Estimating *Giardia* cyst viability using RT-PCR

M. Maux, I. Bertrand, C. Gantzer and J. Schwartzbrod

UMR 7564 UHP-CNRS, Laboratoire de Parasitologie-Virologie, 5 rue A. Lebrun, 54001 Nancy Cedex, France (E-mail: *melinda.maux@pharma.u-nancy.fr*)

Abstract The aim of this work was to evaluate the use of molecular techniques for the detection of viable *Giardia* cysts in the environment to assess public health issues. Three target genes were selected: the heat shock protein gene, *HSP70*, which is expressed in response to stress; the giardin gene, which encodes a structural protein; and, alcohol dehydrogenase E (*ADHE*), a novel gene encoding an enzyme involved in the metabolism of energy. We tested the efficiency of five protocols for the extraction of either genomic DNA or total RNA from *Giardia* cysts: two of these protocols were previously cited in the literature and three consisted of commercial DNA extraction kits. The brands of enzyme were determined according to the primers chosen and the amplification conditions were optimised: 2.5 mM $MgCl_2$, 0.5 mM primers and 60°C for annealing temperature. A semi-nested PCR method and an RT semi-nested PCR procedure were developed to detect mRNA from these three genes and to estimate the viability of *Giardia* cysts.

Keywords *Giardia*; mRNA; RT-PCR; viability

Introduction

The environmental route of transmission (water, soil and food) is important for many protozoan parasites. When assessing the quality of water, soil and food it is essential not only to detect and to identify low concentrations of cysts but also to determine their viability and therefore their potential for causing infection. It is necessary to develop new generic methods that can simply and rapidly determine the viability of such parasites. Except for *in vivo* infectivity, many methods such as *in vitro* excystation (Bingham *et al*., 1979; Rice and Schaefer, 1981; Kasprzak and Majewska, 1983; Hoff *et al*., 1985; Wallis and Wallis, 1986), fluorogenic vital dyes (Schupp and Erlandsen, 1987a; Smith and Smith, 1989; Jarmey-Swan *et al*., 2000) combined with cyst morphology study using DIC microscopy (Schupp and Erlandsen, 1987b; Dowd and Pillai, 1997; Thiriat *et al*., 1998), electrorotation (Dalton *et al*., 2001) and fluorescence *in situ* hybridisation (FISH) (Dorsch and Veal, 2001) have been used to distinguish viable *Giardia* cysts from dead ones. However, the correlation between these assays and the infectivity of cysts in mice is variable (Labatiuk *et al*., 1991; Isaac-Renton *et al*., 1992) and many of the assays are too time consuming to be used routinely.

Many studies have used molecular biology techniques to distinguish viable cysts from dead ones. All of the procedures have relied on the reverse transcription polymerase chain reaction (RT-PCR) to detect mRNA, which can be used to study the expression of the metabolic activity of the microorganism and thus of the viability of *Giardia* cysts. For example, some authors used RT-PCR using primers directed to giardin (Mahbubani *et al*., 1991; Kaucner and Stinear, 1998) or HSP 70 (Abbaszadegan *et al*., 1997; Rochelle *et al*., 1997) mRNA.

We aimed to define a way of detecting mRNA by use of RT-PCR. Firstly, we selected a new target gene from the partially sequenced genome of *Giardia*. This gene encodes the enzyme alcohol dehydrogenase E (ADHE). We also used two other targets described in the literature, both of which encode for non-metabolic proteins: a stress protein HSP 70 and a specific wall protein of *Giardia* cysts, giardin. Secondly, we optimised the amplification

Water Science and Technology: Water Supply **Vol 2 No 3 pp 107–115** © IWA Publishing 2002

conditions for each gene. However, DNA amplification can only be used as a presence/absence test, whereas the reverse transcription of mRNA and amplification of the resulting cDNA with the same primers, can be used to determine whether *Giardia* cysts are viable. Therefore, RT-PCR directed at the mRNA for these three targets was evaluated as a method for estimating the viability of cysts. This method enabled us to detect viable *Giardia* cysts rapidly and specifically with great sensitivity.

