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Virulence of Environmental Urease-Positive and Kanagawa Phenomenon-Negative *Vibrio parahaemolyticus*

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Abstract Fifty-two pathogenic *Vibrio parahaemolyticus* strains were isolated from the environments of Busan and Yeosu, Korea. Forty-three of these strains showed protease activities, whereas 4 strains showed α/β hemolysin activities and 6 strains had urease activities. Their pathogenic factors were not overlapping except one strain, which had both protease and hemolysin activities. The 6 urease-positive strains (*V. parahaemolyticus* YKB4, YKB14, S25, YFB20, YFO21, and YFO22) showed the same biochemical characteristics as a reference strain [*V. parahaemolyticus* KCTC 2471 (urease-negative)], except for urease production. The 6 urease-positive strains showed different urease activities in their culture supernatant during the growth. The urease activity of S25 increased sharply at the late exponential phase, and was the highest at the initial stationary phase and was kept until the late stationary phase. The other 5 isolates, except S25, showed urease activities at the mid-stationary phase and increased steadily until the late stationary phase, when the urease activity was maximal. To compare the degree of virulence of *V. parahaemolyticus* with different pathogenic factors, hemolysin, protease, or urease-positive strains were injected into groups of 10 each of ICR mice (7- to 10-week-old males). The lethal rates of urease-positive *V. parahaemolyticus*, YKB14, YKB4, and S25, were significantly high, being 50, 70, and 80%, respectively. Protease-positive *V. parahaemolyticus* strains FM39 and FM50 showed 40% and 60% of lethal rate, respectively. Hemolysin-positive *V. parahaemolyticus* strains S34 and S72 had no mortality, similar to nonpathogenic *V. parahaemolyticus* FM12.

Key words: Pathogenic *V. parahaemolyticus*, hemolysin protease, urease, virulence

Principal virulence factors of pathogenic *Vibrio parahaemolyticus* are known as hemolysin and protease [25, 38–42]. Hemolysin distroys red blood cells and helps the growth of pathogenic vibrios by supplying ferric ions, while protease invades interstitial tissue space and damages hemorrhagic skin [4, 20, 26, 43]. Hemolysin exhibits β-hemolysis, which is well known for the Kanagawa phenomenon (KP), on a special blood agar plate. It has been suggested that the KP is induced by thermostable direct hemolysin (TDH) that is produced almost exclusively by clinical strains [19, 29, 30, 45].

V. parahaemolyticus has usually been considered to be urease-negative; less than 10% of the strains are positive for urease activity [8, 42, 45, 48]. However, recent reports on clinical strains isolated in Asian countries and Brazil have indicated that the ratio of urease-positive strains among the clinical strains is gradually increasing. [1, 5, 11–13, 16, 18, 24, 31, 33]. Many ureolytic bacteria are pathogenic to animals and humans, and the urease acts as a principal virulence factor in those bacteria [14, 15, 17, 23, 27, 28, 36, 47]. In particular, it is well known that urease is essential for gastric colonization and plays a central role in the pathogenesis of *Helicobacter pylori* infection [6, 7, 9]. Suthienkul *et al.* [44] reported that only 8% of 489 clinical *V. parahaemolyticus* strains were urease-positive. Recently, the KP-negative phenotype of urease-positive *V. parahaemolyticus* has been described in some reports [5, 11–12, 15, 24, 34, 35]. However, studies on urease-positive *V. parahaemolyticus* in Korea are rare.

In the present study, we isolated urease-positive *V. parahaemolyticus* from the environment, and examined the relationship with hemolysin genes and virulence in mice.

Materials and Methods

Media and Kit for Rapid Detection

All media were purchased from Difco Co. (Sparks, MD, U.S.A.) except tryptic soy agar plate (TSA, 5% sheep blood), which was from Micromedia Co. (Daejeon, Korea).

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API 20E kit was obtained from bioMérieux Co. (Marcy l'Etoile, France), and reagents from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Isolation of Urease-Positive *V. parahaemolyticus*

Seawater and fish (Black rockfish, Olive flounder) were collected from Kamak Bay of Yeosu and Gwangan Beach of Busan, Korea between June 2000 and September 2003. The seawater was filtered with millipore membrane filter (0.45 μm pore size, Millipore Co., Bedford, MA, U.S.A.) under vacuum, and 30 g of fish meat and skin were homogenized in 270 ml of phosphate buffer for 2 min with Stomacher (Seward, London, England). The filter and fish homogenate were inoculated and enriched in peptone media (1% peptone, 1% NaCl). The enriched culture spread on thiosulfate citrate bile salt sucrose (TCBS) agar plate and incubated for 24 h at 37°C. Green colonies on TCBS agar plate were selected and examined for their biochemical characteristics with an API 20E kit.

