Disinfection of Human Enteric Viruses in Water by Copper and Silver in Combination with Low Levels of Chlorine

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The efficacy of copper and silver ions, in combination with low levels of free chlorine (FC), was evaluated for the disinfection of hepatitis A virus (HAV), human rotavirus (HRV), human adenovirus, and poliovirus (PV) in water. HAV and HRV showed little inactivation in all conditions. PV showed more than a 4 log_10 titer reduction in the presence of copper and silver combined with 0.5 mg of FC per liter or in the presence of 1 mg of FC per liter alone. Human adenovirus persisted longer than PV with the same treatments, although it persisted significantly less than HRV or HAV. The addition of 700 μg of copper and 70 μg of silver per liter did not enhance the inactivation rates after the exposure to 0.5 or 0.2 mg of FC per liter, although on some occasions it produced a level of inactivation similar to that induced by a higher dose of FC alone. Virus aggregates were observed in the presence of copper and silver ions, although not in the presence of FC alone. Our data indicate that the use of copper and silver ions in water systems may not provide a reliable alternative to high levels of FC for the disinfection of viral pathogens. Gene probe-based procedures were not adequate to monitor the presence of infectious HAV after disinfection. PV does not appear to be an adequate model viral strain to be used in disinfection studies. Bacteroides fragilis bacteriophages were consistently more resistant to disinfection than PV, suggesting that they would be more suitable indicators, although they survived significantly less than HAV or HRV.

Effective disinfection of swimming pool water is required in order to prevent the threat posed to bathers by microorganisms of significant health importance. Outbreaks of enteric viruses (15), including coxsackievirus B5 (27), Norwalk virus (29), and adenovirus (ADV) (34), have occurred as a result of swimming in improperly disinfected recreational waters. Since there are serious problems with the detection of some enteric viruses of public health relevance, such as hepatitis A virus (HAV), human rotavirus (HRV), or Norwalk virus, in environmental samples, most disinfection studies have been conducted with poliovirus (PV) or bacteriophages as models for human enteric viruses (18, 25, 38, 51).

Chlorine is widely used for the routine inactivation of pathogenic microorganisms in water and particularly in swimming pool waters. However, chlorination has been found to contribute to the formation in water of numerous chlorinated organic compounds that are hazardous to human health (5). High chlorine concentrations may also cause irritation of eyes, mucous membranes, and skin, creating considerable discomfort for swimmers (11). Electrolytically generated copper and silver ions have been introduced as a relatively safe and odorless alternative method for water disinfection. Both copper and silver are bactericidal and algicidal and possess fungicidal properties (11, 32, 46, 49, 50). Treatment with copper and silver has been reported to be effective for the removal of bacteriophages and PV from water (51, 52).

In this study, we have compared the behavior of HAV, HRV, ADV, and PV in the presence of low levels of free chlorine (FC) alone or combined with copper and silver ions. Strategies for the monitoring of the efficiency of virus disinfection based on the use of bacteriophages of Bacteroides fragilis as indicators or by employing molecular procedures have been evaluated.

MATERIALS AND METHODS

Viruses and cell cultures. PV 1, strain LSc 2ab, and HRV Ito' p13 were propagated and assayed in BGM and MA-104 cells, respectively, as previously described (8). FRhK-4 cell cultures were used to propagate and assay the cytopathogenic HM-175 strain (courtesy of T. Cromeans, Centers for Disease Control, Atlanta, Ga.) of HAV (14). ADV type 5 was cultivated and assayed in Hep-2 cell monolayers. Bacteriophage B40-8 of B. fragilis was assayed as previously described (44).

Water. The test water used throughout these studies were obtained directly from the Sant Pere Martir well located in Esplugues de Llobregat, Barcelona, Spain, and from laboratory faucets. Water was allowed to flow for several minutes and collected in acid-washed 10-liter high-density polyethylene containers. Water was autoclaved and kept at 4°C. Chemical analyses were performed according to procedures adapted from those in reference 1.

