Genetic relationships between clinical and environmental *Vibrio cholerae* isolates based on multilocus enzyme electrophoresis

M. Farfán, D. Miñana, M. C. Fusté and J. G. Lorén

Author for correspondence: J. G. Lorén. Tel: +34 93 402 44 97. Fax: +34 93 402 18 96.
e-mail: loren@farmacia.far.ub.es

Departament de
Microbiologia i Parasitologia
Sanitàries, Divisió de
Ciències de la Salut, Facultat
de Farmàcia, Universitat de
Barcelona, Avda Joan XXIII
s/n, 08028 Barcelona, Spain

A total of 107 isolates of *Vibrio cholerae*, including 29 strains belonging to serogroup O139, were studied using multilocus enzyme electrophoresis (MLEE) to determine allelic variation in 15 housekeeping enzyme loci. All loci were polymorphic and 99 electrophoretic types (ETs) were identified from the total sample. No significant clustering of isolates was detected in the dendrogram generated from a matrix of coefficients of distances with respect to serogroup, biotype or country of isolation. The mean genetic diversity of this *V. cholerae* population ($H=0.50$) was higher than reported previously. Linkage disequilibrium analysis of the MLEE data showed a clonal structure for the entire population, but not in some of the population subgroups studied. This suggests an epidemic population structure. The results showed that the O139 strains were not clustered in a unique ET, in contrast to previous MLEE studies. This higher genetic variation of the O139 serogroup is concordant with ribotyping studies. The results also confirm that the O139 and O1 ElTor isolates are genetically more closely related to each other than to all the other subpopulations of *V. cholerae* studied.

Keywords: MLEE, linkage disequilibrium, cholera, population genetics, electrophoretic types

INTRODUCTION

Vibrio cholerae is an autochthonous inhabitant of marine and freshwater environments, which is often associated with phyto- and zooplankton (Baumann *et al.*, 1984; Colwell & Huq, 1994). This bacterium is classified, by the composition of its major surface antigen (O), into serogroups, of which there are nearly 200 (Beltrán *et al.*, 1999). Important distinctions within the species are made on the basis of serogroup, production of cholera toxin, which is responsible for severe diarrhoea, and potential for epidemic spread. Only two serogroups of *V. cholerae*, O1 and O139, have been considered as causative agents of cholera, but some strains of these serogroups do not produce cholera toxin and are not involved in epidemics. All the others serogroups, non-O1/non-O139, are more frequently isolated from environmental sources and are associated with sporadic cases of gastroenteritis or extraintestinal

infections. Although occasional strains can produce cholera toxin or other virulence factors, none of them has caused large epidemics (Kaper *et al.*, 1995).

Historically, it seems most probable that cholera emerged after the Neolithic, which began in the Middle East some 10000 years ago, when the adoption of agricultural practices by nomadic groups enabled higher densities of humans to subsist (Byun *et al.*, 1999). There are references to deaths due to dehydrating diarrhoea dating back to Hippocrates and Sanskrit writings (Colwell, 1996). Cholera has been endemic on the Indian subcontinent for centuries. The literature describes the first pandemic spread of cholera outside Asia in 1817 (Blake, 1994). Since then seven pandemics have been recorded. The fifth and sixth were caused by the classical biotype of O1 strains, but the nature of the strains causing the first four pandemics is unknown. In contrast, in 1961 the seventh pandemic started in Indonesia and was due to the ElTor biotype. Recently, in 1992 an epidemic clone of a non-O1 strain with serogroup O139 Bengal caused a large cholera outbreak in Bangladesh and neighbouring countries (Albert *et al.*, 1993; Ramamurthy *et al.*, 1993). Several studies have shown

Abbreviations: AFLP, amplified fragment length polymorphism; ET, electrophoretic type; MLEE, multilocus enzyme electrophoresis; RAPD, random amplified polymorphic DNA.

that the O139 strain is phylogenetically and phenotypically very similar to the O1 ElTor strain, and most probably derived from an O1 seventh-pandemic clone strain by horizontal gene transfer (Bik *et al.*, 1995; Stroher *et al.*, 1997). At the beginning of the outbreak, the O139 strain totally displaced the *V. cholerae* O1 strains, including both classical and ElTor biotypes, which coexisted only in Bangladesh. The subsequent emergence of a new clone of *V. cholerae* O1 ElTor that transiently displaced the O139 strains during 1994 and 1995, and the reemergence in 1996 of *V. cholerae* O139 as the main cause of cholera in Calcutta and its coexistence with the O1 ElTor strains demonstrated temporal changes in the epidemiology of the cholera (Faruque *et al.*, 1997a, b; Mukhopadhyay *et al.*, 1998). The factors that determine the emergence, disappearance or continued presence of particular clones of toxigenic *V. cholerae* are not clear.

The continual emergence of new toxigenic strains of *V. cholerae* and their selective enrichment during cholera outbreaks constitute essential mechanisms for the survival and evolution of *V. cholerae* and the genetic elements that mediate the transfer of virulence genes (Faruque *et al.*, 1998). The molecular mechanisms of cholera pathogenesis are currently being elucidated, as exemplified by the description of the lysogenic filamentous bacteriophage (CTX ϕ), which encodes cholera toxin (Waldor & Mekalanos, 1996), and its receptor, the TCP (toxin-coregulated pilus), which is part of a larger genetic element, the TCP pathogenicity island (Karaolis *et al.*, 1998). These findings may help us to understand the possible origin of new toxigenic clones of *V. cholerae*, and raise the possibility that all strains of *V. cholerae* have the potential to become agents of epidemic cholera (Faruque *et al.*, 1998).

New analysis methods have permitted studies of the genetic variability of *V. cholerae* on a global scale (Faruque *et al.*, 1998). One of them is MLEE, which analyses the electrophoretic mobility differences in multiple enzymes to study divergence of bacterial strains of the same species. Previous studies were primarily concerned with the analysis of strains belonging to the serogroup O1, collected during outbreaks, which were responsible for cholera epidemics and pandemics (Salles & Momen, 1991; Wachsmuth *et al.*, 1993; Evins *et al.*, 1995). More recently, Beltrán *et al.* (1999) applied the MLEE technique to the study of a collection of *V. cholerae* isolates from Mexico and Guatemala; they also included reference strains of all serogroups (toxigenic and non-toxigenic). Moreover, various molecular techniques such as PFGE, RAPD and ribotyping have also been applied in attempts to establish the genetic population structure of *V. cholerae*. Recent work in this field includes the comparative nucleotide sequence analysis of pathogenic isolates (Byun *et al.*, 1999) and the AFLP fingerprinting of clinical and environmental isolates (Jiang *et al.*, 2000). However, the relationship of the pathogenic clones with the environmental isolates remains unclear. In the present study, we analysed by MLEE a collection of *V. cholerae* strains isolated from

several countries, including toxigenic (O1 and O139) and non-toxigenic serogroups from environmental and clinical sources, to determine the population structure of *V. cholerae*.

METHODS

Bacterial strains. This study was carried out on a collection of 100 isolates of *Vibrio cholerae*, from several countries, including toxigenic (O1 and O139) and non-toxigenic serogroups, obtained from clinical and environmental sources (Table 1). Strains were kindly provided by G. B. Nair (National Institute of Cholera & Enteric Diseases, Calcutta, India), M. A. R. Chowdhury (Marine Laboratory, Department of Microbiology, University of Maryland, MD 20740, USA) and M. Talledo (Laboratorio Microbiología y Biotecnología Microbiana, Facultad de Ciencias Biológicas, Universidad Nacional Mayor de San Marcos, Lima, Peru). Six reference strains of *V. cholerae* from the Spanish Type Culture Collection (CECT 514, CECT 569, CECT 652, CECT 655, CECT 658 and CECT 659) and a reference strain of classical O1 *V. cholerae* (ATCC 14035) were also included in the study.

