NOTES

Recovery of Indigenous Viruses from Wastewater Sludges, Using a Bentonite Concentration Procedure
CHRISTOPHER A. TURK, BARBARA E. MOORE, BERNARD P. SAGIK,* AND CHARLES A. SORBER
Center for Applied Research and Technology, The University of Texas at San Antonio, San Antonio, Texas 78285

A distilled water elution-bentonite concentration technique was developed and used to monitor indigenous viruses present in liquid sludges undergoing land application at six field sites.

Viruses adsorbed to or incorporated into sludge solids during wastewater treatment processes are becoming the object of close study as land application of sludges gains widespread use. The possibility of groundwater contamination by infiltration of pathogenic enteric viruses, as proposed by Duboise and co-workers (2) and observed by Wellings et al. (4, 5), is present. Although researchers at an operational sludge disposal site in Illinois concluded that surface water contamination by runoff was not a significant problem (6), the potential for such pollution may exist in other locations. For these reasons, the ability to monitor viruses in sludges is becoming more critical.

The isolation and enumeration of human enteric viruses from domestic sludges are made complex by the fact that two separate problems must be addressed: (i) the dispersion of sludge solids with concomitant viral extraction, followed by (ii) concentration of the released viruses. These procedures must be compatible in that the final volume for plating must reflect a significant reduction in sample size while being without toxicity to in vitro assay systems.

The bentonite adsorption procedure (3) for the concentration of viruses from small volumes of water and wastewater (0.25 to 10 liters) has been used in this laboratory for a number of years. To apply this procedure to sludges, however, it was necessary to find an elution medium that would both actuate viral release and allow reasonable viral recoveries with bentonite. Bitton (1) and Duboise et al. (2) have indicated that reduction of the ionic strength of an elution medium would facilitate the desorption of viruses from various solids. The use of distilled water as an eluant achieved conductivity reduction and made possible subsequent viral concentration by the bentonite procedure.

This note presents an adaptation of the bentonite concentration procedure to sludges. The results obtained by the distilled water elution-bentonite concentration procedure in viral monitoring of liquid sludges undergoing land application at six sites in the United States are reported.

Plaque assays for indigenous and seed viruses were performed with confluent monolayers of HeLa cells grown in 100-mm petri plates. Inocula were added to the plates, and a 45-min virus adsorption period was allowed. Monolayers then were washed for 15 min by the addition of Hanks balanced salt solution containing 500 U of penicillin G and 250 μg of streptomycin per ml. The wash was removed by aspiration, and the monolayers were overlaid with autoclavable modified minimum essential medium (Auto-Pow, Flow Laboratories, Inc.) without phenol red, containing 8% heat-inactivated calf serum, 100 U of penicillin G per ml, 50 μg of streptomycin per ml, 25 μg of gentamicin per ml, and 0.5 μg of amphotericin B per ml. Inoculated plates were incubated at 37°C in a 5% CO₂-in-air atmosphere for 2 days, at which time an additional agar overlay containing 3 μg of neutral red per ml in modified minimum essential basal medium was placed on each plate. At 5 days, plaques were scored and picked for confirmation by passage in homologous cell tube culture. Only plaques which demonstrated a typical cytopathic effect on tube culture passage were reported as plaque-forming units.

Several sludge dispersion techniques, isolating indigenous viruses, were evaluated on primary raw and digested sludges. Sterilized distilled water (conductivity, <2 μS/cm) was obtained from a Corning Mega-Pure glass still and was mixed with equal volumes of the liquid sludge sample. To facilitate viral elution, sludge solids were
dispersed by either sonication, blender homogenization, or mechanical magnetic mixing. Samples to be sonicated were placed in a 500-ml rosette cell, which was submerged in an ice bath, and sonicated at a 60-W measured output (Branson Sonifier model 200) for 5 min. Samples to be homogenized were mixed at high speed in a Waring blender for 3 min. Mechanical mixing was performed in a 2-liter trypsinizing flask containing a stirring bar; the flask was placed on a magnetic stirrer at 4°C, and the contents were mixed at the highest speed possible for 15 min. After dispersion, the solids were removed by centrifuging the sample at approximately 3,000 × g for 10 min. The supernatant (sludge eluate) was collected for subsequent concentration, and the solids were discarded.

