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Epidemiologic Application of a Standardized Ribotype Scheme for *Vibrio cholerae* O1

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A standardized scheme of 27 different *BglI* ribotypes and subtypes of *Vibrio cholerae* O1 strains is proposed on the basis of data from 214 human and environmental strains isolated in 35 countries and 14 U.S. states over the past 60 years. The ribotype patterns obtained are reproducible and stable over time. Seven different but very similar ribotypes (1a to 1g) were observed among 16 strains of the classical biotype. Twenty ribotypes and subtypes were identified among 198 *V. cholerae* O1 strains of the El Tor biotype. Six different patterns were found among the strains causing the current seventh pandemic. Strains of ribotype 8 originated only in central African countries, while those of ribotype 3 originated mainly in Asia and the Pacific Islands. The most widely distributed strains were those of ribotype 6, which was subdivided into three very similar but still distinguishable subtypes. The present Latin American epidemic is caused by strains of ribotype 5. Strains of this ribotype were isolated from several other geographic locations but can be differentiated from the Latin American strains by other molecular methods. Strains associated with two documented environmental reservoirs exhibited three distinct ribotype patterns; those isolated from patients who ate food from the U.S. Gulf waters were all of ribotype 2, while the strains related to the northeast Australian rivers were of ribotypes 9 and 10. Nontoxigenic *V. cholerae* O1 strains originating in Latin America and the U.S. Gulf Coast did not form a specific cluster of ribotypes. Ribotyping in combination with other well-defined methods can assist in epidemiologic investigations, helping to trace the movement of strains and to identify their geographic origins.

In January 1991, cholera appeared in Peru, 100 years after it had disappeared from Latin America; it spread rapidly within the continent at the rate of approximately one new country per month (50, 57). By the end of 1992, 19 countries in Latin America had reported more than 730,000 cholera cases and 6,300 deaths (15). That is more cases than had been reported for the entire world over the past 5 years (15, 56). Epidemiologic surveillance of cholera has been limited in the past by rather insensitive laboratory typing systems, such as biochemical and serologic identification, which give little strain-discriminatory information (28, 51). The epidemic strains are generally of the O1 serogroup, and there are only two biotypes, classical and El Tor, and two serotypes, Inaba and Ogawa (6, 36). Chicken erythrocytes and bacteriophages for biotype determination are often difficult to obtain, and biotype markers such as hemolytic activity have been previously shown to change over time (4). Recently, investigators reported that the switch from one serotype to another may be based on point mutations in the *rfbT* gene of the *Vibrio cholerae* O1 strains (47). These data plus previous reports of serotype conversion in patients and experimentally infected animals make this strain marker epidemiologically less valuable (23, 43, 46).

Molecular techniques, such as plasmid profiles, and restriction fragment length polymorphisms (RFLPs) of genomic DNA have also been of limited value in identifying individual strains of *V. cholerae* O1. El Tor strains of *V. cholerae* rarely harbor plasmids useful as epidemiologic markers, and it may be difficult to interpret the RFLP patterns because of the large number of restriction fragments generated from bacterial chromosomal DNA. Although classical biotype strains did contain certain plasmid DNA and an integrated vibriophage, VcA1, plasmid and VcA1 RFLP patterns did not differentiate individual biotype strains (17). Multilocus enzyme electrophoresis and pulsed-field gel electrophoresis appear to be more discriminating or of certain epidemiologic value (5, 16, 53).

A recent development in DNA analysis, ribotyping, is based on analysis of the RFLPs of rRNA genes (48). rRNA sequences are highly conserved and are present as multiple copies in the bacterial genome of all bacteria (38). Bacterial 16S and 23S rRNA, suitably labeled, can be used as a probe, and the pattern of the hybridization of restriction endonuclease-digested chromosomal DNA with such a probe could be used to characterize bacterial species or strains and to differentiate between otherwise phenotypically indistinguishable pathogens (48). Ribotyping has been successfully used for subserogroup characterization of microorganisms belonging to different genera and species, such as *Salmonella* spp. (3), *Campylobacter* spp. (29), *Haemophilus* spp. (45), *Yersinia enterocolitica* (8), *Staphylococcus* spp. (18), *Shigella sonnei* (25), *Neisseria meningitidis* (55), and others (24). Koblavi et al. have shown that the pattern of the rRNA RFLPs can also be used to type strains of *V. cholerae* O1 (31). We propose a typing scheme based on differences in the *BglI* rRNA RFLP patterns of *V. cholerae* O1 strains. We present data on 214 *V. cholerae* O1 strains isolated worldwide over the past 60 years. The use of a standardized scheme for typing would allow public health laboratories to follow the movement and to identify the origins of *V. cholerae* O1 strains. Such information might well be useful to public health authorities in designing and implementing prevention and intervention strategies based on that information.

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MATERIALS AND METHODS

Bacterial strains. A total of 214 strains of *V. cholerae* O1 were used in this study; 16 strains were of the classical biotype, and 198 strains were of the El Tor biotype. Of these, 77 strains were collected in 14 Latin American countries, mainly during the recent cholera epidemic (1991 to 1992), and 137 strains, isolated worldwide over the last 60 years, were from the strain collection of the Foodborne and Diarrheal Diseases Branch, Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, Atlanta, Ga. Twenty-four of the 214 strains were nontoxigenic (Table 1; also see Table 3). There were 162 human and 52 environmental isolates. Strains were identified and characterized by biochemical tests and serotyping with the group- and type-specific antisera and by polymerase chain reaction for detection of the A subunit of the cholera toxin gene to verify toxigenicity (22). All of the strains were stored frozen or in liquid nitrogen.

DNA purification. (i) DNA extraction by phenol procedure. Isolates were grown overnight in 5 ml of Luria-Bertani broth at 37°C. Subsequently, 3 ml of the broth was transferred to 15-ml-volume polypropylene tubes (Becton Dickinson, Lincoln Park, N.J.) and centrifuged at 8,000 × *g* for 5 min at 4°C. Supernatant was discarded, and the pellet was resuspended in 10 ml of STE (100 mM Tris-HCl [pH 8], 10 mM EDTA [pH 8], 150 mM NaCl) before being centrifuged again at 8,000 × *g* for 5 min at 4°C. The pellet was resuspended in 2 ml of 10× TE (100 mM Tris-HCl [pH 8], 10 mM EDTA [pH 8]), and 4 μl of lysozyme (100 mg/ml; Boehringer Mannheim, Indianapolis, Ind.) was added to achieve a final concentration of 200 μg/ml in the sample. Samples were incubated at room temperature for 10 min, and 200 μl of 10% sodium dodecyl sulfate (SDS) was added to a final concentration of 1%. Incubation in a water bath at 65°C for 15 min followed; 40 μl of proteinase K (10 mg/ml; Boehringer Mannheim) was added to a final concentration of 200 μg/ml. Samples were incubated again in a water bath at 65°C for 1 to 2 h. Extraction with phenol was performed as described earlier (44). The upper aqueous layer was transferred to a new tube, and 150 μl of 3 M NaCl was added to a final concentration of 300 mM. DNA was precipitated with 2 volumes (3 ml) of cold 95% ethanol and transferred to a clean 1.5-ml-volume Eppendorf tube. DNA was washed twice with 1 ml of cold 95% ethanol (−20°C) and centrifuged at 13,000 × *g* for 2 min. Excess ethanol was drained, and the pellet was dried at 37°C for 30 to 45 min before being resuspended in 1 ml of sterile water and stored at −20°C.