Preparation of lysates for electrophoresis. Each isolate was grown overnight at 35 °C in Trypticase Soy Broth (TSB), in a shaker. The cells were harvested by centrifugation (7000 g for 15 min at 6 °C), suspended in TE-NADP buffer (Tris/HCl 10 mM, EDTA 1 mM and NADP 0.5 mM, pH 6.8) and lysed by freezing and thawing. The cell extracts were obtained after three repeated freezing–thawing cycles (at –20 °C for 12–24 h and at 36.6 °C for 5 min) and centrifugation at 110000 g for 25 min at 6 °C. Aliquots of supernatant were transferred to Eppendorf tubes and stored at –40 °C until use. Protein was measured by the Lowry method, with bovine serum albumin (Sigma) as a standard.

Electrophoresis and specific enzyme staining. Nondenaturing vertical polyacrylamide gel electrophoresis was used for all the enzymes. The acrylamide concentration in the gels depended on the enzyme studied (10% continuous polyacrylamide gels and 10%/8% or 8%/5% discontinuous polyacrylamide gels). Tris/HCl 0.8 M (pH 8.8) buffer was used in continuous gels and Tris/HCl 0.125 M (pH 6.8) stacking buffer and Tris/HCl 0.4 M (pH 8.8) resolving buffer were used in discontinuous gels. Tris/glycine 0.19 M (pH 8.3) buffer was used for the electrode compartments. Gels were used within 24 h of preparation and run at 7 °C. A constant voltage, depending on the acrylamide concentration of the gel, was applied until the bromophenol blue band reached the bottom of the gel. All strains were run at least twice to confirm their genotype.

The staining of the gels to reveal specific enzyme activity was performed following Selander *et al.* (1986), except in the case of catechol 2,3-oxygenase (Gibson, 1971; Kataeva & Golovleva, 1990). The following 15 enzymes were assayed: glucose-6-phosphate dehydrogenase (G6P), isocitrate dehydrogenase (IDH), alanine dehydrogenase (ALD), NAD-dependent glyceraldehyde-phosphate dehydrogenase (GP1), malate dehydrogenase (MDH), fumarase (FUM), aspartate dehydrogenase (ASD), leucine aminopeptidase (LAP), malic enzyme (ME), esterase (EST), catechol 2,3-oxygenase (C23O), nucleoside phosphorylase (NSP), xanthine dehydrogenase (XDH), phosphoglucose isomerase (PGI) and 6-phosphogluconate dehydrogenase (6PG). For each enzyme, distinct mobility variants were designated as electromorphs and numbered in order of increasing migration towards the anode.

Table 1. Characteristics of the bacterial isolates used in this study and their allele profiles at each locus

ET	No. of strains	Reference isolates	Allele at enzyme locus															Serogroup/biotype	Source*	Country
			G6P	IDH	ALD	GP1	MDH	FUM	ASD	LAP	ME	EST	C23O	NSP	XDH	PGI	6PG			
1	1	TM35123-77	4	3	1	1	1	3	2	7	1	1	2	3	1	4	1	non-O1/non-O139	E	Brazil
2	2	TM34162-77	4	3	1	2	1	3	2	4	1	1	2	3	1	4	1	non-O1/non-O139	E	Brazil
		TM9024-79	4	3	1	2	1	3	2	4	1	1	2	3	1	4	1	non-O1/non-O139	E	Brazil
3	1	TM52479-78	4	3	1	2	1	2	1	5	2	2	1	3	1	4	6	non-O1/non-O139	E	Brazil
4	1	TM23256-79	3	3	1	1	1	2	1	5	2	2	1	3	1	2	1	non-O1/non-O139	E	Brazil
5	1	TM19225-79	3	1	1	5	1	4	2	5	2	2	1	3	4	2	3	non-O1/non-O139	E	Brazil
6	1	GGPB10	3	3	1	2	1	2	1	5	2	2	1	3	5	3	5	non-typed	U	Brazil
7	1	TM1705-82	3	3	1	2	1	5	2	5	2	2	2	3	1	3	1	non-O1/non-O139	E	Brazil
8	3	TM48733-82	4	3	1	3	2	3	2	7	1	1	2	3	1	4	1	non-O1/non-O139	E	Brazil
		TM11079-80	4	3	1	3	2	3	2	7	1	1	2	3	1	4	1	O1 ElTor	E	Brazil
		CO484	4	3	1	3	2	3	2	7	1	1	2	3	1	4	1	non-O1/non-O139	E	India
9	1	TM1187-83	3	3	1	2	1	4	2	5	3	2	1	3	4	3	1	non-O1/non-O139	E	Brazil
10	1	CT10738-92	3	3	1	2	1	6	2	5	3	2	1	3	4	3	3	non-O1/non-O139	E	Brazil
11	1	CT10834-92	3	3	1	2	1	4	0	5	2	2	1	3	4	2	5	non-O1/non-O139	E	Brazil
12	1	CT12009-92	3	4	1	2	3	4	3	2	5	2	2	3	1	3	1	non-O1/non-O139	E	Brazil
13	1	GGPB29	3	3	1	1	1	5	2	5	3	2	1	3	1	2	1	non-typed	U	Brazil
14	1	GM30-90	3	2	1	2	3	3	3	3	4	2	3	3	1	3	3	non-O1/non-O139	E	Brazil
15	1	GM31-90	4	3	1	1	1	3	2	7	3	1	2	3	1	4	1	non-O1/non-O139	E	Brazil
16	1	GM33-90	3	3	1	1	1	2	1	5	2	2	1	3	1	2	5	non-O1/non-O139	E	Brazil
17	1	GM34-90	3	3	1	2	1	2	1	5	2	2	1	3	1	2	1	non-O1/non-O139	E	Brazil
18	1	GM35-90	4	3	1	2	1	3	2	1	3	1	2	3	1	4	1	non-O1/non-O139	E	Brazil
19	1	GM36-90	4	1	1	2	1	3	2	7	3	1	2	3	4	4	1	non-O1/non-O139	E	Brazil
20	1	GM37-90	3	3	1	2	1	5	2	5	3	2	1	3	1	3	1	non-O1/non-O139	E	Brazil
21	1	GM38-90	2	3	1	2	1	4	2	1	3	2	3	3	1	4	1	non-O1/non-O139	E	Brazil
22	1	CT7606-93	6	3	1	3	2	3	2	7	1	1	1	3	4	4	1	O1 ElTor	E	Brazil
23	1	CT9995-93	4	3	1	1	1	3	2	7	3	1	1	3	4	4	1	O1 ElTor	E	Brazil
24	1	GGPB44	4	2	1	1	1	3	2	7	1	1	2	3	1	4	1	non-typed	U	Brazil
25	2	TM207832-78	4	3	1	2	2	3	2	4	1	1	2	3	1	4	1	O1 ElTor	E	Brazil
		ALVC-92-0344	4	3	1	2	2	3	2	4	1	1	2	3	1	4	1	O1 ElTor	C	Peru
26	1	CT25016-91	6	3	1	3	2	3	2	7	1	1	1	3	4	4	3	O1 ElTor	E	Brazil
27	1	CT7021-94	6	3	1	2	2	3	2	3	1	1	2	3	4	4	1	O1 ElTor	E	Brazil
28	1	TM16457-78	3	3	1	1	1	4	1	5	3	2	1	3	1	3	5	O1 classical	E	Brazil
29	1	CT7649-94	4	3	1	2	2	3	2	7	1	1	2	3	1	4	1	O1 ElTor	E	Brazil
30	1	CTMARM12-92	4	3	1	1	1	3	2	1	3	1	2	3	1	4	3	O1 ElTor	E	Brazil
31	1	CTMACM11-92	3	3	1	2	2	4	2	6	3	1	2	3	1	4	1	O1 ElTor	E	Brazil

Table 1 (cont.)

