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Waterborne Viruses and F-Specific Coliphages in Mixed-Use Watersheds: Microbial Associations, Host Specificities, and Affinities with Environmental/Land Use Factors

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ABSTRACT From the years 2008 to 2014, a total of 1,155 water samples were collected (spring to fall) from 24 surface water sampling sites located in a mixed-used but predominantly agricultural (i.e., dairy livestock production) river basin in eastern Ontario, Canada. Water was analyzed for viable F-specific DNA (F-DNA) and F-specific RNA (F-RNA) (genogroup I [GI] to GIV) coliphage and a suite of molecularly detected viruses (norovirus [GI to GIV], torque teno virus [TTV], rotavirus, kobuvirus, adenovirus, astrovirus, hepatitis A, and hepatitis E). F-DNA and F-RNA coliphage were detected in 33 and 28% of the samples at maximum concentrations of 2,000 and 16,300 PFU · 100 ml^{-1}, respectively. Animal TTV, human TTV, kobuvirus, astrovirus, and norovirus GIII were the most prevalent viruses, found in 23, 20, 13, 12, and 11% of samples, respectively. Viable F-DNA coliphage was found to be a modest positive indicator of molecularly detected TTV. F-RNA coliphage, unlike F-DNA coliphage, was a modest positive predictor of norovirus and rotavirus. There were, however, a number of significant negative associations among F-specific coliphage and viruses. F-DNA coliphage densities of >142 PFU · 100 ml^{-1} delineated conditions when ~95% of water samples contained some type of virus. Kobuvirus was the virus most strongly related to detection of any other virus. Land use had some associations with virus/F-specific coliphage detection, but season and surface water flow were the variables that were most important for broadly delineating detection. Higher relative levels of detection of human viruses and human F-RNA coliphage were associated with higher relative degrees of upstream human land development in a catchment.

IMPORTANCE This study is one of the first, to our knowledge, to evaluate relationships among F-specific coliphages and a large suite of enteric viruses in mixed-use but agriculturally dominated surface waters in Canada. This study suggested that relationships between viable F-specific coliphages and molecularly detected viruses do exist, but they are not always positive. Caution should be employed if viable F-specific coliphages are to be used as indicators of virus presence in surface waters. This study elucidates relative effects of agriculture, wildlife, and human activity on virus and F-specific coliphage detection. Seasonal and meteorological attributes play a strong role in the detection of most virus and F-specific coliphage targets.

KEYWORDS viruses, F-specific coliphages, pollution, indicators, surface water, microbial source tracking

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Viruses such as norovirus (NoV), rotavirus (RV), hepatitis A virus (HAV), hepatitis E virus (HEV), adenovirus (AdV), and astrovirus can be excreted in high numbers in human and/or animal waste (1). These viruses can contaminate surface waters via wastewater discharge, runoff and drainage from farming operations, and leakage from faulty septic systems (2–8). With ever-increasing anthropogenic stresses and pressures on surface water resources, identifying the driving factors that govern fecal pollution in open watershed systems and implementing management practices/strategies designed to ultimately reduce infection risks are becoming more crucial (9, 10). Important in this regard is the determination of the source of fecal pollution via biomarkers and host-specific pathogens (e.g., human, wildlife, and livestock), the seasonality of pathogen occurrence, and waterborne pathogen linkages to environmental/land use factors (11–14).

As it is not yet practical to ubiquitously test for the presence of all waterborne pathogens in a timely and tractable manner, water impairment guidelines are defined on the basis of levels of fecal indicator organisms, such as *Escherichia coli*, fecal coliforms, or enterococci (9). However, viruses often survive longer in water and can be more resistant to environmental stress than fecal indicator bacteria (FIB). Moreover, infectious viruses have been recovered at critical levels from surface waters that have met FIB impairment criteria (15). Therefore, for human infectious viruses, other types of indicators with preferred characteristics, depending on the application, will be required (9, 16–21).

