 1-4 – amplicons obtained from DNA isolate received from 10 cysts; paths 5-8 – amplicons obtained from DNA isolate received from 50 cysts; paths 9-12 – amplicons obtained from DNA isolate received from 100 cysts; paths 13-16 – amplicons obtained from DNA isolate received from 500 cysts; paths 17-20 – amplicons obtained from DNA isolate received from 1,000 cysts; paths 21, 22 and 23 – amplicons obtained from positive controls; path 24 – negative control.

DISCUSSION

Giardia intestinalis (*G. duodenalis* or *G. lamblia*) is a common protozoan parasite that infects a wide range of mammalian hosts. In humans it is one of the most frequently identified protozoan parasites causing gastrointestinal disease worldwide (Olson *et al.*, 2004; Caccio *et al.*, 2005). Light microscopy or immunofluorescence assay has been used to identify *G. intestinalis* in most laboratories. However, these techniques might not be sensitive enough to detect cysts in environmental samples (Rochelle *et al.*, 1997; Haque *et al.*, 2007). Recently, a few PCR-based techniques have been developed for detection and genotypic characterization of *G. intestinalis* (Yong *et al.*, 2002; Muller *et al.*, 2008; Ajjampur *et al.*, 2009; Tashima *et al.*, 2009). In water samples *G. intestinalis* cysts occur in very low concentration (Kaucner & Stinear, 1998), so the recovery of this pathogen DNA during DNA extraction becomes important.

There is not much data in the available literature relevant to initial treatment of cysts before the DNA extraction (Mahbubani *et al.*, 1991; Sulaiman *et al.*, 2004) despite the *Giardia* cysts are characterized with high resistance on external influence (Carranza *et al.*, 2002; Sunderland *et al.*, 2007). *Cryptosporidium* oocysts have similar features, because before the DNA extraction they are subjected to different actions in order to destroy their wall (Sluter *et al.*, 1997; Harris & Petry, 1999; Jiang *et al.*, 2005). *Giardia* cysts are oval in shape and range in size from 6 to 10 μ m. The cyst wall varies from 0.3 to 0.5 μ m in thickness that is formed by an outer filamentous layer and an inner membranous layer including two membranes that enclose the periplasmic space (Adam, 2001). The biochemical composition of the cyst wall is composed of carbohydrates, in the form of N-acetyl galactosamine polymers (Lujan *et al.*, 1997), and cyst wall proteins (CWPs) (Lujan *et al.*, 1995; Sun *et al.*, 2003). The presented studies have shown that the extraction of DNA from *Giardia* cysts not preceded with any actions that could destroy the cyst wall is not

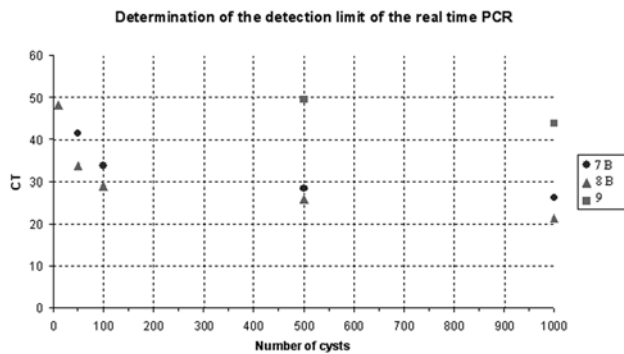


Fig. 3. – Determination of the DNA-based detection limit of the real-time PCR after three protocols: 7 B (3x boiling (100 °C/2 min) and freezing in liquid nitrogen (2 min), QIAamp DNA Stool Kit, QIAGEN, incubation with proteinase 56°C/night), 8 B (boiling – 100 °C/2 min - and freezing in liquid nitrogen - 3x/2 min, QIAamp DNA Tissue Mini Kit, QIAGEN, incubation with proteinase 56°C/night), 9 (boiling – 100 °C/2 min - and freezing in liquid nitrogen - 3x/2 min - FastDNA SPIN Kit for Soil, MP Biomedicals; modified protocol. Average of triplicate thresholds cycle (CT) values are presented. Standard deviation was too small to be depicted.

