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Pathogenic *Vibrio* spp. Isolated from the Gwangan Beach of Busan in 2003

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A total of 52 pathogenic *Vibrio* strains was isolated from the Gwangan Beach during summer in 2003. The isolated vibrios were composed of 6 different species: *V. parahaemolyticus*, *V. cholerae* non O1, *V. fluvialis*, *V. vulnificus*, *V. alginolyticus*, and *V. mimicus*. *V. parahaemolyticus* was most predominant as 46% (24/52), *V. cholerae* non O1 was the second with 23% (12/52), and *V. fluvialis* was the third with 17% (9/52). Among the isolated strains, 22 strains showed hemolytic, proteolytic or ureolytic activity. Eight strains showed both hemolysin and protease activities, and either 6 strains showed only hemolysin activities and 7 strains only protease activities. Only one strain of *V. parahaemolyticus* isolates showed urease activity. The urease-positive *V. parahaemolyticus* strain (*V. parahaemolyticus* S25) showed the same biochemical characteristics as the reference strain, *V. parahaemolyticus* KCTC 2471 (urease-negative) except for urease production. To compare the degree of virulence of *Vibrio* strains having different pathogenic factors, hemolysin, protease, or urease-positive strains were injected into groups of 10 each of ICR mice (7- to 10-week-old male). The lethal rate of urease-positive *V. parahaemolyticus* S25 was significantly high, being 70%. Protease-positive strains showed 40-60% of lethal rate. Hemolysin-positive strains showed no mortality, similar to non-pathogenic *V. parahaemolyticus* KCTC 2471 and *V. parahaemolyticus* FM12.

Key words: Pathogenic *Vibrio*, Hemolysin, Potease, Urease

Introduction

Many seafood poisoning accidents caused by pathogenic vibrios outbreak during summer, because people enjoy eating *hoe* (sliced raw fish) in Korea. It has been reported that pathogenic vibrios can produce several toxins including cytolysin (Gray and Kreger, 1985), protease (Kosary and Kreger, 1985), phospholipase (Edward et al., 1984), siderophore (Larsen, 1984), hemolysin (Miyamoto et al., 1969; Honda et al., 1985; Ichinose et al., 1987), and urease (Oberhofer and Podgore, 1982; Honda et al., 1992, Kaysner et al., 1994; Suthienkul et al., 1995) etc. There have been many research papers about the distribution, physiological characteristics, and hemolysin (a representative pathogenic factor) of *Vibrio* spp. (Lee and Choi, 1973; Kim et al., 1987, 1990, 1997) in Korea. In our country, *V. parahaemolyticus* and *V. vulnificus* have been known as the most important species because they frequently cause seafood poisoning between 1970' and 1980', and they have proteolytic enzymes like enterotoxin and hemolysin (Dotevall et al., 1985; Honda et al., 1985; Ichinose et al., 1987; Chang and Shinoda, 1994; Kim et al., 1997). In recent years, *V. cholerae* non O1 has been detected frequently (Seong, 1997; Park et al., 2002, 2003), while it was known to be rare in Korea. *V. cholerae* non O1 has been studied for its pathogenicity in many other countries, and is designated as a food poisoning bacterium in Japan (Pal et al., 1992; Russell et al., 1992; Dalsgaard et al., 1995; Saha et al.,1996). These results showed that *Vibrio* spp. was variable in environments. Therefore, it is important to examine the distribution of pathogenic vibrios in environment to monitor the food poisoning accident by pathogenic vibrios. In present study, we isolated pathogenic *Vibrio* spp. from seawater of the Gwangan Beach of Busan from May to September in 2003, and examined pathogenic factors and the infection effect

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in ICR mice.

Materials and Methods

Media and kit for rapid detection

All media were purchased from Difco Co. (Sparks, MD, USA) except for tryptic soy agar plate (TSA, 5% sheep blood), which was from Micromedia Co. (Daejeon, Korea). API 20 E kit was obtained from bioMrieux Co. (Marcy I'Etoile, France), and reagents from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of pathogenic *Vibrio*

Seawater was collected from the Gwangan Beach of Busan, Korea between May and September in 2003. Isolation of pathogenic *Vibrio* was coducted by the mothod of Park et al. (2002, 2003).

Pathogenicity test in mice

The culture of pathogenic vibrios (1 h at 37℃) was centrifuged (6,000×g, 30 min), and rinsed twice with phosphate buffered saline (PBS, pH 7.0). Finally the precipitate was resuspended to 10^7-10^8 cfu/mL with PBS. The cell suspension (0.5 mL) was intraperitoneally into groups of 10 each of ICR mice (7- to 10-week-old male) (Starks et al., 2000). The mice were observed for up 48 h postinfection with recording of deaths or moribund animals.