F-specific coliphages have been identified as indicators of fecal contamination, as they primarily infect coliform bacteria present in the mammalian gut (9, 16, 18, 22–24). The F-specific coliphage group is composed of multiple groups of bacteriophages that infect bacteria via the F-specific pili (F-pili) and contain members with single-stranded DNA genomes (F-specific DNA [F-DNA] coliphages) or RNA genomes (F-specific RNA [F-RNA] coliphages). The enumeration and characterization of viable F-specific coliphage are relatively inexpensive and rapid. While F-RNA coliphages have been well characterized and have the added potential of discriminating between human and animal sources, much less is known about the distribution, survival, and ecology of F-DNA coliphages (17, 24–27). The F-DNA coliphages belong to the *Inoviridae* family and appear to have a higher genetic relatedness as a group than the F-RNA coliphages (27). F-RNA coliphages belong to the *Leviviridae* family, which is subdivided into the genera *Levivirus* and *Allolevirus*, and 4 serologically and phylogenetically separate genogroups (28, 29). F-RNA genogroups I (F-RNA GI) and II (F-RNA GII) belong to the *Levivirus* genus, while F-RNA genogroups III [F-RNA GIII] and IV [F-RNA GIV] are part of the *Allolevirus* genus. Genogroups I and IV are generally associated with animals [animal F-RNA] (but genogroup I has been isolated in sewage), and genogroups II and III are predominantly associated with humans [human F-RNA] but have been isolated from swine and chicken excrements (17, 28, 30–32). Different rates of survival at different temperatures for subgroups of F-specific coliphages have been reported in surface water (27, 30, 33, 34). In addition, the numbers and shedding frequency of F-specific coliphages shed by individual hosts can be highly variable, which can result in variability in detection and density in surface waters when fecal contamination occurs (35–37).

Adenoviruses (AdV) are gaining attention as a potential viral indicator as they are ubiquitous in sewage and surface water and are more resistant to disinfection from UV treatment than other viruses (19). AdV have a double-stranded DNA genome and display great diversity, with 7 species and 52 serotypes, where AdV types 40 and 41 are typically associated with gastroenteritis in children (38). Detection of AdV can be used for fecal source tracking in water, as human, porcine, bovine, and avian AdV are host specific (1, 39). AdV infections in animals are either asymptomatic or associated with mild enteric or respiratory diseases (1). Although many types of human AdV (HAdV) can be detected using cell culture, they vary with serotype and cell line and are laborious and time-consuming to measure in water (40). HAdV are readily detected and quantified in surface waters using PCR methods (40–43). With molecular detection techniques,

it is possible to detect nucleic acids of many human-pathogenic viruses, such as NoV, HAV, HEV, and others that are difficult or impossible to cultivate, although no conclusions about virus infectivity can be made from these techniques. Molecular detection methods have opened up research opportunities to explore the potential utility of other viruses as indicators of fecal contamination.

Torque teno virus (TTV) is a potential viral indicator of fecal contamination, as it is frequently shed by humans and animals (44–47). TTV is a small virus classified in the *Anelloviridae* family with a high-genetic-diversity DNA genome (44, 45, 48). TTV is associated with persistent and transient infections, such as acute gastroenteritis, but is also excreted by healthy individuals, so it is not clear if TTV is part of the normal gut flora (19, 48–50). Some studies have demonstrated that TTV is very stable in the environment and more resistant to decontamination treatments than AdV (51, 52). However, as for several enteric viruses, there is no cell line supporting TTV replication *in vitro*.

Further information and data on the associations between indicator viruses and human-pathogenic viruses in impacted surface waters will be required before such viral indicator approaches are employed broadly. A positive correlation between the concentrations of F-RNA genogroup II and HAdV was reported in river water of an urban area in France, while a significant correlation was not detected between genomic copies of HAdV and culturable coliphages, F-specific coliphages, or FIB in urban rivers of California (42, 53). A meta-analysis revealed that F-specific coliphages can be good indicators for viral pathogens (21). When 5 pathogens and 8 commonly used indicators were compared, a positive correlation was only found for F-specific coliphages and AdV but not for other comparisons (21). The U.S. Environmental Protection Agency (USEPA) provides an expansive review of the use of coliphages as a fecal pollution indicator organism, but several studies therein found insignificant and negative coliphage-virus associations (54). Evidence was also provided therein supporting potential relationships between coliphages found in water and human health.

