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Studies on Bacterial Diversity and *Vibrio harveyi* Distribution Associated with Diseased fugu (*Takifugu rubripes*) in Northeastern China

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LQ and YS contributed to the conception of the study. ZJ analyzed the data and wrote the manuscript. QG and WL helped in analysis of data and preparation of manuscript. LR, NP and GY helped in data analysis.

Key words
***Takifugu rubripes*, Bacterial diversity, *Vibrio harveyi*.**

ABSTRACT

This study investigated the bacterial diversity and *Vibrio harveyi* distribution associated with diseased fugu (*Takifugu rubripes*) in northeastern China from January to December in 2014. The main clinical signs included fin ulceration, skin darkness, hepatohemia and intestinal hydrops. Totally, 104 diseased live fish were collected and 70 strains isolated from naturally diseased *T. rubripes*. Most isolates were obtained in May, September and December. The isolates were identified through 16S rRNA gene sequence analysis and *Vibrio* spp.-specific PCR amplification, followed by pathogenicity determination. Results showed that the isolates belonged to 10 genera, including *Vibrio* (72%), *Staphylococcus* (9%), *Pseudomonas* (4%), *Bacillus* (4%), *Vagococcus* (3%), *Shewanella* (3%), *Planococcus migula* (4%), *Exiguobacterium* (1%), *Enterobacter* (1%) and *Kocuria roseus* (1%). *Vibrio* spp. and *Vibrio harveyi* were the predominant genus and species, respectively. In addition, challenge tests demonstrated that 13 out of 70 isolates were strongly pathogenic and identified as *V. harveyi*. This study illustrated that *V. harveyi* could be considered as main pathogen. These investigation results would provide useful information for disease prevention in *T. rubripes* culture.

INTRODUCTION

The fugu (*Takifugu rubripes*) distributes widely in Asia including China, Korea and Japan. *T. rubripes* is an anadromous and economically important fish in China (Gao *et al*., 2011). Recently, the consumption demand for *T. rubripes* is increasing for the tender flesh, delicious tasty and high abundance of protein, and the price soars in China (Liu *et al*., 2017). Artificial breeding developed quickly along with them since the wild resources have been declined. Especially after year 2016, it became a prosperous industry in North China such as Liaoning Province due to the open artificial breeding allowed by National Government. However, diseases caused by virus, bacteria and parasites remain a limiting factor for aquaculture production and cause great economic losses in the development of artificial cultivation.

Recently, bacterial diseases have been mainly reported in *T. rubripes*. *Edwardsiella tarda* belonged to gliding bacteria can cause ascites. *Vibrio harveyi* can cause symptoms of skin ulceration (Zhang, 2002; Wang *et al*., 2008). *Vibrio ichthyoenteri* and *Vibrio penaeicida* can cause congestion of fins and other symptoms (Zhang *et al*., 2009). In addition, *Streptococcus* has been reported to cause skin darkening, head white turbidity and other symptoms (Du, 2003). Most studies have focused on the isolation and identification of some pathogens until now, no more information is available for the periodic distribution and diversity of bacterial pathogens in cultured *T. rubripes*. In order to gain deeper insight into the bacterial disease epidemiology in cultured *T. rubripes* in northeastern China, the diversity of bacterial pathogens associated with disease outbreaks in *T. rubripes* was carried out from January to December in 2014. These results will provide valuable reference and guidance for the diseases prevention in *T. rubripes* aquaculture industry.

MATERIALS AND METHODS

Sampling

The naturally diseased *T. rubripes* were collected from Daheishi farm (farm A) and Zhuanghe farm (farm B) located in Liaoning Province each month from January to December in 2014 (Fig. 1). Body weight of the diseased

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fish was 150 g-200 g. The clinical symptoms included fin ulceration, abdominal redness, splenomegaly, hepatohemia and renomegaly (Fig. 2). Totally, 104 diseased but not dead fish were collected and all samples were transported to our laboratory at 4 °C within 24 h for further analysis.

Fig. 1. Diseased *Takifugu rubripes* sampling farms located in Liaoning Province. A, Daheishi farm; B, Zhuanghe farm.

Bacterial isolation

Diseased fish were washed three times with sterile physiological saline (PS), and then dissected with a scalpel under aseptic conditions. The bacteria were isolated from liver, spleen, kidney, heart, blood, eye, intestine, visceral and ulceration of *T. rubripes* with typical symptoms, inoculated on the tryptic soy agar (TSA) (Hopebio, Qingdao, China) medium with 2% NaCl, and cultured at 28°C for 24 to 72 h. While the prominent isolation ratio of a strain was more than 15% based on the morphological characterization, the isolate would be considered as prominent strain (Li *et al.*, 2010). All prominent strains were subcultured, purified and preserved at –80°C in nutrient broth (NB) supplemented with 15% (v/v) glycerol and 2% NaCl. A total of 70 predominant strains were isolated from the diseased *T. rubripes* (Table I).

