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SCIENCE DIRECT
Research in Microbiology 157 (2006) 508–512
Research in Microbiology
www.elsevier.com/locate/resmic

Molecular epidemiology and origin of cholera reemergence in Italy and Albania in the 1990s

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Received 21 June 2005; accepted 23 November 2005
Available online 25 January 2006

Abstract

In 1994 a cholera epidemic occurred in Italy and Albania after more than a decade of case absence. To investigate genotypic characteristics and the origin of the epidemic strains, 110 *Vibrio cholerae* O1 El Tor isolates from Italy and Albania were studied by randomly amplified polymorphic DNA analysis (RAPD), *Bgl*I ribotyping, and pulsed-field gel electrophoresis (PFGE) of genomic DNA. The Italian and Albanian strains were all ribotype 6 and their RAPD and PFGE patterns were identical as well. These findings indicated that the 1994 isolates belonged to the same clone and that the clone was part of the larger global spread of epidemic ribotype 6 strains, which started in southern Asia in 1990.

Keywords: Cholera; Molecular epidemiology; Europe; *Vibrio cholerae*

1. Introduction

Cholera is a worldwide disease with an estimated incidence of more than five million cases per year, most of which occur in Asia and Africa, with 8% of cases requiring hospitalization [9]. Despite primarily affecting developing countries, cholera remains a serious public health problem for some developed countries.

Since 1817, seven distinct pandemics have occurred. No information exists concerning the *Vibrio cholerae* strains that gave rise to the first four pandemics, whereas the sixth and presumably the fifth were caused by *V. cholerae* O1 of the classical biotype. The ongoing seventh pandemic, the most extensive in distribution and duration, began in 1961 and was caused by *V. cholerae* O1 of the El Tor biotype [8,9]. During the sixth pandemic (1899–1923), cholera outbreaks occurred in the south of Italy in 1910 and 1911 and in the Balkans, including Albania, in the period from 1910 to 1922 [18].

El Tor cholera outbreaks first occurred in the southern regions of Apulia and Campania in 1973; there were a dozen cases in Sardinia in 1973 and 1979 [1]. Since then, except for sporadic imported cases, no cholera was detected in Italy until 1994, when the disease reappeared in Apulia in the same year as in the Balkans [10,25]. A number of molecular typing techniques have been employed to investigate the genomic diversity among the isolates of the seventh pandemic [11,17]. However, little information is available about genetic relatedness and origins of El Tor strains implicated in outbreaks in Europe. In this study, we employed randomly amplified polymorphic DNA (RAPD) assays, *Bgl*I ribotyping, and pulsed-field gel electrophoresis (PFGE) to characterize and compare 110 *V. cholerae* O1 strains isolated in Italy and Albania during the year 1994.

2. Materials and methods

2.1. Bacterial strains, growth conditions and genomic DNA extraction

A total of 110 *V. cholerae* O1 isolates collected from the cholera outbreaks which occurred in Italy and Albania in 1994

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0923-2508/$ – see front matter
doi:10.1016/j.resmic.2005.11.013

Table 1
Origin and RAPD cluster type of 110 *V. cholerae* O1 El Tor strains isolated in Italy and Albania

Biotype/country (no. of isolates)	Place of isolation[a]	Month/year of isolation	Source/ref.	No. of isolates	RAPD cluster type[b]
Epidemic isolates					
El Tor/Apulia, Italy (18)	Bari	Oct/1994	Patient	7	I
	Casamassima	Oct/1994	Patient	2	I
	Mola	Oct/1994	Patient	1	I
	Barletta	Nov–Dec/1994	Patient	2	I
	Bari	Oct/1994	Vegetables	2	I
	Bari	Oct/1994	Sewage	4	I
El Tor/Albania (92)	Kuçove	Sep/1994	Patient	3	I
	Elbasan	Sep–Nov/1994	Patient	19	I
	Tirana	Sep/1994	Patient	9	I
	Librazhd	Sep/1994	Patient	7	I
	Lushnje	Sep–Oct/1994	Patient	3	I
	Berat	Sep–Oct/1994	Patient	12	I
	Peskhopi	Sep–Oct/1994	Patient	2	I
	Fier	Sep–Nov/1994	Patient	22	I
	Kavaje	Nov/1994	Patient	4	I
	Lezhe	Oct/1994	Patient	1	I
	Kruje	Oct/1994	Patient	1	I
	Peqin	Oct–Nov/1994	Patient	8	I
	Tepelene	Oct/1994	Patient	1	I
Reference strains					
El Tor/Apulia, Italy (4)	Bari	1973	Patient	4	II
El Tor/CIRPS 1001	Somalia	1985	[14]	1	II
El Tor/E7946	Bahrain	1978	[13]	1	II
Classical/569B	India	1948	[12]	1	III
Classical/O395	India	1964	[12]	1	III

