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Polymerase Chain Reaction Detection of *Pseudoloma neurophilia*, a Common Microsporidian of Zebrafish (*Danio rerio*) Reared in Research Laboratories

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One of the most prevalent pathogens found in zebrafish (*Danio rerio*) research facilities is the microsporidian parasite *Pseudoloma neurophilia*. Infections occur primarily in the spinal cord and are associated with emaciation and scoliotic changes. It is unclear why *P. neurophilia* is so widespread among research colonies, although transfer of infected animals and eggs between laboratories is a likely contributor. In addition to preventing the spread of this pathogen among facilities, it is desirable to have parasite-free fish for use in experiments. Therefore we have developed a polymerase chain reaction (PCR)-based diagnostic test for *P. neurophilia*. Compared with conventional diagnostic methods, PCR diagnosis is rapid, allows for screening of large numbers of fish, and can be applied to eggs, water filtrates, biofilms, and other samples. Using PCR primers specific to the small subunit ribosomal DNA of *P. neurophilia*, the test was consistently capable of detecting 10 spores per reaction and often as few as 0.1 spore per reaction, and it did not cross-react with other selected microsporidian species from fish. We recommend this PCR diagnostic assay for use by the research community to determine the presence (or absence) of *P. neurophilia* in colonies and for screening fish shipped between facilities, especially when parasite-free fish are required for experiments. Furthermore, we currently are using this PCR method to investigate the potential role of vertical transmission in the spread of *P. neurophilia*.

Abbreviations: PCR, polymerase chain reaction; rDNA, ribosomal DNA; SSU, small subunit

There has been a dramatic increase in the number of laboratories using zebrafish (*Danio rerio*) to study mechanisms of vertebrate embryogenesis and disease.[11,22,23] In support of these endeavors, the Zebrafish International Resource Center was established at the University of Oregon in 1998 to act as a central repository for wild-type and mutant strains of zebrafish and for materials and information about zebrafish research. As part of this Center, we offer a diagnostic service to the research community.

Pseudoloma neurophilia (Microsporidia) is the most common and widespread parasite that we have encountered in zebrafish and has been found in 53% (34 of 64) of the research laboratories that have submitted fish to our diagnostic service since 1999. First reported in 1980 in France,[7] the parasite only recently was described as *P. neurophilia* by Matthews and colleagues.[18] Infection is localized in the spinal cord and ventral nerve roots, but it may disseminate to the somatic muscle, where it is associated with severe, multifocal myocytolysis and myositis. Such infections often are associated with severe emaciation (referred to as 'skinny disease') and scoliotic changes that are regularly observed in zebrafish colonies.[18]

The sharing of various strains of zebrafish among research facilities may explain why the parasite is so widespread in the research community. Fortunately, improved practices for moving fish between facilities minimize the introduction of pathogens, and many facilities use quarantine procedures and egg disinfection[29] before fish are transferred to main water systems. Introduction of the parasite via water supplies seems unlikely, as most zebrafish facilities use dechlorinated and filtered city water, which we presume to be parasite-free. *P. neurophilia* has been found in ovaries and occasionally eggs, thereby leading Kent and Bishop-Stewart[17] to suggest that vertical transmission or contamination of progeny may partially be responsible for the spread of the parasite. Transovarial transmission via infected eggs is common among microsporidia of invertebrates[10] and has been suspected for some fish microsporidia.[8,24,25] Furthermore, transmission to progeny by sexual contamination (for example, ovarian fluid and associated debris) in which the parasite is not within the egg itself has been suggested for *Loma salmonae*[8] and other pathogens of fish, such as the infectious hematopoietic necrosis virus of salmonids.[5,14]

Detection of *P. neurophilia* can be accomplished via preparation of wet mounts of the central nervous system that have been carefully dissected from infected fish. However, histologic examinations are preferred, because removal of the spinal cord from zebrafish is tedious and laborious. Fluorescent stains for chitin have been used to detect spores of many microsporidia,[27] and we found the Fungi-Fluor stain (Polysciences, Warrington, PA) to be more sensitive than histology for detecting the infection in tissue sections.[17] Still, these methods of detection are not easily applied to screening of individual eggs or larval fish, nor do they reliably detect presporogonic stages of the parasite. Therefore, rapid and versatile methods of pathogen detection are desirable for use in the zebrafish research community.

