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Occurrence of and Sequence Variation among F-Specific RNA Bacteriophage Subgroups in Feces and Wastewater of Urban and Animal Origins

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F-specific RNA bacteriophages (FRNAPH) have been widely studied as tools for evaluating fecal or viral pollution in water. It has also been proposed that they can be used to differentiate human from animal fecal contamination. While FRNAPH subgroup I (FRNAPH-I) and FRNAPH-IV are often associated with animal pollution, FRNAPH-II and -III prevail in human wastewater. However, this distribution is not absolute, and variable survival rates in these subgroups lead to misinterpretation of the original distribution. In this context, we studied FRNAPH distribution in urban wastewater and animal feces/wastewater. To increase the specificity, we partially sequenced the genomes of phages of urban and animal origins. The persistence of the genomes and infectivity were also studied, over time in wastewater and during treatment, for each subgroup. FRNAPH-I genome sequences did not show any specific urban or animal clusters to allow development of molecular tools for differentiation. They were the most resistant and as such may be used as fecal or viral indicators. FRNAPH-II's low prevalence and low sequence variability in animal stools, combined with specific clusters formed by urban strains, allowed differentiation between urban and animal pollution by using a specific reverse transcription-PCR (RT-PCR) method. The subgroup's resistance over time was comparable to that of FRNAPH-I, but its surface properties allowed higher elimination rates during activated-sludge treatment. FRNAPH-III's low sequence variability in animal wastewater and specific cluster formation by urban strains also allowed differentiation by using a specific RT-PCR method. Nevertheless, its low resistance restricted it to being used only for recent urban pollution detection. FRNAPH-IV was too rare to be used.

Water contamination by pathogens is a major public health problem. Microorganisms affecting water quality are mainly excreted in feces, and water contamination is caused by discharge of fecal waste of animal origin (e.g., from runoff after rain, farms, and slaughterhouses [SH]) or human origin (e.g., from raw or treated urban wastewater) into the environment. Enteric pathogens include bacteria, protozoa, and enteric viruses (1). The last have been identified in many cases of waterborne and food-borne diseases in developed countries (2).

The virological quality of water is usually indirectly estimated from the number of fecal indicator bacteria, such as *Escherichia coli* and enterococci (3). However, these indicators have serious limitations, especially for the estimation of viral pollution. First, it has been largely demonstrated that viruses are more resistant than bacteria in the environment; therefore viral outbreaks may still be identified even in the absence of indicator bacteria (4, 5). Second, conventional indicators are present in human and animal feces, and they do not provide information about the origin of fecal contamination (6). Yet, this parameter is essential because the reservoir of many waterborne pathogens, especially viruses, is human (7). Finally, the direct estimation of viral pollution (e.g., noroviruses and hepatitis viruses) by molecular tools could be challenging, not only because of the low concentration in the matrix but also because of the difficulty in interpreting positive results without any information about infectivity. In this context, F-specific RNA bacteriophages (FRNAPH) may be useful tools in a number of situations.

FRNAPH are nonenveloped single-stranded RNA viruses, 20 to 30 nm in diameter, belonging to the *Leviviridae* family. They are classified in two genera, *Levivirus* and *Allolevivirus*, each contain-

ing 2 subgroups: FRNAPH-I (MS2-like) and -II (GA-like), which form the genus *Levivirus*, and FRNAPH-III (Qbeta-like) and -IV (SP-like), which form the genus *Allolevivirus* (8). These phages reside in the gut of warm-blooded animals and are characterized by their capacity to infect bacteria producing F pili, like *E. coli*. They get excreted in feces at high frequency and concentrations (9). Considering that they are present in large amounts in wastewater, they may be used as an indicator of viral pollution. These phages can be detected both by rapid culture and by molecular methods (reverse transcription-PCR [RT-PCR]), another advantage compared to pathogenic viruses, which can often only be detected by molecular techniques. FRNAPH are similar in size, shape structure, and genome makeup to the main waterborne pathogenic viruses (e.g., norovirus and hepatitis A virus), and they are often used as surrogates to assess the behavior of enteric viruses in water during treatment (10, 11). Finally, it is assumed that the distribution of the 4 FRNAPH subgroups can be used to dif-

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ferentiate human from animal fecal contamination. Indeed, FRNAPH-I and -IV are generally associated with animal pollution, whereas FRNAPH-II and -III make up the majority in human wastewater (12, 13). For this reason, subgroup distribution has been widely studied to determine the origin of fecal contamination in river water (14, 15), shellfish (16, 17), and sediments (18).

However, there are two main limits to the use of these indicators to track the origin of fecal pollution. First of all, the distribution of phage subgroups is not absolute. (i) FRNAPH-III, generally associated with humans, has been isolated in high proportions in swine wastewater (12, 19), (ii) FRNAPH-I has been detected in municipal wastewater (20), and (iii) even if FRNAPH-II seems to be highly associated with humans (21), it has also been found in animal feces (22). The second limitation is due to the variable survival rates of FRNAPH in the environment. Indeed, FRNAPH-I and -II are less affected by environmental stresses (23–26), thereby being often found in surface waters, regardless of the source of pollution (27–29). As a consequence, this may cause misinterpretation of the subgroup distribution when estimated by infectious-phage isolation.

The aim of this work was to evaluate the potential of FRNAPH as a tool to differentiate human from animal fecal pollution. We explored in particular the possibility of minimizing both drawbacks described above. The first objective was to determine whether genome sequencing within each subgroup could allow better differentiation between animal and human fecal contamination as recently suggested (20, 30). To achieve this, we isolated by culture FRNAPH strains from samples of known origin (animal feces, urban wastewater, and slaughterhouse wastewater). The strains were subjected to genotyping, and the maturation protein gene was sequenced with the aim to design more-specific RT-PCR methods for human and animal phages. The second objective was to determine whether direct genome detection by RT-PCR could reduce the bias caused by differences in the survival rates of each subgroup in the environment. For this purpose, we compared the persistence of infectious phages with that of the genomes of each subgroup over time and assessed the impact of water treatment on these parameters.

MATERIALS AND METHODS

Animal feces samples. Forty-seven fecal samples were collected from different areas in northwestern France from May to October 2014. The samples were stool from cattle ($n = 10$), swine ($n = 8$), sheep ($n = 9$), horses ($n = 10$), chickens ($n = 5$), and ducks/geese ($n = 5$). All samples were collected in sterile polypropylene containers and kept at -20°C during transport.

After thawing, 4 g of stool was mixed with 28 ml of phosphate-buffered saline (PBS) for 3 min in a DT-50 tube with Ultra-Turrax tube drive (IKA-Werke GmbH & Co. KG, Staufen, Germany). Three milliliters of the mixture (stool concentration of 0.125 g/ml) was removed for RNA extraction and treated as described below. A volume of 14.5 ml of PBS–0.3% peptone was added to obtain a final concentration of 0.1% peptone (final stool concentration of 0.083 g/ml). The suspension was mixed for 1 min and kept in ice for 3 h. Finally, 6 ml of chloroform was added to 20 ml of the stool suspension, and after centrifugation ($2,000 \times g$ for 5 min) the supernatant was collected for culture.