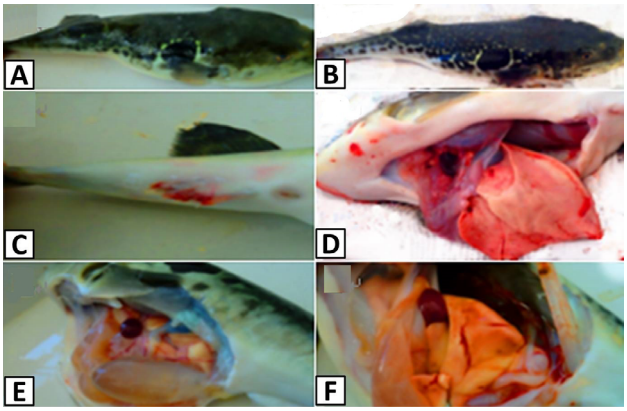

Fig. 2. Main clinical symptoms of naturally and artificially diseased *Takifugu rubripes*. A, black skin; B, fin ulceration; C, fin bleed; D, hepatohemia; E, spleenomegaly; F, intestinal hydrops.

Table I.- Bacterial isolates from diseased *Takifugu rubripes* from January to December in 2014.

Strain No.	Sampling date	Clinical signs	Bacterial origin	Sampling site
2HWH001	January	Fin ulceration, liver redness, splenomegaly	Fin ulceration	A
2FRX001	January	Fin ulceration, intestinal hydrops	Fin ulceration	A
2HWH018	January	Fin ulceration, black skin, hepatohemia	Fin ulceration	B
2PTQ001	February	Fin ulceration	Fin ulceration	A
2DXQ001	February	Fin ulceration, hepatohemia	Fin ulceration	A
2PTQ002	March	Fin ulceration, intestinal hydrops	Fin ulceration	A
2PTQ003	March	Fin ulceration, white feces	Fin ulceration	A
2HWH010	March	Fin ulceration, abdominal redness	Fin ulceration	B
2HWH021	April	Fin ulceration, hepatohemia	Fin ulceration	B
2PTQ004	April	Fin ulceration, intestinal hydrops	Fin ulceration	A
2RZH001	April	Fin ulceration	Fin ulceration	A
2HWH006	May	Fin ulceration	Fin ulceration	A
2HWH003	May	Fin ulceration	Fin ulceration	B
2HWH007	May	Fin ulceration, white feces	Fin ulceration	A
2HWH009	May	Fin ulceration, jejunum, gallbladder swelling	Fin ulceration	A
2RZH002	May	Fin ulceration, intestinal hydrops	Fin ulceration	A
2MG001	May	Fin ulceration, hepatohemia, gallbladder dark	Fin ulceration	A
2HL001	May	Fin ulceration, white feces	Fin ulceration	A
2HL002	May	Fin ulceration	Fin ulceration	A

Strain No.	Sampling date	Clinical signs	Bacterial origin	Sampling site
2CLH001	May	Fin ulceration, hepatohemia	Liver	B
2CLH002	May	Fin ulceration	Fin ulceration	B
2WX001	May	Fin ulceration, hepatohemia, gallbladder dark	Fin ulceration	B
2HJ001	May	Fin ulceration, gallbladder dark	Fin ulceration	B
2HJ002	May	Fin ulceration, white feces	Fin ulceration	B
2HWH002	June	Fin ulceration, intestinal hydrops	Fin ulceration	A
2HWH019	June	Fin ulceration, white feces, visceral anemia	Visceral	B
2RZH003	June	Fin ulceration, gallbladder dark	Fin ulceration	A
2RZH004	June	Fin ulceration, hepatohemia	Liver	A
2HJ003	June	Fin ulceration, black skin, visceral anemia	Visceral	A
2HJ004	June	Fin ulceration, gallbladder dark	Fin ulceration	A
2HWH022	July	Fin ulceration, white feces	Fin ulceration	A
2HWH017	July	Fin ulceration, black skin, intestinal hydrops	Fin ulceration	A
2HWH008	July	Fin ulceration, hepatohemia, gallbladder dark	Fin ulceration	B
2RZH005	July	Fin ulceration, black skin, visceral anemia	Visceral	A
2HJ005	July	Fin ulceration	Fin ulceration	A
2HWH023	August	Fin ulceration	Fin ulceration	A
2HWH024	August	Fin ulceration, liver anemia, renomegaly	kidney	A
2HWH014	August	Fin ulceration, hepatohemia, renomegaly	Liver	A
2HWH005	August	Fin ulceration	Fin ulceration	A
2HWH004	October	Fin ulceration	Fin ulceration	B
2HWH005	May	Fin ulceration	Fin ulceration	A
2CLX003	August	Fin ulceration	Fin ulceration	A
2CLH004	August	Fin ulceration, black skin, visceral anemia	Visceral	A
2CLX005	August	Fin ulceration, black skin, visceral anemia	Visceral	B
2PTQ005	August	Gallbladder dark, intestinal hydrops	Intestine	B
2PTQ006	August	Fin ulceration	Fin ulceration	B
2HWH013	September	Fin ulceration, gallbladder dark	Fin ulceration	A
2HWH012	September	Fin ulceration, gallbladder dark	Fin ulceration	B
2RZH006	September	Fin ulceration	Fin ulceration	A
2YB001	September	Fin ulceration, black skin, visceral anemia	Visceral	A
2HJ006	September	Gallbladder dark, intestinal hydrops	Intestine	A
2HJ007	September	Fin ulceration, hepatohemia	Liver	A
2RZH007	September	Fin ulceration	Fin ulceration	B
2HWH015	October	Fin ulceration, black skin, visceral anemia	Visceral	A
2HWH004	October	Fin ulceration	Fin ulceration	A
2HWH011	October	Fin ulceration, black skin, visceral anemia	Visceral	B
2RZH008	November	Fin ulceration, gallbladder dark	Fin ulceration	A
2YB002	November	Fin ulceration, black skin, visceral anemia	Visceral	B
2XW001	November	Fin ulceration	Fin ulceration	B
2XW002	November	Fin ulceration	Fin ulceration	B
2HWH016	December	Fin ulceration, hepatohemia	Liver	B
2HWH020	December	Gallbladder dark, intestinal hydrops	Intestine	B
2MH001	December	Fin ulceration, gallbladder dark	Fin ulceration	A
2MH002	December	Fin ulceration	Fin ulceration	A
2JDB001	December	Fin ulceration, hepatohemia	Liver	A
2JDB002	December	Fin ulceration, gallbladder dark	Fin ulceration	A
2JDB003	December	Fin ulceration, hepatohemia	Liver	A
2CG001	December	Fin ulceration	Fin ulceration	A
2HJ008	December	Fin ulceration, gallbladder dark	Fin ulceration	B
2HJ009	December	Fin ulceration, hepatohemia	Liver	B
2YB003	December	Fin ulceration	Fin ulceration	B

