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Development of a Two-Color Fluorescence In Situ Hybridization Technique for Species-Level Identification of Human-Infectious *Cryptosporidium* spp.▽

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A two-color fluorescence in situ hybridization assay that allows for the simultaneous identification of *Cryptosporidium parvum* and *C. hominis* was developed. The assay is a simple, rapid, and cost-effective tool for the detection of the major *Cryptosporidium* species of concern to public health.

Cryptosporidium (*Apicomplexa*) is a genus of protozoan parasites with species and genotypes that infect humans, domesticated livestock, companion animals, and wildlife worldwide (5, 6, 14, 15, 20, 23). The majority of cases of cryptosporidiosis in humans are caused by *Cryptosporidium parvum* or *C. hominis* (8, 10, 19, 24), although rare cases due to species such as *C. meleagridis*, *C. felis*, or *C. canis* have been reported (8, 9, 11–13, 17, 18, 22). The specific identification and characterization of *Cryptosporidium* species are central to the control of this disease in humans and a wide range of animals.

One of the most widely adopted techniques for the identification of microorganisms in complex microbial communities is fluorescence in situ hybridization (FISH) using rRNA-targeted oligonucleotide probes (2–4). This method relies on the hybridization of synthetic oligonucleotide probes to specific regions within the rRNA of the organism. While FISH has been applied for the detection of *Cryptosporidium* oocysts in water samples (21), no FISH probes that successfully differentiate *C. hominis* from *C. parvum* have been reported.

We have reported previously on the design of a species-specific probe, Cpar677, that detects *C. parvum* (1). In this study, we report on the design and validation of a *C. hominis* species-specific probe, Chom253. Together, the two probes were used here for the development of a two-color, microscopy-based FISH assay for the simultaneous detection of *C. parvum* and *C. hominis*.

***C. hominis* probe design and validation.** An alignment of 18S rDNA sequences of *Cryptosporidium* species obtained from the GenBank database was used to design a probe specific for *C. hominis* (Chom253, TCA CAT TAA TTG TGA TCC). The resulting Chom253 probe differs from the *C. parvum* 18S sequence by 2 nucleotides and from the *Cryptosporidium* horse genotype 18S sequence by 1 nucleotide. The Chom253 probe was synthesized with Cy3 attached (Proligo, Australia), and the probe specificity was tested against a panel of eight *Cryptosporidium* species (*C. parvum*, *C. hominis*, *C. andersoni*, *C. muris*, *C. meleagridis*, *C. felis*, *Cryptosporidium* cervine genotype, and *Cryptosporidium* rabbit genotype) by use of the FISH protocol developed for *C. parvum* (1). Specificity studies revealed that Ch253-Cy3 hybridized with *C. hominis* and *Cryptosporidium* rabbit genotype oocysts. Fluorescent signals were not observed with any of the other species examined. Hybridization of the *C. hominis*-specific probe to the *Cryptosporidium* rabbit genotype was due to the probe target region being 100% homologous between the rabbit genotype and *C. hominis*.

Two-color FISH for differentiation of *C. parvum* and *C. hominis*. A two-color FISH assay for the simultaneous identification of *C. parvum* and *C. hominis* was developed using Cpar677-Cy3 and Chom253-fluorescein isothiocyanate (FITC) and the FISH protocol reported for *C. parvum* (1), except that the hybridization buffer contained both of the oligonucleotide probes at a final concentration of 1.5 mmol liter^{-1} each. The assay was validated using 50 human fecal samples positive for *Cryptosporidium*, and oocysts were isolated from feces by use of a purification procedure described previously (1). FISH results were compared to PCR-restriction fragment length polymorphism (RFLP) results by use of a DNA extraction method, an 18S rDNA nested PCR, and an RFLP analysis described previously (1). In the dual assay, of the 50 isolates examined, 23 tested positive with Chom253, with a bright-green fluorescence signal observed (Fig. 1A), indicating the presence of *C. hominis*. The remaining 27 isolates tested positive with Cpar677, resulting in a bright-red fluorescence signal (Fig. 1B), indicating the presence of *C. parvum*. PCR-RFLP analysis, using VspI, confirmed our FISH results, as demonstrated by specific banding profiles. The results confirmed the strong correlation between FISH and PCR-RFLP observed previously (1).

