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Occurrence, Diversity, and Pathogenicity of Halophilic *Vibrio* spp. and Non-O1 *Vibrio cholerae* from Estuarine Waters along the Italian Adriatic Coast

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The occurrence, diversity, and pathogenicity of *Vibrio* spp. were investigated in two estuaries along the Italian Adriatic coast. *Vibrio alginolyticus* was the predominant species, followed by *Vibrio parahaemolyticus*, non-O1 *Vibrio cholerae*, and *Vibrio vulnificus*. By using a biochemical fingerprinting method, all isolates were grouped into nine phenotypes with similarity levels of 75 to 97.5%. The production of toxins capable of causing cytoskeleton-dependent changes was detected in a large number of *Vibrio* strains. These findings indicate a significant presence of potentially pathogenic *Vibrio* strains along the Adriatic coast.

Estuarine and freshwater environments both represent the critical reservoirs of *Vibrio* species (6, 12, 21). The prevalence of pathogenic vibrios appears to be influenced by the physicochemical features of the environment (9, 22), whereas factors influencing the production and the activity of their toxins remain to be defined. It is known that a high bacterial density is correlated with coastal eutrophication, and it has been suggested that *Vibrio* spp. are among the major causative agents of acute diarrheal diseases (8, 10, 14, 18, 19, 23).

To evaluate the health risk associated with the presence of potentially pathogenic vibrios, we investigated the virulence of *Vibrio* strains isolated during warm weather months (April to October 1995) from two estuaries (Metauro and Foglia rivers) along the Northwest Adriatic coast. All isolates were also phenotyped with a high-resolution biochemical fingerprinting system to evaluate the intraspecies diversity.

Procedures and sampling sites, as well as in vitro assays to evaluate the virulence of isolated strains, were described in an earlier parallel study on *Aeromonas* occurrence in the same coastal area (13).

Bacteriological analysis. Enrichment for potentially pathogenic *Vibrio* species and viable bacterial counting were performed by the membrane filtration technique. Water volumes of 0.1, 1, 10, and 100 ml (for counting) and 500 ml (for enrichment) were filtered through 0.45-μm-pore-size filters (Millipore). All filters except those used for the enrichment were placed on thiosulfate-citrate-bile-sucrose (TCBS; Oxoid) agar plates with 2% NaCl and incubated at 37°C for 16 to 18 h. The number of viable *Vibrio* isolates was estimated as CFU 100 ml of water^{-1}. The mean number of bacteria in each water sample was estimated according to the method of Bolinches et al. (5). The primary and secondary enrichments into alkaline-peptone-water (APW) for *Vibrio cholerae* detection were performed as suggested previously (3, 14).

***Vibrio* identification and typing.** *Vibrio* strains were identified by colony shape and pigmentation on TCBS, Gram staining, cytochrome-oxidase and catalase activities, motility, and susceptibility to O/129 vibriostatic agent (10- and 150-μg disks; Oxoid). Only oxidase-positive, gram-negative, vibriostatic susceptible colonies were selected for biochemical tests according to the classical procedures. The API 20E and ID32 system (bioMerieux) and the additional test recently suggested by Alsina and Blanch (1, 2) were used for *Vibrio* identification at the species level. Further, biovars of *Vibrio cholerae* strains were defined by using O1 polyvalent antisera (57142; Sanofi Diagnostic Pasteur). Moreover, *V. parahaemolyticus* strains were tested on Wagatsuma agar for their hemolytic activity (Kanagawa phenomenon [20]). All *Vibrio* strains were further typed by using the Phene Plate (PhP) system (17) specifically developed for typing *Vibrio* species (15). *V. alginolyticus* COD.18 and *V. parahaemolyticus* COD.66 (kindly provided by D. Ottaviani, IZS, Ancona, Italy) were included as reference strains.

A Spearman rank correlation, a Wilcoxon matched-pair signed-ranks nonparametric test, and χ^2 analyses were used for statistical analyses.

