Annals of Microbiology, **58 (1)** 141-146 (2008)

Phenotypic and genetic diversity of *Vibrio alginolyticus* strains recovered from juveniles and older *Sparus aurata* reared in a Tunisian marine farm

Mejdi SNOUSSI[1]*, Hafedh HAJLAOUI[1], Emira NOUMI[1], Stefania ZANETTI[2], Amina BAKHROUF[1]

[1]Laboratoire d'Analyse, Traitement et Valorisation des Polluants de l'Environnement et des Produits, Department of Microbiology, Faculty of Pharmacy, Rue Avicenne, Monastir 5000, Tunisia; [2]Dipartimento di Scienze Biomediche, Sezione di Microbiologia Sperimentale e Clinica, Universita di Sassari, Viale San Pietro 43/b, 07100 Sassari, Italy

Received 31 October 2007 / Accepted 28 January 2008

Abstract - This study characterises 43 *Vibrio alginolyticus* strains associated with diseased juveniles and older fish of *Sparus aurata* reared in a marine hatchery installed along the seacoasts of Monastir (Centre of Tunisia). *Vibrio alginolyticus* strains were isolated using the TCBS modified agar plates and the biochemical activities were tested using the API 20 E strips. The exoenzymes production and antibiotics susceptibility were also investigated. The ERIC-PCR was used to evaluate the genetic diversity of these strains. *Vibrio alginolyticus* was isolated from seawater (n = 16), juveniles with white nodular skin lesions (n = 9) and from all the internal organs of the older fish presenting a large and deep lesions in the muscle and with a necrotic eyes (n = 18). Most of the studied *V. alginolyticus* strains were β-haemolytic, hydrolyze the DNA and produce many exoenzymes such as lecithinase, caseinase, amylase and lipase. All tested strains were resistant to at least three antimicrobial agents. The ERIC-PCR profiles among the isolated bacteria were generally heterogeneous, showing a high polymorphism and suggesting independent circulation with some evidence of cross-transmission.

Key words: *Vibrio alginolyticus*, exoenzymes, antibiotic susceptibility, ERIC-PCR.

INTRODUCTION

The gilthead sea Bream (*Sparus aurata* Linnaeus, 1758), is an important species for Mediterranean hatchery thanks to its high economic value and its ability to adapt to environmental in salinity and temperature (Divanch and Kentouri, 1983). The low survival in larval stages and bacterial diseases represents the major problems affecting this species in the hatchery over the word. Bacterial diseases occur in cultured fish and are responsible for mass stock mortality in all the fish farms throughout the Mediterranean waters (Bakhrouf *et al.*, 1995; Bordas *et al.*, 1996; Zanetti *et al.*, 2000; Tulay and Gülşen, 2002; Ben Kahla-Nakbi *et al.*, 2006). The live prey (*Artemia* and Rotifers) represents the route of *Vibrio alginolyticus* to gilthead sea bream larvae and can cause severe losses in the marine hatchery (Snoussi *et al.*, 2006).

Some vibrios species such as *V. cholera*e, *V. parahaemolyticu*s, and *V. vulnificus* are serious human pathogens transmitted by contaminated water and food (Wachsmuth *et al.*, 1994; Finkelstein *et al.*, 2002). *Vibrio alginolyticus* was associated with gastroenteritis in immunocompromised patients (Reina *et al.*, 1995) and cause extra-intestinal diseases (Gahrn-Hansen and Hornstrup, 1994; Gomez *et al.*, 2003).

* Corresponding author. Phone: +21673461000; Fax: +21673461830; E-mail: snmejdi@yahoo.fr

Several methods have been used for identification and molecular typing of the clinical and environmental *Vibrio alginolyticus* strains and others vibrios (Fabiano *et al.*, 2004). The enterobacterial repetitive intergenic consensus (ERIC-PCR) technique is frequently used to type *Vibrio parahaemolyticus* (Marshall *et al.*, 1999; Ashraf at *al.*, 2001), *Vibrio alginolyticus* (Ben Kahla-Nakbi *et al.*, 2006) and other Gram-positive bacteria (Chaieb *et al.*, 2005).

The purpose of this study was to characterise the *Vibrio alginolyticus* associated with infection of gilthead sea bream juveniles and older fish, to study their biochemical activities, exoenzymes production, antimicrobial susceptibility and their genetic diversity using ERIC-PCR method.

