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FEATURE ARTICLE

A Perspective: Molecular Detections of New Agents in Finfish—Interpreting Biological Significance for Fish Health Management

Theodore R. Meyers*
Alaska Department of Fish and Game, Commercial Fisheries Division, Post Office Box 115526, Juneau, Alaska 99811, USA

Nora Hickey
Northwest Indian Fisheries Commission, 6730 Martin Way East, Olympia, Washington 98516, USA

Abstract

The increased sensitivity of advanced molecular techniques greatly exceeds the sensitivities of traditional detection methods for infectious agents. This sensitivity causes difficulty in interpreting the biological significance of such detections in fish (and shellfish), especially when the agent(s) cannot be cultured in the laboratory. In the Pacific Northwest, including Canada and Alaska, molecular detections of "new" (unknown or known but discovered in a different geographic location or fish host) potentially infectious agents in fish have received extensive media attention and misinterpretation that call for resource agencies to change current fish health surveillance practices or policies to include these agents. Fish health specialists from several of these agencies and organizations (see Acknowledgments) advise that any policy changes should be made only after further investigations to avoid wasting resources to conduct surveillance for organisms that are not significant to fish health or for noninfectious genetic material that does not represent a viable agent. Molecular detection is not proof of agent viability within or on host tissues and requires further investigation regarding the agent's ability to replicate and evidence that the agent causes substantial risk of disease to exposed fish populations. This document provides examples of molecularly detected agents causing public concern that were accompanied by little or no data to provide context and assessment of biological significance, highlights important questions to be answered regarding these detections, and provides a suggested pathway of investigative criteria to determine viability and pathogenicity of such agents that are necessary for consideration of any changes to aquatic animal health practices and policies.

Research using new nucleic acid-based technologies has advanced our understanding of infection and disease in a wide variety of species ranging from humans to fish. These technologies include the polymerase chain reaction (PCR) and its derivatives (quantitative PCR [qPCR]; high-throughput real-time qPCR) and a variety of DNA and RNA sequencing methods (Mokili et al. 2012), including metagenomic analysis of microbial communities (Filipa-Silva et al. 2020). Investigative surveillance studies, many of which are designed to discover novel infectious agents, use these molecular methods to detect and characterize previously known and unknown genetic sequences of infectious agents in fish, including detection of known protozoan parasites in shellfish (Burreson 2008). Although much of this work has been extremely important, detection by these molecular methods alone does not provide sufficient evidence of biological importance, especially when the agent is new (see Definitions) and causation of significant disease has not been established (Mokili et al. 2012). A major problem for aquatic animal health managers is insufficient scientific evidence of whether such detections merit changes in current management policy. The common public perception, often amplified by the media, is that any detection of a new agent is a threat to the fishery resource. Such threats

*Corresponding author: ted.meyers@alaska.gov
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would then require aggressive management, potentially at the expense of routine fish health surveillance or other efforts having larger benefits for aquatic animals.

We present the following information in our discussion: definitions of terms used in the context of this subject area; referenced examples of various agents detected by molecular methods in which initial reporting caused excessive public concern from misrepresented risk that lacked corroborating information; importance of data to corroborate detection; questions raised by molecular detections of new agents that require more investigation regarding potential risk to fish health; the investigative criteria that would provide science-based management decisions for these agents and the decision-making pathways as illustrated by Figure 1; and final conclusions.

DEFINITIONS IN CONTEXT OF DISCUSSION

Because the definitions of certain terms used throughout the following discussion can be ambiguous, depending on reader background, we define them here before proceeding.

A. *Agent*: Potentially infectious organism or virus.
New agent: Previously undescribed or novel agent, which could include an *emerging* agent that could not be detected by less-sensitive diagnostic methods or that

