Genetic characterization of *Vibrio cholerae* strains by inter simple sequence repeat-PCR

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Abstract

The utility of inter simple sequence repeat-PCR (ISSR-PCR) assay in the characterization and elucidation of the phylogenetic relationship between the pathogenic and nonpathogenic isolates of *Vibrio cholerae* is demonstrated. A total of 45 *V. cholerae* strains including 15 O1 *El Tor*, nine O139 and 21 non-O1/non-O139 strains were analyzed using eight ISSR primers. These primers, which are essentially simple sequence repeats (SSR) with additional nonrepeat bases at the 5′ or 3′ end, amplify genomic regions interspersed between closely spaced SSRs. Neighbor-joining analysis showed that the strains belonging to the same serogroup clustered together with the exception of one O1 and two O139 strains. The absence of pathogenicity islands in these strains, as confirmed by PCR, suggested their non-O1/non-O139 origin. Thus the ISSR-PCR-based phylogeny was consistent with the classification of *V. cholerae* based on serological methods. A finer resolution of the clustering of the toxinogenic O1 *El Tor* and toxinogenic O139 subtypes was obtained by ISSR-PCR analysis as compared with the *E*nterobacterial *R*epetitive *I*ntergenic *C*onsensus sequences-based PCR analysis for the same set of strains. Thus, it is proposed that ISSR-PCR is an efficient tool in phylogenetic classification of prokaryotic genomes in general and diagnostic genotyping of microbial pathogens in particular.

Introduction

The gram-negative bacterium *Vibrio cholerae* has been identified as the main causative agent of the acute dehydrating diarrhoeal condition called cholera. Among more than 200 O-antigen serogroups that have been identified and characterized, only two serogroups -O1 and O139 – are known to cause the disease, which can be epidemic, endemic or pandemic in nature (Shimada *et al.*, 1994). The strains belonging to the O1 serogroup are further classified into two biotypes, namely the Classical and *El Tor* biotypes, which can be differentiated by biochemical and genetic traits (Kaper *et al.*, 1995). Other *V. cholerae* serogroups, which are not associated with epidemics or pandemics, are collectively referred to as the non-O1/non-O139 serogroup (Albert & Nair, 2005). The toxinogenic O1 and O139 strains contain the Vibrio Pathogenicity Island (VPI) and CTXΦ bacteriophages, but 95% of non-O1/non-O139 *V. cholerae* strains do not harbour these elements. The toxinogenic strains are believed to have evolved from ancestral nontoxinogenic *V. cholerae* strains upon acquisition of the VPI and CTXΦ filamentous bacteriophages by horizontal gene transfer (Hacker *et al.*, 1997). Several studies have shown that O139 strains are phylogenetically and serotypically very similar to the O1 *El Tor* strains (Ramamurthy *et al.*, 1993; Bik *et al.*, 1995; Stroeher *et al.*, 1995; Dalsgaard *et al.*, 1998), supporting the event of horizontal gene transfer between the O1 and nontoxinogenic O139 serogroups. Reports on non-O1/non-O139 strains as the causative agents of sporadic cases of a cholera-like disease (Morris, 1994) and isolated outbreaks (Bagchi *et al.*, 1993) also point towards the emergence of newer variants of toxinogenic *V. cholerae* from non-O1/non-O139 serogroups.

In view of the propensity of O1 and O139 to cause global epidemics and pandemics, an understanding of the genetic structure of microbial populations is relevant to track the source and spread of epidemics, to manage the spread of resistance to drugs and to improve the efficacy and safety of genetically engineered microorganisms intended for environmental applications.

Unlike biochemical and serological methods, molecular tools for genotyping of strains are more accurate in terms of

