Rev. Sci. Tech. Off. Int. Epiz., 2016, **35** (1), 259-269

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

F. Le Roux [1, 2]*

(1) Ifremer, Unité Physiologie Fonctionnelle des Organismes Marins, ZI de la Pointe du Diable, CS 10070, F-29280 Plouzané, France
(2) Sorbonne Universités, Université Pierre-et-Marie-Curie, Centre national de la recherche scientifique, Unité mixte de recherche 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, CS 90074, F-29688, Roscoff Cedex, France
*Corresponding author: fleroux@sb-roscoff.fr

Summary
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.

Introduction

The increases 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, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16). Several lines of evidence 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 *V. salmonicida* and *V. anguillarum* (17). In France, several *Vibrio* species have recently been associated with massive losses in the oyster industry (18, 19, 20). In Spain, *Photobacterium damselae* is associated with diseases of cultured fish species (20) and *V. vulnificus* with haemorrhagic septicaemia in eels (21). Both fish pathogens deserve special attention because they are able to cause septicaemia in humans. Finally, evidence has also been gathered linking *Vibrio* infections (e.g. *V. coralliilyticus*) to increasing mass mortality of benthic corals (e.g. *Paramuricea clavata*) in the north-west Mediterranean Sea (22). In this context, the development of operational tools to identify and detect emerging pathogens is essential for zoosanitary monitoring of cultivated species as well as for facilitating studies on wild animal populations.

doi: http://dx.doi.org/10.20506/rst.35.1.2431

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

Vibrio infections of invertebrates are widely documented (for review see [23]). However, compared with human pathogens, little is known about their mode of pathogenesis. Generally, 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 the 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 house bacteria that can lead to misinterpretations of the results obtained (24).

The use of gnotobiotic systems (i.e. animals cultured in axenic conditions or with a known microflora) is a promising tool to detect and identify pathogenic strains and to extend our understanding of the mechanisms involved in host–microbe interactions (for review see [25]). Most studies performed so far have used axenic *Artemia franciscana* as a test organism. This system has allowed investigation of several aspects of crustacean–*Vibrio* interactions, such as colonisation (of pathogenic or probiotic isolates), biofilm formation, toxicity of exoenzymes and infection route (26, 27, 28, 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 strains tested in this way is less controversial than in adult animals (30). Generally, bacterial isolates are incubated directly with larval cultures and mortalities appear rapidly, from one to ten days after infection. These tests can be miniaturised and therefore enable the screening of large numbers of isolates. However, because several strains are pathogenic only for certain developmental stages (for example, *V. penaeicida* in the shrimp *Litopenaeus stylirostris* [31]), this procedure cannot be used as a broad test for virulence.

Putative pathogens are frequently selected by injection of bacterial suspensions into the animals. The results are obtained quickly because mortality often appears in a few days. The recent development of specific-pathogen-free (SPF) and standardised spats of *Crassostrea gigas* (32, 33) has made possible high-throughput screening of oyster pathogenic isolates by injection (18, 19). However, injection techniques do not reflect the natural route of infection, thus precluding other processes (e.g. chemotaxis, colonisation) that may be involved in the infection process (Fig. 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 colonised 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 (35), oyster (36), clam (37) and octopus (38). 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 (39). Thus, monitoring 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 (40) 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 provide a better understanding of the mode of transmission and primary target tissues or organs for these pathogens.

The lack of knowledge concerning the pathogenic mechanisms of vibrios in marine animals is in part a consequence of the absence of standardised 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* models, the standardisation of animal hatching and attempts to mimic the natural route of infection seem to be the most promising.

Cellular and/or molecular tests for evaluating potential virulence

In light of the limitations of current techniques of experimental infection in non-domesticated animals, the development of cellular and/or molecular tests to evaluate

