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Molecular Subtyping of *Vibrio cholerae* O1 Strains Recently Isolated from Patient, Food and Environmental Samples in Spain

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Nineteen *Vibrio cholerae* O1 strains isolated in Spain from patient, food and environmental samples in the period 1990-1992 were characterized by detection of cholera toxin by enzyme immunoassay, detection of cholera toxin gene by polymerase chain reaction, and by biotyping, ribotyping and pulsed-field gel electrophoresis. Ten isolates were toxigenic and were further characterized by multilocus enzyme electrophoresis. Molecular subtyping methods allowed precise differentiation between isolates, indicating their geographic origin. Isolates associated with the ongoing seventh pandemic were distinguishable from those associated with the present Latin American epidemic. All isolates from the environment and seafood were nontoxigenic, and were genetically different and more diverse than toxigenic isolates. The data suggest that a focus of endemic cholera does not exist in Spain, and that the analyzed nontoxigenic *Vibrio cholerae* O1 isolates from imported seafood were not a threat to public health.

The present seventh cholera pandemic, caused by *Vibrio cholerae* O1 of the Eltor biotype, started in 1961 in Indonesia. To date, over 100 countries have been affected worldwide (1-8). However, the global cholera epidemiology has changed drastically over the past few years. In January 1991, after almost 100 years of absence in Latin America, an explosive cholera epidemic started in Peru. It swept through South America at the rate of one country per month. As of December 31, 1992, over 750,000 cholera cases and 6,000 deaths had been reported in all Latin American countries except Uruguay (9). The Latin American epidemic, and an average of 40,000 cholera cases reported annually by 30 to 40 countries worldwide, make it likely that sporadic cases and outbreaks of cholera will continue in Europe. Spain is in a more vulnerable position than many other European countries because it is close to areas with epidemic cholera in northern Africa and has traditionally strong cultural and economic relationships with Latin American countries.

Molecular techniques, such as the polymerase chain reaction (PCR) (10), multilocus enzyme electrophoresis (MEE) (11), ribotyping (12), sequencing of the B subunit of the cholera toxin gene (*ctx*) (13), and pulsed-field gel electrophoresis (PFGE) of the total genomic DNA (T.J. Barrett et al.; 92nd Annual Meeting of the American Society for Microbiology, 1992, Abstract C-198), have allowed identification of unique strains and determination of their geographic origin.

In order to investigate the eventual presence of a cholera focus in Spain and the possibility of spreading of the cholera into Europe via travellers and/or contaminated food coming from cholera endemic areas, we used molecular subtyping methods to analyze *Vibrio cholerae* O1 isolates obtained from patients with diarrhea and from food and environmental reservoirs in Spain during the last three years.

Materials and Methods

Bacterial Isolates. Nineteen *Vibrio cholerae* O1 isolates obtained in five Spanish cities in the period 1990-1992 were studied. Seven isolates were from cholera patients with history of recent travel to areas with endemic cholera, and two were from apparently indigenous cholera cases. Five isolates were from environmental

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samples collected in three Spanish provinces, and another five were isolated from large prawns imported from Ecuador (Table 1).

Species confirmation and serotyping were done at the Enteric Section of the National Center for Microbiology, Instituto de Salud Carlos III, Madrid, according to standard methods (14) and using commercial antisera (Diagnostic Pasteur Productions, France). Phage typing and biotyping using previously described methods (14, 15) and genetic characterization of the isolates were carried out at the Foodborne and Diarrheal Diseases Branch, Centers for Diseases Control and Prevention (CDC), Atlanta, GA, USA.

Toxin Detection. Cholera toxin gene was detected by PCR with amplification of a 564 base pair segment of the A subunit of the cholera toxin gene (*ctxA*) (10). Isolates negative in the PCR were also tested for cholera toxin production by the GM_1 enzyme immunoassay (EIA) (16).

Ribotyping. Total genomic DNA of each isolate was purified and digested with *BglI* (Gibco BRL, USA). Southern blotting of the restricted fragments was followed by hybridization with a digoxigenin-labeled complementary DNA (cDNA) probe for 16S and 23S ribosomal RNA (rRNA). Designation of restriction fragment length polymorphisms of rRNA genes (rRNA RFLPs) or ribotype were done according to a previously published typing scheme (17).