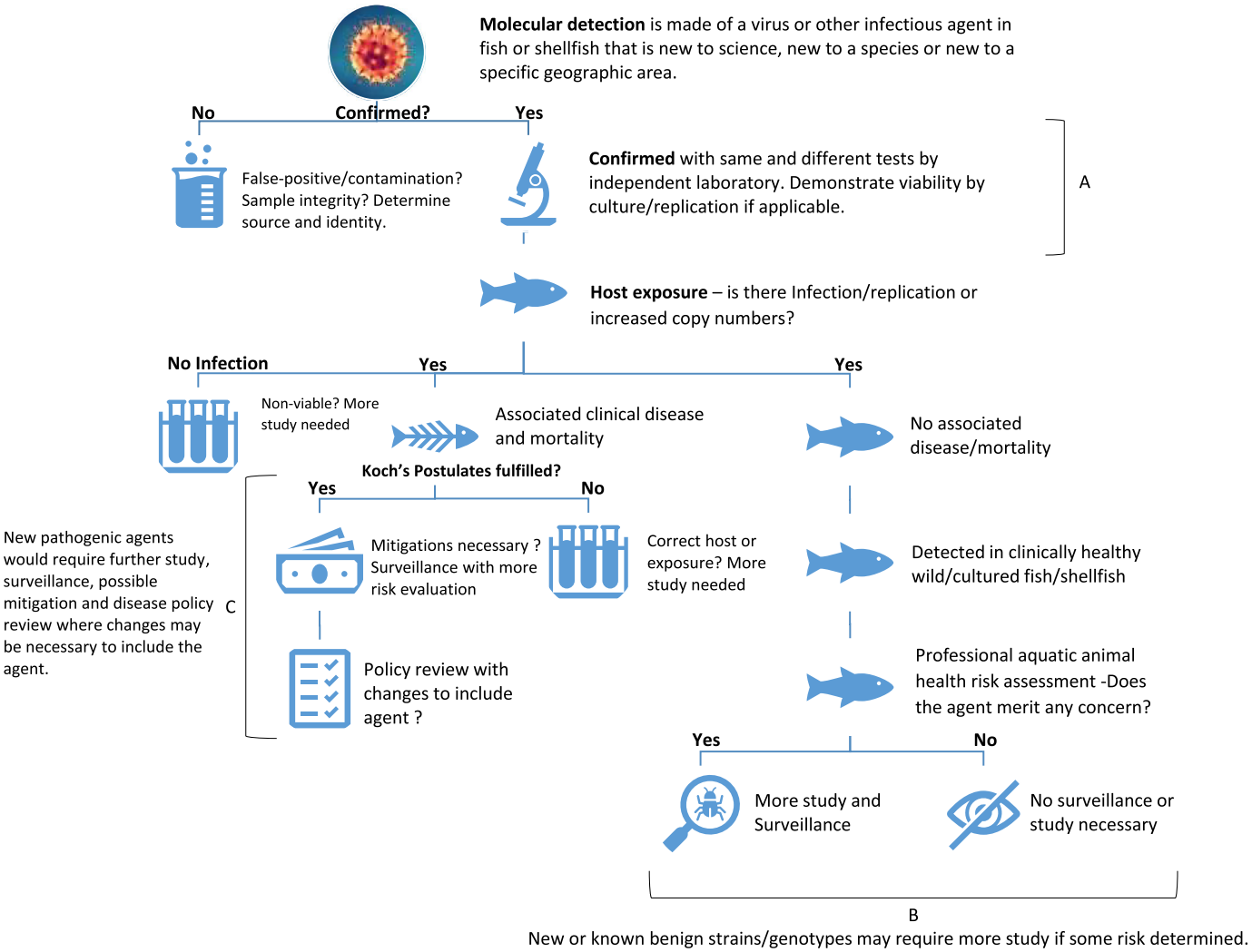

FIGURE 1. Decision pathways **(A–C)** based on results from investigative criteria for determining whether a newly detected infectious agent is pathogenic or benign and warrants specific fish health management: (A) detection of agent is confirmed with same and different tests by an independent laboratory, and viability is demonstrated by culture (if applicable) or replication of copy numbers in the host; (B) detected in clinically healthy wild and cultured fish or shellfish with no associated disease or mortality, while new or known benign strains/genotypes may require more study if some disease concern is determined by health risk assessment; and (C) new agent fulfills Koch's postulates by producing clinical disease and mortality in wild and laboratory-exposed hosts. New pathogenic agents would require further study, surveillance, possible mitigation, and disease policy review, where changes may be necessary to include the agent.

has emigrated to a new area with range extension of the host due to warming seawater temperatures; or an agent that is known but discovered in a different geographic location or fish host, sometimes referred to as an *exotic* agent.

Benign agent: Agent that causes no apparent or known harm to the host; unconfirmed as a pathogen.

Commensal: Symbiotic relationship in which the commensal (agent) gains benefits from the host, which receives neither benefit nor harm.

B. *Harm*: In this discussion, "no harm" indicates no mortality and no other debilitating physiological process that significantly affects fish health or known sustainability of the fish population. Harm could also include negative effects on product trade, product quality, or the physiological health of other aquatic animal species if the agent is capable of infecting other hosts and causing disease.

C. *Surveillance*: Ongoing systematic collection, collation, analysis, and interpretation of animal health data, with the timely dissemination of information to those who need to know so that any management action may be taken. Many surveillance efforts, as mentioned in cited studies, may not satisfy all aspects of this definition.

D. *Validated assay*: Validation is a multi-staged process encompassing assay development, optimization, analytical performance on the bench, and diagnostic performance to ultimately establish the fitness (specificity, sensitivity, repeatability) under the conditions in which the assay will be employed. Few diagnostic tests for aquatic animal pathogens have undergone complete validation as described by the World Organization for Animal Health (OIE 2009) and others (Purcell et al. 2011; Laurin et al. 2018).

