High Prevalence of *Vibrio cholerae* Non-O1 Carrying Heat-Stable-Enterotoxin-Encoding Genes among *Vibrio* Isolates from a Temperate-Climate River Basin of Central Italy

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***Vibrio* spp. of clinical interest from the Arno River basin (Tuscany, Italy) were investigated in this study. *Vibrios* were isolated from 70% of water samples. *Vibrio cholerae* non-O1 was the most prevalent species (82% of isolates), followed by *Vibrio mimicus* (10%) and *Vibrio metschnikovii* (8%). Recovery of vibrios was correlated with temperature, pH, and various indicators of municipal pollution. None of the 150 *Vibrio* isolates carried *ctx*-related genomic sequences, whereas 18 (14.6%) of the 123 *V. cholerae* non-O1 isolates and 1 (6.7%) of the 15 *V. mimicus* isolates carried *sto* alleles. These findings indicate that considerable circulation of *sto*-positive vibrios may occur in temperate-climate freshwater environments.**

Vibrios are inhabitants of aquatic environments (9, 16). They occasionally infect humans, causing intestinal or extraintestinal diseases. The most prevalent diseases caused by vibrios are those that are well reported, including cholera and other forms of acute gastroenteritis. In addition, there may be many other vibrio-caused diseases that go unreported (4, 16, 19, 24, 43). Some of the virulence determinants of *Vibrio* spp. are well characterized. In particular, cholera toxin (CT) and other toxins encoded by the *ctx* genetic element, as well as heat-stable enterotoxin (ST) encoded by the *sto* gene, are clearly established determinants of diarrhea associated with infection by *Vibrio cholerae* and *Vibrio mimicus* (21, 27, 33, 36).

Understanding the ecology of *Vibrio* spp. of clinical interest and tracing virulence determinants in environmental isolates are important issues, and several studies addressing these subjects have been performed in various geographic areas and with samples from various environmental sources (7, 11–15, 18, 20, 29, 31, 33, 34). However, little information is available for temperate-climate freshwater environments (2, 5, 22, 23, 26, 39). Moreover, screening for enterotoxin production has often been restricted to CT, not including other *ctx*-encoded enterotoxins or ST.

The Arno River basin (central Italy) is one of the major Italian river basins. Several towns, the largest of which is Florence, are located along the river. Wastewaters from the main municipal sewage treatment plants and several industries, as well as rainwater overflow, are discharged into the river.

The aims of this study were as follows: (i) to monitor *V. cholerae* and other vibrios of clinical interest in freshwater of the temperate-climate Arno River basin; (ii) to analyze the relationships between recovery of vibrios and various abiotic or

biotic water parameters, including indicators of pollution; and (iii) to screen the isolates for genes encoding known enterotoxins.

Sampling procedure. A total of 172 water samples were collected at 15 different stations in the Province of Florence. Nine stations were on the Arno River, and six stations were on the main tributaries (Fig. 1). Samples were obtained monthly from January to December 1995 at 11 stations and from March to December 1995 at the other 4 stations (Fig. 1). The sampling area is a nontidal freshwater environment at least 54 km upstream of the river estuary (Fig. 1). Na⁺ and Cl[−] concentrations are in the ranges 1.3 to 1.9 mM and 0.9 to 4.1 mM, respectively, without substantial seasonal or geographic variations (1).

Abiotic and biotic parameters in the Arno River basin. Temperature, pH, and dissolved oxygen (O₂) were determined in situ with a portable pH meter (model SA-720; Orion Research, Boston, Mass.) or a portable oxymeter (model 4002-F; Syland Scientific GmbH, Heppenheim, Germany). Total organic carbon (TOC) content, ammonia (NH₄⁺) content, and fecal coliform (FC) count were determined in 106 of the 172 samples according to the following schedule: monthly from the five most-representative sampling stations (Figline, Varlungo, Porto di Mezzo, Capraia, and Fucecchio [Fig. 1]), bimonthly from the other four stations along the Arno River, and bi-monthly or quarterly from the stations along the tributaries. TOC was measured with a 2001 System II apparatus (Astro International, League City, Texas). NH₄⁺ content was measured by Nessler colorimetric assay (3). FC count was determined by the most-probable-number method (3). The Wilcoxon rank sum test (42) was used to compare median values of abiotic and biotic parameters from different areas.