Results and Discussion

Distribution of pathogenic *Vibrio* species in the Gwangan Beach

Fifty two strains of pathogenic vibrios were isolated from seawater of the Gwangan Beach in Busan from May to September in 2003. They were classified into 6 species: *V. parahaemolyticus*, *V. cholerae* non O1, *V. fluvialis*, *V. vulnificus*, *V. alginolyticus*, and *V. mimicus* (Table 1). *V. parahaemolyticus* was the most predominant (46%, 24/52) followed by *V. cholerae* non O1 (23%, 12/52) and *V. fluvialis* (17%, 9/52). The detection rate of other species were very low as 3% (6/52) for *V. vulnificus* and *V. alginolyticus*, and 1% (2/52) for *V. mimicus*.

Park et al. (2002) isolated 6 pathogenic *Vibrio* species: *V. parahaemolyticus*, *V. cholerae* non O1, *V. alginolyticus*, *V. vulnificus*, *V. hollisae* and *V. fluvialis* from seawater of the Gwangan Beach in 2001. Furthermore, they reported 7 *Vibrio* species including *V. mimicus* from same place in 2002 (Park et al., 2003). *V. alginolyticus* was predominant species in 2001. However, *V. parahaemolyticus* was most dominant frequency in 2002 (Park et al., 2003) and in 2003 (in the present study). Until recently, *V. cholerae* non O1 was known to be rare in Korea, but its detection rate is increasing.: 16% (11/68) in 2001 (Park et al., 2002), 22% (12/54) in 2002 (Park et al., 2003), and 23% (12/52) in 2003 (in the present study). From these results, the detection rates of pathogenic *Vibrio* species were variable year after year, while kinds of pathogenic *Vibrio* species isolated from the Gwangan Beach were nearly constant during recent 3 years.

Pathogenic factors of *Vibrio* isolates

Hemolysin, protease and urease activity were examined with the culture supernatant of pathogenic *Vibrio* isolates. Of 52 pathogenic *Vibrio* strains, 22 strains showed the enzymatic activities (Table 2). Eight strains showed both hemolysin and protease activities, 6 strains showed only hemolysin activities, and 7 strains only protease activities. Only one strain of *V. parahaemolyticus* showed urease activity. Park et al. (2002, 2003) reported that 67% (31/46) of pathogenic *Vibrio* isolates in 2001 (Park et al., 2002), and 64% (25/39) in 2002 (Park et al., 2003) from Gwangan Beach were protease-positive. In the present study, 68% (15/22) of pathogenic *Vibrio* strains showed protease activity.

Table 1. *Vibrio* species isolated from seawater of the Gwangan Beach, Busan, Korea, 2003

	May	June	July	Aug.	Sept.	Numbers of the isolated *Vibrio* spp. (%)
V. parahaemolyticus	8	4	2	4	6	24(46)
V. cholerae non O1	2	-[1]	5	2	3	12(23)
V. fluvialis	-[1]	2	1	3	3	9(17)
V. vulnificus	-	-	-	3	-	3(6)
V. alginolyticus	1	1	-	1	-	3(6)
V. mimicus	-	-	-	-	1	1(2)
	11	7	8	13	13	52(100)

[1]Not detected.

Table 2. Pathogenic factors of *Vibrio* spp. isolated from seawater of the Gwangan Beach in Korea, 2003

Strains (a/b)		Phenotypic pathogenicity		
		Hemolysin	Protease	Urease
V. parahaemolyticus (6/24)	S8	-	+	-
	S9	-	+	-
	S19	-	+	-
	S25	-	-	+
	S34	+ (β)	-	-
	S72	+ (β)	-	-
V. cholerae non O1 (10/12)	S7	+ (β)	+	-
	S10	+ (β)	+	-
	S31	+ (β)	+	-
	S35	+ (β)	+	-
	S37	+ (β)	+	-
	S38	-	+	-
	S39	+ (β)	+	-
	S48	-	+	-
	S68	-	+	-
	S70	-	+	-
V. fluvialis (5/9)	S28	+ (α)	+	-
	S32	+ (α)	+	-
	S45	+ (α)	-	-
	S51	+ (α)	-	-
	S75	+ (α)	-	-
V. alginolyticus (1/3)	S42	+ (α)	-	-

Hemolysin, protease, and urease activity were confirmed on tryptic soy agar (5% sheep erythrocyte), 10% skim milk agar plate, and urea broth, rescpectively. a: numbers of strains having phenotypic pathogenic factors; b, numbers of pathogenic *Vibrio* isolates, α, α-hemolysis; β, β-hemolysis; +, detected; -, not detected.

Six strains of 10 *V. cholerae* non O1 isolates and 2 strains of 6 *V. parahaemolyticus* isolates showed β-hemolysis, and all of 5 *V. fluvialis* isolates and one *V. vulnificus* strain showed α-hemolysis on TSA plates (5% sheep erythrocyte). Previously, Park et al. (2002, 2003) reported that 74% (34/46) of pathogenic *Vibrio* isolates in 2001 and 64% (25/39) in 2002 showed/ hemolysis. In the present study, 64% (13/22) of pathogenic isolates were hemolysin-positive. From above results, we could find that hemolysin and protease are common pathogenic factors in pathogenic *Vibrio* species.

