Three-color Cytometry for the Simultaneous Detection of the Cryptosporidium Species Contributing to the Majority of Human Cryptosporidiosis

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***Abstract*-Cryptosporidium is a protozoan parasite which is the most common non-viral cause of diarrhea worldwide. Currently, there are twenty recognized species of Cryptosporidium and among which, C. parvum and C. hominis are the species primarily infecting humans. FISH utilises fluorescently-labelled complementary DNA oligonucleotide probes that target specific sequences of cellular rRNA for direct identification of microorganisms. This work describes the successful development of such probes for the specific detection of the zoonotic species, C. parvum and the human host-specific species, C. hominis. The specificity of the probes was established by testing them against a range of target and non-target Cryptosporidium species using an optimised FISH assay. Validation of the C. parvum–specific probe and C. hominis-specific probe was carried out by a comparison of FISH with PCR-RFLP analysis of the 18S rRNA gene from Cryptosporidium isolated from human infections. Additionally, the C. parvum-specific probe synthesised with Cy3 dye and the C. hominis-specific probe synthesised with Cy5 dye were used in combination with the Cryptosporidium-specific monoclonal antibody CRY104-FITC, for the development of a 3-colour FISH assay that can allow identification of both C. parvum and C. hominis species simultaneously. This assay was developed and validated by using both epi-fluorescence microscopy and flow cytometric technology. Species-level identification by three-color cytometry and PCR-RFLP targeting the 18S rRNA gene was identical. The potential exists for the assay to be implemented into routine immunofluorescence detection methods for Cryptosporidium.**

***Keywords*-Cryptosporidium; Fluorescence in Situ Hybridization; Monoclonal Antibodies; Flow Cytometry; Three-color**

I. INTRODUCTION

Cryptosporidium is a waterborne protozoan parasite that is a major cause of diarrheal illness worldwide [9, 15]. While twenty species have been described affecting a broad host range, the human host-specific species *C. hominis* and the zoonotic species *C. parvum* are responsible for the majority of human cryptosporidiosis cases [24]. Species identification of the parasite plays a vital role in management of the disease and is primarily carried out through DNA sequencing or PCR and RFLP typing targeting the 18S rRNA gene [23, 14].

Fluorescence in situ hybridization (FISH) is a fluorescence technique that enables taxonomic identification of individual microorganisms through the use of rRNA targeted probes [5]. The technique is based on the hybridization of synthetic oligonucleotide probes to specific regions within the ribosome of the target organism and is considered a powerful tool for taxonomic, ecological, diagnostic and environmental studies in microbiology [4]. While epi-fluorescence and confocal microscopy are most commonly used for image analysis following FISH, combinations of flow cytometry (FCM) and rRNA detection by in situ hybridization have been reported [13, 16].

The first application of FISH for fluorescent labelling of *Cryptosporidium* oocysts was described over a decade ago [11]. Recently, we reported on the development of two species-specific DNA FISH probes for the differentiation of *C. hominis* and *C. parvum* in a single assay [2, 3]. In the present study, we further modified the DNA FISH assay to encompass 3-colors for the identification of *C. parvum* and *C. hominis* in infected human fecal samples. We evaluated a range of different fluorochromes based on their fluorescence spectra, their fluorescence signal intensity following hybridization to oocysts and instrument compatibility for both epi-fluorescence microscopic (EFM) or FCM analysis of multi-colour labelled samples. In each case, we utilized two species-specific FISH probes and a monoclonal antibody specific to the genus *Cryptosporidium.*

II. FLUOROCHROMES EVALUATED FOR USE IN A THREE-COLOUR ASSAY

FISH probes that specifically target *C. parvum* (Cpar677) and *C. hominis*-like species (Chom253) were utilized as described previously [2]. After *in situ* hybridization, oocysts were post-stained with an IgG1 monoclonal antibody (CRY104)

DOI:10.5963/PHF0202007

and examined by EFM & FCM. Each fluorochrome was evaluated, with their excitation/emission spectra and suitability for the available instrumentation or applicability in FISH described in Table I.

