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Investigation of the 1994–5 Ukrainian *Vibrio cholerae* epidemic using molecular methods

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SUMMARY

Thirty-seven *Vibrio cholerae* and four non-cholera *Vibrio* isolates from Ukraine, including strains from the epidemic of 1994–5, were analysed by molecular methods. Results from PFGE and ribotyping indicated that all Ukrainian toxigenic *V. cholerae* were closely related to each other and to an isolate from a patient from Pakistan. A non-toxigenic river water strain obtained during the height of the epidemic was more distantly related to these *V. cholerae* strains, while the *Vibrio parahaemolyticus* isolates and *Vibrio alginolyticus* isolate were not closely related to *V. cholerae* or each other. ERIC- and REP-PCR allowed the differentiation of strains identical by other methods. The results obtained confirm that the epidemic Ukrainian strains are most closely related to seventh pandemic strains from Asia and support a hypothesis that the Ukrainian epidemic of 1994–5 was caused by toxigenic environmental strains surviving since the time of the 1991 Ukrainian epidemic or before.

INTRODUCTION

Cholera is a severe diarrhoeal disease characterized by its unpredictability and tendency to cause sudden explosive outbreaks [1]. *V. cholerae* O1 has been responsible for seven of the eight cholera pandemics, each arising in Asia and spreading throughout the world [2]. Seventh pandemic strains continue to cause disease throughout the world [2]; recently there has been a marked increase in the incidence of cholera world-wide, especially in parts of Eastern Europe and South America [3, 4].

Sixth pandemic *V. cholerae* O1 classical strains, as well as seventh pandemic and US gulf coast *V. cholerae* O1 El Tor isolates, may be independently derived from environmental clones [5] by acquisition of a virulence cassette containing the genes for cholera toxin (*ctxA* and *ctxB*), the zonula occludens toxin (*zot*) and the accessory cholera enterotoxin (*ace*). This may have occurred through lysogenic conversion by a bacteriophage carrying these genes [6]. While the organism responsible for the seventh cholera pandemic is one of four currently distinguishable types of *V. cholerae* O1 El Tor in the world [7–11], it is clear

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that geographically and temporally distinct isolates of this organism can display genetic variations resulting from genome rearrangement and genetic exchange, leading to a low level of clonality [2, 5]. The seventh cholera pandemic can therefore be thought of as a simultaneous occurrence of several local epidemics, each caused by a separate clone of *V. cholerae* O1 El Tor [12].

Molecular techniques have been useful for the study of the microbiology and epidemiology of recent cholera epidemics. Detection of rRNA gene restriction patterns (ribotyping) has been very helpful for differentiating clones of *V. cholerae* responsible for waves of epidemics in Asia and Africa [12, 13], South and Central America [14], and world-wide [15]. Ribotyping has also been useful for epidemiological studies of environmental *V. cholerae* strains [16, 17], and a standard ribotyping scheme has been developed [18]. Analysis of genomic DNA by pulsed-field gel electrophoresis (PFGE) has been used to characterize *V. cholerae*, generally allowing finer distinctions among epidemic strains than ribotyping [14, 16, 19, 20]. Several investigators have assessed differences between strains using restriction fragment length polymorphisms of the cholera toxin (*ctx*) gene [11, 13, 21–25] and the insertion sequence IS*1004* [26]. Finally, arbitrarily primed PCR [27, 28] and PCR of enterobacterial repetitive intergenic consensus (ERIC) sequences [29] have also been used for strain characterization.

Two recent epidemics in Ukraine, in 1991 and 1994–5, were caused by *V. cholerae* O1 El Tor. Epidemiological, biological, and biochemical characteristics of these organisms suggested that the epidemic in 1994–5 resulted from the introduction or reintroduction into the Ukrainian population of a toxigenic strain of cholera from an environmental source [30]. In this investigation, we have used DNA-based typing methods to further refine our understanding of the 1994–5 epidemic. The results obtained support an environmental source for the epidemic strain and suggest that some genotypic changes may have occurred during the epidemic.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study were collected from patients and the environment during epidemics of cholera in Ukraine in 1991 and 1994–5, and are described fully in an accompanying paper [30]. Isolation and identification of bacteria from clinical samples was originally done by the local hospital laboratory. Patient isolates of *Vibrio* spp., including *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus*, were collected from hospitals in Mikolajiv, Odessa and Kirovograd by V. V. Alekseenko and stored at the Laboratory of Cholera Infection, Kiev Research Institute. Upon arrival at the Laboratory Centre for Disease control in Ottawa, isolates were grown on thiosulphate-citrate-bile salts–sucrose (TCBS) agar to confirm identity of the organism. Biotypes were confirmed by means of Mukerjee bacteriophage sensitivity, polymyxin B sensitivity, and the Voges–Proskauer reaction, while serotypes were confirmed by slide agglutination using polyvalent rabbit antiserum. All isolates were stored on maintenance media and were cultured in brain heart infusion broth or on nutrient agar plates (Difco Laboratories, Detroit, MI, USA) for 18 h at 37 °C for subsequent manipulations. A summary of isolates and their characteristics is found in Table 1.

Detection of cholera toxin and the *ctx* gene

Cholera toxin in bacterial culture supernatants was detected using the VET-RPLA kit (Unipath Ltd., Basingstoke, UK) according to the manufacturer's directions. Bacteria were tested after overnight growth at 37 °C in both buffered peptone water and AKI medium. PCR for detecting a 564-bp region of the *ctx*A gene was done using the CTX2 (CGGGCAG-ATTCTAGACCTCCTG) and CTX3 (CGATGATC-TTGGAGCATTCCCAC) primers according to the protocol of Fields and colleagues [31], except that the reactions were carried out for 35 cycles. The presence of amplified DNA was demonstrated by electrophoresis of reaction products through 0·8 % agarose, ethidium bromide staining, and visualization with long-wave ultraviolet light. Patient isolate 93-0608 from Pakistan, previously characterized as cholera toxin positive, was used as a positive control.

Enterobacterial repetitive intergenic consensus sequence (ERIC)- and repetitive extragenic palindromic (REP)-PCR

DNA was obtained by lysis of bacteria with sodium dodecyl sulphate (SDS) and phenol-chloroform extraction [32]. Repetitive primer PCR was done using

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Table 1. *Description of* Vibrio *spp. used in this study*