Disinfection. All containers used in these experiments were soaked in 12.5% nitric acid, rinsed with distilled water, and autoclaved prior to use. Glassware used for systems involving FC were kept for over 15 h in a solution of 0.8 mg of FC per liter to satisfy the chlorine demand. FC solutions of 0.2, 0.5, 1.0, and 4.5 mg/liter were prepared from a stock solution of sodium hypochlorite (5%). FC concentrations were determined by the N,N-diethyl-p-phenylene diamine method (1), using a test kit (Aquamerck 11735; Merek, Darmstadt, Germany). Copper and silver ions were generated electrolytically in 1.3 liters of water at room temperature, using a Tarn-Pure Electronic Pool Purity unit (model UTP-2; Tarn-Pure USA, Las Vegas, Nev.). The unit was operated with continuous stirring for a period long enough to achieve concentrations of approximately 700 μg of copper and 70 μg of silver per liter. Occasionally, concentrations as high as 1,200 μg of copper and...
120 μg of silver per liter were produced and assayed. Copper levels were estimated by the cuprizone reaction, using a test kit (Aquaquant 14414; Merck). At the beginning and end of each experiment, samples were collected to be analyzed for copper and silver ions by induced coupled plasma (Jarrell, Franklin, Mass.), as specified in reference 2.

The test systems normally consisted of 500 ml of autoclaved well or tap water containing 0.2, 0.5, or 1.0 mg of FC per liter alone or combined with 700 μg of copper and 70 μg of silver per liter. For each disinfection system, autoclaved water without any disinfectant was used as a control. Experiments were performed at least in triplicate at 25 ± 2°C and pH 7.5 ± 0.2. Purified viruses were added to the various disinfection systems, and at predetermined time intervals 3-ml samples were taken and neutralized with 14.6% sodium thiosulfate and 10% sodium thioglycolate (49).

Survival of the viruses in the test waters systems was determined by calculating the log_{10} (N/N_{0}), where N_{0} is the titer of the virus at the time zero and N is the titer at a given contact time t. Virus enumerations were performed by calculating the most probable number of cytopathogenic units per milliliter by infecting cell monolayers grown in 96-well microtiter plates. Eight wells were infected for each dilution, and 10 μl of inoculum was added to each well. The analysis of variance test (42) was used to determine significant differences between disinfection systems and between behaviors of viral strains.

EM. Samples from each experiment were analyzed for the presence of viral aggregates by transmission electron microscopy (EM), after staining with 2% phosphotungstic acid (KPTA) at pH 6.5, in a Hitachi MT-800 transmission electron microscope.

Molecular hybridization. Hybridization with a digoxigenin-11-UTP-labeled cDNA probe corresponding to 2,078 nucleotides of the 5′ end of the HAV genome was used to evaluate the presence of HAV nucleic acid in the disinfected samples, as described elsewhere (17). This probe was obtained by HindIII digestion of a cDNA of the complete HAV genome (courtesy of S. Feinestone, National Institutes of Health, Bethesda, Md.) cloned in Escherichia coli HB101 with the pGem1 plasmid (13). Nucleic acid was extracted from the samples by heating at 60°C for 30 min in the presence of 100 μg of proteinase K per ml and treatment with phenol-chloroform-isoamyl alcohol (25:24:1; vol/vol/vol) and then was spotted onto nylon membranes (GeneScreen Plus; Dupont, Itisa, Madrid, Spain) by using a manifold apparatus with vacuum suction. After a 3-min exposure to UV light, the filters were baked for 1 h at 80°C.

RESULTS

Table 1 shows the physicochemical characteristics of the well and tap water used in these studies. Since no substantial differences between the disinfection data for tap water and well water were observed, only results obtained with tap water are shown.

