Recovery and Enumeration of *Cryptosporidium parvum* from Animal Fecal Matrices

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Accurate quantification of *Cryptosporidium parvum* oocysts in animal fecal deposits on land is an essential starting point for estimating watershed *C. parvum* loads. Due to the general poor performance and variable recovery efficiency of existing enumeration methods, protocols were devised based on initial dispersion of oocysts from feces by vortexing in 2 mM tetrasodium pyrophosphate, followed by immunomagnetic separation. The protocols were validated by using an internal control seed preparation to determine the levels of oocyst recovery for a range of fecal types. The levels of recovery of 10^2 oocysts from cattle feces (0.5 g of processed feces) ranged from 31 to 46%, and the levels of recovery from sheep feces (0.25 g of processed feces) ranged from 21% to 35%. The within-sample coefficients of variation for the percentages of recovery from five replicates ranged from 10 to 50%. The ranges for levels of recovery of oocysts from cattle, kangaroo, pig, and sheep feces (juveniles and adults) collected in a subsequent watershed animal fecal survey were far wider than the ranges predicted by the validation data. Based on the use of an internal control added to each fecal sample, the levels of recovery ranged from 0 to 83% for cattle, from 4 to 62% for sheep, from 1 to 42% for pigs, and from 40 to 73% for kangaroos. Given the variation in the levels of recovery of oocysts from different fecal matrices, it is recommended that an internal control be added to at least one replicate of every fecal sample analyzed to determine the percentage of recovery. Depending on the animal type and based on the lowest approximate percentages of recovery, between 10 and 100 oocysts g of feces^{-1} must be present to be detected.

The protozoan parasite *Cryptosporidium parvum* is a major cause of waterborne diarrheal illness in human and animal populations. The transmissive form of this organism, the oocyst, frequently finds its way into surface waters, either directly through sewage discharge or indirectly in runoff from land grazed by livestock or wildlife. Oocysts present in animal fecal deposits on land are clearly responsible for many event-related increases in the concentrations of in-stream pathogens in watersheds (3, 8). However, there are significant knowledge gaps regarding the mechanisms and magnitude of pathogen transport and the effects of environmental factors on oocyst inactivation (7). In particular, there have been few attempts to predict the fate of oocysts deposited on land by using quantifiable parameters. The responsible authorities are advocating adequate watershed management as a fundamental requirement for reducing pathogen risks to drinking water quality, and the United States Environmental Protection Agency is seeking estimates of total maximum daily loads for watershed pathogens (18). The quantification of transport mechanisms and environmental inactivation for key pathogens should enable models to be constructed that can be used to predict source water quality in order to better manage the factors that govern pathogen transport in watershed environments. Accurate quantification of initial oocyst loading in a watershed is an essential starting point for such models.

Livestock, such as cattle, sheep, and pigs, are susceptible to infection by *C. parvum*. Although cryptosporidiosis is mainly confined to young individuals, low-level asymptomatic infections in postweaned and adult cattle have been reported (6), with up to 10^4 oocysts per g of feces excreted (17). In addition, postparturient ewes may shed increased but low concentrations of *C. parvum* oocysts (100 to 5,700 oocysts g^{-1}) (21).

The techniques that have been traditionally developed and optimized for enumeration of *Cryptosporidium* oocysts in water are generally not suitable for enumeration of oocysts in animal feces. This is due to the large quantities of particulate and fibrous material present in feces. Additionally, the presence-absence techniques used in clinical microbiology for the examination of feces (i.e., fecal smears) are not sufficiently sensitive or quantitative (2). The efficiency of techniques for enumeration of microorganisms in matrices such as soil, sediment, and feces is dependent upon adequate separation and recovery of the microorganisms from the matrix particles.

The need for adequate separation of the target organisms from particulate material has long been recognized by workers attempting to quantitatively recover bacteria from sediments and soils (12, 16, 22). One of the main procedural problems encountered is the masking of the stained bacteria by particulate material on microscope slides. Consequently, the bacteriological approaches have largely formed the basis for the dispersion of oocysts from matrices such as feces and soil and have generally included physical homogenization (blending, vortexing, or sonication) and/or the use of chemical dispersing agents.

