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Pulsed-Field Gel Electrophoresis-Based Molecular Typing Reveals a Shift in the Major Type of *Vibrio cholerae* O1 Isolated in Korea

KIM, SEONGHAN, JUNYOUNG KIM, YEONHO KANG, YONGKEUN PARK[1], AND BOKKWON LEE*

Division of Enteric Bacterial Infections, Centers for Infections Disease, Korea, National Institute of Health, Seoul 122-701, Korea
[1]Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

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Abstract *Vibrio cholerae* O1 El Tor isolates (n=242), collected in Korea between 1991 and 2002, were classified by NotI-digested pulsed-field gel electrophoresis (PFGE). The major types were A1 before 1998 and B after 1999, among both domestic and imported cases. The prevalent PFGE types among domestic cases were consistent with the prevalent types among imported cases in the same year. These results suggest a close relationship between the domestic and imported cases of cholera in Korea.

Key words: *Vibrio cholerae*, pulsed-field gel eletrophoresis

Since the beginning of the seventh cholera pandemic caused by *Vibrio cholerae* O1 El Tor, most cases of cholera in Korea were restricted mainly to cases imported from countries in which cholera is endemic, especially those of southeast Asia. Recently, however, imported cases have gradually decreased, and domestic cases of the disease in patients with no recent experience of travel abroad have increased and frequent outbreaks of cholera have occurred (Table 1). Domestic cases of cholera were reported for three consecutive years from 1995 to 1997, in the same region [5, 8]. In 2001, 142 cases occurred within a month during an epidemic outbreak in the southeastern part of Korea. Therefore, the possibility that *V. cholerae* O1 exists in the coastal regions of Korea was raised.

Pulsed-field gel electrophoresis (PFGE) analysis is the most powerful tool with which to analyze genetic relationships among *V. cholerae* strains [2]. In this study, *V. cholerae* O1 El Tor strains isolated in Korea were analyzed by antibiotic susceptibility test and PFGE-based molecular typing to elucidate the origins of these strains in domestic cases of disease. A total of 242 *V. cholerae* O1 strains, isolated from 1991 to 2002 in Korea, were examined: 141 strains from domestic cases, 92 strains from imported cases, and 9 strains isolated from the environment in Korea.

Table 1. Distribution of domestic cholera cases by year in Korea.

Year	No. of cases	Patient residence (No. of patients)
1991	113	Chungnam (59), Jeonbuk (22), Gyeongnam (19), Seoul (10), Busan (3)
1995	68	Incheon (Gangwha) (25 (11)), Chungnam (25), Gangwon (6), Gyeongbuk (5), Gyeonggi (4), Daejeon (2), Busan (1)
1996	2	Gangwha (2)
1997	10	Gangwha (10)
1999	3	Jeonnam (3)
2001	142	Gyeongbuk (90), Daegu (20), Gyeongnam (18), Busan (6), Gyeonggi (3), Seoul (1), Ulsan (1), Chungnam (1)

Susceptibilities to the antibiotics ampicillin (AM, 10 μg), chloramphenicol (C, 10 μg), doxycycline (DOX, 30 μg), gentamicin (GM, 10 μg), kanamycin (K, 30 μg), nalidixic acid (NA, 30 μg), streptomycin (S, 10 μg), tetracycline (TET, 30 μg), and trimethoprim/sulfamethoxazole (SXT, 23.75 μg/1.25 μg) were determined using the disk diffusion method [7]. *Escherichia coli* ATCC25922 was used as the control strain.

To identify class I integrons, primers qacEΔ1 (5'-ATC GCA ATA GTT GGC GAA GT-3') and sul1 (5'-GCA AGG CGG AAA CCC GCG CC-3'), which are specific for the 3' conserved segment (CS), were used to amplify the 3' CS region of class I integrons by polymerase chain reaction (PCR), as described previously [1]. To identify SXT elements, primers that produce a 592-bp internal fragment of the integrase of the SXT element, sxt1 (5'-GCT GGA TAG

*Corresponding author
Phone: 82-2-380-1462; Fax: 82-2-352-4767;
E-mail: kkingsh@chol.com

GTT AAG GGC GG-3') and sxt2 (5'-CTC TAT GGG CAC TGT CCA CAT TG-3') were used [3].

