Virulence, antimicrobial and heavy metal tolerance, and genetic diversity of *Vibrio cholerae* recovered from commonly consumed freshwater fish

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Abstract

*Vibrio cholerae* is a leading waterborne pathogen worldwide. Continuous monitoring of *V. cholerae* contamination in aquatic products and identification of risk factors are crucial for assuring food safety. In this study, we determined the virulence, antimicrobial susceptibility, heavy metal tolerance, and genetic diversity of 400 *V. cholerae* isolates recovered from commonly consumed freshwater fish (*Aristichthys nobilis*, *Carassius auratus*, *Ctenopharyngodon idellus*, and *Parabramis pekinensis*) collected in July and August of 2017 in Shanghai, China. *V. cholerae* has not been previously detected in the half of these fish species. The results revealed an extremely low occurrence of pathogenic *V. cholerae* carrying the major virulence genes *ctxAB* (0.0%), *tcpA* (0.0%), *ace* (0.0%), and *zot* (0.0%). However, high incidence of virulence-associated genes was observed, including the RTX toxin gene cluster (*rtxA-D*) (83.0–97.0%), *hlyA* (87.8%), *hapA* (95.0%), and *tlh* (76.0%). Meanwhile, high percentages of resistance to antimicrobial agents streptomycin (65.3%), ampicillin (44.5%), and rifampicin (24.0%) were observed. Approximately 30.5% of the isolates displayed multidrug resistant (MDR) phenotypes with 42 resistance profiles, which were significantly different among the four fish species (MARI, *P* = 0.001). Additionally, tolerance of isolates to heavy metals Hg²⁺ (65.3%), Zn²⁺ (30.3%), and Pb²⁺ (12.0%) was observed. The enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR)-based fingerprinting of the 400 *V. cholerae* isolates revealed 328 ERIC-genotypes, which demonstrated a large degree of genomic variation among the isolates. Overall, the results of this study support the need for food safety risk assessment of aquatic products.

Keywords *Vibrio cholerae* · Virulence · Antimicrobial susceptibility · Heavy metal tolerance · Genotyping · Freshwater fish

Introduction

*Vibrio cholerae* can cause cholera, a severe diarrheal disease that can be quickly fatal if untreated and is typically transmitted via contaminated water and person-to-person contact (Baker-Austin et al. 2018). It was estimated that *V. cholera* caused roughly 2.9 million cases of cholera and 95,000 deaths annually worldwide between 2008 and 2012 (Ali et al. 2015). The bacterium is found growing in aquatic environment or aquatic products such as crustaceans and fish (Vezzulli et al. 2010). Previous studies highlighted the link between cholera outbreaks and the consumption of raw, undercooked, or mishandled fish products contaminated by *V. cholerae*. For instance, consumption of dried fish was significantly associated with the 1997 cholera epidemic in a rural area (Ifakara) in southern Tanzania (Acosta et al. 2001). It has also been reported that the fresh fish imported from Nigeria contributed to the domestic cholera in Germany in 2001 (Schurmann et al. 2001).
Since 2002, China has become the largest producer and exporter in the world for fishery products, which accounted for 62.6% (69,012,500 tons) of the global amount in 2016. Freshwater aquaculture is an important component of Chinese fishery production, and accounted for 48.4% of the total fishery output value in China (Zhang et al. 2018). C. idellus (known as grass carp) is the most important freshwater cultured fish with the largest production in China, and its total output exceeded 5,676,235 tons in 2015 (Jia et al. 2016). C. auratus (known as crucian carp) has been cultured in China for several hundred years (Jing et al. 2016), and recently played an important role in the international fish trade with a total output of 2,912,258 tons in 2015 (Jia et al. 2016). A. nobilis (known as bighead carp) and P. pekinensis (known as white bream) are also two of the most dominant fish species in freshwater aquaculture in China. Therefore, continuous monitoring of V. cholerae contamination in freshwater fish is imperative for food safety control.

Two major virulence genes that encode cholera toxin (CT) and toxin coregulated pilus (TCP) have been identified in epidemic V. cholerae strains of serotypes O1 and O139. These toxin genes are carried by a lysogenic filamentous bacteriophage (CTX prophage) that can integrate into V. cholerae chromosomes (Waldor and Mekalanos 1996). The non-epidemic V. cholerae strains, referred to non-O1/O139, can cause sporadic episodes of diarrhea and gastrointestinal infection (Austin 2010; Ceccarelli et al. 2015). The genes encoding virulence-associated factors that contribute to the pathogenicity of V. cholerae include the zonula occludens toxin (zot) (Preeprem et al. 2014), accessory cholera enterotoxin (ace) (Briquaire et al. 2017), RTX toxin gene cluster (rtxA-D) (Lin et al. 1999), El Tor hemolysin (hlyA) (Ruenchit et al. 2017), thermolabile hemolysin (thl) (Fiore et al. 1997), hemagglutinin protease (hapA) (Halpern et al. 2003), and two morphologically distinct types of pili, namely, mannose-sensitive hemagglutination (MSHA) pili (msHA) (Moorthy and Watnick 2004) and putative type IV pilus (pil) (Fullner and Mekalanos 1999).

Antimicrobial agent treatment can effectively control outbreaks and prevalence of infectious diseases caused by pathogenic microorganisms. However, the inappropriate usage of antimicrobial drugs in aquaculture contributed to the development of antimicrobial-resistant bacteria and imposed potential threat upon human health due to the dissemination of antimicrobial resistance (Woolhouse and Farrar 2014). The aquatic environment is a reservoir of V. cholerae and might be an important source of resistant strains (Baron et al. 2017). Numerous previous studies have been focused on the detection of V. cholerae from various aquatic environments. For instance, Sulca et al. reported that two V. cholerae isolates from Lima (Peru) seawater were resistant to 12 antimicrobial drugs, including ampicillin (AMP), penicillin, amoxicillin, nitrofurantion, kanamycin (KAN), amikacin, aztreonam, ciprofloxacin, gentamicin (CN), co-trimoxazole, ceftazidime, and nalidixic acid (Sulca et al. 2018). Bhuyan et al. also reported that 107 V. cholerae strains originated from different aquatic environment (including river water, canal water, pond water, and hand-pump water) in India showed varying degrees of resistance to AMP, co-trimoxazole, nalidixic acid, polymyxin-B, streptomycin (STR), ciprofloxacin, and tetracycline (TET) (Bhuyan et al. 2016). Antimicrobial-resistant V. cholerae isolated from aquacultured animals such as shrimps and shellfish have also been reported. For example, He et al. analyzed 42 V. cholerae isolates recovered from shrimp collected in 2013 and 2014 in Shanghai, China, and found that 33.3%, 21.4%, 19.1%, 9.5%, and 9.5% of the isolates were resistant to rifampicin (RIF), STR, KAN, AMP, and TET, respectively. Moreover, 25 isolates (59.5%) had MDR phenotypes (He et al. 2015).

