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Viability staining of *Cryptosporidium* oocysts and *Giardia* cysts combined with flow cytometry

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Abstract

The incorporation of flow cytometry as an additional purification step has improved the detection method for *Cryptosporidium* oocysts and *Giardia* cysts in water. Flow cytometry allows separation of (oo)cysts from interfering debris particles present in water concentrates and thus enables the application of fluorogenic vital dyes such as propidium iodide (PI) to assess (oo)cyst viability. Staining with DAPI (4',6-diamidine-2-phenylindole-dihydrochloride) and PI, followed by cell sorting with the Becton Dickinson FACSort flow cytometer, resulted in microscopic preparations in which the readability of vital staining was excellent. Sorting had no effect on oocyst viability and both PI positive and negative oocysts were sorted equally efficiently. Sample processing steps (centrifugation, sonification, flotation, washing) did not affect oocyst viability. Clarification of membrane filters, on to which oocyst were sorted by dehydration, in order to enable the use of DIC microscopy, had a strong negative effect on oocyst viability. All sample processing steps caused a considerable decrease in *Giardia* cyst viability, indicating that the current detection method can only detect the presence of *Giardia* cysts; it cannot give information on their viability. Extension of the detection method with the DAPI/PI stain provides information on *Cryptosporidium* oocyst viability. The information is limited, since DIC microscopy cannot be used to examine whether DAPI and PI negative oocysts have contents and should be considered viable or dead. Due to the limitation of vital staining assays, other methods to assess oocyst viability are still needed. Cell culture techniques may prove valuable tools in the near future.

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Samenvatting

Flow cytometrie als extra zuiveringsstap heeft de detectiemethode voor *Cryptosporidium* oöcysten en *Giardia* cysten in water verbeterd. Door middel van flow cytometrie kunnen (oö)cysten gescheiden worden van het meeste debris dat in waterconcentraten aanwezig is. Door verwijdering van storende debris deeltjes, kan flow cytometrie de toepassing van fluorogene vitale kleurstoffen om de levensvatbaarheid van (oö)cysten vast te stellen mogelijk maken. Vaststellen van levensvatbaarheid is van groot belang, omdat alleen levende (oö)cysten invloed op de volksgezondheid kunnen hebben.
Een protocol voor levensvatbaarheidskleuring van *Cryptosporidium* oöcysten met DAPI (4',6-dimidine-2-fenylindol-dihydrochloride) en propidiumjodide (PI) en van *Giardia* cysten met PI werd ontwikkeld. (Oö)cysten met kapotte membranen kleuren rood met PI en worden als dood beschouwd. DAPI accentueert de interne structuren binnen een (oö)cyste. DIC (Differential Interference Contrast) microscopie kan gebruikt worden om informatie te verkrijgen over de inhoud van oöcysten die zowel DAPI als PI buiten de cel houden. De membraanfilters waarop de oöcysten gesorteerd worden, storen echter het gebruik van DIC. Een protocol dat opheldering van membraanfilters door dehydratie met alcohol en glycerol beschrijft, waardoor toepassing van DIC mogelijk zou worden, werd getest. Tevens werd onderzocht of de DAPI/PI kleuring en dehydratie van membraanfilters te combineren waren met flow cytometrie. Daarnaast werd het effect van verschillende monster opwerk stappen (centrifugeren, sonificeren, floteren op Percoll-sucrose, wassen, flow cytometrie en dehydratie van membraanfilters) op de levensvatbaarheid van (oö)cysten bestudeerd.
Kleuren met DAPI/PI gevolgd door flow cytometrie resulteerde in microscopische preparaten waarin de levensvatbaarheidskleuring uitstekend af te lezen was. De Becton Dickinson FACSort flow cytometer sorteerde PI positieve en PI negatieve oöcysten evengoed en sorteren had geen effect op de levensvatbaarheid van de oöcysten. Ook centrifugeren, sonificeren, floteren en wassen hadden geen effect, maar dehydratie had een negatief effect op de levensvatbaarheid. Oöcysten op gedehydreerde membraanfilters waren vaak vervormd. In oöcysten suspensies was het gemiddelde verlies aan levensvatbaarheid na dehydratie 34 %. Alle monster opwerk stappen veroorzaakten een aanzienlijk verlies in levensvatbaarheid bij *Giardia* cysten.
Deze resultaten geven aan dat de DAPI/PI kleuring informatie geeft over de levensvatbaarheid van *Cryptosporidium* oöcysten, maar dat deze onvolledig is omdat DIC microscopie niet toegepast kan worden. Er blijkt tevens dat de hier gebruikte detectie methode slechts de aanwezigheid van *Giardia* cysten in monsters water aantoont en geen informatie geeft over de levensvatbaarheid van de gedetecteerde cysten omdat die door de methode beïnvloed wordt. Andere methoden, zoals bijvoorbeeld celkweek technieken die de infectiviteit van oöcysten bepalen, zijn waarschijnlijk, eventueel in combinatie met flow cytometrie, meer geschikt om vast te stellen of (oo)cysten uit milieumonsters een risico vormen voor de volksgezondheid .