ET	No. of strains	Reference isolates	Allele at enzyme locus															Serogroup/biotype	Source*	Country
			G6P	IDH	ALD	GP1	MDH	FUM	ASD	LAP	ME	EST	C23O	NSP	XDH	PGI	6PG			
32	1	MX121	3	1	1	2	1	4	1	5	2	2	1	3	1	2	5	non-O1/non-O139	F	Mexico
33	1	MX129	3	3	1	1	1	2	1	5	2	2	2	1	1	3	1	non-O1/non-O139	F	Mexico
34	1	MX134	4	3	1	1	1	3	2	7	1	2	1	3	1	2	5	non-O1/non-O139	F	Mexico
35	1	MX157	4	3	1	1	2	3	2	2	1	1	2	3	1	4	1	non-O1/non-O139	F	Mexico
36	1	MX158	3	3	1	2	1	5	1	5	3	2	1	3	1	3	1	non-O1/non-O139	F	Mexico
37	1	MX159	4	3	1	3	2	3	2	2	1	1	2	3	1	3	1	non-O1/non-O139	F	Mexico
38	1	AT01	4	2	1	2	1	3	2	4	1	1	2	3	1	4	1	non-O1/non-O139	C	India
39	1	CO391	4	3	1	2	1	5	2	5	3	2	1	3	1	2	5	O139	F	India
40	1	CO407	3	3	1	2	1	4	2	5	3	2	2	3	1	3	1	O139	F	India
41	1	CO487	4	1	1	2	1	3	2	4	3	1	2	3	4	4	1	O1 ElTor	C	India
42	1	CO870	3	4	1	1	1	2	1	3	2	2	1	3	4	3	5	O1 ElTor	E	India
43	1	SG24	3	1	1	2	1	3	2	1	1	2	1	3	1	4	3	O139	C	India
44	1	CECT514	6	3	1	2	2	7	2	2	3	1	1	3	6	4	2	O1 classical	F	UK
45	1	CECT569	6	1	1	2	2	7	2	1	1	1	1	3	4	4	1	O1 classical	C	India
46	1	CECT652	6	3	1	2	2	7	2	2	3	1	2	3	1	4	1	O1	C	Unknown
47	1	CECT655	4	3	1	2	2	7	2	2	3	1	2	3	5	4	1	non-O1/non-O139	E	Bangladesh
48	1	CECT658	6	3	1	3	2	3	2	2	1	1	2	3	4	4	1	non-O1/non-O139	E	Bangladesh
49	1	CECT659	6	3	2	3	2	3	2	2	1	1	1	3	1	4	4	non-O1/non-O139	E	Bangladesh
50	1	1-7/31	4	3	1	2	2	3	2	2	1	2	1	3	1	4	1	O1 ElTor	E	USA
51	1	6-3/6	3	3	1	1	1	6	2	3	1	2	2	3	1	4	1	O1 ElTor	E	USA
52	1	NT330	4	3	1	2	2	3	2	2	1	1	2	3	5	5	1	O139	C	India
53	2	CO402	6	3	1	3	2	3	2	2	1	1	1	3	4	4	1	O139	F	India
		SO30	6	3	1	3	2	3	2	2	1	1	1	3	4	4	1	O139	C	India
54	1	CO414	6	3	2	3	2	3	2	2	1	1	1	3	1	4	3	O139	F	Unknown
55	1	NT642	6	3	1	3	2	4	2	2	3	1	2	3	1	4	1	O139	C	India
56	1	BO1	6	3	1	3	2	3	2	2	1	1	1	3	1	4	1	O139	F	India
57	1	ATCC14035	6	3	1	2	2	3	2	2	1	2	1	3	0	4	1	O1 classical	C	UK
58	1	2030 H	1	3	1	2	1	3	2	3	3	2	1	3	4	1	5	non-O1/non-O139	E	USA
59	1	NT329	6	3	1	3	2	3	2	2	1	1	1	3	1	4	4	O139	C	India
60	2	25872	6	3	1	2	2	3	2	2	1	1	1	3	5	4	1	non-O1/non-O139	E	Unknown
		CO406	6	3	1	2	2	3	2	2	1	1	1	3	5	4	1	O139	F	India
61	1	BLO9	6	3	1	2	2	3	2	3	1	1	1	3	1	4	3	O1 ElTor	C	Bangladesh
62	1	MDO90	4	3	1	1	2	3	2	3	1	1	1	3	1	4	1	O139	C	India
63	1	SO29	6	3	1	4	2	3	2	2	1	1	1	3	4	4	1	O139	C	India
64	1	VO6	4	3	1	3	2	3	2	2	1	1	1	3	0	4	1	O139	C	India
65	1	BO2	4	3	1	1	2	6	2	3	1	1	1	3	4	4	1	O139	F	India
66	1	CO404 NT656	6	1	1	3	2	3	2	4	1	1	0	1	1	5	2	O139	F	India

Table 1 (cont.)

ET	No. of strains	Reference isolates	Allele at enzyme locus															Serogroup/biotype	Source*	Country
			G6P	IDH	ALD	GP1	MDH	FUM	ASD	LAP	ME	EST	C23O	NSP	XDH	PGI	6PG			
67	1	CO417	6	3	1	3	2	3	2	2	1	1	1	3	5	4	1	O1 ElTor	F	India
68	1	3/06/02	4	2	1	1	1	3	2	5	1	1	1	3	1	4	1	non-O1/non-O139	F	USA
69	1	5-6/37	4	2	1	2	2	3	2	3	1	1	1	3	1	4	3	O1 classical	E	Tanzania
70	1	1196/78	3	4	1	2	1	6	2	1	1	2	1	2	5	2	6	O1 classical	E	USA
71	1	CO403	4	3	1	2	2	3	2	3	1	1	1	3	4	4	1	O139	F	India
72	1	2076/29	3	2	2	2	1	4	2	3	1	2	2	3	1	3	1	non-O1/non-O139	E	Unknown
73	1	3-6/69	4	2	1	2	1	3	2	3	1	2	0	3	1	5	2	O1 ElTor	E	USA
74	1	MEX332	4	3	1	3	2	3	3	4	1	1	2	3	1	4	0	O1 classical	C	Mexico
75	1	NM4392	3	3	1	2	1	5	2	4	1	2	2	3	1	3	0	O1 ElTor	C	Unknown
76	1	329	4	3	1	2	2	3	2	3	1	1	2	3	1	4	1	O139	C	Unknown
77	1	34-1	4	1	2	2	3	3	3	1	4	2	1	3	2	4	2	non-O1/non-O139	E	Unknown
78	1	1-7/69	3	3	1	2	1	6	2	3	1	2	1	3	1	2	4	O1 ElTor	E	USA
79	1	MEX445	3	4	1	2	1	2	1	1	2	1	1	3	4	4	1	O1 classical	C	Mexico
80	1	NM286	5	3	1	3	2	4	2	4	1	2	2	3	0	5	0	O1 ElTor	C	India
81	1	653/36	3	4	1	2	1	4	2	1	1	2	2	3	0	4	0	O139	C	India
82	1	25873	4	3	1	1	2	3	2	3	1	1	1	3	4	4	4	non-O1/non-O139	E	Unknown
83	1	CO396 NT646	6	3	1	2	2	3	2	1	1	2	1	3	1	4	3	O139	C	India
84	1	3-6/3	3	3	1	2	1	4	2	7	1	2	2	3	1	4	1	non-O1/non-O139	E	USA
85	1	6-3/2	3	2	1	2	1	4	2	3	1	2	2	3	1	4	1	O1 ElTor	E	USA
86	1	6-3/50	3	3	1	2	3	3	3	4	4	2	2	3	0	4	1	non-O1/non-O139	E	USA
87	1	NPO388	3	2	1	2	3	3	3	3	4	2	0	3	1	0	0	O139	C	India
88	1	CO416	3	2	1	2	3	3	3	1	4	2	2	3	2	3	1	O1 ElTor	F	India
89	1	NT648	6	3	1	2	2	3	2	7	1	1	0	2	3	5	2	O139	C	India
90	2	NPO390	6	3	1	3	2	3	2	3	1	1	1	3	1	4	1	O139	C	India
		BO4	6	3	1	3	2	3	2	3	1	1	1	3	1	4	1	O139	F	India
91	1	SO19	3	3	1	2	1	3	2	7	1	2	2	3	1	3	6	O139	C	India
92	1	CO415	6	3	1	2	2	3	2	2	1	1	2	3	1	4	1	O139	F	India
93	1	MOD084	6	3	1	2	2	3	2	3	1	1	2	3	0	4	1	O139	C	India
94	1	NT638	6	3	1	3	2	3	2	2	1	1	2	3	1	4	1	O139	C	India
95	1	1074-78	3	4	1	2	1	1	1	3	3	2	1	3	1	4	3	O1 classical	E	USA
96	1	CO418	6	3	1	1	2	3	2	3	1	1	1	3	1	4	1	O139	F	India
97	2	ALVC-91-0803	4	3	1	2	2	3	2	7	1	1	1	3	1	4	1	O1 ElTor	C	Peru
		ALVC-91-1019	4	3	1	2	2	3	2	7	1	1	1	3	1	4	1	O1 ElTor	C	Peru
98	1	ALVC-92-0534	6	3	1	3	2	3	2	1	1	1	2	3	1	4	1	O1 ElTor	C	Peru
99	1	ALVC-93-0024	4	3	1	1	2	3	2	4	1	1	2	3	1	4	1	O1 ElTor	C	Peru

