Genomics and mollusc pathogens: trends and perspective

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Global mollusc production is continuously increasing and reached 12.8 million tonnes in 2004 representing 28.2 percent of the total world production valued at US$ 9.4 billions. However, cultivated molluscs may suffer from severe mortality outbreaks. Among the possible causes is the occurrence of infectious diseases due to a variety of pathogens including protozoan parasites, bacteria and viruses. Preventing and controlling diseases has become a priority for aquaculture sustainability. However, molluscs are unique in terms of health management. Molluscs like other invertebrates, lack a true adaptative immune system. Vaccination can not be used to protect them against pathogens. Moreover, molluscs are usually reared in the open sea which strongly limits the use of drugs. Finally, pathogen transfers via movements of aquatic organisms appear to be a major cause of epizootics. In this context, a better knowledge of pathogen taxonomy and the development of molecular diagnostic tools are needed.

The taxonomic history of various mollusc pathogenic agents has known controversy last decades. However, molecular biology in recent years allowed clarify some points. Molecular detection assays for pathogens infecting molluscs are being developed at an increasingly rate. Molecular techniques are now moving from development in specialised laboratories for research purposes, to routine application and are expected to be increasingly used in pathogen monitoring programs. The DNA based diagnosis tools need however validation, specificity definition and further development.

Key Words: Pathogens, molluscs, genomics, phylogeny, diagnosis

Introduction

The rapid development of mollusc aquaculture last decades demonstrates the potential of this economic sector to meet the challenge of food supply and to generate foreign exchange in association to employment development. However, infectious diseases remain a major concern for mollusc aquaculture and may result in massive losses. Management of infectious diseases appears as a main priority for aquaculture sustainability. Molluscs including bivalves are unique in terms of health management. There is a few of tools to control and reduce the impact of pathogen agents. Molluscs are invertebrates lacking specific immune response. They only develop innate immunity. As molluscs do not possess lymphocytes and do not produce antibodies, vaccination can not be used to protect them against infectious diseases. Moreover, the use of chemotherapy is highly restricted for an activity usually occurring in the open sea. The most relevant ways to fight against infectious diseases in molluscs are (i) the control of animal transfers based on suited diagnosis tools, (ii) the genetic selection in order to obtain more resistant animals, and (iii) the reduction of the impact of pathogens based on a better knowledge of their biology. In all cases, exact identification of pathogens and specific diagnosis tools are needed. Approaches based on molecular biology and biotechnology appears as well suited to obtain such results.

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Molecular taxonomy of mollusc pathogens

The taxonomic history of various pathogenic agents infecting molluscs including viruses, bacteria and protozoan parasite has known controversy last decades. However, molecular biology in recent years al-

lowed clarify some points for some of them.

Protozoan diseases

The detection of dense cytoplasmic structures called haplosporosmes in microcell parasites (i.e. *Boanamia ostreae*, *B. exitiosa*, *Mikrocytos roughleyi*, *M. mackini*) suggested that these parasites belong to the phylum Haplosporidia whose members are defined by their spores.[72] However, spores have never been detected in microcell parasites including *Bonamia ostreae*. In this context, these parasites have previously not been assigned with certainty to any group. Recent molecular phylogenetic analyses [13,16,61,62] allow the inclusion of the genus *Bonamia* in the phylum Haplosporidia. Sequencing of the SSU rRNA and actin genes in *B. ostreae*, *B. exitiosa* and *Mikrocytos roughleyi* demonstrated that species of *Bonamia* constitute a monophyletic clade within the haplosporidian taxa (e.g. not as a basal clade), as sister taxa to *Minchinia* spp. However, *M. mackini* is not related to *Bonamia* and it is not a member of the Haplosporidia.[15,38] As a result, the genus *Mikrocytos* appeared to be a polyphyletic genus. Moreover, *M. roughleyi* was reassigned to the genus *Bonamia*.[56]

The taxonomic history of *Perkinsus marinus* has known a long period of controversy. However, the situation has recently become more settled through DNA sequencing. *Perkinsus marinus* is the aetiological agent of Dermo disease affecting the eastern oyster, *Crasssostrea virginca*, in the USA. The parasite was originally described as a fungus *Dermocystidium marinum*.[50] Recently, molecular characters were used for molecular phylogenetic analyses. The small subunit RNA (SSUrRNA) gene, the actin gene and the tubulin gene from *Perkinsus marinus* were sequenced and compared to the corresponding sequences from other protists for taxonomic purpose. Data supported that *Perkinsus* species were closely related to the dinoflagellate clade.[62,69] Although DNA sequencing has facilitated examination of taxonomic affiliations of *Perkinus* species, the question is always under debate.

