Evidence that PCR diagnostics underestimate infection prevalence of *Ichthyophonus* in Yukon River Chinook salmon.

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Epidemiologists study the transmission of disease in populations by measuring the frequency of both disease and death from the disease using diagnostic tests to distinguish individuals who are sick from those who are healthy. The best way to know with certainty that an organism is present is to see it! In the case of *Ichthyophonus* this can be accomplished by several well established diagnostic techniques: 1) It can be seen directly by examining fresh fish tissues under a microscope, 2) It can be seen in stained histology slides or 3) It can be seen in culture where it grows from infected tissues. As with any diagnostic tool, none of these methods is 100% accurate, but some are significantly more accurate than others. The greatest source of error results from sampling fish with low levels of infection (e.g. very few parasites per unit of tissue examined). Consequently, direct tissue examination and histology are most accurate in measuring infection prevalence when the number of parasites is high, while they are more likely to miss some positive infections when the number of parasites is low. In vitro culture has an advantage in that it uses a relatively large piece of suspect tissue, thus increasing the probability of having a parasite cell present in the sample. In addition, the parasite replicates in culture, thereby increasing the number of organisms and thus the probability of seeing the parasite.

The advent of highly sensitive molecular techniques in recent years has led a number of investigators to use them as diagnostic tests for the detection of low levels of infectivity and estimating the infection prevalence in a population. One such technique is PCR (polymerase chain reaction), which detects the presence of nucleic acid (DNA or RNA) by amplifying very small amounts of existing nucleic acid. Although this technique is extremely sensitive for detecting nucleic acids it has met with criticism as a suitable method for evaluating infection prevalence. If PCR is so exquisitely sensitive how could it not be ideal for detecting infection prevalence in a population?

First, what is meant by “prevalence” (not to be confused with “incidence”)?

"*Prevalence is defined as the number of infected fish present in the population at a specific time, divided by the number of fish in the population at that time*". Implicit in this definition is that the host species is infected with a live pathogen.

Second, what is PCR?
"*In molecular biology, the **polymerase chain reaction** (**PCR**) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of a particular DNA sequence*".

Specifically missing from the definition of PCR is the need for a whole or intact organism to be present. In fact, PCR is used extensively to identify the organism of origin when only a few cells or even just fragments of DNA are present in the sample e.g. blood cells to distinguish between human and animal origin; DNA on ancient stone weapons to identify animals or humans that were killed by the weapon; contaminated food to identify the microbial contaminant (*E. coli*). The strength of the PCR procedure is its ability to identify very small amounts of DNA without the need for the donor organism to be present.

Dr. Patricia Stanley expressed concern over the misuse of this highly sophisticated technique in a letter to the *American Journal of Infection Control*, where she points out why the presence of DNA/RNA in a system does not prove the presence of viable, infectious or potentially pathogenic organisms. Her letter points out that surfaces that had been disinfected with heat or chemical agents had no viable or infectious organisms present, yet their residual DNA could be detected by PCR (Appendix I).

Similarly, controlled studies by Birch et al. (2001) compared PCR with traditional culture methods and found that non-viable (e.g. dead) bacteria could be found on sterilized surfaces for up to 30 hours post death, and suggested caution when using molecular techniques to evaluate viability of microbes (Appendix II). Similar results were obtained by Sheridan et al. (1998), who used heat and alcohol killed *E. coli* as a test organism (Appendix III).

Even though PCR cannot determine if an organism is alive or dead, or is merely a contaminant, a number of reports have been published in the scientific literature describing the use of PCR as a method of determining infection prevalence in wild populations. With few exceptions, these studies were not able to accurately determine false negatives because of reliance on wild-collected hosts whose history of infection was unknown.

One such group of studies involved the evaluation of malaria infections in wild birds. Concern over the accuracy of these prevalence reports prompted USGS personnel to conduct a controlled study to evaluate two PCR methods as diagnostic tools for identifying sub clinical (aka chronic) infections in birds (Jarvi et al. 2002). In this study the authors experimentally infected birds with malaria, sampled them to confirm that they were infected, then reinfected them a second time. Malaria is an organism, like *Ichthyophonus*, that infects highly vascularized tissues, becomes sub clinical, lasts for the life of the host and is often almost impossible to microscopically detect in the blood. Once the birds in this study became chronic carriers (sub clinical), they were subjected to several diagnostic testing methods, including PCR. The findings were that PCR was only able to detect 61-84% of the known infected birds while serology detected 98%, leading the authors to suggest that the technique significantly underestimates the true prevalence of infection in wild populations (Appendix IV).

