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COMPARISON OF *CRYPTOSPORIDIUM*-SPECIFIC AND *GIARDIA*-SPECIFIC MONOCLONAL ANTIBODIES FOR MONITORING WATER SAMPLES

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Abstract—Routine detection of *Cryptosporidium* oocysts and *Giardia* cysts depend on immunofluorescence assays (IFA) employing fluorescently labeled monoclonal antibodies. Commercially available mAbs used for the detection of *Cryptosporidium* oocysts are of the IgM or IgG3 subclass, whilst those used for *Giardia* analysis are of IgM and IgG classes including IgG1. These mAbs suffer from non-specific binding to detrital particles present in environmental samples resulting in high levels of background fluorescence. New mAbs of the IgG1 subclass to *Giardia* and *Cryptosporidium* selected primarily for water analysis have recently become available. These antibodies exhibited lower levels of non-specific particulate binding compared with commercially available antibodies. The degree of background fluorescence observed following mAb staining of particles that were not oocysts or cysts varied between the water types analysed. © 1999 Elsevier Science Ltd. All rights reserved

Key words—*Cryptosporidium*, *Giardia*, water testing, monoclonal antibodies, flow cytometry, detection

INTRODUCTION

The protozoan parasites *Cryptosporidium* and *Giardia* are a common cause of diarrhoeal disease in humans and animals (Adam, 1991; O'Donoghue, 1995). These parasites have a low infectious dose, with as few as 30 *Cryptosporidium* oocysts being capable of causing infection (Smith and Rose, 1990; Dupont *et al.*, 1995). *Cryptosporidium* oocysts and *Giardia* cysts are environmentally robust and can survive in aquatic environments for months. Both cysts and oocysts are resistant to standard chlorination disinfection used for drinking water treatment (Robertson *et al.*, 1992; Karanis *et al.*, 1993). Many surface waters are often contaminated by oocysts from agricultural run off due to infected stock and by cysts through sewage effluent and wildlife (Madore *et al.*, 1987; Ongerth, 1989; LeChevallier *et al.*, 1991a,b). The robust nature of these protozoans has meant that *Cryptosporidium* and *Giardia* are a serious concern to the water industry.

Several waterborne outbreaks of *Cryptosporidium* and *Giardia* have now been documented, many occurring from water processed by modern water treatment plants (Badenoch, 1990), whilst others have been associated with public swimming pools (Kebabjian, 1995). Many water utilities now routinely monitor surface water for the presence of these pathogens and in many countries regulatory requirements are being introduced.

Most currently used methods depend on immunological techniques both for oocyst and cyst detection (e.g. IFA) and purification (flow cytometry and cell sorting or immunomagnetic separation (IMS) (Vesey and Slade, 1991; Campbell *et al.*, 1993; Vesey *et al.*, 1994). Monoclonal antibodies applied in these assays need to be highly specific. Problems exist as they bind to extraneous particles within water concentrates. The result is high levels of background fluorescence, which obscure microscopic evaluation, making identification of cysts and oocysts extremely difficult and time consuming (Vesey and Slade, 1991; Campbell *et al.*, 1993; Vesey *et al.*, 1993). In our laboratory we have found that non-specific binding of mAbs also makes purification using flow cytometry and IMS less efficient. Little work has been carried out on the selection of the most suitable antibodies for water analysis.

Most currently used antibodies to *Giardia* and *Cryptosporidium* have been developed for clinical detection. With *Cryptosporidium*, standard immunisation protocols produce almost exclusively mAbs of the IgM class. IgMs are multimeric mAbs of lower affinity (Roitt *et al.*, 1996), that tend to bind non-specifically to extraneous material producing high

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Table 1. *Cryptosporidium*-specific monoclonal antibodies evaluated

Antibody	Antibody type	Supplier
CRY104	IgG1 FITC conjugate	MUCAB[a]
CRY26™	IgM FITC conjugate	MUCAB[a]
CRY212	IgM FITC conjugate	MUCAB[a]
Crypto-Cel	IgM FITC conjugate	CelLabs, Australia
Crypto-a-glo™	IgM FITC conjugate	Waterborne, U.S.A.
Immucell™	IgG3 FITC conjugate	Immucell, Portland. U.S.A.
Hydrofluor™ Combo[b]	IgM Not conjugated	Meridian, ENSYS, U.S.A.

