

MOLECULAR EPIDEMIOLOGY

A molecular and phenotypic study of *Vibrio cholerae* in Iran

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Vibrio cholerae is again the subject of attention on account of the current increase in the world-wide incidence of cholera. In this study, 200 clinical isolates of *V. cholerae* serotypes O1 and non-O1, non-O139, were collected from different provinces in Iran. The isolates were subjected to biochemical analysis, antibiogram, PCR of toxin genes, plasmid profile, ribotyping and pulsed-field gel electrophoresis (PFGE). The analysis of plasmid content showed that 33–96% of *V. cholerae* isolated from different provinces carry a large plasmid. PCR analysis of *V. cholerae* O1 showed that the genes encoding cholera toxin (*ctx*), toxin co-regulated pilus (*tcp*), accessory cholera enterotoxin (*ace*) and zonula occludens toxin (*zot*) were present in 55–97% of isolates in different provinces. Restriction fragment length polymorphism (RFLP) of *Bgl*I-digested DNA probed with five oligonucleotides revealed three different ribotype patterns in isolates of *V. cholerae* O1. The ribotype pattern B21 of *V. cholerae* O1 El Tor was found to be the predominant pattern in the isolates studied. *V. cholerae* non-O1, non-O139 isolates showed a single ribotype pattern. PFGE analysis also showed 10 different patterns amongst the isolates, 9 of which were in *V. cholerae* O1. Overall, the analysis of polymorphism of ribotypes and PFGE patterns of the isolates showed that the provinces in Iran were affected by a limited number of clones of *V. cholerae* O1 and non-O1, non-O139 strains.

Introduction

Seven widespread pandemics of cholera have been recorded in which *Vibrio cholerae* O1 biotype El Tor has spread to many Asian countries since its emergence in Indonesia in 1961 [1].

Studies of the molecular evolution of strains of *V. cholerae* isolated in Peru [2, 3], India [4] and Thailand [5] have shown that *V. cholerae* O1 undergoes genetic changes relatively frequently. *V. cholerae* serotype O139 emerged in India [6] and spread to countries such as Thailand [7] and Pakistan [8], demonstrating the ability of *V. cholerae* to mutate and spread rapidly. Furthermore, horizontal gene transfer between different serotypes of *V. cholerae* is believed to have resulted in new variants such as *V. cholerae* O139, which is considered to be the result of gene transfer between O1 and non-O1 serogroups [9]. Therefore, the study of genetic changes of *V. cholerae* is important in under-

standing the epidemiology and evolution of the cholera bacteria.

Despite the importance of *V. cholerae* as a cause of diarrhoea in Iran, there is little published information on cholera in that country. This study reports the molecular characterisation of *V. cholerae* O1 biotype El Tor as well as non-O1, non-O139 isolates from infected patients in different provinces in Iran during 1999–2000. Restriction fragment length polymorphism (RFLP) of the 16S and 23S rRNA genes (ribotyping), pulsed-field gel electrophoresis (PFGE), analysis of plasmid content and PCR of the toxin genes were all used for genotypic characterisation of the isolates. The antibiotic susceptibility patterns of the isolates were also examined, because of increasing reports of antibiotic resistance in strains of *V. cholerae* O1.

Materials and methods

Specimen collection

In all, 200 isolates of *V. cholerae* were obtained in 1999 and 2000 from patients suspected of having cholera. The samples were collected in different provinces in

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Iran, including Tehran, Sistasan and Baluchastan, Kerman, Kermanshah, Kuzhastan and Kashan provinces. The specimens were collected on sterile swabs, which were then placed in Cary-Blair transport medium [10]. Alkaline peptone water was used for the enrichment of *V. cholerae*, which was then isolated on thiosulphate-citrate-bile salt-sucrose (TCBS) agar (Sanofi Diagnostic Pasteur, Marnes-La-Coquette, France).

Biochemical analysis and serotyping

Biochemical identification and serotyping were performed by standard procedures [11]. Isolates were serotyped with monospecific sera purchased from Eurobio (Les Ulis, France). The isolates were identified as either *V. cholerae* O1 biotype El Tor with serotypes of Ogawa, Inaba, or non-agglutinable with O1 and O139 serotypes recognised as non-O1, non-O139 *V. cholerae*.

Antibiotic susceptibility testing

The antibiotic susceptibilities of isolates were tested by the standard disk technique [12]. The following antibiotic disks were used (mg): doxycycline (30), chloramphenicol (30), trimethoprim/sulphamethoxazole (25), gentamicin (10), tetracycline (30), erythromycin (15), oxytetracycline (30), ciprofloxacin (5), furazolidone (100) and streptomycin (10). The antibiotic disks were purchased from Difco Laboratories (Detroit, MI, USA).

