ABSTRACT

F-specific RNA coliphages are prevalent in sewage and other fecal wastes of humans and animals. There are four antigenically distinct serogroups of F' RNA coliphages and those predominating in humans (groups II and III) differ from those predominating in animals (groups I and IV). Hence, it may be possible to distinguish between human and animal wastes by serotyping F' RNA coliphage isolates. Because serotyping requires scarce antiserum reagents, we investigated genotyping of F' RNA coliphages using synthetic oligonucleotide probes as an alternative approach to distinguishing these four groups. Oligoprobes I, II, III, IV, A, and B, were selected to detect group I, II, III, IV, I + II, and III + IV phages, respectively. Methods for plaque transfer onto candidate membrane filters and fixation of genomic nucleic acid on the membranes were optimized. The oligoprobes were end labelled using digoxigenin, followed by DNA-RNA hybridization and colorimetric, immunoenzymatic detection. Of 203 confirmed isolates of F' RNA coliphages in environmental samples of water, wastes, shellfish and animal feces, 99.5 and 96.6% could be identified into each group by serotyping and genotyping,
respectively. Probes A & B correctly identified 100% of isolates. For each group (I, II, III, IV), serotyping identified 37, 116, 3, and 46 isolates and genotyping identified 37, 109, 4, and 46 isolates, respectively. Based on these results, this method for genotyping F' RNA coliphages appears to be practical and reliable for identifying isolates in field samples.
ACKNOWLEDGEMENTS

I would like to thank Dr. Mark D. Sobsey for his guidance and support throughout my study at the Department of Environmental Sciences and Engineering. I am also grateful to Dr. Pfaender for his time and positive criticism during the preparation of this report.

A special thanks is deserved by Dr. Y.S. Carol Shieh for her invaluable assistance and technical support. And also thanks Dr. J. van Duin for his kindness to give oligonucleotides.

I am indebted to Doug Wait and my colleagues for their useful help and friendship, espically for David A. Battigelli for his aid in discussing and proofreading this report.

Finally, I appreciate my family and wife, Choi-Iok Wong, without whose support and encouragement this study would be difficult to reach.
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INTRODUCTION

Water and food contaminated by feces may contain a great variety of gastro-intestinal (enteric) viruses, which are major agents of food- and water-borne outbreaks of gastroenteritis (69) and other diseases such as infectious hepatitis (41). In fact, the apparent increase in outbreaks of viral gastroenteritis has been attributed to contamination of water and food (23), particularly in outbreaks of infectious hepatitis viruses (42, 54, 62) and Norwalk virus gastroenteritis (9, 50). Monitoring the virological quality of water and food would be useful; however, monitoring for enteric viruses in water and food is difficult allow and expensive and some epidemiologically important enteric viruses are unculturable or inefficiently cultured. For this reason, there is a need to find reliable indicators for detecting fecal contamination containing enteric viruses and other pathogens in water, shellfish and other foods.

Because no consistent and reliable relationships between fecal indicator bacteria (fecal coliforms) and enteric viruses have been demonstrated (31), enteric bacteriophages have been recommended for the role of enteric
virus indicators (17). F-specific (F') RNA coliphages may be good indicators for monitoring the virological quality of water and food because they superficially resemble enteroviruses, caliciviruses, and hepatitis A virus. They also are consistently present in a variety of wastewaters, sufficiently persistent in the environment (21 and 22), and resistant to treatment processes to be acceptable indicators. In fact, one prototype F' RNA coliphage, MS2, has been shown to be very persistent in water and shellfish and more resistant to disinfection by ultraviolet irradiation, chlorine dioxide and monochloramine treatment than other agents, including hepatitis A virus, enterovirus Coxsackievirus B5, E coli, and Klebsiella pneumoniae (5 and 8).

F' RNA coliphages are comprised of serogroups I through IV. Strains isolated from human feces are usually groups II and III, while groups I and IV are usually found in animal feces (43). The risk of viral gastroenteritis and hepatitis in humans from ingestion of fecally contaminated water and food is considered higher from human feces than from animal feces, since enteric viruses from animal sources do not cause human illness (27). Hence, determining the sources of fecal contamination is important. Serotyping F' RNA coliphages may help distinguish human fecal contamination from animal fecal contamination. However, antisera for the F' RNA coliphages are not readily available
and some isolates are difficult to serotype. Some isolates have been found to be neutralized by two heterologous antisera (10 and 14-16). Hence, in this study, we developed a more specific and accessible method of grouping F' RNA coliphages by nucleic acid hybridization using oligonucleotides probes and compared this method with serotyping.
OBJECTIVES

1. Develop a molecular technique (genotyping) for typing F' RNA coliphages
   - Select oligonucleotides on the basis of homology with each group phage and test for their specificity and sensitivity.
   - Optimize plaque hybridization conditions and procedures, involving denaturing solutions, types of membranes, hybridization solutions, and hybridization temperatures to achieve adequate detection limits.

2. Serotype and genotype original field isolates (unpurified isolates) of F' RNA coliphages
   - Test isolates from agar plates without purification before serotyping and genotyping to speed up and simplify detection procedures.
   - Evaluate genotyping and compare with serotyping.

3. Distinguish between animal feces and human feces from environmental samples by grouping F' RNA coliphages.
LITERATURE REVIEW

1. Outbreaks of food-borne and waterborne viral diseases

The overall significance of viral agents to the risk of food-borne and waterborne viral gastroenteritis is poorly quantified due to the lack of surveillance, and the underestimation of outbreaks of viral gastroenteritis caused in part by the lack of simple routine techniques to detect and confirm the viral etiology. Fecal contamination of drinking, bathing and irrigation waters, raw shellfish and other foods, including those contaminated by ill food handlers is responsible for increasing outbreaks of viral gastroenteritis (23). The human enteric viruses, which comprise more than 100 different viruses, are considered as major causes of food-borne and waterborne viral gastroenteritis. They typically infect the cell lining of the alimentary tract and are excreted in large amounts from the feces of infected persons (48).

The viruses mostly often implicated in waterborne and foodborne disease are hepatitis A virus, enteric Non A-Non B hepatitis virus, small, round, structured gastroenteritis viruses, Norwalk agent and Snow Mountain agent, rotaviruses,
caliciviruses, astroviruses and parvoviruses that are responsible for recognized water-borne and shellfish-associated viral illnesses (65).

A. Hepatitis viruses

Waterborne and shellfishborne outbreaks of hepatitis A have been documented and they continue to occur in the USA and the rest of the world. In November 1982, use of untreated water from a single spring caused 73 cases of hepatitis A in Meade County, Kentucky (7). In the USA, from 1983 to 1987, hepatitis A rates increased from 9.2 cases/100,000 population to 14.4 cases/100,000. Hepatitis A rates occurred in males through waterborne were consistently 20% higher than in females. 42% of outbreaks of hepatitis A had unknown risk factor, and only 3% was confirmed to associate with food or water (54). In China, hepatitis A virus caused a huge outbreak in Shanghai between January and February, 1988 (18, 57 and 68). At least 300,000 cases of acute hepatitis were reported in two months, and they were confirmed as being associated with consumption of raw clams. Hepatitis A virus was isolated from clams collected from the market and sea bed, which was apparently seriously contaminated by sewage.

