Pulsed-field Gel Electrophoresis Typing of Multidrug-resistant *Vibrio parahaemolyticus* Isolated from Various Sources of Seafood

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***Vibrio parahaemolyticus* (*V. parahaemolyticus*), one of the most important foodborne pathogens in many maritime Asian countries, is frequently associated with the consumption of seafood. Thirty eight strains of *V. parahaemolyticus* were isolated from seafood in Hebei province of China. Resistance to 13 antibiotics was determined using broth microdilution methods. These strains were typed by pulsed-field gel electrophoresis (PFGE) technique with short pre-processing following *SfiI* digestion and a typing scheme was generated. The 38 strains were grouped into 5 types with 71% pattern similarity. All the type E were isolated from Shijiazhuang, Baoding and Langfang and simultaneously resistant to ampicillin, sulfisoxazole, streptonigrin and vancomycin, suggesting a clonal relationship between these strains. The data of antimicrobial susceptibility test and PFGE profiles in this study showed a good correlation among antimicrobial susceptibility test, PFGE profiles and geographic distribution.**

Key words—— *Vibrio parahaemolyticus*, pulsed field gel electrophoresis, antimicrobial resistance

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INTRODUCTION

Vibrio parahaemolyticus (*V. parahaemlyticus*) is a halophilic Gram-negative bacterium that inhabits warm estuarine waters worldwide.[1,2] It is one of the most common cause of seafood-associated bacterial gastroenteritis in maritime Asian countries which is often associated with eating raw or inadequately cooked or any food contaminated by handling raw seafood or contaminated water.[3] The storage and consumption of seafood may determine the horizontal transmission and enriching of *V. parahaemolyticus* strains, which is a major public-health problem. As a huge amount of antibiotics will be invested in the process of aquaculture seafood, this may cause *V. parahaemolyticus* of seafood has a certain antibiotic resistance. This gives great difficulties to the treatment of diseases caused by *V. parahaemolyticus.* Therefore, the study of relationship between antimicrobial resistance and rapid identification of *V. parahaemolyticus* especially with molecular techniques will contribute to food-borne disease control, as well as to epidemiological analysis.

Several molecular typing methods were developed for *V. parahaemolyticus*, for example, polymerase chain reaction (PCR),[4] random amplified polymorphic DNA,[5] restriction fragment length polymorphism (RFLP),[6] ribotyping[7] and pulsed-field gel electrophoresis (PFGE).[8] The PFGE is a technique to understand the relatedness of different clones of pathogenic bacteria. PFGE separates large DNA fragments created by digestion of total genomic DNA with restriction endonucleases that cut DNA infrequently. PFGE has higher discriminating ability and reproducibility than PCR, RFLP and ribotyping, so it has been applied in characterizing clinical[4] and foodborne outbreaks of *V. parahaemolyticus*.[9] Usually, PFGE is used to characterize foodborne bacteria, along with antimicrobial susceptibility testing.[10] In this study, the technique of PFGE was used to investigate the possibility of developing a rapid, molecular identification and subtyping scheme for characterization of antimicrobial resistant *V. parahaemolyticus* isolates, which can yield useful to trace possible seafood borne outbreaks of *V. parahaemolyticus* and to assess epidemic spreads of infections diseases. The objectives of the study were to investigate the relationship of *V. parahaemolyticus* strains isolated from various sources using short pre-procession PFGE technique.

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MATERIALS AND METHODS

Bacterial Strains —— A total of 38 strains of *V. parahaemolyticus* were isolated from various seafood sources in Hebei province of China in 2006 (Table 1). The strains were inoculated onto thiosulfate–citrate–bile salt–sucrose agar with or without prior enrichment culture in alkaline peptone water, and were incubated at 37°C for 18 to 24 hr. The strains were identified as *V. parahaemolyticus* by standard biochemical tests.[11] The cultures were stored at −80°C in Luria-Bertani broth (Becton Dickinson Diagnostic, Franklin Lakes, New Jersey, U.S.A.) −3% NaCl containing 20% glycerol.