E. *Viable agent*: As used in the discussion, viable means infectious and able to replicate within host tissues. This definition includes viruses, which, depending on point of view, are considered nonliving strands of DNA and RNA. However, based on phylogenomic analysis of three-dimensional protein folds among eukaryotic organisms and viruses, investigators have shown evidence that viruses could be considered as a form of life that evolved from multiple ancient cells. These data have allowed construction of a universal tree of life that includes viruses (Nasir and Caetano-Anollés 2015).

EXAMPLES OF NUCLEIC ACID-BASED DETECTIONS OF INFECTIOUS AGENTS CAUSING PUBLIC CONCERN IN THE ABSENCE OF OTHER CORROBORATING EVIDENCE OR SIGNIFICANT DISEASE

Aquatic animal health managers represent a broad group of North American state, federal, provincial, tribal, academic, and private agency aquatic animal health and veterinary professionals using validated scientific methods and best practices to guide management decisions. These decisions become problematic when molecular discoveries of new, potentially infectious agents are publicized without supporting evidence of their effect on fish health or, in the case of known agents, whether their detection can be corroborated. These diagnostic findings generally receive broad media attention whereby this incomplete information is often exaggerated to be of high concern for fish health, resulting in public advocation for resource agencies to include these agents in current fish health surveillance or policies. Absent from the overall discussions have been the necessary investigative criteria and decision pathways (illustrated in Figure 1) for addressing the questions raised by these molecular discoveries that determine whether policy changes should be considered.

Failure to corroborate the molecular discovery of a known disease agent in a new geographic area was exemplified in 2005, when there was an unexpected detection of *Myxobolus cerebralis* (*Mc*) by qPCR in the tissues of clinically normal Rainbow Trout *Oncorhynchus mykiss* from a hatchery in Anchorage, Alaska (Arsan et al. 2007). This salmonid parasite causes whirling disease and has been detected in the USA since 1958 (Hoffman 1990) but not previously in Alaska. This molecular detection occurred despite negative findings from decades of surveillance by the Alaska Department of Fish and Game, with no observance of clinical whirling disease or parasite spores by the U.S. Fish and Wildlife Service (USFWS) after performing standard tissue digest methods on more than 2,000 wild Rainbow Trout from 26 watersheds in the Bristol Bay area during the 1998–2004 National Wild Fish Health Survey (USFWS, unpublished database). The positive results with high cycle threshold (Ct) values (32–39) from the PCR study (Arsan et al. 2007) could not be corroborated by molecular testing at another laboratory or by conventional diagnostic methods. This failure to verify the presence of *Mc* suggested that the original PCR-positive findings were falsely positive or that detectable nucleic acid in the watershed was transient and the parasite never became established. Continued molecular testing of sentinel Rainbow Trout also failed to detect the parasite, but intense public concern resulted in the alteration of the Alaska stocking policy for all fish stocks from that hatchery for several years afterwards. Despite the absence of any corroboration of the initial molecular detection, Alaska has since been considered part of the geographic range of *Mc*, illustrating a common problem with unsubstantiated detections: they remain in the public record as test-positive despite the inability of additional testing to validate the original positive result or show any significant fish health risk. This and other examples discussed are justification for having standardized criteria for interpretation of

molecular detections regarding fish health concerns and to direct the necessary decision path (Figure 1) toward the most logical management for a specific agent.

