DETECTION AND COUNTING OF *CRYPTOSPORIDIUM PARVUM* IN HCT-8 CELLS BY FLOWCYTOMETRY

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Summary:

The objective of the present study was to evaluate flowcytometry analysis (FCA) as a tool for rapidly and objectively estimating the percentage of cells infected with *Cryptosporidium parvum* in an *in vitro* model. We compared the results to those obtained with immunofluorescence assay (IFA) and evaluated the intra-assay variability of both assays and the inter-assay variability of IFA. Human ileocecal adenocarcinoma cells (HCT-8) were infected with different doses of excysted oocysts. After 24 hours, cells were analysed by FCA and by IFA using a monoclonal antibody that recognises a *C. parvum* antigenic protein and a lectin that binds with glycoproteins present in the parasitophorous vacuoles. The coefficient of variability in terms of the percentage of infected cells was lower for FCA (i.e., 13-14 %) than for IFA (i.e., 27-38 % when performed by a single operator and 19-22 % when performed by three operators), suggesting that FCA is more accurate, in that it is not subject to operator expertise. FCA also has the advantage of allowing the entire culture to be examined, thus avoiding problems with heterogeneity among microscopic fields. In light of these results, this method could also be used to test new anti-*Cryptosporidium* drugs.

KEY WORDS : *Cryptosporidium parvum*, HCT-8 cells, flowcytometry, monoclonal antibody, *in vitro*.

Résumé : DÉTECTION ET COMPTAGE DE *CRYPTOSPORIDIUM PARVUM* EN CULTURE CELLULAIRE (HCT-8) PAR CYTOMÉTRIE DE FLUX

Le développement de la culture in vitro *de* Cryptosporidium parvum *pose le problème de la quantification des cellules infectées par une méthode rapide et objective. Dans ce travail, une analyse en cytométrie de flux (CF) a été mise au point pour détecter et quantifier les cellules infectées par* C. parvum. *Des cellules d'un adénocarcinome iléo-caecal humain (HCT-8) ont été infectées* in vitro *par des doses différentes d'oocystes sporulés. Après 24 h de culture, les cellules ont été analysées en CF et par immunofluorescence indirecte (IFI) en utilisant un anticorps monoclonal dirigé contre une protéine de* C. parvum *et une lectine s'attachant à des glycoprotéines présentes dans les vacuoles parasitophores. Les coefficients de variation du nombre de cellules infectées étaient compris entre 13 et 14 % pour une CF sur toute la culture cellulaire, entre 27 et 38 % pour une détection en IFI sur 20 champs microscopiques par un seul opérateur et entre 19 et 22 % pour une détection en IFI sur les mêmes 20 champs microscopiques par trois opérateurs. Les faibles coefficients de variation obtenus par la cytométrie de flux suggèrent que cette méthode est plus précise et moins dépendante des compétences de l'opérateur que l'IFI.*

MOTS CLÉS : Cryptosporidium parvum, *cellules HCT-8, cytométrie de flux, anticorp monoclonal,* in vitro.

INTRODUCTION

Cryptosporidium parvum constitutes a major cause of diarrhoeal disease in many species of mammals, including humans (Fayer *et al.*, 1997; O'Donoghue, 1995), yet little is known about the basic biology of this organism, and no specific effective therapy for *C. parvum* infection exists. For many years, the study of *C. parvum* and of effective drugs has been hampered by the lack of specific *in vitro* systems and a rapid method for evaluating the infection rate in a host cell population. However, in recent years, cell cultures for studying the life cycle and metabolic requirements of *C. parvum* have been developed (Arrowood *et al.*, 1994; Griffiths *et al.*, 1994; Upton *et al.*, 1994a; Upton *et al.*, 1994b; Upton *et al.*, 1995), and it has been proposed that these cell cultures be used instead of animal models for pharmacological screening (Woods *et al.*, 1995; Yang *et al.*, 1996; Deng & Cliver, 1998). Furthermore, several studies have quantified *C. parvum* infection *in vitro* using immunofluorescence assay (IFA) (Slifko *et al.*, 1999), enzyme-linked immunosorbent assay (Woods *et al.*, 1995), or real-time polymerase chain reaction (Fontaine & Guillot, 2002).

