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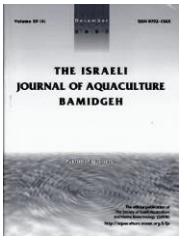

Recovery of *Listonella anguillarum* from Diseased Pufferfish (*Takifugu rubripes*) in China

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Keywords: pufferfish; *Listonella anguillarum;* pathogen; infection

Abstract

In May 2016, there was a disease outbreak among pufferfish (*Takifugu rubripes*) in a marine farm in Tianjin municipality, with the cumulative mortality rate reaching 45% within 7 days from the beginning of the outbreak. The main symptoms of the disease were abdominal and anal swelling of the diseased fish. Significant damage was also observed histopathologically in the intestine, liver, and kidney. The strain H008 was isolated from diseased kidneys, and the challenge test revealed that the same disease was diagnosed in pufferfish which suffered similarly high mortality rates. Using physiological biochemical tests combined with 16S rDNA sequence analysis, the strain was identified as *Listonella anguillarum*. Antimicrobial susceptibility tests showed that H008 was resistant to 10 of 15 antimicrobial agents tested. To the best of our knowledge, this is the first report of *L. anguillarum* causing disease in pufferfish.

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Introduction

Pufferfish, also called Fugu or Takifugu, are a famous culinary delicacy in China. As a result of their palatable and nutritious flesh (Gao et al., 2011), high market price (Kikuchi et al., 2009), and successful techniques for intensive cultivation (Fu, Lin & Lin 2005), pufferfish have become one of the most extensive and economically important maricultured fish in China, with an annual output of 28,592 tons (Guo and Zhao, 2016). However, disease is now one of the biggest threats to pufferfish aquaculture, resulting in significant economic losses.

In May 2016, an outbreak of disease was reported in pufferfish in a marine industrial recirculation aquaculture system at a fish farm located in Tianjin city with a cumulative mortality of 45% within 7 days. The causative agent was isolated and identified as *Listonella anguillarum* by morphological and biochemical characteristics, and phylogenetic analysis of 16S rDNA gene sequences. To the best of our knowledge, this is the first report of isolation of *L. anguillarum* from diseased pufferfish.

Materials and Methods

Fish.

Diseased pufferfish, 13-17 cm long, were collected from a marine fishery in Tianjin City. Dying fish were loaded into oxygen bags and quickly sent to laboratories for diagnosis and pathogen isolation. Healthy pufferfish, 6–10 cm long, with no signs of disease were provided by another marine fishery in Tianjin City.

Pathogen isolation.

After repeated external swabbing of the fish with 70% alcohol, three diseased fish were dissected under aseptic conditions, and small samples from the kidney, liver, and spleen from each fish were streaked and inoculated on 2216E and TCBS plates. The samples were cultured at 28°C for 48 h. Single colonies of the dominant bacteria were selected for further purification. Samples of fins, gill, mucus, and visceral tissue from three diseased fish were examined under a microscope for parasites.

Pathogen identification.

Purified single colonies were plated on 2216E and TCBS plates to enable observations of colony morphology. Physiological and biochemical assays were performed using standard methods (Dong and Cai, 2001). Purely cultured bacterial 16S rRNA sequences were PCR amplified using primers (27F) 5′-AGAGTTTGATCCTGGCTCAG-3′ and (1492R) 5′-TACGGCTACCTTGTTACGCTT-3′ (Lane, 1991). The reaction procedure was as follows: pre-denaturation at 95°C for 6 min, followed by denaturation at 94°C for 1 min; renaturation at 55°C for 1 min; extension at 72°C for 2 min. The procedure was repeated for 30 cycles, followed by incubation at 72°C for 6 min. The amplified products were sequenced by Sangon Biotech (Shanghai). The sequencing results were compared with the gene fragments in the NCBI database that were registered in GenBank for homology, and a phylogenetic tree was constructed using MEGA 4.1.

Artificial infection.

