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Co-occurrence of viral and bacterial pathogens in disease outbreaks affecting newly cultured sparid fish

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Summary. Several microbial disease outbreaks in farm stocks of newly cultured sparid fish species, such as common seabream, redbanded seabream, and white seabream, were recorded from 2004 to 2006. This study describes the isolation and characterization of the potential causative agents, either bacteria or viruses, of these outbreaks. The isolated bacterial strains were characterized according to traditional taxonomical analyses and sequencing of a 16S rDNA fragment. Most bacteria were identified as *Vibrio* spp. and *Photobacterium damselae* subsp. *damselae*. The development of cytopathic effects (CPE) on different fish cell lines, the application of specific nested-PCR tests for infectious pancreatic necrosis virus (IPNV), viral nervous necrosis virus (VNNV) and viral hemorrhagic septicemia virus (VHSV), and subsequent sequence analyses were used for virus detection and identification. VNNV, related to the striped jack neural necrosis virus (SJNNV) genotype, and VHSV, related to the genotype Ia, were the only viruses detected. VNNV was isolated from the three fish species under study in five different outbreaks, whereas VHSV was isolated from common seabream and white seabream during two of these outbreaks. IPNV was not detected in any case. [**Int Microbiol** 2007; 10(3):193-199]

Key words: *Vibrio* spp. · *Photobacterium damselae* subsp. *damselae* · Virus: VNNV, VHSV · sparid fish · co-occurrence of pathogens

Introduction

The *Sparidae* family comprises a diverse group of fish species, some of which are of great interest in aquaculture. Although gilt-head seabream (*Sparus aurata* L.) is the most economically important fish species of this family cultured in Southern Europe, new extensive studies carried out on the reproductive cycle, nutrition, and growth of other sparid species, such as redbanded seabream (*Pagrus auriga* Valenciennes), common seabream (*Pagrus pagrus* L.), and white seabream (*Diplodus sargus* L.), have led to their culture at an experimental scale. However, as a consequence of the intensive culture of these fish species, several microbial diseases outbreaks have been recorded [16].

Members of the *Vibrio* genus and *Photobacterium damselae* subsp. *piscicida* are the major bacterial pathogens affecting cultured marine fish in southern Europe [2,3,17, 39]. Viral infections of cultured marine fish from this geographical region are predominantly due to infectious pancreatic necrosis virus (IPNV), viral nervous necrosis virus (VNNV), and lymphocystis disease virus (LCDV) [1,5,7,12, 28]. The aim of this study was to detect and identify the

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potential microbial pathogens that affected three newly cultured sparid fish species in the course of seven microbial disease outbreaks recorded from 2004 to 2006.

Materials and methods

Fish sampling. Diseased specimens of redbanded, common, and white seabream were collected in an experimental fish farm located in southern Spain during several disease outbreaks occurred from 2004 to 2006. The mortality caused by these outbreaks ranged from 5% in outbreak 7 to 94% in outbreak 6. Affected or moribund specimens were killed with an overdose of MS-222 in sea water (65 mg/ml, final concentration; Sigma Chemical Co., St Louis, MO, USA) and immediately processed for bacteriological and virological analyses.

Bacteriological analyses. Samples collected from the eyes, brain, spleen, liver, and kidneys of the dead specimens were seeded on tryptic soy agar and broth (Difco, Detroit, MI, USA) supplemented with 1.5% NaCl (TSAS, TSBS), marine agar (Cultimed Panreac Química SA, Barcelona, Spain), thiosulfate-citrate-bile salt-sucrose agar (TCBS, Biolife, Milan, Italy), and TCBS supplemented with 1.5% NaCl (TCBS-1). The inoculated media were incubated at 22ºC for 2–5 days. The isolates were subjected to taxonomical analyses according to *Bergey's Manual of Determinative Bacteriology* [14]. The API 20E system (BioMérieux, Madrid, Spain) was used to confirm the biochemical characterizations.

