Vol. 103: 133–139, 2013
doi: 10.3354/dao02572

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published March 26

Identification and characterization of *Vibrio harveyi* associated with diseased abalone *Haliotis diversicolor*

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ABSTRACT: Mass mortality of farmed small abalone *Haliotis diversicolor* occurred in Fujian, China, from 2009 to 2011. Among isolates obtained from moribund abalones, the dominant species AP37 exhibited the strongest virulence. After immersion challenge with 10^6 CFU ml^{-1} of AP37, abalone mortalities of 0, 53 and 67% were induced at water temperatures of 20°C, 24°C, and 28°C, respectively. Following intramuscular injection, AP37 showed a low LD_{50} (median lethal concentration) value of 2.9×10^2 CFU g^{-1} (colony forming units per gram abalone wet body weight). The LT_{50} (median lethal time) values were 5.2 h for 1×10^6 CFU $abalone^{-1}$, 8.4 h for 1×10^5 CFU $abalone^{-1}$, and 21.5 h for 1×10^4 CFU $abalone^{-1}$. For further analysis of virulence, AP37 was screened for the production of extracellular factors. The results showed that various factors including presence of flagella and production of extracellular enzymes, such as lipase, phospholipase and haemolysin, could be responsible for pathogenesis. Based on its 16S rRNA gene sequence, strain AP37 showed >98.8% similarity to *Vibrio harveyi*, *V. campbellii*, *V. parahaemolyticus*, *V. alginolyticus*, *V. natriegens* and *V. rotiferianus*, so it could not be identified by this method. However, multi-locus sequence analysis (MLSA) of concatenated sequences, including the *rpoD*, *rctB*, *gyrB*, *toxR* and *pyrH* genes, identified strain AP37 as *V. harveyi*. Phenotypic characters of AP37 were identified by API 20E. In antibiotic susceptibility tests, strain AP37 exhibited susceptibility to 7 antibiotics and resistance to 13. This is the first report of a *V. harveyi*-related species being linked with the mass mortality of adult abalone *H. diversicolor* in southern China.

KEY WORDS: *Small abalone* · Vibriosis · Characterization · Virulence · Multi-locus sequence analysis · MLSA

INTRODUCTION

China is one of the key abalone-producing countries in the world, with 5000 and 3000 t per annum produced in the mainland and Taiwan, respectively (Flores-Aguilar et al. 2007, You et al. 2010). Small abalone *Haliotis diversicolor* is naturally distributed along the coastal waters of East Asia from Japan to the Philippines (Lindberg 1992), and is a commercially important species cultured along the coast of southern China (Cai et al. 2008). Since late 2000, small abalone farmers have experienced mass mortality in grow-out ponds (You et al. 2010), leading to considerable damage. Bacterial infection is likely to be an important factor in this mortality (Nicolas et al. 2002, Handlinger et al. 2005, Sawabe et al. 2007). Different types of *Vibrio* infections leading to abalone death over a wide geographical area have been reported, including *V. alginolyticus*, *V. parahaemolyticus* and *V. vulnificus* isolated from *H. diversicolor* (Wang et al. 1999, Liu et al. 2000, Cai et al. 2006), *V. carchariae* (*V. harveyi*) from *H. tuberculata* (Jean-Louis et al. 2002, Nicolas et al. 2002), and *V. fluvialis* and *V. harveyi* from *H.*

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discus hannai (Li et al. 1998, Sawabe et al. 2007, Austin 2010).

Vibrio spp. are widely distributed in coastal and estuarine waters, and inhabit eukaryotic marine hosts, including molluscs, shrimps and zooplankton, at high densities throughout the world (Thompson et al. 2007). Recently, *V. harveyi* and related species have been recognized as some of the most significant causative agents of disease in marine-reared vertebrates and invertebrates, particularly shrimp and fish (Austin & Zhang 2006, Cano-Gomez et al. 2011). Due to genetic and phenotypic similarities, discrimination among members of the Harveyi clade of vibrios remains difficult, and this has led to misidentifications and inaccurate estimations of a species' involvement in certain environments (Lin et al. 2010, Hoffmann et al. 2012). Some studies have suggested that this frequent misidentification could undervalue the importance of the Harveyi clade among marine shellfish and fish pathogens (Thompson et al. 2007). Therefore, in this study, a polyphasic approach for the precise identification of *Vibrio* was applied with biochemical profiling and multi-locus sequence analysis (MLSA) of the 16S rRNA, *rpoD*, *rctB*, *gyrB*, *toxR* and *pyrH* genes. To understand the virulence of this pathogenic strain, *in vitro* and *in vivo* virulence tests were established, including experimental challenge and investigation of the production of extracellular factors.