Antimicrobial Susceptibility Testing —— The antimicrobial minimum inhibitory concentrations (MICs) for the *V. parahaemolyticus* isolates tested were determined with the Sensititre automated an-

Table 1. Sources, Areas and Characteristics of the *V. parahaemolyticus* Strains

Strain no.	class	Source	Date	Area	Resistance pattern												
					AMP	SUL	SXT	CHL	GEN	TOB	CIP	OFL	NOR	CFP	CRO	CEP	FT
BD074	F	fish	2006.07.10	Baoding	R	R	S	S	S	S	S	S	S	S	S	S	S
BD076	F	mussel	2006.07.10	Baoding	R	R	R	S	S	S	S	S	S	S	S	S	I
BD078	F	mussel	2006.07.10	Baoding	R	R	R	S	S	R	I	S	S	S	S	S	S
BD080	F	fish	2006.07.10	Baoding	R	R	R	S	S	S	S	S	S	S	S	S	I
BD077	F	fish	2006.07.10	Baoding	R	R	R	S	S	I	S	S	S	I	I	I	I
BD079	F	fish	2006.07.10	Baoding	R	R	R	S	S	S	I	S	S	S	S	S	I
BD081	F	shrimp	2006.07.10	Baoding	R	R	S	S	S	I	S	S	S	S	S	S	I
BD091	O	shrimp	2006.07.10	Baoding	R	R	R	S	S	S	S	S	S	I	I	S	R
SJZ082	F	fish	2006.07.11	Shijiazhuang	R	R	R	S	S	I	I	S	S	I	R	S	I
SJZ091	O	mussel	2006.07.11	Shijiazhuang	R	R	R	S	S	S	S	S	S	I	S	I	R
QH056	F	oyster	2006.07.16	Qinhuangdao	R	S	S	S	S	I	I	S	I	I	S	I	R
QH059	F	fish	2006.07.16	Qinhuangdao	R	S	S	S	I	S	S	S	S	S	S	S	I
QH061	O	shrimp	2006.07.16	Qinhuangdao	R	S	S	S	S	R	I	S	S	I	S	S	I
QH064	O	oyster	2006.07.16	Qinhuangdao	R	S	S	S	S	S	S	S	S	S	S	S	R
QH065	O	Sea urchin	2006.07.16	Qinhuangdao	R	S	S	S	S	S	S	S	S	S	S	S	I
QH068	O	fish	2006.07.16	Qinhuangdao	R	S	S	S	R	R	S	S	S	I	I	I	R
QH069	O	Sea-lrchin	2006.07.16	Qinhuangdao	R	S	S	S	S	S	S	I	S	S	S	S	I
QH070	O	fish	2006.07.16	Qinhuangdao	R	S	S	S	I	I	S	I	S	R	S	I	R
CD055	F	mussel	2006.07.10	Chengde	R	S	R	S	I	I	I	S	S	S	S	S	S
CD061	F	crab	2006.07.10	Chengde	R	S	S	S	I	I	S	S	S	I	S	S	R
CD063	F	fish	2006.07.10	Chengde	R	S	S	S	S	I	I	S	S	S	I	S	I
CD068	F	scallop	2006.07.10	Chengde	R	R	S	S	S	I	S	S	S	I	S	S	S
CD069	F	mussel	2006.07.10	Chengde	R	R	S	S	S	I	S	S	S	I	S	S	S
CD070	F	mussel	2006.07.10	Chengde	R	R	S	S	S	S	I	S	S	S	S	S	S
LF073	F	Sea-lrchin	2006.07.23	Langfang	R	R	S	S	I	R	I	S	S	I	R	R	R
LF075	F	clam	2006.07.23	Langfang	R	R	S	S	I	R	S	S	S	S	S	I	R
LF077	F	fish	2006.07.23	Langfang	R	R	I	S	S	I	S	S	S	S	S	S	S
LF079	F	clam	2006.07.23	Langfang	R	R	R	S	S	R	S	S	S	I	I	I	R
LF080	F	mussel	2006.07.23	Langfang	R	R	I	S	I	I	I	S	S	I	S	S	R
HD072	F	fish	2006.07.20	Handan	R	S	S	S	I	I	S	S	S	S	S	S	S
HD073	F	fish	2006.07.20	Handan	R	S	S	S	I	R	S	S	S	S	S	S	I
HD075	F	fish	2006.07.20	Handan	R	S	S	S	S	S	S	S	S	S	I	I	R
HD077	F	crab	2006.07.20	Handan	R	S	S	S	S	R	S	S	S	S	S	S	S
HD082	F	fish	2006.07.20	Handan	R	S	S	S	I	R	S	S	S	S	S	I	I
HD079	F	mussel	2006.07.20	Handan	R	S	S	S	I	I	S	S	S	I	S	S	I
HD080	F	fish	2006.07.20	Handan	R	S	S	S	S	R	S	S	S	S	S	S	R
HD078	F	mussel	2006.07.20	Handan	R	S	S	S	I	R	S	S	S	I	S	S	I
HD081	F	fish	2006.07.20	Handan	R	S	S	S	S	R	S	S	S	S	S	I	I