[a]Macquarie University Centre for Analytical Biotechnology.[b]The Hydrofluor™ Combo kit contains both *Cryptosporidium* and *Giardia* specific mAbs.

levels of background fluorescence compared with IgG1. While IgG1 mAbs identifying *Giardia* have been available previously, until recently no IgG1 mAbs had been available for identifying *Cryptosporidium*.

Recently a simple method has been developed to quantitatively assess the level of non-specific binding of a particular mAb in a particular water type (Vesey *et al.*, 1997a). Briefly, positive control samples of each pathogen are stained with mAbs specific to *Cryptosporidium* or *Giardia* and the level of fluorescence monitored using flow cytometry. Water concentrates free of the protozoan parasites are then similarly stained and evaluated for the presence of fluorescent extraneous material. Here we use this method to evaluate some commercially available *Cryptosporidium*-specific and *Giardia*-specific mAbs of the IgG1, IgG3 and IgM subclasses, for the level of non-specific binding produced following staining. The most suitable mAbs for water analysis were then evaluated for their specificity within different water types.

MATERIALS AND METHODS

Cryptosporidium oocysts and Giardia cysts

Cryptosporidium parvum oocysts were isolated from naturally infected calves as previously described (Vesey *et al.*, 1997a). The oocysts were heat inactivated at 65°C for 15 min. *Giardia lamblia* cysts were obtained formalin fixed from Waterborne (New Orleans, U.S.A.). Cysts and oocysts were stored in 0.01 M phosphate buffered saline (150 mM NaCl, 15 mM KH_2PO_4, 20 mM Na_2HPO_4, 27 mM KCL, pH 7.4 ± 0.2) (PBS) (Oxoid, Sydney, Australia) at a concentration of 1×10^6 oocysts ml^{-1} and stored at 4°C.

Cryptosporidium-specific and Giardia-specific antibodies

Seven *Cryptosporidium*-specific and four *Giardia*-specific mAbs were evaluated in this study. The supplier, fluorescent label and class of antibody are described in Tables 1 and 2.

Concentrated water samples

Samples (10 l) of untreated surface water were collected from sites around Australia and concentrated by flocculation (Vesey *et al.*, 1993) or filtration (Ongerth and Stibbs, 1987). A composite untreated water sample was prepared by mixing aliquots of samples from a range of different sites. The sample was centrifuged at 13,000 rpm for 10 min and the pellet resuspended in PBS. The volume of the sample was adjusted so that 100 μl of concentrate was equivalent to 5 l of original untreated water. Samples were also prepared from different water types: river, effluent, slow sand filtered and filtered backwash. All samples were pre-filtered through a 38-μm stainless steel mesh filter prior to use.

Sample preparation

Fluorebrite™ 6 μm beads (Polysciences, Warrington, PA) were used to standardise the assay. The beads were added at a concentration of 2.3×10^5 beads/ml into bovine serum albumin (BSA, Sigma Chemical Co., St Louis, U.S.A.) (4% w/v) in 0.01 M PBS and azide (0.05% v/v). The same volume of beads was then added to every sample, making it possible to monitor the volume of concentrate analysed, by the number of beads detected. The volume of water concentrate analysed for each sample was then standardised by producing a result per bead analysed. Sample buffer for staining samples consisted of BSA (1% w/v) Tween 20 (0.05% v/v) in 0.01 M PBS.