MATERIALS AND METHODS

Isolation of bacterial strain AP37

Small abalone *Haliotis diversicolor* samples were collected during 3 different outbreaks from the same culture pond at Zhangpu, Fujian, China, in late May 2009 to 2011. The mean temperature of the sea water was 26 ± 1°C. The moribund abalones were surface sterilized with 70% alcohol and then washed 3 times with sterilized double-distilled water. Ulcerative areas of foot muscle, intestinal tract and hepatopancreas were thoroughly homogenized. The crude homogenate was centrifuged at 600 × g for 2 min. The supernatant containing bacteria was plated on marine 2216 agar (MA) medium and thiosulphate citrate bile sucrose agar (TCBS) at 28°C for 5 d. Predominant and morphologically different colonies were selected and streaked on nutrient agar to obtain pure cultures.

The dominant species were cultivated overnight at 28°C on MA agar and liquid medium for morphological and biochemical characterization. A bacterium capable of making an extended colony without a rounded edge on the agar plate was recorded as positive for swarming motility (Sawabe et al. 2007). Cell morphology was observed with a transmission electron microscope (TEM, Hitachi, JSM-6390LV). The negative staining technique was used to add contrast to strains with phosphotungstic acid (PTA) as the staining solution (Horne & Wildy 1979).

Virulence tests

Small abalone *Haliotis diversicolor* samples (shell length 47.80 ± 2.25 mm; wet body weight 10.09 ± 0.21 g) were reared temporarily in a 40 l plastic tank provided with constant aeration and 100% daily water exchange for about 1 wk. Abalones were fed with thin *Gracilaria* sp. every day; excrement and remaining feed were siphoned off every day. They were maintained at a salinity of 30‰, pH 8.1 to 8.3 and a dissolved oxygen content of 7.8 ppm. Afterwards, healthy abalones were segregated and maintained at a density of 30 abalones per tank. Inoculum for the pathogenicity experiment was raised in liquid MA and serially diluted in normal saline (NS, 0.85% NaCl). Bacteria were enumerated in a haemocytometer slide and further confirmed with viable counts.

In order to find the most virulent isolate, a preliminary virulence screening by oral inoculation was performed with about 10^5 CFU per abalone. During the oral inoculation test, pipette tips were used to prevent scratching of the digestive tract. A pipette tip containing bacteria was slowly inserted into the oral cavity to a depth of 0.5 to 1.0 cm. Successful inoculation resulted in no overflow and the abalone could swallow the injected liquid during the injection. The most virulent strain (AP37; see Table S2 in the supplement at www.int-res.com/articles/suppl/d103p133_supp.pdf) was selected and its pathogenicity was further examined. A group of 10 abalones was challenged for 10 d with an AP37 suspension of 10^6 CFU ml^{-1} by bath immersion. The water temperature during the experiment was controlled at 20°C, 24°C and 28°C (starting temperature: 19 to 20°C, rising 2°C every day). Each assay was conducted in triplicate. Based on the results of the above experiments, concentrations of 10^3 to 10^7 CFU per abalone in 50 µl NS were inoculated intramuscularly into the foot muscle using a syringe. Triplicates of 10 abalones each were used for each inoculation level. After bacterial challenge, the experimental and control abalones were kept in 40 l tanks at 28°C with continuous aeration. Mortalities were recorded every 15 min in the first hour post-inoculation and every 1 h

until the 6th hour. Subsequent monitoring was continued every 12 h for a total period of 7 d, and every sign and symptom was recorded. Moribund abalones were sampled for bacterial re-isolation in MA medium. The LD_{50} values of those pathogens which caused abalone mortality within 7 d were calculated by the normal method (Wardlaw 1985). All control groups were treated with sterile NS.