[a] Geographic distribution of sites is shown in Fig. 1.
[b] Single RAPD patterns characteristic of each cluster type are shown in Fig. 2.

were analyzed (Table 1). *V. cholerae* O1 strains from Albania were identified by the Institute of Public Health of Tirana and sent for further characterization to the National Reference Center for enteric pathogens of the Istituto Superiore di Sanità, Rome. Antimicrobial susceptibility (by disk diffusion method) and phenotypic characterization of strains were performed as previously described [4,7,22]. Disks loaded with 10 μg ampicillin, 30 μg chloramphenicol, 30 μg doxycycline, 30 μg kanamycin, 10 μg streptomycin, 10 μg spectinomycin, 30 μg tetracycline, 25 μg sulfametoxazole and 5 μg trimethoprim were used. Four *V. cholerae* O1 El Tor strains isolated in Italy (Apulia) during the 1973 outbreak, *V. cholerae* O1 classical strains 569B and O395 [3], El Tor strains E7946 [14] and CIRPS 1001 [13] were included for comparison. Original stock cultures of isolates were kept in 20% glycerol Luria–Bertani broth at −70 °C. Strains were grown in Luria–Bertani broth at 37 °C and genomic DNA was extracted by the cetyl trimethylammonium bromide method [16].

2.2. RAPD assay (ERIC1, ERIC2, VCR1, VCR2, ATX1, ATX2)

PCR reactions were performed in a volume of 20 μl containing 50–100 ng of total DNA, 3 mM $MgCl_2$ and 5 units of the *Taq* polymerase Stoffel Fragment (Perkin–Elmer). dNTPs (Pharmacia) were used at a final concentration of 200 μM. The primers used were: ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′), selected from enterobacterial repetitive intergenic consensus sequences [21]; VCR1 (5′-CAGCTCCTTAGGCGGGCGTTAG-3′) and VCR2 (5′-ACAGTCCCTCTTGAGGCGTTTG-3′), selected from conserved regions of *Vibrio cholerae* repeat elements [2]; ATX1 (5′-AAGCGATTGAAAGGATGA-3′) and ATX2 (5′-CCGCGAGTGCTTGGTTAG-3′), respectively corresponding to *ctx*B gene sequence nt 249–266 [15] and *cep* gene sequence nt 100–83 on the CTXϕ phage genome of *V. cholerae* [24].

All primers were synthesized commercially and purified through reverse phase chromatography by Invitrogen Life Technologies. Primers were employed separately at a final concentration of 1 μM per reaction and PCR conditions were the same for each of the six primers. PCR conditions were as follows: 94 °C for 2 min; 5 cycles of 1 min at 92 °C, 1 min at 40 °C and 10 min at 72 °C; followed by 30 cycles of 1 min at 92 °C, 1 min at 52 °C and 10 min at 72 °C; followed by 10 min at 72 °C. A ramp of 1 °C/s was programmed from annealing to extension temperatures. All reactions were performed in duplicate, and results were found to be reproducible.

2.3. Gel electrophoresis and computer analysis of RAPD patterns

PCR products were separated in 2% (w/v) agarose gel (Bio-Rad), 45 mM Tris–borate, 1 mM EDTA buffer, at 60 V and at a temperature of 14 °C. Agarose gels were stained with ethidium

bromide at a final concentration of 0.5 μg/ml. Images from gels were converted into a digital form by the gel-doc 2000 photo documentation system (Bio-Rad). PCR-Marker 50–2000 bp was purchased from Sigma-Aldrich. Computer diagrams were generated using the Diversity Database Fingerprinting Software (Bio-Rad).