Pulsed Field Gel Electrophoresis. PFGE was performed using a CHEF-DR II system (BioRad Laboratories, USA), as previously described (18). Total genomic DNA was restricted with *NotI* (New England Biolabs, USA), according to the technique of Barrett et al. (T.J. Barrett et al.; 92nd Annual Meeting of the American Society for Microbiology, 1992, Abstract C-198). Southern blotting and hybridization with the cDNA probe for 16S and 23S rRNA as described for ribotyping were performed on selected isolates.

Multilocus Enzyme Electrophoresis. All toxigenic isolates were assayed according to the procedure of Chen et al. (11). The allelic variation at 16 enzyme loci was analyzed and the isolates classified into multilocus enzyme electrophoretic types (ETs) as described by Wachsmuth et al. (19).

Antimicrobial Susceptibility. The disc diffusion method of the NCCLS (20) was used to determine susceptibility of the isolates to the following antimicrobial agents (disc content in brackets): chloramphenicol (30 µg), trimethoprim-sulphamethoxazole (1.25/23.75 µg), tetracycline (30 µg), doxycycline (30 µg), erythromycin (15 µg), furazolidone (100 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), ampicillin (10 µg), sulfisoxazole (250 µg), streptomycin (10 µg) and kanamycin (30 µg) (Becton Dickinson, USA). Trimethoprim-sulphamethoxazole and sulfisoxazole zones indicating an obvious 80 % diminution in growth were recorded as endpoints.

Results

Toxigenic Isolates. Ten *Vibrio cholerae* O1 isolates were toxigenic. Nine of these isolates were from patients with clinically diagnosed cholera; seven patients had a history of recent travel in cholera endemic areas, but two patients from Seville had no history of travel in cholera affected areas. One toxigenic isolate was from sewage obtained during the outbreak in Ceuta (Table 1). All isolates were of the Eltor biotype and were nonhemolytic; nine isolates were of the Ogawa serotype, and a single isolate was of the Inaba serotype. Identical antimicrobial susceptibility patterns were observed in all isolates; the isolates were susceptible to all antimicrobial agents assayed, with the exception that intermediate susceptibility was observed to erythromycin and streptomycin. Eight isolates had identical ribotypes (ribotype 5, Figure 1), PFGE patterns (pattern A, Figure 2) and electrophoretic types (ET 3). A single isolate from a patient with a history of

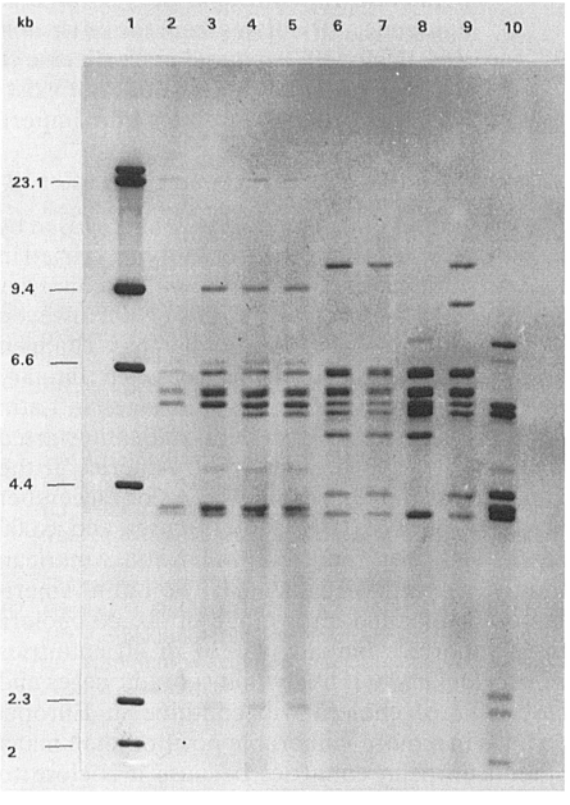

Figure 1: *BglI* ribotypes of *Vibrio cholerae* O1 isolates from Spain. Lanes 2 to 5 represent toxigenic isolates and lanes 6 to 10 nontoxigenic isolates. Lane 1: DNA marker, lambda phage restricted with *Hind*III (Boehringer Mannheim, Germany); lane 2: isolate F 751 (ribotype 5); lane 3: ribotype 5 control strain (human origin) from Guatemala, 1992; lane 4: isolate F 750 (ribotype 3); lane 5: ribotype 3 control strain (human origin) from the Philippines, 1963; lane 6: isolate F 761 (ribotype 16); lane 7: ribotype 16 control strain (water origin) from the USA, 1991; lane 8: isolate F 758 (ribotype a); lane 9: isolate F 765 (ribotype b); lane 10: isolate F 766 (ribotype c).

