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Diversity of DNA sequences among *Vibrio cholerae* O139 Bengal detected by PCR-based DNA fingerprinting

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Abstract

Vibrio cholerae O139, a causative agent of a large epidemic of cholera-like illness, has suddenly emerged and spread widely over several months. To investigate the characteristics unique to O139, traditional typing techniques for *V. cholerae*, such as biochemical characteristics, antibiotic susceptibility and detection of toxin production, were performed, with the result that 145 O139 strains, except for two O139 strains isolated from Argentina and Germany, were indistinguishable from O1 strains. Thus, in order to clarify the genetical relatedness among O139 strains, and between O139 and O1 strains, the RAPD (random amplified polymorphic DNA) DNA fingerprinting method was undertaken. Although the RAPD arrays in five O139 isolates from Vellore with one arbitrary primer were slightly different from the other O139 strains, the RAPD patterns of the 145 forty-five O139 strains except for two O139 strains from Argentina and Germany were quite similar to each other, but were different from those of O1 strains, indicating that those O139 epidemic strains are closely related to each other regardless of their place of isolation. Furthermore, the RAPD patterns of the O139 strains resembled those of El Tor strains rather than classical strain, and a small change in the RAPD pattern of O139 strains occurred during subculture for 200 generations. These results taken together suggested that O139 *V. cholerae* have emerged from a common origin associated with the El Tor strain.

Keywords: Polymerase chain reaction; Random amplified polymorphic DNA; Arbitrary primer; Fingerprinting; *Vibrio cholerae*

1. Introduction

Until recently, it has been believed that only O1 *Vibrio cholerae* had epidemic potential, with non-O1 strains being limited to sporadic cases and small outbreaks. However, an unprecedented event in the history of cholera was witnessed in October 1992 in the Indian subcontinent with the emergence of a novel toxigenic strain of *V. cholerae*. This strain does not agglutinate with O1 antiserum but possesses the potential to cause explosive outbreaks of cholera-like illness and to spread swiftly. First detected as the cause of a large outbreak of cholera-like illness in Madras, this strain was isolated a month

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later from other parts of India including the cities of Madurai, Vellore and Calcutta, and, in mid-January, 1993, similar strains of *V. cholerae* that did not agglutinate with O1 antiserum were shown to be responsible for an epidemic of cholera in Bangladesh [1]. Shimada et al. [2] assigned these strains to a new serogroup, O139, with the synonym Bengal as they were first isolated from areas along the coast of the Bay of Bengal. The O139 serogroup of *V. cholerae* has now spread into Thailand [1], Nepal, Pakistan and Malaysia [3]. Very recently, *V. cholerae* O139 has been isolated from patients with a cholera-like illness in Japan [4], Germany and Argentina [5], countries far from the Bay of Bengal, indicating that there is a risk of *V. cholerae* O139 spreading very quickly all over the world. Thus, it is important from the view point of public health to elucidate how *V. cholerae* O139 spread so swiftly and widely. In addition, it is important to clarify the genetic relatedness among those O139 *V. cholerae* and to understand changes in genetic structure and the evolutionary trends of microbial populations.

Arbitrarily primed PCR (also called RAPD for random amplified polymorphic DNA) [6,7] is one of the most promising methods for distinguishing individual bacterial strains and estimating nucleotide sequence diversity. This method is more sensitive than other similar methods, for example restriction fragment length polymorphisms (RFLP), and has many advantages from the viewpoints of e.g. economics, simplicity, reproducibility and time [8,9]. It uses single oligonucleotides of an arbitrarily chosen sequence to prime DNA synthesis at low stringency from pairs of sites to which the oligonucleotide is matched or almost matched. This generates strain-specific arrays of amplified DNA fragments. The formation of these arrays does not depend on prior knowledge of the nucleotide sequence, nor is it affected by DNA modifications that complicate typing by restriction endonuclease digestion of genomic DNA. Because the RAPD method is PCR-based, only nanogram quantities of DNA are required and the DNA does not need to be double-stranded, highly purified, or of high molecular mass. These features make the RAPD method especially useful for organisms that grow slowly, or for which culturing large volumes is costly or hazardous.

