Use of Polyelectrolytes and Electron Microscopy for Detection of Viruses from Stool

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Insoluble polyelectrolytes (PE60) were used for the concentration of viruses from stool specimens, confirming the results of Wallis et al. (1969). Ten percent suspensions inoculated with poliovirus type 3 were used in these experiments. A small number of stool specimens from patients naturally infected with enteroviruses were also tested. Preferential adsorption of viruses to PE60 was maximum at a pH range of 4.5 to 6.0. The elution of the adsorbed viruses was optimal at pH 8.5. Other parameters were also investigated. Electron microscopy was used successfully to detect the eluted viruses.

Although under special circumstances enteroviruses isolation rates may be high (6, 7), they are frequently less than 10% (3, 5), partly due to poor specimen selection but also to low virus concentrations and heavy contamination. These factors along with the presence of organic debris are also responsible for the difficulties encountered with direct electron microscopy (2).

The technique first reported by Wallis et al. (10) could overcome both of these difficulties by using insoluble polyelectrolytes (PE60) which selectively adsorb viruses from stool suspension and the adsorbed viruses could then be eluted into a small volume of sterile eluant. The use of insoluble PE60 for adsorbing viruses from clinical stool specimens has not yet been reported.

In this study, experiments were aimed at evaluating the use of insoluble PE60 for concentrating viruses from stool specimens experimentally inoculated, as well as those obtained from patients infected with enteroviruses. Eluted virus has also been examined by electron microscopy.

MATERIALS AND METHODS

**Virus.** Poliovirus type 3 (Sabin strain) was used for experimental inoculation of stool suspensions. The virus pool was prepared and titrated in second passage green monkey kidney cells (GMK).

**Cells and medium.** Primary GMK cells were grown in medium 199 containing 10% fetal calf serum (FCS) and were used in second passage. The cells, medium 199, and FCS were purchased from Grand Island Biological Co., New York, N.Y.

**Virus assays.** Virus preparations were titrated in GMK cells in tubes, and the 50% tissue culture infective dose was calculated by the "Karber" method (4). Some of the clinical stool specimens were titrated by plaque assay and, in these cases, the overlay medium consisted of 0.6% Noble agar (Difco) in medium 199 with 1.0% FCS and 25 mM MgCl₂ (1, 9).

**Insoluble PE60.** PE60 was kindly provided in the form of 100-mesh powder by Monsanto Co., St. Louis, Mo. It is an insoluble cross-linked copolymer of isobutylene maleic anhydride.

PE60 was freshly prepared for use by the method of Wallis et al. (10) by suspending the required amount in sterile distilled water, shaking it for 30 to 40 min, and then centrifuging at 2,000 rev/min for 5 min. The supernatant fluid was discarded, and the packed PE60 was washed twice with sterile distilled water.

**Stool specimens.** For experimental inoculation, stool was obtained from healthy individuals, and a 10% suspension was prepared in saline. The suspension was initially centrifuged at 2,000 rev/min for 15 min, and the supernatant fluid was adjusted to pH 4.0 with 1 N HCl. The sample was chilled for 15 min at −20°C and again centrifuged at 4,000 rev/min for 1 hr. The supernatant at this stage was usually clear and suitable for use, but in some cases recentrifugation was needed. According to the method of Wallis et al. (10), the clear supernatant was used for virus inoculation and for treatment with PE60. Ten clinical stool specimens from which enteroviruses had been isolated during the year 1971 were kindly supplied by the Virus Laboratory, Central Public Health Laboratory, Toronto, Ontario, Canada. Only five specimens contained enough material to permit the preparation of 10 ml or more of 10% suspension, and therefore, only these five were used for adsorption and elution experiments. All 10 specimens were
titrated to determine the concentration of virus present.