Assays for supernatant and bacterial cytotoxicity and for bacterial adhesion. *Vibrio* spp. were inoculated in brain heart infusion broth supplemented with 0.5% NaCl and grown in 25-ml flasks incubated at 37°C for 18 h with agitation at 150 rpm. For the supernatant cytotoxicity assay, cell-free filtrates were prepared by centrifugation (3,000 rpm) at 4°C for 30 min, with subsequent filtration of the supernatants through a 0.45-μm-pore-size filter (Millipore). The filtrates were either refrigerated prior to use or stored at −80°C. For both the adhesion and cytotoxicity assays, exponentially growing bacteria were washed three times in phosphate-buffered saline and resuspended in serum-free Dulbecco modified Eagle medium (DMEM).

For the supernatant cytotoxicity assay, *Vibrio* strains found to be stable in the biochemical tests were selected. CHO cell monolayers were maintained in DMEM containing 10% fetal calf serum. Serial doubling dilutions of filtrates starting from 1:2 were tested for 24 h as previously described (13).

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TABLE 1. *Vibrio* CFU and *Vibrio* species isolated on TCBS medium or APW medium from the Metauro and Foglia estuaries between April and October 1995

Month	Vibrio CFU (100 ml^{-1})		No. of isolates		Origin (medium)	No. (%) of strains							
						V. alginolyticus		*V. parahaemolyticus*		*V. cholerae* non-O1		*V. vulnificus*	
	Metauro	Foglia	Metauro	Foglia		Metauro	Foglia	Metauro	Foglia	Metauro	Foglia	Metauro	Foglia
April	5.0×10^2	3.0×10^3	3	4	TCBS	2 (67)	2 (50)						
					APW	1			1		1		
May	1.0×10^2	3.0×10^3	4	7	TCBS	2 (50)	4 (57)		1 (14)				
					APW	2	2						
June	2.5×10^3	5.0×10^4	5	11	TCBS	5 (100)	9 (82)				1 (9)		
					APW						1		
July	1.1×10^{14}	1.0×10^6	6	8	TCBS	2 (33)	1 (13)				1 (13)		
					APW	1	2	2	2	1	2		
August	1.0×10^6	3.0×10^7	12	11	TCBS	3 (25)	4 (36)	2 (17)	1 (9)				
					APW	5	2		4	2			
September	3.0×10^5	2.0×10^6	5	5	TCBS	4 (80)	2 (40)						2 (40)
					APW	1	1						
October	1.5×10^5	1.0×10^6	8	14	TCBS	4 (50)	6 (43)	2 (25)	1 (7)				
					APW	2	7						
Total			43	60		34 (79)	42 (70)	6 (14)	10 (17)	3 (7)	6 (10)		2 (3)

Bacterial adhesion and cytotoxicity were tested on HEp-2 cells growing in DMEM containing 10% fetal calf serum. Bacteria were added to HEp-2 cells at a multiplicity of infection of 100 in serum-free DMEM and incubated for 1 h 30 min at 37°C. Cells were then washed three times in serum-free DMEM to remove nonadherent bacteria, fixed in methanol, and stained with May-Grunwald-Giemsa. When assessed by light microscopy (LM), a sample was considered cytotoxic

