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Effect of Alum on Free-Living and Copepod-Associated *Vibrio cholerae* O1 and O139

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The effects of alum [$KAl(SO_4)_2$] on free-living and copepod-associated *Vibrio cholerae* O1 and O139 were investigated by using plate counts and immunofluorescence direct viable counting (DVC). Growth of alum-treated cells in 0.5‰ Instant Ocean seawater was inhibited, i.e., no growth was obtained on Luria-Bertani (LB) agar or thiosulfate-citrate-bile salt-sucrose (TCBS) agar. However, a significant number of the inhibited cells maintained viability, as measured by DVC. In comparison, a significant number of *V. cholerae* organisms associated with zooplankton, most of which were crustacean copepods, were viable but nonculturable, with only a small number of cells retaining culturability on LB and TCBS agar. Both DVC and viable plate counts (CFU) were significantly greater for *V. cholerae* O1 and O139 associated with zooplankton than for *V. cholerae* in water alone, i.e., without copepods. It is concluded that alum is an effective coagulant but not an effective killing agent for *V. cholerae* and that association with copepods offers protection for *V. cholerae* O1 and O139 against alum and chlorine treatments.

Vibrio cholerae O1 and O139, causative agents of epidemic cholera, are recognized as autochthonous to the aquatic environment. Their association with zooplankton has been documented in a series of studies, including microcosm experiments, the results of which have shown that attachment to living copepods remarkably promotes survival of *V. cholerae* (11). Furthermore, results of field studies carried out in regions where cholera is endemic and employing molecular genetic methods for detection have documented that *V. cholerae* O1 is present throughout the year in the aquatic environment of such regions. However, during interepidemic periods, *V. cholerae* exists predominantly in a viable but nonculturable (VBNC) state, thereby posing an unrecognized potential threat to public health (9).

Since cholera has long been known to be transmitted via water, safe drinking water is essential in regions where cholera is endemic. Water can be treated by boiling, chlorination, or chemical flocculation followed by sedimentation and chemical disinfection. Potassium aluminum sulfate [$KAl(SO_4)_2$], commonly known as alum, is used to purify drinking water in many developing countries, including Bangladesh. Recently, the effectiveness of alum in decontaminating household water has been reported (13, 16). That is, the chemical is recommended for treating tank, pond, or well water to obtain water safe for drinking, as well as water for oral rehydration solutions during cholera outbreaks (1). However, the effectiveness of chemical treatment of water depends on environmental conditions. For example, chitin, a major component of crustacean exoskeletons, has been shown to protect *V. cholerae* O1 from acidic action during movement through the stomach (15). In addition, chitin can protect *V. cholerae* O1 at low temperatures for long periods (2). Because it appeared possible that such protection could occur under selected environmental conditions, this study was undertaken to investigate whether *V. cholerae* is killed or merely enters the VBNC state after treatment with alum. Since VBNC cells of *V. cholerae* can maintain pathogenicity (5) and are capable of producing clinical symptoms of cholera, as has been demonstrated in human volunteer trials (6), the effect of alum treatment on both free-living cells and cells attached to copepods was investigated.

Zooplankton samples, the majority of which were crustacean copepods, were collected from ponds and lakes in College Park, Md., using a 100-μm-mesh-size plankton net. Samples were allowed to settle at room temperature for 2 h to remove sedimentable material. Copepods were separated from the water with a 100-μm-mesh-size net and were washed with 4 liters of filter-sterilized (0.22-μm pore size) 0.5‰ Instant Ocean (IO) (Aquarium Systems, Mentor, Ohio), which provided controlled environmental conditions in the microcosms throughout the experiments. The salinity employed in the studies reported here was approximately that of brackish waters used as a source of drinking water in the delta regions of countries where cholera is endemic, such as Bangladesh.

Washed cells of *V. cholerae* suspended in sterile IO water were added to flasks containing live copepods (approximately five copepods per ml) to a final concentration of 10^8 cells per ml and 0.5‰ total salt content. Nearly 95% of the copepods remained alive throughout the experiments, as determined by monitoring of mobility. All microcosms were maintained at room temperature overnight at the initiation of each experiment to allow ample time for attachment of the bacterial cells to the copepods.