Biochemical analysis and antibiotic susceptibility test

Physiological and biochemical analyses of strain AP37 were performed in triplicate by employing API 20E commercial kits according to the manufacturer's instructions (ATB system, bioMérieux). Tests were conducted to study the sensitivity of strain AP37 to 30 antimicrobial drugs. The bacteria were inoculated onto MA medium and the discs (TianHe Pharmaceuticals) were placed 2 cm apart. After 16 h growth, the diameter of the inhibition zone around the discs was measured. Based on the size of the inhibitory zone around each disc, the sensitivity was graded into 3 ranks, resistant, intermediate and sensitive, according to standards suggested by the National Committee for Clinical Laboratory Standards (NCCLS 1998).

MLSA using the 16S rRNA, *pyrH*, *rpoD*, *gyrB*, *rctB* and *toxR* genes

To characterize strain AP37 at the molecular level, MLSA of 16S rRNA, *rpoD*, *rctB*, *gyrB*, *toxR* and *pyrH* genes was carried out. Bacterial genomic DNA of AP37 was extracted according to a method described previously (Sambrook et al. 1989). The PCR primers used for the amplification and sequencing of these genes have been described previously (DeLong 1992, Pascual et al. 2010). Amplified products were examined by agarose gel electrophoresis and ligated into pMD19-Tclone (TaKaRa Biotechnology). The cloned sequences were then sequenced (Sangon Biotech). The gene sequences were checked for similarities by using BLAST in the NCBI database (www.ncbi.nlm.nih.gov/BLAST/). The sequences were aligned using ClustalX and phylogenetic trees were constructed using the neighbour-joining method and maximum parsimony (MEGA ver. 4.0) (Kumar et al. 2001). The robustness of each topology was checked by 1000 bootstrap replications.

All gene sequences presented in this study have been deposited in the EMBL database under the accession numbers FN554614 (16S rRNA), JQ898678 (*rpoD*), JQ898677 (*rctB*), JQ898680 (*gyrB*), JQ898679 (*toxR*) and JQ898681 (*pyrH*).

Enzymatic and haemolytic properties of strain AP37

Six major enzymes (protease, lipase, agarase, amylase, cellulase and alginate-degrading enzyme) of strain AP37 were analysed to investigate the activities of extracellular products, as described previously (Zhao et al. 2012). All plates were prepared in 3 replicates and incubated at 28°C overnight. For the haemolytic assay, bacterial liquid and supernatant from AP37 cultures grown for different time periods were used to saturate sterilized paper discs (Whatman, 6 mm). The paper discs were then placed on the surface of the blood agar plates which were pre-saturated with 5% rabbit erythrocytes. The plates were examined for the presence of haemolytic zones after incubation at 28°C for a period of 48 h. In addition, the biomass of the AP37 cultures grown for different time periods was determined by spectrophotometry and viable counting using standard cultivation methods.

RESULTS

Isolation of the bacterial strain AP37

A total of 25 bacterial strains were isolated from the moribund abalones in 2011 (Table S1 in the supplement at www.int-res.com/articles/suppl/d103p133_supp.pdf). Among these isolates, *Vibrio harveyi*-related strains were more abundant, on the basis of their morphological characteristics, and made up the main cultivated bacterial group (up to 24%, 6/25). In order to find the most virulent strain, preliminary haemolytic assays and pathogenic challenges were performed. Compared with the other potential pathogens isolated from *Haliotis diversicolor*, such as *Pseudoalteromonas* sp. AP1, *V. shilonii* AP4, *V. parahaemolyticus* AP10, *V. chagasii* AP13 and *V. alginolyticus* ATCC 33787, strain AP37 had the highest pathogenicity (Table S2).

Based on single colony morphological appearance, AP37-related colonies appeared to be the dominant species. Colonies had a circular form, undulated margins and convex elevation. They appeared white on MA and yellow on TCBS plates. AP37 grew well at a temperature range of 20 to 35°C. The cells of strain

AP37 were comma-shaped rods, and 1.5 to 3.0 µm in length and 0.52 to 0.75 µm in width (Fig. S1). Under the TEM, bacterial flagella appeared to be structurally simple helical filaments. At the base of the filament, a hook structure with complex basal architecture was embedded in the bacterial cell wall. With increasing growth time, more and more particulate material seemed to be secreted into the outer membrane of the cells.