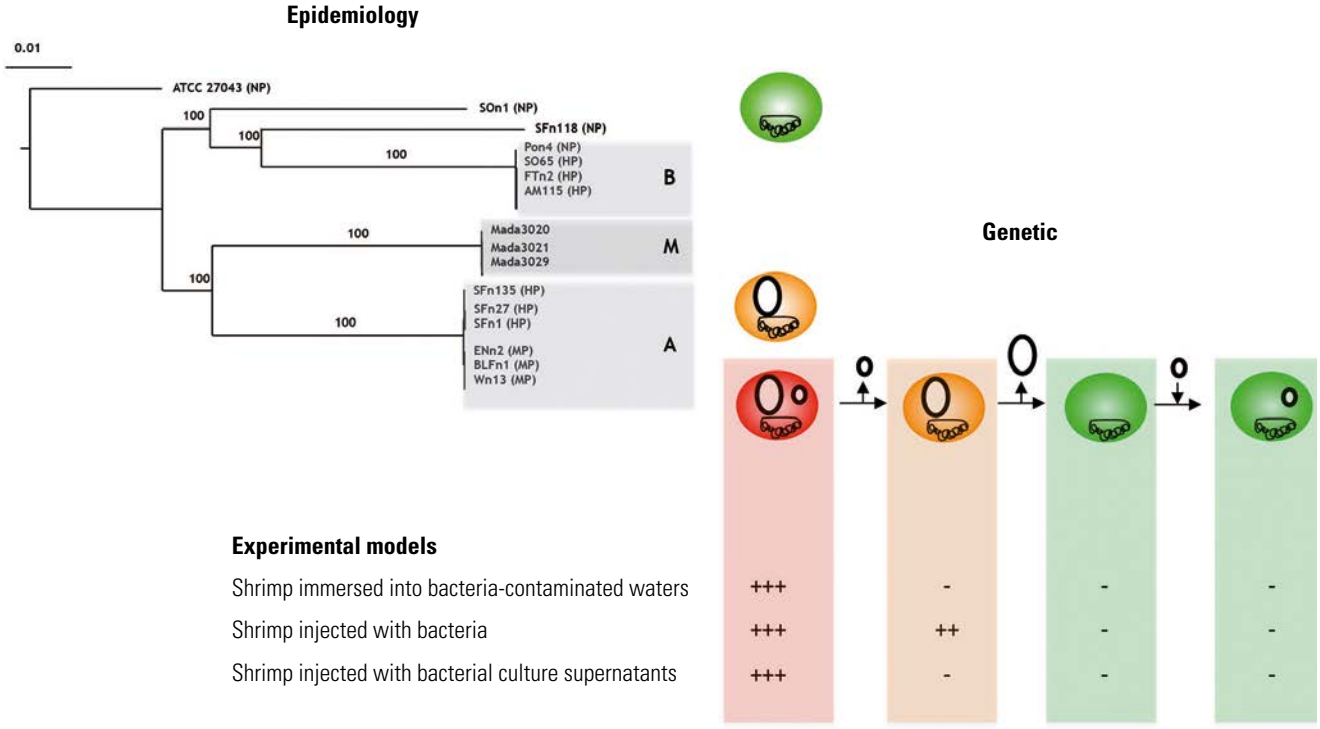


Fig. 1

Phylogeny, genetic and experimental infection models for *Vibrio nigripulchritudo*

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 three experimental models: shrimp transiently immersed in bacteria-contaminated waters, shrimp intramuscularly injected with bacteria, and shrimp intramuscularly injected with bacterial culture supernatants; this yielded a distinction among highly, moderately and non-pathogenic strains (HP in red, MP in orange and NP in green). Each contemporary lineage is composed of nearly identical strains, but comparative genomics has allowed differentiation of genetic elements specific to shrimp pathogenesis of varying severity (34). In clade A, the highly pathogenic phenotype coincides with the presence of two plasmids: pB1067 (11 kilobase pairs [kb]) and pA1066 (240 kb). The role of each replicon was investigated genetically using plasmid cured HP derivatives. In the immersion model, only strains containing both pA1066 and pB1067 were virulent, while in the 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 models in the results obtained 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 (34, 35)

the potential virulence of strains is necessary. Such bioassays could include descriptions of host alterations that define the virulence mechanisms involved in pathogenesis. However, in these systems, it is difficult to establish a list of specific clinical disease characteristics for four main reasons:

i) it is necessary to analyse a large number of samples to investigate any one specific observation

ii) the absence of external clinical signs frequently leads to the observation/description of post-mortem lesions

