J. Protozool. Res., 10, 155 - 165 (2000)

Bench Scale Experiments to Evaluate the Usefulness of Sucrose Flotation Techniques for Separation of *Cryptosporidium* Oocysts from Water

A. KIMURA [1], P. KARANIS [2], E. MALTEZOS [3] AND H. M. SEITZ [2]

[1] *Laboratory of Medical Zoology, Osaka Prefectural Institute of Public Health, 1-3-69 Nakamichi, Higashinari-ku, Osaka 537-0025, Japan,* [2] *University of Bonn, Institute for Medical Parasitology, Sigmund-Freud-Str. 25, D-53127 Bonn, German and* [3] *Department of Internal Medicine, Democritos University of Thrace, P. B. 107, 68100 Alexandroupolis, Greece*

Received October 25, 1999 and Accepted June 30, 2000

Keywords: *Cryptosporidium parvum*, modified sucrose flotation technique, discontinuous sucrose gradients, separation of oocysts, water borne disease.

ABSTRACT

Several series of bench scale experiments were performed to evaluate the usefulness of well established sucrose flotation techniques for separation of *Cryptosporidium* oocysts from water samples under the various conditions.

Firstly, fresh (one month old) and aged (36 month old) oocysts of *C. parvum* were spiked into distilled water and separated by the modified sucrose flotation technique or by the discontinuous sucrose gradients. The mean recovery rate of fresh oocysts by the modified sucrose flotation technique was higher (87.2%) than by the discontinuous sucrose gradients (68.4%). In contrast, the mean recovery rates of aged oocysts by two methods were significantly lower. The recovery rate using different initial concentration of oocysts showed no significant differences in recovery between each of them. The usefulness of the modified sucrose flotation technique for the separation of oocysts from raw water samples was also evaluated. The recovery rate of oocysts from low and moderate turbidity water (ntu=0.8 and 15.4) was nearly 80%, however the recovery rate from high turbidity water (ntu=350) was significantly lower (60.7%).

From these results we assume that the modified sucrose flotation technique is

still useful as a fast one-step, simple to perform and inexpensive method for the separation of *Cryptosporidium* oocysts in environmental water samples.

INTRODUCTION

Cryptosporidium parvum was identified firstly in 1907 and it was primarily associated with infections in animals (Tyzzer 1907). The sporulated oocysts is the dormant stage of the parasite and the only exogenous stage, it survives for a long time in the environment. Infection of appropriate hosts by *Cryptosporidium* occurs directly via the faecal-oral route, mainly by water or food contaminated with oocysts. After the oocysts are ingested, four sporozoites excyst from oocysts and parasitize epithelial cells of the hosts intestine. After multiplication and development the newly produced oocysts in the infected host are excreted in the feces. Current (1985; 1986) described two types of oocysts in the life cycle of *C. parvum*; the thin walled oocysts (20% of the whole) responsible for the autoinfection of the host and the thick walled oocysts (80% of the whole) which leave the body by the feces to widespread in the environment.

C. parvum has been recognized to be one of the most important waterborne pathogens for industrialized countries. Between 1993 and 1996, this parasite caused 23 waterborne outbreaks in USA and United Kingdom (Fricker and Crabb 1998; Smith and Rose 1998). Other outbreaks have been reported from Canada (Bell et al. 1993; Wallis et al. 1996) and Japan (Kuroki et al. 1996; Yamazaki et al. 1997).