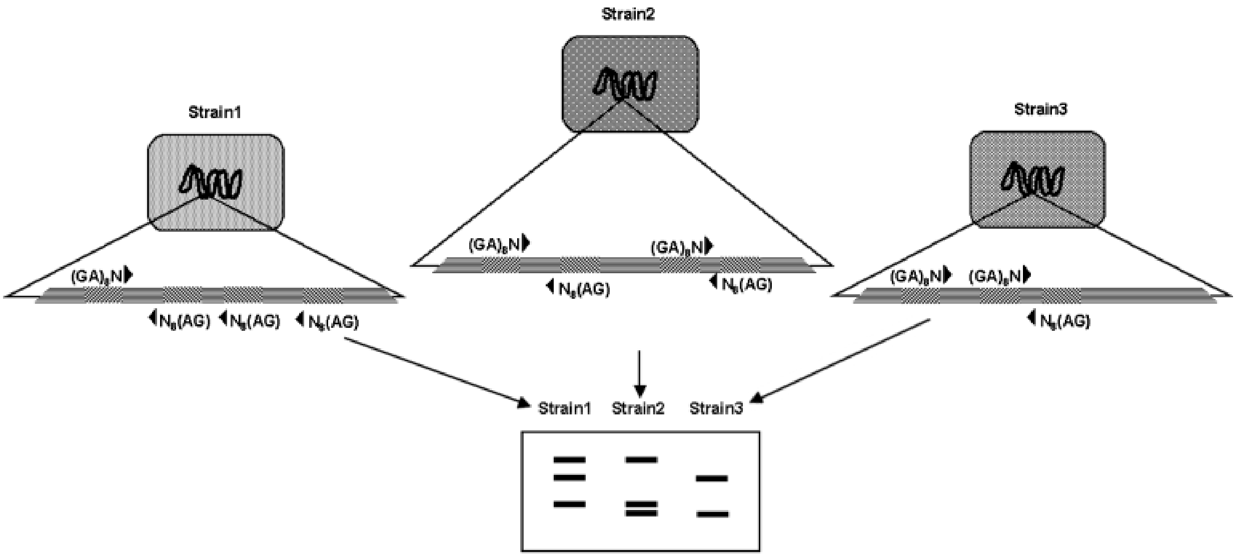

Fig. 1. A schematic representation of the ISSR-PCR assay. $(GA)_n$ repeats arranged closely and in inverted fashion (boxes with diagonal lines) among nonrepeat regions in the genome are targets for the single primer $(GA)_8$ N▶. The variation in numbers and position of $(GA)_n$ repeats in the genomes of the three representative strains result in differential amplicon profiles.

identifying sublineages of microorganism that would enhance the sensitivity of epidemiological investigations. Various molecular techniques such as pulse field gel electrophoresis (PFGE) (Safa *et al.*, 2005), randomly amplified polymorphic DNA (RAPD) (Makino *et al.*, 1995), amplified fragment length polymorphism (AFLP) (Jiang *et al.*, 2000), repetitive extragenic palindromic PCR (REP-PCR) (Shangkuan *et al.*, 1997), Enterobacterial Repetitive Intergenic Consensus sequences-based PCR (ERIC-PCR) (Versalovic *et al.*, 1991), ribotyping (Bhanumathi *et al.*, 2002), Multi Locus Sequence Typing (MLST) (Lee *et al.*, 2006) and, finally, comparative genomics (Beres *et al.*, 2006) have been applied in attempts to characterize and establish the genetic population structure of microorganisms. Difference in sensitivity and accuracy of different methods reflects upon the need for more efficient genotyping techniques in the microbiologists' tool kit.

The presence of simple sequence repeats (SSR) in prokaryotes is well documented (Gur-Arie *et al.*, 2000), and some SSRs show extensive length polymorphisms (Yang *et al.*, 2003; Sreenu *et al.*, 2006). Successful use of PCR-based SSR amplification followed by amplicon size determination to analyze the spread of microbial pathogens has been reported for *Haemophilus influenzae* and *Candida albicans* (Bretagne *et al.*, 1997; van Belkum *et al.*, 1997). Inter simple sequence repeat-PCR (ISSR-PCR) technique also exploits the genome-wide distribution of SSRs. Single oligonucleotide primers containing a frequently occurring SSR motif with a stretch of arbitrary nucleotides, anchored at either the 5′ or 3′ end, initiate PCR amplification of genomic segments flanked by inversely oriented, closely spaced repeats (Fig. 1). The PCR products thus generated reveal multilocus profiles, which could be revealed on agarose or polyacrylamide gels (Zietkiewicz *et al.*, 1994). ISSR-PCR analysis is technically simple and requires no prior genomic sequence information. The method yields highly reproducible results and generates abundant polymorphisms in eukaryotic systems. To date, many studies have been reported on using ISSR markers for identification or genotyping of variants in agriculturally important plants like wheat, rice and recently in insects (Nagaoka & Ogihara, 1997; Reddy *et al.*, 1999; Nagaraju *et al.*, 2002), but until now the feasibility of employing ISSR markers for diagnostic purpose or for fingerprinting of microbial pathogens has not been addressed.

In the present study, the utility of ISSR-PCR as a novel method of genotyping of *V. cholerae* has been demonstrated. The results show that as a diagnostic tool, ISSR-PCR could be invaluable in disease diagnosis, recognizing outbreaks of infection, and detecting the source and spread of infection.

