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Diagnosis of vibriosis in the era of genomics: lessons from invertebrates

Le Roux Frederique [1, 2, *]

[1] IFREMER, Unite Physiol Fonct Organismes Marins, ZI Pointe Diable, CS 10070, F-29280 Plouzane, France.
[2] Univ Paris 06, Univ Paris 04, CNRS,UMR 8227,CS 90074, Integrat Biol Marine Models,Stn Biol Roscoff, F-29688 Roscoff, France.

* Corresponding author : Frédérique Le Roux, email address : fleroux@sb-roscoff.fr

Abstract :

Global changes linked to increases in temperature and ocean acidification, but also to more direct anthropogenic influences such as aquaculture, have caused a worldwide increase in the reports of Vibrio-associated illnesses affecting humans and also animals such as shrimp and molluscs. Investigation of the emergence of Vibrio pathogenesis events requires the analysis of microbial evolution at the gene, genome and population levels, in order to identify genomic modifications linked to increased virulence, resistance and/or prevalence, or to recent host shift. From a more applied point of view, the elucidation of virulence mechanisms is a prerequisite to devising prophylactic methods to fight infectious agents. In comparison with human pathogens, fairly little is known about the requirements for virulence in vibrios pathogenic to animals. However, the advent of genome sequencing, especially next-generation technologies,the possibility of genetically manipulating most of the Vibrio strains, and the recent availability of standardised animals for experimental infections have now compensated for the considerable delay in advancement of the knowledge of non-model pathogens such as Vibrio and have led to new scientific questions.

Keywords : Ecology, Emergence, Evolution, Marine invertebrate, Vibriosis, Virulence

Increase in sea surface temperature and ocean acidification linked to global change and human activities such as aquaculture, have caused a worldwide increase in the reports of *Vibrio*-associated diseases, with ecosystem-wide direct and indirect effects on humans and marine animals (For a review see 1). In Europe, several studies have reported the presence of human pathogens (e.g., *V. parahaemolyticus*, *V. cholerae* non-O1/non-O139*)* in shellfish as well as in coastal and estuarine waters (2-16). Several lines of evidences suggest that these infections are increasing and tend to follow regional climatic trends with outbreaks typically following episodes of unusually warm weather. In northern Europe, the salmonid farming industry is constantly threatened by pathogens, such as *Vibrio salmonicida* and *V. anguillarum* (17). In France, several *Vibrio* species have been associated with recent and massive losses in the oyster industry (18, 20). In Spain *Photobacterium damselae* is associated with diseases of cultured fish species (20) and *V. vulnificus* with hemorrhagic septicaemia in eels (21). Both fish pathogens deserve special attention since they are able to cause septicemia in humans. Finally, evidence has also been gathered linking *Vibrio* infections (e.g. *Vibrio coralliilyticus*) to increasing mass mortality of benthic corals (e.g. *Paramuricea clavata*) in the NW Mediterranean Sea (22). In this context, the development of operational tools to identify and detect emergent pathogens is essential to zoosanitary monitoring of cultivated species as well as to inform and enhance studies on wild animal populations.

Experimental challenge still remains the only way to determine the virulence of an isolate

Vibrio infections of invertebrates are widely documented (for review see 23). However, compared to human pathogens, little is known regarding the mode of pathogenesis. More often, the virulence of a given strain must be assessed by experimental challenge. However, analysis of the data generated by this approach is hampered by the variability of the physiological state and a heterogeneous genetic background of the animals, combined with a continuously changing environment (food, temperature, salinity, pollutants). Even more disturbing is the fact that invertebrate tissues naturally housing bacteria that can lead to misinterpretations of obtained results (24).

The use of gnotobiotic systems (i.e. animals cultured in axenic conditions or with a known microflora) is a promising tool to diagnose pathogenic strains and extend our understanding of the mechanisms involved in host-microbe interactions (for review see 25). Most studies performed so far have used axenic *Artemia fransciscana* as a test organism. This system has allowed for the investigation of several aspects of crustacean-*Vibrio* interactions, such as colonization (of pathogenic or probiotic isolates), biofilm formation, toxicity of exoenzymes, and infection route (26-29). However as a heterologous system, this model may exclude species-specific pathogens and specific virulence mechanisms.