We report here the use of RAPD to detect DNA sequence diversity among *V. cholerae* isolates and compare the patterns with various *V. cholerae* O139 and O1 strains from different regions. The data presented here also indicate that the RAPD method is feasible for investigating the possible origins of *V. cholerae* O139 infection.

2. Materials and methods

2.1. Strains used in this study

The *V. cholerae* O139 strains used in this study are listed in Table 1. One-hundred-and-fifteen isolates from India, 20 isolates from Bangladesh, eight isolates from Thailand, two isolates from Germany, and one isolate from Argentina were kind gifts from Dr. G.B. Nair, National Institute of Cholera and Enteric Disease, India; Dr. M.J. Albert, International Center for Diarrhoeal Disease, Bangladesh; Dr. M. Kusum, NIH, Thailand; Dr. R. Stephan, Institute of Robert Koch, Germany; and M. Rivas, Instituto Nacional de Microbiologia "Dr Carlos G Malbràn", Argentina, respectively. Another two isolates and an O1 clinical isolate (biotype El Tor) VC9302 from Japan were from stocks in the Saitama Institute of Public Health, Japan. Standard strains of *V. cholerae* O1, 569B (classical biotype) and MAK757 (biotype El Tor) were kind gifts from Dr. H. Kurazono, Faculty of Medicine, Kyoto University, Japan. All the isolates were biochemically and serologically confirmed as *V. cholerae* belonging to the O139 serogroup by using specific antiserum raised against O139 [2].

2.2. RAPD fingerprinting and gel electrophoresis

PCR-based fingerprinting was carried out in a volume of 25 μl containing 50 ng of *V. cholerae* total DNA, 3 mM $MgCl_2$, 20 pmol of primer, 1 U of Taq DNA polymerase (Promega), 250 μM each of dCTP, dGTP, dATP and dTTP (Pharmacia) in 10 mM Tris · HCl (pH 8.3), 50 mM KCl and 0.1% Triton X-100, under a drop of mineral oil. A thermal Cycler (Perkin Elmer type PJ2000) was used for amplification. The program for amplification was four cycles of (94°C, 5 min; 36°C, 5 min; and 72°C, 5 min), 30 cycles of (94°C, 1 min; 36°C, 1 min; and

72°C, 2 min), followed by a final incubation at 72°C for 10 min. To examine the RAPD patterns using total DNAs from O139 strains after cultivation for 200 generations, the following program for amplification was used; 45 cycles of (94°C, 2 min; 38°C, 2 min, 72°C, ramp 3 min; 72°C, 2 min), and a final incubation at 72°C for 10 min. After PCR, 10–20-μl aliquots of product were electrophoresed in 2% agarose gels, followed by ethidium bromide staining and photography under UV light. In the RAPD tests, three kinds of 10-nucleotides (10-nt) primers, AP40 (5′-CCGCAGCCAA-3′), AP42 (5′-AACGCGCAAC-3′), and AP46 (5′-GAGGACAAAG-3′), were used [10]. Total DNA preparation for RAPD tests was purified as previously described [10].

