Stool sample storage conditions for the preservation of *Giardia intestinalis* DNA

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Stool is chemically complex and the extraction of DNA from stool samples is extremely difficult. Haemoglobin breakdown products, such as bilirubin, bile acids and mineral ions, that are present in the stool samples, can inhibit DNA amplification and cause molecular assays to produce false-negative results. Therefore, stool storage conditions are highly important for the diagnosis of intestinal parasites and other microorganisms through molecular approaches. In the current study, stool samples that were positive for Giardia intestinalis *were collected from five different patients. Each sample was stored using one out of six different storage conditions [room temperature (RT), +4ºC, -20ºC, 70% alcohol, 10% formaldehyde or 2.5% potassium dichromate] for DNA extraction procedures at one, two, three and four weeks. A modified QIAamp Stool Mini Kit procedure was used to isolate the DNA from stored samples. After DNA isolation, polymerase chain reaction (PCR) amplification was performed using primers that target the* β-giardin *gene. A* G. intestinalis*-specific 384 bp band was obtained from all of the cyst-containing stool samples that were stored at RT, +4ºC and -20ºC and in 70% alcohol and 2.5% potassium dichromate; however, this band was not produced by samples that had been stored in 10% formaldehyde. Moreover, for the stool samples containing trophozoites, the same* G. intestinalis*-specific band was only obtained from the samples that were stored in 2.5% potassium dichromate for up to one month. As a result, it appears evident that the most suitable storage condition for stool samples to permit the isolation of* G. intestinalis *DNA is in 2.5% potassium dichromate; under these conditions, stool samples may be stored for one month.*

Key words: *Giardia intestinalis* - DNA isolation - storage conditions - stool

Giardia intestinalis (syn. *Giardia lamblia*, *Giardia duodenalis)* is the most common intestinal protozoan in the world. This parasite can infect humans and a variety of other members of the animal kingdom, including mammalian, avian and reptilian species (Thompson 2002, Appelbee et al. 2005). The life cycle of *G. intestinalis* includes two different forms: the infective cyst, which is resistant to many environmental conditions, and the trophozoite, which colonises the intestinal lumen, but does not invade the mucosa (Eckmann & Gillin 2001). *G. intestinalis* are shed as cysts in faeces and the faecal-oral route is the main path of infection for this parasite. Contaminated water, food and fomites are generally effective vehicles for transmitting the infection. The traditional microscopy of stool samples, direct fluorescent antibody tests and enzyme-linked immunosorbent assays are still commonly used techniques for the diagnosis of giardiasis. However, the sensitivity of these methods may be inadequate for detecting low levels of infection (Johnston et al. 2003, Nantavisai et al. 2007), identifying different *G. intestinalis* genotypes or assemblages or discriminating among genetic variations of *G. intestinalis* (Amar et al. 2004, Bertrand et al. 2005).

The isolation of DNA from samples is often an early and routine step in many analytical processes, such as the detection of bacteria, viruses, fungi and parasites, the diagnosis of diseases or genetic disorders, forensic investigation and historical identification (Van Belkum & Hays 2008). DNA extraction is the separation of DNA from any type of cell or sample for a particular purpose. DNA extraction follows a series of steps. In particular, the DNA extraction begins with the lysis of the cell that contains the DNA of interest; this lysis is followed first by the removal of all proteins from the DNA and then by the final step of precipitating the DNA (Van Belkum & Hays 2008). DNA is normally obtained from samples of skin, tissue, cerebrospinal fluid, blood, urine, sputum, stool, or formalin-fixed embedded tissue. However, stool samples have a particularly complex structure. DNA isolation from stool samples is hampered by the inhibitory compounds that are present in faeces, which include the degradation products of haemoglobin, bilirubin, bile acids and mineral ions. Frequently, stool samples can produce failed attempts to isolate DNA and false-negative amplification reactions (Kostrzynska et al. 1999, Nantavisai et al. 2007).

Storage time and conditions are also important factors in the isolation of DNA from stool samples. The DNA of trophozoites, which are particularly vulnerable to environmental factors, can rapidly degenerate. Therefore, either DNA from fresh stool should be extracted as quickly as possible, or the stool in question should be appropriately stored. Stool storage conditions are highly important for the use of molecular approaches to diagnosing intestinal parasites and other microorganisms. The objective of this work was to determine the best storage conditions for the isolation of DNA from stool samples containing *G. intestinalis*.