Experimental challenge in larvae seems to give reproducible results, and as a consequence, the pathogenic status of tested strains is less controversial than in adult animals (30). Generally, bacterial isolates are incubated directly with larval cultures and mortalities appear rapidly, i.e. from 1-10 days after infection. These tests can be miniaturized and therefore permit the screening of large numbers of isolates. However, because several strains are pathogenic only from certain developmental stages (for example *Vibrio penaeicida* in the shimp *Litopenaeus stylirostris*, see 31), this procedure cannot be used as a broad test for virulence.

Putative pathogens are frequently selected by injection of bacterial suspensions in the animals The results are obtained quickly since mortality often appears in a few days. The recent development of specific-pathogen-free (SPF) and standardized spats of *Crassostrea gigas* (32, 33) has made high-throughput screening of oyster pathogenic isolates by injection possible (18, 19). However, injection techniques do not reflect the natural route of infection, thus precluding other factors (e.g. chemotaxis, colonisation etc.) that may be involved in the infection process (Figure 1). Furthermore, these laboratory analyses do not capture the complexity of infection occurring in the natural environment. For instance, oysters are typically injected with a single bacterial strain, whereas in the environment, animals are typically colonized by a diverse assemblage of *Vibrio* species (19, 30).

Experimental infection by immersion has gained success in demonstrating the virulence of some *Vibrio* strains towards shrimp (34), oyster (35) clam (36) and octopus (37). However, this method frequently does not result in any mortality at all. This lack of reproducibility may be due to the fact that the ability of vibrios to induce disease in the wild may depend on associations with other bacteria or attachment to other organisms and particles. For instance, a recent study has reported that marine aggregates facilitate retention of nanoparticles (including bacteria) by suspension-feeding bivalves (38). Thus, monitoring of animals in an environment in which bacteria are not simply in a planktonic form may yield a more accurate understanding of the factors that contribute to virulence. Identifying the microhabitats of pathogens (39) may facilitate further development of an experimental infection model representative of the natural route of infection (e.g. through the use of adapted polymeric substrates and or cellular vectors). Such an approach would also allow for a better understanding of the mode of transmission and primary target tissues or organs for these pathogens.

The lack of knowledge concerning the vibrio pathogenesis in marine animals is in part a consequence of the absence of standardized models for in vivo studies. Indeed, with no inbred animal lines, the genetic background of the experimental animals is heterogeneous. Furthermore as bacteria naturally infect invertebrates, the diversity of the natural microflora may also contribute to a lack of reproducibility. Of the recent work aiming to improve the in vivo model, the standardization of animal hatching and attempts to mimic the natural route of infection determination seem to have the most promising perspectives.

Cellular and/or molecular tests to evaluate potential virulence

In light of the difficulties faced by current techniques of experimental infection in non-domesticated animals, the development of cellular and/or molecular tests to evaluate the potential virulence of strains is necessary. The development of such bioassays could be informed by descriptions of host alterations to define the virulence mechanisms involved in pathogenesis. However, in these systems, it is difficult to establish a specific clinical table of disease characteristics for four main reasons: 1) it is necessary to analyze a high number of samples to investigate any one specific observation; 2) the absence of external symptoms frequently leads to the observation/description of post-mortem lesions; 3) the size of the pathogen is inhibitory to detection; and 4) invertebrates are already colonized by commensal community of microbes. Thus, specific molecular tools, such as *in situ* hybridization and fluorescent-labeled bacteria, should be developed to describe the infection route and eventual specific localization and migrations of *Vibrio* pathogens. GFP-labeled *Vibrio* have already been used to investigate *Vibrio*-invertebrate interactions at both organismal and cellular levels (Figure 2) (40-42).

Standardized *in vitro* assays are also necessary to screen for distinct activities covered by the generic term virulence, such as adherence and cytolytic effects. As marine invertebrate cell lines are not yet available, experiments aiming to understand interactions between host cells and pathogenic *Vibrios* have thus far relied mainly on primary lines of hemocytes (41, 43). However, the use of such a cell population has drawbacks. The hemocyte population is made of distinct cell types that have yet to be fully characterized. Moreover, each animal provides a limited number of cells (about 10^6), and experiments using this hemocyte procedure would require the pooling of hemocytes from different individuals with heterogeneous genetic backgrounds in order to achieve the required sample size. Additionally, during an experiment of sufficient duration, bacteria that naturally occur in the hemolymph will proliferate. Interestingly, a recent report has described the use of primary cell cultures from abalone gills to investigate the *V. harveyi*-abalone interaction (44). Also, heterologous cell lines, such as Bge (mollusk from fresh water) or NIH3T3 (mouse fibroblastic cell lines), have been used to describe the cytopathic effects of a metalloprotease expressed by an oyster pathogen (45). However, due to osmolarity restrictions, such an approach is still limited to extracellular product analyses.

