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Differentiation between Viable and Dead *Cryptosporidium* Oocysts Using Fluorochrome Staining

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The use of nucleic acid staining with a fluorochrome dye to differentiate viable and dead (heat-killed) *Cryptosporidium* oocysts was assessed. The specificities (percentage of unstained viable oocysts) and sensitivities (percentage of stained dead oocysts) of the seven tested dyes (SYTO-17® and SYTO-59® to 64®) ranged from 65 to 76% (average 71%) and 83 to 95% (average 91%), respectively. SYTO-59 and SYTO-17 imparted greater color (4+) intensity than the other dyes (2+ or less). Of these two dyes, SYTO-17 exhibited more brightness and slower discoloration and was selected for use in further experiments. The optimum staining time for SYTO-17 at 37°C was one hour or more (sensitivity of 96%). Dye concentrations of 20 and 30 μM resulted in maximal color intensity, and no further improvement was observed with further increases in dye concentration. Staining a mixture of viable and dead oocysts (1:1 ratio) with 20 μM dye at 37°C for one hour yielded the expected results (approximately 50%), but no remarkable increase in the percent staining with time (up to 8 hours) was observed. In this study, no ghost oocysts were observed. The present study indicated that the fluorogenic nucleic acid dye SYTO-17 could be used to discriminate between live and dead *Cryptosporidium* oocysts.

INTRODUCTION

Cryptosporidium is a coccidian parasite that is widely distributed in nature, and it is one of the important causes of diarrheal disease in man, both in developing and developed countries. The illness is self-limiting in immunocompetent individuals but may be life-threatening in young children and immunocompromised individuals (4,8,14). Infection is acquired by the ingestion of viable oocysts released (in millions) in the feces (15) of infected humans and animals (4,8,14). Many outbreaks associated with surface water (11,14) and deep borehole water (18) contamination have been reported, and the worst outbreak occurred in Milwaukee (USA) in 1993 (11). *Cryptosporidium* oocysts can escape water treatment systems and enter distribution systems, as they are resistant to the chemical agents used in water treatment systems.

Considering this public health problem, various preventive measures and oocyst detection methods have been developed for use in public water works facilities (14). However, the mere detection of *Cryptosporidium* oocysts in drinking water does not indicate their infectivity, as only viable oocysts cause infections. To differentiate between viable and dead oocysts, various methods have been described, including the inclusion/exclusion of vital dyes (propidium iodide (PI) and 4,6-diaminodino-2-phenylindole (DAPI)) (3), *in vitro* oocyst excystation (13), parasite morphology analysis (6), uptake/exclusion of fluorochrome dyes (2,10), animal infectivity (7), tissue culture (16) and reverse transcriptase-polymerase chain reaction (RT-PCR) (9,17). Of these methods, however, only the animal infectivity method provides direct evidence of the ability of oocysts to cause infection, but this method involves a large number of experimental animals.

In an effort to improve cost-effectiveness and simplicity, we used viable and heat-killed oocysts to examine the relevance of seven nucleic acid stains (SYTO-17 and SYTO-59 to 64) to discriminate viable oocysts from dead oocysts (2,10).

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MATERIALS AND METHODS

1. Preparation of *Cryptosporidium* oocysts

Calf feccal specimens were collected from Kinashi Farm, Kobayashi, Miki City, Hyogo Prefecture, Japan, with the permission of the owner of the farm. *Cryptosporidium* oocysts recovered from calf feces by immunomagnetic separation (Dynabeads, Dynal, Oslo, Norway) were used in this study. The number of oocysts was assessed in a counting chamber (CSTI counter, Funakoshi, Tokyo, Japan). A stock oocyst suspension containing 1 x 10^7 oocysts/ml was prepared in phosphate-buffered saline (PBS), and a working suspension of 1 x 10^6 oocysts/ml was prepared from the stock suspension. Oocysts were used within a week of their recovery. A 1.5-ml suspension was boiled for 10 min to kill the oocysts (Table I).