Virulence tests

Strain AP37 caused significant mortality of abalones by oral inoculation and intramuscular injection. After 10 d immersion challenge, AP37 induced 0%, 53% and 67% abalone mortality at water temperatures of 20°C, 24°C and 28°C, respectively (Table S3). No mortality was observed in the control groups challenged with 50 µl NS. After intramuscular injection ($\geq 10^5$ cells abalone^{-1}), 100% mortality was observed. Strain AP37 had an LD_{50} of 2.9×10^2 CFU g^{-1} (abalone wet weight). All abalone samples died within 5 h when challenged with $\geq 10^7$ cells abalone^{-1}, so we were unable to determine the LT_{50} for this dose. The other LT_{50} values obtained for strain AP37 were 5.2 h for 1×10^6 CFU abalone^{-1}, 8.4 h for 1×10^5 CFU abalone^{-1} and 21.5 h for 1×10^4 CFU abalone^{-1} (Table 1). The infections mostly started 3 h post-inoculation at 10^7 CFU per abalone, and increased with time. The external clinical symptoms of abalones *Haliotis diversicolor* were reduced appetite, dark pigmentation, insensitivity to the environment, prostrate swimming movements, outward-protruding radula and pustule formation at the site of injection (Fig. S3). After 3 to 5 h of the pathogenicity tests, the tank water exhibited abundant mucus bubbles.

Table 1. Effect of strain AP37 on challenged healthy abalones *Haliotis diversicolor* (n = 30). NS: normal saline. Wet body weight of each abalone was 10.09 ± 0.21 g

Challenge dose (CFU abalone^{-1})	Number of mortalities	Percentage of mortalities	LT_{50} (h)
1×10^8	30	100%	–[a]
1×10^7	30	100%	–[a]
1×10^6	30	100%	5.2
1×10^5	30	100%	8.4
1×10^4	26	87%	21.5
1×10^3	12	40%	–[b]
NS	0	0	–[c]

[a]Samples were all dead within 5 h; unable to determine the LT_{50}
[b]The percentage of mortalities was less than 50% in 7 d; unable to determine the LT_{50}
[c]No mortality was observed

Biochemical analysis and antibiotic susceptibility testing of strain AP37

Standard physiological and biochemical plate and API 20E analyses (Table S4) showed that AP37 colonies were Gram-negative, lactose-positive, 3 to 4 mm in diameter after overnight incubation at 28°C and had a mucoid aspect. They were positive for the production of phospholipase, indole, and gelatinase, and negative for the production of β-galactosidase arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S, urease, tryptophane deaminase and acetoin.

The antibiotic resistance test showed that strain AP37 was susceptible to 7 of the 30 antibiotics tested based on inhibition zones. However, it was resistant to tetracycline, doxycycline, midecamycin, zanocin, ciprofloracin, penicillin, prostaphlin, ampicillin, carbenicillin, amikacin, kanamycin, clindamycin and vancomycin, and it varied in its sensitivity to other drugs.

Enzymatic and haemolytic properties of strain AP37

To understand the potential virulence of AP37, the enzymatic and haemolytic properties were determined. The strain was found to produce extracellular protease, phospholipase and haemolysin. The analysis of growth and haemolysin production by strain AP37 showed that the initial haemolytic activity immediately after inoculation (0 h) may be attributed to haemolysin in the inoculum (Fig. S2). Haemolysin production began in the early log phase of growth as can be seen by the rise in activity at 2 h in MA broth, and continued through the log phase to maximal levels at about 5 h. These results indicate that haemolysin is a primary growth product. A significant level of haemolysin production occurred during the first 24 h of growth, but then the haemolysis activity sharply decreased. However, the growth of *Vibrio* sp. AP37 was still in the stationary phase at this point.

Identification of strain AP37

A nearly complete 16S rRNA gene sequence (1501 bp) for strain AP37 was obtained and subjected to comparative analysis. The BLAST result showed that its closest cultured relatives were *Vibrio harveyi*

(GQ180186, 99%), *V. rotiferianus* (JQ670739, 99%), *V. alginolyticus* (JX221045, 98%), *V. parahaemolyticus* (JN108879, 98%) and *V. natriegens* (AJ874352, 98%). Phylogenetic analysis of the 16S rRNA sequence showed that strain AP37 is distantly related to several species of the Harveyi clade, including some pathogenic *V. harveyi* isolated from other moribund abalones (Fig. 1A). Nevertheless, analysis of 5 other housekeeping genes showed that they have >99% homologies with *V. harveyi*. Phylogenetic analysis of concatenated sequence alignments (including *rpoD*, *rctB*, *gyrB*, *toxR* and *pyrH* genes) yielded a fully resolved and highly concordant tree topology (Fig. 1B), indicating that isolate AP37 should be identified as *V. harveyi*. The neighbour-joining trees generated from the *rpoD*, *rctB*, *gyrB*, *toxR* and *pyrH* genes support the MLSA tree of concatenated sequences (Fig. S4).

