APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1981, p. 164-168
0099-2240/81/010164-05$02.00/0

Vol. 41, No. 1

# Distribution of Ribonucleic Acid Coliphages in Animals

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To determine the distribution pattern of ribonucleic acid (RNA) coliphages (classified by serological groups I through IV) in animal sources, we isolated RNA phages from (i) feces samples from domestic animals (cows, pigs, horses, and fowls), some other animals in a zoological garden, and humans, (ii) the gastrointestinal contents of cows and pigs, and (iii) sewage samples from treatment plants in slaughter houses. These samples were then analyzed serologically. The concentration of RNA phages in the first and second kinds of material was fairly low (10 to $10^3$ plaque-forming units per original phage sample), whereas that in the third kind of material was fairly high ($10^3$ to $10^5$ plaque-forming units per original phage sample). Concerning the group types of the RNA phages in the first and second kinds of material, human feces contained RNA phages of groups II and III almost equally, the gastrointestinal contents of pigs included those of groups I and II equally, and the feces or gastrointestinal contents of other mammals other than humans and pigs had those of group I exclusively. In the third type of material, we found mostly group I phages with a minor fraction of group II phages. Thus, the prominent features of the distribution pattern of RNA phages are the predominance of groups III and II in humans and the predominance of group I in animals.

To elucidate the ecology of bacteriophages, we have made extensive efforts to determine the geographical distribution of ribonucleic acid coliphages (RNA phages) in sewage collected from domestic drainage in several countries, since we believe this to constitute one of their natural habitats (16). These phages were classified into four groups (I through IV) on the basis of various biological and physicochemical properties (9, 13-17). Analysis of such sewage samples revealed several distinctive features in the distribution pattern of RNA phages in the global Asian area. That is to say, the most prevalent RNA phages in mainland Japan (north of Kyushu) are group II phages, whereas group III phages are predominant in the southern part of Japan (south of Amamiohshima Island) and Southeast Asia (at least in Taiwan, the Philippines, Singapore, and Indonesia). We therefore propose a border line between Kyushu and Amamiohshima Island in the geographical distribution of RNA phages in the domestic drainage of South and East Asia (6-8, 10-12).

In the course of our systematic surveys, we found that the feces of several animals represented suitable sources for the isolation of RNA phages besides sewage (12). At the same time, we observed that gnotobiotic mice established with male strains of *Escherichia coli* could support the propagation of RNA phages in their intestine throughout our experiments (at least for 3 months). These results suggest that the gastrointestinal tract of mammals constitutes one of the natural habitats of RNA phages when suitable host bacteria are present. No significant differences in fecal and rectal counts of RNA phages were observed in the same experiments (2). Thus, fecal counts appear to reflect closely the rectal counts of RNA phages.

In an attempt to clarify the distribution pattern of RNA phages in animals and to determine the extent of the contribution of RNA phages from animal sources to the propagation and transmission cycles of RNA phages in their natural habitats, we investigated the amounts and kinds of RNA phages in animal sources such as feces, gastrointestinal contents, and sewage from slaughter houses.

## MATERIALS AND METHODS

**Bacterial strains.** *E. coli* K-12 strains A/λ ($F^+$), Q13 (ribonuclease I negative, Hfr), and W3110 ($F^-$) were used as host strains for the isolation and preparation of RNA phages.

**Media.** The PG (peptone glucose) medium used for the collection of samples, isolation of RNA phages, and dilution of phage and antiphage sera and the PGYC medium (PG medium supplemented with 0.25% yeast extract and 0.01 M $CaCl_2$) used for the preparation of crude phage lysate were as described previously (1).

**Antiphage sera.** Preparation of antiphage rabbit sera was carried out according to the method of Sak-

urai et al. (16). Antiphage sera of groups I (MS2, BO1, and JP501), II (GA, BZ13, TH1, KU1, and JP34), III (Qβ, VK, ST, and TW18), and IV (SP, FI, TW19, TW28, MX1, and ID2) were employed for the serological grouping of newly isolated RNA phages.

**Collection of samples and preparation of original phage samples.** Approximately 1 g of fresh feces or gastrointestinal contents of individual cows or pigs was taken from the Tama or the Shibaura slaughter house (Tokyo) and suspended in 5 ml of PG medium by using an attached spoon in a small, sterilized plastic tube. The fresh feces of many animals listed in Table 1 were collected from Tokyo Tama Zoological Park. The fecal samples of fowls and horses were obtained from a poultry farm and a stable in the suburbs of Tokyo, respectively. Most of the human feces were collected by taking advantage of the opportunities for examining the stools of healthy individuals in Tokyo. Approximately 1 g of human or animal feces was suspended in 5 ml of PG medium, as in the case of cows and pigs. Samples of raw sewage (about 5 ml) or sludge (about 1 g) were also taken from the sewage treatment plants of slaughter houses and mixed with or suspended in 5 ml of PG medium. The materials so obtained were treated as soon as possible with 0.5 ml of chloroform to kill bacteria and centrifuged to remove bacterial debris and certain other precipitates. The supernatant fractions were utilized as the original phage samples.