A second example of uncorroborated molecular detection of a known disease agent that was new to the Pacific Northwest (PNW) region also resulted in unnecessary surveillance efforts by USFWS and state management authorities in Washington and Alaska. These efforts were based on detections of infectious salmon anemia virus (ISAV) by reverse transcription qPCR (RT-qPCR) in healthy salmonids from British Columbia (BC), Canada, in 2011 (Simon Fraser University 2011; Kibenge et al. 2016). Infectious salmon anemia virus is responsible for anemia and other blood disorders causing losses of farmed Atlantic Salmon *Salmo salar* from Norway, Scotland, the Faroe Islands, Chile, Maine, and the east coast of Canada (Amelfot et al. 2015; Nylund et al. 2019). Despite the absence of confirmatory findings to establish ISAV presence in the PNW, intense public pressure resulted in national legislative action for a risk analysis conducted through the National Aquatic Animal Health Task Force. That response directed the aforementioned state and federal authorities to plan and conduct surveillance for the virus (Amos et al. 2014). Contrary to the initial positive results from Canadian researchers (Simon Fraser University 2011; Kibenge et al. 2016), 3.5 years of extensive efforts by several U.S. agency and university laboratories yielded no molecular evidence of ISAV in more than 4,900 samples of wild and cultured Pacific salmon representing five species present in the PNW (Gustafson et al. 2018). A subset of these samples was again tested for ISAV using three additional ISAV real-time RT-PCR assays, including some of the unvalidated assays (see Definitions) reported to have produced positive results in fish from BC. These samples also were test-negative for ISAV (Purcell et al. 2018) and were supported by other evidence, including (1) decades of viral surveillance by U.S. and Canadian agencies with no observance of infectious salmon anemia disease and (2) negative RT-qPCR results from over 8,000 BC salmonids that were tested during 2012–2013 by the Canadian Food Inspection Agency (CFIA 2014). Additional insight for absence of the virus was provided by earlier studies in which Pacific salmon were found to be relatively resistant to ISAV infection (Rolland and Winton 2003). Unfortunately, continued public concern regarding the initial molecular detection caused changes to occur in regional fish disease policies prior to completion of this surveillance effort. This included a proactive ISAV contingency plan (Amos et al. 2014) initiated through the Pacific Northwest Fish Health Protection Committee that developed an agreement regarding how resource agencies would respond to suspected and confirmed ISAV-positive molecular test results. Despite the overwhelmingly positive success of agency cooperation, the amount of agency effort required to plan, coordinate, and report on such a survey cannot be overstated. In the final analysis, there was no past or present credible rationale for resource agencies to routinely conduct active surveillance programs for ISAV in the PNW region of the USA and Canada (CFIA 2014; Gustafson et al. 2018).

Other examples of nucleic acid detections of new and known viruses in healthy Pacific salmon that caused needless public concerns include (1) parvovirus from Fraser River, BC, Sockeye Salmon *Oncorhynchus nerka* (Miller et al. 2011); (2) piscine orthoreovirus (PRV), the agent of heart and skeletal muscle inflammation (HSMI; Palacios et al. 2010; Wessel et al. 2017), in salmon from the PNW (CBC News 2012); and (3) more recently, the molecular detection of a novel arenavirus, reovirus, and nidovirus from juvenile wild and farmed Chinook Salmon *Oncorhynchus tshawytscha* and Sockeye Salmon in BC (Mordecai et al. 2019). As discussed in more detail below, these cases when first reported were accompanied by little or no corroborating evidence that the viruses cause significant disease in Pacific salmon populations.

The genetic detection of a parvovirus sequence in liver, brain, and gill tissues was reportedly associated with a mortality-related gene expression profile or genomic signature (mortality-related signature [MRS]) also sequenced from tissues of tagged fish, potentially causing the collapse of the 2009 Sockeye Salmon returns to the Fraser River (Drews 2011; Miller et al. 2011; K. Miller, unpublished information presented at a Department of Fisheries and Oceans Canada workshop, April 2011). The initial work referred to the parvovirus sequence as endogenous and indicated that it was likely causing significant salmon disease. However, several years later, experimental infection studies failed to show a relationship of the viral sequence to the MRS and the viral genetic material did not replicate or cause Sockeye Salmon mortality (DFO 2018).

The first reported detection of PRV nucleic acid by RT-PCR from PNW salmon in 2012 by investigators caused public concern and suggested that the virus was new to North America (Brend 2016). However, fish pathologists at the BC Ministry of Agriculture had detected the virus in 2010 from farmed salmon, causing no disease (G. Marty, BC Animal Health Centre, unpublished data). Subsequent studies of archived tissue samples provided sequencing evidence (GenBank accession numbers MT506522–MT506523) that PRV-1 was present in BC steelhead (anadromous Rainbow Trout) as early as 1977, prior to establishment of the salmon pen farming industry (Marty et al. 2015; Siah et al. 2020). The necessary information from laboratory host exposure studies regarding pathogenicity differences among PRV strains had not yet been obtained. Subsequent extensive molecular testing completed in connection with the aforementioned ISAV surveillance work established that the virus genotype in the PNW (PRV-1a) was endemic in several wild and hatchery stocks of Pacific salmon (Kibenge et al. 2013; Purcell et al. 2018). Necessary laboratory studies were eventually completed

