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Arbitrarily Primed PCR To Type *Vibrio* spp. Pathogenic for Shrimp

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A molecular typing study on *Vibrio* strains implicated in shrimp disease outbreaks in New Caledonia and Japan was conducted by using AP-PCR (arbitrarily primed PCR). It allowed rapid identification of isolates at the genospecies level and studies of infraspecific population structures of epidemiological interest. Clusters identified within the species *Vibrio penaeicida* were related to their area of origin, allowing discrimination between Japanese and New Caledonian isolates, as well as between those from two different bays in New Caledonia separated by only 50 km. Other subclusters of New Caledonian *V. penaeicida* isolates could be identified, but it was not possible to link those differences to accurate epidemiological features. This contribution of AP-PCR to the study of vibriosis in penaeid shrimps demonstrates its high discriminating power and the relevance of the epidemiological information provided. This approach would contribute to better knowledge of the ecology of *Vibrio* spp. and their implication in shrimp disease in aquaculture.

Vibriosis is a major disease problem in shrimp aquaculture, causing high mortality and severe economic loss in all producing countries (5, 17, 21). *Vibrio* spp. are most often considered opportunistic pathogens in shrimp, but primary disease caused by highly virulent strains has also been reported (9, 13, 27). On the basis of phenotypic data, the major species causing vibriosis in shrimp are *Vibrio alginolyticus*, *V. anguillarum*, *V. harveyi*, and *V. parahaemolyticus* (14, 17, 18).

In New Caledonia (South Pacific), shrimp aquaculture is based on the complete cycle of *Penaeus stylirostris* in a semi-intensive farming system in earthen ponds. Located between latitudes 19°S and 23°S, New Caledonia has a tropical oceanic climate with a hot season from mid-November to mid-April. The average minimum and maximum morning water temperatures in shrimp ponds are 20.5°C in July and 28.2°C in February. Since 1993, shrimp farms have been affected by a disease, named syndrome 93, causing mass mortality with a significant decrease in yields and survival rates. Mortality episodes frequently occur during the southern winter, from mid-May to mid-September. Moribund prawns display a wide spectrum of clinical signs, including disoriented swimming, lethargy, weakness, and abnormal coloration of the body and appendages. High numbers of bacteria belonging to the genus *Vibrio* are systematically isolated from diseased shrimp hemolymph, revealing bacterial septicemia (20). Based on phenotypic and genotypic studies (ribotyping and DNA-DNA hybridization) (6), the species involved in syndrome 93 belong to the genospecies *V. alginolyticus*, *V. harveyi*, *V. nigripulchritudo*, and *V. penaeicida*. Since 1994, *V. penaeicida* and *V. nigripulchritudo* were the most frequently isolated, and their high pathogenicity was demonstrated in an in vivo experimental infection system in *P. stylirostris* (11).

Phenotype-based identification of marine bacteria relies on time-consuming techniques that have limited discriminating power (1, 2). The current genomic approaches used for the identification and the typing of *Vibrio* strains, such as DNA-DNA hybridization and ribotyping (1, 13, 23), are useful for taxonomic studies and identification to the subspecies level. However, reliable tools for strain differentiation are essential for studying epidemiology and pathogenicity. Arbitrarily primed PCR (AP-PCR) generates fingerprints that can be used to compare microorganisms at the species level and within a species with high discriminating power (30, 31). This method, which has successfully been applied to numerous bacterial species and strains (19, 24, 25, 32), is very reliable, does not require any previous knowledge of DNA sequences in the genome to be analyzed, and needs much less DNA than current molecular genotyping methods. The purpose of this study was to characterize and differentiate the *Vibrio* isolates involved in syndrome 93 by using fingerprints obtained with AP-PCR with regard to their geographic origins and the zootechnical practices used at the corresponding shrimp farms.

MATERIALS AND METHODS

Bacterial strains. Both reference strains and wild-type isolates were cultured in accordance with standard procedures (1).

(i) Reference strains. Four type strains, from the Collection of the Pasteur Institute (Paris, France), representing the major species previously identified in syndrome 93 mortality outbreaks were included in this study (Table 1): *V. penaeicida* KH-1^T (which was isolated from *Penaeus japonicus* in Japan), *V. alginolyticus* CIP 103336^T (= ATCC 17749), *V. harveyi* CIP 103192^T (= ATCC 14126), and *V. nigripulchritudo* CIP 103192^T (= ATCC 27043).