Given the general inadequacy of conventional techniques (1, 20) and the poor recovery of low oocyst concentrations from feces and soil (9), it was deemed necessary to attempt to improve the procedure. The objective of the study described in this paper was to identify a means of reliably and effectively

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recovering and enumerating oocysts present in a range of animal fecal matrices based on previous approaches used for bacteria in soils and/or feces.

MATERIALS AND METHODS

Reagents. Tetrasodium pyrophosphate (TSPP) (2 mM) (1, 12, 19), phosphate-buffered saline (PBS) (10 mM) (12), Tris-Tween 80 (50 mM Tris and 0.5% [vol/vol] Tween 80) (9, 11), and deionized water (MilliQ) were used as dispersing agents. The pHs of the four dispersing solutions were 9.2, 7.2, 9.4, and 7.6, respectively. Disodium EDTA was used at a concentration of 1 mM. All chemicals were purchased from Sigma Aldrich Pty. Ltd. (Castle Hill, New South Wales, Australia).

Sources of oocysts and immunofluorescent antibody (IFA) stains. *C. parvum* oocysts (10^6 oocysts) (Camden isolate, New South Wales, Australia) purified from calf feces by sucrose and CsCl gradient centrifugation were obtained from BTF Decisive Microbiology (North Ryde, New South Wales, Australia). Various oocyst concentrations were obtained by dilution of this stock preparation in MilliQ water. These oocysts were used in the sample pretreatment experiments and stained with the fluorescein thiocyanate (FITC)-labeled monoclonal antibody Crypt-a-Glo (Waterborne Inc., New Orleans, La.). In all subsequent experiments, oocysts were stained with FITC-labeled monoclonal antibody EasyStain (BTF Decisive Microbiology) according to the manufacturer's recommended procedure. It has been reported that different commercially available antibodies generally recognize the same or similar epitopes on the oocyst wall (13). Although these antibodies are specific for the genus *Cryptosporidium* (but not *C. parvum*), it is not known if they detect all known *Cryptosporidium* or *C. parvum* strains. ColorSeed *C. parvum* oocysts (gamma irradiated, Texas Red labeled, and quantified by flow cytometry; BTF Decisive Microbiology) were used as an internal control (at a concentration of 100 ± 1 oocysts per sample).

Sample pretreatment. The *Cryptosporidium*-negative status of cattle fecal samples was determined by immunomagnetic separation (IMS) and IFA staining. Three *Cryptosporidium*-negative cattle fecal samples were mixed thoroughly with a sterile disposable wooden tongue depressor and composited in roughly equal proportions. One-gram portions of the feces composite were weighed into 24 centrifuge tubes (50 ml) and 24 stomacher bags. Twelve of the tubes and 12 bags were seeded with 100 *C. parvum* oocysts suspended in 100 μl of MilliQ water. The other 12 tubes and 12 bags were seeded with 10^4 *C. parvum* oocysts suspended in 100 μl of MilliQ water. The seeded feces were allowed to equilibrate overnight at 4°C. Twenty milliliters of each dispersant was added to each of three tubes and three bags of seeded feces at the two seed levels. The tubes were vortexed (maximum speed; model SVM1; Selby Biolab, Clayton, Victoria, Australia) for 2 min, and the bags were stomached (double bagged; Stomacher 80; Seward Ltd., London, United Kingdom) for 5 min. The contents of the stomacher bags were washed twice with 10 ml of the appropriate dispersant, and the rinse solutions were transferred to 50-ml centrifuge tubes. The fecal slurries were held at room temperature for 30 min and centrifuged at 2,500 × g for 10 min. Each supernatant was aspirated down to a volume of approximately 5 ml. The fecal pellet was resuspended in the remaining overlying liquid by vortexing. The resulting slurry was sieved through a piece of coarse metal mesh (approximately 2.5 cm square; pore size, approximately 1.5 mm), which was facilitated by using a wooden tongue depressor, and rinsed into a second clean 50-ml tube. The volume was adjusted to 20 ml with MilliQ water, the solution was mixed thoroughly, and 10 ml was transferred by pipette into a Leighton tube. The oocysts were enumerated by IMS by using anti-*Cryptosporidium* Dynabeads kits (Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions but with the following modification: in order to produce cleaner preparations and thereby facilitate the counting of oocysts, an extra washing step was incorporated into the normal IMS procedure. After the 60-min mixing step, 10 ml of 0.2% (vol/vol) Tween 80 in PBS was added to each tube, and the tubes were returned to the rotator for 5 min.