LCDC number	Source	Location	Date	Disease outcome	Cholera toxin*
V. cholerae					
93-0608	Patient	Pakistan	1993	Recovered	+
94-0457	Patient	Peru	1994	Recovered	+
95-0743	Patient	Odessa	28/08/91	Recovered	+
95-0745	Fish	Odessa	09/10/93	n.a.†	+
95-0760	Patient	Mykolajiv	18/09/94	Fatal	+
95-0762	Patient	Mykolajiv	19/09/94	Fatal	+
95-0755	Patient	Mykolajiv	20/09/94	Fatal	+
95-0775	River water	Mykolajiv	20/09/94	n.a.	+
95-0780	Sea water	Mykolajiv	20/09/94	n.a.	+
95-0773	Sea water	Mykolajiv	21/09/94	n.a.	+
95-0776	River water	Mykolajiv	21/09/94	n.a.	+
95-0777	River water	Mykolajiv	21/09/94	n.a.	+
95-0779	Sea water	Mykolajiv	21/09/94	n.a.	+
95-0772	Sewage	Mykolajiv	22/09/94	n.a.	+
95-0754	Patient	Mykolajiv	24/09/94	Fatal	+
95-0759	Patient	Mykolajiv	24/09/94	Fatal	+
95-0763	Patient	Mykolajiv	24/09/94	Fatal	+
95-0761	Patient	Mykolajiv	27/09/94	Fatal	+
95-0765	Patient	Mykolajiv	30/09/94	Recovered	+
95-0766	Patient	Mykolajiv	30/09/94	Recovered	+
95-0768	Patient	Mykolajiv	30/09/94	Recovered	+
95-0769	Patient	Mykolajiv	30/09/94	Recovered	+
95-0767	Patient	Mykolajiv	01/10/94	Recovered	+
95-0770	Patient	Mykolajiv	01/10/94	Recovered	+
95-0764	Patient	Mykolajiv	02/10/94	Recovered	+
95-0756	Patient	Mykolajiv	06/10/94	Fatal	+
95-0757	Patient	Mykolajiv	06/10/94	Fatal	+
95-0771	Sewage	Mykolajiv	16/10/94	n.a.	+
95-0758	Patient	Mykolajiv	20/10/94	Fatal	+
95-0781	Patient	Kirovograd	1994	Recovered	+
95-0749	Patient	Mykolajiv	1995	Recovered	+
95-0750	Patient	Mykolajiv	1995	Recovered	+
95-0751	Patient	Mykolajiv	1995	Recovered	+
95-0746	Patient	Mykolajiv	1995	Recovered	+
95-0747	Patient	Odessa	1995	Recovered	+
95-0753	Patient	Odessa	1995	Recovered	+
95-0752	River	Odessa	1995	n.a.	−
V. alginolyticus					
95-0742	Patient	Odessa	1995	Recovered	−
V. parahaemolyticus					
95-0744	Patient	Odessa	1995	Recovered	−
95-0748	Patient	Odessa	1995	Recovered	−
95-0778	Patient	Odessa	1995	Recovered	−

* PCR and the VET-RPLA tests were done as described in the Materials and Methods. The presence of a 564-bp amplicon after agarose electrophoresis and staining with ethidium bromide constituted a positive PCR results.
† n.a., not applicable.

either the ERIC1R (ATGTAAGCTCCTGGGGATTCAC) or the REP1R-I (IIIICGICGICATCIGGC) primers by the method of Versalovic and colleagues [33] in a Perkin–Elmer Cetus DNA Thermal Cycler 480. Following an initial denaturation (94 °C, 5 min), genomic DNA was amplified through 35 cycles of

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denaturation (94 °C, 2 min), annealing (REP 40 °C, 1 min; ERIC 52 °C, 1 min), and extension (72 °C, 1 min), with a single final extension (72 °C, 10 min). PCR products were electrophoresed directly on 1·5% agarose gels, stained with ethidium bromide, and analysed using a Bio-Image image acquisition system. UPGMA analysis was accomplished using Bio-Image Whole Band Analyzer software to facilitate grouping of isolates and comparison of the groups identified. Selection of the ERIC1R and REP1R-I primers was based on the results of preliminary studies using different combinations of primers, as well as on data showing that PCR amplification with ERIC1R alone yielded less complex amplification patterns [33]. This use of this primer set produced an optimal number of bands for differentiating closely related isolates while minimizing background and faint bands.

RFLPs of *ctx*A and rRNA genes

ctx-RFLP was done using a 564-bp *ctx*A probe essentially according to the method of Wachsmuth and colleagues [11, 23], while ribotyping was done according to the method of Popovic and colleagues [18], but with modifications as outlined below. Genomic DNA was restricted with *Hin*dIII or *Bgl*I and fragments were separated on a 0·8% agarose gel. DNA in the gel was blotted to a Hybond™-N+ membrane (Amersham Life Science Inc., Oakville, Ontario) in a Stratagene PosiBlot 30-30 Pressure Blotter (PDI Bioscience Inc., Aurora, Ontario) and crosslinked to membranes with ultraviolet light. Membranes were prehybridized in DNA Hybridization Buffer (Gibco BRL, Burlington, Ontario) for 1 h at 65 °C, after which denatured digoxigenin-11-dUTP-labelled probes were added directly to the hybridization buffer and allowed to hybridize ON at 65 °C. Blots were subjected to two 10 min room temperature washes with 20 × SSC containing 0·1% sodium dodecyl sulphate (SDS), one 15 min wash at 65 °C with 1 × SSC containing 0·1% SDS, and one 15 min wash at 65 °C with 0·1 × SSC containing 0·1% SDS. After incubation of blots for 30 min at room temperature in 5% Blocking Reagent (Boehringer–Mannheim Canada, Laval, Quebec) dissolved in TBS (25 mM Tris, 3 mM KCl, 137 mM NaCl), blots were developed with anti-DIG-alkaline phosphatase conjugate (Boehringer Mannheim Canada, Laval, Quebec). Hybridized probes were detected using CDP-*Star*™ substrate (Boehringer–Mannheim Canada, Laval, Quebec) according to the manufacturer's instructions, followed by exposure to Hyperfilm-MP photographic film (Amersham Life Science Inc., Oakville, Ontario).

The 564-bp *ctx*A fragment generated by PCR as outlined above was labelled with digoxigenin-11-dUTP using the DIG PCR Labelling Mix (Boehringer–Mannheim) in a second PCR reaction with the same primers. The *rrn*B probe from plasmid pKK3535 [34] was labelled with digoxigenin-11-dUTP using a DIG-High Prime labelling kit (Boehringer–Mannheim Canada, Laval, Quebec) according to the manufacturer's instructions. Supercoiled DNA Ladder (Gibco BRL, Burlington, Ontario) used as a size standard was linearized with *Pvu*II and labelled with Fluorescein High Prime reagent (Boehringer–Mannheim Canada, Laval, Quebec) according to the manufacturer's directions. Bound ladder probe was detected using anti-fluorescein-alkaline phosphatase (Boehringer–Mannheim Canada, Laval, Quebec) in the same manner as digoxigenin-labelled proves.

Pulsed-field gel electrophoresis

PFGE was done according to the protocol of Böhm and Karch [35] with the following modifications. One loopful of overnight bacterial culture was suspended in 10 mM Tris/HCl containing 5 mM EDTA (TE buffer), pH 8·0 to give an OD_{600} of 0·1. This bacterial suspension (0·1 ml) was mixed with 0·9 ml of 1% low melting point agarose and transferred to disposable plug molds (Bio-Rad laboratories Ltd., CA). Cells were lysed at 52 °C for 48 h in 4 ml of 50 mM Tris/HCl + 50 mM EDTA, pH 9·0, containing 1·0% *N*-laurolysarcosine (wt/vol) and 1 mg/ml proteinase K. Agarose plugs containing DNA were washed three times with TE buffer, pH 7·5, containing 1 mM phenylmethlysolfonylfluoride (PMSF) for a minimum of 5 min per wash, then three times with TE buffer, pH 7·5, for a minimum of 20 min per wash. Agarose-embedded DNA was digested with 80 U of *Not*I overnight at 37 °C; digestion was stopped by incubating plugs for 10 min at 65 °C. Melted plugs were mixed with 50 μl dye mixture (0·25% bromthymol blue, 0·02% xylene cyanol FF in distilled water) and added to 1% agarose gels. Electrophoresis was for 17 h at 200 V with a 2–20 s linear ramp time, followed by a further 9 h at 200 V with a 20–30 s linear ramp time in a Bio-Rad CHEF-DR™ II electrophoresis system. Gels were cooled to 14 °C throughout the run.