Antibody concentrations

The optimal concentration or dilution of each antibody to be evaluated (Tables 1 and 2) was firstly determined by flow cytometry. Serial dilutions for mAbs of unknown protein concentration were set-up between 1:20 and 1:1,280 in appropriate sample buffer as described by the manufacturer. Those mAbs of known protein concentrations were set-up as serial dilutions between 8 and 0.5 μg/ml. An aliquot (100 μl) of each dilution was added to seeded samples. Seeded samples consisted of 50 μl water concentrate and 50 μl of the oocyst or cyst seed. Samples were incubated at room temperature for 30 min and then analysed using flow cytometry. Data were analysed to determine which concentration of antibody produced the

Table 2. *Giardia*-specific monoclonal antibodies evaluated

Antibody	Antibody type	Supplier
G203	IgG1 FITC conjugate	MUCAB[a]
Giardia-Cel	IgM ITC conjugate	CelLabs, Australia
Giardia-a-glo™	IgG1 FITC conjugate	Waterborne, U.S.A.
Hydrofluor™ Combo[b]	IgG Not conjugated	Meridian, ENSYS, U.S.A.

[a]Macquarie University Centre for Analytical Biotechnology.[b]The Hydrofluor™ Combo kit contains both *Cryptosporidium* and *Giardia* specific mAbs.

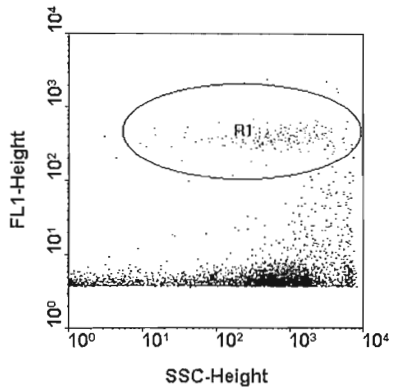

Fig. 1. Flow cytometric analysis of *Cryptosporidium* oocysts seeded in a 50 μl composite water concentrate. The fluorescent oocyst population within R1 (FL1 parameter) is clearly separated from autofluorescent and extraneous particles bound non-specifically to the antibody. The optimal concentration of mAb to use for water analysis needs to define a clear separation between stained oocysts (R1) and background fluorescence as seen in this figure. Note the side scatter parameter (SSC) spreading by the oocyst population when analysed within a water concentrate.

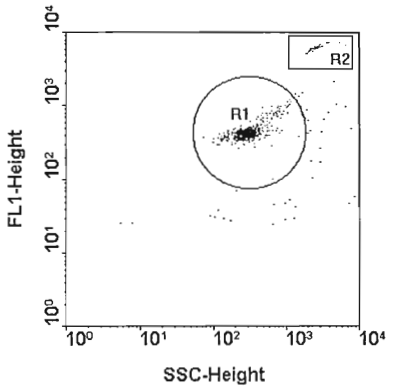

Fig. 2. Flow cytometric analysis of a positive control sample of fluorescently stained *Cryptosporidium* oocysts with Cry26. A default elliptical region (R1) is centered on the main population of pure fluorescent oocysts for the antibody. A rectangular region is defined around the bead standard population (R2). This procedure is repeated for each antibody evaluated.

greatest separation between the immunofluorescent positive control (oocyst/cysts) population and the background fluorescent particles detected within water concentrates (Fig. 1).

Staining water samples

Following optimisation of antibody concentrations, each mAb was diluted to the appropriate concentration in sample buffer. Positive controls were prepared consisting of 50 μl of the respective seed. Water samples to be tested were prepared in triplicate and consisted of 50 μl water concentrate. Each mAb was added to controls and water samples to a total volume of 150 μl in sample buffer and mixed. After incubation at room temperature for 30 min, samples were seeded with aliquots (20 μl) of the Fluorbrite℗ bead standard. Samples were then mixed by vortexing for 5 s and then analysed.

The Hydrofluor mAb kit contains unconjugated *Cryptosporidium*-specific and *Giardia*-specific antibodies as well as an anti-mouse FITC conjugated antibody. The reagents were used as recommended in the manufacturer's instructions.

Flow cytometry

Comparison of *Cryptosporidium* and *Giardia* mAbs was carried out using a Becton-Dickinson FACScan flow cytometer (Becton-Dickinson, Lane Cove, NSW, Australia). Sheath fluid consisted of undiluted Isoton II (Lab Aids Pty, Narrabeen, NSW, Australia). Detectors used were side angle light scatter (SSC) vs green fluorescence (FL1 detector). Voltages of detectors were set at 300 mV for SSC and 450 mV for detectors 1, 2 and 3.