* C, clinical; E, environmental; F, faecal; U, unknown.

Displacement of the electromorphs was expressed in terms of relative electrophoretic mobility with respect to the bromophenol blue band. Electromorphs of an enzyme were equated with alleles at the corresponding structural gene locus. Absence of enzyme activity was attributed to a null allele, and designated as 0. Distinct combinations of alleles over the 15 loci assayed were named as electrophoretic types (ETs).

Data treatment. Genetic diversity for a locus was calculated according to Nei (1978). The probability that two isolates differ at the j th locus is $h_j = (1 - \Sigma p_{ij}^2) n / (n - 1)$, where p_{ij} is the frequency of allele i at locus j and n is the number of isolates. The mean genetic diversity, H , is the arithmetic mean of h_j for m loci. Genotypic diversity was calculated as $G = 1 - \Sigma g_j^2$, where g_j is the frequency of the j th genotype (ET). Clustering of data obtained by MLEE was performed with the PHYLIP package (Felsenstein, 1993) from a matrix of coefficients of distances by the unweighted pair-group method for arithmetic averages (UPGMA). Distance between pairs of ETs was calculated as the proportion of loci at which dissimilar electromorphs occurred. The cophenetic correlation coefficient was calculated using NTSYS-pc, version 1.80 (Rohlf, 1993). Multilocus linkage disequilibrium was estimated on the basis of the distribution of allelic mismatches between pairs of bacterial isolates among all the loci examined. The ratio of the observed variance in mismatches (V_o) to the expected variance at linkage equilibrium (V_e) provides a measure of multilocus linkage disequilibrium that can be expressed as the index of association (I_A); $I_A = (V_o/V_e) - 1$ (Brown *et al.*, 1980; Maynard Smith *et al.*, 1993). For populations in linkage equilibrium, $V_o = V_e$ and I_A is not significantly different from zero, whereas values of I_A greater than zero indicate that recombination has been rare or absent. To determine whether V_o is significantly different from V_e in any sample, a Montecarlo procedure was generated by randomly sampling alleles, without replacement, according to their respective frequencies at each locus (Fusté *et al.*, 1996). Computer programs written by T. S. Whittam (Selander *et al.*, 1986) and J. G. Lorén were used to calculate V_o and V_e and to perform the Montecarlo randomization.

Estimates of the genetic differentiation between subpopulations were obtained by using Nei's genetic distance (Nei, 1972). Phylogenetic analyses were performed with the PHYLIP package by using the neighbour-joining method. The reliability of the cladograms obtained (Fig. 3) was determined by bootstrapping as follows. Gene frequencies were randomly selected, with replacement, to produce 100 replicated data sets of the same size as the original data sets. New Nei's distance matrices were then calculated and subjected to the neighbour-joining method. Comparison of the 100 bootstrapped cladograms generated was made using the Consensus program of the PHYLIP package.

RESULTS

ETs and genetic diversity

From a collection of 107 isolates of *V. cholerae* analysed by MLEE, 99 ETs were identified. The genotypic diversity (G) for all samples was 0.9872. All the enzyme loci studied were polymorphic, and the number of alleles ranged from two (ALD and EST) to seven (FUM, LAP, XDH and 6PG) (Table 2). The mean number of alleles per locus was 4.8. A small number of null alleles was observed (17 among the 99 ETs), distributed over 4 of the 15 enzymes assayed. Genetic diversity ranged from

0.08 for the least polymorphic loci (ALD and NSP) to 0.83 for the most polymorphic locus (LAP), with a mean genetic diversity (H) of 0.50 (Table 2). (A comparison of genetic diversity between the different sample sets is shown in Table 5.)

Genetic relationships among multilocus genotypes

The genetic relationship among the 99 ETs is shown in Fig. 1. The cophenetic correlation coefficient of the total sample was $R = 0.81$. The shortest genetic distance observed between ETs (0.06) corresponds to a single locus difference. No significant clustering among isolates was detected according to their serogroup, biotype or country of isolation. All but six of the total ETs are represented in two major divisions in the dendrogram, designated as divisions I and II, which diverge at a genetic distance of 0.6. Division II is constituted by 34 ETs, most of which are environmental non-O1/non-O139 strains. In division I we have designated two subgroups: Ia, which includes a large number of environmental strains, and Ib, with a greater number of clinical strains. The third group, division III, diverges at a genetic distance of 0.65. This is the deepest lineage found in the dendrogram and is clearly differentiated from all the other strains. This lineage is represented by six ETs (77, 12, 14, 87, 86, 88) without any apparent relationship between them.

Table 4 shows the 30 pairs of ETs that differ at a single enzyme locus. The loci that occur most frequently in this analysis are GP1 (seven pairs of ETs) and LAP (six pairs of ETs); none of these 30 pairs of ETs differ in the loci EST, FUM, NSP or PGI. In addition, we identified 15 pairs of ETs which are formed by strains of the same serogroup: seven pairs of O139 strains, five of non-O1/non-O139 and three of O1 ElTor isolates. In four of these cases, the pairs of ETs are represented by strains from a different country of isolation. On the other hand, the other pairs of ETs are formed by strains of different serogroups, except O1 classical.

Linkage disequilibrium analysis

Allele mismatch distribution among 99 ETs is shown in Fig. 2. Our population of *V. cholerae* presents a unimodal allele mismatch distribution, which suggests that it has a panmictic structure (Whittam, 1995). The complete set of isolates and population subsets was analysed for multilocus linkage disequilibrium (Table 5). The index of association (I_A) found for the total sample was 1.25 ± 0.14 , which suggested that this population of *V. cholerae* presents a significant level of linkage disequilibrium. Table 5 also shows the results obtained for several population subsets studied. When we considered divisions of the dendrogram, serogroups, sources and geographical origin of the strains, all subsets showed I_A values differing significantly from zero, except division II and III, O1 classical and USA/Mexico subgroups, which exhibited I_A values of 0.41, 0.08, 0.64 and 0.42, respectively.

