

Evaluation of *VcA* VNTR as a strain-typing and phylogeny study method of *Vibrio cholerae* strains

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SUMMARY

This study attempted to examine the relatedness between RAPD–PCR, PFGE and *VcA* VNTR results with those of conventional phage typing of *V. cholerae* strains and to evaluate *VcA* VNTR as an indispensable molecular-typing tool that accomplishes the urgent need for effective epidemiological surveillance. All the O1 El Tor strains were predominantly clustered into phage type T27 with the new phage-typing scheme. Using RAPD–PCR, a total of 69 O1 El Tor strains were grouped under 16 different electrophoretic patterns. A total of 33 pulsotypes were identified in these strains by PFGE. *VcA* VNTR revealed high *VcA* polymorphism in all *V. cholerae* strains incorporated in this study. Our results underline the considerable potential of *VcA* VNTR analysis as a tool for molecular typing of *V. cholerae*.

Key words: Molecular epidemiology, *Vibrio cholerae*, VNTR.

INTRODUCTION

Vibrio cholerae, a Gram-negative γ -proteobacteria, is the causative agent of cholera disease and is acquired through the ingestion of contaminated food or surface water. Globally 1·3 billion cases of acute diarrhoea occur in children aged <5 years annually [1]. Only two serogroups namely, O1 and O139 are recognized as pathogenic and responsible for epidemic and pandemic cholera [2]. Similarly non-O1/non-O139 serogroups of *V. cholerae* may cause sporadic diarrhoeal episodes with mild to severe morbidity [2–4].

Studies on phages of *V. cholerae* have been of historical interest. Among several typing methods, phage

typing is one of the most important and useful methods for the identification and differentiation of *V. cholerae* strains. A phage-typing scheme was developed by Basu & Mukerjee for *V. cholerae* O1 biotype El Tor [5] and later a new phage-typing scheme was developed for O1 [6]. Subsequently, a further phage-typing scheme specific for *V. cholerae* O139 was developed at the National Institute of Cholera and Enteric Diseases (NICED) [7]. As a WHO collaborating centre for diarrhoeal diseases research and training, NICED is a national reference laboratory and receives annually between 700 and 1000 strains of *V. cholerae* from different parts of India and abroad for biotyping, serotyping and phage typing [8]. Phage-typing methods can test large numbers of strains rapidly. It is a cost-effective and a simple laboratory method that does not require any sophisticated equipment. Because of host specificity it offers the basic information on identification, biotyping and discrimination of strains [9].

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Increasingly, laboratory diagnosis, identification and epidemiological surveillance of cholera are based on genotypic characteristics rather than phenotypic characteristics (biochemical and antigenic properties), including antibiotic-resistance testing, biotyping, serotyping and phage typing. Conventional typing methods can not accurately predict the epidemiological potential of *V. cholerae* strains [10] due to low discriminatory power. The use of genome-based bacterial identification and typing would reveal an epidemic relationship and the association of certain clones with potential sources of contamination [11–15]. Several DNA-based methods, e.g. detection of virulence genes, pulsed-field gel electrophoresis (PFGE) and random amplification of polymorphic DNA (RAPD) are now extensively used for typing of *V. cholerae* strains. Following the advent of DNA-based strain typing methods, there is need for a suitable strain typing method that must be rapid, cost-effective and with high discriminatory power.

Variable number of tandem repeats (VNTR) has been demonstrated to be superior in its fine-tuning discriminatory power for some enteric pathogens. This method is also applied in *V. cholerae* genomes by PCR amplification and size determination of simple sequence repeats and DNA sequencing of mononucleotide repeat regions identified in the *V. cholerae* genomes [16–18]. These molecular loci themselves became known as VNTR regions and are now well-established targets for ancestry analysis [19]. The existence of slipped-strand mispairing as a basic molecular mechanism of DNA variation in VNTR regions is generally accepted [20]. The repeat number indicates the Mendelian inheritance, may vary within a species, and represents a unique genetic feature of an individual [21]. The availability of the full genome sequence of *V. cholerae* enables computerized analysis of the presence of repetitive DNA with VNTR-like potential. In this study, the *VcA* locus has been selected as it has already been employed in identifying various strains of the cholera pathogen. We evaluated the potential of *VcA* VNTR analysis as a tool for genotyping of *V. cholerae*.

METHODS

Bacteriology

A total of 125 *V. cholerae* isolates were included in this study. These strains were collected from different geographical regions of India and abroad in different

time-frames and sent to NICED for confirmation, biotyping, serotyping and phage typing (Table 1). Sixty-seven strains of O1 El Tor, 29 of *V. cholerae* O139, 23 non-O1/non-O139 and six O1 classical strains were included. *V. cholerae* O1 biotype El Tor strains MAK 757 (ATCC 51352) and N16961, *V. cholerae* O1 biotype classical strains CL-154 and O395 and *V. cholerae* O139 strain NPR-4 were used as controls [5, 6, 22, 23]. All these strains were maintained in nutrient agar stabs and kept in the dark, at the Vibrio Phage Reference Laboratory, NICED. All strains of *V. cholerae* were identified and confirmed by using standard techniques [24]. A single colony of *V. cholerae* from nutrient agar was inoculated into Mueller–Hinton broth and incubated under stationary conditions for 4 h at 37 °C. The cultures were then plated on Mueller–Hinton agar plates containing 50 µg/ml (122·34 USP unit/ml) of polymyxin B sulphate (Sigma, USA) and incubated at 37 °C overnight [22]. *V. cholerae* O1, O395 and MAK 757 were used as negative and positive controls, respectively. These strains were subsequently serotyped by using polyvalent O1, monospecific Ogawa and Inaba antisera (Difco, USA). This study is a continuation of our previously published work where 26 strains (PL-1 to PL-26) were analysed by antibiogram, phage typing, PFGE and RAPD–PCR [9].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disk diffusion method with commercially available disks (HiMedia, India). All the *V. cholerae* strains were examined for resistance to ampicillin (10 mg), azithromycin (15 mg), ceftriaxone (30 mg), chloramphenicol (30 mg), ciprofloxacin (5 mg), co-trimoxazole (25 mg), furazolidone (100 mg), norfloxacin (10 mg), streptomycin (10 mg) and tetracycline (30 mg) according to criteria of the National Committee for Clinical Laboratory Standards [25]. Strains with intermediate zone of inhibition were interpreted as resistant [26]. The American Type Culture Collection (ATCC) strain *E. coli* (ATCC 25922) was used as a standard quality control.