Materials and methods

SSR analysis

The whole genome sequence of *V. cholerae El Tor* strain N16961 was analyzed for the abundance and distribution of SSRs using the prokaryotic microsatellite database, MICdb (http://210.212.212.7/MIC/index.html) (Sreenu *et al.*, 2003). The two chromosomes VC (chromosome 1, 2.9 Mb) and VCA (chromosome 2, 1.1 Mb) were screened for perfect SSRs, which were repeated a minimum of three times.

Bacterial strains

A total of 45 *V. cholerae* strains including O1, O139 and non-O1/non-O139 strains, obtained from the culture collections of the International Centre for Diarrhoeal Diseases Research, Bangladesh (ICDDR, B), Dhaka, was streaked on the differential and selective TCBS agar plates (Kobayashi *et al.*, 1963). Of these, 21 belonged to the Non-O1/Non-O139 serogroup (strains numbers N-1, N-2, N-3, N-7, N-8, N-9, N-11, N-12, N-13, N-14, N-17, N-19, N-20, N-21, N-22, N-27, N-28, N-33, N-35, N-36, N-37), 15 to O1 serogroup (G-1, G-3, G-4, G-5, G-6, G-7, G-8, G-9, G-10, G-12 G-18, G-22, G-26, G-30, G-32) and nine to O139 serogroup (B-3, B-4, B-12, B-18, B-20, B-22, B-27, B-36, B-38).

Preparation of genomic DNA

Genomic DNA was prepared by a modification of a method described elsewhere (Chakraborty *et al.*, 2001). A single bacterial colony was inoculated in 10 mL of Luria–Bertani (LB) broth with 0.5% NaCl (w/v) and incubated in a rotary shaking incubator at 37 °C and 200 r.p.m. for 16 h. Overnight-grown bacterial cells were pelleted and resuspended in 2 mL of extraction buffer [100 mM Tris HCl (pH 8.0), 50 mM NaCl, 50 mM EDTA, 1% sodium dodecylsulphate (SDS)] to which lysozyme was added at a final concentration of 1 mg mL^{-1} and incubated at 37 °C for 1 h. To this lysate, proteinase K was added at 100 µg mL^{-1} concentration and incubated at 37 °C for 2 h and was extracted with a phenol/chloroform/isoamyl alcohol (25 : 24 : 1) mixture. To the aqueous supernatant, equal volume of isopropanol was added and pelleted. The pellet was washed with 70% ethanol, air-dried and suspended in TE buffer (10 mM Tris HCl, 1 mM EDTA).

ISSR primers

Eight ISSR primers including five di- and three trinucleotide repeats with a stretch of arbitrary nucleotides anchored at either 3′ or 5′ ends were used in the study (Table 1). All of these primers except (GA)$_8$T were designed in house at the Centre for DNA Fingerprinting and Diagnostics (CDFD while the sequence of (GA)$_8$T was obtained from NAPS, University of British Columbia (UBC).

ISSR-PCR

ISSR-PCR amplification was carried out in a volume of 10 µL containing 1 × PCR buffer, 0.1 mM each of dCTP, dGTP, dTTP, dATP, 2.5 mM MgCl$_2$, 0.8 µM primer, 1.0 U of Taq DNA polymerase (MBI Fermentas) and 20 ng of template DNA. All the PCR amplifications were carried out on a PE 9700 (Perkin Elmer Corp) thermal cycler using the following reaction conditions: initial denaturation at 94 °C for 3 min and 35 cycles of 94 °C for 1 min, 50 °C for 30 s and 72 °C for 2 min followed by a final extension of 72 °C for 10 min. The PCR products were analyzed on a 2.5% Metaphor agarose gel (0.5 × TAE buffer, 80 V). The gels were stained with ethidium bromide and documented using the Molecular Imager Gel Dc XR system (Bio-Rad Laboratories).

