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ORIGINAL ARTICLE

Virulence properties of *Aeromonas* spp. from modified-atmosphere- and vacuum-packed milk fish (*Chanos chanos* Forsskal, 1775)

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Abstract The study was undertaken to determine the pathogenic potential of *Aeromonas* spp.; *Aeromonas hydrophila* (18 isolates), *Aeromonas veronii* biovar *sobria* (15 isolates), and *Aeromonas veronii* biovar *veronii* (28 isolates) isolated from milk fish packed under modified atmosphere and vacuum and stored at 0–2 °C. The isolates were identified by biochemical methods and checked for the presence of 11 virulence genes; *ahh1*, *aerA*, *act*, *ast*, *alt*, *asaI ahpA*, *ahpB*, *gcat*, *lip*, and DNase. All the virulence genes were present in 39 % of the *A. hydrophila* isolates. Virulence-associated phenotypes were present in most of the isolates. The antibiotic resistance patterns of the *Aeromonas* spp. from fish revealed that all the isolates were resistance to ampicillin and penicillin. In addition, resistance of the isolates to cephalothin (86.7 %), carbenicillin (62.2 %), and erythromycin (40 %) was also observed. To our knowledge, this is the first report of isolation and characterisation of pathogenic *Aeromonas* spp. from modified atmosphere packed fish in India. The results of this study add to our knowledge of the ecology of this organism in packed fish, and may in the future help prevent infections in humans.

Keywords *Aeromonas* spp. · Chanos chanos · Modified atmosphere · Virulence factors

Introduction

Aeromonas spp. has emerged as an important human pathogen due to being found in suspected food-borne outbreaks (Altwegg et al. 1991) and the increased incidence of *Aeromonas* isolation from patients with gastroenteritis and traveller's diarrheoa (Hanninen et al. 1995). Members of the *Aeromonas* genus are widely prevalent and are frequently isolated from various foods, mainly seafood (fish, clams, shrimps, etc.) (Hanninen et al. 1997; Alavandi and Ananthan 2003; Ravishankar et al. 2008) and clinical samples (Pin et al. 1995; Sinha et al. 2004; Ivani et al. 2007). Three *Aeromonas* species, *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas veronii* biotype *sobria* are more frequently isolated from patients suffering from diarrheoa, and the role of these species in diarrheoa is well established.

Aeromonas-associated gastroenteritis has been reported after consumption of raw fish in Norway (Granum et al. 1998), seafoods in Japan (Tanaka et al. 1992), and shrimp cocktail in Switzerland (Altwegg et al. 1991). In India, *Aeromonas*-associated diarrhoea has been reported from Bombay, Calcutta, Goa, Vellore, Pondicherry and Chennai (Alavandi and Ananthan 2003).

Several studies have reported the detection and characterization of virulence factors in *Aeromonas* spp. isolated from freshwater fish, raw and smoked rainbow trout (*Oncorhynchus mykiss*) and salmon (*Salmo salar*) from Spain (González-Rodríguez et al. 2002), Tilapia (*Oreochromis niloticus*) from Mexico (Castro-Escarpulli et al. 2003), and pond-raised catfish (*Ictalurus punctatus*) from the Unites States (Nawaz et al. 2010). The *Aeromonas* contamination can occur prior to harvest, during harvest and processing operations, distribution, storage, and preparation of the product. Milkfish, *Chanos chanos* (Forsskål, 1775) is one of the major fish species in Southeast Asian aquaculture, with a total production of 594,787 mt in 2005, which had risen to 1,043,935 mt in 2013 (FAO 2016). The shelf life of chilled raw milk fish is short due to the rapid growth of Gram-negative microorganisms. Strong potential spoilage activities

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of *A. hydrophila*, *A. veronii* biovar sobria in fish and prawn were reported, confirming their contribution to the deterioration of iced wild freshwater fish (González-Serrano et al. 2001) and farmed freshwater prawn (Lalitha and Surendran 2006).