The methodology currently used to detect *Cryptosporidium* in water (Musial et al. 1987; Rose et al. 1988; LeChevallier et al. 1991; Wallis et al. 1996; Karanis et al. 1998) is based on the method primarily developed for the detection of *Giardia* in water (Jakubowski and Ericksen 1979; Jakubowski 1984). The most commonly used technique is the U. S. Environmental Protection Agency (EPA) cartridge filter/ immunofluorescence method for concentration and identification of *Cryptosporidium* oocysts in environmental samples. It has been designed to meet monitoring reqauirements of the EPA and consists of four major steps: a) filtration of water to concentrate oocysts, b) elution of filter material, c) separation of oocyst from debris, and d) finally microscopic analysis for the identification of *Cryptosporidium* oocysts in a pellet resulting from the sample preparation. Yet, there are still many problems with this method, because recovery of oocysts from water may be affected by all of the preparation steps for several reasons. Naturally occurring debris such as clays and

algae in water and also chemicals added to the water during the treatment process may affect the concentration of parasites by this detection method. Turbidity, caused by debris usually occurring in water, results in additional interference and loss of parasites during the preparation of the water sample (LeChevallier et al. 1995; Fricker 1995). Not only water quality, but also the biological properties of the parasite may affect the effectiveness for *Cryptosporidium* oocysts separation resulting in the decrease of the entire recovery efficiency of oocysts in water samples. There are strong evidences that the sources of the isolate, age of oocysts, storage media, and counting techniques may affect the results of the analytical procedure (Klonicki et al. 1997; Karanis, unpublished data).

For the separation of oocysts from debris in water, sucrose flotation or percoll-sucrose flotation technique have been commonly used (Musial et al. 1987; Rose et al. 1988; LeChevallier et al. 1991; Wallis et al. 1996; Karanis et al. 1998). A number of oocysts must be lost in this separation step because of the factors mentioned above, however the actual recovery efficiencies of the sucrose flotation technique in several conditions (e.g. specific gravity of sucrose, age of oocysts, water quality) are still unclear. The purpose of our study was: 1) to evaluate the sucrose flotation techniques in bench scale experiments for the recovery of *Cryptosporidium* oocysts from water, 2) to show possible variability in the recovery of oocysts from water samples using fresh and aged oocysts, 3) to assess the usefulness of sucrose flotation technique to separate oocysts from water with variable turbidity.

MATERIALS AND METHODS

Source and isolation of oocysts

C. parvum oocysts were isolated from fecal samples of infected calves and have been stored in 2.5% potassium dichromate solution at 4°C. Oocysts were purified by discontinuous sucrose gradients technique (Arrowood and Sterling 1987). The oocysts used for the experiments were 1-5 months old and 36 months old. The oocysts were identified and confirmed by phase contrast microscopy and by a commercial immunofluorescence assay (IFA) kit (Cellabs Pty. Ltd., Sydney, Australia). The numbers of oocysts were counted by a Neubauer hemocytometer.

Trials for the recovery of oocysts in distilled water using the discontinuous sucrose gradients

2.04×10^5 of one month old or 36 months old oocysts were suspended in 5 ml distilled water and laid onto sucrose discontinuous solution in a 50 ml centrifugation tube (Greiner Labortechnik) as described by Arrowood and Sterling (1987). After a centrifugation at 1,500*g* for 30 min at 4°C without brake (Heraeus Sepatech, Varifuge 3.0 R), the middle layer from 15 ml to 7.5 ml which contained the interface of two different gravity of sucrose solutions was pooled into a 50 ml centrifugation tube to evaluate the recovery of oocysts. The upper layer from 25 ml to 15 ml, and the lower layer from 7.5 ml to the bottom of the tube which contained sediment were also pooled to be examined for possible presence of oocysts. All pooled layers were diluted with distilled water and centrifuged at 3,000*g* for 20 min at 4°C with brake. After the centrifugation, the supernatant was aspirated till 1 ml and the pellet was stored at 4°C.

