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Novel PCR-based genotyping method, using genomic variability between repetitive sequences of toxigenic *Vibrio cholerae* O1 El Tor and O139

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ABSTRACT

A novel genotyping method for toxigenic *Vibrio cholerae* O1 El Tor and O139 was developed. The method was designed to amplify DNA sequences "sandwiched" between any given pair of repetitive sequences, "V. cholera repeats (VCR)", in highly polymorphic "integron island" of ca. 125 kb in the small chromosome of toxigenic *V. cholerae* so that the resultant PCR amplicons would present with a strain-specific electrophoretic pattern. The VCR-targeted PCR assay (VCR-PCR) for 37 strains of toxigenic *V. cholerae* O1 El Tor and O139 revealed that the O1 strains isolated before 1990 showed distinct clonality whereas those isolated after 1990 could be separated into two clones, one consisting of strains isolated from South American countries and another of those from other countries. By contrast, O139 strains were genotypically homogenous regardless of the geographic origin or time of isolation. VCR-PCR therefore would be a robust but rapid method for genotypic differentiation of toxigenic *V. cholerae* O1 El Tor and O139 strains and to recognize strains with epidemic potential.

1. Introduction

Vibrio cholerae is a gastrointestinal pathogen that causes "cholera", one of the most notorious enteric diseases with serious morbidity and mortality worldwide. It is estimated that the disease causes the deaths of over 100,000 people worldwide every year and infects mostly children between 1 and 5 years old [1,2]. Not every *V. cholerae* strain but those that belong to serogroups O1 and O139 producing an enterotoxin, cholera toxin (CT), are reported to cause the disease [3]. To our knowledge, the world has experienced 7 major pandemics of cholera since the early 19th century [4]. The first 6 pandemics were caused by toxigenic strains belonging to the classical biotype of serogroup O1, which originated India, while the seventh pandemic was caused by those belonging to El Tor biotype, a newly emerged O1 biotype that originated in the Celebes Islands of Indonesia in 1961. The pandemic of O1 El Tor was limited to Asian countries in the 1960s but spread to African countries in the 1970s and further to the countries of both American continents from 1990 onward. In 1992, another serogroup of toxigenic *V. cholerae*, O139, emerged to cause large outbreaks in the Bay of Bengal region [5].

Many researchers have employed various DNA fingerprinting or genotyping methods to reveal any epidemiological link among *V. cholerae* isolates but with limited success. For example, Raychoudhuri et al. [6] performed ribotyping and pulsed-field gel electrophoresis (PFGE) on *V. cholerae* O1 and O139 strains isolated from various sources at different times but found it very difficult to differentiate the clonality of isolates due to their rather homogenous chromosomal DNA profile. Other workers performed PCR-based DNA fingerprinting methods, such as amplified fragment length polymorphism [7], arbitrarily primed PCR [8], and randomly amplified polymorphic DNA analysis [9] on *V. cholerae* isolates and reported that the methods were useful to differentiate the clonality of the isolates. However, these methods were often found to yield too few or too many phylogenetic signals with poor reproducibility. More recently, Chokesajjawatee et al. [10] developed a novel PCR-based DNA fingerprinting method (ERIC-PCR) targeting repetitive genetic elements for *V. cholerae*, and made the point that the method permits both phylogenetic inference and clonal differentiation of individual *V. cholerae* strains.

Meanwhile, our preliminary work employing representative difference analysis (or "genomic subtraction") on whole genomic DNAs of two *V. cholerae* O1 El Tor isolates, whose origins are

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geographically and temporally distant, revealed that subtracted DNAs were mostly ORFs in the so-called "integron island" (data not shown). The integron island was reported to be in the small chromosome of *V. cholerae* El Tor N16961, ca. 125 kb in size, consisting of 216 ORFs with more than 140 repetitive sequences, referred to as "V. cholera repeats (VCR)" [11]. Our blast analysis of DNA sequences of the integron islands of various *V. cholerae* strains [i.e., N16961, NCTC 8457, V52, MAK757, B33, MO10] available in Genbank confirmed that the integron island is highly polymorphic with scattered distribution of VCRs. This is mostly, if not all, due to a multitude of deletions or replacements of genetic elements between the VCRs. The accumulating evidence led us to develop an assay of amplified fragment length polymorphism (referred to as "VCR-targeted PCR assay [VCR-PCR]" hereafter), revealing genetic polymorphism of the integron island of toxigenic *V. cholerae* O1 El Tor and O139 strains.