**Virus concentration with PE60.** The technique reported by Wallis et al. (10) for concentrating viruses from stool was employed in these studies. Most of the parameters for adsorption and elution were tested by using 10-ml volumes of stool suspension. A constant concentration of 35 mg of PE60 per 10 ml was used. In all experiments, a sample from the inoculated stool suspension was taken before addition of PE60 to determine the virus content. All virus-PE60 suspensions were stirred for 1.5 hr at 25 C with a magnetic stirrer unless mentioned otherwise. After stirring, the mixtures were centrifuged at 2,000 rev/min for 5 min, and the supernatant was collected for titration of unadsorbed virus. The pelleted PE60 was then treated with eluant. The effect of pH on adsorption was tested by varying the pH values from 4.0 to 8.0 in separate experiments; the virus PE60 pH being adjusted with either 1 N HCl or 0.2 M NaOH using a pH meter. Five stool suspensions from clinical specimens were tested in 10-ml volume with 35 mg of PE60 at pH 5.25. The elution of adsorbed virus was carried out by using 0.1 M NaOH, with 10% FCS at pH 8.5. The eluent is similar to that reported by Wallis et al. (11) with slight change in pH and molarity. The pH was adjusted after mixing PE60 and eluent. The elution was carried out at 37 C for 10 min with vigorous shaking. The mixture was then centrifuged at 2,000 rev/min for 10 min, and the supernatant was collected for virus assay. The effect of different volumes of eluant on the efficiency of elution was also tested.

**Electron microscopy.** A 4-ml amount of eluted virus from experiments containing 10^9 infectious virus particles per ml was centrifuged at 6,000 x g for 30 min in a Spinco ultracentrifuge. The supernatant fluid was recentrifuged at 38,000 x g for 1 hr. The pellet was suspended in 0.1 ml of distilled water. One drop of the virus suspension was placed on a Formvar-carbon-coated copper grid (400 mesh) and negatively stained with 2% sodium phosphotungstate at pH 7. Grids were examined in a Philips EM300 electron microscope at a magnification varying from 25,000 to 110,000, with double-condenser illumination at accelerating voltage of 60 kv. Photographs were taken on Kodak Electron Microscope film.

**RESULTS**

**Adsorption of viruses as related to pH.** Differences in pH significantly affected the efficiency of poliovirus type 3 adsorption to PE60. Maximal adsorption was achieved within a pH range of 4.5 to 6.0, with the peak adsorption of 88% at pH 5.25; adsorption beyond this pH range was poor (Fig. 1).

**Adsorption of viruses as related to PE60 concentration.** In two separate series of experiments, 25 and 35 mg of PE60 were tested with 10 ml of suspension. The efficiency of adsorption achieved in these two experiments was 82 and 88%, respectively. A statistical analysis by t test indicated that there is no significant difference between these two values; however, we preferred the use of 35 mg of PE60, as it was easier to handle during centrifugation and other procedures.

**Adsorption as related to temperature.** In a series of experiments, the effect of temperature on adsorption was determined. A minimum of four experiments was carried out at each of three temperatures and gave an adsorption rate of 46% at 4 C, 88% at 25 C, and 53% at 37 C.

**Adsorption as related to volume.** Recovery of viruses from suspension with low virus concentration may require the use of large volumes of fluid. Experiments were therefore performed by using 100 ml of stool preparation with a constant concentration of 100 mg of PE60. Results showed an average adsorption of 85% (Table 1). The volume was further increased to 400 ml, and 200 mg of PE60 was used for adsorption, which resulted in an average recovery of 79% (Table 2). Both results show that efficient adsorption could be

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**Fig. 1. Effect of pH on the adsorption of poliovirus type 3 to PE60 (35 mg) from stool suspension (10 ml) at 25 C. Each point represents the average value of four or six experiments.**

**Table 1. Adsorption of poliovirus from 100 ml of stool suspension to 100 mg of PE60 at pH 5.25 and elution at pH 8.5 with phosphate (5 ml)**

| Log 10 titer of original (per ml) | Log 10 titer of supernatant (per ml) | Adsorbed (%) | Log 10 titer of eluted (per ml) | Eluted (%) |
|----------------------------------|-------------------------------------|---------------|---------------------------------|------------|
| 3.0                              | 2.3                                 | 80            | 4.3                             | 89         |
| 4.8                              | 4.0                                 | 83            | 5.8                             | 60         |
| 3.5                              | 2.8                                 | 80            | 4.5                             | 62         |
| 4.3                              | 2.8                                 | 97            | 5.3                             | 56         |
| Avg                              | 85                                  |               | 58                              | 67         |
achieved from larger volumes, which also required proportionately less PE60. The virus recoveries at the two different volumes did not differ significantly.