The primary objective of this study was to determine the degree of correlation among viable F-specific coliphages and a suite of molecularly detected human and animal enteric viruses (i.e., AdV, TTV, HAV, HEV, astrovirus, NoV genogroup I [GI] to GIV, rotavirus [RV], and kobuvirus) in surface water of several watersheds in a mixed-use, but predominantly agricultural river basin in eastern Ontario, Canada. A secondary objective was to determine the seasonality and environment/land use factors associated with these virus and F-specific coliphage targets.

RESULTS

Occurrence of F-coliphage and viruses in water. F-DNA and F-RNA coliphage were detected in 33 and 28% of the water samples, respectively. The prevalence of F-DNA and F-RNA coliphage was greater than that of any specific virus (Table 1). The maximum numbers of F-DNA coliphage were 8-fold lower than the maximum numbers of F-RNA coliphages, where F-RNA GI was detected most frequently (21%), while F-RNA GIV was not detected in any water sample. Just over half of the samples (55%) exhibited detections for any virus. Human TTV and animal TTV were detected in 20 and 23% of the samples, respectively, while astrovirus, NoV GIII (often associated with bovines), and kobuvirus were detected in more than 10% of the samples and AdV 40/41 in 3% of the samples.

Table 2 shows the levels of detection of any virus, animal virus, human virus, and F-specific coliphage in water samples for individual sample sites. For all the sample sites, the average percentages of detection of any virus, animal virus, human virus, F-RNA or F-DNA coliphage, F-DNA coliphage, human F-RNA coliphage, and animal F-RNA coliphage in water were 55, 36, 42, 53, 33, 15, and 79%, respectively. Some notable observations include site 12 on the mixed-use Little Castor River, which had the highest detection by percentage of all sites for any virus (67%) and human virus (52%), while the stream draining from a forested catchment with no human or agricultural upstream land uses (site 24) had the lowest percentages of any virus (35%) and animal

TABLE 1 Summary of virus detections and maximum F-specific coliphage densities

Coliphage or virus[c]	No. of detections	% detections	No. of nondetections	% nondetections	Maximum PFU · 100 ml^{-1}[a]
F-DNA	383	33	786	67	2,000
F-RNA	330	28	835	72	16,300
Human F-RNA	161	14	1,004	86	16,300
Animal F-RNA	248	21	917	79	925
F-RNA GI	247	21	918	79	16,300
F-RNA GII	118	10	1,047	90	925
F-RNA GIII	53	5	1,112	95	450
F-RNA GIV	0	0	1,165	100	ND
Hepatitis E (HEV)	20	2	1,135	98	NA
Hepatitis A (HAV)	18	2	1,014	98	NA
Astrovirus	83	12	638	88	NA
Norovirus GI (NoV GI)	48	4	1,107	96	NA
Norovirus GII (NoV GII)	57	5	1,098	95	NA
Norovirus GIII (NoV GIII)	89	11	755	89	NA
Norovirus GIV (NoV GIV)	59	7	785	93	NA
Rotavirus (RV)	23	2	1,132	98	NA
Human torque teno virus (TTV)	236	20	919	80	NA
Animal TTV	260	23	895	77	NA
Adenovirus 40/41 (AdV 40/41)	28	3	1,004	97	NA
General adenoviruses (general AdV)	22	2	1,010	98	NA
Kobuvirus	16	13	107	87	NA
Any virus[b]	634	55	521	45	NA

[a]ND, not detected (below detection limit); NA, not applicable.
[b]F-specific coliphage not included.
[c]See Materials and Methods for virus group definitions.

virus (15%) detection. The percentages for F-RNA and F-DNA coliphage detection were greatest at site 14 and lowest at site 24 (forested catchment). Site 14 is located on a surface drainage network draining crop fields and is an area associated with a suite of municipal sewage treatment lagoons (the degree of hydrological connectivity of the drainage channel with the lagoons is unknown). The lowest percentages of human F-RNA coliphage detection were found at site 22 and site 24. Site 22 was located immediately downstream of a protected stream channel/riparian zone that received primarily agricultural drainage but, interestingly, has been a site subjected to septic system leakage inputs (6, 39, 55). The lowest animal F-RNA coliphage detection was at site 22. The highest level of animal F-RNA coliphage detection was observed at site 24 and site 10 (a site immediately downstream of a cow pasture where cattle had access to the water course [56]).

