PERSPECTIVE

Perspectives of diagnostic approaches for mollusc diseases

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Abstract

Surveillance of mollusc diseases is routinely performed by histology and PCR. Efforts made in research and development of DNA-based diagnostic methods currently offer a broad panel of probes and tests. These methods have the theoretical advantages of high sensitivity and high specificity and possible rapid screening of molluscs for the presence of a targeted pathogen. However, validation and standardisation of these tests are still needed. In recent years, the European Union Reference Laboratory (EURL) for mollusc diseases has developed and validated a range of duplex Taqman® PCR assays aiming at facilitating the detection of bivalve pathogens notifiable to the EU. These assays offer good performances and allow better monitoring and investigation of the epidemiology of these different pathogens. Complementary to these PCR assays, new diagnostic approaches based on the use of passive sensors, Magnetic Beads (MBs), electrochemical biosensors, MALDI-TOF MS or Next Generation Sequencing (NGS) have been developed and tested notably in the context of the European project H2020 VIVALDI. The interest of these tools to better detect, characterise and monitor known pathogens is presented and discussed. These new developments will require further assessment, standardisation and validation before being available for routine diagnostics. Additionally, they will certainly not replace but rather complement diagnostic tools currently used to ensure alertness to emerging diseases.

Introduction

The shellfish industry is a major contributor to global aquaculture production and has a significant social impact. However, diseases are among the main threats to the sustainability of this industry. Considering that molluscs are usually grown in open farms, treatment and vaccine-like approaches cannot be used and eradication is almost impossible. Consequently, the control of mollusc diseases relies mostly on preventive measures which require reliable diagnostic methods (Arzul, 2018; Carnegie et al., 2016).

Surveillance of mollusc diseases is routinely performed by histology and PCR. When outbreaks of mortality occur, histology is indicated as a front-line method if no specific pathogens are suspected and no presumptive diagnostic methods can be used. When a pathogen is detected and identified using light microscopy, confirmatory methods

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(e.g. sequencing, transmission electron microscopy and/or molecular probes) are usually used to overcome the lack of specificity of histology.

The efforts in research and development of DNA-based diagnostic methods currently offer a broad panel of probes and tests. These methods offer the theoretical advantages of high sensitivity and high specificity and possible rapid screening of molluscs for the presence of a targeted pathogen. However, while moving from development in research laboratories to routine application in disease monitoring programmes, validation and standardisation of these tests are still needed (Carnegie et al., 2016). A combination of diagnostic methods and the selection of appropriate tests urgently calls for the establishment of sensitivity and specificity values.

Complete and transparent reporting of key elements of diagnostic accuracy studies for infectious diseases in aquatic animals benefits end-users of these tests, enabling the rational design of surveillance programs, the assessment of test results from clinical cases and comparisons of diagnostic test performance (Gardner et al., 2016). A checklist that specifies critical information that should be reported for test accuracy studies in aquatic animals, regardless of species, has been established. These Standards for Reporting of Diagnostic Accuracy guidelines for aquatic diseases (STRADAS-aquatic) was developed and refined by an expert group of 14 transdisciplinary scientists based on the STRADAS-paratuberculosis checklist (Gardner et al., 2011).

Since these last years, the European Union Reference Laboratory (EURL) for mollusc diseases has developed and validated a range of duplex Taqman® PCR assays aiming at facilitating the detection of bivalve pathogens notifiable to the EU. These assays include Real-Time PCR for the concurrent detection of *Marteilia refringens* and *Bonamia* sp. (Canier et al., 2020), Real-time PCR for the detection and typing of *M. refringens* as well as the detection of *Bonamia ostreae*/*B. exitiosa* (http://www.eurl-mollusc.eu/SOPshttp://www.eurl-mollusc.eu/SOPs). These assays offer good performances and allow better monitoring and investigation of the epidemiology of these different pathogens.