2. Materials and methods

2.1. Strains used

A total of 37 *V. cholerae* strains, consisting of 19 toxigenic O1 El Tor strains, which included *V. cholerae* N16961, the whole genomic DNA sequence of which is available from GenBank (NC_002506.1; *V. cholerae* O1 biovar El Tor strain N16961 chromosome II, complete sequence), and 18 toxigenic O139 strains were used in this study as described in Table 1. These strains were isolated from various sources between 1977 and 2006 and their taxonomic identity as *V. cholerae*, serotypes of O1 El Tor or O139, and presence of the CT gene were determined and confirmed by following the molecular methodology described by Lipp et al. [12]. As presented in Table 1, the strains could be classified into 4 groups as follows: i) 23 strains isolated clinically from patients outside Japan; ii) 7 strains isolated from patients in Japan with recent overseas travel; iii) 5 strains isolated from patients in Japan without recent overseas travel; and iv) 2 strains isolated from food imported from overseas. All strains were grown in Luria-Bertani broth at 37 °C for 18–20 h without shaking, and the cells were collected for subsequent DNA extraction.

2.2. DNA preparation

Genomic DNA from each bacterial isolate was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The purity and amount of DNA in each preparation were determined spectrophotometrically by measuring the absorbance at 260 nm.

Table 1
List of toxigenic *Vibrio cholerae* strains used.

Strain No.	Serogroup, biotype		Serotype	Year isolated	Where isolated	Source or origin	Patient then traveled to: (if applicable)
Isolates from patients outside of Japan							
N16961	O1	El Tor	Inaba	1971	Bangladesh	Patient	Unknown
1010	O1	El Tor	Inaba	1991	India	Patient	Unknown
1009	O1	El Tor	Ogawa	1991	India	Patient	Unknown
1005	O1	El Tor	Ogawa	1992	Peru	Patient	Unknown
1006	O1	El Tor	Ogawa	1992	Bolivia	Patient	Unknown
1008	O1	El Tor	Ogawa	1995	Indonesia	Patient	Unknown
1011	O1	El Tor	Ogawa	1996	Mongolia	Patient	Unknown
11	O1	El Tor	Ogawa	1997	Thailand	Patient	Unknown
14	O1	El Tor	Ogawa	1997	Thailand	Patient	Unknown
19	O1	El Tor	Ogawa	1997	Singapore	Patient	Unknown
39	O1	El Tor	Ogawa	1997	China	Patient	Unknown
5	O1	El Tor	Ogawa	1997	Thailand	Patient	Unknown
58	O1	El Tor	Ogawa	1997	Philippine	Patient	Unknown
81	O1	El Tor	Ogawa	1997	Thailand	Patient	Unknown
184–93	O139	–	–	1993	Bangladesh	Patient	Unknown
21–93	O139	–	–	1993	India	Patient	Unknown
515–95	O139	–	–	1993	Bangladesh	Patient	Unknown
63–93	O139	–	–	1993	India	Patient	Unknown
1010–94	O139	–	–	1994	Denmark	Patient	Unknown
298–95	O139	–	–	1994	Thailand	Patient	Unknown
387–94	O139	–	–	1994	Korea	Patient	Singapore
1008–96	O139	–	–	1996	China	Patient	Unknown
199–98	O139	–	–	1998	India	Patient	Unknown
Isolates from Japanese patients with recent overseas travel records							
1056–93	O139	–	–	1993	Japan	Patient	Thailand & India
236–93	O139	–	–	1993	Japan	Patient	India
495–93	O139	–	–	1993	Japan	Patient	Nepal
294–94	O139	–	–	1994	Japan	Patient	Thailand
435–94	O139	–	–	1994	Japan	Patient	Thailand
895–94	O139	–	–	1994	Japan	Patient	Bangladesh
234	O139	–	–	2004	Japan	Patient	China
Isolates from Japanese patients without recent overseas travel records							
1001	O1	El Tor	Ogawa	1977	Japan	Patient	None
1002	O1	El Tor	Inaba	1978	Japan	Patient	None
1003	O1	El Tor	Inaba	1989	Japan	Patient	None
1004	O1	El Tor	Ogawa	1991	Japan	Patient	None
252	O139	–	–	2006	Japan	Patient	None
Isolates from food imported to Japan							
Q5	O1	El Tor	Inaba	1989	Japan	Frozen fish imported from Indonesia	
1180–93	O139	–	–	1993	Japan	Soft shell turtle imported from Bangladesh	