(ii) Wild-type isolates. Fifty-three field isolates from New Caledonia were selected as representative of the strains involved in outbreaks of vibriosis (syndrome 93) from January 1994 to June 1995 (Table 1). Forty-five were isolated from diseased shrimp during three mortality peaks that occurred between March and June 1995 in four different shrimp farms (Fig. 1). The eight remaining strains are representative of previous outbreaks. In order to compare pathogenic *V. penaeicida* isolates from New Caledonia with others field strains, three Japanese *V. penaeicida* strains (KO-1, KT-1, and PD-A) isolated from diseased *P. japonicus* shrimp were included in the studied set. New Caledonian field strains were isolated from hemolymph of diseased shrimp with vibriosis as the only or major morphotype. A single strain was conserved for each individual shrimp. All of the strains were isolated on Marine Agar 2216F (Difco Laboratories, Detroit, Mich.), except strain F14 (TCBS Agar; Difco). Identification to the species level

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TABLE 1. *Vibrio* sp. reference strains and field isolates used in the present study

Organism or source and isolate name, isolation date,[a] and origin	Identification
Reference strains	
V. alginolyticus CIP 103336[T][b]	
V. harveyi CIP 103192[T]	
V. nigripulchritudo CIP 103195[T]	
V. penaeicida KH-1[T]	
Japanese field strains	
V. penaeicida KO-1, 1986, Japan	
V. penaeicida KT-1, 1986, Japan	
V. penaeicida PD-A, 1986, Japan	
New Caledonian field strains	
Aquamon farm	
AQ61,[c] March 1994, NC[d]	*V. alginolyticus*[e]
AQ66,[f] March 1994, NC	*V. alginolyticus*[e]
AQ102, June 1995, NC	*V. penaeicida*
AQ103, June 1995, NC	*V. penaeicida*
AQ104, June 1995, NC	*V. penaeicida*
AQ105, June 1995, NC	*V. penaeicida*
AQ106, June 1995, NC	*V. penaeicida*
AQ107, June 1995, NC	*V. penaeicida*
AQ109, June 1995, NC	*V. penaeicida*
AQ110, June 1995, NC	*V. penaeicida*[e]
AQ111, June 1995, NC	*V. penaeicida*
AQ112, June 1995, NC	*V. penaeicida*[e]
AQ113, June 1995, NC	*V. harveyi*
AQ114, June 1995, NC	Not identified
Aquamer farm	
AM23, October 1994, NC	*V. penaeicida*[e]
AM101, May 1995, NC	*V. penaeicida*
AM102, May 1995, NC	*V. nigripulchritudo*
AM107, May 1995, NC	*V. penaeicida*
AM108, May 1995, NC	*V. penaeicida*
AM109, May 1995, NC	*V. nigripulchritudo*
AM111, May 1995, NC	*V. penaeicida*
AM112, May 1995, NC	*V. penaeicida*
AM113, May 1995, NC	*V. penaeicida*
AM114, May 1995, NC	*V. nigripulchritudo*
AM115, May 1995, NC	*V. nigripulchritudo*
AM116, May 1995, NC	*V. penaeicida*
FAO farm	
F1, May 1995, NC	*V. penaeicida*[e]
F2, May 1995, NC	*V. penaeicida*
F5, May 1995, NC	*V. penaeicida*
F6, May 1995, NC	*V. penaeicida*
F11, May 1995, NC	*V. penaeicida*
F14,[g] May 1995, NC	*V. penaeicida*[e]
F15, May 1995, NC	*V. penaeicida*
F24, May 1995, NC	*V. penaeicida*
F25, May 1995, NC	*V. penaeicida*
Sea Farm	
SF5, January 1994, NC	*V. alginolyticus*[e]
SF100, March 1995, NC	*V. penaeicida*[e]
SF101, March 1995, NC	*V. penaeicida*
SF113, March 1995, NC	*V. penaeicida*
SF116, March 1995, NC	*V. penaeicida*
SF121, March 1995, NC	*V. penaeicida*
SF122, March 1995, NC	*V. penaeicida*
SF125, March 1995, NC	*V. penaeicida*[e]
SF126, March 1995, NC	*V. penaeicida*
SF127, March 1995, NC	*V. penaeicida*
SF140, May 1995, NC	*V. penaeicida*[e]
SF143, March 1995, NC	*V. penaeicida*[e]
Sodacal farm	
SO27,[h] October 1994, NC	*V. nigripulchritudo*[e]
SO38, May 1995, NC	*V. nigripulchritudo*[e]
SO65, May 1995, NC	*V. nigripulchritudo*[e]
Webuihoone farm	
W1,[h] April 1994, NC	Not identified
W9, April 1994, NC	Not identified
W11, April 1994, NC	*V. harveyi*[e]

