Detection of *Cryptosporidium molnari* Oocysts from Fish by Fluorescent-Antibody Staining Assays for *Cryptosporidium* spp. Affecting Humans[▽]

Rona Barugahare, Michelle M. Dennis, Joy A. Becker, and Jan Šlapeta*

Faculty of Veterinary Science, University of Sydney, Sydney, New South Wales 2006, Australia

Received 16 November 2010/Accepted 5 January 2011

Three direct fluorescent-antibody staining assay kits for the detection of zoonotic *Cryptosporidium* species were used to detect *Cryptosporidium molnari* from Murray cod, and the cryptosporidia were characterized by using small-subunit (SSU) ribosomal DNA (rDNA). To facilitate rapid diagnosis of infection, this study demonstrated that all three kits detected fresh *C. molnari* and two kits detected formalin-fixed oocysts.

Cryptosporidiosis is a waterborne disease caused by protozoan parasites of the genus *Cryptosporidium* (Apicomplexa). It has debilitating effects in both humans and production animals and is characterized by diarrhea and general symptoms of gastrointestinal disease (5, 7). Two genotypes are the most common causes of human cryptosporidiosis, and these are the zoonotic *C. pestis* (or the *C. parvum* “bovine genotype”) and the human-specific *C. hominis* (or the *C. parvum* “human genotype”) (8, 19–21). Direct fluorescent-antibody staining assays are routinely used for diagnosing gastrointestinal cryptosporidiosis in humans and domestic mammals and are considered the gold standard because of their high sensitivity and specificity (11, 12, 16).

There is a great diversity of *Cryptosporidium* species and genotypes recorded from mammals, birds, reptiles, and amphibians as well as fish (8, 19). Cryptosporidiosis is an emerging disease in both wild and farmed fish in numerous countries worldwide (9, 14, 18). Expensive, time-consuming, and labor-intensive histopathology and PCR have been used to diagnose cryptosporidiosis (14, 23). The aim of this study was to apply human direct fluorescent-antibody staining assays for *Cryptosporidium* to detect the fish parasite. We tested three commercially available monoclonal antibody (MAb)-based assays for their capacities for detecting fresh and formalin-fixed oocysts of *Cryptosporidium molnari* in fish.

Sample collection and processing. All samples for *C. molnari* detection were from a grow-out farm for Murray cod (*Maccullochella peelii peelii*). Thirty-four 6-month-old fish (70 to 100 mm in length) that were smaller than normal and exhibiting spiral swimming were collected from a single tank. Twelve fish were frozen at -20°C , and 22 were preserved in 10% neutral buffered formalin. For the latter group, histopathological examination (hematoxylin and eosin [H&E])

revealed the presence of *C. molnari* in the stomachs of 21 of 22 (95%) of the fish.

Fish stomach dissection. The stomachs were dissected from the frozen fish ($n = 12$), and mucosal scrapings were taken (approximately 50 mg). An aliquot of the scraping was placed directly onto a microscope slide for testing, and the remainder was mixed with 10% neutral buffered formalin (0.5 ml) in an Eppendorf tube and thoroughly homogenized using a vortex.

Direct fluorescence assay kits. Three fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* sp. MAbs in direct fluorescent-antibody staining assay kits for diagnosis of human cryptosporidiosis were used according to the manufacturers' instructions. Test 1 was the Crypto Cel immunofluorescence (IF) test (Cellabs, Brookvale, New South Wales, Australia), test 2 was the BTP *Giardia/Cryptosporidium* combined antigen detection kit (Biotech Trading Partners, CA, and Banksia Scientific, Bulimba, Queensland, Australia), and test 3 was the MeriFluor *Cryptosporidium/Giardia* test (Meridian Bioscience, OH, and Immuno, St. Peters, South Australia, Australia). Slides were examined on an Olympus BX60 microscope equipped for FITC fluorescence (maximum excitation wavelength, 490 nm; mean emission wavelength, 530 nm) and with an Olympus DP70 camera. Under fluorescent light, the positive controls showed bright green spherical *Cryptosporidium* oocysts (i.e., 4 to 5 μm in diameter).

