Veterinaria Italiana, 2011, **47** (3), 371-378

Detection of *Vibrio splendidus* and related species in *Chamelea gallina* sampled in the Adriatic along the Abruzzi coastline

Marina Torresi[(1)], Vicdalia A. Acciari[(1)], Annamaria Piano[(2)], Patrizia Serratore[(2)], Vincenza Prencipe[(1)] & Giacomo Migliorati[(1)]

Summary

Vibrio species are an important and widespread component of marine microbial communities. Some *Vibrio* strains are potentially pathogenic to marine vertebrates and invertebrates. The aim of this study was to identify vibrios, in particular *Vibrio splendidus* and related species, isolated from clams (*Chamelea gallina*) collected along the coasts of the Abruzzi region from May to October 2007. The isolates obtained were phenotyped and classified as belonging to the genus *Vibrio*. The strains underwent biochemical testing in accordance with Alsina's scheme for *V. splendidus* identification. Molecular analysis of the 16S-23S intergenic space region and *recA* gene was used to identify *V. splendidus* and related species. All the samples examined were found to contain halophylic *Vibrio* species, with *V. alginolyticus*, *V. splendidus*-related species and *V. mediterranei* most commonly found. A polymerase chain reaction of the 16S-23S intergenic space region and sequencing of the *recA* gene from isolates confirmed that phenotyping of *Vibrio* species is not sufficient to distinguish between different species. Differentiation of the highly related species among *V. splendidus*-related clusters remains an important issue. In this regard, our data suggests sequencing the *recA* genes was far more discriminatory than sequencing 16S rDNA for this purpose.

Keywords

Abruzzi, Adriatic Sea, *Chamelea gallina*, Italy, *RecA*, *Vibrio splendidus*, *Vibrio* spp.

Introduction

The *Vibrio* genus currently comprises about 81 species of Gram-negative, non-spore-forming halophylic bacilli which are widespread in estuaries and marine environments (18).

Various species have been implicated in outbreaks of human gastrointestinal infections following the consumption of bivalve molluscs or raw or undercooked seafood. *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* are the species most often isolated from gastro-intestinal infections in humans.

Other species are potential aetiological agents of infectious diseases in both vertebrate and invertebrate marine animals. Among these, *V. splendidus*, *V. alginolyticus*, *V. harveyi*, *V. lentus*, *V. salmonicida* and *V. anguillarum* have been repeatedly reported as the cause of infection. *V. splendidus* is a ubiquitous member of marine microbial communities. It is not a human pathogen, but some strains have shown pathogenicity in numerous marine species, including bivalves (10) and teleost fish (8). It has also been isolated in several cases of oyster and scallop larva mortality in France

(1) Istituto 'G. Caporale' Teramo, via Campo Boario, 64100 Teramo, Italy
m.torresi@izs.it, g.migliorati@izs.it
(2) Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale, Università di Bologna, viale Vespucci, 2, 47042 Cesenatico (FC), Italy

© Istituto G. Caporale 2011

(12) and turbot larva mortality in Spain (9). Recent studies conducted by the University of Bologna's Department of Veterinary Public Health and Animal Pathology and the Marine Research Centre in Cesenatico postulated that some cases of mortality in the clam *Chamelea gallina* in the Adriatic might be associated with the presence of *V. splendidus* (16).

Identification of *Vibrio* species requires molecular and genetic tests that enable the differentiation of highly similar species that cannot be distinguished by biochemical analysis alone. The literature also indicates that 16S rDNA sequencing is not sufficiently discriminatory and that the *recA* gene, coding for a multifunctional protein involved in the homologous recombination and repair of DNA, is a valid alternative phylogenetic marker for the identification of *Vibrio* spp. (17).

Studies conducted over the last 10 years have demonstrated significant levels of *Vibrio* spp. in the seas around Italy and especially along the northern and central Adriatic coastline and the coast of the Ionian Sea (3, 4, 6, 14, 22). However, to date, no similar study has been performed on the waters along the Abruzzi coast.

The aim of our study was to detect and isolate *Vibrio* spp. in samples of *C. gallina* harvested from the sea along the Abruzzi and Molise coastline, to reveal the distribution of *Vibrio* species, especially strains from the important group of *V. splendidus*-related species.