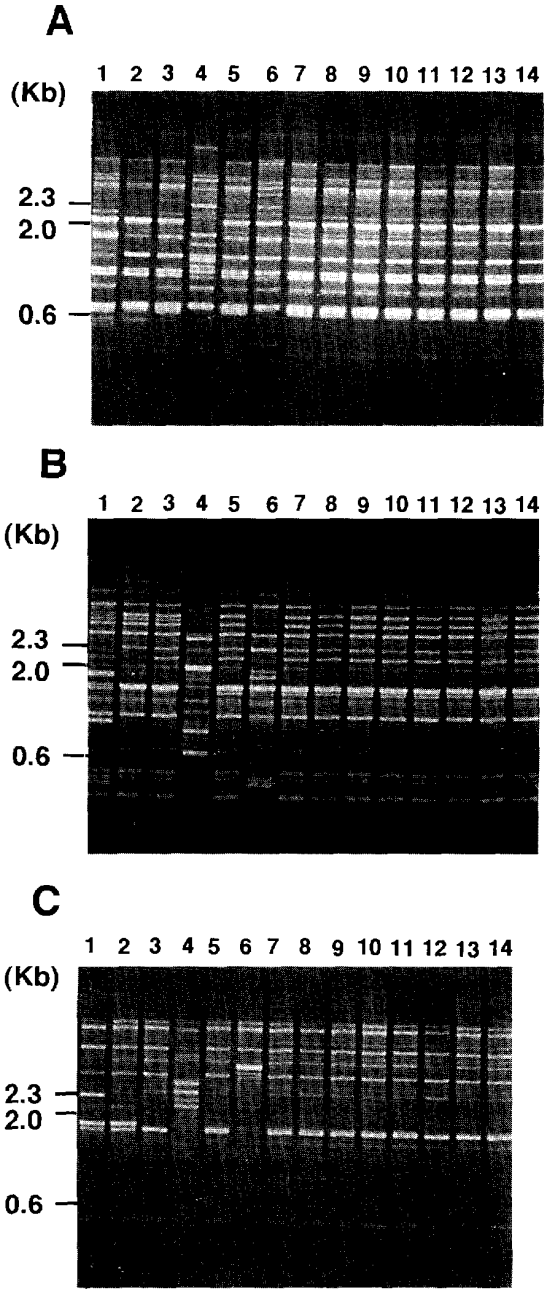

Fig. 1. Comparison of the RAPD patterns of representative *V. cholerae* O139 and O1 strains. Three kinds of arbitrary primers, AP40 (A), AP42 (B) and AP46 (C) were used for PCR. Lanes: 1, 569B (O1 biotype classical); 2, MAK757 (O1 El Tor); 3, VC9302 (O1 El Tor); 4, 394 (O139; from Argentina); 5, 495 (O139; from Japan); 6, 1033 (O139; from Germany); 7, 1034 (O139; from Germany); 8, AL3703 (O139; from Bangladesh); 9, TH166 (O139; from Thailand); 10, MO6 (O139; from Madras); 11, VO10 (O139; from Vellore); 12, SG16-1 (O139; from Calcutta); 13, MDO6 (O139; from Madurai); 14, K111 (O139; from Japan).

2.3. Other procedures

For detection of toxin genes, parts of the toxin gene (*ctxA*) and the zonula occludens toxin gene (*zot*) were amplified by the PCR method [4]. In addition, all strains were biochemically tested for cholera toxin production using the BT-test 'Nissui' CT (Nissui Co. LTD., Japan) [4]. According to the procedure recommended by the National Committee for Clinical Laboratory Standards, antimicrobial disk susceptibility tests were carried out using ampicillin, chloramphenicol, kanamycin, tetracycline, trimethoprim-sulfamethoxazole, polymyxin B, streptomycin, and a vibriostatic O/129 chemical reagent.

3. Results

3.1. Various characteristics of V. cholerae O139 isolates

To investigate the characteristics unique to *V. cholerae* O139, traditional typing techniques were carried out on O139 and O1 strains. Although antibi-

otic-resistance patterns among those strains were not generalized (data not shown), biochemical characteristics and toxin production of 145 O139 strains, except for strains 394 and 1033, isolated from Argentina and Germany, respectively, were the same as those of O1 isolates, indicating that those O139 strains could not be distinguished from O1 strains using traditional typing techniques as reviewed by Albert [17]. Since strain 394 could not ferment mannose, and strains 394 and 1033 do not produce cholera-toxin as tested by biochemical and genetic methods (data not shown), both are distinguishable from other O139 strains.