Associations between F-specific coliphage and viruses in water. Fig. 2A shows significantly higher percentages of detection of kobuvirus, astrovirus, and AdV 40/41 when F-RNA coliphage and F-DNA coliphage were not detected in a sample. The reverse was true for human TTV and any TTV (there were higher detection percentages associated with F-RNA and/or F-DNA coliphage detection). For just F-DNA coliphage, these same relationships held true except for significantly higher levels of detection of animal TTV when F-DNA coliphage was detected in a water sample, and there was no significant difference in the levels of detection of kobuvirus with respect to F-DNA coliphage (Fig. 2B). For F-RNA coliphage only, significant detection associations were found for a vast array of viruses, but 7 out of the 9 significant associations indicated lower levels of virus detection when F-RNA coliphage was present in a water sample (Fig. 2C). Animal F-RNA coliphage associations with groups of viruses were only significant among kobuvirus and animal TTV, both negatively (Fig. 3A). Regarding human F-RNA coliphage detection, significant associations were found among human

TABLE 2 Sample site characteristics and detection of grouped viruses and F-specific coliphages at sampling sites

Site	Site characteristics							Virus detection				F-specific coliphage detection				
	Description	Upstream contributing area (km²)	Agriculture (% land)	Urban/ developed (% land)	Forest (% land)	Wetland (% land)	Other (% land)	No.	Any virus (%)	Animal virus (%)	Human virus (%)	No.	F-RNA or F-DNA (%)	F-DNA (%)	Human F-RNA (%)	Animal F-RNA (%)
1	WTP intake	2,371	52	2	42	3	2	47	57.4	40.4	44.7	48	52.1	39.6	12.5	79.2
2	Main river	2,371	52	2	42	3	2	42	54.8	38.1	35.7	41	51.2	36.6	12.2	80.5
3	Agricultural stream	<5	67	3	30	0	1	46	43.5	26.1	32.6	46	69.6	37.0	13.3[a]	73.3[a]
4	Tributary	724	51	5	39	2	3	45	53.3	37.8	44.4	46	52.2	32.6	19.6	80.4
5	Tributary	81	65	2	30	1	2	72	59.7	45.8	27.8	74	63.5	48.6	14.9	81.1
6	Tributary	176	54	1	43	1	1	72	55.6	36.1	36.1	75	53.3	36.0	16.0	85.3
7	Main river	1,216	48	1	47	4	1	46	54.3	32.6	47.8	46	58.7	37.0	21.7	80.4
8	Main river	1,413	49	1	46	3	1	46	56.5	37	50	49	61.2	34.7	19.1[b]	70.2[b]
9	Tributary	54	72	1	23	4	1	72	45.8	37.5	29.2	75	56.0	34.7	14.7	76.0
10	Tributary	68	75	2	19	3	1	43	62.8	44.2	51.2	43	48.8	32.6	16.3	88.4
11	Main river	1,548	51	1	44	3	1	45	62.2	35.6	46.7	45	51.1	31.1	24.4	80.0
12	Tributary	96	67	2	29	1	2	46	67.4	41.3	52.2	49	55.1	30.6	20.4	77.6
13	Tributary	88	66	2	30	1	2	45	60.0	44.4	44.4	46	56.5	34.8	10.9	84.8
14	Agricultural stream	<5	69	2	25	0	5	21	57.1	42.9	38.1	20	85.0	50.0	45.0	80.0
15	Agricultural stream	<5	90	0	10	0	0	39	64.1	43.6	51.3	39	53.8	41.0	5.1	74.4
16	Tributary	739	51	5	40	2	3	44	63.6	38.6	47.7	44	47.7	25.0	9.1	72.7
17	Main river	1,450	50	1	45	3	1	45	64.4	37.8	51.1	45	57.8	35.6	17.8	71.1
18	Agricultural stream	<5	90	0	10	0	0	69	50.7	37.7	33.3	71	56.3	29.6	4.2	69.0
19	Agricultural stream	<5	89	0	11	0	0	47	53.2	27.7	44.7	46	39.1	19.6	15.2	84.8
20	Agricultural stream	<5	90	0	9	0	1	64	45.3	26.6	31.3	66	36.4	18.2	7.6	81.8
21	Agricultural stream	<5	77	0	21	0	1	40	57.5	37.5	50	39	43.6	23.1	15.4	82.1
22	Agricultural stream	<5	90	0	10	0	0	39	51.3	28.2	35.9	39	53.8	28.2	2.6	66.7
23	Agricultural stream	<5	90	0	10	0	0	34	52.9	35.3	44.1	32	50.0	37.5	9.7[c]	77.4[c]
24	Forest stream	<5	0	0	100	0	0	46	34.8	15.2	28.3	45	22.2	17.8	2.2	91.1