Summary

The incorporation of flow cytometry as an additional purification step has improved the detection method for *Cryptosporidium* oocysts and *Giardia* cysts in water. By flow cytometry (oo)cysts can be separated from most of the debris that is present in water concentrates. Through removal of interfering debris particles, flow cytometry could enable the application of fluorogenic vital dyes to assess (oo)cyst viability.Viability of (oo)cysts is of particular interest, since only viable (oo)cysts may have impact on public health.
We developed a protocol for viability staining of *Cryptosporidium* oocysts with DAPI (4',6-diamidine-2-phenylindole-dihydrochloride) and propidium iodide (PI) and of *Giardia* cysts with PI. (Oo)cysts with broken membranes stain red with PI and are considered dead. DAPI highlights the internal structures of (oo)cysts. DIC (Differential Interference Contrast) microscopy can be of use in providing information on the contents of oocysts that exclude both DAPI and PI. The membrane filters onto which the oocysts are sorted, however, hamper the use of DIC. A protocol which describes the clarification of membrane filters by dehydration with ethanol and glycerol, which could enable the use of DIC, was tested. The compatibility of the DAPI/PI staining and clarification protocol with flow cytometry was studied. We also studied the effect of various sample processing steps (centrifugation, sonification, Percoll-sucrose flotation, washing, cell sorting by flow cytometry and dehydration of membrane filters) on (oo)cyst viability.
Staining with DAPI and PI followed by cell sorting with the Becton Dickinson FACSort flow cytometer resulted in microscopic preparations in which the readability of vital staining was excellent. The FACSort sorted PI positive and PI negative *Cryptosporidium* oocysts equally efficient and sorting had no effect on oocyst viability. Centrifugation, sonification, flotation and washing did not affect oocyst viability, but dehydration of membrane filters had a negative effect on oocyst viability and morphology. Oocysts on dehydrated membrane filters were often deformed, and in oocyst suspensions the average viability loss was 34 %. All sample processing steps caused a considerable decrease in *Giardia* cyst viability.
These results imply that the DAPI/ PI stain provides information on *Cryptosporidium* oocyst viability, though the information is limited since DIC cannot be used. The results also indicate that the detection method we used merely detects the presence of *Giardia* cysts in water samples, but does not provide information on their viability. Other methods, such as cell culture techniques which determine infectivity of oocysts, may prove, possibly in combination with flow cytometry, more suited to determine whether environmental oocysts are a risk for public health.

1. Introduction

The current detection technique for *Cryptosporidium* oocysts and *Giardia* cysts in water, consisting of filtration of large volumes of water, elution of the captured particles, concentration and purification by centrifugation and flotation, staining with fluorescein isothiocyanate (FITC) labelled monoclonal antibodies (MAb) directed towards the (oo)cysts wall and detection by epifluorescence microscopy, has been substantially improved by the incorporation of flow cytometry as an extra purification step prior to microscopy (Vesey et al, 1993; Vesey et al, 1994; Schets et al, 1995; Medema et al, 1998).

Water concentrates contain huge numbers of debris particles which interfere with fluorescence microscopy, making it extremely tedious and inaccurate. (Oo)cysts may be obscured and weakly fluorescing (oo)cysts may go undetected because of background fluorescence caused by the debris. With the aid of fluorescence activated cell sorting (FACS), (oo)cysts can be separated from most of the debris. Preparations for microscopic confirmation thus obtained contain far less interfering particles and are much easier to examine. Samples analysed with a flow cytometer are more often found positive and higher numbers (oo)cysts are detected (Schets et al, 1995; Medema et al, 1998). With flow cytometry, larger sample volumes can be analysed.

Viability of (oo)cysts is of particular interest, since only viable (oo)cysts may have impact on public health and low numbers of oocysts already give a significant probability of infection. Methods to determine (oo)cyst viability, like in vitro excystation, cell culture, animal infectivity and staining with vital dyes, are hardly or not applicable on concentrated water samples directly because of large amounts of debris particles and low (oo)cyst concentrations. In spite of the improvement by the use of flow cytometry, water concentrates purified by flow cytometry still contain to few (oo)cysts and to much debris: application of animal infectivity models or in vitro excystation assays remains impractical. The application of vital dyes in combination with flow cytometry, on the contrary, seems promising.

DAPI (4',6-diamidine-2-phenylindole-dihydrochloride) is an AT-selective DNA stain which is used to visualize nuclear material. In combination with monoclonal antibody staining it can aid in the recognition of *Cryptosporidium* oocysts. DAPI is not a vital stain, but it highlights the presence of internal structures: the sporozoite nuclei fluoresce sky-blue under UV light. Some morphological intact oocysts have walls that are impermeable to DAPI; a pre-incubation treatment (best results with acidified Hanks' Balanced Salt Solution for 1 h at 37 °C (Robertson et al, 1993)) which alters the permeability of the oocyst wall and allows DAPI to enter the oocyst, decreases the percentage of morphological intact, DAPI negative oocysts

(Campbell et al, 1992). In *Giardia* cysts DAPI highlights the nuclei which fluoresce sky-blue under UV light (Thiriat et al, 1998).

Cells with disrupted or broken membranes are the only ones reported to stain with propidium iodide (PI) (Campbell et al, 1992). *Cryptosporidium* oocysts with broken membranes that stain with PI and fluoresce from bright red solely in the sporozoite nuclei to diffuse red within the oocyst , are considered dead. PI stained *Giardia* cysts can fluoresce bright red throughout the complete cyst with more intense fluorescing nuclei, or fluorescence may be restricted to the nuclei; PI stained cysts are considered dead.

Comparison of a *Cryptosporidium* viability assay, combining the inclusion of DAPI and the exclusion of PI, with a 4-hour in vitro excystation assay, resulted in an observed correlation of 99.7 % for oocysts from various sources (Campbell et al, 1992). Pre-incubation treatment with acidified Hanks' Balances Salt Solution resulted in increased excystation and an increase in the proportion of oocysts that included DAPI, but the proportion of oocysts that stained with PI did not change (Robertson et al, 1993), indicating that oocyst viability was not affected.