The objective of the present study was to evaluate flowcytometry analysis (FCA) as a tool for rapidly and objectively estimating the percentage of *C. parvum*-infected cells in an *in vitro* model. We compared the results to those obtained with IFA and evaluated the intra-assay variability of both assays (i.e., the variability among replicates analysed by a single operator) and the inter-assay variability of IFA (i.e., the variability among replicates analysed by three operators).

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MATERIALS AND METHODS

In vitro culture

Human ileocecal adenocarcinoma cells (HCT-8) were cultured in RPMI 1640 (Hyclone) supplemented with 5 % foetal calf serum (FCS, Hyclone), 200 mM L-glutamine (Sigma), 1 % sodium pyruvate (Sigma), 5 % penicillin, and 5 % streptomycin, and they were maintained in tissue-culture flasks in a 5 % CO_2 atmosphere at 37° C and 85 % humidity. When the cells reached 80-90 % confluence, they were detached by incubating them in PBS with 0.05 % Trypsin and 0.02 % EDTA (Euroclone) at 37° C in the CO_2 atmosphere for 5-8 minutes. The cell suspension was vigorously and repeatedly pipetted using a 200 µl pipette tip until the cells were separated. The cells were then centrifuged at 500 x*g* for 10 minutes; the pellet was resuspended in the maintenance medium (Upton *et al.*, 1995).

Excystation of *Cryptosporidium parvum* oocysts

Cryptosporidium parvum oocysts (isolate code ISSC4) were obtained from experimentally infected calves after faeces purification by sucrose and Percoll® density gradients (Rossi *et al.*, 1990). For excystation, the oocysts were resuspended in 10 mM HCl and incubated at 37° C for 10 minutes, according to a previously published protocol (Gut & Nelson, 1999). Briefly, the suspension was centrifuged at 3,000 x*g* for five minutes, and the pellet was resuspended in 2 mM sodium taurocholate (Sigma) in PBS and incubated at 15° C for 10 minutes and followed by a 5-8 minutes incubation at 37° C. The level of excystation was 90 % when 2-3 week-old oocysts were used and decreased when using older oocysts; thus 2-3 week-old oocysts were used throughout the study. This protocol allows for the synchronous excystation of at least 10^8 oocysts in 1.5 ml microfuge tubes. For a smaller quantity of oocysts, the tubes are pre-coated with bovine serum albumin to reduce sporozoite loss. The excystation mix was centrifuged at 1,000 x*g* for two minutes. The excysted oocysts were resuspended, aliquoted in the growth medium, and transferred into a Petri dish.

Before the infection with excysted oocysts, the HCT-8 cells were plated in Petri dishes at 1×10^6 cells/100 mm diameter; the FCS concentration was increased to 10 %, and the cells were grown to 80-90 % confluence. The HCT-8 cells were infected with 0.1, 0.5, 1, 2, 3, 5, 10, and 20 excysted oocysts per cell. After 24 hours of incubation, we were able to observe a direct correlation between the infective dose and the quantity of infected cells, because parasites of the first generation grown *in vitro* were not yet able to infect other cells (Slifko *et al.*, 1999).

Indirect immunofluorescence assay

HCT-8 cells were plated on tissue culture glass slides (Falcon) and grown to 80-90 % confluence. HCT-8 monolayers were then infected with 0.1, 0.5, or 1 excysted oocyst per cell. After 24 hours of incubation, the cells were fixed in 4 % formaldehyde in PBS for 20 minutes and treated with 1 % Triton X-100 in PBS for 10 minutes and then with 2 % BSA in PBS for 30 minutes. The fixed cells were incubated at room temperature for 30 minutes with 0.5 µg/ml of a monoclonal antibody that recognises the SA35 antigen, which has been identified in the sporozoite stage of *C. parvum* (Tosini *et al.*, 1999) and also in the endocellular stages (unpublished data), and with 0.5 µg/ml of a lectin (VVL-biotin) (Vector Laboratories, B-1235) that binds glycoproteins present in the parasitophorous vacuoles (Gut & Nelson, 1999). Samples were then incubated with 1 µg/ml of goat anti-mouse FITC and streptavidin-PE at room temperature for 30 minutes. As negative controls, non-infected cells and *C. parvum*-infected cells incubated with FITC and PE conjugates were used. Slides were observed under epifluorescence microscopy at 400 X magnification. The level of infection was expressed as the mean number of parasitophorous vacuoles per field out of a total of 20 fields (which correspond to 14 % of the total well). Each experiment was performed in triplicate. For the inter-assay test, each experiment was carried out by three different operators.