The threshold was set at green fluorescence detector 1 (FL1). Compensation was set at FL1–FL2 45% (orange–red fluorescence detector). Positive samples containing cysts or oocysts for each mAb were analysed first. Approximately 1,000 events were collected and an ellipse region (R1) of default size was defined around the centre of the oocyst or cyst population (Fig. 2). A rectangular sort region (R2) was defined around the respective bead standard used. Once sort regions were defined, a sample of water concentrate was analysed to allow the operator to increase or decrease the discriminator until extraneous particles detected fluoresced just below R1 (Fig. 3). When all regions were defined 5,000 events were collected for every sample. It was important that R1 be moved to the centre of the oocyst or cyst population for each antibody evaluated as fluorescence varied between mAbs.

Data analysis

Data analysis was carried out using LYSIS II software obtained from Becton-Dickinson (Lane Cove, NSW, Australia). Bivariate dot-plots defining SSC vs FL1 were used for analyses (Figs 2 and 3). Regions were defined as above. To enumerate the number of beads detected, a sort rectangle (R2) was defined around the bead population. This region was determined by analysis of a control sample consisting of beads and oocysts/cysts (Fig. 2). The specificity of each mAb was calculated in terms of a non-specific binding ratio by dividing the number of events collected in R1 by R2.

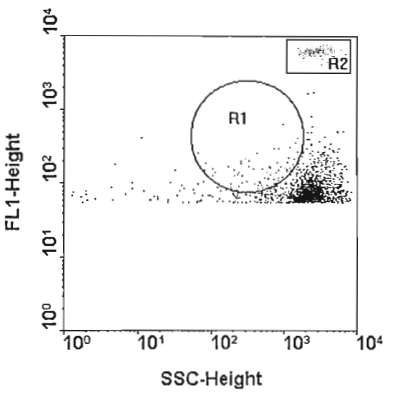

Fig. 3. Flow cytometric analysis of the composite water sample stained with Cry26, containing neither oocysts nor cysts. The regions (R1 and R2) are default from Fig. 2. The particles within the oocyst or cyst region (R1) contain both autofluorescent and non-specifically bound particles. The number of particles within R1 are divided by the number of beads analysed (R2) to produce the non-specific binding ratio.

Statistical analysis

Data interpretation was performed using Microsoft Excel® 4.0 and the Analysis Toolpack® Add-in. The hypothesis that the means from different antibodies were equal was tested using analysis of variance (ANOVA). Using a significance level of 5% ($p < 0.05$), the critical values for the *F*-statistic were calculated and compared to that obtained from the ANOVA. The hypothesis that means for different water samples by the most specific mAbs (Tables 5 and 6) were equal to the unstained samples were tested using both the Students *t*-test and by ANOVA, with significance levels of 5% ($P < 0.05$).

RESULTS

When concentrated water samples were stained with mAbs directed to either *Cryptosporidium* or *Giardia* there was a significant ($p < 0.05$) increase in the number of fluorescent particles observed with the stained, compared with unstained water samples for all mAbs, except Cry104 and G203 (Tables 3 and 4). Differences were observed between antibodies with respect to the background immunofluorescent staining, with increases in fluorescent particles detected depending on mAb type.

The IgG1 antibodies Cry104 and G203 produced no significant increase in the levels of background fluorescence detected following staining of the composite water concentrate. *Cryptosporidium*-specific antibodies of the IgM or IgG3 subclass produced significantly higher levels ($p < 0.05$) of binding to extraneous particles in water (Table 3). Staining with the *Giardia*-specific antibody G203, which was selected specifically for water analysis by screening using flow cytometry, resulted in significantly less fluorescent background particles than when staining with antibodies produced by conventional methods (Table 4).