Staining with DAPI and PI provides information on the viability of *Cryptosporidium* oocysts that include DAPI and exclude PI : they are considered viable. When PI is included oocysts are considered dead. However, when both dyes are excluded, the oocysts may be empty, and considered dead, or have contents and are potentially infectious. The latter may become DAPI positive upon biochemical triggers (Robertson et al, 1993), but not all oocysts with contents do. By means of Differential Interference Contrast (DIC) microscopy further information on the contents of these oocysts can be acquired. For *Giardia* DIC microscopy can also be used as a tool in assessing cyst viability (Schupp and Erlandsen, 1987b). The ultra structure of polycarbonate membrane filters onto which (oo)cysts are sorted by flow cytometry, however, strongly interferes with DIC microscopy making it unfit for use. The use of cellulose acetate filters and clarification of these filters by dehydration is reported to enable the use of DIC (LeChevallier et al, 1995).

Fluorescein di-acetate (FDA) can also be used for viability staining of *Giardia* cysts: it enters the cysts by diffusion and in metabolic active cysts it is catalysed by non-specific esterases resulting in the release of free fluorescein, which accumulates in living cells. Injured cells with disrupted membranes cannot accumulate the fluorescein. FDA positive cysts fluoresce intensely green (Schupp and Erlandsen, 1987a). When the FDA/PI double staining was compared to in vitro excystation of *Giardia* cysts, it was shown that FDA positive cysts did not always excyst and the number of FDA positives thus overestimated cyst viability. Some FDA negative and PI negative cysts that did not excyst, were FDA positive upon repeated staining and may have contained living sporozoites which were unable to initiate excystation

under in vitro conditions (Smith and Smith, 1989). PI positive cysts never excysted (Smith and Smith, 1989) and were never able to cause infections in a neonatal mouse assay (Schupp and Erlandsen, 1987a).

This report describes the introduction and the development of laboratory protocols for viability staining and DIC microscopy of *Cryptosporidium* oocysts and *Giardia* cysts. The effect of various steps in sample processing on (oo)cyst viability was studied and the viability of naturally occurring (oo)cysts in environmental samples was determined.

2. Materials and methods

2.1 Sources of *Cryptosporidium* oocysts and *Giardia* cysts

Purified oocysts of a cervine/ovine *Cryptosporidium parvum* strain were obtained from Moredun Animal Health Ltd, Edinburgh, Scotland. Purified *Cryptosporidium parvum* oocysts from infected calves were kindly provided by Dr. J. Peeters (National Institute of Veterinary Research, Brussels, Belgium). Purified *Giardia lamblia* cysts of a human isolate maintained in gerbils, were obtained from PRL Dynagenics, Phoenix, USA.

2.2 Sampling and sample processing

Samples of river water (river Meuse, 200 l) and samples of reservoir water (2000 l), were collected by using a centrifugal pump and wound polypropylene cartridge filters (Schets et al, 1995). After sample collection, filters and housings were placed on melting ice and transported to the laboratory where they were stored at 2-8 °C. Filters were processed within 24 to 72 h according to the method described by LeChevallier et al (1991) with minor alterations (Schets et al, 1995). Sample processing included centrifugation, sonification, flotation on Percoll-sucrose and washing. The obtained concentrates were stained with vital dyes and monoclonal antibodies within 48 h.

2.3 Viability staining

2.3.1 DAPI and PI staining of *Cryptosporidium* and *Giardia*

The incorporation of the viability staining method as described by Campbell et al (1992), in our laboratory resulted in the procedure described below. The application of this procedure on *Cryptosporidium* suspensions gave reproducable results and when used in survival studies the DAPI/PI stain correlated well with *in vitro* excystation (Medema et al, 1997).

Concentrates were siphoned down to approximately 200 µl and pellets were gently resuspended; to pre-treat the oocysts wall before staining, 800 µl acidified Hanks' Balanced Salt Solution (aHBSS: 200 µl 1 M HCl added to 20 ml Hanks' Balanced Salt Solution (HBSS, GibcoBRL no. 24020-091, Life Technologies), pH 2.75) was added; incubation was for 1 hour at 37 °C. The concentrates were then washed twice with 900 µl HBSS and

siphoned down to 100 µl. Pellets were gently resuspended and 10 µl DAPI (Boehringer no. 236 276, 2 mg/ml in methanol) and 10 µl PI (Sigma no. 25535-16-4, 1 mg/ml in 0.01 M phosphate buffered saline (PBS), pH 7.2) were added. Concentrates were gently vortexed (about 3 sec.) and incubated at 37 °C in the dark for 2 hours. 50 µl of each FITC conjugated monoclonal antibody specific for *Cryptosporidium* (Detect IF cryptosporidium, Shield Diagnostics, Dundee, UK) or *Giardia* (Giardia-Cel IF test, Cellabs Diagnostics, Brookvale, Australia) was added in the last 30-45 min of the incubation period. After staining the concentrates were washed twice with 900 µl HBSS. Supernatants were aspirated to 100 µl. Pellets were gently resuspended. Stained concentrates were filtered through 35 µm mesh filters to remove sand and other large particles. Staining vials were washed twice with 100 µl HBSS, the washing fluid was also filtered through the 35 µm mesh filters. Finally about 250-300 µl stained concentrate was ready for flow cytometric analysis.