MATERIALS AND METHODS

Samples. Juveniles of *Sparus aurata* (weight: 7 g and length: 8 cm) and older fish (weight: 220 g and length: 20 cm) were obtained from a Tunisian farm installed along the Mediterranean Sea coasts (Monastir, Centre of Tunisia) during the summer 2006. The juveniles were reared in a basin with a depth ranging from 1 to 1.5 m and with a capacity of 143 juveniles/m^3. Older fish were hatched in a second basin with a depth ranging from 0.5 to 1 m and with a density of 33 fish/m^3. Juveniles and older *Sparus aurata*

fish were fed with artificial and commercial diets. The seawater salinity was 37‰ and the temperature was ranging from 20 to 25 °C. The clinical signs of vibriosis touching juveniles in this hatchery included darkened body colour, white nodular skin lesion and sudden death. In the infected older fish, the visceral organs were haemorrhagic, the intestine was usually spotty and there are haemorrhages in the skeletal muscle. The ulcer was very deep and necrotic. Focal haemorrhages were also seen on the surface of the heart and the gills are usually pale (Austin *et al.*, 1993).

Biochemical characterisation of isolated strains. A group of ten juveniles and ten older fish were used in this study. Bacteria were isolated from white nodular skin lesions associated with juveniles, water entry flow and from the internal organs of chronically diseased older fish (Gills, spleen, liver and kidney). All samples were cultured on alkaline peptone water (1% NaCl, pH 8.6) and incubated at 30 °C for 18 to 24 h.

A loopful of the enrichment culture was streaked onto Thiosulphate-citrate-bile salt-sucrose modified agar used for the selective isolation of *V. parahaemolyticus* (TCBS modified agar, Scharlau Microbiology, Spain). After 18 to 24 h of incubation at 30 °C, yellow colonies were randomly selected then subcultured on Tryptic soy agar (TSA, Difco, Spain) supplemented with 1% NaCl and identified using morphological, physiological and the various biochemical tests included in API 20 E strips (Bio Mérieux, Marcy l'Étoile, France). In this study, the identification procedure of environmental *Vibrio alginolyticus* strains using the API 20 E strips was modified in order to inoculate a loopful of a bacterial suspension prepared on sterile saline water (2.5% NaCl). All strains tested with this modification and using the API Web software were successfully identified with a high level of acceptance. *Vibrio alginolyticus* strains were stored frozen at −80 °C in Luria-Bertani broth supplemented with 2% NaCl and 15% glycerol.

Exoenzymes production. For exoenzymes productions, all strains were tested for lipase on a medium including Tween 80, haemolysin (Human blood agar, BIO-RAD, France) and DNA hydrolysis (DNase Agar, Scharlau Microbiology). The enzymes amylase, caseinase and lecithinase were detected on media prepared with Phosphate Buffer Saline (PBS) supplemented respectively with 0.5% starch, 5% skim milk powder and 5% egg yolk emulsion were tested as described previously by Hörmansdorfer *et al*. (2000) and modified by Snoussi *et al.* (2006). After incubation up to 72 h at 37 °C, the formation of a clear zone caused by protein degradation is considered a positive test (Zanetti *et al.*, 2000).

Antibiotic susceptibility. The antibiotic susceptibility was determined using the disc diffusion method. After incubation at 30 °C for 18 to 24 h, the diameter of the inhibition zone was determined according to the Comité de la Société Française de l'Antibiogramme (Cavallo, 2006) and followed the recommendations of the NCCLS (2002) guidelines. The antibiotics tested on Mueller Hinton agar plates supplemented with 1% NaCl were selected amongst those used for the treatment of *Enterobacteriaceae* or *Vibrio cholerae* human infection as described by Ottaviani *et al.* (2001) and Ripabelli *et al.* (2003). Antimicrobial discs (Oxoid, England) were used at concentration of: chloramphenicol (30 µg); erythromycin (15 UI); ceftazidim (30 µg); nalidixic acid (30 µg); tetracycline (30 UI); ampicillin (10 µg); gentamycin (10 UI); cefotaxime (30 µg) and ciprofloxacin (5 µg).

Disk diffusion susceptibility test against the vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine (O/129, Oxoid) was performed on Mueller Hinton agar plates supplemented with 1% NaCl using 150 µg diagnostic disk as described by Elliot *et al.*, (1992).

Enterobacterial repetitive intergenic consensus polymerase chain reaction. All isolated *Vibrio alginolyticus* strains were grown overnight at 30 °C on TSA plate supplemented with 1% NaCl. Total DNA was extracted according to the scheme described by Sambrook *et al.* (1989) and stored at −20 °C until required.