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After staining with ethidium bromide, banding patterns were digitized and analysed as before.

RESULTS

Thirty-eight patient and environmental isolates obtained during the course of the 1994–5 Ukrainian cholera epidemic were selected for genetic analysis. In addition, a single patient isolate from the 1991 cholera outbreak in the Crimea and two control strains from the L.C.D.C. culture collection that originated from cases in Pakistan and Peru were included for comparison (Table 1). These isolates included 37 *V. cholerae* O1 El Tor isolates, 3 *V. parahaemolyticus* isolates, and a single *V. alginolyticus* isolate (Table 1). All patient isolates, including the *V. parahaemolyticus* and *V. alginolyticus* strains, were associated with severe cholera symptoms. The only isolates that were negative both for the *ctx* gene by using PCR and for cholera toxin production in the VET-RPLA assay were the three *V. parahaemolyticus*, the single *V. alginolyticus*, and an environmental *V. cholerae* isolate (95-0752) obtained from a river near Odessa in 1995 (Table 1). The absence of the *ctx* gene in isolated 95-0752 differentiated this strain from other environmental *V. cholerae* obtained during the epidemic and suggested it may be unrelated to these epidemic isolates.

All toxigenic *V. cholerae* O1 El Tor isolates had identical *Bgl*I ribotype patterns (Fig. 1*a*, *b*, lanes 5–11 and 13–18). The non-toxigenic *V. cholerae* O1 El Tor river isolate 95-0752 was quite different from the toxigenic *V. cholerae* (Fig. 1*a*, *b*, lane 12) while the *V. alginolyticus* and *V. parahaemolyticus* isolates were more similar to each other than to any of the *V. cholerae* isolates (Fig. 1*a*, *b*, lanes 1–4). Both the Pakistani isolate (93-0608) and the Peruvian strain (94-0457) were identical to Ukrainian toxigenic disease and environmental isolates.

PFGE patterns of *Vibrio* isolates from the 1994–5 and 1991 Ukrainian epidemics revealed that most *V. cholerae* were very closely related (Fig. 2*a*, *b*). The largest identical group of isolates, designated 1a in the dendrogram (Fig. 2*a*, *b*, lanes 3, 7, 8, 10, 13), contained 27/34 patient and environmental Ukrainian toxigenic *V. cholerae* strains as well as an isolate obtained from a patient from Pakistan (93-0608). Group 1b (Fig. 2*a*, *b*, lanes 5, 15) was closely related to group 1a and again contained both patient and environmental isolates as well as the *V. cholerae* isolated from a fish near Odessa immediately before the Ukrainian epidemic (95-0745; Fig. 2*a*). Members of group 1b had a one band difference in their PFGE patterns compared with members of group 1a. Patient isolates 95-0755 and 95-0765 (Fig. 2*a*, *b*, lane 9) produced PFGE patterns slightly different from both groups 1a and 1b, exhibiting one and two band differences, respectively. This level of variation could have arisen during the course of the epidemic. Only strains 94-0457 (Fig. 2*b*, lane 12), which was isolated from a patient form Peru, and 95-0752, which was a non-toxigenic strain isolated from river water near Mykolajiv, had patterns somewhat different than the main group, differing by four and five bands, respectively, from group 1a (see Fig. 2*a*). These isolates were most likely unrelated to the epidemic strains. As expected, the PFGE patterns for the *V. alginolyticus* (Fig. 2*a*, *b*, lane 2) and three *V. parahaemolyticus* (Fig. 2*a*, *b*, lanes 4, 14) isolates were very different from each other and from the *V. cholerae* isolates.

Isolate 95-0743, from a patient in Odessa in 1991, exhibited PFGE and ribotype patterns identical to the majority of toxigenic *V. cholerae* from the 1994–5 epidemic (Figs. 1, 2; Tables 2, 3), suggesting that strains surviving from the 1991 epidemic may have caused the 1994–5 epidemic. Alternatively, the 1994–5 epidemic may have been caused by the re-introduction of an Asian endemic clone, represented by isolate 93-0608, from a source outside Ukraine.

ERIC-PCR allowed further differentiation of isolates. Only bands larger than 600 bp were seen consistently in repeated analyses, so only the areas of the PCR patterns containing fragments of 600 bp and larger were used for further analysis. These patterns were reproducible for all isolates both times the PCR was done.

Fourteen ERIC-PCR patterns were seen among the toxigenic Ukrainian isolates. Isolates within group 1 differed from the most common pattern (1a) by only one or two bands, while isolates from other groups were progressively more different with increasing genetic distance on the dendrogram (Fig. 3*a*, *b*). Isolates from group 1a contained patient and environmental isolates from different geographical locations and different years, and also contained the Pakistani isolate (93-0608). The Peruvian strain (94-0457) appeared to be relatively closely related to toxigenic Ukrainian isolates, while the Ukrainian patient isolates 95-0750 and 95-0770 clustered more distantly from the main group of strains (Fig. 3*a*).

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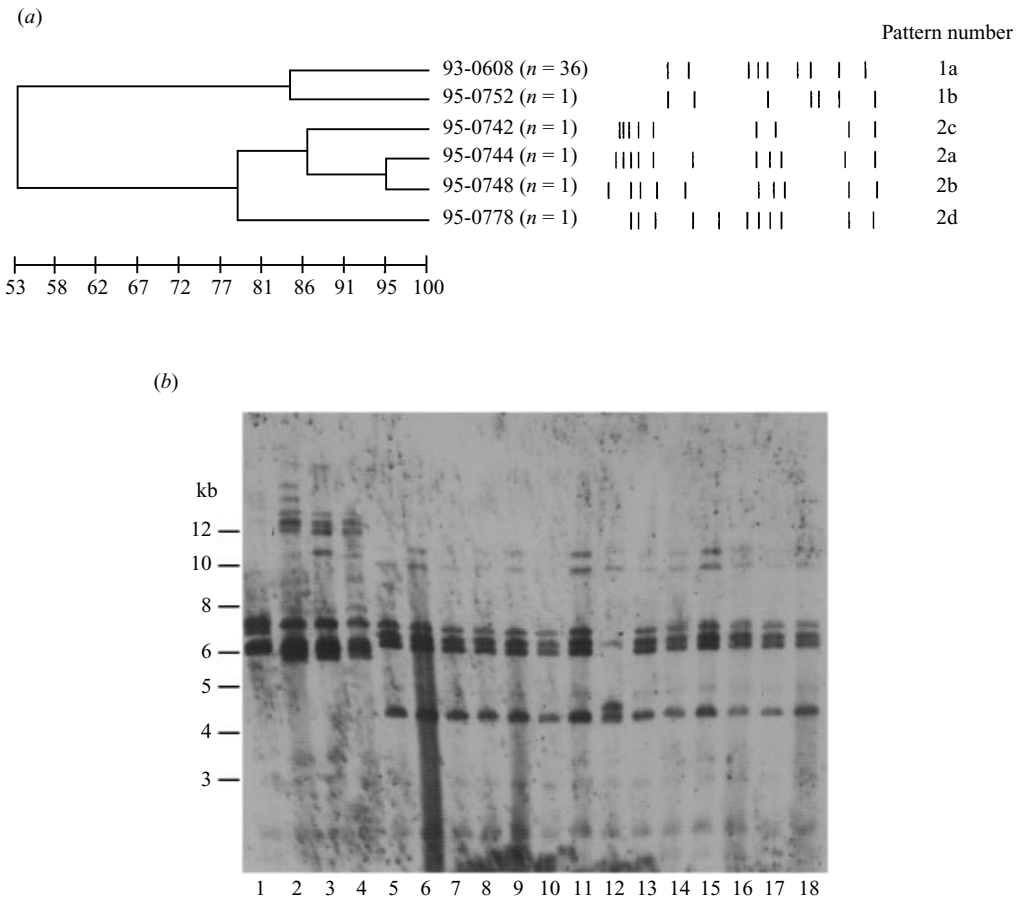

