Molecular phylogenetic analysis of *Vibrio cholerae* O1 El Tor strains isolated before, during and after the O139 outbreak based on the intergenomic heterogeneity of the 16S-23S rRNA intergenic spacer regions

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We have cloned, sequenced and analysed all the five classes of the intergenic (16S-23S rRNA) spacer region (ISR) associated with the eight *rrn* operons (*rrn*a-*rrn*h) of *Vibrio cholerae* serogroup O1 El Tor strains isolated before, during and after the O139 outbreak. ISR classes 'a' and 'g' were found to be invariant, ISR-B (ISRb and ISRe) exhibited very little variation, whereas ISR-C (ISRc, ISRd, and ISRf) and ISRh showed the maximum variation. Phylogenetic analysis conducted with all three ISR classes (ISR-B, ISR-C and ISRh) showed that the pre-O139 serogroup and post-O139 serogroup O1 El Tor strains arose out of two independent clones, which was congruent with the observation made by earlier workers suggesting that analyses of ISR-C and ISR-h, instead of all five ISR classes, could be successfully used to study phylogeny in this organism.

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1. Introduction

Vibrio cholerae, a Gram-negative bacterium, is responsible for severe epidemics of cholera. Seven pandemics of cholera have been recorded since 1817, the seventh one beginning in 1961 and is still with us (Faruque *et al* 1998). About 200 different serogroups of this organism have been identified till date (Yamai *et al* 1997) of which serogroup O1 were the sole causative agent of the disease cholera till 1992. The others, collectively known as non-O1 strains are associated with sporadic cases of gastroenteritis and extra-intestinal infection, but lack endemic and pandemic potential. *V. cholerae* serogroup O1 strains are divided into two biotypes, classical and El Tor. Classical biotype was responsible for the first six pandemics of cholera (Faruque *et al* 1998). *V. cholerae* El Tor biotype strains although made its appearance in 1906 (Gotschlich 1906), caused only a small number of cases till 1960. Since early sixties El Tor biotype strains slowly exceeded the classical infection and from mid-sixties completely replaced the classical strain from the global endemic scene. This marked the beginning of the seventh pandemic (Faruque *et al* 1998) and is still with us with El Tor biotype strains as the major causative agent. In 1992 a novel toxigenic strain of *V. cholerae*, which did not agglutinate with the O1 antiserum, emerged as a new causative agent replacing the existing serogroup O1 El Tor strains. The disease soon assumed an endemic proportion and it was thought to mark the beginning of the eighth pandemic (Ramamurthy *et al* 1993). The new causative agent was identified as belonging to a hitherto unknown serogroup O139, synonym Bengal. However,

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Abbreviations used: ISR, Intergenic spacer region; MP, maximum parsimony; NJ, neighbour joining; PCR, polymerase chain reaction.