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Received 25 October 2011
Accepted 21 August 2012

SUBJECTS, MATERIALS AND METHODS

Samples and storage conditions - In this study, *G. intestinalis*-positive stool samples were collected from five different patients at the Erciyes University Faculty of Medicine and Department of Medical Parasitology; these patients volunteered to participate in this study. Three of the stool samples that were obtained contained more *Giardia* cysts than *Giardia* trophozoites, whereas the remaining two samples contained more *Giardia* trophozoites than cysts. A 200 mg *G. intestinalis*-positive stool sample was divided into 24 portions and each sample was stored at one of six different conditions [room temperature (RT), +4ºC, -20ºC, 70% alcohol, 10% formaldehyde or 2.5% potassium dichromate] for DNA extraction at one, two, three and four weeks of storage.

DNA isolation - Before the DNA isolation was performed, the stool samples were washed three times in sterile distilled water. In accordance with the manufacturer's instructions, a modified QIAamp Stool Mini Kit (Qiagen, USA) was used to isolate DNA from the stored samples. All of the centrifugation steps were performed at RT (20-25ºC) and 14,000 rpm. A quantity of 1.4 mL of ASL buffer was added to the washed stool samples, which were then heated at 95ºC for 5 min. Subsequently, 100 µL of buffer AE was directly pipetted onto the membrane of the QIAamp spin column and the column and buffer were incubated for 2 min at RT. The resulting DNA was eluted, centrifuged twice at full speed and then stored at -20ºC until it was used in the polymerase chain reaction (PCR) amplifications.

PCR - Prior to the PCR amplifications, the DNA concentration of each sample was measured using a Nanodrop (ACTGene ASP-3700, USA) instrument. After this measurement, PCR was performed with primers that targeted the *β-giardin* (*bg*) gene; in particular, these primers were known as G367 (5'CATAACGACGCCATCGCGGCTCT CAGGAA3') and G759 (5'GAGGCCGCCCTGGATCTT CGAGACGAC3') (Cacciò et al. 2002). The PCR amplifications were each performed in a 25 µL volume that contained 12.5 µL 2x PCR Master mix (Vivantis, Malaysia), 2 µL of each primer (at 20 pmol) and 5 µL of genomic template DNA. These reactions were performed in a PCR Labcycler (SENSQUEST, Germany). The following PCR cycle was used: an initial denaturation at 95ºC for 5 min, 35 subsequent cycles of denaturation at 94ºC for 30 sec, annealing at 65ºC for 30 sec and extension at 72ºC for 1 min and a final extension at 72ºC for 7 min. Negative controls for the amplification reaction mixtures that contained only the PCR reaction reagents and sterile distilled water were prepared. PCR products from all of the amplifications were visualised by electrophoresis on a 1.5% agarose gel containing ethidium bromide.

RESULTS

DNA was isolated from all of the stool samples and analysed with a Nanodrop instrument prior to PCR amplification (Table I) and the samples that contained a minimum DNA concentration were stored in 10% formaldehyde. For stool samples that contained more *Giardia* cysts than trophozoites, storage in 10% formaldehyde for one day or longer resulted in the absence of *G. intestinalis*; however, a *G. intestinalis*-specific 384 bp band was obtained from all of the stool samples with cysts that were stored at RT, at +4ºC, at -20ºC, in 70% alcohol or in 2.5% potassium dichromate for a month (Figure, Table II).

Two samples contained more *Giardia* trophozoites than cysts. The *G. intestinalis*-specific 384 bp band was obtained from all of the stool samples that were stored in 2.5% potassium dichromate. The presence of *G. intestinalis* DNA was not observed in the stool samples that had been stored for one, two, three or four weeks in any of the other examined conditions (Figure, Table II).

DISCUSSION

Compared with the genotyping methods that are available for other protozoan pathogens, the existing genotyping techniques for *Giardia* spp are not particularly advanced. The small subunit ribosomal RNA (ssu-rRNA), bg, glutamate dehydrogenase, elongation factor 1-alpha, triose phosphate isomerase and GLORF-C4 genes (Cacciò & Ryan 2008) have been used for *Giardia* spp genotype analysis; recently, the inter-genomic rRNA spacer region has also been utilised for this purpose (Lee et al. 2006). Among the aforementioned genes, two of the most commonly used regions for genotype analysis are the small ssu-rRNA and the bg (Faubert 2000, Cacciò et al. 2002).

