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Attachment of *Vibrio cholerae* Serogroup O1 to Zooplankton and Phytoplankton of Bangladesh Waters

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Vibrio cholerae **serogroup O1, the causative agent of cholera, is capable of surviving in aquatic environments for extended periods and is considered an autochthonous species in estuarine and brackish waters. These environments contain numerous elements that may affect its ecology. The studies reported here examined physical interactions between *V. cholerae* O1 and natural plankton populations of a geographical region in Bangladesh where cholera is an endemic disease. Results showed that four of five clinical *V. cholerae* O1 strains and endogenous bacterial flora were attached preferentially to zooplankton molts (exuviae) rather than to whole specimens. One strain attached in approximately equal numbers to both exuviae and whole specimens. *V. cholerae* O1 also attached to several phytoplankton species. The results show that *V. cholerae* O1 can bind to diverse plankton species collected from an area where cholera is an endemic disease, with potentially significant effects on its ecology.**

Vibrio cholerae serogroup O1 causes human enteropathogenic disease (cholera) in temperate and tropical climates (2, 7, 27). In regions of Bangladesh, cholera is an endemic disease and occurs in a seasonal pattern (7). In such epidemics, the aquatic environment appears to be an important vector in transmission of cholera. The incidence of *V. cholerae* O1, coupled with epidemiological data, shows that contaminated surface and household waters are associated with human infections (24). These observations have stimulated research to define the effect(s) of aquatic habitats on the ecology and pathogenicity of *V. cholerae* O1 and its survival under various physicochemical conditions (3, 5, 13, 16, 21, 22, 26, 28). However, very little is known of interactions between *V. cholerae* O1 and biotic components of water.

A variety of biological surfaces in water can bind bacteria. Bacteria associated with surfaces have been shown to survive in aquatic environments for longer times than suspended forms (14, 20), possibly as an adaptation to the stressful effects of low nutrient levels (6). Surfaces commonly encountered by aquatic bacteria are those of plankton, microscopic plants and animals that dominate the microfloras of aquatic ecosystems. In many instances, bacteria, including *Vibrio* spp., are found attached to their surfaces (10, 12, 17, 23) and as part of their gut floras (23). We hypothesize that an important aspect of the ecology of *V. cholerae* O1 in cholera-endemic regions of Bangladesh may involve a relationship with plankton, supporting previous hypotheses that interepidemic reservoirs of *V. cholerae* O1 in Bangladesh are influenced by seasonal plankton blooms that accompany cholera epidemics (11).

The present study investigated attachment of *V. cholerae* O1 to endogenous zooplankton and phytoplankton of Bangladesh waters and whether attachment occurred with specific plankton species and their anatomical structures.

Plankton specimens were collected from a river adjacent to a sewage outfall near the Matlab (Bangladesh) hospital and from two local ponds near Matlab in April 1987. Plankton were collected during daylight hours, with the exception of one night collection of river water. Samples were obtained by towing a 64-μm-mesh nitex plankton net, fitted with a 250-ml bucket, through the top 1 to 2 m of surface water. Specimens were rinsed from the collection bucket, suspended in approximately 250 ml of homologous water (prefiltered through 64-μm-mesh nitex), and transferred to the laboratory at ambient temperature. In some experiments, surface waters were collected in a 20-liter carboy and plankton were separated in the laboratory.

Five *V. cholerae* O1 isolates (VC1, VC2, VC3, VC4, VC5) were cultured from diarrheal stools of Bangladesh patients by the Microbiology Branch of the International Centre for Diarrhoeal Disease Research, Bangladesh. The attachment properties of these isolates were tested following culture in high-nutrient medium (tryptic soy broth), since *V. cholerae* O1 is rapidly multiplying in human feces when it enters Matlab waters. VC1, VC2, and VC3 were classical biotypes; VC4 and VC5 were El Tor. *V. cholerae* O1 isolates were grown at 35°C on tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing 1% NaCl for 18 h, incubated until mid-exponential-growth phase in tryptic soy broth (Difco) containing 1% NaCl, washed with 3 20-ml volumes of filter-sterilized homologous water at 3,000 × *g*, and then adjusted to approximately 10^7 CFU/ml of water.