the serogroup O139 disappeared in the next year and a new clone of the serogroup O1 El Tor biotype once again became the major causative agent (Sharma *et al* 1997a). In September 1996 *V. cholerae* O139 strains staged a resurgence and now shares this locale with the serogroup O1 El Tor strains. It thus appears that clonal shifts in *V. cholerae* represent a fundamental characteristic of cholera dynamics. Attempts to understand the genetic relatedness of these clones received global attention and concern. It was reported earlier from our laboratory that the O139 strains have evolved from El Tor strains as suggested by ribotyping as well as RFLP analyses of the CTX genetic element (Sharma *et al* 1997a) and the progenitor strains were identified (Pajni *et al* 1995). It was also reported that the El Tor strains isolated before, during and after the O139 era belonged to different ribotypes (Sharma *et al* 1997a). This study had also stated the origin of pre-O139 El Tor and post-O139 El Tor strains from two independent parental strains or clones (a clone refers to bacterial isolates sharing a common origin). However, ribotypes are simply a tool to probe the overall genetic relatedness among strains; comparative genomic sequencing provides the most definitive measure of relatedness among strains. The ribosomal RNA (*rrn*) genes and the transfer RNA (*tRNA*) genes being highly conserved in the bacterial and archaebacterial kingdoms are the ideal candidates for evolutionary studies and have been used extensively for this purpose (Gurtler 1999). However, compared to these, variation is even more in the (16S-23S) ribosomal RNA intergenic spacer region (ISR) between multiple genomic copies. This variation has been used by a number of researchers for bacterial evolutionary studies (Gurtler 1999; Rumpf *et al* 2000). Hence in the present study, the phylogeny of the *V. cholerae* O1 El Tor strains was constructed on the basis of the sequence of the most variable region of the ribosomal operon, the ISR. Before the publication of *V. cholerae* genomic sequence, analysis of three ISR sequences among single representatives of *V. cholerae* O1 classical, O1 El Tor, O139, O22 and O31 strains, and three representatives of non-O1/non-O139 *V. cholerae* strains was conducted to determine the interserotype and intercistronic variations (Chun *et al* 1999). Publication of the *V. cholerae* genomic sequence (Heidelberg *et al* 2000) led to the identification of eight ISRs corresponding to the eight different *rrn* operons. In this present report we selected all the eight ISRs for comparative sequence analysis to study microheterogeneity among O1 El Tor strains isolated before, during and after the genesis of the *V. cholerae* O139 strains and to determine their evolutionary relationships. The O139 strain AS231, which reappeared in 1996, is also included in this study for better evaluation of our technique to establish clonality (see § 3). Based on this study we report here the intra-biotype and inter-biotype variations amongst O1 El Tor strains and O139 strains as well as the evolutionary relationships among the above-mentioned strains.

2. Materials and methods

2.1 *Bacterial strains, media and growth conditions*

V. cholerae strains used in this study are given in table 1 and were obtained from National Institute of Cholera and Enteric Diseases, Kolkata. The strains were routinely grown and maintained as described before (Sharma *et al* 1997a).

2.2 *DNA isolation and polymerase chain reaction amplification*

Chromosomal DNA was isolated from bacterial cultures as described before (Kar *et al* 1996). The ISRs were amplified from the strains on a PERKIN ELMER GeneAmp 2400 system using a pair of oligonucleotides, the forward primer 16SP1 (5′ TGGGGTGAAGTCGTAACAAGG 3′) and the reverse primer 23SP2 (5′ TCTGACTGCCCAGGCATCC 3′). Chromosomal DNAs isolated from the different *V. cholerae* strains (table 1) were used as tem-

Table 1. *V. cholerae* strains used in this study.

Group	Strains	Serogroup, serotype, biotype	Place and year of isolation	Reference
Strains isolated				
I. Before O139 outbreak	VC1	O1, El Tor	Calcutta, India, March 1992	Sharma *et al* 1997a
	VC3	O1, El Tor	Calcutta, India, April 1992	Sharma *et al* 1997a
II. During O139 outbreak	CO327	O1, El Tor	Calcutta, India, September 1993	Sharma *et al* 1997a
	CO427	O1, El Tor	Calcutta, India, November 1993	Sharma *et al* 1997a
III. After O139 outbreak	CO458	O1, El Tor	Calcutta, India, March 1994	Sharma *et al* 1997a
Resurgent O139 strains	AS231	O139	Calcutta, India, (August–September) 1996	Sharma *et al* 1997b
Strain used for genome sequencing	N16961	O1, Inaba, El Tor	Bangladesh 1975	Kaper *et al* 1984

plates. The reaction was performed in a total volume of 50 µl containing 40 pmol of each primer, 200 µM (each) deoxynucleoside triphosphate (dNTP), and 2 units of Taq polymerase (Roche Applied Science) in 1X reaction buffer. Annealing was carried out at 55°C for 45 s and extension was at 72°C for 1 min.

