Bacterial Diseases In Coldwater Aquaculture: Tracking Pathogens

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ABSTRACT

With intensified salmonid aquaculture in fresh and salt water, diagnosis and treatment of microbial diseases became a priority for economic and fish health protection reasons. Fish health management decisions need to consider the detection method used as well as the nature of the pathogen and its routes of transmission in assessing the risk of disease. Molecular diagnostic procedures are increasingly used to detect bacterial pathogens in advance of stress-related disease outbreaks. Methods such as PCR can substantially alter the level at which a pathogen is detected in fish populations. Epidemiological fingerprinting methods have been used to imply sources of infections and routes of transmission of bacterial pathogens in aquaculture and in the environment. However, interpreting these results requires knowledge of the specific pathogen and consideration of factors such as vaccination, which can alter pathogen ecology. Diversification of aquaculture species presents new potential opportunities for bacterial pathogens - and new fish health concerns.

Introduction

Diseases in aquaculture are multifactorial. The presence of a pathogen alone is not sufficient to bring about a disease outbreak. Sniezko [17] illustrated the interactions between pathogen, host and environmental factors as circles, with disease occurring in the area of overlap where a pathogen encounters predisposing host and environmental conditions. Aquaculture intensifies many host and environmental stresses. The influence of such factors as rearing densities, water quality, fish handling and nutrition in the disease process has received considerable study. However, the ability to detect pathogenic organisms in carrier fish or in other reservoirs in the aquatic environment remains an important component of fish health management. Recent technique advancements, reviewed by Leong [9], allow pathogens to be detected at low thresholds and permit a greater diversity of environmental sources to be examined. Changing methods can alter our views about the prevalence, ecology and epidemiology of bacterial pathogens of salmonid fish, and this is the subject considered here.

Culture-based monitoring

Routine health screening of fish populations indicates the incidence and level of pathogens in apparently healthy populations. The agent of the disease is detected, generally in the absence of evident pathological signs in the animals. The results of laboratory testing then need to be interpreted in light of what might be considered a “normal load” - if any - of the

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organism in fish, and whether all strains of the bacterium are viewed as being virulent. Knowing just what the test has detected is a significant part of this evaluation.

Screening programs for bacterial pathogens of salmonid fish have emphasized culture-based methods, which have the advantage of detecting live - and presumably infective - pathogens, which are then available for further study. The corollary is that screening programs have focused on detecting rapidly growing pathogens with relatively simple nutrient requirements, such as *Aeromonas salmonicida* (furunculosis), *Yersinia ruckeri* (enteric redmouth disease) and *Vibrio anguillarum* (vibriosis). Kidney tissue samples streaked onto plates of a medium such as trypticase soy agar is the basis for testing in procedures outlined by the Canadian Fish Health Protection Regulations or the American Fisheries Society Fish Health "Blue Book" [18].

The success of detecting target organisms by culture procedures can be altered by modifications of the basic technique. For example, recognition of colonies of *A. salmonicida* is enhanced when tyrosine or Coomassie Brilliant Blue are added to the plating agar, while addition of serum increases isolations of atypical forms of *A. salmonicida*. Enriching kidney tissue samples by incubation in broth for two days prior to streaking plates or testing additional organs can substantially increase the number of carrier fish detected in an infected population [4, 3]. Sampling gills and surface mucus enhances detection of *A. salmonicida* in carrier fish, as does prior stressing of the fish to be tested with increased water temperature or hormone injections [3].

Alternate test procedures

Some bacterial pathogens are not so readily cultured, yet the diseases they produce make them important monitoring targets. Culture of the bacterial kidney disease organism, *Renibacterium salmoninarum*, requires specialized media and may take 12 weeks or more for colonies to be evident [7]. *Piscirickettsia salmonis*, a recently-described pathogen of salmonids in Chile, must be grown in fish cell lines [6]. For such bacteria, alternate immunological and genetic methods are essential for monitoring purposes. Such methods are useful and powerful, but they detect particular antigenic structures or nucleic acid sequences, and only by implication a living pathogen.