Fecal samples from milk-fed calves and piglets were defatted by adding 15 ml of diethyl ether to the feces diluted in 35 ml of TSPP. After mixing by inversion of the tubes, the fecal slurries were immediately centrifuged at 2,500 × g. The supernatants including the fat and diethyl ether were aspirated off, and the residual pellets were washed by resuspension in MilliQ and centrifugation (which was repeated twice).

In optimization experiments with pig feces, 20 ml of EDTA (final concentration, 1 mM) was used as the dispersant in place of TSPP.

Oocyst enumeration. After IMS, the captured oocysts were dissociated from the beads by using two 50-μl portions of 0.1 M HCl and were neutralized with 10 μl of 1 M NaOH in 1.5-ml centrifuge tubes. The suspensions were passed through a membrane filter (diameter, 3 mm; pore size, 0.8 μm; Millipore Australia Pty. Ltd., North Ryde, New South Wales, Australia). The oocysts retained on the surface of the membrane were stained with 4′,6′-diamidino-2-phenylindole (DAPI) and FITC-labeled monoclonal antibody according to the manufacturer's instructions. The membranes were mounted on glass slides, and each entire coverslip was scanned at a magnification of ×250 by fluorescence microscopy (excitation at 450 to 490 nm; long pass [LP] emission at 520 nm; Nikon Optiphot-2 with EFD-3 fluorescence attachment). The identity of *Cryptosporidium* oocysts was confirmed at a magnification of ×400 by the presence of DAPI-stained nuclei (excitation at 340 to 380 nm; LP emission at 425 nm).

Levels of recovery were calculated by determining the number of seed oocysts expressed as a percentage of the mean number of oocysts counted in duplicate 100-μl aliquots of the seed suspension or, for the samples seeded with ColorSeed oocysts, the number of ColorSeed oocysts expressed as a percentage of the manufacturer's stated concentration.

Validation of recovery method. Feces from 18 individual animals were collected from sheep, pigs, cattle, and calves at two different farms. Each sample was mixed, and clumps were broken up by using a tongue depressor. Five replicate portions of each fecal type (0.5 g for cattle and pigs; 0.25 g for sheep and kangaroos) were weighed into 50-ml centrifuge tubes. One hundred ColorSeed oocysts were added to each of five replicates for each feces sample and processed as described above; the only difference was that after centrifugation, the fecal pellet was resuspended in the overlying liquid and sieved and the entire sample was rinsed with MilliQ water into Leighton tubes. MilliQ water was used to adjust the total volume in each tube to approximately 10 ml.

Animal fecal survey. On two separate occasions (in April and June; Australian fall and winter) fecal samples were collected from animals at a number of sites in the Sydney drinking water supply watershed, including five farms, an intensive piggery, and a protected area (accessible by native animals but not by livestock). On each occasion, 15 fresh fecal samples were collected from each of the following animal groups: adult pigs, juvenile pigs, adult cattle, juvenile cattle, adult sheep, and adult eastern grey kangaroos (*Macropus giganteus*). These animals are the most prevalent livestock species and the most prevalent large native mammal in southeastern Australian watersheds. Since lambing occurs during winter in the study region, juvenile sheep feces were collected only on the second occasion (nine fecal samples, three composites).