[a] The isolation date corresponds to the date of isolation from the field for field strains.
[b] T indicates type strain.
[c] Underlined strains were isolated on marine agar from diseased cultured *P. stylirostris* hemolymph containing more than 100 CFU of the monomorphous bacterial strain per drop of hemolymph.
[d] NC, strain isolated on marine agar 2216E (Difco Laboratories) from cultured *P. stylirostris* hemolymph in New Caledonia.
[e] Strain previously identified by DNA-DNA hybridization and ribotyping (6).
[f] Isolated in marine broth from diseased cultured *P. stylirostris* hemolymph.
[g] Isolated on TCBS agar from diseased cultured *P. stylirostris* hemolymph.
[h] Major strain isolated on marine agar from apparently healthy cultured *P. stylirostris* hemolymph.

was done by using phenotyping tests (Biotype 100, Api System; BioMérieux, Marcy l'Etoile, France). In addition, selected strains (Table 1) were subjected to DNA-DNA hybridization and ribotyping in a parallel taxonomic study (6).

Geographical and zootechnical data. The shrimp farms included in the study are located on the southwest coast of New Caledonia (Fig. 1). Aquamon (AQ isolates), FAO (F isolates), and Sea Farm (SF isolates) are located on Saint Vincent Bay. Sodacal (SO isolates) and Aquamer (AM isolates) are located on Moindou Bay, 50 km north of Saint Vincent Bay. Webuihoone (W isolates) is located 150 km north of Moindou Bay. Aquamer is located at the Moindou estuary; there is evidence that it recycles some of the outlet water from Sodacal, as well as its own. Postlarvae stocked in the ponds originate from two separate hatcheries. Aquamer and Sodacal usually stock postlarvae from the Mara hatchery, located close to Sodacal on Moindou Bay, whereas Aquamon, FAO, and Sea Farm usually stock postlarvae from the Montagnes hatchery, located near Aquamon on Saint Vincent Bay, but there are many transfers of postlarvae between

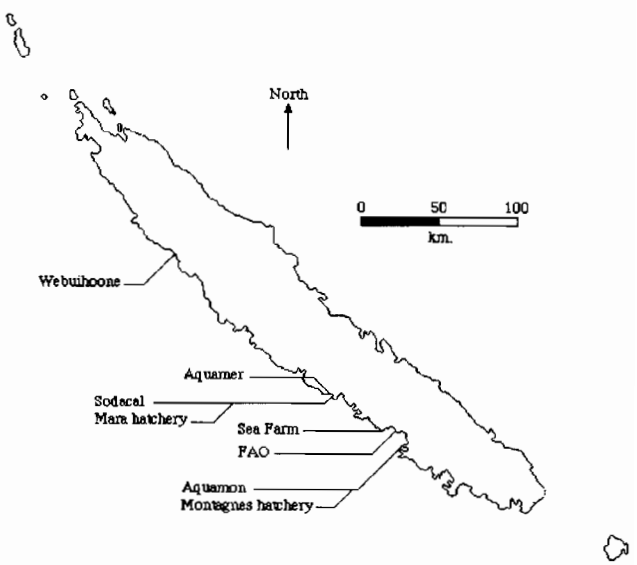

FIG. 1. Schematic map of New Caledonia showing the locations of the shrimp farms and hatcheries discussed in this report.

these two bays. A preliminary field survey did not find either *V. penaeicida* or *V. nigripulchritudo* strains in any postlarval stock.

Extraction of bacterial genomic DNAs. Vibrio strains were cultured in tryptic soy broth (BioMérieux) supplemented with 2% NaCl (Sigma Chemical Co., St. Louis, Mo.) at 30°C with continuous shaking until the stationary phase of growth was reached. DNAs were extracted and purified by two different methods. (i) Cultures (50 ml) were harvested by centrifugation at 10,000 × *g* for 10 min. The resultant pellets were lysed with a 1% sodium dodecyl sulfate (SDS)–1-mg · ml^{-1} proteinase K solution, and the bacterial nucleic acids were extracted by a phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) mixture as described by Brenner et al. (4). Extracted DNAs were resuspended in 1× Tris-EDTA buffer. (ii) DNAs were also extracted by using silica particles and guanidinium isothiocyanate lysis buffer in accordance with the 2-h method described by Boom et al. (3) from small culture aliquots (3 to 5 ml).