The strain H008 was cultured on 2216E plates at 28°C for 48 h. A single colony was then picked into 2216E liquid medium and cultured at 28°C and 150 r/min for 24 h followed by centrifugation at 6000 g/min for 10 min. The bacteria were collected and their concentration adjusted to 10^7, 10^8, and 10^9 CFU/mL using sterile PBS. Then, 120 healthy pufferfish were randomly divided into four groups with 30 in each group. Groups 1, 2, and 3 were intraperitoneally injected with 0.1 mL of the 10^7, 10^8, or 10^9 CFU/mL bacterial suspension, respectively. Group 4 was injected with 0.1 mL of sterile PBS as the control. The fish were cultured in tanks filled with 150 L of sea water held at a constant temperature of 25°C and with 24 h continuous oxygenation. The fish were closely observed for 15 days and any dead fish were removed immediately. Bacteria were re-isolated from the liver and kidneys of dying fish. The isolates were then identified by physiological and biochemical reactions.

Pathological section.

To observe any pathological changes in the tissues and organs of diseased fish, intestine, liver, kidneys and spleen were removed from dead fish and fixed with neutral formaldehyde; tissues from healthy fish were used as controls. Fixed tissues were

processed by routine histological procedures; 5-μm-thick tissue sections were stained with hematoxylin and eosin (H&E).

2.6 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing.

The susceptibility pattern of isolate H008 was tested using a previously published method (Bauer et al., 1966). Briefly, 0.1 mL of a 10^8 CFU/mL H008 suspension was plated on Mueller Hinton agar (MHA) plates. Then, 15 types of drug susceptibility test paper [ampicillin (10 μg), streptomycin (10 μg), chloromycetin (30 μg), kanamycin (30 μg), norfloxacin (10 μg), tetracycline (30 μg), doxycycline (30 μg), enoxacin (10 μg), ciprofloxacin (5 μg), gentamycin (10 μg), erythromycin (15 μg), tobramycin (10 μg), florfenicol (30 μg) vancomycin (30 μg), and roxithromycin (15 μg)] were attached to each plate. The diameter of the inhibition zone was then measured after the samples had been cultured at 28°C for 48 h to determine the sensitivity of H008 to these drugs.

Results

The main symptoms exhibited by the diseased fish were decreased appetite and abnormal swimming. They were often found on the surface of water reacting slowly to sound. The diseased fish also showed excess mucus, and tissues on the body surface were affected showing partially rotten fins, and abdominal and anal swelling (Fig 1A). Ascites was observed in the form of swollen gallbladders, inelastic intestines, and pale livers (Fig 1B).

Fig. 1. Clinical signs of a diseased pufferfish *Takifugu rubripes*. Congestive body surface with partially rotted fins and anal swelling (A). Ascites and gallbladder enlargement (B).

Parasites were not detected in gills, fins, mucus, or visceral tissue from diseased fish. One strain was isolated from kidneys, namely H008. After 48 h incubation at 28°C on 2216E plates, the colonies were round, moist, smooth, light yellow and opaque with neat edges and diameters of approximately 1.5–2 mm. On TCBS medium, colonies appeared yellow after 24-h culture and then gradually turned blue-green. The biochemical characteristics are summarized in Table 1.

Table 1. Comparison of phenotypic characteristics of isolate H008

Identified item	*Result*	*Identified item*	*Result*
Gram staining	-	H_2S production	-
Motility	+	Methyl red test	+
Growth on 0% NaCl	-	Gelatin	+
Growth on 1% NaCl	+	Starch	+
Growth on 3% NaCl	+	Glucose	+
Oxidation/fermentation	F	Lactose	-
Voges–Proskauer	+	Maltose	-
Oxidase test	+	Sucrose	+
Catlase	+	Mannose	+
Indol production	+	Mannitol	+

Note: "+", positive; "-", negative.

