Monitoring of Low-Level Virus in Natural Waters

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The insoluble polyelectrolyte technique for concentrating virus is extended to extremely low virus levels. The effectiveness of this method employing a coliphage T2 model is a constant 20% over a range of virus levels from $10^0$ to $10^{-4}$ plaque-forming units/ml. The efficiency of the method is dependent upon pH control during the concentration phase. Although the study was initiated to develop a method for quantitating the effectiveness of water and wastewater treatment methods for the removal of viruses from waters at low concentrations, the potential of the technique for efficient monitoring of natural waters is apparent.

Major epidemics have been attributed to waterborne viruses. Horsfall (9) indicates that the average person in the United States is afflicted by viral diseases during 10% of his life.

Over 100 identifiable enteric viruses are known to be excreted in the feces of man. In the past 20 years, at least 70 strains of viruses have been recovered from water and municipal wastewater. Clark et al. (3) reported that 39% of all the samples of chlorinated effluents of conventional treatment analyzed contained viruses.

One of the most difficult problems associated with virus and water supply has been the inability to detect quantitatively the presence of virus at low titers. This problem is even more significant for surface waters which carry suspended particulate and colloidal materials.

Both the Committee on Environmental Quality Management of the Sanitary Engineering Division, American Society of Civil Engineers (5) and the Committee on Viruses in Water of the American Water Works Association (4) have indicated that considerable improvements in detection techniques must be achieved before reliable information regarding the presence or absence of virus in water supplies can be determined.

Many methods for the concentration of viruses from large quantities of finished or raw water have been proposed. These methods include: the gauze pad method, hydro-extraction, ultra-centrifugation, electrophoresis, adsorption on inorganic salt complexes, ultrafiltration, membrane chromatography, two-phase polymer separation, and adsorption on an insoluble polyelectrolyte (1, 2, 6, 10, 12). All of these methods except the use of insoluble polyelectrolytes encounter logistical difficulties when used to analyze large volumes of samples (i.e., more than a few liters) or when the water contains significant amounts of suspended organic and inorganic solids.

This paper reports the initial results of the use of insoluble polyelectrolyte to concentrate extremely low virus levels. Since bacteriophages, rather than enteric viruses, were used in this part of the investigation, the results merely serve as a model. Extensions of these studies employing enteric viruses are to be published soon. The results are similar to the model study reported below.

MATERIALS AND METHODS

Suspending media. All experiments reported herein were conducted in either phosphate-buffered saline (PBS) at pH 7.2 or natural surface water. Surface water was obtained from Town Lake, a shallow man-made impoundment of the Colorado River at Austin, Texas. Town Lake receives storm drainage, urban runoff, and some rural runoff in addition to the releases of six other man-made lakes upstream on the Colorado River.

Virus. The coliphage T2 was employed throughout this study. Stock cultures were prepared by the plate method by using Tryptose phosphate broth (Difco) solidified by 1% agar. Plaque assays omitting bottom layer agar were used to titrate the virus (10). A 0.2-ml amount of a 24-hr-old Escherichia coli B culture in 7.5 ml of Tryptose phosphate agar was inoculated with samples ranging from 0.1 to 0.5 ml. Plaques were counted after 15 hr of incubation at 37°C.

Virus concentration method. The insoluble polyelectrolyte technique for concentrating virus suspensions employed in these studies was described previously by Wallis et al. (13) and Grinstein et al. (8). The polyelectrolyte PE 60 (Monsanto Co., St. Louis, Mo.) was used in the water-washed form in all experiments. A constant concentration of 100 mg of the polyelectrolyte per liter was used and all samples were...
stirred mechanically for 1 hr. The pH was adjusted to the desired level with 1 N HCl immediately after the addition of the polyelectrolyte.

Recovered polyelectrolyte was suspended in 5 ml of borate buffer (pH 9), to which was added 10% calf serum for 1-liter samples and 8 ml of borate buffer-calf serum for 5-liter samples. For all low-level studies, the total available eluate was plated for plaque counting.

Radioactive virus. Coliphage T2 labeled with ³H-thymidine was prepared in M9 medium (7). The lysate was clarified by centrifugation at 2,500 × g for 15 min at 4 C. The virus was concentrated from the supernatant fluid by high-speed centrifugation in the cold (21,000 × g for 60 min). The pellet was allowed to resuspend overnight in one-tenth the original volume of phosphate-buffered saline. After an additional low-speed centrifugation, 2 ml of virus was layered on a discontinuous sucrose gradient (4 ml each of 37.5, 33.8, 30.0, 26.3, 22.5, 18.8, and 15%) and centrifuged in the cold at 21,000 × g for 45 min. The virus banded at the interface between the 18.8 and 22.5% sucrose layers. It was removed in 4 ml of sucrose with a Pasteur pipette. The plaque titer was 1.5 × 10⁶ PFU/ml, and the radioactivity was 2.8 × 10⁶ counts per min per ml as determined by counting in Cabosil with a Nuclear-Chicago scintillation counter, model Mark I.

