

Isolation and antibiotic susceptibility profile of *Vibrio cholerae* isolated from catfish (*Pangasius hypophthalmus*)

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Abstract

To date, cholera has cycle the world seven times through the seven pandemic cycles that has affected tens of millions of people. The objective of this study was to determine the presence and density as well as the antibiotic resistance profile of *Vibrio cholerae* isolated from catfish (*Pangasius hypophthalmus*). From the combination of the Most Probable Number-Polymerase Chain Reaction-plating on TCBS agar methods, *V. cholerae* was detected in 32 samples and *V. cholerae* O139 was detected in 7 samples, with a density ranging between <3.0 to 75.0 MPN/g and <3.0 to 9.3 MPN/g respectively. The results obtained in this study indicate that *V. cholerae* will continue to be a major healthcare burden, as the pathogen can be transferred from the aquatic environment to the catfish and the consumption of catfish by humans will present a route of exposure to *V. cholerae*. Proliferation of antibiotic and multiantibiotic resistant bacteria is a public health threat worldwide. Results of antimicrobial susceptibility investigation of *V. cholerae* isolates collected from catfish reported in this study will establish an important baseline data. All the *V. cholerae* isolates were multiantibiotic resistant towards the ten antibiotics tested, including 6 isolates that were resistant to all ten antibiotics and 38 antibiotic resistance patterns. The MAR index values of 0.2 to 1.0 indicate that the isolates were exposed to high risk sources in the environment. Taken together, the information on the prevalence and antibiotic resistance of *V. cholerae* indicate that catfish consumption presents a potential risk to human health and highlight the need for ongoing epidemiological and antimicrobial resistance surveillance.

1. Introduction

Vibriosis is caused by bacteria belonging to the genus *Vibrio*, and it is one of the most prevalent infectious diseases in fish and shellfish species, cultivated in marine and estuarine environments (Woo and Bruno, 2011). Various studies have been carried out on seafood and findings concerning the distribution of pathogenic *Vibrio* in shellfish and fish are well documented (Gopal *et al.*, 2005; Roque *et al.*, 2009; Terzi *et al.*, 2009; Collin and Rehnstam-Holm, 2011; Yu *et al.*, 2013). In China, freshwater catfish, such as yellow catfish (*Pelteobagrus fulvidraco*); southern catfish (*Silurus soldatovi meridionalis*,

Chen); Zhengchuan catfish (*Silurus soldatovi meridionalis*, Chen ♂ × *Silurus asotus* ♀); brown bullhead catfish (*Ictalurus nebulosus*); and channel catfish (*Ictalurus punctatus*), are the most widely cultured catfish. However, in Malaysia the *Pangasius hypophthalmus* is more commonly sold in the market. As a tropical country, Malaysia is a favourable environment for the growth of *Vibrio* spp., but most people are unaware of the potential danger of *Vibrio* infection associated with consumption of catfish as well as the infection due to finning injury, typically during occupational or accidental exposure while handling the catfish. To underscore this hazard, our report here described the detection and quantification of *Vibrio cholerae* in catfish.

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Efforts to detect *Vibrio* species of public health concern in various types of food samples have met with various degrees of success due to the availability of various culture-based and culture-independent methods. *Vibrio* spp. are known worldwide as virulent pathogens that can quickly cause necrotising soft-tissue infection and life threatening bacteraemia in people with underlying chronic disease. Today, the society is put in danger similar to the pre-antibiotic era due to the worldwide incidences and increased of bacterial resistance to antimicrobial therapy over the past decade. The World Alliance against Antibiotic Resistance (WAAR) has painted a bleak scenario in which the antibiotics being used now may lose their effectiveness over the next few years due to a combination of both used in medication as well as in agricultural practices, leading to the emergence of resistant bacterial strains. Therefore, the need to monitor the multiple antibiotic resistance among various pathogenic bacteria becomes the utmost importance to protect and promote global public health. This study focused on the detection and the determination of antibiotic resistance among *Vibrio cholerae* isolated from catfish.

2. Materials and methods

2.1 Sampling

A total of 100 catfish samples were randomly purchased from local supermarkets in Serdang, Selangor, Malaysia. They were transported immediately to the laboratory and analysed upon arrival.

2.2 Most probable number (MPN) procedure

The MPN procedure described by Bacteriological Analytical Manual was employed with modifications (Kaysner and DePaola, 2004). Briefly, 25 g portion of the sample was weighed approximately into a sterile stomacher bag and plunged with 225 ml of Tryptic Soy Broth (TSB) (Bacto™, France) with 1% NaCl (Merck, Germany) and blended in a stomacher for one minute. The stomached mixture was diluted tenfold for three successive times, and pre-enriched at 37°C for 18 to 24 hours prior to MPN analysis. For MPN analysis, 1 ml of each dilution tube was transferred into three tubes set containing 9 ml of APW and further incubated at 37°C for 18 to 24 hours. After the incubation period, turbid MPN tubes were then streaked onto Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar (Merck, Germany). Presumptive flat yellow colonies on TCBS agar plates were picked and confirmed by Polymerase Chain Reaction (PCR).