**Elution of viruses from PE60.** The viruses could be eluted from PE60 with phosphate buffer as reported by Wallis et al. (11); however, the average efficiency of elution was only 53% from 10 ml. A higher molarity of the same eluant at pH 8.5 resulted in a recovery rate of 67%, which is about 30% higher than those obtained with the recommended eluant. The average values were calculated from five experiments (Fig. 2).

A similar pattern of elution was observed when higher concentration of PE60 was used for larger volumes of stool suspension. The results showed an average recovery of 67% from 100 ml and 58% from 400 ml of suspension. Details are given in Tables 1 and 2, respectively. There was no statistical difference between the three recoveries which were 67, 67, and 58% ($P > 0.2$).

**Adsorption of clinical specimens.** The virus was recovered from clinical stool specimens by the use of PE60 technique, but the efficiency was less than that achieved with experimental suspension. An average recovery of 69% was obtained (Table 3). In one instance no virus was adsorbed.

**Recovery of viruses from clinical specimens.** Viruses adsorbed to PE60 from clinical specimens could be eluted with phosphate buffer (11). Elution was poor, and an average of 36% was obtained (Table 3).

**Detection of eluted virus by electron microscopy.** Electron microscope observation of the eluted virus showed that both empty and full particles were present. The virus particles were evenly distributed, and it was possible to see two or three particles in most of the fields. There was no difficulty in recognizing virus particles, since the preparative method gives a clear background. One empty and one full particle are shown in Fig. 3.

**DISCUSSION**

The results reported here confirmed the findings of Wallis et al. (10) that enteroviruses in stool could be selectively adsorbed to and eluted from PE60. It also demonstrated the effectiveness of PE60 for concentrating viruses from stool. In our experiments, the efficiency of adsorption was reproducible and was not statistically different from that reported by the above authors. We observed a broad pH range (4.5 to 6.0) for optimal adsorption which is also similar to that reported by Wallis et al. (10) for sewage. This is in contrast with the results of Sorber et al. (8) who indicated a very narrow pH range, around 5.25, for T2 phage adsorption to PE60.

The recovery of poliovirus from PE60 was not as efficient as reported by Wallis et al. (10).

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**Table 2. Adsorption of poliovirus from 400 ml of stool suspension to 200 mg of PE60 at pH 5.25 and elution at pH 8.5 with phosphate (5 ml)**

| Log 10 titer of original (per ml) | Log 10 titer of supernatant (per ml) | Adsorbed (%) | Log 10 titer of eluted (per ml) | Eluted (%) |
|----------------------------------|-------------------------------------|--------------|---------------------------------|-----------|
| 3.3                              | 2.8                                 | 68           | 4.8                             | 56        |
| 3.8                              | 2.8                                 | 90           | 5.5                             | 70        |
| 4.5                              | 3.8                                 | 80           | 6.0                             | 47        |
| Avg                              |                                     | 79           | 6.0                             | 58        |

**Fig. 2. Elution of virus from PE60 (35 mg) with different volumes of eluant (0.1 M Na$_2$PO$_4$ containing 10% fetal calf serum at pH 8.5) at 37°C.**

**Table 3. Assay of virus from positive stool specimens and the use of PE60 for concentration at pH 5.25 and elution at pH 8.5 (3 ml) with phosphate**

| Specimen no. | Type of virus isolated | Wt (g) | Virus titer Log 10 (per g) | Adsorbed to PE60 (%) | Eluted from PE60 (%) |
|--------------|------------------------|--------|---------------------------|----------------------|----------------------|
| 2622/71      | Coxsackie B2           | 1.0    | 4.0                       | 68                   | 47                  |
| 2216/71      | Coxsackie B5           | 1.0    | 4.5                       | 69                   | 25                  |
| 2933/71      | Coxsackie B5           | 0.6    | 4.8                       | NT*                  | 25                  |
| 2352/71      | Coxsackie B5           | 1.2    | 5.0                       | 69                   | 47                  |
| 2617/71      | Coxsackie B5           | 0.4    | 650 PFU*                  | NT                   | 0                   |
| 1731/71      | Coxsackie B5           | 1.0    | 4.8                       | 68                   | 47                  |
| 2747/71      | Echovirus 9            | 0.4    | 100 PFU                   | NT                   | 0                   |
| 2756/71      | Coxsackie A9           | 1.2    | 2.9                       | 0                    | 0                   |
| 1715/71      | Coxsackie B4           | 0.6    | 400 PFU                   | NT                   | 0                   |
| 3122/71      | Coxsackie B5           | 0.2    | 500 PFU                   | NT                   | 36                  |
| Avg          |                        |        |                           |                      |                     |

* NT, Not tested because of insufficient quantity of specimen.
* PFU, Plaque-forming units.

