Original Article

Molecular Typing of *Vibrio vulnificus* Isolated from Clinical Specimens by Pulsed-Field Gel Electrophoresis and Random Amplified Polymorphic DNA Analysis

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SUMMARY: Primary *V. vulnificus* septicemia has been continuously reported in Korea. We analyzed the molecular diversity of *V. vulnificus* strains isolated from clinical specimens using pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) analysis. We analyzed a total of 23 *V. vulnificus* strains isolated from 22 patients between 1992 and 1997. RAPD analysis was performed using five random primers, and we obtained chromosomal DNA restriction patterns with *NotI* and *SfiI* by PFGE. Two isolates from one patient disclosed similarity value 1.0 by RAPD and had an indistinguishable pattern when analyzed with PFGE, which indicated that they were the same strains. The remaining 22 isolates from 22 patients could be separated into 5 different molecular types in RAPD analysis. The range of similarity values among the isolates was wide (0.29-0.81). Of 22 strains, four strains could not be typed in repeated trials by PFGE with *NotI* and *SfiI*. The PFGE patterns of 18 strains showed a remarkable polymorphism consisting 12-19 fragments (20-870 kb). These results show that *V. vulnificus* strains isolated from Korea are genetically diversified for the most part.

INTRODUCTION

Vibrio vulnificus is a virulent *Vibrio* species associated with wound infection after exposure to contaminated sea water and with primary septicemia following consumption of contaminated seafood (1). *V. vulnificus* infection has broken out on the western and southern coastal areas of Korea every summer since 1979. The clinical presentations in Korean patients have been mostly primary septicemias, which were preceded by eating raw or undercooked seafood in patients with preexisting hepatic diseases (2, 3). Cases of wound infection have been very rare.

Serotyping has been frequently used to differentiate *V. vulnificus* strains in Korea: most of *V. vulnificus* isolates have a 04 serotype (4). But these *V. vulnificus* isolates disclosed varying degrees of biochemical reaction profiles as established by the ATB 32GN system (API systems, La Balme-Les Grottes, France) (5). The diversity of *V. vulnificus* isolates from clinical specimens in Korea was noted in our previous studies on restriction fragment length polymorphism (RFLP) (6), cellular fatty acid (CFA) compositions (7) and random amplified polymorphic DNA (RAPD) assay (8). Hoi et al. (9) and Arias et al. (10) described ribotyping, RAPD-PCR and amplified fragment length polymorphism for the intraspecific differentiation and characterization of *V. vulnificus*.

This study was based on 23 *V. vulnificus* strains from clinical specimens in 22 Korean patients during the last 5 years and a reference strain (ATCC 33815). This study was made primarily to confirm the genetic diversity of *V. vulnificus* isolates using pulsed-field gel electrophoresis (PFGE) analysis, with these results then compared with those of RAPD analysis.

MATERIALS AND METHODS

***V. vulnificus* strains:** A total of 24 *V. vulnificus* strains were analyzed in this study. Test strains consisted of 23 strains (Nos. 1-23) from patients of Chonnam University Hospital (CUH; Kwangju, Korea) (Table 1) and a *V. vulnificus* ATCC 33815 reference strain. Strains 1 and 2 were isolated from the blood and bulla of one patient. All strains except the reference strain were isolated from clinical specimens (blood and/or bulla) during the years 1992 to 1997 at CUH. Blood and bulla specimens taken from patients were primarily cultured using a BACTEC blood culture system with blood and McConkey agar plates (BBL Microbiology System, Cockesville,

Md.) at 36°C for 18 to 24 hr. The identification of isolates was accomplished using commercial identification systems (API 20E and ATB 32GN systems, La Balme-Les Grottes, France) and conventional biochemical tests, including a salt tolerance test. All strains were maintained on modified salt water-yeast extract agar.

RAPD analysis: *V. vulnificus* genomic DNA was extracted by the phenol/chloroform method. Twenty primers having 10-mer nucleotides (Operon 10-mer kit A; Operon Technologies, Atlanta, Ga.) were screened for their suitability in generating reproducible fingerprints that allowed discrimination among *V. vulnificus* isolates. Five suitable primers were selected: OPA-01, 5'-CAGGCCCTTC-3'; OPA-03, 5'-AGTCAGCCAC-3'; OPA-04, 5'-AATCGGGCTG-3'; OPA-11, 5'-CAATCGCCGT-3'; OPA-16, 5'-AGCCAGCGAA-3'. The protocol of RAPD-PCR has been previously described (11).

