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Comparison of Immunomagnetic Separation and Flocculation to Isolate *Cryptosporidium spp.* Oocysts from Drinking Water Samples

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Introduction

Cryptosporidiosis is a common cause of diarrheal disease world-wide, often affecting children and immunocompromised persons. The infectious stage of the parasite is the oocyst which is excreted in great numbers in the faeces of infected hosts. Infection occurs by the faecal-oral route either directly from person to person, or indirectly through contaminated vectors like water (1) and food (2). The oocysts are extremely robust and can withstand a range of disinfectant procedures, including those commonly used for water treatment (3, 4). Several waterborne outbreaks of cryptosporidiosis have been documented, including a massive waterborne outbreak in 1993 in Milwaukee, USA, with more than 403 000 cases (1, 5–7). The numerous outbreaks of waterborne cryptosporidiosis led to considerable interest in monitoring water for the presence of oocysts. However, environmental monitoring for these parasite is made problematic by their small size (4–6 μm), their relative low concentrations in most waters, their inability to augment their numbers by culture and their difficulty in identifying the oocysts amongst the vast number of other particles. Moreover, different laboratory experiments reveal that within every step of current analytical procedures substantial numbers of oocysts are lost, and that the more steps that are included the greater are the losses that can be expected. Most techniques available at present are inefficient, costly, tedious and time-consuming (8–12). Therefore, there is a great need to improve the methodology for detecting these parasites in water.

The purpose of the present study was to develop a simple and cost-effective method to detect *Cryptosporidium spp.* oocysts in drinking water on a routine basis.

Materials and methods

Oocysts

Cryptosporidium parvum oocysts were obtained from one naturally infected calf (two weeks of age). Faecal samples were taken rectally and analysed by modified Ziehl-Neelson staining (13). To confirm *Cryptosporidium* positive results, samples were stained with a fluorescent monoclonal antibody (direct immunofluorescent staining (DIF) with Monofluo Kit *Cryptosporidium;* Sanofi Pasteur) and identified under an epifluorescence microscope with 400 × magnification (Nikon Optiphot). Oocysts were purified from faecal samples according to *Schweizer* (14) by density gravity centrifugation, and stored in PBS containing 100 U of penicillin per ml and 100 µg streptomycin sulphate per ml at 4° C. Oocysts were enumerated by means of a hemocytometer (Neubauer Improved; Brand, Germany).

Standard oocyst suspension

A stock suspension of oocysts with approximately 80 oocysts per 50 µl was prepared for use in seeding experiments and 14 microtubes were filled with 50 µl of this suspension. Nine tubes were chosen by chance to establish the exact concentration of the stock suspension by direct immunofluorescent staining using a fluorescent monoclonal antibody (Crypt-a-Glo Waterborne Inc. USA, article no. A400FL), and the other five tubes were used for seeding experiments. Oocysts were 10 months of age at time of use.

Water concentration by flocculation

Water samples were concentrated by flocculation with aluminium sulphate, as this method resulted in good recovery rates (14–16) and comparative studies (results not shown) suggested that it was superior to calcium carbonate flocculation. A modification of the flocculation method developed by *Schweizer* (14) was used and is described briefly below. 40 ml of 10 % aluminium sulphate were added to the water sample (20 l) while stirring well and the pH was adjusted to 5.8–5.9 with 1 M HCl. The flocculate was allowed to sediment at room temperature for 4 h, then the supernatant was aspirated by vacuum pump. The sediment was divided into 250 ml centrifuge bottles, and the vessel was rinsed with 50 ml 0.1 % Tween 80 and 100 ml demineralized water. The rinsing solutions were added to the centrifuge bottles. After centrifugation at 1500 x g for 10 min, the supernatant was discarded, and 40 ml citrate buffer (42 g citron acid monohydrate and 88.2 g tri-sodium-citratedihydrate ad 500 ml demineralized water; pH = 4.7) were added. The bottles were well shaken on the vortex, the contents transferred to one conical centrifuge tube and left at room temperature overnight to dissolve the gelatinous precipitate of aluminium hydrox-

ide. The next morning, 20 ml 0.1 % Tween 80 was added, and the oocyst suspension well shaken and centrifuged for 10 min at 1500 x g. The supernatant was aspirated by vacuum pump and the pellet containing the oocysts retained.