Phage-typing procedure

The phage typing was performed by the standard methodology of our laboratory [6, 23, 27]. MAK 757 (ATCC 51352) and NPR-4 [23] were used as control for O1 and O139 strains, respectively. Additionally,

φ149 of classical group IV and El Tor group V phage were used to differentiate the biotype of *V. cholerae* strains.

RAPD

A modified method from the study of Murray & Thompson [28] was used for DNA extraction. RAPD-PCR fingerprinting was carried out with 25 ml of reaction mixture containing 2.5 ml of 10 μl PCR buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.0), 15 mM MgCl₂ and 0.01 % gelatin], 20 ng genomic DNA, 20 pmol primer 1281 (5'-AACGCGCAAC-3') [29], 1 U AmpliTaq DNA polymerase and 2.5 ml of 25 mM dNTP for 45 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min in a Peltier Thermal Cycler (PTC-200; MJ Research, USA). After PCR, 10 ml of product were electrophoresed in 2 % agarose gel containing 0.5 mg/ml ethidium bromide and photographed under UV light using the Gel Doc 2000 documentation system (Bio-Rad, USA). The 1 kb DNA ladder (Sigma, USA) was used as the molecular size standard.

PFGE

Genomic DNA was prepared in agarose plugs as described previously [30]. *NotI* [5'-GCGGCCGC-3'] (Takara, Japan) digested inserts of *V. cholerae* were applied to a contour-clamped homogenous electric field in a CHEF Mapper System (Bio-Rad) using 1 % PFGE grade agarose in 0.5× Tris-borate-EDTA buffer for 40 h 24 min at 14 °C. Run conditions were generated by autoalgorithm mode of CHEF Mapper that separates the DNA size range of 20–300 kb. After electrophoresis, the gel was stained in distilled water containing 1.0 mg ethidium bromide/ml for 30 min, destained in distilled water for 15 min and photographed under UV light using the Gel Doc 2000 documentation system (Bio-Rad). A DNA size standard (PFGE ladder; New England Biolabs, USA) was used as the molecular size standard.

PFGE gel images were retrieved and aligned to generate a composite image containing the banding profiles of all the isolates. The image was analysed with Quantity One 1-D Analysis Software (version 4.4.0, Bio-Rad) to detect the relatedness of isolates. Bands ranging from 34.4 to 543.2 kb were considered for the construction of a dendrogram. Pattern profiles were compared by using Dice coefficient and UPGMA (unweighted pair-group method with arithmetic

mean) clustering with a 4 % tolerance window. A dendrogram showing the hierarchical representation of the level of linkage between the isolates was drawn to predict the degree of clonal relatedness.

Vca VNTR

DNA extraction of pure cultures was prepared by a method modified from the work of Murray & Thompson [28]. For crude and fast DNA extraction a suspension of 10⁹ cells in distilled water was heated in boiling water for 15 min, then immediately chilled on ice for 10 min and centrifuged at 10 000 rpm. The supernatant was immediately used for amplification. To amplify a DNA fragment containing *Vca*, two specific primers, 5'-TCT TCT TGC GCT TCT TGA CC-3' (direct) and 5'-TCA TCA AGA TGC ACG ACA CA-3' (reverse) were used [21]. The amplification product containing 23 repeated units was expected to be 222 nt in size in the control strain N16961. PCR mixtures contained 2.5 μl of 10× PCR buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.0), 15 mM MgCl₂ and 0.01 % gelatin], 50 ng of template DNA, 10 μM concentrations of each primer, 2.5 μl of 2.5 mM dNTP and 0.2 U of AmpliTaq DNA polymerase (Takara) in a total volume of 25 μl. The reaction was carried out in a Peltier Thermal Cycler (PTC-200; MJ Research) as follows: 94 °C for 5 min; followed by 30 cycles of 1.5 min at 94 °C, 1.5 min at 50 °C and 1.5 min at 72 °C. All PCR amplification products were analysed by agarose gel (1.5 %) electrophoresis.

PCR products were purified using a QIAquick PCR purification kit (Qiagen, USA). Portions (50 ng) of the purified product were sequenced on both strands using a BigDye terminator cycle sequencing kit (Applied Biosystems, USA) and loaded onto an ABI 3100 Genetic Analyzer sequencer (Applied Biosystems) according to the manufacturer's instructions. Analysis of the results was done by using the sequence analysis software Lasergen software suit (DNAStar Inc., USA).

Nucleotide sequences of *Vca* regions of all the *V. cholerae* strains incorporated in this study were analysed with DNADIST 3.5c to detect the relatedness of the isolates. This program can use a neighbour phylogenetic tree interface to compute distance matrix from nucleotide sequences. Sequence profiles were compared by this program using the neighbour-joining method. A dendrogram showing the hierarchical representation of the level of linkage between

Table 1. Summarized data on year of isolation, geographical locations, phage types, PFGE types, RAPD types and VcA VNTR types of *V. cholerae* strains incorporated in this study

