Quantitation of *Giardia* Cysts and *Cryptosporidium* Oocysts in Fecal Samples by Direct Immunofluorescence Assay

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The lack of quick, simple, and sensitive quantitative tests has impeded studies on infection patterns and treatment of *Giardia* spp. and *Cryptosporidium* spp. A quantitative direct immunofluorescence assay (FA) using a commercial FA kit was developed and evaluated. Recovery rates of the FA for *Cryptosporidium* oocysts in calf feces seeded with 1,000, 10,000, 100,000, and 1,000,000 oocysts per g were 14.8, 40.8, 84.2, and 78.2%, respectively. Interassay coefficients of variation were 10.6 to 47.1%. Recovery rates of the FA for *Giardia* cysts in feces seeded with 1,000, 10,000, and 100,000 cysts per g were 76.4, 96.9, and 89.6%, respectively. Interassay coefficients of variation were 7.4 to 22.1%. By comparison, recovery rates of *Giardia* cyst by sucrose gradient flotation were only 20.5, 51.2, and 42.9%, respectively. Counts of cysts-per-gram obtained by sucrose gradient flotation with samples from calves, lambs, and ewes were only 49.1 to 54.8% of those obtained by the FA. Zinc sulfate flotation detected only 36.4% of infections when there were ≤1,000 cysts per g. The quantitative FA offers a useful technique for epidemiological and control studies of these two parasites.

Despite the pathogenic importance of *Cryptosporidium* spp. and *Giardia* spp., there have been few studies on infection intensities (daily oocyst or cyst output or numbers of oocysts or cysts per gram of feces) in infected humans or animals. Such information would be useful for understanding the epidemiology of these two parasites. Unavailability of suitable quantitative detection methods is partially responsible for the lack of quantitative studies.

The only quantitative tests available are hemacytometer counts for *Cryptosporidium* oocysts and sucrose gradient flotation for *Giardia* cysts. The former was used in the estimation of experimental and natural bovine *Cryptosporidium parvum* infections (4) and experimental murine *C. muris* infection (15). The latter was used in the estimation of *Giardia* infection in laboratory animals and ruminants (5, 11, 12, 17, 20). Both methods have low sensitivities. Furthermore, hemacytometer counts cannot differentiate *Cryptosporidium* oocysts from yeast cells, and sucrose gradient flotation cannot be used in animals with high fecal fat content, such as humans and suckling animals. Some semiquantitative methods have also been used in the detection of *Cryptosporidium* infection (2, 14, 19). The lack of suitable quantitative tests has impeded studies on infection patterns and treatment of these two parasites.

Recently, an indirect immunofluorescence assay (IFA) was used to estimate the intensity of *Cryptosporidium* infection in AIDS patients (13). Although no detailed evaluation of the assay was conducted, the theoretical sensitivity was about 32,000 oocysts per ml of feces, an improvement on the hemacytometer method. We earlier developed a quantitative direct immunofluorescence assay (FA) with a theoretical sensitivity of 100 *Cryptosporidium* oocysts and 100 *Giardia* cysts per g of feces. This new test was evaluated for recovery rates and compared with conventional *Giardia* detection methods (sucrose gradient flotation and zinc sulfate flotation).

MATERIALS AND METHODS

Quantitative FA. The quantitative FA was developed by using the commercial MERIFLUOR *Cryptosporidium-Giardia* kit from Meridian Diagnostics, Inc., Cincinnati, Ohio. A 2-g fecal sample was mixed with about 10 ml of distilled water. The fecal suspension was then filtered through one layer of cheesecloth and washed with an additional 30 ml of water. After transfer to a 50-ml centrifuge tube, the suspension was centrifuged at 1,500 × g for 10 min. The supernatant was decanted, and the pellet was dissolved to a total of 4 ml by addition of water. After thorough vortexing, 20 µl of the suspension was pipetted onto a treated FA slide and used to make a fecal smear. After staining with the FA kit, the entire smear was searched at a magnification of ×100 under a fluorescence microscope for *Cryptosporidium* oocysts and *Giardia* cysts. Identification was confirmed at a magnification of ×400. Numbers of oocysts or cysts per gram of feces were obtained by multiplying the total number of oocysts or cysts on the entire smear by 100. When the number of oocysts or cysts per gram of feces was above 40,000, the fecal suspension was further diluted 5 to 100 times.