2.4. Ribotyping

Ribotyping was carried out according to Popovic et al. [19]. 10 μg of genomic DNA of each strain was digested with 10 U of *Bgl*I for 2 h at 37 °C in a water bath. Restriction DNA fragments were separated by horizontal electrophoresis through a 0.8% agarose gel in TAE buffer at 60 V for 16 h. Digoxigenin-labeled DNA molecular weight marker III (Boehringer, Mannheim, Germany) was included. DNA was transferred to a nylon membrane, positively charged (Boehringer, Mannheim, Germany) by the method of Southern [23]. *Escherichia coli* 16S and 23S rRNA (Roche Molecular Biochemicals, Indianapolis, IN) was reverse-transcribed into cDNA with avian reverse transcriptase and labeled with hexanucleotides containing digoxigenin (DIG)-dUTP according to the manufacturer's instructions (Boehringer, Mannheim, Germany). Hybridization solution with 50% formamide was prepared according to manufacturer (DNA labeling and detection kit, Boehringer, Mannheim, Germany). The membrane was hybridized under high stringency (42 °C with 50% formamide and washes in 0.1× SSC and 0.1% SDS at 68 °C). Hybridized DNA restriction fragments were detected by chemoluminescence as recommended by the manufacturer (Boehringer, Mannheim, Germany).

2.5. PFGE analysis

Total genomic DNA was prepared by the method of Mahalingam et al. [12]. Before restriction enzyme digestion, agarose blocks were equilibrated for 2 h in 200 μl of the appropriate buffer (5 mM Tris–HCl, 1 mM $MgCl_2$, 10 mM NaCl, 0.1 dithiothreitol for *Not*I; or 1 mM Tris–HCl, 1 mM $MgCl_2$, 5 mM NaCl, 0.1 dithiothreitol for *Sfi*I). Fresh buffer containing *Not*I at 8 U/μg of DNA or *Sfi*I at 10 U/μg of DNA was then added and the blocks were incubated overnight at 37 °C for *Not*I and 50 °C for *Sfi*I. PFGE of inserts was performed by using the contour-clamped homogeneous electric field method on a CHEF-DR III system (Bio-Rad, Milan, Italy) in gel of 1% agarose in 0.5× TBE buffer for 28 h (*Sfi*I) or 29 h (*Not*I) at 200 V at a temperature of 14 °C with the following pulse times: *Sfi*I, 5 s (8 h), 15 s (10 h) and 25 s (10 h); *Not*I, 6 s (8 h), 15 s (10 h) and 25 s (11 h). A PFGE marker I λ-ladder (Boehringer, Mannheim, Germany) was included in the first lane of the gel. Computer analysis of PFGE patterns was performed by the Bionumeric software (Applied Maths, St. Martens Latem, Belgium) using Dice coefficient and the unweighted pair group method of average (UPGMA).

3. Results

Genomic typing of 110 *V. cholerae* O1 strains isolated in the Italian region of Apulia and in Albania in 1994 (Table 1, Fig. 1) was first performed by RAPD fingerprinting. RAPD patterns were generated by six single primers selected from enterobacterial repetitive consensus (ERIC) sequences, *V. cholerae* repetitive (VCR) sequences, and sequences of the *V. cholerae* filamentous phage CTXϕ. Each strain was characterized by an individual RAPD cluster type represented by the combination of the six single RAPD patterns (Fig. 2).

The El Tor strains isolated in Apulia and Albania in 1994 exhibited the same cluster type of RAPD patterns, E9/E12/V2/V5/A1/A8, designated RAPD cluster type I. The four El Tor strains isolated in Apulia in 1973 showed a different RAPD cluster type, E1/E14/V2/V3/A2/A8, designated RAPD cluster type II. Differences between the RAPD cluster type I and II consisted of seven DNA amplicons which distinguished the four RAPD patterns (Fig. 2) generated by primers ERIC1 (patterns E1 and E9), ERIC2 (patterns E12 and E14), VCR2 (patterns V3 and V5), and ATX1 (patterns A1 and A2). *V. cholerae* O1 classical strains produced a third RAPD cluster type, E1/E11/V13/V19/A9/A5,