The primer used for ERIC-PCR was the ERIC 2 primer. Each PCR reaction contain 25 µl mixture: 3 µl of genomic DNA, 5 µl of green GO *Taq* buffer (5x), 200 µM of each deoxynucleoside triphosphates (dNTP), 25 µM of the ERIC2 primer (5'-AGTAAGTGACTGGGGTGAGCG-3') and 1 U of GO *Taq* DNA polymerase (Promega, USA). ERIC 2-PCR was performed using a thermal cycler (Peltier Thermal Cycler, MJ. Research, INC, USA). Genomic DNA was amplified through 35 cycles with an initial denaturation step (95 °C for 30 s), hybridisation (52 °C for 45 s) and annealing at 72 C for 1.5 min. Five microlitres of the PCR products were analysed on 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) then visualised under UV transilluminator and photographed using Gel Doc XR apparatus (Bio-Rad, USA). The Bench Top pGEM (Promega) was used as a DNA molecular weight marker.

RESULTS

Biochemical characterisation of isolated strains

Yellow colonies isolated from TCBS modified agar identified as Gram-negative motile fermentative rods, producing enzymes like catalase and oxidase, susceptible to vibriostatic compounds O/129 (150 µg/disk) and swarming colonies on TSA 1% NaCl were characterised biochemically using API 20 E strips.

Most strains (41/43) were Voges-Proskauer and lysine decarboxylase positive. Five strains were ornithine decarboxylase positive. However, all strains were negative for arginine dihydrolase and ONPG test. All the strains grew in peptone water prepared respectively with 3, 8 and 10% of NaCl, produced acids from glucose, sucrose and mannitol but no acids were produced from arabinose, lactose, rhamnose, lactose, melibiose, sorbitol and inositol. Sixteen strains were amygdaline positive.

Antibiotic susceptibility

The antibiotic susceptibility of the *Vibrio alginolyticus* strains showed wide resistance to the previously tested antibiotics (Table 1). Most strains 37/43 were resistant to at least six antibiotics. Height strains isolated from seawater (strains 20, 22, and 23), liver (strain 31 and 33), blood (strain 37), kidneys (strain 30) and white nodular skin mucus (strain 9) were resistant to all antibiotic tested in this study. *Vibrio alginolyticus* strains were resistant to ampicillin (98%), to gentamycin (84%), to erythromycin and tetracycline (88%), and to cefotaxim (86%).

TABLE 1 - Biochemical characteristics and ERIC-PCR patterns of the 43 *Vibrio alginolyticus* identified from seawater, juveniles and older *Sparus aurata* reared in the hatchery isolated during summer 2006

Culture number	Biotype	Origins		Profile of resistance*										ERIC-PCR profile**
				C	E	CAZ	AN	TE	CB	AM	GM	CTX	CIP	
1	4145124	Juveniles		–	–	–	–	–	+	+	+	–	+	A
2	4347125			–	+	–	+	+	+	+	+	–	+	B
3	4147125			–	+	+	–	–	+	+	+	–	+	A
4	4047124			–	+	+	–	+	+	+	+	+	–	A
5	4147125			–	+	+	+	+	+	+	+	+	–	C
6	4047124			–	+	+	–	+	+	+	+	–	–	D
7	4247125			–	+	+	–	+	+	+	+	+	+	D
8	4047124			–	+	+	–	+	+	+	+	+	+	D
9	4147125			+	+	+	+	+	+	+	+	+	+	E
10	4147125	Seawater		–	+	–	–	+	+	+	+	+	+	F
11	4147125			–	+	–	–	–	+	+	+	–	+	F
12	4147125			–	+	–	–	+	+	+	+	–	+	F^{-1}
13	4147125			+	+	+	+	+	–	–	+	–	+	G
14	4247125			–	+	+	–	+	+	+	+	–	+	H
15	4047124			–	+	+	–	+	+	+	+	+	–	I
16	4246125			–	+	+	–	+	+	+	+	+	+	I
17	4145124			–	+	+	+	+	+	+	+	+	+	I^{-1}
18	4147124			–	–	+	–	–	+	+	–	+	+	J
19	4047124			–	+	+	+	+	+	+	+	+	+	K
20	4145124			+	+	+	+	+	+	+	+	+	+	L
21	4145124			–	+	+	+	+	+	+	–	+	+	M
22	4145124			+	+	+	+	+	+	+	+	+	+	N
23	4145124			+	+	+	+	+	+	+	+	+	+	O^{-1}
24	4145124			–	+	–	–	–	+	+	–	–	–	O
25	4147124			–	–	+	–	+	+	+	–	+	+	O
26	4047124	Older fish	Spleen	–	–	–	–	+	+	+	–	–	–	P
27	4047124			–	+	+	–	+	+	+	+	+	+	P^{+1}
28	4047124			–	+	+	+	+	+	+	+	+	+	P
29	4047124			–	+	+	–	+	+	+	+	+	+	Q
31	4047124		Liver	+	+	+	+	+	+	+	+	+	+	S
32	4247125			–	+	+	+	+	+	+	+	+	+	T
33	4047124			+	+	+	+	+	+	+	+	+	+	U
34	4147125		Gills	+	–	+	+	+	+	+	+	+	+	S
35	4047124			–	+	+	+	+	+	+	+	+	+	V
36	0046124			–	+	+	+	+	+	+	+	+	+	W
37	4147125		Blood	+	+	+	+	+	+	+	+	+	+	W
38	4047124			–	+	–	+	+	+	+	+	+	+	W
39	4047124			+	+	–	–	+	+	+	–	–	+	W
40	4147125			–	+	–	+	+	+	+	+	–	+	W
41	4047124			–	+	–	+	+	+	+	–	+	+	W
30	0046124		Kidney	+	+	+	+	+	+	+	+	+	+	R
42	4147125			–	+	+	+	+	+	+	+	+	+	Q
43	4047124			+	+	+	+	+	+	+	+	–	+	X
Resistant strains (%)				26	88	49	56	88	98	98	84	70	86	