Table I. Comparison of the abilities of seven fluorescent nucleic acid dyes (SYTO) to stain *Cryptosporidium parvum* oocysts

Dye (SYTO)	Untreated oocysts			Heat-killed oocysts*		
	No. of oocysts	No. of oocysts (%)		No. of oocysts	No. of oocysts (%)	
	examined	unstained	stained	examined	unstained	stained
17	143	100（70）	43 (30)	156	11 (7)	145 (93)
59	179	132（74）	47 (26)	143	7 (5)	136 (95)
60	192	147（76）	45 (23)	139	18 (13)	121 (87)
61	118	84（71）	34 (29)	113	7 (6)	106 (94)
62	161	110（68）	51 (32)	127	16 (13)	111 (87)
63	213	159（75）	54 (25)	122	18（17）	101 (83)
64	100	65（65）	35 (35)	139	16 (13)	132 (95)

* Oocysts were killed by boiling for 10 min and then stained at 37°C.

2. Fluorochrome staining

Commercially available, fluorogenic nucleic acid dye solutions (Thermo Fisher Scientific, Tokyo, Japan), including SYTO-17 and SYTO-59 to 64, were used in this study. The working dye solutions (concentration of 100 μM) were prepared by diluting the dyes 1:50 in PBS. The staining of the oocysts was performed as described by Belosevic et al. (2). An oocyst suspension (50 μl; approximately 5 x 10^4 oocysts) was mixed with 12.5 μl of working dye solution (final concentration of 20 μM) in a 1.5-ml microtube, covered with aluminum foil (to protect the samples from light), vortexed and incubated at 37°C for one hour. A counting chamber was loaded with the oocyst suspension, and the suspension was observed using a fluorescence microscope (BX60, OLYMPUS, Tokyo, Japan: 488–568 nm). For combined staining, the SYTO-17-stained oocysts were stained with a FITC (fluorescein-isothiocyanate)-labeled anti-*Cryptosporidium* oocyst antibody (10 μl) (Crypto-Cel, Cellabs, Brookvale, Australia) for 1 hour at 37°C. The SYTO-17 dye was diluted (5, 10, 20, 30 and 50 μM) and used to stain dead oocysts for different time periods (0.5, 1, 2, 4, 5, 6 and 8 hours) and at different temperatures (4, 20 and 37°C). The DAPI/PI (4,6'-diamino-2-phenylindole/propidium iodine) staining of oocysts was performed by using a method described by Ozawa et al. (12).

3. Discrimination of non-infective oocysts from infective oocysts

Microscopic examination of the oocysts was performed using differential interference contrast (DIC) and fluorescent microscopy. Clearly stained oocysts were considered dead (non-infective), whereas unstained oocysts were considered viable (infective). The color intensity of the stained oocysts was characterized into four grades from 1+ (very faint staining) to 4+ (dark staining). The number of dead oocysts was calculated as follows: percent dead = (number of stained oocysts counted / total number of oocysts - number of ghost oocysts) x 100 (2).

4. Others

No humans or non-human primates were used in this study, and no samples were collected from endangered or protected species.

RESULTS

1. Specificity and sensitivity obtained from different dyes

In this study, the relevance of fluorogenic nucleic acid staining for the discrimination of viable *Cryptosporidium* oocysts from dead oocysts was evaluated using viable and heat-killed oocysts. The specificities (percentage of unstained viable oocysts) of the seven stains ranged from 65 to 76%. In contrast, the sensitivities (percentage of stained dead oocysts) ranged from 83 to 95% (Table I). The results revealed that the specificity (average 71%) was reduced compared with the sensitivity (91%) of each dye (Table I). Of the seven dyes, SYTO-59 and SYTO-17 had more intense color (4+), whereas the other five dyes exhibited a lower intensity of (2+) or less. SYTO-17 had relatively brighter color and slower discoloration than SYTO-59. Due to the slow discoloration, the SYTO-17 dye was used in the following experiments.

2. Optimal condition for SYTO-17 staining

Of the different durations and temperatures used for dead oocyst staining, one hour or more at 37°C was the best staining condition for SYTO-17, with sensitivities of 75 to 96% (Table II). Based on this finding, a staining time of one hour at 37°C was applied in subsequent experiments. Final SYTO-17 concentrations of 20 and 30 μM resulted in maximal staining, and no further improvement in oocyst staining was observed with further increases of dye concentration (Table III).