V. cholerae has been isolated from approximately 30 fish species belonging to nine different orders within the Actinoptygii class (Halpern and Izhaki 2017). The bacterium was found to reside in healthy fish intestines (Halpern and Izhaki 2017). For instance, V. cholerae was detected from the intestines of ten freshwater fish species collected in Israel in 2008, including Astatotilapia flaviijosephi, Barbus longiceps, Carasobarbus canis, C. idella, Cyprinus carpio, Mugil cephalus, Myrirptis murdjan, Oreochromis aureus, Sarotherodon galilaeus, and Tilapia sp. and Tilapia zilli (Senderovich et al. 2010). Recently, V. cholerae was also isolated from four freshwater fish species (C. auratus, C. idella, Cyprinus carpio, and Hypophthalmichthys molitrix) collected in Chengdu, China (Li et al. 2017). Laviad-Shitrit et al. reported that 95.8% of V. cholerae isolates (non-O1/O139) (n = 48) derived from fish intestines showed high minimal inhibitory concentration (MIC) (MIC90 of 16 μg/mL) to doxycycline (Laviad-Shitrit et al. 2018).

Water contaminated with heavy metals may enhance selection for antibiotic resistance and vice versa (Baker-Austin et al. 2006; Matyar 2012). The commonly detected heavy metals in the environment include chromium (Cr), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), nickel (Ni), and lead (Pb) (Wuana and Okieimen 2011). Nevertheless, very little information is available concerning the tolerance of V. cholerae to heavy metals to date. In our prior study, our data revealed that V. cholerae isolates Chn64, Chn86, Chn91, Chn92, and Chn108 derived from the surface water of Yangtze River Estuary in Shanghai, China, showed high levels of tolerance to Hg, Cd, and Cu; Hg, Cd, and Cu; Hg, Cd, Pb, and Cu; and Hg, Cd, Zn, Pb, and Cu; as well as Hg, Cd, and Pb, respectively. Meanwhile, the isolates Chn64, Chn91, Chn92, and Chn108 were also resistant to AMP; AMP and RIF; and AMP and RIF; as well as AMP, sulfamethoxazole, and STR, respectively (Song et al. 2013). We hypothesized that there could be high incidence of antimicrobial and/or heavy metal–resistant V. cholerae in freshwater fish.
In this study, we investigated the virulence, antibiotic, and heavy metal tolerance of 400 V. cholerae strains isolated from four commonly consumed freshwater fish (A. nobilis, C. auratus, C. idellus, and P. pekinensis) sampled in July and August of 2017 in Shanghai, China. Additionally, we also obtained and compared fingerprinting profiles of the 400 V. cholerae isolates using the ERIC-PCR assay to address their phylogenetic relatedness for better understanding of genome evolution of the bacterium.

Materials and methods

Sample collection

The four commonly consumed freshwater fish were sampled in July and August of 2017 from two largest fish markets located in Shanghai, China, including the Jiyan Aquatic Market (31° 19’ 57.61” N, 121° 10’ 53.05” E) and Jiangyang Aquatic Market (31° 21’ 25.90” N, 121° 26’ 50.68” E). Forty fish samples comprising of A. nobilis (n = 10), C. auratus (n = 10), C. idellus (n = 10), and P. pekinensis (n = 10) were collected into sterile sealed bags (Nanjing Maojie Microbial Technology Co., Ltd., Nanjing, China), and immediately transported in ice boxes (700 × 440 × 390 mm) to the laboratory in Shanghai Ocean University, Shanghai, China, for analysis.

Isolation of V. cholerae

V. cholerae was isolated and identified in accordance with the instructions of the Chinese Government Standard (SN/T 1022-2010) and the Bacteriological Analytical Manual of the US Food and Drug Administration (8th Edition, Revision A, 1998) as described previously (Song et al. 2013). Briefly, 25 g of each fish intestine sample was rinsed with 225 mL sterile 1× phosphate buffer saline (PBS, pH 7.4–7.6, Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., Shanghai, China), and then homogenized for 2 min using a stomacher (Bagmixer 400 W, Interscience, Saint Nom la Bretèche, France). Serial tenfold dilutions were prepared up to 1:105 dilution, and 100 μL of each dilution was spread on thiosulfate citrate bile salts sucrose (TCBS; Beijing Land Bridge Technology Co., Ltd., Beijing, China) agar plates, which were incubated at 37 °C for 24 h. Yellow, flat, and shiny colonies that were 2 to 3 mm in diameter on the TCBS agar plates were picked out for further analysis.

Identification of V. cholerae

V. cholerae isolates were also identified by biochemical tests, including the arginine dihydrolase test and the esculin hydrolysis test (Choopun et al., 2002; Thornley 1960) using the double-arginine hydrolase medium (pH 6.8, 3.0% NaCl) and the esculin medium (pH 7.3, 3.0% NaCl) (Muwei Biotechnology Co., Ltd., Shanghai, China), respectively. After inoculation, the former medium was covered with sterile mineral oil (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., Shanghai, China), and then incubated at 37 °C for 24 h. Appearance of a red color was considered as a positive reaction, while the blackening of the latter medium indicated a positive reaction. V. cholerae isolates that were detected negative in the two tests, showing deep yellow and brown, respectively, were picked out for the further identification by the PCR assay and DNA sequencing analysis.