Upstream of Florence, the median value of dissolved O₂ was significantly higher and those of TOC, NH₄⁺, and FC count were significantly lower than those recorded downstream (Table 1), indicating an overall higher level of municipal pollution in the river downstream of Florence. The median values of temperature and pH were not significantly different for the two

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FIG. 1. Map of the Arno River basin showing the locations of the sampling stations. Sampling stations: 1, Figline; 2, Rignano; 3, Rosano; 4, Varlungo; 5, Ponte a Signa; 6, Porto di Mezzo; 7, Capraia; 8, Marcignana; 9, Fucecchio; 10, Pontassieve; 11, Mantignano; 12, Signa; 13, Carmignano; 14, Montelupo; and 15, Isola. Samples were obtained monthly from January to December 1995 at all stations except those marked by an asterisk. Samples were obtained monthly from March to December 1995 at the latter stations. The numbers reported for each sampling station are the frequencies (percentages) of samples positive for vibrios. Numbers in parentheses are the frequencies (percentages) of *stx*-positive *Vibrio* isolates from each station, calculated from the total number of isolates from that station. The map scale is 1:1,200,000.

areas. The pH values were consistently in the neutral to alkaline range (Table 1).

Isolation of *Vibrio* spp. from the Arno River basin. Screening for *Vibrio* spp. in water samples was performed as follows. Samples (200 ml each) were inoculated into alkaline peptone water (200 ml at 2× concentration; Biolife, Milan, Italy) and incubated at 37°C for 24 h. A loopful (using a 10-μl calibrated loop) of the surface pellicle of each enrichment culture was subcultured on a plate of thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco Laboratories, Detroit, Mich.). Four similar subcultures were also performed starting from 10-fold serial dilutions of the surface pellicle fraction. After incubation at 37°C for 24 h, one colony per plate (if the colonies had the same morphology) or more (if differences in colony morphol-

ogy were evident) were subjected to presumptive identification by the following tests: gram staining, presence of oxidase, string test, susceptibility to O/129, production of lysine and ornithine decarboxylase and of arginine dihydrolase, and growth properties on TSI agar (24). *Vibrio* isolates were further characterized with the API 20E commercial identification system (Bio-Mérieux, Montalieu Vercieu, France), using 0.85% NaCl as the diluent. Duplicate API 20E profiles were obtained for each isolate. O serotyping was performed by slide agglutination with a commercial anti-*V. cholerae* O1 antiserum (Biogenetics Diagnostics, Padova, Italy). Quality control of culture media and reagents for identification was performed with the following strains: *V. cholerae* O1 569/B (25), *V. cholerae* O1 CCUG 13123, *V. cholerae* non-O1 VCC023 (17), *V. mimicus*

TABLE 1. Abiotic and biotic parameters measured at the various sampling stations

Geographic area of sampling station	Median value (interquartile range) [<i>n</i> ^a]					
	Temp (°C)	pH	O ₂ concn (mg/liter)	TOC concn (mg/liter)	NH ₄ concn (mg/liter)	FC count (MPN ^b /100 ml)
Arno River basin	15.5 (8.5–20.0) [172]	7.8 (7.7–7.9) [172]	8.2 (6.1–9.8) [172]	5.8 (4.1–9.6) [106] ^c	0.9 (0.1–2.2) [106] ^c	10,150 (2,300–93,000) [106] ^c
Florence						
Upstream	13.0 (8.0–19.0) [59]	8.0 (7.8–8.1) [59]	9.7 (8.7–10.5) [59]	4.4 (3.5–5.5) [41]	0.1 (0.1–0.1) [41]	4,300 (1,500–12,000) [41]
Downstream	17.0 (9.0–20.0) [113]	7.7 (7.6–7.9) [113]	6.7 ^d (4.9–8.9) [113]	8.0 ^d (5.6–11.6) [65]	1.9 ^d (0.9–4.1) [65]	23,000 ^d (4,000–460,000) [65]

^a *n*, number of samples analyzed.

^b MPN, most probable number.

^c These parameters were measured in 106 of 172 samples (see Materials and Methods for details on the sampling procedures).

^d *P* < 0.00005 (according to Wilcoxon rank sum test for comparison of upstream and downstream data).

TABLE 2. Results of Cochran-Armitage test for relationships between various abiotic and biotic parameters and the frequency of isolation of vibrios in the Arno River basin

Vibrio species isolated	No. of water samples (pos./neg.) ^a	P value for relationship ^b					
		Temp ↑	pH ↑	O ₂ ↓	TOC ↑	NH ₄ ⁺ ↑	FC ↑
<i>Vibrio</i> spp.	121/51 81/25	0.00005	0.0078	0.00005	0.0004	0.0160	0.0202
<i>V. cholerae</i> non-O1	104/68 71/35	0.0006	0.0001	0.00005	0.00005	0.00005	0.00005
<i>V. mimicus</i>	15/157 8/98	0.0001	0.3860	0.0616	0.5223	0.2881	0.8743
<i>V. metschnikovii</i>	12/160 8/98	0.4082	0.0020	0.0117	0.0306	0.0065	0.0131
sto positive	19/153 15/91	0.1763	0.0589	0.0001	0.0627	0.0801	0.0363

^a No. of water samples positive for vibrio/no. of water samples negative for vibrio.