Strain no.	Year of isolation	Geographical location	Biotype	Serogroup	Serotype	Basu & Mukerjee phage type	New phage type	O139 phage type	PFGE		RAPD type	VcA VNTR repeat number
									Pulsotype	Subtype		
PL-1	2004	Goa	El Tor	O1	Ogawa	UT	UT	UT	A	A8	TIII	16
PL-2	2004	Goa	El Tor	O1	Ogawa	UT	UT	UT	A	A8	TIII	16
PL-3	2004	Orissa	—	O139	—	UT	UT	T1	B _x	B _x 2	TI	27
PL-4	2004	Orissa	—	O139	—	UT	UT	T1	B _x	—	TVIII	21
PL-5	2004	Orissa	—	O139	—	UT	UT	T1	E _x ; A†	A8†	TI	15
PL-6	2004	Chennai	El Tor	O1	Ogawa	T2	T27	UT	E	—	TI	13
PL-7	2004	Chennai	El Tor	O1	Ogawa	T2	T27	UT	A	A8	TI	19
PL-8	2004	Surat	El Tor	O1	Ogawa	T2	T27	UT	A	—	TIV	19
PL-9	2004	Surat	El Tor	O1	Ogawa	T2	T27	UT	A	—	TIV	15
PL-10	2004	Delhi	El Tor	O1	Inaba	T2	T27	UT	A	A7	TI	17
PL-11	2004	Madurai	El Tor	O1	Inaba	T2	T27	UT	A	A8	TII	19
PL-12	2004	Delhi	El Tor	O1	Inaba	T2	T27	UT	A	A8	TI	19
PL-13	2004	Delhi	El Tor	O1	Inaba	T2	T27	UT	A	—	TI	14
PL-14	2004	Delhi	El Tor	O1	Inaba	T2	T27	UT	A	A8	TI	15
PL-15	2004	Madurai	El Tor	O1	Inaba	T2	T27	UT	A	—	TI	15
PL-16	2004	Madurai	El Tor	O1	Inaba	T2	T27	UT	A	—	TV	20
PL-17	2004	Madurai	El Tor	O1	Inaba	T2	T27	UT	A	—	TII	20
PL-18	2002	Delhi	—	O139	—	UT	UT	T1	A _x	—	TVI	19
PL-19	2002	Nagpur	—	O139	—	UT	UT	T1	A _x	—	TI	14
PL-20	2002	Udaipur	—	O139	—	UT	UT	T1	A _x	A _x 1	TI _x	13
PL-21	2002	Chennai	—	O139	—	UT	UT	T1	A _x	—	TI _x	14
PL-22	2002	Nagpur	—	O139	—	UT	UT	T1	A _x	—	TII _x	13
PL-23	2002	Baroda	—	O139	—	UT	UT	T1	A _x	—	TI	16
PL-24	2002	Miraj	—	O139	—	UT	UT	T1	A _x	—	TIII _x	16
PL-25	2002	Surat	—	O139	—	UT	UT	T1	A _x	—	TIV _x	16
PL-26	2002	Delhi	—	O139	—	UT	UT	T1	A _x	—	TIV _x	23
PL-27	2005	Austria	—	O12	—	UT	UT	UT	B _y	—	TIX _y	9
PL-28	2005	Austria	—	O37	—	UT	UT	UT	A _y	—	TX _y	8
PL-29*	Control	MAK 757	El Tor	O1	Ogawa	T2	T27	UT	H	—	TXVI	13
PL-30	2005	Austria	—	O12	—	UT	UT	UT	C _y	—	TXI _y	12
PL-31	2005	Austria	—	O12	—	UT	UT	UT	D _y	—	TXII _y	22

Table 1 (cont.)

Strain no.	Year of isolation	Geographical location	Biotype	Serogroup	Serotype	Basu & Mukerjee phage type	New phage type	O139 phage type	PFGE		RAPD type	<i>Vca</i> VNTR repeat number
									Pulsotype	Subtype		
PL-32	2005	Austria	—	O12	—	UT	UT	UT	E _Y	—	TXIII _Y	20
PL-33	2005	Austria	—	O37	—	UT	UT	UT	A _Y	—	TXIV _Y	21
PL-34	2005	Austria	—	O37	—	UT	UT	UT	A _Y	—	TXV _Y	21
PL-35	2005	Austria	—	O12	—	UT	UT	UT	E _Y	—	TXVI _Y	7
PL-36	2000	Bangladesh	El Tor	O1	Ogawa	T2	T27	UT	A	A3	TI	19
PL-37	2000	Bangladesh	El Tor	O1	Ogawa	T2	T27	UT	A	—	TI	22 + 14
PL-38	2000	Bangladesh	El Tor	O1	Ogawa	T2	T25	UT	A	—	TI	8
PL-39	2000	Bangladesh	El Tor	O1	Ogawa	T2	T27	UT	A	A9	TI	17
PL-40	2000	Bangladesh	El Tor	O1	Ogawa	T2	T27	UT	A	—	TI	16
PL-41	2000	Bangladesh	El Tor	O1	Ogawa	T2	T25	UT	A	A5	TI	16
PL-42	2000	Bangladesh	El Tor	O1	Ogawa	T2	T27	UT	A	A9	TI	18
PL-43	2000	Bangladesh	El Tor	O1	Ogawa	T2	T27	UT	A	A1	TI	20
PL-44	2000	Bangladesh	El Tor	O1	Ogawa	T2	T25	UT	A	—	TI	14
PL-45	1996	Brazil	El Tor	O1	Ogawa	T2	T25	UT	A	A6	TIX	16
PL-46	1996	Brazil	El Tor	O1	Ogawa	T2	T25	UT	B	—	TVIII	16
PL-47	1996	Brazil	El Tor	O1	Ogawa	T2	T25	UT	A	A6	TIX	16
PL-48	1996	Brazil	El Tor	O1	Ogawa	T2	T25	UT	A	A4	TVIII	17
PL-49	1996	Brazil	El Tor	O1	Ogawa	T2	T25	UT	A	A8	TVIII	16
PL-50	1996	Brazil	El Tor	O1	Ogawa	T2	T25	UT	A	A2	TVIII	20
PL-51	1996	Brazil	El Tor	O1	Ogawa	T2	T27	UT	A	A2	TIX	21
PL-52	1996	Brazil	El Tor	O1	Ogawa	T2	T25	UT	A	A2	TIX	16
PL-53	1996	Brazil	El Tor	O1	Ogawa	T2	T25	UT	A	A2	TIX	15
PL-54	1998	Chennai	El Tor	O1	Ogawa	T2	T27	UT	A	A3	TVII	21
PL-55	1998	Maharashtra	El Tor	O1	Ogawa	T2	T27	UT	A	A3	TX	15
PL-56	1998	Karnataka	El Tor	O1	Ogawa	T2	T27	UT	A	A3	TI	24
PL-57	1998	Goa	El Tor	O1	Ogawa	T2	T27	UT	A	A3	TI	20
PL-58	1998	Gujrat	El Tor	O1	Ogawa	T2	T27	UT	A	A3	TI	20
PL-59	1998	Chennai	El Tor	O1	Ogawa	T2	T27	UT	A	A3	TI	21
PL-60	1998	Maharashtra	El Tor	O1	Ogawa	T2	T27	UT	A	A3	TX	13
PL-61	1998	Chennai	El Tor	O1	Ogawa	T2	T27	UT	A	A3	TI	21
PL-62	1998	Chennai	El Tor	O1	Ogawa	UT	UT	UT	A	A3	TI	21
PL-63	1970	Kolkata	Classical	O1	—	UT	UT	UT	J	J1	TXI	14
PL-64	1971	Assam	Classical	O1	—	UT	UT	UT	K	—	TXI	16
PL-65	1971	Assam	Classical	O1	—	UT	UT	UT	J	—	TXI	15
PL-66	1971	Assam	Classical	O1	—	UT	UT	UT	J	J2	TXII	15
PL-67	1980	Nagpur	El Tor	O1	Ogawa	T2	T27	UT	A	A8	TI	15
PL-68	1970	Patna	Classical	O1	—	UT	UT	UT	J	—	TXI	13
PL-69	1970	Delhi	Classical	O1	—	UT	UT	UT	J	J3	TXI	14
PL-70*	Control	CL-154	Classical	O1	—	UT	UT	UT	J	—	TXI	23