The oligonucleotide primers (VHMF and VHA-AS5, Table 1) targeting the V. cholerae-specific gene lolB were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The lolB gene with an expected amplicon size of 516 bp was amplified according to the method described previously (Lalitha et al. 2008) with slight modification. Briefly, PCR reaction mixture contained 8 μL of DNase/RNase-free deionized water (Tiangen Biotech Co., Ltd., Beijing, China), 10 μL of 2× Taq Master Mix (Novoprotein Technology Co., Ltd., Shanghai, China), 0.5 μL of each primer (VHMF and VHA-AS5), and 1 μL of DNA template. PCR was carried out under the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min. All PCR reactions were performed in a Mastercycler® pro PCR thermal cycler (Eppendorf, Hamburg, Germany). Amplicons were analyzed by electrophoresis with a 2.0% agarose gel, and visualized and recorded using a UVP EC3 Imaging system (UVP LLC, Upland, CA, USA). V. cholerae GIM 1.449 (lolB+) (Guangdong Culture Collection Center, Guangzhou, China) was used as a positive control strain.

Bacterial 16S ribosomal RNA (rRNA) gene was also amplified using universal bacterial primers 27F and 1492R (Weisburg et al. 1991). A 25-μL reaction mixture contained 12.5 μL of 2× Taq Master Mix (Novoprotein Technology Co., Ltd., Shanghai, China), 1.25 μL of each primer (27F and 1492R), and 1 μL of DNA template. The thermal cycler was used at the following setting: initial denaturing at 94 °C for 3 min, followed by 30 cycles of denaturing at 94 °C for 40 s, annealing at 55 °C for 45 s, elongation at 72 °C for 2 min. The PCR products were analyzed as described above. The PCR products were purified and sequenced by Shanghai Sangon Biological Engineering Technology.
and Services Co., Ltd. (Shanghai, China). DNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) (available online: https://www.ncbi.nlm.nih.gov/).

Genomic DNA extraction

*V. cholerae* isolates were cultured in Luria-Bertani (LB) broth (Beijing Land Bridge Technology Co., Ltd., Beijing, China) (pH 8.5, 3.0% NaCl) overnight at 37 °C. Bacterial lysate was prepared according to the method described previously (Letchumanan et al. 2015). Briefly, 5 μL of bacterial culture was heated in 50 μL of ultrapure water at 95 °C for 10 min, and then transferred on ice for cooling. After centrifugation at 12,000 rpm for 5 min, the resulting supernatant was used as DNA template for PCR assays.

Detection of virulence and virulence-associated genes

The major virulence genes (*ctxAB* and *tcpA*) (Kumar et al. 2010; McGrath et al. 2006) and virulence-associated genes (*ace*, *zot*, *rtxABCD*, *hapA*, *hlyA*, *tlh*, *mshA*, and *pilA*) (Singh et al. 2002; Tulatorn et al. 2018) were detected by the PCR assay. PCR reactions were performed as described above, but with different annealing temperatures and elongation times based on melting temperatures of primer pairs and predicted sizes of PCR products. The primers were synthesized as described above.

Table 1 Oligonucleotides used in this study

| Primer | Sequence (5’→3’) | Amplicon size (bp) | Reference |
|--------|------------------|--------------------|-----------|
| VHMF   | TGGGAGCAGCGTCCATTGTG | 516                | Lalitha et al. (2008) |
| VHA-AS5| CAATCACACCAAGTCTCCTG  | ~1540              | Weisburg et al. (1991) |
| 27F    | GAGGATTTGATCCTGGCTCAG | 778                | McGrath et al. (2006) |
| 1492R  | TACGGCTACCTTTGTTACGAC  | 675                | Kumar et al. (2010) |
| ctxAB-F| TGAATTTAAGACGACTGCTTGT | 316                | Singh et al. (2002) |
| ctxAB-R| GGTATTCTGCACAAATACTCAG | 947                | Tulatorn et al. (2018) |
| tcpA-F | ATGCAATTATAAAAAACAGCTTTAAG | 977                | Rivera et al. (2001) |
| tcpA-R | TTAGCTGTTACCAAATGCAACAG | 400                | This study |
| tcpB-F | TTTCTGCTACAGCCTCTTCTT | 437                | This study |
| tcpC-R | ATCATAGAGCCTTTTCTGCTGAAA | 334                | This study |
| tcpD-F | CGCCCAATGATACAGAAGTCAG | 274                | This study |
| tcpD-R | AAAGCTACAGGCACTCCCAACG | 393                | Kumar et al. (2010) |
| thl-F  | CGGATGTTGCGGCAAGGAAAT | 207                | This study |
| thl-R  | CGTGACGGCTGATCGAAAT | 227                | This study |
| hlyA-F | CCAATGTGGATGGCGCGCGGAC | 189                | Rivera et al. (1995) |
| hlyA-R | TTTCTGCGCCGCGGTGTCGCG | 189                | This study |
| hapA-F | CGTGATGATCCCCGATAGGTTC | 189                | This study |
| hapA-R | CGTGACGGCTGATCGAAAT | 189                | This study |
| pilA-F | GGGATGTCATTCCTCTCAAC | 189                | This study |
| pilA-R | CCTAATGACCTGATGCT | 189                | This study |
| mshA-F | CGCTATGACTTCCGCTCAG | 189                | This study |
| mshA-R | TACCAACAGCAGTTCGAC | 189                | This study |
| ERIC1R | ATGAAGGCTCCTGGGACTCAC | 189                | This study |
| ERIC2  | AAGTAAGGCTGACTGCTGGGAGCG | 189                | This study |

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**Antibiotic susceptibility and heavy metal tolerance assays**

*V. cholerae* isolates were measured for in vitro susceptibility to ten antimicrobial agents (Oxoid, UK) according to the method described previously (Hu and Chen 2016; Tang et al. 2014). Ten antimicrobial agents were AMP, CHL, KAN, RIF, SPT, STR, TET, TM, and sulfamethoxazole plus trimethoprim (SXT). Tolerance of *V. cholerae* isolates to eight heavy metals NiCl₂, CrCl₃, CdCl₂, PbCl₂, CuCl₂, ZnCl₂, MnCl₂, and HgCl₂ (Analytical Reagent, Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) was also determined according to the method described previously (Hu and Chen 2016). *Escherichia coli* strains ATCC25922 and K12 (Institute of Industrial Microbiology, Shanghai, China) were used as quality control strains in antibiotic and heavy metal resistance tests, respectively (Matyar 2012; Song et al. 2013).