RESULTS

Recovery of T2 from PBS. Experiments were carried out to determine the per cent virus recovered as a function of the number of plaque-forming units (PFU) of T2 added to the PBS. The range studied was from 10⁻³ PFU/ml to 10⁴ PFU/ml of T2 in 1-liter volumes of PBS. The results are presented in Fig. 1 and demonstrate a relatively constant recovery rate, especially at levels of or above 10⁻¹ PFU/ml of added virus. Significant variability from predicted levels was encountered at extreme dilutions as indicated by the data shown in Fig. 2. It can be seen, however, that there were only two dilutions at which the expected virus recovery was not achieved. In these cases, total calculated virus counts expected in a 5-liter quantity of PBS were 2.7 and 3.3 PFU and, although no virus was recovered, the adjusted pH of the PBS was greater than 0.5 units above optimum for recovery. The effectiveness of this technique for recovery of serially diluted low-level virus suspensions at a constant pH of 5.25 can be seen in Fig. 3.

Recovery as affected by pH. Minor differences in pH significantly affected the efficiency of T2 recovery. A series of experiments was conducted to evaluate this variable. A known amount of virus was added to several 1-liter containers, 100 mg of PE 60 added, and, while being mixed, the pH was adjusted to various levels by 0.25-pH unit increments. Figure 4 illustrates a typical run where the optimal pH for T2 recovery is seen to cover a very narrow range. Shifting ± 0.3 pH units from the optimum can result in a significant reduction in recovery efficiency. This is in contrast with the results of Wallis et al. (13) who indicated a much broader range for optimal recovery of poliovirus by this method.

Efficiency of polyelectrolyte recovery. It was observed that only about 25% of added T2 was recovered from the polyelectrolyte. It was possible that all of the virus might not attach to the polyelectrolyte. The recovery of T2 bacteriophage from 5-liter samples of PBS as compared to expected number of virus.
electrolyte during the mixing phase of the concentrating technique. Where the filtrate was successively reconcentrated under ideal pH conditions, 22% of the coliphage was recovered on the first polyelectrolyte addition and no additional virus was detected on subsequent concentration attempts. Under less favorable conditions, where pH was 4.75 as compared to an optimum of 5.25, only 1.2% of the initial phage titer was recovered on the second concentration attempt, whereas 15% was recovered during the initial concentration.

Further, after initial elution from the polyelectrolyte, the polyelectrolyte was resuspended in borate buffer and plated directly. Less than 2% of the added phage were found, an expected value, since 0.5 ml of the initial 5 ml of borate remained with the polyelectrolyte.

Tracer studies. In a further attempt to determine the fate of unrecovered virus, phage isotopically labeled with ³H-thymidine were used. This stock was concentrated from PBS with PE 60 as in other experiments (Table 1). Essentially all of the phage deoxyribonucleic acid-associated radioactivity was attached to the polyelectrolyte. Twenty eight per cent of the ³H was recovered in the eluate, whereas 56% remained attached to the polyelectrolyte after borate elution. Infectivity plating indicated that 15% of the initial titer of viable phage was recovered in the eluate, and only 1.5% of the viable virus remained associated with the polyelectrolyte after elution.

Recovery of coliphage from raw water. Numerous attempts to recover indigenous coliphage from raw Town Lake water were made. Recoveries from volumes of 3.5, 5, and 20 liters indicated coliphage titers varying from $10^{-3}$ to $8 \times 10^{-3}$ PFU/ml. Table 2 includes some of the chemical, physical, and biological characteristics of the lake water of three typical samples during the period of these experiments.

Effect of suspended solids. Quantities of lake water were filtered through a glass-fiber filter (Reeve Angel 934 AH) to remove suspended solids, and 10 coliphage per ml were added. In

FIG. 3. Recovery of serially diluted T2 bacteriophage from 1-liter volumes by the polyelectrolyte technique.

FIG. 4. Effect of pH on the recovery of coliphage T2 by the insoluble polyelectrolyte technique.

TABLE 1. Results of tracer studies to determine the fate of unrecovered virus

| State of polyelectrolyte-virus complex | Radioactivity | Total viable virus |
|---------------------------------------|--------------|--------------------|
|                                       | Net counts/ min | Per cent of total | PFU | Per cent of total |
| Polyelectrolyte + coliphage...         | 16,000       | 1.5 $\times 10^{10}$ | 100 |
| Eluate (with coliphage)...            | 4,400        | 2.3 $\times 10^{9}$  | 15.3|
| Polyelectrolyte after elution of coliphage... | 8,900 | 2.2 $\times 10^{8}$  | 1.5 |

* Amount of coliphage added.
Table 2. Some chemical, physical, and biological characteristics of Town Lake water

| Sample | pH | Specific conductance (µmhos/cm) | Alkalinity (mg/liter as CaCO₃) | Chemical oxygen demand (mg/liter) | Filterable solids (mg/liter) | Standard plate count at 35°C (organisms/100 ml) | Coliform (organisms/100 ml) | Coliform: coliphage ratio |
|--------|----|---------------------------------|--------------------------------|---------------------------------|-----------------------------|-----------------------------------------------|-----------------------------|--------------------------|
| 1 (5 liter) | 8.3 | 12 | 8 | 5,000 | 600 | 1.5 \times 10^{-2} | 4,000 |
| 4 (20 liter) | 8.2 | 570 | 160 | 10 | 10 | 13,000 | 3,100 | 8.0 \times 10^{-3} |
| 6 (20 liter) | 8.2 | 560 | 160 | 740 | 7.0 \times 10^{-3} | 1,100 |

* All analyses except those outlined in the text were performed according to Standard Methods for the Examination of Water and Wastewater, 12th edition, American Public Health Association, 1965, New York.