The samples were stored at 4°C and processed as soon as possible (certainly within 2 weeks of collection) by using the protocol outlined above. A total of 10 composite samples were prepared for each animal group (except juvenile sheep, for which three composite samples were prepared) by combining three samples from animals of approximately the same age from the same location in approximately equal proportions. A total of 63 animal fecal composite samples representing samples from 189 individual animals were analyzed. The concentration of oocysts in each of the composite samples was determined by the methods described above, and the concentration for each animal feces type is shown in Fig. 1. Juvenile pig and kangaroo feces samples were analyzed by using the protocols for calf and sheep feces, respectively. Three replicates were analyzed for each composite sample. In addition, 100 ColorSeed oocysts were added to one of the replicates for each sample, and the level of recovery of these oocysts was determined simultaneously. Oocyst concentrations were expressed per gram (dry weight) of feces.

Data analysis. Data manipulation and Student's *t* tests were carried out by using Microsoft Excel 2000. The data were analyzed further by using the SAS Statistical Software package (version 6.12; SAS Institute Inc.). Analysis of variance was performed with the sample pretreatment data to determine significant differences for different dispersion treatments. The post hoc Student-Newman-Keuls test was used to carry out pairwise comparisons of the various treatments. A *P* value of <0.05 was considered significant.

RESULTS

Table 1 shows the effects of various combinations of physical and chemical treatments on the recovery of 10^2 and 10^4 oocysts from cattle feces and the associated variabilities. Analysis of variance showed that there was a significant difference in the levels of oocyst recovery when different dispersants were used and also between the level of recovery of 10^2 oocysts and the level of recovery of 10^4 oocysts. There were no significant differences between the levels of recovery after stomaching and vortexing, and there were no significant interactions between

Sheep/Kangaroo   Cattle/Pig   Calf/Piglet

Break up
with tongue   0.25 g   0.5 g   0.5 g
depressor

Seed 1 replicate with ColorSeed™ & equilibrate overnight at 4°C

Add 20 ml sodium pyrophosphate   Add 35 ml sodium pyrophosphate

Vortex for 2 min

Stand for 30 min

Centrifuge 3500 rpm for 10 min   Add 15 ml diethyl ether for 2 min

Centrifuge 2000 rpm for 10 min

Aspirate supernatant   Aspirate supernatant & resus-
pend in Milli-Q water (x2)

Sieve, rinsing with approx. 7 ml Milli-Q water

Mix & pipette into Leighton tubes — rinse tube with Milli-Q to make up to 10 ml
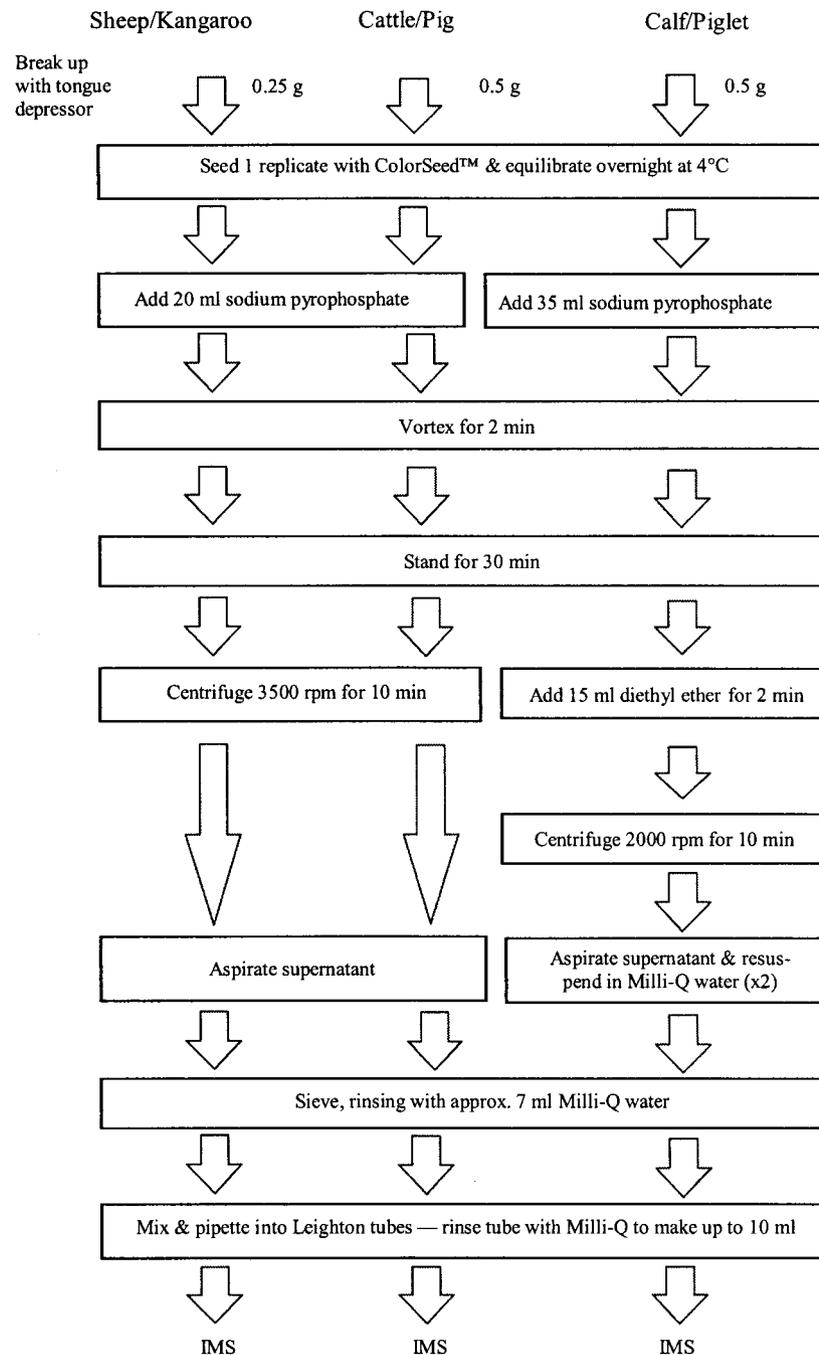
IMS   IMS   IMS