* C: chloramphenicol (30 µg), E: erythromycin (15 µg), CAZ: ceftazidim (30 µg), AN: nalidixic acid (30 µg), TE: tetracycline (30 µg), CB: carbenicillin (100 µg), AM: ampicillin (10 µg), GM: gentamycin (10 µg), CTX: cefotaxim (30 µg), Cip: ciprofloxacin (5 µg); + = resistant, –: sensible.

** apices (–1 or +1): profile with only one band in difference.

ERIC-PCR patterns

Figure 1 represents the patterns obtained with ERIC 2 primer tested with *V. alginolyticus* strains isolated from juveniles with white nodular skin lesions, from seawater and from internal organs of diseased older fish, respectively.

All strains showed reproducible patterns consisting of 4 to 10 bands ranging from 150 pb to 1537 pb estimated using the Gel Pro Analyzer 4.0 software. The phylogenetic analysis of patterns generated by ERIC-PCR (Fig. 2) based on UPGMA method (Jaccard's coefficient) has shown that *V. alginolyticus* strains exhibit high amount of heterogeneity not only according to the location but also within the same samples. Twenty-four genotypes were identified: five from juveniles (A to E), ten from seawater (F to O) and nine genotypes were associated with the internal organs of the older *Sparus aurata* (P to X). Below 75% of homogeneity, clustering of 32 different fingerprint patterns was identified. Each cluster contains from 2 to 6 strains. In this study we found that *V. alginolyticus* strains isolated from the same origin were genetically different as they were grouped in different clusters.

DISCUSSION

This study represents the first attempt to characterise *Vibrio alginolyticus* strains harvested from diseased juveniles of *Sparus aurata* with white nodular skin lesions reared in the Tunisian sea coasts (Monastir, Centre of Tunisia) by both phenotypic and genotypic methods. There is a little data available on phenotypic characteristics and antimicrobial susceptibility of *Vibrio alginolyticus* isolated from *Sparus aurata* hatcheries in Tunisia (Snoussi *et al.*, 2006) and from diseased old fish (Ben Kahla-Nakbi *et al.*, 2006). A new data, based on the ERIC-PCR fingerprinting of 43 *Vibrio alginolyticus* strains isolated from seawater, diseased juveniles and older gilthead sea bream fish included in this study, was created.

TCBS modified agar was used to research *Vibrio alginolyticus* from all samples. The API 20E strips were used successfully to identify *Vibrio alginolyticus* strains recovered from environmental origin with a high degree of identification (100% of strains recovered from TCBS modified agar). These strips were used to identify both clinical and

