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Detection of *Cryptosporidium parvum* in Horses: Thresholds of Acid-Fast Stain, Immunofluorescence Assay, and Flow Cytometry

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Feces collected from three asymptomatic horses and seeded with *Cryptosporidium parvum* oocysts (10^1 to 10^6/g of feces) were evaluated by acid-fast staining (AF), an immunofluorescent antibody (IFA) technique, and flow cytometry. The thresholds of detection were 5×10^5 oocysts/g of feces for the IFA and AF techniques and 5×10^4 oocysts/g for flow cytometry.

Recent outbreaks of gastrointestinal disease caused by *Cryptosporidium parvum* from contaminated water sources have resulted in significant morbidity and mortality in human populations (5, 6). The sources of contamination in these waterborne outbreaks have not been elucidated, resulting in increased public health concern regarding the role of domestic and wildlife species in environmental contamination and the implementation of policies limiting equine access to watershed areas (4–6, 8, 10). Our understanding of the epidemiology of environmentally derived *C. parvum* oocysts is complicated, however, by the variability among the results obtained by the diagnostic methods. There is no "gold standard" of detection reported for equine hosts, and the sensitivity and specificity of the various coprodiagnostic tests differ considerably (3, 10). Before the prevalence of *C. parvum* shedding by equine species into the environment can be evaluated, the threshold of detection of the coprodiagnostic tests used on horse fecal samples must be evaluated.

The purpose of this study was to establish the threshold of detection of the two most commonly utilized methods of equine cryptosporidiosis detection and to compare these thresholds to the level of detection achieved by flow cytometry. Formed fecal material seeded with *C. parvum* oocysts was used to evaluate the usefulness of these techniques for epidemiological studies in asymptomatic equine populations which may be impacted by governmental legislation.

Five grams of fresh, formed stool was collected from each of 3 asymptomatic adult horses. Each fecal sample was placed in a 10% neutral buffered formalin solution in a 1:3 stool/formalin ratio and refrigerated. The absence of oocysts in the samples was confirmed by acid-fast staining (AF) of direct smears of the fecal material from each horse (BBL TB Kinyoun acid-fast stain kit; Becton Dickinson Microbiology Systems, Cockneysville, Md.).

Calf-passaged cryptosporidial oocysts (10^8) preserved in formalin solution were obtained from the laboratory of Charles Sterling (University of Arizona, Tucson). Tenfold dilutions of the oocyst suspension were made in phosphate-buffered saline (PBS; pH 7.4) and added to aliquots of the preserved fecal slurries to make nine sample concentrations for each horse fecal suspension. The final concentrations of the inoculated aliquots were 10^1, 10^2, 10^3, 5×10^3, 10^4, 5×10^4, 10^5, 5×10^5, and 10^6 oocysts/g of feces. Oocyst suspension volumes were less than 200 μl, so there was a negligable change in total fecal volume. Three aliquots from each horse's fecal slurry remained uninoculated, serving as negative controls. All inoculated samples and the uninoculated controls were stored at 4°C.

Prior to AF, large fibrous material was removed from 1-ml aliquots of each fecal suspension (diluted to 10 ml with distilled water) by filtration through gauze. The filtered aliquots were centrifuged at $2,000 \times g$ for 10 min, and the supernatants were decanted. Each pellet was resuspended to 1 ml of distilled water. A 100-μl aliquot of each fecal suspension was smeared on a slide, allowed to air dry, and stained by using a Kinyoun acid-fast stain kit in accordance with the manufacturer's (Becton Dickinson) instructions.

Stained slides of each inoculated fecal sample and uninoculated controls were evaluated at magnifications of ×500 and ×1,000. Each slide was evaluated in a cross-hatch pattern, with all fields being examined in a single longitudinal and horizontal pass across the slide. All acid-fast particles of the correct morphology and dimensions (4 to 6 μm in diameter) were recorded. The microscopist was blinded to the concentrations of inoculated oocysts.