Table 2. Allele frequencies and genetic diversities at 15 enzyme loci in 99 ETs of *V. cholerae*

Enzyme locus	Max. no of alleles	Frequency of each allele								h^*
		1	2	3	4	5	6	7	0	
G6P	6	0.010	0.010	0.354	0.343	0.010	0.273			0.69
IDH	4	0.081	0.101	0.758	0.061					0.41
ALD	2	0.960	0.040							0.08
GP1	5	0.202	0.586	0.192	0.010	0.010				0.58
MDH	3	0.465	0.475	0.061						0.56
FUM	7	0.010	0.081	0.606	0.152	0.061	0.051	0.040		0.60
ASD	4	0.121	0.798	0.071					0.010	0.35
LAP	7	0.121	0.222	0.212	0.101	0.192	0.010	0.141		0.83
ME	5	0.596	0.121	0.222	0.051	0.010				0.58
EST	2	0.556	0.444							0.50
C23O	4	0.515	0.424	0.020					0.040	0.56
NSP	3	0.020	0.020	0.960						0.08
XDH	7	0.636	0.020	0.010	0.202	0.061	0.010		0.061	0.55
PGI	6	0.010	0.111	0.172	0.647	0.051			0.010	0.54
6PG	7	0.626	0.051	0.111	0.040	0.091	0.030		0.051	0.59
Mean genetic diversity, H										0.50

* h , genetic diversity.

Genetic distances within population subgroups

The clustering of strains considering serogroups and geographical origin from values of genetic distances between population subgroups is shown in Fig. 3. The cladogram of the geographical subpopulations showed that the strains from the USA, Brazil and Mexico were more genetically related to each other than to the isolates of *V. cholerae* from India and Peru. On the other hand, the cladogram of the serogroup/biotype subpopulations confirmed that the O1 ElTor and O139 strains were genetically more related to each other than to other serogroups.

DISCUSSION

Nowadays *V. cholerae* is a well-defined species based on biochemical tests and DNA homology studies (Baumann *et al.*, 1984), though it is a highly heterogeneous group with respect to its pathogenic potential: only two out of the nearly 200 serogroups defined at present, O1 and O139, have been associated with epidemics (Kaper *et al.*, 1995). Therefore many aspects of the ecology of *V. cholerae* and its relationship to the pathogenesis and epidemiology of cholera remain unknown (Faruque *et al.*, 1998).

Bacterial population genetics is the study of the natural variability of bacterial populations and has led to the formulation of theories to account for this diversity (Maynard Smith *et al.*, 1993). Classical and molecular methods have continuously been used to study the population diversity of *V. cholerae* and have been extensively applied to characterize different serogroups

and biotypes (Baumann *et al.*, 1984), ribotypes (Karaolis *et al.*, 1994), RAPD fingerprint types (Rivera *et al.*, 1995) and insertion sequence fingerprint types (Bik *et al.*, 1996). These methods have shown a considerable amount of variation in *V. cholerae*. MLEE constitutes the classical methodology in the study of bacterial population genetics (Selander *et al.*, 1986). By using MLEE data we can obtain estimates of genetic and genotypic diversity and estimates of the frequency of recombination in natural populations. These aspects of *V. cholerae* population biology are of great importance to molecular epidemiological studies of cholera.

Previous MLEE studies with *V. cholerae* have shown a limited genetic diversity among toxigenic strains of this bacterium. Pathogenic isolates from various sources all showed identical electrophoretic profiles or differed in only a few loci (Salles & Momen, 1991; Wachsmuth *et al.*, 1993; Evins *et al.*, 1995). Some authors indicate that the sixth-pandemic, the seventh-pandemic and the US Gulf isolates are three independent clones (Kaper *et al.*, 1982; Wachsmuth *et al.*, 1993; Karaolis *et al.*, 1994, 1995; Evins *et al.*, 1995). In a recent study, it has been postulated that all strains of the O139 serogroup belong to a unique ET; similarly the toxigenic O1 ElTor isolates cluster in only three ETs (Beltrán *et al.*, 1999).

Our results show that when MLEE methods were applied to a large collection of isolates of diverse origin including serogroup O1 (classical and ElTor biotypes), O139 and non-O1/non-O139 strains, the different *V. cholerae* populations showed a high degree of genetic variation. The number of ETs found in our work does

Fig. 1. For legend see facing page.

Table 3. Groups of strains with identical genotype

ET	No. of strains	Reference isolates	Serogroup/biotype	Source*	Country
2	2	TM34162-77	non-O1/non-O139	E	Brazil
		TM9024-79	non-O1/non-O139	E	Brazil
8	3	TM48733-82	non-O1/non-O139	E	Brazil
		TM11079-80	O1 ElTor	E	Brazil
		CO484	non-O1/non-O139	E	India
25	2	TM207832-78	O1 ElTor	E	Brazil
		ALVC-92-0344	O1 ElTor	C	Peru
53	2	CO402	O139	F	India
		SO30	O139	C	India
60	2	25872	non-O1/non-O139	E	Unknown
		CO406	O139	F	India
90	2	NPO390	O139	C	India
		BO4	O139	F	India
97	2	ALVC-91-0803	O1 ElTor	C	Peru
		ALVC-91-1019	O1 ElTor	C	Peru

* C, clinical; E, environmental; F, faecal.

not coincide with data previously published (Salles & Momen, 1991; Wachsmuth *et al.*, 1993; Evins *et al.*, 1995; Beltrán *et al.*, 1999). From 107 isolates analysed, 99 ETs were found, 84 containing a single strain, six containing two strains and only one containing three strains. Previous studies described a more limited diversity of *V. cholerae* populations, with most isolates clustering in two or three ETs with minimal differences between them, especially when toxigenic strains were included (Salles & Momen, 1991; Wachsmuth *et al.*, 1993; Evins *et al.*, 1995). The estimate for the mean genetic diversity per locus of the total 99 ETs ($H=0.50$) in our study is greater than other values reported for *V. cholerae* (Chen *et al.*, 1991; Salles & Momen, 1991; Wachsmuth *et al.*, 1993; Evins *et al.*, 1995; Beltrán *et al.*, 1999). Likewise, the mean number of alleles per locus (4.8), and the genotypic diversity (0.9872) were higher than those given in similar studies, with the exception of Beltrán *et al.* (1999), who reported a value of 9.5 alleles per locus as an average. Also, the work of Beltrán *et al.* (1999) differed in the number of strains that fell into the same ET.

Differences in the serogroup, geographical origin and year of isolation could explain this diversity. Another factor that may influence the results is the methodology used; some previous studies used starch as a matrix for the gels, whose resolution could be distinct from that of polyacrylamide (Wachsmuth *et al.*, 1993; Evins *et al.*, 1995; Beltrán *et al.*, 1999). Finally, the number and type of enzymes studied are different; most of them are monomorphic and only a few contribute to the genetic diversity. However, in our work all the enzymes were polymorphic with a considerable genetic diversity, thus hampering comparisons between studies.

The dendrogram obtained in the present study shows no association between isolates with regard to serogroup, biotype or geographical origin. The same serogroup is present in several lineages and does not cluster separately from the others (Fig. 1). Similar results were obtained by Beltrán *et al.* (1999), who showed that strains of the same serogroup may belong to two or more divergent ET lineages. Division II has more non-O1/non-O139 strains than other groups. On the other hand, most of the O139 serogroup strains clustered in division I (24 of 29 isolates), while other serogroups seem to be randomly distributed. With regard to the source of the strains studied, division II seems to contain most of the environmental strains, while clinical isolates predominate in the subgroup Ib. Although this clustering did not coincide with previous studies, the value of the cophenetic correlation coefficient obtained ($R=0.81$) falls into the range (0.74–0.90) of most frequently occurring cophenetic correlations reported by Sneath & Sokal (1973).

Unimodal allele mismatch distribution (Fig. 2) and the shape of the dendrogram (Fig. 1) are typical of a panmictic population (Whittam, 1995). However, evidence for clonal proliferation is provided by the multi-locus linkage disequilibrium calculations, which reveal a significant level of association between alleles when the whole population sample is analysed. Values of I_A among all ETs indicated that there is a nonrandom distribution of alleles, which is clear evidence of a clonal population structure, with a significant degree of linkage disequilibrium $I_A = 1.25 \pm 0.14$ (no significant differences were obtained when we considered strains or ETs). Nevertheless, when we analysed data subsets corresponding to divisions of the dendrogram, sero-

Fig. 1. Dendrogram constructed by the UPGMA method, showing genetic relationships among 99 ETs of *V. cholerae* strains. The sources of the strains are indicated by the letters C (clinical), E (environmental), F (faecal) and U (unknown). Asterisks (*) denote ETs that contain more than one strain (see Table 3). The scale indicates genetic distance.