Phenotypic Identification of Pathogenic Factors

Hemolysin and protease activity were identified from clear zone around disks (Toyo 8 mm, Tokyo, Japan), which were loaded with bacterial culture supernatant (40 μl), on TSA plate [35] and 10% skim milk agar plate [32], respectively. Urease activity was verified with color change to pink in Bacto urea broth. Release of ammonia due to urease activity raises the pH, inducing a change of color from yellow to pink in the medium [18].

Urease Activity Assay

The isolates were inoculated in Luria Bertani broth (LB) and cultured with shaking at 37°C. The culture supernatant (7,000 ×g for 20 min) was assayed for urease activity, employing *Escherichia coli* as a urease-negative and *Proteus vulgaris* as a urease-positive reference strain. Each culture supernatant (50 μl) was mixed with 200 μl of UHEP buffer (20 mM HEPES buffer, pH 7.5, 30 mM urea, 1 mM EDTA, 1 mM 2-mercaptoethanol) for 30 min at 37°C. Phenol nitroprusside (1 ml) and alkaline hypochlorite (2 ml) were added to the reaction mixture, and the quantity of ammonia liberated in the reaction mixture was measured by absorbance at 625 nm [46].

Polymerase Chain Reaction to Detect Hemolysin Genes, *tdh* and *trh*

Reagents for polymerase chain reaction (PCR) were obtained from Takara Co. (Ostu, Japan). To detect thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*trh*), VPD and VPR primers were purchased from Takara Co. (Otsu, Japan). The young cultured bacteria were centrifuged at 9,000 ×g for 5 min (HERMLE Z320, National Labnet Co., Woodbridge, NJ, U.S.A.), and resuspended in distilled water. The suspension was heated for 10 min in boiling water and centrifuged. The supernatant was used as a template DNA. The reaction mixture for PCR contained template DNA (2 μl), primers (20 pmols each), dNTPs (0.2 mM each), and Taq DNA polymerase (2 units). PCR was performed for 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C by Gene cycler™ (Bio-Rad Co., Richmond, CA, U.S.A.). The amplified gene was electrophoresed on 1% agarose gel (40 mA, 100 V). The gel was examined with a UV transilluminator (VILBER LOURMAT TFX-20 M, Sigma Co., St. Louis, MO, U.S.A.) after staining with ethidium bromide (EtBr).

Infection of Pathogenic *V. parahaemolyticus* in Mice

Young cultured cells (18 h at 37°C) were centrifuged at 6,000 ×g for 30 min and rinsed twice with phosphate buffered saline (PBS, pH 7.0). The bacterial cells were then resuspended to 10^7–10^8 cfu/ml with PBS. The cell suspension (0.5 ml) was intraperitoneally injected into groups of 10 each of ICR mice (7- to 10-week-old males). The mice were observed for up 48 h postinfection.

RESULTS AND DISCUSSION

Isolation of Urease-Positive *V. parahaemolyticus*

Fifty-two strains of pathogenic *V. parahaemolyticus* were isolated from seawater and fish (Black rockfish and Olive flounder) between June 2000 and September 2003 (Table 1). H strains were isolated in 2001 [40], FM strains in 2002 [39], and S strains in 2003 [37] from seawater of Gwangan Beach of Busan. YKB strains were isolated from seawater of Kamak Bay in 2002, YFB from Black rockfish, and YFO from Olive flounder of Yeosu in 2003 (unpublished). The 52 isolates could produce hemolysin, protease, or urease, and their pathogenic factors were not overlapping, except the H68 strain, which showed both hemolysin and protease activities. Forty-three strains (82%) showed protease activity on skim milk agar plates, while only 4 strains (7%) showed α/β hemolysis, and 6 strains (11%) showed urease activities.

Clinical *V. parahaemolyticus* strains produce hemolysin by *tdh* and/or *trh* [34, 35]. Osawa *et al.* [35] reported that 100 strains (76%) of 132 clinical *V. parahaemolyticus* isolates were tdh^+. In our study, however, 48 strains (92%) of 52 environmental isolates were hemolysin-negative and tdh^-, while 43 strains (82%) were protease-positive. Therefore, we could suggest that environmental *V. parahaemolyticus* had protease as a main pathogenic factor, but not hemolysin.