Table 1: Epidemiologic data and phenotypic and genotypic characteristics of 19 *Vibrio cholerae* O1 isolates from Spain.

CDC isolate no.	Month/ year of isolation	City of isolation	Geographic origin	Source	Biotype	Serotype	Cholera toxin		Ribotype	Electrophoretic type	PFGE[a] pattern
							PCR *ctxA*	EIA GM_1			
F 750	9/90	Almeria	Algeria	human	Eltor	Ogawa	+	ND	3	3	A
F 751	9/90	Ceuta	Morocco[b]	human	Eltor	Ogawa	+	ND	5	3	A
F 752	9/90	Ceuta	Morocco[b]	human	Eltor	Ogawa	+	ND	5	3	A
F 753	9/90	Ceuta	Morocco[b]	human	Eltor	Ogawa	+	ND	5	3	A
F 754	10/90	Ceuta	Morocco[b]	human	Eltor	Ogawa	+	ND	5	3	A
F 755	10/90	Ceuta	Morocco[b]	human	Eltor	Ogawa	+	ND	5	3	A
F 756	11/90	Ceuta	Morocco[b]	sewage	Eltor	Ogawa	+	ND	5	3	A
F 768	8/92	Seville[c]	Seville	human	Eltor	Ogawa	+	ND	5	3	A
F 767	8/92	Seville[c]	Seville	human	Eltor	Ogawa	+	ND	5	3	A
F 757	4/91	Madrid	Peru	human	Eltor	Inaba	+	ND	5	4	B
F 758	5/91	Seville	Seville	marsh	atypical	Inaba	–	–	a	ND	C
F 759	5/91	Seville	Seville	marsh	atypical	Inaba	–	–	a	ND	C
F 760	5/91	Seville	Seville	marsh	atypical	Inaba	–	–	a	ND	C
F 761	6/91	Madrid	Ecuador	prawn	Eltor	Inaba	–	–	16	ND	D
F 762	7/91	Madrid	Ecuador	prawn	Eltor	Inaba	–	–	16	ND	D
F 763	7/91	Madrid	Ecuador	prawn	atypical	Inaba	–	–	16	ND	D
F 764	7/91	Madrid	Ecuador	prawn	Eltor	Inaba	–	–	16	ND	D
F 765	11/91	Madrid	Ecuador	prawn	Eltor	Inaba	–	–	b	ND	E
F 766	7/92	Malaga	Malaga	river	atypical	Ogawa	–	–	c	ND	F

[a] Pulsed field gel electrophoresis using *Not*I.
[b] Associated with an outbreak in Morocco.
[c] Mother and son ate shrimp of unknown origin.
ND: not done.

travel in Algeria differed from those isolates in the ribotype (ribotype 3, Figure 1). Another isolate from a traveller from Peru was of ribotype 5, as were the majority of isolates, but it had a different PFGE pattern (pattern B, Figure 2) and a different ET (ET 4) (Table 1). Southern blotting and hybridization of the PFGE DNA fragments revealed that at least one copy of the rRNA genes of *Vibrio cholerae* O1 isolates of ribotype 5 and ribotype 3 resides on different fragments.

Nontoxigenic Isolates. Nine isolates were found to be hemolytic and non-toxigenic both in the PCR and EIA. Four isolates were of the Eltor biotype and five were atypical: one isolate from Malaga was lysed by both classical IV and Eltor 5 phages, while the other four atypical isolates were not lysed by either phage. Eight isolates were of the Inaba serotype and one was of the Ogawa serotype (Table 1). Their antimicrobial susceptibility patterns were identical to those of the toxigenic strains. Ribotyping revealed four patterns not seen among the toxigenic isolates: ribotype 16, observed in previous molecular biological studies, and three new ribotyping patterns a, b, and c (Figure 1). Four patterns, different from those seen among the toxigenic isolates, were observed on PFGE (PFGE patterns C, D, E, and F, Figure 2).