Isolation of plasmid DNA

Plasmids were isolated by the method of Kado and Liu [13], with some modifications. Briefly, cells were grown in 3 ml of TCS broth overnight to an OD₆₀₀ of 0.8 and were then incubated for 30 min at 56°C at pH 12 for lysis. After lysis, phenol:chloroform:isoamylalcohol (25:24:1) was added and the samples were mixed. The samples were examined by electrophoresis for the presence of plasmids in the supernates.

Gene detection by PCR

DNA was extracted with the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). PCR

was performed in a reaction mixture containing sterile water 35 ml, 10× Taq polymerase buffer 5 ml, 200 mM deoxynucleotide phosphate (dNTP), Taq DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) 2.5 units, DNA template 1 ml and 50 pmol of each primer. The primers were for cholera toxin (*ctxA*), accessory cholera enterotoxin (*ace*), zonula occludens toxin (*zot*) and toxin co-regulated pilus (*tcpA*) (Table 1) [14–17], which were synthesised and purified by Genset (Paris, France). The cycling conditions were as follows: pre-incubation at 94°C for 5 min, 35 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. The amplicons were electrophoresed through agarose 0.8% gel (Appligene, Illkirch, France) and then stained with ethidium bromide. A negative control (reaction mixture without template) and a toxin-positive control (*V. cholerae* O1 strain 0395) were included in each run [18].

Ribotyping

The extracted DNA was cleaved by restriction endonuclease *Bgl*I (Life Technologies, Cergy Pontoise, France) 20 U/ml at 37°C for at least 4 h. The fragments were separated by electrophoresis in an agarose 0.8% gel (Appligene) in Tris-borate buffer (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.3) for 16 h at 1.5 V/cm. The DNA fragments were then transferred to nylon membranes (Hybond N+, USB Life Science) by an alkali blotting procedure with a vacuum blotter (Amersham Pharmacia Biotech). Hybridisation was performed with a probe labelled with digoxigenin-11-dUTP (DIG). The membranes were then visualised by the addition of alkaline phosphatase-conjugated anti-digoxigenin antibody (anti-DIG-AP; Boehringer Mannheim GmbH, Germany) and 5-bromo-4-chloro-3-indolyl phosphate substrate and nitroblue tetrazolium (Research Organics, Cleveland, OH, USA) [19]. Digitisation and interpretation of patterns were done with programs in the Taxotron package (Taxolab, Institut Pasteur, Paris). The membranes were first scanned and the images searched for bands by RestrictoScan. RestrictoTyper was used to interpolate the fragment sizes from migration data [18, 20, 21]. DNA from *Citrobacter koseri* strain CIP 105177 (Collection de l'Institut Pasteur) was cleaved by *Mlu*I

Table 1. Oligonucleotides used for PCR amplification of the toxin genes

Gene	Oligonucleotide	Sequence	Reference
<i>ctxA</i>	ctx 1	5'-CGG GCA GAT TCT AGA CCT CCT G-3'	16
	ctx 2	5'-CGATGATCT TGG AGC ATT CCC AC-3'	
<i>ace</i>	ace 1	5'-TAA GGA TGT GCT TAT GAT GGA CAC CC-3'	15
	ace2	5'-CGT GAT GAATAA AGATAC TCATAG 3'	
<i>zot</i>	zot1	5'-TGG CTT CGT CTG CTG CCG GCG ATT-3'	17
	zot2	5'-CAC TTC TAC CCA CAG CGC TTG CGC-3'	
<i>tcpA</i>	tcp525	5'-AAA GAG CTC GAT CTC CAC TCC GGA AATA-3'	14
	tcp568	5'-GAA TGG AGC AGT(T/A)CC (G/T)TA CAA-3'	

restriction endonuclease (Amersham Pharmacia Biotech) and the fragments were then used as the molecular size standards.