Flowcytometry analysis

HCT-8 monolayers were infected with 0.1, 0.5, 1, 2, 3, 10, and 20 excysted oocysts per cell. After 24 hours of incubation, the cells were detached by treating them with 0.05 % Trypsin and 0.02 % EDTA (Euroclone) at 37° C in a CO_2 atmosphere, for 5-8 minutes. The cell suspension was vigorously and repeatedly pipetted using a 200 µl pipette tip until the cells were separated. The cells were fixed in 4 % formaldehyde in PBS for 20 minutes and treated with 1 % Triton X-100 in PBS for 10 minutes and then with 2 % BSA in PBS for 30 minutes. The fixed cells were incubated with 0.5 µg/ml of anti-SA35 and 0.5 µg/ml of VVL-biotin at room temperature for 30 minutes. Samples were then incubated with 1 µg/ml of goat anti-mouse-FITC and streptavidin-PE at room temperature for 30 minutes. As negative controls, non-infected cells and *C. parvum*-infected cells incubated with goat anti-mouse-FITC and streptavidin-PE conjugates were used. The infected cells were analysed by a Facs Calibur (Becton Dickinson). The instrument was set up to measure forward-angle light scatter (FSC-H), side-angle light scatter (SSC-H), and the fluorescence intensities of anti-SA35-FITC (FL1-H) and aVVL-biotin-streptavidin-PE (FL2-H). For each oocysts/cell ratio, from 15 to 20 replicates were performed.

RESULTS

ANALYSIS OF *C. PARVUM*-INFECTED CELLS BY IFA

The infection level of HCT-8 cells was monitored by IFA using the anti-SA35-FITC and aVVL-biotin-streptavidin-PE. Both SA35 and VVL-biotin allowed infected cells to be distinguished from non-infected cells (data not shown) and the number of infected cells increased with the infective dose. Since only double stained cells are considered positives, the probability to get false positives diminished. Table I shows the results of the intra-assay test, i.e., the variability in the number of parasitophorous vacuoles among the 20 fields examined (by a single operator and for each replicate), as determined by the standard deviation of the mean and the coefficient of variability (CV). The variability (considered as an indicator of the accuracy of the mean value) increased with the increasing dose, with the CV ranging from 27 % for the lowest dose to 38 % for the highest dose. To conduct the inter-assay test, for each replicate, the number of vacuoles in the same 20 fields are counted by three operators. The results (Table I) showed that the CV ranged from 19 % for the lowest dose to 22 % for the highest dose.

ANALYSIS OF *C. PARVUM*-INFECTED CELLS BY FCA

For the morphometric analysis, the sample was analysed with a threshold for FSC-H, in order to select only the HCT-8 population (i.e., discarding debris, free oocysts, and sporozoites). Figure 1 shows the dot plots of the FCA of the FSC-H and SSC-H of non-infected cells (Fig. 1A) and of cells infected with an oocyst/cell ratio of 0.5, 1, 3, and 10 (Figs 1B, C, D, and E, respectively). For the infected cells, the light-

	Intra-assay			**Inter-assay**		
Oocyst/cell ratio at infection	0.1/1	0.5/1	1/1	0.1/1	0.5/1	1/1
Mean number ± SD of parasitophorous vacuoles per microscopic field	4 ± 1.1	33 ± 12	58 ± 22	5 ± 1	26 ± 5	53 ± 12
CV (%)	27	36	38	20	19	22

Table I. – Intra-assay and inter-assay tests for immunofluorescence analysis. Each experiment was performed in triplicate by either one operator (intra-assay test) or by three operators (inter-assay test) to count the number of parasitophorous vacuoles detected in 20 fields. Data are expressed as the mean number ± standard deviation (SD) and coefficient of variability (CV) of the triplicates.