Complementary to these new PCR assays, new diagnostic approaches have been developed and tested notably in the context of the European project H2020 VIVALDI. These innovating approaches are presented below and include the use of passive sensors to investigate the presence of pathogens in the water, the use of magnetic beads coupled with quantitative Real Time PCR to enable detection of OsHV-1 in seawater, the development of an electrochemical biosensor to perform in-field analysis, the development of MALDI-TOF MS database for fast identification of *Vibrio* spp. and the use of a metabarcoding approach to evaluate oyster or mussel pathobiota. Although some of these approaches are currently better suited for research purposes, they present a great potential for the diagnosis of mollusc diseases.

Passive samplers, a powerful tool to detect viruses and bacteria in marine coastal areas

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Monitoring the microbial diversity in the coastal marine environment has become essential to evaluate the impact of global change on human,

animal and ecosystem health. In order to facilitate the detection of viruses and bacteria in the marine environment, a passive sampling system based on the adsorption capacities of different membranes has been developed and tested in laboratory facilities (Vincent-Hubert et al., 2017). Subsequently, this new passive sampling methodology has been tested in the field to detect *in situ* the presence of microorganisms with the objectives to select a type of membrane and investigate whether the amount of nucleic acids increases with the time of exposure.

Over one year, different membranes, nylon, zetapor, and low density polyethylene (LDPE), were displayed for 48 h and two weeks in an estuarine environment downstream from a treatment plant. DNA and RNA suspensions extracted from the membranes were tested by Real Time quantitative RT-PCR and PCR for the detection of the following micro-organisms: the human pathogenic virus norovirus (NoV), the potentially human pathogenic bacteria *Vibrio* spp., *V. alginolyticus*, *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*, the Ostreid herpes-virus type 1 (OsHV-1) pathogenic for oysters, and tracers of microbial and bacteroidal sources targeting faecal contamination.

Whatever the type of membrane and the immersion time, *Vibrio* spp. and tracers of microbial sources were detected and quantified throughout the year, NoV GII was detected and quantified in winter and spring, when gastroenteritis is known to be at a maximum in the human population. In contrast, NoV GI, *V. alginolyticus* and OsHV-1 have rarely been detected. The detection of OsHV-1 coincided with the occurrence of oyster mortality events. Passive nylon-based sensors allowed a better and concomitant detection of NoV GII, *Vibrio* spp. and tracers from microbial sources. Passive sampling coupled with Real Time PCR is a powerful new method for the detection of natural and anthropic viruses and bacteria in seawater. It not only offers interesting perspectives to analyse the diversity of microorganisms and search for emerging pathogens, but could also be used as an early warning system for the prevention of contaminations in oyster farming areas. However, validation and standardisation efforts would be needed prior to the application of passive sampling for the diagnostic and surveillance of these microorganisms.

Using magnetic beads coupled with qPCR and an electrochemical biosensor for the detection of ostreid herpesvirus 1

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Ostreid herpesvirus 1 (OsHV-1) represents one of the major threats to shellfish aquaculture worldwide, particularly to the production of Pacific oysters (*Crassostrea gigas*). Up to now, surveillance of OsHV-1 has relied on the detection of the virus in oysters through histology, *in-situ* hybridisation, PCR and quantitative PCR (qPCR). As a consequence, new tools able to detect the virus in seawater as well as to perform analysis in the field are of great importance to better anticipate the presence of OsHV-1 and ultimately mitigate the effects of the disease.

A novel approach has been developed to capture and detect OsHV-1. The strategy is based on the use of magnetic beads (MBs) coated with

an anionic polymer (which are able to capture virus in general) followed by a qPCR assay specially designed to detect OsHV-1 (Toldrà et al., 2018). MBs have been successfully used to detect OsHV-1 in seawater and oyster homogenates. Additionally, the infective capacity of the captured virus has been demonstrated through experimental infections of naïve oysters and subsequent mortality monitoring. The use of MBs in combination with qPCR has also been shown to pre-concentrate the virus at least 100 times when compared with qPCR alone. In this regard, the application of this new approach in a depuration experiment has shown its ability to pre-concentrate OsHV-1 from seawater, in some cases before oysters get infected. These results open new perspectives to use MBs as early warning tools for OsHV-1 detection.