Fig. 1. Ribotyping of *Vibrio* isolates. The dendrogram (*a*) was constructed using digitized information from agarose gels as outlined in Materials and Methods. Strain numbers of representative isolates are given for each banding pattern, and numbers in parentheses indicate the number of isolates found for each pattern. The scale at bottom represents an arbitrary measure of pattern differences. A representative agarose gel is shown (*b*). The samples included were as follows: lane 1, 95-0742 (*V. alginolyticus*); lane 2, 95-0744 (*V. parahaemolyticus*); lane 3, 95-0748 (*V. parahaemolyticus*); lane 4, 95-0778 (*V. parahaemolyticus*); lane 5, 95-0743; lane 6, 95-0745; lane 7, 95-0746; lane 8, 95-0747; lane 9, 95-0749; lane 10, 95-0750; lane 11, 95-0751; lane 12, 95-0752 (*ctx*-ve *V. cholerae*); lane 13, 95-0753; lane 14, 95-0754; lane 15, 95-0755; lane 16, 95-0756; lane 17, 95-0757; lane 18, 95-0758. Organisms not designated otherwise were toxigenic *V. cholerae*. Sizes of the DNA size standard are indicated as the left of the gel.

This contrasted strongly with results from the other three methods of analysis, in which 95-0750 and 95-0770 were among the largest group of identical isolates (Table 2), and suggests that ERIC-PCR does not reveal taxonomic or evolutionary relationships between strains. All four non-*V. cholerae* isolates were distinct from *V. cholerae*, and only two of the three *Vibrio parahaemolyticus* isolates were distantly related to each other (Fig. 3*a*). Finally, the non-toxigenic river water isolate 95-0752 was only very distantly related to other *V. cholerae* when this analysis was used, in contrast to results with PFGE and ribotyping (Fig. 3*a*, *b*, lane 14).

Interpretation of REP-PCR results was complicated by the presence of a number of low intensity bands that appeared to stain inconsistently. For construction of dendrograms, we therefore used only the brightest bands larger than 500 bp, and were able to obtain reproducible patterns (Fig. 4*a*, *b*). All toxigenic *V. cholerae* from Ukraine clustered separately from the non-toxigenic river water isolate (95-0752) and from the non-*V. cholerae* isolates (Fig. 4*a*). In contrast to PFGE results, the Peruvian isolate (94-0457) was contained within the largest cluster of identical isolates. Isolate 95-0773, obtained from sea water near Mikolajiv, appeared quite different from other toxigenic *V. cholerae* in this analysis (Fig. 4*a*), though it was part of the largest cluster for both PFGE and ribotyping and was closely related to other toxigenic isolates in ERIC-PCR (Fig. 3*a*). All other toxigenic Ukrainian *V. cholerae* could be considered to be quite similar when analysed by REP-PCR (Fig.

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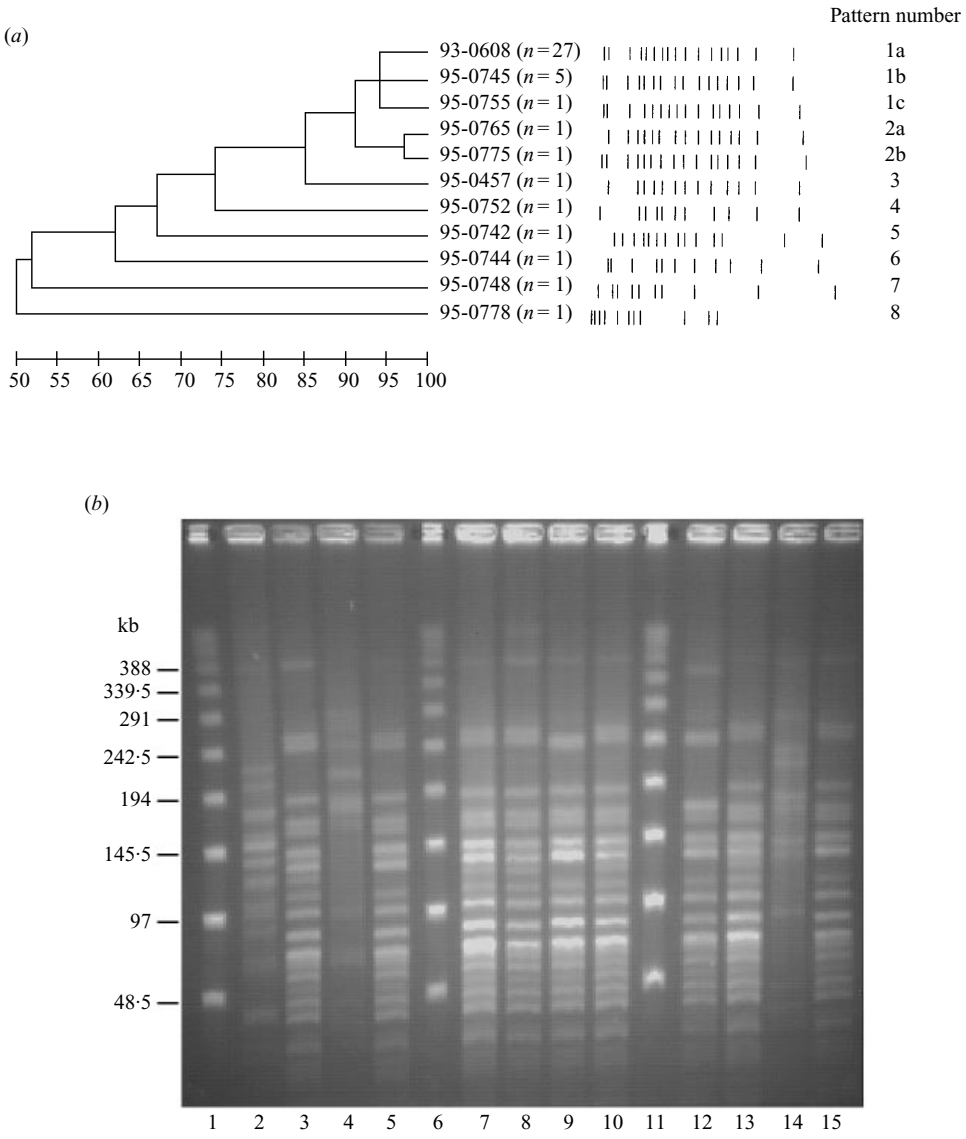