Discussion

The ten toxigenic *Vibrio cholerae* O1 isolates characterized in this study fall into three groups based on the data obtained by the molecular biological methods applied. The first group contains eight isolates of ET 3 and ribotype 5. As previously shown, these molecular markers are typical for the strains causing the present seventh pandemic. Therefore, these eight isolates are assumed not to be associated with the Latin American epidemic. Epidemiological data obtained for six of those isolates confirms these observations; a strong epidemiological association with the occurrence of cholera in Morocco has been established. However, in two cases the origin of the incriminated shrimp consumed by the members of a single family is unknown. A single isolate from a patient returning from Algeria was of the same PFGE pattern and ET as these eight isolates, but it had a different ribotype (ribotype 3). This com-

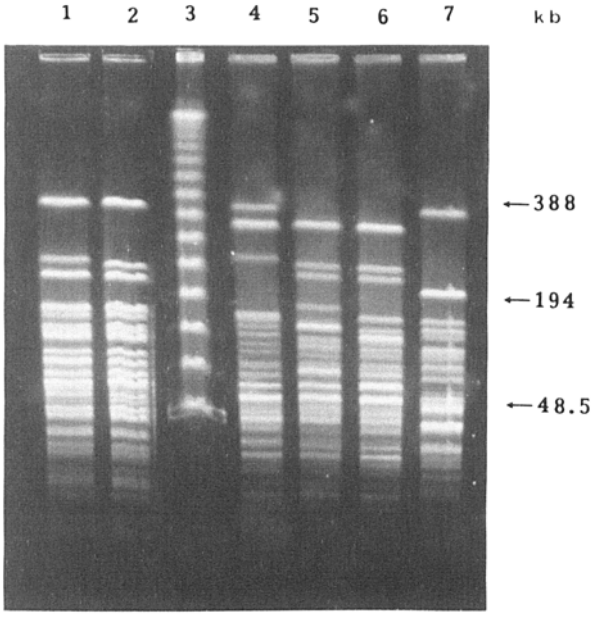

Figure 2: *Not*I pulsed field gel electrophoresis types of *Vibrio cholerae* O1 from Spain. Lanes 1 and 2 represent toxigenic isolates and lanes 4 to 7 nontoxigenic isolates. Lane 1: isolate F 757 (PFGE pattern B); lane 2: isolate F 751 (PFGE pattern A); lane 3: lambda DNA ladder in inCert Agarose gel plugs (FMC BioProducts, USA); lane 4: isolate F 758 (PFGE pattern C); lane 5: isolate F 761 (PFGE pattern D); lane 6: isolate F 765 (PFGE pattern E); lane 7: isolate F 766 (PFGE pattern F).

bination of ET and ribotype is also typical of the present seventh pandemic strains (11). One isolate from a traveller from Peru was ribotype 5 but ET 4, a combination of epidemiologic markers that is characteristic for strains associated with the Latin American epidemic (11, 19). Additionally, this isolate had a PFGE pattern different from that of all the other toxigenic isolates analyzed.

The nontoxigenic isolates analyzed in our study were more diverse than the toxigenic isolates: four distinct ribotypes and four distinct PFGE patterns, different from those of the toxigenic isolates, were observed. One ribotype (ribotype 16), characteristic of the group of isolates from large prawns from Ecuador, has been observed previously in the USA (11). Three ribotypes associated with nontoxigenic isolates from Seville, Madrid and Malaga were observed for the first time (patterns a, b, c). Consequently, nontoxigenic isolates can be divided into four groups. Unlike the toxigenic isolates, the nontoxigenic isolates had both a different ribotype and PFGE pattern within each group. Fortunately, no human cases were reported to be associated with these strains.

Even though the number of strains analyzed is small, our data indicate that Spanish environmental isolates do not represent a focus of toxigenic *Vibrio cholerae* O1. Travel to Latin America or North Africa seems to be associated with cholera infection caused by strains characteristic for the respective areas. The nontoxigenic isolates from imported seafood from Latin America do not appear at present have potential epidemic properties.

Although most of the cholera cases reported in Spain are imported from North Africa, past efforts to prevent importation of cholera through culture of specimens from travellers, or by requiring cholera vaccination or administering antibiotics, have been unsuccessful and disruptive; these measures are now generally discouraged by the WHO. Maintenance of surveillance for cholera in Spain, including investigation of cases to determine the source of infection, and examination of patients' sanitary facilities to evaluate the potential for spread of *Vibrio cholerae* O1 through contamination of food or water, might be the most fruitful way of combatting potential outbreaks (21). Molecular biological methods have made it possible to identify the geographical origin of human isolates and to distinguish strains associated with the present Latin American cholera epidemic from those associated with the seventh pandemic. Such continuous laboratory surveillance can significantly help public health officials in implementing timely and adequate preventive measures.

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