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Pathogenic *Vibrio* spp. Isolated from the Gwangan Beach of Busan, 2002

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Fifty four strains of pathogenic vibrios were isolated from the Gwangan Beach from May to October, 2002. The isolated vibrios were composed of 7 different species: *Vibrio parahaemolyticus*, *V. cholerae* non-O1, *V. alginolyticus*, *V. vulnificus*, *V. hollisae*, *V. fluvialis*, and *V. mimicus*. In the detection rate, *V. parahaemolyticus* was most predominant as 46% (25/54). From the isolated strains, only 25 strains have hemolytic activity or 25 strains only proteolytic activity on agar plates. Eleven strains showed both hemolytic and proteolytic activity. No strains showed urease activity. All strains of *V. parahaemolyticus* did not show hemolytic activity, while *V. cholerae* non-O1 strains showed β hemolytic activity. Kanagawa phenomena of pathogenic vibrios did not accord with hemolytic activity of the culture supernatant at the late log phase. Some strains showed high hemolytic activity despite having proteolytic activity, but some weak hemolytic activities despite having no proteolytic activity.

Key words: Pathogenic vibrios, Hemolytic activity, Proteolytic activity

Introduction

About 40 *Vibrio* species have been known in the world. Of these 12 species are virulent to human (Blake et al., 1980; Davis et al., 1981; Hickman-Brenner et al., 1982; Coffey et al., 1986; Balows et al., 1991; Dalsgaard et al., 1995). Pathogenic vibrios produce various toxins such as cytolysin (Gray and Kreger, 1985), protease (Kosary and Kreger, 1985), phospholipase (Edward et al., 1984), siderophore (Larsen, 1984), urease (Honda et al., 1992; Kaysner et al., 1994; Suthienkul et al., 1995). The major virulence factors are known as proteolytic enzymes like enterotoxin and hemolysin (Dotevall et al., 1985; Ichinose et al., 1987). But there are few studies on other virulence factors except hemolysin in Korea (Kim et al., 1997a; Kim et al., 1997b).

There are many seafood poisoning accidents caused by pathogenic vibrios in summer in Korea. *V. cholerae* prevailed in 1960's in Korea, *V. parahaemolyticus* in 1970's, and *V. vulnificus* in 1980's. Recently *V. cholerae* non-O1 has occurred frequently (Seong, 1997; Park et al., 2002). Seong (1997) detected 8 strains (28%) of *V. cholerae* non-O1 from Gwangan Beach of Busan in 1992, and Park et al. (2002) isolated 11 strains (16%) from Gwangan Beach in 2001.

In present study the authors collected vibrios during summer season to investigate the distribution of pathogenic vibrios in Gwangan Beach of Busan in 2002, and examined enzymatic activities such as hemolysin, protease and urease which are known as virulence factors.

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Materials and Methods

Media and Chemicals

Media used in the study were purchased from Difco Co. (USA) and other reagents from Sigma Co. (USA). Sheep erythrocyte and sheep blood agar plates from Micromedia Co. (Korea), and API 20E kit used for biochemical test from BioMérieux Co. (France).

Isolation of *Vibrio* strains

Seawater was collected from Gwangan Beach of Busan during summer in 2002. The seawater was filtrated with Millipore membrane filter (pore size, 0.45 μm) under vacuum, which was inoculated and enriched in peptone medium (1% peptone, 0.5% NaCl) for 24 hr at 37℃. The enriched culture was spreaded on thiosulfate citrate bile salt sucrose

(TCBS) agar plates. Biochemical characteristics of each colony were examined by API 20E kit. Isolated *Vibrio* strains were cultured in heart infusion broth for 18 hr at 37℃. After centrifugation (7,000×g, 20 min), the culture supernatant was used for enzymatic activity assays.

Enzymatic activity assay

The culture supernatant (40 μL) was spotted onto paper disks (Toyo, 8 mm) on sheep blood agar plates, which incubated for 24 hr at 37℃. Formation of clear zone confirmed Kanagawa phenomenon of hemolytic activity.

