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ORIGINAL ARTICLE

Genotypic Characterization of *Vibrio vulnificus* Clinical Isolates in Korea

Hye Sook Jeong [a,1], Jun Young Kim [b,1], Se Mi Jeon [b], Mi Sun Park [b], Seong Han Kim [b,*]

[a]*Division of Enteric and Hepatitis Virus, Center for Infectious Diseases, Korea National Institute of Health, Osong, Korea.*
[b]*Division of Enteric Bacterial Infections, Center for Infectious Diseases, Korea National Institute of Health, Osong, Korea.*

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Abstract
Objectives: *Vibrio vunificus* is known to cause septicemia and severe wound infections in patients with chronic liver diseases or an immuno-compromised condition. We carried out the molecular characterization of *V. vulnificus* isolates from human *Vibrio* septicemia cases based on pulsed-field gel electrophoresis (PFGE) using NotI and SfiI.
Methods and Results: PFGE was used to characterize a total of 78 strains from clinical cases after NotI or SfiI digestion. The geographical distribution of PFGE patterns for the strains from the southern part of Korea, a high-risk region for *Vibrio* septicemia, indicated that the isolates from southeastern Korea showed a comparatively higher degree of homology than those from southwestern Korea.
Conclusions: We report the genetic distribution of *V. vulnficus* isolated from *Vibrio* septicemia cases during 2000–2004 in Korea. This method has potential use as a subspecies-typing tool for *V. vulnificus* strains isolated from distant geographic regions.

1. Introduction

The pathogenic marine bacterium *Vibrio vulnificus* is a causative agent of food-borne diseases, including life-threatening septicemia, and possibly gastroenteritis in individuals with underlying predisposing conditions such as liver damage, excessive levels of iron, and immuno-compromised conditions [1–4]. Wound infections result from exposure to seawater or from the handling of shellfish contaminated with *V. vulnificus*. Mortality from septicemia is very high (>50%), and death may occur within one or two days after the first signs of illness [2,4–6].

In Korea, *V. vulnificus* infections mostly result from seafood consumption and occupational exposure. Seafood is commonly consumed raw or undercooked.

*Corresponding author.
E-mail: kking@korea.kr

[1]Hye Sook Jeong and Jun Young Kim contributed equally to this study.

Specimens collected from patients as well as food samples from case investigations have previously been subtyped by conventional methods by the Division of Enteric Bacterial Infections at the National Institute of Health.

As found in many other pathogenic bacteria species, *V. vulnificus* comprises a diverse array of strains. These strains exhibit remarkably variable phenotypic and genetic traits, and a wide range of virulence potential. Therefore, proper differentiation and characterization of *V. vulnificus* at the subspecies level is beneficial for epidemiological purposes. Since the first attempt to distinguish *V. vulnificus* into biotypes based on host range [7], biochemical and serological methods that define several phenotypic features have been widely used for intraspecific differentiation of the pathogen [8,9]. Although these early studies provided valuable methods for distinguishing variable phenotypic features, such techniques do not discriminate between all strains and used for primary identification or broad differentiation of the pathogen [8,9].

Several molecular methods have been developed to analyze the intraspecific diversity of *V. vulnificus,* including amplified fragment length polymorphism analysis, random amplification of polymorphic DNA, pulsed-field gel electrophoresis (PFGE), ribotyping [10−14], and multilocus sequence typing [15]. These methods have been applied to differentiate *V. vulnificus* from other *Vibrio* species or to analyze stressed cells of *V. vulnificus*, but have most frequently been used to characterize strains from different sources and regions, and to determine their genetic relatedness.

In this study, we carried out extensive analysis of the genetic variability of *V. vulnificus* isolates from human *Vibrio* septicemia cases, and characterized the seasonal and regional genotypic distribution of *V. vulnificus* strains using PFGE. Through this approach, we aimed to assess the usefulness of PFGE in a longitudinal study of isolates collected from a single defined locality. We report the genetic distribution of *V. vulnficus* isolated from *Vibrio* septicemia from 2000 to 2004 in Korea.

2. Materials and Methods

2.1. Data collection

A retrospective analysis was performed for *V. vulnificus* septicemia cases nationwide from 2000 to 2004, using the *V. vulnificus* database of the Korean National Institute of Health. Because *V. vulnificus* sepsis is categorized as a notifiable disease in Korea, the Division of Enteric Bacterial Infections has collected the demographic data and isolates of all reported cases. Collected isolates were cultured and analyzed for microbial characteristics by molecular subtyping. Based on this collected data, geographic origin and disease occurrence were studied.

