Isolation of Viruses from Sewages and Sewage Sludges in a Sewage Treatment Plant in Northern Part of Japan

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Abstract

Isolation of viruses from sewages and sewage sluges in a sewage treatment plant in Obihiro, Hokkaido, Japan was carried out to grasp the kinetics of viral contamination of sewages during the process of sewage treatment. Inocula of sewage or sewage sludge samples for cell cultures were prepared by precipitation with 10% polyethylene glycol # 6000 and 0.5 M NaCl, and extraction with trichlorotrifluoroethane. This procedure reduced effectively the cytotoxicity of inocula and also eliminated the contamination of microorganisms. Viruses were detected in 83% of raw sewages, 50% of primary sediments, 33% of activated sludges, 17% of chlorinated sewage effluents and 8% of dewatered sludges. The viruses isolated were poliovirus types 1, 2 and 3, coxsackievirus B5 and adenovirus.

Introduction

In Japan, sewage is generally processed by preliminary, primary, secondary (biological) and disinfectant treatments, and in many cases, sewage sludge is digested anaerobically and dewatered before disposal. The reuse of treated sewage or sludge is becoming interesting, and this attempt is practiced in some municipal treatment plants in Japan.

Virus survival during treatments of sewage or sludge has been studied by many workers. These studies have indicated that virus remaining in the sewage or sludge can contaminate surface water, ground water or crops [2,9]. Only a few surveys, however, have been carried out in Japan about the occurrence of viruses in sewage or sludge[5,8]. As the demand for sewage treatment is increasing, the amounts of discharged sewage effluent and then subsequent disposal of sludge also increase concomitantly. It is therefore important to survey virus survival in the sewage treatment systems to consider potential public health hazards associated with viral pollution of the environment. The present report describes the results of a year-long survey of viral contamination of sewages and sludges collected at

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each processing stage of a sewage treatment plant including raw sewage, primary sediment, activated sludge, chlorinated sewage effluent and dewatered sludge for reuse as fertilizer.

Materials and methods

Sampling site.

Sewage samples were collected from a sewage treatment plant in Obihiro City having a population of about 150,000 and being located in northern part of Japan. This plant, which uses the activated sludge process, treats 40,000 m^3 of domestic sewage per day. According to the method of IWASAKI et al.[5], samples of raw sewage (RS), activated sludge (AS) fluid containing 2% of solid, and chlorinated sewage effluent (CSE) were collected by soaking tampons (50 to 100 g) in the sewage flow for 24 hours and about 500 to 1,000 ml of the samples were squeezed from the tampons. About 1,000 ml of primary sediment (PS) fluid containing 5% of solid was pumped out in sterilized bottles. Dewatered sludge (DS) was collected directly from the belt filter. Sampling was undertaken in every month between May, 1979 to May, 1980.

Preparation of the samples for virus isolation.

Organic floculation. The samples were treated using the technique of Glass et al.[3]. PS and AS fluid were centrifuged at 8,000×g for 20 min and the sediments were collected. Twenty grams each of PS, AS and DS were suspended in about 20 ml of distilled water, and the pH was adjusted to 7.0. These suspensions were starting materials for further processing. Four hundred ml of the squeezed liquid samples of RS and CSE were starting materials for further processing. Each sample was mixed with 12 g of beef extract and centrifuged at 10,000×g for 20 min with the exception of CSE. The pH of the supernatants and CSE was adjusted to 3.5 and the resulting flocs were collected by centrifugation at 3,000×g for 10 min. The sediments were dissolved in 3 ml of 0.15 M Na_2HPO_4 and centrifuged at 20,000×g for 15 min. After adjusting pH to 7.2, the supernatants were supplemented with 0.3 to 0.5 ml of antibiotics (20,000 U of penicillin per ml and 20 mg of streptomycin per ml) and used as inocula for cell cultures.

Precipitation with polyethylene glycol # 6000 and sodium chloride, and extraction with trichlorotrifluoroethane.