![FIG. 1. Inactivation of human enteric viruses by FC and copper and silver ions. (A) FC (1 mg/liter). (B) Control without disinfectants. (C) FC (0.5 mg/liter). (D) FC (0.5 mg/liter) plus copper (700 μg/liter) and silver (70 μg/liter). (E) FC (0.2 mg/liter). (F) FC (0.2 mg/liter plus copper (700 μg/liter) and silver (70 μg/liter).](http://aem.asm.org/Downloaded_from/August_27,2017_by/guest)
to acid-washed polyethylene and Pyrex containers were determined. Although over a 48-h period the level of copper in solution decreased significantly faster when the solution was stored in Pyrex than when it was stored in polyethylene, no significant losses were observed within the first 2 h after generation of copper and silver ions. For convenience, Pyrex bottles were used thereafter within 1 h after electrolytical generation of metal ions. The addition of 700 μg of copper and 70 μg of silver per liter did not enhance the inactivation rates observed after exposure to 0.5 mg (Fig. 1C and D) or 0.2 mg (Fig. 1E and F) of FC per liter. ANOVA of inactivation rate data showed that the combination of FC plus copper and silver was not significantly ($P < 0.05$) more effective than FC alone. However, the inactivation of HAV in 0.5 mg of FC per liter plus copper and silver was not significantly ($P < 0.05$) different from the inactivation produced by 1 mg of FC per liter, while the differences between inactivation with 0.5 and 1 mg FC per liter appeared to be significant ($P < 0.05$). The same behavior was observed for ADV after short exposures (5 min). The rates of inactivation for HRV (15 to 120 min), ADV (120 min), and PV (15 min) were significantly ($P < 0.05$) higher with 0.5 mg of FC per liter alone than with 0.2 mg of FC per liter alone, while this pattern disappeared when 0.5 mg of FC per liter alone was compared with 0.2 mg of FC per liter plus copper and silver. A 3 LTR was never observed for HRV or HAV in any experimental system. For ADV a 3 LTR was achieved only after exposure to 1.0 mg of FC per liter; however, PV showed a 3 LTR in all the tested disinfection systems and even showed more than a 4 LTR in the presence of copper and silver combined with 0.5 mg of FC per liter or in the presence of 1 mg of FC per liter alone.

Bacteriophage B40-8 of B. fragilis was added to all the test systems to evaluate its potential role as an indicator of the behavior of human enteric viruses in disinfection studies. This phage was readily inactivated by 1.0 mg of FC per liter, reaching undetectable levels after a 30-min exposure to the disinfectant. In the other disinfection systems, bacteriophage B40-8 was always significantly ($P < 0.05$) more resistant than PV, although it was significantly ($P < 0.05$) less resistant than HAV, HRV, and ADV (Fig. 1C, D, and F). In the presence of 0.2 mg of FC per liter, B40-8 and all the animal viruses, with the sole exception of PV, showed little inactivation (Fig. 1E). The presence of copper and silver ions significantly ($P < 0.05$) enhanced the inactivation of phage B40-8 by 0.2 and 0.5 mg of FC per liter.

The changes in FC and copper-silver levels were monitored throughout these experiments. The concentration of FC dramatically decreased during the disinfection experiments (Fig. 2). Initial FC levels in the disinfection systems averaged 0.94, 0.51, and 0.17 mg/liter, after 30 min, only 79, 35, and 29% of the original FC input, respectively, was detected in these systems. Copper and silver levels were much more stable: 75 and 44% of the initial copper and silver input, respectively, remained in the test water samples after 60 days (Fig. 3).

EM observation of virus suspensions in the presence of 0.2 to 4.5 mg of FC per liter combined with 700 μg of copper and 70 μg of silver per liter showed the generation of virus aggregates (Fig. 4B, D, F, and H). These aggregates were not observed in the presence of 0.2 to 4.5 mg of FC per liter alone (Fig. 4A, C, E, and G).

