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ORIGINAL ARTICLE

Phenotypic and genetic diversity of coexisting *Listonella anguillarum*, *Vibrio harveyi* and *Vibrio chagassi* recovered from skin haemorrhages of diseased sand smelt, *Atherina boyeri*, in the Gulf of Trieste (NE Adriatic Sea)

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Abstract

Aims: This study identified and characterized coexisting Vibrios associated with haemorrhagic skin lesion bearing sand smelt fishes (*Atherina boyeri*) in north-eastern Adriatic Sea.
Methods and Results: Bacteria were isolated from external skin lesions of four samples, and representative morphotypes grown on thiosulfate–citrate–bile salt–sucrose agar were isolated. In total 25 isolates, presumptively assigned to *Vibrio* genus, were biochemically characterized and were grouped in 10 phenotypic profiles. Phenotypes were heterogeneously distributed among the diseased sand smelt analysed; only one phenotype was recovered from all the samples. Sequencing of 16S rRNA was performed to identify representatives of all phenotypes. Phylogenetic analysis using the neighbour-joining method revealed six isolates clustered within the *Vibrio harveyi* group, three clustered with known *Vibrio chagasii* strains and three clustered with *Listonella anguillarum*.
Conclusions: Vibrios with a broad phenotypic variability were found in the external lesions of diseased *A. boyeri*. In total three species of *Vibrio* were identified: *V. harveyi* showed the wider phenotypical and ribotypical heterogeneity while *L. anguillarum* shared similar biochemical characteristics with typical strains.
Significance and Impact of the study: Previously unreported coexistence of potential pathogenic species colonizing diseased *A. boyeri* has ecological as well as epidemiological significance.

Introduction

The sand smelt, *Atherina boyeri* Risso 1810 is a small fish commonly found in the Mediterranean and in the north–east Atlantic from the Azores to the north–western coast of Scotland. It is a short-lived, euryhaline atherinid fish that mainly inhabits coastal and estuarine waters including lagoons, salt marshes and, more rarely, inland waters, over a wide range of salinities from freshwater to hypersaline conditions (Bartulović *et al.* 2006 and the reference therein). In the Gulf of Trieste, *A. boyeri* represent one of the most important commercial species, in particular from November to May when it moves to deeper and warmer areas providing an important trawling fishery resource (Vio *et al.* 1983; Orel and Zamboni 2004).

Bacterial diseases associated with haemorrhagic septicaemia and skin lesions are frequently caused by fish pathogens belonging to *Vibrio* species. The Vibrionaceae family (Baumann and Schubert 1984) is widespread in the aquatic environment throughout the world, especially in marine and brackish waters, tending to be more common during warmer periods, notably when temperatures rise above 17°C; furthermore, many of its species are known to tolerate a wide range of salinities (Wright *et al.* 1996). Vibrios are frequently associated with organisms spanning from microplankton to finfish occupying a variety of ecological niches, such as the human and animal gut, the surface of chitinous organisms, most notably copepods, and the coral mucus layer. Some species are symbionts, whereas others, natural inhabitants of marine,

estuarine and aquaculture systems, are recognized as potential pathogens for various wild and cultured marine invertebrates and fish (Austin and Austin 1999; Paillard *et al.* 2004; Toranzo *et al.* 2005).

In the last 10 years, there have been a growing number of studies aimed at a taxonomic survey of bacteria associated with healthy and diseased marine animals in which the presence of potential pathogenic Vibrios is reported (Gomez-Gil *et al.* 2006; Chimetto *et al.* 2009). In addition, some authors assayed that few strains displayed an individual pathogenicity, whereas quite an important number of strains displayed an enhanced virulence when concomitantly inoculated, suggesting a synergistic action (Cervino *et al.* 2004, 2008; Gay *et al.* 2004). Indeed, recent studies have shown that *Vibrio* species belonging to core group, abundant in the mucus of corals, may cause infections during periods of environmental imbalances (Reshef *et al.* 2006). To date, only one report on infected *A. boyeri* by *Vibrio* is present in the scientific literature (Yiagnisis *et al.* 2007). In their work, Yiagnisis *et al.* (2007) have isolated *Vibrio anguillarum* strains with a unique phenotype, from several fishes collected from two different areas in the NE Aegean Sea.

