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Application of a real-time PCR assay for the detection of *Henneguya ictaluri* in commercial channel catfish ponds

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ABSTRACT: Proliferative gill disease (PGD) in channel catfish *Ictalurus punctatus* is caused by the myxozoan parasite *Henneguya ictaluri*. Prolonged exposure of channel catfish to the actinospore stage of the parasite results in extensive gill damage, leading to reduced production and significant mortality in commercial operations. A *H. ictaluri*-specific real-time (Q)PCR assay was used to determine parasite levels in commercial channel catfish ponds and evaluate the risk of losing fish newly stocked into the system. Previous research has shown the *H. ictaluri* actinospore to be infective for approximately 24 h; therefore, determining the parasite load (ratio of parasite DNA to host DNA) in sentinel fish exposed for 2 separate 24 h periods with a minimum of 1 wk between sampling indirectly represents the rate at which infective actinospores are being released by the oligochaete host and if that rate is changing over time. Alternatively, QPCR analysis of pond water samples eliminates the need for sentinel fish. Water samples collected on 2 separate days, with a minimum of 1 wk between sampling, not only determines the approximate concentrations of actinospores in the pond but if these concentrations are remaining stable. Increases in parasite load ($r = 0.69$, $p = 0.054$) correlated with percent mortality in sentinel fish, as did increases in mean actinospore concentrations ($r = 0.63$, $p = 0.003$). Both applications are more rapid than current protocols for evaluating the PGD status of a catfish pond and identified actinospore levels that correlate to both high and low risk of fish loss.

KEY WORDS: Channel catfish · *Henneguya ictaluri* · Myxozoa · Proliferative gill disease · Real-time PCR

INTRODUCTION

Proliferative gill disease (PGD), caused by the myxozoan parasite *Henneguya ictaluri* (Pote et al. 2000), is one of the most prevalent parasitic infections afflicting the commercial channel catfish *Ictalurus punctatus* industry. The parasite has a 2-host life cycle with development in both the benthic oligochaete *Dero digitata*, and the channel catfish (Burtle et al. 1991, Styer et al. 1991, Pote et al. 2000). Prolonged exposure of channel catfish to the actinospore stage of the organism causes extensive gill damage, resulting in respiratory insult and osmoregulatory distress, which can lead to reduced growth and eventually death (Bowser & Conroy 1985, MacMillan et al. 1989, Wise et al. 2004).

The disease is most common during the spring, with a lower incidence in the fall, when water temperatures are between 16 and 25°C. Significant losses often

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occur when catfish fingerlings are stocked for growout in the spring, or re-stocked after a PGD outbreak, even when resident fish populations show no clinical signs of disease. Currently there are no effective treatments for PGD, but recent research has shown that removing fish from the source of infectious agents results in rapid fish recovery (Wise et al. 2008). These observations led to the development of methods to assess the risk of losing fish to PGD when relocating fish from a pond with an ongoing outbreak or when a severe outbreak necessitates re-stocking a pond.

At present, the approximate concentration of actinospores in the pond environment can be determined through the use of sentinel fish exposures (Wise et al. 2004, 2008). Net-pens, constructed of 5 mm nylon mesh to allow free exchange of water within the confined area, are suspended in the suspect pond and stocked with parasite-free fish. Fish are held in the pond for 7 d, after which they are examined for clinical signs of disease. If PGD is present, parasite-free sentinel fish are re-stocked for an additional 7 d to determine if the severity of the disease has increased or decreased from the first 7 d exposure. After determining the severity and dynamics of the outbreak, decisions on fish stocking can be made. Unfortunately, the use of sentinel fish is labor intensive, requires a source of parasite-free fish and requires a minimum of 2 wk to yield reliable results. Limitations of the sentinel fish protocol demanded the development of a more rapid and efficient method of determining the actinospore concentration in the pond environment.

Real-time polymerase chain reaction (QPCR) assays have been utilized in the quantification of various waterborne parasites from environmental samples, as well as the quantification of bacterial and parasitic agents in fish tissues (Bilodeau et al. 2003, Brinkman et al. 2003, Fontaine & Guillot 2003, Guy et al. 2003, Cavender et al. 2004, Kelley et al. 2004, Chase et al. 2006, Hallett & Bartholomew 2006, Balacázar et al. 2007, Panangala et al. 2007). Recently, a QPCR assay was developed for the quantification of *Henneguya ictaluri* in fish tissues (Griffin et al. 2008).

Filtration methods have been successful in collecting the actinospore stages of *Myxobolus cerebralis* and *Ceratomyxa shasta* from rivers and streams in the Pacific Northwest (Thompson & Nehring 2000, Hallett & Bartholomew 2006). However, there are 2 major hurdles to overcome in order to utilize a filtration method of actinospore quantification in commercial catfish ponds. Primarily, the small size of the target organism and the abundance of phytoplankton, zooplankton and fine particulates in these ponds make filtering pond water difficult and cumbersome as the filter is easily clogged. Secondly, humic substances, often present in environmental samples, are a major source of PCR inhibition, although there have been several methods proposed to separate DNA from these organic inhibitors (Wilson 1997).

The present report describes novel procedures for the isolation and quantification of *Henneguya ictaluri* actinospores from the commercial catfish pond environment. The infective window of the *H. ictaluri* actinospore stage is approximately 24 to 48 h (Wise et al. 2004, 2008). Therefore, quantifying the parasite load in gill tissues of fish exposed to pond water for 24 h indirectly represents the rate at which infective actinospores (actinospores released by the oligochaete host within the past 24 to 48 h) are being released into the environment. Alternatively, correlating the relationship between actinospore concentrations in pond water to disease severity in sentinel fish may indicate the relationship between actinospore concentrations and clinical levels of disease, which would aid in assessing the risk of stocking fish into a pond. Whitaker et al. (2005) developed a PCR protocol for the detection of the actinospore stage in pond water. However, this method is subjective and results are not readily quantifiable.

This paper validates 2 QPCR protocols for the identification and quantification of *Henneguya ictaluri* actinospores in channel catfish ponds and gill tissue. Three trials were conducted to (1) determine the spatial distribution of *H. ictaluri* actinospores in a commercial channel catfish pond based on actinospore concentrations and disease severity in sentinel fish from different locations within the pond; (2) evaluate a pond monitoring protocol based on the determination of *H. ictaluri* actinospore concentrations in pond water; and (3) evaluate a pond-monitoring protocol based on the determination of parasite infection levels in gill tissue following 24 h exposure to a pond.

MATERIALS AND METHODS

Actinospore collection. *Dero digitata* were isolated from sediment collected from a commercial channel catfish pond with an active PGD outbreak determined by the Aquatic Diagnostic Laboratory (ADL) of the Thad Cochran National Warmwater Aquaculture Center, Stoneville, Mississippi (NWAC). The oligochaetes were isolated using protocols described by Pote et al. (1994) and observed for 72 h for shedding of *Henneguya ictaluri* actinospores (Fig. 1).