An electrochemical biosensor based on recombinase polymerase amplification (RPA) has been developed for the detection of OsHV-1 (Toldrà et al., 2020). The combination of an electrochemical readout with an isothermal DNA amplification method is interesting due to the benefits of reducing instrumental requirements and power consumption, thus being more suitable for in situ testing. The strategy consists of a sandwich hybridisation assay with an immobilised capture probe on a miniaturised gold electrode and an enzyme-labelled reporter probe. Apart from the development of the biosensor strategy, efforts have also been focused on demonstrating their applicability in real-life settings. The biosensor has been used to detect OsHV-1 in oyster samples, showing a strong correlation with qPCR and thus demonstrating its reliability. The platform offers great potential to be easily integrated into microfluidic systems to develop compact devices that could be used in the field by end-users and improve decision-making. Both approaches present interesting perspectives for the detection of OsHV-1 and should allow improving the diagnosis of the virus either to investigate mortality outbreaks or establish the status of oyster populations regarding the virus.

Validation of a MALDI-TOF MS database for the fast identification of *Vibrio* spp. potentially pathogenic for marine molluscs.

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In mollusc aquaculture, a large number of *Vibrio* species are associated with high losses in hatcheries-nurseries and in the field. Thus, the development of techniques allowing a rapid detection and accurate identification of *Vibrio* is needed for the surveillance of mollusc bacterial diseases.

Phenotypic, biochemical and molecular techniques based on DNA amplification and sequencing are widely used for the identification of *Vibrio* species in the environment. However these techniques are time consuming and require the use of different markers to differentiate closely related species. In contrast, MALDI-TOF MS (matrix-assisted laser desorption/ionization time of flight spectrometry), is a proteomic based method able to generate a specific proteomic bacteria profile in a few seconds. Considering that existing databases do not include spectra for mollusc pathogenic *Vibrio*, we have created a MALDI-TOF VibrioBase database containing 120 reference spectra of *Vibrio* species potentially responsible for mollusc diseases and belonging

to 25 species: *V. aestuarianus*, *V. cortegadensis*, *V. tapetis* and species of *Coralliilyticus, Harveyi, Mediterranei* and *Orientalis* clades. Each Main Spectra Profile (MSP) was established based on raw spectra obtained from three different media and generated by three collaborating laboratories. Perfect discrimination was obtained for all the tested *Vibrio* species including very closely related ones.

The new VibrioBase library has been validated through a blind test including 100 *Vibrio* strains and involving three laboratories. The majority of the *Vibrio* strains were successfully identified suggesting that MALDI-TOF could be used for the routine diagnosis of mollusc bacteria diseases.

New genetic methods for pathogen detection in bivalves

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Marine bivalves host high microbial diversity and alteration of the microbiota due to stressful conditions and/or environmental changes was previously linked with a condition of compromised health status and susceptibility to diseases. Evaluation of complex microbial communities has been traditionally performed using culture-dependent techniques, but the reduced proportion of bacteria that can be cultured, as well as the inability to reproduce complex microbial environments *in vitro* are limitations that were overcome by deep sequencing techniques. Although widely used, 16S rRNA gene amplicon sequencing might meet some difficulties to highlight the pathogenic component in the vast marine bacterial community associated with bivalves. To overcome this aspect, several oyster samples have been analysed using a gene marker-based protocol and targeting bacterial species and genetic loci known to be associated with bivalve pathogenesis (Figure 1). The dynamics of microbial communities has been investigated by Lasa et al. (2019) from a large number of *C. gigas* samples (525) collected during recurrent mortality episodes in three different sites (Bay of Brest, Ebro delta and Dungarvan bay). Taking advantage of massive parallel sequencing technology, 16S rRNA gene profiling of the microbial community was also obtained on a large number of contrasted (e.g. infected vs not infected) *C. gigas* samples (n=101). In addition, a new target enrichment next-generation sequencing protocol for the selective capturing, sequencing and classification of 884 phylogenetic and virulence markers of the marine microbial pathogenic community in oyster tissues was developed, providing, for the first time to our knowledge, comprehensive high taxonomic resolution analysis of the potential pathogenic microbial community (pathobiota) associated with *C. gigas*.