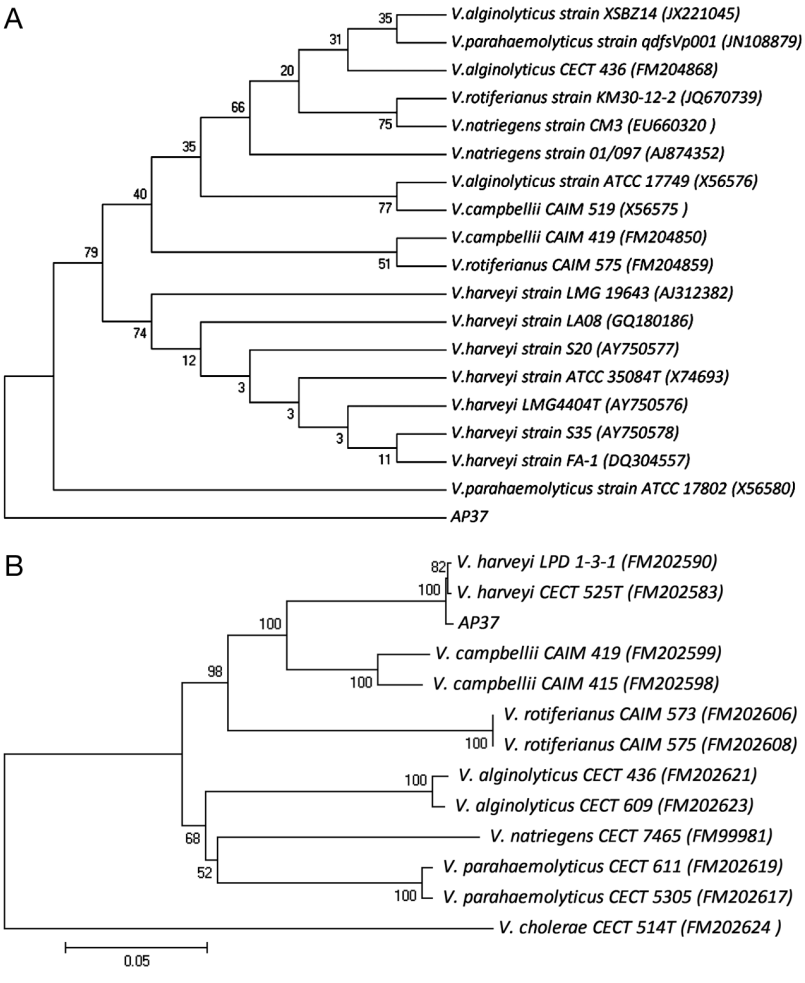

Fig. 1. Phylogenetic reconstructions based on analyses of the (A) 16S rRNA gene using the maximum parsimony method and (B) concatenated *pyrH*, *rpoD*, *gyrB*, *rctB* and *toxR* gene sequences using the neighbour-joining method

DISCUSSION

A mass mortality of small abalones *Haliotis diversicolor* occurred in Zhangpu, Fujian Province, China, in late May from 2009 to 2011. *Vibrio harveyi*-related bacteria, as one group of dominant species, were frequently obtained from the moribund abalones. Although other potential pathogenic species, including *V. parahaemolyticus, V. shilonii*, *V. chagasii* and *Pseudoalteromonas* sp., were also isolated from *H. diversicolor*, virulence tests revealed that they were either avirulent or weakly virulent. Compared with the pathogenic *Vibrio* sp. reported in previous studies of abalones (Liu et al. 2001, Jean-Louis et al. 2002, Nicolas et al. 2002, Lee et al. 2003, Sawabe et al. 2007, Cai et al. 2008), AP37 seemed to exhibit stronger pathogenicity in healthy abalones. The abundance and pathogenicity of this dominant strain in the abalones implicate this bacterium in their mortalities (Nicolas et al. 2002). However, temperature may be an important factor affecting the mortality of farmed small abalones. The zero mortality in the bath immersion at low temperature (≤20°C) and the rising mortality with increasing temperature revealed that abalones are more susceptible to *Vibrio* at higher temperatures, and the outbreak of vibriosis is associated with warm water conditions (Lee et al. 2001, Nicolas et al. 2002). Disease is a complex phenomenon, and infections by microorganisms are one aspect (Sawabe et al. 2007). Once physiological and nutritional imbalances resulting from changes in stressful situations have occurred, mass mortality of aquatic animals may increase as a consequence of disease caused by infection. From this point of view, *Vibrio* AP37 should be considered as an opportunistic pathogen of *H. diversicolor*. The existence of Harveyi clade bacteria including strain AP37 in healthy abalones *H. diversicolor* may perhaps confirm this (Zhao et al. 2012).

