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DETECTION OF *CRYPTOSPORIDIUM PARVUM* AND *GIARDIA LAMBLIA* CARRIED BY SYNANTHROPIC FLIES BY COMBINED FLUORESCENT *IN SITU* HYBRIDIZATION AND A MONOCLONAL ANTIBODY

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Abstract. Wild-caught synanthropic flies were tested for the presence of *Cryptosporidium parvum* and *Giardia lamblia* on their exoskeletons and in their digestive tracks by fluorescent *in situ* hybridization and fluorescein isothiocyanate (FITC)–conjugated monoclonal antibody (MAb) against *Cryptosporidium* and *Giardia* cell wall epitopes. The levels of *C. parvum* were positively correlated with the levels of *G. lamblia*, indicating a common source of contamination. The majority of oocysts and cysts were potentially viable (*C. parvum* = 80% and *G. lamblia* = 69%). More *G. lamblia* cysts occurred on the exoskeleton of the flies than within the digestive tracts; the opposite relationship was observed for *C. parvum*. No genotype other than *C. parvum* G2 was found to be associated with flies. Because filth flies carry viable *C. parvum* oocysts and *G. lamblia* cysts acquired naturally from unhygienic sources, they can be involved in the epidemiology of cryptosporidiosis and giardiasis. Fluorescent oligonucleotide probes used together with FITC-conjugated MAb represent a convenient and cost-effective technique for rapid and specific identification of human-infectious species of *Cryptosporidium* and *Giardia* mechanically transported by flies, and for the assessment of the viability of these pathogens.

INTRODUCTION

Cryptosporidium parvum is an anthropozoonotic protozoan parasite that significantly contributes to mortality of people with various immune system impairments.[1] Diarrheal disease is initiated by a microscopic stage, the oocyst, and a single oocyst can cause infection in immunosuppressed individuals.[2] The 50% infectious dose (ID_{50}) of geographically diverse isolates of *C. parvum* for immunocompetent people ranges from 9 to 1,042 oocysts.[3] *Giardia lamblia* is another anthropozoonotic protozoan parasite that is transmitted via microscopic cysts and causes diarrheal disease worldwide.[4] This species has also been referred to as *G. duodenalis* or *G. intestinalis*.[5] Detection of *C. parvum* and *G. lamblia* in environmental samples is commonly achieved by fluorescein isothiocyanate (FITC)–conjugated monoclonal antibody (MAb) (immunofluorescent assay [IFA]).[6] However, these assays are not specific to *C. parvum* and other species of *Cryptosporidium* can produce a positive IFA reaction.[7]

Refuse and promiscuous-landing synanthropic filth flies are transport hosts for a variety of pathogens of public health importance, as reviewed recently.[8] Outbreaks of foodborne diarrheal diseases demonstrate distinct seasonal patterns which coincide with seasonal increases in abundance of synanthropic flies during warm months of the year.[9,10] Interestingly, the first complete reports on foodborne cases of cryptosporidiosis for the United States provided by the Foodborne Disease Active Surveillance Network (FoodNet) demonstrated a strong seasonal trend.[11,12] The involvement of flies in mechanical transmission of *C. parvum* in experimental settings has been recently reported,[13–15] but it not known if wild-caught filth flies carry this pathogen. Molecular data demonstrated that *C. parvum* can be carried by flies for at least three weeks after elimination of the contamination source.[15] Although *G. lamblia* cysts have been reported in synanthropic flies, the viability of these cysts was not determined.[16]

Fluorescent *in situ* hybridization (FISH) uses fluorescently labeled oligonucleotide probes targeted to species-specific sequences of 18S rRNA.[17] Since rRNA has a short half-life and is only present in high copy numbers in viable organisms, FISH can differentiate between potentially viable and non-viable cells.[18–21] The FISH technique has been developed for specific detection of *C. parvum* and *G. lamblia*.[19–21]

The purpose of the present study was to determine if wild-caught synanthropic filth flies carry anthropozoonotic pathogens such as *C. parvum* and *G. lamblia* and, if so, to determine whether pathogens carried by flies are potentially viable. An additional purpose was to test the suitability and to optimize the protocol of dual labeling, FISH and FITC-conjugated MAb, of *C. parvum* and *G. lamblia* recovered from non-biting flies.

