Inactivation of Animal Viruses during Sewage Sludge Treatment

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Using a previously developed filter adsorption technique, the inactivation of a human rotavirus, a coxsackievirus B5, and a bovine parvovirus was monitored during sludge treatment processes. During conventional anaerobic mesophilic digestion at 35 to 36°C, only minor inactivation of all three viruses occurred. The $k'$ values measured were $0.314 \log_{10}$ unit/day for rotavirus, $0.475 \log_{10}$ unit/day for coxsackievirus B5, and $0.944 \log_{10}$ unit/day for parvovirus. However, anaerobic thermophilic digestion at 54 to 56°C led to rapid inactivation of rotavirus ($k' > 8.5 \log_{10}$ units/h) and of coxsackievirus B5 ($k' > 0.93 \log_{10}$ unit/min). Similarly, aerobic thermophilic fermentation at 60 to 61°C rapidly inactivated rotavirus ($k' = 0.75 \log_{10}$ unit/min) and coxsackievirus B5 ($k' > 1.67 \log_{10}$ units/min). Infectivity of parvovirus, however, was only reduced by $0.213 \log_{10}$ unit/h during anaerobic thermophilic digestion and by $0.353 \log_{10}$ unit/h during aerobic thermophilic fermentation. Furthermore, pasteurization at 70°C for 30 min inactivated the parvovirus by 0.72 $\log_{10}$ unit/30 min. In all experiments the contribution of temperature to the total inactivation was determined separately and was found to be predominant at process temperatures above 54°C. In conclusion, the most favorable treatment to render sludge hygienically safe from the virological point of view would be a thermal treatment (60°C) to inactivate thermolabile viruses, followed by an anaerobic mesophilic digestion to eliminate thermostable viruses that are more sensitive to chemical and microbial inactivations.

The widespread agricultural land application of sewage sludge can be a hazard for animals and humans because of the possible pollution of vegetables, food, or water resources with pathogens or chemicals. In Switzerland the utilization of sewage sludge in agriculture is controlled by legislation regulating the content of bacteria, parasites, and heavy metals, but not of viruses. Within the scope of a Swiss National Research Program we therefore examined the fate of human and animal viruses during various sludge treatment processes. The technique used had been established with bacteriophage F2 (18). In this study we tested a human rotavirus, as the most important causative agent of viral gastroenteritis in infants, and coxsackievirus B5, as a representative of the widespread enteroviruses. The bovine parvovirus strain was selected for its extraordinary thermostability.

The rotavirus and the enterovirus were rapidly inactivated by process temperatures of 55 to 60°C, whereas the parvovirus proved to be more readily inactivated by anaerobic mesophilic digestion.

MATERIALS AND METHODS

Viruses and host cells. The stock solution of the human rotavirus strain Wa (23) and the host cell line MA104 were a gift of H. Brüssow, Nestlé SA, Vevey, Switzerland. Human coxsackievirus B5, obtained by W. Wunderli, Institute of Clinical Microbiology and Immunology, St. Gallen, Switzerland, was isolated from a fecal specimen from a child with meningitis in 1983 and was identified in a plaque inhibition test. The virus in its fifth passage was propagated in HEp-2 cell monolayers using Eagle modified basal medium (Flow Laboratories, Baar, Switzerland) containing 2% fetal calf serum and antibiotics.

The bovine parvovirus strain HADEN (1) was kindly provided by G. Siegl, Institute of Hygiene and Medical Microbiology, University of Bern, Bern, Switzerland. Stock solution of parvovirus HADEN was grown in bovine embryonic lung cells of passage level 2, cultured in Eagles minimal essential medium (AMIMED, Basel, Switzerland) containing 2% heat-inactivated fetal calf serum which was free of bovine parvovirus antibodies. We identified the virus by a plaque inhibition test using a specific antiserum (5). In an indirect immunofluorescence test the strain showed typical intranuclear fluorescence (6). The thermostability of the virus strain at 60°C was tested in phosphate-buffered saline (pH 7.0) supplemented with 5 mM MgSO₄. The virus was obtained by freezing-thawing infected cell cultures three times, followed by centrifugation and filtration through cellulose nitrate filters of 5- and 0.45-μm pore size to remove larger virus aggregates.

The cell lines MA104, HEp-2, and Vero (used for the plaque test with coxsackievirus B5; see below) were free of Mycoplasma spp. as shown by a chemiluminescence assay (3).

Sandwich technique to determine virus inactivation during exposure in reactors. The method for monitoring virus inactivation during sludge treatment processes has been described previously (18). Briefly, the virus was adsorbed to an electropositively charged Zetapor filter membrane with a pore size of 200 nm (AMF, Cuno Div., Meriden, Conn.) sandwiched between two polycarbonate membranes (Nuclepore Corp., Pleasanton, Calif.) with pores of 15-nm size to prevent loss of virus. The three membrane layers were locked within a filter holder of which inlet and outlet connections were removed, leaving openings of about 15 mm in diameter on both sides. The use of polycarbonate membranes without pores enabled us to determine the inactivation effect of temperature separately.