Materials and methods

Samples were taken between May and October 2007. A total of 37 samples of *C. gallina* were taken from the sea along the Abruzzi and Molise coastline. The sampling points were located 500 m, 750 m or 1000 m from the coast. Samples were transported under refrigeration (4°C ± 2°C) and examined within 24 h of sampling. American Type Culture Collection (ATCC) 33125, ATCC 25914 and ATCC 33870 (LGC Promochem, Milan) were used as the *V. splendidus* reference strains.

Detection and biochemical identification of *Vibrio* spp. strains

A total of 25 g of meat and shell liquid were taken from each sample and transferred to a Stomacher® bag containing 225 ml of alkaline peptone water (APW). *Vibrio* spp. were then detected and the isolated strains identified as described in International Organization for Standardization (ISO) 21872-2:2007 (2), with the use of incubation at both 37°C ± 1°C and 25°C ± 1°C, given the stronger growth of *V. splendidus*' at the cooler temperature. Suspect colonies were identified biochemically using Alsina's identification scheme (1).

Polymerase chain reaction

All isolates identified biochemically as *Vibrio* spp. underwent amplification of the 16S-23S intergenic spacer (IGS) region, specific for *V. splendidus*. Those testing positive then underwent *recA* gene amplification. Each isolate cultured on tryptic soy agar containing 2% NaCl for 48 h at 25°C ± 1°C was then suspended in 1 ml of Tris-ethylenediamine-tetraacetic acid (EDTA) (50 mM Tris-HCl pH 8.0-1 mM EDTA pH 8.0), and centrifuged (Eppendorf 5402 rotor F-45-18-11, Milan) at 15 000 g for 5 min at 4°C. The pellet was then reconstituted in 300 µl of Tris-EDTA and heated at 100°C for 15 min to obtain cell lysis. After centrifugation at 15 000 g for 5 min at 4°C the supernatant containing the DNA was transferred to a clean test tube.

The primers (MWG, Ebersberg) listed in Table I were used for amplification of the 16S-23S rDNA IGS region (13) and of the *recA* gene (20).

For IGS amplification, a reaction mixture containing 25 µl of polymerase chain reaction (PCR) master mix (Promega, Madison, Wisconsin), 0.5 µM of each primer and 2 µl of DNA (approx. 100 ng-175 ng) was prepared, for a final total volume of 50 µl. The reaction was performed in an iCycler (Bio-Rad, Hercules, California) thermal cycler in the following conditions: 1 cycle of 10 min at 94°C, 35 cycles of 1 min at 94°C, 1.5 min at 53°C and 2.5 min at 72°C, a final cycle of 10 min at 72°C and storage at 4°C.

© Istituto G. Caporale 2011

Table I
Primers for 16S-23S rDNA intergenic spacer region (IGS) amplification and *recA* gene amplification and sequencing

Gene target	Primer	Sequence (5´→3´)	Length (bp)	Amplicon size (bp)
IGS 16S-23S rDNA	VSPN-F	GATTTAGTTAAAGCCAGAGC	20	240, 294
	VSPN-R	CCTGATAACTGTTTGCCG	18	
recA gene	*recA*-F	TGARAARCARTTYGTTAAAGG	21	837
	recA-R	TCRCCNTTRTAGCTRTACC	19	

For *recA* amplification, a reaction mixture containing 25 µl of PCR master mix, 1 µM of each primer, 0.5 µM of $MgCl_2$ and 2 µl of DNA (approx. 10 ng-175 ng) was prepared, for a final total volume of 50 µl. The reaction was performed in the following conditions: 1 cycle of 10 min at 10°C, 35 cycles of 1 min at 94°C, 1.5 min at 53°C and 2.5 min at 72°C, a final cycle of 10 min at 72°C and storage at 4°C.

PCR products were analysed by electrophoresis in a 1.5% agarose gel (Agarose D-1-LOW EEO, Eppendorf, Milan) containing SYBR® safe 0.25X (Invitrogen, Carlsbad, California). Bands were visualised using a UV transilluminator and photographed using the ChemiImager 5500 program. An AmpliSize™ molecular ruler 50 bp-2 000 bp ladder (Bio-Rad, Hercules, California) was used as a molecular weight marker.

