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Dissemination of a single *Vibrio cholerae* clone in cholera outbreaks during 2005 in Iran

M. R. Pourshafie,[1] B. Bakhshi,[1] R. Ranjbar,[2] M. Sedaghat,[1] N. Sadeghifard,[3]† J. Zaemi Yazdi,[3]‡ M. Parzadeh[1] and J. Raesi[1]

[1]Department of Bacteriology, Pasteur Institute of Iran, Pasteur Avenue, Tehran, Iran

[2]Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

[3]Department of Bacteriology, University of Tehran, Tehran, Iran

Correspondence
M. R. Pourshafie
pour@pasteur.ac.ir

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In this study, 50 *Vibrio cholerae* O1 serotype Inaba isolates were collected during several cholera outbreaks throughout Iran during the summer of 2005. The results of antibiotic susceptibility testing showed that 86, 84, 84 and 82 % of the isolates were resistant to streptomycin, chloramphenicol, co-trimoxazole and tetracycline, respectively. The strains were genotyped using randomly amplified polymorphic DNA (RAPD), PFGE and ribotyping techniques. PCR showed that 100, 98 and 98 % carried the *ctx*, *zot* and *ace* genes, respectively. Biochemical fingerprinting of the isolates using the PhenePlate (PhP) system showed a low diversity index level (0.755), suggesting that the strains were highly homogeneous. Among the strains, 100 and 96 % showed an identical ribotype and PFGE patterns, respectively. The two isolates showing different PFGE patterns also exhibited discrete PhP types. RAPD was able to discriminate the isolates into six distinct groups, suggesting some genetic dissimilarity was present among the strains. These ribotyping, PFGE and PhP techniques revealed the clonal dissemination of a single *V. cholerae* strain throughout Iran in 2005.

INTRODUCTION

Vibrio cholerae O1 causes diarrhoeal disease that afflicts thousands of people annually (Faruque *et al.*, 1998). In the past, the prevalence of *V. cholerae* resistant to antibiotics was low and routine susceptibility testing was not recommended (Dalsgaard *et al.*, 2000). However, reports of *V. cholerae* strains resistant to commonly used antibiotics are appearing with increasing frequency worldwide (Garg *et al.*, 2000, 2001). Outbreaks of cholera due to the El Tor O1 serotype with changeable antibiotic-resistant patterns are occurring periodically in Iran also, causing public health problems.

Previously, we described the clonal diversity of clinical isolates of *V. cholerae* obtained during 1998 (Pourshafie *et al.*, 2000) and 2000 (Pourshafie *et al.*, 2002). Ribotyping analysis of the 16s and 23s rRNA genes of these strains suggested the presence of a predominant *V. cholerae* ribotype in Iran. The present study was designed to characterize isolates obtained during outbreaks in 2005 in several provinces in Iran. We present a detailed study, including genotypic and phenotypic characteristics, of a predominant and new *V. cholerae* strain emerging in Iran, which was not observed in the previous years.

†Present address: University of Medical Sciences of Ilam, Ilam, Iran.

‡Present address: University of Medical Sciences of Yazd, Yazd, Iran.

Abbreviations: CT, common type; PhP, PhenePlate; RAPD, randomly amplified polymorphic DNA.

METHODS

Bacterial isolates. Faecal specimens were collected from patients suspected of having cholera in the summer of 2005 in Iran. The samples were collected with sterile swabs, which were placed in Cary–Blair transport medium. Alkaline peptone water was used for the enrichment of *V. cholerae* and bacteria were then isolated on thiosulphate-citrate-bile salt-sucrose agar plates (Farmer & Hickman-Brenner, 1992). The strains were isolated from different provinces in Iran, including Qom (13 samples), Golastan (14 samples), Zahadan (8 samples) and Tehran (15 samples). Identification of the clinical strains of *V. cholerae* isolates and O serotyping was done by detailed biochemical tests and agglutination by antiserum.

Antimicrobial susceptibility testing. Antibiotic susceptibility was tested by the standard disc diffusion technique according to NCCLS (2001) guidelines with the following antibiotics: gentamicin (10 μg), polymyxin B (300 U), doxycycline (30 μg), ciprofloxacin (5 μg) and tetracycline (30 μg) (purchased from Difco) and streptomycin (5 μg), ampicillin (10 μg), erythromycin (15 μg), furazolidone (100 μg), chloramphenicol (30 μg) and co-trimoxazole (25 μg) (purchased from Becton Dickinson).