For the immunofluorescent antibody assay (IFA), a 5-ml aliquot of each fecal-oocyst suspension and negative control was submitted to a commercial diagnostic laboratory for examination. All samples were evaluated by using a commercially available immunofluorescence kit (Merifluor *Cryptosporidium*/*Giardia* kit; Meridian Diagnostics, Inc., Cincinnati, Ohio) in accordance with the laboratory's standard quality control procedures. The commercial laboratory personnel were also blinded to the concentrations of inoculated oocysts.

Prior to flow cytometry, inoculated samples and uninoculated controls were vortexed to suspend the particles, and 100 μl of each suspension was placed in a 1.5-ml microcentrifuge tube. Large particles and formalin fixative were removed by adding 1 ml of PBS to each microcentrifuge tube, vortexing for 30 s, allowing the contents to settle for 30 s, decanting and centrifuging ($2,000 \times g$ for 5 min) the supernatants, discarding the supernatants after centrifugation, and resuspending each pellet in 200 μl of PBS. The suspended pellet was vortexed and the contents were allowed to settle for 30 s before 150 μl of supernatant were transferred to another microcentrifuge tube.

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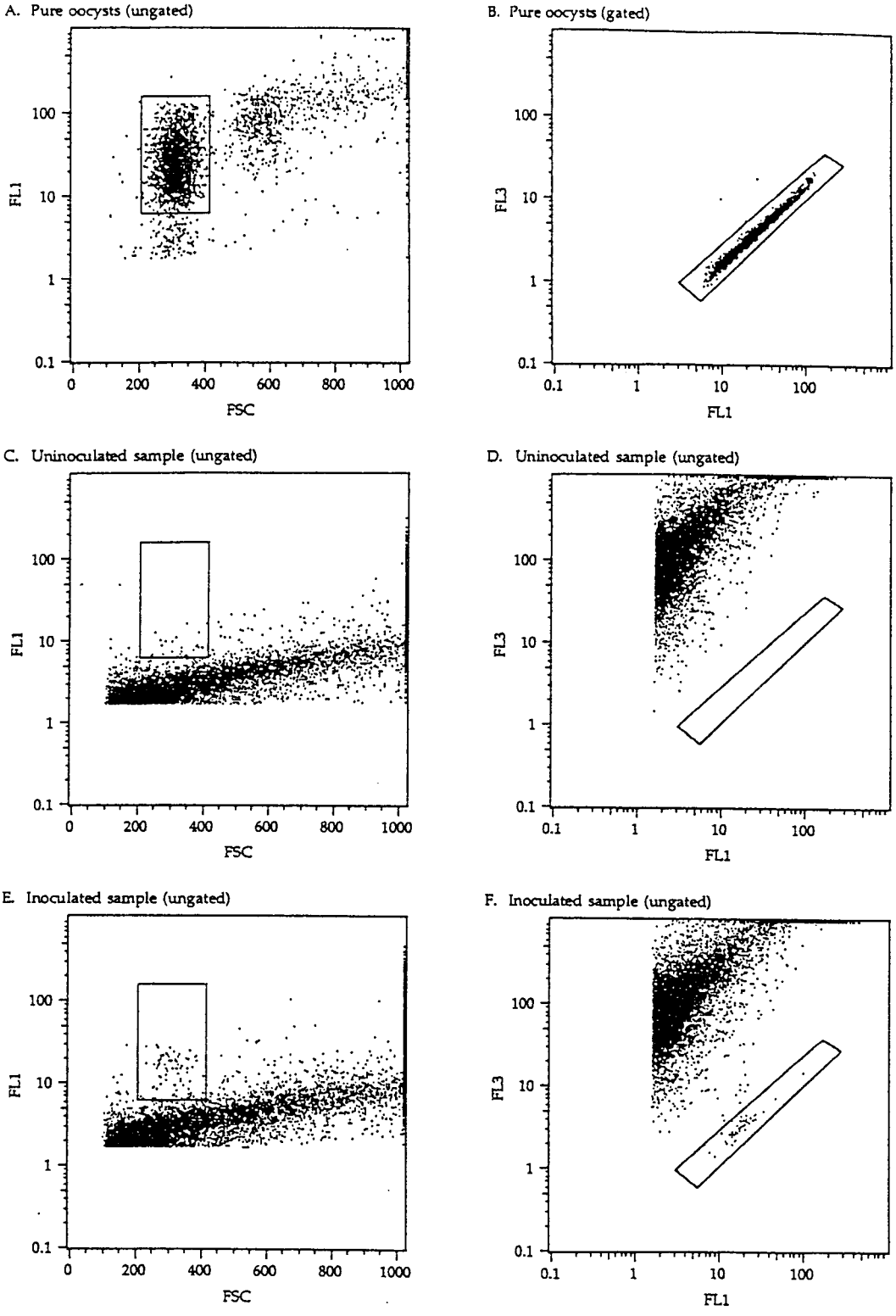