Six urease-positive strains of *V. parahaemolyticus* (YKB4, YKB14, YFB20, YFO21, YFO22, and S25) did not show hemolysin and protease activities. They showed the change of color to red as the bacteria grew, similar to *Proteus*

Table 1. Virulence factors of pathogenic *V. parahaemolyticus* isolated from the environment between 2000 and 2003.

Strains	Hemolysin activity	Protease activity	Urease activity	Strains	Hemolysin activity	Protease activity	Urease activity
H1	-	+	-	FM51	-	+	-
H23	-	+	-	FM52	-	+	-
H26	-	+	-	S8	-	+	-
H27	+(α)	-	-	S9	-	+	-
H39	-	+	-	S19	-	+	-
H43	-	+	-	S25	-	-	+
H47	-	+	-	S34	+(β)	-	-
H50	-	+	-	S72	+(β)	-	-
H51	-	+	-	YKB1	-	+	-
H56	-	+	-	YKB2	-	+	-
H63	-	+	-	YKB3	-	+	-
H65	-	+	-	YKB4	-	-	+
H67	-	+	-	YKB6	-	+	-
H68	+(α)	+	-	YKB8	-	+	-
FM9	-	+	-	YKB10	-	+	-
FM10	-	+	-	YKB14	-	-	+
FM11	-	+	-	YKB15	-	+	-
FM36	-	+	-	YKB17	-	+	-
FM37	-	+	-	YKB18	-	+	-
FM39	-	+	-	YKB19	-	+	-
FM40	-	+	-	YKB20	-	+	-
FM45	-	+	-	YKB21	-	+	-
FM46	-	+	-	YKB22	-	+	-
FM47	-	+	-	YFB20	-	-	+
FM48	-	+	-	YFO21	-	-	+
FM50	-	+	-	YFO22	-	-	+

H strains [40], FM strains [39], and S strains [37] were isolated from seawater of Gwangan beach of Busan from June 2000 to September 2003. YKB strains from seawater of Kamak Bay at 2002, and YFB20 from Black rockfish, YFO21 and YFO22 from Olive flounder at 2003 in Yeosu (unpublished). Hemolysin activity was confirmed on tryptic soy agar (5% sheep erythrocyte), protease activity on 10% skim milk agar, and urease activity in urea broth.
+, positive; -, negative; α,α-hemolysis; β,β-hemolysis.

vulgaris ATCC 6380 (a urease-positive reference strain), in urea broth medium (Table 1 and Fig. 1), and the same biochemical characteristics as a reference strain, *V. parahaemolyticus* KCTC 2471, except urease production

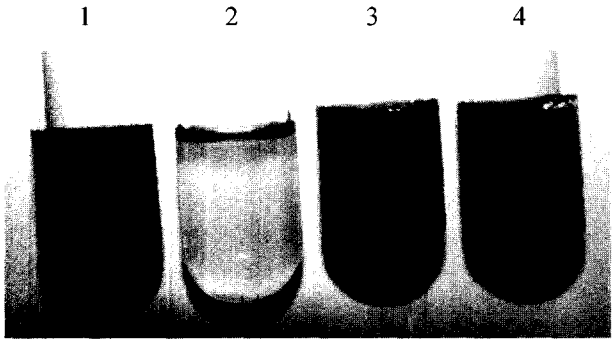

Fig. 1. Identification of the urease-positive *V. parahaemolyticus* in urea broth at 37°C.
1, *Proteus vulgaris* ATCC 6380 (urease-positive reference strain); 2, *E. coli* ATCC 25922 (urease-negative reference strain); 3, *V. parahaemolyticus* YKB4 isolated from Kamak Bay of Yeosu; 4, *V. parahaemolyticus* YKB14 isolated from Kamak Bay of Yeosu.

(Table 2). Until recently, *V. parahaemolyticus* has generally been considered to be urease-negative, however, it has been reported that the proportion of urease-positive strains among the clinical strains is gradually increasing. In the present study, the detection ratio of urease-positive strains was as high as 11%, while urease-positive *V. parahaemolyticus* in Korea has rarely been reported. Our result is in accord with recent reports in other countries [5, 11, 16, 18, 24].