There has been considerable confusion on the taxonomic affinities and phylogenetic placement of the parasite, *Marteilia refringens*, the aetiological agent of Abers disease in the European flat oyster**,** *Ostrea edulis*. The parasite had initially been identified as a fungi.[37] The SSUrRNA gene of *Marteilia refringens* was sequenced and results indicated that the parasite is not closely related to any single eukaryotic phylum.[11] More recently, Cavalier-Smith and Chao[16] suggested that *Marteilia refringens* can be considered as a haplosporidian. In this context, sequencing of other genes of phylogentic interest appears necessary to provide helpful information. Moreover, the question of taxonomic relationship and species delineation between the different *Marteilia* species reported in the literature needs further molecular investigations.[45,48,84]

Bacterial diseases

Bacterial diseases have largely been documented at larval stages in different bivalve species.[42,70] Bacteria belonging to the genera *Vibrio*, *Pseudomonas* and *Aeromonas* were reported in association to mortality outbreaks among bivalve larvae.[39,41,81] Bacteria belonging to genera *Vibrio*, *Nocardia* and *Roseovarius* were also reported to induce diseases and mortality outbreaks in adults and juveniles.[13,28,29,36,40] Although in some cases bacteria are easily identified, the exact identification of several marine *Vibrio* species based on biochemical characters remains difficult. Diversity of hosts and environmental conditions may induce result discrepancies. Phenotypic and genotypic diversity may be analysed as a capability of marine *Vibrio* species to adapt to variable environmental conditions. As a result, gene and genome sequencing need to be taken into account for prokaryotic systematic.[73]

Vibrios are ubiquitous in aquatic ecosystems. Although certain *Vibrio* species ameliorate growth rates in molluscs, other *Vibrio* species influence the health of cultured marine organisms and constitute pathogens or potential pathogens. *Vibrio splendidus*-related species were reported in association with mortality outbreaks of molluscs.[36,40,49,77,83] However through epidemiological studies a high genetic diversity was observed in this group suggesting a polyphyletic nature.[46] Diagnostic biochemical characters failed in species discrimination within this group. DNA gyrase subunit B (*gyrB*) gene sequencing was thus used in order to characterise *V. splendidus*-related isolates from oysters.[46] Although several strain clustered together, they could not be assigned to any known *Vibrio* species.[45] In this context, taxonomic analysis of unidentified isolates based on a polyphasic approach including gene sequencing, fluorescent amplified-fragment length polymorphism (FAFLP) fingerprinting, DNA-DNA hybridisation and biochemical tests were successfully applied by several authors to define new species.[30,46,79,80]

Viral diseases

Although mortality outbreaks have been reported among different mollusc species in association with the detection of viruses, little information is available on their exact affiliation and taxonomic position. Such a lack of data could be explained by a certain inadequacy of diagnosis methods used in laboratories involved in mollusc pathology. The basic method for examination of suspect samples is predominantly histopathology. This technique does not allow the direct detection of viruses. Moreover, since invertebrates lack antibodies, the direct detection of viral agents remains the only possible approach. Finally, the lack of marine mollusc cell lines renders impossible the viral diagnosis based on the detection of cytopathogenic effects in cell cultures.

Irido-like viruses have been reported in two oyster species, *Crassostrea angulata* and *C. gigas*, in France and USA.[19,20,21,22,23,29,30] The properties of these viruses characterise them as members of the Iridoviridae family. However, their exact identification based on molecular characterization has not yet been carried out.