RecA gene sequencing

The sequencing reaction was performed on a mixture containing 2 µl BigDye Terminator v 1.1 sequencing buffer 5X, 1 µl BigDye® ready reaction mix (Applied Biosystems, Foster City, California), 16 nM *recA* primer (Table I) and 1 µl-3 µl purified DNA (QIAquick® gel extraction kit, Quiagen, Hilden), for a final volume of 10 µl. The mixture underwent 25 cycles of 10 sec at 96°C, 10 sec at 58°C and 2.5 min at 60°C.

The reaction products were purified by adding 10 µl nuclease-free water, 50 µl ethanol 96% and 2 µl sodium acetate 3 M. They were then left at room temperature for 15 min and centrifuged at 14 000 g for 20 min. A total of 200 µl of ethanol 70% was added to the pellet. After centrifuging for 5 min, the ethanol was discarded and the pellet was left to dry for 45 min at room temperature. The products were suspended in 25 µl formamide, denatured for 2 min at 95°C and loaded into the automated sequencer ABI PRISM 310 genetic analyser (Applied Biosystems, Foster City, California).

Sequence analysis

Nucleotide sequences for each isolated strain were corrected using the ProSeq program (version 3.0; [en.bio-soft.net/format/ProSeq.html]) and aligned using Clustal_X (21). The software program 'Molecular evolutionary genetics analysis' (version 4.0; [www.megasoftware.net/mega4/mega.htm]) was used for genetic analysis and study of the phylogenetic relationships between the various species encountered, by constructing phylogenetic trees based on p-distance with the neighbour-joining statistical method. Sequences were compared against those in the GenBank database (www.ncbi.nlm.nih.gov/genbank/) using the online 'Basic local alignment search tool nucleotide (BLASTn)' program (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Results

As was to be expected, *Vibrio* spp. were readily recovered from all 37 samples examined. A total of 131 isolates were identified by biochemical methods as belonging to the *Vibrio* genus. Species identification was also possible for 100 strains using Alsina's scheme. Biochemical analysis revealed *V. alginolyticus* to be the most frequently isolated species, followed by *V. splendidus* biovar II (Fig. 1).

Phenotyping identified 28 strains of *V. splendidus* biovar II and one strain of *V. splendidus* biovar I, with the bacterium under study being found in 46% of harvested samples. Amplification of the 16S-23S IGS

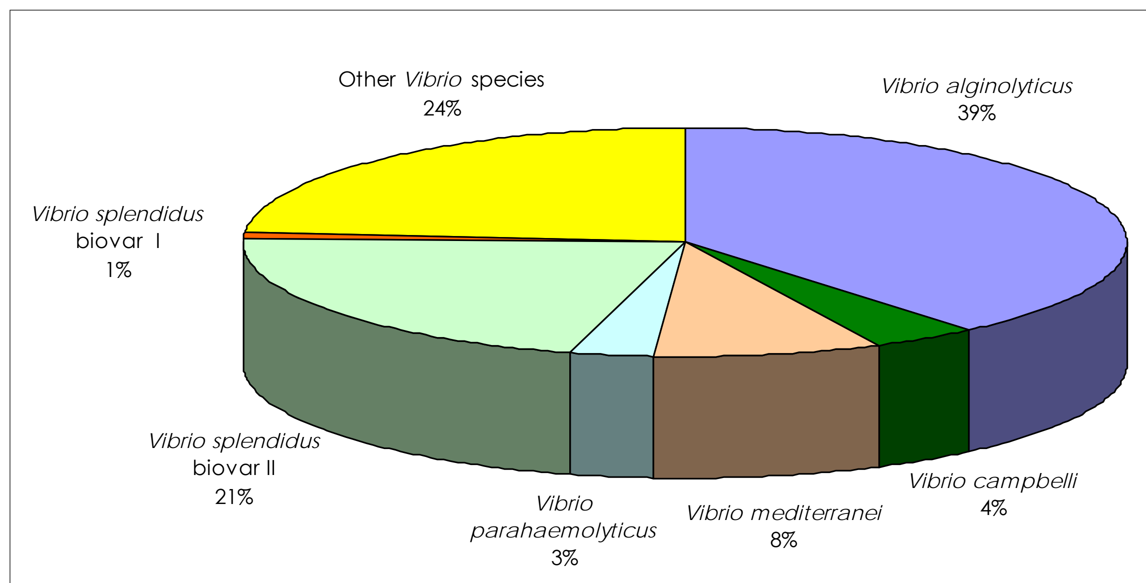

Figure 1
Percentages of the various *Vibrio* species isolated by biochemical analysis

using *V. splendidus*-specific primers for the 131 isolated strains demonstrated that 25 of the 29 strains found positive on biochemical testing were likely to be true positives and 98 of the 102 found negative by these methods were likely to be true negatives.

