Short communication

Vibrio harveyi causes disease in seahorse, *Hippocampus* sp.

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A mass mortality among cultured seahorses, *Hippocampus kuda* and *Hippocampus* sp., occurred in spring 1998 in Tenerife, Spain. Seahorses were held together with tropical shrimps, *Stenopus hispidus*, in glass aquaria supplied with 1000 L^{-1} of sea water at 25 °C. The water supply was conducted between different tanks that contained various marine species, such as octopus, *Octopus vulgaris*, starfish, *Asterias rubens*, sea-urchin, *Paracentrotus lividu*, greater weever, *Trachinus draco*, grouper, *Epinephelus guaz* and Canarian shrimp, *Lismata amboiens*. None of these species was affected, including the shrimps that shared aquaria with the seahorses. Mortalities of seahorses were very high (more than 90%), and fish died in 3−5 days after the first clinical signs appeared. Moribund seahorses were microbiologically analysed and subsequently, chloramphenicol was used as a bath (30 mg L^{-1}) to control the outbreak. The mortality decreased after a few days of antibiotic treatment.

Diseased seahorses presented clinical signs similar to vibriosis: external haemorrhages, and haemorrhagic liver and ascitic fluid accumulation in the intestinal cavity. A bacterium identified as *Vibrio harveyi* was obtained in pure culture from samples of skin haemorrhages, mouth and liver of all moribund seahorses. The aim of this study was to characterize the *V. harveyi* strains isolated from diseased seahorse, and to confirm its pathogenicity by means of experimental infection.

Samples from skin haemorrhages, mouth and liver were analysed by streaking a piece of aseptically obtained tissue onto tryptone-soy-agar supplemented with 1% NaCl (TSA-1) and incubating at 25 °C for 24–48 h. Pure cultures were obtained from all samples. The isolated strains were Gram-negative rods, motile, oxidase- and catalase-positive, sensitive to the vibriostatic agent O129 at 150 μM and fermentative. The isolates were first characterized by API 20NE (BioMérieux, S.A. France) strips, which gave the same profile in all cases (7474445), identified by the database APILAB Plus (BioMérieux) supplied by the manufacturer as *V. vulnificus*, with a probability of 95.1%. Further identification was achieved by colony hybridization as previously described (Biosca, Amaro, Larsen & Pedersen 1997), using an alkaline phosphatase-labelled oligonucleotide DNA probe (VVAP) specific for *V. vulnificus*, constructed from a portion of the *V. vulnificus* haemolysin–cytolysin (*hlyA*) gene sequence (Wright, Miceli, Landry, Christy, Watkins & Morris 1993). Positive and negative controls used were *V. vulnificus* ATCC 27562 and *V. cholerae* ATCC 14035, respectively. All isolates were negative in colony hybridization experiments, which indicated that they were misidentified as *V. vulnificus*. Identification was continued by testing additional biochemical characteristics as described by Biosca, Oliver & Amaro (1996). On the basis of the results obtained, the seahorse isolates were identified as *V. harveyi*. They were almost identical to the type

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strain of *V. harveyi*, except for growth at 12 °C and luminescence (Table 1). *Vibrio harveyi* is a synonym of *V. carchariae* (Pedersen, Verdonck, Austin, Austin, Blanch, Grimont, Jofre, Koblavi, Larsen, Tiainen, Vigneulle & Swings 1998), which is recognized as a fish pathogen (Yii, Yang & Lee 1997). The present strains differed from the type strain of *V. carchariae* in swarming, production of urease, growth with 8% NaCl and at 40 °C, and the utilization of sucrose, arabinose, D-mannitol and L-citruline.

Cultures grown on TSA-1 were suspended in sterile phosphate buffered saline (PBS) at pH 7.2 and DO 600 nm was adjusted to 0.4. Aliquots of 0.1 mL of this suspension were spread onto Mueller–Hinton agar (Oxoid, Basingstoke, UK), and antimicrobial sensitivity tested using antimicrobial discs (Becton Dickinson, Pharmaceuticals, NJ, USA). The following drugs were used: tetracycline (30 μg), flumequine (30 μg), chloramphenicol (30 μg), oxolinic acid (10 μg), trimethoprim-sulphametoxazol (25 μg), nitrofurantoin (50 μg), oxytetracycline (30 μg), erythromycin (15 μg), furazolidone (50 μg), gentamicin (10 μg), kanamycin (30 μg) and polymyxin B (300 U). Strains were sensitive to tetracycline, flumequine, chloramphenicol, nitrofurantoin and polymyxin B. Chloramphenicol and flumequine produced the widest inhibition halos in the test plates.