Materials and methods

Parasites

Giardia cysts from faecal samples. Cyst suspensions were obtained from infected patients, hospitalised at the CHU de Brabois, at Nancy and the Legouest Army Teaching Hospital, at Metz, France. Cysts were purified as described by Thiriat *et al*. (1998). Microscopic analysis of the purified *Giardia* cyst suspensions, showed that the contamination level was between 2.3×10^5 and 6.4×10^5 *Giardia* cysts per mL. Purified cysts were stored in PBS pH 7.4 either at 4°C or –80°C prior to DNA or RNA extraction.

Purified Giardia cysts. Purified *Giardia* cysts (concentration = 2.5×10^5 cyst/mL) were purchased from Waterborne Inc. (New Orleans, LA). *Giardia intestinalis* cysts from the human isolate H-3 were passed through gerbils and purified by up to 95–99% in PBS pH 7.4 with antibiotics. The cysts were stored at 4°C.

Fluorogenic dye staining and microscopic examination

Each sample (100 µL) was incubated with DAPI, PI or FITC as described by Thiriat *et al*. (1997). Aliquots (10 µL per slide) were examined under the microscope and the percentage viability was estimated for the entire pellet. Viable *Giardia* cysts were counted and evaluated according to the criteria defined by Thiriat *et al*. (1998).

Disruption of cysts and extractions of nucleic acids

Disruption of cysts and DNA extraction. We tested five different nucleic acid extraction protocols that had been described in publications or DNA extraction kits: Abbaszadegan *et al*. (1997) method, which is based on extracting nucleic acids by carrying out five freeze-thaw cycles; a conventional DNA isolation method involving acid guanidinium digestion and phenol extraction that was adapted from the AGPC method for RNA extraction proposed by Chomczynski and Sacchi (1987). The phenol/chloroform/ isoamyl alcohol phase was equilibrated to pH 8, which is more suitable for DNA extraction. Both protocols were used on pure solutions of *Giardia* cysts. The three DNA extraction kits tested, Wizard® Genomic DNA Purification (Promega), QIAamp® DNA Stool (Qiagen) and NucleoSpin Tissue (Macherey Nagel) kits, purified DNA by use of a lysis reagent and proteinase K.

Disruption of cysts and RNA extraction. We tested the efficiency of the two nucleic acid extraction protocols described in the literature (freeze-thaw cycles and AGPC) and two extraction kits (Qiagen): QIAamp® DNA Stool kit and QIAamp® Viral RNA kit. The combination of QIAamp® DNA Stool kit followed by the QIAamp® Viral RNA kit has been tested too. The sample was first incubated with the lysis buffer from the first kit to disrupt the cysts, followed by incubation with InhibitEX. The second protocol for RNA extraction was then applied to 240 µL of the resulting lysate.

Primers

We tested the ability of 17 primers to generate fragments from three specific *Giardia* genes: 3 primers for HSP 70, 7 primers for giardin and 7 primers for ADHE (Table 1). The specific

primers, designed by Primer Express Software, for the ADHE gene were chosen by use of the complete *Giardia*-ADHE gene sequence (accession number U93353, GenBank) and were tested with a preliminary semi-nested PCR.

Evaluation of semi-nested PCR

Only one semi-nested PCR could be carried out with the HSP 70 primers. Seven of the 23 giardin semi-nested PCR were selected and named Giardin-1 to Giardin-7. Five ADHE semi-nested PCR were performed, named ADHE-1 to ADHE-5 (Table 2).

Reverse-transcription

Firstly, the RNA was treated with several enzymes to ensure that only RNA was amplified, because the extraction protocols are not specific for RNA. Thus, to determine whether DNA, RNA, or both were being amplified, the nucleic acids were either treated with 1 µL RQ1-RNase-free DNase (1 U/µL) (Promega) or 1 µL RNase A (7 U/µL) (Qiagen), or both DNase and RNase A. Samples were incubated for 30 min at 37°C, and then the enzyme was inhibited by heating to 65°C for 10 min.