Copepods to which *V. cholerae* had been added were removed from the microcosms, washed with 4 liters of 0.5‰ sterile IO, and resuspended in glass containers containing alum and chlorine solutions at different concentrations (chlorine was in the form of commercially available Clorox [The Clorox Company, Oakland, Calif.], which contains 5.25% sodium hypochlorite, a chlorine compound for household use as a disinfectant). After incubation for 4 h at 25°C, the copepods were again collected by filtration, resuspended in 10 ml of sterile 0.5‰ IO, and homogenized with a tissue grinder (Wheaton, Millville, N.J.). Culturable cells of *V. cholerae* associated with

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TABLE 1. *V. cholerae* strains used in this study

Strain	Serogroup	Type and source (reference)
V. cholerae ATCC 14035	O1	Clinical
V. cholerae (569B) ATCC 25870	O1	Clinical
V. cholerae 41	O1	Environmental, Brazil (17)
V. cholerae 60	O1	Environmental, Brazil (17)
V. cholerae NT330	O139	Clinical, India (3)

the copepods were enumerated by using Luria-Bertani (LB) agar and thiosulfate-citrate-bile salt-sucrose (TCBS) agar. LB agar, a nonselective agar, was used to support optimal growth of bacteria, while TCBS agar, a highly selective medium routinely used for enumeration and isolation of *V. cholerae*, was used to obtain total culturable counts of *V. cholerae*. After copepods had been removed, filtrates were concentrated by filtration through a polycarbonate membrane (0.22-μm pore size). The cells retained on the membranes were resuspended by placing the membrane upside down in 10 ml of sterile 0.5‰ IO and agitating. Culturable cells of *V. cholerae* in the filtrates were enumerated by plate counts, as described above. An immunofluorescence method, developed recently in our laboratory, in which the direct fluorescent-antibody and direct viable count (DFA-DVC) methods are combined (4), was employed to detect VBNC cells by a modified procedure, details of which are reported elsewhere (8, 14). The effects of different concentrations of alum and chlorine (sodium hypochlorite) on pure cultures of *V. cholerae* (10^6 cells/ml) suspended in sterile 0.5‰ IO were also determined. Five strains of *V. cholerae* O1 and O139, isolated from both environmental and clinical sources, were used in the study (Table 1). After incubation for 4 h at 25°C, *V. cholerae* cells were collected by membrane filtration (0.22-μm pore size) and washed with 20 ml of sterile 0.5‰ IO. Cells retained on the filters were resuspended in 10 ml of sterile 0.5‰ IO and enumerated by both plate count and DFA-DVC.

Effect of alum on pure cultures of *V. cholerae*. As shown in Table 2, only one of four strains of *V. cholerae* O1 was completely inhibited, as demonstrated from results of plate counts on LB agar, by 0.05% alum, the concentration employed being that which was recommended by Ahmad et al. (1) to kill *V. cholerae* in water. In contrast, when cells were exposed to 0.5% alum, growth of all four strains was inhibited, i.e., no growth was obtained on either LB or TCBS agar. Although LB agar provided superior recovery of *V. cholerae*, it is not the medium of choice for isolation of *V. cholerae* because the medium is not selective for *V. cholerae*, allowing overgrowth by other vibrios and unrelated organisms. However, results of DFA-DVC revealed that the majority of the treated cells retained viability, even after treatment with 10 times more alum than the recommended concentration for water treatment (Table 2). All of the strains examined in this study, including *V. cholerae* NT330, which is a toxigenic strain (3) of the recently designated epidemic serogroup O139, exhibited resistance to 0.5% alum, as determined by DFA-DVC (Table 2).

In cases in which 0.5% alum was found not to inhibit growth of *V. cholerae*, i.e., plate counts on LB and TCBS agar of alum-treated cells were very high, and an even larger number of cells were found to be viable, as determined by DFA-DVC (4), cells were exposed for 4 h to solutions of 1% Clorox and 2.5% Clorox added separately to 0.05% and 0.5% alum to determine whether the combined treatment would inactivate *V. cholerae*. Inhibition of growth of *V. cholerae* was achieved, but 1 to 10% of the cells remained viable, i.e., DFA-DVC positive (Table 3).