FIG. 1. Optimized protocols for recovery and enumeration of *C. parvum* oocysts from feces of various animals.

physical and chemical dispersion techniques. As determined by the Student-Newman-Keuls test, dispersion with TSPP gave the highest mean levels of recovery, but given the variation in the experiment, the values could not be unambiguously distinguished from the levels of recovery obtained by using PBS and Tris-Tween. Using MilliQ as a dispersant resulted in significantly lower levels of recovery of oocysts than the levels of recovery when TSPP and PBS were used. Since vortexing involves fewer transfers of the feces than stomaching, the former is more convenient to use. The optimized protocol for cattle feces is shown in Fig. 1 and includes physical dispersion by

vortexing and chemical dispersion by using TSPP. The coefficients of variation for duplicate determinations with the seed doses prepared by dilution ranged from 0.6 to 23%. The numbers of oocysts in the 10^2-oocyst seed doses ranged from 61 to 177 oocysts (mean, 113 ± 33 oocysts), and the coefficient of variation was 29%.

The protocol optimized for recovery of oocysts from cattle feces was used without modification in an attempt to recover oocysts added to sheep, kangaroo, pig, and calf feces and two soils (both sandy clay loams). The mean levels of recovery of 10^2 and 10^4 oocysts are shown in Table 2. With the exception

TABLE 1. Levels of recovery of seeded *C. parvum* oocysts (10^2 and 10^4 oocysts) from cattle feces by various physical and chemical dispersion techniques

Chemical dispersant	Physical treatment	Recovery (mean ± SD; $n = 3$)	
		10^2 oocysts	10^4 oocysts
PBS	Stomaching	23 ± 22	42 ± 7.5
	Vortexing	23 ± 10	57 ± 2.1
TSPP	Stomaching	23 ± 21	48 ± 14
	Vortexing	38 ± 12	56 ± 11
Tris-Tween 80	Stomaching	9.3 ± 4.5	53 ± 13
	Vortexing	6 ± 10	55 ± 13
MilliQ water	Stomaching	2.3 ± 0.6	45 ± 9.5
	Vortexing	7.7 ± 2.9	30 ± 8.1