Comparative analysis of contrasted *C. gigas* samples conducted using both these methods revealed that oysters experiencing mortality outbreaks displayed signs of microbiota disruption associated with the presence of previously undetected potential pathogenic microbial species. Considering its extensiveness and geographic dimension, this study provides a solid background on the structure and dynamics of microbial communities associated to oyster disease outbreaks paving the way to future studies aimed to shed light on mechanisms underlying polymicrobial infections in *C. gigas* and bivalves in general. By providing a wider picture than traditional

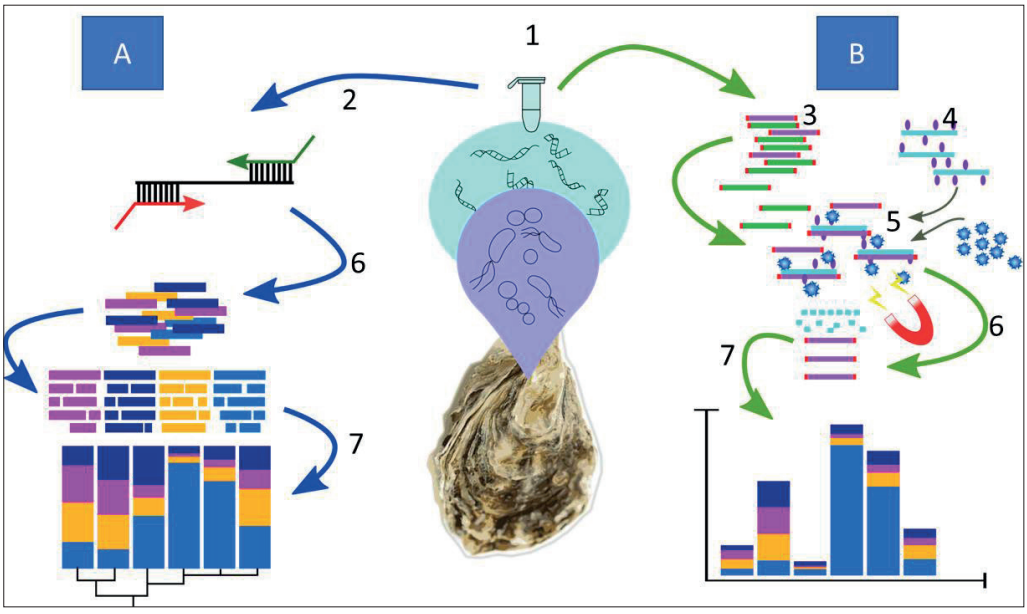

Figure 1. Schematic representation of genetic methods for large scale pathogen detection in bivalves: A) Single-marker taxonomic characterisation of bivalve microbial community; B) Species-specific DNA target enrichment. 1) metaDNA extraction, 2) 16S DNA marker amplification, 3) metaDNA library preparation, 4) species-specific probes design and synthesis, 5) target enrichment via metaDNA-probes hybridisation, 6) high throughput sequencing, 7) bioinformatic analysis for microbial community definition or for target species identification.

Real-Time PCR assays, such deep sequencing approaches allow monitoring bivalve health at the pathobiota scale.

Conclusions

Up to now, monitoring of bivalve diseases has mostly been based on the use of histology and specific tools for the detection of pathogens within their hosts (Barbosa Solomieu et al., 2015). Focusing on pathogens inside their bivalve hosts maximizes the chance to detect them. However, understanding pathogen life cycles and epizootiology is also needed to better monitor and manage associated diseases.

