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Pathogenic Factors of *Vibrio* spp. Isolated from Seawater of Gwangan Beach in Busan

Mi-Yeon Park*, Hyun-Jin Kim, Seung-Tae Choi[1]
Eun-Gyong Oh[2] and Dong-Suck Chang

Department of Food Science and Technology, Pukyong National University, Busan 608-737, Korea
[1]Institute of Genetic Engineering, Changwon National University, Changwon 641-773, Korea
[2]South Sea Fisheries Research Institute, NFRDI, Yeosu 556-823, Korea

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The authors identified 68 *Vibrio* strains from Gwangan beach seawater from June to October in 2001. We identified them as 19 strains of *Vibrio alginolyticus*, 15 strains of *V. vulnificus*, 15 strains of *V. parahaemolyticus*, 11 strains of *V. cholerae* non O1, 7 strains of *V. fluvialis* and just one strain of *V. hollisae*. They showed their typical biochemical characteristics by API 20E kit (bioMérieux), respectively. It was examined whether their cultural supernatants had enzymatic activities such as hemolysin, protease or urease. The 46 strains showed hemolytic activities and/or protease activities. But we could not find any strain which had urease activity. All isolates of *V. cholerae* non O1 showed β hemolysis. The others showed α hemolysis or did not show clear zones on sheep blood agar plates. These results of Kanagawa phenomenon were not always correspondant with hemolytic activities of cultural supernatants at late log phase. Some strains had higher hemolytic activities despite of showing protease activities on skim milk agar plates and in litmus milk media. On the other hand, some strains showed protease activities but did not show hemolytic activities. Therefore we could guess that there were the relationships between hemolysins and proteases produced by pathogenic vibrios.

Key words: *Vibrio* strains, Kanagawa phenomenon, Hemolytic activity, Protease activity, Urease activity

Introduction

While seafood poisoning accidents caused by pathogenic vibrios have been increased year by year, the Korean enjoy eating hoe, sliced raw fish. About 40 *Vibrio* species are known all over the world and 12 species of them are known as pathogen to human. Namely they are *Vibrio cholerae, V. parahaemolyticus, V. vulnificus, V. cholerae* non O1, *V. fluvialis, V. metschnikovii, V. cincinnatiensis, V. hollisae, V. damsela, V. furnissii, V. carchariae* and *V. mimicus* (Blake et al., 1980; Davis et al., 1981; Hickman-Brenner et al., 1982; Coffey et al., 1986; Balows et al., 1991; Honda, 1991; Dalsgaard et al., 1995; Kim et al., 1997b).

It was reported that pathogenic vibrios produced many kinds of toxin such as cytolysin (Wickboldt and Sanders, 1983; Gray and Kreger, 1985), protease (Kosary and Kreger, 1985), phospholipase (Edward et al., 1984), siderophore (Larsen, 1984) and other extracellular toxins as well as hemolysin (Baselaki et al., 1979; Honda et al., 1985). The major pathogenic factors are known as 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., 1997b). While there are a few reports about pathogenicity of urease produced by *V. paraheaemolyticus* (Honda et al., 1992; Honda and Iida, 1993; Honda et al., 1989; Kaper et al., 1984, Kaysner et al., 1994).

*Corresponding author: mypark@mail1.pknu.ac.kr

In Korea, *V. parahaemolyticus* and *V. vulnificus* have been known as most important strains because they frequently caused seafood poisoning accidents. Especially, there were 142 cholera patients in our country in 2001. There are many research papers on the distributions of *Vibrio* spp. or their physiological characteristics in Korea (Lee and Choi, 1973; Kim et al., 1987; Kim et al., 1990; Kim et al., 1997a). But we can rarely find research papers on other pathogenic factors except hemolysin. Therefore we could think it was very important subject to isolate pathogenic vibrios from seawater and clear the relationships among pathogenic factors produced by them.

Then we isolated *Vibrio* species from Gwangan beach seawater and examined their ability to produce pathogenic factors such as hemolysin, protease or urease.

Materials and Methods

Sampling and isolation of *Vibrio* spp.