Each sample subsequently underwent reverse-transcription. The samples (5 µL RNA extract and 1 µL forward primer 10 µM) were heated to 95°C for 3 minutes to denaturate the proteins associated with the nucleic acids. The following reagents were then added: 1X RT

Table 1 Primer sequences and designations

Target (*)	Primer	Sequence (5′–3′)	Position	Reference
HSP 70	HSP70 upst	AGGGCTCCGGCATAACTTTCC	upstream	Abbaszadegan *et al.*,
(X16738)	HSP70 downst	GTATCTGTGACCCGTCCGAG	downstream	1997
	HSP70 inv.	CTTCGGGAACGCCAAGGCCTG	downstream	Rochelle *et al.*, 1997
ADHE	ADG1	GCATGGTCACTTCGGCCTAT	upstream	Defined in this
(U93353)	ADG2	AGAACATGAAGACCGCTGGG	upstream	study
	ADH1	GGTTCACGGCGGTCTTGTA	downstream	
	ADH2	GATGGCAGTGGACGTCGG	downstream	
	ADI1	CACTTGCAGTCCGCAGGAA	downstream	
	ADI2	GAGCTTTTCGCAGGACATCG	downstream	
	ADI3	GGTTCACGGCGGTCTTGTAG	downstream	
Giardin	GGL405-433	CATAACGACGCCATCGCGGCTCTCAGGAA	upstream	Mahbubani *et al.*,
(X85958)	GGR592-622	TTTGTGAGCGCTTCTGTCGTGGCAGCGCTAA	downstream	1991
	GGP510-537	AGCTCAACGAGAAGGTCGCAGAGGGCTT	upstream	
	GGL639-658	AAGTGCGTCAACGAGCAGCTC	upstream	
	GGR789-809	TTAGTGCTTTGTGACCATCGA	downstream	
	GGP751-776	TCGAGGACGTCGTCTCGAAGATCCAG	upstream	
	GGP751-776.i	CTGGATCTTCGAGACGACGTCCTCGA	downstream	

* accession number of complete or partial sequence available from GenBank

Table 2 Semi-nested PCR tested for giardin and ADHE

Target	Name	Fragment size (bp)	Primers for 1st PCR	Primers for 2nd PCR
ADHE	ADHE-1	108	ADG1 and ADH1	ADG1 and ADI1
	ADHE-2	108	ADG1 and ADH1	ADG1 and ADI3
	ADHE-3	108	ADG1 and ADI2	ADG1 and ADH1
	ADHE-4	108	ADG1 and ADI2	ADG1 and ADI3
	ADHE-5	122	ADG2 and ADI3	ADG2 and ADH2
Giardin	Giardin-1	112	GGL 405-433 and GGR 592-622	GGP 510-537 and GGR 592-622
	Giardin-2	141	GGR 789-809 and GGL 639-658	GGL 639-658 and GGP 751-776.inv
	Giardin-3	171	GGP 510-537 and GGR 789-809	GGL 639-658 and GGR 789-809
	Giardin-4	218	GGL 405-433 and GGR 789-809	GGL 405-433 and GGR 592-622
	Giardin-5	298	GGR 789-809 and GGL 405-433	GGR 789-809 and GGP 510-537
	Giardin-6	171	GGR 789-809 and GGL 405-433	GGL 639-658 and GGR 789-809
	Giardin-7	112	GGR 789-809 and GGP 510-537	GGP 510-537 and GGR 592-622

buffer (Perkin Elmer); 250 μM each of the four deoxynucleotide triphosphates (Perkin Elmer); 9 U AMV reverse transcriptase (Promega), 40 U RNasin (Promega), at a final volume of 20 μL. Reverse transcription was carried out at 42°C for 1 h, followed by 5 min at 95°C.

