

Full Length Research Paper

Introducing a simple and economical method to purify *Giardia lamblia* cysts

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Direct microscopic examination of stool to diagnosis giardiasis (wet mount) has low diagnostic value, but immunologic methods (like IFA and especially ELISA) that are based on the determination of parasite antigens in fecal samples (antigen detection) have relatively high sensitivity and specialty. To prepare anti-*Giardia lamblia* antibodies needed to design diagnostic kits as well as parasite culture and other molecular studies, we require purification of the parasite cysts. In this study, we designed a rapid, simple and inexpensive method to purify parasite cysts from fecal samples of the patients suffering from giardiasis. Initially, fecal samples that the presence of *G. lamblia* in them was affirmed by direct microscopic observation of cysts were subjected to various purification methods like one- and two-phase sucrose gradient isolation, percoll-sucrose gradient isolation, and a modified two-phase method run by 0.85 and 1.5 M sucrose. The first procedure contained some contents of bacteria and small particles of feces. In the second and third procedure, bacteria were almost removed and the cysts were intact but the suspension contained some extras and cellulose particles. The recovery rate for modified two-phase method was 1.5×10^4 cysts for each two grams of fecal sample. In this study, by using and comparing with the results of some other studies, we introduce and run a modified method that in fact is a mélange of them with some changes. So this method could be recommended as a fast, advantageous and simple method in purification of *G. lamblia* cysts.

Key words: *Giardia lamblia*, cyst, purification.

INTRODUCTION

Giardiasis is one of the most important gastro-intestinal syndromes with worldwide distribution especially in developing countries (Buret et al., 1990; Sanda and Dorwish, 1996) that is caused by infection with a protozoon, *Giardia lamblia* (Shahabi et al., 2006). This parasite was introduced at the first half of twentieth century as a human pathogen (Keating et al., 1998). It is estimated that about 200 million people are now suffering from giardiasis and although it could involve all age groups, but its incidence is higher in children. It is also the most

prevalent parasite separated from fecal samples in the United States of America (Pickering et al., 2004). The transmitter form of the parasite is its cystic form that could be easily transmitted by water (Shirbazou and Aghamiri, 2000) because the free cysts of this parasite could remain alive for weeks in water (Beaver and Jung, 1993) and standard concentration of chlorine solution used for disinfection and cleaning-up of water would not annihilate them (Adidiss et al., 1992). Parasite enters by feeding contaminated food substances but person-to-person transferring of the cyst is also important in life cycle of this parasite (Garcia et al., 1998; Edvard et al., 1999). This parasite has a high inflammatory capacity and as few as 10 cysts could even cause the disease (Keating et al., 1998). The clinical signs of giardiasis vary

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from an asymptomatic infection to an acute disorder along with intense malabsorption (Mohseni et al., 2007). The symptoms of acute giardiasis may include watery diarrhea, steatorrhea with a stench smell, anorexia, vertigo and a low-degree fever sometimes with trill (Keating et al., 1998; Clyne and Opoulos, 1999). Although, this disorder is benignant, in some cases like in pregnant women and children it could cause severe disease and hospitalization by decreasing fluid reservoirs (Shaw et al., 1987; Fathing et al., 1986). It is reported that in some instances it could even cause gall bladder inflammation and jaundice (Keating et al., 1998) and there is some opinions about the effect of chronic giardiasis on growth of children (Fathing et al., 1986).

Routine method to diagnose giardiasis is direct microscopic examination of stool (Rosoff and Stibbs, 1986; Duque-Beltran et al., 2002). Sensitivity of microscopic method even with several times repeating of each test is just 50 to 70% (Burke et al., 1975, 1977). Sensitivity of this method could actually be lower in the patients with chronic giardiasis, as their cysts are periodically excreted by feces and so decreased. Other examinations like aspiration of duodenum or jejunum as well as small intestine biopsy may have more sensitivity (Sun et al., 1980) but they are not only expensive but also invasive and so are used rarely. Immunologic diagnostic methods like IFA and especially ELISA that are based on fecal detection of parasite antigens are with high sensitivity rate. To gain almost pure antibodies against parasite antigens in order to design such ELISA or IFA kits, initially we have to purify high extents of clean and unmodified parasite cysts. These cysts will then be used as antigens for immunization of animals to gain antibodies. Furthermore, to procure high extents of trophozoite forms in parasite culture, it is necessary to gain a fraction rich of parasite cysts. This rich-cyst fraction in addition to mentioned usages could also be used in molecular studies and genome handlings that require almost pure DNA samples, preparing educational slides, oral immunization of animals to study drug effect on their treatment procedure or drug resistance and epidemiologic studies. So there is a need to a rapid, inexpensive, and simple method that retains the cysts intact, live, and relatively devoid of fecal contaminations during the purification process. The aims of this study were to compare the results of some other studies and introduce a rapid, simple and inexpensive method to purify parasite cysts.

