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Comparison of PCR and Microscopy for Detection of *Cryptosporidium parvum* in Human Fecal Specimens: Clinical Trial

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PCR technology offers alternatives to conventional diagnosis of *Cryptosporidium* for both clinical and environmental samples. We compared microscopic examination by a conventional acid-fast staining procedure with a recently developed PCR test that can not only detect *Cryptosporidium* but is also able to differentiate between what appear to be host-adapted genotypes of the parasite. Examinations were performed on 511 stool specimens referred for screening on the basis of diarrhea. PCR detected a total of 36 positives out of the 511 samples, while routine microscopy detected 29 positives. Additional positives detected by PCR were eventually confirmed to be positive by microscopy. A total of five samples that were positive by routine microscopy at Western Diagnostic Pathology but negative by PCR and by microscopy in our laboratory were treated as false positives. Microscopy therefore exhibited 83.7% sensitivity and 98.9% specificity compared to PCR. PCR was more sensitive and easier to interpret but required more hands-on time to perform and was more expensive than microscopy. PCR, however, was very adaptable to batch analysis, reducing the costs considerably. Bulk buying of reagents and modifications to the procedure would decrease the cost of the PCR test even more. An important advantage of the PCR test, its ability to directly differentiate between different *Cryptosporidium* genotypes, will assist in determining the source of cryptosporidial outbreaks. Sensitivity, specificity, ability to genotype, ease of use, and adaptability to batch testing make PCR a useful tool for future diagnosis and studies on the molecular epidemiology of *Cryptosporidium* infections.

Cryptosporidium is a protozoan parasite that is ubiquitous in its geographic distribution and range of vertebrate hosts (16). Transmission of the parasite is direct, by either the fecal-oral route or the contamination of water supplies with the resistant infective oocyst stage of the life cycle. In humans and many other mammals, *Cryptosporidium* is recognized as a significant pathogen, primarily as a cause of acute, severe diarrheal illness. It is also one of the few parasitic infections that is becoming more prevalent, and outbreaks, which are now common (12), may vary in size from a few individuals to several hundred thousand (10).

Conventional methods for identification include examination of fecal smears with acid-fast stains such as Ziehl-Neelsen (18), which are commonly used by diagnostic facilities. Conventional microscopy, however, is time-consuming and tedious and requires experienced microscopists to accurately identify the oocysts (5, 8). In addition, the detection limits of conventional diagnostic techniques have been reported to be as low as 50,000 to 500,000 oocysts per gram of feces (19). Immunologically based detection methods have been developed for use in both clinical and environmental monitoring. However, antigenic variability within clinical isolates of *Cryptosporidium* (6) can result in some infections remaining undetected, and there are conflicting reports as to the sensitivity of immunodetection methods over microscopy (1, 8).

Recent research has demonstrated that humans are susceptible to infection with at least two distinct, apparently host-adapted genotypes of *Cryptosporidium* (human and calf) (3, 15). Diagnostic tests which can differentiate between human and animal isolates of *Cryptosporidium* will therefore be of particular benefit in outbreak situations such as that in British Columbia (2), in which determination of the source of infection is important in limiting transmission. As part of our activities at the World Health Organisation Collaborating Centre (WHO CC) for the Molecular Epidemiology of Parasitic Infections, we have recently developed sensitive PCR primers for the diagnosis of *Cryptosporidium* (15) which directly differentiate between human and bovine genotypes of *C. parvum* on the basis of the size of the PCR product. The aim of this study was to compare PCR detection of *Cryptosporidium* with conventional microscopic detection of *Cryptosporidium* in order to determine the usefulness and practicality of PCR-based detection methods for clinical diagnosis of *Cryptosporidium.*

MATERIALS AND METHODS

Specimens. Fecal samples collected from individuals referred by general practitioners to Western Diagnostic Pathology for testing for enteric parasites were used in the study. A total of 511 fecal samples were included in this trial. Specimens were tested blindly by Western Diagnostic Pathology and the laboratories of WHO CC at Murdoch University in appropriately sized batches for each procedure. Fecal samples were stored at 4°C without preservatives and were processed within 1 to 2 weeks.