Oocyst recovery efficiency following FISH. The recovery efficiency of the assay was evaluated with polycarbonate filter membranes by performing FISH using Cpar677-Cy3 on a precise number of *Cryptosporidium* oocysts. Precise numbers of *C. parvum* oocysts (105 ± 12) were prepared for recovery determination by use of fluorescence-activated cell sorting (7). Five groups of triplicate samples underwent hybridization on mem-

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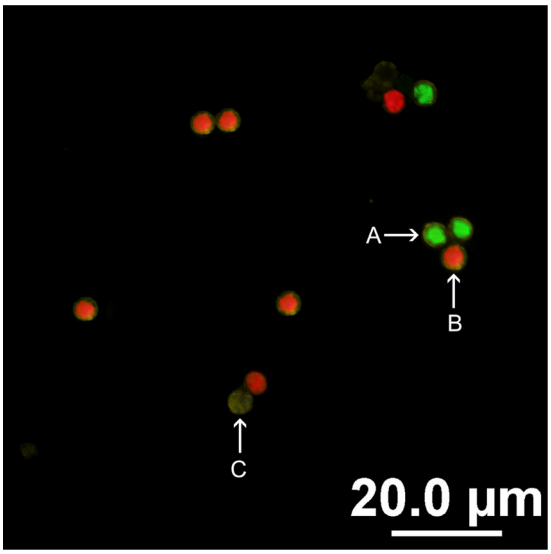

FIG. 1. Two-color FISH assay for the identification of *C. parvum* and *C. hominis* oocysts, carried out using Cpar677-Cy3 and Chom253-FITC. (A) Positive hybridization of *C. hominis* oocysts was revealed by bright-green fluorescence. (B) Positive hybridization of *C. parvum* oocysts was revealed by bright-red fluorescence. (C) A lack of fluorescence confirms FISH-negative oocysts.

branes according to the vacuum manifold technique previously described by Davies et al. (16). Each triplicate set of five samples was processed on the same day. Counterstaining of oocysts with CRY104-FITC enabled the quantification of oocysts to be assessed microscopically. The mean oocyst recovery efficiency for each of the three groups and the overall efficiency were 76.7% ± 14.9%, 88.5% ± 8.6%, 80.9% ± 2.1%, and 82.1% ± 5.9%, respectively. When the data were analyzed by using a two-sample *t* test (based on the assumption that data for both populations [before and after FISH] were normally distributed and the assumption that the population standard deviations for both were the same), no significant differences in oocyst recovery were detected for these groups ($P = 0.985$). The levels of recovery increased significantly by use of this method ($P < 0.001$) compared to recoveries obtained when FISH was not performed according to the vacuum manifold technique (16).

Mixed-species detection. To evaluate the sensitivity of the two-color assay for the detection of mixed infections, precise numbers of *C. parvum* and *C. hominis* oocyst seeds were also prepared. Triplicate samples containing a total of 200 oocysts were used, and percentages of *C. parvum* to *C. hominis* oocysts prepared were 10:90, 25:75, 50:50, 75:25, and 90:10. The two-color assay was then performed as described above and quantified using epifluorescence microscopy. Simultaneous detection of 80.6% ± 3.1% of *C. parvum* and *C. hominis* oocysts occurred at all ratios tested. In each case, as few as 10 *C. parvum* and *C. hominis* oocysts were detected using the two-color assay (Table 1).

Our two-color FISH assay, based on species-specific probes for *C. parvum* and *C. hominis*, can distinguish between the two major species involved in human infections. We have shown here that species-specific FISH is a reliable alternative to PCR and RFLP for rapid identification within a 3-h time frame. In summary, the potential to detect and identify pathogenic *Cryptosporidium* species in clinical, water, and environmental samples within a 3-h time frame demonstrates that FISH offers an alternative to traditional molecular diagnostic methods that utilize PCR.

TABLE 1. Recovery of mixed-species suspensions of *C. parvum* and *C. hominis* oocysts subjected to the two-color FISH assay[a]

Ratio (%) of *C. parvum* to *C. hominis* oocysts	Mean % recovery following two-color FISH	
	C. parvum	*C. hominis*
10:90	6.1 ± 2.2	80.7 ± 5.8
25:75	18.7 ± 5.1	68 ± 4.3
50:50	40.5 ± 5.0	41.1 ± 4.8
75:25	69.2 ± 4.1	18.8 ± 3.2
90:10	82.3 ± 4.9	6.8 ± 2.0

[a] *C. parvum*- and *C. hominis*-specific probes were used for the detection of viable oocysts. Oocysts were quantified using epifluorescence microscopy.

