MECHANICAL TRANSPORT AND TRANSMISSION OF CRYPTOSPORIDIUM PARVUM OOCYSTS BY WILD FILTH FLIES

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Abstract. Over the course of six months wild filth flies were collected from traps left for 7–10 days in a barn with or without a calf shedding Cryptosporidium parvum Genotype 2 oocysts in diarrheic feces. The oocysts of C. parvum transported on the flies’ exoskeletons and eluted from their droplets left on visited surfaces were infectious for mice. The mean number of oocysts carried by a fly varied from 4 to 131, and the total oocyst number per collection varied from 56 to approximately 4.56 × 10^3. Fly abundance and intensity of mechanical transmission of infectious C. parvum oocysts were positively correlated, and both increased significantly when an infected calf was in the barn. Molecular data showed that the oocysts shed by infected calves were carried by flies for at least 3 weeks. Filth flies can acquire infectious C. parvum oocysts from unsanitary sites, deposit them on visited surfaces, and therefore may be involved in human or animal cryptosporidiosis.

INTRODUCTION

Cryptosporidium parvum is a zoonotic and anthropoontic protozoan parasite that primarily infects the intestinal epithelium. Extraintestinal infections are common in people with impaired immune systems where the parasite significantly contributes to mortality. The infectious stage, the oocyst, is transmitted via the fecal-oral route; food and water-borne infections are common. As few as 30 oocysts can cause infection in an immunocompetent person, and it is thought that a single oocyst can induce disease in immunocompromised and immunosuppressed individuals. Two genotypes of C. parvum have been identified by molecular techniques: The “animal-adapted” or “zoonotic” genotype (Genotype 2) is transmissible among cattle and humans, whereas the “human-adapted” genotype (Genotype 1) is thought to cycle exclusively within the human population.

Refuse and promiscuous-landing synanthropic filth flies are recognized transport hosts for a variety of parasites of public health and veterinary importance. These include the coccidian parasites Sarcocystis spp., Toxoplasma gondii, Isospora spp., and Eimeria tenella. Cryptosporidium parvum infections are particularly prevalent in pre-weaned cattle. Cattle manure is a recognized source of C. parvum oocysts and is also a favored breeding place, food source, and landing site of filth flies. The potential role of mechanical vectors other than flies in dissemination of infectious C. parvum oocysts in the environment and their role in the epidemiology of cryptosporidiosis has been intensively discussed. Flies come into direct contact with manure, and small particles readily adhere to their exterior surfaces. However, the role of flies as transport hosts for C. parvum oocysts has been only minimally elucidated. Under laboratory conditions house flies (Musca domestica) mechanically transported C. parvum oocysts and preliminary observations indicate that this may also occur in a natural situation. Also, under laboratory conditions dung beetles excreted and carried on their external surfaces C. parvum oocysts acquired from manure.

The purpose of the present study was to determine if wild, promiscuous-landing synanthropic filth flies can serve as mechanical vectors for C. parvum oocysts under natural conditions and, if so, whether the oocysts transported and transmitted by these flies are infectious.