TABLE II FLUOROCHROMES (DYES) EVALUATED FOR LABELLING CRYPTOSPORIDIUM-SPECIFIC OLIGONUCLEOTIDE PROBES AND A MONOCLONAL ANTIBODY CRY104 FOR DEVELOPMENT OF A THREE-COLOR MICROSCOPY AND/OR FLOW CYTOMETRY ASSAY

Dyes	Abs/Em	+Instrument Compatibility	FISH Compatible
Pacific Blue	410 nm/ 455 nm	EFM & FCM	# Unknown
Alexa Fluor 405	402 nm/ 421 nm	EFM & FCM	Yes
FITC	494 nm/ 518 nm	EFM & FCM	Yes
Alexa Fluor 488	495 nm/ 519 nm	EFM & FCM	Yes
Phyco-erythrin [PE]	565 nm/ 575 nm	EFM & FCM	*No
Cy3	550 nm/ 570 nm	EFM	Yes
Cy5	649 nm/ 670 nm	EFM & FCM	Yes

+ Compatibility with available instrumentation only.
# Pacific Blue [Zenon mouse IgG labelling kit] is based on 6, 8-difluoro-7-hydroxycoumarin; its suitability for FISH was unknown.
* PE is a fluorescent protein that is too large to enter the cell for *in situ* hybridization.

A. Fluorochrome Selection for Multi-colour FISH Analysis by EFM

Based on the water laboratories perspective, the preferred choice of fluorophore colours for differentiating *Cryptosporidium* species by means of a 3-color FISH-microscopy assay are red, green and blue (John Watkins, CREH Analytical Ltd, pers comm.). The *Cryptosporidium*-specific monoclonal antibody, CRY104 targets Cryptosporidium spp and is often utilised conjugated with FITC (green emission). Hence, the 2 fluorophores to be conjugated to the 2 species-specific probes were required to possess blue and red emission spectra. Cy3 (red emission), belonging to the family of cyanine dyes, was reported to be an effective fluorophore for labelling FISH probes in terms of photo stability and brightness [23]. Due to the lack of conventional blue-emitting fluorophores as labels for FISH probes, Alexa Fluor 405 (blue emission) belonging to the Alexa Fluor class of fluorochromes was chosen as the label for Cpar677 and Cy3 was chosen as the label for Chom253. Microscopic analysis of Alexa Fluor 405 hybridized samples revealed that no significant fluorescent labelling was achieved (data not shown).

Due to the lack of a reliable fluorophore with blue emission, it was decided to trial CRY104-PE, Chom253-Cy3 and Cpar677 with a green-emitting fluorophore. Since FITC is not comparable to Cy3 in terms of brightness and stability, it was decided to opt for the best green fluorophore available for labelling oligonucleotides. Alexa Fluor 488 dye was reported to be the best fluorescein (FITC or FAM) alternative available that claimed to be 6-7 times brighter than fluorescein (based on publications, results) [7]. Alexa Fluor 488 was conjugated to Cpar677 and used in the 3-color FISH assay. Interestingly, Cpar677-Alexa Fluor 488 cross-reacted with *C. hominis* oocysts in addition to hybridizing with *C. parvum* oocysts. The non-specific staining of the Cpar677-Alexa Fluor 488 probe, could possibly be attributed to the chemistry of the Alexa Fluor 488 dye, which in some way compromises the stringency in the hybridization buffer, leading to non-specific staining.

Recently, a simple and rapid way of labelling antibodies with a range of different fluorophores has been through the application of the Zenon mouse IgG labelling kits Invitrogen, Australia. CRY104 was conjugated to Pacific Blue using this kit. The antibody conjugation was successful, which was revealed by the oocyst wall being stained blue in colour when *Cryptosporidium* oocysts were post-stained with CRY104-Pacific Blue (data not shown). However, when CRY104-Pacific Blue was used to post stain multi-colour FISH hybridised samples, the blue fluorescence due to CRY104 significantly diminished to the extent that no blue fluorescence due to the antibody staining could be observed. This observation implies that the antibody conjugation needs to be optimised such that it can be used effectively for post-staining FISH hybridized samples.

As a result of the above unsuccessful attempt at conjugation of CRY104 to a blue dye, the following combination of fluorophores were tested in multi-colour *Cryptosporidium* FISH assays: CRY104-FITC (Ex: 494 nm, Em: 525 nm), Cpar677-Cy5 (Ex: 649 nm, Em: 670 nm) and Chom253-Cy3 (Ex: 552 nm, Em: 565 nm). The fluorescence spectra of these dyes could be clearly differentiated. Cy3, FITC and Cy5 were assigned to red, green and blue, respectively on the basis of the RGB colour model. It is important to note that the Cy5 derivative that fluoresces in the near infrared is detectable only with a CCD camera. Concomitant to the selection of the fluorescent label, care was taken in the choice of the right optical filters for the detection of the respective dyes. Optical filters with a high transmission in the emission spectrum of the dye and a strong and sharp blocking of the excitation light that would enable even the detection of weak signals were chosen. These fluorochromes proved to be an effective choice of labels for the probes and CRY104, when samples were analysed using an epi-fluorescence microscope.