3. Discrimination of non-infective oocysts from infective oocysts

The results indicated that the optimal staining condition was as follows: SYTO-17 dye concentration of 20 μM for one hour at 37°C. When a mixture of viable and dead oocysts (1:1 ratio) was stained, approximately half of the oocysts were stained as expected, and the percentage of staining did not markedly increase despite prolonging the staining (up to 8 hours) (Table IV). Figure 1 shows the same oocysts observed by different methods, namely DIC (upper left) and immunofluorescence staining. All oocysts were stained by indirect fluorescent antibody (IFA) (upper center), and only four of them stained positive for SYTO-17 (upper right) (dead oocysts). In this study, no ghost oocysts were observed. Photo of lower left showed the DAPI positive oocyst and four distinct nuclei in are clearly seen.

Table II. Effects of staining period and the temperature used to heat-kill* the *Cryptosporidium parvum* oocysts when staining with a fluorescent nucleic acid dye (SYTO-17)

Staining period (hour)	Temperature (°C)	Total number of oocysts examined	No. of oocysts (%)	
			unstained	stained
0.5	4	113	26 (23)	87 (77)
	20**	103	34 (33)	69 (67)
	37	102	26 (25)	76 (75)
1	4	114	23 (20)	91 (80)
	20	105	29 (28)	76 (72)
	37	115	5 (4)	110 (96)
2	4	105	14 (13)	91 (87)
	20	114	9 (8)	105 (92)
	37	104	4 (4)	100 (96)
4	4	117	20 (17)	97 (83)
	20	125	6 (5)	119 (95)
	37	154	6 (4)	148 (96)

*Oocysts were heat-killed by boiling for 10 min. **Oocysts were stained at room temperature.

Table III. Effect of the fluorescent nucleic acid dye (SYTO-17) concentration on the staining efficiency of heat-killed *Cryptosporidium parvum* oocysts*

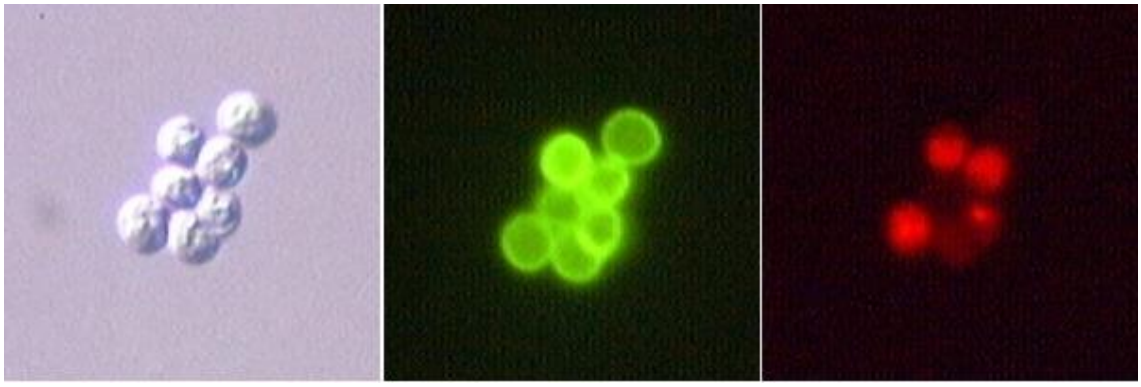
Dye concentration (μM)	Total number of oocysts examined	No. of oocysts (%)	
		unstained	stained
5	186	67 (36)	119 (64)
10	121	24 (20)	97 (80)
20	130	6 (5)	124 (95)
30	131	6 (5)	125 (95)
50	135	8 (6)	127 (94)

* Oocysts were stained at 37°C for one hour.

Table IV. Staining with a solution containing both untreated and heat-killed* *Cryptosporidium parvum* oocysts (1:1) by a fluorescent nucleic acid dye (SYTO-17)

Staining period (hours)	No. of oocysts			Stained (%)
	examined	stained	unstained	
0.5	145	72	73	50
1	138	64	74	46
2	173	61	112	54
4	147	80	67	54
5	150	80	70	53
6	134	79	55	59
8	129	72	57	56

● Oocysts were heat-killed by boiling for 10 min and stained at 37°C.