A 16S rDNA sequence of strain H008 (1506 bp) was obtained by PCR amplification. The alignment analysis in the NCBI database showed the strain to have the highest similarity (99%) to *Listonella anguillarum* strain TL1 (AY662305), with the samples differing in only one base. A phylogenetic tree was constructed by matching 16S rDNA genes of *Vibrio* sp. registered in GenBank. The results showed that strain H008 clustered with *L. anguillarum* (Fig. 2). Thus, combined with physiological and biochemical results, H008 was identified as *L. anguillarum.*

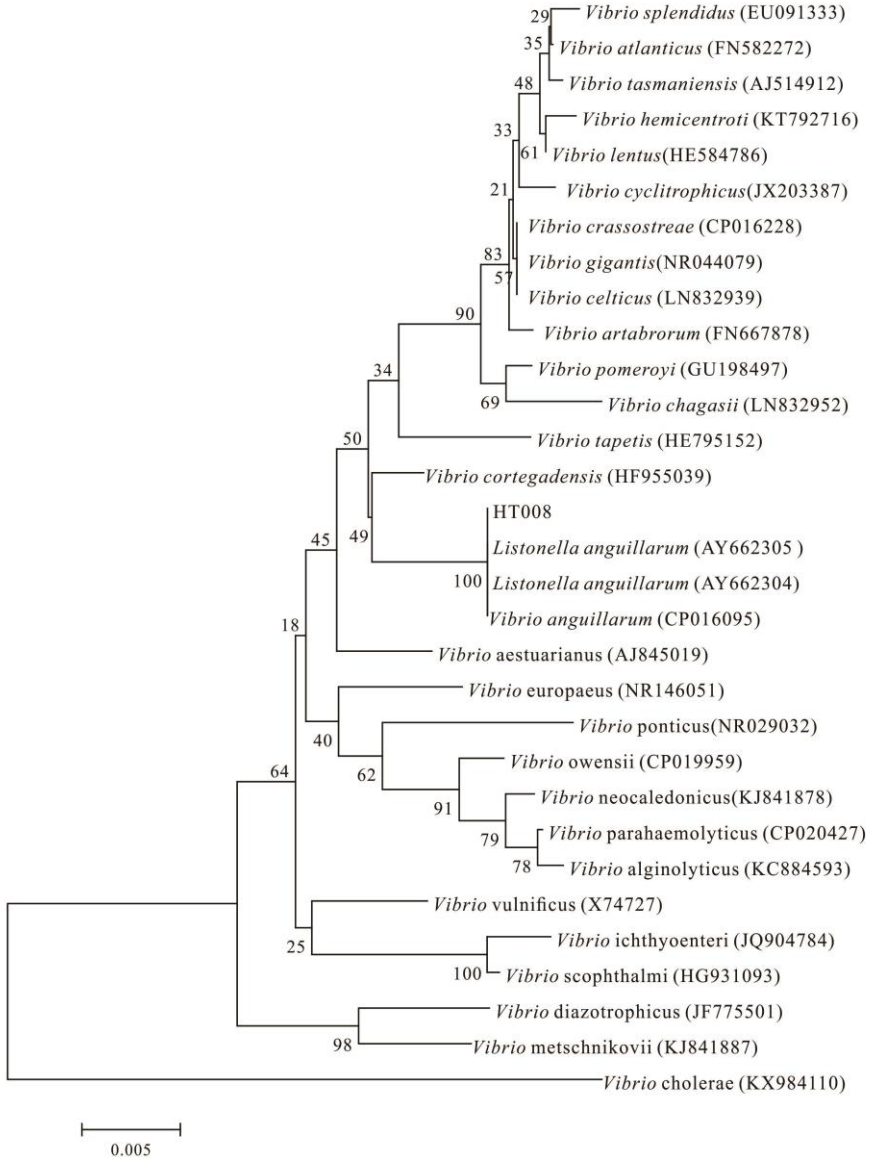

Fig. 2. Phylogenetic tree based on 16S rDNA gene sequences available in GenBank. Accession numbers of all strains are given in brackets.