Table 1. List of primers used in the study

Locus	Primer sequence	Annealing temperature (°C)
ToxR	F: 5′ GATTAGGCAGCAACGAAAGC 3′ R: 5′ GATGAAGGCACACTGCTTGA 3′	55
ToxS	F: 5′ ATTTTGGACTGCCATTCTCG 3′ R: 5′ ACGCATCGTTGCTAACCCTA 3′	60
TcpA	F: 5′ GACTAAGGCTGCGCAAAATC 3′ R: 5′ CTTCCTGGTGCAATGGACTT 3′	55
TcpB	F: 5′ GCACAAGGAGAGATGCACAA 3′ R: 5′ ACCGTGTAAATCAGCCCAAG 3′	55
TcpH	F: 5′ ACTCCCGATGCGACAAAAA 3′ R: 5′ TTGTGAGTAGTCGGGAATCAA 3′	55
TcpI	F: 5′ CTTGCGTGCATCATTACGTT 3′ R: 5′ CGACTGCTTTATCGCGAAGT 3′	55
TcpP	F: 5′ AATATCATCCTGCCCCCTGT 3′ R: 5′ TTGGATTGTTATCCCCGGTA 3′	55
ToxT	F: 5′ AAAAATTGCTTGGTTAGTTATG 3′ R: 5′ TCAAAATCATCCGATTCGTTC 3′	55
CtxA	F: 5′ TCAGACGGGATTTGTTAGGC 3′ R: 5′ ATGATGAATCCACGGCTCTT 3′	55
CTX left junction	rig1: 5′ CACGCTACGTCGCTTATGT 3′ tlc3: 5′ GGGAATGTTGAGTTCTCAGTG 3′	55
CTX right junction	rtxA2: 5′ GCGATTCTCAAAGAGATGC 3′ ctxB3: 5′ CCGCAATTAGTATGGCAA 3′	55
VPI left junction	VPI5: 5′′ GTGAATCTTGATGAGACGC 3′ VPI8: 5′ GCCATTGGGTAAGTAGC 3′	55
VPI right junction	VPI9: 5′ CCAATCCTTTGTGACGTTC 3′ VPI10: 5′ GGAAATCAGGAAGGTCAAAC 3′	55
ERIC	F: 5′ ATG TAA GCT CCT GGG GAT TCA C 3′ R: 5′ AAG TAA GTG ACT GGG GTG AGC G 3′	52
(GA)$_8$T	5′ GAGAGAGAGAGAGAGAT 3′	50
(CA)$_7$C	5′ GCTAGTGCTCACACACACACACAC 3′	50
C(GA)$_7$	5′ GACGATACGAGAGAGAGAGAGA 3′	50
(GA)$_8$C	5′ GAGAGAGAGAGAGAGACGG 3′	50
(ATG)$_4$GA	5′ ATGATGATGATGGACT 3′	50
GC(GCC)$_4$	5′ TGAGCGCCGCCGCCGCC 3′	50
TA(CAG)$_4$	5′ AAATACAGCAGCAGCAG 3′	50
T(GA)$_8$	5′ TGTAATGAGAGAGAGAGAGAGA 3′	50

Detection of virulence genes

Using the sequence information available on the NCBI database, the primers were designed for the following virulence genes: *TcpA*, *TcpP*, *TcpH*, *TcpB*, *TcpI*, *ToxT*, which constitute the pathogenicity island *CtxA*, which codes for a cholera toxin subunit A and for the regulatory *ToxR* and *ToxS* genes. Primer pairs across the left and right junctions of the VPI – VPI5/VPI8 and VPI9/VPI10, respectively, and Cholera Toxin island (CTX) – rig/tlc3 and rtxA2/ctxB3, respectively, were also used for validating their absence (Boyd *et al.*, 2000) (Table 1). PCR amplifications were performed in a reaction volume of 10 μL containing a final concentration of 1 × PCR buffer, 0.1 mM each of dCTP, dGTP, dTTP, dATP, 1.5 mM $MgCl_2$, 1.0 μM primer, 1.0 U of Taq DNA polymerase (MBI Fermentas) and 20 ng of template DNA. All the PCR reactions were carried out in a PE 9700 (Perkin Elmer Corp) thermal cycler using the following reaction conditions: the initial denaturation of 94 °C for 3 min and 35 cycles of 94 °C for 30 s, annealing at respective temperatures for 30 s and 72 °C for 1 min followed by a final extension of 72 °C for 10 min. The PCR products were separated on a 2.0% metaphor agarose gel stained with ethidium bromide.

ERIC-PCR

ERIC-PCR amplification was performed as reported earlier (Rivera *et al.*, 1995) in a reaction volume of 10 μL containing 1 × PCR buffer, 0.1 mM each of dCTP, dGTP, dTTP, dATP, 2.5 mM $MgCl_2$, 1.0 μM primer, 1.0 U of Taq DNA polymerase (MBI Fermentas) and 20 ng of template DNA. All the PCR reactions were carried out on a PE 9700 (Perkin Elmer Corp) thermal cycler using the following reaction conditions: with the initial denaturation of 94 °C for 5 min and 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 5 min followed by a final extension of 72 °C for 10 min. The PCR products were analyzed on a 1.5% metaphor agarose gel stained with ethidium bromide.

Data analysis

Specific ISSR-PCR and ERIC-PCR amplicons that were prominent and reproducible in two independent reactions were selected and scored for the presence as 1 or absence as 0. The sizing of the amplicons was carried out using the QUANTITY ONE software of the BioRad Gel Documentation system, against a size standard. Genetic distance was calculated from the data using WINDIST software of the WINBOOT package with the NTSYS format, and the genetic distance has been used for constructing dendrogram using the Neighbor-joining option of MEGA 3.2 software package (Kumar *et al.*, 1994).