Trials for the recovery of oocysts in distilled water using the modified sucrose flotation technique

The modified sucrose flotation technique was performed as described by Current (1990) with minor modifications. 2.04×10^5 of one month old oocysts or 36 months old oocysts were suspended in 5 ml of distilled water and mixed well with 30 ml of 1.3 specific gravity sucrose solution in a 50 ml centrifugation tube. Then 5 ml of distilled water were carefully laid over the sucrose solution and centrifuged at 1,500*g* for 20 min at 4°C without brake (Heraeus Sepatech, Varifuge 3.0 R). After the centrifugation, the upper layer from 40 ml to 30 ml which contained the interface of water and sucrose was pooled into a 50 ml tube. The lower layer from 30 ml to bottom which contained sediment was also pooled. All pooled layers were diluted with distilled water and washed by centrifugation at 3,000*g* for 20 min at 4°C with brake. After centrifugation, the supernatant was aspirated till 1 ml and the pellet was stored at 4°C.

Trials for the recovery of oocysts in distilled water with variable initial concentration using the modified sucrose flotation technique

2.26×10^4, 2.34×10^3 and 1.67×10^2 of four months old oocysts were suspended in 5 ml of distilled water and the modified sucrose flotation technique was performed as described above to evaluate the recovery of oocysts.

Trials for the recovery of oocysts in raw water with different turbidities using the modified sucrose technique

2.20 x 10^4 of five months old oocysts were suspended in 5 ml of three raw water samples with different turbidities which were collected from a river (ntu=350), from a lake at 15 ml depth (ntu=15.4) and at 40 m depth (ntu=0.8). The modified sucrose flotation technique was performed as described above to evaluate the recovery of the oocysts.

Evaluation of the results

Each series of experiments was performed at five separate trials. Numbers of oocysts in resulting pellets were counted in a Neubauer hemocytometer under phase contrast microscope. The significance of differences between each mean recovery rate of experiment was expressed at a p value in Man-Whitney U test.

RESULTS

The mean recovery rate of fresh oocysts (one month old oocysts) in distilled water using the modified sucrose flotation technique was higher (87.2%) than using the discontinuous sucrose gradients (68.4%) (Table 1). The statistical comparison by U test indicated a difference between them ($p<0.1$). For aged oocysts (36 months old oocysts), the mean recovery rate was higher (48.1%) using the discontinuous sucrose gradients than using the modified sucrose flotation technique (39.6%) (Table 1), however there was no statically difference ($p>0.1$). Using both techniques, the mean recovery rates of aged oocysts was significantly lower than fresh oocysts ($p<0.05$ in both techniques) (Table 1). Using the discontinuous sucrose gradients, 30.6% if fresh oocysts and 15.3% of aged oocysts were found in the lower part of the layer with sediment. Using the modified sucrose flotation technique, 11% of both fresh and aged oocysts were found in the lower part of the layer with sediment (data not shown).

Table 1. Recovery of *Cryptosporidium* oocysts using the discontinuous sucrose gradients or the modified sucrose flotation technique.

Age of Oocysts* (month)	Discontinuous sucrose gradients		Modified sucrose flotation technique	
	Mean recovery rate (SD)	Range	Mean recovery rate (SD)	Range
1	68.4 (12.9)	47.1 – 78.8	87.2 (13.1)	66.3 – 96.2
36	48.1 (5.9)	39.8 – 55.3	39.6 (12.7)	32.0 – 62.1

*2.02 x 10^5 oocysts were spiked into 5 ml water.

Table 2. Recovery of *Cryptosporidium* oocysts in variable initial concentration using the modified sucrose flotation technique.

No. of Oocysts	Mean recovery rate (SD)	Range
2.26×10^4	83.1 (14.0)	70.9 – 106.2
2.34×10^3	72.3 (17.5)	47.0 – 95.7
1.67×10^2	73.3 (41.1)	20.4 – 116.8

*3 months old oocysts were used.

The mean recovery rate using different initial concentrations of 2.26×10^4, 2.34×10^3 and 1.67×10^2 of three month old oocysts in distilled water by the modified sucrose flotation technique was 83.1%, 72.3% and 73.3%, respectively (Table 2). There were no significant differences between each recovery rate ($p>0.1$).