Relative hemolytic activity was determined with 1% sheep erythrocytes (Tang et al., 1994). Culture supernatant (10 μL) was diluted with 10mM Tris-HCl buffer (pH 7.5, 140 mM NaCl, 0.01% bovine serum albumin, 0.04% sodium azide) and reacted with the same volume of 1% sheep erythrocytes for 30 min at 37℃. After centrifugation (3,000×g, 5 min) the amount of hemoglobin released from disrupted erythrocytes was determined at 540 nm with spectrophotometer (HACH 4000, USA). Distilled water was used for the value of perfect destruction of sheep erythrocytes. Hemolytic activity was calculated as percentage to the value of perfect destruction of erythrocytes. Proteolytic activity was checked with the formation of clear zone around paper disks (Toyo, 8 mm) loaded with 40 μL of culture supernatant on 10% skim milk agar plate (Kosary and Kreger, 1985). Urease activity was checked with red color change in urea broth medium (Suthienkul et al., 1995).

Results and Discussion

Pathogenic vibrios

Fifty four strains of pathogenic vibrios were isolated from seawater of the Gwangan Beach in Busan from May to October in 2002. They were classified into 7 species: *V. parahaemolyticus*, *V. cholerae* non-O1, *V. alginolyticus*, *V. vulnificus*, *V. hollisae*, *V. fluvialis* and *V. mimicus* (Table 1).

Among the isolated strains, *V. parahaemolyticus* showed the highest detection rate corresponding to 46% (25/54). As summarized in Table 1, other strains showed detection rates as 22% (12/54) for *V. cholerae* non-O1, 17% (9/54) for *V. alginolyticus*, 8% for (4/54) for *V. vulnificus*, 4% (2/54) for *V. hollisae*, 1% for *V. fluvialis*, and 1% for *V. mimicus*.

When we studied in 2001, we identified 6 pathogenic *Vibrio* species: *V. parahaemolyticus*, *V. cholerae* non-O1, *V. alginolyticus*, *V. vulnificus*, *V. hollisae* and *V. fluvialis* from seawater on the Gwangan Beach (Park et al., 2002). However, *V. mimicus* was not detected. From the present study, *V. parahaemolyticus* and *V. alginolyticus* was continuously detected from May to October, corresponding with our results studied in 2001 (Park et al., 2002). *V. vulnificus* was detected as 3 strains in August and just one in October, while *V. vulnificus* was continuously detected from June to October in 2001 (Park et al., 2002). Interestingly, previous study reported that it was detected only in August from the seawater (Kim et al., 1990). *V. cholerae* non-O1 was known to be rare in Korea, but it showed high detection rates in 2001 (16%) (Park et al., 2002) and 2002 (22%) (in the present study).

Virulence factors of isolated vibrios

We have characterized the pathogenicity of the isolated strains by testing enzymatic activities such as hemolysin, protease and urease against the culture

Table 1. Isolation of *Vibrio* species from seawater of the Gwangan Beach in Korea, 2002

	May	June	July	Aug.	Sept.	Oct.	Numbers of the isolated *Vibrio* spp. (%)
Vibrio parahaemolyticus	2	8	1	1	9	4	25 (46)
Vibrio cholerae non-O1	-*	1	3	5	3	-	12 (22)
Vibrio alginolyticus	1	1	4	1	2	-	9 (17)
Vibrio vulnificus	-	-	-	3	-	1	4 (8)
Vibrio hollisae	-	1	1	-	-	-	2 (4)
Vibrio fluvialis	-	-	1	-	-	-	1 (2)
Vibrio mimicus	1	-	-	-	-	-	1 (2)
	4	11	10	10	14	5	54 (100)

*not detected

Table 2. Pathogenic factors produced by *Vibrio* spp. isolated from seawater of the Gwangan Beach in Korea, 2002