Slaughterhouse samples. Four wastewater samples were collected from a cattle slaughterhouse (CattleSH-1 to -4) and from a swine slaughterhouse (SwineSH-1 to -4) located in eastern France. The first site generates a wastewater flow of 450 m^3/day when slaughtering 250 animals. The second site processes 30 to 90 swine per day. Wastewater samples

were collected in sterile glass bottles and kept at 4°C for less than 3 h prior to analysis.

Urban sewage samples. Five urban sewage samples were collected at the entry of a wastewater treatment plant (WWTP-1 to -5) receiving 80,000 m^3/day from an urban area in eastern France, with a population of approximately 260,000. All samples were collected in sterile glass bottles and kept at 4°C for less than 3 h prior to analysis. Another sample was collected from a smaller WWTP located in northwestern France (WWTP-6), receiving wastewater from 8,500 inhabitants. The sample was kept at -20°C during transport.

Finally, in order to isolate the most resistant phages, 2 samples (WWTP-4 and WWTP-5) were stored at 4°C in the dark, in glass bottles, and analyzed over time. Two effluent samples were also collected from the first WWTP, whose processes include pretreatment, primary treatment by sedimentation, secondary treatment by activated sludge, and tertiary treatment for dephosphorylation.

Infectious FRNAPH enumeration and phage plaque isolation. FRNAPH were detected and enumerated by the double-agar-layer technique according to International Organization for Standardization (ISO) standard 10705-1 (31). *Salmonella enterica* serovar Typhimurium WG49 (NCTC 12484) was used as the host strain (32), and kanamycin and nalidixic acid were added to obtain a final antibiotic concentration of 100 $\mu\text{g}/\text{ml}$. Culture was performed directly from 1 ml of several dilutions of wastewater and stool suspensions in 90-mm-diameter petri dishes. For each dilution, 2 assays were performed. Viral concentration was expressed in PFU per milliliter after an 18-h incubation period. For low-concentration samples (WWTP effluents or aged samples), enumeration was performed twice from 5 ml of wastewater in 150-mm-diameter petri dishes to lower the detection limit to 0.1 PFU/ml. Several phage plaques were isolated from the positive samples, collected with tips, and suspended in 1 ml of PBS–5% glycerol. After brief vortexing, the suspension was filtered through sterile Acrodisc syringe filters (pore size, 0.22 μm ; Pall Life Sciences, Ann Arbor, MI). RNA was then extracted as described below.

RNA extraction. To detect genomes in the samples, extraction was performed directly from 1 ml of wastewater (WWTP or SH). For the stool samples, 3 ml of the suspension prepared as previously described (stool concentration of 0.125 g/ml) was mixed with 1 ml of chloroform and centrifuged ($2,000 \times g$ for 5 min). Extraction was then performed from 1 ml of the supernatant. It was carried out by adding 3 ml of Isol-RNA lysis reagent (5 PRIME GmbH, Hilden, Germany) to the samples, followed by gentle agitation for 10 min on a tube roller at 70 rpm (Starlab International GmbH, Hamburg, Germany). After addition of 1 ml of chloroform, the mixture was stirred vigorously by hand for 15 s and centrifuged ($2,000 \times g$ for 2 min) in 15 ml of Phase Lock Gel Heavy (5 PRIME GmbH, Hilden, Germany). RNA was extracted from the supernatant using NucliSens magnetic extraction reagents (bioMérieux, Marcy l'Etoile, France) on NucliSens MiniMAG according to the manufacturer's recommendations. RNA was concentrated in 100 μl of elution buffer and stored at -80°C in 1.5-ml DNA LoBind tubes (Eppendorf, Hamburg, Germany).

RNA from phage plaques was extracted using NucliSens EasyMag (bioMérieux, Marcy l'Etoile, France) from 50 μl of phage suspension, eluted in 100 μl of buffer, and stored at -80°C .

Real-time RT-PCR. For the direct detection of genomes, RNA suspensions were subjected to the RT-PCR method proposed by Wolf et al. (22) with some modifications. Separate reactions were carried out for each subgroup. Reverse transcription was performed using SuperScript III reverse transcriptase (Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations from 7.5 μl of RNA using 20 pmol of reverse primer, 100 U of SuperScript III, and 20 U of RNasin (Promega, Madison, WI) in a 20- μl reaction volume. PCR was then carried out from 5 μl of cDNA with TaqMan universal PCR master mix (Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations in a 25- μl reaction volume, with reverse and forward primers at a concentration of 1 μM and the probe at a concentration of 0.3 μM . PCR amplifica-

TABLE 1 Primer sequences used for sequencing reactions

Target virus	Primer sequence (5'–3')	Sense	Position	Product length (bp)
FRNAPH-I	GGG GTC CTG CTC AAC TTC CT	+	17–36 ^b	1,407
	ACC CCG TTA GCG AAG TTG CT ^a	–	1404–1423 ^b	
FRNAPH-II	CAT GCC GTT AGG TTT AGN TGA	+	89–109 ^c	1,280
	GTA CCG CCA TTA TCG ACG AG	–	1349–1368 ^c	
FRNAPH-III	GTG TCC GAY TGG AAG GAR CT	+	941–960 ^d	405
	CAT GAT CDA ATT GAC CCA AWG	–	1325–1345 ^d	

^a Primer selected from a previous study (33).^b Genome location of primers based on GenBank accession number NC_001417.2, MS2 phage.^c Genome location of primers based on GenBank accession number NC_001426.1, GA phage.^d Genome location of primers based on GenBank accession number NC_001890.1, Qbeta phage.

tion was performed at 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C on a StepOne Plus real-time PCR system (Life Technologies, Carlsbad, CA).

Infectious phage genotyping was performed using phage plaque RNA extracted by a 1-step multiplex quantitative RT-PCR using the primers and probes designed by Wolf et al. (22). The reaction was performed with the RNA UltraSense one-step quantitative RT-PCR system (Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations from 2 µl of RNA with 20 U of RNasin (Promega, Madison, WI), each primer at a concentration of 0.3 µM, and the probes at a concentration of 0.15 µM in 20-µl reaction volume. The reaction was carried out on a StepOne Plus real-time PCR system (Life Technologies, Carlsbad, CA) at 55°C for 60 min (reverse transcription) and 95°C for 5 min (hot start), followed by 40 cycles of 15 s at 95°C (denaturation) and 40 s at 58°C (annealing/extension). The primers and probes proposed by Ogorzaly and Gantzer (33) were also tested for the detection of some phage strains under the same conditions.