and indicated that the virus was infectious for Pacific salmon but caused no mortality. Other experimental results suggested that there were cellular pathologies associated with the virus (Di Cicco et al. 2018). However, the overall scientific consensus was that the northeastern Pacific variant of PRV-1a is not a significant disease-causing agent in Pacific salmonids (Garver et al. 2016a, 2016b; Zhang et al. 2019; Purcell et al. 2020; Polinski et al. 2021), contrary to the misinformation claiming that the virus is a threat to wild fish populations and resource sustainability (Noor 2021). The scientific information continues to support the conclusion that endemic PRV-1a in the PNW should be considered as posing a low risk (not zero) to Pacific salmon, thus requiring no significant changes to agency fish health policies (Meyers 2017). However, to be clear, PRV-1 in Norway, where HSMI was first described in 1999 (Kongtorp et al. 2004), diverged into two genetic lineages based on mutations and/or genome segment reassortment (Dhamotharan et al. 2019). One lineage (PRV-1b) is associated with HSMI disease in farmed Atlantic Salmon. The second lineage (PRV-1a) was present in Norway as early as 1988, prior to HSMI disease emergence, and is hypothesized to be a lower virulence subgenotype. The PRV-1 strains from the North American Pacific coast and the Faroe Islands are more closely related to the hypothesized low-virulence PRV-1a subgenotype not associated with clinical HSMI. Out of an abundance of caution, agencies in Washington State and BC have restricted the importation of salmonid eggs from the North Atlantic into the northeast Pacific since the inadvertent transport of the higher virulence subgenotype of PRV from the North Atlantic (Dhamotharan et al. 2019) may present a significant risk to Pacific salmon populations.

The most recent discovery of an arenavirus, reovirus, and nidovirus resulted from metatranscriptomic sequencing in mostly healthy wild and farmed juvenile salmon around Vancouver Island, Canada (Mordecai et al. 2019). The reovirus was detected only from farmed Chinook Salmon, including both live and dead fish, and was classified as belonging to the genus *Aquareovirus*. These are ubiquitous viruses in various freshwater and marine fish species that, depending on the virus strain and host species, range from nonpathogenic to association with variable clinical disease (Kibenge and Godoy 2016). Aquareoviruses currently found in the PNW are not considered to be significant salmonid pathogens (Makhsous et al. 2017) and they require no regional fish health regulation by several state and federal member agencies of the Pacific Northwest Fish Health Protection Committee as reflected in the "Model Comprehensive Fish Health Protection Program" (Pacific Northwest Fish Health Protection Committee, unpublished document, revised in 2007; see https://pnfhpc.wordpress.com/portfolio/publications/). The nidovirus, also found in both farmed and wild Chinook Salmon (although less so in wild fish), was detected only in apparently healthy fish. The arenavirus, found in healthy wild Chinook and Sockeye salmon, was also associated with cellular pathologies in some of the moribund and dead farmed Chinook Salmon, including viral RNA presence in red and white blood cells accompanied by anemia and microscopic lesions in the gills, liver, and kidney (Mordecai et al. 2019). Additional information regarding viral significance to fish health (Figure 1B, C), particularly for the arenavirus, is necessary before there can be meaningful evaluation of these viruses by fish health managers.

IMPORTANCE OF CORROBORATING DATA

The sensitivity and overwhelming scientific importance of molecular detection methods for determining the compositions of complex environmental microbiomes (Mokili et al. 2012) as well as discovering new infectious agents and pathogens in animal populations cannot be overstated. However, many of these assays were designed for research and have not gone through the validation process regarding fitness in sensitivity and specificity. Regarding detection of infectious agents, the necessary follow-up evaluations on the biological importance of the initial test-positive results require significant efforts and time before being made available, and in some cases such evaluations have not been conducted before media reporting and public awareness. Viruses and their sequences can be ubiquitous in animal hosts, including fish (Filipa-Silva et al. 2020), and some may not be fully functional viral genomes. In other cases, the sequences encode viruses that are nonpathogenic or harmless commensals and are benign or sometimes beneficial to healthy hosts (Roossinck 2011). An important consideration is that most nucleic acid-based detection does not indicate agent viability but requires only a piece of the DNA or RNA (Stanley 2003).

These and other important corroborating test data are necessary information for the lay public and the media and for resource managers who must make and defend their decisions based on science rather than uninformed opinion. The sections below provide essential questions to be answered and a suggested framework of criteria for the stepwise investigation of potential new pathogens discovered by molecular methods that would be required to make rational policy decisions.

ESSENTIAL QUESTIONS TO ANSWER

The following questions must be answered to establish whether the molecular discovery of a new virus or other infectious agent poses significant disease risk to fishery resources.