SSU rDNA gene amplification and sequencing. Nucleic acid was extracted from a gastric scraping (~ 20 mg) that contained *C. molnari* by using the FastDNA soil kit with a Fast Prep-24 (MP Biomedicals, Australia); the speed setting used was 6.0 for 40 s. Nested PCR amplification of a fragment of small-subunit (SSU) ribosomal DNA (rDNA) was applied according to the method of Ryan et al. (17). A PCR product of approximately 550 bp was directly sequenced at AGRF (Westmead, New South Wales, Australia) and analyzed by using CLC Main Workbench 5.5 software (CLC bio, Denmark).

Sequence analysis of SSU rDNA. The obtained sequence of the *C. molnari* SSU rDNA was 99.3% (526/530 nucleotides [nt]) identical to that of *C. molnari* in the gilthead sea bream (*Sparus aurata*) from Spain (15) and 99.8% (404/405 nt) iden-

* Corresponding author. Mailing address: McMaster Building B14, Faculty of Veterinary Science, University of Sydney, Sydney, New South Wales 2006, Australia. Phone: 61 2 9251 2025. Fax: 61 2 935 17348. E-mail: jan.slapeta@sydney.edu.au.

[▽] Published ahead of print on 14 January 2011.

FIG. 1. Direct fluorescent-antibody staining of *Cryptosporidium molnari* oocysts. A fresh oocyst (A) and an oocyst fixed in formalin for 7 days (B) are shown. FITC-conjugated anti-*Cryptosporidium* sp. MAbs (Crypto Cel IF test; Cellabs, Australia) were used. The oocysts exhibit the typical semicircular longitudinal suture in the oocyst wall. Bar, 5 μ m.

tical to that of *C. molnari* in the butter bream (*Monodactylus argenteus*) from Spain (23).

Rapid detection of *Cryptosporidium molnari* oocysts. Test 1 (the Crypto Cel IF test) detected oocysts in 92% (11/12) of the fish samples, which corresponds to the 95% prevalence determined by histology. The oocysts of *C. molnari* were spherical and 4 to 5 μ m in diameter, and they occasionally showed an incomplete suture line of the oocyst wall (1) (Fig. 1A). Three fresh fish stomachs tested positive for *Cryptosporidium* by test 1 (Crypto Cel IF Test) as well as test 2 (BTP *Giardia/Cryptosporidium* combined antigen detection kit) and test 3 (MeriFluor *Cryptosporidium/Giardia* test) (Table 1). Oocysts labeled with test 3 in fresh samples fluoresced weakly and were difficult to visualize against the counterstain. Formalin fixation for 24 h did not reduce the FITC fluorescence of *C. molnari* oocysts by test 1 (4 of 4 samples testing positive) and test 2 (4 of 4 samples testing positive) (Table 1). Shorter fixation (5 min, 30 min, 2 h, or 6 h; 3 samples each) did not reduce FITC labeling for *C. molnari* oocysts by test 1. Test 3 did not label *C. molnari* after formalin fixation for 24 h (0/4 samples), although structures similar in size and shape to *C. molnari* oocysts were observed, and they had a yellow fluorescence. Formalin fixation for 4 (1/1 sample), 7 (6/6 samples), and 9 (2/2 samples) days had no effect on the oocyst labeling using test 1 (Fig. 1B), and formalin fixation for 7 (3/3 samples) days had no effect on labeling using test 2. Material fixed for 63 days appeared to have weaker fluorescence than fresh oocysts with test 1 (1/1 sample) and test 2 (1/1 sample), and this was weaker than the bright FITC fluorescence of the positive control. Test 3 did not label *C. molnari* oocysts that had been fixed for 7 (0/3 samples) and 63 (0/1 sample) days (Table 1).

Conclusions. We successfully labeled *C. molnari* by using existing direct fluorescence assays designed for diagnosis of human cryptosporidiosis. These assays can be readily used to diagnose outbreaks of clinical cryptosporidiosis in farmed freshwater fish, as demonstrated here for the Murray cod samples with comparable sensitivity results with histology. Selection of the appropriate assay is important for accurate diagnosis because (i) one of the three tests did not label oocysts consistently after formalin fixation, (ii) the tests had weak labeling of *C. molnari* oocysts 63 days postfixation, and

(iii) formalin fixation is a very common technique for preserving samples for disease investigation.