Aeromonas spp. also possess several virulence factors, including enterotoxins, aerolysin, and haemolysins, (Carnahan et al. 1988; Sha et al. 2002; Janda and Abbott 2010), that mediate clinical infections, including acute diarrheal disease in humans. Little is known about the virulence gene associations in *Aeromonas* isolates from fish. Recently, interest in modified atmosphere packaging of fish has increased due to extension of shelf life, which facilitates distribution of fish outside current markets (Gould 2000; Lalitha et al. 2005). In India, information on the genotypic characteristics of aeromonads isolated from fish is scant. The aim of the present study was to determine the virulence gene associations in *Aeromonas* strains isolated from packed milkfish steaks during storage at 0–2 °C to assess their pathogenic potential.

Materials and methods

Processing of fish sample

Milkfish, *Chanos chanos* Forsskal, 1775 (40 kg) collected from brackish water ponds situated at Cochin, Kerala (India), with average body length of 43 ± 2 cm and average weight of 600 ± 5 g were used in the present study. On arrival at the laboratory, fishes were beheaded, gutted, washed with chilled potable water and cut into steaks of thickness 2.5 cm with a sharp sterilized knife. The dressed fish steaks were divided into five lots. The steaks in the first, second and fourth lots were not given any treatment. Food grade preservatives such as potassium sorbate (0.5 % w/v) and citric acid (0.2 % w/v) were used in combination for treatment (Lalitha et al. 2005). In the third and fifth lots, steaks were given a dip treatment in chilled potassium sorbate-citric acid solution for 20 min, then drained well. Steaks were then packed in individual pouches aerobically, under vacuum (VP) and modified atmospheres (MAP) in 12-μ polyester laminated with 75-μ low-density polyethylene pouches with dimensions of 15 × 20 cm, having good barrier properties towards water vapour, O_2 and CO_2. The dressed fish steaks with preservative treatment were packed under vacuum and modified atmosphere. Lot 1 (control air pack—CAP) was sealed using an impulse heat-sealing machine; Lot 2 (control vacuum packed—CVP) and Lot 3 (treated vacuum packed—TVP) were vacuum packed at −1 bar pressure using a vacuum sealing machine (Model QS 400 VD, Sevanna Electrical Appliances Pvt. Ltd., Kerala, India); Lot 4 (control modified atmosphere packed—CMAP) and Lot 5 (treated modified atmosphere packed—TMAP) were packed under MA 60 % CO_2 and 40 % O_2 (Ravishankar et al. 2008) by maintaining the gas to produce a ratio of 2:1 using a Dynopack 500 VG machine (Dynopack, Kristiansand, Norway). Immediately after packing, the packs from each of the five lots were divided into two batches; one batch was stored under chilled condition (0–2 °C) and the other at a slightly higher temperature (8 °C).

Isolation of *Aeromonas hydrophila* and *Aeromonas* spp.

Milk fish samples drawn from each lot at regular intervals were screened for *Aeromonas hydrophila* and *Aeromonas* spp. A 25-g portion of the fish was weighed aseptically and transferred to a stomacher bag, 225 mL sterile physiological saline was added, and the suspension was homogenized for 60 s with a stomacher (Lab Blender 400; Seward Medical, Singapore). Serial dilutions of fish homogenates were prepared. For enumeration of *Aeromonas* spp., 0.1 mL of serial dilutions was spread plated on to Starch ampicillin agar (SAA, Hi Media Laboratories, Mumbai, India) containing 10 μg/mL ampicillin. Plates were incubated at 28 °C for 48 h based on the method of Palumbo et al. (1985). Amylase positive yellow to honey-coloured colonies were scored as presumptive *Aeromonas* spp.