All isolates were subcultured to axenic cultures for further DNA sequence analyses. A 16S rDNA fragment was amplified using the universal primers 63f and 1387r [22]. PCR tests were carried out following the protocol described previously by Labella et al. [16]. The 16S rDNA gene sequences were analyzed using Seqman *v*5.53 (DNASTAR) and subsequently in a BLAST search of the GenBank database in order to retrieve the most closely related sequences.

Virological analyses and cell culture. Pools of nervous tissue (eye and brain) and internal organs (liver, kidneys, and spleen) were processed for virological studies following the procedures described by several authors [1,13]. Homogenates of nervous tissue were analyzed on the SSN-1 cell line, and pools of internal organs were inoculated onto TV-1, BF-2, and SAF-1 cells. Inoculated cells were incubated at 20ºC and examined daily for the development of cytopathic effects (CPE) up to 20 days post-inoculation (p.i.). Samples that did not cause CPE after a second passage on the respective cells were considered negative for the presence of infective viral particles. Only positive samples were submitted for RT-PCRs and subsequent nested-PCRs for IPNV, VNNV, and viral hemorrhagic septicemia virus (VHSV) identification. Viral titers from positive samples were calculated as the viral dilution infecting 50% of the cell cultures, and expressed as tissue-culture infective dose $(TCID)_{50}$/ml.

Virus identification by RT-PCR and nested-PCR analyses. The High Pure RNA Isolation Kit (Roche Applied Science, Barcelona, Spain) was used to extract total RNA from those inoculated cells showing early CPE in a second passage. RT-PCRs were done in a single tube using the SuperScript One-Step RT-PCR kit (Invitrogen, Paisley, UK).

Cells inoculated with nervous tissues were tested for the presence of VNNV using primers that amplified a 426-bp fragment within the T4 region [24]. RT-PCR was carried out according to the manufacturer's instructions and the following profile: 30 min at 45ºC, 2 min at 94ºC, 35 cycles of 15 s at 94ºC, 30 s at 58ºC, and 30 s at 68ºC, with a final step of 10 min at 68ºC. A primer pair designed by Olveira and Dopazo (unpublished data) was used to amplify a 156-bp internal fragment (forward primer, F21: 5´ GATTTCGTTCCATTCTCTTG 3´; reverse primer, R31: 5´ AGTGTCTCCAGCTTTCTTCT 3´). Nested-PCR tests were carried out in a 50-µl reaction mixture consisting of 22.5 pmol of both primers, 200 µM dNTPs, 1× PCR buffer, 2.5 U *Taq* DNA polymerase (Roche Applied Science), and 5 µl of the RT-PCR products. The nested-PCR profile was as follows: 5 min at 94ºC, 35 cycles of 30 s at 94ºC, 30 s at 58ºC, and 30 s at 68ºC, with a final step of 10 min at 68ºC.

Cells inoculated with pooled internal organs were tested for the presence of VHSV, as described by Lopez-Vazquez et al. [19], and for IPNV. Primers used for IPNV detection (forward primer, F2: 5´ GTGCTGGCCACAAACGACAAC 3´; reverse primer, R2: 5´ AATTGGTCTGCCGTTCCTA 3´) amplify a 599-bp fragment within the pVP2 nucleotide sequence. Internal primers for the IPNV nested-PCR (forward primer, R21: 5´ ACACTGGTGGCCTACGAGAAGATG 3´; reverse primer, R22: 5´ GGTCGTACTTGCCATAGCGTGTCAC 3´) yield a 127-bp fragment.

The IPNV RT-PCR profile was as follows: one step of 30 min at 45ºC, 2 min at 94ºC, followed by 35 amplification cycles at 94ºC for 15 s, annealing at 55ºC for 30 s, and 68ºC for 1 min. RT-PCR was concluded by an extension period of 10 min at 68ºC. Nested-PCRs were carried out in a 50-µl reaction mixture consisting of 22.5 pmol of each primer, 200 µM dNTPs, 1× PCR buffer, 2.5 U *Taq* DNA polymerase (Roche Applied Science), and 5 µl of the RT-PCR products. The nested-PCR profile was as follows: 5 min at 94ºC, 35 cycles of 30 s at 94ºC, 30 s at 55ºC, and 30 s at 68ºC, with a final step of 10 min at 68ºC.