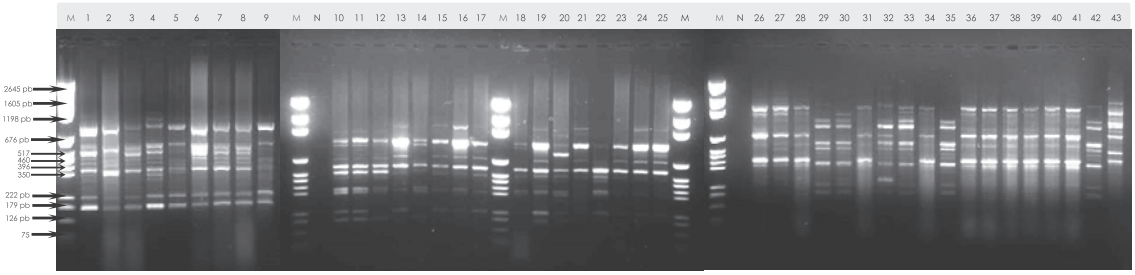

FIG. 1 - DNA fingerprints generated on 1% agarose gel by ERIC2-PCR analysis of *Vibrio alginolyticus* strains isolated from white nodular skin lesions associated with juveniles of Gilthead sea bream weighting 7 g (lanes 1 to 9), from seawater (lanes 10 to 25) and from different internal organs of infected older fish (lanes 26 to 43). Lanes as follows: (M) pGEM DNA molecular weight marker and (N) negative control. Lanes 1 to 43: *Vibrio alginolyticus* strains.

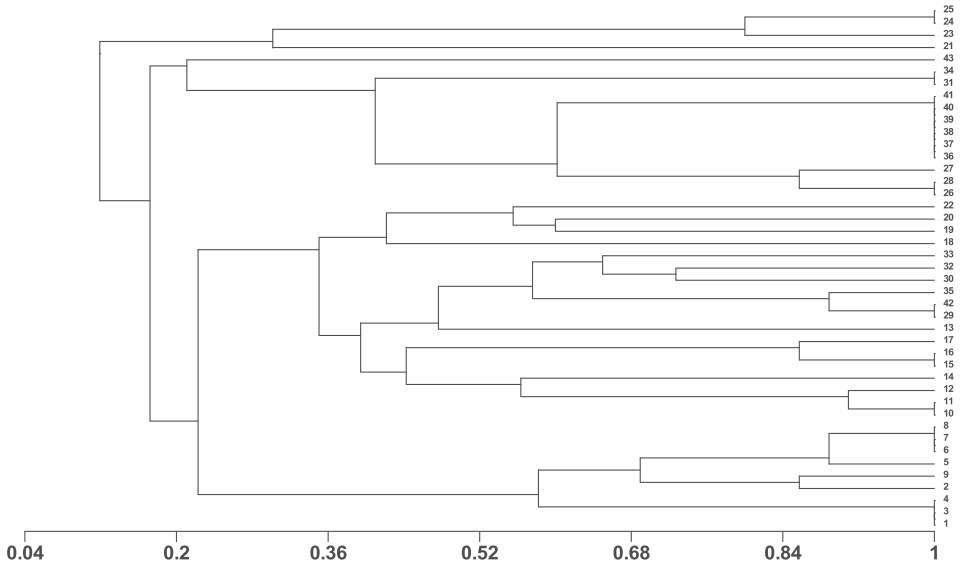

FIG. 2 - Dendrogram based on the unweighted pair group method of arithmetic averages and Jaccard's correlation coefficient on the basis of ERIC 2 patterns for the 43 strains of *Vibrio alginolyticus* isolated from seawater, juveniles with white nodular skin mucus and from different internal organs of old diseased fish. Numbers on the horizontal axis indicate the percentage of similarity.

environmental *Vibrio* spp. strains (Overman *et al.*, 1985; Elliot *et al.,* 1995). Several studies have demonstrated that these miniaturized biochemical tests, especially API 20 E and 20 NE were used with success to identify bacteria belonging to *Vibrionaceae* family isolated from different environmental biotopes (Croci *et al.*, 2001, López-Torres and Lizárraga-Partida, 2001, O'Hara *et al.*, 2003).

Good identification of large environmental *Vibrio* strains was obtained when applying the biochemical keys proposed by Alsina and Blanch (1994a, 1994b) and the same scheme modified by Ottaviani *et al.* (2003). Species level can be confirmed using molecular tools as demonstrated by Di-Pinto *et al*. (2006) when they described a collagenase-targeted multiplex-polymerase chain reaction (m-PCR) for the identification of *Vibrio alginolyticus*, *Vibrio cholerae* and *Vibrio parahaemolyticus* isolated from shellfish samples enriched in alkaline peptone water.

In this study all isolated strains from juveniles with white nodular skin mucus produced several enzymatic activities such as amylase, lecithinase, and caseinase and were lypolytic and were able to hydrolyze the DNA. These results were the same signalled by Balebona *et al*. (1998). These activities allow to vibrios strains the faculty to adhere to the epithelial cells of the juveniles of *Sparus aurata*, to brook the first barrier of natural defence and to colonise all internal organs inducing external haemorrhages, necrotic eyes, deep ulcers, haemorrhagic liver, pale kidney and splenomegaly (Colorni *et al.*, 1981; Paperna, 1984; Austin *et al.*, 1993).