A. *Is there a possibility of false positives and/or contamination? Can results be corroborated?* Because PCR methods and other molecular assays are extremely sensitive, occasional false positive results can be expected from

human error, contamination of samples or reagents within the field or laboratory, and cross-reactions with other substances in the samples. If the tissue tested has an external surface, the test also does not exclude contamination by environmental nucleic acid that is not present within the tissues but originates from the ambient water column or other external source. Contamination of fish tissues can also result from contact with tissue culture fluids or other stabilizers containing animal serum that may result in the detection of mammalian viral nucleic acid in other molecular assays, such as metatranscriptomic sequencing that requires further effort to subtract from the desired target messenger RNA sequences. Polymerase chain reaction assays detecting multiple gene targets can provide greater certainty when interpreting results, but discrepancies in those results can lead to nonspecific detection that can be incorrectly reported as positive (Public Health England 2020). Furthermore, each PCR assay will have a different limit of detection, or the maximum Ct (generally 35–40 cycles) at which the lowest concentration of a given agent can be reliably and consistently detected. Excessive cycling increases the opportunity for nonspecific amplification and errors. Selection of the proper maximum threshold also varies with each assay type regarding methods, reagents, targeted genomes, etc.; therefore, Ct values cannot be directly compared between assays of different types (Public Health England 2020).

All molecular tests used as management surveillance tools should be validated for fitness in specificity and sensitivity, including other proper development, optimization, and standardization for the intended purpose (Burreson 2008; OIE 2009; Purcell et al. 2011). The PCR result by itself is only presumptive evidence that further corroboration is required. Amplicon sequencing, confirmation by another technique (e.g., in situ hybridization), a different PCR method, or detection by an independent laboratory increases confidence in the finding to help rule out the potential for contamination and false positives.

B. *New or previously undetected?* Based on metagenomic analyses of environmental samples, it has been estimated that there are 1.2×10^{30} to 2.5×10^{31} viruses on the planet, with <1% discovered in the field of virology (Mokili et al. 2012). Therefore, PCR detection of an unculturable agent is insufficient evidence that it is "new" or exotic because this does not exclude that the agent may have been present previously before the use of PCR as a surveillance tool. This has been the case for nonpathogenic PRV-1. During decades of viral surveillance of hatchery and wild fish, the virus was never detected by cell culture and clinical HSMI disease was not observed. Polymerase chain reaction testing demonstrated that PRV-1 was widespread in western North America (Purcell et al. 2018). Additionally, PRV-1 RNA was shown to be present in archived fish tissues from the PNW since the late 1970s (Marty et al. 2015; Siah et al. 2020). Piscine orthoreovirus 1 produced inclusion bodies and virus particles that were morphologically similar to those produced by erythrocytic inclusion body syndrome (EIBS) virus, which also could not be isolated in cell culture. First reported in the PNW in the early 1980s, EIBS causes anemia in Coho Salmon *O. kisutch* and other Pacific salmon (Piacentini et al. 1989). Whether these two viruses are the same or otherwise related has not been established, but a second PRV strain (PRV-2), not reported in western North America, has also been associated with EIBS in Japanese farmed Coho Salmon (Takano et al. 2016).

C. *Infectious virus or fragmented endogenous viral sequence?* As previously stated, molecular detection can indicate the presence of foreign agent nucleic acid but generally does not determine infectious viability (Stanley 2003; Burreson 2008; Mokili et al. 2012). This may not be entirely true when genetic materials of a particular agent are detected at a low Ct, indicating high loads. For example, detection of viral RNA in humans from the severe acute respiratory syndrome coronavirus 2 at a low Ct of 25 has been regarded as presenting a high risk of infectivity, with virus viability in cell culture occurring for 70% of the samples (Jaafar et al. 2021). Conversely, detection of viral RNA at a high Ct value presents a lower risk of infectivity, where a Ct of 35 produced less than 3% of the samples with replicating virus in cell culture. This is dependent on whether the sample was taken during early versus late infections; early infection will result in more virus replication, while late infection generally results in more nonviable RNA (Jaafar et al. 2021).

The use of "viability PCR" is another technique in which the photoreactive dye propidium monoazide is added to the sample, followed by exposure to intense visible light that binds with and insolubilizes DNA or RNA free in the sample or present in the dead target organisms or noninfectious virus. The nucleic acid of viable agents is protected by intact cell membranes (cellular organisms) or undamaged capsid proteins (viruses) and is the only nucleic acid available for extraction and detection by PCR (Karim et al. 2015).

Polymerase chain reaction and sequencing detections could also represent endogenous viral nucleic acid, particularly for suspected retroviruses and other RNA viruses that could include bornavirus and filovirus in mammals and bunyavirus, orthomyxovirus, reovirus, rhabdovirus, and flavivirus in insects (Gilbert and Feschotte 2010; Holmes

2011). Suspect DNA viruses could also include double-stranded hepadnavirus in birds and single-stranded DNA viruses, such as circoviruses and parvoviruses, in mammals (Gilbert and Feschotte 2010; Holmes 2011). When viral RNA or DNA is integrated into the germline DNA of animal host cells, it is passed on to future generations in Mendelian fashion as an endogenous provirus or as other genetic material (Holmes 2011). Excluding the retroviruses, these endogenous viral sequences have no role in viral replication. They are highly mutated and typically comprise only fragments of the viral genome; therefore, they cannot give rise to infectious virus but can play an active role in the evolution of host genomes (Holmes 2011). About 5–8% of the human genome consists of mobile genetic elements, which include endogenous proviruses and other fragmented sequences (Katzourakis and Tristem 2005). Fish represent 50% of the total vertebrate species on earth and, therefore, it is likely that they would host an enormous diversity of yet-undiscovered RNA viruses (Zhang et al. 2018). This would include endogenous retroviruses that have already been reported in fish (Basta et al. 2009) but also likely includes these other endogenous, noninfectious viral sequences, which are abundant in healthy mammals, birds, and insects (Holmes 2011).