2.3 *Cloning and selection of clones*

The polymerase chain reaction (PCR) amplified ISR fragments were purified using the QIAquick PCR purification kit (QIAGEN) and ligated to pGEM T Easy vector (pGEM T Easy Vector System, Promega) according to the instructions of the manufacturer. *Escherichia coli* DH5*a* cells (Raleigh *et al* 1998) were transformed with the recombinant plasmids and the recombinants were selected according to the standard blue-white cloning procedure (Sambrook *et al* 1989). From about 150 colonies per strain, plasmids were purified using the QIAGEN plasmid mini kit (QIAGEN).

2.4 *DNA sequencing*

The ISR fragments of the recombinant plasmid were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer, France) and an ABI 377 automated DNA sequencer using the M13 forward and reverse primers.

2.5 *Phylogenetic analysis*

Nucleotide sequences for a particular ISR were aligned using Clustal W (Thompson *et al* 1994). Phylogenetic trees of the ISR sequence alignments were constructed using the various programs in PHYLIP [Phylogeny Inference Package (Version 3·6b)] (Felsenstein 2004) and the robustness of the tree topology were tested with the bootstrapping option (SEQBOOT) in PHYLIP. The methods used were neighbour-joining (NJ) (Saitou and Nei 1987), and maximum parsimony (MP). The evolutionary distance matrices were generated with the Cantor and Juke coefficient by the DNADIST program. Phylogenetic inferences based on the distance matrix (NEIGHBOR) and parsimony (DNAPARS) algorithms were applied to the alignments. In case of both trees, the best tree or the majority-rule consensus tree was selected using the consensus program (CONSENSE) of PHYLIP. The trees were visualized and drawn using the TREEVIEW software (Page 1996). The phylogenetic trees for ISR-B, ISR-C, and ISRh of the *V. cholerae* strains were rooted using the 16S-23S ribosomal RNA intergenic spacer region type 3 (accession number AF114746), type 2 (accession No. AF114744), and type 1 (accession No. AF114747) respectively of *Vibrio mimicus* strain RC5 as outgroups.

2.6 *Nucleotide accession numbers*

The GenBank accession Nos of the ISR nucleotide sequences determined in this study were AY616118, AY616119, AY616120, AY616121, AY616122, AY616123, AY616124, AY616125, AY616126, AY616127, AY616128, AY616129, AY616130, AY616131, AY616132, AY616133, AY955252 and AY955253.

3. Results and discussion

3.1 *PCR amplification of the ISR and sequencing*

The ISR known to exhibit a significant degree of variation in sequence and length as discussed above, were subjected to detailed study to pinpoint the intra-biotype variations amongst the El Tor strains. Chromosomal DNAs prepared from the different El Tor strains isolated before, during and after the O139 outbreak were subjected to PCR amplification with two primers, 16SP1 and 23SP2, flanking 16S-23S rDNA as described under methods. PCR fragments generated were separated by electrophoresis on 1·5% agarose gel. All strains under study yielded identical band pattern for the different O1, El Tor as well as the O139 isolates. There were two major bands of about 600 bp and 500 bp, and one minor band of around 750 bp (figure 1) together representing eight ISRs (see below). The PCR product was purified, cloned into pGEM-T Easy vector, transformed into *E. coli* DH5*a* cells and the recombinant plasmids amplified and recovered as described in § 2.

For identifying the positive clones carrying the three types of ISR inserts, recombinant plasmids containing