The 29 strains that produced amplicons of the expected size were then subjected to automated sequencing of the *recA* gene. A gene fragment of approximately 720 bp, corresponding to 68% of the coding region, was amplified and sequenced. The dendrogram produced with the *recA* gene sequences revealed that all isolates except one formed part of the cluster known as the *V. splendidus*-related group showing *recA* sequence similarity between 87% and 100%. This cluster was split into two main sub-clusters, as follows:

- the first, containing the *V. chagasii* reference strains AJ580874 and AJ580875, included 11 of the strains isolated in this study, and it has showed *recA* sequence similarity between 96% and 100%
- the second, containing the *V. splendidus* reference strain AJ580878 and related species (*V. cyclitrophicus*, *V. lentus*, *V. kanaloe*, *V. pomeroyi*, *V. tasmaniensis*), included the remaining 17 strains and it showed *recA* sequence similarity ranging from 90.4% to 100%.

The only strain not included in the *V. splendidus*-related group (GenBank Accession No. JF432064) was found at the edge of the cluster, as no sequences with a nucleotide similarity of above 82% were found in the database consulted. The phylogenetic tree for the 29 *V. splendidus*-related strains isolated during this study, *V. splendidus* ATCC 33125, 25914, 33870 and 16 reference strains from the GenBank (*V. hollisae* AJ580906, *V. chagasii* AJ580874, *V. chagasii* AJ580875, *V. cholerae* AJ580897, *V. cyclitrophicus* AJ580877, *V. fortis* AJ580871, *V. harveyi* AJ580866, *V. lentus* AJ580879, *V. lentus* AJ580880, *V. kanaloae* AJ580882, *V. mimicus* AJ580896, *V. parahaemolyticus* AJ580864, *V. pomeroyi* AJ580876, *V. splendidus* AJ580878, *V. tasmaniensis* AJ580881 and *V. vulnificus* AJ580890) constructed using the neighbour-joining (N-J) method based on p-distance, is shown in Figure 2.

Discussion

Except for the failure to isolate *V. harveyi*, the biochemical analysis of the distribution of *Vibrio* spp. concurs with previous studies, which report *V. alginolyticus*, followed by *V. harveyi*, *V. mediterranei* and *V. splendidus* II

