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Original article

Influence of selected stool concentration techniques on the effectiveness of PCR examination in *Giardia intestinalis* diagnostics

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Abstract

Giardia intestinalis is a widespread parasitic protozoa which has great significance as a public health threat. Molecular diagnostics of stool sample can be unreliable because of the presence of inhibitors of enzymatic reactions. The aim of this study was to determine the effectiveness of selected pre-treatment methods of fecal samples for further PCR-based diagnostics of *G. intestinalis*, and the effect of each component of pre-treatment solutions on PCR reactions. Seven stool concentration techniques were compared. The results showed that the most efficient concentration method for stool sample preparation for detection of *G. intestinalis* by PCR is centrifugal flotation with Percoll (with saturated NaNO₃ as the flotation solution). This method is relatively inexpensive, less labor-intensive, and suitable for epidemiological monitoring and clinical investigations.

Key words: *Giardia intestinalis*, PCR, molecular diagnostics, protozoa, stool concentration techniques, pre-PCR processing

Introduction

Giardia intestinalis is an important worldwide pathogen causing diarrhea in man and many species of animals including livestock, pets and wildlife (Lane and Lloyd 2002, Zygnier et al. 2006, Bajer et al. 2008, Feng and Xiao 2011). The parasite has two stages in its life cycle: infective cyst and trophozoite. The major sources and routes of cyst transmission are contaminated water and food or direct fecal-oral contact (Adam 2001). In humans, infection can lead to diarrhea, greasy stools, flatulence and abdominal cramps.

While a high percentage of cases are asymptomatic, infection in young children may be the cause of poor cognitive function and stunted growth (Berkman et al. 2002). Basic diagnostic methods for *G. intestinalis* are relatively simple with a commercial test widely available involving light microscopy examination of stool samples, ELISA assays, or immunofluorescence antibody test (IFAT). However, only the molecular characterization of *G. intestinalis* genotypes provides a guarantee of accurately identifying organisms and assessing zoonotic transmission. In addition, molecular diagnostics is more sensitive in comparison to stan-

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dard methods and provides the possibility of detecting low levels of infection (Guy et al. 2003, Read et al. 2004, Plutzer et al. 2010, Solarczyk et al. 2010).

To date, seven assemblages of *G. intestinalis* have been identified. However, only assemblages A and B infect humans and are potentially zoonotic (Read et al. 2004, Lebbad et al. 2010). Unfortunately, there are a number of drawbacks which can cause the amplification reaction to fail, including inhibitors and low quantity and/or quality of acquired nucleic acids. Various mechanisms of action of PCR inhibitors have been described, including the influence of some reagents on nucleic acids during sample treatment, impediment of primer annealing, impact on polymerase efficiency and stability (Schrader et al. 2012). The heterogeneous nature of feces offers challenges in the application of PCR to sensitive detection of pathogens. Complex polysaccharides, bile salts, lipids, hemoglobin degradation products, urate, polyphenolic compounds, and heavy metals are potential inhibitory substances which can be present in feces. In addition, certain inorganic compounds, which are components of sample pretreatment solutions, also have inhibitory effects on PCR reaction (Al-Soud and Radstrom 1998, Staufer et al. 2008, Schrader et al. 2012). Such issues have to be taken into consideration when optimizing diagnostic procedures. Therefore, the aims of the present study were to: 1) determine the capacity of various pre-treatment methods of fecal samples in PCR-based diagnostics of *G. intestinalis*; 2) determine the effect of each component of pre-treatment solutions on PCR reactions; and 3) compare the sensitivity of fecal sample pre-treatment methods for *G. intestinalis* cyst detection by microscopic examination.

Materials and Methods

Source of purified cysts (stock solution)

G. intestinalis viable cysts, produced by passage of the human strain H3 (assemblage B) of *G. intestinalis* through Mongolian gerbils, were obtained from Waterborne Inc. (New Orleans, La., US). According to the product description, cysts were purified by the producer with the use of sucrose and Percoll® density gradient centrifugation and stored in phosphate-buffered saline with penicillin, gentamicin and Tween 20. The number of cysts in the stock solution was determined in the laboratory by using a Fuchs-Rosenthal chamber. Thus, precise numbers of cysts were determined.

Stool Specimens

Stool samples were collected from European shorthair cats (*Felis catus*). Fecal samples (1 g), free of parasites, were spiked with the following number of cysts: 20000, 10000, 5000 and 2000 and stored at 4-8°C until further analysis.