Identification of Vibrios harvested from the marine environment is often imprecise and time-consuming as it implies many biochemical and physiological tests. Notably, a number of diagnostic laboratories still rely on the presumptive identification of *Vibrio* species by means of phenotypic tests (Alsina and Blanch 1994), but discriminating these species remains a hard task for taxonomy because of the huge variability of diagnostic features among species (Gomez-Gil *et al.* 2004; Thompson *et al.* 2004; Fabbro *et al.* 2010). Abundant examples in the literature illustrate the difficulties of correctly identifying *Vibrio* strains pointing out ambiguous identifications, possibly due to the methodological limitations. An alternative explanation for the difficulty in identifying these strains is attributed to the plasticity of the *Vibrio* genomes with hybridization events in the marine environment leading to soft species boundaries (Fraser *et al.* 2007). Also, infections by phages may contribute to changing phenotypes (Vidgen *et al.* 2006).

No information is currently available regarding potential fish pathogenic Vibrios in the productive North Adriatic Sea. As *A. boyeri* has an anadromic behaviour and moves in different coastal environments throughout the year, it could harbour and carry a commensal microbiota attached to external surfaces and at the same time spread potential pathogens. It is therefore important to detect and analyse potentially harmful microbes associated with diseased animals to be able to identify potential pathogens when they arise.

In order to implement current information on potential fish pathogens, in the present study we biochemically and genotypically characterized for the first time coexisting *Vibrio* species isolated from skin lesions of diseased *A. boyeri*.

Materials and Methods

A shoal of *A. boyeri* that appeared disoriented, swimming in an abnormal way and presenting skin lesions was observed in June 2008 in a coastal area in the Gulf of Trieste (45°44′28″N; 13°40′08″E – surface water temperature, 22°C). Moribund *A. boyeri* were captured alive, transferred into tanks with surface seawater (5 l) and transported to the laboratory within 20 min. Diseased fish presented necrosis of the fin and haemorrhagic lesions localized on the flank, around the eyes and the head (Fig. 1); death occurred within 1 h after catching.

Samples from the external lesions of four fishes were collected aseptically with a bacteriological loop and streaked onto thiosulfate–citrate–bile salt–sucrose (TCBS) and glutamate–starch–phenol red (GSP) agars (Oxoid, Basingstoke, UK); the plates were incubated at 25°C for 1 day.

Representative morphotypes that grew on the plates were picked and purified on the same selective media. Pure cultures were cultivated on 0·9% NaCl tryptone soy agar (Oxoid, Basingstoke, UK) to perform classical phenotypic tests; to confirm the typical traits of the *Vibrio*

Figure 1 *Atherina boyeri*. Haemorrhagic lesions localized around the eyes and the head.

genus, the isolates were tested for Gram staining, oxidase, 3% NaCl tolerance, fermentative degradation of dextrose, nitrate reduction, motility, growth at 37°C and 44°C and growth under anaerobic conditions (in 0·9% NaCl TSA tubes overlaid with mineral oil) (Elliot *et al.* 1995). The fermentative degradation of dextrose was tested on ZOF medium: Marine ZoBell 0·3% agar at pH 7·6 ± 0·2, with 0·01% phenol red and 1% dextrose added after sterilization (Lemos *et al.* 1985). Finally, commercially available miniaturized systems API 20E and API 20NE (bioMérieux, Marcy l'Etoile, France) were utilized to biochemically characterize the isolates presumptively assigned to *Vibrio* genus. Incubation time and temperature were maintained within the limits prescribed by the supplier.