PFGE

V. cholerae O1 isolates were subjected to PFGE as described previously [22, 23]. Briefly, bacterial cells were embedded in low melting-point agarose (BioRad Laboratories, Richmond, CA, USA) and lysed with lysis buffer (Sarkosyl 1% in 0.5 M EDTA, pH 8.0, containing lysozyme and proteinase K). The DNAs were then digested with 20 U of *NotI* (5'-GCGGC CGC-3') restriction endonuclease (Amersham, Arlington Heights, IL, USA) at 37°C. PFGE was performed with an agarose 1% gel in Tris-borate-EDTA buffer 0.5% with a CHEF-DR II system (BioRad) under the following conditions: 6 V/cm at 14°C for 22 h at a field angle of 120°. The electrophoresis was performed with switch times of 15–25 s for 3 h and 8–25 s for 19 h [5]. The DNA size standard was a λ ladder consisting of concatemers starting at 48.5 kb (Biolabs, Beverly, MA, USA).

Results

Bacterial specimens

Table 2 shows the distribution of *V. cholerae* serotypes isolated from different provinces; 171 (86%) of the isolates were identified as *V. cholerae* O1 biotype El Tor. Of the serotypes that could be identified, 158 (92%) were Ogawa and 13 (8%) were Inaba. Most Inaba strains were found in the eastern province near the border with Pakistan. *V. cholerae* non-O1, non-O139 represented 29 (14%) of the total isolates, most of which were found in the central province of Iran.

Distribution of the amplified toxin genes

With the specific primers, the *V. cholerae* isolates were analysed by PCR for the presence of *tcp*, *ctx*, *ace* and *zot* genes. Table 2 also shows the most and least detected amplified genes in *V. cholerae* O1 isolates as the following: *tcp* (73% and 66%), *ctx* (97% and 81%), *ace* (78% and 55%) and *zot* (92% and 67%),

respectively. In only one central province, Kashan, were *V. cholerae* non-O1, non-O139 strains predominant, and the PCR results showed either the absence of the toxin genes or only a few positive results.

Antibiotic resistance and conjugation experiments

Except for one province, the antibiotic resistance study showed significant resistance to trimethoprim/sulphamethoxazole and streptomycin, and to a lesser extent to furazolidone, amongst the isolates of *V. cholerae* O1. Except for erythromycin and streptomycin, no antibiotic resistance was found in isolates from the province of Kashan, where all of the isolates were identified as *V. cholerae* non-O1, non-O139 (Table 3).

A large plasmid of *c.* 100 kb was found in the plasmid-positive *V. cholerae* O1 isolates. The percentage plasmid contents of *V. cholerae* isolates from different provinces are shown in Table 3. The greatest and least percentages of plasmids in isolates from the different provinces were 96% and 33%, respectively. Plasmids were conjugated between a *V. cholerae* donor and an *Escherichia coli* K12 recipient. In some cases the transconjugated plasmids were shown upon conjugation to pass the antibiotic resistance to the recipient. The co-transfer of trimethoprim/sulphamethoxazole and streptomycin resistance from Iranian *V. cholerae* isolates to an *E. coli* recipient was also observed, even in the absence of detectable plasmids in the isolates.

Genotyping analysis

Fig. 1 shows the ribotypes of *V. cholerae* isolates from Iran that were digested with *BglII* restriction endonuclease, resulting in four different restriction patterns. Lane 1 is the representative of non-O1, non-O139 *V. cholerae* isolated in the province of Kashan in Iran. This pattern was representative of 29 (14%) isolates of non-O1, non-O139 strains. Lane 2 was recognised as the ribotype pattern B15, in our database, consisting of six bands of 13.0–4.4 kb; 12% of *V. cholerae* O1 isolates showed this pattern. Analysis of 171 *V. cholerae* O1 isolates showed lane 3, identified in our database as B21, to be the predominant ribotype pattern with 81% of the cases. This pattern contained

Table 2. Distribution of serotypes isolated in different provinces in Iran and percentage of amplified genes in the *V. cholerae* isolates

Province	Number of isolates	Percentage of isolates carrying toxin gene and of serotype					
		<i>tcp</i>	<i>ctx</i>	<i>ace</i>	<i>zot</i>	Ogawa Inaba	non-O1, non-O139
Tehran	58	73	92	78	92	100	0
Kashan	29	8	9	0	0	0	100
Khuzastan	60	69	81	58	79	97	1.5
Kerman	44	66	97	55	67	100	0
Kermanshah	4	70	95	62	75	100	0
Sistan & Baluchastan	5	70	90	75	80	0	100

Table 3. Antibigrams of *V. cholerae* isolates in Iran, 1999–2000

Province	Number of isolates	Antibiotic resistance (%)										Plasmids (%)*
		C	CIP	DOX	E	OXT	SXT	T	GM	F	S	
Tehran	58	0	0	0	0	100	100	0	0	100	100	96
Kashan	29	0	0	0	100	0	0	0	0	0	100	62
Khuzastan	60	1.6	0	0	0	95	97	27	0	34	100	52
Kerman	44	2.4	0	4.8	2.4	2.4	100	4.8	0	100	100	75
Kermanshah	4	0	0	0	0	100	100	0	0	100	100	33
Sistan & Baluchastan	5	0	0	0	0	0	100	0	0	100	100	50

C, chloramphenicol; Cip, ciprofloxacin; Dox, doxycycline; E, erythromycin; Oxy, oxytetracycline; SXT, trimethoprim/sulphamethoxazole; T, tetracycline; GM, gentamicin; F, furazolidone, S, streptomycin.