The significance of this PCR-malaria study lies in the fact that the authors knew with certainty how many of the experimental animals were infected (100%) before the PCR tests were conducted, thus allowing them to accurately evaluate the sensitivity of PCR in detecting the proportion of infected individuals (aka “infection prevalence”).

A similar study on *Ichthyophonus* infection prevalence was conducted on Yukon River Chinook salmon (Whipps et al. 2006). In this study the “sensitivity” and “specificity” of PCR for detecting infection prevalence was evaluated over a two-year period (Appendix V). To determine “sensitivity” the true *Ichthyophonus* infection prevalence of Yukon River Chinook salmon was determined using in vitro culture of tissues and histological evaluation of tissues. These values were then compared with results obtained using PCR. The results of this study showed that PCR detected 25% of known lightly infected muscle samples and 64% of known lightly infected heart samples. Heavily infected muscle was accurately detected 79% of the time and heavily infected heart was detected 98% of the time, thus showing a low level of sensitivity for detecting known infected individuals (e.g. false negatives), especially those with low-level infections. These accuracy values are similar to those obtained from malaria-infected birds (previous paragraph). The “specificity” of the test was able to confirm known *Ichthyophonus*-negative fish 94-100% of the time – that is, very few false positives.

As an example of how easily an infection of *Ichthyophonus* can be missed by PCR analysis is seen in Appendix VI. Three salmon with gross (e.g.

visible) *Ichthyophonus* lesions on their heart were simultaneously sampled for both histology and PCR. The three examples show that infections that are both visible to the naked eye and microscopically confirmed to be *Ichthyophonus*, can be totally missed by PCR analysis.

Conclusion: Based on the above studies it is clear that PCR is not a suitable diagnostic tool for evaluating either viable pathogens within an infected host, or infection prevalence within a population of wild fish. Any data from studies that use PCR as the sole diagnostic method for evaluating infection prevalence should be considered suspect unless confirmed by a more reliable diagnostic technique.

Citations

Birch, L., C.E. Dawson, J.H. Cornett, J.T. Keer (2001). A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. Letters in Applied Microbiology 33(4): 296-301.

Jarvi, S.I., J.J. Schultz, C.T. Atkinson (2002). PCR diagnostics underestimate the prevalence of avian malaria (*Plasmodium relictum*) in experimentally-infected passerines. J. Parasitology 88(1): 153-158.

Sheridan, G.E.C., C.I. Masters, J.A. Shallcross, B.M. Mackey (1998) Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. Applied Environmental Microbiology 64(4): 1313-1318.

Rapids Research Center - Yukon River (2006) http://www.rapidsresearch.com/html/*Ichthyophonus*_disease.html

Whipps, C.M., T. Burton, V.G. Watral, S. St-Hilaire, M.L.. Kent (2006). Assessing the accuracy of a polymerase chain reaction test for *Ichthyophonus* hoferi in Yukon River Chinook salmon *Oncorhynchus tshawytscha*. Diseases of Aquatic Organisms 68: 141-147.

Appendix I.

AJIC letters to the Editor

It is *AJIC*'s policy that authors are given an opportunity to respond to any letters pertaining to their article if they choose to do so.

The presence of nucleic acids does not demonstrate the presence of living microbes

To the Editor:

I find it very disturbing that some researchers are evaluating the efficacy of microbicidal methods (eg, heat or chemical treatment) by using nucleic acids as their assay tool. The assumption is that the presence of nucleic acid (deoxyribonucleic acid [DNA] or ribonucleic acid [RNA]) in a system indicates the presence of viable, potentially infectious organisms. Modern molecular techniques are wonderful tools for rapidly detecting microorganisms, especially ones that are difficult to culture (eg, human hepatitis B virus, human hepatitis C virus, *Mycobacterium tuberculosis*). However, the inappropriate application of these methods can lead to incorrect, misleading conclusions since the assays don't reliably distinguish between living and dead cells.[1] A convincing in vivo study supporting this view was done by Deva et al[2] who used polymerase chain reaction (PCR) to detect duck hepatitis B virus DNA on laparoscopes. The DNA was present in approximately constant amounts both before and after disinfection with glutaraldehyde. However, endoscopes disinfected for ≥5 minutes did not transmit infection. In another study[3], the biocides 96% ethanol and 4% paraformaldehyde were used to preserve 2 species of bacteria. After 3 months of storage, the RNA, including the labile messenger RNA (m-RNA), was intact and detectable. In the unpreserved controls, however, the RNA had significantly degraded and some types had become undetectable. The mode of action of the preservatives/biocides is not well understood, but they probably inactivate or inhibit nucleases that would degrade the nucleic acids. A study by Birch et al[4] employed heat-killed cells which were confirmed to be dead by standard culture methods. On the other hand, by using nucleic acid amplification techniques (PCR, reverse transcription-PCR [RT-PCR], nucleic acid sequence-based amplification [NASBA]), the researchers could demonstrate the presence of residual DNA and m-RNA for up to 30 hours (maximum time tested) post death.

