Effect of Bovine Manure on *Cryptosporidium parvum* Oocyst Attachment to Soil

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The objective of this work was to assess the effect of dilute bovine manure (1.0% and 0.1%) versus that of no manure on attachment and subsequent detachment of *Cryptosporidium parvum* oocysts to soil. Manure enhanced the attachment of oocysts to soil particles; the maximum attachment was observed with 0.1% manure. Oocyst attachment was partially reversible; maximum detachment was observed with dilute manure. These results indicate that oocyst attachment to soil is substantially affected by bovine manure in a complex manner and should have implications for how oocysts may be transported through or over soils.

There is concern regarding the contamination of surface waters and groundwaters with *Cryptosporidium parvum*. Several waterborne outbreaks of cryptosporidiosis have occurred in the past decade, the most severe in Milwaukee, WI, where over 400,000 people were infected (15). Dairy/beef animals are widely considered to be a major source of *C. parvum* oocysts. Based on a survey of 7,369 calves from 1,103 dairy farms (located in 28 states), Garber et al. (10) concluded that virtually all herds with >100 cows are infected with *C. parvum*. Neonatal calves are particularly susceptible to infection and can excrete several billion oocysts if they develop cryptosporidiosis. Recent studies indicate that postweaned and adult, asymptomatic cattle also excrete oocysts (9). Although excretion rates (oocysts/gram feces) are lower, the total number of oocysts excreted may still be substantial due to the quantity of feces produced.

Watershed monitoring studies indicate that contamination can occur via surface transport of oocysts from manures applied to land or fecal excretion or via vertical transport via preferential flow to groundwater (e.g., karst groundwater). Hansen and Ongerth (11) and Ong et al. (19) reported that oocyst concentrations downstream of dairy/beef operations were greater than those upstream, while Kuczynska et al. (14) documented the presence of oocysts in karst groundwater in a region devoted primarily to cattle grazing. Brush et al. (5) and Harter et al. (12) have described the leaching of oocysts through sands and sediments as convective dispersion transport in conjunction with sorption-desorption processes. Laboratory studies have also demonstrated the potential for oocyst runoff (2) or leaching (16, 17), although oocyst numbers in runoff or leachate were dramatically attenuated by soils.

Most studies have been conducted with purified oocysts in distilled water or oocysts in calf diarrhea. However, purified oocysts or oocysts from calf diarrhea are a highly unlikely source for water contamination. A much more likely scenario is oocyst contamination from the application to land of calf manures mixed with adult cow manure or fecal deposition on pastures by calves. Oocysts must first be "released" from the manure matrix before they can be transported to surface water or groundwater. Bradford and Schijven (3) have investigated the release of oocysts from dairy cow manure during simulated rainfall. They observed that oocyst numbers were highly correlated with turbidity (i.e., from fiber or microbial biomass), indicating that oocyst "release" was concomitant with manure dissolution.

Bovine manure is a complex matrix consisting of microbial biomass, dietary fiber, bedding materials, urine, and fecal mucus. The single largest component of manure is fiber, both dietary and from bedding (composed of cellulose, hemicellulose, and lignin). Van Kessel and Reeves (21) reported that the fiber content of dry manure was ca. 55% (mean value for 107 manures), although values were variable depending on management practices. Based on visual inspection, bovine manure contains a wide range of fiber sizes, from microscopic to macroscopic. The second largest component of manure is microbial biomass plus sloughed intestinal cells. According to Salo (20), this accounts for ca. 30% of dry fecal matter. The remaining dry matter is predominantly inorganic material (21). Manures also contain glycoproteins (i.e., mucus), which account for up to 40% of total N (1). Based on a mean total N content of ca. 4% (21), manure can contain up to ca. 1.5% mucus.

Manure can potentially affect oocyst attachment to soils in a variety of ways. Attachment or adhesion of oocysts to dietary or bedding fiber in manure prior to application to land may inhibit attachment to soil particles. The difficulties reported by many researchers in extracting oocysts from manure/feces (for examples, see references 6, 13, and 22), particularly at low concentrations, suggest that there is some association between oocysts and manure fiber. Alternatively, the high microbial populations in manure may compete for attachment sites on soils, thereby preventing oocyst attachment. Finally, the presence of mucus may serve to "bind" oocysts to soil particles, fiber particulates, or both. The objective of this study was to assess the effect of bovine manure on the oocysts' initial attachment to and subsequent detachment from soil particles.