Seawater was drawn 20 times from June to October 2001 from the Gwangan beach in Busan, Korea. The seawater was filtrated with millipore membrane filter (pore size 0.45 μm) under vaccum. The membrane filter was inoculated and enriched in peptone medium (1% peptone, 0.5% NaCl). The enriched culture was spreaded on thiosulfate citrate bile salt sucrose (TCBS) agar plates and incubated for 24 hrs at 37℃. The separate colonies on TCBS agar plates were examined their biochemical characteristics by API 20E kit (bioMérieux, France) and identified as *Vibrio* species with homology 95% over.

The *Vibrio* species were cultured in heart infusion broth for 18 hrs at 37℃. After centrifugation (7,000 ×g, 20 min.), the cultural supernatants were used for enzymatic activity assays.

Media and kit for rapid detection

All media used in this study were Difco Co. (USA). Sheep erythrocyte for hemolytic activity assay and sheep blood agar plate used for confirmation of Kanagawa phenomenon were purchased from Micromedia Co. (Korea). API 20E kit used for biochemical test was purchased from bioMérieux Co. (France). Other reagents were purchased from Sigma Co (USA).

Confirmation of Kanagawa phenomenon

The cultural supernatants prepared from *Vibrio* species were spreaded on sheep blood agar plates and incubated the plates for 24 hrs at 37℃. After incubation, the results was observed with formations of clear zones.

Hemolytic activity assay

The hemolytic activity was determined with 1% sheep erythrocytes (Shinoda et al., 1985).

The cultural supernatants were diluted serially with 10 mM Tris-HCl buffer (pH 7.5, 140 mM NaCl, 0.01% bovine serum albumin and 0.04% sodium azide). Each diluted solutions (1 mL) were reacted with the same volume of the 1% sheep erythrocytes for 1 hr 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). Each hemolytic activity was calculated as the percentage to the value of perfect destruction of erythrocytes. For perfect destruction of erythrocytes, distilled water was used instead of 10mM Tris-HCl buffer solution.

Measurement of protease activity

The proteolytic activity was determined on skim milk agar plates and in litmus milk media.

Paper disks (Toyo, 8 mm) were soaked sufficiently with cultural supernatants prepared from vibrios isolated. The disks were loaded on the skim milk agar plates and incubated the plates for 24 hrs at 37℃. Protease activity was checked with formation of clear zones around colonies.

On the other hand, vibrios were inoculated in litmus milk media and incubated for 48 hrs 37℃ and their protease activities were checked with clearing of medium and dissolution of clot by digestion of casein.

Results and Discussion

From Gwangan beach seawater, 68 *Vibrio* strains were isolated from June to October 2001. *V. alginolyticus* took up 19 strains (28%) and *V. vulnificus* and *V. parahaemolyticus* were 15 strains (22%), respectively. *V. cholerae* non O1, *V. fluvialis* and *V. hollisae* were 11 strains (16%), 7 strains (10%) and only one strain (1%), respectively (Table 1).

Table 1. *Vibrio* species isolated from seawater of Gwangan beach, Busan Korea, 2001

Strains	June			July					August				September					October			Numbers of *Vibrio* spp. isolated
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	20th	
Vibrio alginolyticus	−	1	6	−	4	2	2	2	1	−	−	−	−	1	−	−	−	−	−	−	19
Vibrio vulnificus	−	1	2	1	1	2	−	−	1	−	−	1	2	2	−	−	1	1	−	−	15
Vibrio parahaemolyticus	−	1	−	−	1	−	1	2	−	−	1	−	1	1	2	1	1	1	2	−	15
Vibrio cholerae non-O1	−	−	−	−	−	−	−	−	2	−	2	−	−	−	3	2	2	−	−	−	11
Vibrio fluvialis	−	−	−	−	−	−	−	1	1	−	−	−	−	−	−	2	2	1	−	−	7
Vibrio hollisae	−	−	−	−	−	−	−	−	1	−	−	−	−	−	−	−	−	−	−	−	1
Total	11			19					10				23					5			68

It was reported that *V. vulnificus* was deteced only in August from the seawater of Gwangan beach by Kim et al. (1990). While Lee and Choi (1973) reported that *V. parahaemolyticus* was isolated from May to November and *V. alginolyticus* was isolated all the year round from seawater at the coast of Busan.

In our study, *V. vulnificus* was determined continuously from Jun to October. *V. alginolyticus, V. vulnificus* and *V. parahaemolyticus* were isolated frequently on June, and *V. cholerae* non O1, *V. fluvialis* and *V. hollisae* were identified from August to September. Especially *V. cholerae* non O1 which has been rarely detected in Korea was isolated 11 strains (16%).