The molecular characterization by partial 16S rRNA gene sequence analysis was performed on 12 representatives of phenotypes. Genomic DNA was extracted from exponential cultures (0·9% NaCl TSbroth at 37 ± 1°C for 24 h) by means of Dneasy™ Tissue kit (Qiagen, Dusseldorf, Germany) according to manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the extracts was carried out to identify a portion of the 16S rRNA gene by a modification of the touchdown protocol (Don *et al.* 1991) using the universal primer 27F and Eubacterial-specific primer 1492R (Lane 1991), which do not select against *Vibrios* (Thompson *et al.* 2004 and references therein). Amplifications were performed on an Eppendorf Gradient Mastercycler in 50-μl volumes containing 1 μl of extracted nucleic acid, 0·4 μl HotMaster Taq DNA polymerase 5 U μl^{-1} (Eppendorf, Hamburg, Germany), 5 μl HotMaster Buffer with 25 mmol l^{-1} Mg^{2+} (Eppendorf), 1 μl deoxynucleotide triphosphate (Eppendorf dNTP Mix 10 mmol l^{-1}) and 5 μl each primer (10 μmol l^{-1}). An initial 94°C denaturing step for 2 min was followed by 30 cycles of amplification (20 s at 94°C; 10 s annealing starting at 65°C for the first cycle reducing by 0·5°C per cycle to 50°C; 45 s extension at 72°C) and a final 10-min extension at 72°C.

The PCR amplification products were electrophoresed in 0·8% agarose gel and visualized with ethidium bromide staining (0·5 μg ml^{-1}) on an ultraviolet transilluminator to confirm amplification of the desired DNA fragments. PCR products were purified using a QIAquick PCR Purification kit (Qiagen®) according to manufacturer's instructions. The 16S rDNA nucleotide sequences were determined by ABI Prism® BigDye™ dye-terminator chemistry (Applied Biosystems – Life Technologies, Carlsbad, CA) at the 'BMR Genomics' facility at Padova University (http://www.bmr-genomics.it). Sequences were aligned to known sequences in the GenBank database using BLAST (Altschul *et al.* 1990). To identify 16S rDNA chimeras and other sequencing anomalies, all sequences were analysed with the software tool PINTAIL (Ashelford *et al.* 2005). Multiple sequence alignments were performed using CLUSTAL_X 2·0·11 software, and a phylogenetic tree constructed by the neighbour-joining method and drawn with CTREE ver. 1 software (Archer and Robertson 2007). The gene sequences determined in this study are deposited in GenBank under the accession numbers GU120672-GU120684.

Results

Several different colony types grew on the TCBS agar while bacterial growth was not observed on GSP agar plates. A total of 39 pure cultures were obtained: 14 were not assigned to *Vibrio* genus because of negative response to oxidase (and for this reason they were discarded); 25 were Gram negative, were oxidase positive, were 3% NaCl tolerant, reduced nitrates, grew in anaerobic conditions, fermented glucose and were fast growing at 25°C (colonies appeared after 1 day); 21 of 25 isolates grew also at 37°C but none of them at 44°C.

The 25 isolates showed different cultural and biochemical characteristics resulting in 10 phenotypic profiles (PhtI to PhtX) (Table 1). Phenotypes could be separated into four groups on the basis of arginine dihydrolase and lysine- and ornithine decarboxylase reactions: ADH+, LDC−, ODC− and ADH−, LDC+, ODC− were made up of two phenotypical profiles each, while ADH−, LDC+, ODC+ and ADH−, LDC−, ODC− include three profiles each; isolates belonging to ADH−, LDC−, ODC− group did not grow at 37°C. The occurrence of phenotypes among the analysed samples is reported in Table 2; only phenotype I was present in all samples tested.

The molecular characterization by partial 16S rRNA gene sequence analysis was performed on 12 isolates, recovered from two samples (S1 and S2), corresponding to one isolate from each of the 10 phenotypes, except for PhtI and PhtVI of which two isolates were selected. The 16S rDNA sequences classified all isolates in the genus *Vibrio* when compared with the online GenBank database, with identity scores ranging between 99 and 100%. Phylogenetic analysis using the neighbour-joining method revealed six of the isolates to be clustered within the group of *Vibrio* species called the *V. harveyi* group (Reichelt *et al.* 1976), also referred to the core group of the *Vibrio* genus (Dorsch *et al.* 1992). Three isolates clustered with known *Vibrio chagasii* strains, and the remaining three isolates clustered with known strains of *Listonella anguillarum* (Fig. 2).