**ERIC-PCR assay**

Strain taxonomy was determined by ERIC-PCR with the primer set ERIC1R and ERIC2 (Rivera et al. 1995) (Table 1). A 20-μL reaction mixture contained 10 μL of 2× Taq Master Mix (Novoprotein Technology Co., Ltd., Shanghai, China), 1 μL of each primer, and 2 μL of DNA template. The ERIC-PCR was performed under the following conditions: denaturation at 95 °C for 30 s, annealing at 52 °C for 1 min, and extension at 65 °C for 8 min. Following 32 reaction cycles, reaction mixtures were further incubated at 65 °C for an additional 16 min. Six microliters of each amplicon were electrophoresed at 100 V for about 45 min on a 1.0% agarose gel. Amplified DNA fragments were visualized and recorded as described above.

**Statistical analysis**

Data analysis was performed using the SPSS statistical analysis software version 17.0 (SPSS Inc., Chicago, USA). The multiple antimicrobial resistance index (MARI) of an isolate is defined as \( \frac{a}{b} \), where \( a \) represents the number of antibiotics to which the isolate was resistant, and \( b \) represents the number of antibiotics to which the isolate was susceptible (Krumperman 1983). One-way analysis of variance (ANOVA) followed by appropriate post-hoc test (Tukey) was performed to determine significant differences between the four different fish samples and MARI of resistant isolates, and \( P < 0.05 \) was considered statistically significant. DNA banding patterns generated by the ERIC-PCR were analyzed using the BioNumerics 7.6 software (Meacham et al. 2003). All the PCR fingerprinting profiles were assigned arbitrary designations, and quantitative differences among the profiles were defined using the Dice coefficient. Cluster analysis was carried out based on the unweighted pair group with arithmetic averages (UPGMA) using a position tolerance of 0.5. The single numerical index of discrimination (D) was based on the probability that two unrelated strains sampled from the test population will be placed into different typing groups. This probability can be calculated by Simpson’s index of diversity (Simpson 1972).

**Results**

**Prevalence of *V. cholerae* in the fish species**

In this study, a total of 3716 yellow single colonies recovered from the 40 freshwater fish samples were randomly picked out from the selective TCBS agar plates for further identification. Approximately 84.0% (3123/3716) of these colonies were tested negative in either the double-arginine hydrolase test or the esculin hydrolysis test. Moreover, they were detected positive for the *V. cholerae*-specific gene *lolB*, which is highly conserved for *V. cholerae* (Lalitha et al. 2008). The results were confirmed by DNA sequencing and analysis based on amplicons of the *lolB* and 16S rRNA genes. Additionally, 64.8% (2022/3123) of the *V. cholerae* isolates originated from the Jiayang aquatic market, and 35.3% (1101/3123) from the Jiayan aquatic market. Furthermore, approximately 28.7% (n = 897), 20.7% (n = 645), 29.5% (n = 922), and 21.1% (n = 659) of the isolates were recovered from the *C. auratus*, *A. nobilis*, *P. pekinensis*, and *C. idellus* samples, respectively.

**Virulence and virulence-associated genes in the *V. cholerae* isolates**

Pure culture of randomly selected 100 *V. cholerae* isolates recovered from each species of the four commonly consumed freshwater fish was analyzed and reported in this study. All the isolates were detected positive for the *V. cholerae*-specific *lolB* gene, but negative for the toxin genes *ctxAB*, ace, zot, and tcpA (Fig. 1). In contrast, high occurrence of the virulence-associated genes *rtxABCD* (83.0%, 97.0%, 95.8%, and 95.5%, respectively), *hlyA* (87.8%), *tlh* (76.0%), and *hapA* (95.0%) was observed in the 400 *V. cholerae* isolates, whereas low percentages of the *pilA* (0.8%) and *mshA* (0.8%) genes were detected (Fig. 1).

As illustrated in Fig. 1, the *V. cholerae* isolates recovered from the four freshwater fish species had similar toxic genotypes; the most of which were featured with the *rtxA*, *tlh*, *hlyA*, and *hapA* genes (75.0–100.0%), except a lower percentage of *rtxA* gene in *A. nobilis* (61.0%). However, the *pilA* gene was only detected from two isolates derived from *C. auratus* and one from *A. nobilis*. The *mshA* gene was only present in three isolates, which were recovered from *A. nobilis*, *C. idellus*, and *P. pekinensis*, respectively.
Antimicrobial resistance profiles of the *V. cholerae* isolates

We determined antimicrobial susceptibility in vitro of the 400 *V. cholerae* isolates to ten antimicrobial agents, and the resulting data were illustrated in Fig. 2 (Table S1). Approximately 15.3% of the isolates were susceptible to all the ten antimicrobial drugs evaluated. Moreover, most isolates were also sensitive to CN (98.3%), CHL (98.0%), and SPT (95.0%). In contrast, the STR resistance was the most predominant (65.3%) among the *V. cholerae* isolates, followed by AMP (44.5%) and RIF (24.0%). Approximately 72.3%, 39.5%, and 34.3% of the isolates also exhibited intermediate susceptibility to KAN, RIF, and TET. The resistance trend of the 400 *V. cholerae* isolates was STR > AMP > RIF > TM > SXT > KAN > TET > SPT > CHL > CN.

Our data also revealed different antimicrobial-resistant profiles for the *V. cholerae* isolates recovered from different fish species (Fig. 3). The isolates from *C. idellus* had a higher proportion of resistance to STR (75.0%) than those isolates recovered from *C. auratus* (67.0%), *P. pekinensis* (65.0%), and *A. nobilis* (54.0%). About half of *A. nobilis* (54.0%) and *C. auratus* (53.0%) isolates were also resistant to AMP, which were higher than those isolates from *C. idellus* (38.0%) and *P. pekinensis* (33.0%). Meanwhile, the TM, SPT, and SXT-resistant *V. cholerae* isolates from *C. auratus* were 34.0%, 5.0%, and 28.0%, respectively, which were higher than those observed in *A. nobilis* (9.0%, 2.0%, and 8.0%), *P. pekinensis* (19.0%, 3.0%, and 14.0%), and *C. idellus* (16.0%, 4.0%, and 15.0%). Additionally, a few isolates from *C. idellus* and *C. auratus* were also resistant to CN and or CHL, whereas none of the isolates from the other two fish species were resistant to these two drugs.