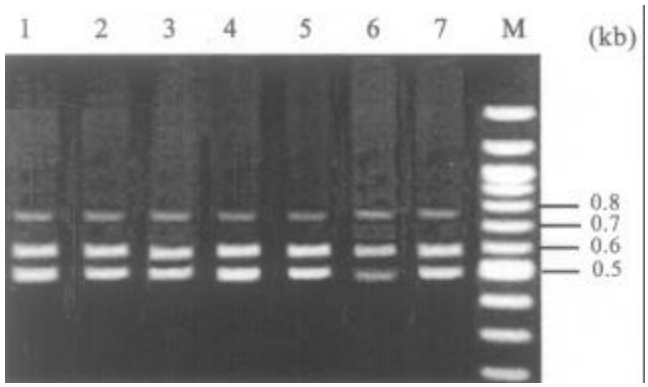

Figure 1. Electrophoresis with a 1·5% agarose gel of PCR-amplified 16S-23S rRNA intergenic spacer regions of *V. cholerae* strains with primers 16SP1 and 23SP2. Lanes 1, N16961; 2, VC1 (before O139); 3, VC3 (before O139); 4, CO327 (during O139); 5, CO427 (during O139); 6, CO458 (after O139); 7, AS231 (resurgent O139); 8, 100 bp DNA marker. The fragment sizes of the marker are given to the right of the figure.

different ISR amplicons were initially screened by digesting with *Eco*RI, and those plasmids that gave inserts of sizes around 750 bp, 600 bp, and 500 bp were identified. However, it was difficult to identify these clones with respective *rrn* operons because of the presence of ISRs with same size but different sequences (see below).

Analysis of the published genome sequence of *V. cholerae* El Tor N16961 strain revealed the presence of eight *rrn* operons, *rrn*a to *rrn*h, each with their corresponding ISRs. Thus the genome was found to contain a total of eight ISRs. We named them according to the *rrn* operons as ISRa to ISRh (table 2). Analysis of the published ISR sequences for the strain N16961 revealed that ISRs for *rrn* operons b and e were identical and so were the ISRs for *rrn* operons c, d and f. Hence the sequence analysis revealed only five sequence classes as a, b/e, c/d/f, g and h and these will be henceforth referred to as ISRa, ISR-B, ISR-C, ISRg and ISRh respectively. Also the size of the ISRs for *rrn* operon a, and b/e was almost identical (table 2). Thus only direct sequencing could identify the clones. To identify various ISRs, each having nearly similar size but varying in sequence, from the pool of recombinant clones for any particular strain, T-track analysis was performed. For this the insert contained in each of the 150 plasmids per strain (as given in methods) was sequenced by dideoxy chain termination method (Sambrook *et al* 1989), but using only the reaction for thymidine (T) residue. Plasmids having similar pattern of the T residue (T track) were grouped together as representing only one type of ISR. Various ISRs so identified were then subjected to automated DNA sequencing followed by Blast analysis (Altschul *et al* 1990). Blast analysis with the complete nucleotide sequence of each ISR for each clone was performed individually with the genomic sequence of the chromosome I of *V. cholerae* El Tor strain N16961. This led to the identification of all the five ISRs belonging to *rrn* operons a (ISRa), b/e (ISR-B), c/d/f (ISR-C), g (ISRg) and h (ISRh) for a total of six strains (VC1, VC3, CO327, CO427, CO458, AS231). ISRs of *rrn* operons a and g, for all the above six strains, had identical sequences to the *rrn* operons a and g of the sequenced *V. cholerae* El Tor strain N16961 respectively and were thus excluded from further analysis.

Table 2. List of *rrn* operons and ISRs of *V. cholerae* El Tor strain N16961 (Kaper *et al* 1984).

rrn[a] Operon	Corresponding ISR[b]	Size in bp (as deduced from N16961 whole genome sequence)
*rrn*a	ISRa	427
*rrn*b	ISRb	433
*rrn*c	ISRc	512
*rrn*d	ISRd	513
*rrn*e	ISRe	433
*rrn*f	ISRf	512
*rrn*g	ISRg	714
*rrn*h	ISRh	688

[a]Ribosomal RNA; [b]Intergenic spacer region.

