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Insufficiency of the Kanagawa hemolytic test for detecting pathogenic *Vibrio parahaemolyticus* in Shanghai, China

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Abstract

We evaluated the Kanagawa hemolytic test and *tdh* gene test for accuracy in identifying pathogenic *Vibrio parahaemolyticus* isolates in Shanghai. One hundred and seventy-two *V. parahaemolyticus* isolates were collected from diarrhea patients, freshly harvested sea fish, or fresh water samples. Statistical data for the Kanagawa hemolytic test and *tdh* gene test were compared. There were 83.51% isolates (81/97) from patients and 22.22% isolates (10/45) from sea-fish positive for the *tdh* gene. However, none of 30 isolates from fresh water samples were *tdh*-positive. Positive Kanagawa hemolytic tests were obtained in 88.66%, 46.67%, and 76.67% of isolates, which were from patients, sea fish, and fresh water samples, respectively. Positive rates of the Kanagawa hemolytic tests and the *tdh* gene tests were significantly different in isolates from those 3 sources ($P < 0.001$). The *tdh* gene test showed higher specificity than the Kanagawa hemolytic test on identifying pathogenic *V. parahaemolyticus* isolates in Shanghai, China.

Keywords: *Vibrio parahaemolyticus*; Sea fish; Fresh water; Pathogenicity; Kanagawa hemolytic test; *tdh* gene

1. Introduction

Vibrio parahaemolyticus is a Gram-negative, halophilic, facultative anaerobic bacterium. It is distributed widely in marine and estuarine environments. It can be carried by various sea creatures, such as sardines, mackerel, and scallops (Su and Liu, 2007). It also can be isolated from fresh water. About 5% of natural *V. parahaemolyticus* isolates are pathogenic to humans. Only pathogenic *V. parahaemolyticus* can induce diarrhea symptoms in persons who have ingested it orally with food. With widespread consumption of seafood, this bacterium has accounted for most cases of food poisoning in Japan, North America, and Southeast Asia (Daniels et al., 2000).

Most of clinical isolated *V. parahaemolyticus* can produce a well-defined, β-type hemolytic zone on a Wagatsuma blood agar plate, which is known as the Kanagawa phenomena (KP) (Liu, 2003; Miyamoto et al., 1969; Sakazaki et al., 1968). However, only 0.3–3% of nonpathogenic environmental isolates are KP positive (Miyamoto et al., 1969; Nordstrom et al., 2007; Sakazaki et al., 1968). Although *V. parahaemolyticus* can be isolated from many sea-fish products (30–60%), it is unnecessary to strictly prohibit any isolation of *V. parahaemolyticus* from seafood supplies to guarantee the food safety for sea-fish consumption. Otherwise, we should take a strict control on the isolation of pathogenic *V. parahaemolyticus*. Thus, in some countries, for example, China, the KP test has been widely used in food-quarantine practice for screening those pathogenic *V. parahaemolyticus* strains on a routine basis.

Thermostable direct hemolysin (TDH) is an identified mammalian enterotoxin, regarded as a major virulence factor for *V. parahaemolyticus* (Chun et al., 1975; Sakurai et al., 1973). TDH related-hemolysin, thermolabile hemolysin, and urease have also been defined as enterovirulence factors for this bacterium (Honda et al., 1988; Hondo et al., 1987). However, *trh* gene (12.0–40.74%) (Bej et al., 1999; Vongxay et al., 2008b) is markedly less prevalent than the

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tdh gene (63.6–92.2%) in *V. parahaemolyticus* populations (Iida et al., 1998; Shirai et al., 1990). Thus, the presence of the *tdh* gene could be a better marker than *trh* gene for predicting pathogenic *V. parahaemolyticus* (Honda et al., 1988; Hondo et al., 1987; Kaper et al., 1984).

Considering the good repeatability of gene test, KP test is relatively difficult to ascertain. Repeatability of KP test is affected by pH variability, concentration of NaCl, and types of erythrocyte used in culture medium (Chun et al., 1975; Nishibuchi et al., 1985). Previous studies have reported the inaccuracy of the KP test for identifying pathogenic *V. parahaemolyticus* isolates in certain circumstances (Taniguchi et al., 1985). Also, the constitution rate of pathogenic *V. parahaemolyticus* in populations distributed in different continental locations can affect the positive rate of KP test. For example, the difference of serotype distribution of *V. parahaemolyticus* in Shanghai, Japan, and United States (Nair et al., 2007; Shi et al., 2007; Vongxay et al., 2008a) can hint the different constitution of pathogenic *V. parahaemolyticus* in those bacteria populations.