In this study, approximately 30.5% (*n* = 122) of the isolates exhibited MDR phenotypes (Ling et al. 1983), which varied depending on the fish species. The strains isolated from *C. auratus* showed the highest occurrence of MDR (42.0%), followed by 34.0%, 31.0%, and 15.0% from the *C. idellus*, *P. pekinensis*, and *A. nobilis*, respectively. The values of MARI of the 400 *V. cholerae* isolates ranged from 0.00 to 0.70, indicating varying degrees of exposing to the antimicrobial agents evaluated. Forty-two different resistance patterns with a significantly different MARI (> 0.20) were observed. Two isolates recovered from *C. idellus* had the highest MARI of 0.70, and displayed resistance to seven of the ten antimicrobial agents tested. Additionally, the mean MARI values for the *V. cholerae* isolates recovered from *C. auratus*, *A. nobilis*, *P. pekinensis*, and *C. idellus* were 0.24, 0.16, 0.18, and 0.21, respectively, suggesting a significantly different antibiotic resistance profiles for the *V. cholerae* isolates recovered from different fish species. The isolates from *C. idellus* had a higher proportion of resistance to STR (75.0%) than those isolates recovered from *C. auratus* (67.0%), *P. pekinensis* (65.0%), and *A. nobilis* (54.0%). About half of *A. nobilis* (54.0%) and *C. auratus* (53.0%) isolates were also resistant to AMP, which were higher than those isolates from *C. idellus* (38.0%) and *P. pekinensis* (33.0%). Meanwhile, the TM, SPT, and SXT-resistant *V. cholerae* isolates from *C. auratus* were 34.0%, 5.0%, and 28.0%, respectively, which were higher than those observed in *A. nobilis* (9.0%, 2.0%, and 8.0%), *P. pekinensis* (19.0%, 3.0%, and 14.0%), and *C. idellus* (16.0%, 4.0%, and 15.0%). Additionally, a few isolates from *C. idellus* and *C. auratus* were also resistant to CN and or CHL, whereas none of the isolates from the other two fish species were resistant to these two drugs.

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resistant *V. cholerae* population in the four fish species ($P = 0.001$).

**Heavy metal tolerance profiles of the *V. cholerae* isolates**

Tolerance of the 400 *V. cholerae* isolates to eight heavy metals was also determined (Table 2). The maximum MICs observed in the tested isolates were 3200 $\mu$g/mL for Pb$^{2+}$; 1600 $\mu$g/mL for Cr$^{3+}$, Mn$^{2+}$, and Ni$^{2+}$; 800 $\mu$g/mL for Cd$^{2+}$, Zn$^{2+}$, and Hg$^{2+}$; and 400 $\mu$g/mL for Cu$^{2+}$, when compared with the quality control strain *E. coli* K12 (Malik and Aleem 2011). Many isolates were also tolerant to Hg$^{2+}$ (49.3%), Zn$^{2+}$ (30.3%), and Pb$^{2+}$ (12.0%), and a few isolates resistant to Cd$^{2+}$ (4.8%), Cr$^{3+}$ (1.5%), and Ni$^{2+}$ (0.3%). In contrast, all the isolates were non-resistant to Cu$^{2+}$ and Mn$^{2+}$. The tolerance trend of the 400 *V. cholerae* isolates was Hg$^{2+}$ > Zn$^{2+}$ > Pb$^{2+}$ > Cd$^{2+}$ > Cr$^{3+}$ > Ni$^{2+}$ > Cu$^{2+}$ = Mn$^{2+}$.

As shown in Fig. 4, the *V. cholerae* isolates recovered from the four freshwater fish species had different heavy metal tolerance profiles. About half of the isolates from *C. auratus*

![Table 2](image)

| Heavy metal | Number of isolates with a maximum observed MIC ($\mu$g/mL) | Resistance |
|-------------|----------------------------------------------------------|------------|
|             | 3.125 | 6.25 | 12.5 | 25 | 50 | 100 | 200 | 400 | 800 | 1600 | 3200 | No. | (%) |
| Cd$^{2+}$   | 1     | 1    | 5    | 25  | 153 | 196 | 13  | 6   |     |      |      | 19  | 4.8 |
| Cr$^{3+}$   |       |      |      |     |     |     |     |     |     |      |      | 6   | 1.5 |
| Cu$^{2+}$   |       |      |      |     |     |     |     |     |     |      |      |     | 0.0 |
| Hg$^{2+}$   | 80    | 123  | 113  | 69  | 12  | 2   | 1   |     |     |      |      | 197 | 49.3|
| Mn$^{2+}$   |       |      |      |     |     |     |     |     |     | 107  | 251  | 16  | 22  | 4   | 0   | 0.0 |
| Ni$^{2+}$   | 1     | 1    | 15   | 320 | 60  | 2   | 1   | 1   | 1    |      |      | 1   | 0.3 |
| Pb$^{2+}$   |       | 1    | 1    | 350 | 47  | 1   |     |     |      |      |      | 48  | 12.0|
| Zn$^{2+}$   |       |      |      |     |     |     |     |     |     |     |      |      |     | 121 | 30.3|

*Minimal inhibition concentration of the standard quality control strain *E. coli* K12*
P. pekinensis (53.0%), and C. idellus (50.0%) showed resistance to Hg\textsuperscript{2+}, and a lower percentage from A. nobilis (30.0%). Approximately 47.0% and 35.0% of the isolates from P. pekinensis and C. idellus were tolerant to Zn\textsuperscript{2+}, respectively, while 20.0% and 19.0% from A. nobilis and C. auratus tolerant to this heavy metal, respectively. Moreover, the isolates from C. auratus showed the highest proportion of Pb\textsuperscript{2+-}resistance (23.0%), followed by 17.0% from P. pekinensis, 7.0% from A. nobilis, and 1.0% from C. idellus. The highest percentage of Cd\textsuperscript{2+-}resistant isolates (12.0%) was observed from P. pekinensis, followed by 5.0% from C. idellus and 2.0% from C. auratus, but all of the isolates from A. nobilis were susceptible to this heavy metal. It is noteworthy that all of the Cr\textsuperscript{3+-} and Ni\textsuperscript{2+-}resistant isolates (6.0% and 1.0%) were recovered from C. auratus and A. nobilis, respectively.