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There is a significant benefit in being able to rapidly determine the presence of microorganisms in the medical environment both in vivo and in vitro. In some instances, detection of nucleic acids by modern molecular techniques such as PCR is a promising method for such analyses. However, when applied to the evaluation of biocidal processes, these techniques lead to erroneous conclusions and are not a substitute for standard cultural methods.

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Awareness of tuberculosis among primary care physicians in low-incidence areas

To the Editor:

In spite of the CDC's continuous tuberculosis (TB) control efforts, primary care physicians (PCPs) are reluctant to treat TB because of the toxic effects of anti-TB drugs. They are not familiar with the CDC's recommendation for protein purified derivative (PPD) testing or treatment of latent and active TB.

A 37-year-old Asian Indian male presented to his PCP with a right neck mass for 1-month duration. The patient had no constitutional symptoms. The patient came to United States 12 years ago; he lived in New Jersey until moving to Missouri last year. He visited India for 3 weeks, 5 months prior to his visit. He had negative PPD results in 1995. A computer tomography scan of the neck showed multiple right supraclavicular

Appendix II

A comparison of nucleic acid amplification techniques for the assessment of bacterial viability

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ABSTRACT

Aims: The ability to determine the presence and viability status of bacteria by molecular methods could offer significant advantages to the food, environmental and health sectors, in terms of improved speed and sensitivity of detection.

Methods and Results: In this study, we have assessed three amplification techniques, PCR, RT-PCR and NASBA, for their ability to detect nucleic acid persistence in an *E. coli* strain following heat-killing. NASBA offered the greatest sensitivity of the three methods tested. The presence of residual DNA and mRNA could be detected by PCR and NASBA, respectively, for up to 30 h postdeath, by which time cell death had been confirmed by culture methods. Thus a single quantitative measurement based on nucleic acid amplification did not permit unequivocal determination of cell viability.

Conclusions, Significance and Impact of the Study: The correlation between cell viability and persistence of nucleic acids must be well characterized for a particular analytical situation before molecular techniques can be substituted for traditional culture methods.

Appendix III

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Detection of mRNA by Reverse Transcription-PCR as an Indicator of Viability in *Escherichia coli* Cells

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The relationship between the detection of mRNA and cellular viability in *Escherichia coli* was investigated in cells killed by heat or ethanol. Reverse transcription-PCR (RT-PCR) methods were developed for detecting mRNA from *rpoH*, *groEL*, and *tufA* genes. mRNA from all three genes was detected immediately after the cells had been killed by heat or ethanol but gradually disappeared with time when dead cells were held at room temperature. In heat-killed cells, some mRNA targets became undetectable after 2 to 16 h, whereas after ethanol treatment, mRNA was still detected after 16 h. In contrast, 16S rRNA was detected by RT-PCR in all samples containing dead cells and did not disappear during a subsequent incubation of 16 h at room temperature. Of the different types of nucleic acid, mRNA is the most promising candidate for an indicator of viability in bacteria, but its persistence in dead cells depends on the inactivating treatment and subsequent holding conditions.

Appendix IV

J. Parasitol., 88(1), 2002, pp. 153–158

PCR DIAGNOSTICS UNDERESTIMATE THE PREVALENCE OF AVIAN MALARIA (*PLASMODIUM RELICTUM*) IN EXPERIMENTALLY-INFECTED PASSERINES

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ABSTRACT: Several polymerase chain reaction (PCR)-based methods have recently been developed for diagnosing malarial infections in both birds and reptiles, but a critical evaluation of their sensitivity in experimentally-infected hosts has not been done. This study compares the sensitivity of several PCR-based methods for diagnosing avian malaria (*Plasmodium relictum*) in captive Hawaiian honeycreepers using microscopy and a recently developed immunoblotting technique. Sequential blood samples were collected over periods of up to 4.4 yr after experimental infection and rechallenge to determine both the duration and detectability of chronic infections. Two new nested PCR approaches for detecting circulating parasites based on *P. relictum* 18S rRNA genes and the thrombospondin-related anonymous protein (TRAP) gene are described. The blood smear and the PCR tests were less sensitive than serological methods for detecting chronic malarial infections. Individually, none of the diagnostic methods was 100% accurate in detecting subpatent infections, although serological methods were significantly more sensitive (97%) than either nested PCR (61–84%) or microscopy (27%). Circulating parasites in chronically infected birds either disappear completely from circulation or to drop to intensities below detectability by nested PCR. Thus, the use of PCR as a sole means of detection of circulating parasites may significantly underestimate true prevalence.