2.2. Bacterial isolates

Thiosulfate citrate bile salts sucrose agar was used as a selective medium for isolation of *V. vulnificus*. For primary identification, the isolates were characterized by standard physiological and biochemical tests with an API 20E kit (bioMerieux, SA, France). Polymerase chain reaction (PCR) amplification of the *vvhA* gene, specific to *V. vulnificus*, was also used to identify the isolates.

2.3. PFGE

Genetic relatedness between isolates recovered from individuals with *V. vulnificus* sepsis was investigated using PFGE as described by Gautom with some modification [16]. Lysed plugs were digested with NotI and SfiI (New England Biolabs, Boston, MA, USA), and PFGE was performed in 1% agarose gels in 0.5 × Tris−borate−EDTA buffer at 14°C using a CHEF mapper apparatus (Bio-Rad, Richmond, CA, USA) at 6 V/cm. Linearly ramped switching times were 3.5−50 seconds for 22 hours (used NotI) or block 1 second, 2−10 seconds for 13 hours and block 2, 20−25 seconds for 6 hours (used SfiI). PFGE banding pattern analysis was performed using BioNumerics software (version 4.6; Applied Math, Kortrijk, Belgium). Analysis of banding patterns was performed using the Dice coefficient with a 1.0% tolerance for the band migration distance. Clustering of the patterns was performed using the un-weighted pair-group method with arithmetic averages.

3. Results

3.1. Prevalence of *V. vulnificus* infection

The annual number of reported cases and the estimated prevalence of *V. vulnificus* infection (per 10^6 persons) in Korea from 2000 to 2004 are shown in Figure 1. *V. vulnificus* infections increased between 2000 and 2003, with the greatest number of patients associated with this infection reported in 2003 ($n = 80$). Most reported cases (>70%) occurred in residents of the southern part of Korea. The prevalence of *V. vulnificus* demonstrated clear seasonal peaks during summer. Nearly all cases occurred from July to September, when the recorded temperature of surface seawater was between 22°C and 25°C. Therefore, the frequency of *V. vulnificus* infections was potentially related to the temperature of surface seawater. It is theorized that climatic changes associated with global warming correlates with increasing potential of *V. vulnficus* infection in Korea.

3.2. Prevalence of *V. vulnificus* patterns analyzed by PFGE

Several restriction enzymes that are commonly used for PFGE, including BglI, NotI, SfiI, SmaI, and XbaI,

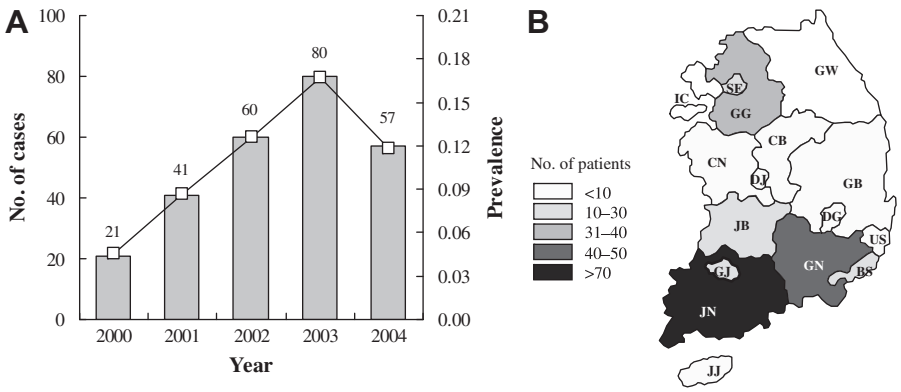

Figure 1. (A) Estimated prevalence (per 10^6 populations) and the annual number of cases of *V. vulnificus* infection reported from 2000 to 2004 in Korea. The line and triangles represent the prevalence, whereas the bars represent the number of cases. (B) Geographic distribution of *V. vulnificus* infection between 2000 and 2004. For each department, the shade of gray indicates the number of *Vibrio* septicemia patients. BS = Busan; CB = Chungcheongbuk-do; CN = Chungcheongnam-do; DG = Daegu; DJ = Daejeon; GB = Gyeongsangbuk-do; GG = Gyeonggi-do; GJ = Gwangju; GN = Gyeongsangnam-do; GW = Gangwon-do; IC = Incheon; JB = Jeollabuk-do; JJ = Jeju-do; JN = Jeollanam-do; SE = Seoul; US = Ulsan.

were initially tested in a preliminary study (data not shown). Of the 78 strains of *V. vulnificus* examined in this study, 76 were successfully characterized by PFGE after genomic DNA digestion with NotI and SfiI. PFGE analysis yielded 15−24 reproducible DNA fragments, ranging in size from approximately 40−640 kb. Use of both NotI and SfiI enzymes allowed differentiations of the 76 isolates into 68 restriction types, with similarities as low as 57.1% and 57.9%.