(ii) Guanidium thiocyanate extraction procedure. The guanidium thiocyanate extraction procedure was performed as described earlier by Pitcher et al. (39).

(iii) DNA purification. DNAs obtained by both methods were purified as described earlier (40). Briefly, a spin column was prepared by piercing the bottom of a 1.5-ml-volume Eppendorf tube with a 25-gauge needle; 25 μl of siliconized glass beads (200 to 300 μm in diameter) and 1 ml of CL6B slurry (60% CL6B in TE; Pharmacia, Uppsala, Sweden) were added. A spin column was placed in another 1.5-ml-volume Eppendorf tube and centrifuged at 600 × *g* for 5 min. The column was then transferred to a new 1.5-ml-volume Eppendorf tube. One microliter of RNase (10 mg/ml; Boehringer Mannheim) to a final concentration of 100 mg/ml and 5 μl of the 10× loading buffer (30% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol) were added to 100 μl of the extracted DNA, and samples were then incubated in a water bath at 65°C for 15 min. The total volume (100 μl) of the DNA sample was added to the prepared spin column and centrifuged at 600 × *g* for 5 min. Collected DNA was stored at −20°C. Alternatively, Quick Spin Columns (Boehringer Mannheim) were used according to the manufacturer's instructions.

Restriction endonuclease digestion of chromosomal DNA. The amount of DNA in each sample was determined by the minigel method (44). Preliminary chromosomal digestion experiments were conducted with seven restriction enzymes (*Bgl*I, *Bgl*II, *Hin*dIII, *Nci*I, *Nco*I, *Pvu*II, and *Xba*I; 2 μl of each enzyme; New England BioLabs, Inc., Beverly, Mass.) and DNA from five *V. cholerae* O1 strains to determine the optimal enzyme for ribotyping. Approximately 2 μl of DNA of each strain was digested with 2 μl of *Bgl*I (10 U/μl) for a total of 2 h at 37°C in a water bath.

Electrophoresis and Southern blotting of restricted DNA fragments. Following restriction, samples were heated at 65°C for 10 min and cooled on ice for 5 min. DNA fragments were separated by horizontal electrophoresis through a 0.8% agarose gel at 60 V for 16 h. Molecular size standards, ladder and lambda (GIBCO BRL), were included in the last lane of each gel. Gels were stained with ethidium bromide (1 μg/μl) for 30 min and photographed with type 55 Polaroid film (ISO 50; Polaroid Corporation, Cambridge, Mass.). DNA was transferred to a nylon membrane (20 by 20 cm) (MSI, Westboro, Mass.) by the method of Southern as modified by Kiehlbauch et al. (29).

Preparation of digoxigenin-labeled cDNA probe for 16S and 23S RNA genes. *Escherichia coli* 16S and 23S rRNA (Boehringer Mannheim) was used to prepare the digoxigenin-labeled cDNA by a reverse transcription reaction with a mixture containing 2 μl of 10× primers from the Genius 2 kit (Boehringer Mannheim), 4 μl of 5× reaction buffer for reverse transcriptase (250 mM Tris-HCl [pH 8.3], 250 mM KCl, 50 mM $MgCl_2$, 5 mM spermidine; Promega, Madison, Wis.), 0.25 μl of rRNA (4 μg/μl; Boehringer Mannheim), and 10.75 μl of deionized, distilled, sterile H_2O. The mixture was heated at 68°C for 5 min and allowed to cool slowly to room temperature; 2 μl of 10× deoxynucleoside triphosphates (Boehringer Mannheim) and 3.0 μl of avian myeloblastosis virus reverse transcriptase (5 to 10 U/μl; Promega) were added, mixed, and incubated at 42°C for 1 h. The probe was precipitated by 2 μl of 4 M LiCl and 50 μl of cold 95% ethanol and centrifuged at 13,000 × *g* for 15 min. The supernatant was removed, and the pellet was washed twice in 100 μl of 70% ethanol and dried for 15 min under vacuum. The pellet was redissolved in 20 μl of TE, transferred into a tube containing 10 ml of hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% blocking reagent [Boehringer Mannheim], 0.1% lauroylsarcosine [wt/vol], 0.002% SDS [wt/vol]), and stored at −20°C for at least a year.

Preparation of digoxigenin-labeled molecular size standards. A 1-kb ladder (1 μl; GIBCO BRL) and *Hin*dIII restriction digest fragments of lambda phage DNA (1 μl; GIBCO BRL) were digoxigenin labeled with the Genius Kit 2 (Boehringer Mannheim) by following the manufacturer's recommendations for the random priming procedure. The labeled DNA was precipitated with 2 μl of 4 M LiCl and 60 μl of cold ethanol and held at −70°C for at least 30 min or at −20°C for 2 h and then centrifuged for 5 min at 13,000 × *g*. Pelleted DNA was washed with 100 μl of cold 70% ethanol and dried under vacuum for 15 min. It was dissolved in 50 μl of TE (pH 8) and transferred to a tube with 10 ml of the hybridization solution; 10 μl of that solution was added to 10 ml of hybridization solution containing the cDNA probe.