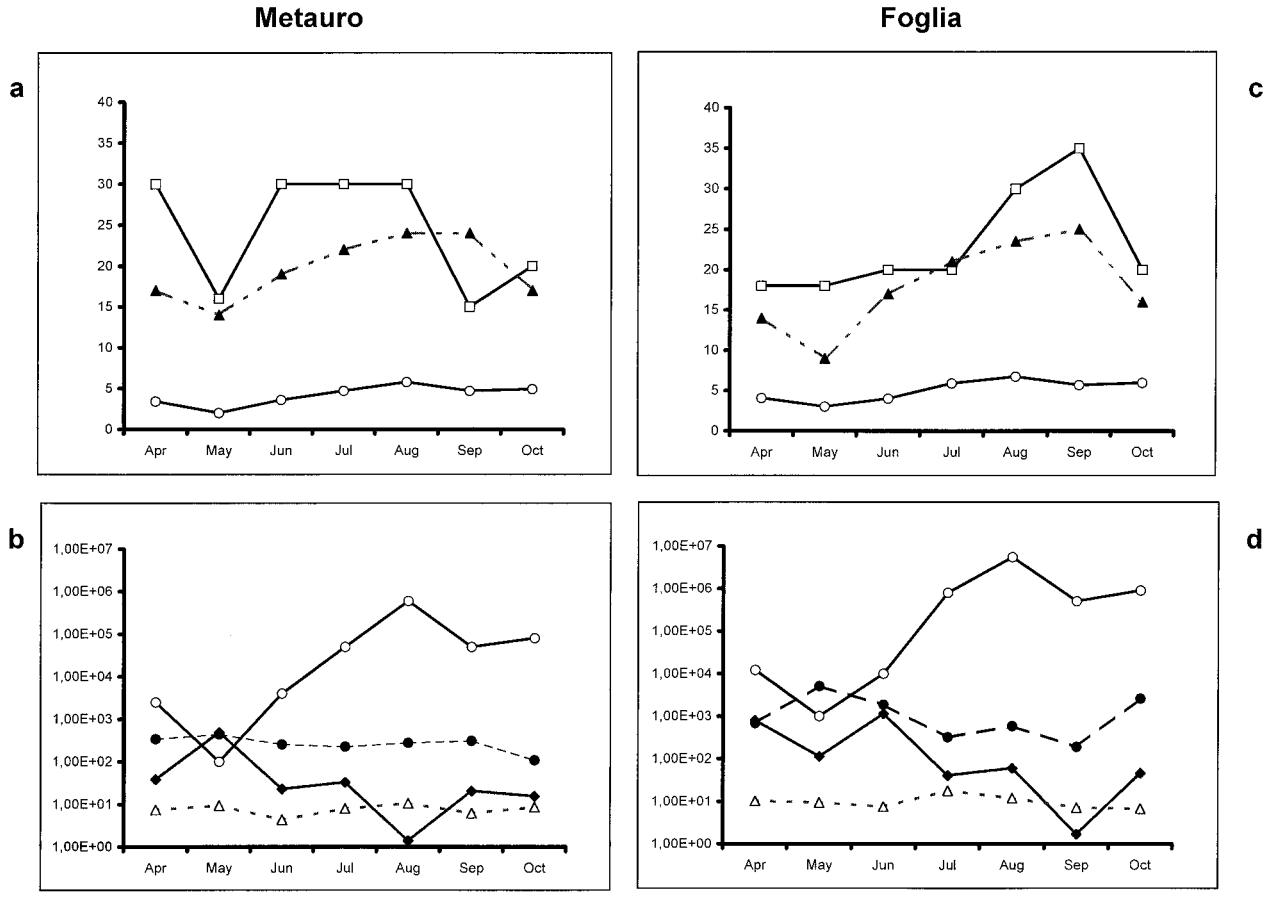
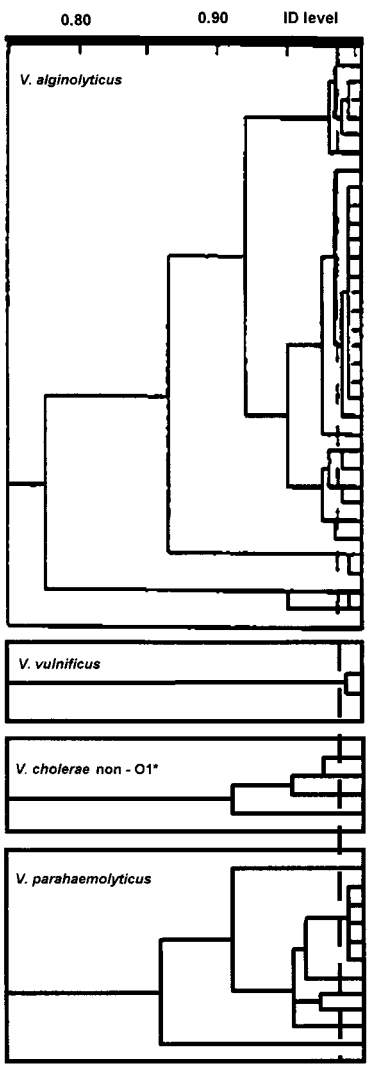