*Percentage of *V. cholerae* isolates that contain plasmids.

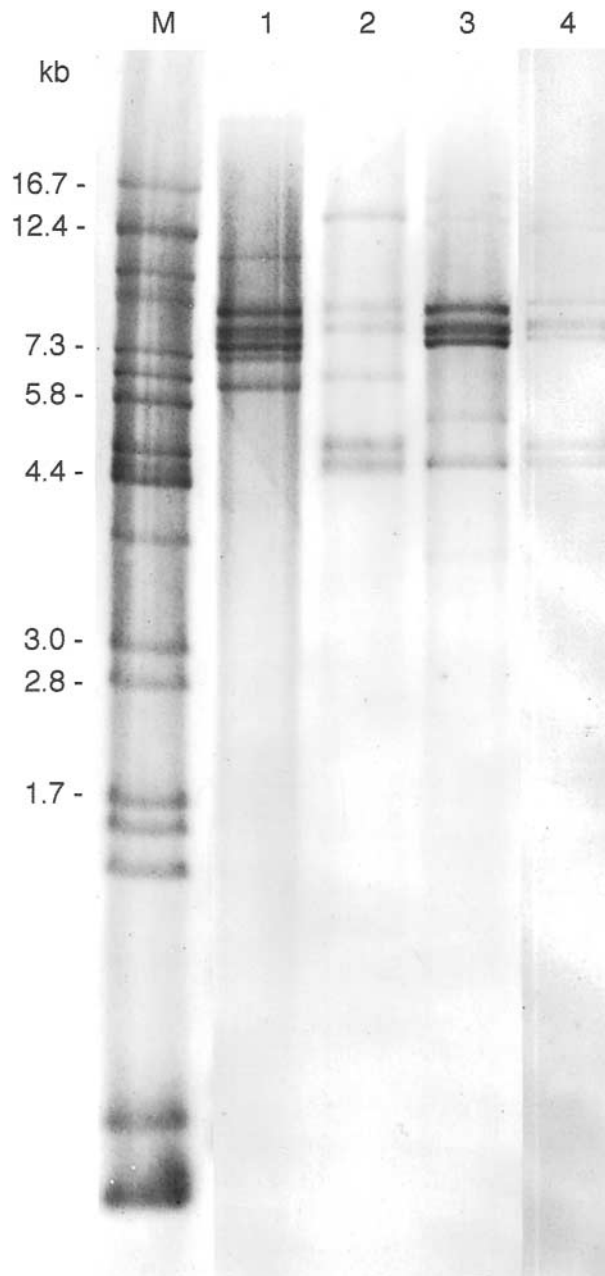


Fig. 1. Ribotype analysis of *V. cholerae* DNA digested with *Bgl*I restriction endonuclease. Numbers on the left indicate the molecular size of the bands of *Citrobacter koseri* strain DNA (CIP 105177) cleaved with *Mlu*I (mol. wt marker, **M**). Lane **1**, representative of non-O1, non-O139 *V. cholerae* isolates; **2–4**, representatives of *V. cholerae* O1 isolates.

seven bands, ranging from 13.0 to 4.5 kb. Lane 4 was also a ribotype pattern of *V. cholerae* O1, with bands similar to lane 3 but with a difference at the lower part of the gel, which showed two bands with molecular sizes of 4.5 and 4.7 kb. This pattern constituted 7% of the total *V. cholerae* O1 isolates.

Fig. 2 shows the representatives of *NotI*-digested PFGE patterns of *V. cholerae* isolates from Iran. PFGE analysis distinguished 10 different patterns amongst the isolates. Pattern 1 is representative of the non-O1, non-O139 isolates and the rest of the lanes are representative of *V. cholerae* O1 isolates.