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The procedure of loading rotavirus and parvovirus onto the filter membrane was the same as for bacteriophage F2 (18). Membranes of 450-nm pore size were used for rotavirus because of its larger particle size. For adsorption, coxsackievirus B5 virions had to be pelleted by ultracentrifugation in a Beckman SW 50.1 rotor at 33,000 rpm for 1.5 h and then suspended in phosphate-buffered saline (pH 7.3) before the final dilution in loading buffer.

The elution technique was the same as described previously (18), except that the eluate was passed a second time through the same membrane to increase the elution efficiency.

**Virus assays.** Eluates were tested for infectious virus as follows. The rotavirus was assayed by the 50% tissue culture infectious dose method in tissue culture tubes on a roller drum. Assay procedures were similar to those described by Sato et al. (14). Titration of coxsackievirus B5 was performed in 24-well cell culture plates by means of a PFU method in Vero cells. The carboxymethylcellulose (no. 27649; BDH Chemicals Ltd., Poole, England) overlay used (8) was supplemented with 25 mM MgCl$_2$ and 100 μg of DEAE-dextran per ml (12). After incubation at 37°C for 3 days, the plates were fixed with phosphate-buffered Formalin and stained with 0.5% crystal violet in double-distilled water. For the quantification of infectious bovine parvovirus we applied the plaque technique of Durham and Johnson (5), using EBLC grown in EEM with 30 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and incubating the plates without CO$_2$. For all virus assays, medium and overlay were supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml). In each plate, the overlay for the plaque test of bovine parvovirus contained econazole (1 μg/ml) (24).

**Field experiments.** As with bacteriophage F2, all field experiments were performed at the sewage treatment plant of Altenrhein, Switzerland (18). Virus inactivation could be studied during various sludge treatment processes, i.e., anaerobic mesophilic digestion, anaerobic thermophilic digestion, aerobic thermophilic fermentation, and pasteurization. The process parameters at the time of experiments are listed in Table 1.

In all experiments, total and thermal inactivation were determined by exposure of two sandwich devices with 15-nm-pore membranes and two sandwich devices without membrane pores for each time point.

Statistical analysis was carried out as described previously (18).

**RESULTS**

**Applicability of the sandwich technique for animal viruses.** The sandwich technique for measuring the inactivation of viruses in a given environment was developed with bacteriophage F2. Only minor modifications were necessary to adapt the method for the human and animal virus strains used in this study. Coxsackievirus did not adsorb to Zetapor membranes unless the cell culture medium was removed by ultracentrifugation. Perhaps a cell protein or another component of the medium competed with the virus for adsorption sites on the membrane. A similar phenomenon was observed by Guttman-Bass and Catalano-Sherman (9) for poliovirus, which did not adsorb in the presence of humic acid.

Because of the relatively low titer of animal viruses, it was important to elute a maximum of the adsorbed virus. Therefore, the same elution volume was passed through the filter membrane twice, since experiments with bacteriophage F2 had indicated that in this way virus could be recovered with greater efficiency (18).

Under these conditions 100% of rotavirus (5 × 10$^5$ 50% tissue culture infectious doses per filter) and coxsackievirus particles (5 × 10$^6$ PFU per filter) and about 95% of parvovirus particles (10$^5$ PFU per filter) adsorbed to the membrane. Elution efficiency as related to the virus content of the loading suspension was 83% for rotavirus, 80% for parvovirus, and more than 100% (probably due to disaggregation of virus particles by the elution medium) for coxsackievirus.

**Field experiments in the sewage treatment plant.** The results of the five experiments are summarized in Table 2. The virus load per filter was the same as stated above except for Fig. 3A (2 × 10$^5$ PFU per filter).

**Anaerobic mesophilic digestion.** Among the four processes tested, the conventional sludge treatment by anaerobic mesophilic digestion was the least effective. The infectivity of all viruses examined decreased less than 1 log$_{10}$ unit/day (Table 2). With regard to total inactivation, parvovirus was two to three times more sensitive (Fig. 3A) than rotavirus (Fig. 1) and coxsackievirus (Fig. 2) and about as sensitive as bacteriophage F2. For coxsackievirus B5, temperature (36°C) appeared to be the main inactivation factor (Fig. 2), whereas the inactivation of rotavirus and parvovirus was probably due to additional factors present in the sludge (Fig. 1 and 3A).

**Anaerobic thermophilic digestion.** The process temperature of 54 to 56°C led to a rapid inactivation of rotavirus and coxsackievirus. The $k'$ values for these two viruses represent a lower limit, since titers fell below the detection limit within the duration of the experiment (Table 2). Under the same conditions the titer reduction for the heat-stable parvovirus was very small, but attributable to thermal inactivation alone (Table 2; Fig. 3B).