B. Fluorochrome Selection for Multi-colour FISH Analysis by FCM

The combination of fluorochromes used for labelling probes and CRY104 for microscopic analysis of hybridized samples

DOI:10.5963/PHF0202007

was not suitable when samples were to be analysed by flow cytometry. A BD FACSAriaI (BD Biosciences, Australia) flow cytometer was used for the analysis of FISH stained *Cryptosporidium* samples and Cy3 dye was not a compatible fluorochrome for analysis by this flow cytometer. Hence, a different combination of fluorochromes, those that are compatible for flow cytometric analysis by FACSAriaI instrument was chosen. PE (Phycoerythrin, Ex 488 nm, Em 575 nm) was selected as an alternative fluorochrome, compatible for analysis. For successful flow cytometric analysis of 3-color FISH samples, CRY104 was conjugated to PE, Cpar677 was conjugated to Cy5, and Chom253 was conjugated to FITC (Table II). The fluorescence spectra of PE, Cy5, and FITC can be differentiated clearly with not much spectral overlap observed.

TABLE II FINAL THREE COLOUR ASSAY FLUOROCHROME (DYE) COMBINATION FOR ANALYSIS BY EPI FLUORESCENCE MICROSCOPY AND FLOW CYTOMETRY

Reagents	Dyes Used for Microscopic Analysis	Dyes Used for Flow Cytometric Analysis
*IgG1 Mab CRY104	FITC	PE
[A] Cpar677 Probe	Cy5	Cy5
[B] Chom253 Probe	Cy3	FITC

* CRY104-FITC was supplied from BTF, Sydney, Australia.
[a]Cpar677; (TCATATACTAAAATATATAGTAATAT)
[b]Chom253; (TCACATTAATTGTGATCC)

III. THREE-COLOUR CYTOMETRIC ANALYSIS FOR IDENTIFICATION OF C.PARVUM OR C.HOMINIS OOCYSTS

Oocysts from fifty-one human fecal samples positive for *Cryptosporidium* were purified from feces [19] and identified to the species level using PCR and RFLP targeting the 18S rRNA gene [21]. Identified oocysts were then de-identified and supplied to UNSW for identification by Macquarie University as part of a continuing epidemiological investigation. All FCM analysis was performed using a FACSAriaI flow cytometer (BD Biosciences, Sydney, Australia) equipped with three lasers: a violet (405 nm), solid state (488 nm) and a HeNe air-cooled laser (633 nm) for excitation. Rainbow QC beads (BD Biosciences) were used for instrument calibration and sheath fluid consisted of undiluted Osmosol (Lab Aids Pty Ltd, Narrabeen, NSW, Australia). Positive and negative controls consisting of both *C. hominis* and *C. parvum* oocysts were used for instrument set up (Fig. 1).

Detectors used were side scatter (SSC-A) with the voltage set at 225 V, forward scatter (FSC-A) set at 100 V, while the fluorescent detectors varied between 300-400 V. The assay combined 488 nm laser excitation and analysis of green fluorescence (FITC, Alexa 488) detectable in FL1 530/30 nm with orange fluorescence (PE) detected using FL2. While the 633 nm laser was used to detect the red fluorescence (Cy3) detected in FL3 and compensation was set: PE-FITC at 30.0 and FITC-PE at 4.0. The acquisition threshold was set to 5,000 in FSC-H and the flow rate was adjusted for the analysis of 100-150 events per second. For analysis, control preparations were analysed first on a bivariate dotplot of SSC-H versus FL2, the threshold was adjusted to remove non-specific detrital particles and a polygonal region (R1) was defined around the centre of the fluorescing oocyst population (Fig. 1A). A datafile containing 5,000 events were collected for every sample and recorded. Data analysis was carried out using FACSDiva$_{TM}$ software (BD Biosciences). Two flow cytometric histograms were used for the species analysis with the fluorescent intensities of FITC (*C. hominis*) or Cy5 (*C. parvum*) presented on the x-axes and the oocyst counts on the y-axes (Fig. 2A and B). Both histograms were gated on the oocyst population presenting in R1 enabling non-oocyst particles to be removed from the analysis.