© Istituto G. Caporale 2011

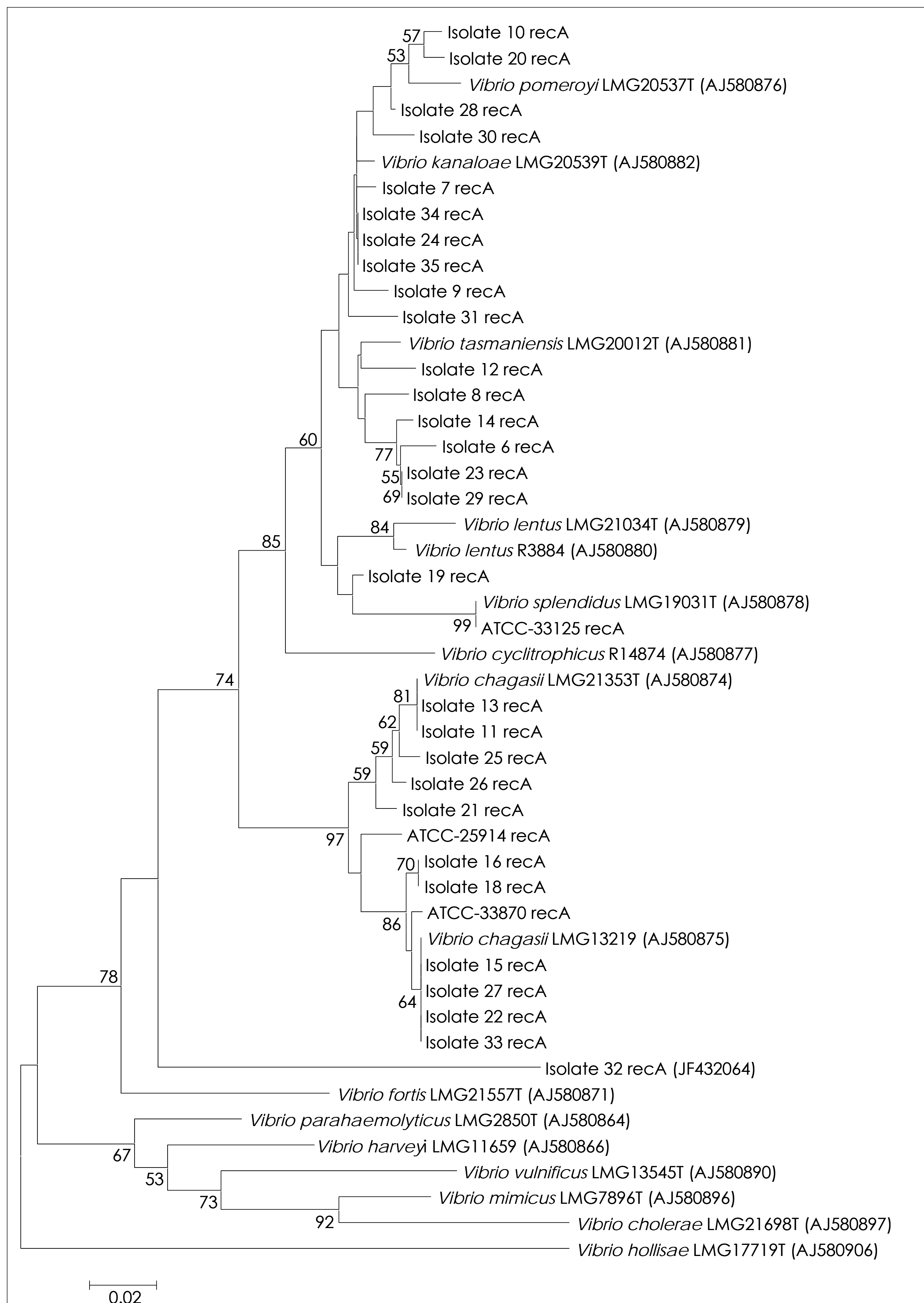

Figure 2
Phylogenetic tree obtained by alignment of *recA* gene sequences against the strains isolated in this study and comparative sequences from GenBank
These sequences are preceded by the accession number
The dendrogram was constructed with the neighbour-joining method based on p-distance
Bootstrap values above 50% after 1 000 simulations are shown in correspondence with the nodes

© Istituto G. Caporale 2011

among the most frequently isolated species (5, 6, 14). The frequency of *V. splendidus* II strains isolated in our study was greater than that seen along the coast of the Marches region or the Gulf of Taranto, but less than that found along the Galician coast in Spain.

A comparison of the results from isolates identified to species level by both phenotyping and molecular analysis revealed that biochemical analysis produced four false positives and four false negatives for *V. splendidus*.

Further confirmation of the complexity of the *V. splendidus*-related group was provided in that most isolates for which PCR amplification of the 16S-23S IGS had produced amplicons positive for *V. splendidus* were subsequently identified by *recA* gene sequencing as *V. chagasii*, *V. pomeroyi*, *V. tasmaniensis* or *V. kanaloe*. The *V. splendidus* reference strains ATCC 25914 and ATCC 33870 were found to be part of the sub-cluster containing the *V. chagasii* reference strains AJ580874 and AJ580875, not the sub-cluster containing the *V. splendidus* reference strain AJ580878.

Our higher detection of *V. splendidus* strains and failure to isolate *V. harveyi* may be due to the focus on *V. splendidus* isolation as the target of our study, as well as the use of sampling points at a depth where the temperature is more suited to *V. splendidus* than to other species, which are more easily found at higher temperatures. Indeed, a study of the coastal waters of the Conero Riviera in Italy found that at temperatures between 15°C and 20°C, *V. splendidus* II and *V. mediterranei* were the most common species encountered, whereas *V. harveyi* was isolated less frequently (14). Further studies are thus needed to assess the relationship between the physical and chemical properties of the sampling area and the *Vibrio* species present.

The finding of false negatives and false positives further confirms that phenotyping provides insufficient information to identify different species in the *V. splendidus*-related group, validating the thesis is that it is almost impossible to distinguish between many of the currently known *Vibrio* species, especially if closely related, on the base of biochemical testing alone (19).