of the calf A sample and the soils, the mean levels of recovery of oocysts were very low, and ≤10% of 10^2 and 10^4 oocysts were recovered from each feces type. The levels of recovery of oocysts from feces with the protocol optimized for cattle but with some modification are also shown in Table 2. The recovery of oocysts from calf B feces was significantly improved by defatting ($P < 0.05$). In contrast, defatting failed to improve the recovery of oocysts from pig feces, as did both addition of EDTA and reducing the amount of pig feces processed from 0.5 to 0.1 g. Reducing the amount of kangaroo feces processed from 0.5 to 0.25 g increased the recovery of oocysts from this feces type ($P < 0.05$). Based on these results, the optimized protocols for pig, calf, kangaroo, and sheep feces are shown in Fig. 1.

In order to validate the optimized protocol for each animal feces type and to determine the associated variability, the protocols developed were used to recover 100 ColorSeed oocysts from five replicate portions of feces from individual animals of various types. The mean levels of recovery of ColorSeed oocysts from five replicates are shown in Table 3. The mean levels of recovery of ColorSeed oocysts from six cattle fecal samples ranged from 31 to 46%, and the within-sample coefficient of variation ranged from 12 to 50%. For six sheep feces samples, the range of mean levels of recovery was 21 to 35%, and the

TABLE 2. Levels of recovery of seeded *C. parvum* oocysts (10^2 and 10^4 oocysts) from various animal feces and soil by the protocol optimized for cattle feces with modifications for each animal type as described in the text

Feces type	Protocol modification	% Recovery (mean ± SD; $n = 3$)	
		10^2 oocysts	10^4 oocysts
Pig	None	0	10 ± 2.6
Pig	Defatted	2.0 ± 2.5[a]	ND[b]
Pig	0.1 g	1.7 ± 0	2.9 ± 0.9
Pig	EDTA added	0	ND
Sheep	None	7 ± 6.5	3 ± 2.5
Calf A	None	69 ± 36	71 ± 27
Calf B	None	3.2 ± 3.0	ND
Calf B	Defatted	53 ± 17	ND
Kangaroo	None	1.7 ± 2.1	ND
Kangaroo	0.25 g	36 ± 13	75 ± 4.2
Soil 1	None	46 ± 2.9	ND
Soil 2	None	42 ± 12	ND

[a] $n = 5$.
[b] ND, not determined.

TABLE 3. Levels of recovery of 100 ColorSeed oocysts from different individuals for various animal feces types by the optimized procedure[a]

Sample	% Recovery (mean ± SD; $n = 5$)[b]	Coefficient of variation (%)
Cattle 2	36 ± 6.2	17
Cattle 3	40 ± 4.6	12
Cattle 4	46 ± 23.2	50
Cattle 5	36 ± 6.0	17
Cattle 6	31 ± 9.4	30
Cattle 7	36 ± 6.4	18
Calf 10	47 ± 8.7	19
Calf 3	44 ± 10.5	24
Calf 15	32 ± 6.4	20
Calf 16[c]	9.2 ± 3.8	41
Sheep 1	35 ± 5.0	14
Sheep 2	34 ± 4.9	14
Sheep 3	22 ± 5.5	25
Sheep 5	22 ± 2.2	10
Sheep 6	31 ± 12.5	40
Sheep 7	21 ± 4.6	22
Pig 4	0	
Pig 2	0	

[a] See Fig. 1.
[b] Recovery of oocysts from 0.5 g (cattle and pigs) or 0.25 g (sheep and kangaroos) of feces.
[c] Not defatted.

within-sample coefficient of variation ranged from 10 to 40%. The mean levels of recovery of oocysts from defatted calf feces ranged from 32 to 47%. One sample was not defatted, and the mean level of recovery for this sample was 9.2%. The coefficients of variation within the samples ranged from 19 to 41%. The protocol used failed to recover any oocysts from the two pig feces samples, but the recovery of oocysts from the two soils was relatively good (levels of recovery, 42 and 46%).