effective (protocols 1-3) (Table II). Effectiveness of DNA extraction with the use of each kit after extraction with each treatment was measured by semi-nested PCR product of β -giardin gene fragment and C_T values of real time PCR of the SSU rRNA gene of *G. intestinalis*. The β -giardin gene is considered unique to *Giardia* spp., effectively eliminating the chance of cross-amplification of host or other no target templates (Caccio *et al.*, 2002). Gene encoding 18S rRNA is often used as a genetic marker for the detection of *G. intestinalis* because of the possibility of designing primers complementary to its sequences, specific only for this species (Rochelle *et al.*, 1997; Haque *et al.*, 2007, Nantavisai *et al.*, 2007; Castro-Hermida *et al.*, 2008).

Comparison of efficiency of various *G. intestinalis* undiluted cysts treatments prior to DNA extraction with the use of three kits have shown that the best results were obtained after extraction of DNA with QIAamp DNA Tissue Mini Kit (T kit), preceded with triple liquid nitrogen/water bath in 100 °C for 2 minutes and with proteinase K digestion all night long (protocol 8 B). The alternating application of boiling and freezing in the temperature of -70 °C before the extraction did not cause the destruction of cysts cell wall and the extraction occurred to be not effective (protocols 4-6), whereas sonication gave satisfactory results (protocols 10-12) but worse than in case of applying the alternating boiling and freezing in liquid nitrogen (protocols 7-9) (Table II). Besides, the usage of liquid nitrogen enabled to prepare more number of samples dependent on the capacity of container in which they were frozen, whereas the appliance for sonication allowed to subject to ultrasounds

only single samples what lengthened the time of preparing samples for DNA extraction. Time of treatment with proteinase K was also very important, only the all night long incubation guaranteed the effective extraction of DNA from *Giardia* cysts (Table II). The extraction of DNA from undiluted solution including *Giardia* cysts with T kit and St kit occurred to be equally effective (protocols 7 B and 8 B) (Table II), but isolation from smaller number of cysts was more effective in case of using T kit (protocol 8 B) (Table III). It may result from fact that St kit is designed for isolation of DNA from stool and cysts present in water environment may be partly removed with pollutions and inhibitors. So kit occurred to be completely ineffective, but it was used without a FastPrep Instrument recommended by producer, which is an integral part of the kit because of its high price. Similar trials were carried out earlier in order to extract the DNA from oocysts of *C. parvum* and the effects were very similar to those in case of *G. intestinalis* (Adamska *et al.*, 2010). The detection limit, defined as the lowest numbers detected in 100 % cases, was 100 cysts per 200 μ l when effectiveness was evaluated with nested PCR and 50 oocysts with real time PCR after extraction DNA with T kit (Table III). We used small number of cysts, so the extraction efficiency can be lower and detection limit can be smaller than in case of using large number of cysts.

When higher sensitivity of PCR is required, nested PCR is often used, which involves two reactions. The product of the first reaction is used as a template for the second reaction. In this way, all irregularities should take place in the first reaction. The second one, starting amplification from a reasonably high amount of the template should be already fully effective. Sensitivity of detection of *G. intestinalis* has been further improved by using nested PCR (Caccio *et al.*, 2002). Unfortunately, conventional nested PCR is not very suitable for routine diagnostics usage because of the risk of contamination by short amplified DNA fragments (Abravaya *et al.*, 1997). That risk may be eliminated by applying the real time PCR. In the presented work, we have applied a TaqMan real time PCR protocol for sensitive detection of *G. intestinalis*, which development was carried out by Haque *et al.* (2007). Thus, PCR can be monitored continuously, in closed tubes, without the need of any subsequent, discontinuous analysis and without electrophoresis. This version of method seems to be very useful for the sensitive detection of DNA of *G. intestinalis*, especially when our studies have shown that TaqMan real time PCR method characterizes with higher sensitivity than typical PCR reaction (Table III). Results of our comparative studies have shown that all stages preceding the molecular detection of *G. intestinalis* are equally important and materially influence on the final effect.

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