Fig. 2. PFGE of *Vibrio* isolates. The dendrogram (*a*) was constructed using digitized information from agarose gels as outlined in Materials and Methods. Strain numbers of representative isolates are given for each banding pattern, and numbers in parentheses indicate the number of isolates found for each pattern. The scale at bottom represents an arbitrary measure of pattern differences. A representative agarose gel is shown (*b*). The samples included were as follows, with isolates other than toxigenic *V. cholerae* designated and PFGE pattern numbers in parentheses: lane 1, size standard; lane 2, 95-0742 (*V. alginolyticus*; pattern 5); lane 3, 95-0751 (pattern 1a); lane 4, 95-0778 (*V. parahaemolyticus*; pattern 8); lane 5, 95-0761 (pattern 1b); lane 6, size standard; lane 7, 95-0767 (pattern 1a); lane 8, 95-0760 (pattern 1a); lane 9, 95-0765 (pattern 2a); lane 10, 95-0743 (pattern 1a); lane 11, size standard; lane 12, 94-0457 (pattern 3); lane 13, 95-0746 (pattern 1a); lane 14, 95-0748 (*V. parahaemolyticus*; pattern 7); lane 15, 95-0772 (pattern 1b). Sizes of the DNA size standard are indicated at the left of the gel.

4*a*), though the relationships between groups of strains are different from those seen with other methods of analysis. The 1991 patient strain (95-0743) was again more closely related to the fish strain (95-0745) than to either the Pakistani or Peruvian strains.

Two isolates obtained on 6 October 1994 from twin adults who died from cholera (95-0756 and 95-0757) were differentiated by both ERIC- and REP-PCR (see Table 2; Fig. 3*b*, lanes 19, 20; Fig. 4*b*, lanes 19, 20). This may suggest that both techniques are capable of measuring rapid changes in bacterial populations, that the two siblings were infected independently with closely related strains, or that the techniques are subject to artifacts. The fact that two independent

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Table 2. *Typing of* Vibrio *isolates from Ukraine using molecular methods*

Isolate number	Ribotype	PFGE type	ERIC type	REP type	*ctx*-RFLP band size (kb)*
V. cholerae					
1994, patient isolates from Mykolajiv, fatal					
95-0754	1a	1a	2a	3b	16·6
95-0755	1a	1c	1a	1b	16·6
95-0756†	1a	1a	2d	1b	16·6
95-0757†	1a	1a	2a	1a	16·6
95-0758	1a	1a	1b	2a	16·6
95-0759	1a	1a	1a	1a	16·6
95-0760	1a	1a	1a	1a	16·6
95-0761	1a	1b	1a	1a	17·3
95-0762	1a	1a	1c	2a	16·6
95-0763	1a	1a	2a	2a	16·6
1994, patient isolates from Mykolajiv, recovered					
95-0751	1a	1a	1a	1b	16·6
95-0764	1a	1a	1a	1a	16·6
95-0765	1a	2a	2c	1a	17·3
95-0766	1a	1b	2e	1a	17·3
95-0767	1a	1a	2b	1a	16·6
95-0768	1a	1a	2b	1a	16·6
95-0769	1a	1a	2b	1a	16·6
95-0770	1a	1a	5b	1b	16·6
1995, patient isolates from Mykolajiv, recovered					
95-0746	1a	1a	1a	2b	16·6
95-0749	1a	1b	2a	2a	17·3
95-0750	1a	1a	5a	1b	16·6
1994, patient isolate from Kirovograd, recovered					
95-0781	1a	1a	3b	1a	16·6
1991, patient isolate from Odessa, recovered					
95-0743	1a	1a	1d	2d	16·6
1995, patient isolates from Odessa, recovered					
95-0747	1a	1a	2a	2c	16·6
95-0753	1a	1a	1a	1b	16·6
1993, patient isolate from Pakistan, recovered					
93-0608	1a	1a	1a	3a	16·6
1993, patient isolate from Peru, recovered					
94-0457	1a	3	4	1a	17·3
1994, sewage isolates from Mykolajiv					
95-0771	1a	1a	1a	3a	16·6
95-0772	1a	1b	1a	1b	17·3
1994, river water isolates from Mykolajiv					
95-0775	1a	2b	1a	1b	17·3
95-0776	1a	1a	1a	1a	16·6
95-0777	1a	1a	1a	1a	16·6
1994, sea water isolates from Mykolajiv					
95-0773	1a	1a	2f	5	16·6
95-0779	1a	1a	1a	4	16·6
95-0780	1a	1a	3a	2a	16·6
1993, fish isolate from Mykolajiv					
95-0745	1a	1b	2a	2a	17·3
1995, river water isolate from Mykolajiv					
95-0752	1b	4	9	6	—
V. alginolyticus, patient isolate					
95-0742	2c	5	6	7	—

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Table 2. (*Cont.*)

Isolate number	Ribotype	PFGE type	ERIC type	REP type	*ctx*-RFLP band size (kb)*
V. parahemolyticus, patient isolates					
95-0744	2a	6	7a	9a	—
95-0748	2b	7	8	9b	—
95-0778	2d	8	7b	8	—

* All *V. cholerae* isolates except 95-0752 were *ctx* positive by PCR. *V. alginolyticus* and *V. parahemolyticus* isolates did not contain the *ctx* gene.
† Strains were from 36-year-old twins, and were isolated on the same day.

Table 3. *Summary of types of* V. cholerae *determined using molecular methods*

Pattern*	Strains
1a:1a:1a:1a:16·6	95-0759, 95-0760, 95-0764, 95-0776, 95-0777
1a:1a:1a:1b:16·6	95-0751, 95-0753
1a:1a:1a:3a:16·6	95-0608, 95-0771
1a:1a:1a:4:16·6	95-0779
1a:1a:1a:2b:16·6	95-0746
1a:1a:1b:2a:16·6	95-0758
1a:1a:1c:2a:16·6	95-0762
1a:1a:1d:2d:16·6	95-0743
1a:1a:2a:1a:16·6	95-0757†
1a:1a:2a:2a:16·6	95-0763
1a:1a:2a:2c:16·6	95-0747
1a:1a:2a:3b:16·6	95-0754
1a:1a:2b:1a:16·6	95-0767, 95-0768, 95-0769‡
1a:1a:2d:1b:16·6	95-0756†
1a:1a:2f:5:16·6	95-0773
1a:1a:3a:2a:16·6	95-0780
1a:1a:3b:1a:16·6	95-0781
1a:1a:5a:1b:16·6	95-0750
1a:1a:5b:1b:16·6	95-0770
1a:1b:1a:1a:17·3	95-0761
1a:1b:1a:1b:17·3	95-0772
1a:1b:2a:2a:17·3	95-0749, 95-0745
1a:1b:2e:1a:17·3	95-0766
1a:1c:1a:1b:16·6	95-0755
1a:2a:2c:1a:17·3	95-0765
1a:2b:1a:1b:17·3	95-0775
1a:3:4:1a:17·3	95-0457
1b:4:9:6:*ctx*-ve	95-0752

* Ribotype:PFGE:ERIC:REP:*ctx*-RFLP.
† Strains were from 36-year-old twins, and were isolated on the same day.
‡ Isolates were obtained on the same day in Mykolajiv.

techniques differentiated these strains suggests that they are indeed different and that the results obtained were not artifactual. Interestingly, three of five isolates (95-0767, 95-0768 and 95-0769) obtained from patients in Mykolajiv on 30 September and 1 October 1994 had identical ERIC- and REP-PCR patterns (Table 2; Fig. 4*a*), confirming that these methods were capable of grouping temporally and geographically related isolates.

