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USE OF MOLECULAR TOOLS FOR MOLLUSC DISEASE DIAGNOSIS

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Introduction

Recently, an increasing number of research teams are engaged in developing DNA-based diagnostic techniques for mollusc pathogens. These techniques are slowly moving from development in specialised laboratories for research purposes, to routine application and are expected to find an increasing use in routine disease monitoring programs in mollusc aquaculture and in efforts to prevent the spread of pathogens within and between nations. Considering the probable development and interest in these diagnostic techniques, the potential for wide applications in aquaculture and the inherent problems currently associated with their use, it appears necessary to address these issues. The objectives of the workshop, held during the ninth conference of the European Association of Fish Pathologists (19-24 September 1999), were to :

Evaluate needs for molecular diagnostic techniques for the principal diseases of molluscs.

Identify areas where the introduction and use of molecular and related technologies (DNA-based and immunological) are likely to have a significant impact on improving mollusc disease diagnosis.

Review the status of research towards meeting these needs.

Make recommendations for research programs to be developed to produce, standardise and validate molecular diagnostic tools for major mollusc pathogens.

World mollusc aquaculture and detrimental impact of diseases

Aquaculture of molluscs has been growing at an average annual rate of 11% over the past decade, compared to capture fisheries which showed only 1.6% annual growth. In 1997, global mollusc aquaculture production rose to 8.5 million metric tons. Main productions are oysters (36% of the total mollusc production), clams (23%), scallops (15%), and mussels (13%). Recently, annual growth in production has been rapid ; 16% for clams, 20% for abalones, but only 3% for mussels. This growing rate is to be considered as a key point for zoosanitary risks in molluscan aquaculture. In fact, mollusc farming is traditionally based on wild stocks, but natural populations frequently do not satisfy market demand because of overfishing, low value species or disease outbreaks. An answer to this situation has been the introduction of new stocks or species. Introductions of non-native stocks can, however, result in the simultaneous introduction of new pathogens or strains.

Disease outbreaks are recognised as a significant constraint to aquaculture production and trade, affecting both the economic development and socio-economic revenue of many countries. Mollusc farming in many countries faces serious disease problems resulting in significant production losses. Pathogen transfers via movements of aquatic organisms appear to be an important underlying cause of such epizootics. One of the very few ways to reduce the impact of such pathogens on commercially exploited bivalves is to establish effective programs to prevent the transfer of infected stocks. Consequently, a region or country where molluscs are infected with any of these pathogens should not be allowed to export into another area free of this disease. In addition to the routine surveillance of stocks, country imports and abnormal mortalities in mollusc should be examined for the presence of pathogens.

Diagnosis of mollusc diseases and possible development of molecular tools

The effective control of diseases of bivalve molluscs requires access to diagnostic tests that are rapid, reliable and sensitive. Techniques applicable to molluscan pathogens are limited, and most of the investigations are based on histological and ultrastructural examinations. However, many pathogens are difficult to detect and recognise, particularly at low numbers, using such methods. Recent efforts to overcome these problems have led to the development of immunoassays and nucleic acid based diagnostic methods. These techniques offer the advantages of high sensitivity and high specificity, and possible rapid screening of aquatic organisms for the presence of a pathogen. To date, gene probes used in the diagnosis of diseases of cultured molluscs have been for detection of the most economically important pathogens.

A three tier examination procedure is currently recommended by the Office International des Epizooties, which includes surveillance, presumptive and confirmation methods. Surveillance of mollusc diseases is routinely performed by histology. Histology should be used before and beside any other type of examination because this technique provides a large amount of information. Mortality is often associated with the presence of several pathogens in bivalves or loss of condition following spawning, and this can only be determined by histology. When abnormal mortality outbreaks occur, various presumptive diagnostic methods such as tissue imprints, squashes or smears can be used in addition to histology because of the quick answer provided by these techniques. When a pathogen is encountered in the course of routine surveillance or mortality outbreak studies, electron microscopy and/or molecular probes when available may be necessary for specific identification.

