Purpose:

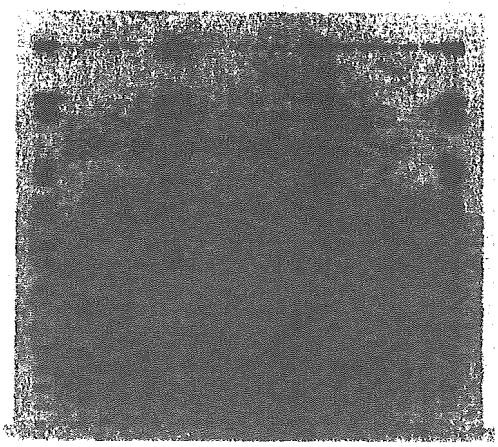
To evaluate the utility of two enzymes for PFGE subtyping of *Campylobacter,* and to generate recommendations for use of PFGE for analysis of *Campylobacter* in outbreaks investigations, and longitudinal studies

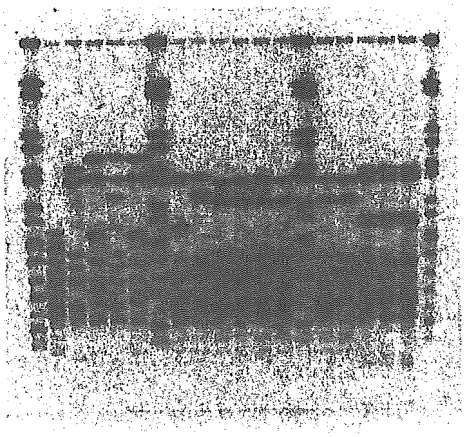
Methods:

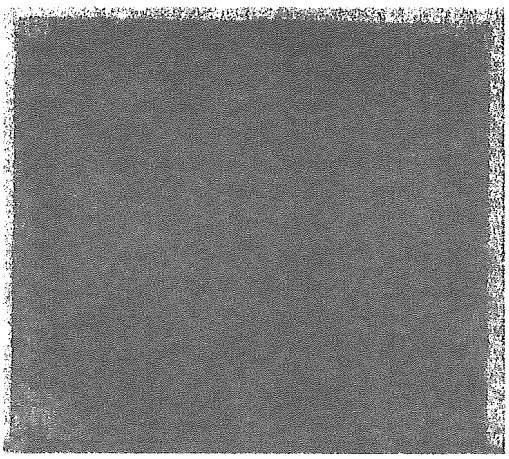
The PulseNet protocol for *Kpn*I digestion of *Campylobacter* was obtained, and available brands of enzyme evaluated on sets of isolates. The PulseNet Aotearoa New Zealand *Campylobacter* database was interrogated to examine *Sma*I and *Kpn*I profiles. Where *Kpn*I profiles were not present, additional subtyping was performed using the optimized protocol.

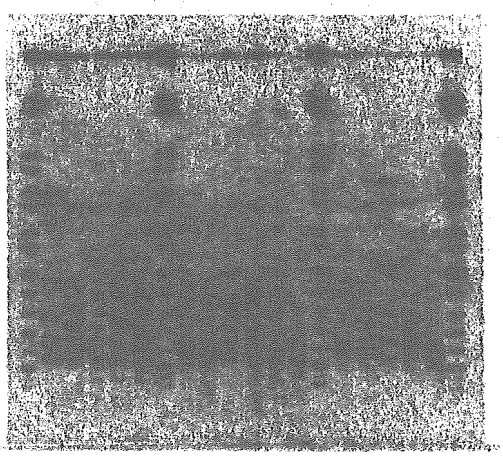
Results:

The PulseNet protocol works well, although use of *Kpn*I from NEB produced often poor results, with consistently better profiles achieved using *Kpn*I enzyme from Invitrogen. Digestions were performed for no longer than 45 minutes using 30 units of enzyme. Each set of images below has same set of plugs digested with NEB enzyme (on left) and Invitrogen enzyme (on the right).



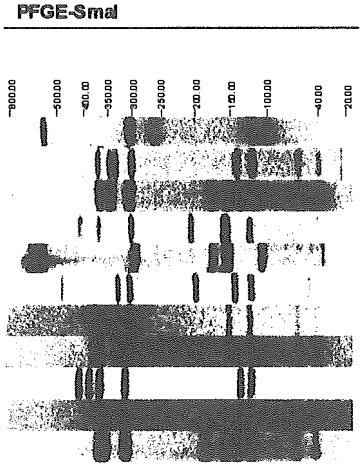






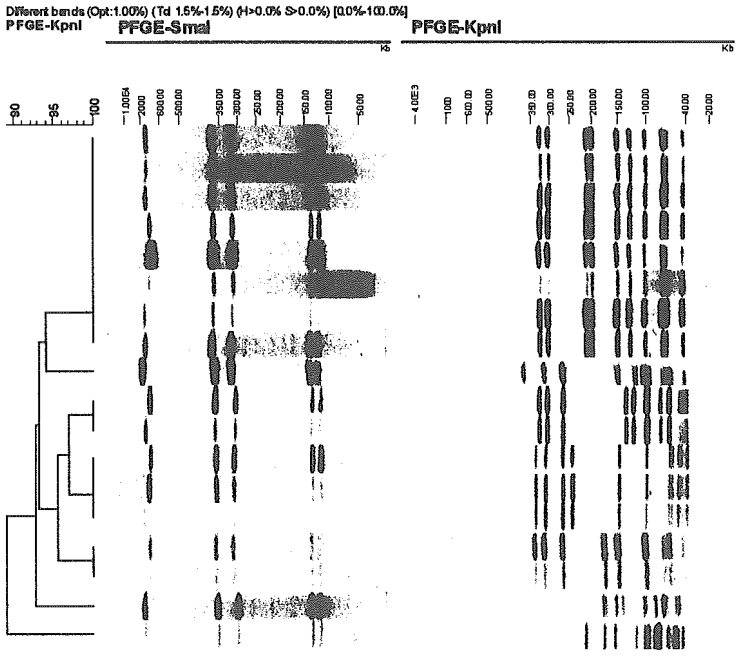
Campylobacter PFGE and the value of digestion with a second enzyme

Analysis of isolates in the PulseNet Aotearoa New Zealand *Campylobacter* database identified 271 different *Sma*I patterns. The most common *Sma*I patterns are presented in Figure 1. In contrast, 101 of the patterns are represented by only a single isolate in the database. Analysis of *Sma*I PFGE subtyping of *Campylobacter* indicates that digestion with *Sma*I alone, is sufficient to show *differences* between most isolates, but in many instances is insufficient to demonstrate *similarity* between isolates. For example the most frequent *Sma*I pattern in the database - Sm0001 consists of only five bands. Some variation is evident within this *Sma*I pattern, but this variation is insufficient to reproducibly differentiate the isolates (Figure 2, left panel). Digestion with *Kpn*I however clearly distinguishes the isolates into clonal groups, which correlate with the Penner serotypes within the Sm0001 group.