FRNAPH sequencing. Amplification was performed with a OneStep RT-PCR kit (Qiagen, Hilden, Germany) on RNA extracted from phage plaques, according to the manufacturer's recommendations. For each subgroup, primers were designed based on consensus sequences retrieved in the GenBank database, National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/GenBank/index.html>). Alignments and consensus sequence determination were performed using Seaview software v.4 (34), and designs were performed with Primer3Plus software (35). Percent sequence similarity was calculated using the ClustalW2 program of the European Bioinformatics Institute (EBI) of the EMBL (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

For FRNAPH-I and -II, the amplified fragment was about 1,300 to 1,400 nucleotides in length, including the entire maturation protein gene (Table 1). For FRNAPH-III, the size of the amplified fragment located on the maturation protein gene was about 400 nucleotides. The amplified RT-PCR products were purified and sequenced by a sequencing service provider (Beckman Coulter Genomics, Takeley, United Kingdom). Nucleotide sequences were aligned using Seaview software v.4 (34), and phylogenetic trees were generated using BioNJ analysis, with distance estimations based on the Kimura two-parameter equation (K2P). Confidence values of branches were calculated by bootstrap analysis using 100 replications. Designs of trees were then performed using Figtree software v.1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Corresponding sequences of FRNAPH available in GenBank were added to the trees. Strains and accession numbers for FRNAPH-I are as follows: 253A, HQ332484.1; 862A, HQ332489.1; DL1, EF107159.1; DL16, EF108464.1; DL52, JQ966307.1; DL54, JQ966308.1; JP501, AF227251.1; L1.G10.6, FJ799473.1; L2.G10.13, FJ799546.1; L3.G40.5, FJ799645.1; M12, AF195778.1; MS2, NC_001417.2; and ST4, EF204940.1. Those for FRNAPH-II are as follows: DL10, FJ483837.1; DL20, FJ483839.1; GA, NC_001426.1; KU1, AF227250.1; and T72, FJ483838.1. Those for FRNAPH-III are as follows: 2010JunDWTP11-13, AB627184.1; 1FR,

JF719735.1; 2FR, JF719736.1; 3FR, JF719737.1; AncP1, AB971354.1; BR12, FJ483842.1; BZ1, FJ483844.1; HL4-9, FJ483841.1; MX1, S81548.1; M11, S81550.1; Qbeta, NC_001890.1; Qbeta_3, GQ153930.1; TW18, FJ483840.1; and VK, FJ483843.1.

Statistical analysis. Analysis of covariance (ANCOVA) was used to test the difference in linearity of each genome and infectious-phage persistence. Statistical analyses were performed using StatEL (AD Science, Paris, France). Differences were considered significant when P was <0.05.

RESULTS

FRNAPH in stool samples. FRNAPH concentration was evaluated after culture by plaque assay and direct genome detection in animal feces. Samples from 7 different species ($n = 47$) were analyzed (Table 2). Analysis of individual stool specimens revealed variable incidences of infectious phages depending on the species. Plaque formation was detected in a total of 9 (19%) fecal samples: no FRNAPH were isolated from cattle feces, but at least 1 sample for the other species was positive. Except for cattle samples, prevalence in fecal specimens was between 10% and 40%, with the highest prevalence in duck/goose and chicken specimens. Concentrations of infectious FRNAPH in stool samples ranged from 1.2×10^1 to 1.9×10^3 PFU/g for swine, 1.6×10^4 to 1.9×10^4 PFU/g for sheep, and 5.1×10^1 to 3.0×10^2 PFU/g for ducks/

TABLE 2 Incidence and concentration of infectious FRNAPH and genomes in feces and wastewater

Sample source	Infectivity		Total genome	
	No. of positive samples/no. taken	Concn range ^a	No. of positive samples/no. taken	Concn range ^a
Feces				
Cattle	0/10		2/10	3.9–4.1
Swine	2/8	1.1–3.3	5/8	3.8–5.8
Sheep	2/9	4.2–4.3	5/9	3.7–6.5
Horses	1/10	1.2	1/10	4.8
Chickens	2/5	3.3–5.0	3/5	3.7–8.9
Ducks and geese	2/5	1.7–2.5	3/5	4.0–5.4
Wastewater				
Urban WWTP	6/6	2.3–4.2	6/6	4.9–5.8
CattleSH	4/4	4.7–5.2	4/4	6.4–6.8
SwineSH	4/4	2.4–4.5	4/4	4.7–6.1

^a Concentrations are expressed in log₁₀ PFU/g of stool or log₁₀ PFU/ml of wastewater.

TABLE 3 Distribution of infectious FRNAPH subgroups isolated from feces and wastewater

		No. of plaques classified as:				
Sample source	No. of plaques picked	FRNAPH	FRNAPH-I	FRNAPH-II	FRNAPH-III	FRNAPH-IV
Feces						
Cattle	0					
Swine	14	13	12			1
Sheep	20	20	20			
Horses	3	1	1			
Chickens	24	24	24			
Ducks and geese	25	24	13	11		
Wastewater						
Urban WWTP	115	106		28	77	1
CattleSH	117	103			100	3
SwineSH	58	52	40			12
Total	376	343	110	39	177	17

geese. The highest concentrations were found in chicken manure, with a maximum of 1.1×10^5 PFU/g.

FRNAPH genomes were more often detected in stool samples than infectious phages. Depending on the species, 10% to 62.5% of the samples were positive, with the highest prevalence in swine feces (Table 2). Ten samples contained genomes, while no infectious phages were found (specimens from 2 cattle, 3 swine, 3 sheep, 1 chicken, and 1 duck). The concentration of genomes was higher than that of infectious phages, with a ratio of between 1.1 and $3.9 \log_{10}$. The average concentration for each species ranged from 1.0×10^4 genome copies (gc)/g (cattle) to 2.7×10^8 gc/g (chickens), with the highest concentrations observed in samples with the largest amounts of infectious FRNAPH.

FRNAPH in wastewater. FRNAPH concentration was also evaluated by plaque assay and direct genome detection in urban wastewater and in cattle slaughterhouse (CattleSH) and swine slaughterhouse (SwineSH) wastewater. All 14 samples were positive for infectious phage (Table 2). The average concentration was 5.7×10^3 PFU/ml in urban wastewater, with the lowest concentration in the sample from the small WWTP located in northwestern France. In CattleSH waters, the average concentration was 1.1×10^5 PFU/ml, and it was about 8.6×10^3 PFU/ml in SwineSH waters.

In all the samples, genomes were found in higher concentrations than infectious phages, with ratios of between 1.5 and 2.6 \log_{10} . The average concentration was 3.3×10^5 gc/ml in urban wastewater, 4.3×10^6 gc/ml in CattleSH wastewater, and 5.6×10^5 gc/ml in SwineSH wastewater.