Semi-nested PCR

Semi-nested PCR was used to enhance the PCR-based detection of *Giardia* cysts. For each semi-nested PCR, segments of between 100 bp and 300 bp of the specific gene (Table 2) were PCR-amplified following the direct extraction of *Giardia* DNA, or the reverse-transcription of extracted RNA.

The reaction mixtures contained 5 μL extracted DNA or 1 μL amplified DNA at a total volume of 50 μL for the second step of semi-nested PCR. The initial PCR conditions tested were as described by Mahbubani *et al*. (1991). They were used in all subsequent analyses to select the most sensitive method for DNA and RNA extraction and, for primer evaluation. The following concentrations of the various reagents were used: 1X PCR buffer (Perkin Elmer); 2.5 mM $MgCl_2$; 200 μM of each of the four deoxynucleotide triphosphates (Perkin Elmer); 0.5 μM primers (GGL 405-433 and GGR 592-622) and 2.5 U of AmpliTaq DNA Polymerase (Perkin Elmer). The amplification parameters were as follows: initial denaturation 3 min at 94°C; followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; final elongation 10 min at 72°C; hold at 4°C.

The PCR-based detection was optimised with genomic DNA extracted with the QIAamp DNA Stool kit. We selected the best primer groups and optimised the amplification parameters. *Taq* polymerase (Qiagen or Perkin Elmer), or HotStar*Taq* polymerase (Qiagen) were used. Both worked with the same conditions of cycle, but the latter needed an initial activation step of 15 min at 95°C.

Detection of amplified products

Following amplification, 6 μL of each PCR product were analysed by electrophoresis on a 2% agarose gel, stained with ethidium bromide (0.6 μg/mL) and visualised under a UV-transilluminator. A 100 bp DNA ladder (Promega) was included as a size marker.

Results and discussion

Comparison of DNA and RNA extraction methods

Two of the extraction protocols came from the literature and were used to extract DNA from a pure solution, whereas the three other protocols tested were kits and were used on faecal samples. The extraction conditions were thus more favourable for the protocols derived from the literature than for DNA extraction kits. Different band profiles were obtained by all five methods and they were not all reproducible, indicating that methods of DNA extraction affect semi-nested PCR (Table 3).

The AGPC method detected at least 4×10^3 cysts, whereas the freeze-thaw protocol gave no positive results. But, the main disadvantages of the guanidin thiocyanate/phenol method were that it is a very hands-on and time consuming method, which is not appropriate when a large number of samples need to be analysed. In contrast, the freezing-thawing treatment can be completed within 30 min and there is little manipulation involved, which minimises the chance of accidentally contaminating the samples during the genomic DNA extraction process and does not use hazardous chemical products. The poor sensitivity obtained with both these treatments suggests that simple DNA extraction cannot be used to prepare templates for amplification, whereas many authors (Abbaszadegan *et al*., 1997; Deng and Cliver, 1999) have shown that the freeze-thaw method is suitable for PCR-based methods. We also investigated the effects of the freeze-thaw cycles followed by guanidin thiocyanate/phenol protocol, but this did not increase the detection sensitivity.

Table 3 Sensitivity of extraction protocols for DNA[a]

	Sensitivity (number of cysts)[b]					
	10^3	10^2	10	1	10^{-1}	10^{-2}
Freeze-thaw	–	–	–	–	–	–
AGPC	+	–	–	–	–	–
Wizard Genomic	+/–	+/–	+/–	–	–	–
NucleoSpin	+/–	+/–	+/–	–	–	–
QIAamp DNA Stool	+	+	+	+/–	–	–

[a] Results are from two sets of experiments
[b] The number of cysts was determined by immunofluorescence

Table 4 Sensitivity of extraction protocols for RNA[a]

	Sensitivity (number of cysts)[b]					
	10^3	10^2	10	1	10^{-1}	10^{-2}
Freeze-thaw	–	–	–	–	–	–
AGPC	–	–	–	–	–	–
QIAamp DNA Stool	+	+	–	–	–	–
QIAamp Viral RNA	+	+	+	–	–	–
QIAamp DNA/RNA	+	+	+	+	–	–