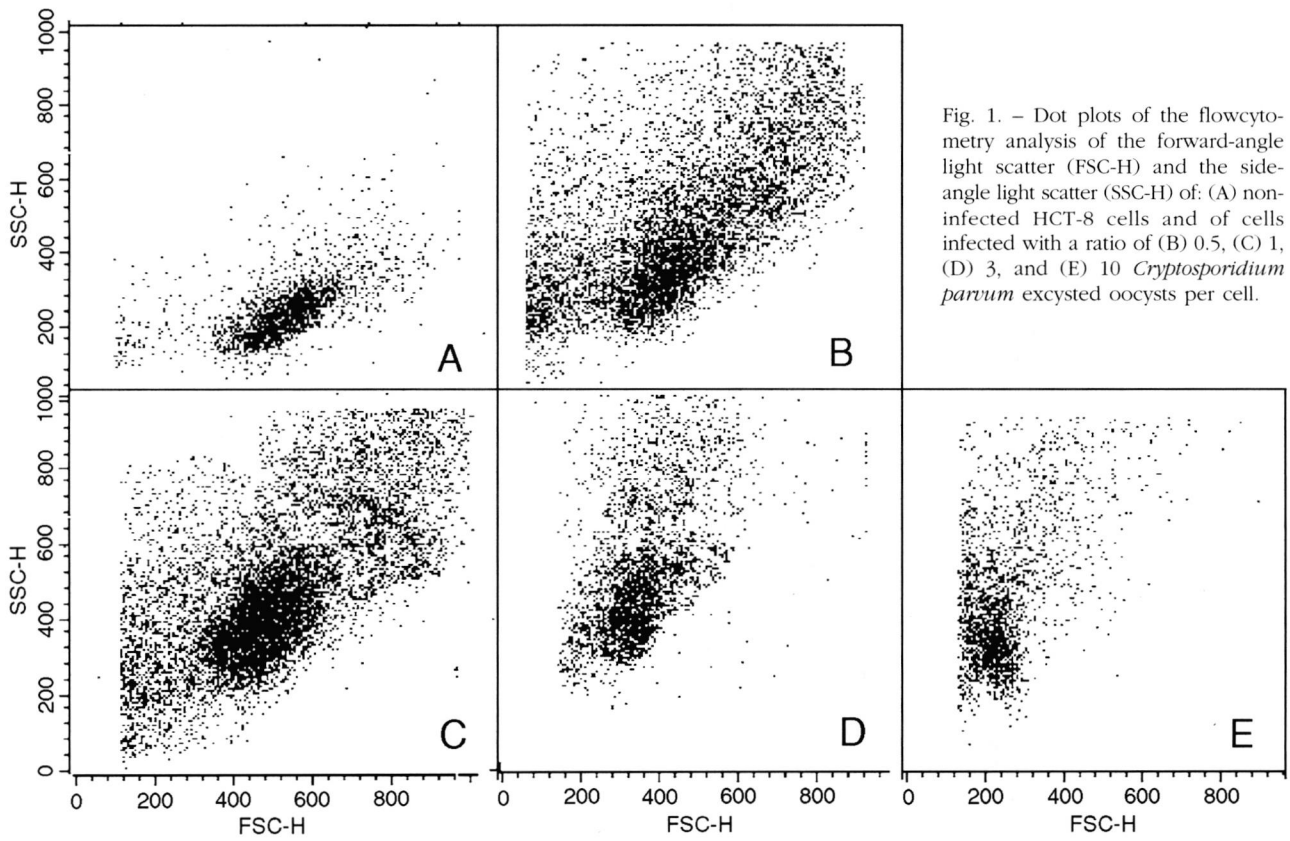

Fig. 1. – Dot plots of the flowcytometry analysis of the forward-angle light scatter (FSC-H) and the side-angle light scatter (SSC-H) of: (A) non-infected HCT-8 cells and of cells infected with a ratio of (B) 0.5, (C) 1, (D) 3, and (E) 10 *Cryptosporidium parvum* excysted oocysts per cell.