2.3.2 FDA and PI staining of *Giardia* cysts

To 50 µl of a cyst suspension 25 µl FDA (Sigma no. F-7378, user strength: 4 µl stock solution (10 mg/ml in acetone) in 1 ml PBS, freshly prepared) and 5 µl PI (1 mg/ml in 0.01 M PBS, pH 7.2) were added. Suspensions were gently vortexed (about 3 sec.) and incubated. Finally, 10 µl wet mounts were made and examined by epifluorescence microscopy.

We varied incubation temperature (37 °C and room temperature) and time (10 min and 2 h) to optimize the procedure, studied the applicability of simultaneously staining with a monoclonal antibody and calculated the correlation between FDA-PI double staining and DIC microscopy in predicting *Giardia* cyst viability.

2.4 Flow cytometry

Flow cytometric analysis was performed on a Becton Dickinson FACSort flow cytometer. The FACSort is equipped with a 15 mW Argon-ion laser operating at 488 nm and it uses an aerosol-free sorting principle with a catcher tube which mechanically captures particles from the flowstream. Samples were analysed at a flow rate of 60 µl/min and the instrument was put in the "recovery" sort mode. Signals used for sorting were forward scatter (FSC) and FITC fluorescence. Instrument specifications and instrument settings are described in more detail in Schets et al (1995) and Medema et al (1998).

Particles were sorted directly onto 1.2 µm pore size, 25 mm diameter polycarbonate membrane filters (Millipore RTTP). To support the polycarbonate filter during the sort, a

5 µm pore size cellulose acetate support filter (Millipore SMWP) was used. Polycarbonate filters were placed on glass slides, embedded in a drop of DABCO (1,4-diazabicyclo[2.2.2] octan, Aldrich-Sigma no. D2780-2)-glycerol mounting medium (2.0 g DABCO, 40 ml PBS, 60 ml glycerol) and covered with a coverslip. Slides were stored at 2-8 °C and examined within 2 weeks by epifluorescence microscopy.

2.5 Membrane filter processing for DIC microscopy

Samples were processed for FACSort analysis and analysed as described above. Particles were, however, not sorted onto polycarbonate filters, but onto 0.2 µm pore size, 25 mm diameter cellulose acetate membrane filters (Sartorius no. 11107); a 0.45 µm pore size polyvinyl filter (Millipore HVLP) was used as a support filter. After sorting the cellulose acetate top filter and the polyvinyl support filter were transferred to a membrane filter holder as a pair. Successively, 1 ml volumes of ethanol/glycerol mixtures (containing 5% glycerol and 10-20-40-80 or 90.2 % ethanol respectively) were filtered through the transferred membrane filter pair to dehydrate the cellulose acetate filter. After dehydration the cellulose acetate filter was transferred to a glass slide with 40 µl DABCO-glycerol medium (2.0 g DABCO in 100 ml pre-heated (37 °C) glycerol, pH 10.0). Accurate care was taken to moisten the entire bottom of the membrane filter. The membrane filter was embedded in an additional 50 µl DABCO-glycerol medium, covered with a coverslip and sealed with colorless nailpolish. Slides were incubated in the dark for 30 min at room temperature to completely clarify the membrane filters. After clarification, slides were stored at 2-8 °C and examined by epifluorescence and DIC microscopy as described in section 2.6 within 2 weeks.

2.6 Microscopy

Preparations were examined by using a Zeiss Axioskop epifluorescence microscope fitted with a x 25 and a x 100 Plan neofluar oil immersion objective. Preparations were screened at x 250 magnification for the presence of *Cryptosporidium* oocysts (spherical shapes with apple green fluorescence of the oocyst wall and a diameter of 3-7 µm) and *Giardia* cysts (oval to spherical shapes with apple green fluorescence of the cyst wall and a size of 5-15 x 8-18 µm). A band pass 450-490 nm blue exciter filter (filter set 09), was used to visualize FITC fluorescence.

Staining with DAPI, PI or FDA, was examined at x 1000 magnification in the same microscope field by using a 365 nm UV-G exciter filter (filter set 02) for DAPI, a 546 nm green exciter filter (filter set 15) for PI and filter set 09 for FDA. DAPI positive (oo)cysts

contained blue fluorescing nuclei, PI positive (oo)cysts fluoresced deep red and FDA positive *Giardia* cysts fluoresced intensely green.
To study internal structures, (oo)cysts were examined at x 1000 magnification with Zeiss DIC optics. DIC positive *Cryptosporidium* oocysts were defined as having contents. Mostly, four banana-shaped sporozoites were visible. DIC negative oocysts contained either a residual body or were empty oocyst walls. *Giardia* cysts were judged according to the criteria described by Schupp and Erlandsen (1987b). They were considerd DIC positive when they had a clearly delineated cyst wall, a clearly visible space between cyst wall and cytoplasm (peritrophic space) and a hyaline appearence of the cytoplasm. Intracellular details should be difficult to discriminate, accept from the axonemes. DIC negative cysts contained easily recognizable cytoplasmic organelles (2-4 nuclei, intracytoplasmic flagellar axonemes) and the cytoplasm had a fine granular appearance. Occasionally an empty cyst wall was seen, which was also recorded as DIC negative.

2.7 Effect of sample processing steps on (oo)cyst viability

Seeding experiments with *Giardia* cyst and *Cryptosporidium* oocyst suspensions with known DAPI/PI staining and DIC characteristics were performed on various sample processings steps to determine if these processing steps had an effect on (oo)cyst viability. Fractions of the samples were seeded with (oo)cyst concentrations high enough to recover at least 1000 (oo)cysts after the processing step, thus enabling omission of membrane filtration when desired.