The squeezed liquid samples of RS and CSE were centrifuged at 10,000×g for 20 min. PS, AS and DS were treated in the same way with the method of organic flocculation. The 400ml of sonicated sludge suspensions were centrifuged at 10,000×g for 20 min. After adjusting the pH, the 400ml each of the supernatants were treated with 12ml of calf serum, 40g of polyethylene glycol #6000 (PEG) and 11.7g of NaCl. After stirring for 60 min, the precipitates formed were allowed to settle at 4 C overnight, followed by centrifugation at 8,000×g for 20 min. The precipitates were suspended in about 20 ml of phosphate buffered saline (PBS). The suspensions were extracted with an equal volume of trichlorotrifluoroethane (TTF) by homogenizing and centrifuging at 4 C. When the aqueous phase was turbid, this extraction was repeated. The aqueous phase was then centrifuged at 90,000×g fof 60 min. The precipitate obtained were suspended in 4 ml of Eagle's minimum essential medium (MEM) containing 1% calf serum or fetal calf serum, 500 U of penicillin per ml, 500 μg of streptomycin per ml and 60 μg of kanamycin per ml. The resulting suspensions were used for inoculation of the cell cultures.

Cell cultures.

HeLa, Vero and BGM cells were used for virus isolation. Vero cells were used during May, 1979 to October, 1979, BGM cells were used during November, 1979 to May, 1980, and HeLa cells were used during June, 1979 to May, 1980. The cells were cultured in Eagle's MEM

containing 10% calf serum and 60 μg of kanamycin per ml. After inoculation of samples, the cells were maintained in the same medium containing 1% calf or fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml and 60 μg of kanamycin per ml.

Virus assay.

The prepared samples were inoculated into four monolayer cultures (0.1 ml per culture) grown in tubes (15×150 mm) or multidish disposo trays (24 holes, 16 mm; Limbro Chemical, Vineland, N. J.). After 1 hour of adsorption at 36 C, 1 ml of maintenace medium was added and incubated at 36 C. When cytopathic effect (CPE) appeared, the cultures were frozen and thawed, and the culture fluids were inoculated into fresh cultures. When the cultures showed CPE again, the sample inoculated into the cultures were judged as virus positive and kept at −20 C for future identification of isolated viruses. The cultures which did not show any CPE during 7 days of incubation or showed cytotoxicity, were passed blindly after freezing and thawing. When CPE appeared in the cultures passed blindly, the cultuer fluids were passed again to fresh cultures and the cultures showing CPE were kept at −20 C. When the cultures passed blindly did not show any CPE, the sample inoculated was judged as virus negative. The isolated virus strains were typed by the neutralization test (NT) against enterovirus serum pools by Piggy-back microtransfer technique[1]. The viruses showing typical CPE of adenovirus were identified by the complement fixation test (CFT) using immune serum against human adenovirus type 2.

Results

Evaluation of the preparation methods for virus isolation.

The samples collected in May, 1979 were treated by the method of organic flocculation and inoculated into vero cells. Cytotoxic effect was observed in the cell cultures inoculated with samples of RS, PS, and AS on the next day of inoculation. When tenfold dilution of RS sample, and fourfold dilution of PS and AS samples were inoculated, the cytotoxicity was not observed but no viruses were isolated from any samples. The method of organic flocculation was used only once since the preparations by organic flocculation showed cytotoxicity as described above.

The method of precipitation with PEG and NaCl, and extraction with TTF was carried out during the period from June, 1979 to May, 1980. Although only AS samples prepared by this method in August and September, 1979 and in February, 1980 showed cytotoxicity in HeLa cells, virus was isolated from the AS sample collected in September, 1979 after blind passage (Table 2). Except for these samples, cytotoxicity and contamination of microorganisms were not observed in any of the other samples. To determine the efficiency of the method of precipitation with PEG and NaCl, and extraction with TTF to recover virus from samples, poliovirus type 2 vaccine strain was added as a marker virus to the samples of RS and CSE, and the samples were treated using the same procedure. The results (Table 1) show that 2 to 9% of the added virus were recovered in PBS suspension before or after treatment with TTF, whereas 0.15 to 1% of the virus was recovered in the final materials used as inocula.

Incidence of viruses in the samples and identification of the virus isolates.