The differences between MAb labeling of *C. molnari* and that of the positive control (zoonotic *Cryptosporidium* spp.) imply vulnerability of the oocyst antigens to formalin, especially the epitope recognized by the MAb in test 3. Formalin progressively cross-links proteins (including antigen epitopes) of the oocyst wall, possibly inhibiting successful labeling with MAbs (2–4, 6, 22). The cross-reactivity of the MAb tests illustrates the distinct evolutionary trajectory of the piscine clade of *C. molnari* and mammalian *Cryptosporidium* spp. (15). The FITC-conjugated anti-*Cryptosporidium* sp. MAb in test 3 was previously shown to label gastric *C. serpentis* from snakes preserved in formalin (12) and gastric *Cryptosporidium* sp. oocysts from farmed *Xenopus laevis* frogs (13). Assuming that the fish *C. molnari* is ancestral to the gastric species and the intestinal zoonotic *Cryptosporidium* spp. (15), the oocyst surface antigen was under selection pressure to become more resilient while the oocyst morphology remained the same.

Traditionally, the diagnosis of cryptosporidiosis in fish has been conducted on samples collected postmortem, often requiring the sacrifice of stock. This study demonstrated that existing direct fluorescent-antibody staining assays detect oocysts of *C. molnari* from fish and therefore have advantages over histology and even PCR by being rapid (1 to 1.5 h turnaround time), cheaper, and simpler to perform. Similar to the case for a rapid medical response, a direct immunofluorescence assay is preferred over virus isolation and real-time reverse transcription-PCR (RT-PCR) for influenza A (H1N1) virus in respiratory specimens (10).

Direct fluorescent-antibody staining assays for *Cryptosporidium* are designed to work on fecal or even water samples after concentration, avoiding the necessity of sacrificing stock. Murray cod are an iconic Australian freshwater fish of high conservation value. Undiagnosed *C. molnari* infections allow the parasite to be moved across catchments or states with live fish movements for restocking programs and aquaculture. Therefore, these tests may potentially be applied to both wild and farmed fish for the purposes of routine health checks, stock quarantine, and epidemiological studies.

Nucleotide sequence accession number. The nucleotide sequence of the SSU rDNA of *C. molnari* in the Murray cod was deposited in GenBank under the accession number HQ585890.

TABLE 1. Detection of fresh and formalin-fixed oocysts of *Cryptosporidium molnari*^a

Test no.	No. of samples positive for <i>C. molnari</i> /total			
	Unfixed	Formalin fixed		
		24 h	7 days	63 days
1	3/3	4/4	6/6	1/1
2	3/3	4/4	3/3	1/1
3	3/3	0/4	0/3	0/1

^a Test 1 was the Crypto Cel IF test, test 2 was the BTP *Giardia/Cryptosporidium* combined antigen detection kit, and test 3 was the MeriFluor *Cryptosporidium/Giardia* test.

This work was supported by the Faculty of Veterinary Science, University of Sydney.