A urease-positive *V. parahaemolyticus* (*V. parahaemolyticus* S25) was isolated in the present study. The urease-positive *V. parahaemolytiucs* S25 did not show hemolysin and protease activity. It showed the change of color to red as the bacteria grow, similar to *Proteus vulgaris* ATCC 6380 (a urease-positive reference strain), in urea broth medium (data not shown) and the same biochemical characteristics as a reference strain, *V. parahaemolyticus* KCTC 2471 except for urease production (Table 3). Park et al. (2002, 2003) could not isolate any urease-positive *V. parahaemolyticus* strain from the Gwangan Beach between 2001 and 2002. However, the proportion of urease-positive *V. parahaemolyticus* strains is gradually increasing in other countries (Huq et al., 1979; Nolan et al., 1984; Honda et al., 1988, 1992; Abbott et al., 1989; Kelly and Stroh, 1989; Norqvist et al., 1990; Honda et al., 1992, Magalhaes et al., 1992; Cai and Ni, 1996), while studies on urease-positive *V. parahaemolyticus* in Korea are rare except for Kim (1999), studies on the virulence of environmental urease-positive *V. parahaemolyticus* KH410 having *tdh/trh* gene. Therefore, it is noted the isolation of the urease-positive *V. parahaemolyticus* S25 in the present study.

Virulence of *Vibrio* isolates having different pathogenic factors in mice

The virulence of urease-positive *V. parahaemolyticus* was investigated by injecting a urease-positive strain (*V. parahaemolyticus* S25), protease- positive strains (*V. parahaemolyticus* FM39 and FM50), hemolysin-positive strains (*V. parahaemolyticus* S34 and S72), and non-pathogenic *V. parahaemolyticus* (KCTC2471 and FM12) into mice. The lethal rate of urease-positive *V. parahaemolyticus* S25 was significantly high, being 70%. Protease-positive strains showed 40-60% of lethal rate. Hemolysin-positive strains had no mortality, similar to non-pathogenic *V. parahaemolyticus* KCTC 2471 and *V. parahaemolyticus* FM12. There are some reports that the urease of *P. mirabilis* can cause pyelonephritis in rat or mouse (Braude and Siemienski, 1960, Aronson et al., 1974; Musher et al., 1975; Jones et al., 1990; Johnson et al., 1993). The active gastritis due to *Helicobacter pylori* is predominantly correlated to its urease (Fujino et al., 1974; Hawtin et al., 1990; Cover and Blaser, 1995). In the present study, the lethal time by urease-positive *V. parahaemolyticus* S25 was within 24 h postinfection, and the strain was recovered from viscera of the injected mice. The inoculated mice generally showed maw swelled out and reddish viscera, compared to the non-inoculated (data not shown).

In the present study, we could isolate a urease-positive *V. parahaemolyticus* strain from the environ-

Table 3. Biochemical characteristics of urease-positive *Vibrio parahaemolyticus* S25 isolated from seawater of the Gwangan Beach in Korea in 2003

Strains \ Reactions	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
V. parahaemolyticus KCTC2471	-	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	+	+
V. parahaemolyticus S25	-	-	+	+	-	-	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+

Biochemical reactions were determined by API 20 E kit.
+, positive reaction; -, negative reaction; ONPG, β-galactosidase; ADH, arginine dehydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; CIT, citrate utilization; H_2S, H_2S production; URE, urease; TDA, tryptophan deaminase; IND, indole production; VP, acetoin production; GEL, gelatinase; GLU, glucose hydrolysis; MAN, mannitol hydrolysis; INO, inositol hydrolysis; SOR, sorbitol hydrolysis; RHA, rhamnose; SAC, sucrose hydrolysis; MEL, mellibiose hydrolysis; AMY, amygdalin hydrolysis; ARA, arabinose hydrolysis; OX, cytochrome-oxidase.

Table 4. Virulence of *Vibrio parahaemolyticus* having different pathogenic factors in mice

Phenotypic pathogenic factor	Strain	Lethal time (h)	Lethal rate (%)
Buffered saline	Control		0
None	*V. parahaemolyticus* KCTC2471		0
	V. parahaemolyticus FM12		0
Urease	*V. parahaemolyticus* S25	6-24	70
Protease	*V. parahaemolyticus* FM39	4-13	40
	V. parahaemolyticus FM50	5-8	60
Hemolysin	*V. parahaemolyticus* S34		0
	V. parahaemolyticus S72		0

FM strains was isolated in 2002 (Park et al., 2003), and S strains in 2003 (in the present study) from seawater of Busan, Korea. Each bacterial suspension (0.5 mL of 10^7-10^8 cfu/mL) was inoculated intraperitoneally in groups of 10 each of ICR mice (7- to 10-week-old male). Each test was duplicate. Lethal rate indicates the ratio of numbers of dead to total 10 mice inoculated.

ment and find the pathogenicity in mice. Therefore, we should study on the urease as a pathogenic factor of *V. parahaemolyticus* and continuously monitor in Korean waters.

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