Amplification products were analyzed by electrophoresis on 1.5% agarose gels, purified with DNA and the Gel Band Purification Kit (Roche Applied Science), and then sequenced (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). The sequences of the VNNV isolates were deposited in the GenBank under accession nos. EF523822–EF523824. Sequence alignment and identity analyses were carried out using the MegAlign program, Clustal method (DNASTAR software).

Results

Description of the fish disease outbreaks. One hundred and two specimens of cultured common seabream, redbanded seabream, and white seabream were sampled from seven outbreaks that occurred during the period 2004–2006. The affected animals presented several external signs of infection, such as exophthalmia, dark skin pigmentation, erratic swimming, corneal opacity, mucus-covered and pale gills, eroded fins, hemorrhagic gills, and necrotic and epidermic ulcers. The most predominant internal symptoms were the presence of a fatty hemorrhagic liver with petechial signs and necrotic areas, splenomegaly, enteritis, pale liver, and abdominal swelling with ascitic liquid.

Bacterial analysis. Isolated bacteria were gram-negative, motile organisms, and cytochrome-oxidase- as well as catalase-positive. The bacteria were identified by physiological and biochemical characterization as well as by the sequencing of a 16S rDNA fragment. Table 1 shows the bacterial species identified (and records the presence of VHSV and VNNV; IPNV was not detected).

Virological analysis on cell cultures. Samples of nervous tissue obtained from common seabream, redbanded seabream, and white seabream caused CPE on SSN-1 cells at

Table 1. Microbial disease outbreaks affecting newly cultured sparid species from 2004 to 2006

Outbreak (season)	Date	Fish	Mortality (%)	Bacterial isolates	Viruses	
					VHSV	VNNV
No. 1 (Spring 2004)	April-June	Redbanded seabream	26	*P. damselae* subsp. *damselae*	–	+
	May-June	Common seabream	26	*V. harveyi, V. fischeri, V. alginolyticus, V. splendidus, V. ichthyoenteri*	–	+
No. 2 (Summer 2004)	July	Redbanded seabream	20	*V. harveyi*	–	–
	August	Common seabream	15	*V. harveyi*, *V. fischeri*, *V. alginolyticus*, *V. splendidus*	+	–
No. 3 (Fall 2004)	October-December	Redbanded seabream	28	*P. damselae* subsp. *damselae*	–	+
No. 4 (Winter 2005)	February	Redbanded seabream	13	*V. splendidus*	–	+
	February	Common seabream	25	*V. splendidus*, *V. ichthyoenteri*	–	+
No. 5 (Spring 2005)	May	White seabream	80	*P. damselae* subsp. *damselae*, *V. fischeri*	–	+
No. 6 (Summer 2005)	August	White seabream	94	*P. damselae* subsp. *damselae*	+	–
No. 7 (Spring 2006)	May	Redbanded seabream	5	*V. harveyi*, *V. ichthyoenteri*	–	+

different days p.i. (2–4, 3–4, and 1, respectively). These CPE included the complete disruption of the cell monolayer by 1–4 days p.i. A titer of 10^4 $TCID_{50}$/ml was obtained.

All cell lines inoculated with internal-organ samples from common seabream and white seabream developed CPE, which were observed earlier on BF-2 cells (2–3 days p.i.) than on TV-1 and SAF-1 cells (4 and 7 days, respectively). Viral titers on BF-2 cells ranged from 10^3 to 10^5 $TCID_{50}$/ml.

