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Virulence Characteristics of Sucrose-fermenting *Vibrio vulnificus* Strains

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We identified 6 sucrose-fermenting *Vibrio vulnificus* strains and examined their virulence characteristics. They were all encapsulated, motile, capable of producing toxins and utilizing transferrin-bound iron, cytotoxic to cultured cells, and virulent enough to kill mice. They could be definitely identified only by genetic identification methods such as PCR, and not by conventional culture-based identification methods such as API 20E (bioMérieux, France). These results indicate that it is essential to adopt genetic approaches as early as possible in order to avoid misdiagnosis of such strains, especially in clinical situations. *(Korean J Lab Med 2010;30:507-10)*

Key Words : *Vibrio vulnificus, Sucrose, Thiosulfate-citrate-bile salts-sucrose agar, PCR, rDNA sequencing*

Vibrio vulnificus causes rapidly progressing fatal septicemia and necrotizing wound infections with a high mortality rate in susceptible individuals [1]. Accordingly, a rapid and appropriate diagnosis is essential for reducing the mortality of *V. vulnificus* infections. Most *V. vulnificus* strains cannot ferment sucrose, and therefore, they form green colonies on thiosulfate-citrate-bile salts-sucrose (TCBS) agar, which is still the most widely used medium for the selection and differentiation of *Vibrio* species [2]. However, it is known that a small percentage (3-15%) of *V. vulnificus* strains can ferment sucrose and form yellow colonies on TCBS agar [3]. Nagao et al. [4] reported 2 cases of sucrose-fermenting (SF) *V. vulnificus* infection via 16S rDNA sequencing. In many clinical laboratories, a selective medium such as TCBS and a rapid identification system such as API 20E and VITEK (bioMérieux, Marcy l'Etoile, France) are generally used first; then, genetic methods such as PCR or rDNA sequencing are used. These facts suggest that SF strains may be misidentified during rapid or traditional culture-based identification processes, although the strains may be virulent enough to cause clinical infections. However, the virulence characteristics of SF strains are yet to be examined. In this study, we first identified 6 SF strains via PCR and 16S rDNA sequencing, and then, we investigated their virulence characteristics.

Through subcultures of our laboratory stock strains, 6 SF strains (Y1-Y6) showing yellow colonies on TCBS agar (BD, Franklin Lakes, NJ, USA) were finally selected. The *V. vulnificus* M06-24/O strain that forms green colonies on TCBS agar was used as the control in this study; it is a highly virulent clinical isolate and is widely used among researchers [5](Fig. 1).

No noticeable differences in colony morphology, salt

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requirement, and antibiotic susceptibility were observed among the 6 SF strains and the control M06-24/O strain (data not shown). They all formed opaque colonies on 2.5% NaCl-Heart Infusion (HI, BD) agar and grew on HI agar containing 4.0% NaCl. They were susceptible to tetracycline and ciprofloxacin, which was assayed by the routine disk diffusion method on HI agars. When their biochemical characteristics were tested by the API 20E test, no remarkable differences in the seven-digit profiles were observed among the 6 SF strains and the M06-24/O strain, with only 1 exception (YES or NO for sucrose fermentation) (Table 1). However, all SF strains could not be definitely identified (around 50% probability) as *V. vulnificus* or were misidentified as *Aeromonas* species by the API 20E test because of their ability to ferment sucrose. Dalsgaard et al. [6] reported that the API 20E test is not adequate for the identification of environmental isolates of *V. vulnificus*. Moreover, Nagao et al. [4] reported that the VITEK 2 system (bioMérieux) can also be insufficient for the identification of SF strains. Accordingly, all these observations indicate that SF strains are apt to be misidentified during the traditional culture-based identification processes.

Genomic DNA was isolated from *V. vulnificus* strains using the G-spin genomic DNA extraction kit (iNtRON Biotechnology, Seongnam, Korea). The genomic DNA was