TABLE I

DNA concentration (ng/µL) before polymerase chain reaction according to storage condition and duration

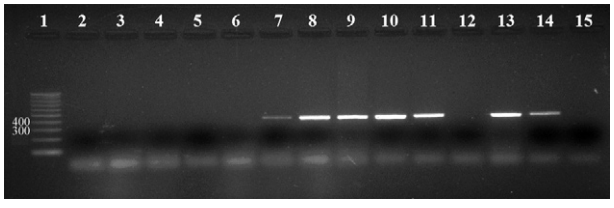
Storage condition	First week min.-max.(average ± SD)	Second week min.-max.(average ± SD)	Third week min.-max.(average ± SD)	Fourth week min.-max.(average ± SD)
Room temperature	27-158.8 (63.54 ± 55.8)	58.7-160.7 (69.9 ± 53.4)	5.7-68.5 (69.8 ± 64.8)	15.3-83.4 (58.5 ± 37.6)
+4ºC	52-146 (86.8 ± 50.9)	64.9-136.7 (89.4 ± 49)	12.6-121.5 (81.4 ± 59.9)	92.3-150 (125.7 ± 29.9)
-20ºC	27-198 (86.6 ± 84.4)	72.6-199.8 (94.4 ± 80.5)	10.5-137.1 (74.5 ± 63.3)	77.4-176 (131.5 ± 49.9)
70% alcohol	8.9-226.9 (108.9 ± 108.6)	176.4-225.7 (139.4 ± 99.8)	10.5-178 (119.5 ± 94.4)	137.5-535 (282.6 ± 219.4)
10% formaldehyde	1.6-14.8 (8.7 ± 6.6)	1.3-5.4 (5.4 ± 4.2)	3.8-5.5 (3.6 ± 1.9)	5.4-8.3 (6.4 ± 1.6)
2.5% potassium dichromate	22.6-82.3 (48.7 ± 24.2)	33-82.8 (52.3 ± 21.6)	17-52.8 (40.7 ± 15)	20-52.3 (36.9 ± 15.3)

SD: standard deviation.

Because sequences of the *Giardia* ssrRNA gene have an unusually high GC content, the amplification of the *Giardia* ssrRNA gene presents technical problems. Moreover, the ssrRNA assay can lack the desired specificity; the use of certain primers has resulted in the amplification of DNA from protozoa that are totally unrelated to *Giardia* (Weiss et al. 1992). The amplification of the *bg* gene is highly specific and no cross-amplification was observed to result from using host, protozoan, fungal, algal or bacterial DNA as templates for PCR amplification. The giardin genes that encode *α1-giardin*, *α2-giardin*, *bg* and *γ-giardin* have been cloned and used for PCR amplification and genotyping (Aggarwal_et al. 1989, Alonso & Peattie 1992, Nohria et al. 1992). Volotão et al. (2007) investigated a total of 87 stool samples and reported that 62 isolates could be amplified through the use of the *bg* gene for both short and long fragments. Because of these *bg-related* advantages, the 384 bp *bg* gene fragment was used in this study as an indicator of the presence of *G. intestinalis* DNA.

Although many methods are available for the diagnosis of *G. intestinalis*, molecular approaches must be developed because these techniques will permit greater diagnostic sensitivity and allow for different *G. intestinalis* genotypes to be distinguished. However, because the basis of molecular methods is DNA extraction, an effective DNA isolation procedure is important for improving the sensitivity of molecular methods. The choice of appropriate samples and sample storage conditions is very important for DNA isolation. In particular, to ensure the effective isolation of DNA from stool samples, these samples must be stored properly. In this study, each examined stool sample was stored under one of six different conditions prior to DNA extractions that were performed over the course of one month. It has been demonstrated that for stool samples containing both cysts and trophozoites, the most suitable storage condition for the isolation of DNA from *G. intestinalis* is the use of 2.5% potassium dichromate. Samples can be stored for one month. Trophozoite structures that are susceptible to environmental conditions may be rapidly damaged at RT, +4ºC, or -20ºC. In addition, although the presence of *G. intestinalis* DNA was not observed in stool samples that contained more trophozoites than cysts and were stored in 70% alcohol, a *G. intestinalis*-specific 384 bp band was obtained from stool samples containing cysts that were stored in 70% alcohol. It is known that the pH levels of 2.5% potassium dichromate and 70% alcohol are 4 and 8.3, respectively. We suggest that the pH or another factor may affect the success of DNA isolation procedures from trophozoite-containing stool samples.