Viral detection by nested-PCR. No RT-PCR products were detected from cells inoculated with nervous tissues when primers designed by Nishizawa et al. [24] for VNNV detection were used. In contrast, the specific nested-PCR yielded a band of 156 bp from samples collected from the three seabream species (Fig. 1). The nested-PCR products from all viral isolates were identical with respect to nucleotide sequence, regardless of host fish species or outbreak. A Blast search analysis confirmed these isolates as VNNV and showed 100% identity between the sequences of the isolates and that of striped jack neural necrosis virus (SJNNV) (GenBank accession no. D30814), and 97.85 ± 0.67% with some isolates of sea bass, gilt-head seabream, and Senegalese sole from the Iberian Peninsula (IBNNV, GenBank accession nos.: AM110723×AM110725, AM110727, AM110729, AM110731, AM110742, AM110745, AM265971-AM265973, AM265976) included in the SJNNV genotype [9]. Lower identities of 64.4–75.4% were determined with the BFNNV (D38635), RGNNV (D38636) and TPNNV (D38637) genotypes (Table 2).

Analyses of cells inoculated with internal organs by a specific RT-PCR for VHSV detection were negative. However, a 301-bp fragment was amplified from pooled internal organs collected from common and white seabream using a VHSV-specific nested-PCR (Fig. 2). Viral isolates from both fish species yielded identical nested-PCR products and were identified as VHSV by a Blast search. Alignment of these sparid viral isolates with different VHSV genotypes showed a nucleotide identity between 70.8 and 100% (Table 3). The highest identity was with strain FR-07/71, belonging to genotype Ia. Neither RT-PCR nor nested-PCR products were obtained when specific primers for IPNV detection were used.

Discussion

Although fish bacterial pathogens have been well-studied [23], the present work is, to our knowledge, the first description of microbial disease outbreaks (which occurred between 2004 and 2006) affecting newly cultured sparid species