MATERIALS AND METHODS

Fly trapping. Filth flies were trapped from April to September, 1999 in Beltsville, Maryland (31°01′76″53′). Ten flying insect traps of the Victor type (Woodstream, Lititz, PA) were baited with rotten fish flesh (located inside perforated 15-mL capacity plastic tubes) and placed inside a barn (approximately 880 m²). The barn was a cinder-block building with a poured concrete floor sealed with epoxy resin and cleaned weekly with a pressurized washer containing soap; insect screens were installed in the windows. No other insect control or trapping devices were present in the barn for the duration of the experiment. Traps were emptied at 7- to 10-day intervals, and flies were counted and identified. The inner surfaces of traps containing fly droplets, i.e., feces, regurgitates, and mechanically dislodged particles, were each eluted by agitation for 20 min with 200 mL of eluting fluid along with, or without, the flies (Figure 1). In some experimental options, flies from each trap were grouped by families, and each group was eluted separately (Figure 1). The resulting eluants were filtered through a cellulose acetate membrane (CAM) (25-mm diameter, 3.0-μm pore size) (Millipore, Bedford, MA). The membrane was processed as described previously, and C. parvum oocysts in the aliquots were identified by the immunofluorescent antibody (IFA) of the MERIFLUOR® test kit (Meridian Diagnostic, Inc., Cincinnati, OH). The oocysts were counted and some of them were measured to assist in identification of C. parvum. Processing of oocysts by the CAM-filter dissolution method does not alter their infectivity. Some aliquots were processed with acid-fast stain (AFS) to assess cellular morphology of the oocysts as an indication of potential viability. The remaining oocyst fractions were stored for 2 wk at 4°C in phosphate-buffered saline (PBS) pH 7.4. After each col-
lection, traps were washed thoroughly with warm water and soap (Backdown, Curtin Matheson Scientific, Inc., Houston, TX) to prevent any carry-over contamination.

Fly identification. Flies were removed from each trap separately, placed into a plastic petri dish and identified with the aid of a binocular microscope to the Family taxon level using morphologic criteria. The Family level was deemed adequate for simplicity and because the trapping procedure selected for species with similar feeding habits within each family.

Exposure of C. parvum-infected calves. During the six-month period a total of four male Holstein calves were experimentally infected with C. parvum, Genotype 2 (Figure 1). Each calf was housed in the barn for approximately 3 wk after oral inoculation with oocysts. Within the barn the calves were held in a metabolic crate with a plastic-coated wire-mesh floor which allowed urine to drain onto the barn floor and diarrheic feces to collect in a flat 1.5 × 3.0 m tray. All diarrheic feces were retrieved daily; C. parvum oocysts were recovered, purified over a CsCl3 gradient, and counted.

Determination of infectivity of fly-derived oocysts. The infectivity of pooled oocysts recovered from fly external surfaces and from the inner surface of the traps that were collected when C. parvum-infected calves were in the barn (Figure 1) was determined by mouse bioassay. Each of four, 9-day-old suckling BALB/c mice was gastrically inoculated with approximately 3.75 × 104 oocysts. The mice were killed 96 hr post-inoculation (PI) and a segment of ileum anterior to the ileocecal junction was fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 5 mm thick sections, and stained with hematoxylin and eosin. Sections were examined for developmental stages of Cryptosporidium by brightfield microscopy and the percent of infected epithelial cells recorded.

DNA extraction, PCR amplification and sequencing of the amplified fragments. DNA was extracted from aliquots of 150 μL of eluants containing debris of exoskeletons of flies trapped during the first six collections, samples 1 through 6, respectively (Figure 1). The samples were extracted without washing with the FastPrep disrupter and the FastDNA kit (Bio 101, Vista, CA) as described previously.

To identify C. parvum oocysts of Genotype 2 shed by infected calves, PCR amplification was performed using three distinct primer pairs to amplify polymorphic regions from the thrombospondin-related adhesive protein C1 (TRAP-C1) and the Cryptosporidium oocyst wall protein (COWP) genes, as well as a variable coding region of Cryptosporidium sp. small subunit ribosomal RNA (SSUrRNA). Polymerase chain reaction primers CPE/CPZ were used to amplify a fragment of 248 base pairs (bp) from the TRAP-C1 and primers CRY12/CRY14 were used to generate a fragment of 357 bp of the COWP C-terminal domain. To amplify the Cryptosporidium sp. SSUrRNA coding region, primers CPBDIAGF/CPBDIAGR were used. Amplification with these primers generate DNA fragments of 435, 431 and 455 bp of C. parvum, Cryptosporidium muris, and Cryptosporidium felis SSUrRNA coding region, respectively. PCR reactions were performed in 50 μL total volumes and the conditions for primers CPBDIAGF/CPBDIAGR and CPE/CPZ were as described previously, except that the annealing temperature for primers CPE/CPZ was 45°C and the reactions were subjected to 40 amplification cycles. Amplification with CRY12/CRY14 was performed as described previously23 with reactions being subjected to 45 cycles. DNA sequencing and analysis of the amplified fragments were carried out as previously described.