Table 4. ETs that differ at a single locus

ET	Serogroup	Country of isolation	Locus	ET	Serogroup	Country of isolation	Locus
1	non-O1/non-O139	Brazil	ME	29	O1 ElTor	Brazil	C23O
15	non-O1/non-O139	Brazil	ME	97	O1 ElTor	Peru	C23O
1	non-O1/non-O139	Brazil	IDH	35	non-O1/non-O139	Mexico	LAP
24	Unknown	Brazil	IDH	99	O1 ElTor	Peru	LAP
2	non-O1/non-O139	Brazil	MDH	48	non-O1/non-O139	Bangladesh	C23O
25	O1 ElTor	Brazil	MDH	53	O139	India	C23O
	O1 ElTor	Peru	MDH				
2	non-O1/non-O139	Brazil	IDH	48	non-O1/non-O139	Bangladesh	XDH
38	non-O1/non-O139	India	IDH	94	O139	India	XDH
4	non-O1/non-O139	Brazil	6PG	49	non-O1/non-O139	Bangladesh	6PG
16	non-O1/non-O139	Brazil	6PG	54	O139	Unknown	6PG
4	non-O1/non-O139	Brazil	GP1	49	non-O1/non-O139	Bangladesh	ALD
17	non-O1/non-O139	Brazil	GP1	59	O139	India	ALD
8	non-O1/non-O139	Brazil	GP1	53	O139	India	XDH
	non-O1/non-O139	India	GP1	56	O139	India	XDH
	O1 ElTor	Brazil	GP1	67	O1 ElTor	India	XDH
29	O1 ElTor	Brazil	GP1	53	O139	India	GP1
19	non-O1/non-O139	Brazil	LAP	63	O139	India	GP1
41	O1 ElTor	India	LAP	56	O139	India	6PG
20	non-O1/non-O139	Brazil	ASD	59	O139	India	6PG
36	non-O1/non-O139	Mexico	ASD	56	O139	India	LAP
22	O1 ElTor	Brazil	6PG	90	O139	India	LAP
26	O1 ElTor	Brazil	6PG	56	O139	India	C23O
22	O1 ElTor	Brazil	LAP	94	O139	India	C23O
53	O139	India	LAP	60	non-O1/non-O139	Unknown	GP1
	O139	India	LAP		O139	India	GP1
25	O1 ElTor	Brazil	LAP	67	O1 ElTor	India	GP1
	O1 ElTor	Peru	LAP	62	O139	India	G6P
29	O1 ElTor	Brazil	LAP	96	O139	India	G6P
76	O139	Unknown	LAP	90	O139	India	GP1
25	O1 ElTor	Brazil	GP1	96	O139	India	GP1
	O1 ElTor	Peru	GP1	92	O139	India	GP1
99	O1 ElTor	Peru	GP1	94	O139	India	GP1
27	O1 ElTor	Brazil	XDH	94	O139	India	LAP
93	O139	India	XDH	98	O1 ElTor	Peru	LAP

groups or geographical origin, some of them (divisions II, III, O1 classical and USA/Mexico strains) showed I_A values less than one (0.41, 0.08, 0.64 and 0.42, respectively). The V_O values of these subgroups were also within the 95% confidence limits of V_E and within the maximum and minimum values of the variance obtained

by the Montecarlo procedure. However, I_A values corresponding to the division III and the O1 classical subgroup should be taken cautiously because of the low number of ETs in these groups (six and nine ETs, respectively). These results are partially concordant with those obtained by Beltrán *et al.* (1999), who found

Fig. 2. Allele mismatch distribution among 99 ETs of the total sample studied.

Table 5. Multilocus linkage disequilibrium analysis of *V. cholerae*

	No. of ETs	Mean no. of alleles per locus	H	V_O	V_E	95 % confidence limits of V_E^*	$I_A^\dagger$	Montecarlo V minimum	Randomization V maximum
Total 107 isolates	99	4.80	0.50	7.13	3.17	2.29–4.04	1.25 ± 0.14	2.54	3.89
Dendrogram									
Division I	59	3.47	0.35	3.91	2.62	1.67–3.56	0.49 ± 0.18	1.87	3.77
Division II	34	3.73	0.44	3.77	2.67	1.41–3.92	0.41 ± 0.24	1.78	3.88
Division III	6	2.33	0.40	2.03	1.88	0.24–3.99	0.08 ± 0.56	0.27	7.52
Serogroup/biotype‡									
O1	35	4.00	0.49	6.54	3.04	1.63–4.44	1.15 ± 0.23	2.15	4.35
O1 ElTor	24	3.60	0.45	7.01	2.94	1.29–4.57	1.39 ± 0.28	1.74	5.03
O1 classical	9	3.33	0.57	4.35	2.65	0.20–5.09	0.64 ± 0.46	0.99	7.56
Non-O1/non-O139	37	4.00	0.51	6.72	3.20	1.75–4.64	1.10 ± 0.23	2.28	4.68
O139	26	3.53	0.40	8.05	2.94	1.34–4.52	1.74 ± 0.27	1.51	5.09
Country of isolation‡									
America§	54	4.26	0.49	6.37	3.10	1.94–4.25	1.05 ± 0.19	2.28	4.09
Brazil	31	3.53	0.49	7.82	3.01	1.53–4.49	1.59 ± 0.24	2.14	4.25
USA/Mexico	20	3.86	0.51	4.49	3.15	1.22–5.08	0.42 ± 0.31	1.71	5.83
India	32	4.06	0.46	8.74	3.05	1.57–4.53	1.86 ± 0.24	1.74	4.61
Source‡									
Environmental	47	4.33	0.52	6.17	3.16	1.89–4.42	0.95 ± 0.19	2.38	4.43
F	22	3.53	0.48	10.16	3.27	1.35–5.18	2.10 ± 0.29	1.84	5.96
C	31	3.67	0.42	6.17	2.96	1.50–4.42	1.08 ± 0.25	1.75	4.63

* Calculated according to Brown *et al.* (1980).

† Index of association $\pm$ SD.

‡ The same ET could be represented in various subgroups when it includes strains of diverse origin, source, serogroup or biotype. See Tables 1 and 3.

§ Considering isolates from Brazil, Mexico, Peru and USA together.

I_A values quite similar to ours (1.248 ± 0.083) for the whole population, and I_A values close to zero when they analysed population subgroups. Therefore, our results do not rule out the possibility that the population of *V. cholerae* studied has a clonal structure, nor do they exclude recombination. We believe that this population has a clonal structure which has not been broken by

genetic recombination. Another possibility is to consider that the population has an epidemic structure (Maynard Smith *et al.*, 1993), in which strains of different origin would coexist with others of identical origin corresponding to an outbreak of disease from a single person in a specific geographical area (e.g. in July, 1994, the massive outbreak of O1 ElTor *V. cholerae* among

Fig. 3. Unrooted phylogenetic trees of different subpopulations from the total sample generated by the neighbour-joining method of clustering of Nei's distances. The bootstrap values for nodes are presented for only those clusters of subpopulations which occurred more than 80% of the time. The scale bars indicate genetic distance.

O1 ElTor strains

O139 strains

Fig. 4. Comparison of the genetic diversity of two subpopulations of the total sample, O1 ElTor and O139. Asterisks (*) denote ETs that contain more than one strain (see Table 3). The scale bars indicate genetic distance.

Rwandan refugees in Goma, Zaire: Sanchez & Taylor, 1997).