The commercial mAbs Immucell® (*Cryptosporidium*-specific) and Hydrofluor® (*Giardia*-specific) were selected for evaluation on non-specific binding following staining different water types. They were the most specific commercially available mAbs thus a comparison with the new mAbs Cry104 and G203 was carried out (Tables 5 and 6). Cry 212 was highly specific but was not chosen for the evaluation on different water types as it is not commercially available. Evaluation of Cry104 revealed no significant increase in background fluorescence for 7 of the 10 samples examined, whilst a substantial increase was observed in all of the same samples stained with Immucell® (Table 5). A similar trend occurred with the *Giardia* mAbs, with G203 producing no significant increase in background fluorescence in 6 of the 10 water types examined compared with Hydrofluor® (Table 6). The hydrofluor mAb is an indirect antibody assay containing both *Cryptosporidium* and *Giardia* specific mAbs, the non-specific binding observed following staining was expected to be higher than single antibody assays, however the antibody was selected as one of the most specific for water analysis.

Statistically different levels ($p < 0.05$) of non-specific binding of particles to antibodies was observed for both *Giardia* and *Cryptosporidium* analysis (Tables 5 and 6). However the variability in non-specific binding following staining with antibodies selected specifically for water analysis (Cry104 and G203) was reduced compared to the other mAbs. Figure 4 shows the level of non-specific binding following immunofluorescent staining of a raw water sample (Sample 8) with 2 *Cryptosporidium*-specific mAbs. Thus, a significant difference in the non-specific binding by mAbs amongst a diverse range of water types was apparent.

Table 3. Comparison of the level of non-specific binding of a composite water concentrate to *Cryptosporidium*-specific mAbs

Antibody	Non-specific binding ratio[a]
CRY104	0.045 ± 0.013
CRY26	0.623 ± 0.023
CRY212	0.112 ± 0.034
Crypto-Cel	0.177 ± 0.034
Crypto-a-glo®	0.980 ± 0.125
Immucell®	0.155 ± 0.013
Hydrofluor®	0.159 ± 0.058
Unstained	0.033 ± 0.010

Results are the means of 3 separate analyses.[a]Arbitrary scale developed for *Cryptosporidium* analysis (see Section 2).Statistical difference ($p < 0.05$) by ANOVA.

Table 4. Comparison of the level of non-specific binding of a composite water concentrate to *Giardia*-specific mAbs

Antibody	Non-specific binding ratio[a]
G203	0.029 ± 0.002
Giardia-Cel	0.163 ± 0.024
Giardia-a-glo®	0.201 ± 0.032
Hydrofluor®	0.044 ± 0.005
Unstained	0.020 ± 0.002

Results are the means of 3 separate analyses.[a]Arbitrary scale developed for *Giardia* analysis (see Section 2). Statistical difference ($p < 0.05$) by ANOVA.

DISCUSSION

The detection of fluorescent-mAb labeled *Cryptosporidium* oocysts and *Giardia* cysts in water concentrates is hampered by the fluorescence of contaminating debris such as algae and mineral particles. The fluorescence of these interfering particles is caused by either naturally occurring autofluorescence (Vesey *et al.*, 1997b) or non-specific binding of the fluorescent mAbs. Previously we reported our investigations into the autofluorescence properties of particles in water samples and detailed how interference from these particles can be minimised by the selection of appropriate fluorochromes (Vesey *et al.*, 1997a,b). In this study we focused on the second source of interfering fluorescence, the non-specific binding of fluorescently labeled mAbs.

Table 5. Evaluation of the level of non-specific binding of selected *Cryptosporidium*-specific mAbs in different water types