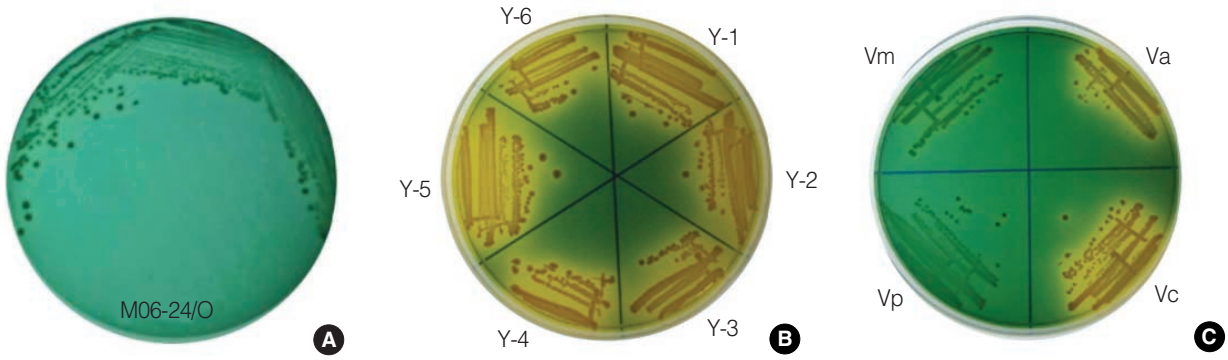

Fig. 1. Sucrose-fermenting *Vibrio vulnificus* strains forming yellow colonies on thiosulfate citrate bile salts sucrose (TCBS) agar. The M06-24/O strain (A), the 6 sucrose-fermenting *V. vulnificus* strains (Y-1 to Y-6; B), and other *Vibrio* species (C), *V. alginolyticus* ATCC17749 (Va) strain, *V. cholerae* Non O1 (Vc) strain, *V. parahaemolyticus* ATCC27519 (Vp) strain, and V. *mimicus* ATCC33653 (Vm) strain, were used. One colony of each strain grown on Heart Infusion agar was picked, streaked on TCBS agar, and incubated at 37℃ for 24 hr.

Table 1. Identification and virulence characteristics of sucrose-fermenting *Vibrio vulnificus* strains

Characteristics	Y-1	Y-2	Y-3	Y-4	Y-5	Y-6	M06-24/O
Source	E	E	C	E	E	E	C
TCBS	Y	Y	Y	Y	Y	Y	G
API20E*	5147025	5047025	5047125	5247025	5147025	5047125	5247105
vvhA	*P*	*P*	*P*	*P*	*P*	*P*	*P*
16S rDNA (%)†	100	99.8	99.9	100	99.9	99.9	100
Colony	O	O	O	O	O	O	O
VvpE	++	++	++	++	++	++	++
VvhA	++	++	++	++	++	++	++
Swarming	++	++	+‡	++	+‡	++	+++
TBI utilization	++	++	++	+‡	+‡	+‡	+++
Cytotoxicity (%)§	101±5	41±4‖	78±4‖	15±2‖	32±2‖	101±3	109±7
Mouse lethality (hr)¶	2.7±0.2	15.8±6.9‖	2.4±0.3	19.5±1.4‖	18.8±0.0‖	18.7±0.0‖	2.0±0.0

*The sixth one of the 7 digits indicates the ability to ferment sucrose; †Sequence homology with the 16S rDNA of the *V. vulnificus* ATCC27562 strain; ‡P<0.05 in ANOVA on ranks; §Mean±SE from triplicate experiments; ‖P<0.05 in one way ANOVA; ¶Mean time required for killing the five mice.
Abbreviations: E, environmental; C, clinical; TCBS, thiosulfate citrate bile salts sucrose; Y, yellow; G, green; O, opaque; TBI, transferrin-bound iron; *vvhA*, *Vibrio vulnificus* cytolysin/hemolysin gene; P, positive PCR band with the expected molecular size; +, weak positive; ++, moderate positive; +++, strong positive.

mixed with the PCR premix (iNtRon Biotechnology) containing the PCR primers 5′-ccgcggtacaggttggcgc-3′/5′-cgccacccactttcgggcc-3′ for the *V. vulnificus* hemolysin A gene (*vvhA*) [7] or 5′-agagtttgatca(c)tggctcag-3′/5′-tacggc(t)taccttgttacgactt-3′ for 16S rDNA [designed for this study]. A total of 30 cycles of PCR were performed in an Exicycler A2060 thermal cycler (Bioneer, Daejeon, Korea) under routine conditions. The amplified PCR products were visualized on a 1.0% agarose gel by staining with ethidium bromide. Sequencing of 16S rDNA was done by the double-strand dideoxy-chain termination method using the ABI 3130*xl* genetic analyzer (Applied Biosystems, Foster City, CA, USA) at the Korea Basic Science Institute. Sequence homologies were searched for in the GeneBank database using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/). The expected PCR product targeting the *vvhA* gene was amplified in all *V. vulnificus* strains (Table 1), but not in other *Vibrio* species such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus*, and *V. mimicus* (data not shown). Furthermore, the 16S rDNA sequences of all SF strains showed homologies of over 99.8% with that of the *V. vulnificus* ATCC27562 reference strain (GenBank accession no. X76333.1) (Table 1). For the final or accurate genetic identification of the *V. vulnificus* strains, especially unidentifiable by conventional bacteriological or biochemical identification methods, 16S rDNA sequencing has traditionally been used [4]. Nevertheless, PCR approaches are likely to be more suitable than 16S rDNA sequencing, especially in clinical settings that require a rapid diagnosis. For the genetic identification of *V. vulnificus* strains by PCR, the *vvhA* gene encoding *V. vulnificus* cytolysin/hemolysin (VvhA) has been being used with no noticeable problems [7].