Microscopy. Microscopic diagnosis of *Cryptosporidium* was performed by Western Diagnostic Pathology with a cold Ziehl-Neelsen stain. Briefly, a drop of

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TABLE 1. Comparison of PCR versus microscopic detection of *Cryptosporidium*

Method	No. of samples examined	No. of positives detected	Sensitivity (%)[a]	Specificity (%)[b]	Strain discrimination
PCR	511	36[c]	100	100	Yes[c]
Microscopy	511	29	83.7	98.9	No

[a] Calculated as follows: [number of true positives/(number of true positives + number of false negatives)] × 100.

[b] Calculated as follows: [number of true negatives/(number of true negatives + number of false positives)] × 100.

[c] The PCR test was able not only to detect *Cryptosporidium* oocysts but also to identify isolate genotypes (15). Of the 36 positives detected in the present study, 30 were of the human genotype and 6 were of the calf genotype.

fecal suspension was placed on a glass slide and spread to form a thin smear (similar to a blood film). Slides were fixed in absolute alcohol for 10 min and then flooded with carbol fuchsin for 1 h. Following washing, the slides were decolorized in 3% acid-alcohol for between 15 s and 1 min, depending on the film thickness. Slides were then washed, counterstained with 1% methylene blue for 4 min, washed and air dried, and examined under 20× and 40× objectives. One slide was reviewed per patient at a rate of 5 min per slide.

PCR detection. PCR diagnosis of *Cryptosporidium* was performed at Murdoch University. Fecal samples were diluted 1 in 4 in phosphate-buffered saline, and 20 μl of this suspension was used for total DNA extraction. A modification of a previously described fecal extraction procedure (15) was employed. Briefly, 20 μl of the fecal suspension was added to 80 μl of 10% polyvinylpolypyrrolidone (PVPP) (Sigma, St. Louis, Mo.) in distilled water and boiled for 10 min (PVPP was added because previous experience in our laboratory has shown that it reduces PCR inhibition). This solution was spun for 30 s, and the supernatant was added to a tube containing 200 μl of Al buffer (Qiagen, Hilden, Germany) and 10 μl of glassmilk (Bio-Rad, Richmond, Calif.). The sample was vortexed, incubated at 72°C for 5 min, and spun for 1 min, and the pellet was washed twice with 700 μl of AW wash buffer (Qiagen). The pellet was then vacuum dried, and the DNA was eluted by adding 50 μl of AE elution buffer (Qiagen), incubating the solution at 72°C for 10 min, spinning for 1 min, and transferring the supernatant to a fresh tube. A 2.5-μl aliquot of the eluate was used for PCR analysis. The primers used in this study had previously been extensively tested for specificity and sensitivity (15). PCR amplification conditions were as previously described (15). Duplicate reactions were run for each sample, one consisting of the test sample and a second reaction mixture containing the test DNA which was spiked with *Cryptosporidium* DNA in order to rule out PCR inhibition. A molecular mass ladder (100-bp; Gibco BRL) and positive and negative controls were used for each batch run.

RESULTS

A total of 511 fecal samples were screened by both microscopy and PCR (Table 1). All 511 fecal samples spiked with positive control *Cryptosporidium* DNA amplified the correct-sized band, indicating that PCR inhibition was not a factor in this trial. PCR analysis identified a total of 36 positives (7% of the test samples). Microscopy detected a total of 29 positives (5.6%) of the 36 identified by PCR. Repeat microscopy in our WHO CC laboratory on PCR-positive but microscopy-negative samples eventually revealed them to be positive by microscopy, although in a number of cases up to seven slides were screened at a rate of 10 min per slide before *Cryptosporidium* oocysts were detected. Routine microscopy at Western Diagnostic Pathology detected five additional positives which were negative by PCR and by microscopy in our laboratory and were considered false positives. Microscopy therefore showed 83.7% sensitivity and 98.9% specificity compared to 100% sensitivity and specificity for PCR (Table 1). The PCR test was also able to directly differentiate between human and calf *Cryptosporidium* genotypes. Six of the 36 positives displayed the calf genotype (approximately 17%), and the remaining 30 samples displayed the human genotype.