Water type	Increase in fluorescent non-specific binding of a 50 μl water concentrate sample following antibody staining		
	Unstained	Cry104	Immucell™
Raw	0.023 ± 0.002	0.023 ± 0.001	3.294 ± 0.327
Backwash	0.023 ± 0.003	0.020 ± 0.006	0.440 ± 0.049
Raw	0.021 ± 0.003	0.020 ± 0.004	0.385 ± 0.016
Backwash	0.022 ± 0.002	0.087 ± 0.038	0.233 ± 0.052
Effluent	0.035 ± 0.006	0.085 ± 0.010	1.801 ± 0.093
Backwash	0.023 ± 0.004	0.277 ± 0.031	0.869 ± 0.154
Raw	0.022 ± 0.006	0.025 ± 0.005	1.391 ± 0.036
Raw	0.036 ± 0.006	0.099 ± 0.013	0.651 ± 0.047
Backwash	0.023 ± 0.004	0.031 ± 0.002	0.156 ± 0.027
Filtered	0.026 ± 0.007	0.027 ± 0.002	0.166 ± 0.015

Statistical difference ($p < 0.05$) by Student t-test and ANOVA, $N = 3$.

Evaluation of the number of fluorescent particles detected in a water concentrate before and after immunofluorescent staining revealed that the majority of mAbs tested produced significantly ($p < 0.05$) higher numbers of fluorescent particles following staining. However the mAbs selected by flow cytometry for water analysis (Cry104 and G203) were highly specific, producing significant increases in background fluorescence in only four of the ten water samples evaluated for G203 (Table 6) and in three of the ten samples for Cry104 (Table 5). A large variation in the number of fluorescent particles detected after staining with different mAbs was observed and can be attributed to the class and development methods used to produce each mAb.

Analysis of different classes of mAbs revealed that staining with IgG1 mAbs generally produced less unwanted fluorescent particles than staining with IgM and IgG3 mAbs. However the selection process used to identify the mAbs also affects specificity in water analysis, with the IgM Cry212 producing lower levels of background when compared with mAbs produced for clinical applications such as faecal analysis (Table 3). This increase in specificity can be attributed to the selection process described by Vesey *et al.*, 1997a for producing this mAb. This method is useful for comparing mAbs from commercial suppliers or for screening suitable mAbs directly from cell lines. This trend was observed following *Giardia* analysis of IgG1 mAbs, with G203 having higher affinity than the IgG1, Giardia-a-glo™ (Table 4).

Variation in the level of non-specific binding of particles to mAbs is largely due to the type of antibody used for the assay. The primary immune response to an antigen induces the production of IgM. They exist as pentameric mAbs containing ten binding sites, thus they have a greater chance of binding non-specifically to particles compared with IgG. The surface of oocysts and cysts contain carbohydrates that induce the production of low affinity IgM and IgG3. IgG1 mAbs are generally produced following repeated exposure to an antigen. Isotype switching occurs from IgM to IgG, resulting in higher affinity mAbs containing two identical binding sites more specific to the antigen against which they are produced (Roitt *et al.*, 1996).

Comparison of different water types revealed a significant difference in the non-specific binding seen in various water types. Different levels of background staining were detected following staining with IgG1 mAbs (Cry104 and G203), however the variability in non-specific binding to different water types was significantly reduced over the other mAbs

Table 6. Evaluation of the level of non-specific binding of selected *Giardia*-specific mAbs in different water types

Water type	Increase in fluorescent non-specific binding of a 50 μl water concentrate sample following antibody staining		
	Unstained	G203	Hydrofluor™
Raw	0.022 ± 0.002	0.048 ± 0.007	0.645 ± 0.010
Backwash	0.022 ± 0.003	0.034 ± 0.005	0.669 ± 0.008
Raw	0.020 ± 0.003	0.025 ± 0.002	0.696 ± 0.022
Backwash	0.021 ± 0.003	0.214 ± 0.094	14.63 ± 4.580
Effluent	0.032 ± 0.007	0.070 ± 0.001	1.021 ± 0.075
Backwash	0.021 ± 0.003	0.081 ± 0.014	10.36 ± 1.547
Raw	0.021 ± 0.006	0.023 ± 0.003	0.062 ± 0.005
Raw	0.031 ± 0.006	0.070 ± 0.022	1.637 ± 0.096
Backwash	0.022 ± 0.004	0.036 ± 0.009	0.111 ± 0.016
Filtered	0.024 ± 0.006	0.024 ± 0.003	0.043 ± 0.004

Statistical difference ($p < 0.05$) by Student t-test and ANOVA, $N = 3$.