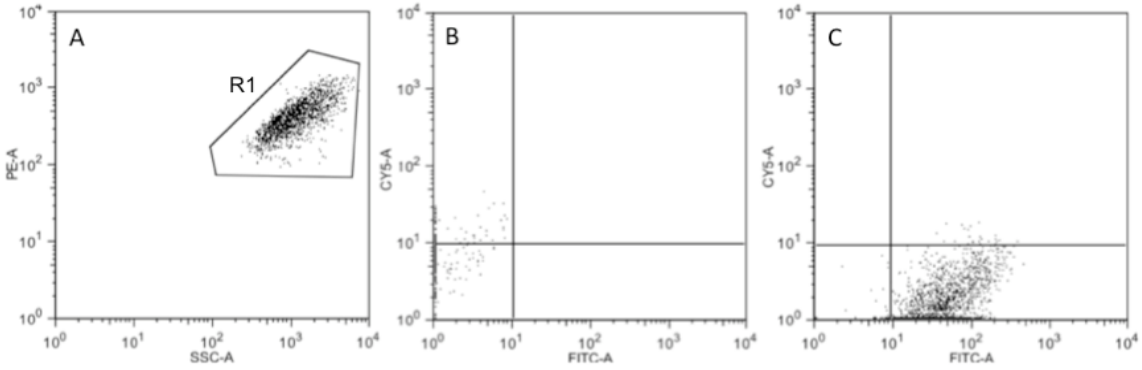

Fig. 1 (A) A bivariate dotplot of side scatter (SSC) versus PE fluorescence (FL2) in arbitrary units, one dot represents a single oocyst or particle. The instrument was adjusted for the detection of labelled oocysts on a second bivariate dotplot of FITC versus Cy5 following positive hybridization to Cpar677-Cy5; (B) or Chom253-FITC; (C) The gated region (R1) was then defined on the oocysts population detected when oocysts were labelled with CRY104-PE (A) and was used for subsequent data analysis

DOI:10.5963/PHF0202007

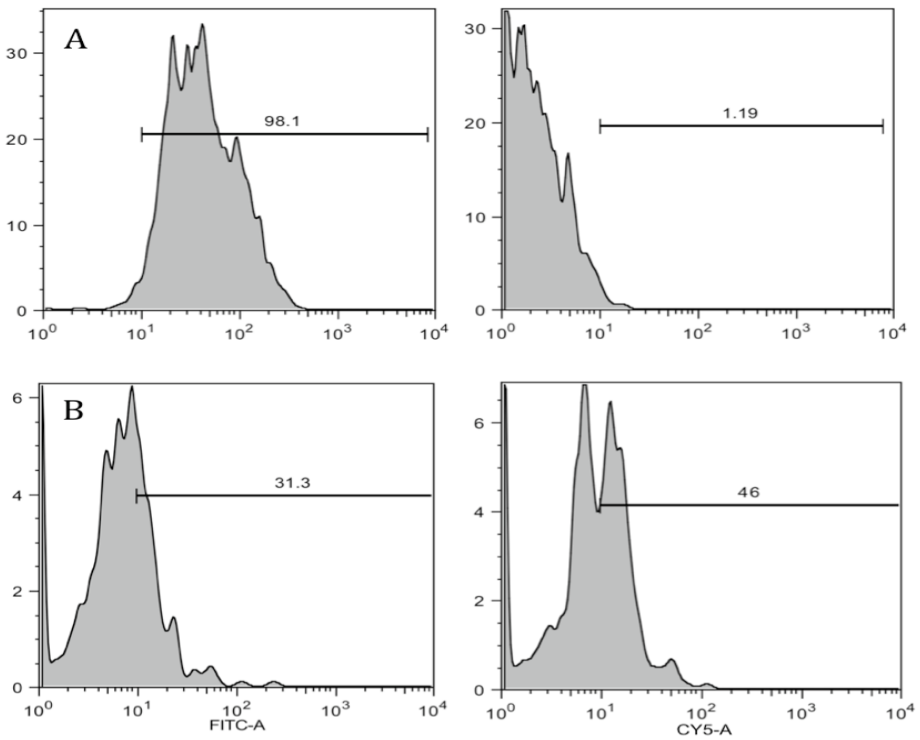

Fig. 2 Three-color data analysis for simultaneous identification of *C. parvum* or *C. hominis* oocysts by FCM- Histograms displaying Cy5 or FITC fluorescence on the x-axes and the oocysts counts on the y-axes were used for species identification: (A) Sample positive for *C. parvum* as observed by hybridization to Cpar677-Cy5 and signal detection on the Cy5 histogram only; (B) Sample positive for *C. hominis* as observed by hybridization to Chom253-FITC and signal detection on the FITC histogram only