Immunomagnetic separation (IMS)

For processing by immunomagnetic separation, the pellet to be separated was washed with 0.1 % Tween 80 and subsequently with PBS (1500 x g for 10 min) Dynabeads anti *Cryptosporidium* (Dynal AS, Norway; article no. 730.01) were used according to the manufacturer's instructions. The separated oocysts were transferred onto one well of a slide, dried, fixed and stained with a fluorescent monoclonal antibody (Crypt-a-Glo) according to *Grimason et al.* (17). The antibody solution was gently aspirated and washed out with oocyst free water. 10 µl of mounting medium (30 ml glycerol and 20 ml PBS and 1 g DABCO (diazabicyclooctane)) was placed onto the slide, covered with cover-slip and sealed with clear nail varnish. The oocysts were identified under an epifluorescence microscope at 400 × magnification on the basis of size (4–6 µm), shape (round to oval), and staining (apple green with a stronger marked outer ring).

Separation by flotation

The method was adapted after *Rose* (11) and *Schweizer* (14). The pellet to be separated was resuspended in 10 ml 0.1 % Tween 80 and well shaken. The flotation medium (Sheather's solution with a specific gravity of 1.19 g ml^{-1}) was carefully laid under the suspension with a syringe. After centrifugation at 1200 x g for 10 min, the supernatant, the interlayer and 2–3 ml of the flotation medium were aspirated with a Pasteur pipette and transferred to a new tube and washed twice with demineralized water and then with 0.1 % Tween 80 at 1500 x g for 10 min. The pellet was carefully transferred to one or two wells, dried, fixed, stained and screened as described following the IMS procedure.

Comparison of separation methods

Water samples (20 litre, tap water from the cantonal Laboratory Canton Basel-Landschaft) were filtered through a nylon mesh (pore size: 41 µm) to capture eventual large particles. Then, the samples were spiked with approximately 80 oocysts (50 µl of standard oocyst suspension) and concentrated by aluminium sulphate flocculation as described. The pellet was resuspended in 4 ml demineralized water and divided in equal halves, which were subsequently either separated by flotation or by immunomagnetic separation and then both samples identified and enumerated (fig. 1). Five such paired isolation experiments were undertaken to establish and to compare the efficiency for recovering oocysts of the two different methods. Results were analysed using the one sided Wilcoxon signed rank test.