Effect of alum on copepod-associated cells of *V. cholerae*. *V. cholerae* attached to copepods remained culturable after treatment with alum for 4 h, as evidenced by growth when inoculated into LB and TCBS agar (Table 4).

The primary mechanism of the bactericidal activity of alum is acidification, as reported by Khan et al. in 1984 (13). From the results of the study reported here, it is concluded that alum is effective in inhibiting growth of free-living *V. cholerae* cells, preventing colony formation, to some extent, due to induction of the VBNC state. However, the same treatment was not effective when copepod-associated *V. cholerae* cells were suspended in alum solutions. These results confirm our earlier observations (7) showing that copepods play an important role in the survival of *V. cholerae* in the environment. We hypoth-

TABLE 2. Effect of alum on free-living *V. cholerae*

Strain and alum concn[a]	No. of *V. cholerae* organisms		
	LB agar (CFU/ml)	TCBS agar (CFU/ml)	DFA-DVC (cells/ml)
V. cholerae O1 569B			
Control	$(2.02 \pm 0.78) \times 10^4$	$(1.02 \pm 0.65) \times 10^3$	$(3.14 \pm 1.01) \times 10^5$
0.05%	$(3.85 \pm 0.46) \times 10^3$	<10	$(1.25 \pm 0.98) \times 10^5$
0.50%	<10	<10	$(1.06 \pm 1.40) \times 10^5$
V. cholerae O1 41			
Control	$(6.65 \pm 0.65) \times 10^4$	$(2.25 \pm 0.85) \times 10^4$	$(6.03 \pm 2.10) \times 10^5$
0.05%	$(5.50 \pm 0.15) \times 10^1$	<10	$(2.75 \pm 0.74) \times 10^5$
0.50%	<10	<10	$(2.16 \pm 0.57) \times 10^5$
V. cholerae O1 60			
Control	$(1.31 \pm 0.52) \times 10^4$	$(2.70 \pm 0.77) \times 10^3$	$(2.59 \pm 0.48) \times 10^5$
0.05%	$(2.50 \pm 2.50) \times 10^1$	<10	$(1.44 \pm 0.64) \times 10^5$
0.50%	<10	<10	$(1.39 \pm 1.77) \times 10^5$
V. cholerae O139 NT330			
Control	$(3.10 \pm 0.67) \times 10^4$	$(2.30 \pm 0.33) \times 10^4$	$(2.59 \pm 0.74) \times 10^5$
0.05%	<10	<10	$(1.44 \pm 0.81) \times 10^5$
0.50%	<10	<10	$(1.39 \pm 0.44) \times 10^5$

[a] Alum solution was added to cell suspensions containing a pure culture of free-living *V. cholerae* cells (10^6/ml) in 0.5‰ IO.

TABLE 3. Effects of alum and hypochlorite on survival of free-living and copepod-associated *V. cholerae* ATCC 14035

Concn (%)[a]		*V. cholerae* cell type[b]	No. of *V. cholerae* organisms			%[c]
Alum	Hypochlorite (Clorox)		LB agar (CFU/ml)	TCBS agar (CFU/ml)	DFA-DVC (cells/ml)	
0.50	2.50	C	<10	<10	$(9.13 \pm 0.38) \times 10^4$	53.71
		F	<10	<10	$(4.00 \pm 1.00) \times 10^2$	4.76
	1.00	C	<10	<10	$(5.83 \pm 0.77) \times 10^4$	34.29
		F	<10	<10	$(6.00 \pm 2.00) \times 10^2$	7.14
0.05	2.50	C	<10	<10	$(4.91 \pm 0.96) \times 10^4$	28.88
		F	<10	<10	$(7.50 \pm 1.50) \times 10^2$	8.93
	1.00	C	<10	<10	$(2.34 \pm 0.49) \times 10^4$	13.76
		F	<10	<10	$(1.40 \pm 0.30) \times 10^3$	16.67
Control	Control	C	$(1.31 \pm 0.29) \times 10^4$	$(4.00 \pm 2.00) \times 10^2$	$(1.70 \pm 0.88) \times 10^5$	
		F	$(6.40 \pm 0.20) \times 10^2$	<10	$(8.40 \pm 0.37) \times 10^3$	