Table II.- Specific primers sequence of *Vibrio* spp.

Vibrio spp.	Genes	Target fragment	Primers (5'-3')	Note
V. harveyi	toxR	382 bp	F:GAAGCAGCACTCACCGAT R:GGTGAAGACTCATCAGCA	Pang *et al*. (2006)
V. parahaemolyticus	Col	271bp	F:GAAAGTTGAACATCATCAGCACGA R:GGTCAGAATCAAACGCCG	Di Pinto *et al*. (2005)
V. anguillarum	rpoN	519 bp	F: GTTCATAGCATCAATGAGGAG R: GAGCAGACAATATGTTGGATG	Tapia-Cammas *et al*. (2011)
V. splendidus	VSFur	223 bp	F: GACGCATATGTCAGACAATAATCAAG R: CTCGAGCTTCTTCGCTTTATGT	Liang *et al.* (2016)
V. alginolyticus	colH	526 bp	F: TCGCGATTGCGACAACATTAACCAGCACTGGCGT R: ACAAACGCATCCACTGATTCTTTCACCGCTGGGGTGA	Xu *et al*. (2017)

Identification of bacterial isolates

Two different methods were used to identify these 70 isolates.

16S rRNA genes sequence analysis

DNA extraction and purification were carried out following the methods of Li *et al*. (2010) with some modifications. The isolates were cultured in TSB with 2% NaCl for 24 h at 28 °C. Cells were harvested by centrifugation (150 × g, 10 min) at 4 °C and the pellets were washed 3 times with distilled water. The pellets were then suspended in distilled water and DNA was extracted following manufacturer's instruction of TIANamp bacteria DNA kit (TIANGEN). The DNA was purified by increasing the DNA washing times with tris-ethylenediaminetetraacetic acid (TE) buffer.

Two universal primers, Eubac 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and Eubac 1492R (5′-TACGGCTACCTTGTTACGACTT-3′) synthesized by Sangon Biotech (Shanghai) were used to amplify bacterial 16S rRNA genes (~1500 bp). Twenty five microliters used in the PCR system included 2.5 µL 10× PCR buffer, 0.5 µL dNTPs (10 mM of each dNTP), 2 µL $MgCl_2 \cdot 6H_2O$ (25 mM), 0.5 µL of each primer (10 µM), 1µL DNA template, and 0.2 µL Taq DNA polymerase (5 U μL^{-1}). The final volume was adjusted with the addition of triple distilled water. The thermal cycle was run in a T3 thermal cycler (Biometra) at 94 °C initially for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, and then 72 °C for 10 min. The PCR products were analyzed by 1% agarose gel electrophoresis and sequenced by Sangon Biotech (Shanghai).

The obtained sequences were aligned and compared with other bacterial 16S rRNA sequences available in GenBank of NCBI database and in EzTaxon server 2.1. According to the results of PCR amplification by *Vibrio* spp.-specific primers and challenge tests (as following), four representative strains (2HWH003, 2HWH017, 2HWH019 and 2HWH020) were selected for further analysis. A phylogenetic tree of these four bacteria was constructed by the neighbor-joining method using the MEGA 5.0 software, and bootstrap analysis with 1000 replicates was adopted to estimate the relative branch support of the tree (Wu *et al*., 2015).

PCR amplification by Vibrio *spp.-specific primers*

Based on the results from 16S rRNA genes sequence analysis, 50 strains were identified to *Vibrio* spp. and they were further identified by PCR amplification with *Vibrio* spp.-specific primers (Table II). Genomic DNA was extracted as described above and PCR amplification were conducted following the procedures (Di *et al*., 2005; Pang *et al*., 2006; Liang *et al.,* 2016; Tapia-Cammas *et al*., 2011; Xu *et al*., 2017). PCR products were examined by 1% agarose gel electrophoresis.

Challenge tests

The above identification results showed that *Vibrio* spp. (72%, *Vibrio* / total isoaltes) were the predominant genus, and *V. harveyi* (48%, *V. harveyi* / *Vibrio* spp.) was the predominant species and could be detected each month. Thus, *V. harveyi* was selected to do challenge tests to investigate the pathogenicity of isolates to *T. rubripes*. Two challenge methods were used to determine the pathogenicity.

