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Inactivation of Norwalk Virus in Drinking Water by Chlorine

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Norwalk virus in water was found to be more resistant to chlorine inactivation than poliovirus type 1 (LSc2Ab), human rotavirus (Wa), simian rotavirus (SA11), or f2 bacteriophage. A 3.75 mg/liter dose of chlorine was found to be effective against other viruses but failed to inactivate Norwalk virus. The Norwalk virus inoculum remained infectious for five of eight volunteers, despite the initial presence of free residual chlorine. Infectivity in volunteers was demonstrated by seroconversion to Norwalk virus. Fourteen of 16 subjects receiving untreated inoculum seroconverted to Norwalk virus. Illness was produced in four of the eight volunteers and in 11 of 16 control subjects. A similar Norwalk virus inoculum treated with a 10 mg/liter dose of chlorine produced illness in only one and failed to induce seroconversion in any of eight volunteers. Free chlorine (5 to 6 mg/liter) was measured in the reaction vessel after a 30-minute contact period. Norwalk virus appears to be very resistant to chlorine which may explain its importance in outbreaks of waterborne disease.

Norwalk virus has recently been recognized as an important cause of waterborne illness (3, 11–13, 15, 20, 21) and is responsible for ca. 23% of reported waterborne outbreaks. Since outbreaks have been reported from municipal water systems, the potential for large epidemics exists as does the need for effective post-contamination treatment of drinking water. Although it is evident that Norwalk virus is readily transmitted via contaminated drinking water, there is only one report of the resistance of Norwalk virus to disinfection in water. In an outbreak at a camp, Norwalk virus transmission was linked to contaminated water which contained 1.0 mg of residual iodine per liter (W. E. Woodward, personal communication). Study of this virus, unlike rotavirus and other enteric viruses, is complicated by the lack of an animal model or a readily available cell culture system. Therefore, to study the inactivation of Norwalk virus in water, volunteers are necessary. This study examined the dose of chlorine required to inactivate Norwalk virus in drinking water.

MATERIALS AND METHODS

Volunteers. Informed consent was obtained from 32 healthy volunteers who had the study explained twice, passed a written exam, and passed both physical and laboratory examinations before participation. The protocol used was reviewed and approved by Committee for the Protection of Human Subjects at the four institutions involved, the University of Texas Medical School at Houston, Baylor College of Medicine, The Methodist Hospital, and the General Clinical Research Center. Groups of eight volunteers were housed for 7 days in the General Clinical Research Center of the Methodist Hospital under "enteric precautions". Before receiving the inoculum, all volunteers provided a stool specimen or rectal swab and a serum specimen. Each volunteer collected all stool specimens while in the hospital. Convalescent serum specimens were collected 1, 2, and 3 weeks after discharge. Volunteers were not screened for antibodies to Norwalk virus before participation.

Norwalk virus inoculum and assay. Three milliliters of a Norwalk virus suspension prepared in veal infusion broth and designated 8FIIa was obtained from A. Kapikian and R. Wyatt of the National Institute for Allergy and Infectious Diseases, National Institutes of Health. This inoculum has been safety tested and used previously in successful volunteer studies (5, 6, 10, 22). Upon receipt, the inoculum was divided into four samples and frozen at −70°C until use. Seroconversion to Norwalk virus was detected by radioimmunoassay (5, 9). Assays were performed under code in the laboratories of N. R. Blacklow (University of Massachusetts Medical School) and G. W. Gary (Centers for Disease Control).

Reference viruses preparation and assay. Two strains of rotavirus (Wa [human] and SA11 [simian], poliovirus type 1 LSc2ab, and bacteriophage f2 were used as reference viruses in the disinfection studies. The rotaviruses were prepared as ×10 concentrates from MA-104 cell cultures and were detected by plaque assay in MA-104 cell cultures as described previously (14). Poliovirus was also a ×10 concentrate prepared from and assayed in cultures of BGM cells (16). The bacteriophage f2 was harvested from a lawn of *Escherichia coli* Hfr bacteria and filtered through a 0.45-μm (pore size) membrane filter. Bacteriophage f2 was detected on the same host bacterium by plaque assay (1). Viruses were not purified further to simulate actual field conditions in which viruses are associated with particulate matter and may be aggregated. Each reference virus was suspended in sterile phosphate-buffered saline. The amount of chlorine required to yield a free chlorine residual of 0.5 to 1.0 mg/liter after 30 min was determined by diluting stock virus in sterile demand-free water, dosing with chlorine, and measuring the residual chlorine after 30 min of mixing. Since only a small amount of Norwalk virus inoculum was available, a mock inoculum of stool suspended in veal infusion broth (6) was used to determine the required dose of chlorine to be added to the Norwalk virus inoculum.