Only two different fragment sizes were detected

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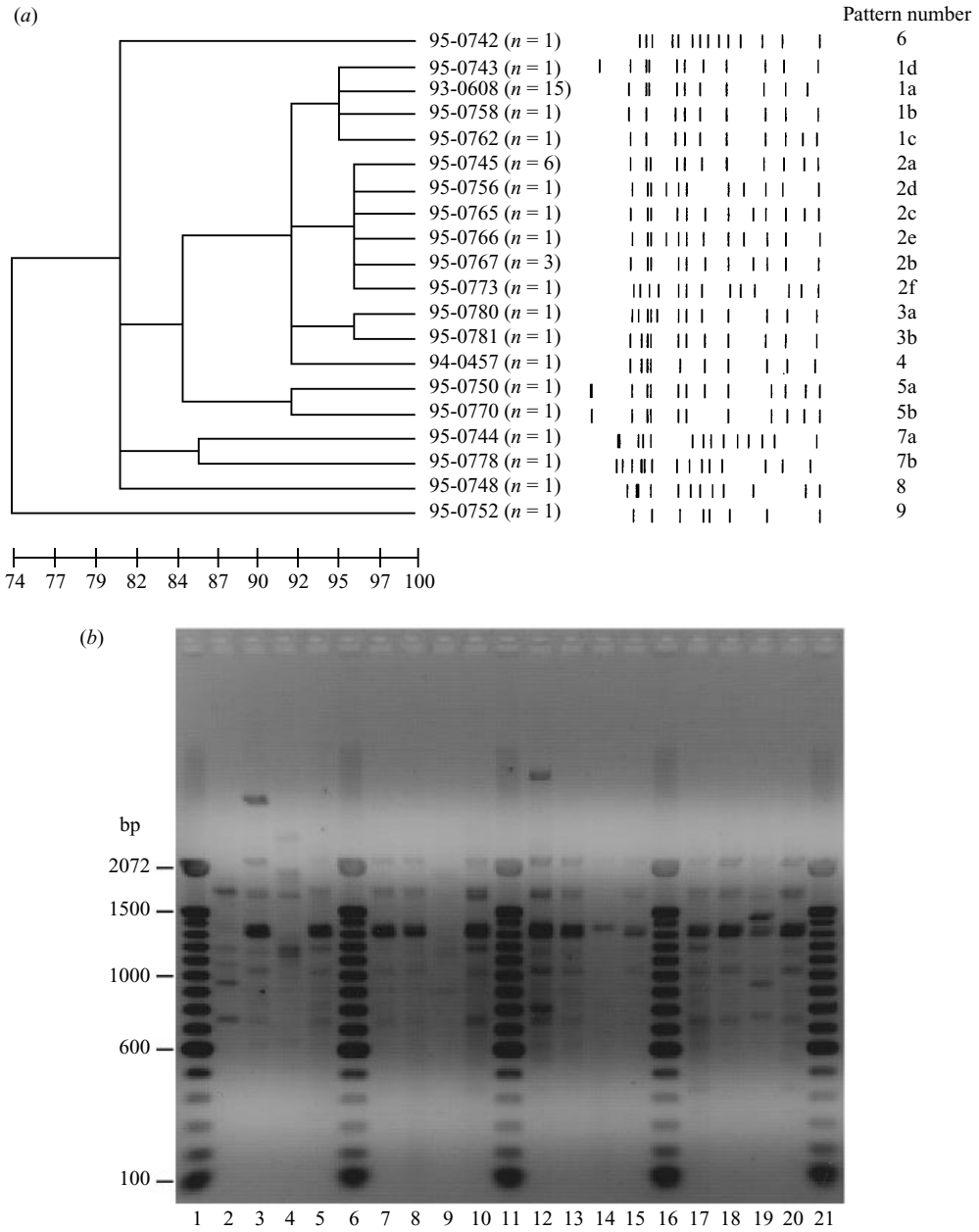

Fig. 3. ERIC-PCR of *Vibrio* isolates. The dendrogram (*a*) was constructed using digitized information from agarose gels as outlined in Materials and Methods. Strain numbers of representative isolates are given for each banding pattern, and numbers in parentheses indicate the number of isolates found for each pattern. The scale at bottom represents an arbitrary measure of pattern differences. A representative agarose gel is shown (*b*). The samples included were as follows, with isolates other than toxigenic *V. cholerae* designated and ERIC pattern numbers in parentheses: lane 1, size standard; lane 2, 95-0742 (*V. alginolyticus*; pattern 6); lane 3, 95-0743 (pattern 1d); lane 4, 95-0744 (*V. parahaemolyticus*; pattern 7a); lane 5, 95-0745 (pattern 2a); lane 6, size standard; lane 7, 95-0746 (pattern 1a); lane 8, 95-0747 (pattern 2a); lane 9, 95-0748 (*V. parahaemolyticus*; pattern 8); lane 10, 95-0749 (pattern 2a); lane 11, size standard; lane 12, 95-0750 (pattern 5a); lane 13, 95-0751 (pattern 1a); lane 14, 95-0752 (non-toxigenic environmental *V. cholerae*; pattern 9); lane 15, 95-0753 (pattern 1a); lane 16, size standard; lane 17, 95-0754 (pattern 2a); lane 18, 95-0755 (pattern 1a); lane 19, 95-0756 (pattern 2d); lane 20, 95-0757) (pattern 2a); lane 21, size standards. Sizes of the DNA size standard are indicated at the left of the gel.

when *Hin*dIII digested *V. cholerae* DNA was reacted with a probe specific for the *ctx* gene (Table 2). The size of DNA detected with probe appeared to correlate with the PFGE pattern of the isolate; the *ctx* gene was present in a 16·6 kb DNA fragment in isolates having PFGE patterns 1a, 1c and 4, while it