Subtype	Number of isolates in the database
Sm0001	96
Sm0050	53
Sm0037	52
Sm0021	48
Sm0028	45
Sm0024	44
Sm0009	41
Sm0046	32
Sm0002	27
Sm0081	23
Sm0036	23

Figure 1. Most common *Sma*I PFGE patterns in the *Campylobacter* database.



Animal	Sm0001	Kp0003	Untypable
Animal	Sm0001	Kp0003	55
Animal	Sm0001	Kp0003	55
Animal	Sm0001	Kp0003T	55
Animal	Sm0001	Kp0003	55
Animal	Sm0001	Kp0003	55
Animal	Sm0001	Kp0003	55
Human	Sm0001	Kp0003	55
Animal	Sm0001	Kp0031T	42
Animal	Sm0001	Kp0006T	Untypable
Animal	Sm0001	Kp0006	Untypable
Animal	Sm0001	Kp0002	27
Animal	Sm0001	Kp0002	27
Animal	Sm0001	Kp0002	27
Animal	Sm0001	Kp0001	42
Animal	Sm0001	Kp0001	42
Human	Sm0001	Kp0013T	25
Animal	Sm0001	Kp0020T	6

Figure 2. Variation within the Sm001 PFGE group

A second example of the value of *Kpn*I digestion to distinguish similar *Sma*I patterns is shown in Figure 3. *Sma*I patterns Sm0036 and Sm0098, although different, appear similar, and could be interpreted as potentially related. However digestion with *Kpn*I shows these are quite different patterns (Figure 3). Conversely Sm0013 and Sm0014 have *Sma*I patterns that are differ by only one band, and digestion with *Kpn*I confirms that these two patterns are similar (Figure 4).

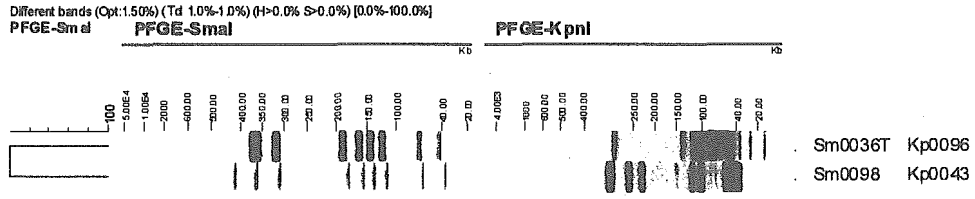


Figure 3. Visually "similar" PFGE profiles can be distinguished with second enzyme

Figure 4. *Kpn*I digestion can confirm the similarity of isolates.

Analysis of defined set of human isolates

To evaluate the potential of two enzymes for epidemiological studies, 183 isolates from two eight week periods, and from the same city were analysed by PFGE. *Sma*I PFGE patterns were generated for all 183 isolates, with 57 different patterns observed (Diversity index = 0.31). Three of the isolates would not restrict with *Kpn*I, but of the remaining 180 isolates, 71 different *Kpn*I patterns were generated (Diversity index = 0.39). When combined, 77 different *Sma*I:*Kpn*I combinations were observed (Diversity index = 0.42). Forty-nine of the isolates produced *Sma*I:*Kpn*I profiles which were observed only once in the study. These clusters showed some correlation with potentially significant demographic factors (2)

Discussion:

In this study the digestion of plugs for no more than 45 minutes with 30 units of *Kpn*I enzyme manufactured by Invitrogen was crucial to reproducible results. In other countries evaluation of enzymes from different manufacturers under local conditions

may be necessary. PFGE analysis of *Campylobacter* isolates should ideally be performed using both *Sma*I and *Kpn*I. Whenever the intention is to demonstrate that two or more isolates are indistinguishable, a second enzyme digestion should be performed. Digestion with *Kpn* Iwas almost as discriminatory as *Sma*I and *Kpn*I combined suggesting that digestion with *Kpn*I alone could be an effective approach - a conclusion also supported by Michaud et al. (3). In addition, the cost of the *Kpn*I enzyme is less than 30% of the cost of *Sma*I, reducing the overall consumable cost of PFGE with *Kpn*I to almost half of PFGE with both *Sma*I and *Kpn*I. However, even among the limited number of isolates in this study, isolates with indistinguishable or similar *Kpn*I patterns can be further subgrouped when analysed with *Sma*I. Internationally, most *Campylobacter* PFGE data has been generated using *Sma*I as the primary enzyme (PulseNet, CampyNet), partly perhaps because *Kpn*I is a more difficult enzyme to achieve reproducible results. *Sma*I digestion while less discriminatory, is sufficient in many cases to demonstrate that isolates are different. We believe that to demonstrate similarity, or that isolates are indistinguishable, digestion with two enzymes is essential, a finding also supported by other researchers (4,5). With sufficient international data, a reconsideration of using *Kpn*I as the primary enzyme could be made in the future, and the useful comparisons with existing data still made.

Reference list:

1) **Anonymous.** 2005. Notifiable and other diseases in New Zealand. Annual Report 2004. ESR Limited. http://surv.esr.cri.nz/surveillance/annual_surveillance.php

2) **Gilpin B, Cornelius A, Robson B, Boxall N, Ferguson A, Nicol C, Henderson T.** 2006. Application of pulsed-field gel electrophoresis to identify potential outbreaks of campylobacteriosis in New Zealand. J Clin Microbiol. 44:406-12.

3) **Michaud, S., S. Menard, C. Gaudreau, and R. D. Arbeit.** 2001. Comparison of *Sma*I-defined genotypes of *Campylobacter jejuni* examined by *Kpn*I: a population-based study. J. Med. Microbiol. **50:**1075-81.

4) Lindmark, H., B. Harbom, L. Thebo, L. Andersson, G. Hedin, B. Osterman, T. Lindberg, Y. Andersson, A. Westoo, and E. Olsson Engvall. 2004. Genetic characterization and antibiotic resistance of *Campylobacter* jejuni isolated from meats, water, and humans in Sweden. J. Clin. Microbiol. 42:700-706.

5) **Saito, S., J. Yatsuyanagi, S. Harata, Y. Ito, K. Shinagawa, N. Suzuki, K. Amano, and K. Enomoto.** 2005. *Campylobacter jejuni* isolated from retail poultry meat, bovine feces and bile, and human diarrheal samples in Japan: Comparison of serotypes and genotypes. FEMS Immunol. Med. Microbiol. **45:**311-9.

Publication list for this work:

1) **Gilpin, Robson, Devane and Cornelius.** Importance of using two enzymes for PFGE analysis of *Campylobacter* isolates. In preparation

Title: PFGE Standardization and molecular epidemiological study of *Vibrio vulnificus.*

Names of researchers; Shukho Kim, Hye Sook Jeong, Junyoung Kim, and Bok-Kwon Lee.

Principal Investigator; Dr. Bok-Kwon Lee.

Affiliation; Div. of Enteric Bacterial Infections, Korea National Institute of Health, Seoul, Korea.

