3.2.18 *Ichthyophonus* Disease (Ichthyophoniasis)

Paul K. Hershberger
U.S. Geological Survey
Western Fisheries Research Center
Marrowstone Marine Field Station
Nordland, WA 98358
phershberger@usgs.gov

A. Name of Disease and Etiological Agent

Ichthyophonus disease (sometimes referred to as ichthyophoniasis, *Ichthyosporidium* disease, and ichthyosporidiosis) is a systemic granulomatous disease caused by the protistan parasite *Ichthyophonus* spp. A lack of distinguishing morphological characteristics and incomplete species descriptions of the causative agent have resulted in nomenclature inconsistencies within the genus; to avoid further confusion, the organism(s) should be referred to generically as *Ichthyophonus* until phylogenetic studies provide an objective basis for speciation.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The geographic range of ichthyophoniasis is generally considered to include marine waters throughout the world; as such, ichthyophoniasis is one of the most widespread diseases of fish. Ecological and economical impacts of ichthyophoniasis are most commonly reported in wild marine fishes (eg. reviewed in McVicar 1999, Kocan et al 2004, Marty et al 2010, Hershberger et al 2010); however, the disease is periodically reported from cultured marine and freshwater species (Gustafson and Rucker 1956, Doriere and Degrange 1960, Erickson 1965, Miyazaki and Kubota 1977, Anonymous 1991, Athanassopoulu 1992, Franco-Sierra 1997, Gavryuseva 2007) where its origin is often traced to the feeding of un-processed tissues from infected marine fishes. Although reports are relatively rare, *Ichthyophonus* infections are periodically documented in free-ranging freshwater fishes (eg. Schmidt-Posthaus & Wahli 2002).

2. Host Species

The host range of *Ichthyophonus* is extremely broad, encompassing more than 80 fish hosts (reported in Spanggaard et al 1994), including 35 marine and 48 freshwater fishes (Reichenbach-Klinke & Elkan 1965). The parasite demonstrates low parasite-host specificity in fish, and reports of natural hosts are likely a reflection of whether a particular fish species has been thoroughly examined (McVicar 1999).

C. Epizootiology

Basic epizootiological understanding of the disease is limited because the predominance of scientific reports consists of case histories and responses to observed epizootics rather than controlled empirical studies. Empirical evidence for vertical transmission does not exist; however, *Ichthyophonus* can sometimes be cultured from the ovaries Pacific herring demonstrating heavy infections. Transmission in piscivorous and scavenger hosts likely occurs through consumption of infected prey (Kocan et al 1999). Horizontal transmission through cohabitation occurs in some species, including cultured rainbow trout

(Gustafson & Rucker 1956). The route of transmission for planktivorous hosts, including Clupeids, remains unclear; laboratory studies have repeatedly failed to establish infections through cohabitation, feeding with food containing *Ichthyophonus* schizonts, or by direct intubation of *Ichthyophonus* schizonts into the stomach of Pacific herring (Hershberger & Gregg unpublished data). Repeated feeding of captive, Atlantic herring with *Ichthyophonus*-spiked mussel and liver tissues resulted in low prevalence of infection ($\leq$ 12%); however, a natural route of infection has not been demonstrated. Spiking invertebrate colonies with *Ichthyophonus* resulted in attachment of the parasite to copepod appendages and invasion of parasite germination tubes into the body of a single *Calanus finmarchicus*; however, feeding of these invertebrates to Atlantic herring did not establish infection (Sindermann and Scattergood 1954). It is interesting to note that schizonts released from the skin of infected herring are infectious when injected into the body cavity of Pacific herring but not when administered orally (Kocan et al 2010). Further research is needed to understand the possible involvement of intermediate hosts and other natural routes of infection. There is some indication that infectivity of the parasite to Pacific herring is inversely related to temperature, with infection prevalence decreasing as temperature increased from 9 to 15°C (Gregg et al 2011).