MATERIALS AND METHODS

Wild fly capture, identification, and processing. Non-biting synanthropic flies were captured in August 2001 at three locations near Raleigh, North Carolina: 1) a North Carolina State University (NCSU) dairy unit, 2) an NCSU waste-processing facility, and 3) grocery store trash dumpsters near Lake Wheeler. The air temperatures ranged from 26°C to 33°C, and the relative humidity ranged from 95% to 100%, as recorded on line by the North Carolina Agricultural Research Station Weather and Climate Network. Flies were captured with a sweep net over surfaces where flies were landing. Flies were placed into labeled plastic bags, transported to the laboratory in a cooler with ice packs, and stored at 4°C until processed.[15] Flies were identified to the family taxon level using morphologic criteria[9,22] and surface-eluted as described previously.[15] After elution, the flies were homogenized in batches of 10 specimens in an Eppendorf tube (VWR, Piscataway, NJ) with a plastic grinding pin fitting the bottom of the tube,[23] and the homogenate (approximately 1 mL) was trans-

ferred to a 15-mL tube. The eluting fluid[24] was added to the homogenate to obtain a volume of 15 mL and decanted after debris sedimented to the bottom, resulting in a volume of approximately 13 mL. The surface eluants (15 mL) and decanted fluids (approximately 13 mL) were separately processed by the cellulose acetate membrane (25-mm diameter, 3.0-μm pore size; Millipore, Bedford, MA) (CAM)-filter dissolution method.[24] Processing of *C. parvum* oocysts by the CAM-filter dissolution method does not alter their infectivity as tested *in vivo* by mouse bioassay,[25] although *in vitro* testing indicates that oocyst infectivity decreases.[26] Surface elution ensures recovery of particles derived from the exoskeleton of the fly, and homogenization releases the particles within the digestive tract.[15] The resulting pellets were stored in 200 μL of 95% ethanol at 4°C[18] until analyzed by FISH and FITC-conjugated MAb.

Oligonucleotide probes. A *C. parvum*-specific oligonucleotide probe, i.e., Cry 1, (5′-CGG TTA TCC ATG TAA GTA AAG-3′) was used.[19] The probe targets the positions between 138 and 160 on the *C. parvum* 18S rRNA[27] and does not hybridize with oocysts of *C. baileyi* and *C. muris*.[19] Oocysts of *C. parvum* with fluorescing internal structures after hybridization tested viable and oocysts without fluoresce were dead.[18,19] Two probes specific for *G. lamblia* (as tested against *G. microti*, *G. ardea*, and *G. muris*) were used; Giar-4 (5′-CGG CGG GGG GCC AAC TAC-3′), and Giar-6 (5′-CGG GGC TGC CGC GGC GCG-3′)[20] were used for all samples. *Giardia lamblia* cysts producing a positive FISH reaction tested viable.[20] All probes were synthesized by the DNA Analysis Facility of the Johns Hopkins University (Baltimore, MD) in 1.0-μM scale, purified by high-performance liquid chromatography, and labeled with a single molecule of a fluorochrome (hexachlorinated 6-carboxyfluorescein).

Monoclonal antibodies. An FITC-conjugated combination of MAb against the cell wall antigens of *Cryptosporidium* and *Giardia* from the MER***IFLUOR***™ *Cryptosporidium*/*Giardia* test kit (Meridian Diagnostic, Inc., Cincinnati, OH) was used.