Summary:

Vibrio vunificus (*V. vulnificus*) is a gram negative and estuarine bacterium commonly found in coastal waters and in association with shellfish and fish. This bacterium is known to cause septicemia and severe wound infections in patients with chronic liver diseases or immuno-compromised condition. In order to present current epidemiological status of *V. vulnificus* cases in Korea, monthly occurrence of clinical and environmental *V. vulnificus* and morbidity and mortality during 2001 to 2005 year were analyzed. For the PFGE standardization of *V. vulnificus* for PulseNet Korea and Asia, we had trial-and-error for the best result with several restriction enzymes and various PFGE running conditions. We also performed comparison of PFGE patterns with 13 clinical isolates and 49 environmental isolates obtained in 2005 year. We used *Not* I restriction enzyme for the digestion of genomic DNA, and the best condition of PFGE for *V. vulnificus* was initial pulsing time: 4.16 sec, final pulsing time: 40 sec, voltage: 6 v/cm, running temperature: 14℃, and running time: 18 hrs with CHEF Mapper system (Bio-Rad Laboratories, CA, USA). In addition to PFGE analysis, we performed RAPD analysis for the fine clustering of the isolates. Interestingly, 12 of 13 clinical isolates clustered into D RAPD type. However, PFGE patterns of total *V. vulnificus* isolates were more various and heterogeneous than RAPD analysis.

Purpose:

1. PFGE standardization of *Vibrio vulnificus* for PulseNet Korea and Asia.
2. Comparison of PFGE patterns of *Vibrio vulnificus* isolated from various origin.
3. Phenotypic and genotypic characterization and epidemiological study for *Vibrio vulnificus* database.

Methods:

Data Collection

A retrospective analysis was performed for *V. vulnificus* sepsis cases nationwide from 2001 to 2005, using the *V. vulnificus* database of the Korean National Institute of Health (KNIH). Because *V. vulnificus* sepsis is categorized as a class 3 notifiable disease in Korea, the laboratory of enteric pathogens in KNIH has collected the demographic data and isolates of the reported cases. Collected isolates were cultured again and analyzed for the microbial characteristics including molecular subtyping. Using these data collected from 2001 to 2005, place of residence and data of disease occurrence were studied.

Bacterial Isolates

The thiosulfate-citrate-bile salts-sucrose (TCBS) agar, as selective media, used for isolation of *V. vulnificus*. For primary identification, the isolates were characterized by conducting standard physiological and biochemical tests with an API 20E kit (Bio Meriux SA, France). PCR amplification of the *vvhA* gene, specific to *V. vulnificus*, was also used for confirmation of the isolates.

Pulsed-Field Gel Electrophoresis for genetic relatedness (PFGE).

The preparation of genomic DNA blocks, digestion with *Xba*I or *Bln*I restriction enzyme and PFGE analysis was carried out as described elsewhere.

The preparation of genomic DNA blocks and digestion with a restriction enzyme were carried out, as described by Gautom and PFGE protocols suggested by CDC. *V. vulnificus* isolates were tested firstly and analysed by using restriction enzymes *Xba* I, *Not* I or *Sfi* I (New England Biolabs, MA, USA). Typing by PFGE of genomic DNA digested with *Not* I was carried out in a CHEF Mapper system (Bio-Rad Laboratories, CA, USA). The PFGE pulsing and running conditions were changed independently for various running times and 6 Volts/cm at 14℃ for the optimization and

standardization. *Salmonella* Braenderup BAA664 was used as a molecular size marker strain. After electrophoresis, the gels were stained with ethidium bromide for 20 minutes and were photographed using Gel Doc 2000 (Bio-Rad Laboratories, CA, USA).

RAPD analysis.

Ten 10-bp oligonucleotide primers (Bioneer, Daejeon, Korea) with G+C contents of 50% were screened for the ability to provide a suitable band pattern with various *V. vulnificus* strains. The primer selected had the following sequence: 5'GGATCTGAAC3'. RAPD-PCR amplification of the DNA was performed using a AccuPower® PCR PreMix (Bioneer). The cycling profile was as follows: one cycle consisting of 94°C for 5 sec, 35 cycles consisting of 94°C for 40 sec, 43°C for 40 sec, and 72°C for 1 sec, and a final cycle consisting of 72°C for 5 sec. The RAPD products were electrophoresed at 100 V for approximately 1 h on a 2.0% agarose gel. A 100-bp plus ladder (Bioneer) was used as a molecular size marker.

Clustering of isolates

PFGE patterns and RAPD profiles of V. vulnificus isolates were visually compared and numbered in sequence according to the molecular sizes of the bands. Coefficients of dice similarity were calculated, and cluster analysis was performed with the unweighted pair group method with arithmetic averages (UPGMA) algorithm in the BioNumerics software (Applied Maths BVBA, Belgium) by using a 1.0% tolerance for the band migration distance.

Results & Discussion:

1. Epidemiological analysis.

The number of *Vibrio vulnificus* isolates from patients was obtained had a clear seasonal peak during the summer months (Figure 1, 2). The greatest frequency occurred in August or September every year. The number of notified *V. vulnificus* sepsis cases was about 80 or more yearly. Mortality of the patients was over 50% (Figure 3).

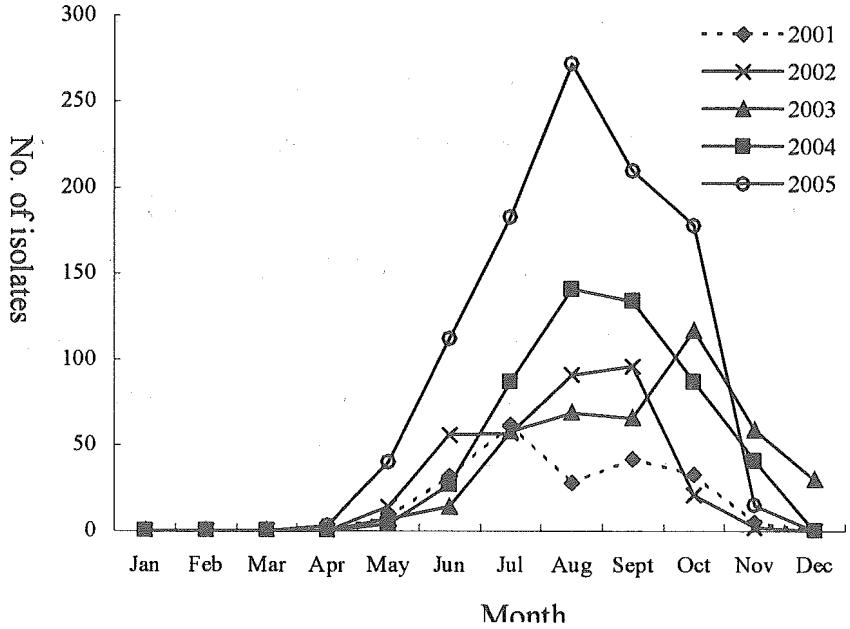


Figure 1. Monthly occurrence of environmental *Vibrio vulnificus* isolates for recent 5 yr in Korea.