PFGE was performed as described by Gautom [4], with modifications. Briefly, for the preparation of plugs, bacterial cells were suspended in cell suspension TE buffer (100 mM Tris and 100 mM EDTA, pH 7.5), and mixed with equal volumes of 1.2% SeaKem Gold Agarose (BioWhittaker, ME, U.S.A.). Plugs were lysed with ES buffer (0.5 M EDTA [pH 9.0] and 1% sodium lauroylsarcosine) containing proteinase K. Lysed plugs were digested with 30 units of NotI (New England Biolabs, Boston, MA, U.S.A.), and PFGE was performed on 1% agarose gel in 0.5× Tris-borate-EDTA buffer at 14°C using a CHEF mapper apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A.) at 6 V/cm with a linearly ramped switching time of 1–25 s for 15 h. To investigate the chromosomal position of the SXT element, chromosomal DNAs were transferred from the PFGE gel to a nylon membrane by the method of Southern [9] and hybridized with a peroxidase-labeled 592-bp SXT integrase fragment with the ECL direct nucleic acid labeling and detection systems (Amersham Biosciences Buckinghamshire, U.K.).

Computer-assisted analysis of the clonal relatedness of the PFGE banding patterns was performed with Fingerprinting II Informatix software (Bio-Rad). Analysis of banding patterns was performed with the Dice coefficient and the patterns were clustered using the unweighted pair group method with arithmetic averages.

All the tested strains were susceptible to AM, C, DOX, GM, K, NA, and TE; 128 strains showed resistance to S, and six strains, all isolated only in 1999, were resistant to S and SXT. A similar pattern has been reported in another study [6]. Although resistance to S was neither characteristic of any specific area from which a strain was isolated nor of the country of import, it correlated closely with the year of isolation and the PFGE type.

The NotI-digested PFGE patterns are summarized in Tables 2 and 3. We categorized NotI-digested PFGE patterns into 13 types, A to L. Those PFGE patterns classified as same type showed more than 85% relatedness between strains (Fig. 1). Types A, B, C, and G were subdivided into 5, 9, 3, and 3 subtypes, respectively.

Among these 242 strains, the most prevalent type was A (64.0%, 155 of 242). Among the 155 type A strains, 113 were subtype A1, which is one of five type A subtypes. The other major type was B (28.9%, 70 of 242 strains). Similar tendencies were observed among both domestic and imported cases.

Until 1998, all 79 domestic cases belonged to type A, and around 90% (71 of 79) of imported cases were also type A. Since 1999, the newly emerged type B prevailed among both domestic (100%) and imported cases (61.5%, 8 of 13), and type A strains were isolated from only five imported cases. Imported strains were isolated from patients who had been to Thailand, Indonesia, The Philippines, Vietnam, or Hong Kong. Subtype A1 strains were isolated

Table 2. PFGE banding patterns of *Vibrio cholerae* O1 domestic isolates examined in this study.

Type	1991	1995	1996	1997	1998	1999	2001	2002	Total
A1	17	27	2	3	-	-	-	-	49
A2	-	26	-	-	-	-	-	-	26
A3	-	2	-	1	-	-	-	-	3
A4	1	-	-	-	-	-	-	-	1
B1	-	-	-	-	-	-	7	2	9
B2	-	-	-	-	-	-	29	-	29
B3	-	-	-	-	-	-	9	-	9
B4	-	-	-	-	-	-	1	-	1
B5	-	-	-	-	-	-	2	-	2
B6	-	-	-	-	-	-	6	-	6
B7	-	-	-	-	-	-	2	-	2
B8	-	-	-	-	-	4	-	-	4
G1	-	-	-	-	*1	-	-	-	1
G2	-	-	-	-	-	-	-	*1	1
G3	-	-	-	-	*1	-	-	-	1
H	-	-	-	*1	-	-	-	-	1
I	-	-	-	-	-	-	*1	-	1
J	-	-	-	-	-	-	-	*1	1
K	-	-	-	-	-	-	*1	-	1
L	-	-	-	-	-	-	-	*2	2
Total	18	55	2	5	2	4	58	6	150

*Strains isolated from environmental specimens.

Table 3. PFGE banding patterns of *Vibrio cholerae* O1 imported isolates examined in this study.

Type	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	Total
A1	–	6	25	22	4	3	1	–	1	2	–	64
A3	–	–	1	1	1	–	–	–	–	–	1	4
A4	–	–	–	–	1	–	–	–	–	–	–	1
A5	3	–	1	–	1	–	1	–	–	–	1	7
B1	–	–	–	–	–	–	–	–	–	4	1	5
B5	–	–	–	–	–	–	–	–	–	1	–	1
B9	–	–	–	–	–	–	–	2	–	–	–	2
C1	2	–	–	–	–	–	–	–	–	–	–	2
C2	2	–	–	–	–		–	–	–	–	–	2
C3	–	–	1	–	–	–	–	–	–	–		1
D	–	–	1	–	–	–	–	–	–	–	–	1
E	–	–	1	–	–	–	–	–	–	–	–	1
F	–	–	1	–	–	–	–	–	–	–	–	1
Total	7	6	31	23	7	3	2	2	1	7	3	92

from tourists who had visited the countries listed above. The subtype A5 strain was isolated from those who had travelled to Thailand, Indonesia, The Philippines, or Hong Kong. Subtypes A3 and A4 were only isolated from patients who had travelled to The Philippines or Thailand. Among the type B strains, two cases each of subtypes B1 and B9 were isolated from travellers to Thailand, three cases of B1 from travellers to The Philippines, and one