**Isolation and grouping of RNA phages by the serological method.** The isolation and grouping of RNA phages by the serological method were carried out as described previously (8, 10, 16). Original phage samples were plated as such or after appropriate dilution to give a suitable number of plaques (less than 20 plaques per plate) for the subsequent single-plaque isolation procedure. Usually, we picked up 5 to 20 plaques per sample in the case of fecal and gastrointestinal materials and 20 to 60 plaques per sample for sewage from treatment plants and analyzed them serologically. A sample which contained one or more coliphages (plaques produced on any one of the three indicator strains) per 0.1 ml of original phage sample was judged to be a coliphage-positive sample. In the same manner, a sample which contained one or more RNA phages (plaques which lysed male indicator strains A/λ or Q13 or both specifically but did not lyse A/λ when ribonuclease was added at 100 μg per plate) per 0.1 ml of original phage sample was judged to be an RNA phage-positive sample.

## RESULTS

**Frequency of appearance of total coliphages and RNA phages in the fecal and gastrointestinal materials.** As shown in Table 1, the frequencies of appearance of coliphages (percentage of coliphage-positive samples in collected materials) in the feces samples collected from many species of birds and mammals in the zoo and from pigs in the slaughter houses were fairly high (74 to 92%), whereas those in feces samples collected from certain domestic animals (fowls, horses, and cows) and humans were very low (10 to 30%). The coliphage concentrations of the former samples showed widely ranging distribution patterns from low to high titers, whereas those of the latter samples had a narrow peak in the lower titer region (Table 2). RNA phages were isolated at a low frequency (0 to 5%) from all the fecal samples examined and at a fairly high frequency (20%) from the gastrointestinal contents of cows and pigs.

As shown in Table 3, the amounts of total coliphages in sewage samples from slaughter houses (including raw and activated wastewater, primary, secondary, and final sludge, and final effluent) were fairly high, ranging from $10^3$ to $10^6$ plaque-forming units per ml. All the sewage samples which we collected contained RNA phage at a relatively high density, representing 5 to 90% of the total coliphages.

**Serological grouping of newly isolated RNA phages.** When two or more RNA phages isolated from the same original sample showed similar inactivation patterns serologically, they were considered to be the same strain. We isolated 527 RNA phage strains (227 strains from feces and gastrointestinal content and 300 strains from sewage from slaughter houses) and analyzed them serologically by the spot test and the plating method (8) using the 18 antiphage sera listed above. All the RNA phages tested were inactivated by at least one of the six standard antisera (MS2, group I; GA, II; JP34, II; Qβ, III; VK, III; and SP, IV) and classified into one of the four known groups.

The RNA phages isolated from foxes, elephants, horses, and cows all belonged to group I, those from pigs belonged to groups I and II, and those from humans belonged to groups II and III. The group I and II phages in pigs and group II and III phages in humans were isolated at almost equal proportions to each other. Thus, group III phages were detected only in samples from humans and group I phages were detected only in samples from animals. About 90% of the RNA phages isolated from sewage samples from slaughter houses belonged to group I, and the remainder (10%) belonged to group II. The same result was obtained at both the Tama and Shibaura slaughter houses. Thus, special relationships appear to exist between RNA phage groups and their host animals, i.e., group I phages with animals, and group III phages with humans.

Based on the low isolation frequency of group IV phages in sewage samples from domestic drainage (7, 8), it can be said that group IV phages were isolated with a relatively high frequency from animal sources although the actual numbers of isolates were very few (five strains).

TABLE 1. *Frequencies of isolation of total coliphages and RNA phages in animal and human sources*

| Source | Material | No. of samples | Coliphage positive[a] | | RNA phage positive[b] | | | RNA phage group | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | No. of samples | % | No. of samples | % | No. of strains[c] | I | II | III | IV |
| Birds (zoo)[d] | Feces | 25 | 23 | 92.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Fowls | Feces | 30 | 9 | 30.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mammals (zoo)[e] | Feces | 97 | 72 | 74.2 | 5 | 5.2 | 5 | 3[f] | 0 | 0 | 2[g] |
| Horses | Feces | 30 | 3 | 10.0 | 1 | 3.3 | 1 | 1 | 0 | 0 | 0 |
| Cows | Gastrointestinal contents | 20 | 13 | 65.0 | 4 | 20.0 | 4 | 4 | 0 | 0 | 0 |
| Cows | Feces | 20 | 6 | 30.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Pigs | Gastrointestinal contents (pooled) | 3 | 3 | 100 | 1 | 33.3 | 3 | 1 | 1 | 0 | 1 |
| Pigs | Large-intestinal contents | 30 | 30 | 100 | 6 | 20.0 | 8 | 3 | 5 | 0 | 0 |
| Pigs | Feces | 11 | 10 | 90.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Humans | Feces | 597 | 140 | 23.5 | 14 | 2.3 | 18 | 0 | 9 | 7 | 2 |
| Total | | 863 | 309 | 35.8 | 31 | 3.6 | 39 | 12 | 15 | 7 | 5 |

