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Short Communication

Potential for cross-contamination of *in vitro* explant cultures initiated from *Ichthyophonus*-infected rainbow trout, *Oncorhynchus mykiss* (Walbaum)

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In vitro culture of pathogens has been used for over a century to identify the causative organisms responsible for a variety of diseases (Koch 1881; Bass & Johns 1912; Dobell & Laidlaw 1926; Diamond 1960; Kocan 1969). For *in vitro* culture to correctly identify pathogenic organisms and/or determine infection prevalence, the parasite should be isolated in pure culture with a high degree of certainty that cross-contamination of cultures has not occurred.

Recently, extensive field studies were conducted on the prevalence of *Ichthyophonus* in several species of fish using *in vitro* explant culture as a method for evaluating infection prevalence (Hershberger, Stick, Bui, Carroll, Fall, Mork, Perry, Sweeney, Wittouck, Winton & Kocan 2002; Kocan, Hershberger & Winton 2004). During the course of these studies, culture medium was sterilized, instruments were disinfected between fish and the investigators had extensive experience with *in vitro* culture techniques. However, because of the high infection prevalences detected, concern was expressed that cross-contamination of cultures may have occurred. Likewise, cross-contamination of cultures has been invoked to explain spurious data obtained in other field studies (Whipps, Burton, Watral, St-Hilaire & Kent 2006).

Ichthyophonus is not difficult to culture, but unlike the laboratory environment, field conditions do not have many of the built-in safeguards that reduce the probability of cross-contamination. To evaluate the probability of cross-contamination under worst-case scenarios, we conducted two studies: a simulated field study and a controlled laboratory study, each designed to evaluate the potential for cross-contamination of cultures.

Conditions encountered during field sampling of wild fish were simulated by initiating explant cultures from heart tissue derived from 48 one-year-old rainbow trout, *Oncorhynchus mykiss* (Walbaum), sampled from three different raceways known to contain *Ichthyophonus*-infected fish. The infection prevalence for each raceway was not known at the time of sampling. To evaluate the probability of cross-contamination, heart tissue was excised from each fish and placed into Eagle's minimal essential medium, supplemented with 5% foetal bovine serum and antibiotics (Hershberger *et al.* 2002) by two experienced technicians using disinfected and contaminated instruments.

Technician 1 disinfected instruments between fish by dipping scissors and forceps in 70% alcohol and wiping them with sterile gauze. Technician 2 used similar instruments for all cultures but did nothing to disinfect them between fish, i.e. no attempt was made to remove body fluids and host tissue from the instruments between fish.

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Table 1 Data from a simulated field-sampling exercise: comparison of *Ichthyophonus*-positive cultures resulting from the use of disinfected and contaminated instruments

Raceway	Disinfected (positive/*n*)	Contaminated (positive/*n*)	% difference
1	20/24	21/24	4.2
2	4/12	4/12	0
3	10/12	10/12	0
Total	34/48	35/48	2.1

All 48 of the fish from the three raceways were processed first by technician 1 using disinfected instruments. After cultures of heart tissue were initiated, each fish was passed to technician 2 who repeated the culture procedure on the remaining heart tissue but without disinfecting instruments between fish. Cultures were microscopically examined for the presence of *Ichthyophonus* for 14 days and infection prevalence resulting from the two techniques compared. The objective of this exercise was to determine if the two protocols resulted in different infection prevalences for the same set of fish originating from a population with unknown infection prevalence – similar to what would occur during a field survey.

Infection prevalence in the field simulation study was 70.8% when disinfected instruments were used and 72.9% when contaminated instruments were used (Table 1). The one additional positive culture (2.1%) was detected in the group initiated with contaminated instruments, which was preceded by an *Ichthyophonus*-infected fish, suggesting, but not confirming, the possibility of cross-contamination.

In the laboratory control study, explant cultures were initiated from heart tissue obtained from 24 700–900 g rainbow trout. Twelve fish were specific pathogen free (SPF) and 12 were experimentally exposed to *Ichthyophonus* by cohabitation with known infected fish for 15 months. None of the exposed fish was clinically positive (i.e. visible lesions) for *Ichthyophonus* at the time of sampling.