Evaluation of inhibition of PCR assay by different components used in cyst recovery method solutions

The second aim of the study was to determine the inhibiting effect of the selected components (used in cyst recovery methods) on PCR. This part of the study was undertaken in 3 variants with various *G. intestinalis* cyst concentrations. For this purpose, different amounts of cysts were added to water and selected component solutions. Three different ratios of compound to cyst water suspension (1:10, 1: 20, 40:1) were used. The final volume of reconstituted suspensions was 200 µl. Compounds used in cyst recovery methods were as follows: diethyl ether, 10% formalin, saturated ZnSO₄, saturated NaNO₃, saturated MgSO₄, saturated NaCl, 25% Percoll solution, saturated sucrose solution.

Methods of cyst isolation and concentration from feces

Water-ether sedimentation (Allen and Ridley 1970)

This method was based on the WHO manual (Ash et al. 1994) previously described by Allen and Ridley (1970) with certain modifications. One gram of each stool sample was mixed with 10 ml redistilled water (Millipore Water Purification System) and filtered through a plastic sieve into a beaker. The stool suspension was then poured into a 15 ml conical centrifuge tube, followed by adding 3 ml of diethyl ether. The suspension was then mixed in a centrifuge tube by shaking vigorously for 30 s and centrifuged (800g x 10 min). The centrifugation resulted in the following four layers: diethyl ether (with fat and debris), plug of debris, water (with debris) and sediment. The three layers from the top were decanted and the sediment was washed by centrifugation (1100g x 10 min) with 13 ml redistilled water, and stored at 4°C until further analysis.

Fülleborn's flotation method (Fülleborn 1920)

One gram of feces was mixed with 7 ml of saturated NaNO_3 and filtered through a plastic sieve into a beaker. The suspended fecal material was placed in a glass tube. After 15 minutes, 200 μl from the top of the solution was removed and placed in 15-ml conical centrifuge tube. Samples were then washed by centrifugation in 13 ml of redistilled water (1100 g x 10 min), and the sediment was stored at 4°C until further analysis.

Fülleborn's flotation method with Willis' modification (Willis 1921)

One gram of feces was mixed with 15 ml saturated NaNO_3 and filtered through a plastic sieve into a beaker. The suspended fecal material was placed in a glass tube up to a convex meniscus and the tube was then covered with cover glass. After 15 minutes the cover glass was placed on a glass slide. The preparation was examined by microscopy and the slide was flushed with redistilled water into a 15-ml conical centrifuge tube and washed by centrifugation (1100g x 10 min) in 13 ml redistilled water. The sediment was stored at 4°C until further analysis.

Method of centrifugal flotation with diethyl ether step – own method (Ether method)

The modified water-ether concentration procedure was initially performed as previously described above. Three volumes of saturated NaNO_3 were added to the sediment and mixed, followed by centrifugation at 1000 g for 10 min. 200 μl from the top of the solution was then removed and placed into a 15-ml conical centrifuge tube. Samples were then washed by centrifugation (1100 g x 10 min) in 13 ml redistilled water. The sediment was stored at 4°C until further analysis.

Method of centrifugal flotation with Percoll® step (Pertoft et al. 1978)

One gram of feces was mixed with 8 ml 25% Percoll solution and filtered through a plastic sieve into a beaker. The suspension was poured into a 15 ml conical centrifuge tube, followed by centrifugation at 1600 g for 5 min (Pertoft et al. 1978, Karamon et al. 2008). Three volumes of saturated NaNO_3 were added to the sediment and mixed, followed by centrifuga-

tion at 1000 g for 10 min; 200 μl from top of the solution was then removed, placed in a new 15-ml conical centrifuge tube and washed by centrifugation (1100g x 10 min) in 13 ml redistilled water. The sediment was stored at 4°C until further analysis.

Method of Weber's centrifugal flotation with formol-ether step (modified) (Weber et al. 1992)

One gram of feces was mixed with 6 ml 10% formalin solution and filtered through a plastic sieve into a beaker. The solution was then poured into a 15 ml conical centrifuge tube, and 3 ml of diethyl ether was added. Before the centrifugation step (800g x 10 min) the centrifuge tube was mixed by shaking vigorously for 30 sec and the tube was opened for pressure reduction. The centrifugation resulted in the following four layers: diethyl ether (with fat and debris), plug of debris, formalin (with debris) and sediment. The top three layers were decanted and the sediment was re-suspended in 5 ml redistilled water, followed by the addition of 5 ml saturated NaNO_3 before centrifugation (500 g x 10 min). This resulted in the following three layers: redistilled water with *G. intestinalis* cysts, saturated NaNO_3 , and sediment at the bottom. The 3.5 to 4 ml of the top layer was removed. The rest of the top layer and 0.5 ml of the NaNO_3 top layer were placed in a 15 ml conical tube and washed by centrifugation (1100 g x 10 min) in 13 ml redistilled water. The sediment was stored at 4°C until further analysis.