Bacterial strains

All presumptive isolates were tested for Gram reaction, cell morphology, catalase and oxidase reactions, motility and oxidation/fermentation test. They were further tested for the most relevant characteristics such as, indole production, esculin hydrolysis, MRVP test, sensitivity to vibriostatic agent O/129, fermentation of carbohydrates such as cellobiose, arabinose, rhamnose and sucrose, decarboxylation of amino acids, of *Aeromonas* spp., then identified to species level using the key schemes proposed by Kirov (2001). API 20NE standardized identification system (bioMérieux, Marcy l'Etoile, France) was used for confirmation of the species. Out of 120 presumptive isolates characterized by biochemical methods, 61 were confirmed as belonging to *Aeromonas* spp. and were identified to the species level as *A. hydrophila* (18 isolates), *A. veronii* biovar sobria (15 isolates) and *A. veronii* biovar veronii (28 isolates)

Determination of virulence-associated phenotypes

Isolates were tested for protease and lipase activity by the plate assay method (Swift et al. 1999; Harrigan 1998). A clear zone around the bacterial colony indicated a positive result. Haemolytic positive isolates were identified by the presence of clear (β-haemolysis) halos around the colonies grown at 37 °C for 24 h on TSA agar (Difco) containing 5 % human blood (Swift et al. 1999). Extracellular nucleases (DNases) were determined on DNase agar plates (BBL Difco) incubated

at 30 ± 1 °C for 24 h. A pink halo around the colonies indicated nuclease activity (Swift et al. 1999). Elastase activity was determined as described by Williams et al. (1988).

Detection of virulence genes by PCR

PCR assays were performed to detect the presence of 11 genes: three haemolysin genes [*ahh*1, *asa*1, aerolysin *aerA* (Wang et al. 2003)], three enterotoxins genes [cytotoxic enterotoxin (*act*) (Kingombe et al. 1999), and cytotonic enterotoxins (*ast*, *alt*)], and five virulence determinants elastase [*ahpB* (Sen and Rodgers 2004), lipases (*lip* and the GCAT), serine protease (*ahpA*) and nuclease (Dnase) (Soler et al. 2002)]. The 16S rRNA gene of *Aeromonas* (Wang et al. 2003) was included as an internal control.

PCR reactions were performed separately for each gene, and the reaction mixture (25 μL volume) contained 1 μM each of forward and reverse primer, 1X PCR buffer (Sigma), 1.5 mM $MgCl_2$, 200 μM each dNTP, 1.25 U *Taq* DNA polymerase enzyme and 1 μL boiled template DNA from LB broth culture of each strain for the *act*, *ast*, *alt*, and *ahpB* genes. While 2 μM of forward and reverse primers were used for amplification of *ahh1*, *aerA*, *asa1*, GCAT, *lip*, *ahpA* and Dnase genes with 3.0 U *Taq* DNA polymerase. The PCR mixture without template DNA was used as a negative control, and a mixture with template DNA from reference strain NCIMB 12840 was used as a positive control. PCR was performed in an Eppendorf Mastercycler (Eppendorf, Germany). Amplicons were visualized after electrophoresis in a 2 % agarose gel stained with ethidium bromide (0.5 μg mL^{-1}).

Antibiotic susceptibility

Antibiotic susceptibility of the isolates to ten antimicrobial agents was determined using antibiotic discs (HiMedia Laboratories). Pure cultures of Aeromonas were enriched in Tryptone Soy Yeast Extract (TSYE) broth at 37 °C for 16–18 h. These cultures were then streaked on Muller Hinton agar plates (HiMedia Laboratories) using a sterile cotton swab. The resistance of all strains to different antibiotics was determined by the disk diffusion method as described (NCCLS 2002). The antibiotics and their concentration were as follows (μg/mL): chloramphenicol (30 μg), ampicillin (25 μg), streptomycin (10 μg), oxytetracycline (30 μg), erythromycin (15 μg), nalidixic acid (30 μg), penicillin G (10 μg), cephalothin (30 μg), carbenicillin (100 μg) and colistin (10 μg). The antibiotic discs were dispensed using a disc dispenser (HiMedia Laboratories) and after 30 min, the plates were incubated at 37 °C for 24 h.