NotI restriction patterns were clustered, and a dendrogram for the strains examined is presented in Figure 2. Strains with similarity indices of 63.5% or more were arbitrarily grouped into three clusters designated NA, NB, and NC. Cluster NB was further subdivided into three subtypes (NB1-3). Most of the strains (60.5%) comprise cluster NB, whereas clusters NA and NC were comprised of 17.1% and 14.5% of the strains, respectively. Strains belonging to other unnamed

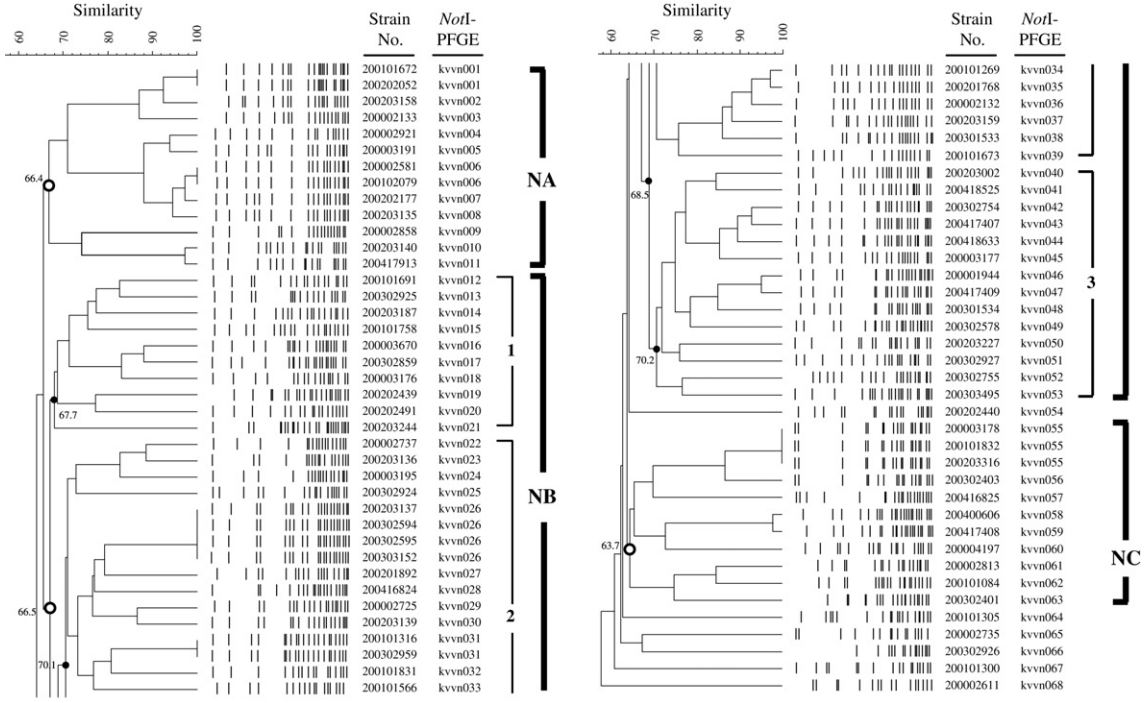

Figure 2. Dendrogram and banding patterns of NotI-digested chromosomal DNA of *V. vulnificus* strains analyzed by NotI-PFGE. Several clusters were grouped as clusters NA−NC, at similarity indices of 63.5%. PFGE = pulsed-field gel electrophoresis.