Vibrio harveyi has been identified as a causative agent of disease in abalones (Lee et al. 2001, Liu et al. 2001, Sawabe et al. 2007). In previous studies of pathogenic bacteria associated with *Haliotis diversicolor* in

China, members of the Harveyi core group have shown high levels of pathogenicity, particularly *V. alginolyticus* and *V. parahaemolyticus* (Liu et al. 2001, Lee et al. 2003). Although the mechanism of pathogenesis is not completely understood, a few virulence-related factors and genes have been identified in *Vibrio*, including genes affecting extracellular products with proteolytic or haemolytic activity (McCarter 2001, Frans et al. 2011). The correlation of protease and phospholipase with the pathogenicity of AP37 identified in this study deserves further research. Haemolysin is another important virulence factor of Vibrionaceae, causing lysis of erythrocytes in the host and thus the release of the intracellular haem (Frans et al. 2011). When compared with the growth curve of strain AP37, it can be seen that the production and activity of haemolysin are dependent on the active growth and nutrient requirements of the bacterial cells. Although potential virulence factors have been detected, the precise pathogenic nature of strain AP37 remains unidentified, and is worthy of future study.

Phenotypic observations of some *Vibrio harveyi* species have shown that they produce green colonies on TCBS agar (Hoffmann et al. 2012). Many biochemical tests have also shown that *V. harveyi* is lysine and ornithine decarboxylase-positive (Thompson et al. 2007). Strain AP37 colonies are yellow-coloured, and lysine and ornithine decarboxylase-negative. In the family Vibrionaceae, sequence similarities for the 16S rRNA gene are ≥97.6% among members of the so-called *Vibrio* core group, which is formed by 6 species, *V. harveyi*, *V. campbellii*, *V. rotiferianus*, *V. parahaemolyticus*, *V. alginolyticus* and *V. natriegens* (Dorsch et al. 1992). Recent studies have shown that the 16S rRNA gene is not adequate for resolution of the 6 species of the core group of the genus *Vibrio*, because the overlap between the intraspecific and interspecific distances is very large (Gomez-Gil et al. 2004, Lin et al. 2010, Pascual et al. 2010). The phylogenetic analysis of the 16S rRNA gene of strain AP37 also confirms this conclusion. Thus, the 16S rRNA gene and biochemical analysis profiles reported here make identification at the species level difficult. However, the phylogenetic analysis of selected genetic markers, including *rpoD*, *rctB*, *gyrB*, *toxR* and *pyrH*, offered a more definitive and discriminative identification. Thompson et al. (2009) reported that when 2 *Vibrio* strains shared more than 95% similarity by MLSA, they were the same species. Therefore, strain AP37 could be identified as a *V. harveyi*-related species.

The antibiotic susceptibility test revealed that strain AP37 is resistant to some antibiotics commonly used in aquaculture farms, for example doxycycline, penicillin and ampicillin. This finding highlights the potential danger of regular use of antibiotics in aquaculture and indicates that these products could increase the virulence of pathogenic *Vibrio* species. Therefore, the analysis of pathogens is necessary to develop prevention and treatment techniques to optimise farming conditions.

Acknowledgements. This research was supported by grants from the National Natural Science Foundation of China (No. 31202014; No. 31101896), Hi-Tech Research and Development (863) Program of China (No. 2012AA10A412). The work described in this article was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments. The authors declare that they have no conflict of interest.

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Editorial responsibility: Mike Hine, Fouras, France

Submitted: June 19, 2012; Accepted: December 12, 2012
Proofs received from author(s): March 13, 2013