FIG. 1. Dot plots (FSC versus FL1 and FL1 versus FL3) of purified *C. parvum* oocysts labeled with a commercially available (Merifluor *Cryptosporidium/Giardia* Kit; Meridian Diagnostics) monoclonal antibody conjugated to FITC (A and B), equine fecal material (C and D), and equine fecal material seeded with antibody-labeled *C. parvum* oocysts (E and F). Boxes indicate acquisition regions for *C. parvum* oocysts.

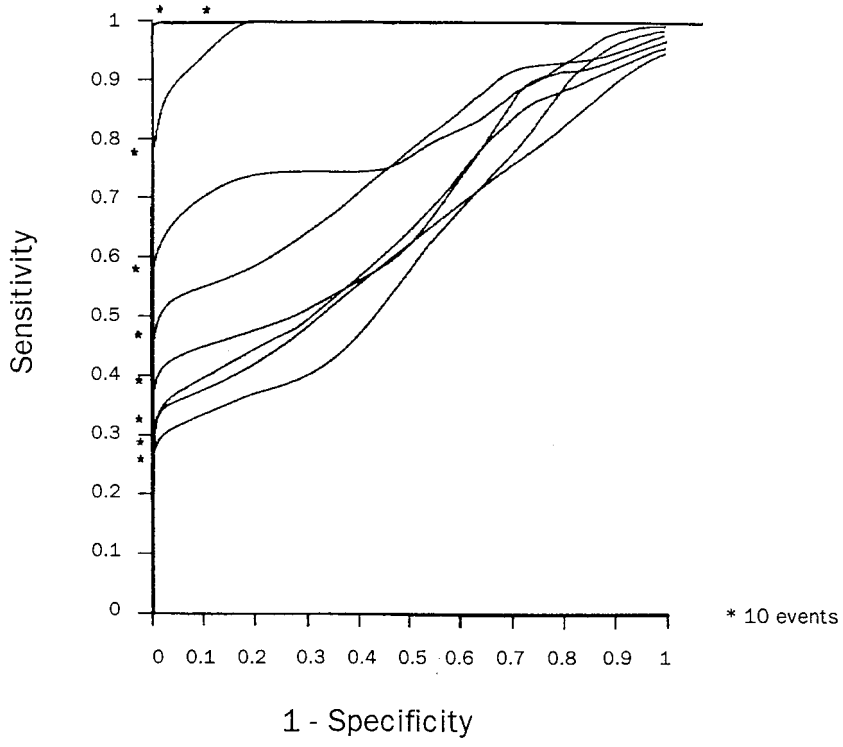

FIG. 2. Receiver-operator characteristic curves generated with acid-fast stain (BBL TB Kinyoun Stain Kit; Becton Dickinson Microbiology Systems) to detect *C. parvum* oocysts in seeded, formed equine fecal samples. Each curve represents the minimum concentration of *C. parvum* oocysts required before a sample is considered positive. Asterisks represent the sensitivity and 1 − specificity values when the observation of 10 or more events is required for a positive test result.

To each prepared sample, 50 μl of a standard oocyst-specific, fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody from a commercial immunofluorescence kit (Merifluor *Cryptosporidium*/*Giardia* kit) was added, and the samples were incubated at 37°C for 1 h. Following incubation, each sample was washed with 1 ml of PBS, the supernatant was decanted, and the pellet was resuspended in 1 ml of PBS. All samples were transferred to 12- by 75-mm polystyrene tubes for flow-cytometric evaluation.