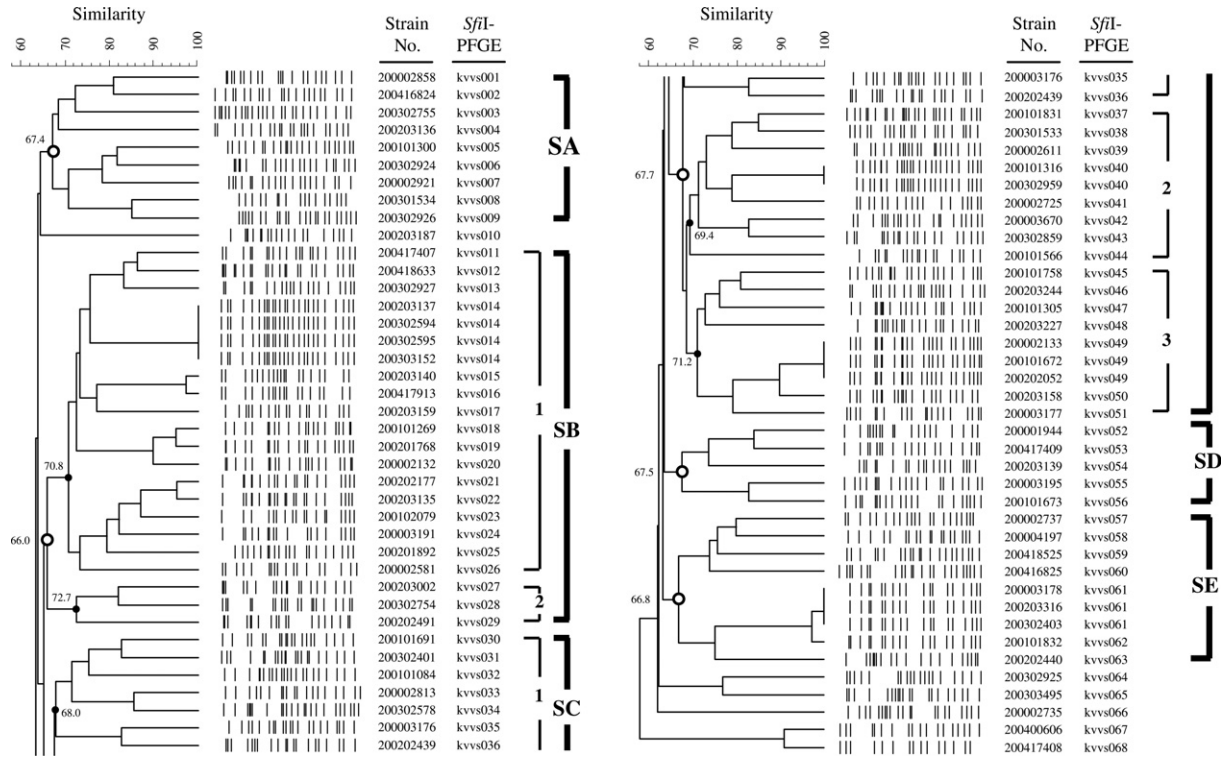

Figure 3. Dendrogram and banding patterns of SfiI-digested chromosomal DNA of *V. vulnificus* strains analyzed by SfiI-PFGE. Several clusters were grouped as clusters SA−SE, at similarity indices of 66.0%. PFGE = pulsed-field gel electrophoresis.

clusters totaled 7.9%. The subtypes (NB1-3) of cluster NB comprised 13.2%, 28.9%, and 18.4% of the strains, respectively.

Strains analyzed by PFGE after SfiI digestion also showed unique banding patterns, and the clustering of SfiI restriction patterns was similar to those analyzed by NotI (Figure 3). Strains with similarity indices of 66.0% or more were also grouped into clusters SA−SE. Clusters SB and SC were further subdivided into subtypes SB1 and 2, and subtypes SC1−3, respectively. Clusters SB and SC were the most abundant groups and comprised 22.9% and 35.5% of the strains, whereas clusters SA, SD, and SE comprised 6.6−11.8% of the strains. Subtypes SB1 and SB2 comprised 25% and 3.9% of the strains, respectively. Subtypes SC1, SC2, and SC3 each comprised 11.8% of the total isolates. Twelve strains were discriminated in a single cluster (similarity >97%) with the SfiI types SB1, SC2, SC3, and SE. The most common PFGE clusters using NotI and SfiI were NB (60.5%) and SC (35.5%), respectively.

The discriminatory powers of PFGE using NotI and SfiI were compared by examining the number of NotI-PFGE types seen among isolates with a particular SfiI-PFGE type and vice versa (data not shown). There was no significant difference between the two distributions. The broad overlap in confidence intervals suggests that the two typing methods have very similar discriminatory abilities for a variety of *V. vulnificus* strains.