Appendix V. (from Whipps et al. 2006)

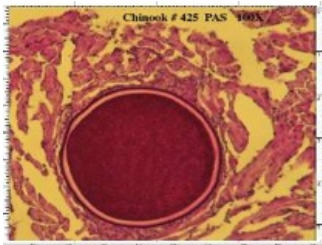
Table 3. Sensitivity and specificity of the *Ichthyophonus hoferi* PCR test on somatic or heart muscle to identify the infection status of fish. The sensitivity of the PCR used on muscle to detect lightly and heavily infected fish was calculated separately. Values are mean % (95% CI)

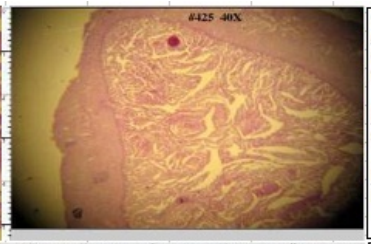
Tissue		Sensitivity			Specificity
		Combined	Light	Heavy	
Somatic					
Tanana	2003	79.4 (62–91)	50.0 (18–83)	88.5 (69–97)	94.0 (82–98)
	2004	50.0 (28–72)	0 (0–44)	76.9 (46–94)	100.0 (95–100)
Emmonak	2004	47.8 (27–69)	25.0 (7–57)	72.7 (39–93)	100.0 (94–100)
Heart					
Tanana	2003	83.3 (67–93)	42.9 (12–80)	93.1 (76–99)	97.9 (88–100)
	2004	78.6 (49–94)	50.0 (14–86)	100.0 (60–100)	100.0 (95–100)
Emmonak	2004	100.0 (80–100)	100.0 (52–100)	100.0 (73–100)	95.8 (87–99)

Sensitivity and specificity. The ability of the PCR test to detect infected and non-infected fish is referred to as the test's sensitivity and specificity, respectively. These were calculated using the following formulas: Sensitivity = (no. of test-positive infected fish)/(no. of truly infected fish); Specificity = (no. of test negative non-infected fish)/(no. of truly non-infected fish).

The 'true' infection status of a fish was determined by examining the results of all other tests performed on the fish with the exception of the PCR test being evaluated. If any of the tissues of a fish was positive by any of the other diagnostic tests used, the fish was categorized as infected with *Ichthyophonus hoferi*. Fish that were negative with all other tests except the one evaluated were considered negative.

Appendix VI (from Rapids Research Center; 2006 data)
http://www.rapidsresearch.com/html/*Ichthyophonus*_disease.html

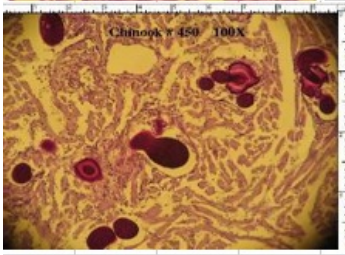


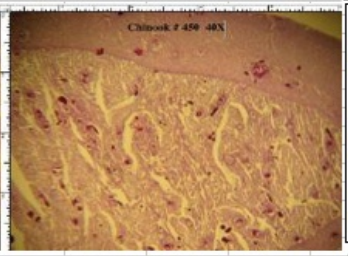


Chinook # 425
At Rapids showed relatively light disease - 15 (approx.) heart spores visable with spleen, liver and flesh all visably negative.

Histology showed lowest level of infection < 1 spore/field

PCR test showed negative for Ichthyophonus infection.

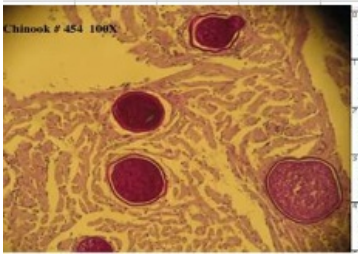


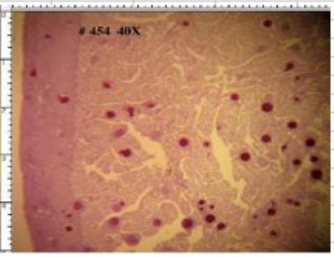


Chinook # 450:
At Rapids showed relatively heavy disease - 200 (approx.) heart spores visable and spleen, liver and flesh all visably diseased.

Histology showed heavy infection showing germination of spores; high level of infection > 10 spores/ field

PCR test showed negative for Ichthyophonus infection.





Chinook # 454:
At Rapids showed relatively heavy disease - 100 (approx.) heart spores visable and liver and flesh visably diseased but no spleen disease present.

Histology showed heavy infection without germination of spores ; high level of infection > 10 spores/ field

PCR test showed negative for Ichthyophonus infection.