Results were recorded by measuring the diameter of the inhibition zones, and compared with the standards for antimicrobial disc susceptibility tests, supplied by the Himedia Laboratories, and were classified as resistant, intermediate, or sensitive

Results

The 61 *Aeromonas* isolates obtained from packed milkfish steaks were identified as follows: *A. hydrophila* (17), *A. veronii* biovar sobria (15) and *A. veronii* biovar veronii (28). Among the three *Aeromonas* species, *A. veronii* biovar veronii (56 %) was the dominant species in vacuum-packed milkfish steaks, followed by *A. hydrophila* (28 %) and *A. veronii* biovar sobria (16 %). In modified atmosphere packed (60 % CO_2:40 % O_2) milkfish steaks, *A. hydrophila* (75 %) constituted the most prevalent species, followed by *A. veronii* biovar sobria (25 %).

Determination of virulence associated phenotypes

The prevalence of five virulence-associated phenotypes was studied in the 61 *Aeromonas* isolates. Lipolytic activity was the most prevalent, being present in all 61 isolates characterized (Table 1). Proteolytic activity was found in all *A. veronii* biovar *veronii* and *A. veronii* biovar *sobria* isolates. However, this activity was prevalent only in 94 % of the *A. hydrophila* isolates.

Haemolysin production was found in the majority of the isolates (94 % in *A. hydrophila*, 96 % in *A. veronii* biovar *veronii*, and 100 % in *A. veronii* biovar *sobria*). Only 50 % of the 18 *A. hydrophila* isolates had elastase activity. In *A. veronii* biovar sobria and *A. veronii* biovar veronii, elastase activity was found in 73 % and 82 % of the isolates respectively. Nuclease activity was present in all the *A. veronii* biovar veronii isolates (100 %). However, this virulence-associated phenotype was found only in 89 % of *A. hydrophila* isolates, and 73 % of *A. veronii* biovar *sobria* isolates.

Table 1 Incidence of phenotypic virulence markers in *Aeromonas* spp. isolated from packed milkfish

Aeromonas spp.	Proteolytic activity	Lipolytic activity	Haemolytic activity	Nuclease activity	Elastase activity
A. hydrophila, *n* = 18	17 (94 %)	18 (100 %)	17 (94 %)	16 (89 %)	9 (50 %)
A. veronii biovar *veronii*, *n* = 28	28 (100 %)	28 (100 %)	27 (96 %)	28 (100 %)	23 (82 %)
A. veronii biovar *sobria*, *n* = 15	15 (100 %)	15 (100 %)	15 (100 %)	11 (73 %)	11 (73 %)

Table 2 Incidence of genotypic virulence markers in *Aeromonas* spp. isolated from packed milkfish

Aeromonas spp.	Enterotoxin genes			Aerolysin / haemolysin genes			Serine protease (*ahpA*) gene	Elastase (*ahpB*) gene	GCAT gene	Lipase (*lip*) gene	DNase gene
	Cytotoxic enterotoxins (*act* gene)	Cytotonic enterotoxin		*ahh1* gene	*aer A* gene	*asa1* gene					
		(*ast* gene)	(*alt* gene)								
A. hydrophila, $n = 18$	17 (94 %)	10 (56 %)	13 (72 %)	18 (100 %)	18 (100 %)	ND[a]	18 (100 %)	10 (56 %)	18 (100 %)	18 (100 %)	18 (100 %)
A. veronii biovar veronii, $n = 28$	25 (89 %)	ND[a]	20 (71 %)	28 (100 %)	ND[a]	ND[a]	28 (100 %)	23 (82 %)	28 (100 %)	28 (100 %)	28 (100 %)
A. veronii biovar sobria, $n = 15$	12 (80 %)	ND[a]	ND[a]	15 (100 %)	ND[a]	13 (87 %)	15 (100 %)	11 (73 %)	15 (100 %)	15 (100 %)	12 (80 %)
Total, $n = 61$	54 (88.5 %)	10 (16.4 %)	33 (54 %)	61 (100 %)	18 (29.5 %)	13 (21 %)	61 (100 %)	44 (72 %)	61 (100 %)	61 (100 %)	58 (95 %)

[a] Not determined

Distribution of virulence genes in *Aeromonas* spp.