Testing of oligonucleotide probes and MAb. *Cryptosporidium parvum* and *G. lamblia* were obtained from experimentally infected calves, and *C. serpentis* was obtained a clinically infected snake (*Pituophis melanoleucus*).[28] *Cryptosporidium serpentis* oocysts were used because the Cry 1 probe has not been tested with this species.[19] Cysts and oocysts were purified by cesium chloride gradient centrifugation[29] and stored at 4°C in phosphate-buffered saline (PBS) pH 7.4. *Cryptosporidium parvum* and *C. serpentis* oocysts were subjected to treatments with 70% formic acid, 70% acetic acid, and 5% dodecyltrimethyl ammonium bromide (DTAB) for 30 min at 80°C to increase oocyst wall permeability.[19] *Giardia lamblia* cysts were not subjected to wall permeabilization treatment because this does not effect the intensity of FISH.[20] In addition *C. parvum* and *C. serpentis* oocysts and *G. lamblia* cysts were subjected to pretreatment with acetone and ethanol as used in the CAM-filter dissolution method.[24] The oligonucleotide probes, i.e., Cry 1, Giar-4, and Giar-6, and FITC-conjugated MAb were tested jointly for their reactivity with *C. parvum*, and *C. serpentis* oocysts and *G. lamblia* cysts under the previously described conditions.[19,20]

Fluorescent *in situ* hybridization and FITC-conjugated MAb. All FISH and FITC-conjugated MAb testing applied to all material recovered from flies, i.e., surface eluant and decanted fluid pellets, was carried out in Eppendorf tubes in a total volume of 100 μL of hybridization buffer[21] at 48°C for one hour.[20] The concentration of each oligonucleotide probe was 1 mM and MAb was diluted 1:1. After incubation, the tubes were centrifuged twice at (8,000 × *g* for two minutes at 4°C and the pellets were resuspended in 100 μL of PBS. Five 20-μL samples were transferred onto five lysine-coated wells (5-mm diameter) on a teflon-coated glass slide (Carlson Scientific, Inc., Peotone, IL) and air-dried. The wells were examined with an Olympus (Melville, NY) BH2-RFL epifluorescent microscope with a dry 60× objective and a BP-490 exciter filter. The number of potentially viable *C. parvum* and dead, i.e., empty, oocysts (oocyst shells) was counted. A similar enumeration procedure was applied to *G. lamblia* cysts with the modification that small amounts of diffuse fluorescing cysts were also scored as dead. Infectious *C. parvum* oocysts and *G. lamblia* cysts obtained from experimentally infected calves were used as positive controls. Negative controls consisted of snap-frozen and thawed *C. parvum* oocysts and *G. lamblia* cysts.

Extraction of *C. parvum* DNA, amplification by a polymerase chain reaction (PCR), and sequencing of the amplified fragments. The DNA was extracted from 20 (seven each from sites 1 and 2 and six from site 3) 150-μL aliquots of eluants containing fly-derived debris using proteinase K digestion and glass bead disruption of the oocysts as described elsewhere.[15,30] The PCR amplification using primers for the *Cryptosporidium* oocyst wall protein (COWP) and small subunit (SSU) rRNA genes[31,32] followed our previously described protocols.[15] Sequencing analysis was performed based on a published protocol[33] with some modifications. Briefly, amplified products from all PCRs were purified using the Strataprep PCR purification kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. Sequencing reactions were analyzed on the Perkins Elmer (Wellesley, MA) ABI 3100 automatic DNA sequencer, and the sequences were assembled using SeqMan II (DNASTAR Inc., Madison, WI).

Statistical analysis. The numbers of *C. parvum* oocysts and *G. lamblia* cysts recovered by the CAM-filter dissolution method was adjusted for the method recovery efficiency of 78.8%.[24] Statistical analysis was carried out with Statistix 4.1 (Analytical Software, St. Paul, MN). The numbers of *C. parvum* oocysts and *G. lamblia* cysts recovered from flies were tested by Wilk-Shapiro ranking plots to determine whether their distribution conformed to a normal distribution and if so, a parametric test, i.e., two-sample t test, was used. The degree of linear association was assessed using Pearson's correlation coefficient (R). Statistical significance was considered to be a $P < 0.05$.