[a] Results are from two sets of experiments
[b] The number of cysts was determined by immunofluorescence

Kits have provided good results on faecal material, and all of them can detect less than 10 cysts (Table 3). However, there were major differences in amplification between the DNA extracted by QIAamp® DNA Stool kit and the DNA extracted with the other two kits. The amplification of DNA extracted either by Wizard® Genomic DNA Purification kit or by NucleoSpin Tissue kit did not provide reliable results (50% of positive results), whereas DNA extracted by QIAamp® DNA Stool kit was detected in all of the experiments, with a sensitivity of less than 10 cysts. The sensitivity was not improved by carrying out freeze-thaw cycles before using the QIAamp® DNA Stool kit. Another disadvantage of Wizard® Genomic DNA Purification kit and NucleoSpin Tissue kit is the time required compared to QIAamp® DNA Stool kit, which can be processed in 50 min. Moreover, the QIAamp® DNA Stool kit is totally suitable for the analysis of faeces because InhibitEX, a PCR inhibitor-absorption resin, is used.

Table 4 shows the results obtained for RNA extractions. With the two protocols taken directly from the literature and despite the numerous conditions tested with pure *Giardia* cysts, no amplified product was obtained. We used RT-PCR to determine whether there was any RNA in the samples extracted by the QIAamp DNA Stool kit. This method gave positive results but was not very sensitive (detection limit ~200 cysts). The use of QIAamp Viral RNA Stool test combined with the first step of the QIAamp DNA Stool kit could successfully detect two *Giardia* cysts. Thus, as for DNA extraction, only kits were suitable for RNA extraction.

Evaluation of primers and semi-nested PCR

We evaluated each target-gene and chose the most suitable combination of three primers. The only combination tested for HSP 70 failed to produce an amplified product of the correct size (Table 5). These results are consistent with the findings of Kaucner and Stinear (1998). Among the five semi-nested PCR investigated for ADHE, the semi-nested PCR named ADHE-5 led to no amplification of a fragment. ADHE-3 produced a specific fragment, but the result was not reproducible, with a difference of two log between the two assays. The sensitivity for ADHE-4 was too weak, with a detection threshold of between 60 and 600 cysts. The remaining semi-nested PCRs ADHE-1 and ADHE-2 produced reproducible specific bands with a sensitivity of about 60 cysts and thus have the potential to be

used to optimise the amplification parameters. All of the primers for the giardin gene allowed the amplification of reproducible specific bands, with sensitivity threshold of between 6 and 60 cysts. Two semi-nested PCR per target-gene have been selected to optimise amplification parameters: Giardin-1 and Giardin-2, ADHE-1 and ADHE-2.

Choice of polymerase

We evaluated the efficiency of three different polymerases to enhance the detection threshold and to limit non-specific amplification products.

The results for Giardin-1 are shown in Figure 1A. In terms of sensitivity, the most suitable polymerase was the HotStarTaq, which allowed the detection of less than six cysts, whereas the other two enzymes had a detection threshold of less than 60 cysts. No non-specific bands were seen with either of the Qiagen enzymes, contrary to the AmpliTaq. However, with Giardin-2 and the two semi-nested PCR ADHE selected non-specific bands remained. Moreover, the results for ADHE-1 (Figure 1B) showed that *Taq* (Qiagen) gave the highest sensitivity threshold, 60 cysts. Similarly, the optimal polymerase for semi-nested PCR ADHE-2 was *Taq* (Qiagen).