D. *Pathogenic or benign?* Additional evidence that determines whether an infectious agent is of concern would require laboratory culture (if culturable), detection by positive immunohistochemically stained tissue sections (i.e., viral protein production), and/or clear evidence of clinical disease in animals from which the positive samples were taken (OIE 2019). Fulfilling Rivers' postulates (Rivers 1937) and Koch's postulates with in vivo virulence assessment modified to accommodate nucleic acid-based detection methods (Fredericks and Relman 1996) is required to ascertain whether any newly discovered agent can infect and cause disease in the original host species as well as other hosts (see Investigative Criteria C and Figure 1C).
E. *Are there different strains or genotypes?* As with PRV and ISAV, comparison with known genotypes may also determine whether the virus could be pathogenic or benign and whether it is indigenous or newly introduced. If benign, there may be no need for further study and surveillance would depend on the interests of individual resource agencies (Figure 1A, B). Therefore, each genotype should be treated as potentially a different virus that may differ in host pathogenicity. Strain differences may apply to other nonviral agents as well.

These questions resulting from molecular detections of new and/or emerging infectious agents in aquatic animals that are lacking additional evidence of biological significance illustrate the need to educate the media, the public, and possibly nonfish health resource managers about the difference between simply discovering the presence of agent sequences using molecular techniques versus demonstrating whether those sequences represent an infectious agent that is capable of physiological harm. Answering these questions forms a rational basis on which to initiate changes in surveillance practices or regulatory actions. Furthermore, the best scientific evidence is never absolute. Media critics often demand that regulatory agencies provide "proof of the negative," such that a virus or other infectious agent poses zero risk. This demanded reassurance is not possible in an uncontrolled natural environment with a diverse assemblage of potential fish hosts.

INVESTIGATIVE CRITERIA AND DECISION PATHWAY FOR DETERMINING PATHOGENICITY AND WHETHER MANAGEMENT IS WARRANTED

The following criteria (A–C) provide decision pathways (Figure 1) for establishing corroborating facts to evaluate whether the discovered molecular presence of an infectious agent is a risk for causing disease in a fish resource; the criteria would also apply to shellfish or possibly to other animal populations. These are the minimum data required before changes could be considered in aquatic animal disease management strategies to reduce potential health threats of newly discovered viruses or other infectious agents detected by molecular methods or any other methods of primary surveillance. However, even for cases in which fish are clinically normal or there is low evidence of disease, a health risk assessment (Arthur et al. 2009) by multi-agency regional fish health professionals should be part of the decision process to ultimately determine whether there is any perceived aquatic animal health concern. Depending on the agent discovered, an important obstacle may be that these investigations will require the passage of time to develop reagents and assays or to obtain results on genetic sequencing and in vivo infection studies, which will prolong the decision-making risk assessment. During the waiting period, which may take weeks, months, or longer, resource agencies will have to respond to the public sector with as much professional information on the issue as is known to help dispel any misinformation from the less-informed sources. Interim management of such an issue would have to be determined on a case-by-case basis, with any emergency action being highly dependent on whether the agent fits the general criteria in C-1 (described below) as associated with significant clinical disease and fish mortality.

A. Criteria to confirm the existence of undescribed, emerging, or exotic viruses and other infectious agents

detected by molecular surveillance methods from fish and shellfish (Figure 1A–C).