Experimental animals and acclimation

Normal *T. rubripes* (mean body weight of 200 g) were obtained from a farm at Dalian, Liaoning Province and acclimated in a tank of static water at temperature of 17

ºC, pH of 8.0, salinity of 29-32 psu and DO higher than 5 mg L^{-1} for 2 weeks prior to the experiments. The tank was provided with aeration and water was exchanged by 30% daily throughout the whole experiment. *T. rubripes* were fed with commercial diets (Tongwei Feeding Company, China) three times daily at 3% of their body weight under a 12 h light/12 h dark cycle.

Intramuscular injection

T. rubripes were randomly divided into 24 tanks with 10 individuals per tank. All 24 *V. harveyi* isolates were incubated in nutrient broth (NB) containing 2% NaCl at 180 rpm in an orbital shaker for 24 h at 28 °C. Bacterial cells were collected by centrifugation at 6000 × g for 10 min at 4 °C, and bacterial suspension (1.0×10^8 cells mL^{-1}) in sea water was prepared by observing optical density at 600 nm (OD_{600}). Each *T. rubripes* was injected with 0.2 mL of bacterial suspensions (1.0×10^5 cells g^{-1} fish) by intramuscular injection at dorsal fin base as experimental groups, and the control group was injected with an equal volume of sterilized sea water. *T. rubripes* were observed daily for 14 days post-bacterial challenge, and all mortalities were recorded. When the cumulative mortality was more than 50% at 14 d, the isolate was considered as pathogenic bacteria.

Immersion infection

Four representative strains (2HWH003, 2HWH017, 2HWH019 and 2HWH020) were selected for immersion tests based on their above virulence investigation. *T. rubripes* were randomly divided into five tanks with 10 individuals per tank, and the fin of each fish was sheared by scalpel under sterile conditions. Then, those wounded fish were immersed in the sea water with final bacterial suspension of 4.4×10^6 cells mL^{-1} for 1 h as experimental groups and fish in control group was soaked in sterilized sea water. The clinical signs and mortalities were recorded within 14 days. All challenge tests were conducted in triplicate.

RESULTS

Clinical symptoms of diseased T. rubripes

During one-year diseases investigation associated with *T. rubripes*, the main symptoms of diseased fish were skin darkening, fin ulceration, liver congestion, splenomegaly, intestinal tract ascites, and gallbladder was deep (Fig. 2).

Bacteria associated with disease

A total of 70 strains were isolated from diseased *T. rubripes* in farms A and B within one year, 45 strains of which were isolated from farm A and 25 strains from farm B. And these 70 strains were identified and characterized to 10 genera by 16S rRNA gene sequence analysis, including *Vibrio* (72%), *Staphylococcus* (9%), *Pseudomonas* (4%), *Bacillus* (4%), *Vagococcus* (3%), *Shewanella* (3%), *Planococcus migula* (4%), *Exiguobacterium* (1%), *Enterobacter* (1%) and *Kocuria roseus* (1%) (Fig. 3A). Based on the results of challenge tests, four representative strains were selected and the phylogenetic tree was constructed using the 16S rRNA gene sequences. Results showed that the four bacterial isolates were clustered into one clade and closed to *V. harveyi* (Fig. 4). Moreover, 50 strains belonged to *Vibrio* spp. were amplified by *Vibrio* spp.-specific primers. The results showed that 24 strains belonged to *V. harveyi* (24 strains of *V. harveyi*/50 strains of *Vibrio* spp. = 48%), 8 strains of *V. alginolyticus* (16%), 5 strains of *V. splendidus* (10%), 2 strains of *V. anguillarum* (4%), 1 strain of *V. parahaemolyticus* (2%), and 10 strains were unidentified to the species level (Fig. 3B).

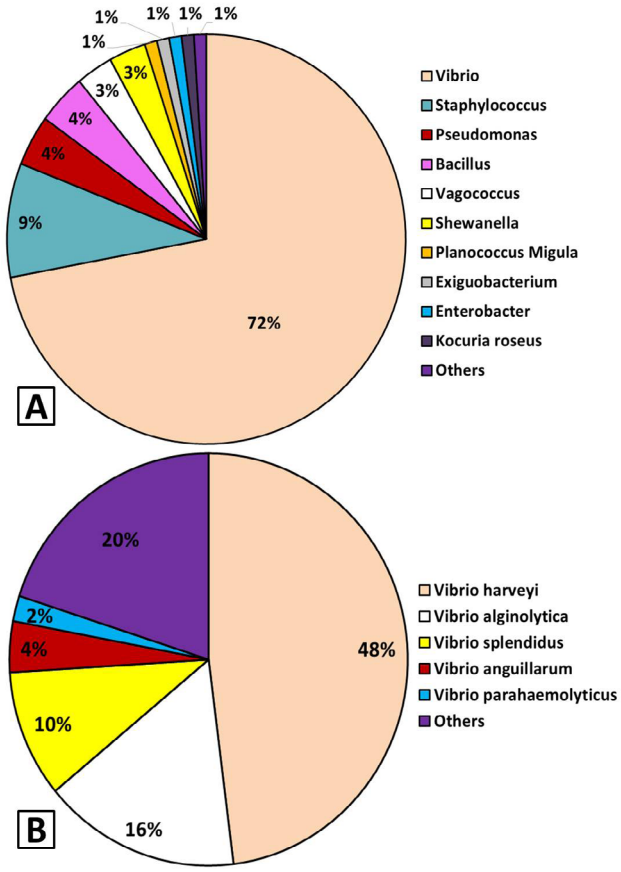

Fig. 3. Bacterial diversity of all the 70 isolates. A, proportion of strains in all the isolates at species level; B, percentage of *Vibrio harveyi* in *Vibrio* genera.