FRNAPH subgroup distribution. Distribution was determined directly in the samples by detection of subgroup genomes and after culture by phage plaque genotyping. Phage plaque distribution in feces and wastewater is shown in Table 3. Among the 376 phage isolates using *S. Typhimurium* WG49 as host (32), 91.2% gave a positive RT-PCR signal for a subgroup ($n = 343$).

The 14 plaques tested from swine feces were typed as 86% ($n = 12$) formed by FRNAPH-I and 7% ($n = 1$) by FRNAPH-IV. As regards sheep and chicken stool specimens, 20 and 24 plaques, respectively, were picked and all were formed by FRNAPH-I. Only 3 phage plaques were isolated from horse feces; 1 was formed by FRNAPH-I, but the other two did not give positive signals for any subgroup. In the 2 duck fecal samples, 25 phage plaques were

typed; the first specimen contained only FRNAPH-I ($n = 8$) and 1 uncharacterized plaque, while the second specimen contained 31% ($n = 5$) FRNAPH-I plaques and 69% ($n = 11$) FRNAPH-II plaques.

One hundred fifteen phages were isolated from the urban wastewater samples. Of the 92% of plaques that were characterized ($n = 106$), 26% were formed by FRNAPH-II ($n = 28$) and 72% by FRNAPH-III ($n = 77$). Among the 117 phages isolated from CattleSH waters, 88% gave positive RT-PCR signals ($n = 103$), among which 100 were FRNAPH-III and 3 FRNAPH-IV. Finally, among the phages isolated from SwineSH waters ($n = 58$), 40 were FRNAPH-I and 12 FRNAPH-IV. All FRNAPH-IV phages were isolated from the same specimen, representing 92% of the phages characterized in this sample.

This distribution was confirmed overall by direct detection of genomes in the samples. Most genomes detected in the stool samples were that of FRNAPH-I, though with some exceptions: 100% of the genomes detected in 1 sheep stool sample were that of FRNAPH-III (1.0×10^5 gc/g), but this sample was negative for infectious phages. The FRNAPH-II genome was detected in the duck fecal sample in which were found infectious FRNAPH-II (2.2×10^5 gc/g, representing 93% of the total genome).

The genomes detected in the urban wastewater samples were mostly those of FRNAPH-II (6.0×10^4 to 2.2×10^5 gc/ml, representing 30% to 61% of the total number of genomes) and those of FRNAPH-III (2.9×10^4 to 4.7×10^5 gc/ml, representing 30% to 67% of the total number of genomes). FRNAPH-I and -IV genomes were detected at lower concentrations (respectively, 7.8×10^3 and 4.1×10^3 gc/ml on average), representing less than 6% of all the genomes detected. With regard to SwineSH wastewater, the FRNAPH-I genome was detected in all the samples (5.1×10^4 to 1.3×10^6 gc/ml). The FRNAPH-IV genome was detected in 1 sample, representing 6% of the total genomes detected in this specimen. Finally, in all CattleSH wastewater samples, the FRNAPH-III genome prevailed (>99%), with an average concentration of 4.3×10^6 gc/ml. The FRNAPH-I genome was found in 3 samples (1.1×10^3 to 1.8×10^4 gc/ml), the FRNAPH-II genome was found in 2 samples (1.5×10^3 to 2.0×10^3 gc/ml), and the FRNAPH-IV genome was found in 1 sample (9.2×10^3 gc/ml).

The presence and isolation of FRNAPH-II and -III strains of animal origin (ducks and cattle) seem particularly interesting for

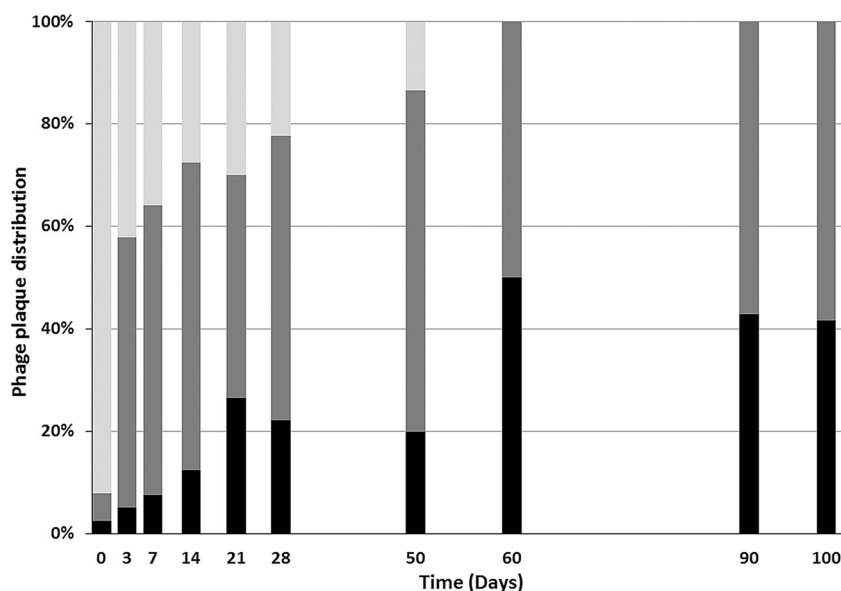


FIG 1 Infectious FRNAPH-I (black), FRNAPH-II (dark gray), and FRNAPH-III (light gray) distribution in urban wastewater. Between 16 and 24 plaques were isolated and typed for each analysis.

evaluating the potential of FRNAPH to track the origin of fecal pollution. These subgroups, generally associated with human pollution, were sequenced and compared with strains isolated from urban wastewater. Conversely, no FRNAPH-I strains were isolated from the raw WWTP samples.

Genome and infectious FRNAPH stability in urban wastewater. The stability of FRNAPH (genomes and infectious phages) was evaluated in wastewater over time and after wastewater treatment. Two raw sewage samples (WWTP-4 and WWTP-5) were analyzed over time. On the first day, infectious FRNAPH concentrations were, respectively, 3.8×10^3 and 8.0×10^3 PFU/ml. Infectious FRNAPH subgroup distribution over time is shown in Fig. 1. Initially, FRNAPH-III prevailed (>90%) in raw wastewater. Its proportion decreased steadily over time and was less than 50% after 3 days. The FRNAPH-II proportion was hidden during the first days by the large number of FRNAPH-III phages, but it was between one- and two-thirds of the total number of infectious FRNAPH after 3 days. Finally, the proportion of FRNAPH-I phages was low during the first days, but it increased steadily to one-half of the infectious phages after 60 days.

While infectivity decreased significantly (time required for 90% loss of infectivity [T_{90}] estimated at day 21.7), genomes were overall more persistent over time (Fig. 2). The FRNAPH-IV genome seemed to be the least resistant, but the rapid achievement of quantification limits due to low concentrations in the original samples prevented a trend from being accurately determined. The genome of FRNAPH-I turned out to be the most resistant under these conditions (decrease of $<1 \log_{10}$ after 100 days), and a significant but low difference between its persistence and that of FRNAPH-II was observed (P value = 0.027, ANCOVA). No marked difference between the persistence of the FRNAPH-II genome and that of FRNAPH-III was observed during the first 60 days (P value = 0.69, ANCOVA). The genomic search approach thus appears more reliable to estimate subgroup distribution, especially in the case of an aged fecal contamination, thereby reducing the survival bias.