**Genetic diversity of the V. cholerae isolates**

The ERIC-PCR was used to investigate genetic diversity of the 400 V. cholerae isolates recovered from the four fish species. The obtained fingerprinting profiles comprised various numbers of DNA bands mainly ranging from 100 to 5000 bp, among which a 500-bp band was shared by all the isolates (Figure S1). Based on the fingerprinting profiles, all the isolates were classified into 328 different ERIC-genotypes, 86.9% (n = 285) of which were assigned as singletons. Approximately 27.0% (n = 77), 26.0% (n = 74), 24.6% (n = 70), and 22.4% (n = 64) of these singletons were derived from A. nobilis, C. auratus, P. pekinensis, and C. idellus, respectively. The UPGMA algorithm grouped all the 328 ERIC-genotypes into 12 distinct clusters at a 22.0% similarity cut-off level (Figure S1). About half (47.8%) of the 400 V. cholerae isolates were grouped into Cluster 5, while 20.5% (n = 82) were distributed into Cluster 6 (10.8%, n = 43) and Cluster 11 (9.8%, n = 39), and the remaining (31.8%, n = 127) were classified into Clusters 1 to 4, 7 to 10, and 12 with percentages in the range from 5.5 to 1.5% (Figure S1). Most isolates had the similarity coefficient of 30.0–90.0%, and the Simpson’s diversity index was 0.9987. These results demonstrated high genetic diversity of the 400 V. cholerae isolates recovered from the four fish species.

On the other hand, approximately 28.8% (n = 115) of the 400 V. cholerae isolates shared 43 ERIC-genotypes (Table S2), most of which were grouped into Cluster 5 (65.1%, n = 28). Among these 115 isolates, approximately 31.3% (n = 23) were recovered from P. pekinensis, followed by 26.1% (n = 30), 22.6% (n = 26), and 20.0% (n = 23) from P. pekinensis, C. auratus, and A. nobilis, respectively. For instance, the most predominant ERIC-genotype vc00124 was derived from C. idellus (5.2%, n = 6), suggesting likely near-present relatives or clonal relatedness. Likewise, the ERIC-genotypes vc00129 (3.5%, n = 4), vc00307 (3.5%, n = 4), and vc00148 (3.5%, n = 4) were derived from the V. cholerae isolates from C. idellus, C. idellus, and A. nobilis, respectively. For instance, the most predominant ERIC-genotype vc00124 was derived from C. idellus (5.2%, n = 6), suggesting likely near-present relatives or clonal relatedness. Likewise, the ERIC-genotypes vc00129 (3.5%, n = 4), vc00307 (3.5%, n = 4), and vc00148 (3.5%, n = 4) were derived from the V. cholerae isolates from C. idellus, C. idellus, and A. nobilis, respectively. Moreover, there were 15 genotypes (34.9%, n = 15) shared by the isolates derived from different fish species, suggesting possible interspecies transmission of V. cholerae. For instance, five isolates shared the identical genotype vc00067, three of which were recovered from C. auratus (C. auratus02-50, C. auratus 02-65, and C. auratus 02-22), and two from A. nobilis (A. nobilis10-63) and P. pekinensis (P. pekinensis 08-05), respectively.
Comparison of the MDR and heavy metal tolerance

The 122 V. cholerae isolates with MDR phenotypes were further analyzed, and the resulting data revealed the great genetic diversity with the Simpson’s diversity index of 0.9970 (Fig. 5). These MDR isolates belonging to 106 ERIC-genotypes were classified into four distinct clusters, designated as Cluster α, β, γ, and δ. The majority of the MDR isolates were grouped into Cluster β (68.0%, n = 83) with 70 ERIC-genotypes, about 43.4% (n = 36), 26.5% (n = 22), 15.6% (n = 13), and 14.5% (n = 12) of which were recovered from C. auratus, P. pekinensis, A. nobilis, and C. idellus, respectively. Cluster α was the second largest cluster (13.1%, 16/122) and consisted of 106 ERIC-genotypes, while the Cluster δ also contained 16 isolates and Cluster γ had only seven MDR isolates. Among the 122 MDR isolates, about half (51.6%, n = 63) were tolerant to one heavy metal, and 16.4% (n = 20) and 5.7% (n = 7) were tolerant to two and three heavy metals, respectively. Different resistance profiles were observed in different phylogenetic clusters. For instance, the Hg/AMP/RIF/STR resistance profile was the most predominant in Cluster α (25.0%, 4/16), followed by the Hg/AMP/STR/TM (18.8%, 3/16) and Hg/AMP/STR/SXT resistance profiles (12.5%, 2/16). In Cluster β, approximately 27.7% (23/83) of the isolates exhibited resistance to Hg/STR/SXT/TM, followed by Hg/AMP/RIF/STR (20.5%, 17/83) and Hg/AMP/STR/TM (18.1%, 15/83). The isolates in Cluster γ had the Hg/AMP/KAN/STR (28.6%, 2/7) and Hg/AMP/KAN/RIF resistance profiles (14.3%, 1/7), while two major resistance profiles Hg/AMP/RIF/STR (31.3%, 5/16) and Zn/AMP/SXT/TM (18.8%, 3/16) were observed in Cluster δ.

Additionally, 89.6% (n = 95) of the 106 ERIC-genotypes were assigned as singletons. Among these singletons, approximately 35.8% (n = 34), 28.4% (n = 27), 22.1% (n = 21), and 13.7% (n = 13) were derived from C. auratus, C. idellus, P. pekinensis, and A. nobilis, respectively. The MDR isolates with the identical ERIC-genotypes had the similar resistance profiles. For instance, two isolates (C. auratus02-25 and P. pekinensis09-76) recovered from C. auratus and P. pekinensis had the identical ERIC-genotype vc00036, and showed similar resistance to five antimicrobial agents (KAN/SPT/STR/SXT/TM) and one heavy metal (Hg) (Table S2).

Taken together, these data demonstrated the considerable genetic diversity of the 122 MDR V. cholerae isolates, as well as the close relatedness of resistance phenotypes between MDR and heavy metals.