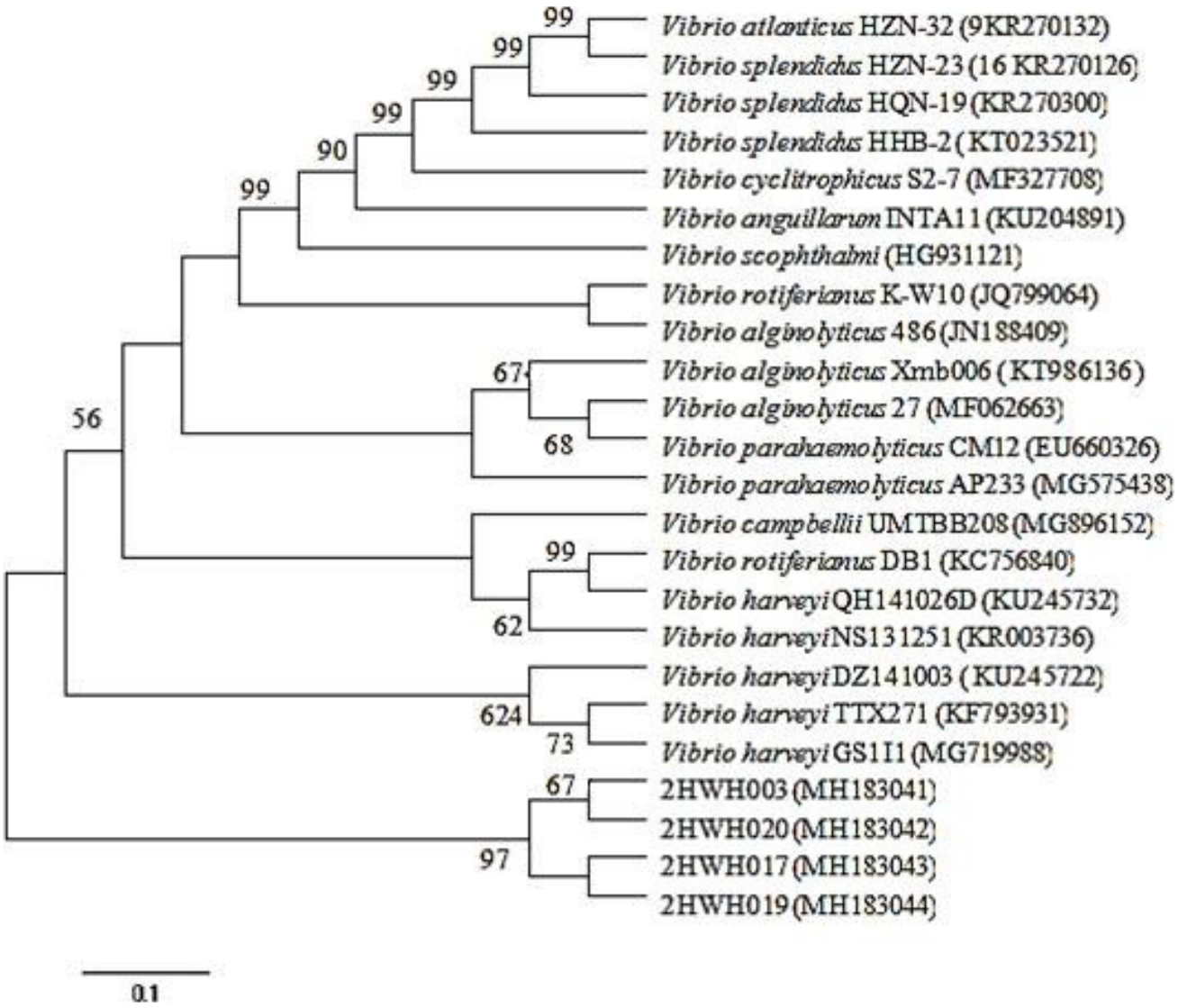

Fig. 4. Phylogenetic trees of the four representative isolates constructed using Neighbor-Joining method in Mega 5.05.

Bacterial diversity was different from farm A to farm B. In farm A, 45 strains were obtained and included 32 strains of *Vibrio* spp. (71%), 4 strains of *Staphylococcus* spp. (9%), 3 strains of *Pseudomonas* spp. (7%), 2 strains of *Vagococcus* spp. (4%), each 1 strain of *Planococcus Migula* spp., *Enterobacter* spp., *Bacillus* spp. and *Kocuria roseus* spp. (2%), respectively (Fig. 5A). Among 32 strains of *Vibrio* spp., 14 strains belonged to *V. harveyi* (44%), 7 strains of *V. alginolyticus* (22%), 2 strains of *V. anguillarum* and *V. splendidus* (6%), 1 strain of *V. parahaemolyticus* (3%) (Fig. 5B). In farm B, 25 strains were obtained, and 18 strains belonged to *Vibrio* spp. (72%), 2 strains of *Staphylococcus* spp., *Shewanella* spp. and *Bacillus* spp. (8%), 1 strain of *Exiguobacterium* spp. (4%), respectively (Fig. 6A). Among 18 strains of *Vibrio* spp., 10 strains belonged to *V. harveyi* (56%), 3 strains of *V. splendidus* (17%) and 1 strain of *V. alginolyticus* (5%), respectively (Fig. 6B). *Vibrio* spp. were the predominant genus and *V. harveyi* was the main species in both farms A and B. Compared with farm A, bacterial diversity in farm B was lower (Fig. 5B).