KP test does not require a lot of facilities to perform. Therefore, it is currently applied widely in many first-line quarantine laboratories. However, only TDH can reflect the real pathogenicity of *V. parahaemolyticus*. Thus, the accuracy of the KP test to identify pathogenic bacterium should be compared with that of the *tdh* gene test. This comparison has not ever been carried out in United States or in Japan on their domestic *V. parahaemolyticus* populations, nor in China.

Shanghai is located on the east coast of China, and >151 800 tons of seafood are consumed annually (Shanghai statistic bureau, 2008). Pathogenic *V. parahaemolyticus* accounted for 63.7% of cases of food poisoning in Shanghai each year (Liu, 2003). Thus, we compared the Kanagawa hemolytic test with the *tdh* gene test to assess its applicability in Shanghai.

2. Materials and methods

2.1. V. parahaemolyticus isolates

A total of 172 sporadic isolates were collected from November 2006 to September 2007 without direct epidemiologic linkage between each other in Shanghai. Ninety-seven of these isolates were from diarrhea patients and were obtained from the Pudong Center for Disease Control and Prevention, Shanghai (clinical isolates). Forty-five isolates were from freshly harvested sea fish, which were purchased from an offshore fishery market in Zhoushan Fishing Ground. Zhoushan Fishing Ground is located in the East Ocean adjacent to the east of Shanghai. Thirty isolates were isolated from fresh water samples from the Jinshan Center for Disease Control and Prevention, Shanghai. All isolates were cultured on thiosulfate-citrate-bile-sucrose agar (TCBS) plates at 37 °C for 18–24 h and then used for testing within 24 h.

V. parahaemolyticus was first identified by its ability of successful growth on TCBS plates containing 3% or 7% NaCl, but no growth on plates with 0% NaCl or with >10% NaCl. Biochemical characteristics of isolates were analyzed by using API 20E test (Biomérieux, Craponne, France). Those isolates were finally identified as *V. parahaemolyticus* with characteristics of ADH−, GEL+, GLU+, LDC+, MAN+, MOB+, NO3+, ODC+, OF-F, ONPG−, OX+, SAC−, and VP−.

2.2. Kanagawa hemolytic test

Colonies ready from TCBS plates were used for KP test. They were streaked on Wagatsuma blood agar plates that contained 7% NaCl, 3 g yeast extract, 10 g peptone, 15 g agar (Oxoid, Cambridge, UK), 5 g K_2HPO_4, 10 g mannitol, 0.001 g crystal violet, 1 L distilled water, and 20% rabbit defibrinated blood. Ubiquitous qualities of these plates were guaranteed by the Shanghai Center for Disease Control and Prevention, P.R. of China. Plates were then incubated for 18–24 h at 35 °C. *V. parahaemolyticus* strain ATCC17802 (American Type Culture Collection, Manassas, VA) was taken as a negative KP control and an early isolate VPJ33 (Shanghai Ocean University, Shanghai, China) was taken as a positive control for this KP test. A well defined, hemolysis zone around and under a single colony was recorded as KP positive (Miyamoto et al., 1969).

2.3. tdh gene test

Genomic DNA was extracted from individual bacterial colonies by using UNIQ-10™ bacterial genome purification kit (SK1202; Sangon Biological Engineering Technology and Service, Shanghai, China), following the manufacturer's instructions. Extracted DNA was then dissolved in ddH_2O at an estimated concentration of 0.02–0.05 μg/μL.

Primers were designed following the reference sequence retrieved from GenBank (accession no. M10069.1). The forward primer was 5′-CCATC TGTCC CTTTT CCTGC C-3′ (nt 330–350). The reverse primer was 5′-CCACT ACCAC TCTCA TATGC-3′(nt 754–735) (Lynch et al., 2005). The expected product was 425 bp in length.

Amplification was performed in a 50-μL volume. Each reaction mixture contained 4 μL DNA template, 0.4 μmol/L each forward and reverse primer, 200 μmol/L dNTPs, 5 μL 10× PCR buffer (100 mmol/L Tris–HCl, pH 8.0, 500 mmol/L KCl, 15 mmol/L $MgCl_2$), 0.025 U rTaq polymerase (TAKARA Taq™; TAKARA Biotechnology, Dalian, China); other volume was fulfilled by ddH_2O. Cycle parameters were set up to an initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min.

Positive and negative controls were included in all reactions. Purified *tdh* PCR products were taken as *tdh*-positive control while DNA-free ddH_2O was taken as *tdh*-negative control.