The flow cytometry methods used and the optical characteristics of the purified oocysts have been previously described (1, 7, 9). The flow cytometer (FACSCalibur, Becton-Dickinson Immunocytometry Systems, San Jose, Calif.) and acquisition software (CellQuest; Becton-Dickinson Immunocytometry Systems) used for evaluation of these samples were different from those previously described, so evaluation of positive and negative controls was done. Flow cytometry was performed with linear amplification of the forward light scatter (FSC) signal (voltage, E00; gain, 3.50) and linear amplification of side light scatter (SSC; voltage, 270). Compensation was not applied during acquisition. The sample stream was set at a low flow rate to avoid excessive clogging of the injection port. Using a sample of stained, pure oocysts, a region based on an FSC versus FL1 plot was set to include over 99% of the oocysts, and only events falling within that region were stored to list mode files. All samples were analyzed in triplicate, and data were collected over a sampling time of 102 s. Between each sample, the sample injection port was flushed with deionized water to prevent cross-sample contamination. Plots of the flow cytometric data were generated by using the FlowJo software (Tree Star Inc., San Carlos, Calif.).

A significant amount of debris with light scattering and FL1 intensity similar to those of the stained oocysts was present in the uninoculated fecal samples (Fig. 1). Since the fecal debris had a higher level of fluorescence emission at a wavelength of >650 nm (FL3) than the FITC-labeled antibody, the fecal debris was distinguished from labeled oocysts by using an FL1-versus-FL3 plot (Fig. 1). Only events falling within the regions defined for pure oocysts by FSC-versus-SSC and FL1-versus-FL3 plots were counted as oocysts in the experimental samples.

To evaluate the flow cytometry and AF results, the sensitivities and specificities of these procedures were calculated for every concentration of inoculated oocysts, using several possible cutoff values as the threshold for a positive test result. These values were plotted on receiver-operating characteristic (ROC) curves (Fig. 2 and 3). Using the ROC curves, a positive result was defined as 10 events falling within the region defined for oocysts for flow cytometry and by visualization of 10 acid-fast structures of the appropriate morphology on two passes across the slide for AF. The flow-cytometric threshold of detection was consistent (i.e., sensitivity = 100%, specificity = 100%) at a concentration of 5×10^4 oocysts/g of fecal material (Table 1). Although positive detection of 10^5 oocysts/g by AF was possible for one of the three inoculated samples, consistent detection (sensitivity = 100%) for AF was achieved with 5×10^5 oocysts/g of fecal material.

The commercial IFA technique was able to detect 5×10^4 oocysts/g of fecal material 67% of the time but did not detect 10^5 oocysts/g. The IFA was slightly more sensitive than the AF (Table 1) but did not perform reliably at oocyst concentrations below 5×10^5/g of feces. The oocyst concentration required for 100% sensitivity (5×10^5 oocysts/g of feces) was the same for IFA and AF. The IFA did not perform as well in our study, as reported elsewhere (2, 7).

It is possible that the higher fiber content of adult herbivore fecal samples relative to human stool samples complicates the