The mean oocyst concentrations and levels of recovery of ColorSeed oocysts for one of the three replicates for a range of animal feces types from the animal fecal survey are shown in Table 4. Oocysts were detected in a number of samples, and the concentrations ranged from <1 to 10^4 oocysts per g (dry weight) of feces. The levels of recovery of ColorSeed oocysts from different samples were highly variable across the animal

TABLE 4. Summary of oocyst concentrations and recovery from samples collected in a watershed animal fecal survey

Fecal source	Concn of oocysts (oocysts g[dry wt]$^{-1}$; $n = 10$)[a,b]		% Recovery of 100 ColorSeed oocysts ($n = 10$)[b]		
	Range	Median	Range	Mean ± SD	Coefficient of variation (%)
Cattle (adult)	<1–5,988	0.5	14–70	40 ± 16	40
Cattle (juvenile)	<1–17,467	23	0–83	25 ± 30	120
Sheep (adult)	<1–152,474	148	4–48	23 ± 16	64
Sheep (juvenile)[c]	<1–641	275	42–62	49 ± 11	22
Pig (adult)	<1–58	0.5	3–24	10 ± 7	70
Pig (juvenile)	<1–770	0.5	1–42	13 ± 12	92
Kangaroo (adult)	<1–39,423	0.5	40–73	54 ± 9	17

[a] The values were not adjusted for recovery.
[b] n is the number of composite samples comprising three individual animal fecal samples.
[c] $n = 3$.

types, ranging from 14 to 70% for cattle feces (adults), from 0 to 83% for calf feces, from 4 to 48% for sheep feces, from 40 to 73% for kangaroo feces, and from 3 to 24% for pig feces.

DISCUSSION

Adsorption of oocysts to particulate material is determined by the hydrophobic and surface charge characteristics of the particles and the oocysts, which are influenced by the ionic strength and pH of the suspension medium. Since in natural environments most particles are negatively charged, the degree of electrostatic repulsion is determined by the ionic strength of the medium. Increasing the ionic strength results in decreased electrostatic repulsion, as well as an increase in hydrophobicity, thereby promoting aggregation of particles. Previous approaches to desorbing oocysts from particulate material have been based on modification of one of these properties so that particle separation is promoted. Surfactants such as Tween 80 increase the hydrophilicity of particles, thereby promoting particle dispersion. Chelating agents such as TSPP (ionic strength, 16 mM) and EDTA form complexes with cations such as Ca^{2+}, Mg^{2+}, and Na^+, thereby decreasing the ionic strength of the medium, which again promotes particle dispersion. MilliQ has a low ionic strength, and in contrast, PBS has a relatively high ionic strength (164 mM); these dispersants promote dispersion and aggregation, respectively. According to this theory, therefore, it should be expected that use of TSPP, Tween 80, and MilliQ should result in better recovery of oocysts from particulate matter than use of PBS results in. The data presented in this paper failed to unequivocally support this conclusion due to the great variation associated with preparation of seed oocysts by dilution. Our decision to include the use of TSPP in the optimized protocol was, therefore, partially based on previous observations that TSPP significantly improved the separation of oocysts from particles in water concentrates (M. Lepesteur, S. Blasdall, and N. J. Ashbolt, unpublished data).

There is great inherent variation associated with preparation of oocyst seed suspensions by dilution. For seed doses on the order of 10^2 oocysts, coefficients of variation may approach 30% for doses prepared by dilution of a stock oocyst suspension (5). It has been reported that for preparation of suspensions having low oocyst densities, flow cytometry provides replicate seed doses with the least variation (4). ColorSeed oocyst suspensions are accurately calibrated suspensions (by flow cytometry) of a given number of gamma-irradiated oocysts that have been labeled with a red fluorochrome and therefore can be distinguished under a microscope from oocysts that are naturally present in a sample by their red fluorescence with excitation at 540 to 580 nm. The use of ColorSeed oocysts as an internal control therefore provides an accurate and convenient means of determining recovery efficiencies for individual samples. It should be noted, however, that a significant but small (approximately 3%) decrease in the recovery of ColorSeed oocysts compared to the recovery of unlabeled oocysts by IMS (Dynal) from water samples has been reported (M. Warnecke, C. Weir, and G. Vesey, submitted for publication).