Non-A, non-B hepatitis (Hepatitis E) is responsible for outbreaks of hepatitis through fecal contamination of
drinking water, and is widespread in the India-asian regions, Africa, and Latin America (47). In China, 9 hepatitis E outbreaks have been documented since 1982, of which five were associated with water and four were associated with food. They occurred in 6 different provinces and they infected large numbers of people (71). Also, in northern Ethiopia, an outbreak of acute hepatitis E infection from water caused 423 illnesses among military men. The outbreak was serologically confirmed as HEV. Protection of water supplies from fecal contamination by chlorination, boiling, and hygiene education were recommended to control hepatitis E transmission (62). The largest reported epidemic of hepatitis E virus, which infected about 79,091 persons in Kanpur, India, occurred from December 1990 to April 1991. Fecal contamination of river water and inadequate chlorination of water in a reservoir were considered to cause this outbreak (42).

B. Norwalk virus and Norwalk-like viruses

Norwalk virus and Norwalk-like viruses are major causative agents for human viral gastroenteritis, and many outbreaks have been reported (23). The Norwalk-like SRSVs are now known to be caliciviruses. Between May 1, 1982 and December 31, 1982, there were 103 well-documented outbreaks associated with eating raw shellfish in New York State. Norwalk virus was the predominant etiologic agent on the
basis of symptoms of the illness, seroconversion, and the presence of IgM antibody to Norwalk virus in sera of affected persons. Clam and oyster samples from 2 of these outbreaks were confirmed to be contaminated by Norwalk virus through radioimmunoassay (34). Secondary transmission of Norwalk virus also occurred. A food handler recovering from illness transmitted Norwalk virus to potato and fruit salads, coleslaw, and tossed salad that caused viral gastroenteritis in 220 persons in 1982 (67). Other waterborne and shellfishborne outbreaks are documented. Sewage-contaminated river water caused a large outbreak of gastroenteritis at a caravan park in southern New South Wales in 1989. A 27-28 nm small round structured virus (Norwalk-like virus) was identified as a causative agent (32). Consumption of raw oysters has caused a number of outbreaks of gastroenteritis around the world that were linked to Norwalk-like viruses (29, 60 and 50). In Quebec, Canada, more than 200 people were infected with a Norwalk-type virus after eating raw oysters in 1991. Low levels of fecal coliforms were detected in the oysters, and a Norwalk-like virus was identified by direct electron microscopy of fecal samples from acutely ill persons.

C. Other enteric viruses

In addition to the hepatitis viruses and the Norwalk-type SRSVs, enteroviruses, adenoviruses, rotaviruses,
reoviruses, and astroviruses are also enteric viruses. Outbreaks of viral gastroenteritis by these viruses may not be as frequent as those by Norwalk virus, Norwalk-like viruses, and hepatitis viruses, but they are still a threat to public health through fecal oral transmission. Several outbreaks of rotavirus gastroenteritis have been reported. In southern Israel, attack rates of 72% and 56% were reported in two sequential outbreaks of gastroenteritis associated with rotavirus and confirmed by rotavirus-specific serum IgG. The age-specific morbidity rate increased with decreasing age in both outbreaks (13). In some outbreaks two or more viruses may be involved. Rotaviruses and astroviruses were detected from an outbreak of viral gastroenteritis in a psychiatric hospital. The duration and symptoms of illness by astrovirus infection were shorter and milder than by rotavirus, but the infection rate of astrovirus was higher than that of rotavirus (36). Although most epidemics of rotavirus gastroenteritis are due to group A, a five-member family fell ill with viral gastroenteritis caused by group C rotavirus in Finland (38).

2. Fecal indicators

2.1 Bacterial indicators

A. Coliforms

Coliforms usually refer to a group of bacteria
belonging to the *Enterobacteriaceae* which ferment lactose and also share other properties. They comprise several genera, including *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, and *Citrobacter*. Total coliforms may not be good fecal indicators, since *Klebsiella*, *Citrobacter*, and *Serratia* are not exclusively intestinal flora and they exist widely in the natural environment. *Citrobacter* normally can be found in fresh vegetables. Fecal coliforms are more associated with fecal contamination due to the exclusion of nonfecal coliforms by increasing the incubation temperature. In general, when contamination is of fecal origin, *E. coli* strains are predominant and may comprise 90% of the total coliforms (26). For these indicators, the relative numbers in samples are considered important because the presence of these microorganisms in water, air, soil and on vegetables and hands can sometimes be detected as a background that is not strictly related to fecal contamination. However, monitoring fecal coliforms in waters probably is insufficient to indicate the presence of viruses. For example, outbreaks of viral gastroenteritis in Florida in Nov. 1993 were associated with consumption of raw oysters which apparently met the fecal coliform standard for shellfish (33).

*E. coli* is considered a more reliable fecal indicator
due to its primary habitat in the intestinal tract of many warm-blooded animals. However, there are several disadvantages for *E. coli* to be a fecal indicator in water. First, it is less resistant than some bacterial pathogens, enteric viruses and protozoan cysts in water. Second, it may be a natural inhabitant of phyllosphere in the tropics. Third, it cannot distinguish between human and animal fecal contamination. This last criterion is important because human fecal contamination is a greater risk to human health from viruses and some other pathogens.

B. *Clostridium perfringens*

Although *Clostridium perfringens* is believed to be only from feces, it may be not a good fecal indicator of recent contamination because it is extremely persistent in the environment due to its spore-forming ability. However, the size of the spores and their great resistance suggest that they may be good indicators of protozoan cysts. *Clostridium perfringens* probably do not distinguish between human and animal fecal contamination.

C. Enterococci

*Enterococci* occur in the human and animal intestine; they usually can grow at extreme conditions (10°C and 45°C, in 6.5% NaCl, and pH 9.6). They were confirmed to be better
indicators than fecal coliforms for swimming associated gastroenteritis and for waterborne diarrheal disease from drinking water (4 and 40). Enterococcus faecalis was found to predominate in human and poultry feces, but Enterococcus durans, Enterococcus hirae, and Enterococcus faecium were not associated with any fecal source (45). Hence, their specificity to detect fecal contamination exclusively is uncertain.

D. Bifidobacterium spp. and Bacteroides spp.

Bifidobacterium spp. are the common flora of the gastrointestinal tract in human, pigs, or occasionally in cattle and sheep. B. breve and B. adolescentis are exclusively associated with human feces. However, Bifidobacterium spp. do not survive well in water, compared with fecal coliforms, and the detection of B. breve and B. adolescentis is not simple because they are anaerobes and require specialized media for growth. They are not as widely used for fecal indicators as fecal coliforms and E. coli.

Bacteroides spp. are the second largest group of organisms in the human intestinal tract, but they also can be found in the rumen of cattle and in the mouth and intestinal tracts of humans and other animals. Because they are strict and fastidious anaerobes, they are very difficult
to detect in environmental samples. Hence, they may not be highly specific for detection of human contamination.

2.2 Bacteriophage indicators

A. Coliphages

Coliphages are considered good fecal indicators for several reasons: (1) they can be detected from the feces of humans and other warm-blooded animals; (2) they are widely distributed in sewage and sewage-polluted waters; (3) they are more resistant in the environment and to treatment processes than bacterial indicators; (4) and detection and enumeration of coliphages is simple, rapid and inexpensive by a plaque assay (17). Unfortunately, the reliability of coliphages as indicators fecal contamination may be questionable due to their heterogeniety, variable detection using different host cells, and lack of correlation with human enteric virus levels (63).