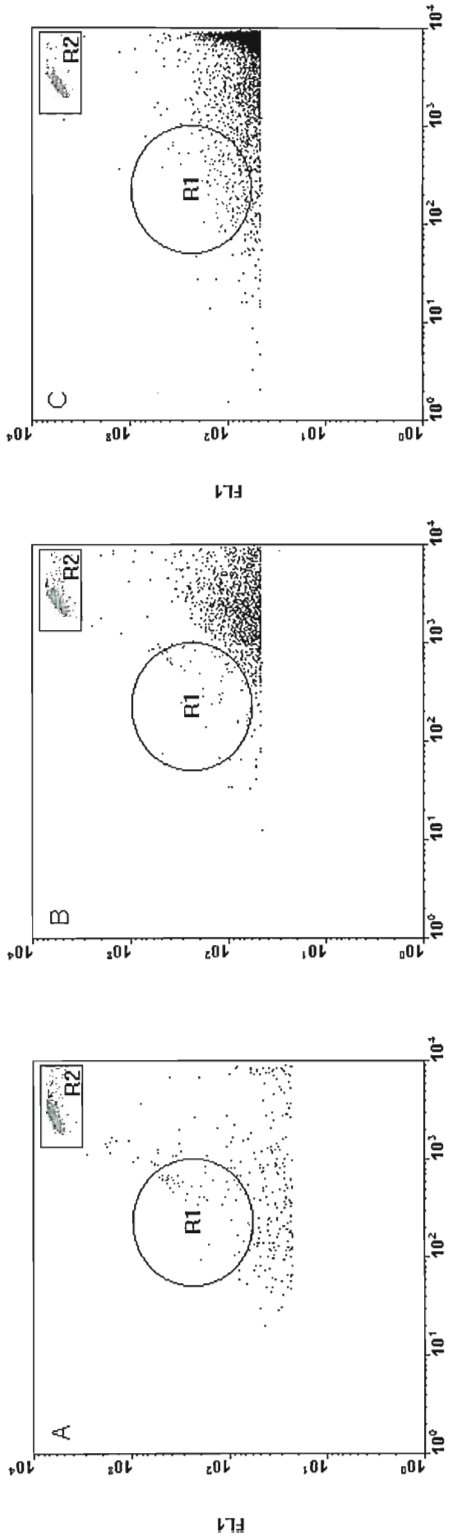

Fig. 4. Flow cytometric analysis of antibody staining of a raw water sample (Sample 8, Table 5) containing no oocysts. (A) Water concentrate only. (B) Water concentrate stained with the *Cryptosporidium*-specific mAb Cry104. (C) Water concentrate stained with the *Cryptosporidium*-specific mAb Immucell®. Note a significant increase in non-specific fluorescent particles following staining by mAbs, with the level of fluorescent background increasing most with the IgG3 Immucell® mAb.

examined. The degree of variation in non-specific binding was most apparent with backwash samples that displayed a range in fluorescence between a ratio of 0.111 and 14.63 for the Hydrofluor® mAb. Thus some water concentrates are sticky and may contain particles that have a much higher chance of binding to certain mAbs. However, for all water types examined, staining with mAbs of the subclass IgG1 resulted in the lowest numbers of non-*Cryptosporidium* particulate staining.

Monoclonal antibodies produced specifically for water analysis have a high affinity for cysts and oocysts over extraneous particles present within water concentrates. Cry104 and G203 analyses have been carried out on non-viable and viable oocysts or cysts and are now routinely used for detecting *Cryptosporidium and Giardia* in water samples. IgG1 types are smaller mAbs that bind specifically to the antigenic sites against which they were produced with little background interference. In the future, production of new IgG1 mAbs to different antigenic sites on cysts or oocysts may enable the development of highly specific assays for *Cryptosporidium* and *Giardia* detection within environmental samples. Further work into examining the specificity of Cry104 and G203 in clinical samples will indicate if they are suitable for testing different types of samples.

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