For attachment assays, plankton samples were added to polypropylene vials (inner diameter, 1 cm) fitted at one end with 64-μm-mesh nitex. Plankton were washed three times by gravity filtration with filter-sterilized homologous water. Vials containing washed plankton were transferred to 24-well sterile cluster plates (catalog no. 3524; Costar, Cambridge, Mass.) containing 0.9 ml of filter-sterilized water and

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TABLE 1. Binding of *V. cholerae* O1 to phytoplankton and zooplankton of Matlab, Bangladesh, waters[a]

Plankton	Degree of binding[b] to:		Sample source
	Whole specimens	Exuviae	
Zooplankton			
Copepods			
Acartia sp.	–	+	River
Acartia chilkaensis	–	+	River
Acartia sewelli	–	+	River
Cyclops sp.	–	+	Pond
Diaptomus sp.	–	NP	Pond
Cladocerans			
Bosmina sp.	–	+	River
Daphnia sp.	–	NP	Pond
Ceriodaphnia sp.	–	+	River
Diaphanosoma sp.	–	+	River
Bosminopsis sp.	–	+	Pond
Rotifers (*Brachionus* sp.)	–	+	River, pond
Phytoplankton			
Volvox sp.	+	+	River
Pediastrum simplex	+	+	River
Unicellular cyanobacteria	+	NP	Pond
Spirulina sp.	–	NP	River, pond

[a] Degree of binding observed for VC1, VC2, VC4, and VC5 isolates. VC3 attached in similar numbers to both whole plankton and exuviae.

[b] –, <10 *V. cholerae* O1 per specimen; +, >100 *V. cholerae* O1 per specimen; NP, not present in sample.

0.1 ml of bacterial suspension, incubated for 60 min at 25°C, and then washed by gravity filtration with 3 10-ml volumes of sample water. Samples were transferred to wells containing 2% formaldehyde, incubated for 15 min at 25°C, and then washed with phosphate-buffered saline (0.13 M NaCl, 5 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.4]). Attached *V. cholerae* O1 were labeled with anti-*V. cholerae* O1 monoclonal antibody (3), washed with phosphate-buffered saline, incubated for 1 h at 25°C with fluorescein-conjugated goat anti-mouse immunoglobulin G (Organon Teknika, Malvern, Pa.), and then rinsed in phosphate-buffered saline. Specimens were transferred to microscope slides fitted with cover glasses and examined by epifluorescent microscopy (Olympus, Lake Success, N.Y.). Surfaces of zooplankton, phytoplankton, and detritus were scored for low (<10), medium (≥10 and ≤100), and high (>100) numbers of attached fluorescent *V. cholerae* O1 cells. In some experiments, samples were not inoculated with *V. cholerae* O1 but were stained with 0.1% acridine orange for 1 min at 25°C to observe bacteria that were representative of the endogenous attached flora.

River and pond water contained diverse plankton species (Table 1). Calanoid copepods, *Acartia* spp., and a *Senecella* sp. were the predominant zooplankton in river water. The third most abundant organism was a cladoceran, a *Bosmina* sp. Other cladocerans, members of the genera *Diaphanosoma* and *Ceriodaphania* were also present, but in lower numbers. One or two species of *Cyclops* and a rotifer (a *Brachionus* sp.) were observed. The predominant phytoplankton in river water were *Spirulina*, *Volvox*, and *Pediastrum* species.

Approximately 90% of the plankton flora of pond water were strains of the genus *Diaptomus*, a calanoid copepod. Most females were gravid, and many copepod nauplii were present. The dominant phytoplankton was a unicellular cyanobacterium. No *Volvox* spp. were observed. The most abundant zooplankton in a separate pond was a *Diaptomus* sp. *Cyclops* spp. were present in lower numbers. A *Bosminopsis* sp. was the dominant cladoceran, with some *Daphnia* spp. Unicellular cyanobacteria and *Spirulina* spp. were the dominant phytoplankton. A *Brachionus* sp. and another unidentified rotifer were present. No *Volvox* spp. were observed.