Statistical analysis. Statistical analysis was carried out with Statistix 4.1 (Analytical Software, St. Paul, MN). Variables were tested by Wilk-Shapiro/Ranking Plots to determine whether their distribution conformed to a normal distribution and if so, parametric tests, i.e., two-sample t-test, and analysis of variance (ANOVA), were used. The degree of linear association between variables was assessed using Pearson’s correlation coefficient (R) and linear regression. Average temperatures at the study area were retrieved from the on-line weather archive for the Beltsville Agricultural Research Center. The numbers of C. parvum oocysts recovered by the CAM-filter dissolution method was adjusted for the method recovery efficiency of 78.8%. Mean values (x̄) were associated with standard deviation (SD), and statistical significance was considered to be a P < 0.05.

RESULTS

Fly trapping. Approximately one third of all flies trapped were alive at the time of retrieval. The total number of flies recovered from 10 traps varied from 4 to 84 (mean ± SD [x̄ = 30 ± 15.4]), with no discernable temporal pattern (Figure 1) or statistically significant relationship to the ambient temperatures (P > 0.05) (Figure 2); however, the area was under drought conditions for much of the summer and this may have affected fly populations. The overall number of flies caught during each of the 3-wk periods when calves were present in the barn (x̄ = 41 ± 4.7) was significantly higher than the number of flies trapped (x̄ = 26 ± 8.7) when
The barn was empty (two-sample t-test; \( t = 4.36, P < 0.05 \)). The mean number of Cryptosporidium oocysts per fly varied from 4 ± 3.1 to 131 ± 10.5; \( \bar{x} = 50 ± 28.9 \) (Figure 2). Overall, \( \bar{x} = 86 ± 10.4 \) Cryptosporidium oocysts per fly were found for the time-periods with C. parvum-infected calves in the barn, and \( \bar{x} = 22 ± 5.4 \) when the calves were absent; the mean values were significantly different (two-sample t-test; \( t = 5.83, P < 0.01 \)). The total number of Cryptosporidium oocysts eluted from flies and the traps varied from 56 to approximately \( 4.56 \times 10^3 \), \( \bar{x} = 1.61 \times 103 \), and was significantly higher while the infected calves were in the barn (\( \bar{x} = 3.21 \times 10^3 \)) compared to collection (\( \bar{x} = 4.64 \times 10^2 \)) carried out during the absence of calf (two-sample t-test; \( t = 5.64, P < 0.01 \)).

Overall, for the six collections when the flies and traps were eluted separately (Figure 1), significantly higher numbers of oocysts were obtained from fly exoskeletons (\( \bar{x} = 830 \)) than from droplets in the traps (\( \bar{x} = 453 \)) (two-sample t-test; \( t = 10.0, P < 0.02 \)). The same relationship was observed for the two collections during which a C. parvum-infected calf was in the barn (Figure 1) (two-sample t-test; \( t = 1.85, P < 0.01 \)). However, when the infected calf was absent during the four collections (Figure 1), a significantly higher number of Cryptosporidium oocysts were eluted from traps than from the external surfaces of the flies (two-sample t-test; \( t = 1.89, P < 0.03 \)). There was a significant positive relationship between the number of Cryptosporidium oocysts carried by the flies on their external surfaces and the number deposited on visited surfaces, i.e., inside the traps (\( F = 4.81, P < 0.05 \)).

Overall, neither the number of Cryptosporidium oocysts per fly nor the total number of oocysts recovered from flies and traps correlate with the number of trapped flies (\( P > 0.05 \)). However, for the time-periods when C. parvum-infected calves were in the barn, the number of fly-and-trap-recovered oocysts correlate with the number of trapped flies (\( R = 0.85, P < 0.02 \)).