TABLE 1. Origins and ribotypes of 214 *V. cholerae* O1 strains

Strain and/or origin (site of isolation and/or reference)	No. of strains	Source	Yr	Ribotype[a]
Classical biotype strains				
India	1	Human	1949	1a
Bangladesh	1	Human	1970	1b
Hong Kong	1	Human	1939	1b
India	1	Human	1941	1b
Iran	1	Human	1960	1b
Japan	1	Unknown	1921	1b
Pakistan	1	Human	1959	1b
Unknown (NIH 404)	1	Unknown	1922	1c
India	1	Human	1941	1c
Bangladesh	1	Human	1972	1d
Burma	1	Human	1961	1e
Bangladesh	1	Human	1960	1e
Bangladesh	1	Human	1980	1e
Pakistan	1	Human	1960	1e
India	1	Human	1940	1f
India	1	Human	1960	1g
U.S. Gulf Coast strains				
Florida (30)	1	Human	1986	2
Georgia[b]	1	Human	1984	2
Louisiana (7)	2	Human	1978–1992	2
Louisiana (33)	1	Sewage	1986	2
Maryland (32)	1	Human	1984	2
Mississippi	1	Human	1984	2
Texas (26, 54)	4	Human	1973–1992	2
Asia (Costa Rica)	1	Human	1992	3
Guinea-Bissau	1	Human	1988	3
Honduras	1	Human	1992	3
Indonesia	1	Human	1961	3
Thailand (Maryland) (13)	1	Human	1991	3
Philippines	1	Human	1963	3
Truk	1	Human	1982	3
Gilbert Islands	1	Human	1977	4
Ribotype 5 strains isolated in Latin America				
Belize	1	Human	1992	5
Bolivia	4	Human	1991	5
Bolivia	3	Water	1991	5
Bolivia	1	Carrots	1991	5
Brazil	1	Human	1992	5
Chile	8	Human	1991	5
Colombia	2	Human	1991	5
Ecuador	2	Human	1991	5
Ecuador	1	Conchas	1991	5
El Salvador[c]	4	Human	1991–1992	5
Guatemala	6	Human	1991–1992	5
Honduras	1	Human	1992	5
Mexico	5	Human	1991–1992	5
Mexico	1	Water	1991	5
Nicaragua	2	Human	1992	5
Peru	13	Human	1991–1992	5
Peru	6	Water	1991	5
Venezuela	1	Human	1991	5
Japan[d]	1	Human	1992	5
Ribotype 5 strains isolated in the United States associated with Latin American epidemic				
South America (Alabama)	11	Oysters	1991	5
South America (Alabama) (34)	7	Ship water	1991	5
Peru (California)	1	Human	1992	5
El Salvador (California)	2	Human	1992	5
South America (California) (14)	3	Human	1992	5
Ecuador (Connecticut)	2	Human	1992	5
Venezuela (Florida)	1	Human	1992	5
Ecuador (Florida)	1	Human	1992	5
Peru (Georgia) (10)	1	Human	1991	5
South America (Louisiana) (34)	1	Ship water	1991	5
Venezuela (Louisiana)	1	Human	1992	5
Brazil (Maryland)	1	Human	1991	5
El Salvador (Maryland)	1	Human	1992	5
Peru (Maryland)	1	Human	1992	5
Ecuador (New Jersey) (11)	2	Human	1991–1992	5
Ecuador (New York) (12)	4	Human	1991–1992	5
Mexico (Texas)	2	Human	1992	5
El Salvador (Texas)	1	Human	1992	5
Ribotype 5 strains isolated worldwide and not associated with Latin American epidemic				
Cambodia (Washington)	2	Human	1992	5
Malawi	2	Human	1990	5
Philippines	1	Human	1987	5
Romania[c]	6	Human	1977–1991	5
Romania	2	Water	1992	5
Thailand (California)	1	Human	1990	5
Thailand (New Jersey)	1	Human	1990	5
Thailand (Maryland) (13)	1	Human	1991	5
Truk	2	Human	1990	5
Uganda	1	Human	1992	5
Vietnam (California)	1	Human	1992	5
Unknown (California)	1	Human	1992	5
Bangladesh	2	Human	1992	6a
Brazil	1	Human	1992	6a
India	1	Human	1990	6a
Mexico	4	Human	1991–1992	6a
Pakistan	1	Human	1992	6a
Romania	2	Human	1991	6a
Hawaii	2	Human	1991	6b
Indonesia (California)	1	Human	1992	6c
Philippines (California)	1	Human	1992	6c
Philippines (Hawaii)	1	Human	1992	6c
Philippines (New York)	1	Human	1992	6c
Texas[b]	1	Human	1992	6c
Guam	2	Human	1988–1990	7
Burundi	2	Human	1992	8
Rwanda	1	Human	1991	8
Uganda	1	Human	1992	8
Zambia	6	Human	1991	8
Australian strains				
Beenleigh (9, 20, 42)	1	Human	1977	9
Burdekin River (9, 20)	1	Human	1984	9
Beenleigh (9, 20, 42)	1	Human	1977	10
Nontoxigenic strains				
Peru	2	Human	1987	11
Mexico	2	Sewage	1983	12
Brazil	1	Sewage	1978	13
Florida	1	Human	1980	14
Louisiana	1	Sewage	1986	14
El Salvador	1	Water	1991	14
Alabama	1	Fish	1991	15
Louisiana	1	Sewage	1991	15
Guatemala	1	Water	1991	15
Alabama	1	Water	1991	16
United States	4	Aquatic birds	1992	17
Bolivia	1	River water	1991	17
Trinidad	1	Water	1992	18
Brazil	1	Human	1992	19

[a] All strains of ribotypes 11 to 19 are nontoxigenic.
[b] Nontoxigenic strain.
[c] One strain nontoxigenic.
[d] Nontoxigenic strain associated with the airline cholera outbreak (14).

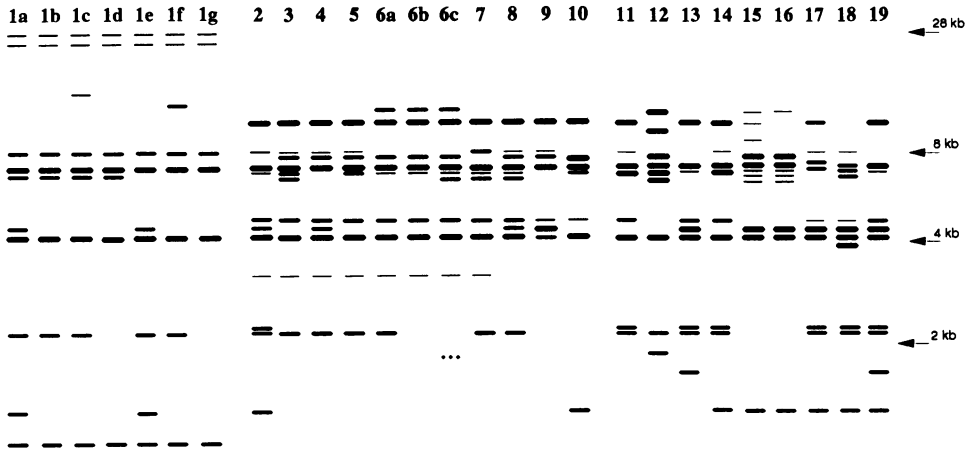

FIG. 1. Schematic presentation of the 27 *BglI* ribotypes among 214 toxigenic and nontoxigenic *V. cholerae* O1 strains. Lane designations correspond to the ribotype designations given in Table 2. The widths of the lines reflect observed intensities of the bands on the membrane. The dotted line indicates the inconsistent presence of a band. Molecular sizes are indicated by numbers on the right.

The remaining digoxigenin-labeled molecular size standards were stored at −20°C.