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Manure samples were collected from the Beltsville Area Research Center dairy farm on two separate occasions. The manure had dry matter contents of ca. 10%. Manure was diluted 10-fold with deionized-distilled (DD) water and blended at high speed for 2 min (10% manure). The manure suspension was subsequently diluted 10-fold with DD water and vortexed for ca. 30 seconds (1% manure). Both 1% and 10% manure suspensions were inoculated with purified *C. parvum* oocysts; purified *C. parvum* oocysts were obtained from infected calves as previously described (8). Oocysts were suspended in distilled water to give a concentration of ca. 10^5 oocysts ml^{-1} manure suspension. Oocyst suspensions were prepared approximately 30 min prior to use.

One milliliter of a 1% or 10% manure-oocyst suspension was added to 9 ml of a 1% (wt/vol) suspension of sandy loam or clay loam soil in DD water, resulting in suspensions consisting of ca. 0.9% soil and either 0.1% manure (0.01% manure solids) or 1.0% manure (0.1% manure solids). Twelve tubes were prepared for each soil and consisted of (i) four tubes with oocysts but without manure, (ii) four tubes with oocysts and 0.1% manure, and (iii) four tubes with oocysts and 1.0% manure. After soil and manure suspensions were combined, the tubes were vortexed thoroughly and incubated for 2 h on their sides with a gentle shaking motion at ca. 8°C. After being shaken the tubes were centrifuged for 10 min at 100 × *g* to pellet the soil-manure, and the top 9 ml of supernatant was removed using a glass pipette. Nine milliliters of distilled water was added to the tubes, and the tubes were vortexed for ca. 30 seconds and then incubated a second time as previously described. Oocysts in supernatants from the first and second incubations were enumerated using an immunofluorescence assay (14).

An additional set of four tubes that consisted of oocysts in DD water (ca. 10^5 oocysts ml^{-1}) without manure or soil was prepared. After the tubes were shaken and centrifuged, oocyst concentrations in the top 9 ml and bottom 1 ml of water were analyzed. Oocyst concentrations were identical in the two water fractions, indicating that centrifugation for 10 min at 100 × *g* was not responsible for the observed differences in oocyst distributions within the tubes.

Two independent experiments were conducted with manure collected at different times. The same experimental procedure was used for both experiments. Since results were generally comparable, data from the first and second incubations have been combined.

Statistical analyses were done with software SPLUS (Math-Soft, 1999). Significance of differences between average values was tested with the Welch modified *t* test. Analysis of variance was applied to see whether soil, manure content, or resuspension was a significant factor affecting the attachment of partition coefficient values.

In the absence of manure, the percentages of oocyst attachment to sandy loam and clay loam soil were 72.0% and 93.1%, respectively; differences were statistically significant ($P \leq 0.001$; Table 1). This is consistent with previous work by Kuczynska and Shelton (13) in which percentages of oocyst recovery from soils were inversely correlated with clay content. Presumably, the increased number of soil particles and/or surface area (per gram of soil) presents a greater number of potential attachment sites. This is also consistent with the results of Atwill et

TABLE 1. Effect of dilute bovine manure on oocyst attachment to soil particles

Treatment	% Attachment[a] to:	
	Sandy loam	Clay loam
Water	72.0 ± 3.6A	93.1 ± 2.9D
Manure		
1.0%	86.7 ± 2.2B	93.1 ± 3.8D
0.1%	97.4 ± 1.8C	97.7 ± 2.0C

[a] Values represent the mean ± standard deviation from two experiments ($n = 8$). Different letters following values indicate statistical significance of differences between average values evaluated at the 0.05 significance level.

al. (2), who observed an ca. 2- to 3-log (99 to 99.9%) reduction in oocysts transported per meter of loam or sandy loam soil. This contradicts the findings of Dai and Boll (7), who observed no attachment of oocysts to clay particles. However, the maximum soil concentration tested in their experiments was 2 mg ml^{-1}, which occurs only in pristine waters; hence, this has no relevance to agricultural runoff, where typical sediment loads are 1% (10 g ml^{-1}).

In general, manure enhanced the attachment of oocysts to soil particles, indicating that some component of manure facilitated oocyst attachment; collectively, differences were statistically significant ($P \leq 0.001$) (Table 1). The highest attachment percentage (and K_d value) (Fig. 1) was observed with the 0.1% manure suspension, suggesting that there is some optimal concentration of the "facilitating" component in manure between 0 and 1.0%. It is unclear why oocyst attachment decreased with increasing manure concentration. Perhaps the "facilitating" component was offset by higher biomass concentrations in the 1.0% manure suspension which competed with oocysts for soil attachment sites.