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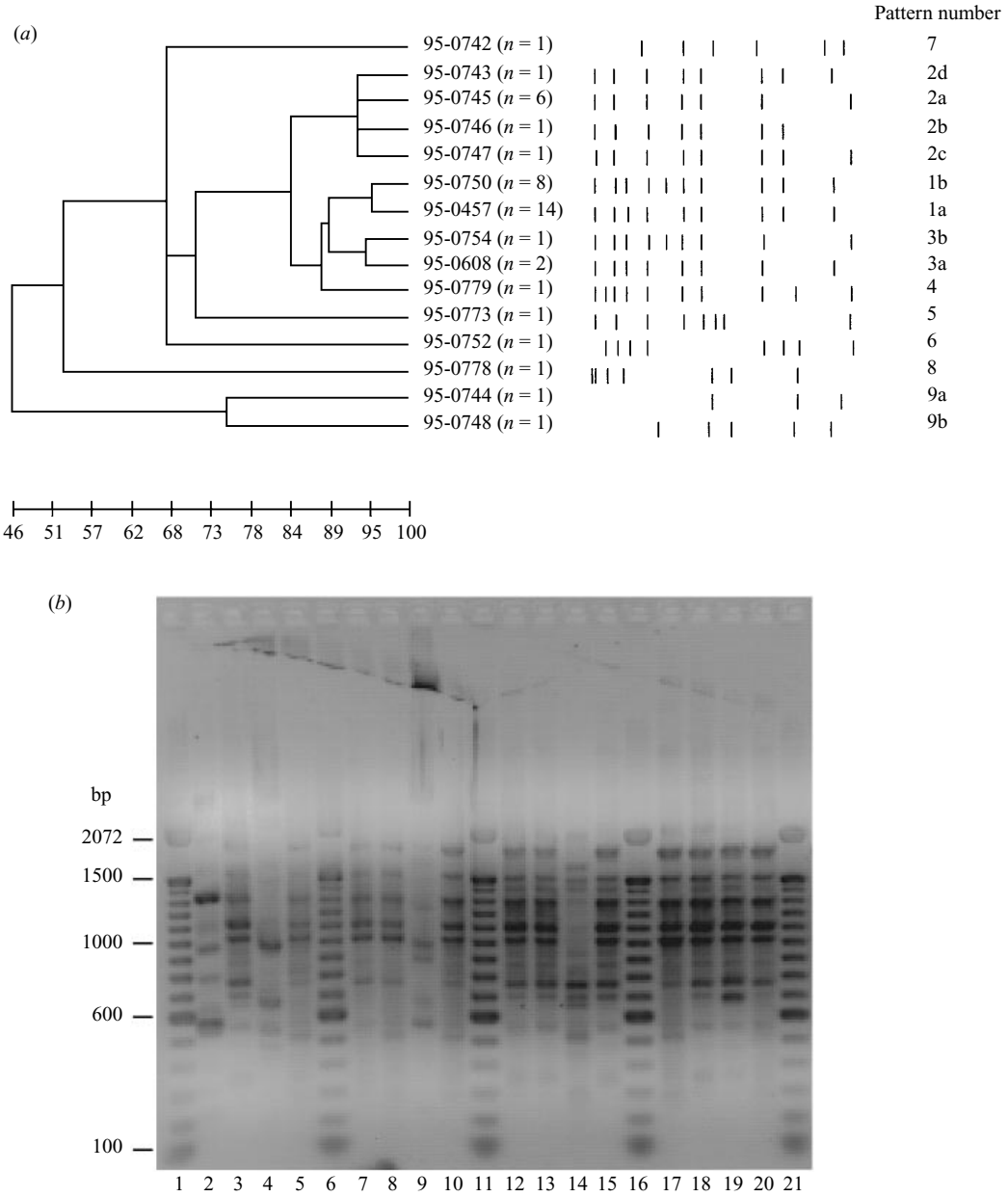

Fig. 4. REP-PCR of *Vibrio* isolates. The dendrogram shown (*a*) was constructed using digitized information from agarose gels as outlined in Materials and Methods. Strain numbers of representative isolates are given for each banding pattern, and numbers in parentheses indicate the number of isolates found for each pattern. The scale at bottom represents an arbitrary measure of pattern differences. A representative agarose gel is shown (*b*). The samples included were as follows, with isolates other than toxigenic *V. cholerae* designated and REP pattern numbers in parentheses: lane 1, size standard; lane 2, 95-0742 (*V. alginolyticus*; pattern 7); lane 3, 95-0743 (pattern 2d); lane 4, 95-0744 (*V. parahaemolyticus*; pattern 9a); lane 5, 95-0745 (pattern 2a); lane 6, size standard; lane 7, 95-0746 (pattern 2b); lane 8, 95-0747 (pattern 2c); lane 9, 95-0748 (*V. parahaemolyticus*; pattern 9b); lane 10, 95-0749 (pattern 2a); lane 11, size standard; lane 12, 95-0750 (pattern 1b); lane 13, 95-0751 (pattern 1b); lane 14, 95-0752 (non-toxigenic environmental *V. cholerae*; pattern 6); lane 15, 95-0753 (pattern 1b); lane 16, size standard; lane 17, 95-0754 (pattern 3b); lane 18, 95-0755 (pattern 1b); lane 19, 95-0756 (pattern 1b); lane 20, 95-0757 (pattern 1a); lane 21, size standards. Sizes of the DNA size standard are indicated at the left of the gel.

was contained within a 17·3 kb fragment in isolates with PFGE patterns 1b, 2a, 2b and 3 (Table 3). The basis for this difference is unknown.

Combining data from all methods resulted in 26 different groups for the 37 *V. cholerae* isolates and four different groups for the *V. alginolyticus* and *V.*

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parahaemolyticus isolates (Tables 2, 3). This is a much higher level of discrimination than could be obtained using any method of analysis alone or a combination of PFGE and ribotyping, which resulted in 7 different groups among the 37 *V. cholerae* isolates.

DISCUSSION

Comparison of ribotypes was the least discriminatory method of typing used in this study. Ribotyping did not differentiate among toxigenic *V. cholerae* isolates, consistent with a previous report that ribotyping does not distinguish Latin American *V. cholerae* strains from other strains associated with the seventh pandemic [19]. It did, however, differentiate toxigenic disease isolates from a non-toxigenic environmental strain (95-0752), complementing differences in antimicrobial resistance seen previously [30]. Though we agree with Desmarchelier and colleagues [8] that it is difficult to compare published photographic and schematic representations of ribotypes, it is clear that the ribotype patterns obtained for the toxigenic isolates were very similar to pattern R11 from *V. cholerae* El Tor Ogawa isolated in Australia [8] and to patterns 6a–c from all over the world in the standardized ribotype scheme of Popovic and colleagues [18]. A number of human isolates from sources world-wide have ribotypes different from those seen in this study [18], suggesting that though the Ukrainian isolates may not be strictly 'clonal', they are at least a relatively homogenous subgroup of the seventh pandemic strain.

PFGE analysis yielded a very homogenous group of strains among the toxigenic Ukrainian isolates. Because the PFGE run conditions were modified from previously published protocols, it is difficult to directly compare results presented here with previously published data. However, it is clear that the patterns obtained in this study are different from any of those obtained by Cameron and colleagues [19] or Dumontier and colleagues [9] for seventh pandemic *V. cholerae* O1 El Tor strains, with most major differences in bands larger than 145 kb. PFGE patterns for all toxigenic *V. cholerae* isolates included in this study were very similar, with a high proportion of identical strains. Dumontier and colleagues [9] also observed a strong similarity in both *Not*I and *Sfi*I PFGE patterns among seventh pandemic strains of *V. cholerae* isolated from Africa and South America, and India, implying that the genome organization of pandemic *V. cholerae* O1 is relatively stable. Similarly, Evins and colleagues [14] found that PFGE, multilocus enzyme electrophoresis (MEE), and ribotype data identified only two distinct strains of toxigenic *V. cholerae* biotype El Tor from 18 different countries in South America, while at the same time non-toxigenic strains showed substantial heterogeneity. It is therefore not possible to interpret the PFGE data alone as evidence for the clonal spread of cholera from a single source into the Ukrainian population. In contrast to the results discussed above and to the postulated 'clonal' nature of seventh pandemic strains in general [15], Cameron and colleagues [19] were able to distinguish 63 *Not*I PFGE patterns among 180 strains of *V. cholerae* from around the world. The Ukrainian strains therefore represent a limited subset of the total diversity of PFGE types available.