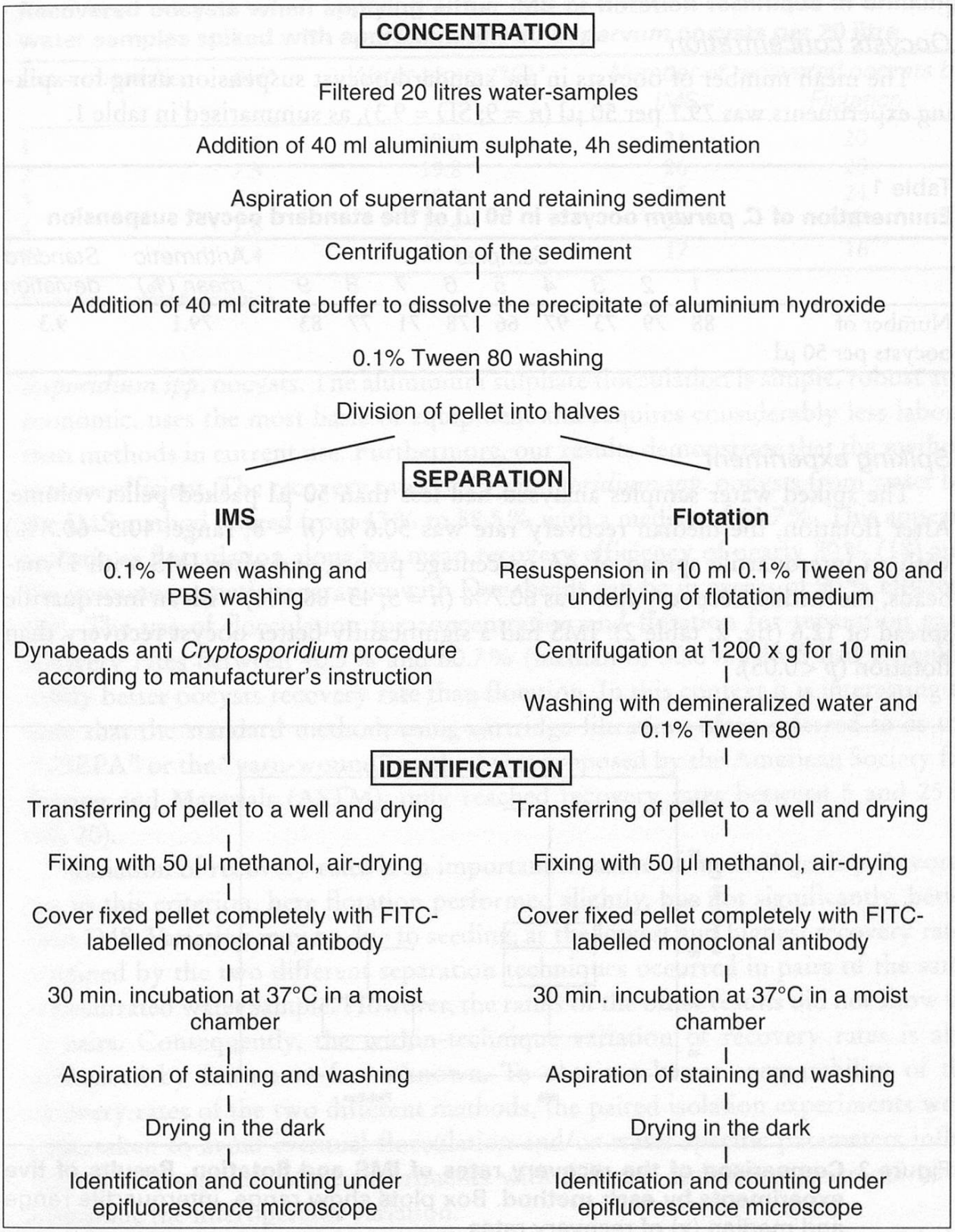

Figure 1 **Procedure for isolating *Cryptosporidium spp.* oocyst from water samples using aluminium sulphate flocculation for concentration, either IMS or flotation for separation, and direct immunofluorescent staining for identification**

Results

Oocysts concentration

The mean number of oocysts in the standard oocyst suspension using for spiking experiments was 79.1 per 50 µl ($n = 9$; SD = 9.3), as summarised in table 1.

Table 1
Enumeration of *C. parvum* oocysts in 50 µl of the standard oocyst suspension

	Samples									*Arithmetic mean (%)*	*Standard deviation*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*		
Number of oocysts per 50 µl	88	79	73	97	66	78	71	77	83	79.1	9.3

Spiking experiment

The spiked water samples analysed had less than 50 µl packed pellet volume. After flotation, the median recovery rate was 50.6 % ($n = 5$; range: 40.5–60.7 %) with an interquartile spread of 7.6 percentage points, and after IMS with Dynabeads, the median recovery rate was 60.7 % ($n = 5$; 43–88.5 %) with an interquartile spread of 12.6 (fig. 2, table 2). IMS had a significantly better oocyst recovery than flotation ($p < 0.05$).

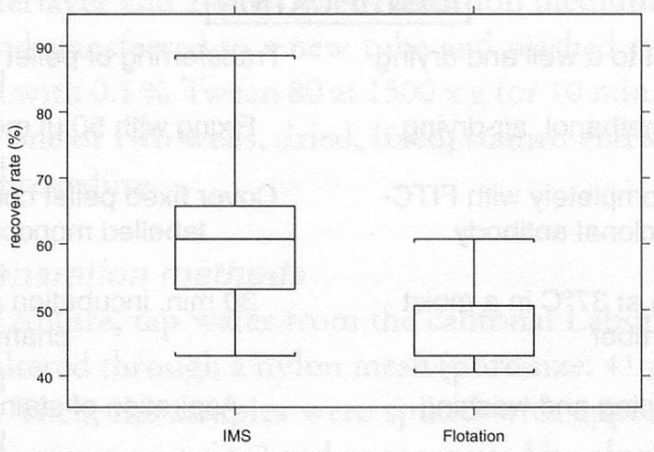

Figure 2 **Comparison of the recovery rates of IMS and flotation. Results of five experiments by each method.** Box plots show range, interquartile range and median (x) of recovery rates