Polymerase chain reaction (PCR) tests afford several advantages over other diagnostic methods in that they are rapid, require very little tissue, can be adapted to screen water sources,

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and detect all life stages of the pathogen. Sensitive and specific PCR tests have been developed for several microsporidia,[13,28] including those infecting fishes.[3,6,8,9,16] The ribosomal DNA gene has been the target used most frequently for such tests, as it can provide DNA sequence useful for taxonomic identification, and the presence of multiple copies of the gene in each nucleus provides multiple templates, allowing for the development of especially sensitive tests. We developed the following sensitive and specific PCR test for *P. neurophilia* as a tool to screen fish for the zebrafish research community and for use in future investigation of the modes of transmission of this common parasite.

Materials and Methods

Sample collection. All fish used in this study were obtained from an ongoing project that was approved by the Oregon State University Institutional Animal Care and Use Committee. For routine screening, 30 zebrafish were obtained from a population exhibiting no clinical signs of microsporidiosis. Fish were euthanized with an overdose of tricane methanesulfonate (Argent Laboratories, Redmond, WA) and processed as follows. With sterile instruments for each fish, the brain and spinal column were removed, discarding the tail, viscera, much of the skeletal muscle, and most of the head. The nervous tissues then were placed in a 2-ml, screw-capped centrifuge tube and stored in a –20 °C freezer overnight, prior to DNA extraction as described later.

Other microsporidians for testing the specificity of the PCR test were obtained as either frozen DNA or ethanol-fixed tissues from our parasite collections at Oregon State University and the University of Oregon. These organisms were *L. salmonae*, *Glugea stephani*, *Nucleospora salmonis*, and *Heterosporis* sp. Previously developed general microsporidian primers,[26] 530f (5′ GTG CCA GCM GCC GCG G 3′) and 580r (5′ GGT CCG TGT TTC AAG ACG G 3′), were used to verify that the DNA from these samples was of sufficient quality for PCR amplification.

Minimum detection limit. Known quantities of spores were required for assessing the minimum detection limit of the PCR test (sensitivity). Briefly, zebrafish exhibiting clinical signs of microsporidiosis (emaciation and malaise) were euthanized (as earlier), and spinal cords were collected. The presence of *P. neurophilia* was verified by observation of spores in wetmount preparations from these tissues. Infected spinal cords were pooled and homogenized in saline, yielding 30 ml of tissue slurry containing free spores. A 300-µl (1%) aliquot of this slurry was saved for subsequent PCR testing.

P. neurophilia spores were purified from the tissue slurry following standard methods of Docker and colleagues.[8] Purified spores were resuspended in 50 µl buffered saline, and spore counts were made using a hemocytometer. Spore suspensions were diluted to solutions of 100, 10, 1, and 0.1 spores per µl for use in PCR reactions. In addition, from the number of purified spores we obtained and counted, we were able to estimate the number of spores per µl in the saved 300-µl aliquot of tissue slurry. From tissue slurry, we prepared solutions of 10, 1, and 0.1 spores per µl for PCR testing.

Primers. Species-specific PCR primers were designed for *P. neurophilia* small subunit ribosomal DNA (SSU rDNA) based on DNA sequence alignment with 48 other closely related microsporidia by using Matthews and colleagues[18] and the basic local alignment search tool[1] on GenBank as a guideline. Several sets of primers were evaluated (data not shown), and the primer pair Pn18S5F (nucleotides 334 through 356), 5′ GAA AAT TAC CGG AGC CTG AAG TC 3′, and Pn18S5R (nucleotides 1121 through 1099), 5′ TTC CCT CTC TCT CCA AAT TTC GG 3′, yielded the best results in our preliminary analyses and were selected for further optimization of the PCR test.