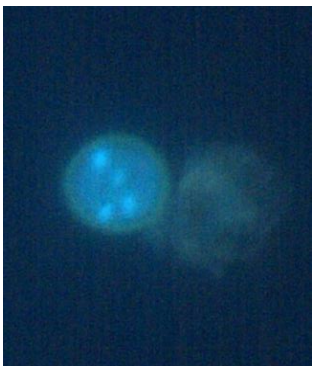


Figure 1. *Cryptosporidium* oocysts. *Cryptosporidium* oocysts (mixture of viable and dead oocysts at a 1:1 ratio) observed by different methods. Seven oocysts were observed using a DIC microscope (upper left). All oocysts were clearly observed by IFA staining (upper center). However, only four oocysts stained positive for SYTO-17 (upper right). Lower left shows DAPI-positive and PI-negative viable oocyst. Four distinct nucleuses in the oocyst are clearly seen.

DISCUSSION

To detect *Cryptosporidium* oocysts, extensive investigations under strict regulations are performed at public water works facilities (14). However, the mere detection of oocysts in the source and/or treated water does not indicate infectivity. Therefore, it is necessary to confirm the viability (infectivity) of the oocysts. The DAPI/PI staining developed to confirm the presence of *Cryptosporidium* oocyst by DAPI staining and to confirm the viability of oocyst by PI staining. However, it is known that the stainability of PI is affected by the temperature of staining or acidified HBSS pretreatment of oocysts. Therefore, it is thought that the reliability of evaluation of viability by PI staining is not necessarily high (5). Thus, we studied the effectiveness of fluorochrome nucleic acid dye staining to discriminate between the live and dead oocysts (2,10).

The specificities of the seven dyes used in this study were low (mean: 71%). It was unclear why an average of 29% of the oocysts stained were live. Physical and biochemical characteristic changes may have occurred in the oocysts (1). In addition**,** some of the oocysts probably died during the recovery process by the immunomagnetic separation method. In contrast, the sensitivity of the procedure was quite high (91%), but it was not 100% even though the oocysts were subjected to heat killing.

Of the seven dyes used in this study, SYTO-59 and SYTO-17 exhibited consistency in oocyst staining with a better intensity (4+) of the heat-killed oocysts than the other five dyes. Belosevic et al. (2) also reported better staining results with SYTO-59 and SYTO-9, but these authors did not include SYTO-17 in their study. However, we did not use SYTO-9 in the present study. As a single staining dye, SYTO-17 stained dead oocysts more brightly and stably than the other dyes. Based on these observations, the SYTO-17 dye was used for dead oocyst staining under different conditions (dye concentration, staining time and temperature). In view of cost-effectiveness, the staining conditions used in this study could be useful for the discrimination of the *Cryptosporidium* oocysts in routine workplaces.

The staining result of the viable and dead oocyst suspensions (1:1 ratio) was as expected. The percent staining, however, did not markedly increase despite the prolonged staining (up to eight hours), thereby indicating that the staining conditions in this study were optimal (positive and negative reactions were clearly distinguishable). In the present study, no ghost oocysts, such as those previously reported by other scientists (2), were observed, which may be due to the use of fresh *Cryptosporidium* oocysts recovered from the feces of calves less than two months old (15).

Single nucleic acid staining is reportedly superior to the *in vitro* excystation technique for the evaluation of the viability of parasites, particularly in the study of infectivity in animals (2). The results of this study confirmed that nucleic acid staining with fluorogenic dyes is a simple, reproducible and effective tool for the detection and discrimination between viable and dead *Cryptosporidium* oocysts in water treatment systems. We need not care about the outbreak of the cryptosporidiosis if the oocyst is not infective. However, in Japan, we have to stop the water supply, if *Cryptosporidium* oocysts are found at the water works facilities or in the distribution creates, regardless of the viability of the oocyst. This countermeasure is sometimes a cause of panic among the public. Therefore, priority should be placed on viability confirmation of the oocysts on watershed management and monitoring. In addition, effort should be paid to reduce the chances of water source contamination as well as installing physical barriers (such as nanofilters) (8) to prevent outbreaks of water-borne cryptosporidiosis.

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