Increasing the annealing temperature from 55°C to 60°C for each semi-nested PCR reaction, did not eliminate the non-specific bands. Similarly, there was no difference when

Table 5 Sensitivity of semi-nested PCR assay for the detection of *Giardia* cysts[a]

Target		Sensitivity (number of cysts)[b]					
		6.10^3	6.10^2	6.10	6	6.10^{-1}	6.10^{-2}
HSP	–	–	–	–	–	–	
ADHE	1	+	+	+/–	–	–	–
	2	+	+	+/–	–	–	–
	3	+	+/–	+/–	–	–	–
	4	+	+/–	–	–	–	–
	5	–	–	–	–	–	–
Giardin	1	+	+	+	–	–	–
	2	+	+	+	–	–	–
	3	+	+	+	–	–	–
	4	+	+	+	–	–	–
	5	+	+	+	–	–	–
	6	+	+	+	–	–	–
	7	+	+	+	–	–	–

[a] Results are from two set of experiments
[b] The concentration of cysts was determined by immunofluorescence

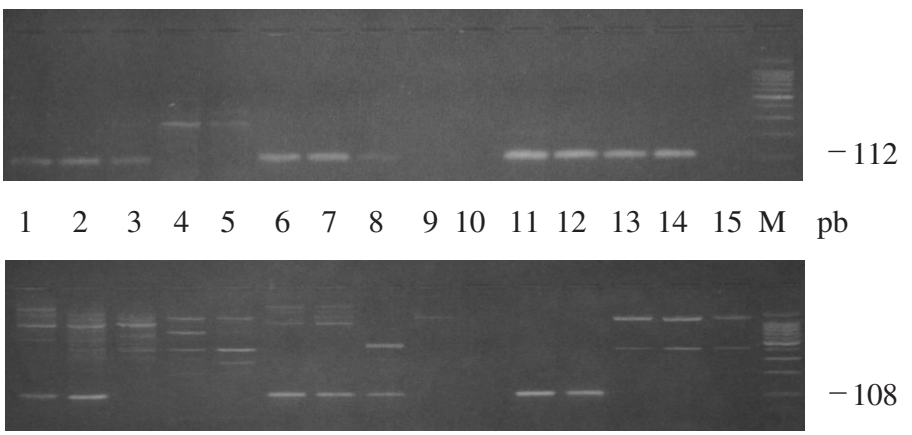

Figure 1 Evaluation of polymerase with between 10^3 and 10^{-1} *Giardia* cysts. **A-** semi-nested PCR Giardin-1; **B-** semi-nested PCR ADHE-1. Lines 1 to 5: amplification with Ampli*Taq* polymerase (Perkin Elmer); lines 6 to 10: amplification with *Taq* (Qiagen); lines 11 to 15: amplification with HotStar*Taq* (Qiagen). Line M: DNA size marker

Table 6 Results of RNase and DNase treatment of nucleic acids extracted from viable *Giardia* cysts

Enzymatic treatment of cyst nucleic acids	RT semi-nested PCR result	Acid nucleic amplified
RQ1-RNase free DNase	+	RNA
RNase 1	+	DNA
RQ1-RNase free DNase + RNase 1	–	no amplification
no treatment	+	RNA and DNA

concentration of primers and $MgCl_2$ was increased. Therefore, the optimal concentrations of $MgCl_2$ and primers were determined as 2.5 mM and 0.5 μM, respectively.

These preliminary steps showed that different amplification conditions are needed according to the target. Thus, only one semi-nested PCR per target was selected for the final optimisation steps. The Giardin-1 semi-nested PCR was performed with HotStarTaq, with an annealing temperature of 60°C, whereas the best conditions for the ADHE-1 were with *Taq* (Qiagen) at 60°C.

Evaluation of RT-PCR

RT-PCR was only performed for giardin and ADHE semi-nested PCR selected after evaluating the primers, with the amplification conditions defined above. RT-PCR was performed on the DNase-treated samples, on the RNase-treated samples, on the samples treated with both RNase and DNase, and finally untreated samples. The viability of *Giardia* cysts was assessed by prior microscopic examination. No amplified product was detected for the ADHE primers, regardless of the semi-nested PCR performed. However, when the giardin gene was used as the target (Table 6) either DNA or RNA could be amplified, depending on the enzyme treatment. No amplification resulted when both enzymes were used because all of the nucleic acids (DNA and RNA) had been degraded. In the DNase-treated samples, the presence of a specific band indicated that the RNA template had been amplified and not the DNA.