Discussion

We have developed an assay which utilizes aluminium sulphate flocculation and Dynabeads for immunomagnetic separation (IMS) for the detection of *Cryp-*

Table 2
Recovered oocysts when applying either IMS or flotation technique in drinking-water samples spiked with approximately 80 *C. parvum* oocysts per 20 litre

Experiment No.	*pH**	*Water temp. (°C)**	*Number of recovered oocysts by*	
			IMS	*Flotation*
1	7.6	19.9	21	20
2	7.5	19.8	26	20
3	7.6	18.2	35	24
4	7.6	18.4	24	17
5	7.4	20.1	17	16

* measured before flocculation

tosporidium spp. oocysts. The aluminium sulphate flocculation is simple, robust and economic, uses the most basic of equipment and requires considerably less labour than methods in current use. Furthermore, our results demonstrate that the method is more efficient. The recovery rates of *Cryptosporidium spp.* oocysts from water by the IMS method ranged from 43 % to 88.5 % with a median of 60.7 %. This appears realistic as flocculation alone has mean recovery efficiency of nearly 80 % (18) and the immunomagnetic separation with Dynabeads can be in excess of 90 % efficient (19). The use of flocculation for concentration and flotation for separation gave recovery rates between 40.5 % and 60.7 % (median of 50.6 %). IMS had a significantly better oocysts recovery rate than flotation. In this context it is interesting to note that the standard method, using cartridge filtration, often referred to as the "USEPA" or the "yarn-wound" method was proposed by the American Society for Testing and Materials (ASTM), only reached recovery rates between 5 and 25 % (10, 20).

Variation of recovery rates is an important measure of method quality. According to this criterion, here flotation performed slightly, but not significantly, better than IMS. Variation may be due to seeding, as the lowest and highest recovery rates obtained by the two different separation techniques occurred in pairs of the same concentrated water sample. However, the ranks of the other results did not show up in pairs. Consequently, the within-technique variation of recovery rates is also influenced by factors so far unknown. To obtain a better comparability of the recovery rates of the two different methods, the paired isolation experiments were undertaken to avoid eventual flocculation and/or water specific parameters influencing recovery rate. More experiments with different technicians are required to determine the interoperator variation.

The aim of any separation technique is to obtain oocysts as pure as possible for identification. *Graczyk et al.* (21) reported that two of 11 commercial laboratories have misdiagnosed *Cryptosporidum* oocysts (from water samples) on the basis of a positive fluorescent reaction with the algea *Oocystis minuta*. IMS selects *Cryptosporidium spp.* oocysts by monoclonal antibody binding whereas flotation selects

by buoyant density. Thus flotation is non-specific and will select all particles with a specific gravity identical to or lower that of the flotation medium. Such particles may impede the detection of oocysts. Furthermore, oocyst density may vary according to viability status and if the oocysts bind to heavier particles they will not be selected (9). Whilst flotation is inexpensive for materials, the increased time required during detection due to the occluding debris increases the cost.

There were also considerable differences in the time required for the two procedures and the difficulty of identifying oocysts under the epifluorescence microscope. For samples separated using IMS, examination of one well of a slide took approximately 15 min, and there were few or no interfering particles. For samples separated using flotation, screening of one well required 25 min. Sometimes it was necessary to screen two wells, owing to interfering background particles and to oocysts hidden under particles. Screening of these samples needed more experience than screening of samples concentrated with IMS.

It is important to note that none of the techniques established so far is specific for *C. parvum*. Currently available monoclonal antibodies for staining and separation are only genus-specific. Clearly, there is a strong need to develop antibodies specific for *C. parvum*. On the other hand, a possibility to overcome the species-specificity would be species-specific primers. The respective methods are available (22, 23), and another study is currently undertaken in our laboratories to explore this approach.