The infected Pufferfish began to die from day three after artificial infection. The main symptoms of the dead fish were abdominal and anal swelling. Different infectious doses resulted in different mortality rates. In group 1, with an infection dose of 10^7 CFU/mL, the cumulative mortality rate of pufferfish was 46.7%. In group 2 and 3, with infection doses of 10^8 CFU/mL and 10^9 CFU/mL, respectively, the cumulative mortality rate was 86.7% and 100%, respectively. In the control group, no pufferfish died (Fig. 3). Two strains, namely H008-1 and H008-2, isolated from the kidneys and spleen of dying pufferfish, had the same physiological and biochemical features as strain H008.

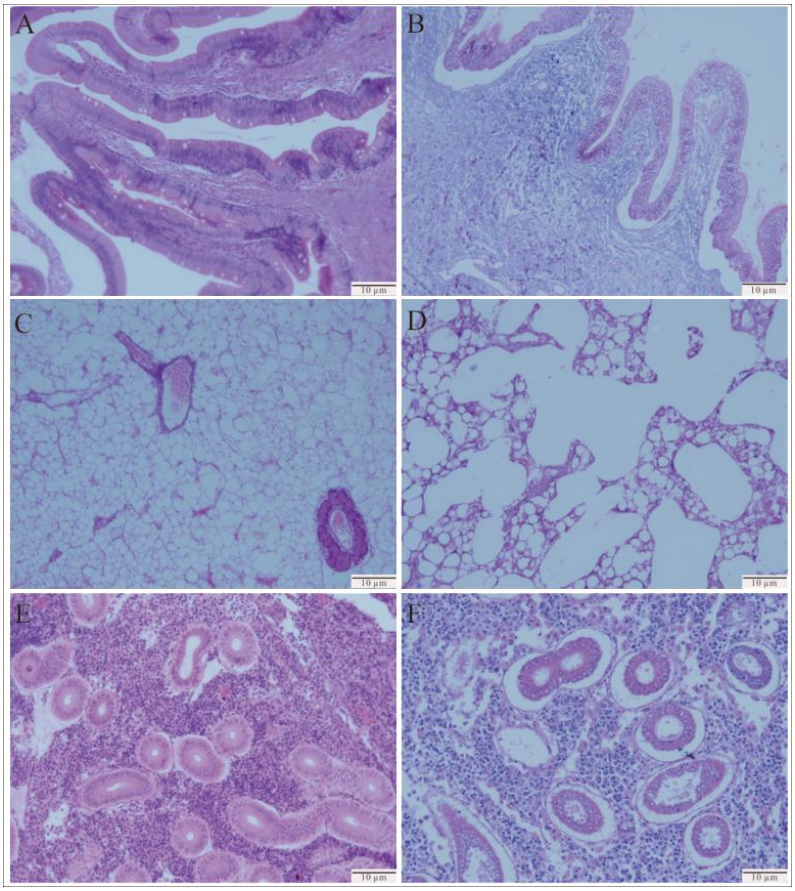

Fig. 3. Histological change in the intestinal tract, liver, and kidney of *Listonella anguillarum*-infected pufferfish *Takifugu rubripes*. (A) Normal intestinal tract. (B) Intestinal tract, showing intestinal villi and mucosal tissue largely rotted and separated from the lining of the intestine, and severely degenerated mucosal epithelial cells. (C) Normal parenchymatous appearance. (D) Diseased liver, showing many irregular vacuolations. (E) Normal kidney appearance. (F) Diseased kidney, showing vacuolar degeneration and necrosis of epithelial cells of renal tubule.

Pathological sections showed necrotic hepatocytes with vacuoles in dead pufferfish and that hepatocytes had been replaced by fat cells (Fig. 4B). Intestinal villi and mucosal tissues had largely rotted and become detached from the lining of the intestine, and mucosal epithelial cells showed severe degeneration (Fig. 4D). Also, epithelial cells from the renal tubule showed vacuolar degeneration and necrosis (Fig. 4F).