2.3 DNA extraction and PCR assay

A well isolated colony was transferred to 2 ml of TSB with 1% sodium chloride (NaCl) and incubated overnight in an incubator shaker. A 1 ml portion of the cultures was centrifuged at 15,000 x g for 1 min. The pellet was re-suspended in 500 µL sterile distilled water and vortexed vigorously. The cell suspension was boiled for 10 min, immediately chilled on ice for 10 min and centrifuged again at 15,000 x g for 1 min. The supernatant containing crude DNA was transferred into a new 1.5 mL tube and 5 µL was used as DNA template in PCR, using the specific primer pairs and the PCR conditions as described by Hoshino *et al.* (1998). The PCR product was analysed by electrophoresis on 2% agarose gel and stained with 0.3 µg/mL ethidium bromide for visualization of the amplicons under UV light gel documentation system (Alpha Imager®, Alpha Innotech).

2.4 Antibiotic susceptibility

The susceptibilities of 65 *V. cholerae* isolates to antibiotics were determined via disk diffusion method (Bauer *et al.*, 1966) that is recommended by CLSI (2006). Briefly, a colony was directly suspended into 1 mL of normal saline inoculum of 0.85% NaCl, and adjusted to 0.5 McFarland standard. The inoculum was swabbed evenly on Mueller–Hinton (MH) agar plate (Merck, Germany) using a sterile cotton swab and left to dry for 3–5 min at room temperature. Antibiotic discs were then placed arranged accordingly onto the plate and subsequently incubated at 37°C overnight. The inhibition zone was measured, and the results were interpreted based on the CLSI recommendation (CLSI, 2010) M45-2A. Ten antibiotic discs (Oxoid, UK) were used: Amikacin (Ak 30), Bacitracin (B 10), Ceftazidime (Caz 30), Chloramphenicol (C 30), Erythromycin (E 15), Gentamicin (Cn 10), Kanamycin (K 30), Norfloxacin (Nor 10), Streptomycin (S 10) and Tetracycline (Te 30).

2.5 Multiple antibiotic resistance index

MAR index is a useful tool that provides an excellent estimation about the origin of contamination. MAR index is calculated as the ratio of some resistance antibiotics to the total number of antibiotics to which isolates are exposed to (Krumperman, 1983).

3. Results and discussion

In many developed and developing countries, incidence of human *Vibrio* illnesses due to seafood consumption continued to increase over the last decade (CDC, 2010; MMWR, 2014), due to three

most commonly reported species, *Vibrio vulnificus*, *Vibrio parahaemolyticus* and *Vibrio cholerae* (WHO, 2001; Baker-Austin et al., 2010). Among the 100 catfish samples randomly collected and analysed, 32 samples were positive for *V. cholerae* and 7 samples were positive for *V. cholerae* O139. The density of the *V. cholerae* and *V. cholerae* O139 in the positive samples ranged between <3.0 to 75.0 MPN/g and <3.0 to 9.3 MPN/g, respectively (data not shown). *V. cholerae* is of paramount health significance in human, particularly strains which produce the cholera toxin, belonging to the O1 serogroup, but non-O1/O139 serogroup strains may also produce toxins and disease. *V. cholerae* has been reported in water used to house or transport ornamental fishes, however, *V. cholerae* is rarely reported as a disease agent in fishes and its role as a fish-borne zoonotic is unclear (Reddacliff et al., 1993; Smith et al., 2012).

Despite the epidemiological relevance and the prevalence of *V. cholerae* in aquatic environment, the presence of this human pathogen is not routinely determined in food sources in many developing countries, despite the facts that the transmission pathway of this pathogen has been traced from fish and shellfish products in the developed countries (Ottaviani et al., 2009; Canigral et al., 2010; Scharer et al., 2011). The data obtained in this study indicated that potentially pathogenic *Vibrios* are present in catfish, a cheaper source of proteins for the lower income group of consumers, though to date, there is a dearth of data concerning the exposure to *V. cholerae* associated with the consumption of catfish in Malaysia. More attention should be given to fishes from aquaculture as the aquatic environment is more supportive to pathogenic bacteria independently of their host than the terrestrial environment, allowing the pathogen to reach high densities around the fishes, which then ingest them or contaminate the fishes during harvesting.