B. B. fragilis phages

B. fragilis phages have been reported as potential fecal indicators. About 10% of human feces tested was found containing B. fragilis phages, which were not found in feces of the other animals and in unpolluted water (58 and 59). Also, they were persistent in the environment and resistant to disinfection treatment (44). B. fragilis phages survived longer than F+ RNA coliphages, poliovirus, HAV, and
rotaviruses in water. However, in other studies only low
titers of B. fragilis phages were detected in estuarine
water and oyster samples when compared with F' coliphages
(10). Hence, it may be difficult to determine recent human
fecal contamination and low level fecal contamination using
B. fragilis phages as indicators. It seems that B. fragilis
phages are human-specific fecal indicators, but more
extensive research on the distribution of B. fragilis phages
is needed to reach a conclusion.
C. F' RNA coliphages

F' RNA coliphages are a group of coliphages that only
attack host cells with F-pili. They can be divided into 4
serogroups (group I, II, III, IV) by neutralization with
antisera that are summarized in Table 1. Group I and group
II belong to major group A due to about 50% homology in
their sequences; groups III and IV are classified as major
group B, and their sequences show about 40% homology.
Among subgroups within a group, they show more than 90% 
homology in sequences except subgroups in group IV (about
70% homology). Several F' RNA coliphages have been reported
as intermediates, which will be neutralized by two or more
antisera, like prototypes JP34, MX1, and ID2.
The distribution of F' RNA coliphages in environmental
waters has been studied since 1973. Tables 2 and 3 show the
serogroups of F' RNA coliphages in human and animal feces and wastewaters. Clearly, only groups II and III were found in human feces and groups I and IV were detected in animal sources with the exception of pigs in which group II also was detected. With this exception, it should be possible to distinguish between human and animal fecal contamination. Furthermore, the wide distribution and high levels of F' RNA coliphages in wastewaters and at least some environmental samples make them possible fecal indicators.

A specific host cell, Salmonella typhimurium (WG49), has been constructed by inserting an E. coli F-plasmid into a Salmonella typhimurium strain (WG45), by Havelaar and colleagues in 1984. Using this host cell, more than 90% of the phages detected in sewage were F' RNA coliphages. Moreover, somatic Salmonella phages were detected at less than 10 pfu/ml by host WG49 host cell and constituted ≤ 5% of the total phages detected.

3. Serotyping and Genotyping

3.1 Serotyping F' RNA coliphages

Serological neutralization with antibodies is a conventional method to serologically group and identify organisms. For serotyping F' RNA coliphages, isolates were neutralized with each group of antiserum by spot tests on
Table 1. F' RNA Coliphage Groups and Serotypes

<table>
<thead>
<tr>
<th>Major group</th>
<th>Group</th>
<th>Prototype Strains</th>
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<tbody>
<tr>
<td>A</td>
<td>I</td>
<td>MS2, f2, R12, JP501</td>
</tr>
<tr>
<td>A</td>
<td>II</td>
<td>GA, SD, TH1, BZ13, KU1, JP34</td>
</tr>
<tr>
<td>B</td>
<td>III</td>
<td>Qβ, VK, ST, TW18</td>
</tr>
<tr>
<td>B</td>
<td>IV</td>
<td>SP, FI, TW19, TW28, MX1, ID2</td>
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</table>

Source: Phage Ecology
Table 2. Distribution Patterns of F' RNA Coliphages in Animal and Human Sources

<table>
<thead>
<tr>
<th>Sources</th>
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<td>Fowls</td>
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1. Furuse, 1987
2. Havelaar, 1986
3. Havelaar, 1990
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<tr>
<th>Country</th>
<th>City</th>
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<th>No. of RNA phages</th>
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<td>Hospital</td>
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<td>-</td>
<td>229</td>
<td>210</td>
<td>8</td>
<td>196</td>
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</tbody>
</table>

1. Furuse, 1987
2. Havelaar, 1990
3. Chung, 1993
male hosts. An isolate was typable if it was neutralized only by a homologous antiserum (no plaque or zone of lysis appeared on host cell lawns). After the spot tests for preliminary classification, each confirmed isolate was subgrouped by plating with or without antisera (Watanabe, 1967). Despite the general success in serologically grouping F' RNA coliphages, this method sometimes gives ambiguous results because there are intermediate phages that do not fit clearly into the four well known serogroups. For example, JP34 phage was neutralized by both anti-MS2 serum and anti-GA serum; MX1 was neutralized by anti-SP and anti-Qβ sera; and ID2 was neutralized partially by each antiserum (14, 15 and 16).

3.2 Genotyping

Recently, genotyping has been studied to classify organisms. Nucleotide sequencing, the restriction fragment length polymorphism assay (RFLPA), and hybridization techniques are the one mostly commonly used for genotyping organisms.

A. Sequence analysis

The principle of sequence analysis for genotyping is that the significance of sequence variation among 5'-untranslated regions or other regions could possibly be used
to classify or genotype organisms. Hepatitis C virus isolates from 62 patients were directly sequenced in their 5'-untranslated regions. These isolates then were classified into type 1 (55%), type 2 (18%), type 3 (19%), and type 4 (6%) through sequence analysis (30). A recent minireovirus clinical isolate was suggested to be a Norwalk-like virus and should be included in the caliciviridae family because it has 52% nucleotide sequence identity with Norwalk virus sequence (35). Moreover, this genotyping method is also very useful for identification of organisms for which sequence data is not available.

B. Restriction fragment length polymorphism assay (RFLPA)

A restriction endonuclease cuts a pure DNA into a consistently reproducible set of fragments that can be separated by gel electrophoresis. Different sources of DNA will form a unique fragment pattern (RFLPA) after treatment with a restriction endonuclease due to its site-specific cleavage. Therefore, it may be possible to distinguish serologically and hence genetically different organisms by this method. For example, after cleavage of total DNA of Staphylococcus aureus by an EcoRI restriction enzyme, gel electrophoresis, Southern transfer, and hybridization with a radiolabeled 16S rRNA probe were performed to characterize Staphylococcus aureus isolates from outbreaks (39). A
restriction fragment length polymorphism assay was carried to analyze reverse-transcribed and PCR-amplified rotavirus gene segment 9 for differentiation of human serotype 3 rotaviruses from animal serotype 3 rotaviruses. The results show that the restriction fragment length polymorphism assay is a useful method to distinguish among interspecies of rotaviruses (64). In another application, rRNA gene restriction patterns (ribotyping) was used for differentiation of *Shigella flexneri* strains (11). In this study, HindIII was the best endonuclease to produce an optimum digestion pattern for differentiating *Shigella* strains. RFLPA may be useful in epidemiological studies when compared with serotyping because of its potentially high degree of specificity in characterizing gentically related organisms. Hence, it is a potentially powerful tool for "molecular epidemiology".

C. Hybridization

Probes for genotyping by hybridization could be oligonucleotides, or a fragment of DNA representing a specific gene obtained by cloning or PCR. In general, target nucleic acids fixed on filters are hybridized with probes and then visualized by colorimetric, fluorescence or radioactive detection methods. This technique has been extensively studied in recent years due to its simplicity,
quickness, sensitivity, and specificity. For example, hybridization tests with a set of 4 DNA probes was capable of classifying enterotoxigenic E. coli strains producing heat stable toxin, or heat labile toxin, or both (46). A liquid-hybridization assay showed that it was more sensitive than the ELISA for typing the Vp4 and Vp7 gene of bovine rotaviruses (49). Oligonucleotide probes were used to type human group A rotavirus, which were specific for each G type of human group A rotavirus. The author pointed out that hybridization could be valuable in typing G types of human group A rotavirus during vaccine trials (53). Probes selected from 5'-untranslated regions of hepatitis C virus were able to identify the four HCV genotypes and their subtypes from 61 PCR-positive Brazilian HCV sera; 33% was identified as type 1, 38% as type 1b, 1.5% as type 2a, and 24.5% as type 3 (56).