Infection can result in one of three outcomes: acute disease and mortality, chronic disease associated with decreased condition and performance, or subclinical infection; there is no indication that infected fish are able to clear the infection once it becomes established. Infected Atlantic herring typically have lower condition factor and gonad weight (Kramer-Schadt et al 2010), and infected Pacific herring demonstrate a reduction in total energy content and energy density relative to uninfected cohorts (Vollenweider et al 2011). The prevalence of infection often increases with host size and age (Hershberger et al 2002, Marty et al 2003, Kramer-Schadt et al 2010).

D. Disease Signs

1. Behavioral Signs

Behavioral signs of ichthyophoniasis are minimal. Infected rainbow trout demonstrate decreased swimming performance (Kocan et al 2006), and the difference in swimming performance is more pronounced at warmer temperatures (Kocan et al 2009). In hatchery conditions, diseased individuals may appear lethargic and consume less food than uninfected cohorts. Infected wild herring may aggregate around the periphery of highly dense schools (Holst 1996).

2. Gross Signs

Externally, few if any gross signs typically appear on most affected hosts, with the notable exception of ‘sandpaper skin’ on clinically diseased Atlantic and Pacific herring. The condition is often most pronounced on the caudal third of the body surface and is caused by large numbers of raised papules under the skin surface. The parasite is eventually released from these papules, leaving pigmented ulcers that resemble flakes of pepper on the skin surface (Figure 1). Heavily infected rainbow trout may demonstrate petechial hemorrhages (Figure 2) on the skin and pigmented ulcers on the ventral surface (Figure 3).

Figure 1. Juvenile Pacific herring demonstrating external signs of ichthyophoniasis including pigmented skin ulcers and general emaciation. This fish died from ichthyophoniasis after experimental laboratory exposure. Photo: P. Hershberger, U.S. Geological Survey.

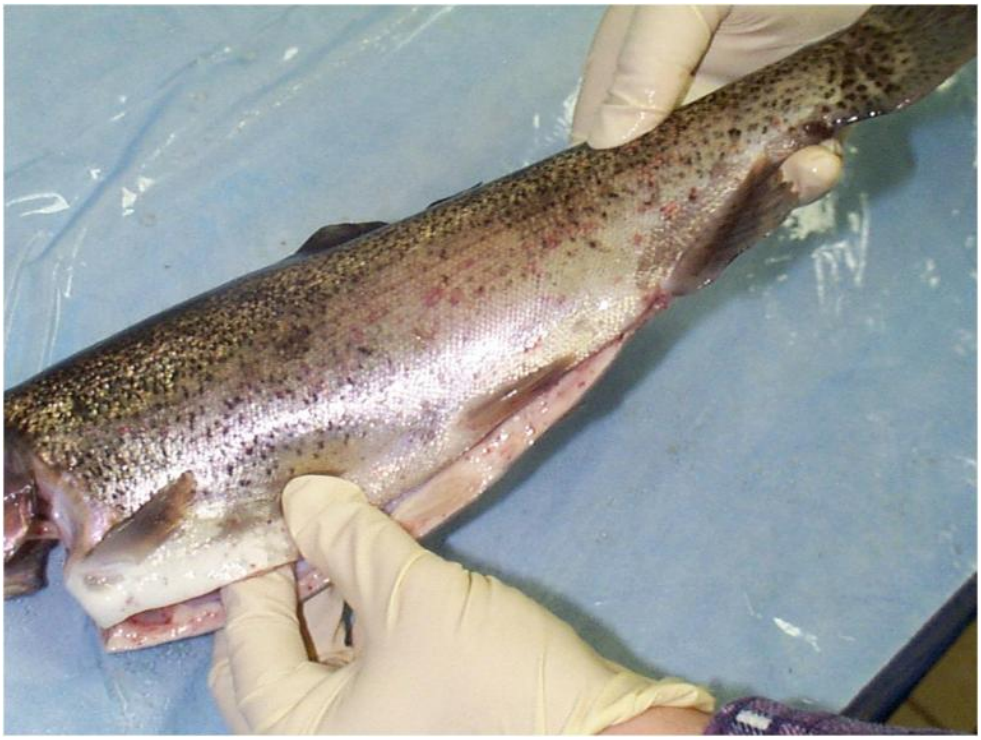

Figure 2. Rainbow trout with ichthyophoniasis demonstrating petechial hemorrhages on the skin surface. Photo: Dr. Scott LaPatra, Clear Springs Foods, Inc.