Secundary effluent was processed by centrifugation, sonification, Percoll-sucrose flotation and washing with PBS with Tween80 and SDS. In one set of experiments, samples were seeded prior to centrifugation and after each processing step a fraction was taken in which (oo)cyst viability was determined. We were thus able to study the cumulative effect of the sample processing steps. In another set of experiments, fractions of samples were seeded just before undergoing the various steps. (Oo)cyst viability was determined directly after performing the step. In the first set of experiments (oo)cyst viability was determined on clarified membrane filters, in the last it was determined in 10 µl wet mounts.

2.8 Effect of FACSort on (oo)cyst viability

With FACSort (oo)cysts are sorted on basis of their size and fluorescence characteristics (the latter applied by staining with specific MAb's). To determine whether FACSort sorts PI positive and PI negative (oo)cysts equally, (oo)cyst suspensions were stained with MAb and

PI and sorted as described above. The ratio PI+/PI- (oo)cysts was determined by epifluorescence microscopy before and after sorting.

To study if (oo)cyst viability was affected by sorting with FACSort, MAb stained (oo)cysts were either DAPI/ PI stained before sorting, or DAPI/PI stained after they were sorted. (Oo)cysts were respectively sorted onto or polycarbonate membrane filters or filtered through polycarbonate membrane filters after DAPI/PI staining. In both situations, the ratio viable/non-viable (oo)cysts was determined by epifluorescence microscopy.

Vesey et al (1997) were able to differentiate intact, partially excysted and excysted (empty) *Cryptosporidium* oocysts on basis of differences in light-scatter properties. On a FSC - SSC (side scatter) dot plot, gated by a region defined in a FITC fluorescence- SSC dot plot, they could clearly distinguish three separate populations.

We subjected *Cryptosporidium* oocyst suspensions to the in vitro excystation protocol described by Robertson et al (1993). Excysted oocyst suspensions were MAb stained and analysed with FACSort using the instrument setting as described in section 2.4. In the FITC-fluorescense-FSC dot plot a region R was defined that enclosed the stained oocysts. A FSC - SSC dot plot, gated by R, was generated. Dot plots of oocyst suspensions that were subjected to the excystation protocol were compared to those of oocyst suspensions that were not subjected to the excystation protocol.

3. Results and discussion

3.1 Viability staining

3.1.1 DAPI and PI staining of *Cryptosporidium* and *Giardia*

Cell sorting with FACSort made staining of *Cryptosporidium* oocysts with DAPI and PI, in addition to staining with MAb, possible. Oocysts from suspensions or from water concentrates were stained before sorting. Sorting removed most of the debris particles that disturbed reading of the DAPI/PI stain in unsorted samples. On the relatively clean membrane filters, the oocysts could be easily detected and with the appropriate microscope filters the DAPI/PI stain was clearly visible.

DAPI staining of *Giardia* cysts gave variable results especially in PI negative cysts: in some cysts the nuclei clearly stained blue under UV light, in others DAPI did not enter the cyst and stained the cyst wall. In most PI positive cysts the cytoplasm was shrunken and fluoresced pale blue under uv light, while other PI positive cysts did not stain with DAPI at all. Pre-incubation treatment in acidified HBSS had no effect.

Staining of (oo)cysts with DAPI and PI on membrane filters after cell sorting gave poor results. DAPI formed large yellow fluorescing crystals which hampered the reading of the DAPI/PI stain in the (oo)cysts.

3.1.2 FDA and PI staining of *Giardia* cyst suspensions

Simultaneous staining with FDA/PI and MAb (for 10 min or for 2 h) resulted in good readability of MAb in FDA negative cysts, but in FDA positive cysts MAb fluorescence was completely masked by FDA fluorescence. Staining at 37 °C (for 10 min or 2 h) caused a strong decrease in cyst viability compared to incubation at room temperature: eg. a 2 hour incubation at room temperature resulted in the detection of 54 % PI positive cysts, whereas a 2 hour incubation at 37 °C gave 96 % PI positive cysts. Viability sightly increased when incubation time was elongated from 10 min to 2 hours. Incubation at room temperature for 2 hours was therefore included in the protocol.

It was observed that cyst viability had to be examined immediately after ending incubation with the vital dyes, since leaving the reaction vessels at room temperature for longer than 10 minutes or placing them in a refrigerator caused a decrease in cyst viability and a change in cyst morphology.

Cyst viability was predicted by both FDA/PI double staining and DIC microscopy. When comparing the results, a correlation of 99.0 % for FDA and DIC and a correlation of 97.8 % for PI and DIC was observed. Correlation for FDA and PI was 98.4 %. From these observations it was concluded that staining with FDA did not give more information on *Giardia* cyst viability than PI staining and DIC microscopy did. Combining this with the disturbing effect of FDA fluorescence on the recognition of MAb stained cysts, we decided to rely on PI and DIC for predicting cyst viability in natural samples.

3.2 Effect of sample processing steps on (oo)cyst viability

Figure 1 shows that successive processing steps had no obvious cumulative effect on (oo)cyst viability. Compared to the seeding suspensions, however, the percentage dead (oo)cysts increased. (Oo)cyst viability in the seeding suspensions was recorded in wet mounts directly after DAPI/PI staining. Seeded fractions, on the contrary, were membrane filtered after DAPI/PI staining and (oo)cyst viability was determined after clarification of the membrane filters.