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| Log 10 titer of original (per ml) | Log 10 titer of supernatant (per ml) | Adsorbed (%) | Log 10 titer of eluted (per ml) | Eluted (%) |
|----------------------------------|-------------------------------------|--------------|---------------------------------|-----------|
| 3.3                              | 2.8                                 | 68           | 4.8                             | 56        |
| 3.8                              | 2.8                                 | 90           | 5.5                             | 70        |
| 4.5                              | 3.8                                 | 80           | 6.0                             | 47        |
| Avg                              |                                     | 79           | 6.0                             | 58        |
We obtained an average recovery rate of 67%, which was in reasonable agreement with their results. However, Sorber et al. (8) reported a recovery rate of only 25%, which was much lower than that reported here. Differences in the nature of poliovirus and bacteriophage might be a contributing factor to the discrepancies in the amounts recovered. However, the differences between recovery rates observed with enteroviruses might reflect only the differences in method used for recovering viruses, that is, centrifugation as opposed to filtration. Wallis et al. (11) reported that filtration enhanced virus adsorption and presumably it would therefore also enhance recovery. In spite of slightly low recovery rates, we did achieve a reasonable virus concentration and confirmed the usefulness of PE60 for this purpose.

The viruses from clinical specimens did adsorb to PE60 and were subsequently eluted from it, but the recovery rate was poor. Further investigation might resolve the difficulty.

A possible application of this technique was indicated by the fact that eluted virus was readily detected by electron microscopy. Empty and full particles were observed, indicating that both types of particles were adsorbed and eluted and that even inactivated virus may be detected by this treatment. In this study, virus could be detected when only $10^4$ infectious particles per ml were present in stool suspensions, in contrast to $10^6$ particles per ml reported by Doane et al. (2) as being the minimum required for direct detection. Centrifugation, clear background, and a high ratio of noninfectious to viable particles might be the contributing factors. Our study indicates that PE60 could be used for concentrating viruses from stool for direct electron microscopy.

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LITERATURE CITED
1. Chaudhary, R. K., and J. C. N. Westwood. 1969. Plaque assay of poliovirus in plastic chambers. Can. J. Microbiol. 15:1301–1303.
2. Doane, F. W., N. Anderson, A. Zhitnew, and A. J. Rhodes. 1969. Application of electron microscopy to the diagnosis of virus infections. Can. Med. Ass. J. 100:1043–1049.
3. Herrmann, E. C., Jr. 1972. Rates of isolation of viruses from a wide spectrum of clinical specimens. Amer. J. Clin. Pathol. 57:188–194.
4. Kärber, G. 1931. Beitrag zur Kollektiv von Beh und lung pharmakologischer Reihenversuche. Arch. Exp. Pathol. Pharmacol. 162:480–487.
5. Kelen, A. F., and N. A. Labzofsky. 1967. Variation in the prevalence of enterovirus infections in Ontario, 1956–65. Can. Med. Ass. J. 97:797–801.

Fig. 3. Electron micrograph of viruses eluted from PE60 and negatively stained with PTA. ×100,000.
6. Parks, W. P., J. L. Melnick, L. T. Queiroga, and H. A. Khan. 1966. Studies of infantile diarrhea in Karachi, Pakistan. I. Collection, virus isolation and typing of viruses. Amer. J. Epidemiol. 84:382-395.

7. Ramos-Alvarez, M., and J. Olarte. 1964. Diarrheal diseases of children. Amer. J. Dis. Child. 107:218-231.

8. Sorber, C. A., B. P. Sagik, and J. F. Malina, Jr. 1971. Monitoring of low-level virus in natural waters. Appl. Microbiol. 22:334-338.

9. Wallis, C., and J. L. Melnick. 1962. Magnesium chloride enhancement of cell susceptibility to poliovirus. Virology 16:122-132.

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

11. Wallis, C., J. L. Melnick, and J. E. Fields. 1971. Concentration and purification of viruses by adsorption to and elution from insoluble polyelectrolytes. Appl. Microbiol. 21:703-709.