Table 1 (cont.)

Strain no.	Year of isolation	Geographical location	Biotype	Serogroup	Serotype	Basu & Mukerjee phage type	New phage type	O139 phage type	PFGE		RAPD type	VcA VNTR repeat number
									Pulsotype	Subtype		
PL-71*	Control	O395	Classical	O1	—	UT	UT	UT	J	—	TXI	23
PL-72	2003	Andhra Pradesh	El Tor	O1	Inaba	T2	T27	UT	A	—	TXIII	20
PL-73	2003	Andhra Pradesh	El Tor	O1	Inaba	T2	T27	UT	A	—	TXIII	20
PL-74	2003	Andhra Pradesh	El Tor	O1	Inaba	T2	T27	UT	A	—	TXIII	20
PL-75	2003	Andhra Pradesh	El Tor	O1	Inaba	T2	T24	UT	A	—	TXIII	20
PL-76	2003	Bombay	El Tor	O1	Inaba	T2	T27	UT	A	—	TXIII	20
PL-77	2003	Bombay	El Tor	O1	Inaba	T2	T27	UT	A	—	TXIII	20
PL-78	2003	Bombay	El Tor	O1	Inaba	T2	T27	UT	A	A4	TXIII	20
PL-79	2003	Bombay	El Tor	O1	Inaba	T2	T27	UT	A	—	TXIII	20
PL-80	2003	Andhra Pradesh	El Tor	O1	Inaba	T2	T27	UT	A	—	TXIII	21
PL-81	2002	Gujrat	—	O139	—	UT	UT	UT	A _X	—	TV _X	22
PL-82	2002	Gujrat	—	O139	—	UT	UT	UT	A _X	—	TV _X	11
PL-83	2002	Gujrat	—	O139	—	UT	UT	UT	B _X	B _X 1	TV _X	26
PL-84	2004	Gujrat	—	O139	—	UT	UT	UT	A _X	—	TV _X	21
PL-85	2004	Gujrat	—	O139	—	UT	UT	UT	C _X	—	TV _X	21
PL-86	2004	Chennai	—	O139	—	UT	UT	UT	B _X	—	TV _X	21
PL-87	2002	Chennai	—	O139	—	UT	UT	UT	A _X	—	TV _X	21
PL-88	2002	Chennai	—	O139	—	UT	UT	UT	A _X	—	TV _X	11
PL-89	2004	Chennai	—	O139	—	UT	UT	UT	B _X	—	TV _X	21
PL-90	2001	Goa	El Tor	O1	Ogawa	UT	UT	UT	C	—	TXIV	16
PL-91	2001	Delhi	El Tor	O1	Ogawa	UT	UT	UT	D	—	TXV	19
PL-92	2001	Bikaner	El Tor	O1	Ogawa	UT	UT	UT	E	—	TXIV	14
PL-93	2001	Delhi	El Tor	O1	Ogawa	UT	UT	UT	A	A7	TXIV	13
PL-94	2001	Goa	El Tor	O1	Ogawa	UT	UT	UT	F	—	TXV	19
PL-95	2001	Bikaner	El Tor	O1	Ogawa	UT	UT	UT	G	—	TVI	16
PL-96	2001	Baroda	NAG	—	—	UT	UT	UT	P _Y	—	TIX _Y	16
PL-97	2001	Baroda	NAG	—	—	UT	UT	UT	Q _Y	—	TIX _Y	16
PL-98	2001	Baroda	El Tor	O1	Ogawa	T2	T27	UT	A	—	TXV	28
PL-99	2006	Delhi	—	O139	—	UT	UT	T1	A _X	—	TVI _X	18
PL-100	2006	Delhi	—	O139	—	UT	UT	T1	A _X	—	TVI _X	19
PL-101	2006	Delhi	—	O139	—	UT	UT	T1	A _X	—	TVI _X	18
PL-102	2006	Delhi	—	O139	—	UT	UT	T1	A _X	—	TVI _X	19
PL-103	2006	Delhi	—	O139	—	UT	UT	T1	A _X	—	TVI _X	18
PL-104	2006	Delhi	—	O139	—	UT	UT	T1	A _X	—	TVI _X	18
PL-105	2006	Delhi	—	O139	—	UT	UT	T1	A _X	—	TVI _X	18
PL-106	2006	Delhi	—	O139	—	UT	UT	T1	A _X	—	TVI _X	18
PL-107*	Control	NPR-4	—	O139	—	UT	UT	T1	D _X	—	TVI _X	23
PL-108	2007	Kolkata	El Tor	O1	Ogawa	T2	T25	UT	A	A7	TXVIII	14
PL-109	2007	Kolkata	El Tor	O1	Ogawa	T2	T16	UT	A	A7	TXVIII	15

Table 1 (cont.)