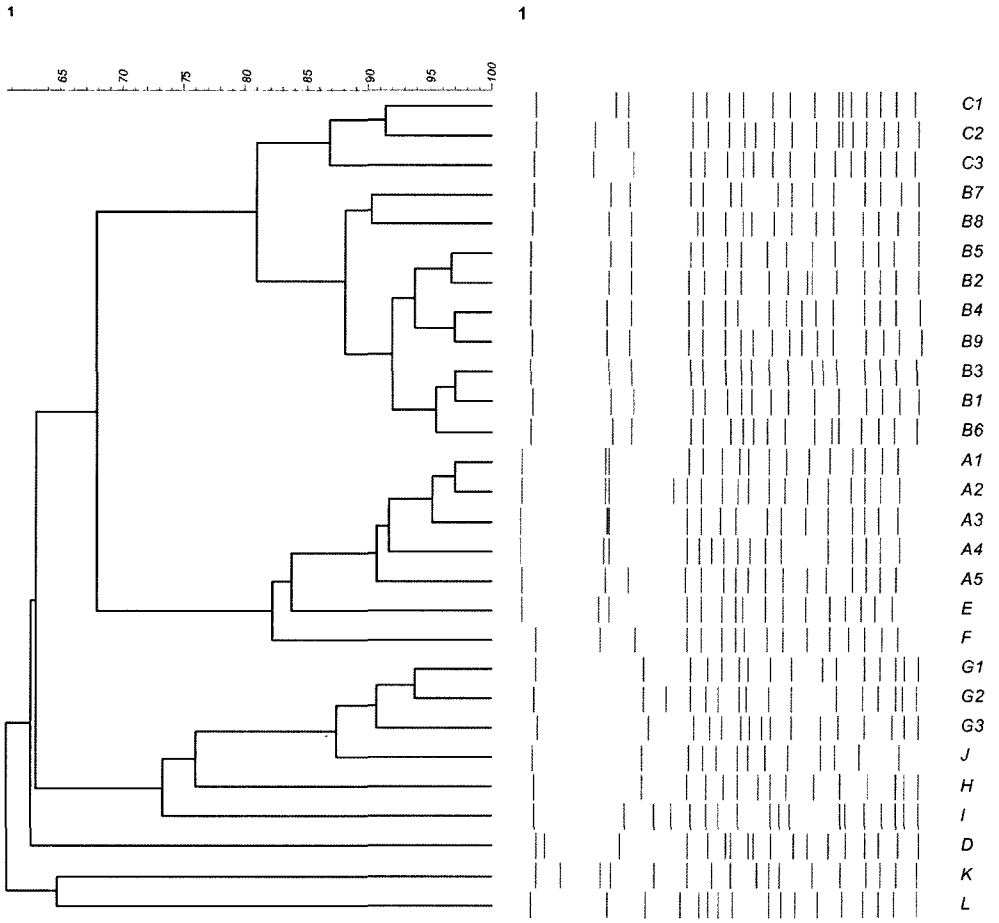

Fig. 1. Dendrogram of NotI-digested PFGE patterns for *V. cholerae* O1 El Tor isolates investigated in this study.