It was observed that *Cryptosporidium* oocysts on dehydrated membrane filters were more ofter deformed showing unnatural folding and a raisin-like appearance, as can be seen on the photographs on page 18. Dehydration of membrane filters was therefore suspected of having a negative effect on oocysts. This was confirmed in separate experiments with (oo)cyst suspensions which showed (Table 1) that the viability of (oo)cysts decreased after clarification of membrane filters. The morphology of *Giardia* cysts as seen by immunofluorescence was not affected by dehydration. Due to the strong effect of dehydration, the possible effect of other sample processing steps was masked.

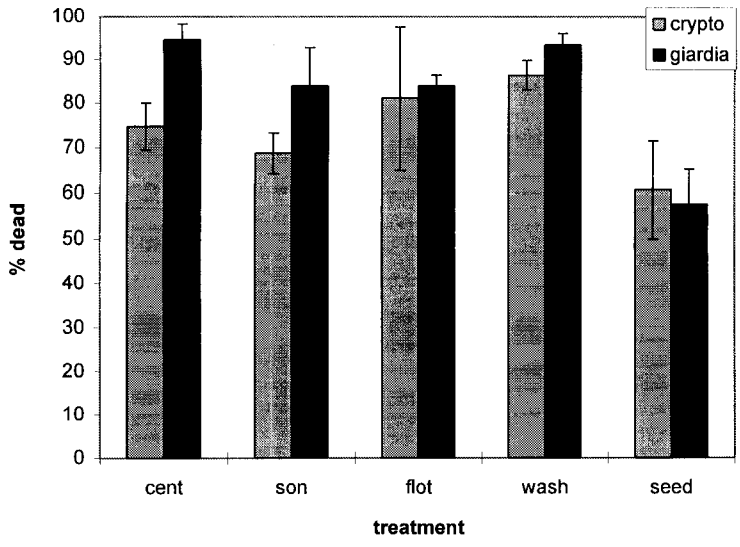


Figure 1: Cumulative effect of centrifugation (cent), sonification (son), percoll-sucrose flotation (flot) and washing with PBS with SDS and Tween80 (wash) on the viability of *Cryptosporidium* oocysts and *Giardia* cysts.

Table 1: Viability of *Cryptosporidium* oocyst and *Giardia* cyst suspensions in wet mounts and on dehydrated membrane filters (mf). Viability was determined by propidium iodide exclusion and DIC microscopy.

isolate	% viable in wet mount	% viable on dehydrated mf
Cryptosporidium		
Belgium (crypt 633)	80	58
Moredun (crypt 799)	55	7
Moredun (crypt 799)	37	5
Giardia		
PRL (crypt 623/98)	72	4
PRL (crypt 623/98)	32	0

Figure 2 shows that individual sample processing steps have very little effect on *Cryptosporidium* oocyst viability. In these experiments viability of both seeding suspensions and seeded fractions was determined in wet mounts, directly after DAPI/PI staining. The viability of *Giardia* cysts decreased after each step, with the effect of sonification being the largest.

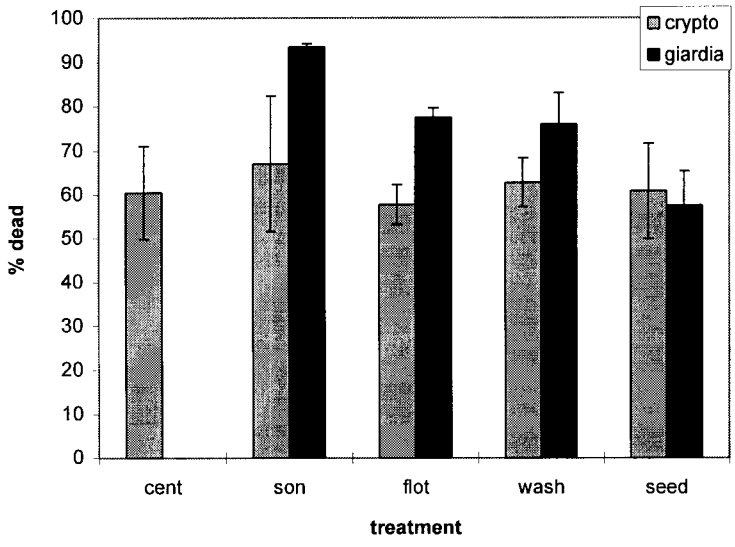
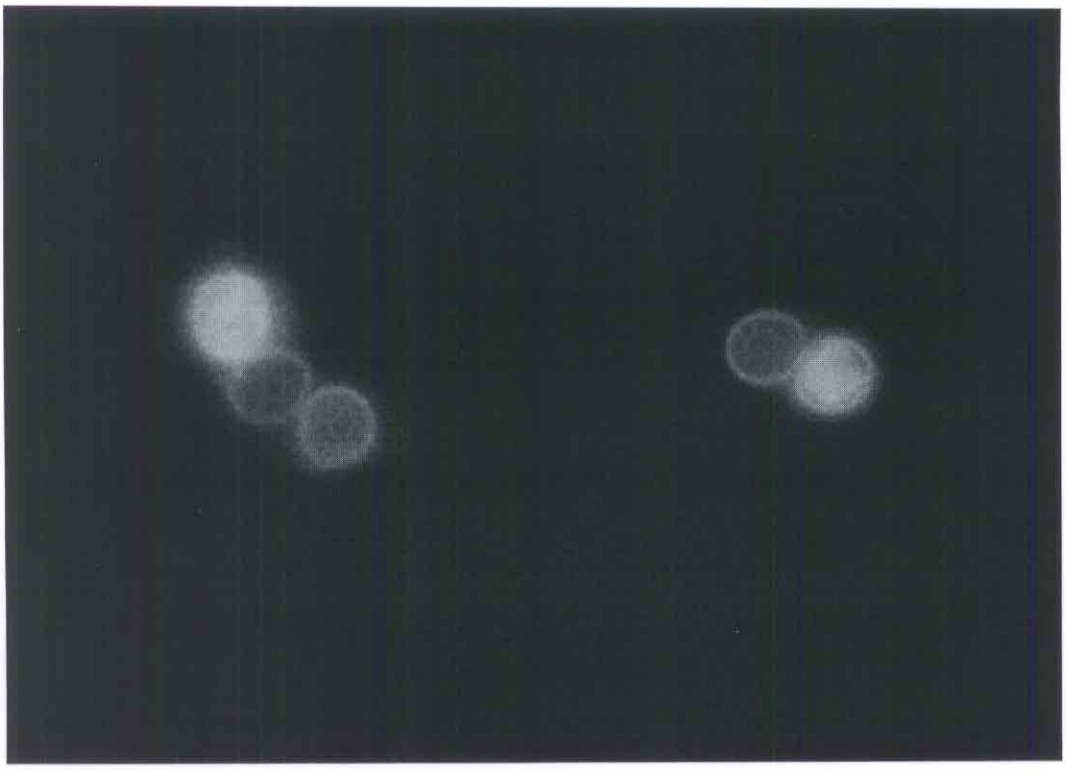