Abbreviation: F, Frozen fresh aquatic products; O, Omophagia aquatic products; AMP, ampicillin; SUL, sulfisoxazole; SXT, sulfamethoxazole-trimethoprim; CHL, chloramphenicol; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; OFL, ofloxacin; NOR, norfloxacin; CFP, cefoperazone; CRO, ceftriaxone; CEP, cephalothin; FT, nitrofurantoin; S, Susceptive; I, Intermediate; R, Resistance.

timicrobial susceptibility system (Trek Diagnostic Systems, Westlake, OH, U.S.A.) according to the manufacturer's instructions, and the results were interpreted according to National Committee for Clinical Laboratory Standards guidelines for broth microdilution methods.[12] *V. parahaemolyticus* isolates were screened for susceptibility to the following 13 antimicrobials: ampicillin, sulfisoxazole, sulfamethoxazole-trimethoprim, chloramphenicol, gentamicin, tobramycin, ciprofloxacin, ofloxacin, norfloxacin, cefoperazone, ceftriaxone, cephalothin, nitrofurantoin. All susceptibility testing was performed in duplicate on separate days.

PFGE—— The strains were cultured in 3 ml of LB-3% NaCl at 37°C with shaking at 160 rpm for 6 to 7 hr. Bacterial cells were harvested from 2 ml of the culture by centrifugation at 12000 × g for 2 min. and were resuspended in 2 ml of cell suspension buffer [10 mM Tris, 50 mM Ethylene Diamine Tetraacetic Acid (EDTA) (pH 8.0), 20 mM NaCl]. The concentration of each cell suspension was adjusted to an optical density of 1.5 to 1.6 at 600 nm with a spectrophotometer (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Agarose plugs were prepared by mixing equal volumes of bacterial suspension with 2% low-melting agarose. The bacterial cells in the agarose plugs were lysed by treatment with lysis solution [1 mg of lysozyme per ml, 10 mM Tris-HCl (pH 8.0)] at 37°C for 4 hr. The plugs were washed two times with 2 ml of sterile water (pre-heated to 37°C) in a 37°C water bath or shaker incubator for 10–15 min with constant agitation and then treated with cell lysis buffer [50 mM Tris-HCl, 50 mM EDTA (pH 8.0), 1% Dodecyl Sulfate Sodium Salt (SDS), 0.1 mg/ml proteinase K] at 54°C for 4 to 5 hr. The plugs were then washed two times with 2 ml of sterile water (pre-heated to 54°C) in a 54°C water bath or shaker incubator for 10–15 min with constant agitation. This was followed by four washes with 2 ml of 10 mM Tris-HCl (pH 8.0) buffer containing 1 mM ethylene diaminetetraacetic acid disodium salt (EDTA) pre-heated to 54°C as described above. After the last wash, 2 ml of TE buffer (room temperature) were added to each tube to serve as storage media for the plugs. The plugs were digested with restriction enzymes immediately or stored in TE buffer at 4°C until needed. Agarose plugs were equilibrated in 200 ul mol/l buffer (restriction enzyme buffer) for 5 min at room temperature and were cleaved in 300 ul M buffer containing 40U *SfiI* enzyme at 37°C for 4 hr. High-molecular-weight restriction fragments were resolved in a 1% agarose gel in a 0.5% Tris–borate–EDTA buffer by using a Clamped homogeneous electric field (CHEF) apparatus (CHEF-DR III; Bio-Rad Laboratories). The running conditions were 6 V for 22.4 hr at 14°C, with 3 to 80 s pulse times.[13] The gels were stained in ethidium bromide (Sigma, St. Louis, MO, U.S.A.), destained in distilled water, and photographed with the Gel Doc system (Bio-Rad Laboratories) after electrophoresis.