Sequence analysis may be useful for distinguishing serological differences in some organisms, but it is impractical to serotype each isolate from field samples using such complicated, expensive and time-consuming procedures. Although RFLPA is considered a good genotyping method, it may not be able to distinguish some serogroups of organisms which have a very high sequence homology. Moreover, This method requires the testing of several
restriction enzymes to obtain an optimum digestion pattern. This procedure will take too much time for practical analyses.

Hybridization with oligoprobes seems to be practical for genotyping field isolates because of its simplicity, quickness, sensitivity and specificity, compared with sequence analysis and RFLPA. In this study, we selected hybridization to genotype F' RNA coliphages on the basis of these several advantages.
MATERIALS AND METHODS

F' RNA coliphage type strains and the host.

Five representative F' RNA coliphage strains, MS2 (group I), GA (II), QB (III), FI and SP (IV) were kindly given by Dr. Furuse (Japan). The host was a genetically constructed strain of Salmonella typhimurium, WG49, containing the E. coli plasmid (F'42 lac :: Tn5) that encodes pilus production (20). This host strain was grown in TYG-broth containing 1.3% tryptone, 0.1% yeast extract, 0.8% NaCl, 0.1% glucose, 0.03% CaCl2-2H2O, 100 mg/l nalidixic acid and 20 mg/l kanamycin sulfate. Broth cultures were prepared in baffled shaker flasks of medium by inoculating with host cells from frozen stock and incubating with shaking at 37°C overnight.

Preparation of environmental samples.

The environmental samples used in this study included sewage, human and animal feces, oysters and seawater samples. Sewage samples were suspended in one-third volume of trichlorotrifluoroethane (Freon), and were vortexed vigorously for 2 min. The emulsion was centrifuged at 5,000 x g for 20 min at 5°C, and the supernatant was concentrated
30-fold by ultrafiltration (Megafuge Centrifuge, Baxter Scientific Products, Heraeu Sepatech GmbH, Osteode, Germany) using YM100 membranes (Amicon, Danvers, MA). Fecal samples were diluted tenfold in phosphate buffered saline (PBS) supplemented with 0.2% Tween 80 and 15% glycerol. Samples were vortexed rapidly and centrifuged at 5,000 x g for 20 min to sediment solids. F+ RNA coliphages were extracted from oysters by homogenizing in an Omni-mixer (Sorvall) with an equal amount (w/v) of nutrient broth containing 0.5% Tween 80, and then centrifuging at 5,500 x g for 20 min to recover the supernatant (8).

**Infectivity assay of F+ RNA coliphages in environmental samples.**

F+ RNA coliphages present in the environmental samples of feces, sewage and oyster extract were detected by infectivity assay using WG49 host lawns for the double agar layer (DAL) plaque assay technique (2). F+ RNA coliphages present in water samples were concentrated and quantified by a direct membrane filtration method (55). One hundred ml water samples were supplemented with 0.05M MgCl₂, and filtered through 0.45 µm cellulose nitrate-acetate membrane filters (type HA, Millipore, Bedford, MA) at a flow rate of 100 ml/3-6 min to adsorb coliphages. The filters were applied face down on TYG agar containing log phase WG49 and
0.3% Tween 80 and 0.03% tetrazolium violet. Plaques were visualized after the plates were incubated at 37°C overnight.

**Isolation of F* RNA coliphages from environmental samples.**

F* RNA coliphages were isolated from assay plates with fewer than 50 plaques and were transferred by aspirating with a micropipette and suspending in 0.5 ml PBS containing 15% glycerol. Isolates were tested with RNase A (type I-A; Sigma, St. Louis, MO) to distinguish between RNA and DNA phages (70). This was done by spotting 5μl suspensions of isolates on TYG top agar containing WG49 host cells and 100 μg/ml RNase. Confirmed RNA coliphages were those which failed to produce plaques (lysis zones) due to the cleavage of RNA by RNase. These F* RNA coliphages were stored at -70°C for serotyping.

**Serotyping of F* RNA coliphages.**

A spot test was carried out to rapidly determine serotypes of the isolates (66). Twenty μl volumes of the undiluted, tenfold and hundredfold dilutions of an isolate were spotted on WG49 lawns on TYG top agar containing one of the five F* RNA coliphage neutralizing antisera. Each antiserum was diluted to achieve about three log_{10} reductions of a prototype phage within the same serogroup before addition to top agar. In each trial of serotyping,
five known F' RNA coliphage strains, each representing a serogroup (I, II, III, IVa, IVb), were included as positive controls in order to evaluate the reliability of this method. After overnight incubation at 37°C, serotypes were determined by scoring coliphage plates exhibiting the presence or absence of clear zones of lysis on the lawns. The absence of a clear zone on a lawn was considered complete neutralization of the phage infectivity by the antiserum in that plate and hence positive identification of phage serotype.

**Plaque-transfer and fixation for hybridization.**

The method for plaque transfer and fixation onto membranes was modified from that of Benton and Davis (6). In brief, 2 µl volumes of environmental isolates and five prototype strains were spotted on lawns of host WG49 and incubated at 37°C overnight. Plates were incubated at 4°C for 30 min and then were covered with nylon membranes to adsorb plaques for 2 min (4 min for the second transfer). The adsorption efficiencies of five different membrane filters were compared: GeneScreen (NEN Research Products, Boston, MA), nylon membranes (Boehringer Mannheim, Indianapolis, IN), Biotrans (ICN Radiochemicals, Inc., Irvine, CA), Biodyne (PALL Biosupport, East Hills, NY), and Zeta-probe blotting membranes (Bio-RAD Laboratories,
Richmond, CA).

Three solutions were compared for the efficiency of denaturation of the plaques transferred to membranes: 1) 0.05 N NaOH (1 min fixation and 1 min neutralization by 0.1 M sodium acetate pH 6.0; 2) 37% formaldehyde for 2 min; and 3) blotting buffer containing 7.5 x SSC (20 x SSC: 3 M NaCl and 0.3 M sodium citrate, pH 7.0) and 4.6 M formaldehyde (heat at 65°C for 15 min). The membranes were then treated to crosslink the denatured nucleic acid by irradiating with UV for 5 min and baking for 15 min in a vacuum oven at 80°C prior to hybridization.