The bacterial diversity was diverse along with months based on one-year survey from two farms. The bacteria were isolated more in May and December, and *V. harveyi* could not be isolated in February and November (Fig. 7A). More bacteria were carried by diseased *T. rubripes* in May and September, followed by June, July and December. In May, *V. harveyi*, *V. alginolyticus*, *Vagococcus* spp. and

Kocuria roseus spp. were isolated more. In September, *V. harveyi*, *V. alginolyticus* and unidentified *Vibrio* spp. and *Bacillus* were isolated. The bacterial diversity was similar in June and July. Apart from February, bacteria could be isolated in other months, and the diversity was sole in January, March, April, June, July and October. *V. harveyi* was the predominant species (Fig. 7).

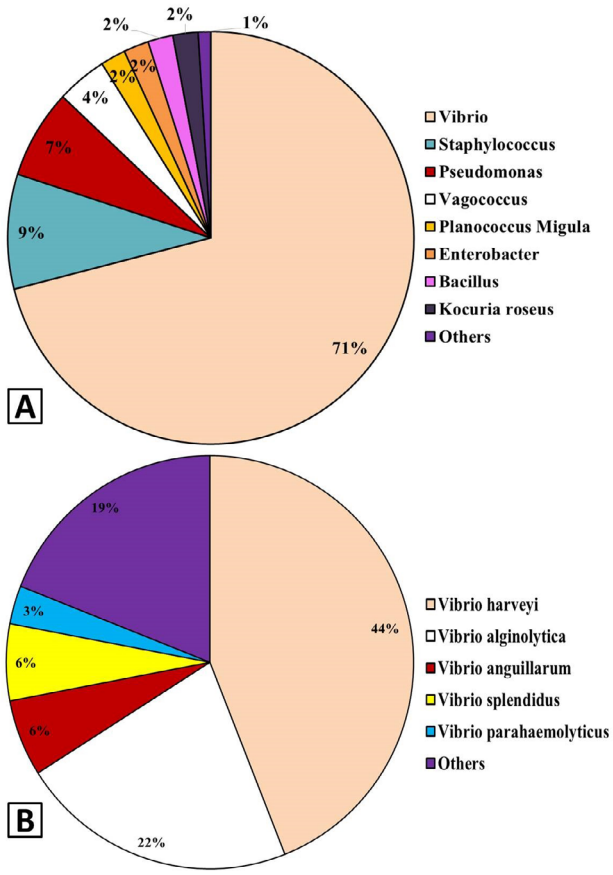

Fig. 5. Bacterial diversity of 45 isolates obtained from farm A. A, proportion of strains in all the isolates obtained from farm A at species level; B, percentage of *Vibrio harveyi* in *Vibrio* genera in farm A.

V. harveyi *distribution*

V. harveyi could be isolated from both two farms throughout year. *V. harveyi* could mainly be isolated in January, May, June, July, August, September and October in farm A. In addition, *V. harveyi* could almost be isolated each month in farm B except February, August and November.

Challenge tests

The clinical signs of diseased *T. rubripes* infected naturally and artificially by intramuscular and wounded immersion were similar, including skin darkening (Fig. 2A), fin ulceration (Fig. 2B), fin bleed (Fig. 2C), liver congestion (Fig. 2D), splenomegaly (Fig. 2E) and intestinal hydrops (Fig. 2F). The mortality was observed at 2 dpi (days post infection) in most bacterial injection groups. No clinical signs and death were noted in control group. Thirteen strains were determined to be virulent with 14-d cumulative mortalities of more than 50%, which were numbered as strains 2HWH001, 2HWH002, 2HWH003, 2HWH004, 2HWH005, 2HWH008, 2HWH010, 2HWH011, 2HWH012, 2HWH013, 2HWH017, 2HWH019 and 2HWH020, respectively (Table III). The virulence was different from strains. Among them, strain 2HWH020 showed highest virulence with cumulative mortality of 80% by intramuscular injection and 50% by wounded immersion. Strain 2HWH003 as a pathogenic isolate was lowest virulent to *T. rubripes*, and the cumulative mortality was 70% by intramuscular injection and 10% by wounded immersion (Table III).