Sample 1 was collected from the lake after several months of essentially no precipitation in the watershed, whereas sample 4 was collected 3 days after a 1-inch rainfall, the first since sample 1 was collected. Sample 6 was collected 8 days later.

parallel, containers of unfiltered lake water were dosed with identical amounts of coliphage. Both series were concentrated at pH values ranging from 5.0 to 7.0. Maximal recovery was about 20% of the initial titer in both cases although the filtered water had an optimal recovery of pH 5.5, whereas that of the unfiltered water was 5.25. The difference appears to be related directly to the amount of HCl required to overcome the buffer capacity of the suspended solids.

**DISCUSSION**

This report has demonstrated the effectiveness of an insoluble polyelectrolyte for concentrating extremely low levels of coliphage from artificial as well as natural waters. At optimal pH for the coliphage, recoveries were reliable and reproducible at about 20 to 25% of added virus. Of equal significance is the demonstrated ability of the PE 60 to adsorb essentially 100% of the virus in the suspension at the dose and mixing time employed. For the coliphage used in this study, pH control is critical if results are to be reproducible.

One of the major purposes for undertaking this study was to find a method for quantitating the effectiveness of water and wastewater treatment methods for the removal of viruses from waters at concentrations at which they can be expected to occur naturally. The technique described satisfies that need. In addition, its potential as a monitoring technique for low-level virus appears great.

It is recognized that the largest sample of water handled in this investigation was 20 liters. Experimental data indicate that the results can be applied to any manageable volume. At this time, further investigations are proceeding with the purpose of simplifying the mechanical problems associated with handling several hundred liters of water, a requirement for the effective monitoring of viruses from finished potable waters. Volumes of 5 and 10 liters would seem to be large enough for effective monitoring of most raw water.

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**LITERATURE CITED**

1. Anderson, N. G., G. B. Cline, W. W. Harris, and J. G. Green, 1967. Isolation of viral particles from large fluid volumes. In G. Berg (ed.), Transmission of virus by the water route. Interscience Publishers, Inc., New York.

2. Bier, M., G. C. Bruckner, F. C. Cooper, and H. E. Roy. 1967. Concentration of bacteriophage by electrophoresis. In G. Berg (ed.), Transmission of viruses by the water route. Interscience Publishers, Inc., New York.

3. Clarke, N. A., G. Berg, P. W. Kahler, and S. L. Chang. 1964. Human enteric viruses in water: source, survival and removal. In Advances in water pollution research. Proc. Int. Conf., London, 1962, vol. 2. Pergamon Press, London.

4. Committee Report (N. A. Clarke, Chairman). 1969. Viruses in water. J. Amer. Water Works Ass. 61:491-494.

5. Committee Report, 1970. Engineering evaluation of virus hazard in water, J. Sanitary Eng. Div. (ASCE), 96(s):111-161.

6. Duf, M. G. 1970. Isolation of ether-resistant enterovirus from sewage: methodology. Appl. Microbiol. 19:120-127.

7. Eisenstark, A. 1967. Bacteriophages. In K. Moromosch and H. Koprowski (ed.), Methods in virology, vol. 1. Academic Press Inc., New York.

8. Grinstein, S., J. L. Melnick, and C. Wallis. 1970. Virus isolations from sewage and from a stream receiving effluents of sewage treatment plants. Bull. W. H. O. 42:291-296.

9. Horsfall, F. L. 1957. Virus diseases. Public Health Rep. 72:905.
10. Johnson, J. H., J. E. Fields, and W. A. Darlington. 1967. Recovery of viruses from water by polyelectrolytes. Nature (London) 213:665–667.

11. Rizvi, S., and P. D. Nova. 1963. Bacteriophage plaque-count assay and confluent lysis on plates without bottom agar layer. Nature (London) 200:1324–1326.

12. Shuval, H. J., S. Cymbaiesta, B. Fatal, and N. Goldblum. 1967. Concentration of enteric viruses in water by hydroextraction and two-phase separation. In G. Berg (ed.), Transmission of viruses by the water route. Interscience Publishers Inc., New York.

13. Wallis, C., S. Grinstein, J. I. Melnick and J. E. Fields. 1969. Concentration of viruses from sewage and excreta on insoluble polyelectrolytes. Appl. Microbiol. 18:1007–1014.