Strain no.	Year of isolation	Geographical location	Biotype	Serogroup	Serotype	Basu & Mukerjee phage type	New phage type	O139 phage type	PFGE		RAPD type	<i>VcA</i> VNTR repeat number
									Pulsotype	Subtype		
PL-110	2007	Kolkata	El Tor	O1	Ogawa	T2	T24	UT	A	A7	TXVIII	15
PL-111	2007	Kolkata	El Tor	O1	Ogawa	T2	T25	UT	A	A7	TXVIII	15
PL-112	2007	Kolkata	El Tor	O1	Ogawa	T2	T25	UT	A	A7	TXVIII	14
PL-113	2007	Kolkata	El Tor	O1	Ogawa	T2	T25	UT	A	A7	TXVIII	15
PL-114	2007	Kolkata	El Tor	O1	Ogawa	T2	T24	UT	A	A7	TXVIII	14
PL-115	2007	Kolkata	El Tor	O1	Ogawa	T2	T27	UT	A	A7	TXVIII	14
PL-116	2007	Kolkata	El Tor	O1	Ogawa	T2	T27	UT	A	A7	TXVIII	15
PL-117	1992	Brazil	—	O26	—	UT	UT	UT	J _Y	—	TI _Y	17
PL-118	1992	Brazil	—	O26	—	UT	UT	UT	G _Y	—	THI _Y	12
PL-119	1992	Brazil	—	O26	—	UT	UT	UT	J _Y	—	TI _Y	10
PL-120	1992	Brazil	—	O26	—	UT	UT	UT	K _Y	—	THII _Y	5
PL-121	1994	Brazil	—	O26	—	UT	UT	UT	L _Y	—	THII _Y	15
PL-122	2004	Brazil	—	Non-O1/ Non-O139	—	UT	UT	UT	M _Y	—	TIV _Y	10
PL-123	2004	Brazil	—	Non-O1/ Non-O139	—	UT	UT	UT	N _Y	—	TIV _Y	15
PL-124	2004	Brazil	—	Non-O1/ Non-O139	—	UT	UT	UT	O _Y	—	TV _Y	10
PL-125	2004	Brazil	—	Non-O1/ Non-O139	—	UT	UT	UT	F _Y	—	TI _Y	10
PL-126	2004	Brazil	—	Non-O1/ Non-O139	—	UT	UT	UT	F _Y	—	TI _Y	13
PL-127	2004	Brazil	—	Non-O1/ Non-O139	—	UT	UT	UT	G _Y	—	THI _Y	14
PL-128	2004	Brazil	—	Non-O1/ Non-O139	—	UT	UT	UT	H _Y	—	TVI _Y	18
PL-129	2004	Brazil	—	Non-O1/ Non-O139	—	UT	UT	UT	I _Y	—	TVII _Y	23
PL-130*	Control	N16961	El Tor	O1	Inaba	T2	T27	UT	I	—	TXVII	23

UT, Untypable.

* These strains were used as control strains in this study.

† This *V. cholerae* O139 strain had a similar PFGE type as the El Tor strain.

Table 2. *Antimicrobial susceptibility pattern of V. cholerae strains incorporated in this study*

Antibiotics	% of susceptible strains (no. of strains screened)			
	<i>V. cholerae</i> O1 El Tor (67) susceptible	<i>V. cholerae</i> O1 classical (6) susceptible	<i>V. cholerae</i> O139 (29) susceptible	<i>V. cholerae</i> non-O1/ non-O139 (23) susceptible
Ampicillin	0	0	0	0
Azithromycin	92.75	100	65.51	43.47
Ceftriaxone	33.34	0	65	0
Chloramphenicol	85.50	83.33	96.55	100
Ciprofloxacin	73.91	100	100	95.65
Co-trimoxazole	68.11	100	100	100
Furazolidone	59.42	66.66	97.10	100
Norfloxacin	85.50	100	96.55	100
Streptomycin	31.89	100	86.20	100
Tetracycline	85.50	83.33	93.10	95.65

the isolates was drawn to predict the degree of clonal relatedness.

RESULTS

Antibiotic sensitivity profile

All the O1 El Tor and O139 isolates were uniformly resistant to ampicillin (Table 2). All O1 classical and non-O1/non-O139 strains were resistant to ampicillin and ceftriaxone. In total, 68.1% *V. cholerae* O1 El Tor isolates were resistant to streptomycin, whereas a higher level of susceptibility was observed in O139, classical and non-O1/non-O139 isolates against this antibiotic. In the current study, all the *V. cholerae* strains were not uniformly susceptible to tetracycline. Higher susceptibility was seen in all strains except non-O1/non-O139 tested with azithromycin. All strains showed high susceptibility against ciprofloxacin, norfloxacin, chloramphenicol, co-trimoxazole, furazolidone, ciprofloxacin and chloramphenicol.

Phage-typing results

Phage-typing results are given in Table 1. During the study period all the O1 El Tor strains were clustered into phage type T2 with Basu & Mukerjee's scheme. It was evident from Table 2 that type T27 was the predominant type (56.7%). The phage types other than type T27 did not follow any regular pattern and appeared scattered in different parts of the world. Except for type T27 other phage types were type T25 (22.3%), type T24 (4.4%), type T16 (1.4%) and type

T4 (1.4%). Out of 67 strains, 12.1% were untypable with the new typing scheme. The remaining 29 O139 isolates (68.9%) from different geographical regions of India were grouped as type T1 and 31.0% were untypable with the O139 typing scheme. All O1 classical and non-O1/non-O139 strains were untypable with both the O1 and O139 typing scheme.