Antibiotic susceptibility analysis is a useful typing method for some pathogens (Rudolph *et al.*, 1998). Seven of the ten antibiotics tested against *Vibrio alginolyticus* strains were previously used by Ottaviani *et al.* (2001). In their study, Ottaviani and colleagues showed that salt concentration has an effect in the response of "non- cholera vibrios" (NCVs) tested against 27 antibiotics. They recommended testing antimicrobial susceptibility on Mueller-Hinton plates with 1% NaCl. Multiple resistance to several antibiotics (ampicillin, carbenicillin, tetracycline and nalidixic acid) tested founded in this study where previously described (Ottaviani *et al.*, 2001; Zanetti *et al.*, 2001; Snoussi *et al.*, 2006). In the present study, high rates of resistance were noted for ciprofloxacin, cefotaxime and chloramphenicol comparing the results described by the same researches.

Ben Kahla-Nakbi *et al.* (2006) founded that 12/34 strains (35.29%) of *Vibrio alginolyticus* isolated from internal organs and external lesions of diseased gilthead sea bream and sea bass from two Tunisian farms were resistant to tetracycline whereas we founded in the present study that 88% of strains were resistant to the same chemotherapeutic agent. This high percentage of resistance can be explained by the large use of this antibiotic causing a increasing of strains resistance in marine biotopes.

The ERIC2-PCR was repeated twice for all strains tested and we founded a high level of typable bacteria (100% of typability) and reproducible patterns were observed for all tested strains. Though, the intensities of some of the bands generated occasionally varied somewhat. In 1999, Marshall and colleagues used the ERIC1-PCR primer to investigate the genetic diversity of *Vibrio parahaemolyticus* strains and obtained a reproducible pattern and demonstrate that this technique can be used to characterise the *Vibrio parahaemolyticus* populations in the environment. The same researches founded that the ERIC-PCR is a technique of choice when a single method is used for typing *Vibrio parahaemolyticus.* Coupled to ribotyping, the ERIC-PCR has a best discrimination index and typing ability. Analysis of *Vibrio alginolyticus* strains indicates that this species is genetically heterogeneous and there was a high level of genetic diversity among strains irrespective of their source of recovery. Moreover, *V. alginolyticus* strains originated from the same sample (seawater) were heterogeneous as they fell into different clusters.

The high intraspecific heterogeneity obtained by ERIC2-PCR can be explicated by the fact that juveniles and infected older fish harbour many *V. alginolyticus* genotypes and with a possible existence of the same genotype in different origins. Studying the genetic heterogeneity of *V. alginolyticus* strains isolated from different cultured fish in Spain using ribotyping method performed with *Hind*III enzyme, Zorilla *et al*, (2003) demonstrated that from the 34 strains tested, 23 ribotypes were distinguished confirming that these bacteria belonged to different clonal lineages. In a recent study, Ben Kahla-Nakbi *et al.* (2006) used ERIC2-PCR fingerprinting method to type 34 strains of *Vibrio alginolyticus* isolated from internal organs and external lesions of diseased gilthead sea bream and sea bass weighing from 20 to 125 g and reported a high level of genomic diversity (19 genotypes) with patterns including 2 to 7 bands and there is no correlation between genotypes and season of recovery was signalled.

Molecular characterisation methods were commonly used to study the genomic heterogeneity of both clinical and environmental strains belonging to *Vibrionaceae* family. In this work, we found that ERIC2-PCR represents quick tools to generate information on intraspecific differences in environmental *Vibrio alginolyticus* strains.

The present study indicates that the genetic variation can occur even within the *alginolyticus* strains originating from the same sample in the *Sparus aurata* farming systems and confirm the opportunistic character of *Vibrio alginolyticus* strains explained by the high heterogeneity of these strains associated with different stage of the life cycle of *Sparus aurata*. These data allow as developing a long time monitoring programs to study the implication of this bacterium in the high mortality in this farm and to explain the interaction between this bacterium and the fish body.

REFERENCES

Alsina M., Blanch A.R. (1994a). A set key for biochemical identification of environmental *Vibrio* species. J. Appl. Bacteriol., 76: 79-85.

Alsina M., Blanch A.R. (1994b). Improvement and update of a set of key for biochemical identification of key for biochemical identification of *Vibrio* species. J. Appl. Bacteriol., 77: 719-721.

Ashraf A.K., McCarthy S., Wang R.F., Cerniglia C.E. (2001). Characterization of United States outbreak isolates of *Vibrio parahaemolyticus* using enteriobacterial repetitive intergenic consensus (ERIC) PCR and development of a rapid PCR method for detection of O3:K6 isolates. FEMS Microbiol. Lett., 206: 209-214.