Urban WWTP effluents ($n = 2$) were analyzed to estimate the impact of water treatment on infectious FRNAPH and genome concentration. The average concentration of infectious FRNAPH in effluents was found to be 33.2 PFU/ml. Among the plaques typed ($n = 28$), 86% were formed by FRNAPH-I and the rest by FRNAPH-II. The genome of FRNAPH-I was detected at concentrations up to 1.1×10^3 gc/ml. Genomes of other subgroups were not detected, mainly because of the low volume analyzed. During wastewater treatment, the infectivity decreased by 2.3 \log_{10} units and the genome copies decreased by 1.0 \log_{10} unit for FRNAPH-I. As FRNAPH-II and -III genome concentrations in effluents were below the detection limit, losses during water treatment were, respectively, at least 2.0 \log_{10} and 1.9 \log_{10} .

The change in the FRNAPH subgroup distribution over time in urban wastewater and the analysis of effluents allowed FRNAPH-I strains of urban origin to be isolated; these were compared with animal strains by sequencing.

FRNAPH sequencing. Sequencing was performed on FRNAPH isolated by culture from samples of different origins. A two-phase strategy was used. First, we sequenced a representative panel of the supposed animal subgroup (FRNAPH-I) isolated from animal samples and the supposed human subgroups (FRNAPH-II and -III) isolated from urban wastewater. Then, we sequenced most FRNAPH-II and -III strains isolated from animal samples on the one hand and most FRNAPH-I strains isolated from urban samples on the other hand. Altogether, we sequenced the entire maturation protein genes of 158 FRNAPH-I and 70 FRNAPH-II strains, as well as part of those of 92 FRNAPH-III strains.

FRNAPH-I strains of animal origin were isolated from feces or slaughterhouse (SH) waters. Group formation during sequence comparison appeared to be sample dependent, and each stool sample formed a robust grouping (Fig. 3). In each stool sample, FRNAPH-I sequences presented a high degree of similarity (>98.6%). However, no relationship between sequences isolated from different animals of the same species was observed. For example, the percentage of identity between the

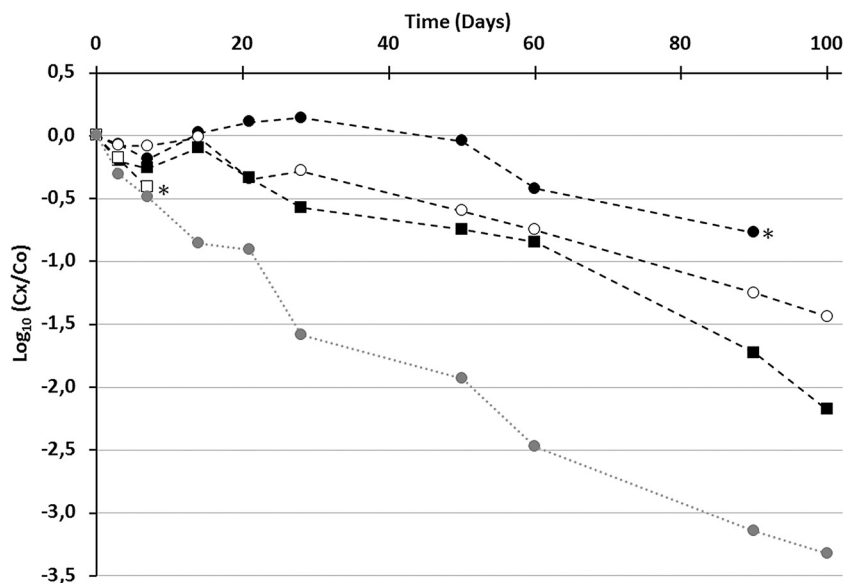


FIG 2 Persistence of FRNAPH-I (black circles), FRNAPH-II (white circles), FRNAPH-III (black squares), and FRNAPH-IV (white squares) genomes, as well as of infectious FRNAPH (gray circles) in urban wastewater kept at 4°C in the dark. *, quantification limit; Cx, concentration at the indicated time; Co, initial concentration.

2 sheep samples was only $90.7\% \pm 0.3\%$; it was $92.8\% \pm 0.4\%$ between the 2 chicken samples, and it was $94.5\% \pm 0.3\%$ between the 2 duck samples. FRNAPH-I strains isolated from swine feces were not grouped with any strains from SwineSH wastewater samples, but they were close to isolates from the Chicken-2 sample ($>98.7\%$ similarity). More variability was observed in SwineSH wastewater samples (93.2% similarity in SwineSH-2 and only 90.9% in SwineSH-4). Groupings were again sample dependent, except for 1 strain isolate from SwineSH-2, which was close to the group formed by strains from SwineSH-1 ($98.0\% \pm 0.2\%$ similarity).

FRNAPH-I strains of urban origin were isolated from WWTP samples. They were present in low density in urban wastewater but showed high resistance; therefore urban strains could be isolated from aged samples or effluents. Sequences were spread out over the entire phylogenetic tree obtained with animal FRNAPH-I strains. No relationship appeared between them, and some isolates were close to strains of animal origin. For example, many strains isolated from urban WWTP effluent 1 were close to strains from SwineSH-4, and all grouped with prototype phage MS2. In this cluster, percent sequence variation was only 0.7% .

FRNAPH-II strains of human origin were isolated from urban wastewater. Percent sequence identity between them ranged from 82.3% to 100% . The isolated strains could be grouped into several clusters (Fig. 4). No sample-dependent grouping was observed, and the FRNAPH isolated from WWTP-6 (urban WWTP located in another geographical area) were present in all clusters.

All FRNAPH-II strains isolated from animals were sequenced. They all originated from a duck fecal sample and were grouped into a cluster with strains coming from urban wastewater. Even if urban strains and duck strains were close, with a maximum similarity of 98.7% in this cluster, FRNAPH-II strains from the duck sample formed a separate grouping because all isolates exhibited identical sequences. All previously

mentioned FRNAPH-II strains were detected by using multiplex RT-PCR as proposed by Wolf et al. (22), but the singularity of the duck strains was confirmed by testing the primer and probe design described by Ogorzaly and Gantzer (33). This PCR assay targeted the 3' end of the replicase gene and allowed most urban FRNAPH-II strains to be detected, leaving out strains isolated from duck feces. More precisely, out of the 6 urban strains belonging to the cluster formed with the duck strains, half gave a typical positive RT-PCR signal, while the others were not detected. Out of the 10 strains tested from the T72 cluster, 70% gave a typical positive signal, 1 gave a late signal, and 2 were uncharacterized. Finally, out of the 10 strains tested from other clusters, 100% were detected.

