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Distribution of Ribonucleic Acid Coliphages in Raw Sewage from Treatment Plants in Japan

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To determine the transmission cycle of ribonucleic acid (RNA) coliphages in their natural habitats, we investigated the distribution patterns of RNA phages in raw sewage collected from treatment plants in various localities in Japan. Most of the sewage samples contained group II and III phages. Samples from treatment plants in Sapporo, Tokyo, and Toyama contained appreciable amounts of group I phages in addition to the group II and III phages. As a whole, raw sewage from treatment plants in Japan contained RNA phages of the three groups in the ratio 1:2:5, group I/II/III. Based on the distribution patterns of RNA phages in sewage from domestic drainage in Japan proper (group II/III, 3:1), in animal feces and sewage from slaughter houses (mostly group I), and in human feces (group II/III, 1:1), it can be reasonably said that group I phages tend to be introduced from animal sources and group II and III phages tend to be introduced from human sources. Raw sewage from treatment plants in Japan consists mainly of human feces, sewage from domestic drainage, and industrial wastewater, and, in part, from slaughter houses. In fact, sewage from slaughter houses together with that from human sources flowed into the treatment plants of Tokyo as far as we could confirm.

Ribonucleic acid (RNA) coliphages have been classified into four groups (I through IV) according to their serological and physicochemical properties (7, 11).

In the course of our systematic surveys on RNA phages, we chose sewage samples from domestic drainage and feces of certain domestic animals and of humans as sources for the isolation of such phages. Analysis of these three types of material revealed a unique distribution pattern of RNA phages in each material examined. That is, the sewage from domestic drainage in Japan proper contained group II and III phages in a 3:1 ratio (4, 6); the sewage from slaughter houses, feces of domestic animals, and feces of animals in zoological gardens contained group I phages exclusively; and the feces of humans contained group II and III phages almost equally, although the frequencies of isolation of these phages in human and animal feces were fairly low (2 and 6%, respectively) as far as we could determine (9). Thus, in general, group I phages were considered to be of animal origin, and group II and III phages were thought to be of human origin.

In this connection, it was of interest to investigate the distribution patterns of RNA phages in raw sewage from treatment plants. Raw sewage from treatment plants consists mainly of human feces, sewage from domestic drainage, and industrial wastewater, and, in part, of sewage from slaughter houses, and would be expected to contain mostly group II and III phages, with a minor fraction of group I phages.

MATERIALS AND METHODS

Media. Peptone-glucose medium (5 g of NaCl, 20 g of peptone, 2 g of glucose, 1,000 ml of water, pH 7.4) was used for collection of sewage samples, isolation of RNA phages, and dilution of phage and antiphage sera; peptone-glucose medium supplemented with 0.25% yeast extract and 0.01 M $CaCl_2$ was used for the preparation of crude phage lysate.

Bacterial strains and antiphage sera. Bacterial strains (*Escherichia coli* K-12 strains Aλ [F^+], Q13 [Hfr], and W3110 [F^-]) and antiphage sera against phages MS2 (group I), GA and JP34 (II), Qβ and VK (III), and SP (IV) were the same as those described previously (1, 6, 8).

Collection of samples and preparation of original phage samples. Approximately 10 ml of raw sewage was collected in small sterilized plastic tubes from each of the treatment plants listed in Table 1 and brought back to our laboratory under normal temperature conditions within 2 days, except for the samples from Recife (Brazil), New York (N.Y.), and Würzburg (West Germany). Two milliliters of the raw sewage was mixed with the same volume of peptone-

glucose medium, treated with 0.5 ml of chloroform to kill bacteria, and subjected to low-speed centrifugation (Sorvall GLC 1) to remove bacterial debris and certain other precipitates. The supernatant fractions so obtained were stored in a cold room (4°C) and used as original phage samples.

Isolation and grouping of RNA phages by the serological method. The isolation of RNA phages was performed as described previously (8). Each 0.1 ml of original phage sample was plated on *E. coli* strains A/λ (F^+), Q13 (Hfr), and W3110 (F^-), and single plaques on the individual plates were picked up, suspended, and stirred vigorously in 2 ml of peptone-glucose medium with 0.5 ml of chloroform. The samples were left standing for several minutes, after which the supernatant fraction was used for further analysis. In the present study, we picked up 30 to 70 plaques per sample. These phage stocks were subjected to spot tests on *E. coli* strains A/λ, Q13, W3110, and A/λ with ribonuclease (100 μg/plate). Phages which lysed strain A/λ or Q13, or both, but did not lyse strains W3110 and A/λ with ribonuclease were picked up, purified several times by the single-plaque isolation method, and stocked in a cold room (4°C) as RNA phages. RNA phages infect specifically male host strains and are sensitive to ribonuclease treatment.