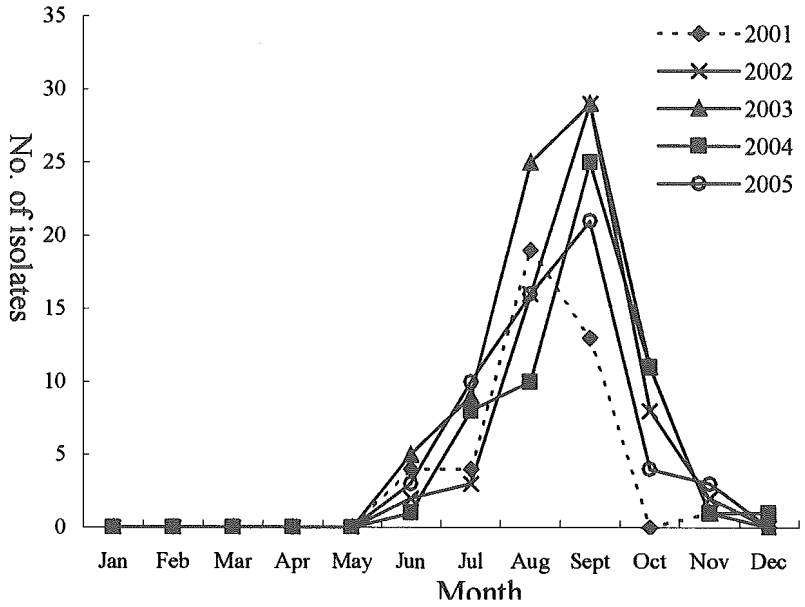


Figure 2. Monthly occurrence of *Vibrio vulnificus* sepsis for recent 5 yr

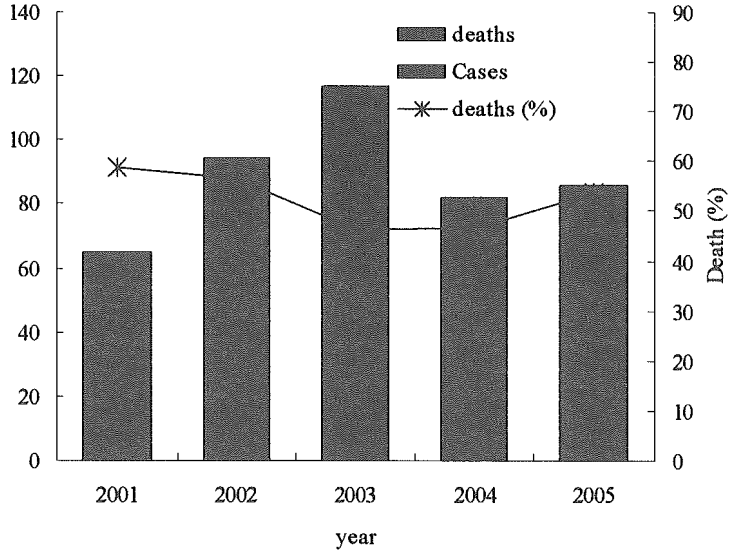


Figure 3. Occurrence and lethality of *Vibrio vulnificus* sepsis for recent 5 yr

The *Vibrio vulnificus* sepsis was 295 reports from 2001 to 2005. We received 81 (27.5%) reports of *Vibrio vulnificus* sepsis from Jeonnam, 48 (16.3%) from Gyeongnam, 33 (11.2%) from Gyeonggi (Figure 4).

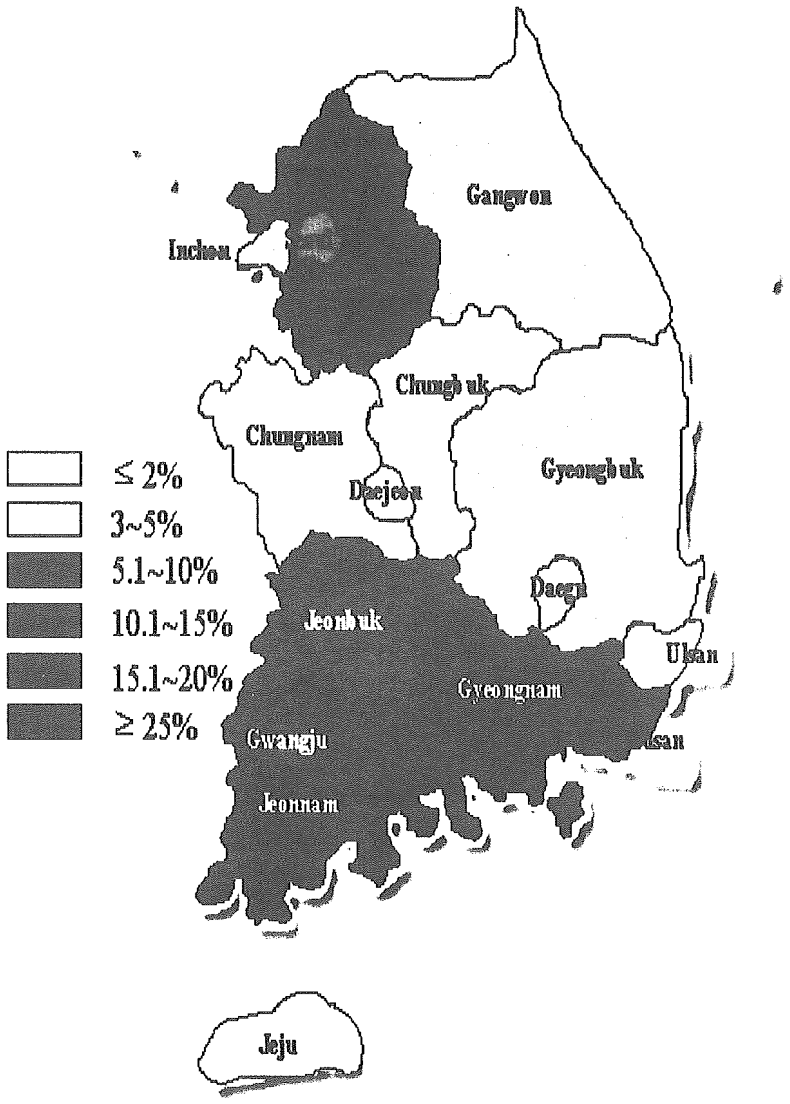


Figure 4. Map of *Vibrio vulnificus* sepsis for recent 5 years.

2. PFGE

We performed comparison of PFGE patterns with 13 clinical isolates and 49 environmental isolates obtained in 2005 year (Table 1). We used *Not* I restriction enzyme for the digestion of genomic DNA, and the best condition of PFGE for *V. vulnificus* was initial pulsing time: 4.16 sec, final pulsing time: 40 sec, voltage: 6 v/cm, running temperature: 14℃, and running time: 18 hrs with CHEF Mapper system (Bio-Rad Laboratories, CA, USA) (Figure 5).