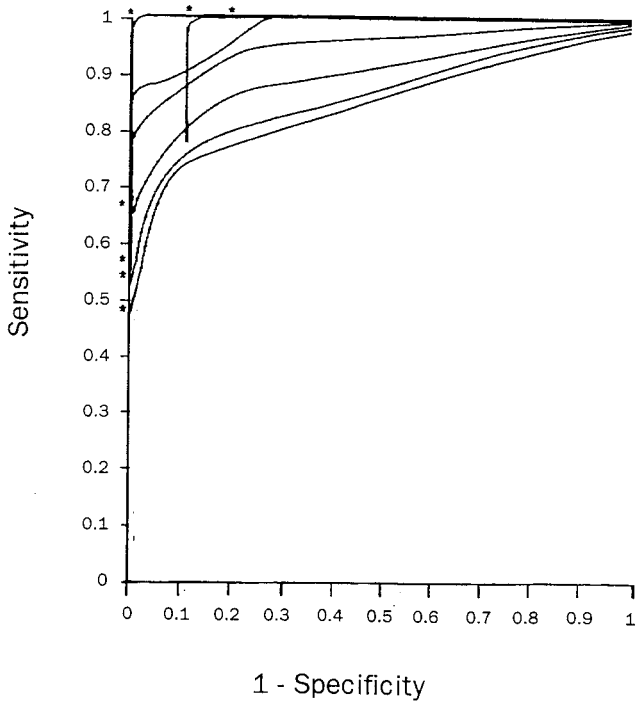

FIG. 3. Receiver-operator characteristic curves generated by flow cytometry (FACSCalibur; Becton Dickinson Immunocytometry Systems) to detect *C. parvum* oocysts in seeded, formed equine fecal samples. Each curve represents the minimum concentration of *C. parvum* oocysts required before a sample is considered positive. Asterisks represent the sensitivity and 1 − specificity values when the observation of 10 or more events is required for a positive test result.

detection of oocysts by the IFA technique. Improved detection of oocysts has been observed when stools are watery and contain little fecal debris in comparison to formed stools (9). It is possible that some samples in our study contained more fecal debris than others, thereby preventing adequate visualization of oocysts by IFA.

The increased fiber content of equine feces relative to that of human stools did not seem to affect the threshold of detection by AF, however. Our level of detection of 5×10^5 oocysts/g of feces is consistent with the level reported for formed human samples (9). One important consideration, however, is the cutoff value for AF-positive structures used in our study. Since fecal debris and some yeasts may also stain red (2, 3, 10), ROC curves for the AF results were utilized to obtain the best cutoff value for this study. In previous studies which did not report positive result cutoff values, high rates of false-positive results for the AF were noted (2, 9). Although our counting technique was not quantitative, allowing a low level of background uptake of stain (<10 AF-positive structures of the correct size and morphology) resulted in improved reliability of AF results in this study.

TABLE 1. Sensitivity and specificity of AF, IFA, and flow cytometry (FC) for detection of *Cryptosporidium* oocysts in equine fecal samples with various concentrations of oocysts as the threshold for a positive sample

Lowest concn considered positive (no. of oocysts/g of feces)	Diagnostic test result (sensitivity/specificity)		
	AF	IFA	FC
10^1	0.26/1.0	0.29/1.0	0.44/1.0
10^2	0.29/1.0	0.33/1.0	0.50/1.0
10^3	0.33/1.0	0.38/1.0	0.57/1.0
5×10^3	0.39/1.0	0.8/1.0	0.67/1.0
10^4	0.53/1.0	0.53/1.0	0.80/1.0
5×10^4	0.58/1.0	0.67/1.0	1.0/1.0
10^5	0.78/1.0	0.67/0.93	1.0/0.89
5×10^5	1.0/0.97	1.0/0.93	1.0/0.87

Flow cytometry represented the most sensitive method of detection with these samples, detecting 5×10^4 oocysts/g of fecal material, which is consistent with other reports (1, 7). Flow cytometry was 10 times more sensitive than the IFA and AF techniques. Again, the high fiber content of equine fecal samples may have limited the sensitivity of the flow cytometry, since temporary disruptions of flow by large fecal particles may decrease the number of events observed by reducing the sample volume evaluated over a 102-s time interval. It is unknown whether a time-based sampling protocol is the most appropriate, for the volume of sample suspension necessary to detect small numbers of oocysts is unknown. It is possible, however, that increased sensitivity may be achieved in flow cytometry by using a volume-based sampling protocol.

Tests which prove suitable for the diagnosis of acute cryptosporidial disease in horses may not exhibit adequate sensitivity for the detection of oocyst shedding in asymptomatic carriers, which is necessary for epidemiological research. In this study, the superior sensitivity of FC compared to AF and IFA suggests that this method may be preferable for epidemiological studies. For the diagnosis of acute cryptosporidiosis, however, AF represents the most efficient method for veterinary clinical practices, for it is the simplest to perform.

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