Results and discussion

ISSR-PCR profile brings about the variation in the frequency of SSR repeats among different strains of *V. cholerae*. In the *V. cholerae* genome, mono-, di- and trinucleotide repeats are the most abundant (Fig. 2). Among dinucleotide repeats, CG/GC (CG and GC being complementary, are considered as one group) repeats are the most abundant followed by AG/CT and, TGA/TCA is the most frequent followed by TGC/GCA among the tri-nucleotide repeats (Fig. 2). For the present study, a total of eight anchored ISSR primers that included five di- and three tri-nucleotide repeat containing primers (Table 1) were chosen based on the SSR abundance allowing for a better coverage of the whole genome and scorability of the marker profile on a metaphor agarose gel electrophoresis. For considering a marker as polymorphic, the absence of an amplified product in at least one strain in a pairwise strain comparison was treated as the criterion.

All eight primers used in this study produced a total of 221 bands ranging from 190 to 2500 bp and all of them were found to be polymorphic, with 100% reproducibility (Table 2). A representative ISSR-PCR profile generated by primer $(GA)_8C$ (Fig. 3) and its comparison with the ERIC-PCR profile (Fig. 4) revealed the level of informativeness of ISSR markers. The 5′-anchored dinucleotide primer $(GA)_8T$ generated the least number of 16 markers as against the highest number of 37 markers generated by 3′ anchored

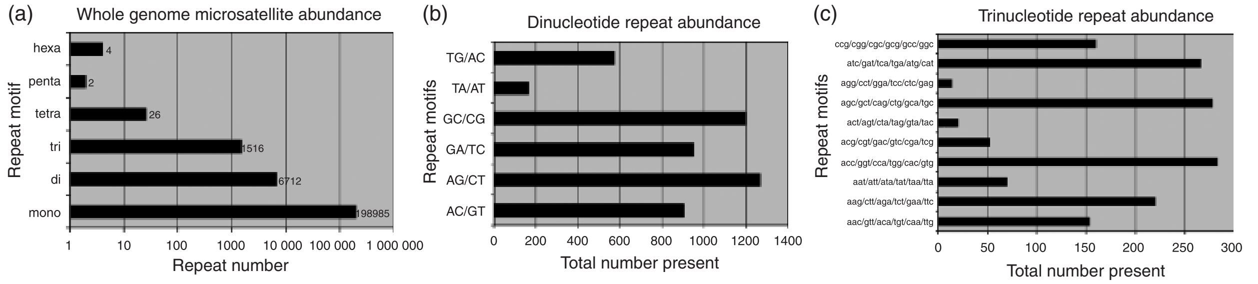

Fig. 2. (a) Whole genome profile of microsatellite abundance in *Vibrio cholerae*: Motifs ranging from mono- to hexa-nucleotides repeating a minimum of three times were considered. The numbers at the top of the bars are the actual number of the respective SSR type present in the genome. (b) Whole-genome profile of dinucleotide repeat abundance in *Vibrio cholerae* (complementary repeat motifs are grouped into one class, for e.g. CA/GT etc). (c) Whole-genome profile of tri-nucleotide repeat abundance in *V. cholerae* (the three different possible tri-nucleotide repeat units and their complementary motifs are grouped into one class for e.g. TGA/TCA, GAT/ATC, ATG/CAT etc).

Table 2. Amplicon profile generated by the eight ISSR primers

Primer code	Amplicon size range (bp)	No. of markers	No. of polymorphic markers	Markers specific to strains of (bp)					
				O1	O139	O1 and O139	non-O1/ non-O139	O1 and non-O1 non-O139	O139 and non-O1 non-O139
(ATG$_4$GA	250–1700	25	25	1700	–	–	250, 400	–	950
(CA)$_7$C	230–2500	29	29	–	–	360	250, 300, 340	470	230
CGA)$_7$	190–1300	32	32	–	–	–	550	470	230
(GA)$_8$C	300–2000	27	27	–	580	350, 520	980	–	–
(GA)$_8$T	420–2400	16	16	900	–	1000, 2400	500, 1250	–	–
GC(GCC)$_4$	200–1900	26	26	–	–	–	1300	1900	–
TA(CAG)$_4$	350–2000	37	37	–	–	450, 520	380, 500	–	–
T(GA)$_8$	190–1900	29	29	–	800	590	550	620, 750, 950	700