Bentonite adsorption was accomplished by adding 200 mg of bentonite clay (Fisher Scientific Co.) per liter, previously expanded by vigorously mixing 1 g of bentonite in 100 ml of distilled water for 1 h, to the sludge eluate. Calcium chloride was added to give a final concentration of 0.02 M, the pH was adjusted to 6.0 with 1 N HCl, and the sample was mixed for 30 min. The bentonite was recovered by centrifuging the sample at 3,000 × g for 10 min. Pellets resulting from the elution and concentration of 1 liter of digested sludge were suspended in 50 ml of tryptose phosphate broth, pH 7.3 (Difco Laboratories), whereas 80 ml of tryptose phosphate broth was used to elute the bentonite from 1 liter of primary raw sludge. The elution was performed by sonicating the pellet and eluant to homogeneity at 60 W for 3 min in a 250-ml rosette cell submerged in an ice bath. Before being assayed, samples were centrifuged at 8,000 × g for 30 min. These conditions yielded a recovery of 74% for poliovirus 1 (Chat) from seeded primary sludge eluates.

Experimental results shown in Table 1 suggest that the efficiency of viral release from sludge, using distilled water, depended to some degree on the method of solids disruption, as well as on the type of sludge. Based on the recovery of indigenous viruses, it was concluded that distilled water elution-blender homogenization provided maximal viral release. This procedure then was applied to the recovery of indigenous enteric viruses from domestic wastewater sludges undergoing land application (Table 2). Dispersal of liquid sludge solids in distilled water by blender homogenization was followed by viral concentration from sludge eluates by viral adsorption onto bentonite clay.

Table 2. Indigenous viruses recovered from liquid sludges

<table>
<thead>
<tr>
<th>Field site</th>
<th>Sludge Type</th>
<th>Distilled water-bentonite adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TSS (mg/liter)</td>
</tr>
<tr>
<td>1</td>
<td>Primary</td>
<td>61,500</td>
</tr>
<tr>
<td>2</td>
<td>Primary</td>
<td>44,800</td>
</tr>
<tr>
<td>3</td>
<td>Primary</td>
<td>68,500</td>
</tr>
<tr>
<td>4</td>
<td>Digest</td>
<td>50,300</td>
</tr>
<tr>
<td>5</td>
<td>Lagooned</td>
<td>22,400</td>
</tr>
<tr>
<td>6</td>
<td>Digest</td>
<td>16,900</td>
</tr>
<tr>
<td></td>
<td>Lagooned</td>
<td>50,700</td>
</tr>
</tbody>
</table>

* Samples (1 liter each) were dispersed with 1 liter of distilled water. Solids were removed by centrifugation, and the supernatant was concentrated. The concentrates were assayed on HeLa cell monolayers. TSS, Total suspended solids; PFU, plaque-forming units. Concentration factors were calculated by dividing the initial sludge volume by the final plating volume.

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onstrated by the recovery of indigenous virions from primary raw sludges during laboratory testing and from anaerobically digested and lagooned sludges during the field monitoring portion of this study.

The viral levels reported for treated wastewater sludges must be considered as minimal concentrations as the use of HeLa cells in a plaquing procedure limits virus isolations largely to polioviruses and coxsackie B viruses. These findings serve as a reminder that, regardless of the theoretical efficacy of treatment processes, actual sludge handling practices do not result in complete viral removal or inactivation in domestic wastewater sludges. Proper site selection and land use management, therefore, are crucial at sludge application sites.

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LITERATURE CITED