The presence of amplified product following the RT semi-nested PCR, after each treatment, was indicative not only of the presence of *Giardia* acid nucleic in the extracts, but also of the viability of the *Giardia* cysts. The giardin primers GGL 405-433, GGR 592-622 and GGP 510-537 were thus suitable for the amplification of RNA isolated from *Giardia* cysts. The sensitivity limit of the viability test was less than two *Giardia* cysts, whereas for the absence/presence test it was about 6 cysts.

Conclusion

There is a great interest in the development of methods for the detection of viable *Giardia* cysts, as viable cysts represent the infective stage of this microorganism. This work led to the development of a method to detect *Giardia* cysts based on the semi-nested PCR with a choice of target genes (HSP 70, giardin or ADHE). Our results indicate that the quality of the amplification depends on major factors such as the nucleic acid extraction protocol and the DNA polymerase used. Moreover, the choice of polymerase and amplification conditions relate to the primers used. Finally, parameters for the amplification of both giardin and ADHE were well defined, but no amplification product was obtained for HSP 70. There are still some major factors which are preventing us from amplifying this target gene. PCR amplification from the *Giardia*-specific genes detected both live and dead cysts. Thus, to assess the viability of *Giardia* cysts, we used reverse transcription to detect mRNA. This enabled us to define the best RT-PCR conditions for the giardin gene, but not for the ADHE gene. Further experiments must be performed to assess the capacity of a such method to identify the viable *Giardia* cysts treated with different conditions. There are two ways to discriminate between live and dead cysts: by detecting the stress-induced synthesis of the

target mRNA, or by measuring the amounts of PCR-amplified fragments produced from the target mRNA before and after induction. Further experiments must be also performed to validate this viability assay with others target genes.

These molecular techniques provide an attractive, rapid and simple method that can be used to detect viable *Giardia* cysts in a variety of environmental samples.

Acknowledgements

This work was funded by a E.U. International Co-operation with developing countries (INCO-DC) contract number: ERB 3514 PL 972471.

References

Abbaszadegan, M., Huber, M.S., Gerba, C.P. and Pepper, I.L. (1997). Detection of viable *Giardia* cysts by amplification of Heat Shock-induced mRNA. *Appl. Environ. Microb.*, **63**(1), 324–328.

Bingham, A.K., Jarroll, E.L., Meyer, E.A. and Radulescu, S. (1979). Induction on *Giardia* excystation and the effect of temperature on cyst viability as compared by eosin-exclusion and in vitro excystation. In: *Proceedings of National symposium on waterborne transmission of giardiasis*, W. Jakubowski and J.C. Hoff (ed.), U.S.E.P.A., September 18–20 ,1978, Cincinnati, Ohio, 217–229.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**(1), 156–159.

Dalton, C., Goater, A.D., Pethig, R. and Smith, H.V. (2001). Viability of *Giardia intestinalis* cysts and viability and sporulation state of *Cyclospora cayetanensis* oocysts determined by electrorotation. *Appl. Environ. Microbiol.*, **67**(2), 586–590.

Deng, M. and Cliver, D.O. (1999). Rapid DNA extraction methods and new primers for randomly amplified polymorphic DNA analysis of *Giardia duodenalis*. *J. Microbiol. Methods*, **37**(2), 193–200.

Dorsch, M.R. and Veal, D.A. (2001). Oligonucleotide probes for specific detection of *Giardia lamblia* cysts by fluorescent *in situ* hybridization. *J. Appl. Microbiol.*, **90**(5), 836–842.

Dowd, S.E. and Pillai, S.D. (1997). A rapid viability assay for *Cryptosporidium* oocysts and *Giardia* cysts for use in conjunction with indirect fluorescent antibody detection. *Can. J. Microbiol.*, **43**(7), 658–662.