[a] $n = 45$.
[b] $n = 47$.
[c] $n = 31$.

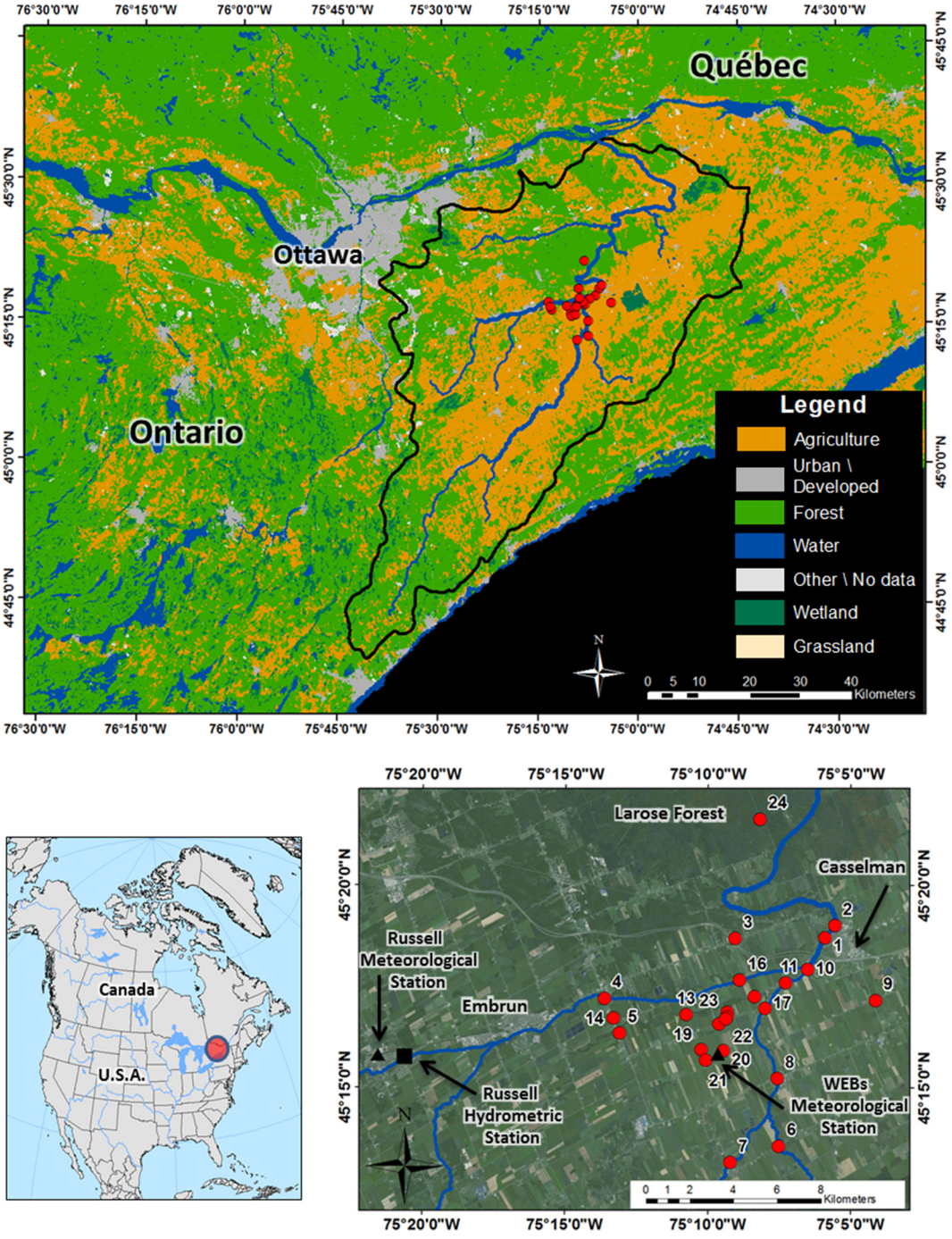

FIG 1 Map of study site. Top, land cover of the South Nation river basin year 2013. This panel was adapted from that in reference 103. Bottom right, the location of the water sample sites. Bottom left, location of study area in North America.

TTV, AdV 40/41, and human viruses; however, these detection relationships were all negative (Fig. 3B).

Table 3 shows that a majority of the significant ($P \leq 0.05$) associations among quantitative F-specific coliphage and virus detection/nondetection were negatively related. That is, F-specific coliphage average rank sums were lower when viruses were detected in a sample than when viruses were not detected in a sample. Positive associations, however, included (i) F-RNA coliphage versus NoV (GI, GII, and GIV) and RV, (ii) F-DNA coliphage versus any, human, or animal TTV, and (iii) F-DNA coliphage versus any, human, or animal virus.