Freshly released *Henneguya ictaluri* actinospores were collected and suspended in 20 ml of nuclease-free H_2O and gently stirred with a magnetic stir bar. The number of actinospores μl^{-1} was determined by counting the number of actinospores in 10 separate 10 µl samples. Eight samples with 25, 100 and 1000 *H. ictaluri* actino-

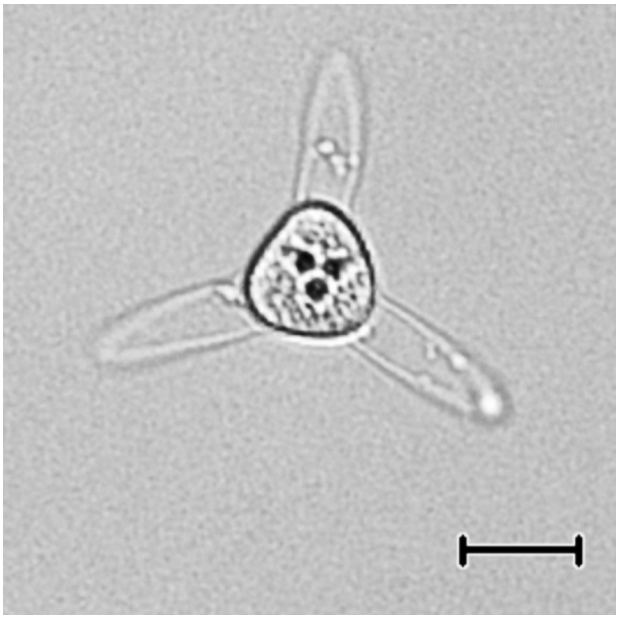

Fig. 1. *Henneguya ictaluri.* Aurantiactinomyxon type actinospore. Scale bar = 20 µm

.spores each were collected using a volumetric pipette and placed in individual Powersoil® DNA Isolation Kit microbead tubes (Mo Bio Laboratories). Eight samples of 1, 5 and 10 actinospores each were individually collected using a dissecting microscope and a fine glass pipette and also placed directly in individual Powersoil® DNA Isolation Kit microbead tubes.

Collection and processing of water samples. Water samples (2 l) were collected at arm's length (approximately 0.5 m) below the surface of the pond and processed within 24 h of collection. Samples were divided into four 500 ml subsamples, transferred to 1 l centrifuge bottles (each 1 l centrifuge bottle contained 500 ml of water) and centrifuged for 20 min at 9500 × *g* in a Sorvall RC-6 centrifuge (Thermo Fisher Scientific). The supernatant from each subsample was removed and the pellet resuspended in 50 ml of distilled water, transferred to a 50 ml conical centrifuge tube and centrifuged at 7000 × *g* for 10 min. The supernatant was again removed and the pellet was resuspended in 1.5 ml of nuclease-free H_2O, transferred to a 1.8 ml microcentrifuge tube and stored at −80°C until DNA isolation could be performed. Centrifuge bottles were washed vigorously 3 times with distilled water between samples.

Validation of water sampling protocol. Water collected from a commercial channel catfish pond with no previous history of PGD was separated into eleven 500 ml water samples. *Henneguya ictaluri* actinospores were collected from isolated *Dero digitata* and enumerated. Eight samples of 100 actinospores were processed directly by the Powersoil DNA isolation kit. An additional 8 samples of 100 actinospores were added directly to 500 ml (200 actinospores l^{-1}) of pond water. The remaining pond water samples had no actinospores added and served as negative controls. All pond water samples were then processed according to the previously described protocol.

DNA extraction. DNA was isolated from actinospore aliquots using the Powersoil® DNA Isolation Kit (Mo Bio Laboratories) and resuspended in 30 µl of Puregene® DNA hydration solution (Gentra Systems). Genomic DNA from pond water samples was also isolated using a Powersoil® DNA Isolation Kit but following the protocol suggested for wet samples. Genomic DNA isolated from the pond water subsamples was pooled, concentrated according to the manufacturer's suggested salt precipitation protocol and resuspended into 30 µl of Puregene® DNA hydration solution.

Genomic DNA from fish tissues was isolated using a slight modification of the Puregene® DNA Isolation Kit (Gentra Systems). Gill clips (40 to 80 filaments) taken from a left gill arch were placed in 600 µl of cell lysis solution from the Puregene® DNA Isolation Kit (Gentra Systems) and initially incubated for 10 min at 95°C prior to the addition of 3 µl of Proteinase K (20 mg ml^{-1}). Samples were then incubated at 55°C until all tissue homogenate had dissolved. The remainder of the DNA isolation was carried out according to the manufacturer's suggested protocol for animal tissues. After drying by vacuum centrifugation, the purified DNA was resuspended in 100 µl of Puregene® DNA Hydration Solution (Gentra Systems) and quantified using a Nanodrop® spectrophotometer and accompanying software v 3.2.1 (Nanodrop Technologies)

Standard design. Target regions of the small subunit (SSU) ribosomal DNA (rDNA) genes were amplified from the genomic DNA of *Henneguya ictaluri* actinospores and specific pathogen free (SPF) channel catfish by use of *H. ictaluri*-specific PCR primers (Whitaker et al. 2001, Pote et al. 2003) and generic eukaryotic SSU rDNA primers (Elibol-Fleming 2006), respectively (Table 1). The 25 µl PCR reaction mixtures contained 2.5 µl of 10× reaction buffer (10 mM Tris, 50 mM KCl, pH 9.0, 4.0 mM $MgCl_2$), 5 nM of each dNTP, 10 pM of each primer, 0.5 units of Takara® Hot Start *Taq* Polymerase (Takara Bio USA), 2 µl of DNA template and nuclease-free water to volume. The PCR was carried out on a PTC-100 Thermal Cycler, (Global Medical Instrumentation) programmed for 1 cycle of 95°C for 10 min, 50°C for 1 min, and 72°C for 2 min followed by 35 cycles of 92°C for 1 min, 50°C for 15 s, and 72°C for 15 s and a final extension cycle of 72°C for 5 min. The PCR amplification products were analyzed by electrophoresis on a 1.5% agarose gel and stained (Gelstar® nucleic acid stain, Cambrex) to confirm the presence of a single PCR product.