Hybridization with digoxigenin-labeled cDNA probe and detection. Nylon membranes with immobilized DNA restriction fragments were pretreated with hybridization solution in a sealed plastic bag (25 by 30 cm) for 1 h. Ten milliliters of the hybridization solution containing the digoxigenin-labeled cDNA probe and molecular size standards was boiled for 10 min and cooled on ice for 5 min. Prehybridization solution was then discarded and replaced by hybridization solution. Membranes were hybridized overnight at 68°C in a water bath. DNA restriction fragments which hybridized with the digoxigenin-labeled probe were detected colorimetrically with an alkaline phosphatase-labeled anti-digoxigenin Fab fragment, as recommended by the manufacturer of the Genius Kit 3 (Boehringer Mannheim).

RESULTS

Evaluation of the extraction procedure. There were no apparent differences in ribotyping patterns whether DNA was extracted by the guanidium thiocyanate or the phenol method. The DNA obtained by the first method was stable for only 3 to 4 weeks, while DNA extracted with phenol was stable for more than a year when stored at −20°C.

Assessment of the suitability of different restriction endonucleases. DNA isolated from five strains of different origins was digested with a test panel of seven different restriction enzymes, and *BglI* gave optimal patterns of rRNA genes regarding the number, diversity, and distribution of the bands, thus allowing for good discrimination (data not shown).

Stability and reproducibility of *BglI* restriction patterns. Ribotype patterns were stable and no differences were recorded for DNA from two strains (2164-78 and C 6709, proposed ribotype reference strains 2 and 5, respectively) that were ribotyped 10 times each, from four separate DNA extracts of a single-colony growth, cultured from the original frozen stock culture. The remaining 25 ribotype reference strain patterns differed only in overall intensity and degree of background primarily associated with the differences in the concentration of the DNA in the analyzed samples. All ribotype reference strains were tested at least four times, and all study strains were tested at least twice. Repeat experiments were often performed in a blinded fashion.

Stability of the digoxigenin-labeled cDNA probe. The digoxigenin-labeled cDNA probe was stable for at least 1 year when stored at −20°C and was reused up to five times without any changes in the intensities of the hybridized bands.

***BglI* ribotype patterns.** Among the 214 *V. cholerae* O1 strains analyzed, 27 different *BglI* rRNA gene restriction patterns were observed. The chromosomal fragments containing rRNA genes ranged in size from 1 to 28 kb. All strains contained common fragments of 4.1 and 6.2 kb. Among the 198 *V. cholerae* O1 strains of the El Tor biotype, there were 20 different *BglI* ribotype patterns. Sixteen classical strains isolated worldwide over 60 years (1922 to 1980) were typed, and seven different patterns were observed. Fragments specific for the classical strains were 1.7, 24, and 28 kb in size (Fig. 1).

Ribotype reference strains and patterns. A schematic representation of the ribotype patterns obtained with *BglI* is presented in Fig. 1. A list of proposed ribotype reference strains with their designations and sources is given in Table 2. Strains were classified according to the similarities in their patterns. All of the classical biotype strains were designated ribotype 1 with subtypes 1a to 1g on the basis of the similarity of their ribotype patterns and the differences between ribotype 1 and the patterns of all El Tor strains. Likewise, ribotype 6 strains were further classified into three subtypes because they possessed a unique pair of bands (10 and 11.5 kb) not seen in any other ribotype. Subcultures of the 27 proposed *V. cholerae* O1 ribotype reference strains will be donated to the American Type Culture Collection.

Epidemiologic application of the ribotype scheme. (i) The classical biotype. A total of 16 strains isolated worldwide over 60 years were assayed, and seven different but very similar ribotypes were observed (Fig. 2). Strains of ribotype 1b were isolated over 50 years.

(ii) Seventh pandemic strains (except for those from the Western Hemisphere and Australia). Strains of ribotypes 3, 4, 5, 6, 7, and 8 were isolated worldwide over 30 years (1963 to 1992) (Tables 1 and 3). Strains belonging to ribotype 6 had a unique pair of bands at 10 and 11.5 kb and were classified into three subtypes (subtypes 6a to 6c). Ten strains belong-

TABLE 2. Proposed *V. cholerae* O1 ribotype reference strains for *BglI* ribotypes 1 to 19: sources and phenotypic and genotypic characteristics

BglI ribotype	Proposed ribotype reference strain	Country	Yr	Source	Biotype	Serotype	*ctxB* genotype[a]	PCR[b] result	ET[c]
1a	9060	India	1949	Human	Classical	Ogawa	I	+	ND
1b	NIH 41	India	1941	Human	Classical	Ogawa	ND	+	ND
1c	NIH 35	India	1941	Human	Classical	Inaba	ND	+	ND
1d	J 139	Bangladesh	1972	Human	Classical	Ogawa	ND	+	ND
1e	B 1307	Bangladesh	1960	Human	Classical	Ogawa	ND	+	ND
1f	569B	India	1940	Human	Classical	Inaba	ND	+	ND
1g	RV 2	India	1960	Human	Classical	Inaba	I	+	ND
2	2164-78	Louisiana	1978	Human	El Tor	Inaba	I	+	2
3	E 9956	Philippines	1963	Human	El Tor	Ogawa	III	+	3
4	E 8260	Gilbert Islands	1966	Human	El Tor	Inaba	III	+	3
5	C 6706	Peru	1991	Human	El Tor	Inaba	III	+	4
6a	C 7754	Romania	1991	Human	El Tor	Ogawa	ND	+	3
6b	C 7861	Hawaii	1991	Human	El Tor	Ogawa	ND	+	3
6c	C 8641	California	1992	Human	El Tor	Ogawa	ND	+	3
7	2560-90	Guam	1990	Human	El Tor	Ogawa	III	+	3
8	2432-88	Rwanda	1988	Human	El Tor	Ogawa	III	+	3
9	2463-88	Australia	1984	Human	El Tor	Inaba	II	+	1
10	2270-77	Australia	1977	Human	El Tor	Inaba	II	+	1
11	2583-87	Peru	1987	Human	El Tor	Ogawa	NT	−	2
12	CM 91-3	Mexico	1983	Sewage	El Tor	Ogawa	NT	−	5
13	1074-78	Brazil	1978	Sewage	El Tor	Ogawa	NT	−	6
14	C 7724	El Salvador	1991	Water	El Tor	Ogawa	NT	−	2
15	C 7641	Guatemala	1991	Water	El Tor	Inaba	NT	−	ND
16	C 7654	Alabama	1991	Water	El Tor	Inaba	NT	−	ND
17	C 7965	Bolivia	1991	River water	El Tor	Ogawa	NT	−	ND
18	C 8108	Trinidad	1992	Water	El Tor	Inaba	NT	−	ND
19	C 8481	Brazil	1991	Human	El Tor	Ogawa	NT	−	ND

[a] *ctxB*, cholera toxin B subunit (37).
[b] PCR, polymerase chain reaction (22).
[c] ET, electrophoretic type by multilocus enzyme electrophoresis (53); NT, nontoxigenic; ND, not done.

ing to ribotype 8 were isolated over the past 2 years from patients in four central African countries.