Discussion

V. cholerae is a leading waterborne pathogen worldwide. Continuous monitoring of V. cholerae contamination in aquatic products and identification of risk factors (e.g., virulence and transmissibility of antimicrobial resistance and heavy metal tolerance) are crucial for assuring food safety. To date, a few studies have been conducted to characterize V. cholerae in fish (Lan and Love 2012; Runft et al. 2014; Senderovich et al. 2010; Traore et al. 2014; Zago et al. 2017). In this study, V. cholerae was isolated and characterized from the four commonly consumed freshwater fish (A. nobilis, C. auratus, C. idellus, and P. pekinensis). To our knowledge, V. cholerae has not been previously detected for A. nobilis and P. pekinensis.

Our data revealed none occurrence of epidemic V. cholerae (ctxAB+ tcpA+) in the fish samples evaluated in this study, consistent with some previous reports showing that neither ctxA nor tcpA is commonly expressed in environmental strains of V. cholerae (Traore et al. 2014; You et al. 2008). Virulence factors associated with the CTX element, such as zot and ace, were also rarely found in V. cholerae isolates of environmental water origin (Akochere and Mbuntcha 2014; Bakhshi et al. 2009). For example, Li et al. isolated 16 V. cholerae strains from water samples collected at aimes of a 2005 cholera outbreak occurred in the Nansha District of Guangzhou in China, and found that all the isolates were negative for either both ctxA and tcpA or all the four genes ctxA, tcpA, ace, and zot, except for one strain that was positive for all four genes (Li et al. 2015). Recently, Zago et al. also reported that none of 53 V. cholerae strains (non-O1/O139) isolated from ornamental fish species in Italy contained ctxA, zot, and ace. Most of the fish originated from South-East Asian countries between 2000 and 2015 (Zago et al. 2017). These reports were consistent with our findings regarding the ace and zot genes detected in the 400 V. cholerae isolates in this study. Previous studies have also shown that V. cholerae strains isolated from the environment have other virulence-associated genes such as rtxA and hlyA (Halder et al. 2017; Kumar et al. 2010). The RTX toxin gene cluster (rtxABCD) is essential for the cytotoxic activity of V. cholerae O1 El Tor strain upon Hep-2 cells in vitro test (Lin et al. 1999). The extracellular pore-forming toxin hemolysin (HlyA) can be produced by biotype El Tor of serogroup O1 and most of the non-O1/O139 strains, and has various biological activities (Benitez and Silva 2016; Gao et al. 2018). Recently, Zago et al. reported that 31.5% (n = 17) and 18.5% (n = 10) of V. cholerae strains isolated from ornamental fish species carried the rtxA and hlyA genes, respectively (Zago et al. 2017). In this study, our data revealed high percentages of the rtxA (83.0%), rtxB (97.0%), rtxC (95.8%), and rtxD (95.5%) genes, as well as the hlyA gene (87.8%) in the 400 V. cholerae isolates recovered from the four fish species. The nth gene-encoding protein has phospholipase and lecithinase activity (Fiore et al. 1997). In this study, the nth gene was detected positive in 76.0% of the 400 V. cholerae isolates. V. cholerae produces at least three morphologically distinct types of pili. Except for the TCP, the MSHA pili of V. cholerae is used to adhere to zooplankton exoskeletons as a survival strategy in the
Fig. 5  The ERIC-PCR fingerprinting profiles of the MDR V. cholerae isolates
aquatic environment (Moorthy and Watnick 2004; Chiavelli et al. 2001). The third type of pili is encoded by a 54-kb pil gene cluster that resembles the tap gene cluster in Aeromonas hydrophila and other type IV-A pilus assembly operons in bacteria (Fullner and Mekalanos 1999). In this study, the mshaA and pilA genes were present in 0.8% and 0.8% of the 400 V. cholerae isolates, respectively. Additionally, the hapA gene encodes a hemagglutinin protease, and plays an important role in V. cholerae interaction with aquatic hosts (Halpern et al. 2003). Previous research has indicated that 98.0% of V. cholerae strains harbored the hap gene irrespective of their source, i.e., clinical or environmental (Hasan et al. 2013). Recently, Jiang et al. reported that all three V. cholerae pathogenicity-related genes isolated from hepatitis B cirrhosis patients in China harbored a source, i.e., clinical or environmental (Hasan et al. 2013). Therefore, the high proportion of resistance of V. cholerae to STR could be facilitated by the selective pressure. Previous studies have also revealed high percentages of AMP-resistant V. cholerae. For example, Thapa Shrestha et al. reported that all the 24 V. cholerae isolates derived from the clinical and water samples were resistant to AMP (Thapa Shrestha et al. 2015). Recently, Ahmed et al. also reported that the AMP-resistant percentage of V. cholerae strains in clinical and aquatic Vibrio spp isolates was 100.0%, suggesting intrinsic resistance of Vibrio spp. to AMP (Ahmed et al. 2018). The AMP-resistant bacteria may be attributed to the abuse of drugs and the inappropriate release of industrial wastes into the environment (Taviani et al. 2008). In this study, the low occurrence of the resistance to CN (0.5%) and CHL (0.8%) was observed in the V. cholerae isolates. It has also been reported that none of the 42 V. cholerae isolates recovered from shrimp collected in 2013 and 2014 in Shanghai, China, were resistant to CHL (He et al. 2015). The low percentage of CHL-resistant isolates may be explained by the drug and its salts and esters (including chloramphenicol succinate) have been banned from the animal breeding industry in China (China Department of Agriculture, Bulletin No.193). TET, sulfonamides, and quinolones are widely used in aquaculture. In this study, the resistance to TET was detected positive in 8.3% of the 400 V. cholerae isolates. It has been reported that about 11% of 550 V. cholerae O1 El Tor strains isolated from the seventh cholera pandemic in China from 1961 to 2010 were resistant to TET (Wang et al. 2012). Recently, Ottaviani et al. also reported a lower incidence of the TET resistance in V. cholerae strains (3 of 42) isolated from the sea food, sea water, and fresh water in Italy (Ottaviani et al. 2018). Additionally, in this study, the high percentages of intermediate susceptibility to KAN (72.3%), RIF (39.5%), and TET (34.3%) may suggest a potential resistance trend for these drugs.