Table 1. Primer and probe sequences. FAM: 6-carboxyfluorescein; BHQ1: Black Hole Quencher-1; HEX: hexachloro-6-carboxy-fluorescein

Name	Direction	Sequence	Source
A1-1	Forward	5′-CAA AAG TTT CTG CTA TCA TTG-3′	Whitaker et al. (2001), Pote et al. (2003)
A1-2	Reverse	5′-AGC GCA CAG ATT ACC TCA-3′	Whitaker et al. (2001), Pote et al. (2003)
18SCCF	Forward	5′-CGG AGA GGG AGC CTG AGA A-3′	Elibol-Fleming (2006)
18SCCR	Reverse	5′-CGT GTC GGG AAT GGG TAA TTT G-3′	Elibol-Fleming (2006)
HITMP	Probe	[FAM]-5′-TCA GCC TTG ATG TTG CCA CCT CA-3′-[BHQ1]	Griffin et al. (2008)
18STMP	Probe	[HEX]-5′-ACC ACA TCC AAG GAA GGC AGC AGG C-3′-[BHQ1]	Elibol-Fleming (2006)

PCR products were purified using a Montage® PCR Centrifugal Filter Device (Millipore) and cloned into a T7 vector using the PCR4-TOPO® cloning kit (Invitrogen). The plasmid clones with SSU rDNA gene inserts from *Henneguya ictaluri* and channel catfish were each grown in culture overnight, and plasmid purification was performed using a Qiagen plasmid mini-prep kit (Qiagen). The plasmid standards were quantified as described above and their concentrations were adjusted to 1.0 ng μl^{-1}. The standards were then serially diluted to serve as positive controls and for generation of standard curves in QPCR analysis.

Determination of actinospore equivalents. Genomic DNA was isolated from the previously mentioned actinospore aliquots using the Powersoil® DNA isolation kit according to the manufacturer's suggested protocol. A standard curve was prepared by analyzing 10 replicates of the enumerated spore samples. Real-time PCR analysis of genomic DNA isolated from *Henneguya ictaluri* actinospore aliquots, as well as genomic DNA isolated from pond water samples was performed using *H. ictaluri*-specific primers (Whitaker et al. 2001, Pote et al. 2003) and a *H. ictaluri*-specific TaqMan probe (Griffin et al. 2008) (Table 1) labeled on the 5′ end with the fluorescent reporter dye, 6-carboxyfluorescein (FAM), and the quencher dye, black hole quencher-1 (BHQ-1) on the 3′ end. The 12.5 µl PCR reactions contained 6.25 µl BioRad IQ® supermix (Bio-Rad), 20 pM of each primer, 0.25 pM of probe, 3 µl of template DNA and nuclease-free H_2O to volume. Amplifications were performed on a BioRad Icycler v3.1® real-time PCR system (BioRad) programmed for 1 cycle of 95°C for 3 min 30 s followed by 40 cycles of 95°C for 30 s, 56°C for 1 min and 72°C for 30 s. Data collection was carried out following the 72°C elongation step at the end of each cycle. The cycle threshold (C_T) was set the same for all runs and sample C_T values were used to generate a standard curve for actinospore equivalents (AE). All samples were run in triplicate with a negative control and a serially diluted plasmid standard which served as both a positive control and a measure of reaction efficiency. The amplification efficiency (E) was estimated by the formula $E = 10^{-1/s} - 1$, where s is the slope of the standard curve (Wong & Medrano 2005) and efficiency estimations between 90 and 110% were considered acceptable. Actinospore equivalents for water samples were determined by comparing the mean of the sample C_T values to the standard curve generated by the analysis of the enumerated spore samples.

Inhibition of QPCR. Pond water samples demonstrating inhibition, evident by sample amplification with a significantly reduced slope compared to the amplification slope of positive controls, were diluted with Puregene® DNA Hydration Solution (Gentra Systems) in 2-fold increments until amplification curves were similar to positive controls.

QPCR analysis of fish tissue. Parasite DNA was amplified from host tissue using the previously described *Henneguya ictaluri*-specific primer and probe combination, while the internal standard (host SSU rDNA) was amplified from genomic DNA using the 18SCCF/18SCCR primers (Elibol-Fleming 2006) and 18STMP labeled on the 5′ end with the fluorescent reporter dye, hexachloro-6-carboxy-fluorescein (HEX), and the quencher dye BHQ-1 on the 3′ end (Table 1). At least 1 ng of total genomic DNA (parasite and host) was added to each reaction and all samples were analyzed in triplicate according to the QPCR protocol described above. To correct for initial template variations between samples, SSU rDNA plasmid standard equivalents were determined for each sample. Sample C_T values were compared to a standard curve based on serially diluted plasmid standards of the amplicons generated by the A1-1/A1-2 (Whitaker et al. 2001, Pote et al. 2003) primers or the 18SCCF/18SCCR, respectively (Table 1). Again, data were considered valid if the reaction efficiency estimations fell between 90 and 110%, as described previously. Data were normalized to the equivalent of the reference gene (host SSU rDNA) and the log of the ratio of parasite standard equivalent (PSE) to host standard equivalent (HSE) was used for comparisons (Griffin et al. 2008).

Gross examination of fish tissues. Approximately 40 to 80 gill filaments were removed from a left gill arch and used for wet mount preparations. The percentage

of gill filaments exhibiting at least one chondrolytic lesion (Fig. 2) was calculated and the mean gill damage for each cage was determined. PGD outbreaks were designated as mild, moderate or severe according to the sentinel fish protocol described by Wise et al. (2004) (Table 2).

Experimental trials. All experimental trials took place in the spring of the year, when water temperatures were within the optimal range for PGD (16 to 25°C). For Trial 1, the spatial distribution of actinospores in a pond was determined during a PGD outbreak. A commercial channel catfish pond (4 ha) with clinical PGD in the resident fish population was divided into a 4 × 4 grid (Fig. 3). Parasite-free fish (n = 15) were held in nylon cages positioned at intersections of the grid for 6 d to determine the effect of cage location on gill damage observed in sentinel fish. On Days 0, 3 and 6 water was collected adjacent to each cage as described above. On Day 6, all surviving fish were euthanized with an overdose (1000 mg l^{-1}) of MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate) (Argent Chemical Laboratories), gills were processed for gross microscopic examination, and the mean percentage gill damage determined for each cage according to Wise et al. (2004, 2008). The cage placed at location D2 was lost and water sampling was not carried out on Days 3 and 6.

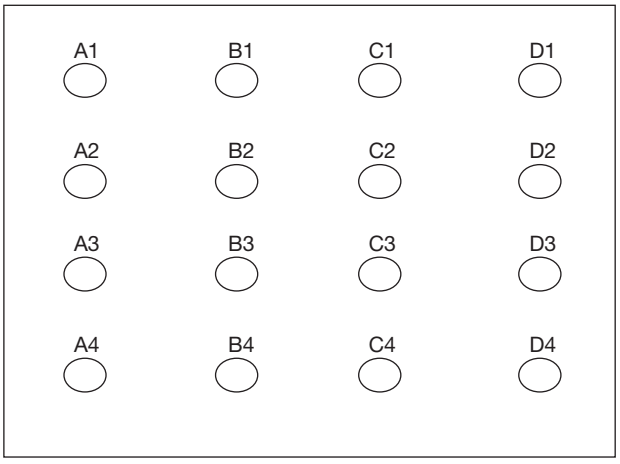

Fig. 3. Cage and water sampling locations in a commercial channel catfish pond diagnosed with PGD in the resident fish population