Figure 3. Cultured rainbow trout with ichthyophoniasis demonstrating open ulcers and pigmented spots on the ventral surface. Photo: Dr. George Savvidis, Veterinary Research Institute of Thessaloniki, Greece.

Internally, gross signs typically appear as white or cream-colored nodular lesions throughout the blood-rich organs, including heart, liver, kidney, and spleen (Figures 4 and 5). The hearts from diseased fishes are often significantly larger and heavier than those from uninfected hosts, presumably as a result of the additional mass contributed by the massive parasite load and granulomatous response (Daniel 1933, Kocan et al 2006). Pigmented lesions occur in the skeletal muscle of heavily infected fishes, periodically resulting in the inability of fishery processors and aquaculture industries to market the affected fillets (reviewed in McVicar 1999; Figure 6).

Figure 4. Rainbow trout with ichthyophoniasis, demonstrating white nodular lesions throughout all internal organs. Photo: Dr. George Savvidis, Veterinary Research Institute of Thessaloniki, Greece.

Figure 5. Macroscopic signs of ichthyophoniasis, including white nodular lesions, throughout the heart of a diseased Chinook salmon. Photo: Stan Zuray, Yukon River Rapids Research Center.

Figure 6. Unmarketable fillets from rainbow trout with heavy ichthyophoniasis infections. Note the pigmented lesions and focal hemorrhages throughout the fillets, with signs becoming more pronounced towards the caudal region. Photo: Dr. Scott LaPatra, Clear Springs Foods, Inc.

3. Microscopic signs

Developmental stages of *Ichthyophonus* are easily observed in fresh squash preparations, tissue explant cultures, and stained histological sections:

- The most commonly observed stage occurs within well-defined host cellular granulomas and consists of a large (10-250µm), thick-walled, multi-nucleate, spherical body that has periodically been referred to as spore, macrospore, resting spore, multinucleate resting spore, and cyst. Hereafter, this stage will be referred to as a schizont, or multinucleate stage that reproduces asexually and produces a number of daughter cells.
- Germination tubes (A.K.A hyphae and pseudohyphae) are typically observed after the infected host has been dead for a period of time. *In vitro* cultures containing schizonts can be stimulated to produce germination tubes through manipulation of culture conditions (Spanggaard et al 1994, Spanggaard & Huss 1996).
- A small, reportedly motile mono-nucleate stage has periodically been referred to as endospore, microspore, amoeboblast, and plasmodium. This stage is presumed to develop into larger schizonts and is speculated to be involved in infectivity (McVicar 1982) and/ or dissemination within an infected host (Spanggaard et al 1995).

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Presumptive diagnosis of *Ichthyophonus* infection is made through a combination of visible internal and external signs, tissue squash preparations, *in vitro* culture of *Ichthyophonus* from infected tissues, schizont germination, and histopathology. The most sensitive diagnostic technique involves *in vitro*

culture from infected tissues, whereby low level infections can be visualized by the growth and multiplication of the parasite in culture medium.

a. Internal and external signs (described above). High intensities of the disease are accompanied by gross signs on internal organs; however, subclinical infections can be easily overlooked if more sensitive diagnostic techniques are not employed.

b. Squash preparations. Spherical schizonts (10-250 µm diameter) can be observed in fresh squash preparations from infected soft tissues. Schizonts are often surrounded by host granulomatous tissues. The technique is most useful for providing partial confirmation of moderate to heavy infection intensities, where clinical signs are present.