Data Analysis—— During PFGE analysis, the *V. parahaemolyticus* isolates were separated according to their PFGE patterns, based on two band differences.[14] The PFGE patterns were compared by means of the Dice coefficient with Fingerprinting DST Molecular Analyst software (Bio-Rad Laboratories), and clustering of strains was based on the unweighted pair group method with averages (a tolerance of 4% in the band position was applied). The computer-assisted analysis was performed according to the instructions of the manufacturer.

RESULTS

Antimicrobial Susceptibility of *V. parahaemolyticus*

The results of *V. parahaemolyticus'* antimicrobial susceptibility are shown in Table 1. As a result, all *V. parahaemolyticus* strains were resistant to ampicillin (Minimal inhibition concentration (MIC) ≥ 32 μg/ml). Nonsusceptibility (include resistant and intermediate resistant) was 44.74% to sulfisoxazole, 31.58% to sulfamethoxazole-trimethoprim, 34.21% to gentamicin, 68.42% to tobramycin, 21.05% to ceftriaxone, 28.95% to cephalothin, 73.68% to nitrofurantoin, 39.47% to cefoperazone and 26.32% to ciprofloxacin. Susceptibility was 100% to chloramphenicol (MIC ≤ 8 μg/ml). Two strains (QH069, QH070) were intermediate resistance to the ofloxacin (MIC = 4 μg/ml), the remainders were all susceptible. Only one isolate collected from Qinhuangdao showed intermediate resistance to the norfloxacin (MIC = 8 μg/ml), the remainders (n = 37, 97.37%) were all susceptible. Strains isolated from the same city had higher similarity than strains collected from different city. For example, strains isolated from Baoding were simultaneously resistant to ampicillin, sulfisoxazole and susceptible to chloramphenicol, gentamicin, ofloxacin, norfloxacin. Strains isolated from Qinhuangdao