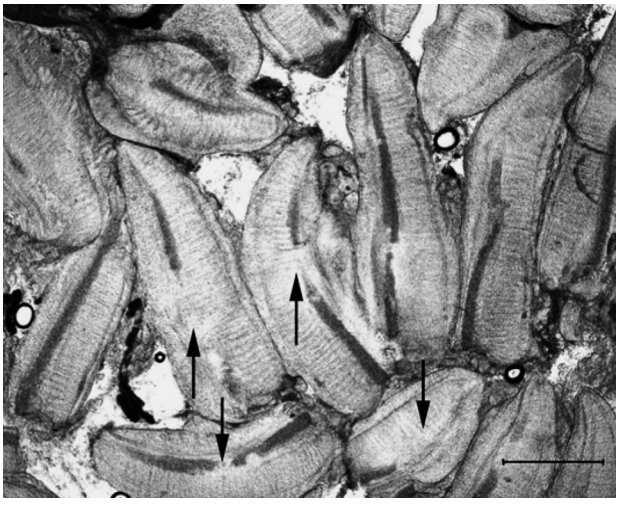

Fig. 2. *Henneguya ictaluri.* Wet mount preparation of gill clips from a channel catfish fingerling with proliferative gill disease (PGD). Foci of chondrolysis characteristic of PGD are evident in the filamental cartilage (arrows). Scale bar = 1 mm

Table 2. Grading scale for *Henneguya ictaluri*-induced lesions (Wise et al. 2004)

Filaments exhibiting cartilage breaks (%)	Score	Effect on fish
1–5	Mild	Little to no effect on fish health
6–15	Moderate	If environmental conditions are optimal, no direct mortalities involved
>15	Severe	Expect mortalities within 2 wk

Trial 2 consisted of 20 replicate 7 d pond trials carried out in 8 different commercial channel catfish ponds (range 3 to 6 ha) with PGD diagnosed in the resident fish populations. Pond water was sampled as described previously on Days 0 and 7 in coordination with the setting out and sampling of cages stocked with sentinel fish. One water sample was collected adjacent to the cage and a second sample was collected at least 100 m from the cage. The mean AE l^{-1} for the 2 locations was determined by QPCR analysis, and inhibition of the QPCR was corrected for by a 1:4 dilution of genomic DNA for all samples. Fifteen parasite-free sentinel fish were held for 7 d in nylon net pens. On Day 7, surviving fish were sampled, euthanized with an overdose of MS-222 and processed for gross microscopic examination.

In Trial 3, 8 replicate 7 d pond trials were carried out in 2 commercial channel catfish ponds diagnosed with clinical PGD in the resident fish population. Parasite-free channel catfish fingerlings were placed in 2 nylon net pens designated A and B (n = 15 fish pen^{-1}). After 24 h, survivors from net pen A were euthanized with an overdose of MS-222 and processed for molecular analysis and gross examination to determine mean percentage gill damage. On Day 6, net pen A was restocked with parasite-free fish (n = 15). On Day 7, surviving fish from net pen A (24 h exposure) and net pen B (7 d exposure) were sampled, eutha-

nized and processed for molecular analysis, and mean percent gill damage was determined by gross examination.

Statistical analysis. All statistical analysis was performed using SAS Software v. 9.1 (SAS Institute). For Trial 1, a 1-way ANOVA and Fisher's least significant difference procedure was used to determine differences in mean percentage gill damage. For Trial 2, the correlation between mean actinospore concentration and mortality in sentinel fish held in the pond for 7 d was estimated by Pearson's product-moment correlation coefficient (r). For Trial 3, parasite standard equivalents determined by QPCR, were normalized to the host SSU rDNA, and the ratio of PSE to HSE was log transformed prior to statistical analysis. Samples in which no parasite DNA was detected by PCR were assigned log values of −8, one order of magnitude lower than the lowest observed value. Multiple comparisons of the data for Trial 3 were performed using Duncan's multiple range test. Pearson's product-moment correlation coefficient was used to estimate the linear relationship between mortality in sentinel fish exposed to the pond for 7 d and the mean PSE to HSE ratios for the 2 separate 24 h exposures.

RESULTS

Sensitivity

The standard curve for the log of known starting numbers of whole spores processed and assayed as per pond water samples was described by the equation $y = -5.1512x + 34.938$ with an $R^2 = 0.9773$ (Fig. 4), where the mean C_T value for a single actinospore was 35.0 (range 33.5 to 36.6). Pond water samples spiked with 100 *Henneguya ictaluri* actinospores had a mean C_T value of 25.31 (range 23.92 to 26.58) compared to 23.9 (22.7 to 25.2) for 100 *H. ictaluri* actinospores processed directly. The C_T values for the spiked pond water samples were equivalent to approximately 80 AE according to the established standard curve, indicating an approximate 20% loss of actinospore DNA during the sampling process. No parasite DNA was detected in the negative controls.

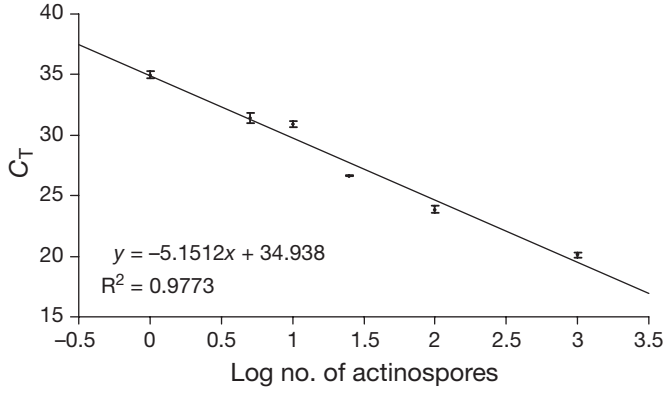

Fig. 4. *Henneguya ictaluri*. Standard curve derived from known numbers of actinospores. DNA was extracted as per pond water samples, showing log of number of spores versus mean (±SEM) real-time (Q) PCR C_T value. Note $\log_{10}$ scale on *x*-axis

Trial 1

When fish were stocked into multiple cages in a pond with a fish population with active PGD, all fish surviving to Day 6 had clinical signs of PGD, and fish in only 1 cage location (A2) had a mean percentage gill damage significantly greater than that of the other cage locations ($p < 0.05$) (Table 3). Survival rates varied by location and mortalities were observed in each cage. Cages from locations D1 and A1 had no surviving fish after 6 d and the cage from location D2 was lost prior to Day 3 sampling. Actinospore equivalent concentrations (AEC) varied by location, but the mean AEC for all locations for all days were within 1 SD of the mean for the entire pond. Mean AEC decreased from the first to the last sampling day for all locations. All pond water samples were positive for *Henneguya ictaluri* DNA. The highest AE were found on the first sampling day and decreased across sampling days (Fig. 5), although the differences in AEC for Day 3 and 6 were not significant ($p < 0.05$). The greatest AEC was ≥249 AE l^{-1}, observed on Day 1, and the lowest AEC was ≥38 AE l^{-1} on Day 6. The mean AEC for all cage locations for all days was ≥94 AE l^{-1}. The average mortality rate for all cages was 48.9% with mean percentage gill damage for surviving fish of 11.7%.