Table 2 showed typical biochemical characteristics of vibrios isolated. *V. cholerae* non O1, *V. alginolyticus* and *V. fluvialis* showed yellow color but *V. vulnificus, V. parahaemolyticus* and *V. hollisae* showed green color on TCBS agar plates (data is not shown).

It was examined whether the cultural supernatants prepared from 68 *Vibrio* strains isolated had enzymatic activities such as hemolysin, protease or urease. The 46 strains of them showed hemolytic activities and/or protease activities (Table 3). Some showed β- or α- hemolysis, but the others did not show hemolysis on sheep blood agar plates. All of *V. cholerae* non O1 showed β hemolysis on sheep blood agar plates. But some of them showed high hemolytic activity but the others showed no hemolytic activities with their cultural supernatants. In case of *V. vulnificus,* some showed high but some showed no hemolytic activities. One of *V. alginolyticus* showed high hemolytic activity but the other one did not show at all. And also *V. fluvialis* showed α hemolysis on sheep blood agar plate but they did not show hemolytic activities with their cultural supernatants.

From above, the results of Kanagawa phenomenon were not always correspondant with hemolytic activities of their cultural supernatant at late log phase.

The proteolytic activities were checked on skim

Table 2. Biochemical characteristics of identified *Vibrio* species

Strains \ Reactions	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
Vibrio alginolyticus	−	−	+	+	+	−	−	−	+	−	+	+	+	−	−	−	+	−	−	−	+
Vibrio vulnificus	+	−	+	+	−	−	−	−	+	−	+	+	+	−	−	−	−	−	+	−	+
Vibrio parahaemolyticus	−	−	+	+	−	−	−	−	+	−	+	+	+	−	−	−	−	−	−	+	+
Vibrio cholerae non-O1	+	−	+	+	+	−	−	−	+	+	+	+	+	−	−	−	+	−	−	−	+
Vibrio fluvialis	+	+	−	−	−	−	−	−	+	−	+	+	+	−	−	−	+	−	−	+	+
Vibrio hollisae	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+

Biochemical reactions were determined by API 20 E system.
+, positive reaction; −, negative reaction; ONPG, β-galactosidase; ADH, arginine dihydrolase; 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, melibiose hydrolysis; AMY, amygdalin hydrolysis; ARA, arabinose hydrolysis; OX, cytochrome-oxidase.

Table 3. Enzymatic activities of cultural supernatants

Strains (isolated No.)	Reactions	Hemolytic activity: Kanagawa phenomenon	Hemolytic activity: Relative hemolytic activity (%)*	Protease activity	Urease activity
Vibrio alginolyticus	5	α	<3.0	+	−
	14	·	79	−	−
Vibrio vulnificus	6	·	85	+	−
	12	α	77	−	−
	13	α	<3.0	−	−
	19	α	79	−	−
	20	α	39	−	−
	34	α	80	−	−
	40	α	95	−	−
	41	α	100	−	−
	42	α	63	−	−
	45	α	<3.0	−	−
	60	α	65	−	−
	64	·	57	−	−
Vibrio parahaemolyticus	1	·	<3.0	+	−
	23	·	<3.0	+	−
	26	·	<3.0	+	−
	27	α	<3.0	−	−
	39	·	<3.0	+	−
	43	·	<3.0	+	−
	47	·	<3.0	+	−
	50	·	<3.0	+	−
	51	·	<3.0	+	−
	56	·	<3.0	+	−
	63	·	<3.0	+	−
	65	·	<3.0	+	−
	67	·	<3.0	−	−
	68	α	<3.0	+	−
Vibrio cholerae non-O1	31	β	50	+	−
	32	β	<3.0	+	−
	37	β	97	+	−
	38	β	<3.0	+	−
	48	β	96	+	−
	49	β	96	+	−
	52	β	65	+	−
	53	β	82	+	−
	57	β	18	+	−
	61	β	30	+	−
	62	β	<3.0	+	−
Vibrio fluvialis	28	α	<3.0	+	−
	35	α	<3.0	−	−
	54	α	<3.0	+	−
	55	α	<3.0	+	−
	58	α	<3.0	+	−
	59	α	<3.0	+	−
	66	α	<3.0	+	−

*Relative Hemolytic activitis were calculated with the percentages of samples to positive control.

milk agar plates and in litmus milk media. All *V. cholerae* non O1 showed protease activities. Most *V. parahaemolyticus* and *V. fluvialis* showed protease activities. One of *V. alginolyticus* showed proteolytic activity but the other one did not. All *V. vulnificus* did not show protease activities.