3.3. Geographical distribution of *V. vulnificus* PFGE patterns

Most cases of *Vibrio* septicemia were in residents inhabiting the southern area of Korea (>70%) (Figure 1B). We compared the PFGE patterns for strains from groups divided into the high-risk southwestern (SWK) and southeastern (SEK) regions of Korea, and the similarity indices of strains in each category were calculated and compared (Table). Isolates from SEK showed a comparatively higher degree of homology than those from SWK. According to PFGE results, the regional distribution of PFGE clusters between SWK and SEK was significantly different. Cluster NC grouped by NotI-PFGE comprised 21.1% and 4.2% of the strains from SEK and SWK, respectively. Moreover, cluster SA and SE grouped by SfiI-PFGE comprised 5.3% and 13.2% of the strains from SEK, respectively, whereas cluster SA and SE consisted of 16.7% and 0% of the strains from SWK, respectively.

3.4. Changes in *V. vulnificus* patterns analyzed by PFGE from 2000 to 2004

Yearly distribution patterns of *V. vulnificus* PFGE clusters analyzed by NotI and SfiI from 2000 to 2004 are shown in Figure 4. Before 2002, the NB2 subtype among the NB clusters by NotI-PFGE was predominant, however its abundance decreased (35−11%) from 2002 to 2004, whereas the NB3 subtype increased (10.4−44.4%). The frequency of the NC cluster

Table. Percentage of *V. vulnificus* strains at selected similarity indices as analyzed by PFGE

Enzyme used	Origin of isolates[a]	Percentage of isolates at similarity level of				
		60%	70%	80%	90%	100%
NotI	Total	99	88	69	40	17
	SEK	96	88	54	21	0
	SWK	100	97	76	45	29
SfiI	Total	99	96	71	29	16
	SEK	96	83	25	21	8
	SWK	100	95	61	37	24

[a]*SEK = southeastern Korea; SWK = southwestern Korea.*

decreased from 16.6% to 5.3% from 2000 to 2002 and dramatically increased to 33.3% in 2004 (Figure 4A).

According to the SfiI-PFGE results, cluster SC was the predominant type in 2000 (38.9%) and 2001 (61.5%), but it subsequently decreased (29.4−0%) from 2002 to 2004. Before 2001, cluster SB was the minor type, but increased to become the dominant strain from 2002 to 2004 (Figure 4B). These results suggest that the distribution of PFGE patterns has changed over the period from 2000 to 2004.

4. Discussion

Residents in Korea are often exposed to marine microorganisms through the consumption of raw or undercooked seafood or during occupational exposure. The number of reported cases of infection attributable to *V. vulnificus* has increased since the first case was reported in 1982. Identification of strains of this species is therefore important in the control of infection, which can be aided by the use of a range of molecular methods that each have distinct advantages. Our study reports an extensive analysis of *V. vulnificus* clinical isolates including genotypic characterization using PFGE. This is a conventional method that has previously been used to determine the genetic diversity of clinical and environmental strains of *Vibrio* spp [17−19]. PFGE is a highly discriminatory and reproducible method, and it has been used to examine the pandemic strains of *V. cholerae* [17,20] and *V. parahaemolyticus* [18]. Since these pandemic strains are mostly derived from a single clone or from closely related clones, and since environmental strains are usually heterogenous [18,21], these clinical strains can be differentiated from their environmental strains by PFGE pattern clustering analyses [18,21]. In contrast, the genomes of both environmental and clinical *V. vulnificus* strains exhibit high intraspecific diversity, as demonstrated by PFGE [12,14] and several other methods [10,13]. Such diversity was further confirmed in our study through the PFGE with NotI or SfiI digestion of clinical strains isolated from distant geographic regions (Figure 5).

Other molecular methods may be useful for correlating genotypes with the phenotypic traits or geographic origins of *V. vulnificus* isolates. RAPD-PCR and ribotyping have been used to reveal the genetic relatedness between Spanish fish farm isolates, revealing