MATERIALS AND METHODS

Fecal samples of the patients referred to Imam Khomeini Hospital, Tabriz, Iran were initially surveyed by direct microscopic examination (wet mount), and the samples chosen to cyst purification were not only with the most contamination rate (more than 8 cysts in each microscopic field with the magnification of $\times 40$) but they were also without any other gastrointestinal parasite or yeast contaminations. By adding distilled water to the chosen samples, homogeneous suspensions were made that then purified

and centrifuged at 500 g for 5 min and the sediments were the subject to purification by the following methods.

One-phase sucrose gradient

This procedure was done based on studies of Rosoff and collaborators (Rosoff and Stibbs, 1986). Briefly, the sediments were solved in 0.5% tween 80 solutions followed by centrifugation at 700 g for 5 min and solving and centrifugation processes were repeated periodically till the surface liquids were cleaned. Then the sediments were collected and subjected to prepare suspensions with proper concentrations by adding distilled water. The suspensions were then decanted into tubes containing equal volumes of 0.85 M sucrose solutions so that two separate phases be created. Then the tubes centrifuged for 20 min at 500 g. So the large surplus particles of feces settled at bottom of the tubes and the content of the thin layers formed between distilled watery and sucrose phases were collected by Pasteur pipette. The mixtures were then centrifuged 2 to 3 times at 500 g for 5 min to expunge sucrose from the mixture. The earned sediments were then solved in distilled water to gain suspensions.

Two-phase sucrose gradient

The method of work in this procedure was based on studies of Walderich et al. (1997). In summary, the sediments were solved in 0.2 M PBS buffer and then periodically centrifuged at 500 g for 5 min and washed until the surface liquids got cleaned. Then the sediments were solved in distilled water and poured into tubes containing equal volumes of 1.5 M sucrose solution to get two separate phases followed by another centrifugation at 1700 g for 10 min. Contents of the mediated phases were collected and centrifuged at 300 g and 4°C for 5 min to eliminate their sucrose. At the second phase of this purification procedure, the obtained sediments were solved in distilled water and the gained suspensions were added to 0.75 M sucrose solution followed by centrifugation at 1700 g for 10 min. So the cysts settled at the bottom of the tubes and cellulose particles aggregated at mediated phase.

Percoll-sucrose gradient

Sucrose and percoll solutions were used as gradient in a study done in 1988 (Stibbs et al., 1988) and the procedure done in that study was used in this part of our procedure. After solving the sediments in distilled water, the gained suspensions were centrifuged at 500 g for 5 min and washed periodically till the surface liquids seemed to be cleaned. The sediments were then solved in distilled water and added to equal volumes of 1 M sucrose solutions followed by centrifugations at 500 g for 10 min. Middle phase contents were sucked and washed 2 to 3 times again with distilled water. In the next step, suspension was added to two solutions of percoll with gravities of 1.05 and 1.09. The tubes were centrifuged at 500 g for 20 min and the contents of the phase created between two percoll layers were collected and washed 2 to 3 times again and finally mixed with distilled water to gain a suspension.

Modified two-phase method

Tween 80 solutions with concentrations of 0.5% were used to solve the sediments. The solutions were then centrifuged at 500 g for 5 min. The solving and centrifugation processes were periodically

repeated till the surface liquids got cleaned. Then the sediments were solved in distilled water and poured on equal volume of sucrose with gravity of 1.5 M followed by centrifugation for 10 min at 1300 g and 4°C. Contents of the mediated phase were collected and washed by 2 to 3 times centrifugation at 500 g and for 5 min. Then they solved in distilled water. The second step of purification was done by adding them on 0.85 M sucrose and centrifugation at 1600 g and 4°C for 10 min. In this step, fecal particles remained from the first step of purification were trapped in the middle phase and the cysts settled at bottom of the tubes.