The preparation of each slide and the performance of the acid-fast stain procedure required about 10 min of a technologist's time. The reading of the slide required an additional 5 min. Interpretation of the acid-fast stain requires considerable expertise on the part of the operator. The cost of reagents per test for the acid-fast stain was very low, approximately $0.30. The cost of technicians' time at a rate of 5 min per slide must be also factored into this figure, and microscopy is not amenable to bulk processing, as the technologist is required to spend a minimum of 5 min per slide irrespective of the number of samples to be screened. The cost per test, including controls for the PCR procedure, was approximately $2.57; however, technologist time would add considerably to these costs (Table 2). The extraction of total DNA, PCR amplification, and subsequent gel analysis required a total of 4.5 h for a single sample plus controls; however, only about 1 h of this time was hands-on time for the technologist. PCR analysis is particularly amenable to bulk processing, and 96 samples can easily be processed in 1 to 2 days. Interpretation of the PCR test was easy, as it was based simply on the presence or absence of a band and the size of the band denoted the genotype of the isolate detected. With large throughput processing (96 samples/batch), the cost per PCR test was reduced to $1.20. A period of approximately two working days was required to process 96 samples, with approximately 11 to 12 h of technician time, although this time could be greatly reduced with improvements to the technique and the use of robotic workstations.

DISCUSSION

We compared conventional microscopy with acid-fast staining with a recently developed PCR test (15) for the detection of *Cryptosporidium* and found microscopy to be considerably less sensitive and less specific than PCR analysis. The PCR test was compared with microscopy instead of with more recently developed immunological methods for a number of reasons. Firstly, microscopical analysis of stained fecal smears is the most widely used method for screening stool samples for *Cryptosporidium* in clinical diagnostic laboratories. Secondly, previous research has shown that immunologically based detection methods are not significantly more sensitive than conventional microscopy (8, 17). In addition, because of differences in the interpretation of results obtained with immunologically based detection methods, as well as antigenic variability of clinical isolates of *Cryptosporidium* (6), microscopy was considered to be a more reliable diagnostic tool for the purposes of this comparative trial.

Because it is sensitive and easy to use, PCR amplification is an obvious choice for improved detection of *Cryptosporidium* from feces. However, fecal constituents such as bilirubin, bile salts, and complex polysaccharides inhibit PCR even when they are present at low concentrations (13, 21). We have therefore developed a simple, fast, and low-cost extraction technique which appears to eliminate most PCR inhibitors present in human feces from the template DNA. We initially tested PVPP, as it had been shown by another group at Murdoch to be useful in removing PCR inhibition in amplifications of fungi

TABLE 2. Cost of PCR versus microscopy for the detection of *Cryptosporidium*

Method	Reagent costs ($/test) for:		Technologist time (min)[a]
	Single test	Batch test[b]	
Microscopy	0.30	0.15	15
PCR	2.57	1.20	60

[a] Time is for a single test plus controls.

[b] Ninety-six tests per batch.

from soil samples (4a). The initial boiling step in PVPP was found to be necessary, as without this step approximately 30% of samples exhibited PCR inhibition (data not shown). Simply diluting the template DNA was not sufficient in all cases and resulted in a reduction of the sensitivity of the assay. A commercially available glassmilk (Bio-Rad) was used in this trial, although it has recently been shown that silica which is of defined size is at least 1,000 times less expensive and as effective as glassmilk quantitatively and qualitatively for purifying plasmid DNA minipreps and recovering fragments from agarose gels (4). Therefore, a 100-mg/ml suspension of silica (Sigma) in 3 M sodium iodide (4) would be an inexpensive and effective alternative to glassmilk.