Polymerase chain reaction. The DNeasy Tissue kit (Qiagen, Valencia, CA) was used to extract DNA from spores or infected tissues according to the manufacturer's protocols with the addition of an overnight freeze–thaw at –20 °C prior to DNA extraction. The PCR was carried out in 25-µl volumes containing 0.2 mM dNTPs, 12.5 pmol each primer, 2.5 mM $MgCl_2$, 1.25 U Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 1× buffer (Qiagen) with 1 or 2 µl extracted DNA. All PCR reactions were performed in an DNA Engine 200 (MJ Research, Watertown, MA). Amplification using the 530f–580r primer pair was conducted using the previously described reaction components with an initial denaturation at 95 °C for 3 min; then 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 90 s; and followed by final extension at 72 °C for 7 min. Reactions using the Pn18S5F–Pn18S5R primers were done using the following conditions: initial denaturation at 95 °C for 3 min; 35 cycles consisting of 94 °C for 30 s, 66 °C for 45 s, 72 °C for 60 s; and then a final extension at 72 °C for 7 min. Products were visualized on a 1.5% agarose gel containing 0.1 µg/ml ethidium bromide run at 100 V for 1 h.

Results and Discussion

Using the PCR primers designed here, we were able to develop a sensitive and specific test for *P. neurophilia*, which yielded a 788-bp fragment with no extraneous reaction products. The PCR primers Pn18S5F and Pn18S5R were designed to be specific to *P. neurophilia* based on DNA sequence alignment of SSU rDNA from 48 other microsporidia, including *Loma salmonae*, a representative of a closely related genus.[18] Indeed, specificity of the PCR test was high, and it did not cross-react with any other piscine microsporidia available to us (Table 1). Although the PCR test did not detect these other microsporidians, we were unable to check the specificity of the PCR primers against all known parasites or against as-yet unknown parasites that might emerge and that could share similar DNA sequence with *P. neurophilia*. Therefore, it is advisable to periodically confirm the infection status in a proportion of fish by using an alternate technique, such as DNA sequencing. This confirmation may be especially important in facilities where the parasite has not been detected previously.

The method used for sample preparation from tissues is important for recovering DNA from resilient microsporidian spores. Indeed, extraction of DNA from intact spores may require physical disruption, as suggested by Müller and coworkers.[20] However, Docker and colleagues[8] obtained equivalent results in detection of *Loma salmonae* when tissues were processed by disruption with silica beads, mechanical homogenization, or proteinase K digestion alone. Because Docker and coworkers[8] found Proteinase K digestion to be the least labor-intensive and less subject to cross-contamination than the other methods tested, we too used Proteinase K digestion that was preceded by an overnight freeze–thaw. This method also was preferred because it required equipment that is available in many laboratories. In addition to using a straightforward method of DNA extraction, we also avoided the use of a nested PCR test. Although a nested PCR test may increase sensitivity,[3] the likelihood of false positives also increases.[4]

The lower limit of detection of this test with DNA originating from infected spinal cords or purified spores was consistently 10 spores or fewer per PCR reaction (Table 2). Detection of a

Table 1. Specificity of ribosomal DNA polymerase chain reaction with microsporidian general primers 530f/580r and *Pseudoloma neurophilia*-specific primers Pn18S5F/Pn18S5R for detection of microsporidia

Species	Product generated with	
	530f/580r	Pn18S5F/Pn18S5R
Pseudoloma neurophilia	+	+
Loma salmonae	+	–
Glugea stephani	+	–
Nucleospora salmonis	+	–
Heterosporis sp. ex. *Perca flavescens*	+	–
Naïve zebrafish	–	–

single spore in a PCR reaction was inconsistent; we obtained positive results from 1 of 4 (25%) reactions originating from spores, and positive results from 2/4 (50%) reactions originating from infected tissue. Occasionally, results were positive for infected tissues diluted to a theoretical 0.1 spores per PCR (2 of 4, 50%; Table 2). Detection of a fraction of a spore is possible, as multiple copies of the target gene occur in each spore. Differences in detection level between purified spores and infected tissues can be explained by a number of factors. For example, our estimate of the number of spores present in the infected tissue 'slurry' was based on the number of spores we purified per tissue volume. The loss of spores during the purification process likely would make our estimates of the number of spores in the tissue low, as tissues may contain developmental stages that were not counted. This difference would increase the apparent sensitivity of the PCR test on tissues as opposed to suspensions of pure spores.