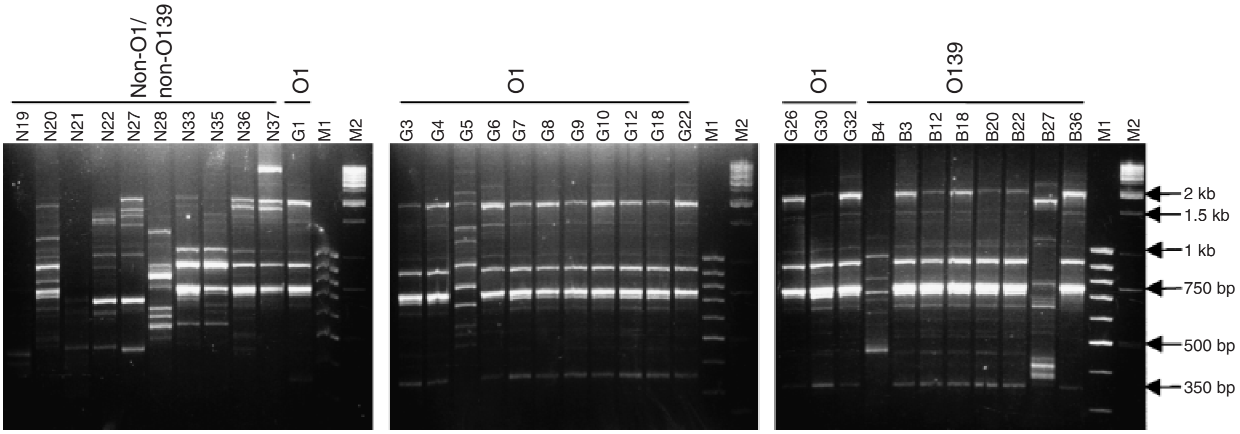

Fig. 3. ISSR-PCR profile for non-O1/non-O139, O1 and O139 strains generated by (GA)$_8$C primer run on 2.5% agarose gel. M1 – 100 bp size marker (MBI Fermentas), M2 – 1 kb size marker (MBI Fermentas). Arrows indicate marker sizes.

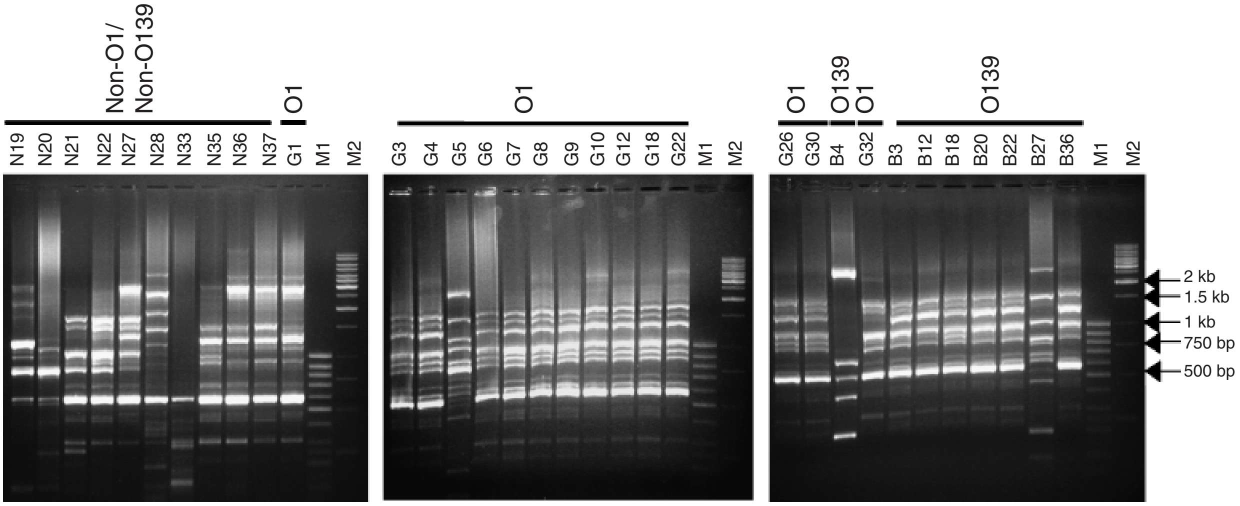

Fig. 4. ERIC-PCR profile of the same set of non-O1/non-O139, O1 and O139 strains used in Fig. 3, run on 1.5% agarose gel. M1 – 100 bp size marker (MBI Fermentas), M2 – 1 kb size marker (MBI Fermentas). Arrows indicate marker sizes.