The level of *V. vulnificus* metalloprotease (VvpE) or cytolysin (VvhA) production was measured as described in a previous study [8] and arbitrarily expressed as follows: weak (+), moderate (++), and strong (+++). All SF strains produced extracellular VvpE and VvhA (Table 1). No appreciable difference in the VvpE and VvhA production levels was observed between the 6 SF strains and the M06-24/O strain. VvpE and VvhA have been extensively studied and have a variety of biological functions, but their pathogenetic roles in *V. vulnificus* infections still remain enigmatic [1]. VvhA and VvpE are not likely to be major contributors to the virulence of *V. vulnificus*. However, VvhA was recently reported to be involved in the partial cytotoxicity by *V. vulnificus*, which can be observed only in the background when the *rtxA* gene encoding the RTX toxin responsible for contact cytolysis is mutated [9]. VvpE was reported to be involved in the swarming motility of *V. vulnificus* that may play important roles in surface adherence, colonization, and mucosal invasion of *V. vulnificus* [10].

As mentioned above, all SF strains exhibited mucoid opaque colonies on HI agar, which is indicative of the presence of capsule (Table 1). All SF strains exhibited swimming motility in semisolid agar containing 0.3% Bacto-agar (BD) and swarming motility on the surface of semisolid agar containing 0.3% Bacto-agar, which is at least indicative of the presence of flagella. The size of the swarming halo varied among the strains and appeared to be small compared to that of the M06-24/O strain. Capsule and motility (or flagella) are authentic decisive virulence factors [1].

To compare the ability of *V. vulnificus* strains to utilize transferrin-bound iron (TBI), the 6 mol/L urea-gel electrophoresis was performed, as described in our previous study [11]. Through relative comparison, the level of TBI utilization was arbitrarily expressed as weak (+), moderate (++), and strong (+++). All SF strains could utilize TBI and shift transferrin bands up from the diferric form to the monoferric form with individual variance (Table 1). Their ability to utilize TBI appeared to be slightly lower compared to that of the M06-24/O strain. In the ferrophilic bacterium *V. vulnificus*, iron-uptake systems are the well-known virulence factors because its pathogenic potential is primarily determined by its ability to survive and multiply within the host where available iron is very low [1, 11].

To compare the cytotoxic activity, HeLa P3(S) cells [12] were treated with *V. vulnificus* strains at a multiplicity of infection of 100 for 2 hr. The activity of lactate dehy-

drogenase in the supernatants was measured using the CytoTox non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA) in accordance with the manufacturer's manual. All SF strains caused cytotoxicity against HeLa P3(S) cells, although their ability to cause cytotoxicity appeared to be variable among the SF strains and to be lower than that of the M06-24/O strain (Table 1). This is indicative of the production of RTX toxin. As reported in a previous study [9], the RTX toxin is the major cytotoxic and virulence factor that affects mouse lethality.

To compare the lethality against mice, *V. vulnificus* strains (1×10^8 cells) were injected intraperitoneally into 8-week-old specific pathogen-free female ICR mice (ORIENT Bio, Seongnam, Korea), and the mice were observed for 24 hr. All SF strains killed all mice within 20 hr, although the mean time required for killing varied among the strains and was more prolonged in the SF strains than in the MO6-24/O strain (Table 1). This observation indicates that *V. vulnificus* SF strains are virulent enough to kill mice. No single virulence factor was consistent with mouse-lethality, which implies that the virulence of *V. vulnificus* is multi-factorial, consistent with previous reports [1].

In summary, *V. vulnificus* SF strains also appear to be virulent enough to kill mice. In conventional identification methods, a selective medium such as TCBS and a rapid identification system such as API 20E and VITEK are generally used first; then, genetic methods such as PCR or rDNA sequencing are used. However, our observations show that in order to avoid the misdiagnosis of virulent SF strains, it is essential to adopt genetic approaches as early as possible in the confirmative diagnosis of virulent SF strains.

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