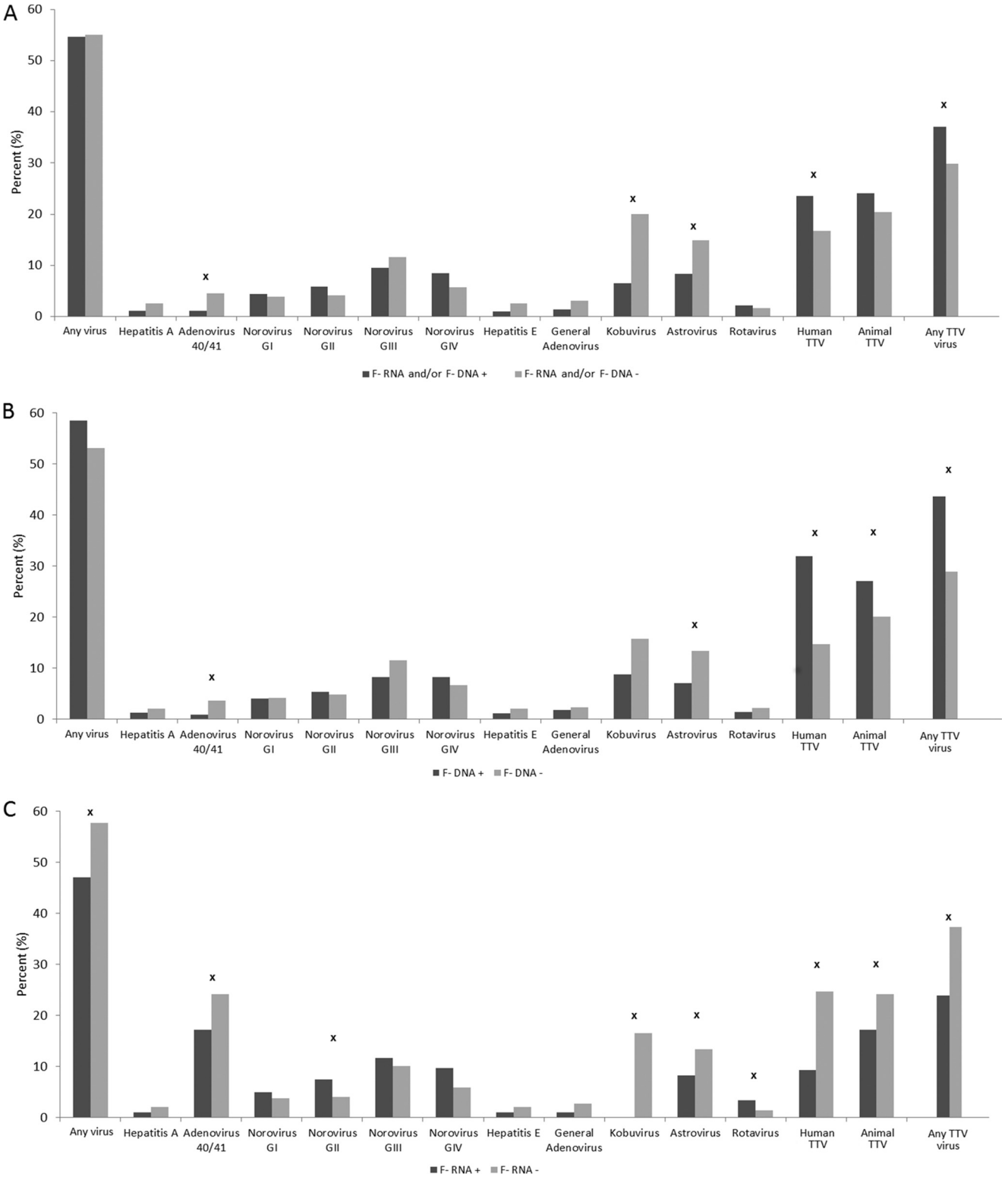

FIG 2 The percentage of specific virus and virus group detections and nondetections in relation to detections and nondetections of F-DNA and/or F-RNA coliphages (A), F-DNA coliphages (B), and F-RNA coliphages (C). x = Fisher's exact test, $P \leq 0.05$. + and − refer to F-coliphage detection and nondetection, respectively.

Classification and regression tree (CART) analyses were used to classify threshold F-DNA coliphage densities above which there would be a higher relative prevalence of viruses (groups). We focused this effort on F-DNA coliphage versus human virus, animal virus, and any virus. F-DNA coliphage, as already underscored, had the strongest