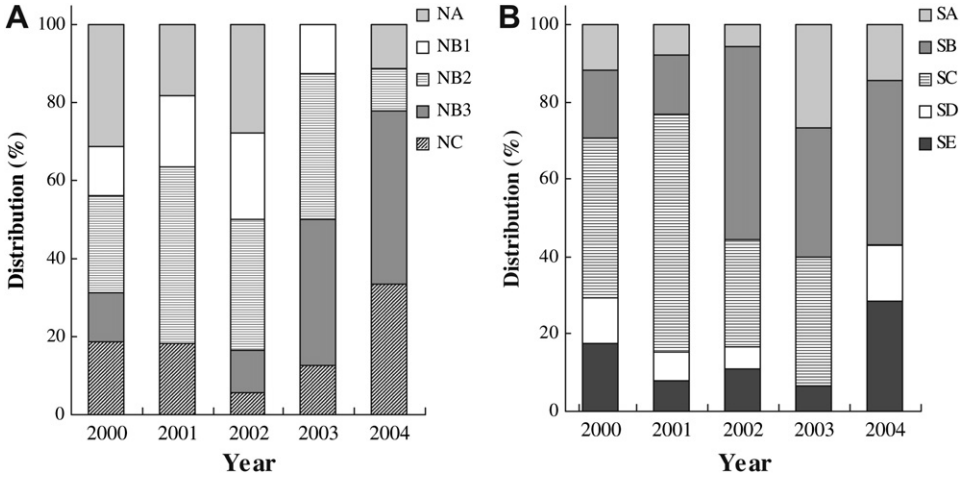

Figure 4. Yearly distributions of PFGE patterns analyzed by NotI (A) and SfiI (B) for *V. vulnificus* strains in Korea from 2000 to 2004.

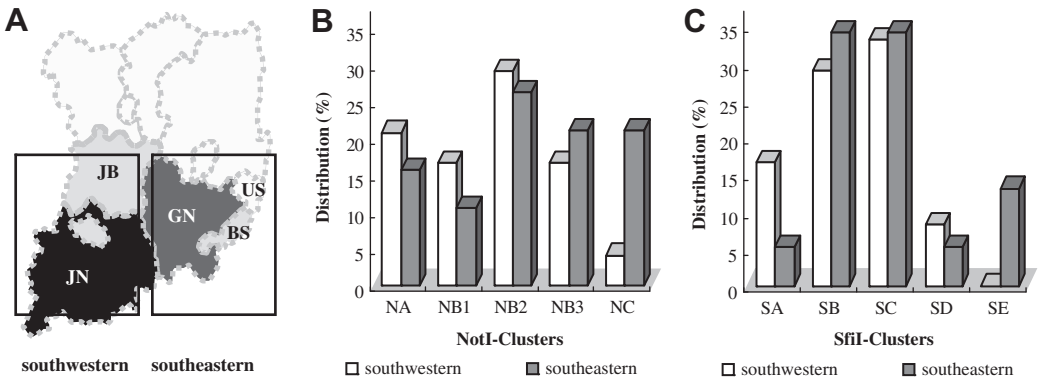

Figure 5. (A) Map of southwestern and southeastern Korea showing sampling locations for clinical isolates of *V. vulnificus*. Regional distribution of PFGE patterns by NotI (B) and SfiI (C) of *V. vulnificus* from 2000 to 2004. BS = Busan; GN = Gyeongsangnam-do; JB = Jeollabuk-do; JN = Jeollanam-do; US = Ulsan.

a close relationship between Spanish eel farm isolates, which are typically homogenous, and Mediterranean isolates of *V. vulnificus* [11]. Compared with RAPD-PCR and ribotyping, PFGE, with its higher degree of discrimination, is often unsuccessful in demonstrating a correlation between genotype and phenotype, and thus it is not frequently used for environmental research.

Since *V. vulnificus* was first recognized as a human pathogen in 1979 [22], great advances have been made in the understanding of the epidemiology this species [23]. *V. vulnificus* is commonly isolated from seawater and seafood in Korea and infections are expected to increase, with more than 100 cases already occurring annually [20,24]. The question then arises as to why more people do not become infected with *V. vulnificus*. Indeed, despite a high degree of phenotypic and genotypic heterogeneity among *V. vulnificus* strains, all putative determinants of virulence are expressed in both clinical and environmental isolates. In another study, we also performed RAPD for the differentiation of clinical and environmental *V. vulnificus* isolates. We found a common PCR product in clinical isolates, which was identified as the *nusA-IF2* gene (data not shown). It is hypothesized that there may be sequence variable region(s) or differences in the *nusA-IF2* sequence between clinical and environmental isolates, and requires further investigation.

In summary, PFGE-pattern clusters showed that clinical strains of *V. vulnificus* from SEK have a higher level of genetic homogeneity compared with those from the southwest. In future studies, we aim to investigate the association between molecular patterns by PFGE and virulence markers. This method may be used for further subspecies typing of *V. vulnificus* strains isolated from distant geographic regions.

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