Ribotyping. Ribotyping was performed as described previously (Pourshafie *et al.*, 2000). Briefly, DNA extracted from *V. cholerae* isolates was cleaved by the restriction endonuclease *BglI* (Roche Diagnostic). The fragments were separated by agarose gel

electrophoresis and then transferred onto nylon membrane using an alkali blotting procedure with a vacuum blotter. Hybridization was performed with probes labelled with digoxigenin-11-dUTP. The membranes were then visualized by the addition of alkaline phosphate-conjugated anti-digoxigenin antibody (Roche Diagnostic), and 5-bromo-4-chloro-3-indolyl phosphate substrate and nitro blue tetrazolium.

PCR for toxin genes. The primers used PCR of genes encoding cholera toxin (*ctx*A), accessory cholera enterotoxin (*ace*) and zonula occludens toxin (*zot*) were as described previously (Pourshafie *et al.*, 2000). DNA was extracted and PCR carried out in a reaction mixture containing 20 µl sterile water, 2.5 µl 10x *Taq* polymerase buffer, 0.3 µl dNTPs (10 mM), 0.5 U *Taq* DNA polymerase, 25 pmol each primer. The cycling conditions were as follows: preincubation at 94 °C for 5 min, 30 cycles of 1 min at 94 °C for denaturation, 1 min at 64 °C for annealing, 2 min at 72 °C for elongation, and incubation at 72 °C for 3 min for final elongation.

Random amplification. The randomly amplified polymorphic DNA (RAPD) technique used the primers ARB11 5′-CTAGGACCGC-3′ and AP-PGO5 5′-AGCCCAGCTATGAAC-3′ (Killgore & Kato, 1994). The cycling conditions for RAPD-PCR were as follows: pre-incubation at 94 °C for 5 min, and 40 cycles of 30 s at 94 °C, 1 min at 40 °C, 72 °C for 1 min, and a final incubation for 10 min at 72 °C.

Biochemical fingerprinting with the PhenePlate (PhP) system. *V. cholerae* isolates were typed with the PhenePlate (PhP) system, a rapid, semi-automated and computerized biochemical method using PhP-RV plates (PhPlate). These microplates are used to measure the kinetics of bacterial metabolism of 11 substrates, which are specifically chosen to differentiate between isolates of *Vibrio*. Briefly, one bacterial colony was inoculated in PhP suspending medium containing 0.011 % (w/v) bromothymol blue (pH 8), 0.05 % (w/v) proteose peptone, 2 % (w/v) sodium chloride and 0.0016 M phosphate buffer, in the first column of the pre-prepared PhP plate. The bacterial suspension was then dispensed to the plate wells containing dehydrated substrates, and incubated for 16, 40 and 64 h. The biochemical fingerprints of isolates were compared pair-wise and the similarity between each pair was calculated as the correlation coefficient. This yielded a similarity matrix and accordingly a dendrogram. The level of identity between the isolates was defined as the mean of the correlation coefficients obtained between these duplicate assays minus two SDs of this mean. All the data processing, including optical readings and calculations of correlation coefficients, as well as clustering and printing of dendrograms, was performed with PhP software (Ansaruzzaman *et al.*, 1996; Kühn *et al.*, 1990).

PFGE. A Pulsenet (www.cdc.gov/pulsenet) standardized protocol was used for subtyping of *V. cholerae* isolates. Briefly, bacteria from the surface of the culture plates were transferred into cell suspension buffer (100 mM tris, 100 mM EDTA, pH 8.0) after which they were adjusted to absorbance values of 0.8–1.0 at a wavelength of 610 nm. Agarose plugs were prepared by mixing equal volumes of the adjusted bacterial suspension with 1.0 % SeaKem gold agarose (Cambrex) and 20 µl proteinase K (20 mg ml^{-1} stock). Cells in the agarose plugs were lysed by treatment with a lysis solution [50 mM tris, 50 mM EDTA (pH 8.0), 1 % sarcosine, 0.5 mg proteinase K ml^{-1}] for 1 h at 54 °C. Washing was performed in six stages, twice with sterile ultrapure water and four times with TE buffer (10 mM tris, 1 mM EDTA, pH 8.0). One section of the plug was equilibrated with an enzyme buffer, placed in 200 µl fresh buffer containing 40 U *Not*I (Roche Diagnostic) and incubated for 4 h. *Salmonella choleraesuis* serotype Branderup H9812 (www.cdc.gov/pulsenet) plugs digested with *Xba*I for 2 h at 37 °C were used as DNA molecular mass size markers. The electrophoresis conditions consisted of a two-block program: block I with an initial switch time (IST) of 2 s to final switch time (FST) of 10 s and a run time of 13 h; block 2 with an IST of 20 s to a FST of 25 s and a run time of 6 h. Both blocks were run with a gradient of 6.0 V cm^{-1} and an included angle of 120° at 14 °C.