Before optimization of the PFGE for *V. vulnificus*, *Xba* I and *Sfi* I restriction enzymes were used to digest genomic DNA, but there were too many bands to analyze them (Figure 6, 7). Based on the *Not* I PFGE patterns, dendrograms were produced. There were few indistinguishable PFGE patterns among total isolates and many various patterns (under similarity 80%)(Figure 8, 9). Even though the isolates were form clinical patients, their PFGE patterns show independent manner (Figure 10). For the molecular epidemiological analysis in specific sites, dendrograms were produced regionally (Figure 11 – 15)

Table 1. The sources of *Vibrio vulnificus* isolates used in this study

Strain	Date of Isolation	Isolation site	Source
Clinical			
05-12004	June/05	Gyeongnam	Blood
05-12354	June/05	Jeonnam	Blood
05-12520	July/05	Gyeongbuk	Blood
05-12561	July/05	Gyeonggi	Blood
05-ente-CI-1	July/05	Gyeongnam	Peritoneal dialysis fluid
05-ente-CI-2	July/05	Gyeongnam	Blood
05-14300	August/05	Chungnam	Blood
05-14301	August/05	Chungnam	Blood
05-14946	August/05	Daegu	Blood
05-17101	August/05	Jeonnam	Blood
05-17102	August/05	Jeonnam	Blood
05-15744	September/05	Jeonbuk	Blood
05-17789	September/05	Incheon	Blood
Environmental			
05-09128	April/05	Jeonnam	Sediment
05-09129	April /05	Jeonnam	Sediment
05-09404	May/05	Busan	Seawater
05-09405	May/05	Busan	Seawater
05-09485	May/05	Gyeongnam	Seawater
05-10066	May/05	Busan	Brackish water
05-11802	June/05	Chungnam	Seawater
05-11847	June/05	Incheon	Clam
05-12123	June/05	Incheon	Sea slug
05-12182	June/05	Gangwon	Seawater
05-12418	June/05	Jeonbuk	Plankton
05-12748	July/05	Jeonnam	Aquarium sea
05-12544	July/05	Jeonbuk	Foul sea
05-12749	July/05	Jeonnam	Aquarium sea
05-12858	July/05	Incheon	Clam
05-13102	July/05	Jeonbuk	Plankton
05-13881	August/05	Ulsan	Seawater
05-14564	July/05	Incheon	Aquarium sea
05-14332	August/05	Jeonbuk	Plankton
05-14938	August/05	Jeonnam	Aquarium sea
05-14939	August/05	Jeonnam	Aquarium sea
05-15557	August/05	Jeonbuk	Plankton
05-15548	August/05	Jeju	Clam
05-15668	August/05	Ulsan	Seawater
05-15677	August/05	Ulsan	Seawater
05-15735	September/05	Daegu	King of clam
05-15866	September/05	Ulsan	Seawater
05-15945	July/05	Chungnam	Seawater
05-15947	July/05	Chungnam	Sediment
05-15952	August/05	Chungnam	Sediment
05-15959	August/05	Chungnam	Seawater
05-16149	September/05	Incheon	Sediment
05-16156	September/05	Jeonbuk	Plankton
05-16945	September/05	Gyeonnam	Sediment
05-16960	September/05	Chungnam	Seawater
05-16963	September/05	Chungnam	Seawater
05-16964	September/05	Chungnam	Seawater
05-17105	September/05	Jeju	Ark shell
05-17106	September/05	Jeju	Ark shell
05-17472	October/05	Jeonbuk	Seawater
05-17473	October/05	Jeonbuk	Seawater
05-17714	October/05	Jeonbuk	Sediment
05-17715	October/05	Jeonbuk	Sediment
05-ente-275	November/05	Incheon	Sediment
05-ente-277	November/05	Incheon	Seawater
05-ente-280	November/05	Incheon	Clam
05-ente-281	November/05	Incheon	Clam
05-15955	August/05	Chungnam	Seawater

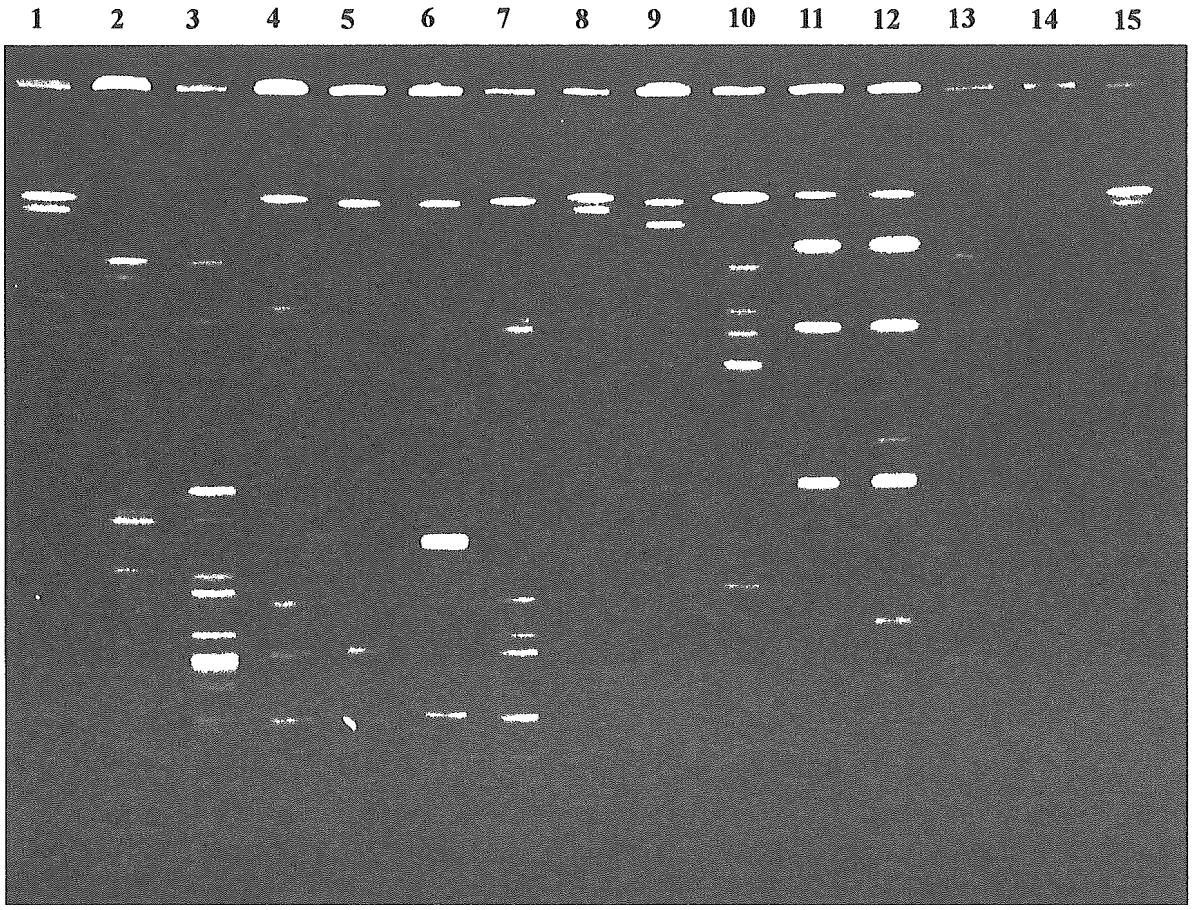


Figure 5. Representative *Not* I digested PFGE patterns of *V. vulnificus* isolates in 2005. Lane 1, 8, 15, *S.* Breanderup ATCC BAA-664; Lane 2, 05-09404; Lane 3, 05-09405; Lane 4, 05-09485; Lane 5, 05-10066; Lane 6, 05-11802; Lane 7, 05-11847; Lane 9, 05-12123; Lane 10, 05-12182 ; Lane 11, 05-12418; Lane 12, 05-12748; Lane 13, 05-12749; Lane 14, 05-12858