Viruses morphologically similar to herpes viruses have also been described in several marine mollusc species. The purification of herpes-like viral particles from *Crassostrea gigas* larvae allowed the extraction of the viral DNA.[43] The genome of the virus was completely sequenced (GenBank number AY509253). The coding potential of the genome sequence was analysed allowing the identification of 132 unique protein-coding open reading frames (ORFs). Although capsids are structurally similar to that of other herpes viruses,[25] amino acid sequence comparisons demonstrate that the virus infecting larval oysters is not closely related to vertebrate herpes viruses. However, a common origin between the virus infecting oysters and vertebrate herpesviruses could be suspected on the basis of the identification of a gene coding the ATPase subunit of the terminase. All herpes viruses express this protein which is involved in packaging DNA into the capsid. Moreover, a protein (ORF30) identified in the genome of the virus infecting oyster larvae has also homologues only in mammalian herpes viruses. These data suggest that herpes viruses of mammals and birds, herpes viruses of fish and amphibians and herpes viruses of invertebrates form three major lineages. The virus infecting oyster larvae was classified as a member of the *Herpesviridae* family under the name ostreid herpesvirus-1 (OsHV-1),[53] and was considered the only member of a new major class of the family *Herpesviridae*.[24,25] OsHV-1 is currently the single representative of what may be a large number of invertebrate herpes viruses. A variant of ostreid herpesvirus-1 (OsHV-1var) was also described in larvae of different bi-

valve species,[3,4] but OsHV-1 and OsHV-1var are considered representatives of a single viral species.

Diagnosis of mollusc pathogens

The effective control of pathogens infecting molluscs requires diagnostic tests that are specific, reliable and sensitive, and that can discriminate between genera and species. For many pathogens of molluscs, available diagnostic techniques have historically been based on histological and ultrastructure examinations. Thus, infectious agents can be diagnosed by applying stained tissue imprints. Histology examination provides also valuable information on the intensity and severity of infection at the individual level, co-infections with different noticeable pathogens as well as potential emerging pathogens and non-infectious conditions. However, the accuracy of diagnosis by means of the 'eye based' methods is highly linked to the experience and the training of the investigator, and the time allocated to the examination. Moreover, pathogens can be difficult to detect and recognise using these techniques, particularly when present in low numbers.

Although histology does not allow identification to the species level for most mollusc pathogens, this technique has extensively been used. In a large number of available reports and publications, pathogen speciation was based on host species and geographic range: ie a parasite presenting features characteristic of the genus *Bonamia* was identified as being *Bonamia ostreae* when detected in flat oysters, *Ostrea edulis,* in Europe. Some data must thus be considered with caution in terms of pathogen identification. In this context, efforts have been made to overcome limitations of microscopy. Molecular detection assays for mollusc pathogens are being developed at an increasingly rate.[66,83] The routine use of molecular based diagnostic tools is however hampered by major concerns. Most of them need formal validation against traditional techniques and testing for their specificity.

The main concern is that molecular tools too often are developed from a few sequences without a good understanding of the overall sequence variability within the species. Taxonomy of mollusc pathogens is still unsettled and the use of small sequences as probes for diagnostic purpose has been very rapid with usually little consideration for the lack of information about their true specificity. Also, DNA probes were most often designed from genes or clusters of genes of phylogenetic significance - such as the SSU rRNA gene for example - which frequently does not reflect the huge diversity in terms of virulence. In other words, not all regions of pathogen DNA are equally useful as targets for molecular detection. Closely related pathogens may present high sequence similarities. The assays often have not been thoroughly tested for inclusivity (detection of all strains of the pathogen) or specificity (cross reaction with any other organism). Finally, molecular tools detect DNA and not necessarily a viable pathogen.

Comparing diagnostic techniques for mollusc pathogen detection

The DNA-based assays need formal validation. They must first be compared to traditional methods. Problems may arise when the new diagnostic test is assumed to be more sensitive and specific than the previous standard. All molecular assays specific for a pathogen should be tested in parallel and validated, and further sensitive diagnostic assays that will clearly discriminate between all "valid" species should be developed.