c. *In vitro* culture from infected tissues. The most sensitive diagnostic technique to detect *Ichthyophonus* infections involves *in vitro* culture of tissue explants. *Ichthyophonus* schizonts and pseudohyphae grow readily in common broth media including Tris or Hepes-buffered Eagles Minimum Essential Medium (MEM) and Leibovitch-15 (L-15) supplemented with 5% fetal bovine serum. Addition of antibiotics in the medium (100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 100 µg ml^{-1} gentamycin) is recommended to decrease the possibility of bacterial contamination; however, the addition of anti-mycotics to the medium, including Amphoterecin-B (Fungizone™), will kill the parasite and should be avoided. Cultures are initiated by placing tissues into broth culture media ($\leq$ 1 part tissue: 5 parts media, w/v), incubated at 15°C, then screened microscopically (100X magnification) for signs of *Ichthyophonus* growth, including the presence of schizonts and germinating stages (Figures 7 & 8). If aspetic technique is not practiced, the cultures can periodically become over-run with yeast and mold (considering the lack of anti-mycotics in the medium); when this happens, the contaminants out-compete *Ichthyophonus* growth and the cultures become un-readable. Therefore, it is advised that cultures should be screened for *Ichthyophonus* 7d after tissue incubation, before any contaminants over-run the cultures. Uncontaminated cultures should be re-screened after 14d to allow optimal time for *Ichthyophonus* growth and detection. The polymerase chain reaction (described below) can be effective at confirming *Ichthyophonus*-positive cultures that become overrun with yeast

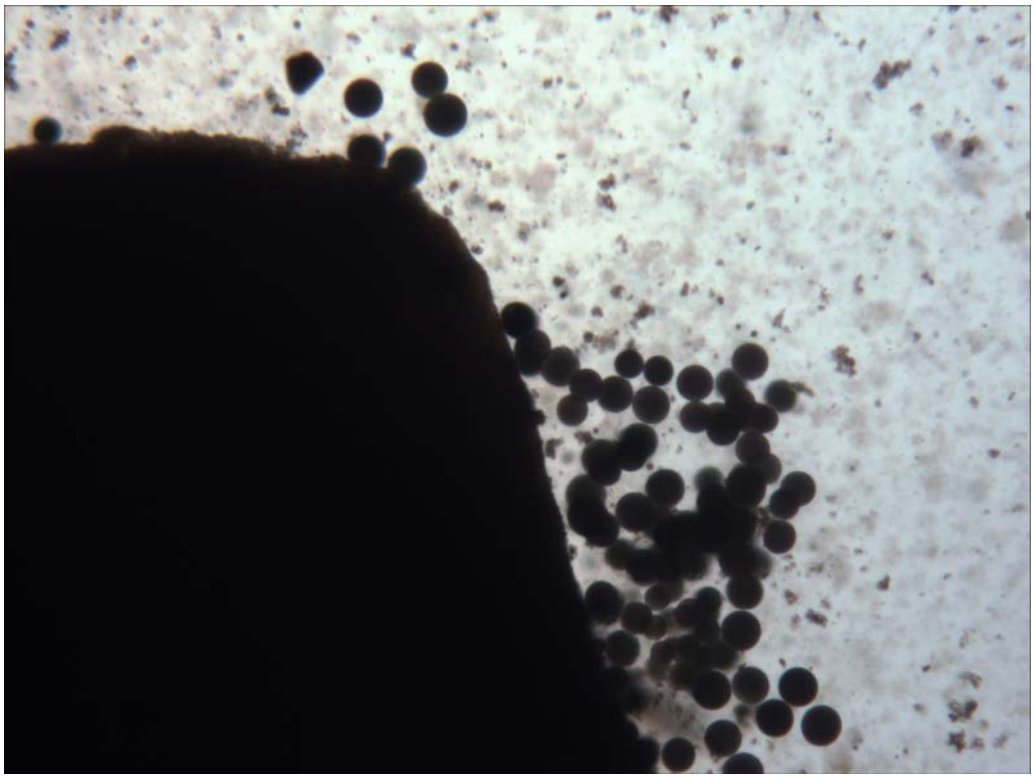

Figure 7. Typical *Ichthyophonus* schizonts in liver explant culture from an infected Pacific herring (40X magnification). Photo: P. Hershberger, U.S. Geological Survey.

d. Schizont germination.