The presence of HAV nucleic acid in disinfected samples was monitored by molecular hybridization with a specific digoxigenin-labeled cDNA probe. Copper and silver ions interfered with the hybridization assay, preventing the generation of signals by the digoxigenin-labeled probe. In the absence of copper and silver ions, viral RNA was consistently detected by hybridization in samples disinfected with 0.2 to 4.5 mg of FC per liter. Figure 5 shows the signals generated by RNA from 10-fold dilutions of $5 \times 10^{4}$ most probable number of cytotoxic pathogenic units (MPNCU) of HAV after disinfection with 4.5 mg of FC per liter. After 30- and 60-min contact times, hybridization signals were produced by samples in which virus detection by cell culture was no longer possible (Fig. 5D3 and E3).

**DISCUSSION**

Well over 100 different virus strains are found in wastewater and may contaminate surface waters used for recreational purposes (37). Among them are viruses such as HAV, Norwalk

![FIG. 2. Change in FC levels over time. Values shown are means with vertical error bars corresponding to one standard deviation.](image-url)
virus, and HRV, the propagation of which in cell cultures poses serious problems (10, 33). Since current standards for recreational waters are based exclusively on the monitoring of the presence of enteroviruses (21) and since PV is readily propagated in vitro, the latter virus is routinely used as model enteric virus in disinfection studies (3). In this paper, we describe the comparative behavior of HAV, HRV, ADV, PV, and bacteriophages of B. fragilis (B40-8) in the presence of low levels of FC alone or combined with electrolytically generated copper and silver ions.

The behaviors of the studied enteric virus strains in the presence of 1 mg of FC per liter were different. HAV and
HRV persisted longer than ADV and PV. Similar levels of virus inactivation have been reported for HAV with 0.5 to 1 mg of FC per liter (35) and for simian RV with 0.75 mg of FC per liter (40). On the other hand, a higher level of virus decay has been reported for HAV with 0.4 mg of FC per liter by Sobsey et al. (41) and for HRV with 0.2 to 0.3 mg of FC per liter by Vaughn et al. (48). These discrepancies may be due to differences existing in the test conditions, i.e., temperature, pH, suspension medium, virus strain, or use of CsCl-purified virus stocks.

There are few data on the inactivation of enteric viruses in water by copper and silver ions. The presence of 400 μg of copper per liter and 40 μg of silver per liter caused a 2.5 LTR of purified PV infectivity after 3 days (51). Maximum contaminant levels allowed in swimming pool waters are 1,000 μg/liter for copper ions and 50 μg/liter for silver ions (31). We observed that exposure to concentrations as high as 1,200 μg of copper per liter and 120 μg of silver per liter alone did not result in a 3 LTR for PV and HAV after 10- and 30-day exposures, respectively (data not shown). The efficacy of the action of reduced levels of FC combined with copper and silver was investigated. Overall, the addition of 700 μg of copper per liter and 70 μg of silver per liter did not enhance the inactivation rates observed for human enteric viruses after exposure to 0.5 or 0.2 mg FC per liter, although on some occasions it produced a level of virus inactivation similar to that induced by a higher dose of FC alone. Yahya and coworkers (51) reported that the addition of at least 0.2 mg of FC per liter to the copper-silver disinfection system was necessary to achieve a 3 LTR of the levels of bacteriophage MS-2. However, when added to the FC system, copper and silver ions did not significantly increase its inactivation capacity. Copper and silver ions have been reported to be effective against PV, although again they did not significantly enhance the rate of inactivation of this virus by FC alone when added to this system (51). It has been reported (49) that E. coli and Streptococcus faecalis numbers were more effectively reduced by the combination of copper and silver (400 and 40 μg/liter, respectively) and FC than by FC alone. In our experiments, the low enhancement of virus inactivation rates induced by the addition of copper and silver ions may be the result of the greater resistance of human enteric viruses to disinfection in general compared with that of waterborne enteric bacteria (3, 7, 24).

Metal ions may inactivate viruses in a number of ways by binding electron donor groups on proteins or nucleic acids (45). Inactivation of biological macromolecules by copper is believed to involve a modified site-specific Fenton mechanism producing hydroxide radicals, which may affect the peptide backbone of the capsid proteins of the virions (39). With respect to silver, Cliver and coworkers (12) observed that some viruses, such as vaccinia virus, were completely resistant to silver, while others, such as influenza virus, were relatively insensitive and others, such as PV, were readily inactivated by the metal. The differential sensitivity of enteric viruses to the presence of metal ions may be due to the fact that some viruses have an inherently more stable molecular structure than others. This is the case for HAV, among other picornaviruses (47).