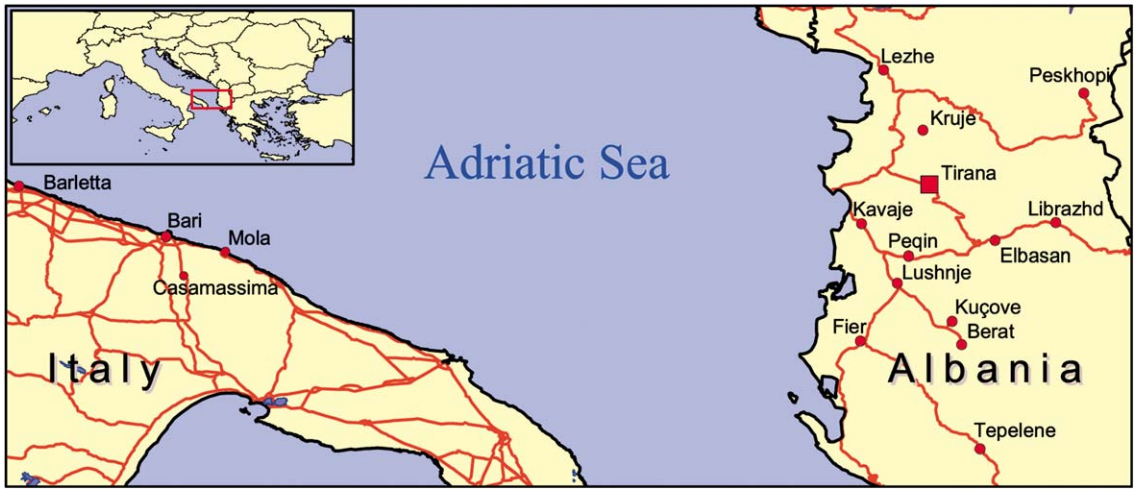

Fig. 1. Geographical distribution of towns affected by cholera in Italy and Albania in 1994.

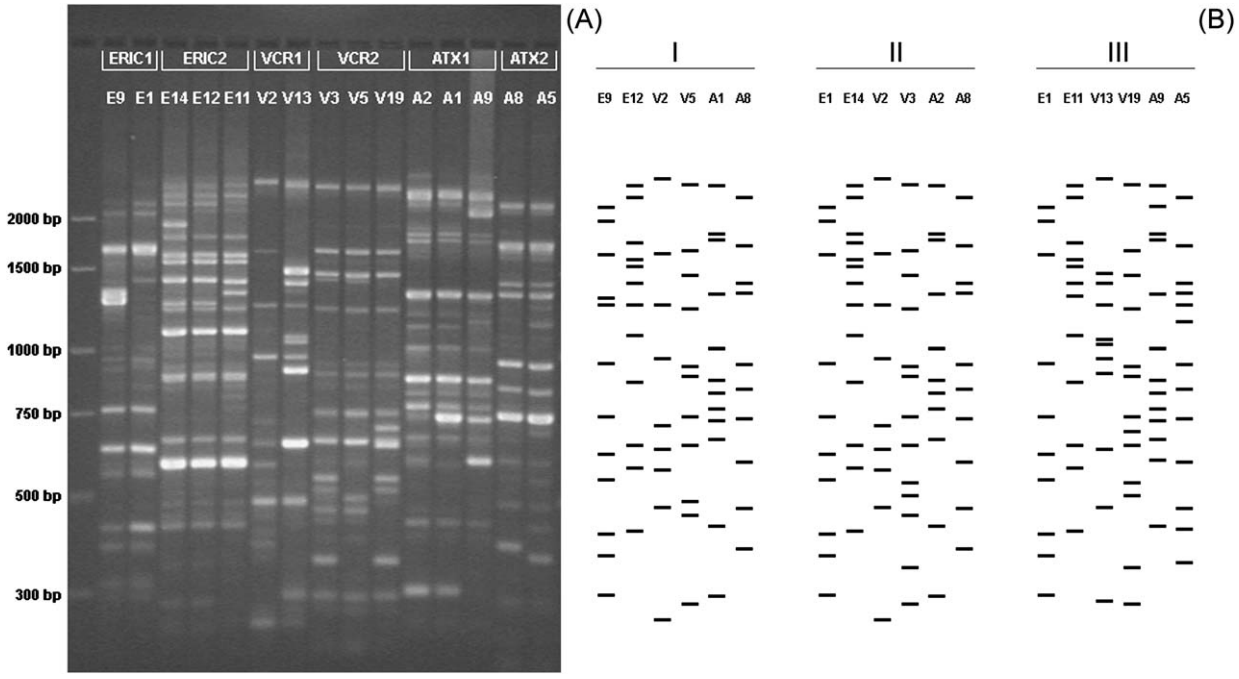

Fig. 2. Genomic typing by RAPD fingerprinting. (A) RAPD patterns of genomic DNA of *V. cholerae* O1 strains generated by six primers. All representative variants of single RAPD patterns are included. Each specific pattern is designated by a letter with a serial number (E1, V2, A1, etc.) and the corresponding primer is reported at the top of the gel (ERIC1, VCR1, ATX1, etc.). Molecular size markers are given on the left. (B) Schematic diagram of RAPD cluster types I, II and III characterizing the different groups of *V. cholerae* O1 strains.