Discussion

In this study, we analysed the phenotypic diversity of 25 *Vibrio* isolates collected from skin lesions of diseased

Table 1 Biochemical and physiological profiles of the isolates

Test	I† (*n* = 4)‡	II (*n* = 2)	III (*n* = 2)	IV (*n* = 2)	V (*n* = 2)	VI (*n* = 3)	VII (*n* = 3)	VIII (*n* = 3)	IX (*n* = 2)	X (*n* = 2)
Yellow colonies on TCBS	+	+	+	−	−	+	+	+	−	+
Indole	+	+	+	+	+	+	+	+	+	−
Swarming (3% NaCl)	+	+	−	−	−	+	−	−	+	−
Growth at (°C)										
37	+	+	+	+	+	+	+	−	−	−
44	−	−	−	−	−	−	−	−	−	−
Gas from glucose	−	−	−	−	−	−	−	−	−	−
Arginine dihydrolase	+	+	−	−	−	−	−	−	−	−
Lysine decarboxylase	−	−	+	+	+	+	+	−	−	−
Ornithine decarboxylase	−	−	−	−	+	+	+	−	−	−
Urease	−	−	−	−	−	−	+	−	−	−
Gelatinase	+	+	+	+	−	+	+	+	+	+
Citrate utilization	+	−	−	−	−	−	−	−	−	−
Esculin	−	−	+	+	+	+	+	+	+	−
Voges–Proskauer	−	−	−	−	−	−	−	−	−	−
Acid from										
Glucose	+	+	−	−	+	+	+	+	+	+
Mannitol	+	+	−	−	+	−	−	+	+	−
Inositol	−	−	−	−	−	−	−	−	−	−
Sorbitol	+	+	−	−	−	−	−	−	−	−
Rhamnose	−	−	−	−	−	−	−	−	−	−
Sucrose	+	+	+	−	−	+	+	+	−	+
Melibiose	−	−	−	−	−	−	−	−	−	−
Arabinose	−	−	−	+	−	−	−	−	−	−

†Phenotype.
‡Number of strains.

Table 2 Distribution of the phenotypic profiles (PhtI–PhtX) among samples (four Atherina boyeri; S1–S4)

	S1† (*n* = 6)‡	S2 (*n* = 6)	S3 (*n* = 7)	S4 (*n* = 6)
PhtI	*	*	*	*
PhtII		*	*	
PhtIII	*		*	
PhtIV		*		*
PhtV		*	*	
PhtVI	*	*		*
PhtVII	*		*	*
PhtVIII	*		*	*
PhtIX		*		*
PhtX	*		*	

†Sample name.
‡Number of strains isolated from each sample.

A. boyeri. Haemorrhages on body surface of diseased fish represent one of the clinical features of vibriosis, which, in the Mediterranean area, was mainly caused by *L. anguillarum* (Toranzo and Baria 1990; Yiagnisis *et al.* 2007 and the reference therein), by *V. harveyi* (Zorrilla *et al.* 2003; López *et al.* 2009) and also by *V. ordalii* (Korun and Timur 2008) or *V. alginolyticus* (Ben Kahla-Nakbi *et al.* 2006; Snoussi *et al.* 2008). Because of the great variability of diagnostic features among *Vibrio* species, in accord with several researchers (Gomez-Gil *et al.* 2004; Thompson *et al.* 2004), molecular analyses were necessary to unambiguously identify the isolates. The number of isolates was reduced with wide phenotypic representation, resulting in ten phenotypes, subsequently genetically identified as *L. anguillarum*, *V. Harveyi* and *V. chagasii* (Table 2).

The genotypically identified *L. anguillarum* isolates corresponded to phenotype I and II that have similar biochemical profiles except for citrate utilization (Table 1); although bacteria belonging to phenotype I (OGS110, OGS111) resulted positive for glucose, mannose, mannitol, maltose, *N*-acetyl-glucosamine, potassium gluconate and malate assimilation (data not shown), isolates genetically characterized shared similar biochemical characteristics with typical *L. anguillarum* strains (Pazos *et al.* 1993).