Trial 2

According to criteria established by Wise et al. (2004), of the 20 different 7 d trials, outbreak severity was classified as mild for 6 ponds, moderate for 5 and severe for 9. AEC varied by sampling day within trials and by trials; however, for each sampling day, AEC between sampling locations were within 1 order of magnitude of each other. Greater AEC were found in the more severe outbreaks. The greatest AEC was ≥153 AE l^{-1}, while the lowest AEC was <1 AE l^{-1}, observed on 3 occasions where no parasite DNA was detected (ND). There was a positive correlation ($r = 0.63$, $p = 0.003$) between mean AEC and percent mortality observed in sentinel fish exposed for 7 d (Fig. 6). Data are summarized in Table 4.

Table 3. *Henneguya ictaluri* infecting *Ictalurus punctatus*. Trial 1 data. Survival and mean percent gill damage of sentinel fish and approximate parasite abundance by cage location by day determined by QPCR analysis. Sentinel fish were held in pond for 7 d; mortality was calculated as the number dead out of 15 fish. Mean percent gill damage calculated for surviving sentinel fish exposed to each location for 6 d. For Days 0 and 3, inhibition was corrected by an 8-fold dilution of template DNA (actinospore equivalents [AE] l^{-1} calculated by a dilution factor of 8). For Day 6, inhibition was corrected by a 4-fold dilution of template DNA (AE l^{-1} calculated by a dilution factor of 4). Mean AE l^{-1}: average of Day 0, Day 3 and Day 6 observations for the given location. Within columns, values with different superscript letters (^{a,b,c}) are significantly different ($p < 0.05$); NA: not applicable, since all fish died

Pond location	Mortality (%)	Gill damage (%)	C_T			AE l^{-1}			
			Day 0	Day 3	Day 6	Day 0	Day 3	Day 6	Mean
A1	100.0	NA	28.4	26.9	27.4	74.3	73.3	57.8	68.5^{c}
A2	86.7	30.0^{a}	27.2	25.9	27.4	127.5	116.6	58.8	101.0^{abc}
A3	13.3	7.3^{b}	27.0	27.5	28.0	138.3	55.9	44.9	79.8^{bc}
A4	6.7	8.5^{b}	26.4	26.9	26.5	183.3	72.5	86.7	114.2^{ab}
B1	20.0	8.9^{b}	26.8	25.6	27.2	152.7	133.0	63.6	116.5^{ab}
B2	73.3	17.0^{b}	28.3	26.5	26.8	77.0	88.2	78.0	81.1^{abc}
B3	20.0	14.6^{b}	27.0	27.6	27.1	138.8	52.3	65.8	85.7^{abc}
B4	33.3	13.5^{b}	26.8	26.8	28.0	149.0	76.9	48.8	91.6^{abc}
C1	26.7	8.1^{b}	25.7	27.3	28.0	249.3	62.7	45.3	119.1^{a}
C2	26.7	9.0^{b}	27.4	27.5	27.4	120.7	57.0	58.8	78.9^{bc}
C3	80.0	9.3^{b}	27.7	25.8	28.4	101.7	122.0	37.4	87.1^{abc}
C4	60.0	10.0^{b}	27.0	26.3	26.1	146.3	97.6	105.3	116.5^{ab}
D1	100.0	NA	26.3	26.6	28.1	189.8	84.9	44.7	106.5^{abc}
D3	46.7	8.4^{b}	27.0	27.0	28.3	145.2	71.2	37.8	85.1^{abc}
D4	40.0	8.0^{b}	27.6	27.2	27.0	108.4	64.0	69.1	80.6^{bc}
Me^{a}n	48.9	11.7	27.1	26.2	26.8	140.15	81.9	60.4	94.2
(SD)	(±32.0)	(±6.7)	(±0.8)	(±0.7)	(±0.7)	(±48.0)	(±26.1)	(±19.4)	(±45.9)

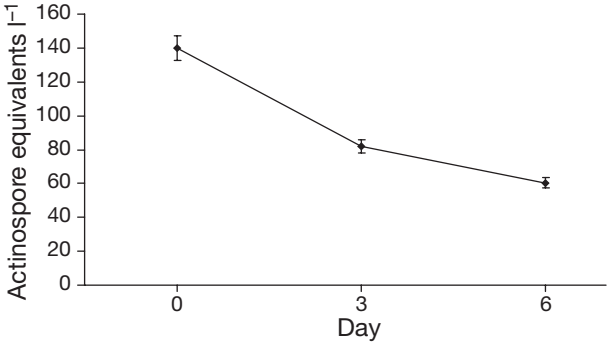

Fig. 5. *Henneguya ictaluri*. Trial 1 data. Mean (±SEM) actinospore equivalents l^{-1} by day for all pond locations

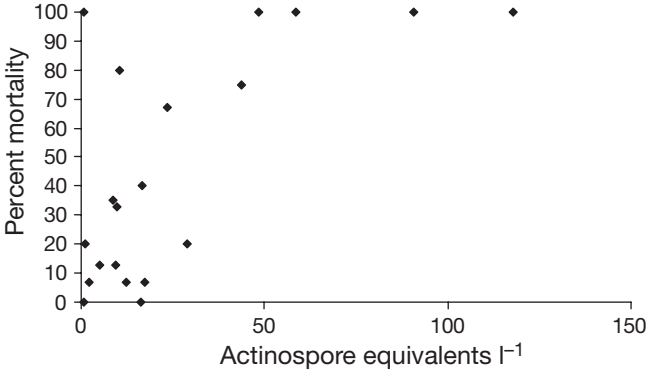

Fig. 6. *Henneguya ictaluri*. Trial 2 data. Positive correlation between mean actinospore equivalents l^{-1} for 2 separate sampling days and percent mortality in sentinel fish exposed to a PGD-positive pond for 7 d ($r = 0.63$, $p = 0.003$)

Trial 3

According to the criteria established by Wise et al. (2004), outbreaks in 5 of the 8 ponds used for Trial 3 were classified as severe, while the outbreaks in the remaining 3 ponds were considered mild. Mean percentage gill damage of fish surviving for 7 d in ponds with severe outbreaks of PGD was 34 and 23%, but only 3, 2 and <1% from ponds with mild PGD. Mortality ranged from 33.3 to 100% in ponds with severe outbreaks of PGD, and 7 to 27% in ponds determined to have a mild outbreak. In ponds identified as having mild outbreaks of PGD within the system there was no gill damage in fish after 24 h exposure to the pond. Conversely, gill damage was observed within 24 h exposure in 2 of the 5 ponds classified as severe (Table 5).