The entire heart was aseptically removed first from each of the 12 SPF fish followed by the *Ichthyophonus*-exposed fish. The bottom one-third of each heart was removed and slices were taken for explant culture. The specimens were arranged such that an *Ichthyophonus*-exposed tissue was followed by *Ichthyophonus*-negative tissue from an SPF fish. In the first sampling, disinfected instruments were used between each heart. In the second sampling, contaminated instruments (not cleaned in any way) were used between each heart. The third sampling repeated what was done in the first and the fourth sampling repeated what was done in the second sampling. This resulted in two cultures initiated with disinfected instruments and two with contaminated instruments, thus ensuring that *Ichthyophonus*-negative tissue from an SPF fish was handled after each *Ichthyophonus*-exposed tissue. In summary, each heart was sampled four times using two different sampling regimes (disinfected and contaminated), resulting in 96 heart explant cultures (Table 2). The question addressed was: 'can cultures be contaminated with *Ichthyophonus* via contaminated instruments?'

No difference in the number of *Ichthyophonus*-positive fish was observed when disinfected and contaminated instruments were compared. A total of 75% (9/12) of the experimentally exposed fish cultured positive for *Ichthyophonus*, while none of the SPF fish cultured positive (Table 2). Of the nine positive fish, six produced four positive cultures from the four slices of heart tissue (4/4), while the remaining three positive fish produced 3/4, 2/4 and 1/4 positive cultures, indicating a non-random distribution of *Ichthyophonus* in the heart tissue.

Based on the findings presented here, we find it highly improbable that *Ichthyophonus* cultures can be cross-contaminated if minimal effort is made to disinfect instruments between fish. The inability of *Ichthyophonus* cells to contaminate instruments may be related to their large size and close association with the host tissue. If cross-contamination of cultures occurred in the field simulation study, a different pattern of positive and negative cultures would be expected in cultures initiated with disinfected and contaminated instruments. Because all positive cultures corresponded between the two groups (with the exception of one additional positive culture), it is reasonable to conclude that the infection prevalence obtained using disinfected instruments approaches the actual infection prevalence of this population.

The single additional *Ichthyophonus*-positive culture initiated with contaminated instruments could have resulted from transfer of *Ichthyophonus* from the previous infected fish, or it may represent the detection of a low-level infection that was not detected in the culture initiated with disinfected instruments. This hypothesis is supported by data from the controlled laboratory study where 33% of known positive fish did not culture positive for all tissue slices obtained from known infected fish.

Table 2 Controlled laboratory study demonstrating no unintentional transfer of *Ichthyophonus* to cultures by means of contaminated instruments

	Instruments			
Fish status	Disinfected – 1	Contaminated – 1	Disinfected – 2	Contaminated – 2
Exposed – 1			+	
Control – 1				
Exposed – 2				
Control – 2				
Exposed – 3	+	+	+	+
Control – 3				
Exposed – 4		+		+
Control – 4				
Exposed – 5	+	+	+	+
Control – 5				
Exposed – 6	+	+	+	+
Control – 6				
Exposed – 7	+	+	+	+
Control – 7				
Exposed – 8				
Control – 8				
Exposed – 9				
Control – 9				
Exposed – 10	+	+	+	+
Control – 10				
Exposed – 11	+		+	+
Control – 11				
Exposed – 12	+	+	+	+
Control – 12				

+ Indicates *Ichthyophonus*-positive cultures; shaded areas indicate cultures with the highest probability for cross-contamination.

This finding emphasizes the importance of controlled studies in interpreting observations derived from field studies.

Of the 12 experimentally exposed fish, nine (75%) cultured positive for *Ichthyophonus*. Of the nine confirmed positive fish, three did not result in positive cultures in all four slices of the same heart tissue, demonstrating that the organism was not uniformly distributed in the heart muscle and/or was present in very low numbers.

Although cross-contamination of *Ichthyophonus* cultures is highly unlikely, it is recommended that sterile technique be practiced and every effort made to disinfect instruments between fish whenever tissues are being cultured.

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