Bacterial pathogenicity is known to be associated with the structural components of the cells (e.g. capsules, fimbriae, LPS, endotoxins, and other cell wall components), or with the active secretion of substances that either damage host tissues or protect the bacteria against host defenses (invasin enzymes, hemolysin, coagulase, toxins). Several virulence factors have already been identified in *Vibrio* pathogenic to invertebrates. A metalloprotease has been demonstrated to be a key factor in the extracellular product (ECP) toxicity in *V. splendidus* (46) *V. aestuarianus* (43)*V. tubiashi* (47) and *V. corallyliticus* (48). The porin OmpU was reported to be an essential virulence factor in the *V. splendidus*-*Crassostrea gigas* interaction (49). More recently, a serine protease was found to be specifically secreted through outer membrane vesicles (OMVs) and shown to participate in the virulence of a *V. splendidus*-related strain (50). DjlA, an inner membrane co-chaperone belonging to the DnaJ family, was shown to be necessary for cytotoxicity of the clam pathogen *V. tapetis* to hemocytes (51). Additionally, from the genomic identification of homologues of genes involved in virulence in other bacterial pathogens, several putative virulence factors have been identified (52). These include potential toxins such as hemolysins, MARTX, proteases, a type VI secretion system, adhesins, as well as genes for siderophore production, transport and utilization. A caveat of this analysis is that the majority of these homologues has been identified on the basis of vertebrate pathogen studies, possibly precluding the discovery of new mechanisms specific to invertebrate pathogens. Furthermore the knowledge of the absence/presence/diversity of these genes does not appear sufficient for the determination of a strain's pathogenicity.

These last few years, few likely virulence mechanisms of vibrios pathogenic for invertebrates have been described at the cellular and molecular level, leading to a better understanding of some pathogenic effects. However to date, detection of these genes is not sufficient to conclude about the pathogenicity of the strain. Microbial pathogenesis is often multifactorial, and pathogens use several biochemical mechanisms operating in concert to produce infections and diseases. In addition, intra and inter-species diversity in virulence mechanisms complicates the development of diagnostic tools to determine the pathogenicity of the strain.

Genome sequence and typing virulent strains

Due to the lack of knowledge of marine bacterial virulence mechanisms and diversity, a "blind" approach has been necessarily employed in order to correlate a genotype (or a group

of related genotypes) with virulence potential. In the last few years, significant progress has been made in understanding the population structure and diversity of the *Vibrio* (53). Despite their enormous microdiversity, these organisms fall into well-defined genetic clusters that have similar resource preferences. These clusters have been hypothesized to correspond to populations that act as cohesive ecological units, i.e. ecological populations (39). However, a link between ecological populations and pathogenicity has not been demonstrated, and it is unclear whether pathogenicity is a trait primarily linked to clones, or to populations comprising a large number of distinct genotypes.

Combining experimental ecology, a high throughput infection assay, and genome sequencing, we were recently able to show that *Vibrio* pathogenicity for oyster spat in France can be ascribed to a cluster of genetically related strains that coincides with a previously defined ecologically cohesive population and to the *V. crassostreae* species delineation (19). Despite a strong clonal frame in the core genome (genes shared by all the strains of the population), the flexible genome was highly diverse with 100-1200 strain specific genes. Genes specific to this population likely reflect the selective pressure associated with population specialization, and we have demonstrated that one of these genes is required for pathogenicity. Thus, in the case of the *V. crassostreae* infection, the functional unit of virulence is the population (or species) and diagnostic tools can be based on taxonomic markers polymorphisms or population specific gene detection (Figure 3).

The genome based phylogeny of *V. aestuarianus* related strains shows that the isolates infecting diseased oysters are clustered into two specific lineages (18). These lineages contain a large majority of the virulent strains. The strong clonal frame in the core genome and absence of strain specific genes within the lineages led us to hypothesize that the common ancestor was pathogenic and that a few modern strains have lost some key virulence factor(s). This has been recently illustrated by the identification of a frameshift in a non-virulent strain, in a gene coding for a histidine kinase (VarS). A knock out of this gene in a virulent strain confirmed its role as a regulator for *V. aestuarianus* virulence. Thus, in the case of *V. aestuarianus,* the functional unit of virulence is the lineage, probably as a consequence of the clonal expansion of a virulent strain (Figure 3). The diagnosis should map taxonomic markers as soon as it leads to the discrimination of the lineage within the species.