Chlorine solution preparation and measurement. Demand-

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TABLE 1. Clinical response to Norwalk virus in volunteers receiving untreated or chlorine-treated inoculum

Chlorine dose (mg/liter)	No. of volunteers with symptoms/no. of volunteers tested (%)				
	Any symptom	Vomiting	Diarrhea	Nausea	Seroconversion
0	11/16 (69)	8/16 (50)	8/16 (50)	7/16 (44)	14/16 (88)[a]
3.75–6.25	5/8 (63)	2/8 (25)	3/8 (38.5)	4/8 (50)	5/8 (63)
10.0	1/8 (12.5)	1/8 (12.5)	0/8 (0)	1/8 (12.5)	0/8 (0)

[a] Results show the first two groups not to be significantly different from each other, but both are significantly different from the last at the 95% confidence level with the Neuman-Keuls studentized range yardstick.

free distilled water and demand-free glassware used in all experiments was prepared as described previously (2). Household bleach (Clorox Co., Oakland, Calif.) diluted 1:200 in demand-free distilled water was used as a stock solution. Chlorine concentration in the stock solution was determined by the amperometric method. Residual chlorine in the test solutions was determined by the DPD (*N,N*-diethyl-*p*-phenylenediamine) method with a commercial kit (LaMotte Chemical Products Co., Chestertown, Md.). Before each experiment, reference chlorine solutions provided by the U.S. Environmental Protection Agency were measured by each method to ensure accurate readings.

Disinfection studies. Virus inocula were added to 2,000 ml of sterile, distilled demand-free water at pH 7.4. The solutions were mixed with a magnetic stirrer throughout the course of the experiment. All experiments were conducted at room temperature, i.e., 25°C. Before being dosed with chlorine, a control sample was taken for virus assay. The desired amount of chlorine was added and mixed thoroughly, immediately after which a sample for residual chlorine was collected (T_0). The reaction was stopped with 0.05 N sodium thiosulfate after 30 min of contact time. The Norwalk virus inoculum was administered to the volunteers within 30 min after treatment. Each volunteer received 250 ml of the solution 2 min after drinking 100 ml of 2% sodium bicarbonate. Volunteers in control groups received untreated inocula handled in the same manner. Reference viruses were assayed in cell culture as described above.

RESULTS

Control groups. Two groups of eight volunteers received the untreated Norwalk virus inoculum. Of those, 11 of 16 developed illness typical of that caused by Norwalk virus, including nausea, vomiting, and diarrhea (Table 1). Serological testing revealed that 14 of the 16 seroconverted to Norwalk virus by detecting a fourfold rise in antibody titer. Thus, three subjects seroconverted in the absence of overt signs or symptons of illness.

Treated groups. Two treatment studies were conducted. In the first study, viruses were treated with 3.75 to 6.25 mg of chlorine per liter to yield a free residual of 0.5 to 1.0 mg/liter, as might be found in a drinking water distribution system. In the second experiment, all viruses were treated with 10 mg of chlorine per liter to simulate post-contamination treatment of a water supply system. One group of eight volunteers received Norwalk virus inoculum which had been treated with a dose of 3.75 mg of chlorine per liter (Table 1). The dose was chosen to yield ca. 0.5 to 1.0 mg of free residual chlorine per liter, as might be expected in a drinking water distribution system. Five of the eight volunteers developed signs of illness, and five of eight seroconverted (Table 1). Although free residual chlorine was detected immediately after dosing, no free residual was detected after 30 min of contact time. With the exception of bacteriophage f2, each of the reference viruses, including a human strain of rotavirus, was completely inactivated (1 to 4 $\log_{10}$ PFU reductions) by similar doses (Table 2). The chlorine demand of each virus preparation was somewhat different as evidenced by the dose required to produce approximately equivalent free residuals at T_0.

Since the first dose of chlorine did not adequately disinfect the Norwalk virus, a higher dose of 10 mg/liter was chosen to simulate post-contamination treatment of a water supply, irrespective of the differences in chlorine demand of the inocula. One of eight volunteers became ill (Table 1) but did not seroconvert to Norwalk virus. Therefore, Norwalk virus was not considered to have induced the illness. As in the first experiment, each of the other virus preparations, with the exception of f2, was inactivated beyond the level of detection (Table 3). Norwalk virus and f2 bacteriophage in this study survived 30 min in the presence of residual free chlorine.