Further presumptive diagnosis of *Ichthyophonus* can be provided by the detection of polymorphic parasite life history stages, including non-septate germination tubes that occur in response to host mortality or *in vitro* culture conditions. After the death of an infected host, *Ichthyophonus* schizonts often produce germination tubes through which the schizont contents migrate and uni- or multi-nucleate daughter cells are released (Figures 8 & 9). Presence of these germination tubes in stained histopathology sections is often considered a confirmatory diagnosis (McVicar 1982). Similarly, confirmatory diagnosis can be made by *in vitro* induction of germination tubes and polymorphic forms by the addition of 1% glucose and reduction of culture pH to 3.5 (Spanggaard et al 1994, Spanggaard and Huss 1996).

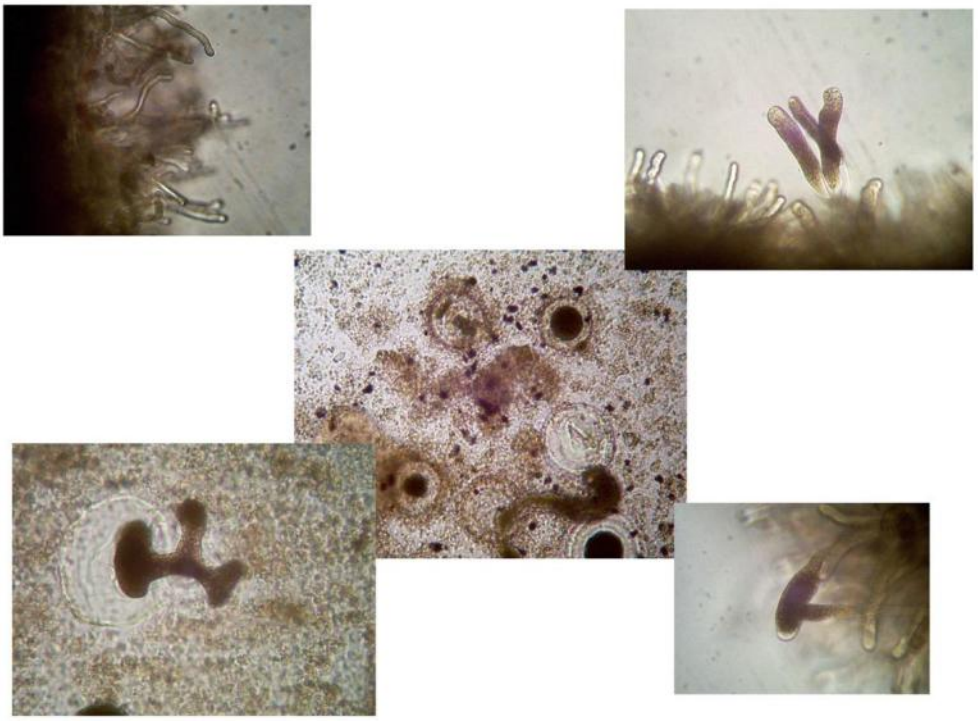

Figure 8. Germinating stages of *Ichthyophonus* observed in tissue explant cultures from infected rainbow trout. Photo: Dr. Scott LaPatra, Clear Springs Foods, Inc.