Grouping of RNA phages by the serological method was carried out as described previously (6, 10). In short, newly isolated RNA phages were subjected to spot tests on strain A/λ or Q13 with ($K = 1$ per plate) or without antiserum of six standard phages (MS2 [group I], JP34 and GA [II], Qβ and VK [III], and SP [IV]).

RESULTS

Density of total coliphages in samples. The densities of total coliphages were fairly high, ranging from 10 to 10^6 plaque-forming units per ml of original phage sample (with many showing 10^2 to 10^4 plaque-forming units per ml), in almost all of the samples collected from treatment plants in various localities in Japan (Table 1). Although we observed a fluctuation in density of the order of 10^2 in surveys of raw sewage at 2-h intervals during the day (data not shown), we usually collected sewage samples from treatment plants in the afternoon. There appears to be a tendency toward somewhat higher phage densities in the summer (10^4 to 10^6 plaque-forming units per ml) than in the winter (10 to 10^3 plaque-forming units per ml).

Density of RNA phages in samples. To obtain as many RNA phages as possible from the raw sewage of treatment plants, we picked up plaques from only A/λ (F^+) and Q13 (Hfr) plates (not from W3110 [F^-] plates) and rejected plaques which showed an apparently deoxyribonucleic acid phage morphology. As a result, the number of RNA phage strains per total number of phages tested does not give the proportion of RNA phages in a given sample. The density of RNA phages in a given sample was determined by subtracting the number of plaque-forming units on ribonuclease containing plates (100 μg/plate) from the total plaque-forming units on ribonuclease-free plates.

Almost all of the samples contained RNA phages at relatively high densities, i.e., 5 to 90% of the total coliphages (with many at 30 to 60%). Thus, RNA phages constituted the predominant phage species in sewage from treatment plants, as in the case of sewage from domestic drainage (2, 3, 6, 8).

Distribution pattern of RNA phages in collected materials. We picked up 3,243 plaques and obtained 1,832 RNA phage strains (Table 1). By serological analysis, these were classified into four groups, (I/II/III/IV, 221:487:1,122:2). Most of the sewage samples contained only group II and III phages. Samples from the treatment plants in Sapporo, Tokyo, and Toyama contained appreciable amounts of group I phages in addition to the group II and III phages. As a whole, raw sewage from treatment plants in Japan contained RNA phages of the three groups in the ratio 1:2:5, group I/II/III. Excluding the data for group I, group II and III phages were isolated at a ratio of 1:1 in Hokkaido and the Tohoku district, but at a ratio of 1:3 south of the Kanto district. Thus, the two prominent features in the distribution patterns of RNA phages in raw sewage from treatment plants in Japan were the predominance of group III phages over group II phages and the existence of group I phages in certain restricted treatment plants (Sapporo, Tokyo, and Toyama).

Continuous surveys over 3- to 5-year periods of the distribution patterns of RNA phages in raw sewage from the Shinkawa (Sapporo), Shibaura (Tokyo), Jonan (Takasaki), and Chubu (Fukuoka) treatment plants (see Table 1) have revealed a continuity of RNA phages in the sample materials, although the presence of RNA phages fluctuated more widely than that in domestic drainage (4).

In addition, raw sewage and sludge collected from treatment plants also contained many RNA phages, and the group types of RNA phages isolated were roughly the same in both materials (data not shown).

DISCUSSION

The most prominent features in the distribution patterns of RNA phages in raw sewage collected from treatment plants in Japan are the predominance of group III phages over group II phages and the existence of appreciable amounts of group I phages as well as group II and III phages, in contrast to the sewage from domestic drainage. Based on the distribution patterns of RNA phages in sewage from domestic drainage

TABLE 1. *Density of total coliphages and the distribution pattern of RNA phages in raw sewage collected from treatment plants of various localities in Japan*