**Nonradioactive oligonucleotide probe selection and labeling.**

The six oligonucleotides ultimately used as probes are summarized in Table 1. Initially, six oligonucleotide probes (I, II, III, IV, A, B) were obtained from Dr. J. van Duin, Leiden University, The Netherlands. Despite systematic and repeated attempts to optimize hybridization conditions for all probes, probes I and II were found to give considerable cross reactivity with group IV phages (FI and SP) at 50°C. Therefore, oligonucleotide sequences for alternate probes I and II were selected by performing a multiple nucleotide sequence alignment analysis using a nucleic acid sequence alignment and analysis software program (MACAW, National Center for Biotechnology
Information, Bethesda, MD). After a multiple alignment against the sequences of MS2, GA, Qβ, and SP phages, oligonucleotides with less homology were selected for probes I and II. Then oligonucleotide analyses were performed for melting temperature (Tm), percent GC content, and most stable hairpin loops and dimers using the computer program Gene Runner (Hastings Software, Inc., N.Y.). Probes III, IV, A, and B which were kindly provided by Dr. J. van Duin, gave acceptable hybridization results with prototype phages. Oligonucleotides were labeled with digoxigenin-dUTP (DIG-dUTP) at the 3'-end by terminal transferase using the Genius 5 Oligonucleotide 3'-End Labeling Kit (Boehringer Mannheim, Indianapolis, IN) according to manufacturer's instructions. After the labeling reaction was completed, ethanol precipitation was performed to remove unincorporated DIG-dUTP from the labeled oligonucleotides, and probes were stored at -20°C for future use.

Hybridization conditions and detection.

The fixed membranes were prehybridized for 2 h at 45°C in prehybridization solution containing 6 x SSC, 5 x Denhardt's solution, 16mM Tri-HCl buffer (pH 8.3), 0.1% SDS and 75μg/ml of salmon sperm DNA. Hybridization was performed in the same solution by adding 5 pmol of DIG-dUTP-labeled probe overnight at 45°C. After hybridization, the
membranes were washed twice in 6 x SSC/0.01% SDS at 37°C for 15 min. Membranes were incubated with an alkaline phosphatase-conjugated anti-DIG antibody at room temperature for 30 min. The unbound antibody conjugate was removed by washing membranes with 100 mM Tris-HCl and 150 mM NaCl, pH 7.

Colorimetric detection was performed by using the substrates of 5-bromo-4-chloro-3-indolyl-phosphate (x-phosphate) and nitroblue-tetrazolium-chloride (NBT) (Boehringer Mannheim, Indianapolis, IN) to visualize DNA-RNA hybrids. Samples containing the target RNAs of the F' RNA coliphages were detected as oligoprobe hybrids by the appearance of a dark-blue color on the membranes after 8-12 hr incubation.

**Data analysis**

Classification results for 203 field isolates of F' RNA coliphages grouped by serotyping and genotyping were compared by two-way analysis of variance (ANOVA), performed using the SAS software (SAS institute Inc., Cary, NC). The p-value was calculated to test for a statistically significant difference between serotyping and genotyping at the 5% level.
RESULTS

Efficiency of plaque transfer/fixation by three denaturing solutions.

An alkaline solution of 0.5 N NaOH has often been used to denature/fix DNA on membranes for plaque hybridization (6). However, to prevent RNA from being hydrolyzed, a 0.05 N NaOH solution was tested in this study to fix/denature RNA on the membranes. The other two denaturing solutions tested were mainly constituted with formaldehyde as described in the Materials and Methods sections. Among the three denaturing solutions, the blotting buffer (7.5 x SSC with 4.6 M formaldehyde) plus heating showed a higher efficiency of denaturation for phages MS2 and GA than the other two denaturing solutions (Figure 1). The signal intensity of the MS2 and GA plaques after hybridization was weak both when 0.05 N NaOH and when 37% formaldehyde were used to fix phage RNA on membranes. For phages Qβ (group III), FI and SP (group IV), the blotting buffer with heating also gave the strongest signal after hybridization with probes III and IV, respectively (figure 2). Formaldehyde, 37%, had the same efficiency of denaturing group IV phages as the
blotting buffer, but less efficiency for denaturing phage Qβ. Clearly, the blotting buffer was the best denaturing solution for denaturation of five prototype F' RNA coliphages tested in this study.

**Efficiency of plaque transfer using five filters.**

Five commercially available membranes were examined for their efficiencies of plaque transfer using the blotting buffer at 65°C to denature F' coliphage RNA. The results of experiments in which the five filters were tested for the five prototype phages using probes against groups I and II phages are summarized in figure 2. Membranes A and B showed a non-specific hybridization reaction, in which a heterologous phage of group II (GA) was moderately recognized by probe I. Membranes C, D, and E gave specific and similar efficiencies in plaque transfer and detection of prototype group I phage MS2 by probe I. All five filters also gave specific and similar efficiencies of plaque transfer and detection of prototype group II phage GA by hybridization with probe II (Fig. 3). Weak signals of plaque hybridization were observed when membrane A was used to transfer/fix Qβ (group III), FI (group IV), and SP (group IV) phages after they were hybridized with probes III and IV, respectively (figure 4). Membrane B gave similar hybridization signals for group IV phages compared with
membranes C, D, and E, but stronger background also was found. Filters C, D, and E gave similar reactions of phages Qβ, FI, and SP with their respective probes. Our observations indicate that membranes C, D, and E are capable of transferring a sufficient amount of F' RNA coliphages to perform reliable and specific plaque hybridization with each probe.

**Hybridization conditions.**

A hybridization solution containing 5 x SSC, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulfate, and 1.0% (w/v) blocking reagent [recommended by The User's Guide for Filter Hybridization; Boehringer Mannheim, Indianapolis, IN] yielded poor sensitivity for this study (data not shown). After colorimetric detection, the strength of the signal from hybridized plaques was weak. However, this problem was solved by using the hybridization solution (6 x SSC, 16 mM Tris-HCl, 0.1% SDS, and 75 μg/ml) with 5 x Denhardt solution (0.1% Ficoll, 0.1% bovine serum albumin fraction V, and 0.1% polyvinylpyrrolidone). As shown by the hybridization results in figures 1 to 4, adequate sensitivity of hybrid detection can be achieved using this hybridization solution.

Hybridization is usually performed at 5-10°C below melting temperature (Tm) (51). The Tm of our
oligonucleotides ranged from 58 to 74°C (Table 1). A high background was observed when the hybridization reaction was carried out at 50°C and 55°C although there was no non-specific hybridization between the probes and targets (figure 5). Two different temperatures, 37°C and 45°C, were then tested in order to optimize the hybridization reactions. At both temperatures, background could be minimized, but at 37°C, non-specific binding occasionally occurred during a long period of color development, especially in probes I and II. The effects of different hybridization temperatures for each probe are summarized in Table 5. Based on these results, the hybridization temperature was set at 45°C to obtain an optimum hybridization condition.

**Specificity of probes.**

Five prototype F\* RNA coliphages and five serotyped isolates from field samples (P1 and P2 from piglet feces, W1 and W2 from surface water, and E1 from secondary effluent) were used simultaneously to test the specificity of each probe. All six probes hybridized only with their specific prototype and field strain group members, and no cross reactions with heterologous groups occurred (figure 3A and 3B). Therefore, all six probes were highly specific in distinguishing each serogroup of F\* RNA coliphages.
For the classification of each environmental isolate, W1 was classified into group I due to positive hybridization reactions with both probes I and A. Based on their positive reactions with homologous probes, isolates E1, W2, P1, and P2 were likewise classified into groups II for E1, III for W2, and IV for P1 and P2 (Figure 3-B). These genotypic identification are the same as those determined by serotyping.