FIG. 1. Viable *Vibrio* spp. $\log_{10}$ of CFU 100 ml^{-1} (○), temperature (▲), and per mille salinity NaCl (□) measured monthly in the Metauro (a) and Foglia (c) estuaries. Viable *Vibrio* spp. $\log_{10}$ CFU 100 ml^{-1} (○), nutrient concentrations as total nitrogen micrograms liter^{-1} (●) and phosphate micrograms liter^{-1} (◆) and as dissolved oxygen milligrams liter^{-1} (△) measured monthly in the Metauro (b) and Foglia (d) estuaries. All abiotic parameters were measured as previously reported by Fiorentini et al. (13).



Similarity coefficient	Strain	PhP-type	Virulence factor: Adhesivness (cells with more then 10 bacteria on the surface)	Virulence factor: Bacterial CT	Virulence factor: Supernatant CT	Virulence factor: Hly§	Sampling: Site	Sampling: Month	Isolation method: TCBS/APW**
	93	S2	+	-	-		F	Oct.	TCBS
	Fo	C1	-	-	-	+	F	Oct.	APW
	Yb	C1	-	-	-	+	F	Apr.	APW
	Fl	C1	-	-	-		F	Oct.	APW
	46	C1	+	-	-	+	M	Sept.	TCBS
	46 a	S1	+	-	-	+	M	Sept.	TCBS
	374 g	S3	+	+	-	+	M	Apr.	TCBS
	414 b	S4	-	+	-	+	F	Jun.	TCBS
	21	C3	-	-	+	+	F	Sept.	TCBS
	25	C3	-	-	+	+	M	Sept.	TCBS
	Mc 2 B	C3	+	-		+	M	Apr.	APW
	57	C3	-	-	-		F	Oct.	TCBS
	61	C3	-	-	-	+	F	Oct.	TCBS
	69	C3	-	-	-	+	M	Oct.	TCBS
	90	C3	+	-	-	+	M	Oct.	TCBS
	Ya	C3	+	+	-	+	F	Apr.	APW
	MA7	C3	-	-	-	+	F	Jun.	APW
	AM6	C3	-	-	-	+	F	Aug.	APW
	AF3 g	C3	-	-	-	+	F	Aug.	APW
	497	C3	+	+	-	+	M	Aug.	TCBS
	MA2	C3	+	-	-	+	M	Jul.	APW
	459	S5	-	-	-	+	M	Jul.	TCBS
	49	C4				+	F	Oct.	TCBS
	56	C4	-	-	-	+	F	Oct.	TCBS
	76 pl	C4	-	-	-		M	Oct.	TCBS
	76 v	C4	+	-	-		M	Oct.	TCBS
	80	C4	+	+	-	-	M	Oct.	TCBS
	AF3vb	C4	-	-	-	+	F	Aug.	APW
	88g	C5	+	+	-	+	F	Oct.	TCBS
	472	C5	+	-	-	+	F	Aug.	TCBS
	457	C7	+	+	-	+	F	Aug.	TCBS
	AM5	S6	+	-	-	+	F	Aug.	APW
	8	C2	-	-	+	-	F	Sept.	TCBS
	10	C2					F	Sept.	TCBS
	FA2	C6	-	+	+	+	F	Jun.	APW
	F e 2	C6	-	+	-	-	F	Apr.	APW
	420	C7	-	+	-	-	F	Jul.	TCBS
	FA7	S7	+	+	+	+	F	Jul.	APW
	58	C4	-	-	-	-	F	Oct.	TCBS
	AM3	C9	-	+	-	-	M	Jul.	APW
	461	C9	-	+	-	-	F	Aug.	TCBS
	485	C9	+	+	-	+	M	Aug.	TCBS
	488	C9	+	+	-	-	M	Aug.	TCBS
	MA1 b	C9	-	-	-	-	M	Aug.	APW
	F f 1	S8	+	+	-	-	F	Apr.	APW
	AF2	S9	-	+	-	-	F	Aug.	APW
	AF5	S10	+	+	-	-	F	Aug.	APW
	AF3v	S11	+	+	-	-	F	Aug.	APW
	86	S12	+	-	-	-	M	Oct.	TCBS