RAPD analysis

Purified genomic DNA isolated from *V. cholerae* isolates was used for DNA fingerprinting by the RAPD method. The results were obtained with primer 1281 (5'-AACGCGCAAC-3') with amplified fragment size ranging from 0.2 kb to 10.0 kb. RAPD-PCR was used as a means to determine the clonal relatedness of isolates by their chromosomal polymorphism. Using RAPD-PCR out of 130 *V. cholerae* strains 69 O1 El Tor strains gave rise to 16 different electrophoretic patterns TI-TX and TXIII-TXVIII (Table 1). RAPD type TI was the most prevalent, and includes 33.3% isolates belonging to both serotypes of Ogawa and Inaba. This was followed by TXIII (13.0%), TXVIII (13.0%), TIX (7.2%) and TVIII (5.7%). Eight *V. cholerae* O1 classical strains were grouped into two types TXI (10.1%) and TXII (1.4%) and that were different from O1 El Tor strains. Only six distinct RAPD types TI_X (6.6%), TII_X (3.3%), TIII_X (3.3%), TIV_X (6.6%), TV_X (30%) and TVI_X (30%) were found in 30 *V. cholerae* O139 strains. Six of the O139 strains were presented as TI (four isolates), TVI (one isolate) and TVII (one isolate) which shared 100% similarity with these RAPD types of O1 El Tor

strains. Much more heterogeneity was observed within 23 *V. cholerae* non-O1/non-O139 strains, i.e. from TI_Y to TXV_Y. RAPD patterns obtained with reference *V. cholerae* strains MAK 757 (PL-29, TXVI), N16961 (PL-130, TXVII), CL-154 (PL-70, TXI), O395 (PL-71, TXI) and NPR-4 (PL-107, TVI_X) were different from each other except CL-154 and O395, while all these strains were dissimilar in their RAPD profile from the rest of the strains included in this study.

Analysis of restriction digestion patterns of PFGE and cluster analysis

To assess the genetic interrelationship of *V. cholerae* strains, 130 *V. cholerae* strains were analysed by PFGE using the restriction endonuclease *Not*I. PFGE patterns were classified basically as proposed by Tenover *et al.* [31]. PFGE of all strains exhibited fragments ranging in size from 34.4 kb to 543.2 kb. A total of 33 pulsotypes were identified in 130 strains typable by PFGE (Table 1). Of the 33 distinctive pulsotypes, nine pulsotypes were represented by *V. cholerae* O1 El Tor (A–I). Pulsotype A was the predominant pulsotype, consisting of 59 O1 El Tor strains and showed nine subtypes (A1–A9) which were closely related to each other. The remaining pulsotypes (B–I) of O1 El Tor strains were distinct from pulsotype A and from each other. Two different PFGE patterns (pulsotypes J and K) were noted in the *V. cholerae* O1 classical strains and pulsotype J comprised of nine O1 classical strains exhibited three subtypes (J1–J3). The PFGE patterns of O1 El Tor and O1 classical were totally different from each other. Of 29 *V. cholerae* O139 strains, 22 appeared to have same banding pattern, pulsotype A_X and this pulsotype showed one subtype A_X1 consisting of one O139 strain. The other pulsotypes detected in O139 strains were B_X (five isolates) with two subtypes B_X1 (one isolate) and B_X2 (one isolate), C_X (one isolate), D_X (one isolate, i.e. control strain NPR-4) and E_X (one isolate). Interestingly, the E_X pulsotype of O139 serogroup gave a similar PFGE pattern as the A8 subtype of *V. cholerae* O1. By PFGE, all non-O1/non-O139 isolates were genetically very diverse, as 17 different patterns (pulsotypes A_Y–Q_Y) were identified in 22 typable isolates by PFGE. The PFGE profiles of non-O1/non-O139 strains differed from each other and from the patterns displayed by the strains belonging to the O1 and O139 serogroups. Furthermore, PFGE patterns A8 and A4 predominated

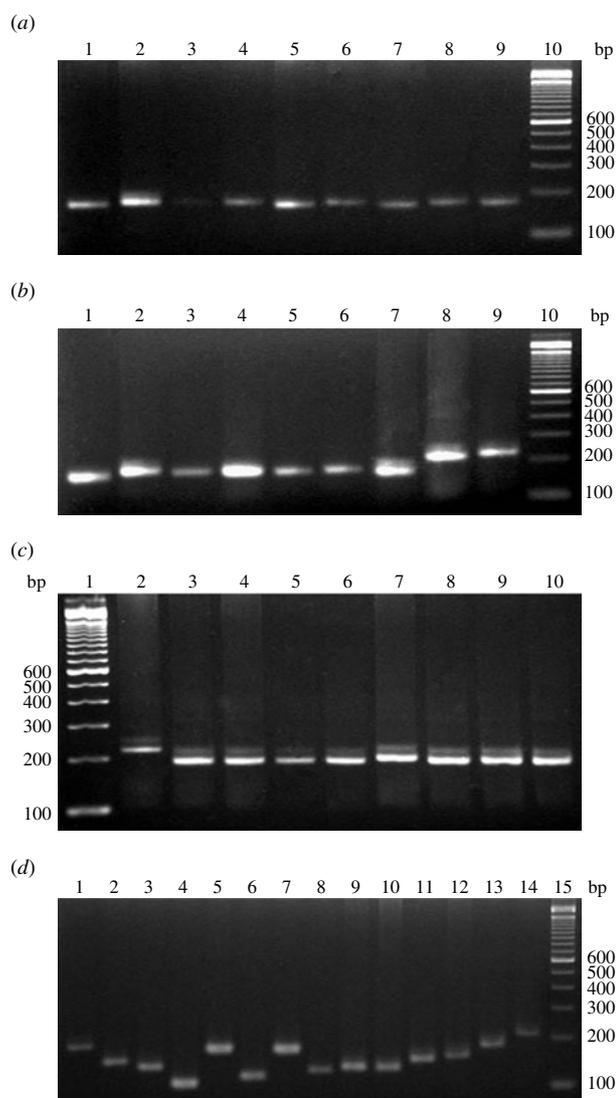


Fig. 1. *VcA* VNTR profiles of representative *V. cholerae* strains of this study. (a) Representative *V. cholerae* O1 El Tor strains: lane 10, 100-bp DNA ladder; lanes 1–9 contain strains PL-108 to PL-116, respectively. (b) *V. cholerae* O1 classical strains: lane 10, 100-bp DNA ladder; lanes 1–4 contain strains PL-63 to PL-66, lane 5 contains O1 El Tor strain PL-67 and lanes 6–9 contain strains PL-68 to PL-71, respectively. (c) Representative *V. cholerae* O139 strains: lane 1, 100 bp DNA ladder; lanes 2–10 contain strains PL-99 to PL-107, respectively. (d) Representative *V. cholerae* non-O1/non-O139 strains: lane 15, 100 bp DNA ladder; lanes 1–14 contain strains PL-117 to PL-130, respectively.

among Ogawa serotypes whereas subtype A was predominant within Inaba serotypes.