Discussion

Most *V. cholerae* O1 isolates from different provinces in Iran were characterised by resistance to multiple antibiotics. The Iranian isolates can be grouped on the basis of their antibiotic resistance patterns into the following categories: (1) SxtFS, isolates resistant to sulphamethoxazole/trimethoprim (Sxt), furazolidone (F) and streptomycin (S); (2) SxtFSO, isolates with additional resistance to oxytetracycline (O); and (3) ES,

non-O1, non-O139 *V. cholerae* isolates resistant to erythromycin (E) and streptomycin (S) only.

Mukhopadhyay *et al.* [24] reported the resistance pattern SxtFACN (A, ampicillin; C, chloramphenicol; N, nalidixic acid) in isolates from the Indian sub-continent. *V. cholerae* O1 isolates from Africa have been reported with the resistance pattern SxtSAPT (P, penicillin; T, tetracycline) [25, 26]. In all, 81% of *V. cholerae* isolates in the present study showed the biochemical reactions of *V. cholerae* O1 biotype El Tor of the seventh pandemic, but their antibiotic susceptibility patterns differed in some respects from a number of the published reports [24, 27, 28]. Although no *V. cholerae* O139 isolates were obtained in this study, the resistance pattern of isolates studied here was similar to the antibiotic resistance in *V. cholerae* O139 carrying a conjugative transposon encoding for resistance to SxtS, as reported by Waldor *et al.* [29]. The reasons for this variation in antibiotic susceptibility patterns between different provinces are unclear. However, *V. cholerae* O1 strains isolated after the O139 epidemic in India have shown increased resistance to various drugs and genotypes that were distinct from O1 strains isolated before and during the O139 epidemic [22],

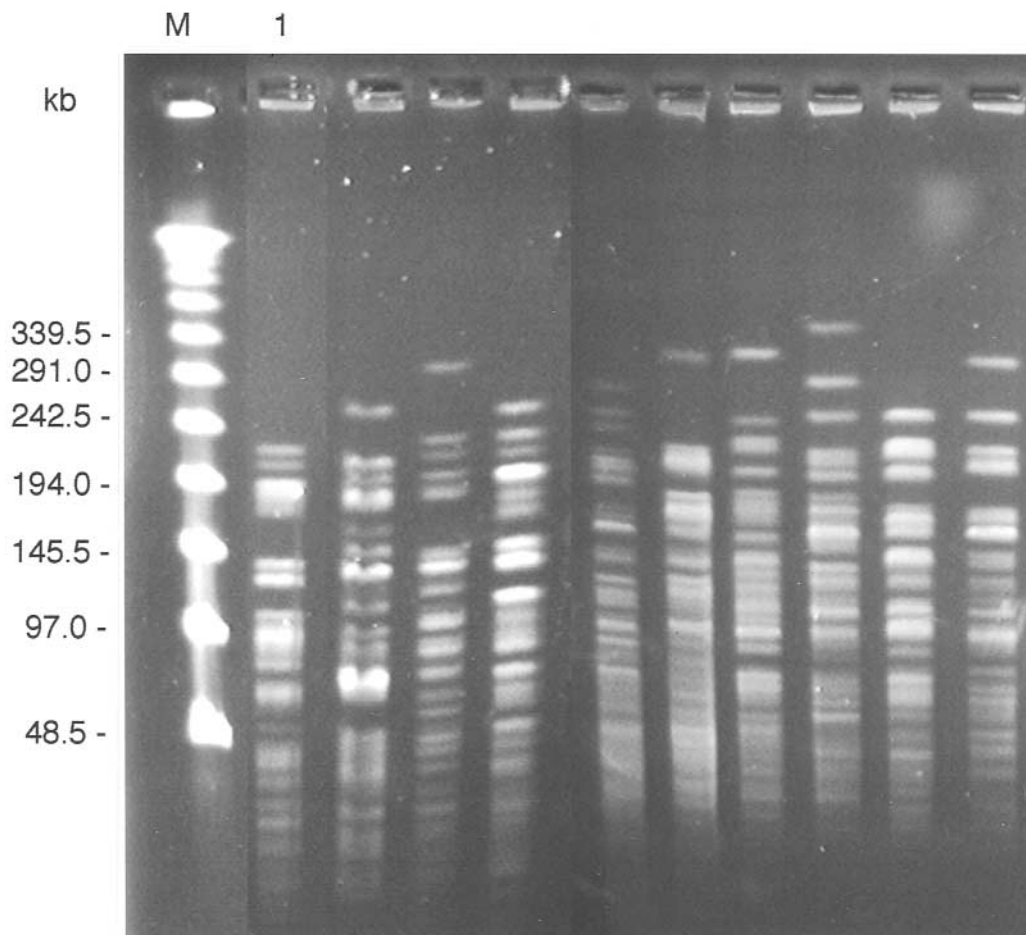


Fig. 2. PFGE fragment patterns of *NotI*-digested total cellular DNAs from *V. cholerae* non-O1, non-O139 (lane 1) and O1 (other lanes) isolates from different provinces in Iran. Size markers (λ ladder) were loaded in lane M. The values are indicated in kb on the left side of the gel.