FRNAPH-III strains of human origin were isolated from urban wastewater. Percent sequence identity between them was $85.3\% \pm 14.7\%$. They were grouped into several clusters (Fig. 5), often associated with one or more known strains available in GenBank. One cluster was formed by a group composed of a large number of isolates ($n = 34$), with most strains already published (TW18, 1FR, AncP1, 2FR, 3FR, Qbeta_3, HL4-9, and 2010JunDWTP11-13). The degree of similarity ranged between 90.0% and 100% for these phages.

Most FRNAPH-III strains originating from animals were also sequenced. They were all isolated from CattleSH wastewater. These strains ($n = 42$) formed a robust cluster with low sequence variations (0% to 1.3%), corresponding to a maximum variation of 5 nucleotides. Among them, 15 isolates had identical sequences and were present in all samples. Similarly, 2 other clone sequences were identified, one in 12 isolates and the other in 7 isolates. These were present in 3 of the 4 samples.

A group formed by 4 FRNAPH strains from the urban WWTP samples was close to the cattle cluster. Percent sequence identity with cattle strains was 94.2% for the most genetically distant ones and 95.8% for the closest ones, but the bootstrap value (100) confirmed the strength of the cattle cluster. As observed for FRNAPH-

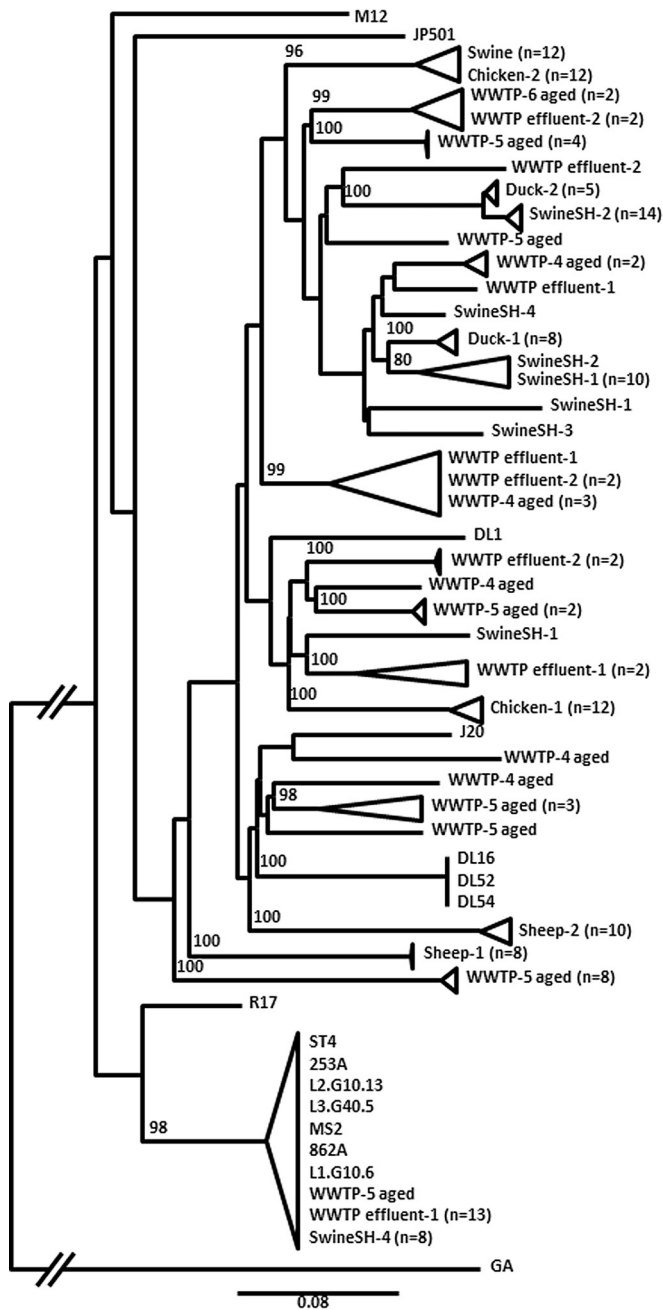


FIG 3 Phylogenetic relationship of FRNAPH-I strains isolated from urban WWTPs and animal slaughterhouse (SH) wastewater or fecal samples based on the maturation protein gene (1,182 nucleotides). Distances were determined by using the K2P model, and the tree was plotted by the BioNJ method. FRNAPH-II GA (NC_001426.1) was used as an outgroup. Only bootstrap values >70% are shown.

II, all FRNAPH-III strains were detected by using the multiplex RT-PCR designed by Wolf et al. (22), but the genetic difference between cattle strains was confirmed by the RT-PCR method proposed by Ogorzaly and Gantzer (33) targeting a coat protein gene region. Using the latter, no FRNAPH strains isolated from the CattleSH samples were characterized, whereas all those tested ($n = 24$), isolated from the urban WWTP samples, including the 4

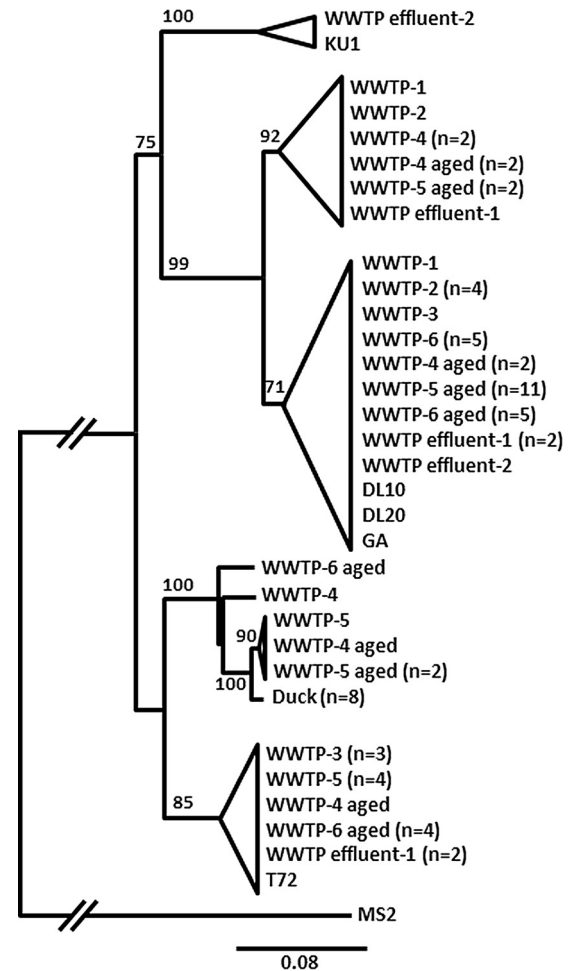


FIG 4 Phylogenetic relationship of FRNAPH-II strains isolated from urban WWTPs and a duck fecal sample based on the maturation protein gene (1,173 nucleotides). Distances were determined by using the K2P model, and the tree was plotted by the BioNJ method. FRNAPH-I MS2 (NC_001417.2) was used as an outgroup. Only bootstrap values >70% are shown.

strains forming the cluster close to the CattleSH group, gave a positive signal for subgroup III.