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REFERENCES

1. **Alagappan, A., N. A. Tujulaa, M. Power, C. M. Ferguson, P. L. Bergquist, and B. C. Ferrari.** 2008. Development of fluorescent in situ hybridisation for *Cryptosporidium* detection reveals zoonotic and anthroponotic transmission of sporadic cryptosporidiosis in Sydney. J. Microbiol. Methods **75:**535–539.
2. **Amann, R., and B. Fuchs.** 2008. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. Nat. Rev. Microbiol. **6:**339–348.
3. **Amann, R., W. Ludwig, and K. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
4. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
5. **Angus, K. W.** 1983. Cryptosporidiosis in man, domestic animals and birds: a review. J. R. Soc. Med. **76:**62–70.
6. **Angus, K. W.** 1990. Cryptosporidiosis in ruminants. CRC Press, Boston, MA.
7. **Bennett, J. W., and M. R. Gauci.** 1999. A comparison of enumeration techniques for *Cryptosporidium parvum* oocysts. J. Parasitol. **85:**1165–1168.
8. **Caccio, S. M.** 2005. Molecular epidemiology of human cryptosporidiosis. Parassitologia **47:**185–192.
9. **Cacciò, S. M., E. Pinter, R. Fantini, I. Mezzaroma, and E. Pozio.** 2002. Human infection with *Cryptosporidium felis*: case report and literature review. Emerg. Infect. Dis. **8:**85–86.
10. **Caccio, S. M., and E. Pozio.** 2006. Advances in the epidemiology, diagnosis and treatment of cryptosporidiosis. Expert Rev. Anti Infect. Ther. **4:**429–443.
11. **Cama, V. A., C. Bern, I. M. Sulaiman, R. H. Gilman, E. Ticona, A. Vivar, V. Kawai, D. Vargas, L. Zhou, and L. Xiao.** 2003. *Cryptosporidium* species and genotypes in HIV-positive patients in Lima, Peru. J. Eukaryot. Microbiol. **50S:** 531–533.
12. **Cama, V. A., R. H. Gilman, A. Vivar, E. Ticona, Y. Ortega, C. Bern, and L. Xiao.** 2006. Mixed *Cryptosporidium* infections and HIV. Emerg. Infect. Dis. **12:**1025–1028.
13. **Chalmers, R., K. Elwin, A. Thomas, and D. Joynson.** 2002. Infection with unusual types of *Cryptosporidium* is not restricted to immunocompromised patients. J. Infect. Dis. **185:**270–271.
14. **Current, W. L.** 1985. Cryptosporidiosis. J. Am. Vet. Med. Assoc. **187:**1334–1338.
15. **Current, W. L., and L. S. Garcia.** 1991. Cryptosporidiosis. Clin. Microbiol. Rev. **4:**325–358.
16. **Davies, C. M., N. Altavilla, M. Krogh, C. M. Ferguson, D. A. Deere, and N. J.**

Ashbolt. 2005. Environmental inactivation of *Cryptosporidium* oocysts in catchment soils. J. Appl. Microbiol. **98:**308–317.
17. **Gatei, W., Y. Suputtamongkol, D. Waywa, R. W. Ashford, J. W. Bailey, J. Greensill, N. J. Beeching, and C. A. Hart.** 2002. Zoonotic species of *Cryptosporidium* are as prevalent as the anthroponotic in HIV-infected patients in Thailand. Ann. Trop. Med. Parasitol. **96:**797–802.
18. **Guyot, K., A. Follet-Dumoulin, E. Lelievre, C. Sarfati, M. Rabodonirina, G. Nevez, J. C. Cailliez, D. Camus, and E. Dei-Cas.** 2001. Molecular characterization of *Cryptosporidium* isolates obtained from humans in France. J. Clin. Microbiol. **39:**3472–3480.
19. **Morgan, U. M., L. Xiao, R. Fayer, A. A. Lal, and R. C. Thompson.** 1999. Variation in *Cryptosporidium*: towards a taxonomic revision of the genus. Int. J. Parasitol. **29:**1733–1751.
20. **O'Donoghue, P.** 1995. *Cryptosporidium* and cryptosporidiosis in man and animals. Int. J. Parasitol. **25:**139–195.
21. **Vesey, G., N. Ashbolt, G. Wallner, M. Dorsch, K. Williams, and D. Veal.** 1995. Assessing *Cryptosporidium parvum* oocyst viability with fluorescent in-situ hybridisation using ribosomal RNA probes and flow cytometry, p. 133–138. *In* W. W. Betts, B. Casemore, D. Fricker, C. Smith, and H. Watkins (ed.), Protozoan parasites and water. Royal Society of Chemistry, Cambridge, United Kingdom.
22. **Xiao, L., C. Bern, J. Limor, I. Sulaiman, J. Roberts, W. Checkley, L. Cabrera, R. Gilman, and A. Lal.** 2001. Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J. Infect. Dis. **183:**492–497.
23. **Xiao, L., and R. Fayer.** 2008. Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. Int. J. Parasitol. **38:**1239–1255.
24. **Xiao, L., and U. M. Ryan.** 2004. Cryptosporidiosis: an update in molecular epidemiology. Curr. Opin. Infect. Dis. **17:**483–490.