Figure 2: Independent effect of centrifugation (cent), sonification (son), percoll-sucrose flotation (flot) and washing with PBS with SDS and Tween80 (wash) on the viability of *Cryptosporidium* oocysts and *Giardia* cysts.

These results indicate that a detection method which includes centrifugation, sonification, flotation or washing may affect the ability of *Giardia* cysts to exclude PI. Sample processing steps influence cyst viability determined by PI exclusion negatively and are likely to have a negative effect on cyst viability determined by other viability assays. The effect on the *Giardia* cyst suspensions we used was obvious, other isolates may react more or less vehemently. Detection methods which alter cyst viability cannot be used to assess viability of naturally occuring *Giardia* cysts.

The detection method does not change *Cryptosporidium* oocyst viability and DAPI/PI staining can be considered as a usefull tool in determining the percentage oocysts that are dead (PI+) or viable (DAPI+PI-). However, both categories will be underestimated, since DIC microscopy cannot be used to provide information on oocysts that exclude both dyes. Dehydration of membrane filters changes oocyst viablity and internal characteristics cannot be determined directly on membrane filters.

A (1 cm = 5 μm)



B (0.5 cm = 5 μm)

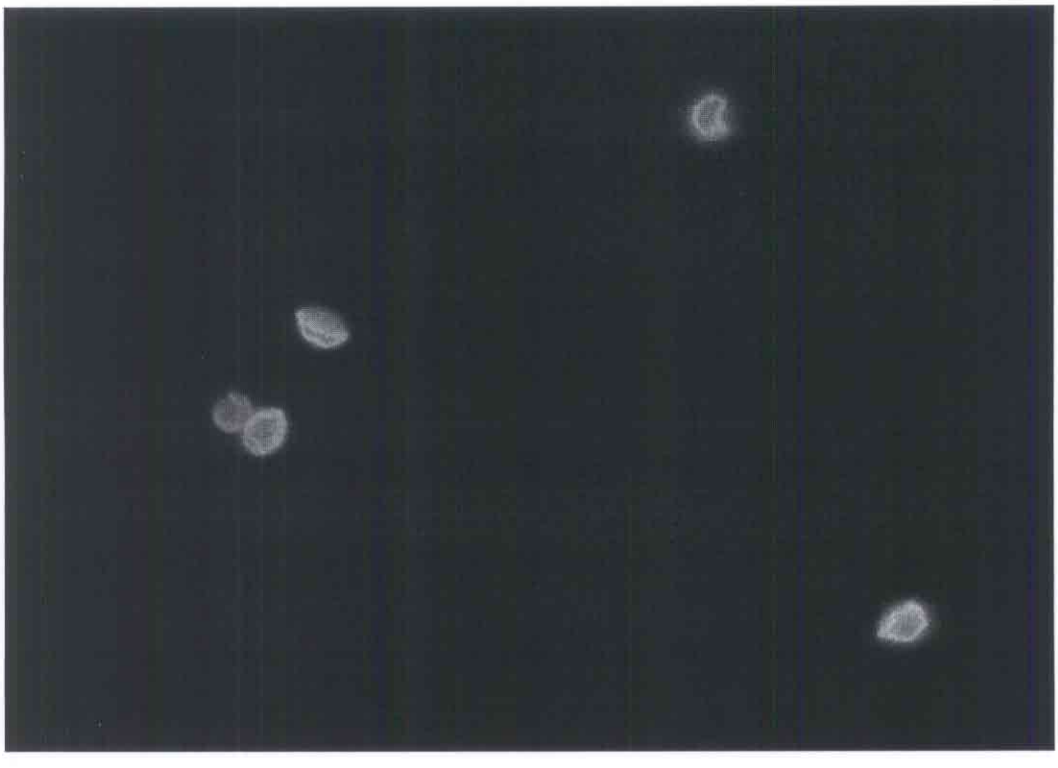


Figure 3: *Cryptosporidium* oocysts in wet mounts (A) and on dehydrated membrane filters (B).