The population structure of *Vibrio nigripulchritudo* related strains highlights a high genetic diversity (54). However virulent strains associated to shrimp disease are found exclusively in specific lineages. Within each lineage, the strains are nearly clonal but only some of them are virulent. Complete sequencing and comparative genome analysis demonstrated that the virulent strains contain specific mobile genetic elements further reported to be necessary for virulence. These elements are different between virulent strains from each lineage but a single toxin is shared by all of them. Thus, in the case of *V. nigripulchritudo,* the functional unit is the strain within lineage and the diagnosis should map the toxin (Figure 3).

To summarize, we have illustrated here the difficulty in developing diagnostic tools based on genotyping methods resulting from the diversity of pathogen evolutionary scenarios within the *Vibrios*. In some cases, pathogens arise from the clonal expansion of a strain via lateral gene transfer. In other cases, virulence seems to be linked to the differentiation of ecological populations or species. It should be noted that none of these data would have been obtained from single gene (more often 16S analyses) which is unfortunately still being used to identify "putative" pathogens in aquaculture.

With the revolution of high throughput technologies, it is now possible to sequence a large collection of genomes, perform population structure analyses at the genome scale, and investigate the functional unit of virulence for each putative pathogen. In addition, comparing the genome of virulent strains or populations with their closest non-virulent phylogenetic neighbors will lead to the identification of virulence markers. Then, genetic analyses will highlight the genes that are necessary for infection – a prerequisite to developing cellular and/or molecular tests to diagnose pathogenicity.

Metagenomic and exploring a polymicrobial disease

Marine invertebrates house abundant communities of microbes, which are increasingly recognized to influence the health of these animals. This association can be beneficial as some microbes provide additional energy sources to the host or prevent the establishment of pathogens. For instance, a recent study showed that about 3% of oyster haemolymph-associated cultivable bacteria displayed antibacterial activity toward vibrio (55). Using a metabarcoding approach, Wegner and coll showed in oyster that microbial communities are stable over time and assemble in a animal's genotype specific manner. However a change of the environment, such as temperature stress, can disrupt such associations and promote dominance of opportunists (56).

In addition two studies support the hypothesis of increased virulence due to microbe interaction in oysters. First, experimental infections have demonstrated that some *Vibrio* strains are moderately virulent when injected into animals individually, and display heightened virulence in mixed experimental infections (57). Second, the analyses of oyster mortality following experimental infection suggest that disease onset can be facilitated by the presence of non-virulent strains (19). Hence although non-virulent strains are not sufficient for pathogenesis, they clearly have some features (as yet undetermined) that contribute either directly or indirectly to pathogenicity mechanisms. One possibility is that non-virulent strains provide resources required by the virulent strains, acting as "cheaters". This phenomenon has been seen in some analyses of siderophore synthesis and utilization (58). An alternate role for the non-virulent strains may be to generate a sufficient bacterial load, either to overcome host defenses or to induce expression of virulence factors that are regulated by quorum sensing (59).

Therefore in the future, the investigation of vibrio pathogenicity for oyster, as for other marine invertebrates, should incorporate metagenomic analysis on the whole microbial community. This approach should lead to correlate spatio-temporal dynamic of populations with cooperative behavior (e.g. quorum sensing, public good) and weapon sharing (e.g. synergic/additional virulence traits; lateral gene transfer).

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Figure 1: *Vibrio nigripulchritudo* is an emerging pathogen of farmed shrimp in New Caledonia and other regions in the Indo-Pacific. Molecular epidemiological studies have suggested that pathogenicity is linked to particular lineages (A, B and M). The pathogenicity has been assessed using 3 experimental models: shrimp transiently immersed into bacteria-contaminated waters, shrimp intramuscularly injected with bacteria, and shrimp intramuscularly injected with bacterial culture supernatants, and yielded the distinction between Highly, Moderately and Non pathogenic strain (HP in red, MP in orange and NP in green). Each contemporary lineage is comprised of nearly identical strains but comparative genomics allowed differentiation of genetic elements specific to shrimp pathogenesis of varying severity (Goudenège et al., 2014). In clade A, the highly pathogenic phenotype coincides with the presence of two plasmids pB1067 (11 kbp) and pA1066 (240kbp). The role of each replicon was investigating genetically using plasmid cured HP derivatives. In the immersion model, only strains containing both pA1066 and pB1067 were virulent, while in bacterial injection model, the wild type strain which contains both plasmids, was more virulent than a strain containing pA1066 alone. When supernatants were injected, toxicity was independent of the presence of pB1067. Collectively, these findings suggest that there may be interactions between factors encoded on the two plasmids. It is likely that differences between results obtained with the models reflect the different host-imposed barriers that are encountered by the bacteria. They may also indicate that there are multiple pathways by which the bacterium impairs shrimp viability (Le Roux et al., 2011; Goudenège et al., 2013).