[a] Cells were exposed for 4 h.
[b] C, copepod associated; F, free living.
[c] Percentage of cells surviving treatment, i.e., DFA-DVC positive.

esize that the chitinolytic activity of *V. cholerae* may create a protective microenvironment (2), whereby the cells are attached to the copepods, embedded in microniches. Furthermore, bacterial cells ingested by copepods are protected from the low pH of the stomach when consumed by humans, by being embedded on the copepod surface and/or in the copepod gut. Since as few as 5 to 10 copepods can harbor an infectious dose of *V. cholerae*, ranging from 10^3 to 10^6 cells/ml, depending on the immunity of the host, and both the O1 and O139 epidemic serogroups may coexist in association with copepods (10), removal of copepods from drinking water by filtration is highly recommended, even after alum treatment.

In summary, it is concluded that alum and chlorine, individually or combined, do not kill *V. cholerae* but, instead, induce a majority of the cells to enter a VBNC state. Furthermore, copepod-associated *V. cholerae* cells appear to be effectively protected from the effects of alum, a chemical recommended for purification of natural waters serving as a drinking water source in regions where cholera is endemic. Even with the addition of chlorine subsequent to alum treatment, up to 90% of *V. cholerae* cells may remain viable and can pose a threat to human health. During times of the year when large numbers of copepods (blooms) occur (9), an increase in the risk of cholera from ingestion of untreated water and even of alum- and/or chlorine-treated water will exist if the water is not filtered. Findings of this study serve as a warning that pretreatment of drinking water with alum, even with the addition of 2.5% Clorox, does not guarantee safe water, without filtration, if *V. cholerae* is present and associated with copepods. A further complication is that water amended with 2.5% Clorox and 0.5% alum may not be acceptable to village populations of some countries like Bangladesh, for reasons of tradition and taste. Also, these levels of chemicals may not be acceptable, if residual concentrations with consistent accumulation are high enough to form potential carcinogens. Use of inadequately treated water should be discouraged, and effective, feasible, and acceptable means of obtaining safe drinking water must be developed. A potential solution to this difficult situation is a simple filtration method, described by Huq et al. (12), followed by chemical disinfection in which lower concentrations of disinfectant will be effective. Thus, an inexpensive, widely available, simple method for treatment of water for drinking purposes will be effective against acute risk of cholera in countries where cholera is endemic and where central distribution of safe drinking water is not available, particularly in disaster situations, such as flooding and weather extremes (e.g., typhoons and monsoons), and this lowered risk ultimately may reduce the epidemic levels of cholera.

TABLE 4. Effect of alum on *V. cholerae* ATCC 14035 associated with copepods

Treatment[a]	No. of *V. cholerae* organisms[b]		
	LB agar (CFU/ml)	TCBS agar (CFU/ml)	DFA-DVC (cells/ml)
Control			
Copepod	$(1.28 \pm 0.73) \times 10^6$	$(9.90 \pm 0.56) \times 10^5$	ND
Filtrate	$(3.34 \pm 0.81) \times 10^4$	$(7.78 \pm 0.29) \times 10^3$	ND
0.5% alum			
Copepod	$(6.58 \pm 0.44) \times 10^5$	$(3.41 \pm 0.73) \times 10^5$	ND
Filtrate	<10	<10	$(8.50 \pm 0.68) \times 10^5$
0.05% alum			
Copepod	$(4.53 \pm 0.22) \times 10^5$	$(3.10 \pm 0.74) \times 10^5$	ND
Filtrate	<10	<10	$(1.05 \pm 0.95) \times 10^6$

[a] Alum solution was added to a suspension containing copepods spiked with *V. cholerae* cells in 0.5‰ IO.
[b] ND, not done.

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