Urease Activity of *V. parahaemolyticus* Isolates

The urease activities of *V. parahaemolyticus* YKB4, YKB14, YFB20, YFO21, YFO22, and S25 were measured with the culture supernatant during the bacterial growth in LB medium at 37°C. The 6 urease-positive strains showed different urease activities in their culture supernatant. The urease activity of *V. parahaemolyticus* S25 increased sharply at the late exponential phase, and was the highest at the initial stationary phase and was retained until the late stationary phase. The other 5 strains showed urease activities at the mid-stationary phase and their activities increased steadily until the late stationary phase, when the activities were maximal. The maximum urease activities of

Table 2. Biochemical characteristics of environmental urease-positive *V. parahaemolyticus* isolates.

Strains \ Reactions	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
Urease-negative *V. parahaemolyticus* KCTC 2471	-	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	+	+
Urease-positive *V. parahaemolyticus* isolates	-	-	+	+	-	-	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+

Biochemical reactions were determined by API 20 E kit.

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 hydrolysis; SAC, sucrose hydrolysis; MEL, mellibiose hydrolysis; AMY, amygdalin hydrolysis; ARA, arabinose hydrolysis; OX, cytochrome-oxidase; +, positive reaction; -, negative reaction.

V. parahaemolyticus isolates were 2 times as high as that of *P. vulgaris*, which is a urease-positive reference strain (Fig. 2). Kim [18] reported that the urease activity of the environmental *V. parahaemolyticus* KH410 was the highest at the late exponential phase and decreased rapidly at the initial stationary phase. The result does not agree with ours.

Generally, urease is constitutively synthesized in some organisms such as *Bacillus pasteuri*, *Sporosarcina ureae*, and *Morganella morganii*, but also induced by the substrate urea in organisms such as *Proteus mirabilis* and those that bear plasmid-encoded ureases, including *Salmonella cubana*, *Providencia stuartii*, and some *E. coli* strains [27]. Kim [18] reported that the urease of environmental *V. parahaemolyticus* KH410 was induced,

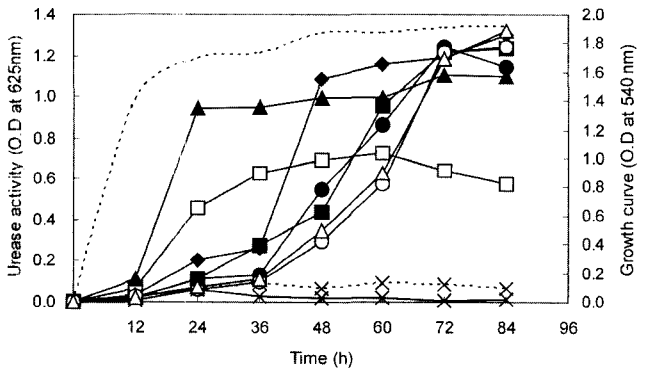

Fig. 2. Changes of urease activity of culture supernatant during the growth of *V. parahaemolyticus*.

Every strain was incubated in Luria-Bertani broth at 37°C. Dotted line with no symbol, bacterial growth curve; dotted ×, *E. coli* ATCC 25922 (a urease-negative reference strain); solid ×, *V. parahaemolyticus* KCTC 2471 (a urease-negative reference strain); open square, *P. vulgaris* ATCC 6380 (a urease-positive reference strain); open circle, *V. parahaemolyticus* YKB4 isolated from Kamak Bay; open triangle, *V. parahaemolyticus* YKB14 isolated from Kamak Bay; closed square, *V. parahaemolyticus* YFB20 isolated from Black rockfish; closed diamond, *V. parahaemolyticus* YFO21 isolated from Olive flounder; closed circle, *V. parahaemolyticus* YFO22 isolated from Olive flounder; closed triangle, *V. parahaemolyticus* S25 isolated from Gwangan Beach.

and that it is an intracellular enzyme. To investigate whether the ureases of *V. parahaemolyticus* YKB4, YKB14, YFB20, YFO21, YFO22, and S25 are intracellular or extracellular enzymes, the cells were disrupted by sonicator (Sonics & Materials Inc., Danbury, U.S.A.). However, the urease activities of the sonicated culture supernatant (30 min on ice at amplitude 10) showed the same values as the untreated culture supernatant (data not shown). Addition of urea to LB medium significantly affected the productivity of urease of *V. parahaemolyticus* isolates (data not shown). These results indicated that the ureases of *V. parahaemolyticus* YKB4, YKB14, YFB20, YFO21, YFO22, and S25 are induced and that they are extracellular enzymes.