* Serotype
** Isolation of *Vibrio* strains from alkaline peptone water (APW) or thiosuphate citrate bile sucrose (TCBS) medium
§ *V. parahaemoliticus* strains were tested on Wagatsuma agar (20)

FIG. 2. Dendrograms showing phenotypic similarity among isolated *Vibrio* strains of different species, their virulence properties, and sites and times of sampling. Abbreviations used: ID level, identity level; Hly, hemolysin; CT, cytotoxin; M and F, Metauro and Foglia estuary, respectively; S1 to S6, single PhP types; C1 to C9, common PhP types.

when at least 50% of cultured cells rounded up, whereas adhesive capacity was expressed as the percentage of cells with more than 10 bacteria on the cell surface. Samples for fluorescence and scanning electron microscopy (SEM) were prepared as previously described (13).

Occurrence of *Vibrio* spp. Counts of viable vibrios, grown on TCBS medium, ranged from 10^2 CFU 100 ml^{-1} (April) to 10^5 CFU 100 ml^{-1} (August) from Metauro estuary and up to ca. 10^6 CFU 100 ml^{-1} (August) from Foglia estuary. Table 1 shows the occurrence of *Vibrio* species in both sampling sites isolated by using TCBS or APW medium. The frequency of all the isolated species did not differ significantly between the two sites ($\chi^2 = 0.534$; $P = 0.766$), except for *V. vulnificus*, which occurred only in Foglia estuary. APW medium used as a secondary enrichment allowed a good recovery of non-O1 *V. cholerae* compared to TCBS medium.

Ecology and diversity of *Vibrio* strains. The Wilcoxon matched-pair signed-ranks nonparametric test computed on abiotic data (N total, phosphate, dissolved oxygen, temperature, salinity, and pH) measured monthly in both sampling sites did not show significant differences, except for the N total concentration, which was significantly higher in the Foglia than in the Metauro estuary (Fig. 1; $P = 0.0425$). This finding coincides with a significantly higher number of *Vibrio* strains