(iii) Strains associated with the Latin American epidemic. A total of 127 strains belonged to ribotype 5; 104 toxigenic strains isolated in 1991 and 1992 were associated with the present Latin American epidemic. Of those, 61 were isolated in 14 Latin American countries, and 43 strains were isolated in the United States. Twenty-four of the U.S. isolates were from persons who travelled to Latin America and became ill there or who ate food bought at local markets in Latin American countries and brought into the United States by the traveler. Eleven strains were isolated from oysters harvested along the U.S. Gulf Coast, and 8 were isolated from the ballast and bilge water of ships arriving from Latin America (Fig. 3). Additionally, 13 toxigenic strains of ribotype 5 were isolated from patients and the environment in five different countries worldwide (Romania, Truk [one of the Federated States of Micronesia], Malawi, Uganda, and the Philippines) and from seven patients in the United States upon their return from Thailand, Cambodia, and Vietnam.

(iv) Strains associated with the U.S. Gulf Coast. Eleven isolates classified as ribotype 2 were associated with the U.S. Gulf Coast over the past 20 years. Ten isolates were from persons with cholera for whom seafood caught in Gulf waters was implicated as the possible source of infection.

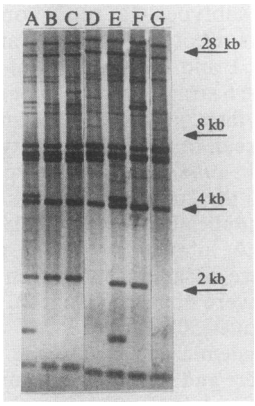

FIG. 2. *BglI* ribotypes of *V. cholerae* O1 strains of the classical biotype. Ribotypes correspond to the reference strain designations given in Table 2. Lanes: A, 1a; B, 1b; C, 1c; D, 1d; E, 1e; F, 1f; G, 1g. Molecular sizes are indicated by numbers on the right.

TABLE 3. Geographic origins of 190 toxigenic *V. cholerae* O1 strains

Geographic origin	No. of strains	No. of strains with the following *BglI* ribotype:									
		1	2	3	4	5	6	7	8	9	10
Asia	34	15		4		7	8				
Africa	14			1		3			10		
Australia	3									2	1
Latin America	108					104	4				
Pacific Islands	6			1	1	2		2			
United States	10		10								
Unknown	15	1		1		8	5				
Total	190	16	10	7	1	124	17	2	10	2	1

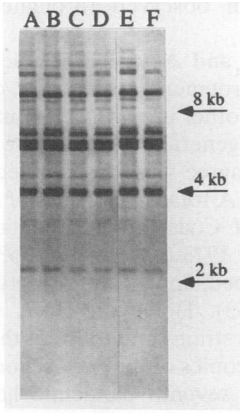

FIG. 3. *BglI* ribotype 5 of *V. cholerae* O1 strains associated with the Latin American epidemic. Explanations of lanes include strain designation, source, country or state, and year of isolation. Lanes: A, C 6706, human, Peru, 1991; B, C 7679, seawater, Peru, 1991; C, C 8233, carrots, Bolivia, 1992; D, C 8034, Connecticut, 1991; E, C 7403, oysters, Alabama, 1991; F, C 7869, ship water, Alabama, 1991. Molecular sizes are indicated by numbers on the right.

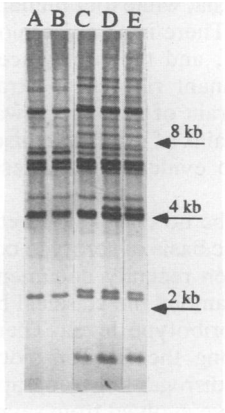

FIG. 4. *BglI* ribotypes 5, 14, and 2 of toxigenic and nontoxigenic *V. cholerae* O1 strains. Explanations of lanes include ribotype, strain designation, source, country or state, and year of isolation. Lanes: A, ribotype 5, toxigenic *V. cholerae* O1, C 6706, human, Peru, 1991; B, ribotype 5, nontoxigenic *V. cholerae* O1, C 7878, human, El Salvador, 1991; C, ribotype 14, nontoxigenic *V. cholerae* O1, 2741-80, human, Florida, 1980; D, ribotype 2, toxigenic *V. cholerae* O1, 2489-86, human, Florida, 1986; E, ribotype 2, nontoxigenic *V. cholerae* O1, 917-84, human, Georgia, 1984. Molecular sizes are indicated by numbers on the right.

(v) Strains isolated in Australia. Two human isolates from Beenleigh (1977) and the Burdekin River area (1984) were ribotype 9; a single strain from a cholera patient in Beenleigh (1977) was ribotype 10.

(vi) Nontoxigenic *V. cholerae* O1 strains. A total of 24 nontoxigenic strains were included in this study; 19 of them were classified as ribotypes 11 to 19. The majority were environmental strains; only four were isolated from humans. Ribotypes 11, 12, 13, 18, and 19 contained nontoxigenic strains from Latin American countries, while ribotypes 14, 15, 16, and 17 contained strains from Latin American countries, the United States, and Africa. Three human isolates from El Salvador, Japan, and Romania were ribotype 5, which is typical of the toxigenic strains causing the present Latin American epidemic. A single nontoxigenic strain from a patient in Georgia belonged to ribotype 2, which contains 10 toxigenic strains associated with the U.S. Gulf Coast (Fig. 4). Also, a single nontoxigenic strain isolated in Texas belonged to ribotype 6c.

DISCUSSION

Epidemiologic studies of cholera have been hindered by the limited discrimination provided by phenotypic characteristics of *V. cholerae* O1 strains. The traditional typing systems based on serotype, antimicrobial susceptibility, phage typing, and physiological properties have major disadvantages and provide limited specificity and/or reproducibility. We propose a standardized scheme of 27 different *BglI* ribotypes based on data from 214 human and environmental strains of *V. cholerae* O1 isolated in 35 countries and 14 U.S. states over the past 60 years. Our data show that the ribotype patterns are reproducible and stable over time and that the digoxigenin-labeled cDNA probe for rRNA is relatively simple to prepare and could be reused at least five times. This scheme applies only to *V. cholerae* O1 strains.

Classical biotype strains. Koblavi et al. demonstrated four different *BglI* ribotyping patterns among 14 classical *V. cholerae* O1 strains from Asia and Africa (31). Our data agree with those of Koblavi et al., but our analysis included isolates from more broadly representative geographic areas and periods. We have observed seven different ribotype patterns among 16 classical strains. Strains of subtype 1b predominate and were isolated in eight Asian and African countries over 50 years. On the other hand, strains from India were represented in six of seven observed subtypes of ribotype 1. These data suggest the wide circulation of several clones of the classical biotype and disagree with previous studies indicating the clonality of this group. An earlier study included all classical strains tested in this study, except for the strains of the 1a subtype, and found that 34 of 42 strains of the classical biotype had identical plasmid profiles and RFLPs of VcA1 genes (17).