RESULTS AND DISCUSSION

Genotyping analysis

Ribotyping was one of the first fingerprinting techniques to be successfully used in the taxonomy of *Vibrio* spp. (Thompson *et al.*, 2004) and a standardized ribotyping scheme has been proposed as a tool for epidemiological studies of *V. cholerae* (Popovic *et al.*, 1993). Fig. 1 shows the ribotype representatives of the isolates obtained during 2005. All strains showed a single ribotype pattern that had not been previously observed in Iran (Pourshafie *et al.*, 2000, 2002). The appearance of this new ribotype pattern suggests that the isolates may have gone through rRNA operon rearrangement (Lan & Reeves 1998).

Representative patterns from RAPD profiling using arbitrary primers are shown in Fig. 2. Six different RAPD patterns were observed among the strains. The RAPD pattern labelled as one was the predominant pattern, containing 36 % of the isolates. This was followed by RAPD patterns five and six with 23 % of the isolates each. The least common RAPD patterns were two and three representing about 4.5 % of the isolates. Further analysis showed several RAPD patterns in isolates that were obtained from a single province. Our data are indicative that PCR amplification of random fragments of genomic DNA using arbitrary 10–15-mer primers might have a substantial discriminatory power.

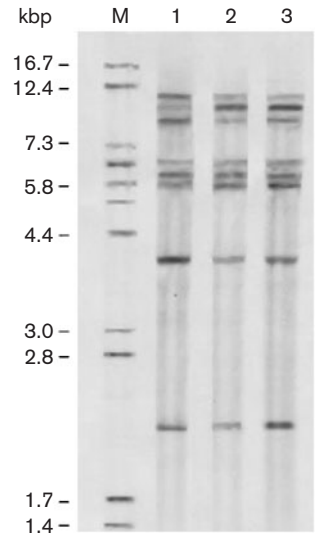


Fig. 1. Ribotype analysis of *V. cholerae* DNA digested with *Bgl*I restriction endonuclease. Molecular marker sizes are indicated on the left. Lanes 1–3 are the representative ribotypes showing a single pattern for all isolates from 2005.

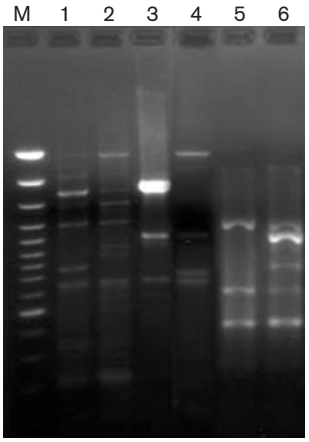


Fig. 2. Representative RAPD patterns, showing six different patterns. M, Marker.

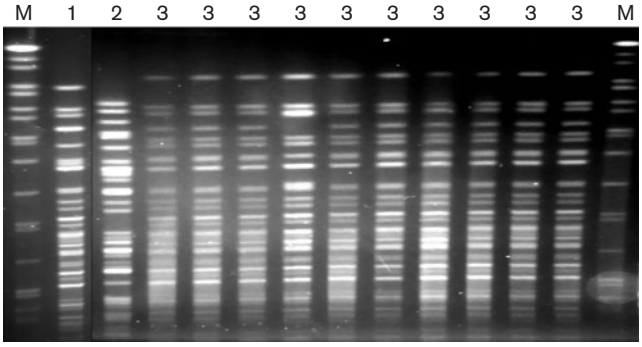


Fig. 3. Representative PFGE patterns of the 50 *V. cholerae* isolates. 1, composed of 1 isolate; 2, composed of 1 isolate; 3, composed of 48 isolates; M, marker.