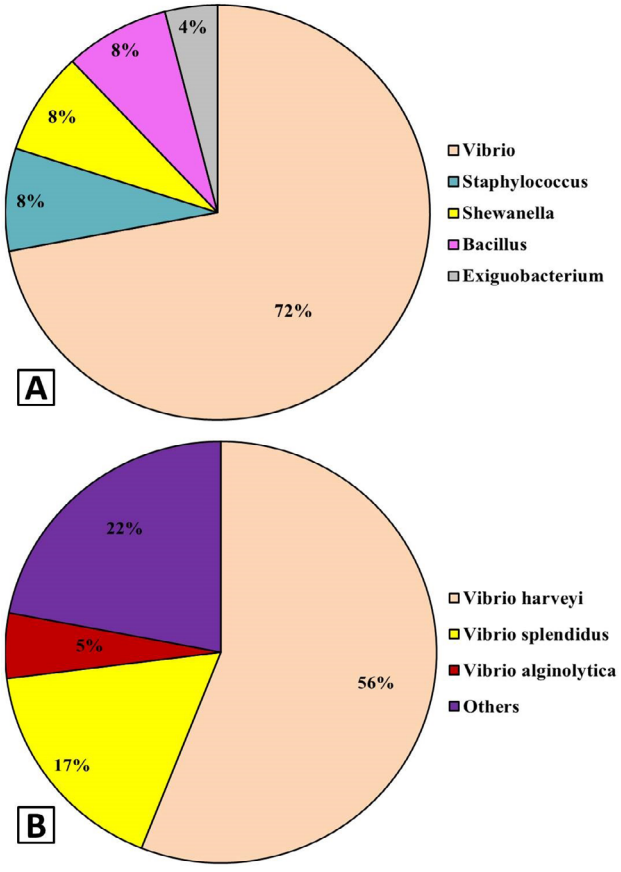

Fig. 6. Bacterial diversity of 25 isolates obtained from farm B. A, proportion of strains in all the isolates obtained from farm B at species level; B, percentage of *Vibrio harveyi* in *Vibrio* genera in farm A.

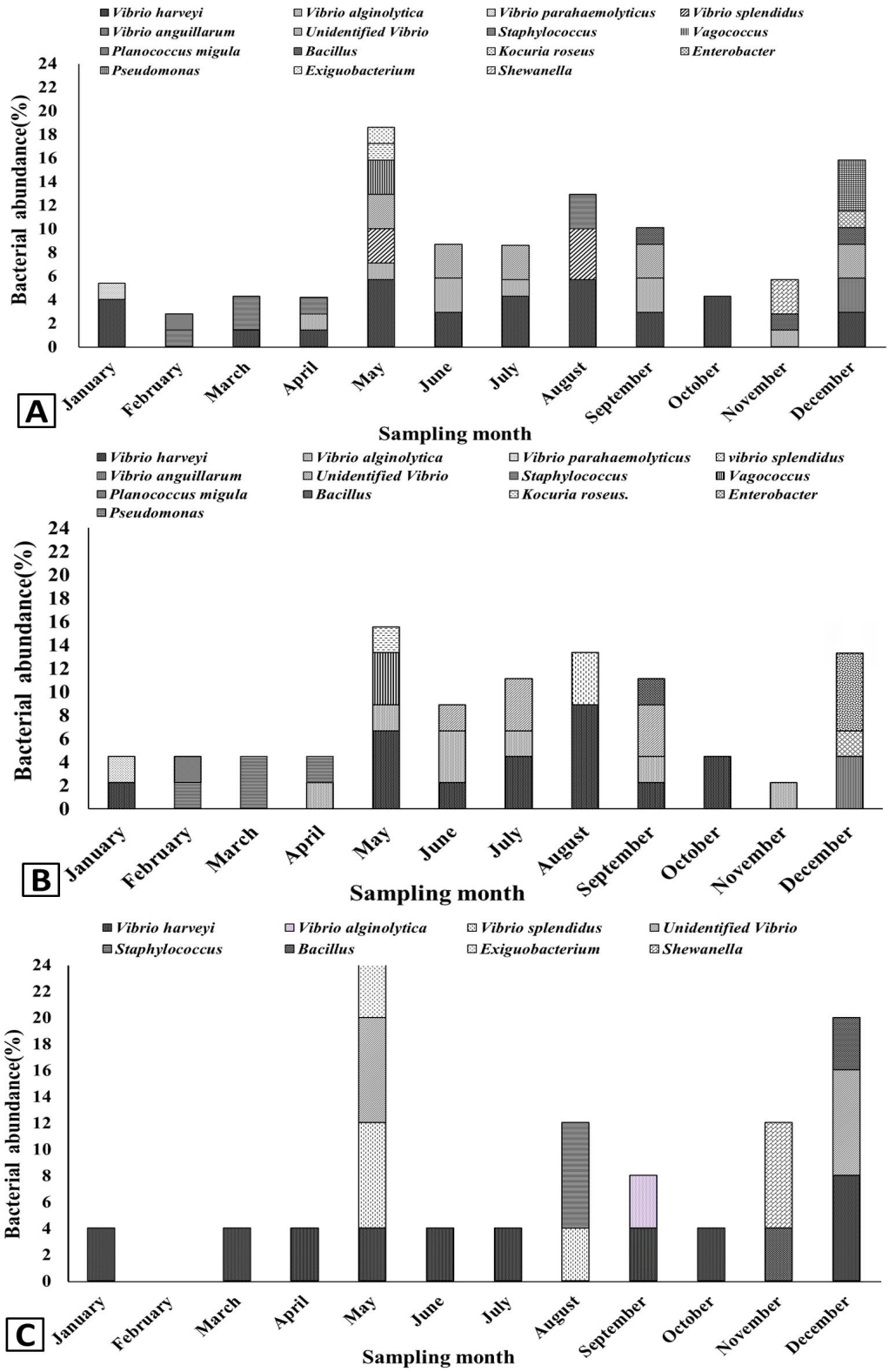

Fig. 7. Time-course bacterial diversity in both farm A and farm B. A, total bacterial diversity at different sampling month; B, bacterial diversity at different sampling month in farm A; C, bacterial diversity at different sampling month in farm B.

Table III.- Results of challenge tests by *Vibrio harveyi* isolates (24 strains).