A variety of PCR primers have been developed for the detection of *Cryptosporidium* in both fecal and environmental samples (14). Few of these primers, however, have been tested on large numbers of clinical or environmental samples or compared with conventional detection methods. In one study PCR was compared with immunofluorescent (IF) antibodies for the detection of *Cryptosporidium* in water samples (7); due to a number of problems, including inhibition, PCR was no more sensitive than immunofluorescence for the detection of *Cryptosporidium*. In another study (11), nested PCR was compared with IF staining, but discrepancies were reported between the two techniques because of PCR inhibition and also presumably because of the antibody binding to empty oocysts which are found routinely in water concentrates (9). In a more recent study, PCR was compared with both auramine phenol and IF staining in bovine feces (20). Immunomagnetic separation was used to purify the oocysts for PCR analysis, and PCR was reported to be several orders of magnitude more sensitive than conventional techniques. However, the test was performed on seeded feces from a limited number of cattle, and when PCR is coupled with immunomagnetic separation, the associated problems with antibody detection, particularly cross-reactivity and antigenic variability between isolates, are reintroduced (6).

Microscopy was cheaper to perform than PCR, but the cost of PCR detection was greatly reduced when a large number of samples was analyzed. With large throughput processing (96 samples/batch), the cost per test was reduced to $1.20. PCR is particularly amenable to automation and large throughput processing, and with bulk buying of reagents and modifications to the technique to reduce hands-on time, such as the use of 96-well plates for PCR setup and amplification, this cost could easily be further substantially reduced, thereby making PCR a more attractive option financially. In this trial, two PCRs were set up for each sample: a test sample and a second sample spiked with *Cryptosporidium* DNA in order to rule out PCR inhibition. However, a nonhomologous internal control could easily be constructed with commercially available kits such as the PCR mimic construction kit (Clontech, Palo Alto, Calif.), which would result in the amplification of a different-sized band with the *Cryptosporidium* diagnostic primers. This internal control could then be used to monitor the success of each PCR and would reduce the assay to a one-tube test, thus rendering it more cost-effective.

For clinical as well as environmental laboratories, both the ability to detect pathogens reliably and the ability to determine the numbers of pathogens present are important. Quantitative PCR techniques such as the Taqman LS-50B PCR detection system (Perkin-Elmer, Foster City, Calif.) have recently been launched, and the test described in the present study could be developed into a quantitative PCR test with this technology; however, the cost of the assay would be significantly higher.

Because of its sensitivity, the assay described in this study has the potential for accurate diagnosis in patients who do not presently know the reason for their diarrhea. This will have considerable advantages in the treatment of immunosuppressed individuals, allowing early diagnosis before the onset of symptoms. In addition, this PCR test is capable of directly differentiating between human- and animal-derived genotypes of *Cryptosporidium* on the basis of the size of the PCR product (15). The potential for zoonotic transmission from livestock and wild-animal reservoirs via environmental contamination is of increasing concern. Thus, the primers used in this study may be valuable in the predictive epidemiology of cryptosporidial infections in humans and livestock.

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REFERENCES

1. **Alles, A. J., M. A. Waldron, L. S. Sierra, and A. R. Mattia.** 1995. Prospective comparison of direct immunofluorescence and conventional staining methods for detection of *Giardia* and *Cryptosporidium* in human fecal specimens. J. Clin. Microbiol. **33:**1632–1634.
2. **Anonymous.** 1996. *Cryptosporidium* outbreak in British Columbia. *Cryptosporidium* Capsule 1, 1–3.
3. **Bonnin, A., M. N. Fourmaux, J. F. Dubremetz, R. G. Nelson, P. Gobet, G. Harly, M. Buisson, D. Puygauthier-Toubas, F. Gabriel-Pospisil, M. Naciri, and P. Camerlynck.** 1996. Genotyping human and bovine isolates of *Cryptosporidium parvum* by polymerase chain reaction-restriction fragment length polymorphism analysis of a repetitive DNA sequence. FEMS Microbiol. Lett. **137:**207–211.
4. **Boyle, J. S., and A. M. Lew.** 1995. An inexpensive alternative to glassmilk for DNA purification. Trends Genet. **11:**8.

4a. **Dobrowolski, D., and P. A. O'Brien.** Unpublished observations.