Austin B., Stobie M., Robertson P.A.W., Glass H.G., Stark J.R. (1993). *Vibrio alginolyticus*: the cause of gill disease leading to progressive low-level mortalities among juvenile turbot, *Scophthalmus maximus* L., in a Scottish aquarium. J. Fish Dis., 16: 277-280.

Bakhrouf A., Jeddi M., Ben Ouada H. (1995). Essai de traitement des vibrioses du loup *Dicentrarchus labrax* dans une zone de pisciculture, à Monastir, Tunisie. Marine Life, 5 (2): 47-54.

Balebona M.C., Andrew M.J., Bordas M.A., Moriñigo M.A., Zorilla I., Borrego J.J. (1998). Pathogenicity of *Vibrio alginolyticus* for cultured gilt-head sea bream (*Sparus aurata*). App. Environ. Microbiol., 64 (11): 4269-4275.

Ben Kahla-Nakbi A., Chaieb K., Besbes A., Zmantar T., Bakhrouf A. (2006). Virulence and enteriobacterial repetitive intergenic consensus PCR of *Vibrio alginolyticus* strains isolated from Tunisian cultured gilthead sea bream and sea bass outbreaks. Vet. Microbiol., 117 (2-4): 321-327.

Bordas M.A, Balebona M.C., Zorilla I., Borrego J.J., Moriñigo M.A. (1996). Kinetics of adhesion of selected fish-pathogenic *Vibrio* strains to skin mucus of gilt-head sea bream. Appl. Environm. Microbiol., 62: 3650-3654.

Cavallo J.D., Chardon H., Chidiac C., Choutet P., Courvalin P., Dabernat H., Drugeon H., Dubreuil, L., Goldstein F., Jarlier V., Leclerq R., Nicolas-Chanoine M.H., Philippon A., Quentin C., Rouveix B., Sirot J., Soussy C.-J. (2006). Communiqué. CSFA - Comité de l'antibiogramme de la société Française de Microbiologie.

Chaieb K., Abbassi M.S., Touati A., Hassen A.B., Mahdouani K., Bakhrouf A. (2005). Molecular characterization of *Staphyloccocus epidermidis* isolated from biomaterials in a dialysis service. Ann. Microbiol., 55: 309-314.

Colorni A., Paperna I., Gordin H. (1981). Bacterial infections in gilt-head sea bream *Sparus aurata* cultured at Elat. Aquaculture, 23: 257-267.

Croci L., Serratore P., Cozzi L., Stacchini A., Milandri S., Suffredini E., Toti L. (2001). Detection of *Vibrionaceae* in mussels and in their seawater growing area. Lett. Appl. Microbiol., 32: 57-61.

Di-Pinto A., Ciccarese G., Fontanarosa M., Terio V., Tantillo G. (2006). Detection of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* in shellfish using collagenase-targeted multiplex-PCR. J. Food Safety, 26: 150-159.

Divanch P., Kentouri M. (1983). Influences des conditions trophiques initiales sur la résorption des réserves lipidiques, la croissance et la survie des larves de daurades, *Sparus aurata*, en élevage extensif. Aquaculture, 35: 43-55.

Elliot E.L., Kaysner C.A., Tamplin M.L. (1992). *V. Cholerae*, *V. parahaemolyticus*, *V. vulnificus* and other *Vibrio* spp., In: Bacteriological Analytic Mannual, Food and Drug Administration, Arlington, AOAC International, pp. 111-140.

Elliot E.L., Kaysner C.A., Jackson L., Tamplin M.L. (1995). *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus* and other *Vibrio* spp. In: FDA Bacteriological Analytical Manual, 8th edn., AOAC International, Gaithersburg, MD, pp. 9.01-9.27.

Fabiano L.T, Iida T., Swings J. (2004). Biodiversity of Vibrios. Microbiol. Mol. Biol. Rev., 68 (3): 403-431.

Finkelstein R., Edelstein S., Mahamid G. (2002). Fulminant wound infections due to *Vibrio vulnificus*. Israel Med. Assoc. J., 4: 654-655.

Gahrn-Hansen B., Hornstrup M.K. (1994). Extraintestinal infections caused by *Vibrio parahaemolyticus* and *Vibrio alginolyticus* at the country of Funen 1987-1992. Ugeskr. Laeger., 156: 5279-5282.