The distribution of 11 virulence genes among 61 *Aeromonas* isolates is shown in Table 2. Haemolysin (ahh1) and aerolysin (*aerA*) genes were present in all the *A. hydrophila* isolates. However, the virulence-associated phenotype, haemolysin production was found in only 94 % of the *A. hydrophila* isolates. The virulence-associated phenotype haemolysin production was found in all the *A. veronii* biovar sobria isolates, as confirmed by amplification of the *asa1* gene fragment that encodes for *A. sobria* haemolysin. In *A. veronii* biovar veronii isolates (100 %), only haemolysin gene *ahh1* was detected.

The *act* gene was detected in 94 % of the *A. hydrophila* isolates, and confers haemolytic and cytotoxic activities in addition to enterotoxic activity. In *A. veronii* biovar veronii and *A. veronii* biovar sobria isolates, its prevalence was 89 % and 80 %, respectively. The cytotonic enterotoxin genes *ast* and *alt* were detected in 56 % and 72 % of the 18 *A. hydrophila* isolates. However, none of the *A. veronii* biovar sobria isolates harbored *ast* and *alt* genes. In *A. veronii* biovar veronii isolates, only the *alt* gene (71 %) was detected.

Lipase genes (*lip*) that alters the plasma membrane of the host, and glycerophospholipid cholesterol acyltransferase GCAT, which functions as a lipase or phospholipase causing erythrocyte lysis by digesting plasma membranes, were found in all 61 *Aeromonas* isolates tested. The metalloprotease (*ahyB*)-encoding (elastase) gene coding for elastolytic activity that plays an important role in invasiveness and the establishment of infection was found only in 44 (72 %) of isolates, while the serine protease-encoding gene (*ahp*A), which is known to activate toxins such as Aerolysin and GCAT was detected in all strains of *Aeromonas* spp. tested. The nuclease (DNase) gene was detected in all the *A. hydrophila* and *A. veronii* biovar veronii isolates, while in *A. veronii* biovar sobria isolates, 80 % carried the DNase gene. This virulence-associated phenotype (nuclease activity) was detected in all the *A. veronii* biovar veronii isolates (100 %). However, this activity was found only in 89 % of *A. hydrophila* isolates and 73 % of *A. veronii* biovar sobria isolates.

The present study revealed that virulence gene associations were found in *A. hydrophila* isolates. Of the 18 *A. hydrophila* isolates, 7 had all ten genes (*ahh1*, *aerA*, *act*, *ast*, *alt ahpA*, *ahpB*, *gcaT*, *lip*, *DNase*) examined. All the *A. hydrophila* isolates carried virulent genes *aerA*, *ahh1*, *ser*, *lip* and *GCAT*. Virulent genes *ahh1*, *ser*, *lip* and *GCAT* were found in all the *A. veronii* biovar veronii and *A. veronii* biovar sobria isolates.

Antibiotic susceptibility of *Aeromonas* spp.

Isolates belonging to three identified *Aeromonas* spp. (*A. hydrophila*, *A. veronii* biovar veronii and *A. veronii* biovar sobria) appeared to have varying levels of susceptibility or resistance to the antibiotics tested. The resistance patterns

obtained with the 61 *Aeromonas* isolates against ten antibiotics are shown in Table 3. All the isolates showed resistance to ampicillin and penicillin. In addition, high resistance to cephalothin (86.7 %), carbenicillin (62.2 %), and erythromycin (40 %) was also noted. The resistance of *A. hydrophila* to colistin was 89 %, whereas very low resistance of 3.3 % and 5.5 % resistance was observed among isolates of *A. veronii* biovar veronii and *A. veronii* biovar sobria. In contrast, all the isolates were susceptible to chloramphenicol, oxytetracycline, nalidixic acid and streptomycin.