Forty-one of the fifty-one isolates analysed were potentially viable, as indicated by an intense red fluorescence signal following hybridization with EUK-Cy3 that targets the 18S rRNA gene of all Eukaryotes [3]. All forty-one isolates were then used for three-color assay identification (Table II). Results revealed twenty-eight samples exhibited positive hybridization with the Cpar677-Cy5 probe (blue fluorescence Fig. 3; FL3 fluorescence Figs. 1 and 2B), indicating the presence of *C. parvum*. Twelve isolates exhibited positive hybridization with the Chom253-Cy3 probe (red fluorescence; Fig. 3) or Chom253-FITC probe (Fig. 2A), indicating the presence of *C. hominis*. Surprisingly, one sample out of the 41 examined hybridized positively with both the Cpar677-Cy5 and the Chom253-FITC/Chom253-Cy3 probes, indicating the presence of both *C. parvum* and *C. hominis* in that fecal sample (Fig. 3). Confirmation of species identified by both approaches revealed all 41 isolates were correctly identified indicating a strong correlation between *Cryptosporidium* species identification by three-color FISH and PCR-RFLP analysis targeting the 18S rRNA gene.

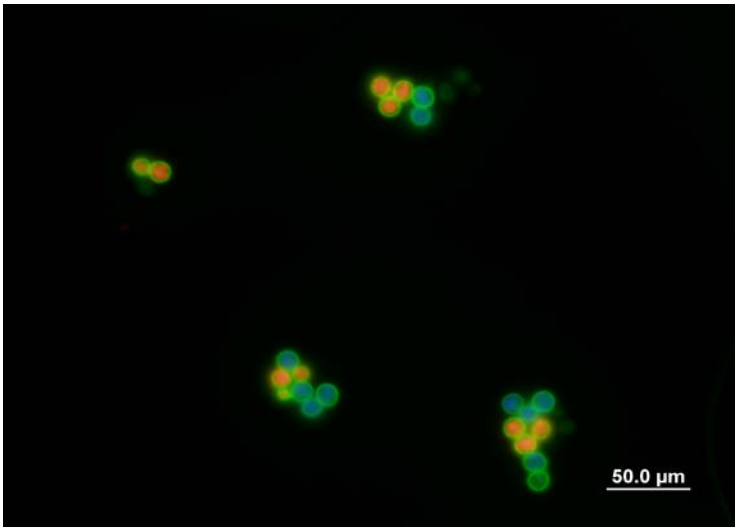

Fig. 3 Three-color FISH assay for the simultaneous identification of *C. parvum* and *C. hominis* oocysts by EFM- The final FISH-EFM fluorochrome combination was CRY104-FITC, Cpar677-Cy5 and Chom253-Cy3. Following hybridization, *C. parvum* oocysts internally fluoresced blue whilst the C.hominis *oocysts* internally fluoresced red. Counterstaining the oocyst cell walls green allowed for all *Cryptosporidium sp* oocysts to be detected Oocysts were examined at 100 X magnification

DOI:10.5963/PHF0202007

IV. CONCLUSIONS

Flow cytometry used in conjunction with monoclonal antibodies, vital dyes and rRNA probes have enabled the detection and characterisation of microorganisms from a diverse range of matrices [8, 10, 17]. In water microbiology, the FCM detection of *Cryptosporidium* oocysts from water samples is not new [19, 20] and in the past we reported on a two-colour method for the detection of *Cryptosporidium* that relied on the use of one antibody conjugated to two different fluorophores [12]. Here, a three colour assay was developed utilizing three independent probes for the differentiation of two *Cryptosporidium* species isolated from fecal samples. By utilizing independent probes, we have shown that FCM detection was possible without the need for signal amplification and allowed high numbers of samples to be analysed in a short time without the operator fatigue associated with manual microscopy.

The novel three-color FISH assay enables simultaneous identification of the two major human infectious *Cryptosporidium* species, *C. parvum* and *C. hominis* by both EFM and FCM. In addition, FISH is a rapid alternative to PCR and RFLP that is approximately 5-fold more cost effective, which can be completed within a 3-hour timeframe [1, 2]. The FISH assay was primarily developed for species-level identification of *Cryptosporidium* oocysts isolated from fecal samples but it has the potential to be adapted for the analysis of *Cryptosporidium* oocysts in water samples such as required by the USEPA method 1623 [6]. In the future, FISH using species specific probes could be used as an alternative tool for the accurate identification of human infectious *Cryptosporidium* species. Such a tool could be applied to both epidemiological and outbreak investigations of *Cryptosporidium*.

ACKNOWLEDGEMENT

This research was funded by the Environmental Biotechnology CRC Pty Ltd, a centre established and supported under the Australian Government's Cooperative Research Centres Program at Macquarie University and the University of New South Wales. Thanks go to Dr Michele Power and Liette Waldron of Macquarie University for the provision of PCR- and RFLP-typed *Cryptosporidium* species for validation in this study.

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DOI:10.5963/PHF0202007

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DOI:10.5963/PHF0202007