Five isolates identified as *V. harveyi* encompassed five different phenotypes (III, IV, V, VI and VII). Such a broad phenotypic variability created a mismatch between genotypical and phenotypical approaches, because *V. harveyi* is typically considered as positive for gelatinase, glucose and mannitol fermentation and negative for urease production and arabinose fermentation; also, the ability of *V. harveyi* to produce ornithine decarboxylase is a key phenotypic feature to separate it from *V. campbelli* (Alsina and Blanch 1994). Finally, all isolates shared the ability

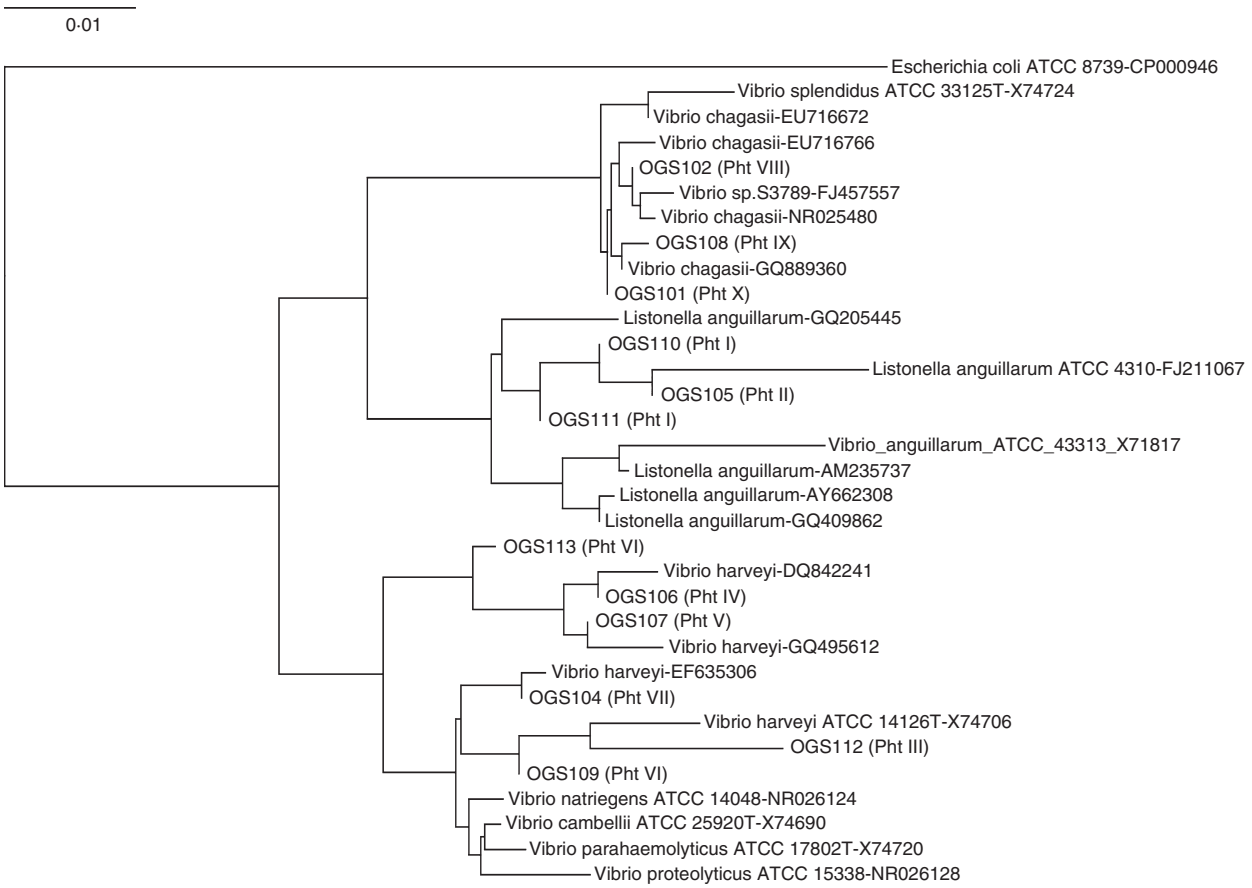

Figure 2 Phylogenetic relationship between isolates and *Vibrio* spp. based on the 16S rRNA gene sequence analysis using the neighbour-joining method. The tree is unrooted with *Escherichia coli* as the outgroup. The scale bar indicates 1% sequence divergence.

to hydrolyse aesculin, an unusual characteristic in *V. harveyi* descriptions previously reported by López *et al.* (2009) and regarding *V. harveyi* isolated from diseased cultured wedge sole (*Dicologoglossa cuneata*) in south–western Spain.