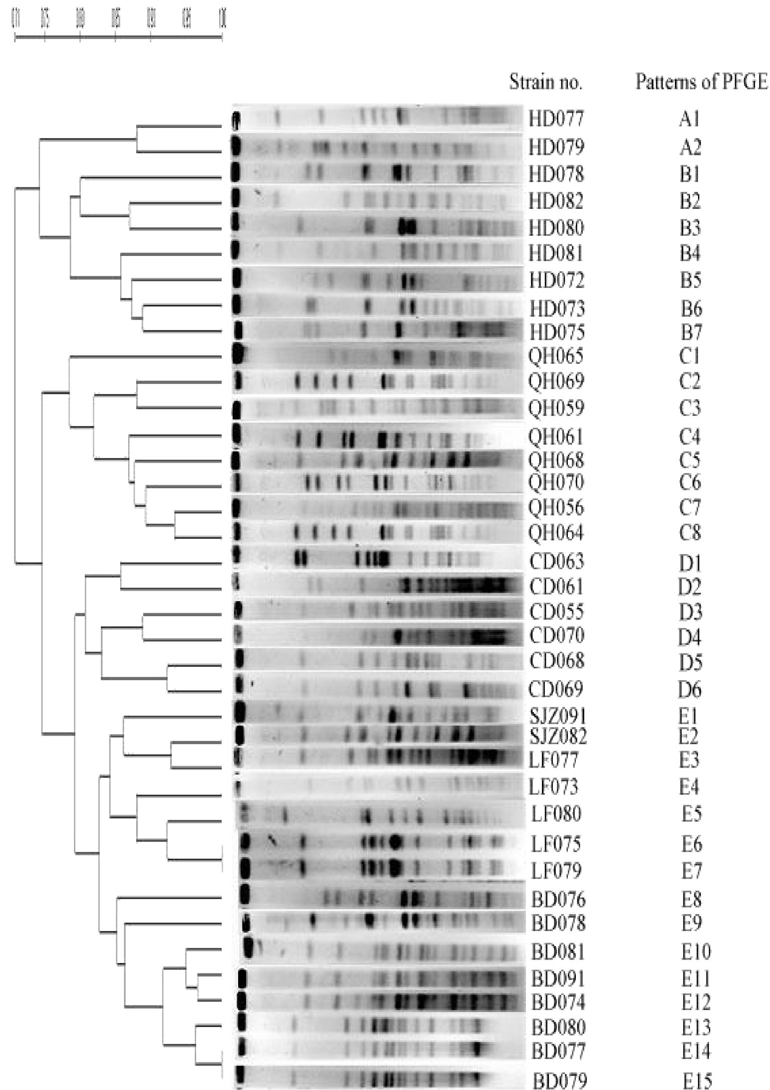

Fig. 1. Dendrogram of Pulsed Field Gel Electrophoresis Patterns of *V. parahaemolyticus* Isolates

The dendrogram was produced according to the unweighted pair-group mean arithmetic method (UPGMA). The serovars of each strain are indicated at the right of each strain designation.

were resistant to ampicillin and susceptible to sulfisoxazole, sulfamethoxazole-trimethoprim, chloramphenicol.

The dendrogram constructed on the basis of *SfiI* PFGE patterns (Fig. 1) were used to compare the banding profiles of the *V. parahaemolyticus* isolates. The PFGE patterns revealed that the 38 strains were grouped into 5 types (A to E) with 71% pattern similarity. The largest PFGE cluster with 80% similarity was E, which included 15 strains from mussel, shrimp, clam and Sea-Irchin samples, from Shijiazhuang, Langfang and Baoding areas. Strains isolated from Handan with 74% similarity contained types A and B. We found that the *V. parahaemolyticus* strains isolated from Handan city were more scattered on the dendrogram than the other strains. For example, 9 isolates collected from Handan city had 74% pattern similarity. 8 isolates originated from Baoding city formed relatively tighter cluster which had 85% pattern similarity. Two groups of isolates (formed by two isolates) showed 100% similarity. One of this group contained strains BD077 and BD079 which were isolated from fish in Handan city. The other group was formed by strains LF075 and LF079 which were isolated from clam in Langfang city. The PFGE pattern also revealed that isolates collected from the same city had higher pattern similarity than strains isolated from different cities.

DISCUSSION

V. parahaemolyticus is a halophilic gram-negative bacterium which naturally inhabits warm seawater and marine animals and frequently causes gastroenteritis, wound infections and septicemia.[15)] *V. parahaemolyticus* was first identified as a cause of foodborne illness in Japan in 1950.[16)] Since then, *V. parahaemolyticus* has been found in many maritime countries from seafood and recognized as an important causative agent of food-poisoning. Especially food-poisoning events caused by *V. parahaemolyticus* have been increasing in recent years, which had raised food safety concerns as a major public-health problem. The best approach to minimize the risk to public-health should classify and comparative analysis of clonal relationships between *V. parahaemolyticus* strains.