Hoff, J.C., Rice, E.W. and Schaefer, F.W. (1985). Comparison of animal infectivity and excystation as measures of *Giardia muris* cyst inactivation by chlorine. *Appl. Environ. Microbiol.*, **50**(4), 1115–1117.

Isaac-Renton, J.L., Shahriari, H. and Bowie, W.R. (1992). Comparison of an in vitro method and an in vivo method of *Giardia* excystation. *Appl. Environ. Microbiol.*, **58**(5), 1530–1533.

Jarmey-Swan, C., Gibbs, R.A., Ho, G.E., Bailey, I.W. and Howgrave-Graham, A.R. (2000). A novel method for detection of viable *Giardia* cysts in water samples. *Water Res.*, **34**, 1948–1951.

Kasprzak, W. and Majewska, A.C. (1983). Infectivity of *Giardia* sp. cysts in relation to eosin exclusion and excystation *in vitro*. *Tropenmed. Parasit.*, **34**(1), 70–72.

Kaucner, C. and Stinear, T. (1998). Sensitive and rapid detection of viable *Giardia* cysts and *Cryptosporidium parvum* oocysts in large-volume water samples with wound fiberglass cartridge filters and reverse transcription-PCR. *Appl. Environ. Microb.*, **64**(5), 1743–1749.

Labatiuk, C.W., Schaefer, F.W., Finch, G.R. and Belosevic, M. (1991). Comparison of animal infectivity, excystation, and fluorogenic dye as measures of *Giardia muris* cyst inactivation by ozone. *Appl. Environ. Microb.*, **57**(11), 3187–3192.

Mahbubani, M.H., Bej, A.K., Perlin, M., Schaefer, F.W., Jakubowski, W. and Atlas, R.M. (1991). Detection of *Giardia* cysts by using the polymerase chain reaction and distinguishing live from dead cysts. *Appl. Environ. Microb.*, **57**(12), 3456–3461.

Rice, E.W. and Schaefer, F.W. (1981). Improved in vitro excystation procedure for *Giardia lamblia* cysts. *J. Clin. Microbiol.*, **14**(6), 709–710.

Rochelle, P.A., De Leon, R., Stewart, M.H. and Wolfe, R.L. (1997). Comparison of primers and optimization of PCR conditions of *Cryptosporidium parvum* and *Giardia lamblia* in water. *Appl. Environ. Microb.*, **63**(1), 106–114.

Schupp, D.G. and Erlandsen, S.L. (1987a). A new method to determine *Giardia* cysts viability: correlation of fluoresceine diacetate and propidium iodide staining with animal infectivity. *Appl. Environ. Microbiol.*, **53**(4), 704–707.

Schupp, D.G. and Erlandsen, S.L. (1987b). Determination of *Giardia muris* cyst viability by differential interference contrast, phase, or brightfield microscopy. *J. Parasitol.*, **73**(4), 723–729.

Smith, A.L. and Smith, H.V. (1989). A comparison of fluorescein diacetate and propidium iodide staining and in vitro excystation for determining *Giardia intestinalis* cyst viability. *Parasitology*, **99**(Pt 3), 329–331.

Thiriat, L., Sidaner, F. and Schwartzbrod, J. (1998). Determination of *Giardia* cyst in environmental and faecal samples by immunofluorescence, fluorogenic dye staining and differential interference contrast microscopy. *Lett. Appl. Microbiol.*, **26**(4), 237–242.

Thiriat, L., Bigot, V. and Schwartzbrod, J. (1997). Evaluation of a procedure for detection of viable *Giardia* cysts in wastewater sludge. *Water Sci. Technol.*, **35**(11–12), 377–380.

Wallis, P.M. and Wallis, H.M. (1986). Excystation and culturing of human and animal *Giardia* spp. by using gerbils and TYI-S-33 medium. *Appl. Environ. Microbiol.*, **51**(3), 647–651.