Levels of parasitic infection, as expressed by the mean log of the PSE:HSE ratio for both 24 h exposures, ranged from –3.2 in the most severe outbreak to –8.0 in the mildest outbreak, where no parasite DNA was detected in sentinel fish from the second 24 h exposure. Mean log PSE:HSE ratio was significantly higher

Table 4. *Henneguya ictaluri* infecting *Ictalurus punctatus*. Trial 2 data. Approximate abundance of *H. ictaluri* actinospores in 20 replicate 7 d trials in 8 commercial channel catfish ponds with varying degrees of proliferative gill disease severity. Sentinel were fish held in ponds for 7 d; percent mortality was calculated as the number dead out of 15 fish, and percent gill damage was calculated from survivors only. Outbreak score according to Wise et al. (2004) (see Table 2). Inhibition was corrected by a 4-fold dilution of template DNA (actinospore equivalents [AE] l^{-1} calculated by a dilution factor of 4). QPCR cycle threshold (C_T) values and approximate spore abundance from water samples. Mean AE l^{-1}: average of Day 0 and Day 7 observations. NA: not applicable, since all fish died; ND: no parasites detected

Replicate	Pond	Mortality (%)	Gill damage (%)	Outbreak score	C_T		AE l^{-1}		
					Day 0	Day 7	Day 0	Day 7	Mean
1	1	66.6	10.0	Moderate	35.4	30.5	≥1.7	≥15.4	≥8.6
2	1	73.3	3.0	Severe	27.0	33.3	≥82.6	≥5.3	≥43.9
3	1	20.0	19.0	Severe	28.6	29.5	≥34.3	≥23.7	≥28.9
4	1	80.0	10.0	Severe	31.7	31.1	≥9.1	≥11.6	≥10.3
5	1	100.0	NA	Severe	37.1	ND	≥1.4	ND	≥0.7
6	1	0.0	2.0	Mild	ND	37.2	ND	≥1.4	≥0.7
7	2	60.0	5.0	Mild	33.3	29.2	≥4.4	≥28.7	≥16.6
8	2	6.7	14.0	Moderate	30.3	32.6	≥17.4	≥7.1	≥12.3
9	2	66.6	9.0	Severe	29.9	30.0	≥27.6	≥19.1	≥23.4
10	2	33.3	4.0	Mild	33.2	31.0	≥6.5	≥12.8	≥9.7
11	2	13.3	1.0	Mild	31.0	33.1	≥12.8	≥5.8	≥9.3
12	3	100.0	NA	Severe	25.3	26.7	≥152.9	≥82.6	≥117.8
13	3	100.0	NA	Severe	26.2	26.7	≥101.4	≥80.1	≥90.8
14	3	100.0	NA	Severe	26.7	29.1	≥82.6	≥34.5	≥58.6
15	3	100.0	NA	Severe	26.7	30.4	≥80.1	≥16.7	≥48.4
16	4	0.0	10.0	Moderate	29.0	33.8	≥29.2	≥3.5	≥16.4
17	5	6.7	9.0	Moderate	29.3	32.9	≥24.8	≥9.6	≥17.2
18	6	20.0	1.0	Mild	ND	34.7	ND	≥2.4	≥1.2
19	7	6.7	6.0	Moderate	36.6	34.9	≥2.2	≥2.1	≥2.2
20	8	13.3	2.0	Mild	36.0	35.3	≥8.5	≥1.7	≥5.1

Table 5. *Henneguya ictaluri* infecting *Ictalurus punctatus*. Trial 3 data. Utilization of a *H. ictaluri*-specific QPCR assay to determine the parasite level in sentinel fish exposed to ponds of varying proliferative gill disease severity for 2 separate 24 h exposures and one 7 d exposure. Sentinel fish were held in ponds for 7 d; percent mortality was calculated as the number dead out of 15 fish. Outbreak score according to Wise et al. (2004) (see Table 2). PSE:HSE: ratio of the parasite standard equivalent (PSE) to host 18S SSU rDNA standard equivalent (HSE) determined by QPCR analysis. Within columns, values with different superscript letters ($^{a,b,c,\cdots}$) are significantly different ($p < 0.05$). NA: not applicable, since all fish died

Replicate	Pond	Mortality (%)	Gill damage (%)			Outbreak score	Log PSE:HSE		
			24 h (1)	24 h (2)	7 d		24 h (1)	24 h (2)	Mean
1	1	100.0	14.0	13.0	NA	Severe	−3.212	−3.667	-3.440^{a}
2	1	100.0	9.0	0.0	NA	Severe	−3.611	−6.167	-5.155^{d}
3	1	60.0	0.0	1.0	34.0	Severe	−4.319	−3.758	-4.038^{b}
4	1	33.3	0.0	0.0	23.0	Severe	−4.025	−5.086	-4.576^{c}
5	1	100.0	0.0	0.0	NA	Severe	−4.690	−4.339	-4.487^{c}
6	2	27.3	0.0	0.0	3.0	Mild	−5.287	−5.198	-5.242^{e}
7	2	6.7	0.0	0.0	2.0	Mild	−5.559	−5.873	-5.716^{f}
8	2	6.7	0.0	0.0	0.0	Mild	−5.402	−8.000	-6.701^{g}

in fish populations considered to have a severe outbreak and there was a positive correlation ($r = 0.70$, $p = 0.054$) between QPCR-determined parasite loads and percent mortality in 7 d sentinel fish exposures (Fig. 7), which becomes more significant when the second replicate from Pond 1 is removed from the data set ($r = 0.83$, 0.021). All trials conducted in ponds classified as having severe outbreaks of PGD within the system had mean log PSE:HSE values greater than −5.2.

DISCUSSION

There was a discrepancy between the theoretical and actual C_T for the 10, 5 and 1 actinospore aliquots. Based on the observed C_T of the 1000, 100 and 25 actinospore aliquots, and given a reaction efficiency of at least 90% (the minimum acceptable efficiency for this study), the corresponding C_T for 10, 5, and 1 actinospores should have been roughly 28, 29 and

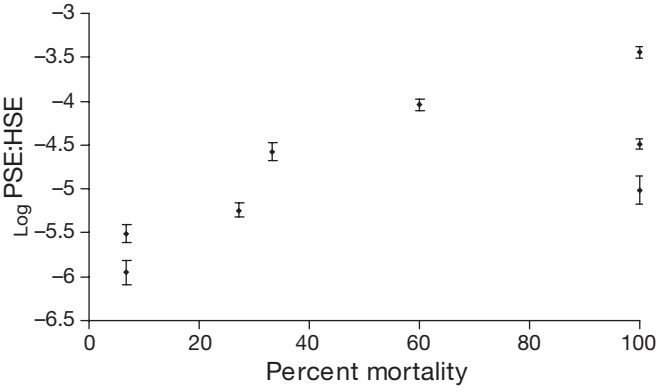

Fig. 7. *Henneguya ictaluri.* Trial 3 data. Correlation between mean (±SEM) parasite level as determined by QPCR in sentinel fish exposed to PGD-positive pond for 2 separate 24 h exposures and percent mortality observed in sentinel fish exposed for 7 d (r = 0.69, p = 0.054). QPCR data is presented in terms of the log of the ratio of mean parasite standard equivalents (PSE) to host standard equivalents (HSE)

31 respectively, rather than the 31, 31.5 and 35 observed in this study. The difference between the theoretical and actual C_T is most likely be attributed to DNA loss during processing. The DNA isolation kit used in this study uses 6 separate supernatant transfer steps as well as a silica membrane binding and elution step. DNA which remains bound to the silica membrane after elution or is retained in the sample tubes during supernatant transfer may be inconsequential for 1000, 100 or 25 actinospore aliquots, but could potentially make up a large portion of the DNA isolated from 10, 5 and 1 actinospores. This could account for the disparity observed between the theoretical and actual C_T obtained from direct processing of actinospore aliquots. Given the consistency of the C_T observed for replicate aliquots, we feel confident that DNA loss through processing is consistent; therefore the generated standard curve is valid for actinospore enumeration.