On average 69, 58, 69, 57, and 72 oocysts per fly were eluted from specimens representing families of Calliphoridae, Muscidae, Sarcophagidae, Anthomyiidae, and Psychodidae, respectively; these values were not significantly different (G-heterogeneity test; \( P > 0.05 \)).

Fly droplets were distributed homogeneously over the internal surface of the traps. Most of the IFA-stained oocysts recovered from flies and traps those in calf feces displayed a similar intensity of fluorescence and morphology. In the AFS, the oocysts derived from flies and the traps displayed non-uniform bright pink coloration and contained characteristic black granules similar to viable and infectious C. parvum oocysts recovered from calf feces. No oocyst shells, i.e., oocysts without cytoplasm, or oocyst ghosts were observed in AFS. The size of the oocysts eluted from flies and the traps collected during the presence of 4 C. parvum-infected calves in the barn conformed to the size of C. parvum oocysts: diameter; 4.2–5.5, \( \bar{x} = 5.1 ± 0.4 \) m (\( n = 30 \)).

**Fly identification.** During the six-month experiment, the vast majority of wild-caught flies belonged to the family Calliphoridae (76.8%), followed by Muscidae (15.4%), Sarcophagidae (3.6%), Anthomyiidae (3.3%), and Psychodidae (0.9%). Calliphoridae were present in all 16 collections, and Muscidae, Sarcophagidae, Anthomyiidae, and Psychodidae in 12, 8, 7, and 3 collections, respectively. Flies from the first four collections (Figure 1) most likely represented overwintering cohorts.

**Exposure of C. parvum-infected calves.** All calves developed clinical infection, and laboratory testing confirmed that each calf was shedding oocysts for approximately 2 wk. The total number of recovered C. parvum oocysts from calves varied from approximately \( 2.5 \times 10^7 \) to \( 2.0 \times 10^9 \) (Figure 1); \( \bar{x} = 6.5 \times 10^7 \), and the mass of diarrheic feces varied from approximately 6.1 to 9.3 kg, respectively; \( \bar{x} = 7.7 \) kg.

**Inf ectivity of fly-derived oocysts.** Cryptosporidium oocysts recovered from fly external surfaces and traps retrieved when C. parvum-infected calves were in the barn induced infection in 3 of the 4 mice. On average 15%, 50%, and
more than 60% of mouse intestinal epithelium cells, respectively, harbored developmental stages of Cryptosporidium.

**Molecular analysis.** Five out of 6 samples were positive by PCR with primers CPE/CPZ (samples 1, 2, 4, 5 and 6). Three of these samples (1, 2, and 6) were also positive with primers CRY12/CRY14. No amplification was obtained with any of the samples using primers CPBDIAGF/CPBDIAGR. DNA sequencing analysis of the PCR products from samples 1, 2, and 6 showed only one type of sequence. They were identical to the respective sequences of the C. parvum Genotype 2 for each locus.

**DISCUSSION**

The involvement of insects in mechanical transmission of Cryptosporidium parvum was discovered only recently. There are only three published reports dealing with insects and C. parvum, two related to flies,22,23 and one to dung beetles.24 The present study, together with the two previous reports involving flies,22,23 demonstrate that filth flies can transport C. parvum oocysts on their external surfaces and in their digestive tracts and can serve as mechanical vectors for the pathogen against which no effective prophylaxis or therapy exists.16

The present study demonstrated for the first time that C. parvum oocysts transported and deposited by filth flies are infectious. This is direct proof that such flies can cause human or animal cryptosporidiosis via deposition of oocysts on visited surfaces. Such epidemiologic involvement however, is difficult to prove as cryptosporidiosis cases that result from fly visitations on food items or raw, pre-processed food products would be classified as foodborne. Foodborne cases of cryptosporidiosis have been extensively documented.37 In poultry (chickens), Eimeria sp. infections resulted from their eating flies carrying infectious oocysts.12