The mean recovery rates of five month old oocysts in three raw water samples with different turbidities are shown in Table 3. Recovery of oocysts in high turbidity water (ntu=350) was lower (60.7%) than in moderate (ntu=15.4) and low turbidity (ntu=0.8) water (79.3% and 84.6%, respectively). There was significant differences between the recovery rate in high and low turbidity waters ($p<0.05$). In low turbidity water, the mean recovery rate corresponded to the mean recovery rate in distilled water which was suspended with the same number of oocysts ($p>0.1$) (Tables 2 and 3).

Table 3. Recovery of *Cryptosporidium* oocysts in water with various turbidities using the modified sucrose flotation technique.

Turbidity (ntu)	Mean recovery rate (SD)	Range
High (350)	60.7 (13.3)	48.6 – 81.8
Moderate (15.4)	79.3 (11.3)	70.9 – 99.1
Low (0.8)	84.6 (17.3)	67.7 – 106.4

*2.2×10^4 oocysts were spiked into 5 ml water.

DISCUSSION

Flotation techniques used for purifying *Cryptosporidium* oocysts in fecal samples had been adapted for separation of oocysts from debris in water samples by combination of sucrose gradients or Percoll-sucrose gradient (LeChevallier et al. 1991; Karanis et al. 1998). The aim of flotation techniques is to separate particles and

debris from oocysts by their diffefence in specific gravity and to arrive of specimen for better visual detection for microscopy. In this study we focused on our trials not only to evaluate the recovery efficiency of sucrose flotation technique but also to examine the factors which may affet the recovery of oocysts during the flotation steps. To evaluate this technique, we used two entirely different ages of oocysts to study the affet of age of parasites to its recovery. In addition we examined the recovery of oocysts under the influences of water turbidity.

The mean recovery rate of one month old (fresh) oocysts in distilled water using the modified sucrose flotation technique was higher than using the discontinuous sucrose gradients (Table 1). In the discontinuous sucrose gradients, nearly three times more fresh oocysts were counted in lower sediment layer compared with the modified sucrose flotation technique. There results demonstrate that mixing the sample with higher specific gravity of sucrose solution yields better recovery rate particularly for fresh oocysts. Using a combination of Percoll-sucrose density gradients, LeChevallier et al. (1995) also demonstrated that recovery of fresh oocysts was improved when a specific gravity of 1.3 was used. Our results suggest that the use of the modified sucrose flotation technique will be better for the separation of fresh oocysts in clean water samples (Table 1).

In contrast to the recovery of fresh oocysts, the mean recovery rate of 36 months old (aged) oocysts was significantly lower and considerable part of them were not found in any layer by both techniques. This is probably that the walls of aged oocysts became fragile because of long-term storage and destroyed by the osmotic pressure of the sucrose solution. Variability in the recovery of oocysts of different age was also reported by Bukhari and Smith (1995). They found that sucrose solution of specific gravity of 1.18 selectively concentrated viable or intact oocysts. Prolonged storage of oocysts affects the viability and infectivity of the parasites has been suggested for other coccidian parasites also. Especially for *Cryptosporidium* it has been suggested that infectivity decreases significantly after two months of storage, but a considerable percentage of oocysts remains viable for up to 12 months (Sherwood et al. 1982; Current 1990). Also in our study, the mean recovery rates of one to five month old oocysts spiked into distilled or low turbidity water showed no difference (from 83.1 to 87.2%). These results suggest that the age of oocysts will greatly affect the whole recovery rate in flotation separation step. Also Klonicki et al. (1997) pointed out that the age of oocysts is one of the most critical factors in

Cryptosporidium research. The same authors criticized that many publications offer only incomplete documentation of important factors (e.g. sources of oocysts, age of oocysts, storage media) which affect the quality of oocyst, resulting in large discrepancy of counting methods. They also found high variation in oocyst recovery using the Percoll-sucrose flotation which explains why the ICR method (Information Collection Rule, EPA method) has shown low precision.