The analysis of the genetic distances for isolates belonging to the six subpopulations that we could establish on the basis of the country of isolation (Fig. 3), showed that strains from Peru cluster in the same group as those isolated from India. This result is fully concordant with the origin of Peru's strains previously determined by Wachsmuth *et al.* (1993). In the same context, we might consider the results obtained with strains isolated in USA and Mexico. USA strains are longer established and remotely related with Mexico's isolates, as Wachsmuth *et al.* (1993) pointed out.

If we consider population subsets referring to serogroup/biotype, there is also a concordance with previous references in relation to the origin of O1 and O139 serogroups (Bik *et al.*, 1995; Strocher *et al.*, 1997). From the analysis of the genetic distances between populations it might be deduced that *V. cholerae* O1 ElTor and O139 originated from a unique splitting event (Fig. 3) and consequently, O139 and O1 ElTor might have gained a similar diversity ($H=0.40$ and $H=0.45$, respectively). Indeed, looking at Fig. 4, we can see that O139 subpopulation strains were distributed in different ETs in the same way as those of O1 ElTor. Nonetheless, the

slight differences in the genetic diversity can easily be explained if we consider the geographical origin of the two sets of strains. O139 are all Indian isolates, whereas O1 ElTor were isolated in several countries (Table 1).

There are two possible explanations for the diversity found among O139. First, the dissociation might have happened a long time ago and consequently both populations have been accumulating diversity lengthwise. Second, the split might have happened recently, but several recombination events have taken place in a diverse genetic background. Neither of these hypotheses agrees with the idea of a unique ET supported by Popovic *et al.* (1995) and Beltrán *et al.* (1999). However, they would explain the diversity we observed in the O139 population.

Other population studies based on the analysis of restriction fragment length polymorphisms in genes for conserved rRNA and cholera toxin (*ctxA*) or in DNA sequences flanking these genes revealed four different ribotypes and four different *ctx* genotypes among 93 strains of *V. cholerae* O139 studied (Faruque *et al.*, 1997b). These results agree with the idea that strains belonging to the O139 serogroup may have emerged from similar serotype-specific genetic changes in more than one progenitor strain of *V. cholerae*. Considering

this background, it is hard to believe that all the O139 strains could belong to a unique ET. Results obtained in our work are in agreement with the hypothesis of a more diverse origin of these strains, a conclusion that can also be reached from ribotyping analysis.

Data from the sequences of some housekeeping genes of *V. cholerae* showed a high degree of similarity when the different strains were compared, especially in clinical isolates. Karaolis *et al.* (1995) determined the DNA sequences of the *asd* (aspartate semialdehyde dehydrogenase) genes from 45 isolates of *V. cholerae*, which included five O139 isolates. Byun *et al.* (1999) sequenced the *mdh* (malate dehydrogenase) and *hlyA* (haemolysin A) genes from environmental and pathogenic strains of *V. cholerae*, including seven strains of the O139 serogroup. Both groups of authors found a high degree of similarity among the pathogenic isolates, suggesting that they are very closely related. These results may appear to disagree with the high diversity found in our work. This contradiction, however, is only apparent. Our results show that *mdh* and *asd* presented a low genetic diversity within the O139 isolates studied (*mdh*, $h=0.32$; *asd*, $h=0.07$). Moreover, the number of O139 strains studied by these authors is low (in our work we studied 29 O139 isolates). Recently, Jiang *et al.* (2000) published a study applying the AFLP method to a collection of clinical and environmental isolates of *V. cholerae*, which supports the idea that pathogenic *V. cholerae* strains have evolved from multiple independent sources like environmental O1 or non-O1 strains. We believe that more extensive areas of the bacterial chromosome should be analysed (as in the case of MLEE) in order to reach a reasonable conclusion about the likely origin of a bacterial population.

The persistence of *V. cholerae* in the environment, for months and probably years, is facilitated by its ability to enter a viable nonculturable state in which its nutrient and oxygen requirements are much decreased (Ravel *et al.*, 1995). It has also been described that *V. cholerae* can bind to chitin in crustacean shells, and colonize the surfaces of algae, phytoplankton, copepods and the roots of aquatic plants such as water hyacinth. One scenario to explain our findings is that during inter-epidemics *V. cholerae* populations might be associated with an ecological reservoir. We can consider that all *V. cholerae* cells constitute a metapopulation integrated by multiple ecological populations. Previous studies have shown that bacterial recombination is too rare to prevent neutral sequence divergence between distinct ecological populations. Moreover, the episodes of periodic selection that purge diversity operate within populations and do not prevent their divergence (Young, 1989). In their reservoir *V. cholerae* clones could diverge from each other. Because the genes for the major virulence factors can be acquired horizontally, we can postulate that each *V. cholerae* cell would have the same probability of being transformed into a toxigenic strain (Beltrán *et al.*, 1999). In the next epidemic episode, some unknown environmental factors might trigger an explosive multiplication of *V. cholerae* populations outside

this reservoir (Epstein *et al.*, 1993; Harvell *et al.*, 1999). This global population would be formed by bacteria from various local populations and include a diversity of toxigenic clones that potentially could infect humans.

ACKNOWLEDGEMENTS

We are grateful to Dr R. Montilla for providing strains for this study and for his helpful comments. We are indebted to Dr J. Palomar for his help in setting up the MLEE technique. We also thank G. B. Nair, M. A. R. Chowdhury and M. Talledo for kindly supplying isolates of *V. cholerae*.

M. Farfán is the recipient of the grant 'Formació en la Recerca i Docència per a alumnes de tercer cicle' from the University of Barcelona. This work was supported by a grant from the Vicerectorat de Recerca of the University of Barcelona.

REFERENCES

- Albert, M. J., Siddique, A. K., Islam, M. S., Faruque, A. S. G., Ansaruzzaman, M., Faruque, S. M. & Sack, R. B. (1993). Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. *Lancet* **341**, 704.
- Baumann, P., Furniss, A. L. & Lee, J. V. (1984). Genus I. *Vibrio*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 518–538. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Beltrán, P., Delgado, G., Navarro, A., Trujillo, F., Selander, R. K. & Cravioto, A. (1999). Genetic diversity and population structure of *Vibrio cholerae*. *J Clin Microbiol* **37**, 581–590.
- Bik, E. M., Bunschoten, A. E., Gouw, R. D. & Mooi, F. R. (1995). Genesis of the novel epidemic *Vibrio cholerae* O139 strain: evidence for horizontal transfer of genes involved in polysaccharide synthesis. *EMBO J* **14**, 209–216.
- Bik, E. M., Gouw, R. D. & Mooi, F. R. (1996). DNA fingerprinting of *Vibrio cholerae* strains with a novel insertion sequence element: a tool to identify epidemic strains. *J Clin Microbiol* **34**, 1453–1461.
- Blake, P. A. (1994). Historical perspectives on pandemic cholera. In *Vibrio cholerae and Cholera: Molecular to Global Perspectives*, pp. 293–295. Edited by I. K. Wachsmuth, P. A. Blake & O. Olsvik. Washington, DC: American Society for Microbiology.
- Brown, A. D., Feldman, M. W. & Nevo, E. (1980). Multilocus structure of natural populations of *Hordeum spontaneum*. *Genetics* **96**, 523–536.
- Byun, R., Elbourne, L. D., Lan, R. & Reeves, P. R. (1999). Evolutionary relationships of pathogenic clones of *Vibrio cholerae* by sequence analysis of four housekeeping genes. *Infect Immun* **67**, 1116–1124.
- Chen, F., Evins, G. M., Cook, W. L., Almeida, R., Hargrett-Bean, N. & Wachsmuth, K. (1991). Genetic diversity among toxigenic and nontoxigenic *Vibrio cholerae* O1 isolated from the Western Hemisphere. *Epidemiol Infect* **107**, 225–233.
- Colwell, R. R. (1996). Global climate and infectious disease: the cholera paradigm. *Science* **274**, 2025–2031.
- Colwell, R. R. & Huq, A. (1994). Vibrios in the environment: viable but nonculturable *Vibrio cholerae*. In *Vibrio cholerae and Cholera: Molecular to Global Perspectives*, pp. 117–133. Edited by I. K. Wachsmuth, P. A. Blake & O. Olsvik. Washington, DC: American Society for Microbiology.
- Epstein, P. R., Ford, T. E. & Colwell, R. R. (1993). Marine ecosystems. *Lancet* **342**, 1216–1219.