RESULTS

The results of the mentioned procedures were compared with each other and with modified method. To determine purity rate of *Giardia* cysts and elimination of additional particles of feces, wet mount slides were prepared for each method and studied under microscope. Furthermore, Neubauer slides were used to distinguish recovery rate of cysts. The suspension earned from one-phase sucrose gradient contained some contents of bacteria and small particles of feces and it seems that 2.5×10^4 cysts could be extracted from each two grams of fecal sample. In two-phase sucrose gradient, bacteria were almost removed and the cysts were intact but the suspension contained some extras and cellulose particles and about 5.10×10^4 cysts were recovered from each two grams of fecal sample. In the third procedure (percoll-sucrose gradient method), the cysts were recovered like two-phase sucrose gradient but with lower recovery rate, as its recovery rate for each two grams of fecal sample was just 3×10^4 cysts. The result for modified two-phase method was a suspension of clean and intact cysts and almost without any contamination that its recovery rate was 1.5×10^4 cysts for each two grams of fecal sample (Table 1).

DISCUSSION

Giardiasis is one of the most widespread gastro-intestinal infections worldwide (Buret et al., 1990; Sanda and Dorwish, 1996). Its trans-contamination is as simple as up taking cystic form of parasite from contaminated water or another person (Shirbazou and Aghamiri, 2000; Akhlaghi et al., 2006). Sensitivity of direct microscopic examination of stool to diagnose giardiasis is low (about 50 to 70%) that is even lower in chronic form of the disorder (Burke et al., 1975, 1977). Biopsy and aspiration of small intestine could gain higher sensitivity rates but this procedures are invasive and so are used infrequently (Jones et al., 1986; Howard et al., 1995). Various sensitivity and speciality of some of these tests along with practical difficulties of their handlings are among restrictive factors in their usage. Immunologic methods like ELISA that have high sensitivity are currently used as powerful serological determination methods in most of the infectious disorders. To access almost pure anti-

bodies against antigens of this parasite in order to design diagnostic kits and pharmaceutical and other scientific studies, parasite cysts have to be purified and recovered live, intact, and unmodified. In the published methods, some chemical materials like formalin, iodine, mercury, and ethers were used for the isolation of protozoa cysts that could affect physical and biological properties of cysts (Blagg et al., 1955; Walderich et al., 1997). Methods like sucrose or percoll-sucrose gradient tests had gained suspensions of cysts that contained surplus fecal particles (Jyothi et al., 1993; Walderich et al., 1997). A special one-phase sucrose gradient method was used in a study to isolate GSA65 specific antigen (Rosoff and Stibbs, 1986) that we used that procedure in the current study as our first method of separation. Other similar methods were used previously in the studies of some other researchers (Visvesvara et al., 1988; Roberts-Thomson et al., 1976).

The results of this method are not satisfactory, because the output of isolation by this method was cysts along with bacteria and additional small particles of feces. Comparing this with a two-phase sucrose gradient method introduced by Walderich and colleague (Walderich et al., 1997) showed that the results of Walderich and its team were more acceptable. Our second method of separation was based on and with similar results of Walderich et al. (1997) method. In this method, although, bacteria were almost completely eliminated but some contents of cellulose and extras remained. In another study done in 1988, sucrose and percoll were used for the isolation of *Giardia* cysts (Stibbs et al., 1988) that was the basis of third separation process in our study. The final suspension in this method had a little amount of extras. In another study that was at 2002 to produce polyclonal antibodies against *Giardia* antigens, the mentioned purification method were used (Duque-Beltran et al., 2002). Percoll solution is very expensive and because of its uncommon usage, is commercially scarce. So this solution is not routinely used in research centers. On the other hand, percoll causes drastic transfigurations on cysts and so its usage is not recommended. As mentioned, each of the aforesaid three methods has some disadvantage, thus, in this study, we compared the results of these methods and introduced the forth method as a simple, inexpensive and high-efficiency one. This method is in fact a combination of first and second methods with some changes that let this method to access worthwhile results. So, we recommend this method as a rapid and inexpensive one to purify cysts of *Giardia* and maybe other parasites like *Entamoeba coli* and *Entamoeba histolytica*.

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