Sixty-five *V. cholerae* isolates were randomly picked from the 37 positive samples were tested for their susceptibility to 10 selected antibiotics. Despite the 7 positive samples detected using PCR assay of the turbid MPN tubes, we were unable to pick any *V. cholerae* O139 isolates on the TCBS agar. The 65 *V. cholerae* isolates were found to be resistant to two or more antibiotics, with 6 isolates from different samples showing resistance towards all 10 antibiotics tested and 38 antibiotic resistance patterns (Table 1). According to Krumperman (1983), a multiple antibiotic resistance (MAR) index values of less than or equal to 2.0 is considered to indicate that the bacterial isolates tested originated from animal in which antibiotics are seldom or never used, but

Table 1. Antibiotic susceptibility patterns and multiple antibiotic resistance index of *V. cholerae* isolated from catfish

Antibiotic resistant patterns	Multiple antibiotic resistance index (MAR)	No. Isolates
EB	0.2	1
KB	0.2	2
TeEB	0.3	5
AkEB	0.3	1
CazEB	0.3	1
TeCazB	0.3	2
NorCnEB	0.4	2
TeAkEB	0.4	6
TeCazEB	0.4	1
TeCEB	0.4	2
TeEKB	0.4	1
TeSEB	0.4	1
CSAkEB	0.5	1
NorCnSKB	0.5	2
NorTeSEB	0.5	2
SCazAkEB	0.5	2
TeAkEKB	0.5	1
TeCSEB	0.5	1
TeSAkEB	0.5	4
TeSCazEB	0.5	1
CnCSEKB	0.6	1
CnTeSAkEB	0.6	1
TeCCazAkEB	0.6	1
TeSAkEKB	0.6	2
NorTeCazAkEB	0.6	1
CnTeSAkEKB	0.7	1
NorCnSCazAkKB	0.7	2
NorTeSCazAkKB	0.7	1
NorTeSCazEKB	0.7	1
NorCnTeCCazAkB	0.7	1
TeCSAkEKB	0.7	1
TeCSCazAkEB	0.7	2
NorCnCSAkEKB	0.8	1
NorCnTeCSEKB	0.8	1
NorCnTeSAkEKB	0.8	1
TeSEBTeAkEB	0.8	1
NorCnTeSCazAkEKB	0.9	1
NorCnTeCSCazAkEKB	1.0	6

Ak: Amikacin (Ak 30); B: Bacitracin (B 10); Caz: Ceptazidine (Caz 30); C: Chloramphenicol (C 30); E: Erythromycin (E 15); Cn: Gentamicin (Cn 10); K: Kanamycin (K 30); Nor: Norfloxacin (Nor 10); S: Streptomycin (S 10); Te: Tetracycline (Te 30)

a MAR index greater than 2.0 indicates that the bacterial isolates originated from high risk sources where antibiotics are widely used. In 1974, a review of human pathogenic bacteria stated that cultured fish

have not been considered to be important vectors of disease to the human consumer but is considered as a minor public health threat (Stevenson and Biol, 1970; Ghittino, 1972; Reasoner, 1974). Today, the scenario has changed, as aquaculture, comprising all forms of culture of aquatic animals and plants in fresh, brackish and marine environments has rapidly grown to be the major food producing sector worldwide. The high MAR index values among the *V. cholerae* isolates in this study is of great concern as fish may serve as both passive and active carriers of this human pathogen, which may have been introduced into the aquatic environment through human sewage and other sources of water pollution. In North America, the consumption of raw molluscan shellfish harvested in waters contaminated with raw or poorly treated human sewage has been implicated in the high numbers of fish-associated human illnesses (Bostock et al., 2010).

Antibiotic resistance of *V. cholerae* was presumably due to the over usage of antibiotics and it is no exceptional in aquaculture for disease prevention. Phuong et al. (2005) reported on the bacterial resistance to antibiotics attributed to the usage of antibiotics in catfish farm and nurseries in the Mekong River Delta. Excessive usage of antibiotics exposes the bacteria to high antibiotic pressure which led to the development of antimicrobial resistance genes. Wastewater treatment plants were also highlighted as the most highly potential formation of antimicrobial resistance due to heavy usage of antibiotic that accumulates in the sewage. This is supported by Ben et al. (2017) who stated that municipal wastewater treatment plant (MWTP) effluents represent an important contamination source of antibiotic resistance which threatens the ecological safety of receiving environments. Antimicrobial resistance occurs mainly due to the development of resistance genes or mutation of existing genes being subjected to environmental stresses and antibiotic pressures. It can also be occurred through horizontal gene transfer, mostly involving plasmid transfer between organisms.

The use of antibiotics will continue to increase in large due to the Earth's population is growing with the increasing number of people needing healthcare. In addition, the uncontrollable usage of antibiotics in animal husbandry and aquaculture will potentially induce the growing numbers of antibiotic resistant pathogens and commensal microorganisms. What makes the situation more dramatic is the globalization of pathogens resistance to antibiotics, for example, the spread of KPC (*Klebsiella pneumoniae carbapenemase*) that synthesizes an enzyme

responsible for the degradation of carbapenem antibiotics (Nordmann et al., 2011). What is more alarming is when bacterial resistance is linked with bacterial virulence factors causing severe impact damage if an outbreak occurs.

Nowadays, we have come to accept the fact that our experience of resistance to antimicrobial agents is nearly as old as our experience of the agents themselves. Currently, measures to protect aquaculture animals from pathogenic bacteria without using antibiotics, using a holistic approach involving the pathogen, host and environment are being developed and tested. However, based on our knowledge on the bacterial resistance, a more realistic technique to control pathogenic bacteria would be the application of biocontrol together with a rational use of antibiotics, to make the industry more sustainable and preserve global public health.

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