Although the virus stocks used throughout these studies were not subjected to equilibrium or rate zonal centrifugation, in order to reproduce the actual conditions of the viruses in the environment, EM observations revealed the absence of virus clumps in all the test samples except samples containing copper and silver ions, which showed virus aggregates. The role of this aggregation in an apparent loss of virus titer after disinfection with copper and silver remains unclear. On the other hand, the extent of virus aggregation strongly influences the survival of viruses under disinfection conditions. It is well documented (4, 5) that viruses persist for longer periods of time when they form aggregates than when they occur as single particles. Divalent and trivalent cations have been reported to induce virus aggregation (22) and to enhance the survival of viruses (5). In our studies, it seems likely that aggregation, probably induced by the presence of Cu²⁺ ions, exerts an influence on the outcome of disinfection of viruses by FC. Other authors (53) have shown a strong influence of aggregation on the chemical disinfection of PV by bromine.

The reduction in FC levels observed may be due to various environmental factors, such as temperature, light, or halogen demand of the sample (11, 20), and could account for the bimodal kinetics observed in the virus inactivation curves. No significant reduction in copper and silver levels was observed during the experiment, as reported elsewhere (49).

Gene probes and other nucleic acid techniques have recently been adapted for the detection of fastidious enteric viruses in environmental samples (8, 16, 19, 28). When molecular hybridization was used to monitor the efficiency of HAV disinfection by FC, viral RNA was consistently detected in disinfected samples, even in those which failed to induce cytopathogenicity in susceptible cell cultures. In previous experiments, this molecular procedure has proved to be sensitive enough to detect 100 infectious units, or 4,000 physical particles of HAV, or 30 pg of homologous RNA (17). By using this methodology, wild-type HAV has been detected in drinking water (7) and sewage (23). A positive hybridization signal proves the presence of encapsidated RNA in the water sample, since free RNA is very labile. Being a strong oxidizer, chlorine can affect the structural integrity of viral particles and consequently can lead to the degradation of nucleic acid (3). In our experiments, viruses inactivated by low levels of FC were still able to yield a positive hybridization signal. In this case, chemical alteration of the nucleocapsids by chlorine produces noninfectious viri-
ons which, however, encapsidate an RNA detectable through gene probes. The possibility that RNAses present in the water samples could also be inactivated by chlorine may contribute to the lack of correlation between cell culture and gene probe assays. It has been pointed out (30) that PCR-based procedures are not adequate to monitor the presence of infectious viruses after disinfection. This observation seems extendable to other molecular techniques, such as hybridization with gene probes.

The behavior of PV in these experiments causes serious concern, since most studies carried out to date to evaluate the effectiveness of virus disinfection have been conducted with PV as a model strain. It can be concluded from data from this and other studies (6) that PV is not an adequate indicator of the behavior of human enteric viruses, such as HAV or HRV, under disinfection conditions.

As an alternative, bacteriophages have been proposed as indicators of viruses because their handling is simple and inexpensive and does not require specialized personnel or sophisticated facilities (26, 44). B. fragilis bacteriophages are among the most promising candidates to be used as surrogate indicators of viruses in disinfection studies (36, 43). In the present study, B. fragilis phages were consistently more resistant to chlorine disinfection than PV, suggesting that they constitute a more suitable model strain than PV itself, although they were significantly less resistant than HAV, HRV, or ADV to inactivation by FC, particularly when combined with copper and silver ions.

On the basis of the evidence presented here it can be postulated that more exhaustive studies are required to ascertain the validity of the use of bacteriophages as virus indicators in disinfection processes and that cell culture-adapted laboratory strains of fastidious enteric viruses should be used to assess the efficiency of virus disinfection procedures in water.

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