Vibrio chagasii isolates differed for sucrose fermentation (forming colonies both yellow and green on TCBS agar), did not produce lysine and ornithine decarboxylase and urease, fermented D-glucose but not rhamnose and arabinose, confirming specific traits of the species first described by Thompson *et al.* (2003); contrarily, phenotype IX resulted swarming, phenotype X did not produce indole but fermented mannitol, and two phenotypes (VIII and X) produced acid from sucrose. In addition, all the strains identified in this study as *V. chagasii* resulted negative for arginine dihydrolase, a trait recorded as variable by Thompson *et al.* (2003). Thus, despite the isolates being clustered in three genetically similar groups, phenotype variability within each cluster, especially for *V. harveyi*-related strains, was so wide to invalidate biochemical identification that still represent the conventional methods used in official laboratory controls.

Phenotypes were heterogeneously distributed among *Atherina* samples except PhI that was identified as *L. anguillarum* in all cases (Fig. 2) and was retrieved from all diseased animals.

Notwithstanding the small number of genetically identified strains that does not allow robust statistics, it is important to note that the coexistence of these three potential pathogenic Vibrios in external lesions of diseased fishes has not been reported so far. In fact, both *V. harveyi* and *L. anguillarum* have been recognized as pathogenic for a wide variety of marine animals (Pazos *et al.* 1993; Gomez-Gil *et al.* 2004; Paillard *et al.* 2004; Austin and Zhang 2006). In particular, Yiagnisis *et al.* (2007) reported for the first time high mortalities of *A. boyeri* in Greece and attested *L. anguillarum* as the causative agent; the microbiological analysis of the moribund sand smelts (head kidney and brain) resulted in the isolation of pure cultures with a single phenotype highly similar to the one we are describing as phenotype I, except for the Voges–Proskauer-positive reaction. Moreover, *V. harveyi* was recently identified as causative bacterium for a tail rot disease of *Sparus aurata* from a hatchery in Malta (Haldar *et al.* 2010); in this study, repre-

sentative isolates corresponded to six ribotypes that caused different percentages of mortality in challenge experiments.

Vibrio chagasii was described only recently (Thompson *et al.* 2003) as a *Vibrio splendidus*-related species on a genotypic basis. Organisms within *V. splendidus* group are known to harbour a great variability in their phenotypic profiles (Macian *et al.* 2001; Thompson *et al.* 2003; Gay *et al.* 2004), which historically have been associated with molluscs and fish mortalities events (Austin and Austin 1999; Le Roux *et al.* 2002; Gay *et al.* 2004). Austin *et al.* (2005) demonstrated that cultures of *V. chagasii* expressed either non- or low virulence in the rainbow trout, but further information is needed to explore in depth the involvement of *V. chagassi* in fish pathologies. Despite the great attention that the *V. splendidus* clade has been receiving in the last decade, no definite biochemical method is available to clearly discriminate species within this group (Macian *et al.* 2001, Thompson *et al.* 2003; Gay *et al.* 2004), and the combination of phenotypic and genotypic characterization is therefore required for the identification of *V. chagassi*.

Although it is not possible to determine which was the aetiological agent within the shoal of fish we have collected, it is important to note that there can be a positive interaction between putative primary and secondary colonizers. The broad variability in metabolic expression and the possible high diversity within one single lesion can be a cooperative strategy adopted by Vibrios rather than an antagonistic ploy. In fact, reports on the enhanced virulence expressed by a consortium instead of a single strain continue to rise (Gay *et al.* 2004; Cervino *et al.* 2008).

These observations might have epidemiological implications because *L. anguillarum*, *V. harveyi* and *V. chagasii* are potential pathogen species that can infect several wild and farmed organisms (Austin and Austin 1999; Toranzo *et al.* 2005; Austin and Zhang 2006), among which *A. boyeri*, being widely distributed in the Mediterranean Sea, might be an important vector in many different environments (from shallow coastal to brackish waters employed for aquaculture). For these reasons, the present study implements the current knowledge on the potential phenotypical and genotypical variability of Vibrios originating even from a single skin lesion affecting the widely distributed sand smelt.

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