In low and moderate turbidity water, the recovery rate of five months old oocysts was nearly 80% and corresponded to the recovery rate of one month old oocysts in distilled water. In contrast, the recovery rate in high turbidity water was significantly lower (Table 3). Fricker (1995) demonstrated that when the sucrose flotation technique was used, the recovery of oocysts from raw water was lower (55%) than from reservoir water or fully treated water (67% and 80%, respectively). LeChevallier et al. (1995) also reported a significant decrease of oocysts recovery from environmental samples in Percoll-sucrose gradient. This occurs presumably because particles in raw water may attach to oocysts altering their sedimentation characteristics or destroy them.

In this study, we directly contaminated small volumes of water (5 ml) with oocysts because we attempted to evaluate only the recovery efficiency of flotation methods without concentrate of oocysts by filtration step. From this reason, initial concentrations of oocysts used in all our trials became higher than expected in the natural contamination level (Karanis et al. 1998). Although the statistical evaluation showed no significant difference of recovery rate between different initial concentrations of oocysts (Table 2), we can not exclude the possibility that the high initial concentrations of oocysts improved the recovery. In this point, further investigation should be carried out using low initial concentrations of oocysts.

Recently new separation method, immunomagnetic separation (IMS) was proposed alternative to flotation techniques (Bukhari et al. 1998). In IMS, the recovery rates of oocysts from water samples of various turbidities (50 to 5000 ntu) ranged from 0.12 to 85% using three commercially available kits (Bukhari et al. 1998). These data indicate the potential ability of IMS for separation of oocysts especially from high turbidity raw water, but the method needs more development for practical use. In addition, commercial kits of IMS are rather expensive to use in daily routine works. For example, Dynabeads anti-*Cryptosporidium* kit (Dynal A.S. Oslo, Norway) is 54,000 Japanese Yen (2,100 Norway-Krone) for 10 samples. We do not

exclude the use of other techniques such as IMS for separation of oocysts from water samples, but at this moment we assume that the modified sucrose flotation technique is still useful as a fast one-step, simple to perform and inexpensive method for the analysis and detection of *Cryptosporidium* oocysts in environmental water samples.

ACKNOWLEDGMENTS

We thank Dipl. – Chem. O. Hoyer from the Wahnbachtalsperrenverband/ Siegburg, Germany for providing waters samples and turbidity measurement.

REFERENCES

Arrowood, M. J. & Sterling, C. R. 1987. Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic percoll gradients. *J. Parasitol. 73:* 314-319.

Bell, A., Guasparini, R., Meeds, D., Mathias, R. G. & Farley, J. D. 1993. A swimming pool-associated outbreak of Cryptosporidiosis in British Columbia. *Can. J. Public Health 84:* 334-337.

Bukhari, Z. & Smith, H. V. 1995. Effect of three concentration techniques on viability of *Cryptosporidium parvum* oocysts recovered from bovine feces. *J. Clin. Microbiol. 33:* 2592-2595.

Bukhari, Z., McCuin, R. M., Fricker, C. R. & Clancy, J. L. 1998. Immunomagnetic separation of *Cryptosporidium parvum* from source water of various turbidities. *Appl. Environ. Microbiol. 64:* 4495-4499.

Current, W. L. 1985. Cryptosporidiosis. *J. Am. Vet. Med. Assoc. 187:* 1334-1338.

Current, W. L. 1986. *Cryptosporidium* its biology and potential for environmental transmission. *Crit. Rev. Environ. Contro. 17:* 21-51.

Current, W. L. 1990. Techniques and laboratory maintenance of *Cryptosporidium.* pp. 31-49. *In:* Cryptosporidiosis of man and Animals, Dubey, J. P., Speer, C. A., Fayer, R. (Eds), CRC Press, Boca Raton, FL.

Fricker, C. R. & Crabb, J. H. 1998. Water-borne Cryptosporidiosis: Detection methods and treatment options. *Adv. Parasitol. 40:* 241-278.