trinucleotide primer TA(CAG)$_4$. Among O1 *El Tor* strains, the primers (GA)$_8$C and (GA)$_8$T generated the highest and lowest levels of polymorphism of 100% and 75%, respectively, and among all the O139 strains, the primers (ATG)$_4$GA, (CA)$_7$C, (GA)$_8$C, (GA)$_8$T, TA(CAG)$_4$ and T(GA)$_8$ generated 100% polymorphism and the lowest

Table 3. Serotype-wise polymorphism profile with the exclusion of the nontoxinogenic O1 and O139 strains

	O1 strains		O139 strains		Non-O1/ non-O139 strains
Primer code	% Polymorphism	% Polymorphism (G-5 excluded)	% Polymorphism	% Polymorphism (B-4 and B-27 excluded)	% Polymorphism
$(ATG)_4GA$	84.61	50.00	100.00	40.00	100
$(CA)_7C$	80.00	61.53	100.00	00.00	100
$C(GA)_7$	86.66	50.00	92.85	00.00	100
$(GA)_8C$	100.00	20.00	100.00	81.81	100
$(GA)_8T$	75.00	28.57	100.00	00.00	100
$GC(GCC)_4$	84.61	75.00	80.00	25.00	100
$TA(CAG)_4$	76.47	38.46	100.00	91.66	100
$T(GA)_8$	83.33	63.63	100.00	00.00	100

polymorphism of 80% was observed with primer $GC(GCC)_4$. The number of polymorphic markers generated and the percentage of polymorphism with each of the primers for toxinogenic O1 *El Tor* and O139 strains decreased drastically, with the exclusion of nontoxinogenic O1 *El Tor* strain G-5 and nontoxinogenic O139 strains B-3 and B-27 (Table 3).

Phylogenetic analysis

The dendrogram generated based on the cluster analysis showed the grouping of O1 *El Tor* strains with O139 strains in a single clade indicating the genetic similarity between these two sets of strains and thus corroborating the hypothesis of evolution of O139 strains from an ancestral O1 *El Tor* strain with the exchange of O antigen by horizontal gene transfer (Calia *et al.*, 1994; Stroeher *et al.*, 1995; Faruque *et al.*, 2003). Furthermore, the finer subclustering within the serogroups reflects on the clonal nature of the strains. For example, the strains N-33 and N-35, N-36 and N-37 grouped into one cluster suggesting their clonal origin. The same was the case with N-1, N-8, N-7 and N-11 strains while the numerous nontoxinogenic singletons suggest their diverse sources of origin (Thompson *et al.*, 2005). In the present study however, one O1 *El Tor* strain (G-5) and two O139 strains (B-3 and B-27) were assigned to the diverse non-O1/non-O139 group, which are nontoxinogenic in nature. Screening for the presence of VPI and CTX islands in these strains resulted negative, consistent with their clustering with the non-O1/non-O139 serogroups (Fig. 5). ISSR-PCR assay thus discriminated toxinogenic and nontoxinogenic strains of *V. cholerae* with a high level of accuracy. The existence of environmental reservoir of strains with pandemic potential (Chakraborty *et al.*, 2000; Nandi *et al.*, 2000) and extensive horizontal gene transfers between strains result in the emergence of new, highly virulent strains of *V. cholerae*. The nontoxinogenic non-O1/non-O139 strains, which are closely related to the toxinogenic O1/O139 strains, could thus be looked at as potential candidates with virulence (e.g. N-33, N-35, N-36 and N-37).