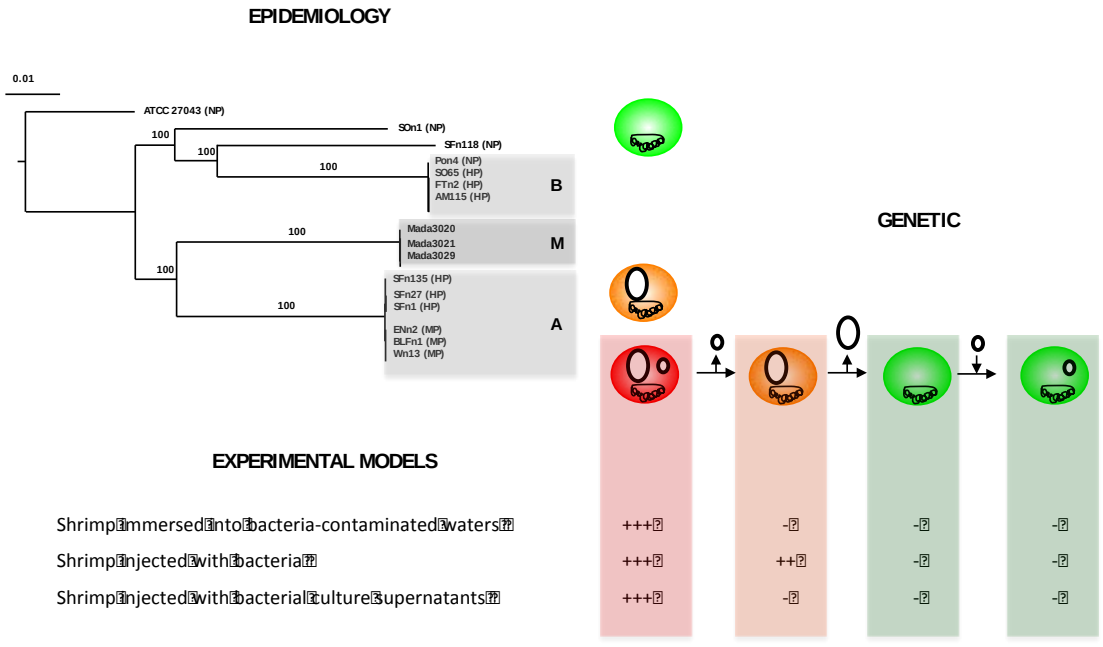

Figure 2: GFP labeled *Vibrio* to investigate interactions with the host at both organismal (A) and cellular levels (B). **A-** *Artemia fransciscana* was immersed into GFP labeled *Vibrio* sp. contaminated waters, fluorescence is visible by transparency in the animal alive. **B-** Hemocyte invasion by *V. crassostreae* strain J2-9. GFP-expressing J2-9 in hemocytes stained with Dapi, and rhodamin-coupled phalloidin (Courtezy from Guillaume Charrière).

A B

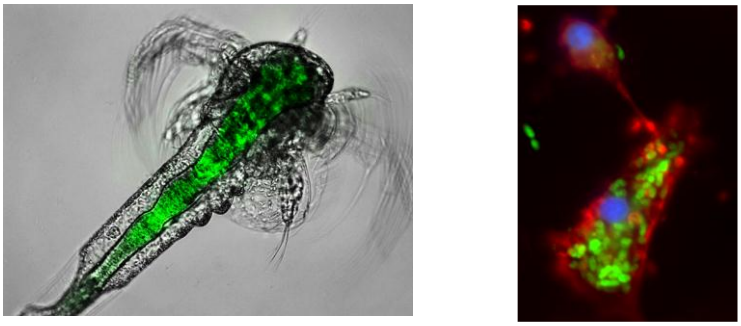

Figure 3: The different functional units of pathogenesis found in *Vibrio.* Pathogenic and non pathogenic strains are indicated in red and green respectively.