AP-PCR. Fingerprinting was performed as described previously (30), with minor modifications. Primers KF (5′-CACACGCACACGGAAGAA-3′), KN (5′-CCTTGCGCGCATGTACATGG-3′), RSP (5′-GGAAACAGCTATGACC ATGA-3′), KZ (5′-CCCATGTGTACGCGTGTGGG-3′), KpnR (5′-CCAAGT CGACATGGCACRTGTATACATAYGTAAC-3′), KG (5′-CACACGCACAC GGAAGAA-3′), and SP (5′-TTGTAAAACGACGGCCAG-3′) were purchased from Genset (Paris, France). Fifty-microliter reaction mixtures were prepared with 100 ng of DNA–1× *Taq* polymerase buffer (100 mM Tris [pH 8.3, 20°C], 500 mM KCl)–$MgCl_2$–0.2 mM each deoxynucleoside triphosphate (Boehringer, Mannheim, Germany)–1 μM single oligonucleotide primer–5 μCi of [^{32}P]dCTP (3,000 Ci/mmol; Amersham International, Amersham, England)–1.25 U of *Taq* polymerase (Amersham).

Amplification reactions were cycled twice in a 96-well GeneAmp 9600 thermocycler (Perkin-Elmer) through a low-stringeney temperature profile and then 40 times through a high-stringency temperature profile as previously described (24). Five microliters of each reaction mixture was combined with 15 μl of 98% formamide dye and heated to 68°C for 15 min; 5 μl of each sample was loaded onto a 4% acrylamide–50% urea sequencing gel with 1× TBE (90 mM Tris-borate, 2 mM EDTA), and electrophoresis was performed at 400 V overnight until the xylene cyanol tracking dye was approximately 10 em from the bottom. pUCBM21 DNA digested by *Hpa*II plus pUCBM21 DNA digested by *Dra*I and *Hin*dIII (Boehringer), pBR328 DNA digested by *Bgl*I and *Hin*fI (Boehringer), and pBR322 DNA digested by *Msp*I (New England Biolabs) were used as molecular size markers. The gel was autoradiographied for 24 to 72 h on Kodak X-Omat X-ray film. Amplicon molecular size was determined by interpolation of the distances of migration (12) of molecular size markers and AP-PCR products. In accordance with Welsh and McClelland (30), only major bands were considered in the analysis as share-derived characters, and this allowed the construction of a key for type grouping of the strains according to the amplicons produced with a given primer (see Table 2).

In order to select primers producing more polymorphism, a first screening of AP-PCR products without [^{32}P]dCTP was performed by using electrophoresis on a 2% agarose gel (NuSieve 3:1, FMC, Rockland, Maine) with 0.5× TBE buffer (3 V/cm for 16 h), followed by ethidium bromide staining.

RESULTS

Primer selection. On the basis of the fingerprints observed on agarose gel, three (RSP, KF, and SP) of the seven primers tested were selected for accurate study with acrylamide-urea sequencing gel.

DNA extraction techniques and reliability. The fingerprints obtained by AP-PCR using the two different techniques of DNA extraction were identical. The only differences, sporadically observed, were due to insufficient quantities of genomic DNA in the amplification mixture, i.e., 50 instead of 100 ng. These variations affected only a few bands in the fingerprints (data not shown). Therefore, concentrations of genomic DNA extracts were carefully monitored by spectrophotometric determination at 260 nm.

Species identification. On the basis of the AP-PCR fingerprints, 54 of the 57 field isolates were identified as belonging to one of the four species for which a reference strain was included in the studied set (Table 1). Examples are shown in Fig. 2 to 4. The remaining three New Caledonian strains (AQ114, W9, and W1) produced fingerprints unrelated to the AP-PCR patterns of the four type strains included in this study, were characterized by the complete absence of any species-specific amplicon, and, consequently, were not identified. For these three strains, phenotypic tests previously performed were inconclusive. Some highly conserved amplicons were found to be

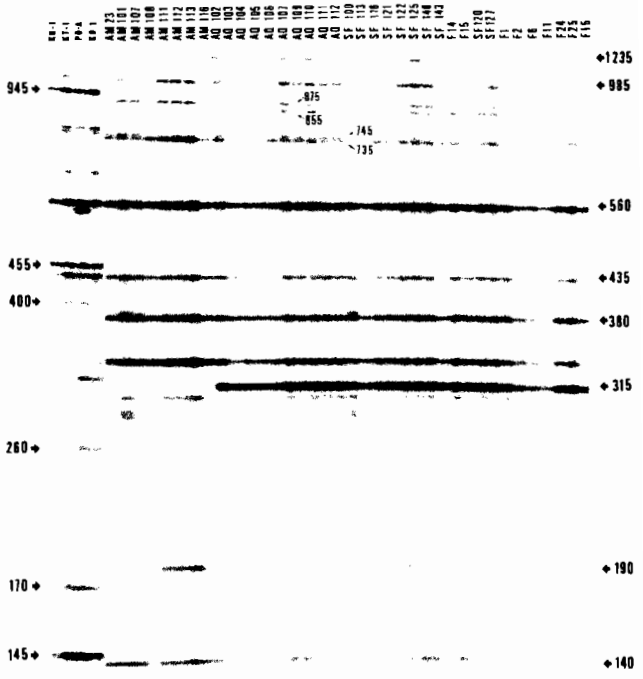

FIG. 2. AP-PCR fingerprints of selected *V. penaeicida* strains produced with primer SP. Shown is an autoradiogram of a denaturing 5% acrylamide gel with TBE. The data for isolates in the upper part are given in Table 1. Molecular sizes (in base pairs) determined as described in Materials and Methods are given on the sides.