**Aerobic thermophilic fermentation.** As with anaerobic

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**TABLE 1. Process and sludge data of the wastewater treatment plant at Altenrhein**

<table>
<thead>
<tr>
<th>Process</th>
<th>Virus</th>
<th>Date</th>
<th>Total solid feed (g/liter)</th>
<th>Organic solids feed (g/liter)</th>
<th>pH of reactor</th>
<th>Reactor temp (°C)</th>
<th>Reactor vol (m$^3$)</th>
<th>Reactor pressure (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic mesophilic digestion</td>
<td>Rotavirus</td>
<td>1 Oct. 1985</td>
<td>3.65</td>
<td>2.33</td>
<td>7.3</td>
<td>36</td>
<td>1.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enterovirus</td>
<td>17 Apr 1986</td>
<td>5.13</td>
<td>2.84</td>
<td>7.25</td>
<td>36</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parvovirus</td>
<td>10 June 1986</td>
<td>5.23</td>
<td>3.13</td>
<td>7.35</td>
<td>36</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>Anaerobic thermophilic digestion</td>
<td>Rotavirus</td>
<td>17 Sept. 1985</td>
<td>4.14</td>
<td>1.67</td>
<td>7.55</td>
<td>54.3</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enterovirus</td>
<td>17 Apr 1986</td>
<td>3.73</td>
<td>1.79</td>
<td>7.65</td>
<td>53.4</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parvovirus</td>
<td>27 June 1986</td>
<td>4.02</td>
<td>1.74</td>
<td>7.85</td>
<td>56.4</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>Aerobic thermophilic fermentation</td>
<td>Rotavirus</td>
<td>17 Sept. 1985</td>
<td>5.44</td>
<td>3.18</td>
<td>6.8</td>
<td>61</td>
<td>7.2</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Enterovirus</td>
<td>29 Apr 1986</td>
<td>4.57</td>
<td>2.86</td>
<td>6.9</td>
<td>60.7</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parvovirus</td>
<td>20 Aug 1986</td>
<td>5.66</td>
<td>3.18</td>
<td>6.75</td>
<td>60.6</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Pasteurization</td>
<td>Parvovirus</td>
<td>15 Oct. 1986</td>
<td>4.71</td>
<td>2.98</td>
<td>6.1</td>
<td>70.5</td>
<td>0.7</td>
<td></td>
</tr>
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</table>
thermophilic digestion, aerobic thermophilic fermentation led to a rapid inactivation of the temperature-sensitive rotavirus and coxsackievirus, whereas the inactivation rate of the parvovirus was low. Inactivation was again mainly attributable to temperature, as shown by similar \( k' \) values for total and thermal inactivation (Table 2). For parvovirus the total inactivation was somewhat lower than the corresponding thermal inactivation, indicating a possible stabilizing effect of the sludge on parvovirus (Fig. 3C).

**Pasteurization.** For obvious reasons, only parvovirus was subjected to pasteurization. In the course of the conventional detention time of 30 min at 70°C the parvovirus lost only 0.7 \( \log_{10} \) unit of its infectivity. The titer reduction was again due to temperature alone, as indicated by identical \( k' \) values for thermal and total inactivation (Table 2). In all treatment processes at elevated temperatures, parvovirus was by far more stable than bacteriophage f2, whereas the stability of rotavirus and coxsackievirus B5 was much below that of the bacteriophage.

**DISCUSSION**

The experiments presented in this paper were carried out with three animal viruses selected for their health significance or their marked heat stability. Thus, the filter sandwich method previously developed with bacteriophage f2, a model virus chosen for easy handling (18), has now been employed successfully to monitor inactivation of viruses that may be considered good indicators for the effectiveness of different sludge treatment processes.

It is well known that mesophilic digestion does not sufficiently reduce the virus content of sludge (2, 4, 13, 15), and this was confirmed in our experiments. In contrast, a thermal treatment (anaerobic thermophilic digestion or aerobic thermophilic fermentation) led to a rapid inactivation of rotavirus and coxsackievirus B5, whereas the heat-stable parvovirus was not inactivated by such treatments. The parvovirus was also resistant to pasteurization, as already described by Leuze et al. (11). Thus our results confirm and
extend previous findings (17, 21, 22). The viruses tested here can be grouped into thermolabile viruses that are comparatively chemoresistant (rotaviruses, enteroviruses) and thermostable viruses that are chemolabile (parvoviruses). In this connection chemoresistance means that viruses are relatively insensitive to chemical factors such as ammonia and detergents (19–21) or to microbial factors (10) present in the sludge.

The parvoviruses, known for their extraordinary heat stability (17), may serve as representatives for other heat-resistant viruses found in sludge, e.g., human hepatitis A virus (7, 16). To render sludge hygienically safe from the virological point of view, a thermal treatment (~60°C) of the sludge to inactivate thermolabile viruses should precede an anaerobic mesophilic digestion to eliminate thermostable viruses that are more sensitive to chemical inactivation.

As shown in Table 2, parvoviruses could be good indicators of thermal inactivation, whereas rotaviruses would seem to be the most suitable indicators of other inactivating factors.

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LITERATURE CITED

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