An in situ hybridisation technique was developed for the detection of *Marteilia refringens*[44] with particular emphasis on confirmation of suspected cases by means of histology. In 2005, Thébault et al published a study[78] focusing on

evaluation of sensitivity and specificity values of in situ hybridisation and histology for the detection of *M. refringens*. They carried out a blind assay of 200 flat oysters (free or not of the pathogen) from three different populations using both techniques. Results were analysed using different methodological approaches. In a first step, histology was considered as the reference method ('gold standard') where sensitivity and specificity were assumed to be unity. The authors used also the maximum likelihood method based on the TAGS V.2.0 program[59] assuming that none of diagnostic techniques was the 'gold standard'. These approaches were completed by a third one using an iterative Markov Chain Monte Carlo (MCMC) technique (Bayesian method). Using this last approach, values of sensitivity and specificity for histology were 0.7 and 0.99, respectively, and 0.9 and 0.99, respectively for in situ hybridisation. This work was the first to provide such information for these diagnostic methods recommended by international standards. Moreover, the authors highlighted that the estimation of sensitivity and specificity for a newly developed diagnostic technique does not require a gold standard.

PCR detection of *Haplosporidium nelsoni* was evaluated using histology as a gold standard reference.[32] This author identified a lack of specificity of the PCR (0.7). However, such an evaluation should be carried out again using other methodological approaches including Bayesian methods in order to either confirm or reject these findings. Recently, a study focusing on detection of *Minchinia* sp. in rock oysters *Saccostrea cuccullata* using DNA probes has been published.[10] The ability of PCR and ISH assays to diagnose infected individuals was compared to histological examination from a sample of 56 oysters. PCR and ISH assays appeared more sensitive with 26 and 29 positive individuals, respectively, versus 14 using histology.

The PCR assay for *Bonamia ostreae* detection[17] has been submitted to several validation tests against histological methods. This assay appears however not species specific and PCR-RFLP analysis must be applied to the amplified products to assess species identity by comparison with the profiles of known species. Marty et al. reported an increased sensitivity of a new real-time PCR as confirmed with histopathology for detection of *Bonamia ostreae* in *Ostrea edulis* cultured in western Canada.[53] Parasite DNA was confirmed in 4 oysters by real-time PCR on paraffin-embedded tissues that was not detected by histopathology.

Quahog Parasite Unknown (QPX) is a protistan parasite that causes disease and mortality in the hard clam Mercenaria mercenaria. PCR primers and DNA nucleotide probes were designed and evaluated for sensitivity and specificity for the QPX organism. A field validation was carried out by Stokes et al. in 2002.76 Two-hundred and twenty-four clams were collected over a 16 month period from a QPX endemic site in Virginia, USA. All individuals were analysed using PCR and histology. The authors demonstrated that the PCR assay was equivalent to histological detection, but only after the initially negative PCR products were reamplified. They pointed out that the failure of PCR to increase detection levels over histology was probably due to the patchy nature of QPX in clam tissues so that the chance of encountering QPX parasites (which are relatively easily detected in histological section due to the intense host response) is about equal in the tissue pieces collected for each assay.

'Candidatus Xenohaliotis califoniensis' is a Rickettsiales-like prokaryote responsible for withering syndrome, a fatal disease of wild and farmed eastern Pacific abalone, *Haliotis* spp. A method of rapidly detecting the pathogen in abalone gastrointestinal tissue has been developed based on the use of Hoechst fluorochrome.[55] Comparison of this method with conventional histological examination was conducted on 109 samples. The fluorochrome method detected 90% of the infections detected by conventional histology with discrepancies

due to false negative diagnosis of low-level infections.

A PCR assay based on the amplification of a part of the rRNA non transcribed spacer (NTS) region of *Perkinsus marinus* has been developed.[52,68] A set of primers for two species specific standard or real-time PCR techniques has also been designed from the internally transcribed spacer sequence (ITS).[6] The NTS PCR assay has been validated against fluid thioglycollate culture.[68] The ITS PCR assay has not been validated against fluid thioglycollate culture. However the ITS primers are recommended over the NTS assay because they are more likely to amplify all *Perkinsus marinus* strains.[6]

PCR, traditional RFTM (Ray's Fluid Thioglycollate Medium), and body burden assays were compared in stout razor clams, *Tagelus plebius*, infected with *Perkinsus chesapeaki*.[12] In two samples, a species specific PCR assay detected no *P. chesapeaki*, whereas the RFTM assay detected 33% and 100%. The body burden assay provided a potential explanation for the discrepancy. In these two samples, the body burden assay estimated average parasite densities of only 0.26 and 0.03 parasites in a ~20 mg piece of tissue – the amount recommended for DNA extration. From this, approximately 50 ng is used as the template in the PCR reaction. In contrast, the amount of tissue used in the RFTM assay is about 200 mg. In another sample with much higher parasite densities, the two methods were comparable. Thus, the small amount of tissue, typically taken from a single tissue type, that is employed in a PCR reaction may limit its usefulness when infections are very light or localized, or both.