1. Molecular detection should use a validated diagnostic test (Burreson 2008; Purcell et al. 2011; Laurin et al. 2018). Some flexibility is acknowledged regarding whether validated tests would be available for undescribed novel agents. Test results should be confirmed by an independent laboratory using the same molecular test and different tests, if available. The same unprocessed or replicate tissue samples should be used, if still available, followed by sequencing to further identify and confirm the agent.
2. Potential for contaminating environmental nucleic acid should be ruled out by reviewing the integrity of the sample collection and type of tissue (external tissues would be suspect); examining the possibility of contamination from external sources, including the laboratory, and whether the same test results are confirmed by another laboratory as above; and corroborative testing of a new tissue sample (or different internal tissue) meticulously collected to exclude contamination from the same affected animal population.
3. Efforts should be made to culture the agent using appropriate media to demonstrate viability and allow further characterization of the agent. In the case of viruses, several cell lines and/or primary cell cultures should be used if no suitable cell lines are available.
4. Whether cultured or not, replication of the agent in the host animal should be confirmed by in vivo laboratory infection studies demonstrating increased titers or copy numbers through qPCR or histological immunoassay methods. Failure to replicate in the host animal would indicate that more investigation is necessary to determine the identity of the detected molecular product. We acknowledge that laboratory infection studies can be very complex regarding the necessary environment and route of exposure to allow agent replication and disease manifestation in the host, which may require repeated attempts to reproduce the necessary experimental requirements. Infection studies also may not be possible in some cases where the necessary host or infectious life stages of the agent are not available.
5. A case definition for the infectious agent should be developed. A case definition establishes specific details for a particular agent, such as (1) nucleic acid sequence and identification of the infectious agent; (2) identification of clinical signs of infection if there are any; (3) temporal characteristics of the infection; and (4) host geographic range of the infection. Some of these details may not be known to develop a comprehensive case definition for undescribed novel agents.

B. Criteria to establish that agents new to fish and shellfish are of low concern and do not require changes in current aquatic animal health management policies (Figure 1B). Subjects of concern may include harm to trade, product quality, salmonid health, or the health of other aquatic animal species if the agent is capable of infecting multiple hosts and causing disease.

1. The agent is not associated with clinical disease or mortality that would be a perceived threat to the sustainability of wild or cultured aquatic animal populations.
2. The agent can be detected in asymptomatic wild and/or cultured aquatic animals.
3. Previous years of nonmolecular surveillance methods of the same aquatic animal stocks have shown no evidence of an idiopathic disease that could be postulated as associated with the agent.

In this case, low risk could be further assessed by aquatic health professionals for wild and/or cultured fish and shellfish based on potential pathogenicity and epidemiology of the specific agent detected. Future surveillance and changes in management policy would depend on whether the aquatic health risk assessment is still low or whether there are circumstances to justify the elevation of risk to high based on prior case definition of the agent detected. If the risk is low, then surveillance would be based on budget and interest to determine host prevalence.

C. Criteria to establish that new fish or shellfish disease agents are of high concern and may require surveillance and/or changes in current aquatic animal health management policies (Figure 1C).

1. The agent is consistently associated with significant clinical disease and aquatic animal mortality that could be a perceived threat to the sustainability of wild or cultured populations.
2. The clinical disease and mortality can be reproducible by experiments in the laboratory with adequate controls that demonstrate that the agent is infectious. This might include animal exposures by cohabitation, immersion, or diet; the same agent can be cultured or detected from infected tissues of the exposed animals; in the case of cohabitation, the exposed animals should have the same disease as the donor animals, with similar high titers or copy numbers corresponding with the severity of disease and occurring in the tissues showing the pathology

as similarly detected in the donor animals; and the agent is not detected from any healthy negative control animals or is detected only at very low copy numbers in those animals (Fredericks and Relman 1996). If the agent is injected by syringe to produce disease rather than more natural routes of transmission, the clinical significance would require further evaluation regarding whether the agent presents a risk of natural infection. Failure to fulfill modified Koch's postulates (Fredericks and Relman 1996) would indicate that the causative agent of the disease has not been identified and will require more investigation.

Short-term mitigating actions determined on a case-by-case basis may be necessary for affected wild or cultured stocks. Such actions could include government financial compensation; destruction of affected cultured stocks and hatchery disinfection; full or partial quarantine on the transport of cultured stocks; or possibly closure of a fishery for a wild stock to reduce aquatic animal losses to the population. Additionally, review of existing surveillance and fish/shellfish health management policies should be done for possible inclusion of a pathogenic agent in long-term aquatic animal health management.

CONCLUSION

Advances in molecular detection methods for infectious agents have been essential to numerous important contributions to the science of aquatic animal health and will continue as such in the future. However, discoveries that are not accompanied by critical information demonstrating viability within the host and the ability to cause disease are only the first steps in the process of data gathering (Middleton et al. 2021). The detection of viral sequences in the tissues of clinically normal fish by itself is insufficient information on which to expend additional effort and resources in making changes to existing surveillance and disease policies of affected resource agencies or other user groups. Regulatory agencies constantly evaluate emerging science to determine whether action is necessary to manage fish health. Additional corroborating test data and overall assessment by fish health professionals are important to provide the necessary facts for the lay public, the media, and, finally, the resource managers, who must make and defend their decisions based on science alone. This document provides a suggested framework for the stepwise investigative criteria required to make those evaluations and decisions.

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ORCID

Theodore R. Meyers https://orcid.org/0000-0003-0782-1912

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