District	City	Treatment plant	Density of total coliphages[a]	No. of phages tested	No. of RNA phage strains	RNA phage group (no.)				Date of collection
						I	II	III	IV	
Hokkaido	Sapporo	Soseigawa	1.3×10^3	70	34	2	30	2	0	27 Sept. 1973
	Sapporo	Soseigawa	5.6×10^3	60	44	0	0	44	0	28 Aug. 1974
	Sapporo	Shinkawa	3.0×10^4	74	68	0	68	0	0	27 Sept. 1973
	Sapporo	Shinkawa	3.9×10^3	75	57	30	0	27	0	28 Aug. 1974
	Sapporo	Shinkawa	6.3×10^4	101	96	17	43	36	0	8 Oct. 1975
	Sapporo	Shinkawa	4.3×10^4	65	56	0	6	50	0	5 Sept. 1976
	Total			445	355	49	147	159	0	
Tohoku	Hachinohe	Asahigaoka	2.4×10^4	45	26	5	21	0	0	24 Nov. 1975
	Hachinohe	Korekawa	1.5×10^4	49	30	0	24	6	0	24 Nov. 1975
	Sendai	Minamigamou	2.0×10^3	92	48	0	2	46	0	11 Aug. 1973
	Sendai	Minamigamou	2.8×10^4	67	3	0	0	3	0	10 Sept. 1974
	Total			253	107	5	47	55	0	
Kanto	Maebashi	Maebashi	4.0×10^4	71	34	0	4	30	0	29 Aug. 1973
	Takasaki	Jonan	4.0×10^4	50	41	0	8	33	0	30 Aug. 1973
	Takasaki	Jonan	4.0×10^4	64	30	0	6	24	0	30 Aug. 1973
	Takasaki	Jonan	3.2×10^5	77	14	0	0	14	0	28 July 1974
	Takasaki	Jonan	1.9×10^3	56	49	4	32	13	0	21 Dec. 1975
	Tokyo	Ochiai	2.0×10^3	104	53	0	4	49	0	19 Sept. 1973
	Tokyo	Ochiai	6.3×10^2	71	40	21	7	12	0	21 Dec. 1973
	Tokyo	Ochiai	1.0×10^3	40	25	18	4	3	0	22 Mar. 1974
	Tokyo	Shibaura	2.0×10^3	78	52	6	24	22	0	19 Sept. 1973
	Tokyo	Shibaura	7.0×10^2	40	31	0	0	30	1	21 Dec. 1973
	Tokyo	Shibaura	7.0×10^2	50	34	21	4	9	0	22 Mar. 1974
	Tokyo	Shibaura	4.5×10^2	98	66	30	3	32	1	27 Dec. 1974
	Tokyo	Shibaura	8.0×10^2	169	92	40	13	39	0	5 Apr. 1975
	Tokyo	Shibaura	1.0×10^4	80	46	2	39	5	0	8 July 1975
	Tokyo	Shibaura	5.0×10^4	108	104	0	17	87	0	21 June 1976
	Tokyo	Shibaura	2.0×10^3	70	49	5	6	38	0	5 Oct. 1977
	Tokyo	Ogasawara	2.0×10^2	16	15	0	0	15	0	5 Nov. 1973
	Choshi	Choshi	2.0×10^5	44	0	0	0	0	0	1 Sept. 1973
	Total			1,286	775	147	171	455	2	
Chubu	Niigata	Funami	1.0×10^4	50	41	0	6	35	0	6 Sept. 1973
	Niigata	Funami	2.9×10^3	82	44	0	0	44	0	14 Sept. 1974
	Niigata	Funami	2.0×10	26	8	0	8	0	0	28 Dec. 1975
	Toyama	Ushijima	8.0×10^4	62	4	1	2	1	0	6 Oct. 1973
	Toyama	Ushijima	8.0×10^2	39	31	14	6	11	0	21 Mar. 1974
	Toyama	Ushijima	7.2×10^3	70	14	0	0	14	0	15 Sept. 1974
	Toyama	Ushijima	1.4×10^2	43	21	2	12	7	0	29 Dec. 1975
	Total			372	163	17	34	112	0	
Kinki	Osaka	Ebie	7.0×10^3	73	47	0	0	47	0	5 Oct. 1973
	Osaka	Nakahama	5.0×10^3	73	69	1	23	45	0	5 Oct. 1973
	Total			146	116	1	23	92	0	
Chugoku	Hiroshima	Eba	1.5×10^5	42	16	0	6	10	0	30 July 1973
	Total			42	16	0	6	10	0	
Kyushu	Fukuoka	Chubu	1.4×10^3	45	2	0	1	1	0	1 Nov. 1973
	Fukuoka	Chubu	2.6×10^5	62	43	0	0	43	0	6 Aug. 1974
	Fukuoka	Chubu	2.2×10^2	27	0	0	0	0	0	3 Jan. 1976
	Fukuoka	Chubu	4.2×10^2	47	38	2	10	26	0	11 Apr. 1976
	Fukuoka	Chubu	1.9×10^6	67	65	0	0	65	0	10 Aug. 1976
	Kurume	Kurume	3.0×10	72	17	0	15	2	0	2 Nov. 1973
	Kurume	Kurume	6.0×10^2	67	30	0	10	20	0	28 Nov. 1973
	Kumamoto	Kumamoto	1.5×10^5	35	11	0	0	11	0	12 Aug. 1976
	Kumamoto	Kusunoki-danchi	4.0×10^5	30	0	0	0	0	0	12 Aug. 1976
	Kagoshima	Kinko	6.0×10^3	67	31	0	23	8	0	19 July 1973
	Kagoshima	Kinko	1.5×10^4	110	7	0	0	7	0	4 Aug. 1975
	Total			629	244	2	59	183	0	
Okinawa	Naha	Naha	2.7×10^3	70	56	0	0	56	0	13 Sept. 1974
	Total			70	56	0	0	56	0	
	Grand total			3,243	1,832	221	487	1,122	2	