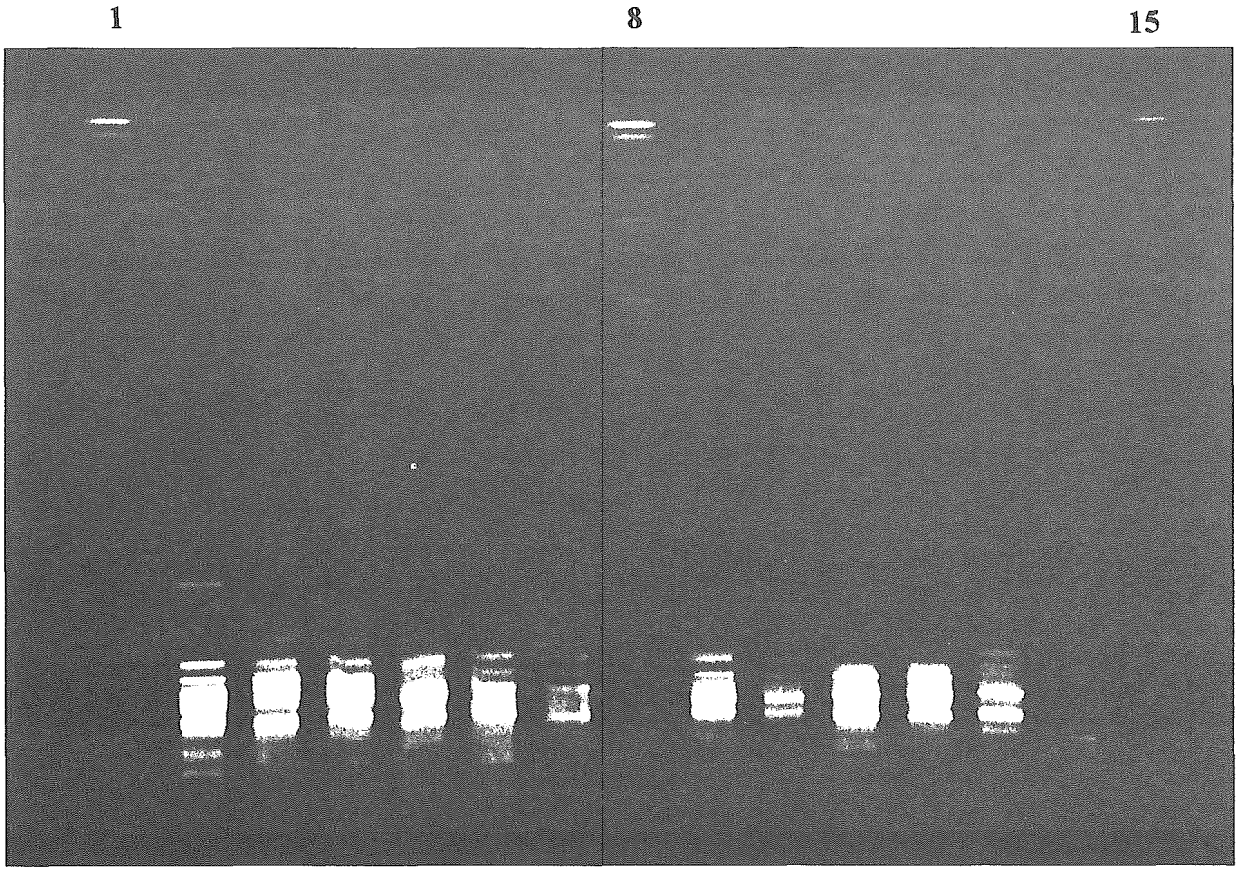


Figure 6. Representative *Xba* I digested PFGE patterns of *V. vulnificus* isolates in 2005. Lane 1, 8, 15, *S.* Breanderup ATCC BAA-664; Lane 2, 05-12748; Lane 3, 05-13102; Lane 4, 05-13881; Lane 5, 05-14939; Lane 6, 05-09128; Lane 7, 05-09129; Lane 9, 05-15945; Lane 10, 05-15947 ; Lane 11, 05-15952; Lane 12, 05-26960; Lane 13, 05-16964; Lane 14, 05-17106