^b ↑, positive relationship; ↓, negative relationship. TOC, NH₄⁺, and FC count data were available for only 106 water samples (see Materials and Methods for details on the sampling procedure). P values < 0.05 were considered statistically significant.

CCUG 13624^T, *Vibrio fluvialis* CCUG 13622^T, and *Vibrio metschnikovii* CCUG 31367^T. The χ^2 test with Yates correction was used to compare frequencies of *Vibrio* isolation. The Cochran-Armitage trend test (8) was used to analyze the relationships between abiotic and biotic parameters and the frequencies of *Vibrio* isolation.

Vibrios were isolated at every sampling station from a total of 121 (70.3%) samples (Fig. 1). One hundred eleven samples yielded isolates of a single species (94 samples with *V. cholerae* non-O1, 6 with *V. mimicus*, and 11 with *V. metschnikovii*), and 10 yielded isolates of two different species (9 with *V. cholerae* non-O1 and *V. mimicus* and 1 with *V. cholerae* non-O1 and *V. metschnikovii*). Of the 104 samples positive for *V. cholerae* non-O1, 85 yielded isolates with a single biochemical profile, and 19 yielded isolates with two different biochemical profiles (either citrate positive or negative, confirmed in the two replicate API 20E assays), resulting in a total of 123 different *V. cholerae* non-O1 isolates. On the whole, these findings are consistent with previous observations concerning other temperate-climate freshwater environments (5, 6, 22, 23, 39–41) and indicate that vibrios are a common component of the microbial ecosystem of the Arno River basin. The absence of species found in other freshwater environments, such as *V. fluvialis* and *Vibrio cincinnatiensis* (5, 39, 41), could reflect true ecological differences or reduced sensitivity of the sampling and detection procedures. More-halophilic *Vibrio* spp. were not found, as expected in these low-salinity conditions in which they would likely lyse (32). The fact that about one-third of samples yielded no vibrio isolates may reflect the occasional occurrence of very low counts of culturable vibrios. However, since a positive control for recovery of *Vibrio* spp. from water samples was not performed, the presence of inhibitory substances in some of the samples cannot be excluded.

Isolation of vibrios was significantly higher at sampling stations located downstream of Florence (79.6 versus 52.5% [$P = 0.0001$]), and it was inversely related to the amount of dissolved O₂ and directly related to pH, water temperature, TOC, NH₄⁺, and FC count (Table 2). When individual species were considered, the above relationships remained valid for *V. cholerae* non-O1 and, except for water temperature, *V. metschnikovii*, whereas isolation of *V. mimicus* was directly related to water temperature only (Table 2). A considerable number of

vibrios were isolated at low temperatures (Fig. 2). Only *V. cholerae* and *V. metschnikovii* were isolated at the lowest temperatures, whereas *V. mimicus* was always found at temperatures of >17°C (data not shown). The relationship observed between isolation of vibrios and water temperature was in overall agreement with previous observations (5, 6, 22, 41). The relationships between vibrio isolation and the various indicators of pollution most likely reflect the fact that municipal

FIG. 2. Frequency of samples positive for vibrios in relation to water temperature. The numbers at the top of each column are the number of vibrio-positive samples to the total number of samples in that temperature range.

FIG. 3. Diagram showing the results of Southern hybridization analysis of the genomic DNAs of the 19 *sto*-positive *Vibrio* isolates. The positions of molecular size markers are shown on the left. The asterisk indicates the *V. mimicus* isolate.

pollution creates high levels of nutrients (as indicated by TOC and NH_4^+ values) which would increase the levels of culturable vibrios especially in these low-salinity conditions (10, 28, 37). The relationship with FC counts, which is not consistent with findings of several studies (5, 12, 20, 23, 34, 41), might merely reflect the fact that polluted water, in addition to high levels of nutrients, also contains fecal bacteria. A similar explanation has been hypothesized by other investigators who also found a relationship between vibrio isolation and FC counts (40).

Prevalence of toxin-encoding genes among *Vibrio* isolates.