The rapid development of industrialization, urbanization, and agricultural modernization during recent decades has resulted in the increasing pollution, such as heavy metals, in both freshwater and marine environment (Devlin 2006; Sun et al. 2018). Heavy metals have been detected from sediments of the fish farming environment (He et al. 2012), which could finally be taken into humans through food chain (Ramirez-Perez et al. 2004; Sawasdee and Kohler 2010). High occurrence of resistance to heavy metals has been reported in Pseudomonas spp isolated from the environment (e.g., marine, river, and agricultural soil) (Malik and Aleem 2011). Nevertheless, very few studies have been conducted to address heavy metal tolerance of V. cholerae originated from the CNCO particularly from fish. In this study, for the first time, our data revealed that 49.3%, 30.3%, and 12.0% of the 400 V. cholerae isolates recovered from the four commonly consumed freshwater fish species were tolerant to Hg\(^{2+}\), Zn\(^{2+}\), and Pb\(^{2+}\), respectively. Xing et al. also reported that in the turbot gastrointestinal metagenome, cobalt-zinc-cadmium resistances were one of the
largest categories of resistance to the toxic compound subsys-


tem (Xing et al. 2013). In this study, about 24.0% of the 400 

V. cholerae isolates were resistant to two or more heavy metals, and one V. cholerae isolate derived from C. auratus was resis-
tant to the highest number of heavy metals (4 of 8). These data 
suggested heavy metal contamination in the aquacultural envi-

riment, which may originate from industrial pollutions, run-

offs from farmlands, or hospital waste that finally ends up in 

rivers and estuaries. Furthermore, the V. cholerae isolates from 

the four fish species had different heavy metal tolerance pat-
tterns, suggesting varying degrees of the exposure to the heavy 

metals evaluated.

The ERIC-PCR has widely been applied to analyze bacte-

rial genotyping in epidemiology (Ranjbar et al. 2010). It has 

also been used for the analysis of clonal diversity and geno-
typic variability of V. cholera strains from aquatic environ-

ment (Goel and Jiang 2011; Rivera et al. 1995). Previous 

studies reported that ERIC-PCR yielded one to eight DNA 

amplicons in the size ranging from 100 to 4000 bp (Rivera 

et al. 1995; Colombo et al. 1997). In this study, the established 

ERIC-PCR condition generated the amplicons mainly ranged 

from 100 to 5000 bp in size. Moreover, in this study, the 400 

V. cholerae isolates were differentiated into 328 ERIC-
genotypes (82.0%), which was more than 179 pulsotypes 

(71.6%) from 250 V. cholerae isolates, and 218 pulsotypes 

(38.4%) from 568 V. cholerae isolates based on NorI-PFGE 

(pulsed-field gel electrophoresis) genotyping technique (Gu 

et al. 2014; Lu et al. 2017). These data demonstrated the 

considerable genetic diversity of the 400 V. cholerae isolates re-

covered from the four freshwater fish species.

Previous research highlighted that novel selective pressure 

from the discharge of heavy metals could have significant 

impact on the environmental selection of antibiotic resistance 
genotypes (Alonso et al. 2001). In this study, comparison of the 

fingerprinting profiles derived from the MDR V. cholerae iso-

lates revealed a close relatedness between the MDR and heavy 

metal resistance phenotypes, suggesting that heavy metal poll-

utation most likely selects for antibiotic resistance and vice 

versa (Baker-Austin et al. 2006). The DNA transfer between 

antimicrobial-resistant bacteria from fish hatcheries and other 

pathogenic bacteria has been reported (Rhodes et al. 2000), 

which imposes potential threat upon human health. Thus, in 

the future studies, continuous monitoring of genetic evolution 

of V. cholerae in fish considered as natural reservoirs and 
vectors of resistance (Laviad-Shitrit et al. 2018) and identifi-
cation of risk factors are crucial for assuring food safety and 

human health.

Conclusions

In this study, for the first time, we isolated and characterized 

V. cholerae from A. nobilis and P. pekinensis, and determined 

heavy metal tolerance profiles of 400 V. cholerae isolates re-
covered from four commonly consumed freshwater fish (A. 
nobilis, C. auratus, C. idellus, and P. pekinensis) collected in 

July and August of 2017 in Shanghai, China. Also, our data 

revealed an extremely low occurrence of pathogenic V. cholerae 
carrying the major virulence genes ctxAB (0.0%), tcpA (0.0%), 

ace (0.0%), and zot (0.0%), as well as potential toxin genes 
mshA (0.8%) and pilA (0.8%). However, high incidence of virulence-associated genes was observed, including the 

RTX toxin gene cluster (rtxA-D) (83.0–97.0%), hlyA (87.8%), 

tlh (76.0%), and hapA (95.0%). Meanwhile, high percentages of resistance to antimicrobial agents STR (65.3%), AMP (44.5%), and RIF (24.0%) were also observed. Approximately 30.5% (122/400) of the isolates 
displayed MDR phenotypes with 42 different resistance pat-
tterns (MAR1 > 0.20), which were significantly different 
among the four fish species (MAR1, P = 0.001). Additionally, tolerance of isolates to heavy metals Hg^{2+} 

(49.3%), Zn^{2+} (30.3%), and Pb^{2+} (12.0%) was observed. 

Approximately 73.8% (90/122) of the MDR isolates were also 
tolerant to heavy metals evaluated. C. auratus was likely ex-
posed to antimicrobial drugs and heavy metals mostly when compared with the other fish species. The ERIC-PCR-based 
fingerprinting of the 400 V. cholerae isolates revealed 328 

ERIC-genotypes, and the MDR isolates were classified into 

106 ERIC-genotypes, which demonstrated a large degree of 

genomic variation among the isolates. Overall, the results in 

this study revealed divergent virulence, resistance phenotypes, 

and ERIC-genotypes of the V. cholerae isolates recovered from 

the four fish species. The increasing resistance of 

V. cholerae imposed potential threat upon human health. 

Therefore, governments particularly in developing nations, 

aquaculture industry, and food consumers should work togeth-
er toward eliminating and controlling the leading waterborne 

pathogen worldwide. In the future research, molecular mech-

anisms underlying the co-selection for antimicrobial and 

heavy metal resistant V. cholerae will be investigated.

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