Discussion

The enteropathogenicity of *Aeromonas* spp. has been attributed to the production of exoenzymes, adhesins and exotoxins such as cytolytic enterotoxin, haemolysin or aerolysin, lipases and proteases (Pemberton et al. 1997). Janda and Abbott (2010) have reported that the virulence factors of *Aeromonas* act multifunctionally and multifactorially. The pathogenicity of *Aeromonas* spp. to humans and fish is considered of high scientific and economic interest (Austin et al. 1998). A PCR and southern hybridization survey by Wang et al. (2003) indicated that all virulent *A. hydrophila* isolates were positive for haemolysin/aerolysin genes (*ahh1* and *aerA*), and they grouped these isolates as genotype 4. Aslani and Hamzeh (2004) reported that, among the different genotypes, haemolysin/aerolysin positive ($hlyA^{+}aerA^{+}$) was the most common genotype in diarrheal isolates. The presence of the cytolytic enterotoxin (*act*) gene is considered to be a characteristic virulence trait in *Aeromonas* spp. (Kingombe et al. 1999), and its presence indicates that these species may have public heath significance.

This is the first study carried out in this country on the distribution of virulence genes in *A. hydrophila*, *A. veronii* biovar veronii and *A. veronii* biovar sobria isolated from vacuum- and modified-atmosphere-packed fish. Interesting in this study there is high prevalence of genes encoding aerolysin/haemolysin and cytotoxic (*act*) and cytotonic (*alt*) enterotoxin genes in *A. hydrophila* isolates. This finding confirms that *A. hydrophila* isolates from vacuum- and modified-atmosphere-packed milkfish possess an extensive array of virulence genes that have been reported in clinical isolates earlier (Wu et al. 2007). In this study, ten *A. hydrophila strains* (56 %) carried both the heat-stable (*ast*) and heat labile (alt) cytotonic enterotoxin genes. Albert et al. (2000) reported *alt* and *ast* genes in 56 % of total diarrheal isolates from children with diarrhea at the Clinical Research and Service Centre of the ICDDR, Bangladesh.

The results of this study revealed that all 18 *A. hydrophila* isolates harbor both *aerA* and *ahh1* genes, and thus belong to genotype 4 of Wang et al. (2003), indicating their pathogenic potential. However, one genotype 4 isolate was non-haemolytic. Wang et al. (2003) also reported 4 % non-haemolytic isolates among *A. hydrophila* genotype 4 isolates. This may be due to the fact that the non-haemolytic isolates carried haemolysin genes that either could not be expressed or had mutations affecting domains responsible for the haemolytic phenotype. In this study, among the 15 *A. veronii* biovar *sobria* isolates, 2 belonged to genotype 2 ($ahh1^{+}$) of Wang et al. (2003), while 13 belonged to genotype 3 ($ahh1^{+}$ asa^{+}) and were haemolytic.

Daling et al. (2007) tested nine strains of *A. hydrophila* isolated from diseased fish or soft-shelled tortoise for the presence of three virulence genes (aerolysin, haemolysin, and extracellular serine protease), and found that all highly virulent *A. hydrophila* strains were $aerA^{+}$ $hlyA^{+}$ $ahpA^{+}$ genotype. Strains with the genotype of $aerA^{-}$ $hlyA^{-}$ $ahpA^{+}$ have middle pathogenicity. The results of the present study revealed that all 18 *A. hydrophila* isolates ($aerA^{+}$ $hlyA^{+}$ $ahpA^{+}$) were virulent based on the above observations. In a study on *A. hydrophila* isolated from faecal samples of diarrheal and asymptomatic healthy persons in Ilam (Iran), Aslani and Hamzeh (2004) found significant correlation between the *aerA*+ *hlyA*+ genotype and diarrhea, and this genotype was the most common genotype in the diarrheal isolates. They

Table 3 Antibiotic resistance of *Aeromonas* spp. isolated from milkfish steaks stored under different atmospheres

Antibiotic (concentration/mL)[a]	Percentage of resistant strains		
	A. hydrophila ($n = 18$)	*A. veronii* biovar *veronii* ($n = 28$)	*A. veronii* biovar *sobria* ($n = 15$)
Erythromycin (15 μg)	46.2	57.1	16.6
Ampicillin (25 μg)	100	100	100
Penicillin G (10 μg)	100	100	100
Carbenicillin (100 μg)	58.3	78.3	50
Cephalothin (30 μg)	92.3	84.6	83.3
Colistin (10 μg)	89	3.3	5.5

[a] All the strains were sensitive to Chloramphenicol (30 μg), oxytetracycline (30 μg), Nalidixic acid (30 μg) and streptomycin (10 μg)

suggested that the *aerA+ hlyA+* genotype could be good predictors of human diarrheal disease.