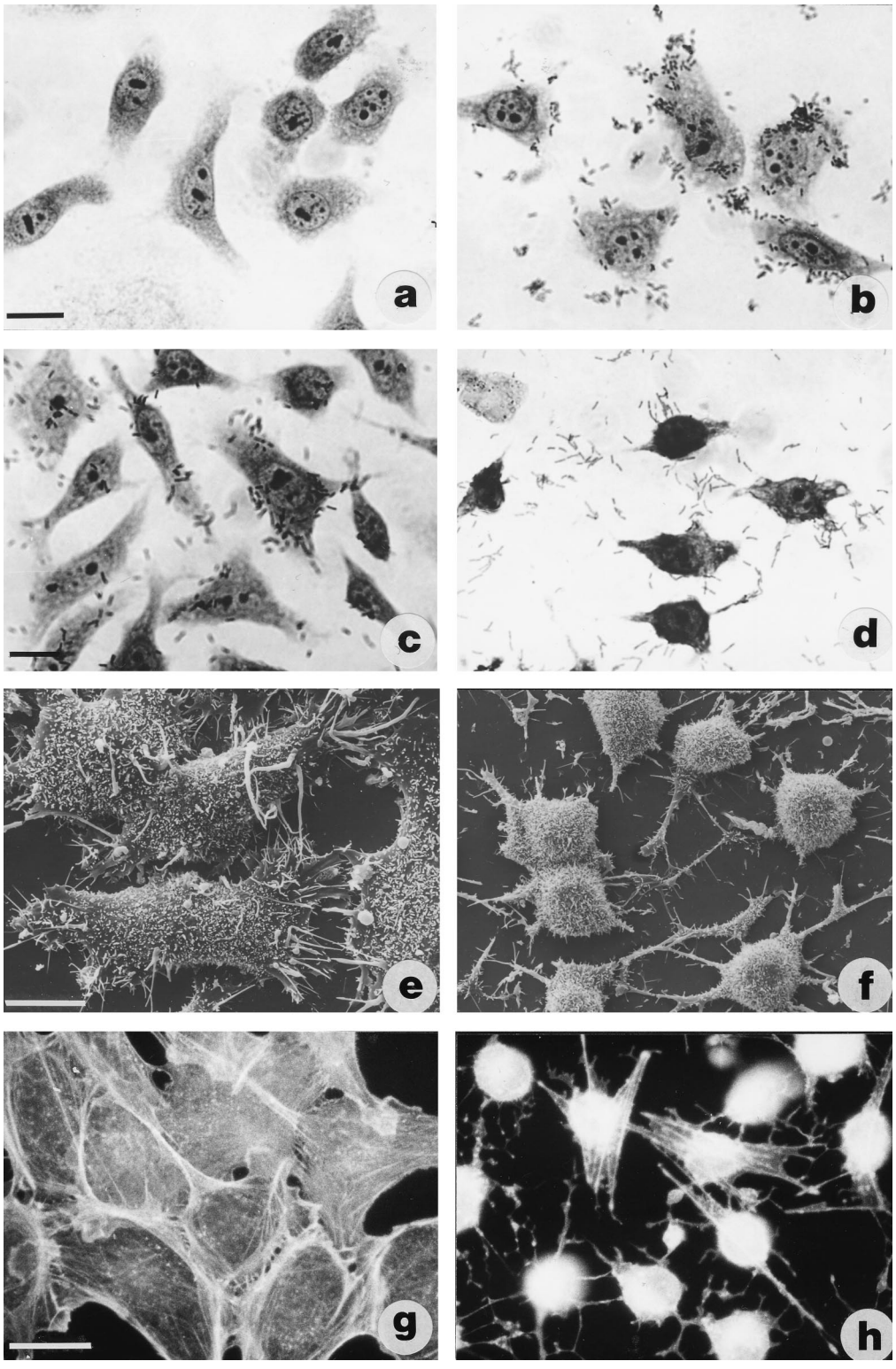


FIG. 3. Phase-contrast micrographs of HEp-2 cells exposed to bacteria (a to d) showing different patterns of adhesion (b and c) or cytotoxicity (d). Note the clustered bacterial distribution on the cells in panel b and the diffuse one in panel c. SEM of control CHO cells (e) and CHO cells after treatment with cytotoxic supernatant (f). Cytotoxicity due either to bacteria or to soluble bacterial products causes cell rounding (d and f). Bar, 10 μm.

recovered from the Foglia estuary compared to the Metauro estuary and with a similar trend (Fig. 1; $P = 0.018$). We observed also a positive correlation between the *Vibrio* occurrence (Fig. 1b and d) and temperature (Fig. 1a and c) throughout the study ($\rho = 0.559$, $P = 0.038$, Spearman coefficients). These results are in agreement with recently reported data concerning the Southern Italian coast (7).