3.2 *Sequence analysis of the ISRs*

Nucleotide sequences for a particular ISR for all strains studied were aligned using Clustal W (Thompson *et al* 1994). It was revealed that the tRNA gene(s) present in any particular ISR for all strains perfectly matched with the tRNA gene(s) in the respective ISR in *V. cholerae* El Tor strain N16961 both in sequence including anticodon and the CCA sequence at the 3′ end as well as in the order of alignment. Identity was observed among the strains irrespective of the serogroup as also of their time of isolation suggesting that the spacer tRNA genes are highly conserved among the strains studied.

The length of ISR-B varied between 431 bp or 432 bp and contained one tDNA coding for $tRNA^{Glu(UUC)}$. Among the strains the sequences differed among each other in 1 to 3 positions leading to high sequence similarity values ranging from 99·3 to 99·7% for intra-serotype and from 99·3 to 100% for inter-serotype comparisons (table 3). Thus this operon appeared to be more or less similar among the six strains of the two serogroups, O1 and O139, studied.

The length of ISR-C ranged from 508 to 515 bp and invariably contained tDNA coding for $tRNA^{Ile(GAU)}$ and $tRNA^{Ala(UGC)}$. The O139 strain As231 had the shortest version (i.e. 508 bp) and the O1 El Tor strain VC1 had the longest version (i.e. 515 bp) among the strains compared (table 4). Compared to VC1 all the other El Tor strains showed a deletion of 6 bases and the O139 strain had 7 bases deleted. The intra-serogroup sequence similarity values ranged from 95·5% to 99·4% and the corresponding inter-serogroup values ranged from 95·7% to 99·8%. Surprisingly for this ISR the sequences of VC1 and VC3, both belonging to strains of the pre-O139 era, differed from each other in 23 positions. However VC3 sequence differed in only one position when compared to the sequence of the O139 strain As231 while VC1 differed in 22 positions under identical conditions. As a result the sequence similarity values of ISR-C for VC3 and As231 were high, being 99·8% but were comparatively lower for VC1 and As231, the value being 95·7%. The sequence similarity value of As231 with CO327 and CO427, the strains isolated during O139 era, was 98·8%. The sequence of ISR-C was found to be same for CO327 and CO427. The ISR-C analysis thus indicates clearly a very close association of the O139 strain with the O1 strain VC3. Also notable is the fact that the post-O139 strain, CO458,

differed from the pre-O139 strains, VC1 and VC3, as also the O139 strain, As231, significantly with sequence similarity values being 95·9%, 98·2% and 98% respectively.

The size of ISRh ranged from 687 to 690 nucleotides and contained three tDNA coding for $tRNA^{Glu(UUC)}$, $tRNA^{Lys(UUU)}$ and $tRNA^{Val(UAC)}$. The ISRh of CO327 and CO427, the strains isolated during serogroup O139 outbreak had 690 bp and had identical sequences while others had 3 to 4 bases deleted in their ISRh (table 5). The intra-serotype and inter-serotype sequence similarity values ranged from 98·8% to 99·5% and 98·7% to 99·1% respectively. The O139 strain As231 differed from the pre-O139 strains VC1 and VC3 in 5 and 2 nucleotide positions respectively thereby showing high sequence similarity values of 99·3% with VC1 and 99·7% with VC3. Significantly the post-O139 strain CO458 exhibited the lower range of sequence similarity values when compared with the pre-O139 strains as also the O139 strain thus following a pattern similar to that obtained for ISR-C sequence comparisons. CO458 differed from VC1 and VC3 in 8 and 7 nucleotides respectively and from AS231 in 9 nucleotide positions thereby showing a sequence similarity value of 98·8% and 98·9% with the pre-O139 strains and 98·7% with the O139 strain. However a close association of CO458 with CO427 and CO327 strains isolated during O139 era could be predicted from the comparatively high sequence similarity value of 99·3%.