Recovery efficiencies of 31 to 46% for adult cattle, 9.2 to 47% for calves, and 21 to 35% for sheep and kangaroo feces were considered acceptable. These levels of recovery represented a considerable improvement compared with a previ-

ously reported mean level of recovery of 10^2 C. parvum oocysts from cattle feces, which was 12.8% as determined by salt flotation (9). Recovery efficiencies between 28 and 95% have been reported for oocyst concentrations of $\geq 10^3$ oocysts g^{-1} for bovine feces as determined by IMS (1, 14, 20). However, there have been few reports of recovery for lower oocyst concentrations with IMS; the only exception is the study of Rochelle and coworkers, who recovered 67% of 496 oocysts added to 1 g of bovine feces (15). The recovery of oocysts from pig feces was not considered acceptable despite various attempts to improve recovery. There were visibly fewer IMS beads captured for pigs feces than for the other types of animal feces. This was thought to be due to competition for the bead binding sites by other particles or ions present in pig feces. However, an attempt to improve recovery by using EDTA to sequester metal ions that may compete for bead binding sites was unsuccessful.

Recent studies have demonstrated that the pH during oocyst capture is an important factor that affects the recovery of oocysts by IMS (10) and that the levels of recovery of oocysts from deionized water at pH 7.0 were significantly higher than those from water that deviated as little as 0.12 pH unit from the optimum. In the present study, deviation of the pH from the optimum during the IMS procedure was not considered to be the cause of poor recovery of oocysts from pig feces, since when it was checked, the pH before and after IMS deviated little from the optimum (data not shown).

In the animal fecal survey the efficiencies of recovery of ColorSeed oocysts from feces by the optimized protocols were highly variable not only in fecal samples from different types of animals but also in feces from the same type of animal. Despite the poor recovery of oocysts from pig feces during the optimization and validation experiments, we decided that pig feces should be included in the survey, and the levels of recovery of ColorSeed oocysts from pig feces collected as part of the animal fecal survey ranged from 3 to 24%. The apparent differences in recovery efficiency were most likely due to differences in the consistency and constitution of the feces from individual animals resulting from variations in feeding regimens and environmental factors at different farms. Given the variation in the levels of recovery of oocysts from different fecal samples even from the same type of animal, it is recommended that an internal control be added to at least one replicate for every fecal sample analyzed. The variation in the level of recovery for different samples was greater than the mean level of recovery observed when the protocols were validated by using different fecal samples. However, given that the fecal samples used for protocol validation were collected from only two farms, one would expect that the variation in the validation samples for a particular type of animal would be lower than that in the survey, in which samples were collected from several different farms. Neither of the former two farms were included in the animal fecal survey as they were not located in the study watershed.

Based on approximate observed lowest recovery efficiencies in the animal fecal survey of 4 and 40% for sheep and kangaroo feces, respectively, and the amount of feces that could be processed (0.25 g), approximately 100 and 10 oocysts would have to be present in each gram (wet weight) of sheep and kangaroo feces, respectively, to be detected by the protocol

shown in Fig. 1. Similarly, for cattle and pig feces, based on observed lowest levels of recovery of 14 and 3%, respectively, and the 0.5 g of feces processed, approximately 14 and 67 oocysts per g of feces, respectively, would have to be present to be detected.

The detection limits for oocyst purification by salt and sucrose flotation techniques coupled with IFA staining and for fecal smears have previously been reported to be on the order of 10^3 oocysts per g of feces (20) and 10^6 oocysts per ml (2), respectively. These techniques are clearly not sufficiently sensitive for enumerating low concentrations of oocysts that may be excreted by a range of asymptomatic infected livestock (6) and native animal species. Animals excreting low concentrations of oocysts, therefore, are not included in estimates of *Cryptosporidium* prevalence and loads in watersheds. Since the animals harboring subclinical infections may outnumber the animals that are severely infected, this may lead to gross underestimates of *C. parvum* loads in watersheds. The protocols reported here enable detection of low concentrations of oocysts ($\leq$100 oocysts per g) in several animal fecal matrices.

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