DISCUSSION

The present work aimed to study F-specific RNA phages (FRNAPH) in stool or wastewater and, in particular, their use as tracers to differentiate urban from animal fecal contamination.

FRNAPH have been used for years as fecal pollution indicators in the environment (36, 37) because of their high prevalence and concentration in stool or wastewater. Our results confirmed this application because FRNAPH were found in very high concentrations in all animal or urban wastewater. Concentrations ranged between 2.3 and 5.2 log₁₀ PFU/ml in wastewater when using the double-agar-layer technique. In comparison, other authors detected infectious FRNAPH concentrations of 2.9 to 4.2 log₁₀ PFU/ml in urban wastewater (15, 38–40), while Schaper et al. (13) observed concentrations ranging from 3.4 to 4.6 log₁₀ PFU/ml in slaughterhouse wastewater. Genome concentrations ranged between 4.7 and 6.8 log₁₀ gc/ml. This type of analysis has been less frequently described in the literature; Wolf et al. (22) observed an

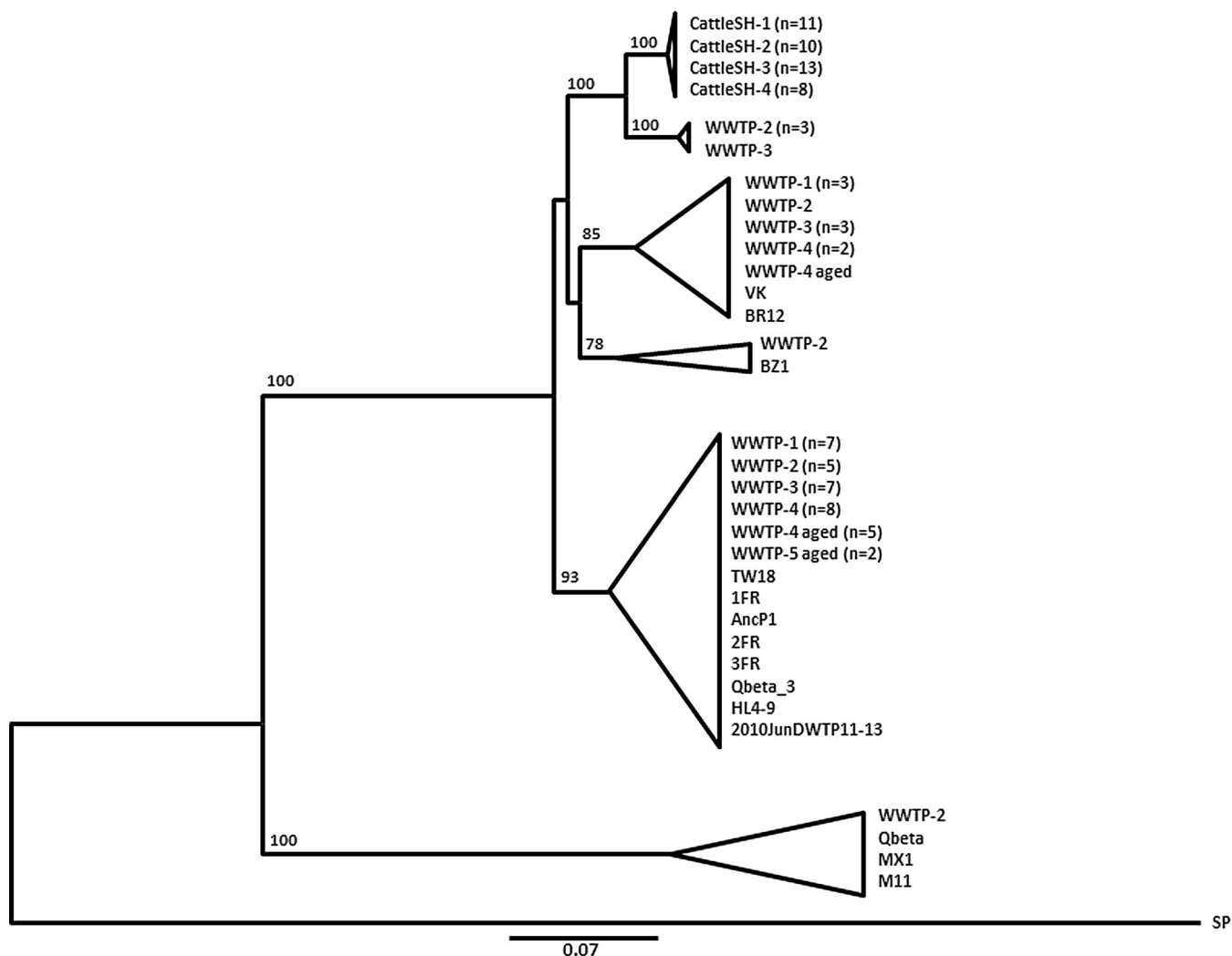


FIG 5 Phylogenetic relationship of FRNAPH-III strains isolated from urban WWTP and cattle slaughterhouse (CattleSH) wastewater based on partial nucleotide sequences of the maturation protein gene (380 nucleotides). Distances were determined by using the K2P model, and the tree was plotted by the BioNJ method. FRNAPH-IV SP (X07489.1) was used as an outgroup. Clusters with bootstrap values >70% are collapsed.

average concentration of $3.5 \log_{10}$ gc/ml in raw urban wastewater, while Hata et al. (40) and Flannery et al. (41) detected only about $2.0 \log_{10}$ gc/ml. In these last two studies, genomes were detected in lower concentration than infectious FRNAPH, which suggests an underestimation of genome concentration. In our samples, the ratio of genomes to infectious phages ranged from 1.5 to $2.6 \log_{10}$.

The prevalence in animal stool samples was found to be low as previously described (12, 42), but, in the case of positivity, concentrations were significant. They ranged between 1.1 and $5.0 \log_{10}$ PFU/g for infectious FRNAPH and between 3.7 and $8.9 \log_{10}$ gc/g for genomes. To sum up, the FRNAPH concentrations obtained in this study are very similar to those reported in the literature.

The main goal of the present work was to investigate the potential of FRNAPH to differentiate between urban and animal pollution. Genotyping before or after culture using the RT-PCR method designed by Wolf et al. (22) clearly confirmed the predominance of FRNAPH-I in stool or wastewater of animal origin and the predominance of FRNAPH-II and -III in raw urban

wastewater, as described in other studies (12, 13, 15, 43, 44). Unfortunately, our results also underlined that FRNAPH genotyping is not sufficient to determine the origin of fecal contamination, since FRNAPH-II and -III were also detected in animal samples. In the same way, the FRNAPH-I genome was detected in urban wastewater. Further knowledge on the variability of sequences within each subgroup according to their animal or urban origin is therefore clearly needed to use FRNAPH as an indicator of the origin of fecal pollution. This was our first objective. We chose to perform sequencing on the maturation protein gene because it shows a high degree of variability (45).