Based on findings from Trial 1, *Henneguya ictaluri* actinospores appear to be relatively evenly distributed throughout commercial catfish ponds over the course of a PGD outbreak. Bellerud et al. (1995) showed that populations of *Dero digitata* are not equally distributed in the pond sediment, and that populations of *D. digitata* within the pond are variable in their rates of actinospore release, making benthic sampling a poor predictor of potential outbreaks. However, Trial 1 showed that although actinospore concentrations varied among sampling sites, actinospore concentrations at most sample sites were within 1 SD of the mean actinospore concentration for the entire pond on the day of sampling. Furthermore, locations in the sampling grid with the highest and lowest concentrations of actinospores varied with time, i.e. pond locations with high and low concentrations of actinospores were not the same from day to day. Homogeneity of actinospore concentrations on a given day and variation in localized high and low concentrations of actinospores from day to day indicates that variations in oligochaete populations and their rate of actinospore release may result in areas with high concentrations of actinospores, but mixing produced by wind, aeration and other physical processes are more significant factors in actinospore distribution than the location of *D. digitata* in the pond sediment. Since channel catfish move freely in the pond and actinospores are mixed in the water, temporary variations in localized actinospore concentrations probably have little effect on the rate of infection among fish in the pond. However, because water samples only capture the approximate actinospore concentrations from a given location at the time of sampling, it is recommended that several water samples be taken from different geographic locations in the pond to better characterize the actual concentration of actinospores. Alternatively, sentinel fish exposures represent a continuous sampling period over time which averages gill damage to infectious actinospores. Therefore, multiple sentinel fish cages in multiple locations are probably not necessary for an accurate estimation of the rate of infection for the pond.

Identification of a potentially lethal outbreak of PGD in a catfish pond is a complicated task facing commercial catfish producers every spring. The use of sentinel fish as described by Wise et al. (2004, 2008) is reliable if the cage system does not physically fail within the sampling period. However, use of sentinel fish cages is time consuming, labor-intensive and requires assistance of trained fish-disease personnel. A major drawback of using sentinel fish to monitor PGD outbreaks is the inability to determine the cause of death of caged fish if all fish die prior to sampling. In the early spring, failure to properly acclimate sentinel fish to ambient pond water temperatures can result in mortalities or predispose fish to other infectious diseases such as saprolegniasis and columnaris, which may be misinterpreted as PGD-related. Death of sentinel fish also frequently occurs in the summer months when algal blooms cause oxygen depletion or algal growth fouls net-pens, restricting water flow to the fish. To prevent oxygen depletion, net-pens are often placed near mechanical aerators, which can result in swift currents flowing through the cage which may exhaust fish to the point of death. This can result in a sampling bias due to post-mortem autolysis, which prevents an accurate evaluation of gill damage in fish that have died prior to sampling. In extreme cases this bias may require the restocking of sentinel fish. In addition,

without an adequate number of fish to be examined, the severity of the outbreak cannot be determined. Another limitation is that the sentinel fish protocol is based on the percentage of filaments with at least one chondrolytic lesion or 'break', and does not take into consideration the number of breaks within each filament. In heavy infections an individual filament may have several breaks, yet it is considered 1 observation, which underestimates disease severity as it is believed each break is associated with infection by an individual actinospore (Wise te al. 2004, 2008). Both QPCR assay protocols eliminate those problems because PGD can be ruled out as the cause of death when the mean water-borne AE l^{-1} or the PSE:HSE ratio in tissues from sentinel fish exposures is relatively low. This was evident in several of the experimental pond trials in which there was greater than 50% mortality of sentinel fish, yet water-borne AE l^{-1} or PSE:HSE ratios were relatively low, indicating that the cause of death may not be directly related to PGD (Tables 4 & 5).

A distinct advantage of quantifying actinospores directly from water samples, compared to using sentinel fish, is that it significantly reduces the time required to determine the presence of this pathogen in commercial catfish ponds. However, since DNA is conserved throughout the life cycle of the parasite, it is possible that DNA detected in pond water samples could also represent myxospore stages not infective to the fish. Even so, the dynamics of the parasite's life cycle leads us to believe that during periods of peak actinospore release, there is a lower prevalence of the myxospore stage in the pond system. Pote et al. (2000) exposed parasite-free channel catfish fingerlings to *Henneguya ictaluri* actinospores and observed the mature myxospore stage in the gills 3 mo post-infection. Given that most outbreaks of PGD occur in the spring (mid-March to mid-June) with a lesser incidence in the fall (mid-September to mid-October), peak concentrations of the myxospore stage in the system would most likely occur 3 mo post-infection. Consequently, the myxospore stage of the organism would be most prevalent mid-summer and mid-winter, and would not coincide with peak concentrations of actinospores observed in the spring and fall. As such, the effect of the myxospore stage on the assay is likely to be negligible.

One difficulty encountered in this research was that ponds used for experimental trials were selected after PGD had been identified through diagnostic case submissions from the ADL. As such, most of the ponds sampled had outbreaks in which mortalities were already decreasing, which may account for the decreases in PSE:HSE ratios and actinospore concentrations from the first to second sampling days in most trials. Further research should be conducted to determine the temporal changes in actinospore abundance to determine year-round prevalence of the organism, as well as diurnal changes in actinospore release to establish the best sampling time for monitoring PGD outbreaks. Such studies would also show the relationship between PGD-induced mortality and various environmental conditions within the pond. The effects of salinity, dissolved oxygen concentration and other environmental variables on PGD-related mortalities also need to be determined.