genospecies specific. (i) With primer SP, *V. penaeicida* was characterized by 335-, 435-, 560-, and 985-bp fragments, *V. nigripulchritudo* was characterized by 225-, 440-, 565-, 940-, and 1,240-bp fragments, *V. harveyi* was characterized by 165-, 310-, 365-, 510-, 745-, and 970-bp fragments, and *V. alginolyticus* was characterized by 230-, 595-, and 745-bp fragments (Fig. 2). (ii) With primer RSP, *V. penaeicida* was characterized by 160-, 210-, 215-, 405-, 770-, and 860-bp fragments, *V. nigripulchritudo* was characterized by 355-, 650-, 675-, and 940-bp fragments, *V. harveyi* was characterized by 150-, 195-, 205-, 270-, 295-, 355-, 365-, 450-, and 540-bp fragments, and *V. alginolyticus* was characterized by 225-, 275-, 450-, 480-, 525-, and 745-bp fragments (Fig. 3). (iii) With primer KF, *V. penaeicida* was characterized by 170-, 220-, 360-, and 725-bp fragments, *V. nigripulchritudo* was characterized by 215-, 250-, 400-, and 825-bp fragments, *V. harveyi* was characterized by 300-, 475-, and 1,065-bp fragments, and *V. alginolyticus* was characterized by 545-, 645-, and 735-bp fragments (Fig. 4).

The New Caledonian *Vibrio* isolates were distributed among the four genospecies as following: isolates AQ61, AQ66, and SF5 were identified as *V. alginolyticus*, isolates AQ113 and W11 were identified as *V. harveyi*, and isolates AM102, AM109, AM114, AM115, SO27, SO38, and SO65 were identified as *V. nigripulchritudo*. The remaining 38 isolates were identified as *V. penaeicida* (as detailed in Table 1). The three Japanese isolates were confirmed to belong to *V. penaeicida*.

Intraspecies differences among *V. penaeicida* strains. Comparative results are summarized in Table 2. Japanese *V. penaeicida* isolates could be discriminated from ones from New Caledonia by using each of the three primers. With primer SP, Japanese *V. penaeicida* strains were characterized by 145-, 170-, 260-, 400-, 455-, 870-, and 945-bp fragments and New

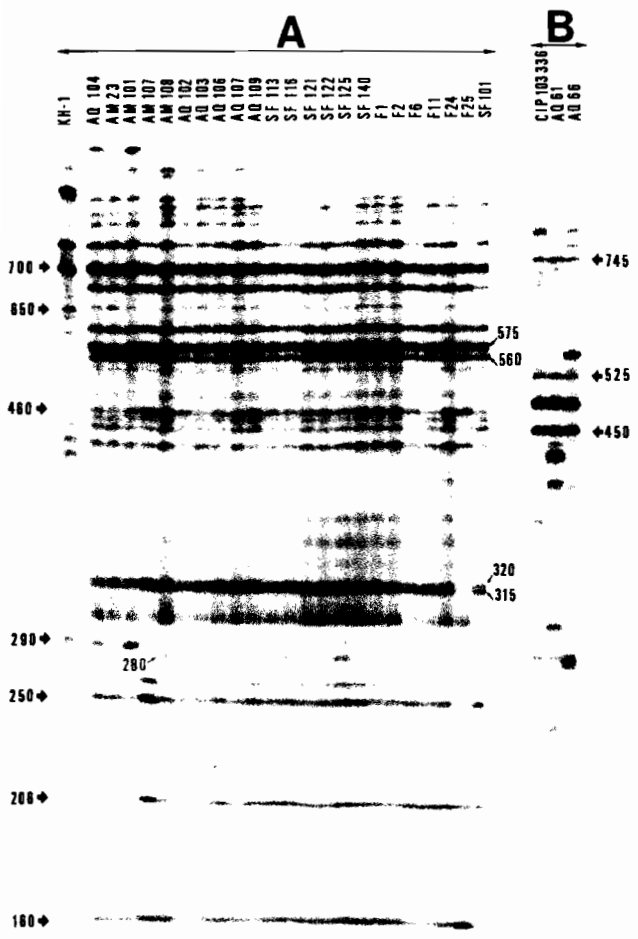

FIG. 3. Autoradiogram of AP-PCR fingerprints of *V. penaeicida* (A) and *V. alginolyticus* (B) produced with primer RSP. The lane assignments for isolates are given in Table 1. Molecular sizes (in base pairs) determined as described in Materials and Methods are given on the sides.