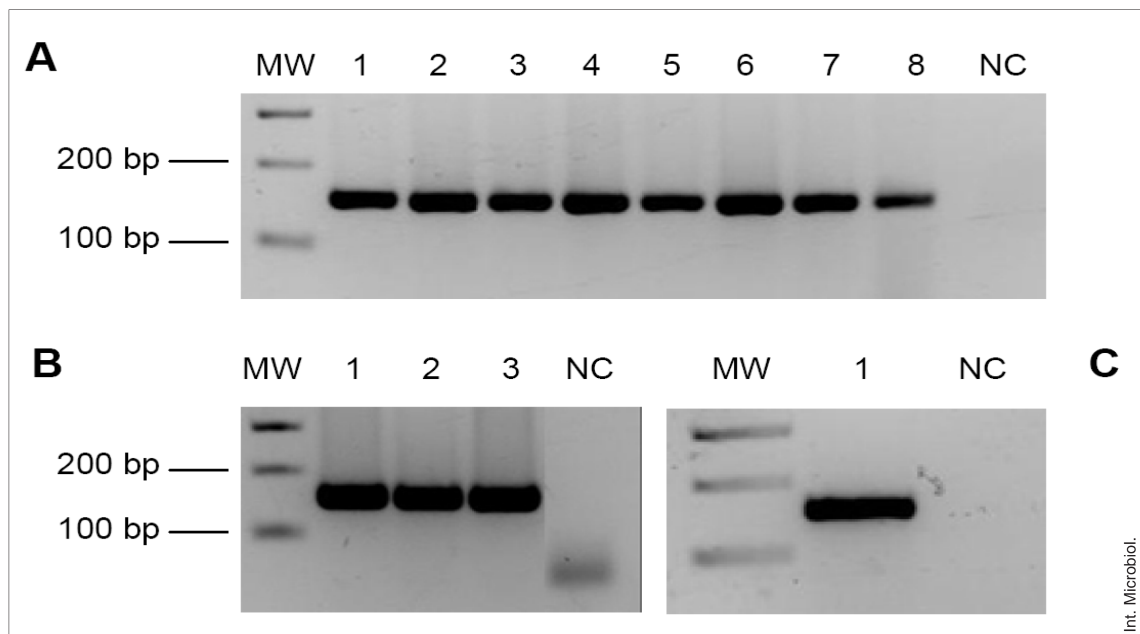

Fig. 1. Amplification of a 156-bp fragment by nested-PCR using primers for the detection of viral nervous necrosis virus (VNNV) in cells inoculated with nervous tissue. (**A**) From common seabream: lanes 1–6 show results from pooled brain and eyes of different affected animals from outbreaks numbers 1 and 4. RNA from a VNNV isolate obtained from Atlantic halibut and a SJNNV isolate was used as positive control (lanes 7 and 8, respectively). (**B**) From redbanded seabream: lanes 1–3 show results from pooled nervous tissues of different affected animals from outbreak numbers 3, 4, and 7. (**C**) From white seabream: Lane 1 is the result from animals affected during outbreak no. 5. MW, molecular weight markers (DNA molecular weight marker XIV, 100 bp ladder). NC, negative control (uninfected cells).

(common, redbanded, and white seabreams). Five *Vibrio* species (*V. harveyi*, *V. fischeri*, *V. alginolyticus*, *V. splendidus* and *V. ichthyoenteri*) were isolated and identified from seven outbreaks affecting three newly cultured sparid fish. Some of these bacterial species are involved in diseases affecting other cultured sparid species, such as gilt-head seabream and common dentex [3,8,30]. The pathogenic *Vibrio* species use different virulence mechanisms, including hemolytic and proteolytic activities, and the production of several toxins (neurotoxins, ADP-ribosyl toxins, and hemolysins) [4,6,18, 32,38]. *V. harveyi* and *V. splendidus* were the two only bacterial species isolated in axenic culture from two outbreaks with moderate fish mortality (outbreaks nos. 2 and 4, respectively, Table 1), whilst several bacterial species were isolated

Table 2. Nucleotide sequence identities of a fragment of the VNNV capsid protein gene. Primer sequences were not considered

	Nucleotide sequence identities (%)				
	Sparid VNNV isolates[a]	IBNNV	SJNNV	BFNNV	RGNNV
IBNNV	97.85 ± 0.67[b]				
SJNNV	100	98.36 ± 0.21			
BFNNV	71.2	69.81 ± 0.11	77.7		
RGNNV	64.4	68.98 ± 0.18	79.0	83.4	
TPNNV	75.4	73.65 ± 0.19	81.6	79.8	79.8

[a]This study.
[b]Mean nucleic acid identity percentage (%) plus its standard deviation.

Table 3. Nucleotide sequence identities of the VHSV nucleoprotein gene fragment. Primer sequences were not considered

	Nucleotide sequence identities (%)				
	Sparid VHSV isolates[a]	FR-07/71[b]	DK 1p12[c]	DK 1p49[d]	GH 30[e]
FR-07/71	100				
DK 1p12	79.2	96.1			
DK 1p49	70.8	86.6	87.5		
GH 30	78.4	87.2	88.4	89	
MAKAH[f]	85.2	86.7	83	85.7	86.6

[a]This study.
[b]Genotype Ia, accession no. AJ233396.
[c]Genotype Ia, accession no. AY356653.
[d]Genotype Ia, accession no. AY356743.
[e]Genotype Ia, accession no. AJ849477.
[f]Genotype Ia, accession no. X59241.

from the other five outbreaks. Immunosupression caused by a primary infection could be the cause of multiple bacterial infections [15].

P. damselae subsp. *damselae* was isolated from the two outbreaks affecting redbanded seabream in 2004, and from two outbreaks affecting white seabream in 2005 (Table 1). *P. damselae* subsp. *piscicida* is frequently isolated from several marine fish species [29]; in contrast, only a few studies have reported the isolation of *P. damselae* subsp. *damselae* from diseased cultured fish [8,16,27,35]. Magariños et al. [21] established that several environmental factors, mainly temperature, can affect the incidence of pasteurellosis in cultured gilt-head seabream. The high water temperature in outbreaks no. 3 and 5 could explain the isolation of *P. damselae* subsp. *damselae* in axenic culture.

The susceptibility of seabream species to these *Photobacterium* subspecies depends on the fish age [25]. Generally, larvae and juvenile fish are more frequently affected by bacterial pathogens. However, in the present study, *P. damselae* subsp. *damselae* was mainly isolated from adult specimens, except in outbreak no. 5.