Detection of *R. salmoninarum* illustrates how changing methods can affect ideas about the prevalence and ecology of the pathogen. Microscopic examination of Gram-stained kidney smears was the standard screening test for the kidney disease bacterium and a substantial level of infection was needed before the small cells were evident among the melanin granules. Indirect and direct fluorescent antibody staining techniques (IFAT and DFAT) made microscopic screening significantly more convenient. It also caused consternation, because the lower threshold level meant many more fish had to be considered carriers of the organism than previously recognized. The eventual result was a reconsideration of *R. salmoninarum* as a notifiable disease agent in fish health reporting. A wide range of immunological techniques have been used to screen for the bacterial kidney disease organism and the levels of detection feasible with them have been compared [16, 15].

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is increasingly used to detect fish pathogens. For *R. salmoninarum*, Brown et al. [2] used primers from the gene for the major surface antigen that is the target of IFAT and other immunological tests. In west coast chinook and coho

salmon populations, high levels of carrier fish are indicated by immunological tests. PCR results suggest that 100% of the fish in some samples were infected [15]. Great Lakes coho and chinook salmon populations were introduced 30 years ago from Pacific stocks. Routine IFAT testing of kidney tissue has detected apparent carrier fish, but few and at low levels of infection. Would PCR reveal a high incidence of the BKD agent in these stocks? In 1997, three groups of spawning salmon were tested by PCR, and the results compared with the routine IFAT result for each fish (Senson P, Lord S, Stevenson RMW, 1998, Abstracts, 48th Annual Meeting, Canadian Society of Microbiology). Sixty chinook salmon were IFAT-negative, but 23% were PCR-positive. Of 229 IFAT-negative coho salmon, 4.8% were PCR-positive. Eighty coho salmon from another location were 12.5% positive by IFAT but 45% positive by PCR. Thus, PCR does increase the number of fish considered to be carriers of *R. salmoninarum,* but does provide enough discrimination to be useful for monitoring. The PCR reaction was also able to *exclude* two IFAT-positive fish as carriers; these were found to have high levels of a cross-reactive organism that was not *R. salmoninarum* (Senson et al., unpublished results).

Validation of test methods

The example given above is one of several attempts to validate a PCR test procedure for bacterial kidney disease by comparing it with the currently used procedure, in the context of a monitoring program [11, 12, 15]. Hiney [8] discusses the issues involved in validating new tests, including the concerns when such techniques are being used to assess presence of pathogens in environmental samples, such as waters and sediments. Information about the detection capability of the method(s) used is essential in designing sampling programs and determining sample sizes that will give statistically reliable measures of fish health.

Health status of wild fish populations

Feral fish populations are most often tested for pathogens in conjunction with spawning and egg-collection programs, as for the PCR studies described above. Stresses associated with spawning may lower immune responses, making pathogens easier to detect. However, this may give a false idea about levels of disease in wild populations at other times. Limited access to wild populations, as well as the expense of the exercise, means that evaluations of pathogens present in wild fish are uncommon. Rarely do assessments include non-cultured species of fish that may be co-habitants or prey for wild salmonids. Baitfish, acquired from natural waters and held for sale, are known to be a potential source of salmonid pathogens [14], but whether these species are infected in the wild, and the extent to which they may transmit diseases to and from aquaculture sites is not known. The expansion of aquaculture to include additional coldwater species, freshwater and marine, makes it necessary to consider not only how susceptible those fish may be to salmonid pathogens, but also whether they act as a reservoir of pathogens, known or unknown.