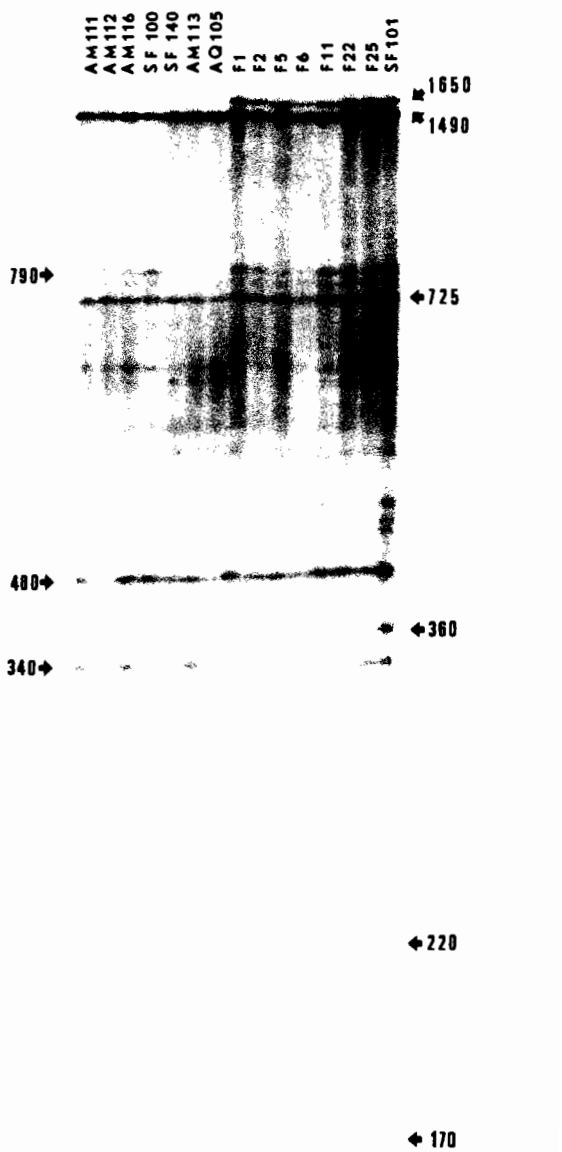

FIG. 4. Autoradiogram of AP-PCR fingerprints of selected *V. penaeicida* field isolates produced with primer KF. The data for isolates in the upper part are given in Table 1. Molecular sizes (in base pairs) determined as described in Materials and Methods are given on the sides.

Caledonian ones were characterized by 140-, 190-, 280-, 285-, 380-, 735-, 745-, and 875-bp fragments (Fig. 2). With primer RSP, Japanese *V. penaeicida* strains were characterized by 185-, 280-, and 650-bp fragments and New Caledonian ones were characterized by 200-, 250-, 315-, 320-, 480-, 560-, 575-, 695-, and 700-bp fragments (Fig. 3). With primer KF, Japanese *V. penaeicida* strains were characterized by 210- and 760-bp fragments and New Caledonian ones were characterized by 340-, 400-, 790-, and 1,490-bp fragments (Fig. 4).

It was also possible to discriminate between the *V. penaeicida* isolates from New Caledonia. With primer SP, New Caledonian *V. penaeicida* was discriminated by 315-, 795-, 855-, and 1,235-bp fragments; fingerprints of the strains originating from Aquamer were missing these fragments (Fig. 2). With primer RSP, New Caledonian *V. penaeicida* was discriminated by a 280-bp fragment; fingerprints of the strains originating from FAO, Aquamer, and SF101 were missing these fragments (Fig. 3). Lastly, with primer KF, the additional presence of a 1,650-bp fragment allowed the individualization of FAO isolates and of 3 (SF101, SF126, and SF127) of the 11 Sea Farm isolates. This discrimination with primer KF was also possible by using the agarose gel-ethidium bromide technique.