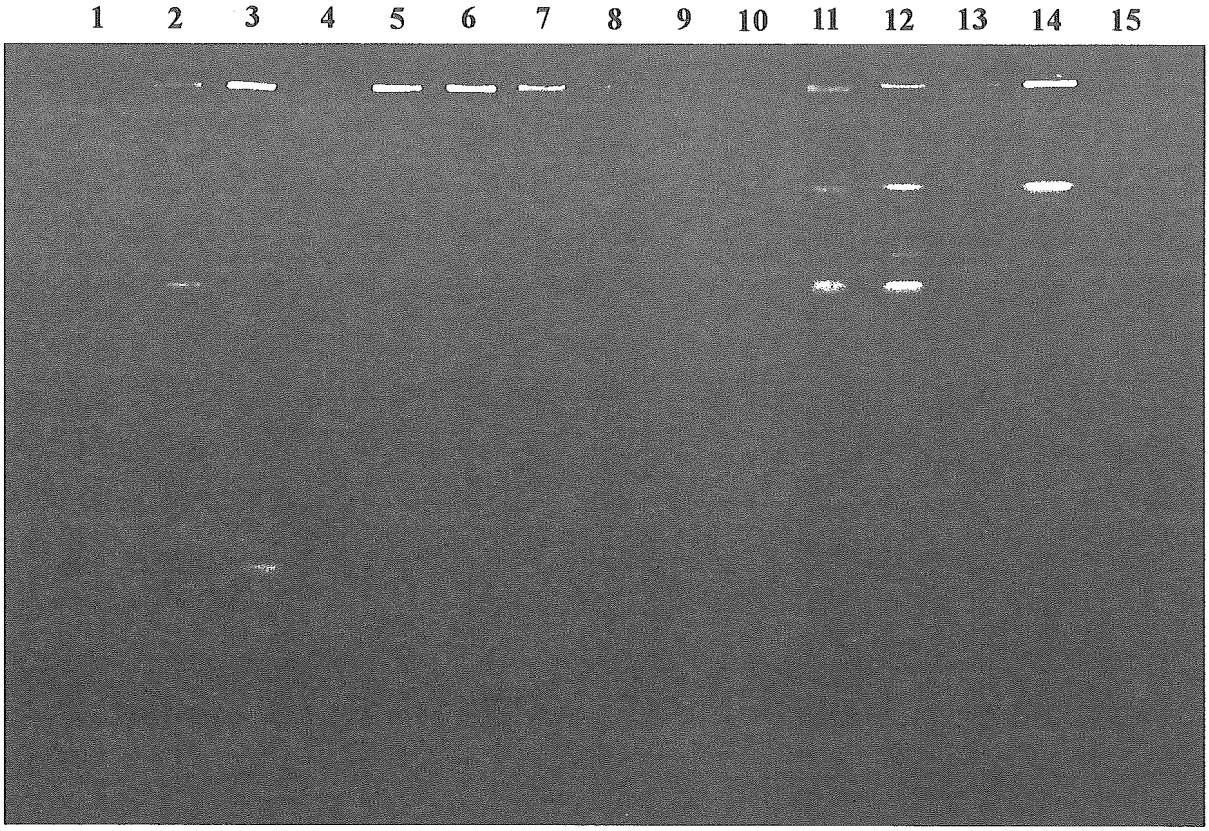


Figure 7. Representative *Sfi* I digested PFGE patterns of *V. vulnificus* isolates in 2005. Lane 1, 8, 15, *S.* Breanderup ATCC BAA-664; Lane 2, 05-12748; Lane 3, 05-13102; Lane 4, 05-13881; Lane 5, 05-14939; Lane 6, 05-09128; Lane 7, 05-09129; Lane 9, 05-15945; Lane 10, 05-15947; Lane 11, 05-15952; Lane 12, 05-26960; Lane 13, 05-16964; Lane 14, 05-17106.

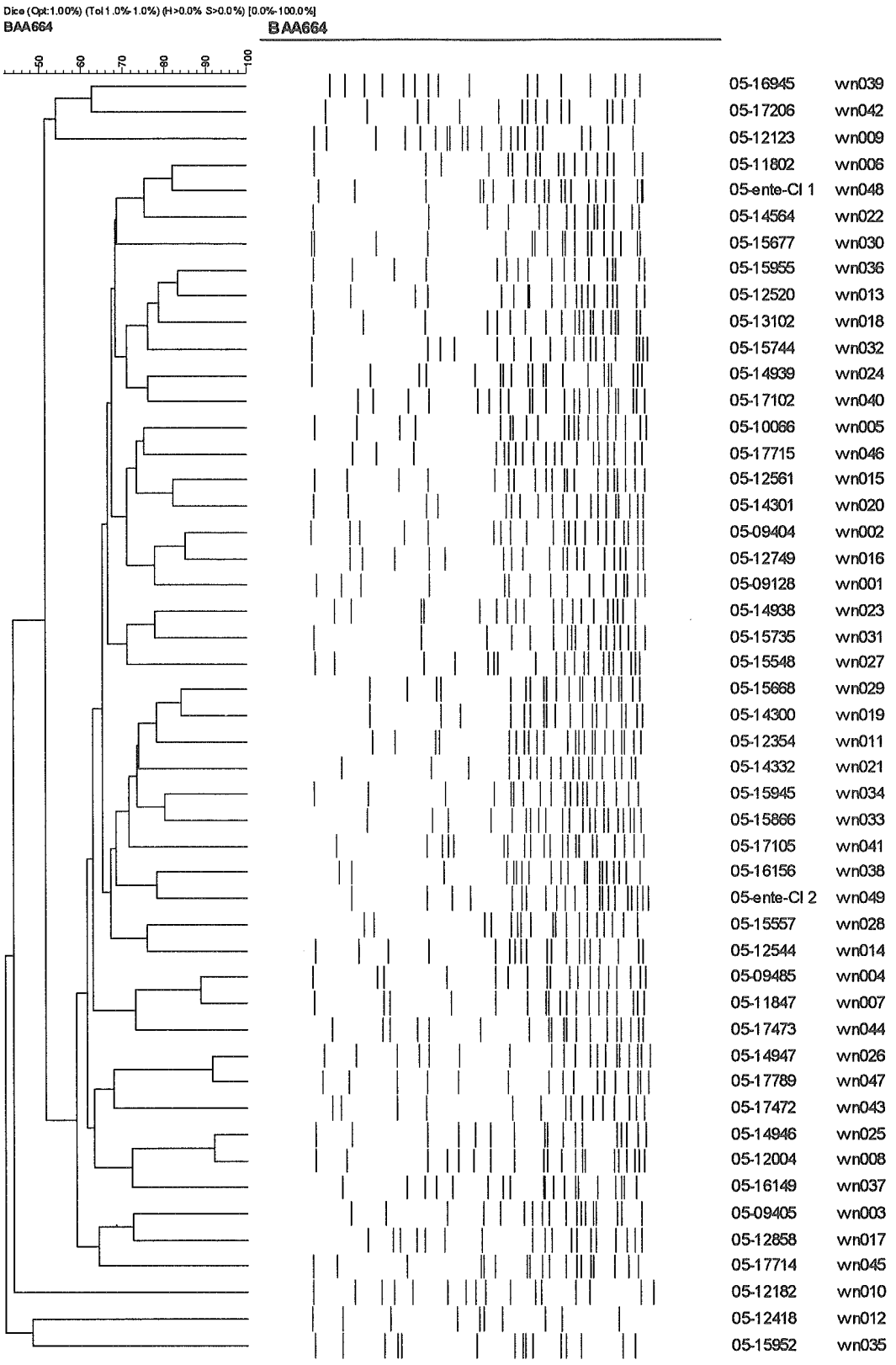


Figure 8. Dendrogram of *Not* I digested PFGE patterns of total *V. vulnificus* isolates in Korea from 2005.