Seventh pandemic strains. Strains causing the current seventh cholera pandemic belong to several different ribotypes (ribotypes 3 to 8). Although seventh pandemic strains belong to the same cholera toxin B subunit (*ctxB*) genotype (37) and electrophoretic type (ET) 3 (53), ribotyping could further differentiate them in general relationship to their geographic origin. Ribotyping could also facilitate identification of the strain involved in a specific outbreak in areas where several *V. cholerae* O1 ribotypes are present simultaneously. Strains of ribotype 8 were isolated only from patients in the central African countries of Burundi, Rwanda, Uganda, and Zambia. These data agree with previous observations that strains of this pattern are most frequently found in Africa (31). *V. cholerae* O1 strains isolated in Truk in 1980 are ribotype 3. However, recently isolated strains are ribotype 5. This does not support the hypothesis of the long-term persistence of a single strain. Rather, it suggests either a simultaneous coexistence of two strains or a new endemic focus (49). Only two strains in this study belong to ribotype 4, and these are the only isolates from Guam; it would be interesting to study earlier and later Guam isolates. Ribotype 6 strains isolated worldwide over the past 2 years were unusual because they have a distinct pair of bands separating them from other seventh pandemic strains. Although only 18 strains in this study were of this ribotype, they exhibited diverse geographic origins. Eight of

them originated in Asia, while four human isolates were from Mexico and Brazil. There is no epidemiologic evidence that they were imported, and their presence in Latin America among the predominant ribotype 5 strains remains unexplained. A single strain of ribotype 3 was isolated in Honduras. As for the strains of ribotype 6a isolated in Brazil and Mexico, there is no evidence that these strains were imported.

There appears to be no correlation between serotype and ribotype. The genetic basis of serotype conversion within *V. cholerae* O1 has been recently documented (47). Although there was diversity among the classical biotype strains, this occurred at the subribotype level. There appears to be a great diversity among the El Tor biotype strains of the seventh pandemic, disregarding geographic differences. El Tor strains might have evolved from several parental strains or clones; the mutation rate in this biotype is high enough to explain worldwide differences.

Latin American epidemic. More than one-half of the strains in this study (127 strains) were ribotype 5; 61 toxigenic human and environmental *V. cholerae* O1 strains originated in 14 Latin American countries. Additionally, 24 strains were from persons in the United States who have developed cholera associated with either traveling to Latin America or eating the seafood from this region. Two of those strains were from patients with cholera associated with consumption of food served on an airline flight from Argentina to Los Angeles (14). The vast majority of *V. cholerae* O1 strains associated with the current epidemic in Latin America belong to ribotype 5; these isolates also belong to ET 4 (53). Ribotype 5 strains were also isolated worldwide over the past 15 years, but these non-Latin American ribotype 5 isolates generally belong to the seventh pandemic ET 3 (53). Initial reports (52) indicated that the Latin American epidemic was an extension of the seventh pandemic, and our data support this. It is difficult to address the differences in electrophoretic type as reported elsewhere (53); identification of the genetic basis for that difference may eventually resolve these issues. Our data do not agree with those of Faruque and Albert (21), who used *Hin*dIII ribotypes to link *V. cholerae* O1 from Bangladesh with those from Latin America. Our experience with *Hin*dIII and other enzymes indicates that they are not as discriminatory as *Bgl*I. Furthermore, Almeida has previously reported that all tested *V. cholerae* O1 strains of both the classical and El Tor biotypes had identical ribotype patterns when *Hin*dIII was used for restriction of *V. cholerae* O1 chromosomal DNA (1).

Isolation of a *V. cholerae* O1 strain of ribotype 5 and ET 4 from a commercial oyster bed in Mobile Bay, Ala., in the summer of 1991 (19) generated concern that the U.S. Gulf Coast shellfish beds were contaminated with the Latin American *V. cholerae* O1 strain. Consequently, eight such strains were isolated from the ballast and bilge water of ships arriving in U.S. Gulf Coast ports from Latin America (34). These data support the hypothesis that the Latin American *V. cholerae* O1 strains were introduced into Gulf Coast waters by ships. These environmental studies do raise the possibility that *V. cholerae* O1 is widely transported by ships; this may be one explanation for the origin of the Latin American epidemic beginning on the coast of Peru. The Latin American epidemic seems to have started with the introduction of a single clone of *V. cholerae* O1. From our observations over the first 2 years and during its introduction to most Latin American countries, it seems that the clone is generally conserved, and no phenotypic or genotypic changes have been observed throughout its geographic spread.

U.S. Gulf Coast and Australia. Cholera has two documented foci of environmental reservoirs, one along the U.S. Gulf Coast and the other in northeast Australia (7, 9, 20, 33, 35, 42). Our data generally support the unique nature of these isolates and agree with their molecular characterization (2, 16, 27, 53). A total of 65 cases of cholera have been related to the Gulf Coast environmental reservoir in the United States since 1973 (2, 16, 27, 41, 53). Eleven of these strains were ribotyped, and all were ribotype 2; they were of a distinct ET 2 (53). However, DNA sequence analysis indicated that these strains and those of the classical biotype have two identical copies of the *ctxB* sequence (37). Other El Tor strains of the seventh pandemic had one copy of a different gene sequence. Likewise, three strains from Australia also have exceptional properties. They have a unique *ctxB* sequence (37) and belong to ET 1 (53).

Nontoxigenic *V. cholerae* O1 strains. Ribotypes 11 through 19 contained nontoxigenic *V. cholerae* O1 strains that originated in Latin America and the U.S. Gulf Coast. These strains did not form a specific cluster of ribotypes and did not contain unique common bands. One nontoxigenic isolate was ribotype 2, identical to the toxigenic U.S. Gulf Coast strains. Additionally, two nontoxigenic Gulf Coast isolates (one human and one environmental) were ribotype 14; patterns of ribotypes 2 and 14 differ only by a single 4.5-kb band. These strains of ribotypes 2 and 14 belonged to the Gulf Coast ET 2. This supports the hypothesis that nontoxigenic isolates constitute a heterogeneous group of organisms, but by multilocus enzyme electrophoresis, some of the nontoxigenic strains are indistinguishable from the toxigenic Gulf Coast clone. That subgroup, in our study designated ribotype 14, could theoretically represent either a precursor or a descendent of the U.S. Gulf Coast toxigenic isolates.

Three nontoxigenic strains were ribotype 5, which contains 124 other toxigenic strains associated with the present Latin American epidemic. One strain was isolated from a cholera patient from El Salvador, and another one was isolated from a patient in Romania. The third strain was from a passenger involved in the cholera outbreak associated with the consumption of airline food (14). A total of 76 passengers were infected, and all other *V. cholerae* O1 isolates were toxigenic; in this study we have ribotyped two of the toxigenic strains from this outbreak, and they were also ribotype 5. These data seem to further support the hypothesis that some nontoxigenic strains may be derivatives of toxigenic *V. cholerae* O1.