Phage isolate purification and mixed phage plaques

Initial phage isolates from field samples were picked and resuspended from plaques without any purification prior to serotyping and genotyping. If isolates gave an ambiguous result (cross reactions with two or more antisera or probes) by either serotyping or genotyping, these initial isolates were purified and tested again. As shown in figure 7, a total of 25 initial isolates from chicken feces gave clear classification results by plaque hybridization, with the exception of isolate C21. Nine of these initial isolates from chicken feces were classified conclusively as group I, and 16 were classified conclusively as group IV. Each of these initial isolates only reacted with probe I or probe IV. These genotyped classifications were the same as the results by serotyping. It should be noted from figure 7 that isolate C21, which reacted strongly with probe I, also
gave a weak cross hybridization with probe IV. This phenomenon is seen clearly in figure 8 in which are shown the results for twelve isolates from chick feces hybridized with probes I and IV. It is interesting to observe that all 12 isolates formed RNA-probe IV hybrids only at the periphery of the plaques, thereby forming an open circle. These same plaques reacted at their interiors to form RNA-probe I hybrids that were a solid circle. One prototype F' RNA coliphage, ID2, also reacted with probe IV at the perimeter of the ID2 plaque and with probe I in the interior of the plaque.

In order to provide further evidence that ID2-like intermediate phages uniquely produce the characteristic hybridization reactions observed with group I and IV probes, dual mixtures of group I phage MS2 and the prototypes of the other groups were tested for their reactivity with group I probe and the probe of their group. When three mixtures of prototype phages (MS2+GA, MS2+Qβ, and MS2+FI) were tested, the results showed that they were detected only by probe I (Fig. 8). We speculated that 13 isolates, including C21, may be ID2-type intermediates because they produced the same hybridization pattern as did ID2. However, upon further testing after purification, they were confirmed to be mixed-populations of groups I and IV F' RNA coliphages.
Serotyping 203 field isolates.

Four kinds of environmental samples, sewage, oysters, surface waters, and feces, were processed by the methods described in the Material and Methods section for the isolation of F' RNA coliphages. Between three and sixty-two coliphages were isolated from each kind of sample, with 203 total isolates (Table 5).

Five antisera, αMS2 (I), αGA (II), αQβ (III), αFI (IV), and αSP (IV), were used to serotype coliphage isolates by the spot neutralization test on host lawns containing one of the five antisera. Isolates were classified into a serogroup on the basis of neutralization with only one antiserum. When isolates could be neutralized by two or more antisera, they were further purified three times and then spot neutralization tested again. Isolates were considered unclassified if they were not neutralized by any of the five antisera. As shown in Table 5, all isolates reacted with antisera and could be classified, with the exception of 1 isolate from surface waters. The majority of field isolates from raw and treated sewage, oysters, surface waters and porcine feces were unequivocally identified as group II phages. However, the phage isolates from chicken feces belonged to either group I or group IV. Thirty-seven isolates from raw sewage, secondary effluent, oysters, and
surface waters gave ambiguous results because they were neutralized completely by anti-GA (group II) serum and neutralized partially by anti-MS2 (group I) serum.

**Genotyping 203 field isolates**

Six phage oligo-probes, group I, group II, group III, group IV, A for both groups I and II, and B for both groups III and IV phages, were used to classify the same field isolates that had been serologically tested. Isolates were classified into phage groups based on hybridization with homologous probes. For example, a group I phage like MS2 would hybridize only with probes I and A, while a group IV phage like FI would hybridize only with probes IV and B. As shown in Table 5, most isolates reacted with an individual group probe (I, II, III, IV) and either with probe A (group I and II) or B (group III and IV) and consistent with the results obtained by serum neutralization tests. The majority of isolates from raw sewage, secondary effluent, and porcine feces were classified by genotyping as group II phages. The F' RNA coliphages from chicken feces were classified definitively by genotyping into groups I and IV, which is consistent with the results of serotyping.

**Comparison of genotyping and serotyping for 203 field isolates**

The ten isolates from raw sewage were classified by
antiserum neutralization into group II although two of these isolates also were partially neutralized by anti-MS2 (group I) serum. The same 10 phage isolates were also classified as group A by hybridization with probe A but not with probe B (Table 6). Based on hybridization testing with probes I-IV, seven of these 10 isolates were classified as group II, but three of these isolates were not identified by hybridization with these probes. Similar results were also observed for isolates from secondary effluent and oyster samples. The majority of these isolates were genotyped as group II but two of 10 isolates from secondary effluent and one of 31 isolates from oysters were not classifiable by using probes I through IV. The classification of isolates by serotyping and by genotyping in porcine and chicken feces yielded identical results in that seventeen and twelve isolates from porcine feces were group II and group IV, respectively, and thirty and thirty-two isolates from chicken feces were classified as group I and group IV, respectively. For isolates from surface waters, fifty-seven could be serotyped and genotyped by hybridization with probes I through IV. The majority of those isolates were group II phages. However, one isolate was classified as group II by serotyping, but was untypable by hybridization with any probe.

Table 7 shows that overall group identification of F
RNA coliphages was 99.5% by serotyping, 96.6% by genotyping with probes I through IV, and 100% by genotyping with probes A and B. The total number of group II phages identifiable through serotyping (116) was higher than through genotyping using probes I through IV (109), and the number of untypable isolates by serotyping (1) was fewer than that by hybridization with probes I through IV (7). However, there was no significant difference between genotyping and serotyping (p<0.05, ANOVA).
Table 4. Nucleotide sequences of oligomers used for probing F⁺ RNA coliphages

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<td>GGCATAGATTCTCCTCTGTAATGCG</td>
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<td>A</td>
<td>AGCCCGATCTATTTTATTGCTTCCAAC</td>
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<td>MS2&amp;GA</td>
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<td>B</td>
<td>TAATTTTGCCATGATCGAATTCAGCCAAAC</td>
<td>Non-translated region &amp; coat proteind</td>
<td>30</td>
<td>74.4</td>
<td>Qβ&amp;SP</td>
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</table>

a: probe I sequence extends from nucleotides 1248-1273 in MS2 phage
   probe II sequence extends from nucleotides 431-457 in GA phage
   probe III sequence extends from nucleotides 27-51 in Qβ phage
   probe IV sequence extends from nucleotides 35-59 in SP phage
   probe A sequence extends from nucleotides 2340-2369 in MS2 phage and from nucleotides 2345-2375 in GA phage
   probe B sequence extends from nucleotides 1327-1356 in Qβ phage and from nucleotides 1409-1438 in SP phage

b: Tm was calculated by the computer program Gene Runner, Hastings Software, Inc., N.Y.

c: five nucleotides locate at maturation protein region

d: twelve nucleotides locate at coat protein region in Qβ phage and 11 nucleotides locates at coat protein region in SP phage.
Table 5. The results of plaque hybridization tested with 4 oligoprobes at different temperatures

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<td>55</td>
<td>High</td>
<td>High</td>
<td>Medium</td>
</tr>
<tr>
<td>IV</td>
<td>37</td>
<td>Medium</td>
<td>Medium</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>High</td>
<td>High</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>High</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>High</td>
<td>High</td>
<td>Medium</td>
</tr>
</tbody>
</table>

*: Sometimes non-specific hybridization was found with a long period (>12hr) of colorimetric detection.
Table 6. Classification of 203 F\(^+\) RNA Coliphages from Environmental Samples by Serotyping and Genotyping