Strain number	14-day cumulative mortality (%)	
	Intramuscular injection	Wounded immersion
2HWH001	100	N
2HWH002	90	N
2HWH003	70	10
2HWH004	80	N
2HWH005	80	N
2HWH006	20	N
2HWH007	20	N
2HWH008	80	N
2HWH009	30	N
2HWH010	70	N
2HWH011	80	N
2HWH012	70	N
2HWH013	90	N
2HWH014	20	N
2HWH015	40	N
2HWH016	30	N
2HWH017	100	20
2HWH018	50	N
2HWH019	80	20
2HWH020	90	50
2HWH021	40	N
2HWH022	40	N
2HWH023	20	N
2HWH024	30	N
Control	0	0

N means no results are given.

DISCUSSION

In agreement with reports by Wang *et al*. (2008), the one-year investigation demonstrated that main symptoms of diseased fish *T. rubripes* were skin darkening, fin ulceration, liver congestion and splenomegaly, respectively. Bacteria could be isolated from all samples tested.

V. harveyi has been considered as one of important bacterial pathogens in sea water aquaculture (Zhou *et al*., 2012; Zhang and Austin, 2000; Ransangan *et al*., 2012; Montero and Austin, 2010) and limits the development of aquaculture seriously. In the present study, *V. harveyi* could be isolated in almost every month except February and could be obtained from both farms A and B. In addition, *V. harveyi* isolates were determined to be pathogenic to *T. rubripes*, and cause main symptoms of fin rot and skin ulceration. Thus, *V. harveyi* can be considered as the main pathogenic bacteria of *T. rubripes* in Liaoning Province, North China. Other researches also demonstrated that *V. harveyi* can cause *T. rubripes* skin ulceration (Won *et al*., 2009; Wu *et al*., 2015) and skin ulcer disease (Shen *et al*., 2017). Two challenge methods were used to determine the pathogenicity of *V. harveyi* isolates. In the challenge tests of *T. rubripes* by intramuscular injection, fin ulceration were not observed as shown in naturally infected fish although higher mortality was recorded. Accordingly, *V. harveyi* showed moderate pathogenicity to tiger puffer when 20% mortality was observed within 6 days post-infection at bacterial concentration of 1.0×10^{8} CFU mL^{-1} (Mohi *et al*., 2010). Thus, the wounded immersion tests were conducted and infected fish showed unfinished fin rot symptoms as naturally infected. It is related with the bacterial infection way as reported by Wang *et al*. (2008). Shi *et al*. (2005) also showed that *V. harveyi* could fail to infect large yellow croaker (*Pseudosciaena crocea*) by intramuscular injection, and the pathogen could successfully infect the organisms by wounded immersion.

Bacterial diversity was various with culture conditions and months. In this study, 45 of 70 strains belonged to 8 genera were isolated from farm A, and another 25 strains belonged to 5 genus were isolated from farm B. The isolation frequency and bacterial diversity in farm A were more various than that in farm B, although both farm A and B are located in Dalian City, Liaoning Province, North China. Their differences might be related with the sea water treatment method. A conventional indoor flow aquaculture system without any seawater treatment is used in farm A, while automated recirculating aquaculture system (RAS) with seawater pre-treated by filtration and sterilization is used in farm B. The over-wintering period of *T. rubripes* is from November of each year to May of the following year, and the temperature is generally 13-16 °C. This study found that less bacteria could be isolated during this period compared to other months. It suggested that the number and type of bacteria might be related to water temperature. The higher temperature is more suitable for bacterial growth. In farm B, water has been treated by drum filters and biological filters to stabilize the environmental conditions. Bacteria number in farm B can be reduced and is obviously lower than that in marine water untreated. In addition, RAS has been reported to enhance the immunity of cultured species (Lin *et al*., 2017; Kikuchi *et al*., 2006; Yanagawa *et al*., 2011; Lin *et al*., 2017). Combined with the present study, the number of diseased fish in farm B was less than that in farm A, and less bacteria could be isolated from farm B than farm A. It suggested that pre-treatment of seawater is more effective in *T. rubripes* aquaculture. The annual bacteria distribution showed that bacteria species increased obviously in May, which might be related with the water temperature raise after over-wintering and lower immunity caused by non-

feeding during the winter. Simultaneously, *V. harveyi* is an opportunistic bacterial pathogen and it grows along with the water temperature. As we know, disease of aquatic organisms is combined with culture environment, hosts and pathogens. Under the lower immunity and more pathogens, it will be easier to be infected and bacterial isolates were obtained more in May in this study. From June to September, *T. rubripes* grows faster at suitable water temperature and no more diseases were detected. However, the bacterial species increased significantly in December, which might be correlated with the culture conditions since *T. rubripes* needs to be transferred from outdoor ponds into indoor tanks for over-wintering. The transfer operation was able to cause body surface injure and intrigue stress, which gave the opportunity to be infected by pathogens. However, the bacterial diversity in December was still lower than that in May due to the lower temperature against bacterial pathogen infection. The above results strongly suggested that some strategies should be taken for disease prevention before/after transfer and post over-wintering.

CONCLUSIONS

A one-year investigation about diseased *T. rubripes* cultured in North China showed that bacteria could be isolated each month, and *V. harveyi* was the main pathogen. *V. harveyi* was isolated more in May and December, suggesting that bacterial diseases should be attracted more attention after over-wintering and before/after transfer.

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Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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