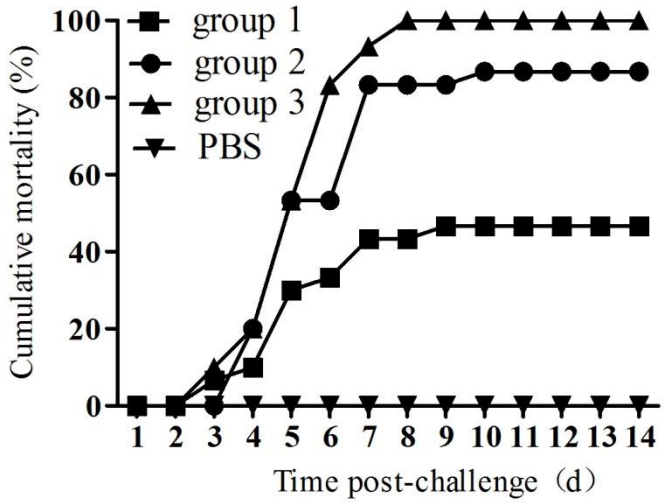

Fig. 4. Cumulative mortalities of healthy pufferfish experimentally infected by isolate H008 with doses of 10^7 (group A), 10^8 (group B), 10^9 (group C) CFU/mL and PBS (group D)

Isolate H008 was tested for susceptibility to 15 antimicrobial agents. The results showed that it was sensitive to tetracycline, florfenicol, and doxycycline; moderately sensitive to streptomycin and ampicillin; and resistant to kanamycin, ciprofloxacin, tobramycin, norfloxacin, chloromycetin, gentamycin, erythromycin, enoxacin, vancomycin, and roxithromycin.

Discussion

Vibriosis is a common disease in aquaculture systems. *V. anguillarum*, *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, and *V. harveyi* can all cause fish vibriosis (Gauger et al., 2006; Kyoung et al., 2009; liu et al., 2011; Paiboon & Kittichon, 2017; Pan et al., 2013). Classical vibriosis is caused by *V. anguillarum* (Toranzo et al. 2005). Various aquatic species can become infected with *V. anguillarum*, including not only marine and freshwater fish, such as turbot *Scophthalmus maximus* and carp *Cyprinus carpio*, but also crustaceans, such as crabs *Eriocheir sinenses* and shellfish (e.g., oysters *Ostrea edulis*) (Bolinches,1986; Chen et al., 2006; Egidius, 1987; Zhang et al., 2009).

V. anguillarum was first isolated in 1909 from eels with what was then called 'red-pest' disease (Bergeman, 1909). In 1985, the strain was re-classified as *Listonella* sp. and renamed *Listonella anguillarum* (MacDonell and Colwell, 1985). In the current study, the isolated strain H008 was identified as *L. anguillarum* based on physiological and biochemical results combined with 16S rDNA sequence analysis. In the challenge test, H008 showed strong pathogenicity against pufferfish, and the symptoms of infected pufferfish were consistent with those of naturally infected fish.

The digestive tract, sputum, and damaged skin are the main ways by which *L. anguillarum* can infect fish (Wang et al., 1998). Since the affected fish were fed with infected dead Pacific sand lance *Ammodytes personatus*, this suggests that these were the source of infection. Replacement of such bait fish could be an effective means to reduce occurrence of this disease.

According to the results of antimicrobial susceptibility testing, pufferfish were treated by oral administration of florfenicol and mortality ceased after 4 days. Isolate H008 showed resistance or moderate resistance to 12 of the 15 drugs tested. These results were similar to those of Zhao et al., who detected the sensitivity of 36 strains of *V. anguillarum* isolated from marine fish to 28 antibiotics. Results showed that all strains were resistant to more than seven antibiotics, and more than 83% of the strains were resistant to more than 12 antibiotics (Zhao et al., 2015). This suggests that most *L. anguillarum* isolates were multi-resistant strains. This could be related to the heavy use of antibiotics in the aquaculture industry (Zhao et al., 2015). Thus, to reduce the use of antibiotics, more research should be directed to other methods of treatment or prevention.

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