A single predominant (96 %) PFGE pattern was observed among the isolates (Fig. 3). Of the 50 *V. cholerae*, only 2 isolates showed distinct and completely different PFGE patterns (Fig. 3, lanes 1 and 2). One of these isolates did not harbour *zot* and *ace* genes (Fig. 3, lane 2). By PCR, all strains carried *ctx* genes, and *zot* and *ace* genes were present in 49 of the 50 isolates.

Antibiotic susceptibility

Widespread antibiotic resistance in *V. cholerae* was unheard of before 1977, but multiple antibiotic-resistant *V. cholerae* have emerged as a major problem worldwide. *V. cholerae* strains resistant to tetracycline, ampicillin, kanamycin, streptomycin, sulphonamides, trimethoprim and gentamicin have been reported frequently (Sack *et al.*, 2004). Table 1 shows the antibiotic susceptibility patterns of Iranian strains isolated in 2005. The highest frequency of resistance was observed with streptomycin (86 %), followed by co-trimoxazole and chloramphenicol (84 % each). Isolates could be grouped on the basis of their antibiotic-resistance patterns into 17 categories (Fig. 4). Resistance to furazolidone (62 %) was only observed in strains from Zahadan province. Resistance to tetracycline was also significant (82 %) (Table 1), in contrast to reports that suggested tetracycline-resistant *V. cholerae* O1 isolates in epidemics or outbreaks are usually uncommon in Malaysia (Chen *et al.*, 2004) and other countries, such as Thailand and Laos where few tetracycline-resistant isolates have been reported (Kondo *et al.*, 2001; Iwanaga *et al.*, 2000). In previous years, however, we also observed that only a small number of the *V. cholerae* isolates in Iran were resistant to tetracycline (Pourshafie *et al.*, 2002).

In the present study, most of the isolates were resistant to streptomycin, co-trimoxazole and chloramphenicol. Resistance to these antibiotics has been shown to be encoded by genes on a conjugative SXT transposon (Hochhut *et al.*, 2001; Amita *et al.*, 2003). It is therefore possible that this transposon was extensively disseminated among our isolates. Furthermore, the PhP analysis showed (Fig. 4) that 19 out of 20 isolates had identical biochemical characteristics [labelled as common type (CT)1] and were resistant to co-trimoxazole, streptomycin and chloramphenicol. Interestingly, these isolates were obtained from different provinces in Iran. This finding may suggest that these strains have vertically acquired the SXT transposon and spread in different geographical areas.