Oocyst/cell ratio at the infection	0.1/1	0.5/1	1/1
Mean percentage ± SD of *C. parvum*-infected cells for the entire culture	3.3 ± 0.5	20.3 ± 2.6	86.0 ± 12.0
CV (%)	15	13	14

Table II. – Intra-assay test on the results of flowcytometry. Mean (15-20 replicates for each oocyst/cell ratio) of the percentage of HCT-8 cells infected with *Cryptosporidium parvum* ± standard deviation (SD) and coefficient of variability (CV) for the entire culture.

scatter pattern varied according to the oocyst/cell ratio, as a result of the cells becoming morphologically more complex when pluri-parasitised (Rosales *et al.*, 1993). Specifically, both the FSC-H and the SSC-H peaked at the ratio of one oocyst per cell (Fig. 1C); they then progressively decreased, suggesting that the infected cells became increasingly granular and then burst (Figs 1D and 1E).

For the evaluation of the infection level by FCA, *C. parvum*-infected cells were labelled in the same way as used for IFA yet after having detached the cells and performing all successive steps in suspension. The results of the intra-assay test (Table II) showed that the percentage of *C. parvum*-infected cells increased with increases in the ratio of excysted oocysts per cell (Table II). Figure 2 illustrates, for a representative sample of HCT-8 cells analysed by FCA, both a dot plot (double fluorescence anti-SA35-FITC and aVVL-biotin-streptavidin-PE) and a histogram plot (single fluorescence anti-SA35-FITC). For the non-infected cells (Figs 2A and 2B), both the dot plot and the histogram plot show only one cell population, which was negative for both FL1 and FL2 (i.e., fluorescence intensity of less than 10^1). At an oocyst/cell ratio of 0.5 (Figs 2C and 2D), two populations were identified: one consisted of non-infected cells, which was negative for both fluorochromes and which represented 80 % of the total population, and the other consisted of infected cells, which was positive for both fluorochromes and which represented 20 % of the total population. At a ratio of one oocyst/cell (Figs 2E and 2F), there was only a population of infected cells. At a ratio of three oocysts/ cell (Figs 2G and 2H), the infected cell population showed a strong increase in fluorescence intensity for both fluorochromes, suggesting that the number of pluri-parasitised cells had increased. At oocyst/cell ratios higher than three (data not shown), the fluorescence intensity had decreased because the high amount of parasites had greatly damaged the cell monolayer, diminishing its ability to support the infection.

Figure 3 shows, for each oocyst/cell ratio, the fluorescence intensity measured as the peak channel of fluorescence, for cells labelled with anti-SA35 compared to those labelled with aVVL-biotin. The peak