It was reported that protease affected hemolytic actvity because it digested hemolysin by proteolytic digestion. By Kim et al. (1997b), it was said that *V. cholerae* non O1 produced hemolysin from initial log phase to late log phase and showed highest hemolytic activity at the late log phase. After that point hemolytic activity showed a sharp fall and the why was because hemolysin was digested by protease produced. But it is not clear up to now that the relationship between hemolysin and protease produced by pathogenic vibrios.

In this study, any way, some vibrios showed high hemolytic activity despite having protease activities. Some *V. cholerae* non O1 showed very high hemolytic activities in spite of having protease activities. In spite of having no protease activity, some of *V. vulnificus* showed high hemolytic activities but the others did not. All *V. fluvialis* having protease activities did not show hemolytic activities at all despite of showing α hemolysis on sheep blood agar plates. Therefore we could think it would have to study these relationships between hemolysin and protease produced by pathogenic vibrios.

While there were recent reports about pathogenicity of urease produced by *V. paraheaemolyticus*, all strains isolated in this study did not show urease activities at all.

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References

Balows, A., W.J. Hausler, Jr., K.L. Herrmann and H.J. Shadomy. 1991. Manual of Clinical Microbiology 4th ed. American Society for Micorbiology, Washington. D.C., pp. 384~395.

Baselaki, V.S., R.A. Media and C.D. Parker. 1979. In vivo and vitro characterization of virulence-deficient mutants

of *Vibrio cholerae*. Infect. Immun., 24, 111~116.

Blake, P.A., R.E. Weaver and D.G. Hollis. 1980. Diseases of human (other than cholera) caused by vibrios. Ann. Rev. Microbiol., 34, 341~367.

Chang, D.S. and S. Shinoda. 1994. Toxin produced by pathogenic vibrios isolated from seafood. Bull. Korean Fish. Soc., 27, 107~113 (in Korean).

Coffey, J.A., R.L. Harris and T.W. Williams. 1986. *Vibrio damsela:* Another potentially virulent marine vibrio. J. Infect. Diseases, 153, 800~802.

Dalsgaard, A., M.J. Albert, D.N. Taylor, T. Shimada, R. Meza, O. Serichantalergs and P. Echeverria. 1995. Characterization of *Vibrio cholerae* non-O1 serogroups obtained from an outbreak of diarrhea in Lima, Peru. J. Clin. Microbiol., 33, 2715~2722.

Davis, B.R., G.R. Fenning, J. Madden, A.G. Steigerwalt, H.B. Bradford, Jr., H.L. Smith, Jr. and D.J. Brenner. 1981. Characterization of biochemically atypical *Vibrio cholerae* strain and designation of a new pathogenic species, *Vibrio mimicus*. J. Clin. Microbiol., 14, 631~639.

Dotevall, H., G. Jonson-Stromberg, S. Sanyal and J. Holmgren. 1985. Characterization of enterotoxin and soluble hemagglutinin from *Vibrio mimicus:* Identify with *V. cholerae* toxin and hemagglutinin. FEMS Microbiol. Lett., 27, 17~22.

Edward, P.D., J.M. Janda, I.A. Frederic and J.B. Edward. 1984. Comparative studies and laboratory diagnosis of *Vibrio vulnificus*, an invasive *Vibrio* sp. J. Clin. Microbiol., 19, 122~125.

Gray, L.D. and A.S. Kreger. 1985. Purification and characterization of an extracellular cytolysin produced by *Vibrio vulnificus*. Infec. Immun., 48, 62~72.

Hickman-Brenner, F.W., J.J. Farmer, R.E. Weaver and D.J. Brenner. 1982. Identification of *V. hollisae* spp. nov. from patients wth diarrhea. J. Clinical Microbiol., 15, 395~401.

Honda, S., S. Matsumoto, T. Miwatani and T. Honda. 1992. A survey of urease-positive *Vibrio parahaemolyticus* strains isolated from traveller's diarrhea, seawater and imported frozen seafoods. Eur. J. Epidemology, 8, 861~864.

Honda, T. 1991. Pathogenesis, genes, and toxins of genus vibrios. Medical Immunology, 21, 313~323.