The 50% lethal dose (LD_{50}) test, with batches of six seahorses per dose, were conducted by intraperitoneal (i.p.) injection as previously described (Alcaide, Amaro, Todolí & Oltra 1999). Seahorse (mean weight 4 g $fish^{-1}$), were injected with 0.05 mL of a bacterial suspension containing 10^7–10^2 cfu mL^{-1} (determined by plate counts on TSA-1), in PBS. Sterile PBS was injected i.p. into seahorses as a control. Mortalities were recorded daily for 14 days,

Table 1 Biochemical and physiological profiles of the isolates and the reference strains determined by API 20NE and conventional tests

Test	Strains (*n* = 12)	*V. harveyi* ATCC 14126	*V. carchariae* ATCC 35084
API code	7434445	7477745	7676645
Sensitivity to O129 (150 μg)	S	S	S
Swarming on TSA-3	–	–	+
Growth on TCBS	Y	Y	Y
Growth at percentage NaCl			
0%	–	–	–
3%	+	+	+
6%	+	+	+
8%	–	–	+
Growth at:			
4 °C	–	–	–
12 °C	–	+	+
28 °C	+	+	+
40 °C	+	+	–
β-Galactosidase (ONPG)	–	–	–
ADH (Thornley's)	–	–	–
ADH (Moeller's)	–	–	–
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
VP test	–	–	–
Indol	+	+	+
Urease	–	–	+
Luminescence	–	+	–
Acid from			
Sucrose, D-mannitol	+	+	+
L-arabinose, D-salicin, rhamnose, inositol, sorbitol	–	–	–
Sole carbon source			
Sucrose, D-mannitol, L-citruline	–	–	+
Ribose, galactose, fructose, cellobiose, piruvate, L-histidine	+	+	+
Dulcitol, D-xylose, inositol, L-lysine	–	–	–
Arabinose	+	+	–
L-serine	10[a]	+	+

[a] Number of positive strains; (+) positive results; (–) negative results; S: sensitive, R: resistant, Y: yellow colonies on TCBS agar; G: green colonies on TCBS agar.

and were only considered positive if the injected strain was recovered from assayed seahorses. The LD$_{50}$ as calculated by the method of Reed & Muench (1938) was 4×10^3 cfu fish^{-1}. Pure cultures of the inoculated strains were re-isolated from liver and skin haemorrhages of moribund seahorse. No mortality was detected in the controls. Clinical signs appeared 12–24 h after i.p. injection and mortalities began 1–7 days post-challenge. The signs observed in challenged seahorses reproduced those observed during the outbreak. This result confirmed the role of *V. harveyi* as the causative agent of the disease.

In the present work, an infectious disease affecting seahorse, *Hippocampus kuda* and *Hippocampus* sp., is described for the first time. The isolates from diseased seahorse had the same morphological and biochemical characteristics, and were identified as *V. harveyi* from comparison of their biochemical characteristics with the type strain of the species. *Vibrio harveyi* is a marine bacterium that causes luminous vibriosis (Zhang & Austin 1999) and is an important pathogen of cultured penaeid shrimp (Lavilla-Pitogo, Baticados, de Cruz-Lacierda & de la Peña 1990; Karunasagar, Pai, Malathi & Karunasagar 1994; Liu, Lee, Yii, Kou & Chen 1996; Montero & Austin 1999). It has also been reported as an opportunistic pathogen of common snook (Kraxberger-Beatty, McGarey, Grier & Lim 1990), and has been isolated from diseased marine fish such as *Acanthopagrus cuvieri* (Saeed 1995), sea bream, *Sparus aurata* (Balebona, Moriñigo, Faris, Krovacek, Mänsson, Bordas & Borrego 1995), and dentex, *Dentex dentex*, cultured on the Mediterranean coast of Spain (Company, Sitjà-Bobadilla, Pujalte, Garay, Alvarez-Pellitero & Pérez-Sánchez 1999). Further studies are in progress to characterize the virulence factors involved in the pathogenicity of *V. harveyi* isolates.

Acknowledgment

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