Cattle manure is a favorite breeding place and food source for filth flies, and barns are sites where flies may breed during the winter or can overwinter.17,18 An important epidemiologic finding of the present study is a positive relationship between the abundance of flies visiting the barn and the number of C. parvum oocysts transported by them. Epidemiologically, it determines a self-intensifying transport of infectious C. parvum oocysts while the oocyst source is available. The biologic basis for this relationship is the fact that cattle feces strongly attract filth flies,17 as evidenced in the present study by a significantly higher abundance of flies during the presence of clinically infected calves. The effectiveness of diarrheic feces in enhancing the transmission of infectious agents by filth flies is much greater than any other substrate because its viscosity increases the efficiency of the fly hairs and bristles in the trapping of suspended particles.21

As cryptosporidiosis can be caused by infectious oocysts of C. parvum deposited by filth flies on visited surfaces, i.e., defecation, regurgitation, or mechanical dislodgement, the proportion of oocysts transported versus deposited is of epidemiologic importance. In the previous study the numbers of C. parvum oocysts deposited by house flies (M. domestica) were lower than the numbers of oocysts eluted from their exoskeletons.22 This finding was explained by the possibility that oocysts trapped by hairs and bristles do not detach easily.22 Dung beetles maintained on cow dung spiked with C. parvum oocysts contained fewer oocysts on their external surfaces than in their intestines and feces.24 An epidemiologically important finding demonstrated for the first time in the present study is a positive relationship between transported and deposited oocysts. Epidemiologically it means that the more C. parvum oocysts that are present on the exoskeleton of filth flies, the more oocysts will be deposited on the surfaces visited by them.

As the barn construction and hygienic maintenance prevented indoor fly breeding, it is thought that the trapped flies came from outside. All flies caught during early spring in the first four collections most likely represented overwintered cohorts. Interestingly, the abundance of these flies and the number of C. parvum oocysts transported by them were comparable to that found in the rest of the season when non-overwintering fly generations were prevalent. Thus, even when environmental conditions do not favor breeding of filth flies, if a source of C. parvum oocysts is present, these oocysts can be transported by overwintered flies at a similar intensity level as in the summer.

Because calves and diarrheic feces strongly attract filth flies,17,18 it is likely that not all flies were captured by the trapping devices when the calves were present in the barn. However, when the barn was empty the flies were more attracted to the baited traps which may explain why C. parvum oocysts were still detected on flies when the source of these oocysts had disappeared. This was supported by molecular data demonstrating that the oocysts of C. parvum Genotype 2 shed by infected calves were carried by flies for at least 3 wk after elimination of the source of these oocysts. However, if these flies leave the barn, i.e., due to the lack of attractants, the oocysts would be carried outside. Epidemiologically it may mean that even under high hygiene standards, C. parvum oocysts acquired in barns by filth flies may be disseminated into the environment.

The biology and ecology of filth flies and their associations with cattle suggest a high potential for mechanical transmission of C. parvum oocysts. The females of such flies can live 15 to 25 days,26 lay up to 6 egg batches,26,36 and produce up to 12 generations in the summer26 and several generations during winter (if indoors).38 Filth flies can travel up to 20 miles,39 their movement is oriented toward sanitary sites,39 and they reside at such sites.17,18 It has been demonstrated previously that wild filth flies carried oocysts of Isospora or Hammondia.11 Because these flies were observed to move constantly between cattle and their manure, it has been postulated that filth flies may play an epizootiologic role in distributing coccidian parasites in the environment.14 The results of the present study support this conclusion with regards to the epidemiology and epizootiology of C. parvum.

The present study demonstrates an intense transport of infectious C. parvum oocysts by flies from a cattle source. Filth flies (the families Muscidae, Anthomyiidae, Sarcophagidae, and Calliphoridae) are synanthropic flies living in close association with man as pestiferous scavengers.13,14,17 Urban outbreaks of diarrheal diseases are closely correlated with an abundance of filth flies, and their control is closely correlated with a decline in diarrheal disease cases.38 Based on the present study we conclude that C. parvum oocysts can be transported by filth flies not only from cattle sources
but from any unhygienic source contaminated with *C. parvum*, i.e., toilets, abattoirs, trash, carcasses, and sewage.

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