[a] Number of samples containing one or more coliphages per 0.1 ml of original phage sample.
[b] Number of samples containing one or more RNA phages per 0.1 ml of original phage sample.
[c] Number of RNA phages exhibiting different serological properties in the same original phage sample.
[d] *Aves* (number of samples in parentheses): Struthiniformes—ostrich (2); Ciconiiformes—black-headed ibis (3), scarlet ibis (3), greater flamingo (3), Chilean flamingo (3); Gilliformes—Japanese pheasant (3), copper pheasant (2); Gruiformes—Manchurian crane (3); Strigiformes—feather-toed scops owl (3).
[e] *Mammalia* (number of samples): Marsupialia—white-throated wallaby (3); Primates—common tree shrew (2), slow loris (3), thick-tailed bushbaby (3), common squirrel monkey (3), douroucouli (3), Japanese macaque (5), Siamang (3), chimpanzee (5), lowland gorilla (2); Lagomorpha—Etigo hare (2); Rodentia—white-cheeked flying squirrel (3); Carnivora—Japanese red fox (3), racoon-like dog (3), Himalayan black bear (3), Japanese black bear (1), Yezo brown bear (3), tiger (3), lion (5), African cheetah (3); Proboscidae—African elephant (3); Perissodactyla—domestic horse (1), Chapman's zebra (2), Malayan tapir (4), Indian rhinoceros (3); Artiodactyla—Japanese wild boar (3), Bactrian camel (4), Yaku Island sika deer (3), reticulated giraffe (3), domestic yak (2), scimitar-horned oryx (3), Japanese serow (2), mouflon (3).
[f] These three strains were isolated from a fox, an elephant, and a horse, respectively.
[g] These two strains were isolated from two tigers.

TABLE 2. *Concentration distribution of total coliphages in animal and human sources*

| Source | Material | No. of samples within range (PFU[a] per ml of original phage sample): | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | <10 | 10 to $\leq 10^2$ | $10^2$ to $\leq 10^3$ | $10^3$ to $\leq 10^4$ | $10^4$ to $\leq 10^5$ | $10^5$ to $\leq 10^6$ | $10^6$ to $\leq 10^7$ | $>10^7$ |
| Birds (zoo)[b] | Feces | 2 | 3 | 3 | 4 | 1 | 3 | 9 | 0 |
| Fowls | Feces | 21 | 8 | 1 | 0 | 0 | 0 | 0 | 0 |
| Mammals (zoo)[b] | Feces | 25 | 15 | 16 | 11 | 9 | 8 | 8 | 5 |
| Horses | Feces | 27 | 2 | 0 | 1 | 0 | 0 | 0 | 0 |
| Cows | Gastrointestinal contents | 7 | 7 | 4 | 1 | 0 | 1 | 0 | 0 |
| Cows | Feces | 14 | 2 | 2 | 1 | 0 | 0 | 1 | 0 |
| Pigs | Gastrointestinal contents (pooled) | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 |
| Pigs | Large intestinal contents | 0 | 0 | 6 | 6 | 0 | 5 | 6 | 7 |
| Pigs | Feces | 1 | 6 | 2 | 1 | 1 | 0 | 0 | 0 |
| Humans | Feces | 457 | 87 | 24 | 15 | 10 | 1 | 2 | 1 |

[a] PFU, Plaque-forming units.
[b] For explanation of animals, see Table 1 footnotes *d* and *e*.

The group IV phages isolated from tigers and pigs belonged to subgroup (d) (TW28), and those from humans belonged to subgroup (b) (FI). Furthermore, it should be noted that the two standard phages of group IV, SP (subgroup a) and FI (b), were isolated initially from apes and humans, respectively (11, 13). Thus, FI could be isolated exclusively from humans.