3.3 *Phylogenetic analysis based on ISR sequences*

Phylogenetic trees were constructed from the ISR-B, ISR-C, and ISRh sequences as described under method. Since the basic tree topology was similar in case of both the NJ and MP reconstructions, only the MP trees for all three ISRs are shown here (figure 2). Phylogenetic trees constructed for ISR-B, ISR-C, and ISRh showed few examples of consistent genealogical relationships among the *V. cholerae* O1 and O139 strains studied and the trees could be broken into two major clades. The number of nucleotide changes between strains was selected as an index for the phylogenomic analyses of the ISR sequence alignments. For the NJ (Saitou and Nei 1987) and MP analysis the tDNA regions were also included as they were found to be homologous among the strains for all three ISRs tested. The O1 El Tor pre-O139 strain VC3 and the O139 strain As231 formed a monophyletic clade (figure 2). The other pre-O139 El Tor strain VC1 was found to group closely with these two strains (figure 2) in spite of differing from the O1 El Tor pre-O139 strain VC3 and the O139 strain As231 by 23 and 22 nucleotide positions respectively for ISR-C (table 4). This possibly suggests that the O139 strain AS231 and the pre-O139 strains VC1 and VC3 originated from a common ancestor. AS231 is a resurgent O139 strain isolated in 1996 and in a temporal sense one would expect a closer resemblance

Table 3. ISR-B sequence similarity values among *V. cholerae* O1 and O139 strains.

		Sequence similarity values*					
Strains	Size (bp)	VCI	VC3	CO327	CO427	CO458	AS231
VC1	432	–	1/432	3/432	2/432	3/432	1/432
VC3	432	99·7	–	3/432	2/432	3/432	0/432
CO327	431	99·3	99·3	–	1/432	2/432	3/432
CO427	432	99·5	99·5	99·7	–	1/432	2/432
CO458	432	99·3	99·3	99·5	99·7	–	3/432
AS231	432	99·7	100	99·3	99·5	99·3	–

*The values in the upper-right triangle indicate nucleotide differences/total number compared. The values in the lower-left triangle indicate the percent similarity values.

Table 4. ISR-C sequence similarity values among *V. cholerae* O1 and O139 strains.

		Sequence similarity values*				
Strain	Size (bp)	VC1	VC3	CO327/CO427	CO458	AS231
VC1	515	–	23/515	19/515	21/515	22/515
VC3	509	95·5	–	7/509	9/509	1/509
CO327/CO427	509	96·3	98·6	–	3/509	6/509
CO458	509	95·9	98·2	99·4	–	10/509
AS231	508	95·7	99·8	98·8	98	–

*See footnote of table 3.

with El Tor O1 strains isolated after the O139 outbreak in 1992. But it was observed that this strain had RFLP for the CTX element identical for those obtained with O139 strains isolated in 1992–1993. It was suggested that the strain AS231 was persisting from the previous O139 epidemic (Sharma *et al* 1997b). Thus, the observed clonal relationship of AS231 with the pre-O139 strains VC1 and VC3 is not surprising and rather lends a strong support for the use of the present technique of the study of intergenomic heterogeneity of the 16S-23S rRNA ISRs to establish clonality in *V. cholerae*.

For all the three ISR regions (B, C and h) studied, CO458 formed a separate monophyletic clade along with the strains CO327 and CO427 isolated during O139 outbreak. Thus our observations based on ISR sequence comparisons clearly proved that pre-O139 and post-O139 strains of *V. cholerae* serogroup O1 El Tor strains arose out of two independent clones, a conclusion that was arrived by earlier investigators (Sharma *et al* 1997a) using independent molecular epidemiological methods.