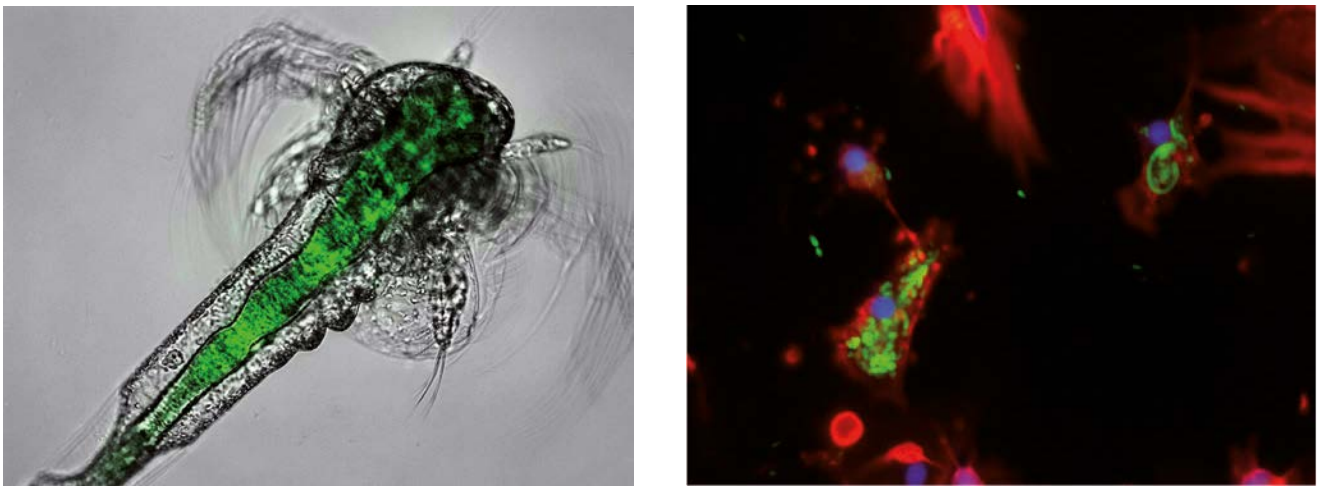
iii) the size of the pathogen makes detection difficult

iv) invertebrates are already colonised by a commensal community of microbes.

Thus, specific molecular tools, such as *in situ* hybridisation and fluorescent-labelled bacteria, should be developed for investigation of the infection route and eventual specific localisation and migration of *Vibrio* pathogens. Green fluorescent protein (GFP)-labelled vibrios have already been used to investigate *Vibrio*–invertebrate interactions at both organism and cellular levels (Fig. 2) (41, 42, 43).

Standardised *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 aimed at understanding the interactions between host cells and pathogenic *Vibrio* species have thus far relied mainly on primary lines of haemocytes (42, 44). However, the use of such a cell population has drawbacks. The haemocyte population is composed of distinct cell types that have yet to be fully characterised. Moreover, each animal provides a limited number of cells (about 106), and experiments using this haemocyte procedure would require the

a) b)



a) *Artemia franciscana* was immersed in water contaminated by GFP-labelled *Vibrio*; fluorescence is made visible by the transparency of live animals
b) Haemocyte invasion by *Vibrio crassostreae* strain J2-9. GFP-expressing J2-9 is seen in haemocytes stained with DAPI (4′,6-diamidino-2-phenylindole) and rhodamin-coupled phalloidin (courtesy of Guillaume Charrière)

Fig. 2
Green fluorescent protein (GFP)-labelled *Vibrio* used to investigate interactions with the host at both organism (a) and cellular levels (b)

pooling of haemocytes 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 haemolymph will proliferate. Interestingly, a recent report has described the use of primary cell cultures from abalone gills to investigate the *V. harveyi*–abalone interaction (45). Also, heterologous cell lines, such as Bge (from a freshwater mollusc) or NIH3T3 (a mouse fibroblastic cell line), have been used to describe the cytopathic effects of a metalloprotease expressed by an oyster pathogen (46). However, because of restrictions caused by differences in osmolarity, such an approach is still limited to analyses of extracellular products (ECPs).

Bacterial pathogenicity is known to be associated with the structural components of the cells (e.g. capsules, fimbriae, lipopolysaccharide [LPS] and other cell wall components), or with the active secretion of substances that either damage host tissues or protect the bacteria against host defences (invasin enzymes, haemolysin, coagulase, toxins). Several virulence factors have already been identified in *Vibrio* species that are pathogenic to invertebrates. A metalloprotease has been demonstrated to be a key factor in the ECP toxicity of *V. splendidus* (47), *V. aestuarianus* (44), *V. tubiashi* (48) and *V. corallyliticus* (49). The porin OmpU was reported to be an essential virulence factor in the *V. splendidus*–*Crassostrea gigas* interaction (50). 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 (51). An inner membrane co-chaperone belonging to the DnaJ family, DjlA, was shown to be necessary for cytotoxicity of the clam pathogen *V. tapetis* to haemocytes (52). Additionally, several putative virulence factors have been identified by the genomic investigation of homologues of genes involved in virulence in other bacterial pathogens (53). These include potential toxins such as haemolysins, multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins, proteases, a type VI secretion system and adhesins, as well as genes for siderophore production, transport and utilisation. A caveat of this analysis is that the majority of these homologues have been identified on the basis of vertebrate pathogenesis studies, possibly precluding the discovery of new mechanisms specific to invertebrate pathogens. Furthermore, knowledge of the absence, presence or diversity of these genes does not appear sufficient for the determination of a strain's pathogenicity.