<table>
<thead>
<tr>
<th>Sources</th>
<th>Total isolates</th>
<th>Neutralized by Antisera against</th>
<th>Hybridized by Oligo-probe of</th>
<th>Hybridized by Oligo-probe of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MS2 GA Q(\beta) SP&amp;P FI GA(^a) MS2 Un(^b) known</td>
<td>I  II  III  IV Un-known</td>
<td>A  B  Un-known</td>
</tr>
<tr>
<td>Raw Sewage(^c)</td>
<td>10</td>
<td>0 8 0 0 2 0</td>
<td>0 7 0 0 3</td>
<td>10 0 0</td>
</tr>
<tr>
<td>2(^c) Effluent</td>
<td>12</td>
<td>0 8 0 0 4 0</td>
<td>0 10 0 0 2</td>
<td>12 0 0</td>
</tr>
<tr>
<td>Oysters(^d)</td>
<td>32</td>
<td>0 24 1 0 7 0</td>
<td>0 30 1 0 1</td>
<td>31 1 0</td>
</tr>
<tr>
<td>Surface Water(^d)</td>
<td>58</td>
<td>7 22 2 2 24 1</td>
<td>7 45 3 2 1</td>
<td>53 5 0</td>
</tr>
<tr>
<td>Piglet(^e)</td>
<td>26</td>
<td>0 14 0 12 0 0</td>
<td>0 14 0 12 0</td>
<td>14 12 0</td>
</tr>
<tr>
<td>Pig(^e)</td>
<td>3</td>
<td>0 3 0 0 0 0</td>
<td>0 3 0 0 0</td>
<td>3 0 0</td>
</tr>
<tr>
<td>Chicken(^f)</td>
<td>62</td>
<td>30 0 32 - 0</td>
<td>30 0 32 0</td>
<td>30 32 0</td>
</tr>
<tr>
<td>Total</td>
<td>203</td>
<td>37 79 3 46 37 1</td>
<td>37 109 4 46 7</td>
<td>153 50 0</td>
</tr>
</tbody>
</table>

\(^a\): isolates were neutralized completely by anti-GA serum and also neutralized significantly by anti-MS2 serum (by spot neutralization test the diameter of spots with anti-MS2 serum was 20% the size of those from plates less without antisera)

\(^b\): no neutralization or hybridization

\(^c\): from Calico Creek, NC

\(^d\): from Calico Creek, NC and New Jersey coastal waters

\(^e\): from swine lagoon, Clinton, NC

\(^f\): from 1993 N.C. State Fair, chickens from throughout the state
Table 7. Summary classification of 203 coliphage isolates by serotyping and genotyping

<table>
<thead>
<tr>
<th>Phage typing by</th>
<th>Type of coliphage</th>
<th>Un-(^a)</th>
<th>Total identified</th>
<th>Total % of classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
</tr>
<tr>
<td>Antisera</td>
<td>37</td>
<td>116(^b)</td>
<td>3</td>
<td>46</td>
</tr>
<tr>
<td>Probes I-IV</td>
<td>37</td>
<td>109</td>
<td>4</td>
<td>46</td>
</tr>
<tr>
<td>Probes A&amp;B</td>
<td>154</td>
<td>49</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\): isolates were not neutralized or hybridized by antisera or probes

\(^b\): total identified isolates of group II were calculated by combining isolates neutralized through anti-GA serum with that through anti-GA/MS2 sera.
Figure 1. Plaque hybridization of F' RNA coliphages fixed by 3 denaturing methods.
Panel A: membranes fixed in 0.05 N NaOH for 1 min and neutralized by 0.1 M sodium acetate for 1 min; panel B: membranes fixed by 37% formaldehyde for 2 min; panel C: membranes fixed by blotting buffer (7.5 x SSC with 4.6 M formaldehyde) for 15 min at 65°C. Hybridization with probe I (left column) and with probe II (right column) are shown.
Figure 2. Plaque hybridization of F' RNA coliphages fixed by 3 denaturing methods.

Panel A: membranes fixed in 0.05 N NaOH for 1 min and neutralized by 0.1 M sodium acetate for 1 min; panel B: membranes fixed by 37% formaldehyde for 2 min; panel C: membranes fixed by blotting buffer (7.5 x SSC with 4.6 M formaldehyde) for 15 min at 65°C. Hybridization with probe III (left column) and with probe IV (right column) are shown.
Figure 3. Plaque hybridization of 5 F' RNA coliphages in 5 filters tested with probes I and II. Zeta-probe (A), Biotrans (B), Biodyne (C), nylon membrane (D), and GeneScreen (E) are shown. Membranes were hybridized with probe I (left column) and with probe II (right column).
Figure 4. Plaque hybridization of 5 F' RNA coliphages in 5 filters tested with probes III and IV. Zeta-probe (A), Biotrans (B), Biodyne (C), nylon membrane (D), and GeneScreen (E) are shown. Membranes were hybridized with probe III (left column) and with probe IV (right column).
Figure 5. Plaque hybridization of 5 prototype F' RNA coliphages tested with probe IV at different temperatures
Figure 6. Plaque hybridization for determining the specificity of 6 probes, and for classifying 5 isolates from environmental samples. Five prototype F' RNA coliphages (MS2, GA, QB, FI, and SP) are shown on the membranes in left column and isolates P1 and P2 from piglet feces, W1 and W2 from surface water, and E1 from 2° effluent are shown on the membranes in right column. Each 2 membranes in each row from row 1 to row 6 was hybridized with probes I, II, III, IV, A, and B, respectively.
Figure 7. Plaque hybridization for the identification of 25 F' RNA coliphage isolates from chicken feces. Positive controls consisting of 5 prototype F' RNA coliphages (MS2, GA, Qβ, FI and ID2) are shown on the top row, and the prototype phage (SP) is shown in the bottom row. Twenty-five isolates (C1-C25) are arranged on the membranes in the order illustrated by the key.
Figure 8. Plaque hybridization of 12 F' RNA coliphage isolates from chicken feces by oligoprobes I and IV. Positive controls consisting of 5 prototype coliphages (MS2, GA, Qβ, FI and ID2) are shown in row 1. Mixed prototype F' RNA coliphages (MS2+GA, MS2+Qβ and MS2+FI) as positive controls are shown on the bottom row. Isolates C33-C50 are shown in the key.
DISCUSSION

Nucleic acid hybridization techniques have been successfully used to classify a variety of microorganisms, including *E. coli* strains (12, 52, 61), rotaviruses (24, 53), hepatitis C virus (56), and herpes simplex virus (37). These hybridization techniques have many advantages over neutralization by antisera for identification and differentiation of microorganisms. However, cross reactions with unrelated phages or other organisms may occur when oligoprobes with common sequences are used for hybridization reactions (1). In developing an oligoprobe hybridization method to group F' RNA coliphages, a number of key technical issues had to be successfully addressed. These were: (1) development of simple and efficient method to isolate target phages for subsequent identification, (2) selection of specific oligoprobes that provide correct identification and (3) development of optimized hybridization reagents and reaction conditions, including choice of blotting membranes, identification of appropriate denaturant and denaturing conditions for transferred plaques and optimal hybridization reaction conditions to achieve specificity and sensitivity.
Efficient isolation of F' RNA coliphages was achieved by using a specific host cell (Salmonella typhimurium, WG49) and by screening field isolates through an RNase test to identify those that contained RNA. The S. typhimurium WG49 host strain has the advantage of yielding a high proportion of F' RNA coliphages and a low proportion of other phages (20). With this approach, more than 90% of the phage isolates were identified as F' RNA coliphages.