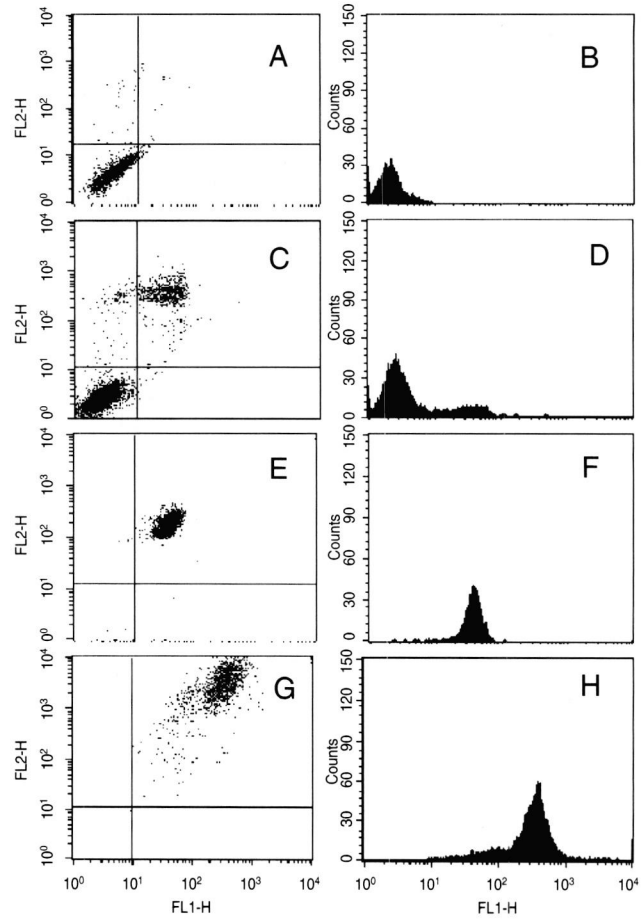

Fig. 2. – Dot plots of the fluorescence of anti-SA35-FITC antibody (FL1-H) and aVVL-biotin-streptavidin-PE (FL2-H) and histogram plots of a single fluorescence (FL1-H). Non-infected HCT-8 cells (A and B); cells infected with 0.5 oocyst/cell (C and D); cells infected with one oocyst/cell (E and F); and cells infected with three oocysts/cell (G and H).

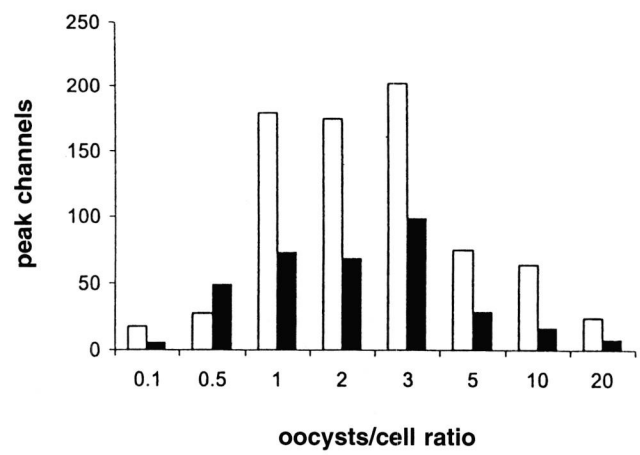

Fig. 3. – Fluorescence intensity (peak channel of fluorescence) at 24 hours after infection for HCT-8 cells infected with excysted oocysts of *Cryptosporidium parvum*, labelled with anti-SA35-FITC (open bars) and aVVL-biotin-streptavidin-PE (black bars), by oocyst/cell ratio.

channels followed a similar pattern when comparing the two labelling methods, although those labelled with anti-SA35 showed higher peak channels, suggesting that anti-SA35 and aVVL-biotin recognise different epitopes with different frequencies. For oocyst/cell ratios of 0.1-1.0, the peak channels progressively increased; they then levelled-off for ratios of 2.0 and 3.0, suggesting that all of the cells were infected. At higher oocyst/cell ratios, the peak channel decreased because the level of infection significantly damaged the cell monolayer, diminishing its ability to support the infection.

DISCUSSION

The extent of parasitism of cells, which is variable over time, is difficult to quantify by IFA, in that parasitised cells usually detach from the substrate and cannot be counted. Furthermore, parasites are not uniformly distributed in the cell monolayer, and a pluri-parasitised cell can be found in contact with non-parasitised cells (Barnes *et al.*, 1998; Gut & Nelson, 1999). For this reason, a high number of arbitrarily chosen fields needs to be analysed when estimating the infection level with IFA. Although our analyses were conducted on 20 fields, the consistently high CV indicates that a greater number is required. The finding that the non-parametric Spearman correlation coefficient was approximately 1 ($r = 0.98$, $P < 0.01$) when comparing IFA to FCA in terms of the level of infection at different oocyst/cell ratios suggests that both methods can be used to evaluate the infection level because they have a similar sensitivity at the oocyst/cell ratio tested. However, the advantage of FCA is that it allows the entire sample to be examined within a short period of time, hence overcoming the problem of heterogeneity within the sample. In fact, in the intra-assay test, the CV was lower for FCA than for IFA. Moreover, the infection level estimated by IFA is subject to the expertise of the operator, as shown by the CV values obtained with the inter-assay test.

In FCA, the cell autofluorescence and the non-specific fluorescence of antibodies are evaluated by subtracting the values detected in non-infected cells from those detected in infected cells. Free oocysts, sporozoites, and debris, which could interfere with the measure of fluorescence of intracellular parasites, can also be excluded. In conclusion, FCA allows the number of infected cells *in vitro* to be analysed and quantified in a short period of time with high reproducibility and without any bias due to operator expertise.

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