In the last few years, a few likely virulence mechanisms of vibrios pathogenic for invertebrates have been described at the cellular and molecular levels, leading to a better understanding of some pathogenic effects. However, to date, detection of these genes is not sufficient to make conclusions 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 a strain.

Genome sequencing and typing virulent strains

Given 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 *Vibrio* species (54). Despite their enormous microdiversity, these organisms fall into well-defined genetic clusters that have similar resource preferences. These clusters have been hypothesised to correspond to populations that act as cohesive ecological units, i.e. ecological populations (40). 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, the author's group was recently able to show that pathogenicity of *Vibrio* 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 100–1,200 strain-specific genes. Genes specific to this population probably reflect the selective pressure associated with population specialisation, and the author and colleagues have demonstrated that one of these genes is required for pathogenicity. Thus, in the case of *V. crassostreae* infection, the functional unit of virulence is the population (or species), and diagnostic tools can be based on polymorphism of taxonomic markers or population-specific gene detection (Fig. 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 the absence of strain-specific genes within the lineages led to the hypothesis 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 (Fig. 3). The diagnostic strategy should therefore map taxonomic markers as soon as it has led to the discrimination of the lineage within the species.

The population structure of *V. nigripulchritudo*-related strains highlights a high genetic diversity (34). However, virulent strains associated with shrimp diseases 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 have demonstrated that the virulent strains contain specific mobile genetic elements further reported to be necessary for virulence. These elements differ among 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 (Fig. 3).

To summarise, the difficulty in developing diagnostic tools based on genotyping methods, which results from the diversity of pathogen evolutionary scenarios within the vibrios, has been illustrated here. 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

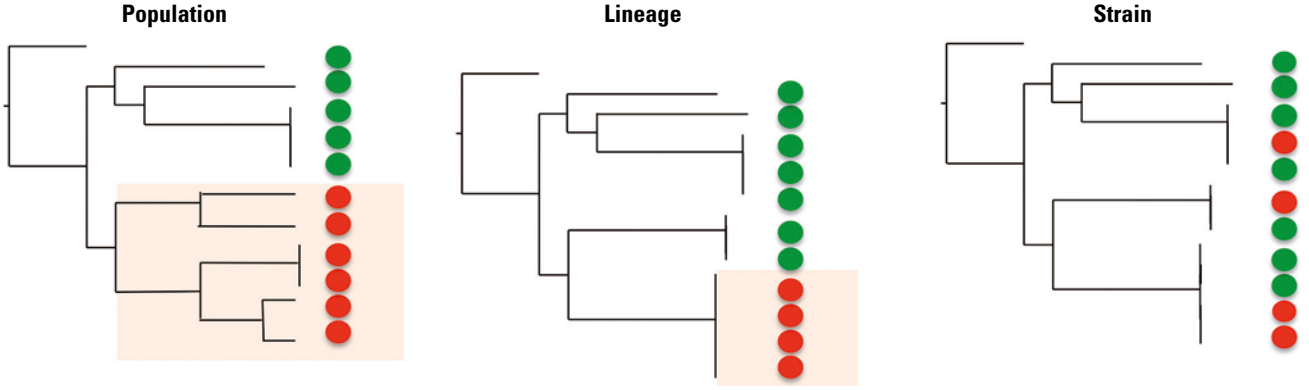


Fig. 3
The different functional units of pathogenesis found in *Vibrio*
Pathogenic and non-pathogenic strains are indicated in red and green, respectively

the differentiation of ecological populations or species. It should be noted that none of these data would have been obtained from single gene (most often 16S ribosomal RNA) analysis, which is unfortunately still being used to identify 'putative' pathogens in aquaculture. With the revolution in 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 genomes of virulent strains or populations with those of their closest non-virulent phylogenetic neighbours will lead to the identification of virulence markers. Subsequent genetic analyses will highlight the genes that are necessary for infection – a prerequisite to developing cellular and/or molecular tests to diagnose pathogenic strains.

Metagenomics used to explore polymicrobial diseases

Marine invertebrates house abundant communities of microbes, which are increasingly recognised as influencing 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 towards vibrios (55). Using a metabarcoding approach in oysters, Lokmer and Wegner showed that microbial communities are stable over time and assemble in a manner that is specific to the animal's genotype. However, a change in the environment, such as temperature stress, can disrupt such associations and promote the 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, but display heightened virulence in mixed experimental infections (57). Second, analyses of oyster mortality following experimental infection suggest that disease onset can be facilitated by the presence of non-virulent strains (19). Therefore, 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 utilisation (58). An alternative role for the non-virulent strains may be to generate a sufficient bacterial load either to overcome host defences or to induce expression of virulence factors that are regulated by quorum sensing (59).