3.2. RAPD analysis of O139 V. cholerae

In order to clarify genetic relatedness among O139 strains and among O139 and O1 *V. cholerae* strains, RAPD tests were performed on total DNA preparations from those strains. Representative results using three suitable primers (AP40, AP42 and AP46) to detect informative arrays of PCR products are shown in Fig. 1. Although many common bands appeared in the RAPD arrays of the strains tested, the RAPD patterns of three O1 strains were distinguishable from each other (Fig. 1, lanes 1–3). However, the RAPD patterns of the nine representative O139 strains were indistinguishable from each other (Fig. 1, lanes 5 and 7–13), suggesting that, regardless of their place of isolation, those O139 strains are closely related to each other. On the other hand, two O139 strains, 394 and 1033, gave rise to unique patterns distinguishable from other *V. cholerae* strains (Fig. 1, lanes 4 and 6). Although O139 strains were distinguishable from O1 strains, especially from a classical biotype strain 569B, the RAPD arrays of O139 strains, except for strains 394 and 1033, comparatively resembled those of El Tor strains (Fig. 1). Interestingly, the RAPD pattern of another O1 El Tor strain, VC9302, which was isolated from travellers in the Philippines at the same time of the outbreak of O139 Bengal, was indistinguishable from those of O139 strains using primers AP40 and AP46 (Fig. 1A and C, lanes 3, 5 and 7–13). However, with primer AP42, an extra band appeared in the VC9302 arrays, distinguishing it from the O139 strains (Fig. 1B, lanes 3, 5 and 7–13).

To further examine the DNA similarity of O139 strains, the RAPD patterns of all O139 strains except for strains 394 and 1033 listed in Table 1 were analysed using the AP40, AP42 and AP46 primers. All the patterns were indistinguishable from each other (data not shown), with the exception of five

Table 1
All O139 strains used in this study

Isolation site	Number of isolates	Month and year of isolation [a]	Origin
Madras, India	26	1992, October	Human
Madurai, India	22	1992, November	Human
Vellore, India	46	1992, October	Human
Calcutta, India	20	1992, November	Human
Bangladesh	20	1993, February	Human or water
Thailand	8	1993, April	Human
Saitama, Japan	1	1993, March	Human
Nagano, Japan	1	1993, July	Human
Germany	2	1992 and 1993	Human
Argentina	1	1992 or 1993	Human

[a] The date of the first isolation of O139 in a given area are shown, but two strains from Germany were isolated independently in 1992 and 1993, and a strain from Argentina was isolated either in 1992 or 1993 [5].

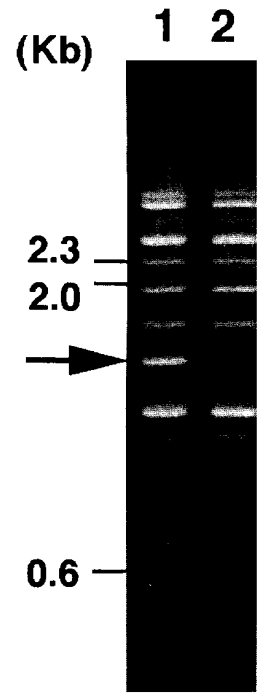

Fig. 2. Representative RAPD patterns of O139 VO1 and VO10 with AP46 primer. The RAPD test was performed using AP46. Lane 1, VO1 and lane 2, VO10.

strains, VO1, VO2, VO3, VO5 and VO8, isolated from Vellore, using AP46. While the RAPD patterns with AP46 in those five O139 strains were identical to each other, they were slightly different from those of the other O139 strains (Fig. 2). These results suggested that the origin of these five O139 strains was different from the other O139 isolates or that some mutations on the chromosome had occurred in O139 strains during the transmission. To investigate the latter possibility, a long passaging of 200 generations was carried out in rich medium using four O139 strains, VO10, MO6, MDO9 and SG16-1, which were isolated from Vellore, Madras, Madurai, and Calcutta, respectively, and RAPD patterns were monitored through the passage. However, significant differences in the RAPD arrays were not noted (data not shown). To further confirm the constancy of RAPD arrays after passaging, we used milder annealing conditions in PCR with the arbitrary primers than was used above (see Materials and methods). The results showed that a single additional faint band appeared only with MDO9 after passaging for 200 generations (Fig. 3, lane 6). This may indicate that mutations occur only rarely on the chromosome of *V. cholerae* in nature.