RESULTS

Fly trapping. The numbers of flies representing families Muscidae, Calliphoridae, Lauxaniidae, and Anthomyiidae were 246, 105, 25, and 25, respectively (Figure 1). Although representatives of all families were found at each site, Muscidae dominated at the dairy (66% of all flies) and waste facility (72% of all flies) sites (Figure 1).

Testing of oligonucleotide probes. *Cryptosporidium serpentis* oocysts did not produce a positive FISH result in any of the trials. Treatments of *C. parvum* oocysts with 70% formic acid, 70% acetic acid, and 5% DTAB resulted in significantly brighter fluorescence in comparison with untreated oocysts.

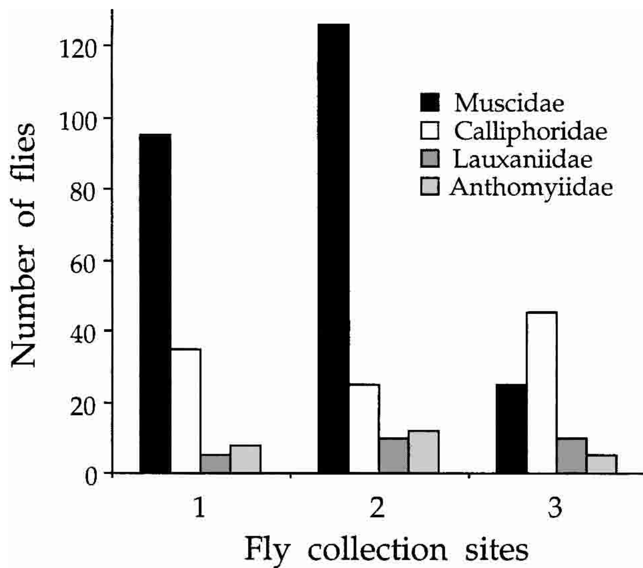

FIGURE 1. Results of trapping in August 2001 of wild synanthropic filth flies at a North Carolina State University (NCSU) dairy unit (site 1), an NCSU waste-processing facility (site 2), and grocery store trash dumpsters near Lake Wheeler (site 3).

However, fluorescence intensity of treated oocysts was similar to the intensity of fluorescence observed for *C. parvum* oocysts pretreated with acetone and ethanol. Pretreatment with acetone and ethanol did not increase fluorescence intensity of *G. lamblia* cysts.

Labeling of fly-recovered material based on FISH and FITC-conjugated MAb. Potentially viable *G. lamblia* cysts were clearly differentiated from nonviable and non-*G. lamblia* cysts by color as a result of FISH and labeling with the MAb (Figure 2). Nonviable cysts were represented by 1) shells with apparently structurally damaged walls (Figure 2C and 2) intact cells with a very small amount of internal structures with diffused appearance (Figure 2B). In comparison, potentially viable intact cysts were filled out completely with cytoplasm without the gap between the internal structures and the wall (Figure 2A).