In a study performed on the virulence factors in 234 clinical and environmental isolates of *Aeromonas* spp., Chacón et al. (2003) concluded that only aerolysin/haemolysin, DNase genes and β-haemolytic activity were prevalent in clinical isolates. In addition, they also found a statistical association between β-haemolytic strains and the combined presence of the aerolysin/haemolysin and serine protease genes, suggesting a role for serine protease in the activation of aerolysin. The results of the present study also revealed that 94 % of the *A. hydrophila* isolates were β-haemolytic, and that all these isolates harboured aerolysin/haemolysin and serine protease genes, thus confirming the pathogenic potential as reported earlier (Chacón et al. 2003).

The three-enterotoxin genes were selected as targets in this study because the cytotoxic enterotoxin, *act/hlyA/aerA* and the cytotonic enterotoxins, *alt* and *ast* have all been implicated as important virulence factors in diarrhoeal disease (Sha et al. 2002). The results of this study confirm the observation made by Chacón et al. (2003) that all β-haemolytic strains of *A. hydrophila*, *A. veronii* biovar veronii, and *A. veronii* biovar sobria possessed aerolysin/haemolysin and serine protease genes. McMahon (2000) reported that proteases and haemolysins of *A. hydrophila* have lower activity when cells were grown at sub-optimal temperature. Gonzalez-Serrano et al. (2002) reported that, at 4 °C, six isolates, including four *A. hydrophila* isolates, one *A. veronii* biovar sobria isolate from freshwater fish, one *A. hydrophila* diaorrheal isolate fulfilled virulence criterion (haemolytic, cytotoxic and enterotoxic), but at 37 °C, and only one *A. hydrophila* isolate from fish was virulent. They concluded that expression of virulence factors is affected by temperature of incubation and is not always related to the presence of haemolytic genes. In this study, *Aeromonas* species characterized were isolated from fish stored at 0–2 °C and they carried genes encoding for haemolysin/aerolysin, cytotoxic enterotoxin and cytotonic enterotoxins, which indicates potential health risk.

There have been many reports of antibiotic resistance of *Aeromonas* spp. isolated from fish (Vivekanandhan et al. 2003; Jacobs and Chenia 2007). The multi-drug resistance profile noted in this study in *Aeromonas* spp. isolated from farmed milkfish indicates a risk to public health. The extensive use of antibiotics in fish farms, either to prevent or to cure fish diseases, has resulted in increasing antibiotic resistance among pathogenic bacteria. The main problem involved in the use of antibiotics against infections in fish is the development of resistance by these bacteria (Mitchell and Plumb 1980). Antimicrobial resistance determinants selected for aquaculture ecosystems may be transmitted to human pathogenic bacteria (Smith et al. 1994). Drug-resistant bacteria present in an aquaculture setting may be transferred to humans by contact with this ecosystem via wound infections, following exposure to contaminated water or fish, or downstream handling of fish for food preparation or consumption of aquaculture fish (Petersen and Dalsgaard 2003).

The results of this study revealed that the three *Aeromonas* spp. (*A. hydrophila*, *A. veronii* biovar sobria, and *A. veronii* biovar veronii) isolated in this study from modified-atmosphere-packed and vacuum-packed milkfish exhibited toxin gene patterns similar to those of clinical strains, highlighting their pathogenic potential to cause human illness. This study also suggests that characterisation of the potential pathogenicity of aeromonads at the genotypic and phenotypic level using an integrated molecular and biological approach is important, considering the multi-factorial nature of the disease the organism causes and the influence of environmental conditions in the expression of putative virulence properties.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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