Five commercial nylon membranes were tested in this study to determine which membranes are efficient for transfer of F' RNA coliphages from zones of lysis on plates. Each membrane may have its optimal conditions for slot and dot blot, southern transfer, and northern transfer based on manufacture's instruction. However, the results of comparative studies showed that each membrane gave a different sensitivity and specificity for plaque (lysis zone) hybridization of F' RNA coliphages with each probe. Three of five membranes (GeneScreen, nylon membranes by Boehringer Mannheim, and Biodyne) were able to give high efficiency for plaque transfer in this study. It is surprising that two of five membranes (Biontrans and Zeta-probe) gave a non-specific hybridization (Figure 3). These results demonstrate that selection of appropriate membranes is a key issue for plaque hybridization of F' RNA coliphage.
Typically RNA is denatured by formaldehyde with heating at 68°C for 15 minutes prior to dot or slot blot (28). In this study, the blotting buffer was adapted to the denaturation of RNA directly on membranes. Such blotting buffer modification for RNA denaturation in plaque hybridization has not been previously reported. This denaturing method allowed us to prepare the plaque-transferred environmental isolates for efficient filter hybridization. In fact, plaques of environmental isolates exhibited high sensitivity in hybridization reactions even after being transferred to nylon membrane filters three times in succession.

Hybridization conditions were optimized to guarantee probe specificity and sensitivity. When a high concentration (5 pmol/ml) of probes, an optimal hybridization temperature of 45°C, and a concentrated washing solution (6 x SSC with 0.1% SDS) were used, all six probes were highly specific and sensitive for the detection and classification of F' RNA coliphages (Figures 1 through 5).

Field isolates of F' RNA coliphages are normally purified before they can be serotyped by spot-tests (16 and 20). However, in this study, plaque isolates from agar plates were not purified before being serotyped and genotyped because the purification of environmental isolates
is costly and laborious. We hoped that serotyping or genotyping F' RNA coliphage field isolates without purification would be an effective and efficient approach to their classification. It was found that the vast majority of F' RNA coliphage isolates (about 87%) would be correctly and unambiguously identified without prior purification. The remaining 13.5% of the isolates still required purification because they were neutralized or hybridized by two or more antisera or probes, respectively. This approach of attempting classification of field isolates without purification took much less time than the conventional method of purifying all field isolates before serotyping. Serotyping unpurified isolates may cause an underestimation of phages of a particular serotype in that a mixed-population of the same group could be typed as a single isolate. However, this would also occur if isolates were purified. If the mixed-population contained more than one group, it could be unclassifiable by serum neutralization spot-tests. In this case the phages of one group will be neutralized by a homologous antiserum, but those of another group still proliferate to lyse host cells and form a clear spot on the agar medium. In principle, it may be possible to detect partial neutralization on the basis of a reduced size of the lysis zone. However, this approach is unreliable because it is difficult to differentiate the
diameter of lysis spots on host cell lawns from separate plates containing one of the five antisera when isolates are a mixed-population.

The potentially ambiguous reactions of serum neutralization spot tests are avoided by using oligoprobe hybridization to classify unpurified isolates. Hybridization reactions with multiple probes are readily recognized as indicative of a mixed-population. In our experiments, thirteen initial isolates from chicken feces were hybridized with probes I, A, IV, and B. These thirteen isolates were then confirmed through a modified plaque assay to contain a mixed-population of group I and IV phages. One antiserum was added to the top agar of the plaque assay medium to suppress a homologous group of phages and thereby separate another heterologous group of phages. Plaques from plates with each antiserum can be picked, spotted and oligoprobed for group identification. In this manner initially unpurified isolates from environmental samples are reliably classifiable by hybridization.

It is noteworthy that seventeen isolates from porcine feces were classified as group II phages, which were not found in any other animal feces. This observation, which is consistent with other reports (22 and 43), suggests that group classification will not always distinguish between human and porcine fecal contamination. Because the dietary
and living conditions of pigs have historically involved sources of human wastes, this may account for the detection of group II phages from pig feces. Once introduced into the pig population these phages may persist because the gastrointestinal physiology and flora of pigs is relatively similar to that of humans.

The overall percentage of F' RNA coliphage classified by serotyping was slightly greater than that by hybridization with probes I through IV, but there was no significant difference between these two methods (p<0.05, ANOVA). However, thirty-seven of 203 isolates were neutralized by both anti-MS2 serum and anti-GA serum. They were classified into group II due to a higher degree of neutralization with anti-GA serum than with anti-MS2 serum. Seven of these thirty-seven isolates could not be genotyped. The morphology of these isolates was very unique in that plaques on WG49 lawns were not identical after five cycles of purification. Several F' RNA coliphages have been reported as serological intermediates between groups I and II and between groups III and IV (14 and 15). Phage JP34 was first classified as a group I phage due to higher neutralization by anti-MS2. In spite of this classification, JP34 was later confirmed as a member of group II by comparing its nucleotide sequences at the 3'-end region to that of other F' RNA coliphages (25). The
nucleotide sequence of JP34 has less than 40% homology with group I but more than 90% homology with group II (3). Moreover, a spontaneous mutant from a group II phage (GA), on which a G²→A transition occurred at the coat protein gene, developed resistance to anti-GA serum and sensitivity to anti-MS2 serum (19). These strains have contributed to the ambiguous results observed through serotyping and such strains may have been detected in this study. However, genotyping through hybridization with oligo-probes would seem to be a useful tool for resolving the problematic serological differences between the group intermediates because specific targets of nucleotide sequences in F' RNA coliphages are be detected.

The total number of group II phages classified through hybridization was seven fewer than through serotyping. We speculate that anti-GA serum may neutralize unrelated serogroup phages or intermediates due to a lack of specificity of the polyclonal antibodies used in the test. Moreover, probe II may not identify all members of group II (SD, TH1, BZ13, KUL, JP34, etc). Among the group II phages, complete nucleotide sequence data are currently available only for phage GA. Probe II was selected from this limited data, and a multiple alignment was performed against the sequences of MS2, Qβ, and SP phages. We are currently investigating the sequence homology of probe II with the
other five prototype members of group II by hybridization studies. Further refinement of the group-specific oligoprobes based on more complete nucleotide sequence information for representative members of each group should result in ever more specific genotyping of the F+ RNA coliphages.
CONCLUSIONS

1. Several brands of positively charged nylon membranes are capable of transferring sufficient amounts of F' RNA coliphages from zones of lysis or plaques to perform nucleic acid hybridization using oligoprobes.

2. The sensitivity of F' RNA coliphage detection by hybridization is best using blotting buffer with heating. Plaques of environmental isolates could be transferred to nylon membrane filters 3 times in succession and still give high sensitivity in hybridization reactions.

3. Serogroup II of F' RNA coliphages predominated in raw and secondary treated sewage and in the oysters and surface waters impacted by discharged secondary effluent.

   - Serogroup IV phages predominated in feces of swine and chickens.

4. Total % identification of F' RNA coliphages by hybridization is somewhat higher than by serotyping: 96.6% by hybridization and 99.5% by serotyping.

   - The results of classification of 203 F' RNA coliphages by serotyping and by genotyping were in close
agreement (95.6% concordance).

- Hybridization helps resolve and correctly classify isolates that are F' RNA coliphage mixtures.

5. Genotyping by oligo-probe hybridization is a practical method for grouping F' RNA coliphages in environmental samples and foods such as shellfish.
References:


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