Jakubowski, W. & Ericksen, T. H. 1979. Methods for the detection of *Giardia* cysts in water supplies. pp. 64-81. *In:* Waterborne transmission of Giardiasis. Jakubowski, W., Hoff, J. C. (Eds), EPA-600/9-79/001, Cincinnati, Ohio, USA.

Jakubowski, W. 1984. Detection of *Giardia* cysts in drinking water-State of the art.

pp. 263-286. *In: Giardia* and Giardiasis. Erlanden, S. L., Meyer, E. A. (Eds), New York, N. Y. Plenum Press, USA.

Jakubowski, W. 1990. The control of *Giardia* in water supplies. pp. 335-353. *In: Giardia* and Giardiasis. Meyer, E. A. (Ed), Elsevier, Amsterdam, New York, Oxford.

Karanis, P., Schoenen, D. & Seitz, H. M. 1998. Distribution and removal of *Giardia* and *Cryptosporidium* in water in Germany. *Wat. Sci. Tech. 37:* 9-18.

Klonicki, P. T., Hancock, C. M., Straub, T. M., Harris, S. I., Hancock, K. W., Alyaseri, Ali Meyer, C. J. & Sturbaum, G. D. 1997. Crypto research: are fundamental data missing? *J. A. W. W. A. 89:* 97-103.

Kuroki, T., Watanabe, Y., Asai, Y., Endo, T., Uni, S., Kimata, I. & Iseki, M. 1996. An outbreak of waterborne cryptosporidiosis in Kanagawa, Japan. *Kansenshogaku-zasshi 70:* 132-140 (in Japanese).

LeChevallier, M. W., Norton, W. D., Lee, R. G. & Rose, J. B. 1991. Detection and treatment of *Giardia* and *Cryptosporidium* in water supplies. AWWA Research Foundation 6666 West Qincy Avenue Denver, CO 80235.

LeChevallier, M. W., Norton, W. D., Siegel, J. E. & Abbaszadegan, M. 1995. Evaluation of the immunofluorescence procedure for detectiojn of *Giardia* cysts and *Cryptosporidium* oocysts in water. *Appl. Environ. Microbiol. 61:* 690-697.

Musial, C. E., Arrowood, M. J., Sterling, C. R. & Gerba, C. P. 1987. Detection of *Cryptosporidium* in water using polypropylene cartridge filters. *Appl. Environ. Microbiol. 53:* 687-692.

Rose, J. P., Kayed, D., Madore, M. S., Gerba, C. P., Arrowood, M. J., Sterling, C. R. & Riggs, J. L. 1988. Methods for the recovery of *Giardia* and *Cryptosporidium* from environmental waters and their comparative occurrence. U. S. Environmental Protection Agency, Cincinnati, Ohaio, USA.

Sherwood, D., Angus, K. W., Snodgrassm, D. R. & Tzipori, S. 1982. Experimental cryptosporidiosis in laboratory mice. *Infect. Immun. 38:* 471-475.

Smith, H. W. & Rose, J. B. 1998. Waterborne cryptosporidiosis. *Parasitol. Today 1: 14-22.*

Tyzzer, E. E. 1907. A sporozoon found in the peptic glands of the common mouse. *Proc. Societ. Esp. Biol. Med. 5:* 12-13.

Wallis, P. M., Erlandsen, S. L., Isaac-Renton, J. L., Olson, M. E., Robertson, W. J. & van Keulen, H. 1996. Prevelence of Girardia cysts and *Cryptosporidium* oocysts

and characterization of *Giardia* spp. isolated from drinking water in Canada. *Appl., Environ. Microbiol. 62:* 2789-2797.

Yamazaki, T., Sasakim, N. & Hori, E. 1997. Clinical features of Japanese children infected with *Cryptosporidium parvum* during a massive outbreak caused by contaminated water supply. *Kansenshogaku-zasshi 71:* 1031-1036 (in Japanese).