A number of molecular biology techniques have been used over the years for determining the clonality of bacterial strains. Of these ribotyping and RFLP of the CTX genetic element (Sharma *et al* 1997a), pulse-field gel electrophoresis and multi-locus enzyme electrophoresis (Karaolis *et al* 1995), sequence analysis of genes namely *mdh* (malate dehydrogenase), *hly*A (hemolysin A), *rec*A, *dna*E (Byun *et al* 1999) and *asd* genes (Karaolis *et al* 1995) have been used to study the clonality of various *V. cholerae* strains. Apart from these, variations in the ISR sequences between bacterial strains have also been widely used for evolutionary studies (Gurtler 1999). The phylogenetic relationship based on $tDNA^{Ile(GAU)}$ and $tDNA^{Ala(UGC)}$ sequences found in ISR-2 of *V. cholerae* has already been emphasized (Chun *et al* 1999), but only three ISRs were identified. Based on the published genome sequence, we report the analyses of five different classes of ISR sequences present in eight *rrn* operons in *V. cholerae* O1 El Tor strains isolated at different time periods. Two ISR classes, ISR-C (identical to ISR-2

Table 5. ISRh sequence similarity values among *V. cholerae* O1 and O139 strains.

		Sequence similarity values*				
Strain	Size (bp)	VC1	VC3	CO327/CO427	CO458	AS231
VC1	687	–	3/687	5/690	8/687	5/687
VC3	686	99·5	–	4/690	7/686	2/686
CO327/CO427	690	99·1	99·4	–	5/690	6/690
CO458	686	98·8	98·9	99·3	–	9/686
AS231	686	99·3	99·7	99·1	98·7	–

*See footnote of table 3.

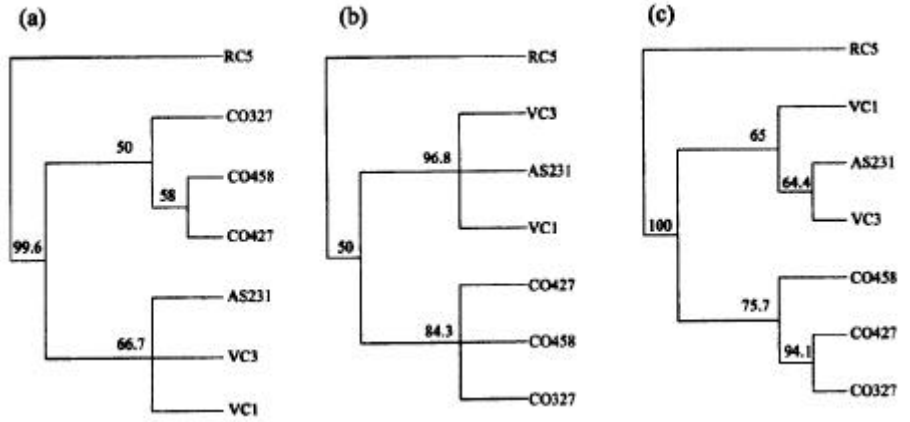

Figure 2. Phylogenies of *V. cholerae* O1 and O139 strains based on ISR-B (**a**), ISR-C (**b**), and ISR h (**c**) sequences. The phylogenetic trees (rectangular cladograms) were constructed using the MP method. The numbers at the nodes indicate bootstrap values (in percentage) retrieved from 1000 replicates for both the parsimony and the neighbour-joining analyses. The phylogenetic trees of ISR h, C, and B of *V. cholerae* O1 and O139 strains were rooted with the type 1, 2, and 3 16S-23S intergenic spacer region of *V. mimicus* (VM) strain RC5 respectively.

above) and ISRh, show significant variations, the remaining three, namely, ISRa, ISR-B and ISRg showed little or no variations at all among the *V. cholerae* strains studied. However, we would like to emphasize that the phylogenetic trees derived from the sequences of all the ISR classes studied, showing both significant as well as little variations, reveal a pattern of clonality of the strains that is congruent with the pattern predicted by earlier workers based on ribotyping and RFLP of the CTX genetic element (Sharma *et al* 1997a). It is thus reasonable to say that analysis of the ISR classes and more precisely of ISR-C and ISRh, showing significant variations instead of all the five ISR classes could serve as a model system for future workers to study the clonality among different *V. cholerae* strains.

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