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Molecular epidemiology of *Vibrio nigripulchritudo*, a pathogen of cultured penaeid shrimp (*Litopenaeus stylirostris*) in New Caledonia

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Abstract

A collection of 57 isolates of *Vibrio nigripulchritudo* from either diseased or healthy shrimp and from shrimp farms environment was studied in order to gain a better understanding of the epidemiology of this pathogen, notably isolated from two distinct shrimp disease complexes. Molecular typing using two different techniques, arbitrarily primed PCR (AP-PCR) and multi-locus sequence typing (MLST), studied together with experimental pathology data allowed a relevant epidemiological insight into this possibly emerging pathogen. Additionally, results obtained with the two molecular typing techniques were congruent and allowed discriminating the strains associated with the "Summer Syndrome" from strains isolated from other contexts, especially the other shrimp vibriosis "Syndrome 93". These results highlight that the "Summer Syndrome" is most probably caused by an emergent clonal pathogen that therefore deserves surveillance and that AP-PCR can satisfactorily be used for that purpose.

Keywords: Vibriosis; Mariculture; AP-PCR; MLST; Typing; Epidemiology; Virulence

Introduction

Shrimp aquaculture has been constantly increasing over the last decades, providing now half of the world shrimp supplies [30]. However, this rapid increase in culture has often been impeded by very severe epizootics [19,34]. Worldwide, viruses are major problems, being responsible for the most spectacular losses among shrimp [23,33]. However, vibriosis is also an important disease among penaeid shrimp [3,21,22,26,36], but this has yet only received little research attention. Therefore, limited knowledge has been gained on these pathologies and little is known concerning the epidemiology of *Vibrio* spp that are pathogenic to farmed shrimp.

New Caledonia (a 450×50 km wide island in the South Pacific between 19°S and 23°S) is a small producer regarding world global trade. However, this industry has gained considerable economic importance in the last three decades, now representing the second major export sector of the country. Actually, the shrimp farming industry has major advantages, namely a tropical oceanic climate, a domesticated *Litopenaeus stylirostris* line reared in closed cycle for almost 25 years, an almost virus-free status, infectious hypodermal and hematopoietic necrosis virus (IHHNV) being the only known virus present and the domestic stock of *L. stylirostris* being resistant to it [40]. Yet this industry

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is based on a unique domesticated but non-indigenous species, *L. stylirostris*. So the emergence of an infectious disease would threaten its profitability and sustainability. In such a context, it is of prime importance to detect the emergence of pathogenic infectious agents as early as possible and to understand the mechanisms of infection in order to be able to control the disease.

Actually, New Caledonian shrimp farming has been affected since 1993 by a cool season vibriosis causing high mortalities in juvenile shrimp reared in earthen ponds, that was shown to be caused by *Vibrio penaeicida* and was named Syndrome 93 [6,12,25,31]. During Syndrome 93 epizootics, a few other *Vibrio* strains could be isolated from moribund shrimp septicemic hemocultures, including *V. nigripulchritudo* [6] that demonstrated to be highly pathogenic by experimental infection in healthy *L. stylirostris* [15]. These pathogenic strains were, at that time, geographically restricted to two adjoining farms within one bay [14] and therefore, zoosanitary recommendations were given in order to minimize the risk of disease spread. The industry's strategy has since been to avoid winter crops, thus limiting the impact of Syndrome 93. However *V. nigripulchritudo* was yet isolated in late December 1997 from moribund shrimp obtained from two ponds at one farm located 50 km south of the original isolation. The organism was also causing an epizootic due to systemic vibriosis [11], but in high water temperature conditions that did not fit the classical Syndrome 93 epidemiology, which typically occurs at lower water temperatures [13,25]. This new disease, which was named "Summer Syndrome" has affected all summer crops on this farm ever since. Since 2002, it also affected a new farm built in the close proximity of the affected one. If it was to affect all shrimp farms, the profitability of the industry would be seriously threatened.

The facts that (i) *V. nigripulchritudo* is associated with two distinct shrimp diseases and (ii) that one of these ("Summer Syndrome") is possibly an emergent disease highlight the need for accurate epidemiological data to gain appropriate knowledge and to propose adequate sanitary surveillance. Phenotypic identification of *V. nigripulchritudo* is easily conducted by traditional methods, but lacks a sufficient discriminating power for epidemiological studies. Therefore, the genetic structure of a New Caledonian *V. nigripulchritudo* collection has been studied using two molecular typing methods over a selection of 58 *V. nigripulchritudo* strains. The two methods used were MLST [24], and AP-PCR [39]. Results were analysed together with virulence patterns of the strains that had been previously determined [15]. Here, we describe the results of this study, compare the two molecular typing techniques used, and propose a surveillance scheme of the potential emergence of this new pathogen for the shrimp aquaculture industry of New Caledonia.

Materials and methods

Bacterial strains and geographical data

Both the type strain and wild-type isolates of *V. nigripulchritudo* were used in this study. *V. nigripulchritudo* CIP 103192^T (= ATCC 27043) was provided by Collection de l'Institut Pasteur. Fifty-seven wild-type isolates, from both clinical and environmental origins, isolated between May 1995 and November 2003 in 13 different farms and 3 hatcheries along almost 300 km of the New Caledonian West coast were included in this study (Table 1). The origin, identification and virulence of these *V. nigripulchritudo* isolates towards healthy *L. stylirostris* were determined previously [6,14,15] (see Table 1).

The shrimp farms and hatcheries included in the study are located on the southwest coast of New Caledonia (Fig. 1).

Clinical *V. nigripulchritudo* isolates originated from septicemic hemocultures associated with Syndrome 93 (= cool season vibriosis; 3 strains), Summer Syndrome (7 strains), and opportunistic vibriosis, i.e. affecting only a few shrimp under adverse pond conditions and lasting only a limited time (9 strains). Other strains were isolated when found (one or very few colonies) in hemocultures from either healthy shrimp (24 isolates) or moribund shrimp dying from a non-bacterial cause (4 strains). Environmental isolates were isolated from pumping water in a Summer Syndrome affected farm (2 isolates), pond water or sediment pore water in the two Summer Syndrome affected farms (7 strains) and from a healthy crab (*Portunus pelagicus*) carapace swab (1 isolate). Identification to the species level was achieved on the basis of phenotyping tests and specific PCR confirmation as described elsewhere [15]. Additionally, *V. penaeicida* strain AM101 [6,13,14], isolated in a Syndrome 93 context was included in this study, as an outgroup for phylogenetic analysis.

Out of these 59 strains, 25 were used for the MLST approach, including *V. nigripulchritudo* type strain and *V. penaeicida* AM101 as an outgroup (see Table 1). All of these 25 strains are deposited in the bacterial collection of the CRB (Centre de Ressources Biologiques, Laboratoire de Génétique et Pathologie, Institut Français de Recherche pour l'Exploitation de la MER [IFREMER], La Tremblade, France).

Culture conditions and extraction of bacterial genomic DNAs

Vibrio strains stored frozen at −80 °C in Marine Broth 2216E (Difco) with 17% glycerol were cultured in accordance with standard procedures [1], i.e. grown in Marine Broth 2216E (Difco) at 30 °C with continuous