Wilke and Robertson (2009) collected seven samples containing *Giardia* cysts and stored these samples under four different conditions (phosphate buffered saline, 5% potassium dichromate, 4% formaldehyde and ethanol). These researchers used real-time PCR to assess seven different samples over a period of three months and stated that their best results with respect to DNA isolation were obtained from samples that had been stored in ethanol or potassium dichromate at 4ºC. In another study, a total of 70 samples (from 60 humans and 10 animals) were stored in 5-10% formaldehyde (the duration of this storage was unclear from the published report of the investigation); these samples were examined and only 44 (from 43 humans and 1 animal) isolates could be amplified from these samples (Minvielle et al. 2008). In addition, Pelayo et al. (2008) investigated a total of 95 samples that were



Agarose gel showing amplifications of polymerase chain reaction of *Giardia intestinalis* which were stored six different conditions. Lane 1: 100 bp Plus DNA Ladder (Vivantis); 2-7: sample contain more *Giardia* trophozoites than *Giardia* cyst; 8-13: sample contain only *Giardia* cyst; 2, 8: from sample stored at room temperature; 3, 9: from sample stored at +4ºC; 4, 10: from sample stored at -20ºC; 5, 11: from sample stored at 70% alcohol; 6, 12: from sample stored at 10% formaldehyde; 7, 13: from sample stored at 2.5% potassium dichromate; 14: positive control; 15: negative control.

TABLE II

Storage duration and condition for DNA isolation of *Giardia intestinalis*

Duration	Storage condition	Number of samples				
		1[a]	2[a]	3[a]	4[b]	5[b]
First week	Room temperature	+	+	+	-	-
	+4ºC	+	+	+	-	-
	-20ºC	+	+	+	-	-
	70% alcohol	+	+	+	-	-
	10% formaldehyde	-	-	-	-	-
	2.5% potassium dichromate	+	+	+	+	+
Second week	Room temperature	+	+	+	-	-
	+4ºC	+	+	+	-	-
	-20ºC	+	+	+	-	-
	70% alcohol	+	+	+	-	-
	10% formaldehyde	-	-	-	-	-
	2.5% potassium dichromate	+	+	+	+	+
Third week	Room temperature	+	+	+	-	-
	+4ºC	+	+	+	-	-
	-20ºC	+	+	+	-	-
	70% alcohol	+	+	+	-	-
	10% formaldehyde	-	-	-	-	-
	2.5% potassium dichromate	+	+	+	+	+
Fourth week	Room temperature	+	+	+	-	-
	+4ºC	+	+	+	-	-
	-20ºC	+	+	+	-	-
	70% alcohol	+	+	+	-	-
	10% formaldehyde	-	-	-	-	-
	2.5% potassium dichromate	+	+	+	+	+

a: sample contain more *Giardia* cyst than *Giardia* trophozoites; *b*: sample contain more *Giardia* trophozoites than *Giardia* cyst.

stored at -20ºC in 70% ethanol for up to 20 months and reported that just 20 isolates could be amplified from their samples. It has been demonstrated that from a total of 147 samples that were stored at 4ºC without preservatives (the duration of this storage was not clearly specified in published reports), only 92 isolates could be amplified (Gómez-Couso et al. 2012). Singh et al. (2009) investigated 45 samples that were stored at -20ºC without preservatives (the duration of this storage was not clearly specified in the published report of this investigation) and observed that only 35 isolates could be amplified from these samples following the storage. Peréz Cordón et al. (2008) also investigated 210 samples that were stored in 2.5% potassium dichromate at +4ºC without preservatives (the duration of this storage was unclear from the published report of this study) and reported that only 16 isolates could be amplified from these samples. Although many of the aforementioned papers had unclear storage times, several of these papers mentioned long storage periods. Samples are generally stored for one month for epidemiological studies. Therefore, our study studied storage times of up to one month.

The efficiency of nested PCR in diagnosing cryptosporidiosis in fresh and formalin-preserved faecal samples has been evaluated in a previously published study. The sensitivity and specificity of this method for assessing fresh and formalin-preserved samples were found to be 100% and 50%, respectively; this result indicated that small quantities of formaldehyde in the samples were inhibiting the PCR reaction, possibly producing false-negative results (Dirim et al. 2009). Thus, in this study, it has been demonstrated that during the course of a storage period of one month, 2.5% potassium dichromate is the most suitable of the examined storage conditions for isolating *G. intestinalis* DNA from stool samples. This study suggests that different forms of microorganisms and other parasites from stool samples should be assessed separately for samples that are stored for longer than one month.

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