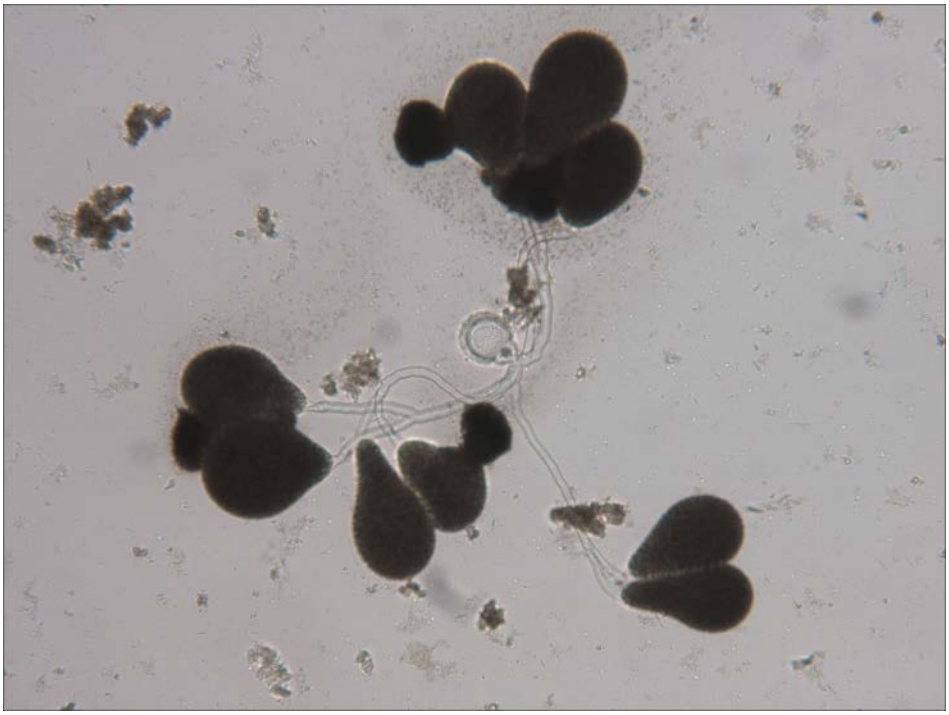

Figure 9. Wet mount of cultured *Ichthyophonus* isolated from Pacific herring. Note the non-septate germination tubes originating from a parent schizont and terminating at club-shaped daughter cells (200X magnification). Photo: P. Hershberger, U.S. Geological Survey.

e. Histopathology.
Ichthyophonus schizonts can be easily observed in stained tissue sections from moderate and heavy intensity infections (Figure 10). The parasite often occurs as single or multiple schizonts inside well-defined host cellular granulomas; although un-encapsulated schizonts are also common throughout infected tissues during various stages of infection. The host granulomatous reaction is easily observed in hematoxalyn and eosin (H&E) stained tissue sections. Polysaccharides on the surface of the parasite stain strongly positive with periodic acid-Schiff (PAS); however, other spherical organisms in the 50-250 µm size range also stain PAS-positive and superficially resemble *Ichthyophonus* schizonts in histological sections. As such, detection of PAS-positive spherical bodies in tissue sections should not be considered confirmatory.

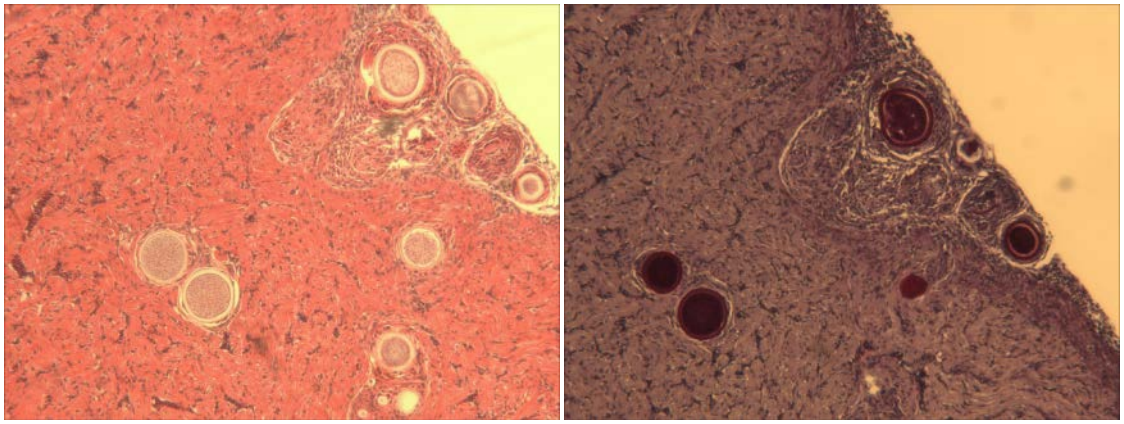

Figure 10. Stained histological sections (100X magnification) of *Ichthyophonus* in the heart of Pacific staghorn sculpin, stained with H&E (A) and PAS (B). Photos: P. Hershberger, U. S. Geological Survey.