[a] Plaque-forming units per milliliter of original phage sample.

in Japan proper (group II/III, 3:1) (4, 6), in animal feces and sewage from slaughter houses (mostly group I), and in human feces (group II/III, 1:1) (9), it can be reasonably said that the group I phages observed in raw sewage from treatment plants tend to be introduced from animal sources (feces of domestic animals and sewage from slaughter houses) and group II and III phages tend to be introduced from human sources (human feces and sewage from domestic drainage). Raw sewage from treatment plants consist mainly of human feces, sewage from domestic drainage, industrial wastewater, and in part, sewage from slaughter houses. In fact, sewage from slaughter houses together with that from human sources flowed into the treatment plants of Tokyo as far as we could confirm.

Group I RNA phages amplified in the alimentary tract of animals (and also presumably in sewage from slaughter houses) probably reinfect and propagate again in the alimentary tract of animals through food and water contaminated with RNA phages excreted from animals in the external environment. Thus, the transmission cycle of group I phages should be complete. The same situation can be expected to occur between humans and the group II and III phages, although the frequencies of isolation of these phages in human feces were fairly low as far as we could determine. Consequently, sewage from treatment plants in Japan contained mostly group III and II phages, with a minor fraction of group I phages, as might be expected from the distribution patterns of RNA phages in the human and animal feces (9) and in sewage from domestic drainage (4, 6). The predominance of group III phages over group II phages in raw sewage from treatment plants in Japan appears to suggest a wider contribution from human feces than from sewage from domestic drainage and a selective amplification of group III phages in the raw sewage.

The distribution patterns of RNA phages in raw sewage collected from treatment plants in several countries are summarized in Table 2. Sewage samples from Recife (Brazil) and Würzburg (West Germany) contained only group I phages, although we were unable to ascertain whether or not the samples included sewage from slaughter houses. Most of the sewage samples contained RNA phages of group II or III, or both, at fairly high densities, comparable to those in the treatment plants of Japan.

In contrast to the preferential relationships observed between RNA phage groups and animal species (group II and III phages with humans, and group I phages with other animals) (9), there appear to be no special phage-host relationships between RNA phage groups and human races. It can be said that (i) the most

TABLE 2. *Distribution patterns of RNA phages in raw sewage collected from treatment plants in various countries*

Country	City	Density of total coliphages[a]	No. of phages tested	No. of RNA phage strains	RNA phage group (no.)				Date of collection	Reference
					I	II	III	IV		
Mexico	Campeche	5×10^2	8	0	0	0	0	0	3 Apr. 1973	5
Saudi Arabia	Kahfuji	1×10^3	43	0	0	0	0	0	March 1973	5
Australia	Sidney	2×10^4	70	10	0	10	0	0	19 Aug. 1973	5
United States	Oakland (Calif.)	10^2–10^8	198	154	0	52	102	0	29 Aug. 1973	5
	Oakland (Calif.)	3×10^2	50	28	1	27	0	0	7 Dec. 1973	5
	Richmond (Calif.)	5×10^4	52	51	0	0	51	0	7 Dec. 1973	5
	New York (N.Y.)	2×10^2	48	45	0	45	0	0	Jan. 1976	Unpublished data
	San Elijo (Calif.)	5×10^3	28	7	0	1	6	0	10 Aug. 1977	Unpublished data
Brazil	Recife	3×10^2	22	4	4	0	0	0	27 Nov. 1975	Unpublished data
The Philippines	Manila	1×10^3	33	3	0	0	3	0	3 Nov. 1976	8
West Germany	Würzburg	5×10^3	58	17	17	0	0	0	June 1978	Unpublished data
	Würzburg (University Hospital)	5×10^4	51	6	0	6	0	0	June 1978	Unpublished data

[a] Plaque-forming units per milliliter of original phage sample.

predominant RNA phage species in raw sewage from treatment plants are those of groups III and II, both in Japan and in several other countries (Table 2); (ii) raw sewage from treatment plants is thought to consist mainly of wastewater of human origin, including human feces, sewage from domestic drainage, and industrial wastewater, both in Japan and in the several other countries listed in Table 2; and (iii) group II and III phages are thought to be primarily of human origin, based on direct analysis of fecal samples (9).

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