Method of Weber's centrifugal flotation with water-ether step (modified Weber water-ether method)

One gram of feces was mixed with 6 ml redistilled water and filtered through a plastic sieve into a beaker. The solution was then poured into a 15-ml conical centrifuge tube, followed by the addition of 3 ml of diethyl ether. Before the centrifugation step (800 g x 10 min) the centrifuge tube was mixed by shaking vigorously for 30 sec and the tube was opened for pressure reduction. The centrifugation resulted in the following four layers: diethyl ether (with fat and debris), plug of debris, water (with debris), and sediment. The top three layers were decanted and the sediment was re-suspended in 5 ml redistilled water, followed by the addition of 5 ml saturated NaNO_3 before centrifugation (500 g x 10 min). This resulted in the following three layers: redistilled water with *G. intestinalis* cysts, saturated NaNO_3 , and sediment at the bottom. The 3.5 to 4 ml of the top layer was removed. The rest of the top layer and 0.5 ml of the top

portion of the NaNO₃ layer were placed in a 15 ml conical tube and washed by centrifugation (1100 g x 10 min) in redistilled water (total volume of suspension was 15 ml). The sediment was stored at 4°C until further analysis

Microscope examination

Ten microliters aliquot of sediment obtained in each of the concentration methods was used for microscope examination (magnification x200-400).

DNA extraction and PCR amplification

Sediment obtained by the stool concentration techniques described above, including part of a sample used in microscopic examination (slides were flushed with redistilled water to the same Eppendorf tube), were subjected to DNA extraction. The resulting total volume of sediment suspension was estimated to be approximately 200 µl. The procedure described below was subsequently applied to examine the influence of selected chemical compounds on amplification efficiency.

The DNA was extracted with QIAamp® DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol (samples were lysed with proteinase K overnight). The extracted DNA was stored at -20°C until PCR assay following the method described by Read et al. (2004). The semi-nested PCR was performed using the primers GDHeF: 5'-TCA ACG TYA AYC GYG GYT TCC GT-3' (first reaction), GDHiF: 5'-CAG TAC AAC TCY GCT CTC GG-3' (second reaction) and GDHiR: 5'-GTT RTC CTT GCA CAT CTC C-3' (first and second reaction). Primers were designed to amplify a 432 bp fragment of the glutamate dehydrogenase locus. Each reaction mixture (25 µl) contained 12.5 pmol of each primer, 0.2 mM of each dNTP (Fermentas), 50 mM of KCl, 10 mM of Tris-HCl (pH 9.0), 1.5 mM of MgCl₂, 0.5 U of *Taq* DNA polymerase (Qiagen GmbH, Hilden, Germany) and 1-2 µl of DNA. Amplification was performed using a TProfessional 48 thermal cycler (Biometra GmbH, Gottingen, Germany) as follows: an initial cycle at 94°C for 2 min; 56°C for 1 min; 72°C for 2min; followed by 55 cycles of 94°C for 30 s; 56°C for 20 s; and 72°C for 45 s. Final extension was done at 72°C for 7 min. Each semi-nested PCR product was subjected to electrophoresis (1.5% Agarose gel stained with ethidium bromide). Negative and positive DNA probes were included in each PCR reaction.

Results

The effects of each component on the efficiency of PCR are summarized in Table 1. The inhibition of PCR amplification capacities was least significant in samples with the addition of diethyl ether, Percoll and NaNO₃ solutions. Relatively good results were obtained using sucrose solution – negative results occurred only for samples spiked with 200 cysts. The most negative impact of formalin and ZnSO₄ on PCR reaction was observed independently of this compound's concentration. Significant inhibition of PCR reaction was also noted with MgCl₂ solution. However, this phenomenon decreased during reduction of the MgCl₂ concentration. Thus, saturated NaNO₃ was used as a flotation solution for further investigations.

Slides of the water-ether concentration method were relatively difficult to read, which resulted in a low sensitivity and significant uncertainty even in the highest level of spiked samples. In addition, molecular investigations were negative at all levels of enrichment, which suggests low purification of the preparations.

Fülleborn's flotation method with Willis' modification was more effective in microscopic investigation. Single *G. intestinalis* cysts were diagnosed in some samples spiked with 5000 cysts per gram. The slides were more reliable than those from the water-ether sedimentation procedure. PCR positive samples were observed among samples spiked with 20000, 10000 and 5000 cysts per gram.