2. Confirmatory Diagnosis
In vitro growth and multiplication of the parasite under suitable culture conditions followed by microscopic detection of *Ichthyophonus* life stages is considered confirmatory. If further confirmation is required, then definitive diagnosis from cultured material should employ the polymerase-chain reaction (PCR) using *Ichthyophonus*-specific primers.

Genomic DNA from *Ichthyophonus* schizonts, pseudohyphae or infected fish tissue can be isolated using standard methods; mechanical homogenization may improve extraction efficiency (Rasmussen et al. 2010). PCR amplification of a 371 bp segment of the small subunit (SSU) rDNA is achieved using primers and PCR conditions described by Whipps et al. 2006:

Ich 7F	5'-GCT CTT AAT TGA GTG TCT AC-3'
Ich 6R	5'-CAT AAG GTG CTA ATG GTG TC-3'

Temperature	Time	Cycles
95°C	3 min	1x
94°C	30 sec	35x
60°C	45 sec	
72°C	60 sec	
72°C	10 min	1x

Products are visualized by conventional agarose gel electrophoresis. Presence of 371 bp band indicates a positive reaction.

Although it is possible to utilize conventional PCR to detect *Ichthyophonus* directly from tissues for surveillance efforts, thereby eliminating the *in vitro* cultivation step, this practice may not yield the most accurate estimate of apparent prevalence in the population, as false negatives can occur at low infection intensities (Whipps et al 2006). *In vitro* culture of infected tissues is currently the most sensitive diagnostic technique and is able to detect low-intensity infections that fail to test positive by conventional PCR, histological examination, or squash preparations (reviewed in Kocan et al 2011). Efforts to validate the sensitivity and specificity of a quantitative PCR (qPCR) reaction are currently underway.

F. Procedures for Detecting Subclinical Infections

The most sensitive diagnostic technique for detecting subclinical infections involves *in vitro* culture of *Ichthyophonus* from tissue explants (described above).

G. Procedures for Detecting Prior Exposure

There is no indication that infected hosts fully clear *Ichthyophonus* infections, so prior exposure is most easily accomplished by determining the current infection status of the host. A strong humoral response develops after *Ichthyophonus* exposure in some hosts (McVicar 1999 & 1982); however the response is poorly characterized there are no standard serological methods for detecting antibodies to *Ichthyophonus*.

H. Procedures for Transportation and Storage of Samples

To obtain the cleanest tissue explant cultures, samples should be collected from recently-dead specimens; however, *Ichthyophonus* schizonts germinate in the host post-mortem, and positive explant cultures can often be obtained from hosts that have been dead several days. It should be cautioned that the quality of explant cultures decreases from hosts that have been dead for extended periods and the potential for yeast and mold contamination is increased if samples were obtained from autolyzing tissues. After collection, explant cultures should be incubated at 15°C prior to microscopic examination for parasite growth. Freeze / thaw cycles can kill *Ichthyophonus* schizonts, rendering tissue explant cultures ineffective on previously-frozen tissues. Although aseptic technique should always be used when collecting tissue samples and dissection tools should be carefully disinfected between specimens, it is worthy to note that cross contamination of explant cultures between specimens is rare, even when tools are not disinfected between fish (LaPatra et al 2008).

Prolonged storage of *Ichthyophonus* samples has been challenging. Parasite schizonts generally do not survive freeze / thaw cycles; however, some researchers have reported limited success recovering *Ichthyophonus* from cultures that were frozen in MEM (pH 3.5) that has been supplemented with 1% glucose and 20% glycerol. The most reliable means of maintaining *Ichthyophonus* for extended periods remains cultivation either:

1) *in vitro* by periodically refreshing the culture medium and cycling pH (Spanggaard et al. 1994) or
2) *in vivo* by maintaining infections within live fish hosts in laboratories that are designed with the appropriate biosafety precautions.

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