Applications of molecular methods

PCR-based detection techniques are particularly useful when applied to samples that cannot be cultured because of the predominance of other organisms, or in which pathogen numbers are close to the detection limit for immunological tests. For example, frozen samples of rainbow trout skin, showing lesions characteristic of “spawning rash” were examined with the bacterial kidney disease PCR primers, confirming an association

suggested by IFAT staining. Application of gene probes and PCR-based methods have also allowed a better picture of possible infection sources and reservoirs in the aquatic environment. For example, survival of *A. salmonicida* in water, sediment, in association with protozoa, or as an unculturable form has been difficult to test by culture-based methods [5]. The use of probes and PCR-based methods makes ecological studies feasible, but the caveat is that non-virulent strains of pathogenic species may also be recognized with the selected probes. In interpreting such studies, the natural diversity within bacterial species needs to be appreciated. This diversity is also the basis of "finger-printing" methods for bacterial isolates, used to track sources of disease outbreaks.

Epidemiological studies of pathogens

Classically, bacteria may be "typed" (i.e. differentiated into infra-subspecific divisions) by such techniques as serology, phage-sensitivity, isoenzyme patterns, plasmid profiles, and antibiotic-resistance patterns. More recently, restriction fragment length polymorphisms, ribotyping, and randomly amplified PCR have provided new ways to differentiate strains. To trace the source of a disease outbreak by fingerprinting procedures, both the technique and its limitations, and the diversity of the pathogen group need to be evaluated. For example, pigmented isolates of *A. salmonicida* are highly uniform by such genetic criteria as restriction enzyme fragment patterns [10], thus pattern differences are substantially more important than similarities in assessing possible transmission history. To test the value of a fingerprinting technique in tracking infections, the procedures need to be evaluated on well-defined isolates of a defined transmission situation, against a sufficient background of other isolates.

Ribotyping was used to analyze a recurring problem with *Y. ruckeri,* which was isolated in 1994 from an Ontario stock of brook trout with increased mortalities (Emch J, Stevenson R 1996, abstract, 21st Annual Eastern Fish Health Workshop). The fish health management response was to destroy the affected fish and disinfect the facility. However, in both 1995 and 1996, routine monitoring again detected serovar 2 *Y. ruckeri* in brook trout stocks at the facility. To assess whether this was a new or continuing outbreak, the ribotypes of these isolates were examined, along with other serovar 2 strains. Hin*dIII* digestion of DNA showed 8 ribotype patterns, of 9-11 bands each, among tested strains. Ten isolates from the outbreak in 1994 and four isolates from 1995 had the same pattern, shared with only two other strains tested, both from Ontario. Two 1995 isolates from brook trout facility and the two 1996 isolates had a different *HindIII* pattern, in which three of the bands had changed. This pattern was shared with 13 of 17 serovar 2 strains from other geographic sources, but only 4 of those also had a Bgl*II* - generated pattern characteristic of all Ontario isolates. The ribotype patterns of these strains is consistent with the main group 1995 isolates being a re-emergence of the previous strain *Y. ruckeri.* The new 1995/96 pattern may represent either a change in the existing strain or a different source, but more information about the rate and causes of pattern changes is needed for interpretation. In this kind of epidemiological study, choice of the restriction enzymes used to cut the bacterial DNA is empirical, based on obtaining readable patterns and the enzyme cost. Analyzing with more than one enzyme adds to the discrimination power but increases the complexity of the data.

Application of fingerprinting methods does demonstrate that we can count on pathogens to keep adapting. Vibriosis vaccination programs in the Bay of Fundy were successful in reducing losses in Atlantic salmon culture. However, the pathogen was also successful, as

the predominant serotype infecting fish changed. Vibriosis vaccines included two O-antigens; *Vibrio anguillarum* O:1 and *Vibrio ordalii* O:2. Strains producing disease in vaccinated fish were *V. anguillarum* O:2, and although the major antigen was similar to that of *V. ordalii*, it was sufficiently different to allow the fish immune defenses to be breached [1, 13]. Change in the nature of a pathogen may result by selection acting on the existing pathogen population, or by one of the wide range of vibrio serological varieties in the marine environment replacing the previously predominant type. In summary, microorganisms have been changing, adapting, diversifying, and venturing for 3.5 billion years, and aquaculture is just a new opportunity.

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