Molecular assays have also been developed and compared for Ostreid Herpes virus 1 (OsHV-1) in bivalve samples.[67] The methods include PCR assays and ISH.[5,47] Three techniques (PCR, ISH and immunochemistry) were used to detect OsHV-1 in 30 normal appearing Pacific cupped oysters.[5] Global agreement between techniques was determined using generalized Kappa.[33] PCR appeared as the most sensitive method for detecting OsHV-1 in adults. In another study[7] using PCR and ISH, attempts were made to screen for OsHV-1 and in 200 fixed, paraffin-embedded oyster samples collected and processed in 1994. The results obtained through this molecular screening of OsHV-1 allowed comparison of the sensitivity of both techniques. Also, histological and TEM observations performed in 1994 were correlated with molecular diagnosis of the virus.

Interlaboratory evaluation

Lack of standardisation of tests and test protocols is a major impediment to the effective implementation of DNA-based methods. Standardisation requires international agreement and cooperation in test selection, practitioner training and laboratory accreditation. Improvements in the reproducibility, validity and comparability of data resulting from accreditation may assist in assessing the suitability of DNA-based methods for detection of listed pathogens.

An important factor that needs to be addressed is the reproducibility between laboratories. The assay procedure not only consists of performing the diagnostic assay but also reproducing the same sensitivity, eliminating false interpretation and implementing contamination control procedures. Studies conducted in parallel with the same isolates in several laboratories would be ideal.

Part of the EU funded project (VINO, FAIR-CT98-4334) was the organisation of a workshop in 2000 at the Ifremer laboratory in La Tremblade (Charente Maritime, France) in order to ensure that common protocols were used for OsHV-1 detection. VINO partners conducted trials using both techniques. The work involved with an evaluation of the reproducibility of both molecular detection techniques through an inter-laboratory analysis of a series of archived frozen and fixed samples. The trial involved four

laboratories routinely conducting mollusk pathogen diagnosis, including Ifremer (La Tremblade) which supplied the reference material (a series of previously analyzed reference samples). Supernatants of ground larvae (15) and seed (15) as well as histology slides (30 histological slides) were sent as positive and negative reference material to each participant of the inter-laboratory assay. Among the 18 samples considered as positive (Ifremer La Tremblade), only 14 samples were found to be positive using two primer pairs by each of the other laboratories. *In situ* hybridization analyses conducted on reference material gave contradictory results. These results may be explained in part by the difficulty in reading HIS slides and underline that tests must be performed by trained staff.

A proficiency test for the detection of *Bonamia ostreae* by PCR is organised by the European Reference Laboratory for Mollusc Diseases in 2008. Seventeen laboratories will be involved. The test will include DNA extraction from gill tissues collected from 30 flat oysters, *Ostrea edulis*, fixed in ethanol and PCR analysis of the 30 samples based on a protocol previously published.[17]

What about diagnostic kits?

Diagnostic tools are generally not standardized and differences can exist in reagents quality and preparation, in controls, as well as in the interpretation of results. Obviously, the use of a "standardized" diagnostic tool for routine analysis should allow the implementation of a calibrated and controlled process in laboratories but also in rearing facilities. The development of commercial kits for the detection of mollusc pathogens appears as an interesting avenue to be explored. Such diagnostic tools may allow laboratories involved with disease surveillance and mollusc producers to stand sentinels and become proactive players in the health management of molluscs. However, to date there are no commercial kits available for the detection of any mollusc pathogens.