As shown in Table 2, the viruses were detected in 10 of 12 (83%) RS samples, 6 of 12 (50%) PS samples, 4 of 12 (33%) AS samples, 2 of 12 (17%) CSE samples, and 1 of 12 (8%) DS samples collected during the preiod from June, 1979 to May, 1980, treated by the method of precipitation with PEG and NaCl, and ex-

Table 1. The efficiency of virus recovery by the PEG-NaCl-TTF method.[a]

Sample	Initial Volume	PBS suspension after PEG-NaCl precipitation		PBS suspension after TTF extraction		MEM suspension used as inoculum	
		Volume	Recovery[b]	Volume	Recovery	Volume	Recovery
Raw sewage I	400 ml	Not tested		20 ml	2%	4 ml	0.15%
Raw sewage II	400 ml	20 ml	2%	Not tested		4 ml	0.8%
Chlorinated sewage effluent	400 ml	20 ml	5%	20 ml	9%	4 ml	1%

a); One ml 1.5×10^7 $TCID_{50}$) of poliovirus type 2 vaccine strain was added asamarker virus to each sample, and the samples were treated by the PEG-NaCl-TTF method as described in the text.

b); Recovery was expressed as pent of input virus.

Table 2. The isolation and identification of viruses from sewages and sludges using the method of precipitation with PEG and NaCl, and extraction with TTF.

mo and yr	Raw sewage		Primary sediment		Activated sludge		Chlorinated sewage effluent		Dewatered sludge	
	HeLa	Vero* BGM	HeLa	Vero BGM	HeLa	Vero BGM	HeLa	Vero BGM	HeLa	Vero BGM
June, 1979	—	—	P−2+	P−2	P−1	—	P−1	—	P−2	—
July, 1979	CB−5	—	CB−5	P−2	—	—	CB−5	—	—	—
August, 1979	CB−5	—	CB−5	—	—	—	—	—	—	—
September, 1979	CB−5	—	CB−5	—	CB−5	—	—	—	—	—
October, 1979	P−2	—	P−2+	—	—	—	—	—	—	—
November, 1979	CB−5	CB−5	—	—	CB−5	—	—	—	—	—
December, 1979	Ad	—	—	—	—	—	—	—	—	—
January, 1980	Ad	—	—	—	—	—	—	—	—	—
February, 1980	Ad	—	—	—	—	—	—	—	—	—
March, 1980		—	—	—	—	—	—	—	—	—
April, 1980	Ad	—	—	—	—	—	—	—	—	—
May, 1980	—	P−3	P−1	—	P−3	P−3	—	—	—	—

P−1, 2, 3, poliovirus type 1, 2, 3, CB−5; coxsackievirus B type 5, Ad; adenovirus.

P−2+; The result of NT indicated that viruses isolated contained poliovirus type 2 and untypable virus. −; virus negative.

* Vero cells were used during June, 1979 to October, 1979 and BGM cells were used during November, 1979 to May, 1980.

traction with TTF. These viruses were identified by NT or CFT. The viruses isolated were poliovirus types 1, 2 and 3 in June, July and October, 1979, and May, 1980; coxackievirus B type 5 in July, August, September and November, 1979. The viruses showing typical CPE of adenoviruses, as evidenced by CFT were isolated from the samples of RS obtained during December, 1979, January, February and April, 1980.

Discussion

The samples of RS, PS and AS prepared by the organic flocculation method showed cyto-

toxicity. This method was used to detect poliovirus in anaerobically digested sludges[3]. The results of this study showed that the organic flocculation method was not suitable for detecting viruses in untreated sewage or sludge. HURST et al.[4] reported a method for detection of enteroviruses in activated sludges using beef extract. By this method, cytotoxicity was avoided by mixing floc of beef extract with 5 volumes of fetal calf serum and using the supernatant as inoculam. In such a precipitation, however, some inactivation of the viruses may occur during exposure to acidic pH. The method of precipitation with PEG and NaCl, and extraction with TTF reduced cytotoxicity in comparison with the organic flocculation method, and could exclude any undesirable acidic precipitation. The results show that this method using PEG, NaCl and TTF is suitable for detecting viruses in untreated sewage or sludge.

The results of a year-long survey of viral contamination of sewages and sludges show that the types of the viruses isolated were poliovirus types 1, 2 and 3 in July, October, 1979 and in May, 1980: coxsackievirus B5 in July, August, September and November, 1979; and adenovirus in December, 1979, January, February and April, 1980. The isolation of polioviruses could be responsible for the administration of live vaccine of the viruses carried out during May and October in this year in Obihiro. The adenovirus was isolated only in winter in the cultures passed blindly. LYDHOLM and NIESEN[6] carried out the isolation of viruses from wastewater. They discussed on the virus assay systems used that tube cultures of HeLa cells with one week of inocubation and two blind passages gave a limitation to the virus types that could be detected, and probably the virus assay systems could not detect reoviruses, most coxsackie A viruses, infectious hepatitis viruses, rotaviruses and the other viruses of non-bacterial gastroenteritis. The virus assay systems used in the present study were similar to their systems except the two blind passages. Therefore, the types of virus isolated in this study, which were poliovirus, coxsackie virus B and adenovirus, were limited.