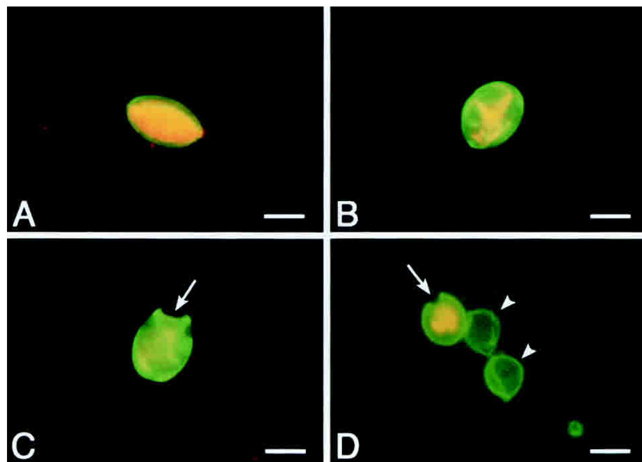

Figure 2. Fluorescent *in situ* hybridization and fluorescein isothiocyanate–conjugated monoclonal antibody images of *Giardia lamblia* cysts (**A**, **B**, and **C**) and *Cryptosporidium parvum* oocysts (**D**) recovered from wild-caught synanthropic filth flies. **A**, Viable *G. lamblia* cyst. **B**, nonviable *G. lamblia* cyst. **C**, *G. lamblia* cyst shell (nonviable cysts) with structural wall damage (**arrow**). **D**, Viable *C. parvum* oocyst (**arrow**) and oocyst shells (nonviable oocysts) (**arrowheads**). Note the structural damage to the oocyst wall and a small gap between the oocyst wall and the internal structures of the viable oocyst. Scale bars = 7 μm in **A**, **B**, and **C** and 4 μm in **D**.

The FISH procedure and labeling with the MAb clearly differentiated potentially viable *C. parvum* oocysts from nonviable and non-*C. parvum* oocysts (Figure 2). Oocysts labeled by FISH and the MAb were predominantly intact, showed a small gap between the oocyst wall and internal structures, and in most of them the sporozoites were visible (Figure 2). In comparison, dead oocysts frequently showed discernable damage to their walls (Figure 2). Rarely, FISH-positive potentially viable oocysts had noticeable ruptures in their walls, which was clearly revealed by staining with the MAb (Figure 2).

No FISH-negative, MAb-negative or FISH-positive, MAb-negative pathogens were observed. Fluorescence of other particles in the samples was not observed except for weak autofluorescence of nonstructural debris.

The numbers of fly-derived potentially viable and nonviable *C. parvum* oocysts and *G. lamblia* cysts are shown in Table 1. The numbers of *C. parvum* showed a positive correlation with the numbers of *G. lamblia* ($R = 0.99$, $P < 0.03$, by Pearson's correlations), indicating a common source of contamination. In general, more *G. lamblia* were found than *C. parvum*, and more viable pathogens were found than nonviable pathogens (Table 1). Viable *C. parvum* oocysts accounted for 80% of all oocysts, and more than 69% of *G. lamblia* cysts were also viable (Table 1). Significantly more *G. lamblia* cysts were found on fly exoskeleton than within the digestive tracts ($t = 32.3$, $P < 0.03$, by two-sample t test); the opposite relationship was observed with *C. parvum* (Table 1). There was a significant positive correlation ($R = 0.73$, $P < 0.01$, by Pearson's correlations) between viable and nonviable pathogens identified in the digestive tracts of flies collected from all three sites for both parasite species.

Polymerase chain reaction and genotyping of *C. parvum*. The PCR amplicons were obtained with DNA extracted from all oocyst isolates by applying three primer pairs for amplification fragments of the COWP and SSU rRNA genes. All fly eluate DNA samples were tested with each primer and

TABLE 1

Cryptosporidium parvum oocysts and *Giardia lamblia* cysts carried by wild-caught synanthropic filth flies and detected by combined fluorescent *in situ* hybridization and fluorescein isothiocyanate–conjugated monoclonal antibody*

Fly collection sites	Pathogen extraction site, number of fly batches	Mean number of pathogen cystic stages per fly					
		C. parvum			*G. lamblia*		
		Viable	Dead	Total	Viable	Dead	Total
1	Exoskeleton, 15	4.1	2.7	6.8	13.8	8.1	21.9
	Gut, 15	11.4	2.0	13.4	3.6	0	3.6
2	Exoskeleton, 18	5.7	1.7	7.4	8.3	5.5	13.8
	Gut, 18	7.5	1.6	9.1	4.5	1.3	5.8
3	Exoskeleton, 8	4.4	2.2	6.6	7.6	3.8	11.4
	Gut, 8	7.0	0	7.0	4.0	0	4.0
Mean total value		6.7	1.7	8.4	7.0	3.1	10.1

* Site 1 = North Carolina State University (NCSU) dairy unit, Site 2 = NCSU waste-processing facility; Site 3 = grocery store trash dumpsters near Lake Wheeler. Flies were processed in the batches of 10 specimens and the numbers of flies are shown in Figure 1.

all amplicons were sequenced. Sequencing analysis of these amplicons has shown that all isolates were uniformly *C. parvum* G2 (Genotype 2) in both loci, the genotype commonly found in humans and animals.