Results of binding experiments showed that VC1, VC2, VC4, and VC5 attached preferentially to moulted zooplankton exoskeletons (exuviae) rather than to whole specimens (Table 1). Exuviae showed high numbers (>100) of *V. cholerae* O1 on body and appendage parts (Fig. 1A), whereas whole specimens typically had few (<10) or no observable *V. cholerae* O1. In contrast, strain VC3 attached in high numbers to both whole zooplankton and exuviae. In general, numbers of *V. cholerae* O1 on individual plankton were greater than 100 or less than 10, with few specimens having between 10 and 100 bound bacteria. Acridine orange stains of uninoculated zooplankton revealed that endogenous populations of bacteria were also attached primarily to exuviae, not to whole specimens. Therefore, *V. cholerae* O1 and endogenous bacteria were bound to similar plankton structures, strengthening the hypothesis that *V. cholerae* O1 is a component of the adherent endogenous microflora of Bangladesh waters. *V. cholerae* O1 was not observed on natural (uninoculated) specimens, possibly because of the inherent limitations on the numbers of specimens that could be examined by microscopy.

High numbers of *V. cholerae* O1 were attached to whole specimens and exuviae of three phytoplankton species (Table 1). Interestingly, *V. cholerae* O1 and endogenous bacteria displayed a consistent focal binding pattern on a *Volvox* sp. (a colonial phytoplankton) which was not observed for other phytoplankton species (Fig. 1B).

It has been reported previously that zooplankton promote the growth of *Vibrio* species (15). Huq et al. (10, 11) showed that the survival of *V. cholerae* O1 is enhanced when it is cultured with laboratory-grown planktonic copepods isolated originally from fresh and estuarine waters. Those authors noted large numbers of *V. cholerae* attached to plankton structures. Other aquatic biota, such as water hyacinths from Bangladesh waters, have also been shown to be colonized by *V. cholerae* and to promote its growth (25).

The mechanisms involved in attachment of *V. cholerae* O1 to plankton were not determined in the present study. It is likely a complex interaction, with physical and chemical requirements for both *V. cholerae* O1 and plankton, including ionic and/or nonionic reactions between lipids, carbohydrates, and proteins. The preferential attachment of *V. cholerae* O1 to exuviae rather than to whole plankton may result from substances exuded by whole plankton that repel bacteria and/or mask sites that are available on exuviae. Likewise, bacteria may form attachment sites for other bacteria.

It is known that during periods of reduced nutrient levels, such as those encountered in aquatic environments, *V. cholerae* O1 and other *Vibrio* spp. undergo physiological and morphological changes. These include the production of novel bacterial proteins and changes in fatty acids (1, 8, 9, 19). As has been shown for other *Vibrio* spp., adherence properties can also be enhanced (6). Importantly, these changes may be related to the viable, nonculturable form of *V. cholerae* O1 described by Colwell et al., which is induced by nutrient-deficient environments (4) and which occurs in high concentrations in Bangladesh waters (3).

A direct relationship between attachment of *V. cholerae* O1 to chitin surfaces and human disease has been proposed