Viruses were detected in 17% (2/12) of CSE samples and 8% (1/12) of DS samples after blind passage. Poliovirus types 1 and 2 were isolated in June, 1979 and coxsackie virus B type 5 was isolated in July, 1979. FUJIOKA and LOH[2] reported that 58% of the chlorinated effluent samples tested were positive for viruses by either the polyelectrolyte 60, polymer two-phase, $Al(OH)_4$ method or the protamine sulfate method. SATTER and WESTWOOD[7] detected viruses in 53% of digested, and 39% of lagoon-dried sludges using the elution with 10% fetal calf serum in saline (pH 7.2). The results of the present study indicate that the PEG-NaCl-TTF method was less efficient for virus isolation from the treated sewages or sludges compared with the other methods reported before. In spite of low virus recovery by the PEG-NaCl-TTF method, viruses were isolated from the CSE and DS samples in this study. The results show that potential viral contamination of CSE discharged into natural waters and DS used as fertilizer can be considered as public health hazards associated with viral pollution of the environment.

References

1) CATALANO Jr. L. W., FUCCILLO D. A., & SEVER J. L. (1968): Piggy-back microtransfer technique. *Appl. Microbiol.* **18**, 1094–1095.

2) FUJIOKA R. S. & LOH P. C. (1978): Recycling of water for irrigation: Persistence of enteroviruses in sewage effluent and natural waters receiving the effluent. *Water, Air and Soil Pollut.* **9**, 213–226.

3) GLASS J. S., Van SLUIS R. J. & YANKO

W. A. (1978): Practical method for detecting poliovirus in anaerobic digester sludge. *Appl. Environ. Microbiol.* **35**,

4) HURST C. J., FARRAH S. R., GERBA C. P. & MELNICK J. L. (1978): Development of quantitative methods for the detection of enteroviruses in sewage sludges during activation and following land disposal. *Appl. Environ. Microbiol.* **36**, 81–89.

5) IWASAI K., YABUUCHI K., YANO K. & SHIBATA T. (1978): Isolation of viruses from sewage. *Clin. Virol.* **6**, 265–269. (in Japanese).

6) LYDHOLM B. & NIELSEN A. L. (1980): Methods for detection of virus in wastewater, applied to samples from small scale treatment systems. *Water Res.* **14**, 169–173.

7) SATTAR S. A. & WESTWOOD J. C. N. (1979): Recovery of viruses from field samples of raw, digested, and lagoon-dried sludges. *Bull. W. H. O.* **57**, 105–108

8) SUGISHIMA S. & HAYASHI E. (1979): Isolation of viruses from sewage. *Jpn. J. Publ. Hlth.* **26**, 17–23. (in Japanese).

9) VAUGHN J. M., LANDRY E. F., BARANOSKY L. J., BECKWITH C. A., DAHL M. C. & DELIHAS N. C. (1978): Survey of human virus occurrence in wastewater-recharged groundwater on Long Island. *Appl. Environ. Microbiol.* **36**, 47–51.

日本北部の一下水処理施設の下水および下水汚泥からのウイルス分離

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摘　　要

北海道帯広市の下水処理施設における下水処理過程でのウイルス汚染の変動を把握するためにウイルス分離を試みた。下水あるいは下水汚泥試料を培養細胞に接種するにあたって，試料をポリエチレングリコール6000と0.5M食塩による沈澱およびトリクロロフルオロエタンによる抽出の前処理を行なった。この方法により接種材料中に含まれる細胞毒性物質および微生物が効果的に除かれた。生下水の83%，初沈汚泥の56%，活性汚泥の33%，塩素処理放流水の17%および脱水汚泥の8%の各試料からウイルスが分離された。分離されたウイルスはポリオウイルス１，２および３型，コクサッキーウイルスＢ５型，およびアデノウイルスであった。

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