DISCUSSION

The present study demonstrates that wild synanthropic flies carry potentially viable anthropozoonotic pathogens (*C. parvum* and *G. lamblia*) naturally acquired from unhygienic sources such as feces, manure, toilets, abattoirs, trash, carcasses, or sewage. Thus, flies can be involved in giardiasis or cryptosporidiosis via deposition of these pathogens on visited surfaces, i.e., food items or raw, pre-processed food products. Since mechanical transfer of pathogens by flies can be achieved through defecation, regurgitation, or mechanical dislodgement,[8,9] the proportion of viable versus dead cysts is of epidemiologic importance. The troubling finding of the present study is that the vast majority of pathogens were viable; thus, capable of infection. Epidemiologic involvement of non-biting flies in cryptosporidiosis and giardiasis is difficult to prove because cases of diarrheal diseases resulting from fly visitations will be classified as foodborne. Foodborne cases and outbreaks of cryptosporidiosis and giardiasis have been extensively documented.[11,12,34]

The biology and ecology of synanthropic filth flies suggest that their potential for mechanical transmission of *C. parvum* and *G. lamblia* is high. The females can live up to a month,[9] lay up to six egg batches,[9] and produce up to 12 generations in the summer[9] and several generations during winter (while indoors).[9] Filth flies can travel up to 20 miles,[35] and their movement is oriented toward unsanitary sites.[9,35]

The results of the FISH applications were largely in accordance with those obtained previously for *C. parvum* and *G. lamblia*.[19–21] The present study used FITC-conjugated MAb together with three fluorescently labeled oligonucleotide probes specific for both pathogen species. Incorporation of FITC-conjugated MAb into the FISH protocol allows (irrespectively of the FISH outcome) observation of the external morphology of pathogens and assessment of any structural damage to their walls. Thus, even pathogens that are FISH positive and theoretically viable[19,20] can be nonviable based on cell wall damage. An advantage of our dual approach is shown in Figure 2D, in which all three *C. parvum* oocysts produced positive IFA reactions when tested according to the current U.S. drinking water regulations.[36,37] However, only one of these oocysts posed a potential public health risk, which was demonstrated exclusively by the combination of the FISH technique with staining with an MAb. Additionally, the dual use of oligonucleotide probes and mAb eliminates the need for labeling the probes with multiple molecules of a fluorochrome.[19]

The FISH technique has multiple major advantages (intensively discussed previously)[19,20] over the PCR and FITC-conjugated MAb, i.e., IFA, used alone. The most important advantage of the FISH technique and the fluorescent MAb used in combination in the present study was the assessment of viability of even a single pathogenic cell. Such resolution is not available, or extremely impractical, in any other technique. For example, a recently developed and highly sensitive reverse transcriptase–PCR for detection of RNA showed that the lowest number of *C. parvum* oocysts that can be assessed for their viability was 10^3.[38] An additional practical feature of the combined protocol is elimination of the need for an extra step for permeabilization of oocyst walls,[19] since permeabilization is achieved through the procedural step of the CAM-filter dissolution method.[24]

When one considers the advantages of the FISH technique for identification of viable *C. parvum* and *G. lamblia*, it is surprising that this technique has not been more widely implemented into screening of a variety of environmental samples. Both pathogens can be transmitted via water and they are of serious concern (particularly *C. parvum*) to the drinking water industry[37,39] which prompted the application of the FISH technique for water testing.[19] However, because of autofluorescent unicellular algae that produce a high background level, the fluorescence of FISH-stained *C. parvum* oocysts was not bright enough to enable their detection in environmental water concentrates.[19] Since only weak autofluorescence of nonstructural debris was observed in the present study, the FISH procedure is a suitable, i.e., convenient and cost-effective, technique for identification of *C. parvum* and *G. lamblia* transported by insect vectors.

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