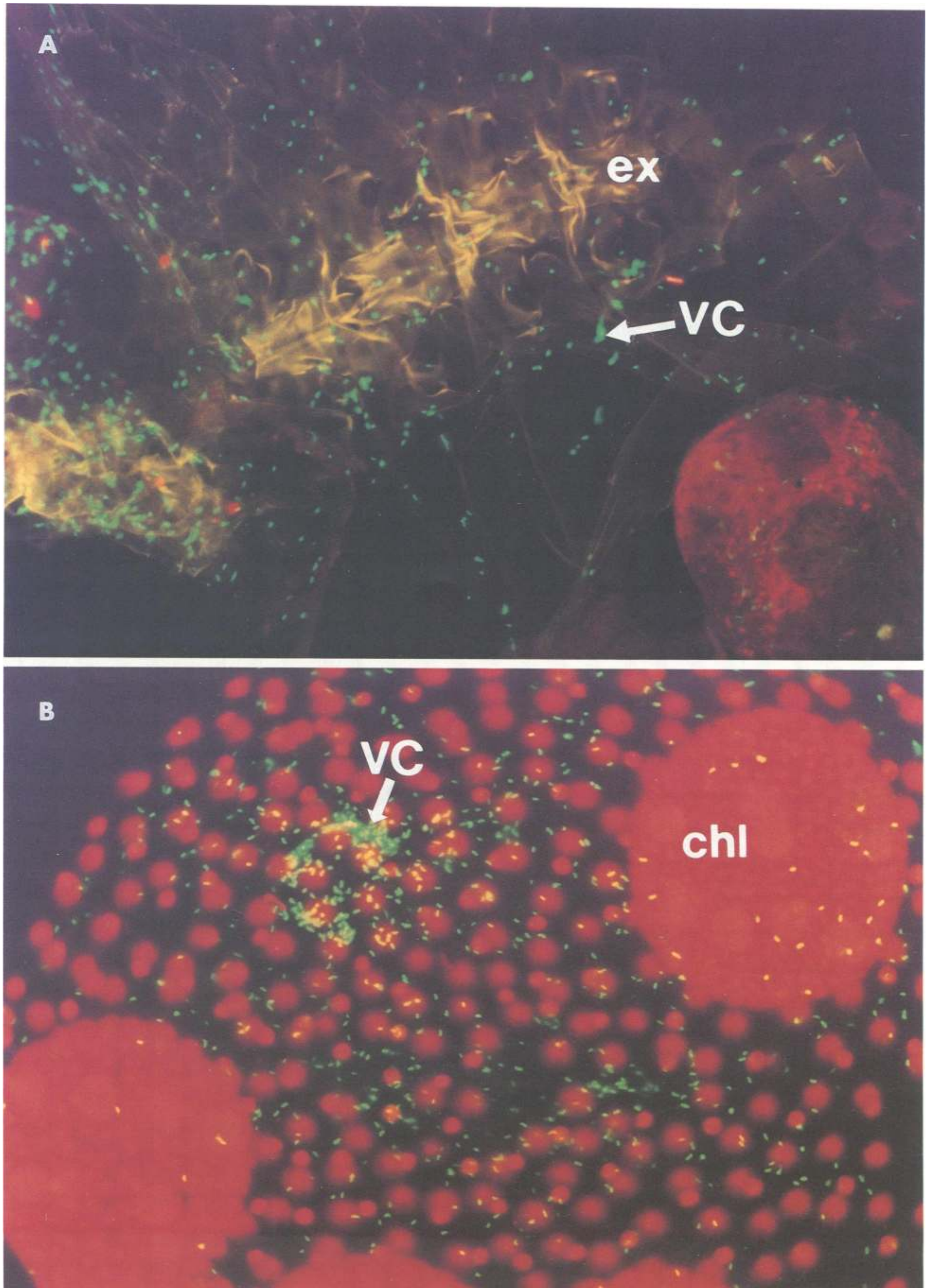

FIG. 1. Fluorescence photomicrography of *V. cholerae* O1 VC1 attached to plankton. (A) Copepod exuviae; (B) *Volvox* species. VC, *V. cholerae* O1 (green); chl, chloroplasts (red); ex, plankton exoskeleton.

by Nalin et al. (18), who showed that chitin protects *V. cholerae* O1 from the lethal effect of low pH. They suggest that chitin may promote pathogenicity of *V. cholerae* O1 by protecting it from the acidic environment of the human gastrointestinal tract. Our experiments show that chitinous surfaces of plankton concentrate *V. cholerae* O1 and may increase the number of *V. cholerae* in a given unit of water. Future experiments will determine if these levels reach an infective dose and if attachment affects the physiology and pathogenicity of *V. cholerae* O1.

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