TABLE 3. *Concentration of total coliphages and the distribution pattern of RNA phages in sewage samples collected from slaughter house sewage treatment plants*

| Place | Date of Collection | Material | Total coliphages (PFU/ml)[a] | No. of RNA phage strains tested | RNA phage group | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | I | II | III | IV |
| Tama (Tachikawa, Tokyo) | June 1975 | Raw wastewater | $4 \times 10^5$ | 4 | 1 | 3 | 0 | 0 |
| | June 1975 | Raw wastewater | $1 \times 10^5$ | 20 | 18 | 2 | 0 | 0 |
| | June 1975 | Activated wastewater | $4 \times 10^3$ | 54 | 47 | 7 | 0 | 0 |
| | June 1975 | Primary sludge | $9 \times 10^3$ | 19 | 18 | 1 | 0 | 0 |
| | June 1975 | Secondary sludge | $3 \times 10^3$ | 60 | 54 | 6 | 0 | 0 |
| | June 1975 | Final sludge | $1 \times 10^3$ | 44 | 42 | 2 | 0 | 0 |
| | June 1975 | Final effluent | $3 \times 10^4$ | 38 | 34 | 4 | 0 | 0 |
| Shibaura (Minato-ku, Tokyo) | October 1977 | Raw wastewater | $4 \times 10^3$ | 7 | 5 | 2 | 0 | 0 |
| | June 1978 | Raw wastewater | $1 \times 10^6$ | 54 | 50 | 1 | 0 | 3 |

[a] Plaque-forming units (PFU) per ml of original phage sample.

## DISCUSSION

The most prominent feature of the distribution pattern of RNA phages in animals is the existence of preferential relationships between RNA phage groups and their host animals. Group III RNA phages could be isolated exclusively from human feces, and group I RNA phages were isolated from feces or gastrointestinal contents of mammals other than humans.

Group II phages are thought to belong primarily to humans, although they were also found in the gastrointestinal contents of pigs. Investigations of sewage samples collected from domestic drainage in Japan indicate that the most prevalent RNA phages in mainland Japan (north of Kyushu) are group II phages (6) and domestic drainage is thought to be principally under the influence of the daily activities of the local people and to be influenced little by contamination from animal feces.

In the present study, we were able to isolate group I phages only from animals such as foxes, elephants, horses, cows, pigs, as well as from sewage from slaughter houses, and not from humans or sewage from domestic drainage. Group I phages are therefore thought to belong primarily to animals. This is supported by the facts that (i) although all the animals tested were bred in zoological gardens or breeding farms and were thought to have ample chance to become contaminated by human phages through their food, they revealed no group III phages, which are thought to be specific to humans, and (ii) preferential propagation of group I phages (MS2) over group III phages (Q$\beta$) was observed in MS2-Q$\beta$ (inoculated with MS2 first and then superinfected with Q$\beta$) or Q$\beta$-MS2 (inoculated with Q$\beta$ first and then superinfected with MS2) double-infection experiments in gnotobiotic mice established with male strain *E. coli* (2).

We were unable to determine whether the group II phages observed in pigs were intrinsic to them or had been introduced from human sources by chance. In any case, pigs appear to be able to support the propagation of RNA phages of group II as well as those of group I under usual breeding conditions. To confirm this, it will be necessary to monitor the fecal titers of phages after inoculating these phages orally.

Dhillon et al. (5) reported an example of a similar "habitat preference" in the virulent phages $\phi$X174 and S13, which contain single-stranded DNA and are closely related. They found that S13 type phages were isolated only from the feces of pigs, whereas $\phi$X174 type phages were isolated only from cow dung.

In contrast with the widespread distribution of RNA phages in sewage from slaughter houses (present result), human sewage treatment plants (manuscript in preparation), and domestic drainage (3, 4, 6–8, 10, 11), RNA phages seem to constitute only a minor fraction of the total coliphages in the feces of humans and animals. Similar results were obtained by Dhillon et al. in a survey of mammalian feces (5).

The fact that raw sewage from human sewage treatment plants contains group I, II, and III phages at a ratio of about 1:2:5 (manuscript in preparation) can be explained fairly satisfactorily by our previous (6) and present results. That is to say, group I phages are derived from animal sources (feces and gastrointestinal contents of several animals and sewage from slaughter houses), and group II and III phages come from human sources (human feces and sewage from domestic drainage), since the raw sewage from human treatment plants consists mainly of human feces, sewage from domestic drainage, and, in part, sewage from slaughter houses. To confirm the special relationships which exist between phage groups and sewage composition, it

will be necessary to analyze sewage samples of known composition from several human sewage treatment plants.

## ACKNOWLEDGMENTS

We express our deep gratitude to Y. Kudo (Department of Microbiology, Tokyo Metropolitan Research Laboratory of Public Health), T. Tenshyo (Tama Meat Inspector's Station, Bureau of Public Health, Toyko Metropolitan Government), M. Hoshi (Tokyo Metropolitan central wholesale market, Meat Market), T. Yoshida and A. Uchiyama (Tokyo Metropolitan Meat Inspector's Office), and A. Komori (Tama Zoological Park) for their generous provision of the samples.

This study was supported in part by a grant from the Waksman Foundation of Japan Inc.

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