DNA is a useful molecule to target for diagnostic procedures because its sequence does not usually vary with the life stage or developmental phase of the pathogen or with the host or tissue location. Not all regions of the DNA, however, are equally useful as targets for probes and/or PCR primers. Closely related organisms have a high degree of sequence similarity, making the development of specific probes and primers difficult. The development of species specific molecular diagnostic tools will be facilitated as sequences for more genes and pathogens become known. Often, however, when DNA sequences have been determined by different laboratories they do not correspond to the same gene or region of a gene, impeding taxonomic studies and sequence comparisons for development of diagnostics (e.g. *Perkinsus atlanticus, P. olseni and P. marinus*). Efforts should be made to obtain the same regions of sequences from related organisms to optimise the chance of developing probes and PCR primers

with the desired specificity. Furthermore, to minimise the possibility of species-specific molecular diagnostics failing to detect a particular strain of a pathogen, as many strains as possible from a wide geographic range should be sequenced. There is a need for international co-operative programs that aim to assess the taxonomic relationships of major pathogens and to determine their geographic range. Some of these programs were recently initiated (e.g. Marteilia project, Mikrocell project).

Some genes, such as the small-subunit ribosomal RNA gene, have been sequenced for some molluscan parasites (e.g. Haplosporidium nelsoni and H. costale) for phylogenetic studies to help clarify the taxonomy and biology of these pathogens. In addition, the rRNA genes are often useful targets for diagnostic tests because there are many copies in the genome, which can help to ensure good sensitivity. The identification and use of virulence genes as targets of diagnostic tools could allow a more relevant assessment of the risk posed by the organism (e.g. *Perkinsus* spp.). However, the differential expression of virulence genes may need to be considered. The identification of such genes has been undertaken in very few cases; however, it appears as a promising field of investigation.

In situ hybridisation is useful to detect light infections or to confirm the identity of a pathogen in a histological section. Therefore it is important that laboratories with appropriately fixed tissues archive this material in case it is needed in the future. Two other issues addressed by in situ hybridisation are mixed infections and latent infections. Consequently, many laboratories or national/regional reference laboratories could adopt in situ hybridisation in the next few years as a confirmatory technique. However, there is a need for standard and validated protocols. These protocols should include negative and positive controls. The latter would provide valuable information on the quality of the DNA molecule in the sample.

The Polymerase Chain Reaction (PCR) is an emerging molluscan disease diagnostic technique that currently should be used in addition to other techniques. Major benefits of PCR techniques include rapid results, high sensitivity and high specificity. In addition, if the sequence is known, amplification of DNA fragments and subsequent sequence analysis can help to identify an unknown pathogen and even confirm infections in a different host. Problems with PCR are that light infections may be missed if tissue is subsampled or inhibitory factors in mollusc tissues may give false negative results. In addition, the extreme sensitivity of PCR may result in a positive result even when a viable pathogen is not present. More research is needed on PCR results in conjunction with other diagnostic techniques before PCR can be recommended as the technique of choice. Again, positive and negative controls as well as internal controls must be included in the protocols. PCR is a potentially useful technique for screening pooled samples of seeds. Reference laboratories must consider the processing of incoming material, as improperly fixed tissues (e.g. in fixative longer than 24-48 hours) are not suitable if PCR is needed later for pathogen identification.

Conclusion

The further development and use of DNA based diagnostic techniques holds promise for efforts to control the introduction of exotic diseases in other geographic areas. Reliable and rapid techniques are needed by national and regional diagnostics laboratories to screen imported fish and shellfish for important pathogens. Therefore, it is of central importance to develop molecular diagnostic techniques for major mollusc pathogens. On the other hand, the routine use of DNA based diagnostic techniques is hampered by a number of problems, which may result in false positive, or false negative results. Efforts must be made to develop, validate and standardise rapid diagnostic techniques for major mollusc diseases and pathogens.