Therefore, in the future, the investigation of *Vibrio* pathogenicity for oysters, as for other marine invertebrates, should incorporate metagenomic analysis of the whole microbial community. This approach should lead to correlation of the spatio-temporal dynamics of populations with cooperative behaviour (e.g. quorum sensing, public good) and weapon sharing (e.g. synergic/additional virulence traits, lateral gene transfer).

Acknowledgements

The author warmly thanks Teagan Mayer (Rebecca Case Lab, University of Alberta, Canada) and Dr Yannick Labreuche for critically reading the manuscript and for helping to edit the English language. This work was supported in part by grants from the Agence Nationale de la Recherche (ANR) (11-BSV7-023-01 'VIBRIOGEN' and 13-ADAP-0007-01 'OPOPOP').

Le diagnostic de la vibriose à l'ère de la génomique : les leçons tirées des invertébrés

F. Le Roux

Résumé
Nous assistons actuellement à un accroissement mondial du nombre de maladies imputables à *Vibrio* rapportées aussi bien chez l'homme que chez des espèces animales comme les crevettes et les mollusques, dû aux changements planétaires liés à l'augmentation des températures et à l'acidification des océans, parmi d'autres influences plus directement associées à l'activité humaine, notamment l'aquaculture. Les études sur la pathogenèse de l'émergence de *Vibrio* recourent à des analyses de l'évolution microbienne tant au niveau génétique et génomique qu'à l'échelle des populations, de manière à identifier les modifications génomiques associées à l'accroissement de la virulence, de la résistance et/ou de la prévalence, ainsi que l'extension récente des spécificités d'hôte. D'un point de vue plus pratique, l'élucidation des mécanismes de virulence est une condition préalable à la conception de méthodes prophylactiques permettant de lutter contre les agents infectieux. Les critères de virulence des *Vibrio* pathogènes pour les animaux sont beaucoup moins connus que ceux des agents pathogènes affectant l'être humain. Néanmoins, l'avènement des techniques de séquençage du génome, en particulier celles de nouvelle génération, la possibilité de soumettre la plupart des souches de *Vibrio* à des manipulations génétiques et la standardisation récente de modèles animaux pour des essais d'infection expérimentale ont permis de rattraper une grande partie du retard des connaissances sur les agents pathogènes non utilisés comme modèles tels que *Vibrio*, en suscitant de nouvelles questions scientifiques.

Mots-clés
Écologie – Émergence – Évolution – Invertébrés marins – Vibriose – Virulence.

Diagnóstico de la vibriosis en la era de la genómica: lo que hemos aprendido de los invertebrados

F. Le Roux

Resumen
Los cambios planetarios ligados no solo al incremento de las temperaturas y la acidificación de los océanos, sino también a factores antrópicos que ejercen una influencia más directa, como la acuicultura, se han traducido en un aumento mundial del número de casos notificados de patologías asociadas a *Vibrio*, ya sea en el ser humano o en animales como camarones o moluscos. Para investigar la aparición de episodios patógenos causados por *Vibrio* es preciso analizar la evolución microbiana a nivel génico, genómico y poblacional con el fin de determinar las modificaciones genómicas relacionadas con un aumento de la virulencia, la resistencia y/o la prevalencia o con un cambio reciente de organismo anfitrión. Desde un punto de vista más aplicado, para concebir métodos profilácticos destinados a combatir a los agentes infecciosos es indispensable dilucidar previamente los mecanismos de la virulencia. En

comparación con los patógenos humanos, poco se sabe acerca de los factores que determinan la virulencia en los vibrios patógenos para los animales. Sin embargo, gracias al advenimiento de la secuenciación genómica, en especial de las técnicas de secuenciación de próxima generación, a la posibilidad de manipular genéticamente la mayoría de las cepas de *Vibrio* y a la existencia desde hace poco tiempo de animales estandarizados con los que proceder a infecciones experimentales, ya se ha podido compensar el considerable retraso que existía en el conocimiento de patógenos que no constituyen «organismos modelo» para la ciencia, como es el caso de *Vibrio*, lo que a su vez ha abierto nuevos interrogantes científicos.

Palabras clave
Ecología – Emergente – Evolución – Invertebrado marino – Vibriosis – Virulencia.

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