The standard Fülleborn method modification gave worse results than the Willis modification. A greater volume of collected suspension resulted in increased dilution of preparation. This fact might be the cause of reduction in the sensitivity of microscopic investigation. Moreover, relatively large volumes of collected suspension caused poorer results in the molecular investigations. This could be due to the occurrence of PCR reaction inhibitors in greater concentration. The minimum level of detection using the PCR method was 10,000 cysts per gram.

By microscopic examination, the method of centrifugal flotation with diethyl ether step (own method) found *G. intestinalis* cysts in 1 gram of stool samples spiked with 20,000, 10,000 and 5,000 cysts. However, the samples seemed to be not fully purified by sedimentation with diethyl ether – transparency of slides was not full. PCR positive samples were observed among samples spiked with a minimum of 10,000 cysts per gram.

Flotation-centrifugation preceded by Percoll density gradient sedimentation allowed PCR-positive results to be obtained at all levels of spiking. Micro-

Table 1 Influence of selected chemical compounds on the efficiency of PCR in *Giardia intestinalis* detection

Ratio (compound/ water with cysts)	Diethyl Ether (99.5%)		Formaline (10%)			ZnSO ₄ (saturated)			NaNO ₃ (saturated)			MgSO ₄ (saturated)			NaCl (saturated)			Percoll (25%)			Sucrose (saturated)			
	1:40	1:20	1:10	1:40	1:20	1:10	1:40	1:20	1:10	1:40	1:20	1:10	1:40	1:20	1:10	1:40	1:20	1:10	1:40	1:20	1:10	1:40	1:20	1:10
No. of <i>Giardia</i> cysts	200	-	+	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	+	+	+	-	-	-
	500	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+
	2000	+	+	+	-	-	-	+	-	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+

Table 2. Efficiency of various concentration methods in detection of *G. intestinalis* cysts by microscopy and PCR.

Methods of isolation	Microscopic results				PCR results*			
	Number of cysts (x10 ³)							
	20	10	5	2	20	10	5	2
Water-ether sedimentation ^a	+/-	+/-	-	-	0/3	0/3	0/3	0/3
Fülleborn method ^b	+	+/-	-	-	2/3	1/3	0/3	1/3
Fülleborn Willis method ^c	+	+	+/-	-	2/3	2/3	1/3	0/3
Ether method (own) ^d	+	+	+	-	1/3	2/3	0/3	0/3
Percoll method ^e	+	+	-	-	3/3	2/3	2/3	1/3
Weber formol-ether ^f	+	+/-	-	-	1/3	1/3	1/3	0/3
Weber water-ether method ^g	+	+	-	-	3/3	2/3	1/3	0/3

* number of positive / number of examined samples

^aWater-ether sedimentation, ^bFülleborn's flotation method, ^cFülleborn's flotation method with Willis' modification, ^dMethod of centrifugal flotation with diethyl ether step – own method, ^eMethod of centrifugal flotation with Percoll® step, ^fMethod of Weber's centrifugal flotation with formol-ether step (modified), ^gMethod of Weber's centrifugal flotation with water-ether step (modified)

scopic investigations were positive for samples spiked to a concentration of $\geq 10\,000$ cysts per gram of stool. Slides were relatively readable and easy to examine, which guaranteed high reliability of the results.

The Weber et al. (1992) method gave only weak results in the microscopic examination. Furthermore, during electrophoresis of PCR products, non-specific products were frequently found. Replacement of formalin with redistilled water resulted in better PCR performance. After this modification, the samples spiked with 20,000 and 10,000 cysts per gram of stool were PCR positive. In both modifications of Weber's method, molecular diagnostics allowed positive results to be obtained for single samples spiked with ≥ 5000 cysts per gram.

Discussion

In the present study, we focused our attention on the effect of concentration methods on efficiency of classic microscopic assay and molecular diagnostics. This type of investigation, although restricted to a few methods, has previously been carried out for *Cryptosporidium* spp. (Webster et al. 1996, Nichols et al. 2006).

To our knowledge, this is the first comparison of fecal concentration methods in relation to their usefulness for *G. intestinalis* microscopic and molecular diagnostics. Because of the potential presence of a low number of invasive forms in the stool during prolonged or asymptomatic infection, using sensitive methods in the diagnostics of these parasites is essential (Robertson et al. 2010).