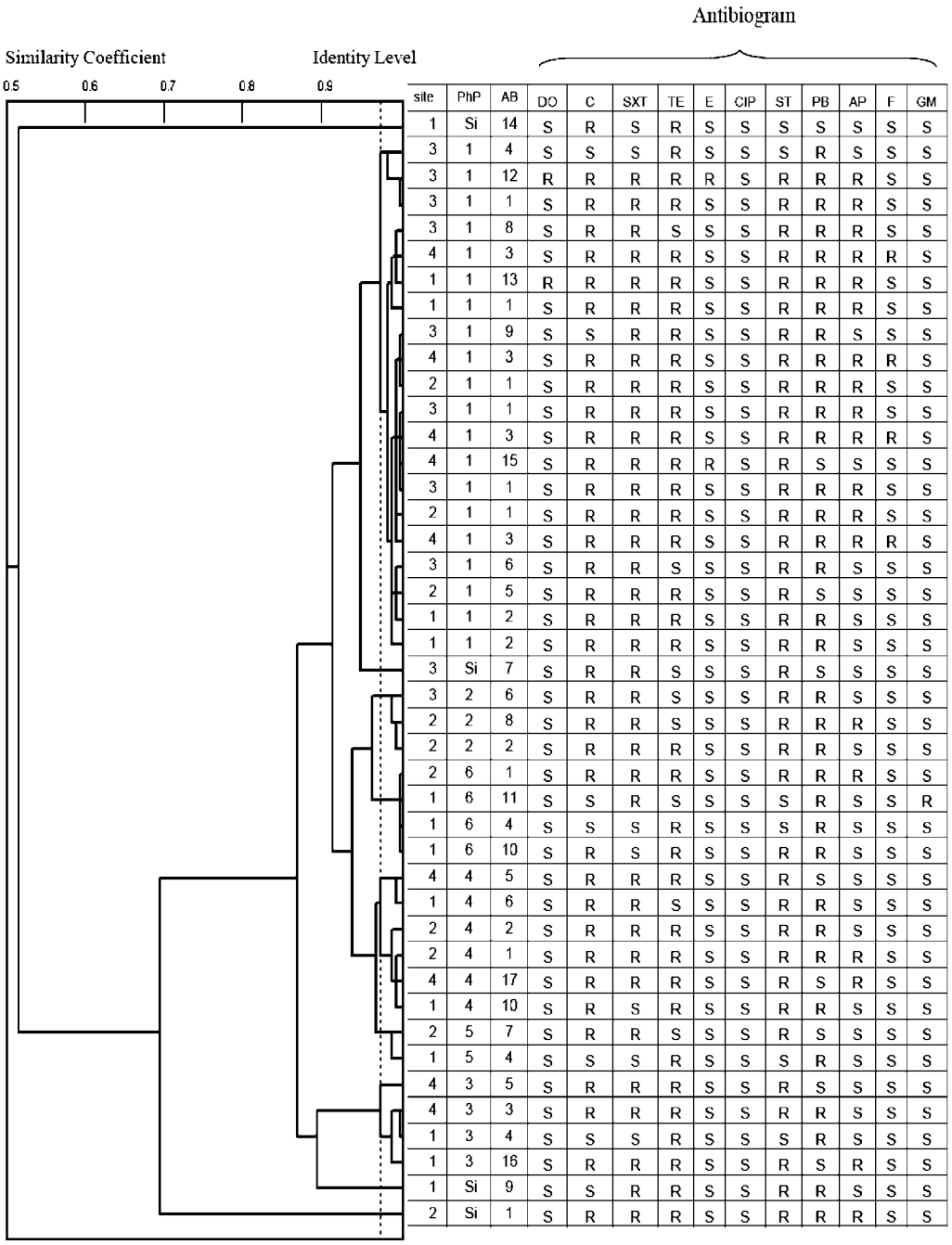
PhP analysis

With an identity level of 0.975, the PhP system showed 1 major cluster comprising 48 isolates (90 %) and 2 isolates

Table 1. Antibiotic-resistance patterns of *V. cholerae* isolated from different provinces in Iran

Definitions of the abbreviations are given in Fig. 4.

Province (no. of strains tested)	C (%)	SXT (%)	TE (%)	E (%)	ST (%)	AP (%)	F (%)	PB (%)	DO (%)	GM (%)
Tehran (15)	66	60	87	0	67	27	0	87	7	7
Qom (13)	100	100	92	0	100	40	0	85	0	0
Golestan (14)	86	93	71	14	93	43	0	71	7	0
Zahadan (8)	100	100	87	0	100	75	62	75	0	0