A dendrogram of 130 *V. cholerae* strains was constructed of nine major well separated clusters (Supplementary Fig. S1, available online). The O1 El Tor, O1 classical, O139 and non-O1/non-O139 strains were constructed separately from their PFGE profiles.

For O1 El Tor strains, the four major clusters were detected and they were closely related. The strains of these clusters were PL-2, PL-8, PL-12, PL-15, PL-77, PL-78; PL-7, PL-9, PL-11, PL-16, PL-80, PL-79, PL-76, PL-116, PL-115, PL-112; PL-114, PL-108, PL-113, PL-111, PL-109, and PL-62, PL-61, PL-60, PL-57, PL-56, PL-55 and PL-54. *V. cholerae* O139 strains revealed two major clusters, one comprised of PL-18, PL-19, PL-21, PL-22, PL-23, PL-25 and PL-26 strains and these strains showed closely similar PFGE profiles with the strains PL-20 and PL-81. The second major O139 cluster with strains PL-99 and PL-101 to PL-106 showed 89% similarity with the first cluster of O139. The O1 classical and non-O1/non-O139 *V. cholerae* strains were in separate clusters.

***Vca* loci distribution within *V. cholerae* genome**

Amplification with the two specific primers for the *Vca* locus revealed high *Vca* polymorphism in all *V. cholerae* strains incorporated in this study. In total, 22 types of *Vca* alleles with a minimum of 5 to a maximum of 28 repeat numbers were found in the *V. cholerae* strains examined; the locus was detected in every strain (Table 1). The TGCTGT repeat number was established for each allele. Allele sizes in all samples were estimated against a molecular-weight marker (Fig. 1) and the results were verified by direct sequencing of the allele found in 130 *V. cholerae* strains of this study. Nucleotide sequences of the 3' terminal of the *Vca* region of all the strains are given in Supplementary Figure S2 (online). The repeat number 16 was the predominant type containing 17 strains (11 O1 El Tor, one O1 classical, three O139 and two non-O1/non-O139). Repeat number 15 was observed in 16 strains, 21 in 14 strains, 13 in eight strains, 19 in nine strains, 14 in 12 strains, 20 in 16 strains, 23 in six strains, eight in two strains, 22 in two strains, 18 in nine strains and 12 in two strains. The three *Vca* repeats, repeat 28 in one strain, repeat 17 in four strains and repeat 24 in one strain were detected only in *V. cholerae* O1 El Tor strain. Out of the 130 *V. cholerae* strains incorporated in this study, only one (PL-37) represented a cell mixture with two *Vca* alleles, 22 and 14. The *Vca* repeats with 11 (two strains), 26 (one strain) and 28 (one strain) repeat numbers were detected only in four *V. cholerae* O139 strains but were totally absent in all other O1 El Tor, classical and non-O1/non-O139 strains. Subsequently, repeat number 9 (one strain), number 4 (four strains) and number 5 (one strain) were

observed only in non-O1/non-O139 strains. Dendrogram analysis of 130 *V. cholerae* isolates (Supplementary Fig. S3, online) showed 11 major well separated clusters with repeats 16, 15, 14, 23, 13, 21, 17, 18, 19, 20 and 10. The *V. cholerae* O139 strains from different parts of India appeared as a separate branch of the dendrogram with 89% of the bands being common.

Comparative study of phenotypic and genotypic methods

Of the 130 *V. cholerae* strains, seven sets of O1 El Tor strains PL-1, PL-2; PL-45, PL-47; PL-57, PL-58; PL-59, PL-61, PL-62; PL-72, PL-73, PL-74, PL-76, PL-77, PL-79; PL-108, PL-112 and PL-11, PL-113, one set of O1 classical PL-70, PL-71 and three sets of *V. cholerae* O139 strains PL-82, PL-88; PL-99, PL-101, PL-102, PL-103, PL-104, PL-105, PL-106 and PL-100, PL-102 which belonged to similar serogroups also gave similar phage type, RAPD type, PFGE profile and same number of repeats in *Vca* VNTR. Under O1 El Tor strains type T27 was the predominant type and included 41 *V. cholerae* strains but showed 12 PFGE types (four pulsotypes and eight subtypes), 12 RAPD types and 13 types of *Vca* alleles. The second major phage type T25 consisting of 15 isolates was differentiated into eight PFGE types (two subtypes and six pulsotypes), three RAPD types and seven types of *Vca* repeats. Three O1 El Tor strains with similar phage type T24 were dissimilar in their PFGE (two types), RAPD (two types) and *Vca* VNTR (three repeats) results. A single strain PL-109 was identical in its PFGE (subtype A7), RAPD (TVIII) profile and *Vca* repeat number [15] with O1 El Tor strains but showed totally different phage type, i.e. T16. Eight *V. cholerae* O1 El Tor strains were untypable with both the O1 typing scheme but molecular-typing methods were able to type them and gave seven types of PFGE (five pulsotypes and two subtypes), three types of RAPD patterns and four types of *Vca* repeats. On the other hand, the eight O1 classical *V. cholerae* isolates exhibited entirely different PFGE (two pulsotypes and three subtypes) and RAPD (TXI and TXII) profile from the rest of the strains in this study but with similar *Vca* repeats. Within *V. cholerae* O139 serogroup 21 strains with phage type T1 were discriminated by their PFGE (four pulsotypes and one subtype), RAPD (five types) and *Vca* VNTR (nine types of *Vca* alleles) profile. Similar results (PFGE, three pulsotypes; RAPD, one

type; and *VcA* repeat, four types) were also seen in the nine untypable O139 strains. Interestingly, five O139 strains PL-3, PL-4, PL-18, PL-19 and PL-23 gave RAPD profiles such as TI, TVIII and TVI similar to O1 El Tor strains. Subsequently, another O139 strain PL-5, exhibited both O1 El Tor PFGE (subtype A8) and RAPD (TI) profiles. In this context 23 *V. cholerae* non-O1/non-O139 strains demonstrated 17 entirely different PFGE pulsotypes and 15 different RAPD types from the remaining *V. cholerae* strains of this study. They produced 15 types of *VcA* VNTR alleles and among them only repeat numbers 5, 10, 12 were found. In the current study, we have also found a geographical relationship between strains of *V. cholerae*. Some sets of strains such as PL-1, PL-2; PL-45, PL-47; PL-59, PL-61, PL-62; PL-76, PL-77, PL-79; PL-72, PL-73, PL-74, PL-75 originated in Goa, Brazil, Chennai, Bombay, and Andhra Pradesh, respectively and were indistinguishable from each other by all the molecular-typing tools. Therefore, these sets of strains were genetically and also geographically related. Interestingly, two large sets of strains PL-99 to PL-106 and PL-108 to PL-116 originated from the same geographical location were genetically similar by PFGE and RAPD but distinguishable by *VcA* VNTR.