PFGE: The PFGE method has been previously described (12) and involved *NotI* and *SfiI* (Boehringer Mannheim GmbH, Mannheim, Germany) restriction enzymes in each strain. Restriction fragments were separated in a 1% fast-lane agarose gel (FMC, Rockland, Maine) in 0.5x TBE (1x TBE is 89 mM Tris base, 89 mM boric acid and 2.5 mM disodium EDTA) by using a CHEF-DR II system (Bio-Rad, Richmond, Calif.). Bacteriophage lambda DNA ladders (FMC, Rockland, Maine) were used as molecular mass standards. DNA fragment sizes were visualized by ethidium bromide (2 µg/ml in water) under UV transillumination. *V. vulnificus* isolates were separated into patterns on the basis of differences in band arrangements. Differences in the presence, absence or intensity of a band among strains were given equal weights.

Analysis of RAPD and PFGE patterns: Statistical analysis of RAPD and PFGE results was performed by the NTSYS-pc (numerical taxonomy system and multivariate analysis system, version 1.50, Applied Biostatistics Inc., Calif.) program to compose the phenogram for the differentiation of *V. vulnificus* strains. The presence or absence of bands was recorded in binary scores (1 or 0) to compose the data matrix that was used by the NTSYS-pc program to produce the dendrogram and genetic similarity (S) values. The genetic similarity was obtained from the distance (D) values using the above program. D is expressed as $1 - 2C_{xy} / (U_x + U_y + 2C_{xy})$ where C_{xy} equals the number of fragments in common to isolates X and Y. U_x and U_y represent the number of unique bands in isolates X and Y. The S value is expressed as $1 - D$.

RESULTS

Molecular typing by RAPD pattern: The isolates were divided

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Table 1. Epidemiological and clinical features of subjects infected with *V. vulnificus*

No.	Date	Sex	Age	Area	Preexisting disease	Source	Clinical manifestation	Prognosis
1	92.9	M	53	Hae-nam	Alcoholic liver ds	Crab	Sepsis, skin lesions	Improved
2	isolated from skin lesion (bulla) of patient 1							
3	93.5	M	32	Kwang-ju	GB stone, cholecystitis	Cockles	Skin lesions	Improved
4	93.8	M	52	Young-Gwang	Alcoholic liver ds	Clams	Sepsis, skin lesions	Death
5	93.8	M	51	Kwang-yang	Alcoholic liver ds	Sea fish	Sepsis, skin lesions	Death
6	94.8	M	40	Dae-chon	Unknown	Clams	Sepsis, skin lesions	Death
7	95.5	M	54	Dam-yang	Liver cirrhosis	Crab	Sepsis, skin lesions	Improved
8	95.7	M	43	Hae-nam	Alcoholic liver ds	Crab	Sepsis, skin lesions	Improved
9	95.7	M	49	Young-am	Alcoholic liver ds	Unknown	Skin lesions	Improved
10	95.8	M	79	Kwang-ju	Alcoholic liver ds	Crab	Sepsis, skin lesions	Improved
11	95.8	M	61	Young-am	Alcoholic liver ds	Sea fish	Sepsis, skin lesions	Death
12	95.8	M	58	Gun-san	Unknown	Unknown	Sepsis, skin lesions	Death
13	95.8	M	76	Sun-chon	Alcoholic liver ds	Crab	Sepsis, skin lesions	Death
14	95.9	M	38	Gang-jin	Alcoholic liver ds	Sea fish	Sepsis, skin lesions	Death
15	95.9	M	65	Bo-sung	Liver cirrhosis	Oyster	Sepsis, skin lesions	Death
16	95.9	M	53	Sun-chon	Liver cirrhosis	Sea fish	Sepsis, skin lesions	Improved
17	96.7	M	55	Jin-do	Alcoholic liver ds	Sea fish	Sepsis, skin lesions	Death
18	97.6	M	54	Sun-chon	Unknown	Unknown	Sepsis, skin lesions	Death
19	97.6	M	56	Bo-sung	Liver cirrhosis	Unknown	Sepsis, skin lesions	Death
20	97.7	M	44	Kwang-ju	Hepatitis (HBV)	Crab	Sepsis, skin lesions	Death
21	97.7	M	59	Bo-sung	Liver ds	Crab	Sepsis, skin lesions	Improved
22	97.8	M	59	Jin-do	Liver ds	Unknown	Sepsis, skin lesions	Improved
23	97.8	M	36	Shin-an	Alcoholic liver ds	Sea fish	Sepsis, skin lesions	Improved

No., number of strains; M, male; ds, disease; GB, gall bladder.