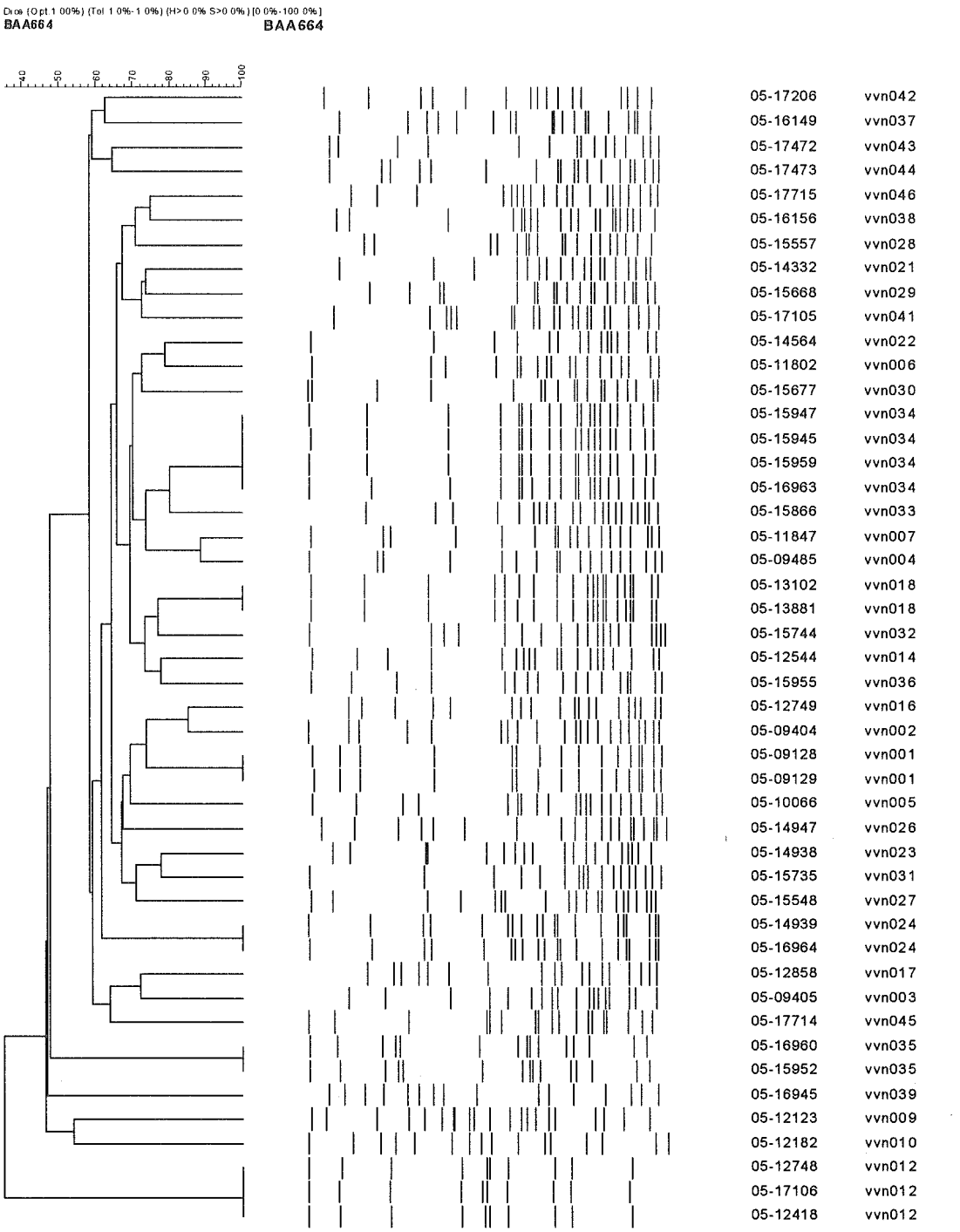


Figure 9. Dendrogram of *Not* I digested PFGE patterns of *V. vulnificus* isolates from Environmental cases in 2005.

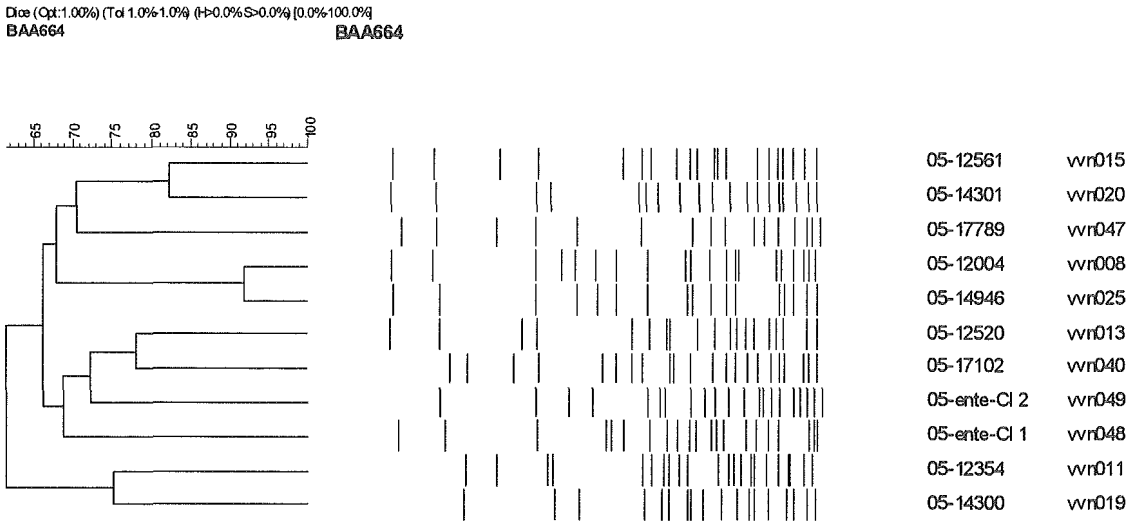


Figure 10. Dendrogram of *Not* I digested PFGE patterns of *V. vulnificus* isolates from Clinical cases in 2005.

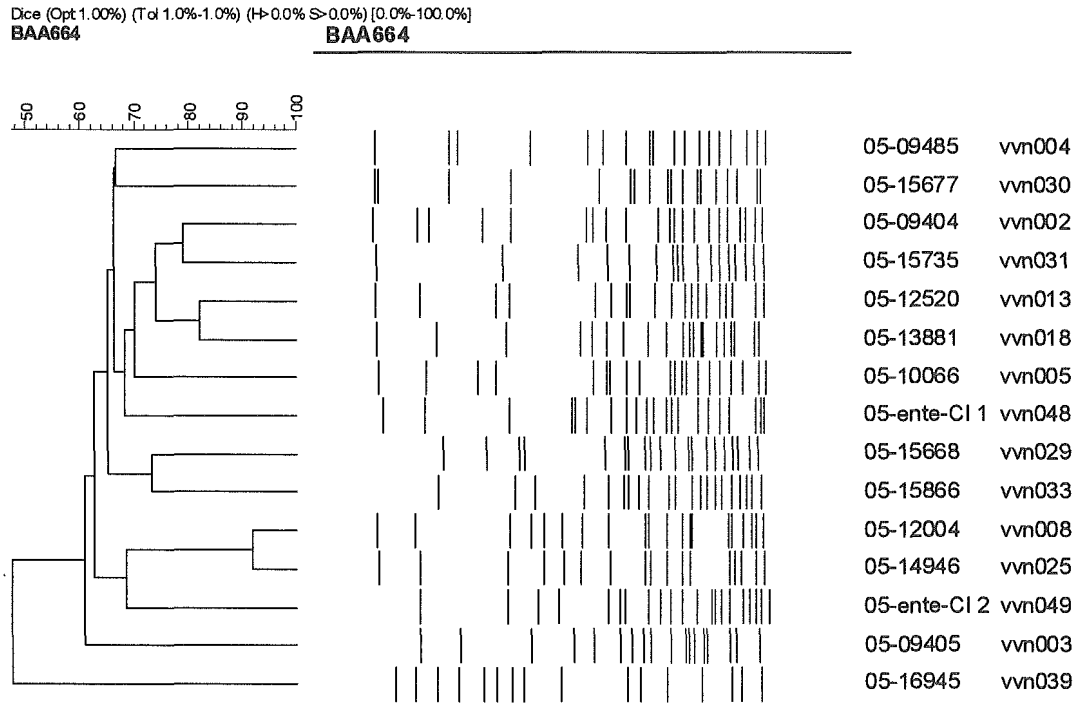


Figure 11. Dendrogram of *Not* I digested PFGE patterns of *V. vulnificus* isolates from Gyeongsang region in 2005.