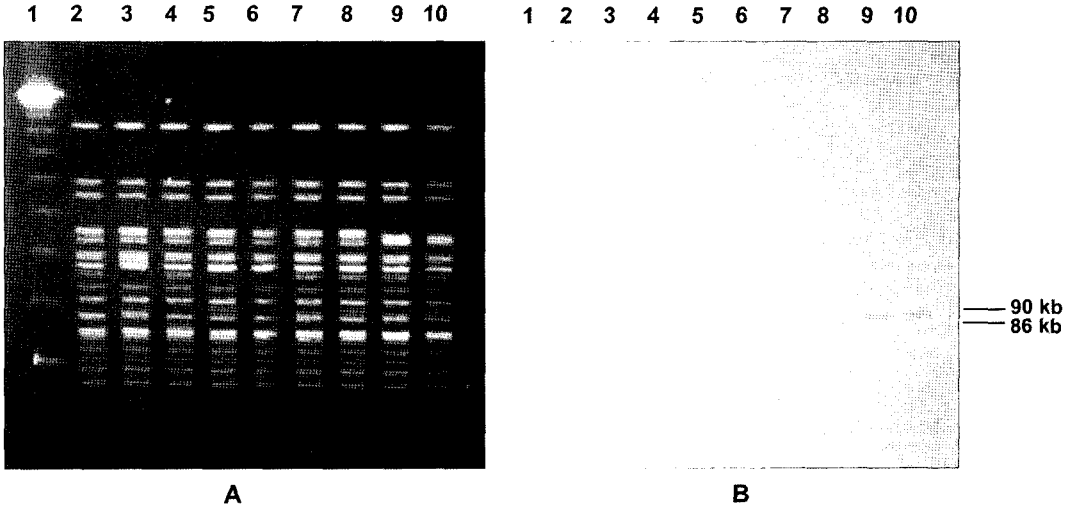

Fig. 2. A. NotI-digested PFGE patterns of *V. cholerae* O1 strains. Lane 1 is a 48.5-kb ladder size marker and lanes 2 to 10 show the subtypes (B1, B2, B3, B4, B5, B6, B7, B8, and B9, respectively) shown in Tables 2 and 3. **B.** Southern blot of same gel shown in panel A after hybridization with a peroxidase-labeled 592-bp SXT integrase fragment.

case of B5 from a traveller to Indonesia. Type C strains were isolated from patients who had been to Indonesia, type D strains from those who had been to The Philippines, and types E and F from those who had been to Thailand.

Domestically, *V. cholerae* O1-infected patients were reported in 1991, 1995–1997, 1999, 2001, and 2002 in various parts of Korea (Table 1). Subtype A1 was the prevalent PFGE type in the outbreaks of 1991, 1996, and 1997. In 1995, subtype A1 was the major type of outbreak in the Gyeongbuk, Incheon, Busan, and Gangwon areas, and A2 was the only type isolated in the outbreaks in the Chungnam and Gyeonggi areas. Subtype A2 was isolated only in 1995, and has never been isolated from imported cases. Because subtype A2 is closely related to subtype A1, differing by only one band, with a computer-calculated relatedness of 96.97%, it is reasonable to presume that subtype A2 evolved from subtype A1 during the outbreaks of 1995. In 1999, subtype B8 was the only type isolated from domestic cases of cholera. In 2001, seven subtypes of the type B strain were isolated (B1 to B7), and subtypes B1, B2, and B3 were the most prevalent. The prevalent types among the domestic cases were consistent with the prevalent types among the imported cases in the same year, and the PFGE genotype correlated with the phenotype evaluated in terms of antibiotic susceptibility. PFGE type A strains (except A5) isolated between 1993 and 2000 showed resistance to streptomycin, and only subtypes B8 and B9 isolates showed resistance to both S and SXT.

Because class I integrons carrying resistance to sulfonamides, trimethoprim, and streptomycin have been reported in *V. cholerae* strains in Vietnam and Thailand [3], we expected the presence of an integron containing gene cassettes encoding SXT- and streptomycin-resistance-related genes. However, no integron was identified in the strains isolated in 1999. In another study, Waldo *et al.* [11] identified an approximately 62-kb self-transmissible, chromosomally integrating genetic element in *V. cholerae* O1 and O139, which they called the SXT element. The SXT element contains genes encoding resistance to sulfonamides, trimethoprim, and streptomycin. In this study, we identified SXT elements in all the *V. cholerae* strains isolated in 1999. Therefore, the SXT element could account for resistance to S and SXT in the 1999 isolates. The SXT probe hybridized with an approximately 86-kb fragment of B8 type and 90 kb of the B9 type isolates in 1999 (Fig. 2), but because the 86-kb fragment also exists in the B2 and B6 type isolates, it is not certain that the presence of SXT elements and their integration into the chromosomes of *V. cholerae* strains isolated in 1999 should generate a different PFGE pattern from those of other *V. cholerae* strains.

When the PFGE patterns of types A and B were compared, they were genetically heterogeneous. These results suggest that a new clone, PFGE type B, might have been introduced into and spread throughout Korea after 1999, and that type B replaced type A as the prevalent PFGE type.

To explain the irregular recurrence of *V. cholerae* O1 infections in Korea, it is reasonable to predict that the Korean ocean environment is contaminated with *V. cholerae* O1, because almost all the sporadic and epidemic *V. cholerae* O1 cases in Korea have occurred in coastal areas. However, a relatedness of less than 62% between strains isolated from the Korean environment and those isolated from domestic and imported cases of the disease is insufficient to support this assumption. Therefore, no clonal relationship between environmental strains and domestic or imported cases can be demonstrated. A second explanation proposes

contact between domestic cholera patients and those patients who have travelled in countries in which cholera is prevalent. This is the most likely explanation, because the most prevalent PFGE and antibiotic-resistant types among the reported domestic cases of cholera are consistent with the prevalent types among imported cases in the years that those reports were made. Despite intensive epidemiological investigation of each domestic cholera case, no clear link has been identified between domestic and imported cases, such as person-to-person contact or contaminated foods. This study shows that PFGE-based analysis is a valuable tool with which to study the relationships among *V. cholerae* O1 isolates in Korea.

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