Concluding, both techniques, IMS using Dynabeads and the flotation separation, were suitable for the analysis of samples of drinking water with few suspended particles, Dynabeads IMS is significantly superior in the analysis of ground and surface water. The new isolation method for *Cryptosporidium spp.* in water samples is efficient and has been successfully applied in commercial analysis of drinking water. Further validation experiments are currently being undertaken under field conditions.

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Summary

A new method to isolate *Cryptosporidium spp.* oocysts in drinking water based on flocculation with aluminium sulphate, an immunomagnetic separation and a fluorescent staining was developed. Recovery efficiency of this method was assessed

and compared to a common method using aluminium sulphate flocculation, sucrose flotation for separation and a fluorescent staining. Experiments were conducted in 20 litres drinking water that has been spiked previously with 80 *C. parvum* oocysts. When immunomagnetic separation technique was used a recovery rate of 61 % was achieved, which was significantly greater ($p < 0.05$) than when the flotation technique was applied (oocyst recovery efficiency 51 %).

Samples purified with the immunomagnetic separation were considerably cleaner than samples prepared by the flotation technique, which improved detection accuracy and reduced operator time. The method suggests a reproducible approach for analysing *Cryptosporidium spp.* oocysts in drinking water which can be undertaken in routine laboratories with microbiological expertise.

Zusammenfassung

Eine neue Methode zur Isolierung von *Cryptosporidium spp.* Oozysten aus Trinkwasser wurde entwickelt. Die Methode basiert auf einer Aluminiumsulfatflockung, einer immunmagnetischen Separation und einer Fluoreszenzfärbung. Die Wiederfindungsraten wurden mit jenen einer Standardmethode (Aluminiumsulfatflockung, Flotation und Fluoreszenzfärbung) verglichen. Die Experimente wurden mit 20 Liter Trinkwasser, inokuliert mit 80 *C. parvum* Oozysten, durchgeführt. Die Wiederfindungsrate der immunmagnetischen Separation lag bei 61 % (Median), was signifikant höher ($p < 0.05$) ist als jene der Flotation (51 %, Median).

Die mit der immunmagnetischen Separation gereinigten Proben waren deutlich sauberer als die mit der Flotationstechnik gereinigten Proben, was zu einer Erhöhung der Genauigkeit und zu einer Reduktion des Zeitaufwandes der Oozystenidentifkation führte. Die neue Methode zur Isolierung von *Cryptosporidium spp.* Oozysten aus Trinkwasser, erlaubt einen einfachen Nachweis, liefert reproduzierbare Resultate und bildet die Grundlage einer Routinemethode, die in Mikrobiologie-Labors mit Standardausrüstungen durchgeführt werden kann.

Résumé

Une nouvelle méthode permettant de détecter des oocystes de cryptosporidies spp. dans de l'eau potable a été mise au point. Cette technique est basée sur la floculation par le sulfate d'aluminium, puis une séparation immuno-magnétique, suivie d'une coloration à la fluorescence. Cette méthode fut comparée à un procédé de mise en évidence standard, alliant la floculation par le sulfate d'aluminium, une flottation et une coloration fluorescente. Les expériences ont été réalisées avec 20 litres d'eau potable, auxquelles furent ajoutés 80 oocystes de *C. parvum*. Avec un taux de recouvrement de 51 % (valeur médiane) pour la technique de flottation, la séparation immuno-magnétique se révéla significativement plus efficace ($p < 0.05$) avec une valeur médiane du taux de recouvrement de 61 %.

Opposés aux échantillons séparés par flottation, la pureté supérieure des échantillons obtenus par la méthode immuno-magnétique, apporte un accroissement de

précision, et un gain de temps pour l'identification des oocystes. Cette nouvelle méthode de séparation des oocystes de l'eau potable permet une mise en évidence aisée, fournit des résultats reproductibles, et constitue la base d'une méthode de routine pour un laboratoire de microbiologie, possédant un équipement standard.

Key words

Cryptosporidium spp., Oocyst, Flocculation, Immunomagnetic separation, Drinking water

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