Environmental DNA (eDNA)-based approaches present considerable advantages to study pathogens outside their host, notably the detection of elusive or non-cultivable organisms (Bass et al., 2015; Mérou et al., 2020). The efficiency of environmental approaches such as passive sensors to reveal the presence of aquatic pathogens in particular viruses (DNA and RNA) in controlled and field conditions has been demonstrated (Vincent-Hubert et al., 2017). Such tools could provide an idea of the micro-diversity including information about the presence of pathogens in mollusc production areas. Passive sensors could also be a non-lethal alternative to monitor the presence of pathogens affecting protected species.

However, further works are needed to improve nucleic acid extraction conditions and validate such approaches. If pathogen RNA detection could be indicative of the presence of live patho-

gens, DNA detection does not allow conclusions to be made regarding the status (live / inactivated/ infectious) of the pathogens. Detecting listed pathogen DNA in the environment raises questions and might have regulatory consequences. Guidelines are needed at the EU level to advise on the decisions that should be taken when detecting pathogen DNA only, especially from environmental samples.

The use of MBs followed by qPCR enabled the capture and pre-concentration of OsHV-1 from oyster homogenate samples and even from seawater. Considering that seawater is the vehicle of transmission of the virus, MBs combined with qPCR could be used as an early warning system in aquaculture facilities. Moreover, an electrochemical biosensor has been confirmed to be a reliable tool to detect OsHV-1 in oyster samples. Although not yet automated, the system offers great potential to be easily integrated into microfluidic platforms to develop compact and hand-held devices that could be used in the field by end-users. Considering that many bivalve pathogens include bacteria in particular of the genus *Vibrio*, investigation of mortality events should systematically include bacteria isolation and characterisation. Efforts have been made in recent years by the EURL for mollusc diseases to harmonise the bacteriological diagnostics including isolation of main Vibrio strains and the detection of some Vibrio species including *V. aestuarianus* in Real Time PCR (https://www.eurl-mollusc.eu/content/download/88657/file/Bacteria%20isolation%20procedure.pdf). However, tools for the quick and accurate identification of bacteria are lacking for molluscs. MALDI-TOF MS is a powerful technique allowing quick and cheap strain identification. A database has been created and includes validated reference spectra for the main Vibrio strains pathogenic to molluscs. It should shortly be widely accessible to the laboratories wishing to identify bacteria isolated from bivalves.

Due to their filter-feeding habit, marine bivalves concentrate a rich and diverse commensal microbiota. Some of these microbial species may be pathogenic. Their pathogenicity partly depends on the host species and age and environmental factors. An increasing number of studies are carried out to characterisae bivalve microbiomes during diseases. This has notably been done in the context of sampling carried out in the frame of the European project VIVALDI (Lasa et al., 2019; Figure 1). Comparative analysis of microbial communities between diseased and healthy animals allowed identification of some differences. Not a single bacterial/virus/protistan species could be pointed as a major agent responsible of a bivalve disease outbreak. Koch's postulates were established to generate evidence that a microorganism is the cause of an infectious disease; however those postulates can't be verified when detected pathogens are non-cultivable. No overall pattern was obvious even when comparing mortality events affecting the same bivalve species. More studies are needed to decipher interactions between pathogens and associated microorganisms and to identify microbiome profile associated with healthy or diseased oysters. Additionally, currently used metabarcoding approaches present some limitations including the difficulty to reach the species level for bacteria and the difficulty to get rid of the host genomic information for eukaryotes. Target enrichment next generation sequencing such as the approaches developed in VIVALDI is an interesting alternative to specifi-

cally detect a wide range of known pathogens and would deserve further validation efforts to be used for the diagnosis of the main bivalve diseases

Finally, eDNA and magnetic beads-based approaches as well as NGS and Maldi-TOF raise interesting perspectives to better detect, characterise and monitor known pathogens. Nevertheless, these new developments will require further assessment, standardisation and validation before being available for routine diagnostics. Additionally, these tools will certainly not replace but rather complement diagnostic tools currently used including general methods such as histopathology, which helps to ensure alertness to emerging diseases (Carnegie et al., 2016). Improving the capacity of diagnostic laboratories to detect known endemic pathogens without losing the capacity to detect emerging ones thus requires continuing to train laboratories in histology and transferring new validated techniques for use on an industry setting.

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