- Evins, G. M., Cameron, D. N., Wells, J. G., Greene, K. D., Popovic, T., Giono-Cerezo, S., Wachsmuth, I. K. & Tauxe, R. V. (1995). The emerging diversity of the electrophoretic types of *Vibrio cholerae* in the Western Hemisphere. *J Infect Dis* **172**, 173–179.
- Faruque, S. M., Ahmed, K. M., Abdul Alim, A. R. M., Qadri, F., Siddique, A. K. & Albert, M. J. (1997a). Emergence of a new clone of toxigenic *Vibrio cholerae* O1 biotype ElTor displacing *V. cholerae* O139 Bengal in Bangladesh. *J Clin Microbiol* **35**, 624–630.
- Faruque, S. M., Ahmed, K. M., Siddique, A. K., Zaman, K., Abdul Alim, A. R. M. & Albert, M. J. (1997b). Molecular analysis of toxigenic *Vibrio cholerae* O139 Bengal strains isolated in Bangladesh between 1993 and 1996: evidence for emergence of a new clone of the Bengal vibrios. *J Clin Microbiol* **35**, 2299–2306.
- Faruque, S. M., Albert, M. J. & Mekalanos, J. J. (1998). Epidemiology, genetics and ecology of toxigenic *Vibrio cholerae*. *Microbiol Mol Biol Rev* **62**, 1301–1314.
- Felsenstein, J. (1993). PHYLIP package, version 3.5. Seattle, WA: University of Washington (<http://evolution.genetics.washington.edu/phylip.html>).
- Fusté, M. C., Pineda, M. A., Palomar, J., Viñas, M. & Lorén, J. G. (1996). Clonality of multidrug-resistant nontypeable strains of *Haemophilus influenzae*. *J Clin Microbiol* **34**, 2760–2765.
- Gibson, D. T. (1971). Assay of enzymes of aromatic metabolism. *Methods Microbiol* **6A**, 463–478.
- Harvell, C. D., Kim, K., Burkholder, J. M. & 10 other authors (1999). Emerging marine diseases – climate links and anthropogenic factors (review). *Science* **285**, 1505–1510.
- Jiang, S. C., Matte, M., Matte, G., Huq, A. & Colwell, R. R. (2000). Genetic diversity of clinical and environmental isolates of *Vibrio cholerae* determined by amplified fragment length polymorphism fingerprinting. *Appl Environ Microbiol* **66**, 148–153.
- Kaper, J. B., Bradford, H. B., Roberts, N. C. & Falkow, S. (1982). Molecular epidemiology of *Vibrio cholerae* in the U. S. Gulf Coast. *J Clin Microbiol* **16**, 129–134.
- Kaper, J. B., Morris, J. G. & Levine, M. M. (1995). Cholera. *Clin Microbiol Rev* **8**, 48–86.
- Karaolis, D. K., Lan, R. & Reeves, P. R. (1994). Molecular evolution of the seventh-pandemic clone of *Vibrio cholerae* and its relationship to other pandemic and epidemic *V. cholerae* isolates. *J Bacteriol* **176**, 6199–6206.
- Karaolis, D. K., Lan, R. & Reeves, P. R. (1995). The sixth and seventh cholera pandemics are due to independent clones separately derived from environmental, nontoxigenic, non-O1 *Vibrio cholerae*. *J Bacteriol* **177**, 3191–3198.
- Karaolis, D. K., Johnson, J. A., Bailey, C. C., Boedeker, E. C., Kaper, J. B. & Reeves, P. R. (1998). A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. *Proc Natl Acad Sci U S A* **95**, 3134–3139.
- Kataeva, I. A. & Golovleva, L. A. (1990). Catechol 2,3-dioxygenases from *Pseudomonas aeruginosa* 2x. *Methods Enzymol* **188**, 115–121.
- Maynard Smith, J., Smith, N. H., O'Rourke, M. & Spratt, B. G. (1993). How clonal are bacteria? *Proc Natl Acad Sci U S A* **90**, 4384–4388.
- Mukhopadhyay, A. K., Basu, A., Garg, P., Bag, P. K., Ghosh, A., Bhattacharya, S. K., Takeda, Y. & Nair, G. B. (1998). Molecular epidemiology of reemergent *Vibrio cholerae* O139 Bengal in India. *J Clin Microbiol* **36**, 2149–2152.
- Nei, M. (1972). Genetic distance between populations. *Am Nat* **106**, 283–292.
- Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small sample of individuals. *Genetics* **89**, 583–590.
- Popovic, T., Fields, P. I., Olsvik, O. & 11 other authors (1995). Molecular subtyping of toxigenic *Vibrio cholerae* O139 causing epidemic cholera in India and Bangladesh, 1992–1993. *J Infect Dis* **171**, 122–127.
- Ramamurthy, T., Garg, S., Sharma, R. & 8 other authors (1993). Emergence of a novel strain of *Vibrio cholerae* with epidemic potential in Southern and Eastern India. *Lancet* **341**, 703–704.
- Ravel, J., Knight, I. T., Monahan, C. E., Hill, R. T. & Colwell, R. R. (1995). Temperature-induced recovery of *Vibrio cholerae* from the viable but nonculturable state: growth or resuscitation? *Microbiology* **141**, 377–383.
- Rivera, I. G., Chowdhury, M. A. R., Huq, A., Jacobs, D., Martins, M. T. & Colwell, R. R. (1995). Enterobacterial repetitive intergenic consensus sequences and the PCR to generate fingerprints of genomic DNAs from *Vibrio cholerae* O1, O139 and non-O1 strains. *Appl Environ Microbiol* **61**, 2898–2904.
- Rohlf, F. J. (1993). Numerical taxonomy and multivariate analysis system, version 1.80. New York: Exeter Software.
- Salles, C. A. & Momen, H. (1991). Identification of *Vibrio cholerae* by enzyme electrophoresis. *Trans R Soc Trop Med Hyg* **85**, 544–547.
- Sanchez, J. L. & Taylor, D. N. (1997). Cholera. *Lancet* **349**, 1825–1830.
- Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N. & Whittam, T. S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol* **51**, 873–884.
- Sneath, P. H. A. & Sokal, R. R. (1973). *Numerical Taxonomy*. San Francisco: Freeman.
- Stroeher, U. H., Parasivam, G., Dredge, B. K. & Manning, P. A. (1997). Novel *Vibrio cholerae* O139 genes involved in lipopolysaccharide biosynthesis. *J Bacteriol* **179**, 2740–2747.
- Wachsmuth, I. K., Evins, G. M., Fields, P. I., Olsvik, O., Popovic, T., Bopp, C. A., Wells, J. G., Carrillo, C. & Blake, P. A. (1993). The molecular epidemiology of cholera in Latin America. *J Infect Dis* **167**, 621–626.
- Waldor, M. K. & Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**, 1910–1914.
- Whittam, T. S. (1995). Genetic population structure and pathogenicity in enteric bacteria. In *Population Genetics of Bacteria*, pp. 217–245. Edited by S. Baumberg, J. P. Young, E. M. H. Wellington & J. R. Saunders. Cambridge: Cambridge University Press.
- Young, J. P. W. (1989). The population genetics of bacteria. In *Genetics of Bacterial Diversity*. Edited by D. A. Hopwood & K. F. Chater. London: Academic Press.

Received 20 December 1999; revised 22 May 2000; accepted 26 June 2000.