Strains (a/b)		Hemolytic activity		Protease activity	Urease activity
		Kanagawa phenomenon	Relative activity (%)		
Vibrio alginolyticus (5/9)	FM 13	α	<3.0	-	-
	FM 16	α	<3.0	-	-
	FM 17	α	<3.0	-	-
	FM 18	α	<3.0	-	-
	FM 19	α	<3.0	-	-
Vibrio vulnificus (4/4)	FM 29	α	91	-	-
	FM 33	α	12	-	-
	FM 35	α	100	-	-
	FM 54	α	95	-	-
Vibrio parahaemolyticus (14/25)	FM 9	-	<3.0	+	-
	FM 10	-	<3.0	+	-
	FM 11	-	<3.0	+	-
	FM 36	-	<3.0	+	-
	FM 37	-	<3.0	+	-
	FM 39	-	<3.0	+	-
	FM 40	-	<3.0	+	-
	FM 45	-	13	+	-
	FM 46	-	<3.0	+	-
	FM 47	-	7	+	-
	FM 48	-	10	+	-
	FM 50	-	<3.0	+	-
	FM 51	-	<3.0	+	-
	FM 52	-	<3.0	+	-
Vibrio cholerae non-O1 (12/12)	FM 14	β	<3.0	+	-
	FM 23	β	<3.0	-	-
	FM 24	β	<3.0	+	-
	FM 25	β	<3.0	+	-
	FM 26	β	<3.0	-	-
	FM 27	β	<3.0	-	-
	FM 28	β	<3.0	+	-
	FM 30	β	<3.0	+	-
	FM 32	β	<3.0	+	-
	FM 42	β	100	+	-
	FM 43	β	<3.0	+	-
	FM 44	β	<3.0	+	-
Vibrio fluvialis (1/1)	FM 21	α	<3.0	+	-
Vibrio mimicus (1/1)	FM 4	β	<3.0	+	-
Vibrio hollisae (2/2)	FM 15	β	<3.0	-	-
	FM 22	β	<3.0	-	-

Relative hemolytic activity is the percentages of hemolysis by cultural supernatant (10 μL) to perfect hemolysis by D.W. a, numbers of pathogenic strains; b, numbers of isolated strains; α, α-type hemolysis; β, β-type hemolysis; +, detected; -, not detected.

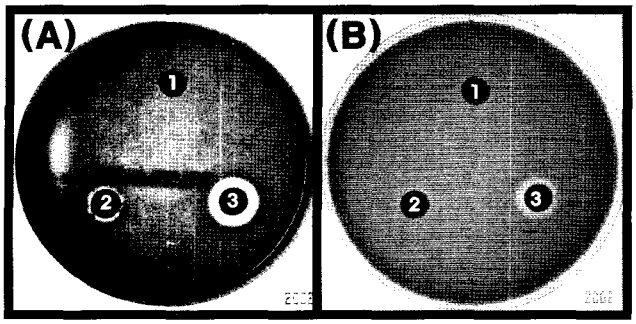

Fig. 1. Qualitative identification of hemolysin and protease produced by *Vibrio cholerae* non-O1 FM 42 isolated from seawater on the Gwangan Beach in Korea, 2002. Hemolytic activity was identified on 5% sheep blood agar plate (A) and proteolytic activity on 10% skim milk agar plate (B). ① 40 μL of D.W.; ② 40 μL of cultural supernatant; ③ 40 μL of crude enzyme precipitated with 60% ammonium sulfate saturation.

supernatant. On the basis of the results from enzymatic acitivity assays for the 54 isolated pathogenic vibrios, 11 strains showed both hemolytic and proteolytic activities, and either 25 strains showed only hemolytic activities or 25 strains only proteolytic activities (Table 2). All of *V. parahaemolyticus* strains isolated in this study did not show urease activity, although there were some reports about pathogenicity of urease produced by *V. parahaemolyticus* (Honda et al., 1992; Kaysner et al., 1994; Suthienkul et al., 1995). Also, all of the isolated *V. parahaemolyticus* strains did not show Kanagawa phenomena on sheep blood agar plate, but 14 strains showed proteolytic activity on skim milk agar plate. All of *V. cholerae* non-O1 strains showed β hemolysis on sheep blood agar plates, while only 9 strains showed proteolytic activity. From the above mentioned results, Kanagawa phenomenon did not always accord with hemolytic activities of the culture supernatant at late log phase in the present study.

Kim et al. (1997a) reported that *V. cholerae* non-O1 produced hemolysin from initial log phase to late log phase, and the highest hemolytic activity was shown at the late log phase. However, the hemolytic activity showed a sharp drop with protease production, and the reason for this drop explained that hemolysin was rapidly digested by protease produced.

Some vibrios showed high hemolytic activities despite having proteolytic activity, while some others showed rare hemolytic activities despite having no proteolytic activity. It is not clear so far that the relationship between hemolysin and protease produced by pathogenic vibrios, and it has to be further studied.

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