Fig. 1. Dendrogram showing estimates of the similarity values among 24 *V. vulnificus* strains by using the summed results of the RAPD assays with the five primers OPA-01, OPA-03, OPA-04, OPA-11 and OPA-16.

into five molecular types (A-E) by RAPD analysis at S value of 0.55; 12 strains (52%) belonged to type B, 5 strains (22%) to type A, 4 strains (17%) to type D and types C and E included one strain each. Strains 1 and 2 isolated from the same patients were assigned to type A. The *V. vulnificus* ATCC 33815 reference strain pertained to type E (Fig. 1). The distribution of genetic S values was 0.29 to

1.00 in all isolates, which represented a wide range of S values. The S value between strain 1 and 2 from one patient was 1.00, which indicated that the two strains were the same at the genetic level.

PFGE: The restriction endonuclease analysis (REA) patterns generated by PFGE after *NotI* digestion gave suitable numbers of well-separated fragments compared to these produced by *SfiI*

digestion. PFGE analysis of the digested DNA yielded 12 to 19 fragments ranging in size from 20 to 870 kb (Fig. 2, 3). The REA patterns were obtained in 20 strains (83%), including the reference strain, but 4 strains failed to get PFGE patterns in spite of the repeated trials. There were complete matches of the DNA fragment profiles in two isolates (strains 1 and 2) from the same patient, so these isolates were considered to be genetically identical. A total of 19 isolates of *V. vulnificus* generated REA patterns which represented 18 different PFGE patterns. Most of the isolates showed differences in three or more DNA bands from each other, and the PFGE pattern of the reference strain also differed from those of the isolates (Fig. 2).

DISCUSSION

We reported the results of RFLP, CFA and RAPD analysis of *V. vulnificus* isolates for the strain differentiation and epidemiological study of *V. vulnificus* infection in Korea (6, 7, 8). These previous studies revealed a high level of genetic diversity and diverse biotypes in the same serotypes of *V. vulnificus* isolates. For that reason, we preferred to use molecular methods and cluster analysis of CFA composition instead of serotyping for our epidemiological study. RAPD and PFGE analysis of *V. vulnificus* strains showed a wide range of S values and a high level genetic diversity in this study. The range of genetic S values among *V. vulnificus* isolates was 0.29 to 0.81 in RAPD analysis.

RAPD analysis is conducted at low annealing temperatures, which

allows for mismatches and thus permits arbitrary primer sequences to bind nonspecifically as well as specifically to the DNA template (13). The resulting profile is thus a combination of artifactual variation mixed with true polymorphism (14, 15). We compared RAPD and PFGE analysis for molecular typing of *V. vulnificus*; our results indicated that PFGE analysis had better discriminative power than did RAPD analysis without the use of many primers, but the typeability of *V. vulnificus* isolates by RAPD and PFGE was 100% and 83% (20/24), respectively.

Buchrieser et al. (16) performed PFGE analysis for differentiation of *V. vulnificus* strains isolated from oysters. A PFGE pattern was not achieved in 20% of the isolates, which was similar to the failure rate in our study. On the study of strain differentiation of 46 *Clostridium difficile*, three strains failed to generate PFGE patterns (17). This phenomenon is explained by the degradation of genomic DNA and methylation of the action site of the endonuclease (16, 18). For the purpose of molecular differentiation of *V. vulnificus*, the combination of PFGE and RAPD analysis is recommended. Nineteen *V. vulnificus* isolates generated REA patterns which represented 18 different PFGE patterns. The isolates showed a difference of three or more DNA bands, so the isolates were considered to be genetically different strains according to the interpretation of PFGE patterns (19).

The isolates were divided into five molecular types (A-E) by RAPD analysis. Strains 1 and 2 showed identical PFGE and RAPD patterns with 1.00 of S values between them, so these organisms were judged to be the same strain. Strains which did not show any restriction fragment belonged to RAPD type D (2 strains), type B (1 strain) and type A (1 strain). They also might be genetically different. Strains 5 and 11, which were isolated in Kwang-yang and Young-am, differed by three bands in PFGE analysis but belonged to RAPD type B with a 0.80 S value. Strains 10 and 22, which differed by three bands in PFGE analysis, represented RAPD type B with a 0.74 S value.

In summary, analysis by PFGE and RAPD of *V. vulnificus* strains that isolated from various sources in Korea disclosed a high level of genetic diversity in general.

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Fig. 2. DNA restriction patterns of *V. vulnificus* as determined by CHEF after cleavage with *Not*I. Lane M, size marker; lanes 1 through 23, clinical isolates; lane 24, *V. vulnificus* ATCC 33815.

Fig. 3. Schematic drawing of PFGE patterns shown in Fig. 2. Lane M, size marker; lane ATCC, *V. vulnificus* ATCC 33815.

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