Estimation of recovery rates. For evaluation of the recovery rate of the new FA, viable *C. parvum* oocysts and *Giardia duodenalis* type cysts isolated from feces of naturally infected calves by the sucrose flotation and sucrose gradient flotation methods (7, 17) were added to formed negative fecal samples from calves. A series of 2-g negative fecal samples were seeded with 1 ml of a suspension containing different levels of *Cryptosporidium* oocysts or *Giardia* cysts. *Cryptosporidium* concentrations of 1,000, 10,000, 100,000, and 1,000,000 oocysts per g and *Giardia* concentrations of 1,000, 10,000, and 100,000 cysts per g were used. There were 10 samples of each concentration. Seeded samples were then examined for *Cryptosporidium* oocysts and *Giardia* cysts by the quantitative FA. Recovery rates were calculated by dividing the number of recovered oocysts or

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TABLE 1. Recovery rates of Cryptosporidium oocysts by the quantitative FA

No. of oocysts seeded/g of feces	Recovery rate (%)	SD	CV (%)
1,000	14.8	7.0	47.1
10,000	40.8	8.5	20.9
100,000	84.2	8.9	10.6
1,000,000	78.2	8.9	11.4

TABLE 3. Comparisons of quantitative FA and sucrose gradient flotation performance in detection of Giardia cysts in clinical samples

Animals	No. of samples	Mean no. of cysts/g detected by:		Mean sucrose/FA ratio ± SD	P value
		FA	Sucrose		
Calves	11	119,318	47,078	0.49 ± 0.26	0.0044
Ewes	8	223,388	122,006	0.53 ± 0.08	0.0236
Lambs	10	396,875	210,809	0.55 ± 0.19	0.0650

cysts per gram with the expected number of oocysts or cysts per gram. Coefficients of variation (CV) were obtained by dividing the standard deviation of the recovery rate by the mean recovery rate.

Performance comparisons between FA and other methods for Giardia detection. The performance of the FA was compared with that of a quantitative sucrose gradient flotation method (17) and a qualitative zinc sulfate flotation method (3). Seeded samples used for evaluation of FA recovery rates were also examined with the sucrose gradient flotation method. Numbers of cysts per gram were obtained by multiplying the total number of cysts in 50 large squares of the hemacytometer by 100. They were then compared with those obtained by the FA. Samples from 11 calves, 10 lambs, and eight ewes naturally infected with *G. duodenalis* were examined for a further performance comparison between the FA and sucrose gradient flotation. In addition, samples from 130 calves (positive and negative) were used for a performance comparison between the FA and zinc sulfate flotation for Giardia detection. Differences in performance between the FA and sucrose gradient flotation were compared with a paired t test.

RESULTS

Recovery rates of the quantitative FA for *Cryptosporidium* oocysts in formed calf feces seeded with 1,000, 10,000, 100,000, and 1,000,000 oocysts per g were 14.8, 40.8, 84.2, and 78.2%, respectively (Table 1). Interassay CV were high when numbers of oocysts in feces were low (47.1 and 20.9% for 1,000 and 10,000 oocysts per g, respectively) but low when numbers of oocysts in feces were high (10.6 and 11.4% for 100,000 and 1,000,000 oocysts per g, respectively). Oocysts were detected in all seeded samples.

Recovery rates of the quantitative FA for Giardia cysts in formed feces seeded with 1,000, 10,000, and 100,000 cysts per g were 76.4, 96.9, and 89.6%, respectively (Table 2). Giardia cysts were found in all seeded samples. Interassay CV were between 7.4 and 22.1%. By comparison, recovery rates of the sucrose gradient flotation with the same samples were 20.5, 51.2, and 42.9%, respectively. The CV of the sucrose gradient flotation were also higher (14.5 to 67.1%). The recovery rates of the FA were significantly higher than

TABLE 2. Giardia cyst recovery rates by the quantitative FA and sucrose gradient flotation

No. of cysts seeded/g of feces	Mean % recovery ± SD (CV [%])		P value
	FA	Sucrose	
1,000	76.4 ± 16.9 (22.1)	20.5 ± 13.8 (67.1)	<0.001
10,000	96.9 ± 16.6 (17.1)	51.2 ± 7.5 (14.7)	<0.001
100,000	89.61 ± 6.67 (7.44)	42.86 ± 6.20 (14.46)	<0.001

those of sucrose gradient flotation at all three levels (P < 0.001).