Gomez J.M., Fajardo R., Patiño J.F., Arias C.A. (2003). Necrotizing fasciitis due to *Vibrio alginolyticus* in an immunocompetent patient. J. Clin. Microbiol., 41 (7): 3427-3429.

Hörmansdorfer S., Wentges H., Neugebaur-Büchler K., Bauer J. (2000). Isolation of *Vibrio alginolyticus* from seawater aquaria. Int. J. Hyg. Environ. Health, 203: 169-175.

López-Torres M.A., Lizárraga-Partida M.L. (2001). Bacteria isolated on TCBS media associated with hatched Artemia cysts of commercial brands. Aquaculture, 194: 11-20.

Marshall F.J., Clarck C.G., Wang G., Mulvery M., Kelly M.T., Johnson W.M. (1999). Comparaison of molecular methods for typing *Vibrio parahaemolyticus.* J. Clin. Microbiol., 37: 2473-2478.

NCCLS - National Committee for Clinical Laboratory Standards (2002). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Tentative standards. M31-T. Document M31-T, Wayne.

O'Hara C.M., Sowers E.G., Bopp C.A., Duda S.B., Strockbine N.A. (2003). Accuracy of six commercially available systems for identification of members of the family *Vibrionaceae*. J. Clin. Microbiol., 41: 5654-5659.

Ottaviani D., Bacchiocchi I., Masini L., Leoni F., Carraturo A., Giammarioli M., Sbaraglia G. (2001). Antimicrobial susceptibility of potentially pathogenic halophilic vibrios isolated from seafood. Int. J. Antimicrob. Ag., 18: 135-140.

Ottaviani D., Masini L., Bacchiocchi S. (2003). A biochemical protocol for the isolation and identification of current species of *Vibrio* in seafood. J. Appl. Microbiol., 95: 1277-1284.

Overman T.L., Kessler J.F., Seabolt J.P. (1985). Comparison of API 20E, API Rapid E and API Rapid NFT for identification of members of the family *Vibrionaceae*. J. Clin. Microbiol., 22: 778-781.

Paperna I. (1984). Review of diseases affecting cultured *Sparus aurata* and *Dicentrarchus labrax*, In: Barnabé G., Billard R., Eds, L'Aquaculture du Bar et des Sparidés. INRA Publisher, Paris, France, pp. 465-482.

Reina J., Fernandez-Baca V., Lopez A. (1995). Acute gastroenteritis caused by *Vibrio alginolyticus* in an immu-nocompetent patient. Clin. Infect. Dis., 21: 1044-1045.

Ripabelli G., Sammarco M.L., McLauchlin J., Fanelli I. (2003). Molecular characterisation and antimicrobial resistance of *Vibrio vulnificus* and *Vibrio alginolyticus* isolated from mussels (*Mytilus galloprovincialis*). Syst. Appl. Microbiol., 26 (1):119-126.

Rudolph K.M., Parkinson A.J., Roberts M.C. (1998). Molecular analysis by pulsed-field gel electrophoresis and antibiogram of *Streptococcus pneumoniae* serotype 6B isolated from selected areas within the United States. J. Clin. Microbiol., 36: 2703-2707.

Sambrook J., Fritsch E.F., Maniatis T. (1989). Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Snoussi M., Chaieb K., Rouabhia M., Bakhrouf A. (2006). Quantitative study, identification and antibiotics sensitivity of some *Vibrionaceae* associated to a marine fish hatchery. Ann. Microbiol., 56 (4): 289-293.

Tülay A., Gülşen T. (2002). Vibriosis in Gilthead Sea Bream (*Sparus aurata* L.) in farms in the Aegean Sea Coast of Turkey. Turkish Journal of Fisheries and Aquatic Sciences, 2: 89-91.

Wachsmuth I.K., Blake P.A., Olsvik O. (1994). *Vibrio cholerae* and cholera. Molecular to global perspectives. ASM Press, Washington, D.C.

Zanetti S., Deriu A., Volterra L., Falchi M.P., Molicotti P., Fadda G., Sechi L. (2000). Virulence factors in *Vibrio alginolyticus* strains isolated from aquatic environments. Ann. Ig., 12 (6): 487-491.

Zanetti S., Spanu T., Deriu A., Romano L., Sechi L.A., Fadda G. (2001). *In vitro* susceptibility of *Vibrio* spp. isolated from the environment. Int. J. Antimicrob. Ag., 17: 407-409.

Zorilla I., Moriñigo M.A., Castro D., Balebona M.C., Borrego J.J. (2003). Intraspecific characterization of *Vibrio alginolyticus* isolates recovered from cultured fish in Spain. J. Appl. Microbiol., 95: 1106-1116.