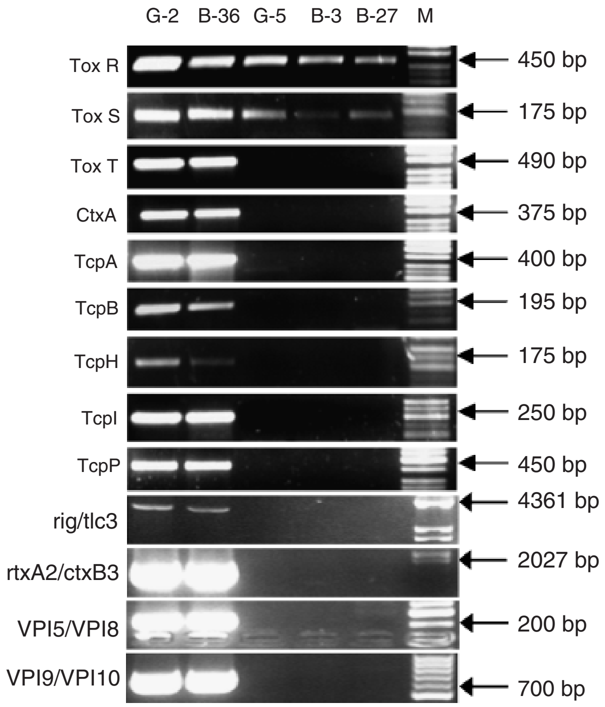

Fig. 5. Amplification profiles of virulence genes *TcpA, TcpP, TcpH, TcpB, TcpI, ToxT* and *CtxA* and regularory genes *ToxR* and *ToxS* of O1 El Tor (G-5) and O139 (B-3 and B-27) strains, which do not cluster with the toxinogenic O1/O139 strains. Primers across junctions of VPI (VPI5/VPI8; VPI9/VPI10) and CTX (rig/tlc3; rtxA2/ctxB3) were also used. Toxinogenic O1 El Tor strain G-2 and toxinogenic O139 strain B-36 are positive controls. Arrows indicate the amplicon sizes.

Comparison of ERIC-PCR and ISSR-PCR profiles

The dendrogram generated using the ERIC-PCR data was similar to that of ISSR-PCR data, with respect to the overall clustering of toxinogenic and nontoxinogenic strains of *V. cholerae* (Fig. 6). However, in ISSR-PCR, within the clade of toxinogenic strains, a distinct subclustering of O139 and O1 *El Tor* strains was observed while no such resolution of clusters was observed in the ERIC-PCR-based dendrogram.

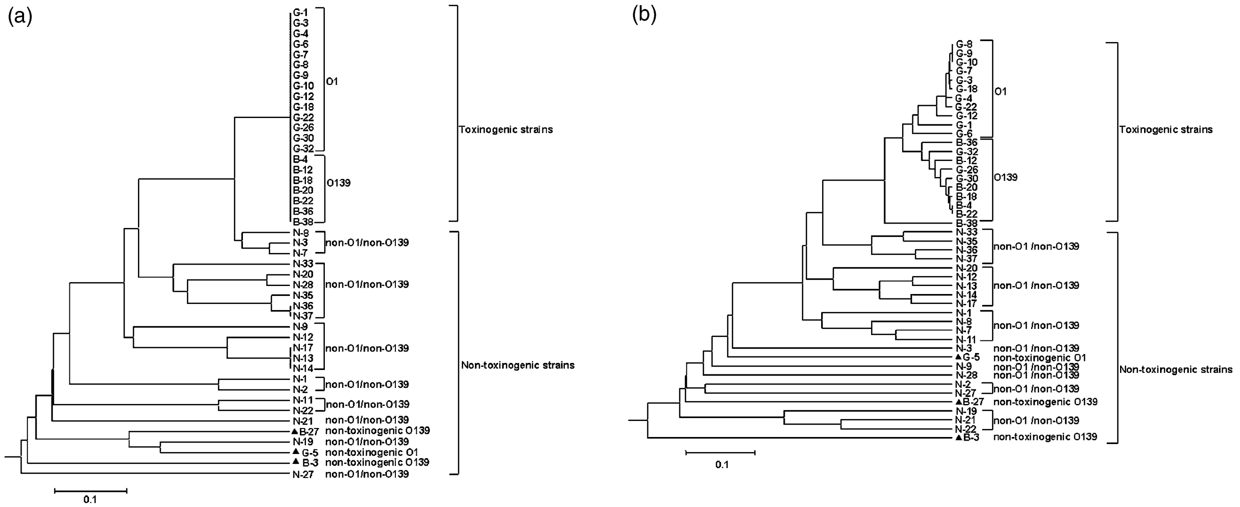

Fig. 6. (a) The ISSR-PCR profile-derived dendrogram illustrating the relationship among 45 strains of *Vibrio cholerae* belonging to O1, O139 and non-O1/non-O139 serogroups. **▲,**marked strains are nontoxinogenic O1/O139 strains clustered with the non-toxinogenic non-O1/non-O139 strains. Bar represents the distance scale. (b) Dendrogram based on ERIC-PCR data illustrating the relationship among 45 strains of *V. cholerae* belonging to O1, O139 and non-O1 non-O139 serogroups. ▲, marked strains are nontoxinogenic O1/O139 strains clustered with the non-toxinogenic non-O1/non-O139 strain. Bar represents the distance scale.

The same was the case with non-O1/non-O139 isolates, the grouping of which is clearly distinct in the ISSR-PCR dendrogram.

Thus, ISSR-PCR is technically simple, robust, high-throughput, universally applicable and cost-effective and could be exploited as an efficient tool in the phylogenetic and molecular epidemiological studies of any prokaryotic genome. Moreover, specific markers for specific pathogens could be identified, standardized and developed as a ready-to-use detection kit.

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Authors contribution

A.R. and V.S. contributed equally to this work.

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