suggesting that continuous genetic re-assortment amongst *V. cholerae* strains is taking place. Overall, several reports have indicated the re-appearance of distinct *V. cholerae* O1 and non-O1 strains in Asia, indicating the importance of genetic monitoring of emerging strains of this organism.

V. cholerae isolates in Iran were characterised by multiple antibiotic resistance coded by a specific plasmid. This observation is in accordance with other reports of the presence of the large plasmid in *V. cholerae* O1 and non-O1 strains [5, 30]. The naturally conjugative plasmid of 100 kb was found in 61% of the isolates of *V. cholerae* O1 from different provinces, suggesting that this plasmid may not have a stable genetic character to remain autonomously in *V. cholerae* species in the cholera outbreaks. Furthermore, the study of co-transfer in the absence of detectable plasmids of SxtS resistance from a *V. cholerae* donor to an *E. coli* recipient provided evidence that the multiple antibiotic-resistant plasmid in the Iranian isolates may be located on a conjugative transposon.

Other investigators [30, 31] have reported the presence of the *ctx* gene in clinical isolates of *V. cholerae* O1. On the other hand, the present study found variable results in PCR analysis of virulence cassette genes in 200 isolates of *V. cholerae*. The differences between the highest and lowest positive PCR-amplified genes in isolates of *V. cholerae* O1 were 8%, 16%, 23% and 25% for *tcp*, *ctx*, *ace* and *zot* genes, respectively. The lower percentage of toxin genes in some provinces in Iran may reflect the deletion of a toxin gene in the virulence cassette of the isolated strains [20, 32, 33]. Although the re-arrangement and copy number of the virulence cassette may play an important role in determining the pathogenicity level of the isolates [34], the presence of a virulence cassette could be variable, as it is subjected to amplification and deletion under the control of the *recA* gene [34]. Moreover, the data obtained in the present study were also in accordance with other reports [35–37], i.e., *V. cholerae* non-O1, non-O139 strains rarely harbour cholera toxin genes or other virulence genes contained in the virulence cassette found in O1 and O139.

The high discriminatory power of PFGE and ribotyping has been used extensively in epidemiological investigations. PFGE and ribotyping of *V. cholerae* O1 isolates from Bangladesh and India have demonstrated that the O1 serotype, which re-appeared after being temporarily displaced by *V. cholerae* O139, may represent a new clone [4, 38, 39]. The suggestion has been made [36, 40] that the new *V. cholerae* ribotype found in India, Bangladesh and Guinea-Bissau could be the cause of new epidemics. Ribotyping with the *Bgl*I restriction endonuclease produced four distinct patterns amongst the *V. cholerae* isolates in this study, three of which were in serotype O1 isolates, and the fourth in non-O1, non-O139 isolates. Ribotype pattern 1 was a

representative of non-O1, non-O139 *V. cholerae* isolates in Iran, which was similar to that reported by Dalsgaard *et al.* [36] in Thailand. Pattern 2 was recognised as *V. cholerae* O1 ribotype B15 in our database. Ribotype 3, known as B21, was a representative of an O1 strain that was found to be the predominant pattern for *V. cholerae* O1 isolated here. A similar pattern has also been reported in isolates from Thailand [5], referred to as type R1, and from isolates in Turkey, Romania and Lebanon [41]. Ribotype pattern 4 has been designated as type R3 by investigators of isolates from Vietnam, India, Thailand and Bangladesh [36, 42].

The results of PFGE have also demonstrated nine and one genotypes for *V. cholerae* O1 and non-O1, non-O139 isolates, respectively, indicating greater biodiversity in isolates of serotype O1. Only one pattern was observed in ribotype and PFGE analysis of non-O1, non-O139 strains, which may be attributable to the fact that the isolates were obtained from an endemic situation.

In summary, these results show that new clones of *V. cholerae*, not found in a previous study [18], have spread in Iran. Furthermore, the data support the genetic diversity of *V. cholerae* in Iran, where this bacterium continues to be an important cause of diarrhoea.

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