DISCUSSION

Few researchers have used molecular biology tools for epidemiological studies on aquaculture-pathogenic marine *Vibrio* sp. isolates. Their genomic diversity was first investigated by using ribotyping (2, 22, 29) or plasmid profiling (10, 22, 26, 29). However, if ribotyping is useful for taxonomic studies or subtyping, its discriminating power can be limited for studying population structures. Plasmid profiling can provide interesting results for *Vibrio* isolates, but the data obtained with this approach are limited to the extrachromosomal genome (15). Lastly, AP-PCR was recently demonstrated as useful for fast

TABLE 2. Distribution of *V. penaeicida* field isolates according to the presence or absence of specific AP-PCR products obtained with primers SP, RSP, and KF[a]

Isolate (origin)[b]	Presence of fragment or fragment cluster:				
	A[c]	B[d]	C[e]	D[f]	E[g]
KO-1 (J)	+	−	−	−	−
PD-A (J)	+	−	−	−	−
KT-1 (J)	+	−	−	−	−
AM23 (NC)	−	+	−	−	−
AM101 (NC)	−	+	−	−	−
AM107 (NC)	−	+	−	−	−
AM108 (NC)	−	+	−	−	−
AM111 (NC)	−	+	−	−	−
AM112 (NC)	−	+	−	−	−
AM113 (NC)	−	+	−	−	−
AM116 (NC)	−	+	−	−	−
AQ102 (NC)	−	+	+	+	−
AQ103 (NC)	−	+	+	+	−
AQ104 (NC)	−	+	+	+	−
AQ105 (NC)	−	+	+	+	−
AQ106 (NC)	−	+	+	+	−
AQ107 (NC)	−	+	+	+	−
AQ109 (NC)	−	+	+	+	−
AQ110 (NC)	−	+	+	+	−
AQ111 (NC)	−	+	+	+	−
AQ112 (NC)	−	+	+	+	−
SF126 (NC)	−	+	+	+	+
SF127 (NC)	−	+	+	+	+
SF100 (NC)	−	+	+	+	−
SF113 (NC)	−	+	+	+	−
SF116 (NC)	−	+	+	+	−
SF121 (NC)	−	+	+	+	−
SF122 (NC)	−	+	+	+	−
SF125 (NC)	−	+	+	+	−
SF140 (NC)	−	+	+	+	−
SF143 (NC)	−	+	+	+	−
SF101 (NC)	−	+	+	−	+
F1 (NC)	−	+	+	−	+
F2 (NC)	−	+	+	−	+
F5 (NC)	−	+	+	−	+
F6 (NC)	−	+	+	−	+
F11 (NC)	−	+	+	−	+
F14 (NC)	−	+	+	−	+
F15 (NC)	−	+	+	−	+
F24 (NC)	−	+	+	−	+
F25 (NC)	−	+	+	−	+

[a] The data for the isolates in the left column are given in Table 1.
[b] J, Japan; NC, New Caledonia.
[c] Includes 145-, 170-, 260-, 400-, 455-, and 945-bp fragments obtained with primer SP, 290- and 650-bp fragments obtained with primer RSP, and 210- and 760-bp fragments obtained with primer KF.
[d] Includes 140-, 190-, 280-, 285-, 380-, 735-, 745-, and 875-bp fragments obtained with primer SP, 200-, 250-, 315-, 320-, 480-, 560-, 575-, 695-, and 700-bp fragments obtained with primer RSP, and 340-, 400-, 790-, and 1,490-bp fragments obtained with primer KF.
[e] Includes 315-, 795-, 855-, and 1,235-bp fragments obtained with primer SP.
[f] A 280-bp fragment obtained with primer RSP.
[g] A 1,650-bp fragment obtained with primer KF.

identification of species and strains of *Vibrio* (19) and another arbitrary amplification method, random amplification of polymorphic DNA (33), was shown to be efficient for the differentiation of the two biotypes of *V. vulnificus* (2). Arbitrary amplification of DNA, which allows analysis of the whole genome, is considered a powerful approach for the study of DNA polymorphism and is usable for the comparison of genomes from eukaryotes (31) or bacteria (25, 32). In this case, AP-PCR can be used for species identification and provides information on intraspecific differences usable for molecular epidemiology studies (25, 32).

Analysis of AP-PCR fingerprints allowed us to categorize a significant set of *Vibrio* field isolates. The epidemiological approach of our study is original in that 35 of 53 field isolates were included on the sole basis of their isolation in high numbers in moribund-shrimp hemolymph during syndrome 93 mortality episodes without any previous analysis of these strains; the 18 others were previously studied by ribotyping (Table 1) (6). AP-PCR allowed identification to the genospecies level when fingerprints of field isolates were compared with those provided by reference strains of genospecies. In addition, complete agreement between AP-PCR and ribotyping data was observed. Fifty of the 53 field isolates were identified as belonging to one of the four genospecies included in the study. The three Japanese isolates exhibited AP-PCR fingerprints which were characteristic of *V. penaeicida*.