Dendrograms (Fig. 2) group the strains by the different PhP types within each *Vibrio* species: the unweighted-linked clustering analysis identified nine clusters of common PhP types (C), which are defined by levels of similarity ranging from 75 to 97.5%. Single phenotypes (S) significantly different from the most common PhP types were also found. A range of diversity from 0.802 to 0.846 was determined among *V. alginolyticus* strains. Of interest, PhP type C7 was found to be common to both *V. alginolyticus* 457 and non-O1 *V. cholerae* 420 collected from Foglia estuary, while all *V. parahaemolyticus* strains belonged to a few different PhP types and elicited a low diversity. Finally, the non-O1 *V. cholerae* strains analyzed yielded a diversity index of 0.822. No evident relationship between the PhP types and the origin of the isolates was found. According to our findings of identical PhP types in both rivers and during different months it is possible to hypothesize that *Vibrio* strains belonging to various species may survive in largely different physicochemical conditions.

Pathogenic properties of *Vibrio* strains. A total of 49 *Vibrio* strains characterized by the PhP system were assayed for their ability to adhere and to induce cytotoxicity in cultured mammalian cells. The 35.5% of *Vibrio* strains recovered from the Foglia estuary and the 66.7% of the strains recovered from the Metauro estuary were able to adhere to HEp-2 cells. A total of 23 strains had adhesive properties to HEp-2 cells (46.9%), 19 strains produced cytotoxic effects on cultured cells (38.8%), and 5 strains were able to release in the supernatant factors or toxins capable of causing cytopathic effects consisting of retraction and rounding up of cultured cells (10.2%). Adhesive and cytotoxic features were contemporaneously present in 45.5% of *V. parahaemolyticus* strains, 25% of non-O1 *V. cholerae* strains, and 18.8% of *V. alginolyticus* strains. Only one non-O1 *V. cholerae* strain showed all of the virulence properties we tested.

Two adhesiveness patterns were detected either by LM or SEM. Figure 3 shows the adhesion patterns of vibrios to HEp-2 cells, either "clustered and localized" (Fig. 3b, *V. parahaemolyticus* 86) or "diffuse" (Fig. 3c, *V. alginolyticus* 46). A large number of *Vibrio* strains isolated from both sampling sites appeared able to produce cytotoxic effects. In particular, 45% of the 49 PhP-typed *Vibrio* strains recovered from the Foglia were cytotoxic, while for strains isolated from the Metauro estuary this percentage fell to 28%. In Fig. 3d the cytotoxic effect of non-O1 *V. cholerae* FA7 can be seen, the HEp-2 cells becoming retracted and roundish within 24 h.

The cellular alterations induced by exposure to bacterial supernatants were investigated. As observed by SEM, CHO cells (Fig. 3e) after overnight exposure to *V. alginolyticus* 21 supernatant showed a significant rounding up, even if the cells remained attached to the substrate by thin protrusions (Fig. 3f). Such a change in cell shape was accompanied by a breakdown of the actin cytoskeleton (Fig. 3g and h). This effect was observed in 7 of 32 (21.8%) *V. alginolyticus* strains. This previously unreported in vitro effect could explain the in vivo pathogenicity elicited by some *V. alginolyticus* strains of clinical interest isolated from wounds and ear infections (4, 11, 16).

The high diversity of strains belonging to the same species, as shown by their phenotypical expression, may explain this strain-specific expression of virulence factors. It still remains unclear which are the most significant environmental factors able to induce a *Vibrio* strain to express a specific virulence for humans.

On the basis of our data, we conclude that toxigenic *Vibrio* strains isolated from the estuarine environment of the Northwest Adriatic coast, where seafood production and consumption and recreational and bathing areas are common, may significantly contribute to the onset of sporadic and epidemic outbreaks of diarrheal disease in humans. A long-term research program for the continuous monitoring and study of this specific coastal pollution is needed as an urgent preventive measure to protect human health.

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