PFGE was done with *Not*I, the enzyme most commonly reported in the literature for *V. cholerae* typing. Use of additional enzymes might improve the resolution of this method and allow differentiation of strains that appear identical. Dalsgaard and colleagues [20] found that, though *Not*I and *Sfi*I gave a suitable distribution of bands for analysis of *V. cholerae* O139 strains, use of either enzyme did not discriminate well between strains. The use of *Cpo*I provided good discrimination in this case.

The results from ribotyping and PFGE together differentiate the toxigenic Ukrainian *V. cholerae* into only six groups (Table 2). *V. alginolyticus* and *V. parahaemolyticus* strains were only distantly related to *V. cholerae* and to each other, confirming the biological relevance of PFGE and ribotype analysis. It is clear that the non-toxigenic river water isolate (95-0752), is less closely related to patient isolates than toxigenic isolates from sewage, river water, sea water, and a fish. This non-toxigenic isolate likely represents endemic environmental *V. cholerae*, while the toxigenic environmental strains were likely introduced into the environment from infected individuals. The Pakistan isolate 93-0608 was identical to a subset of toxigenic isolates from the Ukrainian epidemic, while the isolate from Peru (94-0457) was somewhat different. The PFGE : ribotype pattern of *V. cholerae* isolated from a fish in 1993 (95-0745) was identical to that of a 1994 sewage isolate and three isolates from patients in Mykolajiv. Interestingly, a patient isolate from Odessa in 1991 (95-0743) had the same ribotype and PFGE pattern as the largest clusters of epidemic strains, suggesting that the epidemic strain may have been present in Ukraine prior to the 1991 epidemic

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and survived to cause the 1994–5 epidemic. Since the Ukrainian epidemic strains are very similar to Asian *V. cholerae* El Tor strains and different from an isolate associated with the South American epidemic, Asia was likely the original source of *V. cholerae*. Historical data from previous Ukraine epidemics (C. G. Clark and colleagues, unpublished) supports this hypothesis.

ERIC-PCR appears to be a very sensitive method for differentiating isolates. Twins from whom *V. cholerae* was isolated on the same day had strains with different ERIC patterns (95-0756 and 95-0757), even though the PFGE and ribotype patterns were identical. REP-PCR also differentiated these two isolates, and they had slightly different antibiotic resistance profiles, with 95-0757 having an additional intermediate resistance to cefoxitin [30]. The fact that two *V. cholerae* strains isolated from twins on the same day had different ERIC patterns suggests either that the stability of the distribution of ERIC sequences in the *V. cholerae* genome is not as great as previously thought [29, 33] or that the technique is more susceptible to artifacts than previously thought [29, 36]. Artifacts may arise in ERIC-PCR from non-specific recombination of primers with the DNA template [36]. However, the data presented here strongly suggest that the differences between isolates 95-0756 and 95-0757 are real and that there was a heterogenous mix of *V. cholerae* strains in the environment available for infecting humans and causing disease. The finding that three strains (95-0767, 95-0768 and 95-0769) isolated within 2 days in the same geographical region had identical PFGE, ribotype, ERIC and REP patterns further suggests these characteristics are stable and supports the opinion that the differences between the two isolates from twins were not due to artifacts.

ERIC sequences are located in transcribed regions of the chromosome and have been associated with the haemolysin (*hly*) gene in *V. cholerae* [37]. The chromosomal location of ERIC sequences differs between species, and there is evidence for precise insertion and deletion of these sequences from specific chromosomal locations; partial deletions may also occur [37]. The data presented here suggest that these events may take place during the course of an epidemic, leading to different patterns of PCR products after ERIC-PCR. Alternately, the epidemic may have been caused by a group of pre-existing, closely related *V. cholerae* strains. This latter explanation seems less likely, since the results from PFGE and ribotyping summarized here, as well as epidemiological data [30] seem to indicate the rapid spread of a pathogenic clone of *V. cholerae* into the Ukrainian population.

The resolving power of the ERIC-PCR protocol used here is much finer than that of the protocol used by Rivera and colleagues [29]. Whereas we obtained nine or more bands, Rivera and colleagues obtained between one and eight bands with a diverse collection of *V. cholerae* O1 and non-O1. The protocol used in this study is therefore well-suited to differentiation of closely related strains, while that used by Rivera and colleagues may be better suited to differentiating *V. cholerae* O1 from non-O1 strains and determining the pathogenic potential of these strains.

Results from REP-PCR were more difficult to interpret than those from the other methods used because of higher background and more faint or inconsistent bands. Smearing has been noted before in REP-PCR and can be partly corrected by using inosine-containing primers [33], though use of such primers does not appear to completely solve the problems found in this study. We therefore chose to analyse only regions of the gel showing good reproducibility (see Results). Our results confirmed those of Versalovic and colleagues [33] in that REP-PCR allowed clear distinctions between different bacterial species and strains, while the presence of common bands between strains of a given species allowed grouping of strains.

ERIC- and REP-PCR have excellent reliability and reproducibility when compared to PCR techniques that use arbitrary primers [38]. Comparison of PFGE and ribotype data with that obtained by ERIC- and REP-PCR suggests, however, that the forces driving change are different for the two types of analyses. PFGE and ribotyping relationships appear to agree well with other biologically relevant characteristics such as species, geographical location, and date of isolation. For example, the Peruvian *V. cholerae* isolate 94-0457 is quite different from Ukrainian or Pakistani strains by PFGE and ribotyping, as well as by antibiotic resistance profile [30], as would be expected from published information suggesting that South American *V. cholerae* can be differentiated from other seventh pandemic strains [11]. This strain is less well differentiated from Ukrainian strains by ERIC- and REP-PCR, however, suggesting that (1) changes in ERIC and REP patterns occur independently of changes in ribotype and PFGE pattern, and (2) changes in ERIC and REP patterns occur at a greater rate than changes in PFGE or ribotype

patterns. While this property makes ERIC- and REP-PCR valuable tools for analysing outbreaks and epidemics, it also makes it difficult to ascribe biological relevance to the results. The significance of phylogenetic relationships between strains summarized in dendrograms constructed from ERIC- and REP-PCR data should therefore not be over-emphasized.

Though *ctx*-RFLP analysis has been used extensively to differentiate *V. cholerae*, it does not differentiate strains as well as any other of the other methods used here. Only two sizes of RFLP fragments containing the *ctx* gene were found using *Hin*dIII-digested chromosomal DNA. These appeared to be similar to those found by Yam and colleagues [24] for *V. cholerae* El Tor Ogawa isolates from Hong Kong, but were different from any found by Wachsmuth and colleagues [11] or Desmarchelier and colleagues [8] for a variety of strains. This further supports an Asian origin for the Ukraine epidemic.

Fully pathogenic *ctx*-positive *V. cholerae* can be derived from environmental sources through the acquisition of the virulence cassette [5] present on a bacteriophage [6]. In the case of the Ukrainian epidemic, however, the close genetic relationship of the Ukrainian strains with a 1993 patient isolate from Pakistan argues strongly for the introduction of the Ukrainian epidemic strain from Asia, possibly at some time prior to the 1991 epidemic in southern Ukraine. A review of the history of cholera in Ukraine during the second to sixth pandemics [30] suggests that importation of cholera from Asia is a conserved feature of epidemic spread of cholera in Ukraine. Further work with patient and environmental strains isolated before 1991 could provide clues about the factors influencing the disease potential of the organisms responsible.

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