5. **Garcia, L. S., T. C. Brewer, and D. A. Bruckner.** 1987. Fluorescence detection of *Cryptosporidium* oocysts in human fecal specimens using monoclonal antibodies. J. Clin. Microbiol. **25:**119–121.
6. **Griffin, K., E. Matthai, M. Hommel, J. C. Weitz, D. Baxby, and C. A. Hart.** 1992. Antigenic diversity among oocysts of clinical isolates of *Cryptosporidium parvum*. J. Protozool. Res. **2:**97–101.
7. **Johnson, D. W., N. J. Pieniazek, D. W. Griffin, L. Misener, and J. B. Rose.** 1995. Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. Appl. Environ. Microbiol. **61:**3849–3855.
8. **Kehl, K. S. C., H. Cicirello, and P. L. Havens.** 1995. Comparison of four different methods for the detection of *Cryptosporidium* species. J. Clin. Microbiol. **33:**416–418.
9. **LeChevallier, M. W., W. D. Norton, and R. G. Lee.** 1991. Occurrence of *Giardia* and *Cryptosporidium* spp. in surface water supplies. Appl. Environ. Microbiol. **57:**2610–2616.
10. **MacKenzie, W. R., N. J. Hoxie, M. E. Proctor, S. Gradus, K. A. Blair, D. E. Peterson, J. J. Kazmierczak, D. G. Addiss, K. R. Fox, J. P. Rose, and J. P. Davis.** 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N. Engl. J. Med. **331:**161–167.
11. **Mayer, C. L., and C. J. Palmer.** 1996. Evaluation of PCR, nested PCR, and fluorescent antibodies for detection of *Giardia* and *Cryptosporidium* species in wastewater. Appl. Environ. Microbiol. **62:**2081–2085.
12. **McDonald, V.** 1996. Cryptosporidiosis, p. 256–263. *In* F. E. G. Cox (ed.), The Welcome Trust illustrated history of tropical diseases. The Welcome Trust, London, England.
13. **Monteiro, L., D. Bonnemaison, A. Vekris, K. G. Petry, J. Bonnet, R. Vidal, J. Cabritta, and F. Megraund.** 1997. Complex polysaccharides as PCR inhibitors in faeces: *Helicobacter pylori* model. J. Clin. Microbiol. **35:**995–998.
14. **Morgan, U. M., and R. C. A. Thompson.** PCR detection of *Cryptosporidium*: the way forward? Parasitol. Today, in press.
15. **Morgan, U. M. C., C. Constantine, D. A. Forbes, and R. C. A. Thompson.** 1997. Differentiation between human and animal isolates of *Cryptosporidium parvum* using rDNA sequencing and direct PCR analysis. J. Parasitol. **83:**825–830.
16. **O'Donoghue, P. J.** 1995. *Cryptosporidium* and cryptosporidiosis in man and animals. Int. J. Parasitol. **25:**139–195.
17. **Rodriguez-Hernandez, J., A. Canutblasco, M. Ledesmagarcia, and A. M. Martinsanchez.** 1994. *Cryptosporidium* oocysts in water for human consumption—comparison of staining methods. Eur. J. Epidemiol. **10:**215–218.

18. **Scott, C. D.** 1988. Screening fecal smears for *Cryptosporidium* and *Isospora belli* using a modified Ziehl-Neelsen stain. Aust. J. Med. Lab. Sci. **9:**80–82.
19. **Weber, R., R. T. Bryan, H. S. Bishop, S. P. Wahlquist, J. J. Sullivan, and D. D. Juranek.** 1991. Threshold of detection of *Cryptosporidium* oocysts in human stool samples: evidence for low sensitivity of current diagnostic methods. J. Clin. Microbiol. **29:**1323–1327.
20. **Webster, K. A., H. V. Smith, M. Giles, L. Dawson, and L. J. Robertson.** 1996. Detection of *Cryptosporidium parvum* oocysts in faeces: comparison of conventional coproscopical methods and the polymerase chain reaction. Vet. Parasitol. **61:**5–13.
21. **Widjojoatmodjo, M. N., A. D. C. Fluit, R. Torensma, G. P. H. T. Verdonk, and J. Verhoef.** 1992. The magnetic immuno polymerase chain reaction assay for direct detection of salmonellae in fecal samples. J. Clin. Microbiol. **30:**3195–3199.