site	PhP	AB	DO	C	SXT	TE	E	CIP	ST	PB	AP	F	GM
1	Si	14	S	R	S	R	S	S	S	S	S	S	S
3	1	4	S	S	S	R	S	S	S	R	S	S	S
3	1	12	R	R	R	R	R	S	R	R	R	S	S
3	1	1	S	R	R	R	S	S	R	R	R	S	S
3	1	8	S	R	R	S	S	S	R	R	R	S	S
4	1	3	S	R	R	R	S	S	R	R	R	R	S
1	1	13	R	R	R	R	S	S	R	R	R	S	S
1	1	1	S	R	R	R	S	S	R	R	R	S	S
3	1	9	S	S	R	R	S	S	R	R	S	S	S
4	1	3	S	R	R	R	S	S	R	R	R	R	S
2	1	1	S	R	R	R	S	S	R	R	R	S	S
3	1	1	S	R	R	R	S	S	R	R	R	S	S
4	1	3	S	R	R	R	S	S	R	R	R	R	S
4	1	15	S	R	R	R	R	S	R	S	S	S	S
3	1	1	S	R	R	R	S	S	R	R	R	S	S
2	1	1	S	R	R	R	S	S	R	R	R	S	S
4	1	3	S	R	R	R	S	S	R	R	R	R	S
3	1	6	S	R	R	S	S	S	R	R	S	S	S
2	1	5	S	R	R	R	S	S	R	S	S	S	S
1	1	2	S	R	R	R	S	S	R	R	S	S	S
1	1	2	S	R	R	R	S	S	R	R	S	S	S
3	Si	7	S	R	R	S	S	S	R	S	S	S	S
3	2	6	S	R	R	S	S	S	R	R	S	S	S
2	2	8	S	R	R	S	S	S	R	R	R	S	S
2	2	2	S	R	R	R	S	S	R	R	S	S	S
2	6	1	S	R	R	R	S	S	R	R	R	S	S
1	6	11	S	S	R	S	S	S	S	R	S	S	R
1	6	4	S	S	S	R	S	S	S	R	S	S	S
1	6	10	S	R	S	R	S	S	R	R	S	S	S
4	4	5	S	R	R	R	S	S	R	S	S	S	S
1	4	6	S	R	R	S	S	S	R	R	S	S	S
2	4	2	S	R	R	R	S	S	R	R	S	S	S
2	4	1	S	R	R	R	S	S	R	R	R	S	S
4	4	17	S	R	R	R	S	S	R	S	R	S	S
1	4	10	S	R	S	R	S	S	R	R	S	S	S
2	5	7	S	R	R	S	S	S	R	S	S	S	S
1	5	4	S	S	S	R	S	S	S	R	S	S	S
4	3	5	S	R	R	R	S	S	R	S	S	S	S
4	3	3	S	R	R	R	S	S	R	R	S	S	S
1	3	4	S	S	S	R	S	S	S	R	S	S	S
1	3	16	S	R	R	R	S	S	R	S	R	S	S
1	Si	9	S	S	R	R	S	S	R	R	S	S	S
2	Si	1	S	R	R	R	S	S	R	R	R	S	S

Fig. 4. A UPGMA dendrogram showing the cluster analysis of biochemical fingerprinting data for 50 *V. cholerae* isolates. AB, antibiotic-resistance pattern; DO, doxycycline; C, chloramphenicol; SXT, co-trimoxazole; TE, tetracycline; E, erythromycin; CIP, ciprofloxacin; ST, streptomycin; PB, polymyxin B; AP, ampicillin; F, furazolidone; GM, gentamicin. The sites (provinces) where the isolates were obtained are indicated: 1, Tehran; 2, Qom; 3, Golestan; 4, Zahadan. The PhP type (biochemical phenotype) of each isolate is indicated as belonging to a single type (Si) or a CT numbered from 1–6.

each as a single type (the first and last isolates in the dendrogram) (Fig. 4). Detailed analysis of the UPGMA dendrogram showed six CTs. PhP analysis showed a major CT1 with 20 isolates (46.5 %) followed by CT4 with 6 isolates (14 %). *V. cholerae* strains in the CT1 group exhibited 11 different antibiotic-resistance patterns.

Phenotypic analysis by PhP is based on 11 biochemical tests. None of the isolates metabolized L-arabinose, cellobiose, gentobiose and sorbitol. Most variation in sugar metabolism was observed when gluconate and amylopectin were used as the substrate (data not shown), suggesting that gluconate and amylopectin may play

an important role in differentiation of the *V. cholerae* isolates.

Traditional bacterial typing methods based on phenotypic characterization are often regarded as less discriminatory and having a lower level of reproducibility than genotyping. Nevertheless, the present study showed that the PhP system had a higher discriminatory power than ribotyping. PFGE and the PhP system were able to separate the isolates into three distinct groups, suggesting a similar discriminatory power.

PhP analysis of the isolated strains showed a diversity index of 0.755. Similar diversity indices (0.7) have been reported by other investigators (Ansaruzzaman *et al.*, 1996) with *V. cholerae* from epidemics. It has been suggested that in epidemics, despite the genetic homogeneity of the isolates, there are some biochemical variations among them (Rahman *et al.*, 2006). This could, in part, be the result of the expression of genes involved in the utilization of sugars, which are usually transient and depend on environmental conditions. Such differences could be detected by the PhP system.

In conclusion, three typing methods, PFGE, ribotyping and PhP, indicated the dissemination of a major *V. cholerae* clone in different provinces in Iran. The PFGE and PhP typing methods, but not ribotyping, were able to distinguish two isolates that were significantly diverse in both genotypic and phenotypic characteristics. Furthermore, our results indicated that continuous monitoring of the prevalence of *V. cholerae* strains could be done with a simple, cost-effective and highly discriminatory RAPD technique.

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