Based on the present work, both QPCR assays are able to identify potentially lethal concentrations of *Henneguya ictaluri* actinospores in ponds prior to stocking and have application in preventing PGD-induced mortalities in fingerlings restocked following an outbreak or for identifying a *H. ictaluri*-free environment in which to relocate fish. This work has demonstrated that actinospore abundance is relatively homogeneous throughout the pond, with actinospore numbers decreasing over the course of an outbreak. This corresponds to previous research showing that rates of infection increase to a peak, then decrease gradually over time. Research in this laboratory indicates that once a PGD outbreak begins to subside, it does not recur within the same season. Accordingly, once a pond reaches a safe parasite threshold, the PGD outbreak for that season is most likely over, and the pond can be stocked with little risk of losing fish to PGD (Wise et al. 2004). Although estimates based on this research are conservative, ponds with mean log PSE:HSE ratios less than −5.2 in 24 h sentinel fish exposures or AEC less than 10 actinospores l^{-1} of pond water can be stocked with a lesser risk of losing fish to PGD, provided that parasite levels are decreasing from the first to second sampling period.

This research is the first to isolate and quantify *Henneguya ictaluri* actinospores from the commercial catfish pond environment. Analyses of these data indicate that the determination of parasite load in sentinel fish is a more reliable method of determining PGD severity; however, there are benefits to the water sampling protocol. There were several instances in the second experimental trial where sentinel fish mortality was most likely not attributable to PGD, as evidenced by the low concentrations of actinospores detected in water samples.

These QPCR assays provide an additional resource to be used in conjunction with current diagnostic and management protocols to maximize information provided to catfish producers. Although both methods of determining the prevalence of *Henneguya ictaluri* in catfish ponds identified actinospore levels that correlate to high and low risk of fish loss, these values are not absolute and other variables must also be considered when making management decisions based on the information provided by these assays.

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LITERATURE CITED

Balacázar JL, Vendrell D, de Blas I, Ruiz-Zarzuela I, Gironés O, Múzquiz JL (2007) Quantitative detection of *Aeromonas salmonicida* in fish tissue by real-time PCR using a self quenched fluorogenic probe. Int J Med Microbiol 56: 323–328

Bellerud BL, Pote LM, Lin TL, Johnson MJ, Boyle CR (1995) Etiological and epizootological factors associated with outbreaks of proliferative gill disease in channel catfish. J Aquat Anim Health 7:124–131

Bilodeau AL, Waldbieser GC, Terhune JS, Wise DJ, Wolters WR (2003) A real-time polymerase chain reaction assay of the bacterium *Edwardsiella ictaluri* in channel catfish. J Aquat Anim Health 15:80–86

Bowser PR, Conroy JD (1985) Histopathology of gill lesions in channel catfish associated with *Henneguya.* J Wildl Dis 21:177–179

Brinkman NE, Haughland RA, Wymer LJ, Byappanahalli M, Whitman RL, Vesper SJ (2003) Evaluation of a rapid quantitative real-time PCR method for enumeration of pathogenic *Candida* cells in water. Appl Environ Microbiol 69: 1775–1782

Burtle GJ, Harrison LR, Styer EL (1991) Detection of a triactinomyxid myxozoan in an oligochaete from ponds with proliferative gill disease in channel catfish. J Aquat Anim Health 3:281–287

Cavender WP, Wood JS, Powell MS, Overturf K, Cain KD (2004) Real-time quantitative polymerase chain reaction (QPCR) to identify *Myxobolus cerebralis* in rainbow trout *Oncorhynchus mykiss*. Dis Aquat Org 60:205–213

Chase DM, Elliott DG, Pascho RJ (2006) Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. J Vet Diagn Invest 18:375–380

Elibol-Fleming B (2006) Effects of *Edwardsiella ictaluri* infection on transcriptional expression of selected immune relevant genes in channel catfish, *Ictalurus punctatus*. PhD dissertation, Mississippi State University, Mississippi State, MS

Fontaine M, Guillot E (2003) An immunomagnetic separation real-time PCR method for quantification of *Cryptosporidium parvum* in water samples. J Microbiol Methods 54: 29–36

Griffin MJ, Wise DJ, Camus AC, Mauel MJ, Greenway TE, Pote LM (2008) A real-time polymerase chain reaction assay for the detection of *Henneguya ictaluri* in channel catfish. J Vet Diagn Invest 20:559–566

Guy RA, Payment P, Krull UJ, Horgen PA (2003) Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl Environ Microbiol 69:5178–5185

Hallett SL, Bartholomew JL (2006) Application of a real-time PCR assay to detect and quantify the myxozoan parasite *Ceratomyxa shasta* in river water samples. Dis Aquat Org 71:109–118

Kelley GO, Zaqmutt-Vergara FJ, Leutenegger CM, Myklebust KA and others (2004) Evaluation of five diagnostic methods for the detection and quantification of *Myxobolus cerebralis.* J Vet Diagn Invest 16:202–211

MacMillan JR, Wilson C, Thiyagarajah A (1989) Experimental induction of proliferative gill disease in specific-pathogen-free channel catfish. J Aquat Anim Health 1:245–254

Panangala VS, Shoemaker CA, Klesius PH (2007) TaqMan real-time polymerase chain reaction assay for rapid detection of *Flavobacterium columnare*. Aquacult Res 38: 508–517

Pote LM, Bellerud B, Lin DL, Chenney EF (1994) The isolation and propagation of *Dero digitata* infected with *Auranti-actinomyxon* sp. J World Aquacult Soc 25:303–307

Pote LM, Hanson LA, Shivaji R (2000) Small subunit ribosomal RNA sequences link the cause of proliferative gill disease in channel catfish to *Henneguya* n. sp. (Myxozoa: Myxosporea). J Aquat Anim Health 12:230–240

Pote LM, Hanson LA, Khoo L (2003) Proliferative gill disease. In: Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, Blue Book 5th edn. Fish Health Section, American Fisheries Society, Bethesda, MD

Styer EL, Harrison LR, Burtle GJ (1991) Experimental production of proliferative gill disease in channel catfish exposed to myxozoan-infected oligochaete, *Dero digitata.* J Aquat Anim Health 3:288–291

Thompson K, Nehring RB (2000) A simple technique used to filter and quantify the actinospore for *Myxobolus cerebralis* and determine its seasonal abundance in the Colorado river. J Aquat Anim Health 12:316–323

Whitaker JW, Pote LM, Khoo L, Shivaji R, Hanson LA (2001) The use of polymerase chain reaction assay to diagnose proliferative gill disease in channel catfish (*Ictalurus punctatus*). J Vet Diagn Invest 13:394–398

Whitaker JW, Pote LM, Hanson LA (2005) Assay to detect the actinospore and myxospore stages of proliferative gill disease in oligochaetes and pond water. N Am J Aquaculture 67:133–137

Wilson IG (1997) Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63:3741–3751

Wise DJ, Camus A, Schwedler T, Terhune J (2004) Health management. In: Tucker CS, Hargreaves JA (ed) Biology and culture of the channel catfish. Elsevier, Amsterdam, p 444–502

Wise DJ, Griffin MJ, Terhune J, Pote LM, Khoo LH (2008) Induction and evaluation of proliferative gill disease in channel catfish fingerlings. J Aquat Anim Health 20:236–244

Wong ML, Medrano JF (2005) Real-time PCR for mRNA quantitation. BioTechniques 39:75–85

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