PFGE has high levels of sensitivity, specificity and reproducibility for subtyping foodborne bacterial pathogens which is important to monitor bacterial infectious diseases,[17, 18)] trace infection sources, investigate and identify route of transmission.[19, 20)] In this study, we used PFGE to subtype 38 *V. parahaemolyticus* strains isolated from Hebei province of China and to gain further insight into the geographic source of contamination. According to the PFGE profiles, we demonstrated that the subtyping of *V. parahaemolyticus* strains were associated with geographic areas. For example, Baoding city is further away from Qinhuangdao than Shijiazhuang and Langfang city in the geographic location (Fig. 2). Strains collected from Baoding, Shijiazhuang and Langfang city had higher similarity than strains isolated from Qinhuangdao city. Eight isolates originated from Baoding city formed relatively tighter cluster which had 85% pattern similarity, indicating there may existed certain contamination in Baoding city. Strains with 100% similarity (BD077 and BD079, LF075 and LF 079) in the PFGE profiles were possibility contaminated by the same *V. parahaemolyticus* strains. Strains isolated the same classes of seafood or source, for example, frozen fresh aquatic products, had low similarity. Generally, a good congruence was found between groupings obtained from geographic location and typing obtained by PFGE. As we know that this thesis firstly reported homological analysis of *V. parahaemolyticus* strains isolated from Hebei province of China using PFGE technology. Although *V. parahaemolyticus* separated from seafood in Hebei Province did not caused food-poisoning, it evoked people's attention. In this study, PFGE technology and antimicrobial susceptibility testing were used to analyse *V. parahaemolyticus*. It improved the reliability of pathogen identification and trace infection source, also had great importance to control food-poisoning.

Fig. 2. Map of Hebei Province of China

The data of antimicrobial susceptibility test showed that all strains were simultaneously sensitive to chloramphenicol and resistant to ampicillin. Strains isolated from seafood were resistant at least two antimicrobials and at most seven antimicrobials. A lot of antibiotics have been used by aquaculture industry when feeding seafood in order to avoid the economic losses caused by illness *V. parahaemolyticus* of seafood. This may be the main reason that caused multidrug-resistant *V. parahaemolyticus* in seafood.[21)] Strains isolated from the same seafood samples, for examples fish, were not simultaneously resistant to the same antibiotics except ampicillin. The results of antimicrobial susceptibility test result demonstrated that there was a good relationship between geographic location and resistance. The drug resistance *V. parahaemolyticus* was different with the difference of Chinese geographical positions. For example, Nonsusceptibilities of *V. parahaemolyticus* in Anhui Province of China were 61.00% to ampicillin, 16.30% to ciprofloxacin, 6.30% to sulfamethoxazole-trimethoprim,[22)] *V. parahaemolyticus* in Zhejiang Province of China were 100.00% to ampicillin,

46.00% to ciprofloxacin, 2.00% to gentamycin, 4.00% to tobramycin,[23] *V. parahaemolyticus* in Hebei Province was 100% to ampicillin, 26.32% to ciprofloxacin, 26.32% to sulfamethoxazole-trimethoprim, 34.21% to gentamycin, 68.42% to tobramycin. Meanwhile there were similaritis among different provinces of China, for example, almost all of them were susceptibility to norfloxacin, indicating that norfloxacin may represent a future therapeutic option for caused by *V. parahaemolyticus*. The nonsusceptibilities were high to ampicillin which would cause certain difficulties in treating the diseases.

Combining the data of PFGE and antimicrobial susceptibility test, we noticed that PFGE profiles, resistance, the source of seafood and sampling time had no relationship. However, there was an interesting correlation among the results of PFGE profiles, resistance and sampling places, which indicated that there was a popular trend among seafood in Hebei province. The strains isolated from Baoding, Shijiazhuang and Langfang city were simultaneously resistant to ampicillin, sulfisoxazole, streptomycin which were belonged to type E with 80% pattern similarity. The high similarity showed that the same source of infection or purchase channels might exist among Baoding, Shijiazhuang and Langfang city.

In this study we used PFGE and antimicrobial resistance to trace the possible seafood contamination. To our knowledge, this is the first report of subtyping *V. parahaemolyticus* strains by PFGE with short pre-processing. We developed a rapid (2-day) PFGE protocol for subtyping *V. parahaemolyticus* strains. The quality of the PFGE patterns produced and the discriminatory power obtained with the 2-day standardized PFGE protocol was the same or higher than those obtained with the 3–4-day standardized protocol.[7, 13, 24] The rapid and standardized PFGE protocol described in this study can be useful to detect clusters of food-borne outbreaks earlier than ever before. The PFGE with short pre-processing has high reproducibility and discriminating ability which should be a preferred method for typing of pathogenic strains and useful for epidemiological studies.

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