Polymerase chain reaction tests have inherently low limits of detection, and the detection of 10 spores (sometimes 1 or 0.1) we obtained for *P. neurophilia* is consistent with other studies of fish microsporidia. Using spore number estimates from infected tissues, Docker and colleagues[8] were able to detect as few as 0.01 to 0.001 *Loma salmonae* spores from gill tissue and 0.1 *Nucleospora salmonis* spores from infected kidney tissue.[9] Barlough and coworkers[3] reported that 10 infected lymphocytes were needed for a positive reaction in their PCR test for *N. salmonis*, which may equate to as few as 10 parasites.

Although these estimates usually are based on detection of spores, it is essential to note that PCR can detect stages otherwise undetectable by histology. For example, PCR will detect developmental stages of a parasite early in the infection process, when abundance is low.[16,19] Furthermore, as entire fish or whole spinal cords are used for DNA extraction, our sampling regime is unlikely to overlook *P. neurophilia* infections due to unequal distribution of the parasite, especially during these earlier stages of infection. It is difficult to directly compare our findings from PCR with histology, as whole fish are used for histology, leaving no tissue for PCR. Likewise, removal of the spinal column and brain or use of whole fish for PCR leaves little tissue for histology. Ultimately, the choice of diagnostic technique depends on the goals of each individual study. Histological analysis is more appropriate for evaluating pathologic changes, whereas PCR is better for screening large numbers of samples, subclinical populations, small young fish, water, and other materials.

Presence of *P. neurophilia* within water systems or associated with biofilms has yet to be demonstrated. However, water collection and DNA extraction methods that have been developed for detection of medically important water-borne parasitic protozoa such as *Cryptosporidium parvum*, *Giardia intestinalis*, and other microsporidians[12,21] can be used for *P. neurophilia*. The observation by Kent and Bishop-Stewart[17] of *P. neurophilia* spores

Table 2. Sensitivity of the polymerase chain reaction with Pn18S5F/Pn18S5R primers from infected tissues and purified spores (number positive/number tested)

	Spores per reaction					
	100	10	1	0.1	Naïve zebrafish	Water
Infected tissue	na	4/4	2/4	2/4	0/4	0/4
Purified spores	5/5	5/5	1/4	0/4	0/5	0/5

na, not available.

in ovaries and occasionally eggs of zebrafish raises concerns about spread of this parasite through transplantation of infected eggs. Hogg and colleagues[15] documented vertical transmission of microsporidia in amphipods by using a PCR test, and we currently are investigating this phenomenon with *P. neurophilia* in zebrafish. Until vertical transmission can be verified, screening of eggs by PCR may be unwarranted at this time, although standard quarantine procedures should still be implemented for fish and eggs introduced to research facilities.

The described PCR test may prove useful for screening fish prior to introduction to new facilities. Although pathogen screening protocols are applied routinely to fishes used in aquaculture and rodents used in research, this approach has yet to be adopted by the zebrafish research community. Furthermore, no chemotherapeutant against *P. neurophilia* has yet been developed; therefore monitoring and preventing movement of infected fishes between facilities is preferred to control the spread of the parasite at this time. The number of fish to be tested from an incoming shipment depends on the predicted prevalence within a normal population.[2] In our evaluation of the *P. neurophilia* PCR test on 30 zebrafish from a population exhibiting no signs of clinical disease, we noted a 10% prevalence of the parasite. Therefore, with a predicted prevalence of 10%, between 20 and 30 fish (depending on the size of the population) would need to be tested to have 95% confidence that the parasite is absent from the population.[2]

We provide here a sensitive and specific test for *P. neurophilia* to be used by the zebrafish research community to monitor existing stocks of fish and avoid introduction of this common parasite. The use of PCR in research has become commonplace in most biological research facilities, and equipment is usually available. Therefore, the *P. neurophilia* PCR assay can be performed within individual laboratories by the researchers themselves to minimize costs. With the increasing concern for spread of this prevalent pathogen and the use of pathogen-free stocks of fish for experiments, this PCR assay is an excellent tool for diagnosis and can be adapted to any research situation.

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