DISCUSSION

The intent of this study was to discover a swift, cost-effective and more discriminatory molecular genotyping tool of *V. cholerae* strains that would facilitate the continuous monitoring and extensive epidemiological analysis of cholera outbreaks. For this purpose different molecular-typing methods, RAPD, PFGE and *VcA* VNTR, were used to understand the genetic relatedness between the *V. cholerae* strains isolated from different geographical regions, within the country and abroad, that had different phage types.

An antibiogram of *V. cholerae* strains revealed that 68.1% *V. cholerae* O1 El Tor isolates were resistant to streptomycin, whereas a higher level of susceptibility was seen in O139, classical and non-O1/non-O139 isolates with this drug. Therefore, it appears that one drug, streptomycin, could allow differentiation between the strains belonging to serotypes O1 El Tor, O139 and non-O1/non-O139 of *V. cholerae*. As previously reported, there was no significant correlation between phage types and antibiogram [8].

Phage typing is a conventional typing system, which has been used for decades at NICED, India.

Because of its high host specificity it offers basic information on identification, biotyping and discrimination of strains. Theoretically, strains belonging to a single phage type originate from a single clone. However, in the current study, a high level of heterogeneity was found in RAPD, PFGE, *VcA* VNTR and computer-based analysis of genomic profiles which indicated the clonal diversity of *V. cholerae* O1 and O139 serogroups. In parallel to phage typing, molecular-typing methods should be included for efficient integrated national and international epidemiological research on *V. cholerae*.

Molecular-typing techniques have the potential for studying the origin and interrelatedness of strains of epidemiological settings [1]. PFGE, RAPD-PCR and *VcA* VNTR showed that *V. cholerae* strains belonging to same phage type were genetically heterogeneous except for a few sets of strains. Interestingly, five O139 strains gave RAPD profiles such as TI, TVIII and TVI similar to O1 El Tor strains and another O139 strain PL-5, exhibited both O1 El Tor PFGE (subtype A8) and RAPD (TI) profiles. This indicates that the *V. cholerae* O139 probably originated from O1 ancestry. The clustering of isolates can vary considerably between RAPD and PFGE. The RAPD method does not enable the constitution of a pattern for the discrimination of *V. cholerae* O1 strains. Furthermore, the reproducibility of the reaction conditions poses a problem for routine laboratory use. This inconvenience makes it complicated to create a schematic pattern for use in public health laboratories to identify the different clones for epidemiological investigation.

On the contrary, PFGE has proven to be the best technique for investigating the clonal relatedness amongst enteric pathogens, e.g. *E. coli* O157:H7, *Campylobacter jejuni*, *Vibrio fluvialis*, *Listeria monocytogenes*, *Clostridium perfringens*, *Shigella dysenteriae*, etc. [10, 32–36]. In our previous study, PFGE was more discriminatory than phage typing as the predominant phage type was divided into multiple pulsotypes [9]. PFGE had better typability and concordance with different geographical regions than phage typing as reported earlier from our laboratory [9].

VNTRs are DNA regions consisting of several tandemly repeated oligonucleotides and represent a distinctive hereditary feature of an individual. VNTR has been used by some workers as a strain typing tool for *V. cholerae* by exploiting several VNTR loci [16, 37, 38]. *VcA* VNTR was used in the current study

to type the *V. cholerae* strains. Computer analysis revealed seven potential VNTR loci in the DNA sequences of *V. cholerae* chromosomes 1 and 2 [21]. One of these *VcA*, was chosen in this study. This locus was selected for further analysis by Vodopyanov *et al.* [21], because it has the highest repeat number and thereby allows a greater allele polymorphism in various strains. The fact of being chosen established *VcA* VNTR as a most informative genetic typing tool for *V. cholerae* [21]. However, phylogenetic analysis revealed the clustering of *V. cholerae* strains from different parts of India and abroad, indicating a clonal relationship. Dendrogram analysis of *VcA* VNTR produced 11 well separated clusters with clonal relatedness of *V. cholerae* strains from different geographical regions of India and abroad. *VcA* VNTR detects all genetic variations within the amplified *VcA* fragment while PFGE examines only those that are in specific restriction sites and those that involve large insertions or deletions of DNA. As variation occurs most commonly at the nucleotide level, it could be suggested that the discriminatory ability of *VcA* VNTR is better than PFGE. This fact was also strongly reflected in our results. In particular, results obtained from two batches of strains PL-99 to PL-106 and PL-108 to PL-116 clearly showed the typing abilities of *VcA* VNTR and PFGE. *VcA* VNTR analysis is a rapid, cost-effective, and easy to implement typing method with high discriminatory power. This method also combines genome-wide coverage of loci with different mutation rates, which could serve as an efficient tool for phylogenetic studies and for rapid bacterial typing. The results obtained from our study also exhibited and corroborated this fact and validate *VcA* VNTR as an important genetic typing tool for *V. cholerae*.

NOTE

Supplementary material accompanies this paper on the Journal's website (<http://journals.cambridge.org/hyg>).

DECLARATION OF INTEREST

T.S.B. and M.D. were Senior Research Fellows of the National Institute of Cholera & Enteric Diseases and B.L.S was Deputy Director of NICED at the time of the study. The authors and NICED have no financial interests that are affected by the material in the paper.

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