Counts of Giardia cysts per gram obtained by sucrose gradient flotation were only 49.1 to 54.8% of those obtained by the FA with samples from naturally infected calves, lambs, and ewes (Table 3). Numbers of cysts per gram obtained by FA were higher than those obtained by sucrose gradient flotation with all samples. The FA provided significantly higher mean counts than sucrose gradient flotation in calves and ewes.

Of 130 samples from calves, the quantitative FA revealed that 40 were negative for Giardia infection, 44 had >0 and ≤1,000 Giardia cysts per g, and 46 had >1,000 cysts per g. Zinc sulfate flotation and FA detected comparable Giardia infection rates (45 positives) in samples with >1,000 cysts per g, but at ≤1,000 cysts per g, zinc sulfate flotation detected only 36.4% of infections. One sample was negative by the FA but positive by zinc sulfate flotation.

DISCUSSION

Previously, *Cryptosporidium* oocysts in fecal samples were enumerated by hemacytometer counts (4, 15). This technique is time-consuming and has low sensitivity. A 40-fold dilution of fecal samples (minimal dilution required) and counting of 100 medium squares per sample (taking at least 2 h) gave a sensitivity of 100,000 oocysts per g. The new FA has a sensitivity of at least 1,000 oocysts per g and requires less time per sample.

Even at low infection intensities, the sensitivity of the new FA for *Cryptosporidium* oocysts was much higher than those of previous immunofluorescence methods. Although many previous studies indicated that the FA or IFA was more sensitive and specific than conventional detection methods (1, 6, 9, 10, 16, 18), Weber et al. (21, 22) showed that the thresholds of *Cryptosporidium* oocyst detection in human samples by IFA were 5,000 to 10,000 oocysts per g for watery stool and 10,000 to 50,000 oocysts per g for formed stool. The threshold of oocyst detection by the new FA (1,000 oocysts per g for formed feces) is much lower than these. In the method of Weber et al. (21, 22), a formalin-ethyl acetate stool concentrate method was used prior to the preparation of smears. Improper filter material (gauze), centrifugation force (500 × g), and centrifugation time (2 min) used in the formalin-ethyl acetate technique probably resulted in oocyst loss rather than oocyst concentration (21). The formalin-ethyl acetate method was not used by others in IFA and FA of human samples, despite the use of a similar centrifugation process (1, 9, 10).

The quantitative FA differs from the IFA of Goodgame et al. (13) in that a wash process and larger aliquot are used. Furthermore, the whole smear is examined. As a result, the expected sensitivity for *Cryptosporidium* detection has been

improved from about 32,000 oocysts per ml of feces to 100 oocysts per g of feces. Because the method of Goodgame et al. (13) is an indirect assay, it requires a longer time to process slides.

The quantitative FA is also more sensitive than sucrose gradient flotation for recovery of *Giardia* cysts. In seeded samples, recovery rates of sucrose gradient flotation were between 42.9 and 51.2% when infection intensities were moderate or high. Lower recovery rates were given when intensities were low. This differs from the original study by Roberts-Thomson et al. (17), in which recovery rates of 63 to 66% were obtained, irrespective of infection intensities. Murine feces, *G. muris* cysts, and no samples with low infection intensity, however, were used when they originally developed the method. In both seeded and clinical samples, the quantitative FA yielded cyst-per-gram values almost twice as high as those of the sucrose gradient flotation method in the present study.

The quantitative FA is also more sensitive than zinc sulfate flotation for detection of *Giardia* infection. Zinc sulfate flotation is widely used in veterinary medicine and sometimes in human medicine for *Giardia* diagnosis. An earlier study showed that only 80.9% of infections in beavers and 61.5% of infections in muskrats were identified by zinc sulfate flotation (8). Results from the present study indicated that the performance of zinc sulfate flotation in *Giardia* detection depends on the number of cysts present in fecal samples. Zinc sulfate flotation detected almost all infections when there were more than 1,000 cysts per g but only 36.4% of infections when there were between 100 and 1,000 cysts per g. The zinc sulfate flotation method did identify one infection missed by the FA. Qualitative rather than quantitative determination is another disadvantage of zinc sulfate flotation.

ACKNOWLEDGMENT

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