Microscopy is the most common diagnostic method in clinical parasitology. The same also applies to the diagnostics of intestinal protozoa (Parija and Sirinvasa 1999). Numerous studies analyzing the usefulness of the concentration method for diagnostic parasitic protozoa in stool samples have been published (Weber et al. 1992, Bukhari and Smith 1995, Clavel et al. 1996, Nichols et al. 2006, Karamon et al. 2008). Stool parasitological diagnostics by microscopy offers many advantages over other methods used in parasitology, such as immunodiagnosics and molecular diagnostics. It is a relatively inexpensive and convenient procedure in routine diagnostics. This prompted us to include this technique in our investigation. Comparison of molecular methods and microscopy has previously been performed (Verweij et al. 2003, Schuurman et al. 2007). However, investigators frequently concentrated their work on the study of

efficiency of detection methods, with no assessment of the impact of sample treatment on further microscopic and molecular diagnostics.

In the present study, the centrifugal flotation with diethyl ether step (own method) and Fülleborn's flotation method with Willis' modification were the most efficient concentration protocols for light microscopy diagnostics of *G. intestinalis*. These methods allowed detection of parasites in samples spiked with ≥ 5000 cysts per gram of feces. Slides were relatively easy to read, with consequent measurement reliability. Weaker results in microscopic investigation were achieved by the use of water-ether concentration, Fülleborn's flotation method and Weber's centrifugal flotation method with formol-ether step (modified). These methods were characterized by lower sensitivity, which allowed for cyst detection at a level of ≥ 10000 cysts per gram of feces. In previous studies, investigators using these methods (with slight differences in procedure) achieved a better level of detection; these studies, however, were conducted for another species of parasite (Weber et al. 1992).

Numerous studies concerning the molecular investigation of parasitic protozoa in stool samples have been published (Nichols et al. 2006, Platts-Mills et al. 2012). Studies are frequently designed using a unique method which was motivated by different diagnostic requirements for various protozoa. Furthermore, the presence of PCR inhibitors in stool samples has frequently been associated with the occurrence of sensitivity reduction and may lead to false negative results for PCR (Monteiro et al. 1997, Schrader et al. 2012). Several PCR methods for the detection of *G. intestinalis* have been published. However, there is no standardized procedure (Guy et al. 2004, Read et al. 2004, Lalle et al. 2005). The method used by us (Read et al. 2004) is sensitive, and widely used, which is important for *G. intestinalis* genotyping. In addition, this protocol requires small amounts of thermostable polymerase, what makes this method relatively inexpensive and suitable for large scale investigations.

Due to the negative impact of some chemical compounds on the efficiency of PCR reaction (Favre and Rudin 1996, Al-Soud and Rådström 1998), our investigations concentrated on the impact of the components used in conventional parasite concentration procedures. False-negative results were notable in samples with the addition of 10% formalin, saturated $ZnSO_4$ and $MgSO_4$. The dose-dependent inhibition was also observed for saturated NaCl. Also, the success of amplification in the presence of some chemical compounds was dependent on target DNA concentration.

The main aim of our study was to determine the capacity of various pre-treatment methods of fecal

samples in PCR-based diagnostics of *G. intestinalis*. Previously, some authors have recommended DNA isolation procedures, thermostable polymerases, and/or PCR enhancers suitable for amplification of DNA derived from stool samples (Al-Soud and Rådström 1998, Al-Soud and Rådström 2000, Nantavisai et al. 2007, Stauffer et al. 2008). However, to date there has been no study with direct comparison of the concentration methods of *G. intestinalis* cysts and its effect on molecular investigation. In the present study, only Fülleborn's flotation method and the centrifugal flotation with Percoll® step method allowed parasites to be detected by PCR in samples spiked with 2000 cysts per gram of stool. However, PCR results obtained with samples prepared by Fülleborn's flotation method were unreliable, with a high proportion of false-negative results. Fülleborn's flotation method with Willis' modification resulted in relatively sensitive and consistent results in PCR. However, this procedure is labor intensive on account of the need to flush the sample from the coverslip after flotation. There is also the possibility that during this step some invasive forms of parasite may stay on the coverslip, which can be the cause of false-negative results in downstream applications such as PCR. According to our results, the water-ether concentration method was insufficient for purification of PCR inhibitors from the samples, and it is not recommended as an initial step for further molecular diagnostics of *G. intestinalis* in stool.

In conclusion, this study proposes the useful implementation of some concentration procedures on microscopic and molecular diagnostics of *G. intestinalis*. Furthermore, we also presented the impact of some chemical compounds routinely used in parasitological investigation on PCR diagnostics. According to our data it can be concluded that the most efficient concentration method for stool sample preparation for detection of *G. intestinalis* by PCR is centrifugal flotation with Percoll (with saturated $NaNO_3$ as the flotation solution). This method is relatively inexpensive, less labor-intensive, and suitable for epidemiological monitoring and clinical investigations.

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