Application of the proposed ribotype scheme has allowed us to trace the specific movement of strains, to identify their geographic origins, and to examine diversity among *V. cholerae* O1 strains, such as those from certain environmental reservoirs. Strains causing the present Latin American epidemic have been clearly distinguished from the strains associated with the U.S. Gulf Coast and from other seventh pandemic strains causing cholera worldwide. With the proposed scheme, the use of radioactivity is avoided and the technique is standardized, reliable, and reproducible. More new ribotypes and subtypes will be added to the proposed scheme as more, primarily reference, laboratories start to use ribotyping of *V. cholerae* O1 strains as a supplementary typing method.

REFERENCES

1. **Almeida, R. J.** 1990. Identification and differentiation of pathogenic and epidemic *V. cholerae* O1 strains. Ph.D. dissertation. University of North Carolina, Chapel Hill.
2. **Almeida, R. J., D. N. Cameron, W. L. Cook, and I. K. Wachsmuth.** 1992. Vibriophage VcA-3 as an epidemic strain marker for the U.S. Gulf Coast *V. cholerae* O1 clone. J. Clin. Microbiol. **30:**300–304.
3. **Altwegg, M., F. W. Hickman-Brenner, and J. J. Farmer.** 1989. Ribosomal RNA gene restriction patterns provide increased sensitivity for typing *Salmonella typhi* strains. J. Infect. Dis. **160:**145–149.
4. **Barrett, T. J., and P. A. Blake.** 1981. Epidemiological usefulness of changes in hemolytic activity of *Vibrio cholerae* biotype El Tor during the seventh pandemic. J. Clin. Microbiol. **13:**126–129.
5. **Barrett, T. J., D. N. Cameron, and I. K. Wachsmuth.** 1992. Molecular typing of *V. cholerae* O1 by pulsed-field gel electrophoresis, p. 453. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, D.C.
6. **Benenson, A.** 1991. Cholera, p. 207–225. *In* A. S. Evans and P. S. Brachman (ed.), Bacterial infections of humans. Plenum Medical Book Company, New York.
7. **Blake, P. A., D. T. Allegra, J. D. Snyder, T. J. Barrett, L. McFarland, C. T. Caraway, J. C. Feeley, J. P. Craig, J. V. Lee, N. D. Puhr, and R. A. Feldman.** 1980. Cholera—a possible endemic focus in the United States. N. Engl. J. Med. **302:**305–309.
8. **Blumberg, H. M., J. A. Kiehlbauch, and I. K. Wachsmuth.** 1991. Molecular epidemiology of *Yersinia enterocolitica* O:3 infections: use of chromosomal DNA restriction fragment length polymorphisms of rRNA genes. J. Clin. Microbiol. **29:**2368–2374.
9. **Bourke, A. T. C., Y. N. Cossins, B. R. W. Gray, T. J. Lunney, N. A. Rostron, R. V. Holmes, E. R. Groggs, D. J. Larsen, and V. R. Kelk.** 1986. Investigation of cholera acquired from the riverine environment in Queensland. Med. J. Aust. **144:**229–234.
10. **Centers for Disease Control.** 1991. Importation of cholera from Peru. Morbid. Mortal. Weekly Rep. **40:**258–259.
11. **Centers for Disease Control.** 1991. Cholera—New Jersey and Florida. Morbid. Mortal. Weekly Rep. **40:**287–289.
12. **Centers for Disease Control.** 1991. Cholera—New York. Morbid. Mortal. Weekly Rep. **40:**516–518.
13. **Centers for Disease Control.** 1991. Cholera associated with imported frozen coconut milk. Morbid. Mortal. Weekly Rep. **40:**844–845.
14. **Centers for Disease Control.** 1992. Cholera associated with an international airline flight, 1992. Morbid. Mortal. Weekly Rep. **41:**134–135.
15. **Centers for Disease Control.** 1992. Update: cholera—Western Hemisphere, 1992. Morbid. Mortal. Weekly Rep. **42:**89–91.
16. **Chen, F., G. M. Evins, W. L. Cook, R. Almeida, N. Hargrett-Bean, and K. Wachsmuth.** 1991. Genetic diversity among toxigenic and nontoxigenic *Vibrio cholerae* O1 isolated from the Western Hemisphere. Epidemiol. Infect. **107:**225–233.
17. **Cook, W. L., K. Wachsmuth, S. R. Johnson, K. A. Birkness, and A. R. Samadi.** 1984. Persistence of plasmids, cholera toxin genes, and prophage DNA in Classical *Vibrio cholerae* O1. Infect. Immun. **45:**222–226.
18. **De Buyser, M. D., A. Morvan, F. Grimont, and N. E. Solh.** 1989. Characterization of *Staphylococcus* species by ribosomal RNA gene restriction patterns. J. Gen. Microbiol. **135:**989–999.
19. **DePaola, A., G. M. Capers, M. L. Motes, Ø. Olsvik, P. I. Fields, J. G. Wells, I. K. Wachsmuth, T. A. Cebula, W. H. Koch, F. Khambaty, M. H. Kothary, W. L. Payne, and B. A. Wentz.** 1992. Isolation of Latin American epidemic strain of *Vibrio cholerae* O1 from the U.S. Gulf Coast. Lancet **339:**624.
20. **Desmarchelier, P. M., and C. R. Senn.** 1989. A molecular epidemiological study of *V. cholerae* in Australia. Med. J. Aust. **150:**631–634.
21. **Faruque, S. M., and J. Albert.** 1992. Genetic relations between *Vibrio cholerae* O1 strains in Ecuador and Bangladesh. Lancet **339:**740–741.
22. **Fields, P. I., T. Popovic, K. Wachsmuth, and Ø. Olsvik.** 1992. Use of polymerase chain reaction for detection of toxigenic *Vibrio cholerae* O1 strains from the Latin American cholera epidemic. J. Clin. Microbiol. **30:**2118–2121.
23. **Gangarosa, E. J., A. Sanati, H. Saghari, and J. C. Feeley.** 1967. Multiple serotypes of *Vibrio cholerae* isolated from a case of cholera. Lancet **i:**646–648.
24. **Grimont, F., and P. A. D. Grimont.** 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Ann. Inst. Pasteur/Microbiol. **137B:**165–175.
25. **Hinojosa-Ahumada, M., B. Swaminathan, S. B. Hunter, D. N. Cameron, J. A. Kiehlbauch, I. K. Wachsmuth, and N. A. Strockbine.** 1991. Restriction fragment length polymorphisms in rRNA operons for subtyping *Shigella sonnei*. J. Clin. Microbiol. **29:**2380–2384.
26. **Johnston, J. M., D. L. Martin, J. Perdue, L. M. McFarland, C. T. Caraway, E. C. Lippy, and P. A. Blake.** 1983. Cholera on a Gulf Coast oil rig. N. Engl. J. Med. **309:**523–526.
27. **Kaper, J. B., H. B. Bradford, N. C. Roberts, and S. Falkow.** 1982. Molecular epidemiology of *Vibrio cholerae* in the U.S. Gulf Coast. J. Clin. Microbiol. **16:**129–134.
28. **Kelly, M. T., F. W. Hickman-Brenner, and J. J. Farmer III.** 1991. *Vibrio*, p. 384–395. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
29. **Kiehlbauch, J. A., B. D. Plikaytis, B. Swaminathan, D. N. Cameron, and I. K. Wachsmuth.** 1991. Restriction fragment length polymorphisms in the ribosomal genes for species identification and subtyping of aerotolerant *Campylobacter* species. J. Clin. Microbiol. **29:**1670–1676.
30. **Klontz, K. C., R. V. Tauxe, W. L. Cook, W. H. Riley, and I. K. Wachsmuth.** 1987. Cholera after consumption of raw oysters. Ann. Intern. Med. **107:**846–848.
31. **Koblavi, S., F. Grimont, and P. A. D. Grimont.** 1990. Clonal diversity of *Vibrio cholerae* O1 evidenced by rRNA restriction patterns. Res. Microbiol. **141:**645–657.
32. **Lin, F. C., G. Morris, Jr., J. B. Kaper, T. Gross, J. Michalski, C. Morrison, J. P. Libonati, and E. Israel.** 1986. Persistence of cholera in the United States: isolation of *V. cholerae* O1 from a patient with diarrhea in Maryland. J. Clin. Microbiol. **23:**624–626.
33. **Lowry, P. W., A. T. Pavia, L. M. McFarland, B. H. Peltier, T. J. Barrett, H. B. Bradford, J. M. Quan, J. Lynch, J. B. Mathison, R. A. Gunn, and P. A. Blake.** 1989. Cholera in Louisiana—widening spectrum of seafood vehicles. Arch. Intern. Med. **149:**2079–2084.
34. **McCarthy, S. A., R. M. McPhearson, A. M. Guarino, and J. L. Gaines.** 1992. Toxigenic *Vibrio cholerae* O1 and cargo ships entering Gulf of Mexico. Lancet **339:**624–625.
35. **Miller, C. J., R. G. Feachem, and B. S. Drasar.** 1985. Cholera epidemiology in developed and developing countries: new thoughts on transmission, seasonality and control. Lancet **i:**261–263.
36. **Morris, J. G.** 1990. Non-O1 group *Vibrio cholerae*: a look at the epidemiology of an occasional pathogen. Epidemiol. Rev. **12:**179–191.
37. **Olsvik, O., J. Wahlberg, B. Peterson, M. Uhlen, T. Popovic, I. K. Wachsmuth, and P. I. Fields.** 1993. Three genotypes of cholera toxin in *Vibrio cholerae* O1 strains. J. Clin. Microbiol. **31:**22–25.
38. **Pace, N. R., G. J. Olsen, and C. R. Woese.** 1986. Ribosomal RNA phylogeny and the primary lines of evolutionary descent. Cell **45:**325–326.
39. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
40. **Popovic, T., C. A. Bopp, Ø. Olsvik, and J. A. Kiehlbauch.** 1993. Ribotyping in molecular epidemiology, p. 573–583. *In* D. H. Persing, F. C. Tenover, T. F. Smith, and T. J. White (ed.), Diagnostic molecular microbiology. American Society for Microbiology, Washington, D.C.