Genomic sequences related to the *ctx* genetic element (38) or the *sto* gene (30) were investigated in the 150 *Vibrio* isolates by colony hybridization assay with either a *ctx*-specific probe containing the entire *ctx* genetic cassette or a *sto*-specific probe containing the entire *sto* gene. Hybridization was performed on colonies grown at 30°C on nitrocellulose filters (0.45- μm pore size; Schleicher & Schuell, Dassel, Germany) layered on Columbia blood agar plates. Colonies were lysed in situ by placing filters on paper towels soaked with 0.5 M NaOH–1.5 M NaCl for 20 min. Filters were then neutralized by placing them on paper towels soaked with 0.5 M Tris-HCl–1.5 M NaCl (pH 7.4) for 20 min. After a final wash in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the filters were air dried and baked at 80°C in a vacuum for 90 min. Prehybridization was performed at 65°C for 2 h in 5 $\times$ SSC–0.5% (wt/vol) sodium dodecyl sulfate (SDS)–5 $\times$ Denhardt's solution (35), containing 50 μg of sonicated and denatured salmon sperm DNA per ml. Hybridization was performed at 65°C for 16 h in the same mixture. Washes were performed at 65°C in 5 $\times$ SSC–0.1% SDS once, then in 1 $\times$ SSC–0.1% SDS repeatedly until the washing solution did not contain significant radioactivity (usually three times). The DNA probes used for filter hybridization were PCR amplimers containing either the entire *ctx* cassette or the entire *sto* gene. Genomic DNAs of *V. cholerae* O1 strain 569/B and *V. cholerae* non-O1 strain VCC023 were used as templates to obtain the *ctx*- and *sto*-specific amplimers. The probes were labeled with [α - ^{32}P]dCTP by the random-

priming technique (35), using a commercial kit (Boehringer GmbH, Mannheim, Germany). Colonies of *V. cholerae* O1 569/B and *V. cholerae* non-O1 VCC023 on filters were included as positive controls for hybridization.

None of the 150 isolates were recognized by the *ctx*-specific probe, but 19 (12.7%) were recognized by the *sto*-specific probe. The *sto*-positive isolates included 18 *V. cholerae* non-O1 isolates and one *V. mimicus* isolate, each from a different water sample. The *sto*-positive isolates were found at nine sampling stations throughout almost the entire area analyzed in this study (Fig. 1). The prevalence of *sto*-positive isolates was higher downstream of Florence (15.3 versus 5.1% of all *Vibrio* isolates), although the difference was not statistically significant ($P > 0.2$). The isolation of *sto*-positive vibrios was inversely related to the amount of dissolved O_2 and directly related to the FC count (Table 2). The rate of *sto*-positive vibrios detected in this study was higher than those previously reported in different environmental investigations (5, 7, 15, 18, 29, 31, 33). The above findings indicate that a consistent number of vibrios carrying the *sto* gene can circulate in freshwater environments of temperate-climate areas. This raises a question on the clinical significance of these isolates and suggests that bacteriological analysis of stool samples in these areas should also include a search for enterotoxigenic vibrios. It should be noted that since production of ST by the *sto*-positive isolates was not assayed, the possibility that some of these isolates did not produce the toxin cannot be excluded.

RFLP of the *sto* locus in *Vibrio* isolates. Restriction fragment length polymorphism (RFLP) of the *sto* locus has been reported for *V. cholerae* isolates (15, 31). In the present study, it was investigated by Southern blot hybridization of genomic DNA digested with the restriction endonuclease *Eco*RI, using the same *sto*-specific probe. Southern blotting was performed as previously described (35) by using nitrocellulose filters and the same prehybridization, hybridization, and washing conditions used for colony blotting. Genomic DNA was extracted from *Vibrio* isolates as previously described (25).

In all cases, the *sto*-specific probe hybridized to an appar-

FIG. 4. Distribution of the *sto*-positive *Vibrio* isolates in the study area, according to their Southern blot hybridization profile (see Fig. 3 for profile definition).

ently unique restriction fragment. RFLP of the *sto* locus was observed for different isolates, and five different profiles were distinguished (Fig. 3). The RFLP profiles of serial isolates cultured from the same station were always identical. Interestingly, the *sto*-positive *V. mimicus* profile appeared to be the same as that of a *V. cholerae* non-O1 previously isolated in the same site (Fig. 3), suggesting the possibility of horizontal transfer of genetic material carrying the *sto* locus between the two species. Different overall geographic distributions were observed for isolates with similar and different RFLP profiles (Fig. 4). Although it is not possible to establish the extent of genetic relatedness among different isolates based on the polymorphism of a single locus, the above findings suggest the existence of a clonal heterogeneity among the *sto*-positive *V. cholerae* population of the basin. The facts that *sto*-positive isolates with different genetic profiles were not found at the same site and serial isolates from the same site retained the same genetic profile also suggest a substantial clonal stability and uniqueness of the *sto*-positive vibrio population in different parts of the basin. It would be interesting to confirm this hypothesis by analyzing the isolates for clonality with appropriate techniques.

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