Our analyses confirm that *recA* sequencing is a valid alternative to 16S rDNA gene sequence analysis, which did not enable *Vibrionaceae* identification to the species level for some strains in previous studies (17, 20). In fact, sequencing of this gene enabled differentiation of closely related strains which could not be distinguished by phenotyping or PCR of the 16S-23S IGS, while also highlighting the strong genetic similarity of the strains in the '*V. splendidus*-related' cluster, as reported by other authors (5, 18, 20). The division of the strains into two main clusters comprising the *V. chagasii* reference strains and the *V. splendidus* reference strain and related species, such as *V. lentus*, *V. kanaloe*, *V. pomeroyi* and *V. tasmaniensis*, coincides with the reports of other workers using techniques such as *recA* and *atpA* gene sequencing (18), multi-locus analysis of other targets such as 16S rDNA, *rpoA*, *pyrH* and *recA* (20), and AFLP (5). The sequences of the strains analysed also showed a high level of similarity with those in GenBank.

The high genetic similarity observed between clusters, including *V. splendidus* and *V. chagasii* reference strains, can be explained as the result of their adaptation to the niche in which these species coexist in the same environment (7, 20). As suggested by Cohan (7), natural selection is the main force guiding the evolution of bacterial species, and horizontal transmission of genes among similar species may be a basic evolutionary means in the *Vibrio* genus. Recent studies have revealed numerous cases of horizontal gene transfer in the sense of new acquisitions and displacements of paralogous genes, which in *V. cholerae* involve a significant part of the genome (5.6%) (11).

The strain not included in the *V. splendidus*-related group suggests that this may be a representative of an as yet undescribed species This is in accordance with suggestions that the number of species attributable to the heterogeneous group of the *Vibrio* genus is constantly changing and that the description of new species in years to come is to be expected (15). For instance, molecular analysis of

© Istituto G. Caporale 2011

bivalves in a recent study by Beaz-Hidalgo *et al.* (5) failed to identify the species in 12% of the *Vibrio* strains isolated.

The frequency with which unidentifiable species are detected suggests the need for further studies using an innovative multigene approach in order to study several targets simultaneously, thus increasing the chance of correctly identifying all species of *Vibrio*.

Conclusions

Our study provided evidence suggesting the following:

- bivalves collected along the Italian coastline harbour a diverse and dynamic *Vibrio* spp. community, a fraction of which belongs to *V. splendidus*-related species
- many *Vibrio* spp. are difficult to identify using conventional methods
- *RecA* gene sequencing contributes to more precise identification than that obtained in previous studies based on biochemical analysis or PCR of the 16S-23S IGS region
- a fraction of the *Vibrio* spp. are difficult to classify with *recA* gene sequencing which confirms the need for multiple genetic markers to enhance the discrimination of all currently recognised species of vibrios.

Grant support

This study was conducted as a part of research project funded by the Italian Ministry of Agriculture and Forestry.