All isolates originating from the three mortality peaks that occurred in 1995 were identified as *V. penaeicida* or *V. nigripulchritudo*, except two strains (one *V. harveyi* and one not identifiable). This observation confirms the major epidemiological role of these two genospecies in the pathogenesis of syndrome 93 and demonstrates that several *Vibrio* species and strains were implicated in the pathogenesis of this syndrome, indicating the importance of environmental factors and zootechnical practices. Although *V. penaeicida* was first described (13) in a shrimp vibriosis in Japan, this is the first report on the possible pathological role of *V. nigripulchritudo* in marine aquaculture. The earlier role of *V. alginolyticus* and *V. harveyi* in the pathology could have been surpassed by the more pathogenic strains of *V. penaeicida* and *V. nigripulchritudo* (11). This hypothesis could explain the change in the infection pattern of *Vibrio* species involved in the outbreaks, with a major role of *V. alginolyticus* and *V. harveyi* in the initial episodes (1993 and 1994) followed in 1995 by the increasing impact of strains of *V. penaeicida* and *V. nigripulchritudo*.

The present study, conducted with a significant number of *V. penaeicida* isolates, shows the heterogeneity of these strains according to their geographical origins, since AP-PCR fingerprinting allows clustering of isolates. At a first level, the discrimination between isolates originating from Japan and those from New Caledonia was readily possible whatever the primer used. At a second level, on the basis of the presence or absence of some fragments, the SP fingerprints of the *V. penaeicida* strains isolated in New Caledonia were heterogeneous, with two distinct clusters. Similar results were observed with RSP and KF fingerprints. These two clusters were regarded as topotypes located 50 km apart on Saint Vincent Bay and Moindou Bay, as there was a perfect correlation between the geographical area of origin and a particular fingerprint. These two bays are quite different in ecology, so these two topotypes could have been selected during ecological adaptation to these two respective environments. Moindou Bay is relatively closed, with an important mangrove area, and is probably strongly influenced by aquaculture activities, whereas Saint Vincent Bay is a wide and open bay with few mangrove areas. Within the Saint Vincent Bay topotype, further discrimination was possible that could not be attributed to any geographical, chronological, ecological, or zootechnical differences. However, these investigations would be deepened by phenotypic and virulence studies using representative strains from each cluster. Conversely, the *V. nigripulchritudo* fingerprints were homogeneous whatever the primers used, but these data have to be

confirmed by studying a more comprehensive set of field isolates in order to investigate possible heterogeneity. The results of the present study demonstrate the practical value of this PCR-based strategy in studying marine *Vibrio* isolates for molecular epidemiology purposes. This approach may be applied to the analysis of other marine *Vibrio* strains, especially in aquaculture-pathogenic *Vibrio* species.

All isolates identified as *V. nigripulchritudo* originated from Sodacal and Aquamer, both of which are located on Moindou Bay. Therefore, it seems that pathogenic *V. nigripulchritudo* strains were absent from Saint Vincent Bay at the time of the survey. Despite numerous postlarval transfers between these two bays, there has been neither any contamination by pathogenic *V. nigripulchritudo* in Saint Vincent Bay nor any exchange of *V. penaeicida* topotypes between these two bays, confirming the absence of these pathogenic strains in postlarval stocks. At the time of the survey, both species and derived clusters could be considered geographically restricted. Considering the topotypes and the long persistence of *V. penaeicida* strains in seawater (9), it can be assumed that shrimp ponds were contaminated by the intake water pumped from the bays and that vibriosis must thus be considered a waterborne disease.

This should be confirmed by further ecological studies, but zoosanitary measures should already be taken to avoid possible dissemination of the pathogens. Therefore, shrimp farmers were advised against transfer of live shrimp between the different farms and hatcheries on the different bays. If transfer is necessary, the animals should be treated with antibiotics at the time of transfer, and the water used for the transfer should be sterilized by, as an example, chlorination-dechlorination methods.

Our results demonstrate the practical value of AP-PCR for studying marine *Vibrio* isolates for molecular epidemiology purposes. This approach may be applied to the analysis of other marine *Vibrio* species involved in mariculture pathology. The development of species-specific nonradioactive DNA probes derived from the highly conserved amplicons produced by AP-PCR (16) would simplify identification at the species or subspecies level. In addition, our field results emphasize the importance and the similarity of the respective virulence of *V. penaeicida* and *V. nigripulchritudo* isolates for *P. stylirostris*, concurrently demonstrated by experimental infection studies (11). As virulence plasmids have been reported in *V. anguillarum*, the agent of vibriosis in fish (7, 8, 28), a study aiming to correlate virulence with the plasmid profiles of *V. penaeicida* and *V. nigripulchritudo* isolates is currently in progress in our laboratory.

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