In mollusc hatcheries different diseases are frequently reported in larvae and spat, causing mortalities. Most of these diseases are not notifiable diseases and therefore not subjected to specific control measures under EU or OIE legislation. However, pathogens associated with these mortalities, mainly viruses and bacteria, generate important economic loss and jeopardize the sustainable development of this important socio-economic activity in coastal regions.

The recent Council Directive 2006/88/EC "on animal health requirements for aquaculture animals and products thereof and on the prevention and control of certain diseases in aquatic animals", underlines the necessity for the development of aquaculture in the Community to increase the awareness and preparedness of the competent authorities and aquaculture production business operators with respect to the prevention, control and eradication of aquatic animal diseases. No doubt, an efficient management of the sanitary status of mollusc production implies a significant involvement of the farmers who -unavoidably- are in the front line in the fight against the diseases and can become key players in the control of the pathogens that threaten their livestock. If the latter should become reality, first the efforts of the farmers should be supported by the Authorities and the National laboratories involved in control of disease, and second the farmers should have validated, accurate, easy to use in the field and affordable screening tools at their disposal, which would allow an efficient monitoring of the sanitary status of their production.

Among microbial agents threatening mollusc hatcheries and nurseries, herpes-like viruses have often been detected during mortalities outbreaks in several locations. Consequently, association of OsHV-1 with larval and spat mortalities has motivated the development of specific and sensitive diagnostic methods. According to the OIE International Aquatic Animal

Health Code, OsHV-1 infection is not a notifiable disease and no diagnostic reference method or gold standard method is in force. No cell cultures from marine molluscs are available for virus detection. Detection of this pathogen is usually performed by PCR, real time PCR or in situ hybridization, which requires specialised and expensive laboratory equipment, highly qualified operators, or involve the use of carcinogenic reagents (PCR) and therefore cannot be performed easily, safely, efficiently and/or without generating elevated costs to the producers.

Recently, the collaborative work between Ifremer (La Tremblade, France) and a biotechnology company (SkuldTech, France) has resulted in the development of a mini-array method for OsHV-1 diagnostic as a kit prototype. The aim of this tool is to allow the rapid, secure, cheap and handy screening of oyster samples and preliminary results have reported sufficient analytical and diagnostic specificity and sensitivity. A large scale validation process (interlaboratory assay) will be necessary before the result could be the first mollusc pathogen detection commercial kit.

Conclusion and perspective

There are a number of mollusc pathogens for which DNA-based test methodologies are published. However, in general, further research is required before standardised and validated DNA-based test protocols can be implemented for disease diagnosis and pathogen detection.

Significant pathogens that require long, complex culture or histology-based confirmatory diagnosis are prime candidates for rapid, pathogen-specific diagnostic methods. This applies predominantly to microbial pathogens, but may be equally appropriate for protozoan parasites which are difficult to distinguish morphologically at the light microscope level or which have a diverse host-range. Rapid, pathogen-specific diagnostics would be particularly appropriate for disease management and control when diseases emerge in new geographic locations or host species. However, in the case of molluscs, histology provides a large amount of information and should be used initially, before and together with any other type of examination. The range of tools available and under development show different advantages and disadvantages for a range of different aquatic animal health applications. No one technique shows a replacement advantage over another, and none appear sufficient to merit "stand-alone" application.

Where DNA-based tests are available and/or suitable, the most significant impediment to effective implementation is the lack of standardised methodologies that are validated for specific applications. There is a need for international agreement on methodologies that have been rigorously evaluated and accredited for specific applications in disease diagnosis and pathogen screening. There is also a need to ensure that tests are performed by trained staff with access to standardised reagents and suitably equipped laboratories.

Communication networks of diagnostic practitioners and internationally recognised experts in aquatic animal health should be established and maintained. Activities of the networks should include development of training programs and cooperative programs for test validation and laboratory accreditation. It appears also necessary to identify regions of pathogen genomes that may prove useful for species differentiation. Nevertheless, molecular sequence data is playing an increasingly important role in the identification of mollusc pathogens and requires adequate DNA sequence data at the targeted loci from the same and related species over a wide geographic area in order to develop reliable, accurate and sensitive molecular diagnostic tools. Further researches are required to resolve which DNA sequence differences are consistently significant in the identification of species and how these differences relate to biological parameters that can be used to describe and differentiate between closely related species.

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