No common point between FRNAPH-I sequences from urban wastewater and those of animal origin was observed. The urban FRNAPH-I strains may have a real human origin, or they may come from variety of animals present in the urban area, but in smaller numbers than the 260,000 inhabitants. In any event, detection of FRNAPH-I by RT-PCR targeting the maturation protein gene does not allow distinguishing urban from animal fecal contamination in the environment. FRNAPH-II was never de-

tected in slaughterhouse wastewater either by direct genomic search or after culture. Nevertheless, 11 FRNAPH-II sequences were isolated in a single duck stool sample. All sequences were identical. In addition, if the primers and probe proposed by Wolf et al. (22) were able to detect the duck isolates, the approach proposed by Ogorzaly and Gantzer (33) was not. Indeed, it appears that, in our experiment, the latter RT-PCR allowed specific detection of urban wastewater FRNAPH-II isolates, even if some isolates were missed.

FRNAPH-III strains were more frequently detected in animal wastewater. Forty-two strains were isolated from cattleSH wastewater using RT-PCR as described by Wolf et al. (22). Again, all these sequences clearly clustered together and did not show any overlapping with the urban strains. The RT-PCR method developed by Ogorzaly and Gantzer (33) did not allow detection of these isolates, while it allowed that of the urban strains. The narrow cluster formed by FRNAPH-III isolated from the cattle slaughterhouse and the presence of identical sequences in the samples collected over more than 3 months (December 2013 to March 2014) suggested a specific signature of this slaughterhouse over time. Based on the 5' end of the maturation protein gene, Stewart et al. (30) also observed genetic variations among FRNAPH-III strains isolated from urban WWTPs and from swine lagoons, with specific strains from each swine lagoon.

Practically speaking, the RT-PCR assays designed by Ogorzaly and Gantzer (33) seem more specific for urban FRNAPH-II and -III than those designed by Wolf et al. (22). Such a high degree of specificity is explained by the use of minor groove binder (MGB) probes (46). These results show the bias related to the choice of primers/probes and their spectral detection, but such a choice may be of benefit depending on the strategy pursued. If detection of all FRNAPH is required, the probes designed by Wolf et al. (22) may be more suitable as they provide better sensitivity (18). Their recent design based on all sequences available in GenBank in 2010 may confer the largest spectrum of detection, while Ogorzaly's and Gantzer's FRNAPH-II and -III probes (33) may be preferable for microbial source tracking.

If specificity is an important point, persistence of FRNAPH in the environment is another one. It has been clearly demonstrated in the literature that FRNAPH subgroups have different survival rates in the environment. Their behavior has already been studied using prototypic phages. Yang and Griffiths (24) observed better persistence of infectious MS2 (FRNAPH-I) and GA (FRNAPH-II) than of Qbeta (FRNAPH-III) under various conditions. Muniesa et al. (23) reached the same conclusion by studying naturally occurring infectious FRNAPH in environmental waters (*in situ* conditions). Nevertheless, levels of persistence of the genomes of the subgroups have never been compared in natural waters. Since it is clearly admitted that the viral genome is more resistant than infectious (47–51), our second objective was to study the inactivation rate of infectious FRNAPH and the persistence of each subgroup genome. Two situations were explored: the persistence over time in raw wastewater at 4°C and the impact of a standard wastewater treatment plant. The parameters used during persistence experiments were set to limit the growth of bacteria and yeasts in samples and to be the most favorable for a long survival of FRNAPH.

Even if the genome of FRNAPH-I fell just above the significance threshold as being more resistant than those of FRNAPH-II and -III, on the whole, genomes were more persistent than infec-

tious, with a loss of less than 1.0 log₁₀ after 60 days, versus 2.5 log₁₀ for infectivity. Infectious FRNAPH-I and -II strains had the best survival rates, with a ratio of 1:1 in wastewater after 60 days. Infectious FRNAPH-III strains were the least resistant because, although they were in the majority in raw wastewater (>90%), they were in the minority after 3 days and they were no longer detected after 50 days of incubation.

During water treatment (activated-sludge process), infectious FRNAPH concentration was reduced by 2.4 log₁₀. In WWTPs with similar water treatment systems, De Luca et al. (52) and Hata et al. (40) observed a decrease in the number of infectious FRNAPH of 3 log₁₀, while Lucena et al. (53) noted a reduction of 2 log₁₀ in winter and 3 log₁₀ in summer. In effluents, infectious FRNAPH-I prevailed, with the presence of a few FRNAPH-II strains, but only the FRNAPH-I genome was detectable. Given that the persistence of FRNAPH-I is close to that of FRNAPH-II, it may be supposed that FRNAPH-II strains were physically eliminated during the process. Other studies demonstrated that infectious FRNAPH-I strains showed higher resistance to water treatment using activated sludge, while FRNAPH-II strains were strongly adsorbed on activated sludge but remained infectious (15, 54), which may be explained by different surface properties depending on the subgroup (55).

In our study, the high prevalence of FRNAPH-III before treatment as opposed to that of FRNAPH-I after treatment indicates that the reduction observed was mainly due to FRNAPH-III disappearance. We may hypothesize that the more FRNAPH-III strains there are in raw water, the higher the estimated removal rate will be, and the more FRNAPH-I strains there are, the lower the removal rate will be. For this reason, it may be interesting to perform genotyping before studying removal treatment when FRNAPH strains are used as surrogates.

To conclude, this study has brought forward important considerations when using FRNAPH as a tool to differentiate urban from animal fecal contamination. FRNAPH-I genome sequences did not show any specific clusters of urban or animal origin and thus cannot allow molecular tools to be designed for differentiation. However, as they were the most resistant in wastewater and during water treatment, they may be used as fecal or viral indicators. FRNAPH-II's low prevalence and low sequence variability in animal stool, combined with specific clusters formed by urban strains, allows it to be related to urban pollution by using specific RT-PCR such as the one developed by Ogorzaly and Gantzer (33). Its resistance over time was comparable to that of FRNAPH-I, but its surface properties may have allowed more elimination during activated-sludge treatment, requiring the use of sensitive methods to detect it in the environment. FRNAPH-III's low sequence variability in animal wastewater, combined with specific clusters formed by urban strains, also allows differentiation between urban and animal pollution by using the RT-PCR approach of Ogorzaly and Gantzer (33). However, its very low resistance over time or during water treatment restricts its use to recent fecal contaminations by raw wastewater. FRNAPH-IV was too rare to be used.

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