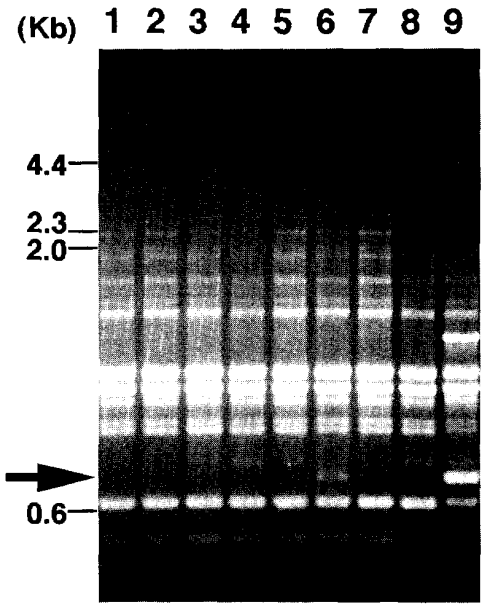

Fig. 3. RAPD analysis of representative *V. cholerae* strains after passaging for 200 generations. The RAPD tests were performed using primer AP46. The RAPD patterns using total DNA samples of representative O139 strains, VO10, MO6, MDO9 and SG16-2, at the starting time are shown in lanes 1, 2, 3 and 4, respectively; those of the four strains after passaging for 200 generations are shown in lanes 5 to 8, respectively. The RAPD pattern of VO1 is shown in lane 9.

4. Discussion

An epidemic of cholera-like disease due to a *V. cholerae* non-O1 serogroup, O139, broke out at Madras, India, in October 1992. Over the next few months, it spread to other cities in India and also to other countries around the Bay of Bengal [1]. Furthermore, O139 *V. cholerae* strains were isolated in countries far from the Bay of Bengal in 1993 [1,3–5]. Since the sudden emergence and swift spread of *V. cholerae* O139 has not been explained clearly, laboratory studies to track the origin and to fingerprint the organisms are interesting and important. In such work, the RAPD tests should be useful, because it is more sensitive and efficient than traditional typing techniques [8,9].

Berche et al. [11] carried out RAPD analysis on O1 and ten O139 strains isolated from one area in India, and showed that the O139 strains were closely related each other. Although that study used only a small number of O139 strains, we have examined about 147 O139 strains isolated from a wide area using the RAPD tests, and confirmed that 145 O139 strains, except for strains 394 and 1033, are very closely related to each other regardless of their place of isolation. We also showed that the RAPD arrays of O139 strains are very similar to those of El Tor strains, especially the seventh pandemic strain VC9302, suggesting that there may be one origin of O139 strains. Similar speculations have been reported by other studies dealing with DNA sequence structures of the cholera toxin gene [12] and of 16S rRNA genes [13], the possession of iron-regulated outer membrane protein profiles [14], the regulation of virulence genes by *toxR* [15,16], the comparison of the patterns of multilocus enzyme electrophoresis [11], etc. (for review, see [17]). One possibility is that El Tor strains mutated and became a new serogroup. In fact, when O139 strains were subcultured for 200 generations in rich medium, one new extra band appeared in one strain upon RAPD analy-

sis (Fig. 3), suggesting that mutations might occur on the chromosome during subculturing. Although the rich medium did not at all mimic the natural environment, mutations could occur at a faster rate during transmission from human to human, or in the natural aquatic environment. Although five strains isolated from Vellore gave rise to a slightly different RAPD pattern from other O139 strains, those strains might be segregated from the Madras strains that were the first isolates of the O139 outbreak.

As another possibility regarding the origin of O139 strains, the hitherto unknown non-O1 *V. cholerae* like O139 strains 394 and 1033, whose RAPD patterns were quite different from those of other O139 strains (Fig. 1), might have been able to cause disease outbreaks by acquiring the necessary virulence genes from El Tor strains. Since non-O1 vibrios have been ignored in the history of cholera infections because they have not been causative agents of cholera illness until now, we may have missed isolating non-O1 strains as the origin of O139 strains. In any case, further detailed epidemiological surveillance of O139 strains like the above Vellore mutated strains is needed to conclude either of the two possibilities. Since it is also possible that new epidemic strains may emerge in the future, laboratory-based global surveillance should be undertaken and continued for the monitoring of vibrios using various typing methods.

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