3.3 Effect of FACSort on (oo)cyst viability

From *Cryptosporidium* oocyst suspensions stained with DAPI/PI before sorting, FACSort sorted PI positive and PI negative oocysts equally efficient: the percentage PI positive and PI negative oocysts hardly changed after sorting, as can be seen in table 2. When oocysts were stained with DAPI/PI after sorting, the percentage PI positive and PI negative oocysts was equal to that before seeding, so FACSort had no effect on oocyst vital staining characteristics.

For *Giardia* cysts, on the contrary, both sorting before and after staining with DAPI/PI resulted in a shift in the percentage PI positive and PI negative cysts: the percentage PI positive cysts increased. An increased percentage PI positive cysts after sorting after DAPI/PI staining, suggests that FACSort may select in favour of PI positive cysts. Since PI positive cysts are not differentiated from PI negative cysts by FITC fluorescence and FSC signals, this is not likely. Sorting before DAPI/PI staining, resulting in an increased percentage PI positive cysts indicates that sorting affects *Giardia* cyst viability.

Table 2: Propidium iodide (PI) positive (PI+) and PI negative (PI-) (oo)cysts in DAPI/PI stained suspensions before and after sorting with FACSort.

isolate	**without** FACSort		**with** FACSort	
	% PI+	% PI-	%PI+	%PI-
Cryptosporidium				
Belgium (crypt 633, 1997)	18	82	13	87
PRL (crypt 420)	91	9	95	5
Belgium (crypt 633, 1998)	16	84	15	85
Giardia				
PRL (crypt 623/98)	55	45	76	24

3.4 Viability of naturally occurring (oo)cysts in river and reservoir water

In 13 river water samples and 13 reservoir water samples *Cryptosporidium* oocysts and *Giardia* cysts were detected, numbers per sample are summarized in appendix 1. Viability of these (oo)cysts was assessed by staining with DAPI and PI. In the river water samples a total of 23 oocysts was detected, of these, 9 (39 %) were both DAPI and PI positive and considered dead. Of 52 % of the detected oocysts no statement on their viability could be made since they were both DAPI and PI negative. In reservoir water a total of 25 oocysts was detected, 19 (76 %) were dead (DAPI+PI+) and the other 6 (24 %) were considered viable, since they were DAPI positive and PI negative. Due to the high percentage oocysts of which the viablity could not be determined, in river water samples, viability results before and after reservoir storage are difficult to compare thus making statements on oocyst inactivation during reservoir storage based on this dataset impossible.

4. Conclusions

- The incorporation of FACSort in the detection method for *Cryptosporidium* and *Giardia* in water, enables the application of DAPI and PI to natural water samples. Due to removal of disturbing debris by FACSort, vital staining characteristics of individual oocysts can easily be detected.
- Staining with DAPI and PI gives limited information on oocyst viability, since DIC microscopy cannot be used to examine whether DAPI and PI negative oocysts have contents or not and should be considered viable or dead.
- Sample processing steps that are part of the current detection method affect the viability of *Giardia* cysts. As a result, with this method merely the presence of *Giardia* cysts can be determined, but no statements can be made on their viability.
- Sample processing does not affect *Cryptosporidium* oocyst viability and due to the limitation of vital staining assays, other methods to assess oocyst viability or infectivity can and need to be used. Cell culture techniques may prove valuable.

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Appendix 1: *Cryptosporidium* oocyst and *Giardia* cyst numbers in river and reservoir water samples

sample	sample code	date	sampled volume (l)	total weight pellet (g)	examined weight (g)	number Crypto	number Giardia	Crypto n/l	Giardia n/l
river	c.437	290395	200	14.48	1.10	0	112	<0.07	7.37
	c.449	050495	201	13.77	1.04	5	165	0.33	17.16
	c.455	120495	200	15.95	1.05	1	48	0.08	3.65
	c.468	190495	200	10.52	0.95	0	79	<0.06	4.37
	c.502	260495	200	18.08	1.02	0	60	<0.09	5.32
	c.484	030595	200	10.07	1.04	7	26	0.34	1.50
	c.497	090595	200	11.69	0.99	10	59	0.59	3.48
	c.504	160595	200	14.31	1.04	0	83	<0.07	5.71
	c.506	300595	205	11.17	0.99	0	134	<0.06	7.38
	c.508	130695	232	16.11	1.08	0	25	<0.06	1.61
	c.510	200695	200	14.59	1.02	2	34	0.14	2.43
	c.512	270695	200	12.03	1.04	1	49	0.06	2.83
	c.529	080895	200	9.02	1.02	0	12	<0.04	0.53
reservoir	c.436	290395	2859	22.57	1.14	0	0	<0.007	<0.007
	c.448	050495	2055	26.92	1.06	0	0	<0.012	<0.012
	c.454	120495	1894	20.15	1.08	2	5	0.020	0.049
	c.467	190495	2000	24.58	1.14	3	3	0.032	0.032
	c.501	260495	2200	22.06	1.14	1	3	0.009	0.026
	c.483	030595	2000	11.14	1.21	0	1	<0.005	0.005
	c.490	080595	2000	9.47	1.10	0	0	<0.004	<0.004
	c.503	160595	2000	10.37	1.04	19	0	0.095	<0.005
	c.505	300595	1991	8.10	0.98	0	0	<0.004	<0.004
	c.507	130695	2160	7.07	1.08	0	0	<0.003	<0.003
	c.509	200695	1952	6.82	1.04	0	1	<0.003	0.003
	c.511	270695	1921	9.84	1.09	0	0	<0.005	<0.005
	c.531	080895	2000	16.00	1.04	0	0	<0.008	<0.008

Appendix 2 Mailing list

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