Table 2
RAPD cluster types, ribotypes, PFGE patterns and antimicrobial resistance patterns of *V. cholerae* O1 strains from Italy and Albania

Country/year	RAPD cluster type (no. of strains tested)	Ribotype (no. of strains tested)	PFGE pattern (no. of strains tested)	Resistance pattern
Apulia, Italy/94	I (18)	6a (18)	A (6)	SPT STR SXT TET TMP (18)
Albania/94	I (92)	6a (80)	A (11)	SPT STR SXT TET TMP (92)
Apulia, Italy/73	II (4)	5 (4)	B (4)	SPT (4)

SPT = spectinomycin; STR = streptomycin; SXT = sulfamethoxazole; TET = tetracycline; TMP = trimethoprim; RAPD = randomly amplified polymorphic DNA; PFGE = pulsed-field gel electrophoresis.

different in more than twenty amplicons from the El Tor cluster types I and II.

The strains isolated in Apulia in 1973 and 1994, and 80 Albanian strains were further characterized by *Bgl*I ribotyping (Table 2). The Italian and Albanian strains isolated in 1994 were all ribotype 6a, while the Italian strains isolated in 1973 were ribotype 5. Of the El Tor strains ribotyped, six Italian strains isolated in 1994 (one strain from each of the different sources and places of isolation; Table 1), 11 Albanian strains isolated in 1994 (one strain from each place of isolation, except Kruje and Tepelene; Table 1) and the four Italian strains isolated in 1973 were also characterized by PFGE of genomic DNA restricted with the two rare-cutting enzymes *Not*I and *Sfi*I. The 1994 strains isolated in Apulia and Albania showed an identical PFGE pattern which was distinct from that of the strains isolated in Apulia in 1973 (Table 2).

In addition to the DNA-based typing results, tests for antimicrobial susceptibility revealed that all the Albanian and Italian strains isolated in 1994 had the same spectrum of antimicrobial susceptibility, being resistant to spectinomycin, streptomycin, sulfametoxazole, tetracycline and trimethoprim and susceptible to ampicillin, chloramphenicol, doxycycline and kanamycin (Table 2). The isolates collected in Apulia in 1973 were resistant only to spectinomycin.

4. Discussion

In 1973, El Tor cholera outbreaks occurred in Italy, with 278 reported cases and 24 deaths. 126 cases were restricted to the Italian region of Apulia. After twenty-one years, in 1994, cholera re-emerged in Apulia with 12 clinical cases reported from October to December. In the same year a total of 2529 cholera cases were notified to WHO from five eastern European countries (Albania, Republic of Moldova, Romania, Russian Federation, and Ukraine), which represented a more than 30-fold increase over the previous year. Albania was the country in the Balkans [25] with the highest number of cases and deaths (626 and 25, respectively).

In this study we investigated the origin of the re-appearance of cholera in Apulia and Albania. Strains of *V. cholerae* O1 El Tor isolated in Apulia and in Albania in 1994 were genotypically characterized by RAPD fingerprinting, ribotyping and

PFGE of genomic DNA. The results demonstrated that the Italian cholera outbreak which occurred in Apulia in 1994 was caused by the introduction of strains derived from the El Tor clone responsible for the Albanian epidemic. The Albanian clone was ribotype 6a and exhibited a uniform type of RAPD fingerprint (type I) and PFGE pattern (pattern A). The 1973 strains from Apulia were ribotype 5 and had clearly distinct characteristics, in both the RAPD and PFGE patterns (type II and B, respectively), from the 1994 strains.

With specific reference to ribotyping, a generally used bacterial typing tool to identify pandemic and endemic waves, ribotype 5 has been isolated worldwide over the past twenty years [19] and thus far has proven to be the predominant ribotype of the seventh cholera pandemic [5,6].

Ribotype 6, which includes subtypes 6a–6c, was relatively infrequent among El Tor isolates during the 1970s and the 1980s. Since 1990, strains of ribotype 6 have increasingly been isolated in India, Bangladesh, Pakistan, Iran, Turkey, Romania and the Middle East [6,20]. In agreement with these epidemiological data, our findings indicate that the El Tor clone responsible for cholera outbreaks in Albania and Italy in 1994 was part of the larger global spread of epidemic ribotype 6 strains.

Acknowledgements

We thank Alessio Fasano for classical strains; Eugenio Bordi for El Tor strains isolated in Apulia in 1973; and Rossana Santacroce and Gianni Ruggieri for technical assistance. This study was supported by grant IC18-CT97-0231 (F.M.) from the European Commission under the INCO-DC program, and, in part, by research funds from the Faculty of Sciences, University of Bari, and the Ph.D. course in Genetics and Molecular Evolution at the University of Bari. Organizational and informatics support was provided by the Scientific Association COMIPA, Roma.

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