DISCUSSION

Data reviewed by Kaplan et al. (11) suggested that Norwalk virus and related 27-nm agents may be responsible for 23% of the waterborne outbreaks of acute gastroenteritis in the United States. Despite its obvious importance, little is

TABLE 2. Inactivation of viruses in water by 3.75 to 6.25 mg of chlorine per liter[a]

Virus	PFU/ml		Chlorine dose (mg/liter)	Residual chlorine (mg/liter)			
	T_0	T_{30}		T_0		T_{30}	
				Free	Total	Free	Total
Rotavirus SA11	2.0×10^4	0	6.25	1.75	4.1	1.1	3.5
Bacteriophage f2	3.0×10^6	1.0×10^4	5.00	1.00	3.9	0.1	2.5
Poliovirus type 1 (LSc2ab)	2.0×10^4	0	3.75	0.75	2.9	0.4	2.0
Rotavirus (human strain Wa)	1.6×10^1	0	3.75	1.55	3.0	1.0	2.4
Norwalk	14/16[b]	5/8[b]	3.75	1–1.5	2.5–3.0	0	1.5–2.0

[a] Virus inocula were treated with a 3.75 to 6.25 mg/liter dose of chlorine, which yielded 0.5 to 1.0 mg of free residual chlorine per liter.
[b] Data shows number of volunteers seroconverted/number tested.

TABLE 3. Inactivation of viruses in water by 10 mg of chlorine per liter[a]

Virus	PFU/ml		Chlorine dose (mg/liter)	Residual chlorine (mg/liter)			
	T_0	T_{30}		T_0		T_{30}	
				Free	Total	Free	Total
Rotavirus SA11	4.5×10^3	0	10	6.45	8.35	5.45	7.35
Bacteriophage f2	3.2×10^4	2.0×10^0	10	5.75	8.10	1.3	7.25
Poliovirus (3/7/84)	1.1×10^3	0	10	5.8	8.1	4.4	6.9
Rotavirus (human strain Wa)	5.0×10^1	0	10	6.3	8.45	5.6	7.55
Norwalk	14/16[b]	0/8[b]	10	5–6	10.0	5–6	8–10

[a] Virus inocula were exposed to 10 mg of chlorine per liter for 30 min at 25°C.
[b] Data show number of volunteers seroconverted/number tested.

known about the environmental transmission, stability, and inactivation of Norwalk virus in water. In fact, the virus has not yet been classified into a known family, and although previously thought to be a parvovirus, recent evidence indicates a relationship to the caliciviruses (8). The apparent lack of information on Norwalk virus is due to the absence of a widely available assay system, the scarcity of reagents, and the need for volunteers to study infectivity. Waterborne outbreaks of illness due to Norwalk virus have been associated with swimming in a lake, drinking contaminated water or ice-containing beverages, septic tank contamination of spring or well water, swimming in a pool with inadequate chlorination, and municipal water systems (3, 11, 12, 17, 20, 21). These data suggest that the survival and migration of Norwalk virus in soil and water systems is similar to that of other enteric viruses.

The results of this study indicate that Norwalk virus present under naturally occurring conditions in a contaminated water supply may be highly resistant to chlorination and that routine chlorination alone cannot be relied upon to inactivate Norwalk virus. The resistance to chlorination exhibited by Norwalk virus in this study is most likely due to the aggregation of virus particles in the inoculum, a condition which reflects actual contamination of a water supply with human wastes. Studies of the inherent resistance of Norwalk virus in monodispersed preparations must await the development of in vitro cultivation systems.

It is also significant that three volunteers seroconverted without apparent signs of illness. In a waterborne outbreak, such persons could serve as "silent" sources of infection. These conclusions are supported by the only report (W. E. Woodward, personal communication) of a waterborne outbreak of Norwalk virus in which disinfection data were recorded before and during the outbreak. In a camp in Maryland, water pumped from a 95-foot (ca. 30-m)-deep well to a storage tank was found to contain 0.7 to 1.0 mg of iodine per liter before and during an outbreak, which affecting ca. 133 persons and suggested that, under field conditions, Norwalk virus also is very resistant to disinfection.

In this study, it was not possible to measure the titer of the inoculum used in terms of infectious units; therefore, the level of disinfection can only be interpreted as inactivation of an infectious dose. Norwalk virus antigen in this inoculum, a 2% stool filtrate, has been detected by radioimmunoassay at dilutions of up to 1:125 (9). The dilution in this study was 1:2,000. The minimum infectious dose of Norwalk virus is unknown, as is the exact nature of the immune response and duration of protection (4, 5, 18).

In addition to Norwalk virus, rotaviruses and hepatitis virus are the most important causes of viral water-borne infection. Based on our data and that of others, Norwalk virus and hepatitis A (7, 19) appear to be more resistant to chlorination than rotavirus. Furthermore, as reported for hepatitis A (19), free residual chlorine levels of 0.5 to 1.5 mg/liter only partially inactivated the virus suspension. It appears that Norwalk virus as well as hepatitis virus are somewhat more resistant to chlorine than are other enteroviruses.

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