41. **Popovic, T., Ø. Olsvik, P. A. Blake, and K. Wachsmuth.** Cholera in the Americas: foodborne aspects. J. Food Protect., in press.
42. **Rao, A., and B. A. Stockwell.** 1980. The Queensland cholera incident of 1977. 1. The index case. Bull. W.H.O. **58:**663–664.
43. **Sack, R. B., and C. E. Miller.** 1969. Progressive changes of *Vibrio* serotypes in germ-free mice infected with *Vibrio cholerae*. J. Bacteriol. **99:**688–695.
44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Sarafian, S. K., T. C. Woods, J. S. Knapp, B. Swaminathan, and S. A. Morse.** 1991. Molecular characterization of *Haemophilus ducreyi* by ribosomal DNA fingerprinting. J. Clin. Microbiol. **29:**1949–1954.
46. **Sheehy, T. W., H. Sprintz, W. S. Augerson, and S. B. Formal.** 1966. Laboratory *Vibrio cholerae* infection in the United States. JAMA **197:**99–104.
47. **Stroeher, U. H., L. E. Karageorgos, R. Morona, and P. A. Manning.** 1992. Serotype conversion in *V. cholerae* O1. Proc. Natl. Acad. Sci. USA **89:**2566–2570.
48. **Stull, T. I., J. J. LiPuma, and T. J. Edlind.** 1988. A broad spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. J. Infect. Dis. **152:**280–285.
49. **Swerdlow, D. L., P. Effler, K. Aniol, S. J. Manea, K. Iohp, K. D. Greene, C. A. Bopp, E. K. Pretrick, and P. A. Blake.** 1991. Re-emergence of cholera in Chuuk: a new endemic focus?, abstr. 530, p. 187. Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 1991. American Society for Microbiology, Washington, D.C.
50. **Tauxe, R. V., and P. A. Blake.** 1992. Epidemic cholera in Latin America. JAMA **267:**1388–1390.
51. **Wachsmuth, I. K.** 1986. Molecular epidemiology of bacterial infections: examples of methodology and investigations of outbreaks. Rev. Infect. Dis. **8:**682–692.
52. **Wachsmuth, I. K., C. A. Bopp, P. I. Fields, and C. Carillo.** 1991. Difference between toxigenic *V. cholerae* O1 from South America and U.S. Gulf Coast. Lancet **337:**1097–1098.
53. **Wachsmuth, I. K., G. M. Evins, P. I. Fields, Ø. Olsvik, T. Popovic, C. A. Bopp, J. G. Wells, C. Carillo, and P. A. Blake.** 1993. The molecular epidemiology of cholera in Latin America. J. Infect. Dis. **167:**621–626.
54. **Weissman, J. B., W. E. DeWitt, J. Thompson, C. N. Muchnick, B. L. Portnoy, J. C. Feeley, and E. J. Gangarosa.** 1975. A case of cholera in Texas, 1973. Am. J. Epidemiol. **100:**487–498.
55. **Woods, T. C., L. O. Helsel, B. Swaminathan, W. F. Bibb, R. W. Pinner, B. G. Gellin, S. F. Collin, S. H. Waterman, M. W. Reeves, D. J. Brenner, and C. V. Broome.** 1992. Characterization of *Neisseria meningitidis* serogroup C by multilocus enzyme electrophoresis and ribosomal DNA restriction profiles (ribotyping). J. Clin. Microbiol. **30:**132–137.
56. **World Health Organization.** 1991. Cholera in 1990. Weekly Epidemiol. Rec. **66:**133–137.
57. **World Health Organization.** 1992. Cholera in the Americas. Weekly Epidemiol. Rec. **67:**33–38.