References

1. Alsina M. & Blanch A.R. 1994. A set of keys for biochemical identification of environmental *Vibrio* species. *J Appl Bacteriol*, **76**, 79-85.
2. Anon. 2007. ISO/TS 21872-2. Microbiology of food and animal feeding stuffs. Horizontal method for the detection of potentially enteropathogenic *Vibrio* spp. Part 2: Detection of species other than *Vibrio parahaemolyticus* and *Vibrio cholerae*. International Organization for Standardization, Geneva, 24 pp.
3. Baffone W., Pianetti A., Bruscolini F., Barbieri E. & Citterio B. 2000. Occurrence and expression of virulence-related properties of *Vibrio* species isolated from widely consumed seafood products. *Int J Food Microbiol*, **54**, 9-18.
4. Barbieri E., Falzano L., Fiorentini C., Pianetti A., Baffone W., Fabbri A., Matarrese P., Casiere A., Katouli M., Kühn I., Möllby R., Bruscolini F. & Donelli G. 1999. Occurrence, diversity, and pathogenicity of halophilic *Vibrio* spp. and non-O1 *Vibrio cholerae* from estuarine waters along the Italian Adriatic coast. *Appl Environ Microbiol*, **65**, 2748-2753.
5. Beaz-Hidalgo R., Cleenwerck I., Balboa S., De Wachter M., Thompson F.L., Swings J., De Vos P. & Romalde J.L. 2008. Diversity of vibrios associated with reared clams in Galicia (NW Spain). *Syst Appl Microbiol*, **31**, 215-222.
6. Cavallo R.A. & Stabili L. 2002. Presence of vibrios in seawater and *Mytilus galloprovincialis* (Lam.) from the Mar Piccolo of Taranto (Ionian Sea). *Water Res*, **36**, 3719-3726.
7. Cohan F.M. 2004. Concepts of bacterial diversity for the age of genomics. *In* Microbial genomes (C.M. Fraser, T.D. Read, & K.E. Nelson, eds). Humana Press Inc., Totowa, New Jersey, 175-194.
8. Farto R., Montes M., Pérez M.J., Nieto T.P., Larsen J.L. & Pedersen K. 1999. Characterization by numerical taxonomy and ribotyping of *Vibrio splendidus* biovar I and *Vibrio scophthalmi* strains associated with turbot cultures. *J Appl Microbiol*, **86**, 796-804.
9. Gatesoupe F.J., Lambert C. & Nicolas J.L. 1999. Pathogenicity of *Vibrio splendidus* strains associated with turbot larvae, *Scophthalmus maximus*. *J Appl Microbiol*, **87**, 757-763.
10. Gómez-León J., Villamil L., Lemos M.L., Novoa B. & Figueras A. 2005. Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from aquacultured carpet shell clam (*Ruditapes decussatus*) larvae associated with mass mortalities. *Appl Environ Microbiol*, **71**, 98-104.
11. Koonin E.V., Makarova K.S. & Aravind L. 2001. Horizontal gene transfer in prokaryotes: quantification and classification. *Annu Rev Microbiol*, **55**, 709-742.

© Istituto G. Caporale 2011

12. Lacoste A., Jalabert F., Malham S., Cueff A., Gelebart F., Cordevant C., Lange M. & Poulet S.A. 2001. A *Vibrio splendidus* strain associated with summer mortality of juvenile oyster *Crassostrea gigas* in the Bay of Morlaix (North Brittany, France). *Dis Aquat Organ*, **46**,139-145.
13. Lee S.K.Y., Wang H.Z., Law S.H.W., Wu R.S.S. & Kong R.Y.C. 2002. Analysis of the 16S-23S rDNA intergenic spacers (IGSs) of marine vibrios for species-specific signature DNA sequences. *Mar Pollut Bull*, **44**, 412-420.
14. Masini L., De Grandis G., Principi F., Mengarelli C. & Ottaviani D. 2007. Research and characterization of pathogenic vibrios from bathing water along the Conero Riviera (Central Italy). *Water Res*, **41**, 4031-4040.
15. Noguerola I. & Blanch A.R. 2008. Identification of *Vibrio* spp. with a set of dichotomous keys. *J Appl Microbiol*, **105**, 175-185.
16. Serratore P., Cattani O., Vitali G., Bosi S., Milandri S., Buda D. & Zoffoli S. 2001. Factors influencing mortality in *Chamelea gallina* populations: identifications of associated bacteria and their metabolic effects. *Ital J Biochem*, **50** (1-2), 62-63.
17. Thompson C.C., Thompson F.L., Vandemeulebroecke K., Hoste B., Dawyndt P. & Swings J. 2004. Use of *recA* as an alternative phylogenetic marker in the family *Vibrionaceae*. *Int J Syst Evol Microbiol*, **54**, 919-924.
18. Thompson C.C., Thompson F.L., Vicente A.C.P. & Swings J. 2007. Phylogenetic analysis of vibrios and related species by means of *atp*A gene sequences. *Int J Syst Evol Microbiol*, **57**, 2480-2484.
19. Thompson F.L., Lida T. & Swings J. 2004. Biodiversity of vibrios. *Microbiol Mol Biol Rev*, **68**, 403-431.
20. Thompson F.L., Gevers D., Thompson C.C., Dawyndt P., Naser S., Hoste B., Munn C.B. & Swings J. 2005. Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Appl Environ Microbiol*, **71**, 5107-5115.
21. Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. & Higgins D.G. 1997. The Clustal_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*, **25**, 4876-4882.
22. Vernocchi P., Maffei M., Panciotti R., Suzzi, G. & Gardini F. 2007. Characterization of Mediterranean mussels (*Mytilus galloprovincialis*) harvested in Adriatic Sea (Italy). *Food Control*, **18**, 1575-1583.

 © Istituto G. Caporale 2011