Soil-manure-oocyst pellets were resuspended in freshwater and incubated a second time to assess the tendency for detachment. K_d values, defined as the ratio of oocysts attached to soil (oocysts g^{-1}) to oocysts in solution (oocysts ml^{-1}) are presented for both incubations in Fig. 1. Note that total oocyst numbers decreased between incubations due to removal of suspended oocysts after the first incubation. Analysis of variance showed that manure content and resuspension both significantly affected K_d values ($P < 0.001$). Average K_d values before and after resuspension were not significantly different in sandy loam and clay loam in the absence of manure. However, resuspension significantly decreased average K_d values in the presence of manure ($P < 0.05$), indicating that oocyst attachment was readily reversible. The magnitude of detachment was consistently greater with the 0.1% manure suspension than with the 1.0% manure suspension. A higher percentage of oocysts initially attached to soil particles, and remained attached after resuspension, in the presence of 0.1% manure.

Although the precise mechanism(s) of oocyst attachment is unclear, previous research is suggestive. Brush et al. (4) examined the impact of different purification methods on oocyst surface charge. They observed that purification using milder methods yielded oocysts with a neutral surface charge, while purification using harsher methods yielded oocysts with a net negative surface charge. They concluded that oocysts in the
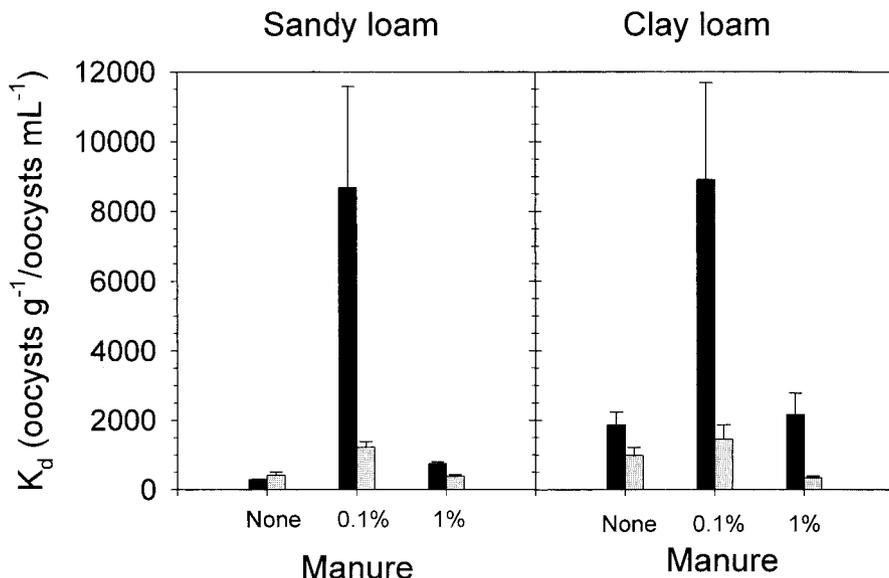
Sandy loam   Clay loam

FIG. 1. Effect of dilute manure versus no manure on oocyst attachment to sandy loam or clay loam soil. ■, K_d values for first incubation (initial attachment); □, K_d values for second incubation (detachment). Bars represent standard errors ($n = 8$).

environment likely have minimal surface charge. Brush et al. (4) also investigated the "stickiness" of oocysts by examining their adhesion to inert polystyrene beads. They observed 50 to 90% adhesion in solutions with ionic strength typical of agricultural runoff (<5 mM). This is consistent with previous research documenting that the oocyst outer wall is composed largely of glycoproteins (18), which promote adhesion. In the current study, oocysts were purified via sucrose flotation and discontinuous CsCl centrifugation. Although the surface charge of oocysts was not measured, the use of a mild purification method likely produced oocysts with minimal surface charge. In addition, manure and soil solutions were prepared using DD water, giving a low-ionic-strength solution. Consequently, our data are consistent with those of Brush et al. and suggest that adhesion is the predominant mechanism of oocyst attachment to soil particles.

In conclusion, these results indicate that oocyst attachment to soil is substantially affected by bovine manure in a complex manner. The extent of oocyst attachment to soil particles and the tendency to remain attached appear to be correlated with manure dilution. Consequently, rates of manure dissolution control not only oocyst "release" but also transient attachment to soil particles. Further research is needed to more thoroughly elucidate the impact of manure dissolution on oocyst transport to surface waters or groundwaters from manure applied to land or fecal deposition.

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