Some recent studies have indicated that the outbreak of gastroenteritis has been caused by a type of KP-negative *V. parahaemolyticus* [10, 12, 34, 35]. The relatively rare urease-positive phenotype of *V. parahaemolyticus* is always associated with the possession of the *trh* gene [24, 34, 35, 44], making urease production a reasonably good clinical diagnostic marker for virulent (*trh*$^+$) *V. parahaemolyticus* [44]. Osawa *et al.* [35] identified only 10 urease-positive of 132 *V. parahaemolyticus* isolates from patients and from suspected food. Of the 10 strains, there were 3 strains having both *tdh* and *trh*, 3 strains having *tdh* only, 2 strains having *trh* only, and 2 strains having none. To examine the presence of *trh*/*tdh* in the 6 environmental urease-positive *V. parahaemolyticus* isolates, PCR was conducted with VPD (specific for *tdh*) and VPR (specific for *trh*) primers. No strains, however, had *trh* or *tdh* (data not shown). Therefore, these results led us to conclude that urease-positive *V. parahaemolyticus* YKB4, YKB14, YFB20, YFO21, YFO22, and S25 isolated in this study were not related to the hemolysin gene.

Pathogenesis of Infection by Environmental Urease-Positive *V. parahaemolyticus* Isolates in Mice

The virulence of urease-positive *V. parahaemolyticus* was then investigated by injecting urease-positive strains (*V.*

Table 3. Effect of *V. parahaemolyticus* infection on different pathogenic factors in mice.

	Urease			Protease		Hemolysin		None	Control
Strains	YKB4	YKB14	S25	FM39	FM50	S34	S72	FM12	Buffered saline
Mortality (%)	70	50	80	40	60	0	0	0	0
Lethal time (h)	4.5-11	9.0-12	6.0-24	4.0-13	5.0-8.0				

FM strains [39] and S strains [37] were isolated from seawater of Gwangan Beach of Busan. YKB strains were isolated from Kamak Bay of Yeosu (unpublished). Bacterial suspension (0.5 ml of 10^7-10^8 cfu/ml) was inoculated intraperitoneally into groups of 10 each of ICR mice (7- to 10-week-old male). Each test was done duplicate. Mortality indicates the ratio of numbers of dead to total 10 mice inoculated.

parahaemolyticus YKB4, YKB14, and S25), protease-positive strains (*V. parahaemolyticus* FM39 and FM50), hemolysin-positive (*V. parahaemolyticus* S34 and S72), and nonpathogenic *V. parahaemolyticus* (FM12) into mice. Thus, the cell suspensions (0.5 ml of 10^7-10^8 cfu/ml) were injected i.p. in 10 each of 10 ICR mice (7- to 10-week-old males). The lethal rates by urease-positive *V. parahaemolyticus* YKB14, YKB4, and S25 were significantly high, being 50, 70, and 80%, respectively. Protease-positive *V. parahaemolyticus* FM39 and FM50 showed 40% and 60% lethal rates, respectively. However, hemolysin-positive *V. parahaemolyticus* S34 and S72 had no mortality, similar to nonpathogenic *V. parahaemolyticus* FM12 (Table 3).

Bacterial urease plays a central role in the pathogenesis, such as urolithiasis (stone formation), catheter encrustation, pyelonephritis, ammonia encephalopathy, hepatic encephalopathy, and inactivation of complement [27]. Several investigators reported that the urease of *P. mirabilis* could cause pyelonephritis in rat or mouse [2-3, 14-15, 21-23, 28]. Unlike *P. mirabilis, Staphylococcus saprophyticus* is a frequent cause of UTI (urinary tract infection) in young, sexually active women [36]. The active gastritis due to *H. pylori* is predominantly correlated to its urease [6, 8, 9]. Our results on the virulence of urease-positive *V. parahaemolyticus* strains in mice indicated that lethal times were within 24 h postinfection, and that the vibrio strains were recovered from viscera of the injected mice. The inoculated mice generally showed maw swelled out and reddish viscera, compared to non-inoculated (data not shown). The virulence of urease-positive *V. parahaemolyticus* was much stronger than those of hemolysin or protease-positive strains.

In conclusion, the author suggests that we should have a new understanding of the urease of pathogenic *V. parahaemolyticus*, and therefore, be able to elucidate its pathogenic mechanism *in vivo/in vitro* as well as its relationship with other pathogenic factors.

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