Honda, T. and T. Iida. 1993. The pathogenicity of *Vibrio parahaemolyticus* and the role of thermostable direct hemolysin and related hemolysins, Rev. Med. Microbiol., 4, 106~113.

Honda, T., Y. Ni and T. Miwatani. 1989. Purification of a TDH-related hemolysin producted by a Kanagawa phenomenon-negative clinical isolate of *Vibrio parahaemolyticus* O6:K46. FEMS Microbiol. Lett., 48, 241~245.

Honda, T., M. Arita, T. Takeda, M. Yoh and T. Miwatani. 1985. Non O1 *Vibrio cholerae* produces two newly identified of toxins related to *Vibrio parahaemolyticus* hemolysin and *Escherichia coli* heat-stable enterotoxin. Lancet., II, 163~164.

Ichinose, Y., K. Yamamoto, N. Nakasone, M.J. Tanabe, T. Takeda, T. Miwatani and M. Iwanaga. 1987. Enterotoxicity of El Tor like hemolysin of *Vibrio cholerae* non O1. Infect. Immun., 55, 1090~1093.

Kaper, J.B., R.K. Campen, R.J. Seilder, M.M. Baldini and S. Falkow. 1984. Cloning of thermostable direct or Kanagawa-phenomenon associated hemolysin of *Vibrio parahaemolyticus*. Infec. Immun., 45, 290~292.

Kaysner, C.A., C. Abeyta, P.A. Trost, W.E. Hill and M.M. Wekell. 1994. Urea hydrolysis can predict the potential pathogenicity of *Vibrio parahaemolyticus* strains isolated in the Pacific Northwest. Appl. Environ. Microbiol., 60, 3020~3022.

Kim, S.H., M.Y. Park, U.K. Park, Y.M. Kim and D.S. Chang. 1997a. Physiological and ecological characteristics of hemolytic vibrios and development of sanitary countermeasure of raw fisheries foods. 3. Growth factor and antibiotic susceptibility of *Vibrio cholerae* non-O1 and FM-3 isolated from seawater. J. Korean Fish. Soc., 30, 550~555 (in Korean).

Kim, S.H., M.Y. Park, Y.E. Lee, M.H. Cho and D.S. Chang. 1997b. Characteristics of hemolysin produced by *Vibrio cholerae* non-O1 FM-3 isolated from seawater. J. Kor. Fish. Soc., 30, 556~561 (in Korean).

Kim, S.M., U.K. Park, M.Y. Park, Y.M. Kim and D.S. Chang. 1997c. Physiological and ecological characteristics of hemolytic vibrios and development of sanitary countermeasure of raw fisheries foods 2. Physiological and psychrotrophic characteristics of *Vibrio mimicus* SM-9 isolated from seawater. J. Fd Hyg. Safety, 12, 9~14 (in Korean).

Kim, Y.M., B.H. Lee, S.H. Lee and T.S. Lee. 1990. Distribution of *Vibrio vulnificus* in seawater of Kwangan beach Busan, Korea. Bull. Korean Fish. Soc., 22, 385~390 (in Korean).

Kim, Y.M., I.S. Shin and D.S. Chang. 1987. Distribution of *Vibrio vulnificus* the coast of South Korea. Bull. Korean Fish. Soc., 20, 591~600 (in Korean).

Kosary, M.K. and A.S. Kreger. 1985. Production and partical characterization of an elastolytic protease of *Vibrio vulnificus*. Infect. Immun., 50, 534~540.

Larsen, J.L. 1984. *Vibrio angillarum:* Infection of temperature, pH, NaCl concentration and incubation time on growth. J. Appl. Bact., 57, 237~246.

Lee, W.J. and W.K. Choi. 1973. *Vibrio parahaemolyticus* in Korean coastal waters 3. The distribution of vibrio flora in the public beach of Busan city. Bull. Korean Fish. Soc., 6, 20~26 (in Korean).

Shinoda, S., S. Miyoshi, H. Yamanaka and N. Miyoshi-Nakahara. 1985. Some properties of *Vibrio vulnificus* hemolysin. Microbiol. Immunol., 29, 583~590.

Wickboldt, L.G. and C.V. Sanders. 1983. *Vibrio vulnificus* infection. Case and update since 1970. J. AM. Acad. Dermatol., 9, 243~251.