VNNV was the most frequently detected virus (5 out of 7 outbreaks) in the three fish species studied. VHSV was detected in only two outbreaks (nos. 2 and 6, summer 2004 and 2005), whereas IPNV was not detected in any of the samples tested. In Spain, VNNV was previously recorded from sea bass, gilt-head seabream, and Senegalese sole [9,34,37]. The only report regarding VHSV detection from farmed marine fish is a preliminary characterization carried out in the Iberian Peninsula [López-Vázquez C, et al. (2003) 11th Int. Conf. of the EAFP, St Julians, Malta, Abstract Book, P-138].

The cell line used for the replication of VNNV in the present study was SSN-1 [7]. The majority of the isolates from farmed fish in southern Europe are classified as RGNNV genotype [10,33]. However, our analyses of the nested-PCR products obtained from the sparid viral isolates showed 100% identity with isolates included into the SJNNV genotype. This result supports the presence of SJNNV isolates in the Iberian Peninsula, as previously described by Thiery et al. [37] and Cutrin et al. [9]. These authors proposed the existence of a SJNNV subtype, called IBNNV, including isolates from the Iberian Peninsula, either from the Atlantic or Mediterranean coast.

The causative agent of viral hemorrhagic septicemia, VHSV, has been traditionally propagated on a variety of well-established fish cell lines [20]. All the cell lines tested in

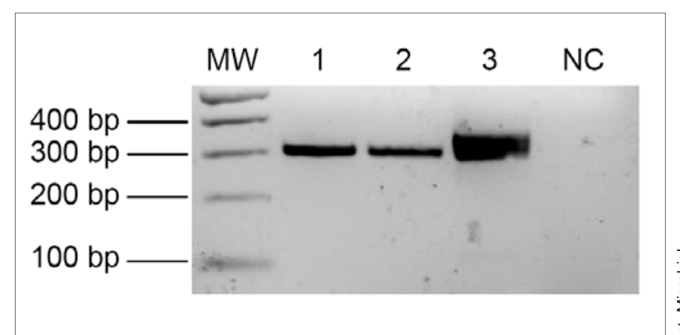

Fig. 2. Amplification of a 301-bp fragment by nested-PCR using primers for the detection of viral hemorrhagic septicemia virus (VHSV) in cells inoculated with pooled internal organs. Lane 1, result from white seabream affected during outbreak no. 6; lane 2, result from common seabream from outbreak no. 2; lane 3, positive control. MW, molecular weight markers (DNA molecular weight marker XIV, 100 bp ladder). NC, negative control (uninfected cells).

this study were susceptible to VHSV isolated from internal organs, but CPE were firstly developed on the BF-2 cell line. Previously, Pérez-Prieto et al. [26] also reported a high permissiveness of these cells to VHSV, and Lorenzen et al. [20] proposed BF-2 and RTG-2 cell lines for the isolation of VHSV, based on a comparison of several cell lines. Sequencing of the nucleoprotein gene fragments obtained by nested-PCR from the sparid viral isolates showed 100% identity with genotype Ia, which has been associated with rainbow trout farmed in continental Europe and with a single isolate from farmed turbot [31,36]. In a preliminary study, Lopez-Vazquez et al. [11th Int. Conf. EAFP, Malta, cited] also reported the inclusion of a marine VHSV isolated from sole in the Iberian Peninsula into this genotype. Even though genotype Ia mainly comprises isolates from freshwater rainbow trout, the close genetic relationship between marine and freshwater VHSV isolates in genotype I suggests the marine origin of VHSV in rainbow-trout farms [11,34].

In short, we detected the occurrence of several potential viral and bacterial pathogens in newly cultured sparid fish species. The main microorganisms isolated were *Vibrio* spp., *P. damselae* subsp. *damselae*, VNNV related to the SJNNV genotype, and VHSV related to genotype Ia. Our results suggest that performing simultaneous virus and bacteria analyses should be considered, and that microbial co-infections may occur in farmed fish.

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