REFERENCES

1. Alvarez-Pellitero, P., and A. Sitjà-Bobadilla. 2002. *Cryptosporidium molnari* n. sp. (Apicomplexa: Cryptosporidiidae) infecting two marine fish species, *Sparus aurata* L. and *Dicentrarchus labrax* L. *Int. J. Parasitol.* **32**:1007–1021.
2. Arnold, M. M., et al. 1996. Effects of fixation and tissue processing on immunohistochemical demonstration of specific antigens. *Biotech. Histochem.* **71**:224–230.
3. Battifora, H., and M. Kopinski. 1986. The influence of protease digestion and duration of fixation on the immunostaining of keratins. A comparison of formalin and ethanol fixation. *J. Histochem. Cytochem.* **34**:1095–1100.
4. Bonnin, A., J. F. Dubremetz, and P. Camerlynck. 1991. Characterization and immunolocalization of an oocyst wall antigen of *Cryptosporidium parvum* (Protozoa, Apicomplexa). *Parasitology* **103**:171–177.
5. Chalmers, R. M., and A. P. Davies. 2010. Minireview: clinical cryptosporidiosis. *Exp. Parasitol.* **124**:138–146.
6. Entrala, E., Y. Sbihi, M. Sanchez-Moreno, and C. Mascaro. 2001. Antigen incorporation on *Cryptosporidium parvum* oocyst walls. *Mem. Inst. Oswaldo Cruz* **96**:233–235.
7. Fayer, R. (ed.). 1997. *Cryptosporidium* and cryptosporidiosis. CRC Press, Boca Raton, FL.
8. Fayer, R. 2010. Taxonomy and species delimitation in *Cryptosporidium*. *Exp. Parasitol.* **124**:90–97.
9. Gabor, L., et al. Cryptosporidiosis in intensively reared Barramundi (*Lates calcarifer*). *J. Vet. Diagn. Invest.*, in press.
10. Ganzenmueller, T., et al. 2010. Comparison of the performance of direct fluorescent antibody staining, a point-of-care rapid antigen test and virus isolation with that of RT-PCR for the detection of novel 2009 influenza A (H1N1) virus in respiratory specimens. *J. Med. Microbiol.* **59**:713–717.
11. Garcia, L. S., A. C. Shum, and D. A. Bruckner. 1992. Evaluation of a new monoclonal-antibody combination reagent for direct fluorescence detection of *Giardia* and *Cryptosporidium* oocysts in human fecal specimens. *J. Clin. Microbiol.* **30**:3255–3257.
12. Graczyk, T. K., M. R. Cranfield, and R. Fayer. 1995. A comparative assessment of direct fluorescence antibody, modified acid-fast stain, and sucrose flotation techniques for detection of *Cryptosporidium serpentis* oocysts in snake fecal specimens. *J. Zoo Wildl. Med.* **26**:396–402.
13. Green, S. L., D. M. Bouley, C. A. Josling, and R. Fayer. 2003. Cryptosporidiosis associated with emaciation and proliferative gastritis in a laboratory-reared South African clawed frog (*Xenopus laevis*). *Comp. Med.* **53**:81–84.
14. Murphy, B. G., D. Bradway, T. Walsh, G. E. Sanders, and K. Snekvik. 2009. Gastric cryptosporidiosis in freshwater angelfish (*Pterophyllum scalare*). *J. Vet. Diagn. Invest.* **21**:722–727.
15. Palenzuela, O., P. Alvarez-Pellitero, and A. Sitjà-Bobadilla. 2010. Molecular characterization of *Cryptosporidium molnari* reveals a distinct piscine clade. *Appl. Environ. Microbiol.* **76**:7646–7649.
16. Rimhanen-Finne, R., H. L. Enemark, J. Kolehmainen, P. Toropainen, and M. L. Hanninen. 2007. Evaluation of immunofluorescence microscopy and enzyme-linked immunosorbent assay in detection of *Cryptosporidium* and *Giardia* infections in asymptomatic dogs. *Vet. Parasitol.* **145**:345–348.
17. Ryan, U., et al. 2003. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. *Appl. Environ. Microbiol.* **69**:4302–4307.
18. Sitjà-Bobadilla, A., F. Padros, C. Aguilera, and P. Alvarez-Pellitero. 2005. Epidemiology of *Cryptosporidium molnari* in Spanish gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.) cultures: from hatchery to market size. *Appl. Environ. Microbiol.* **71**:131–139.
19. Šlapeta, J. 2009. Centenary of the genus *Cryptosporidium*: from morphological to molecular species identification, p. 31–50. *In* M. G. Ortega-Pierres et al. (ed.), *Giardia* and *Cryptosporidium*: from molecules to disease. CAB International, Wallingford, United Kingdom.
20. Šlapeta, J. 2006. *Cryptosporidium* species found in cattle: a proposal for a new species. *Trends Parasitol.* **22**:469–474.
21. Xiao, L., R. Fayer, U. Ryan, and S. J. Upton. 2004. *Cryptosporidium* taxonomy: recent advances and implications for public health. *Clin. Microbiol. Rev.* **17**:72–97.
22. Yu, J. R., S. P. O'Hara, J. L. C. Lin, M. E. Dailey, and G. Cain. 2002. A common oocyst surface antigen of *Cryptosporidium* recognized by monoclonal antibodies. *Parasitol. Res.* **88**:412–420.
23. Zanguee, N., et al. 2010. Identification of novel *Cryptosporidium* species in aquarium fish. *Vet. Parasitol.* **174**:43–48.