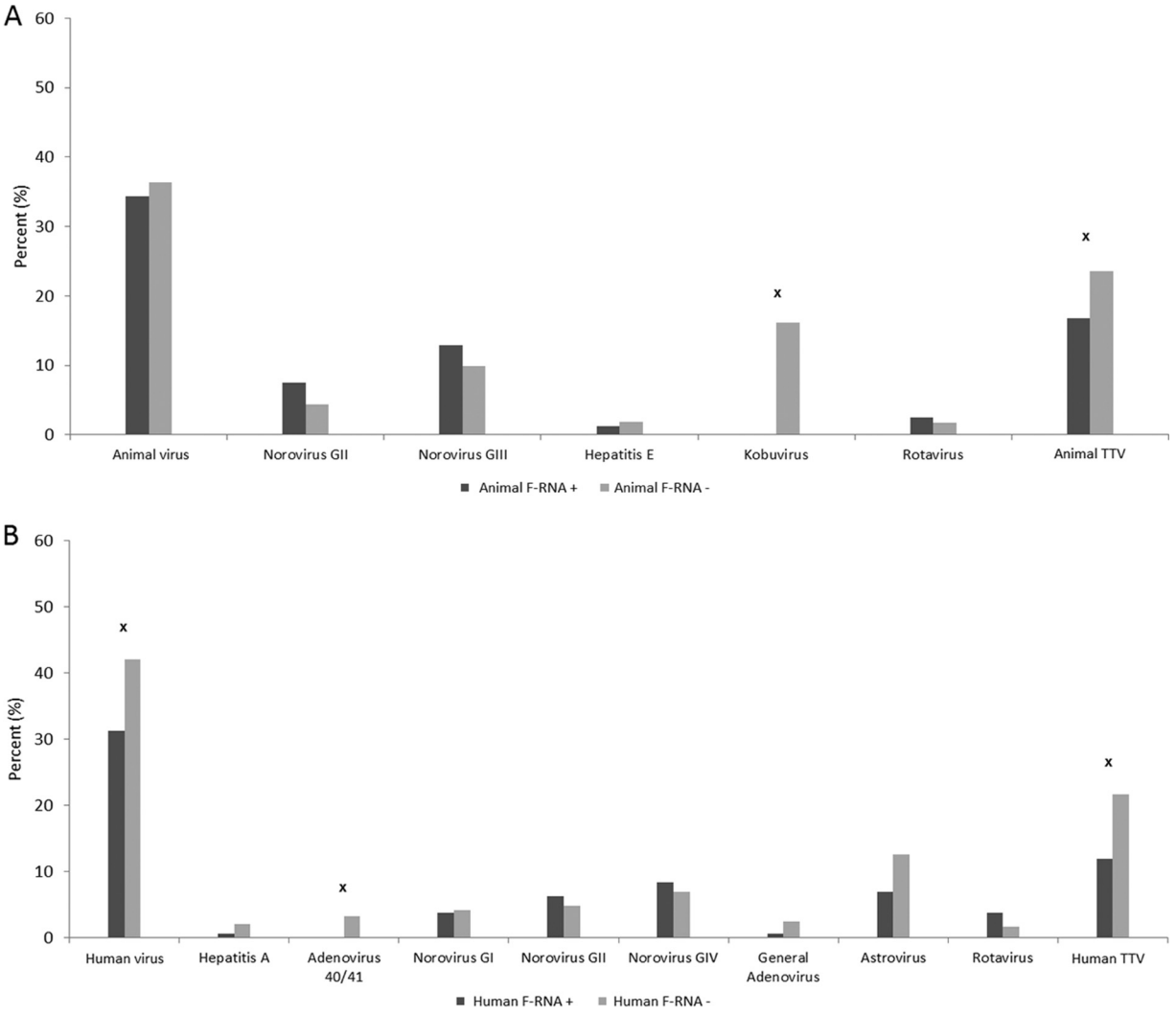

FIG 3 The percentage of specific virus and virus group detections and nondetections in relation to detections and nondetections of animal F-RNA coliphages (A) and human F-RNA coliphages (B). x = Fisher's exact test, $P \leq 0.05$. + and − refer to F-coliphage detection and nondetection, respectively.

positive associations with these virus groups, in relation to F-RNA coliphage. Table 4 indicates that a grand proportion of virus detections were associated with lower F-DNA threshold groupings ($\sim\leq$88 PFU · 100 ml^{-1} for human virus and $\sim\leq$142 PFU · 100 ml^{-1} for animal and any virus), but the greatest classification percentages of virus-positive samples were above threshold values (percentages of virus positivity were 66, 87, and 95% for animal, human, and any virus, respectively), albeit the numbers of samples for these groups were small in relation to the total number of virus detections.

Intervirus associations in water. Table 5 indicates that for HEV, there were a suite of significant Fisher's exact test results, primarily with NoV, but Phi coefficients, while all positive, were all low. Low Phi coefficients were also found for HAV. Astrovirus versus any virus had a modest Phi coefficient of 0.41. NoV GI and GII were associated with a suite of significant Fisher's exact test results; however, the associated Phi coefficients were predominantly positive but low. Interestingly, NoV GIII versus any virus had a modest positive Phi coefficient of 0.40. This could be important considering that NoV GIII is often associated with bovine fecal pollution, bovines being the dominant livestock in the region. Both animal TTV and human TTV versus any virus had modestly positive Phi coefficients (0.46 to 0.49). The strongest Phi coefficient belonged to kobuvirus versus any virus, at 0.51.

TABLE 3 Significant ($P \leq 0.05$) Mann-Whitney *U* test results associated with F-specific coliphage densities in samples where a virus was or was not detected[a]

Virus grouping variable	Quantitative F-specific coliphage target	F-specific coliphage rank sum for virus detection group	F-specific coliphage rank sum for virus nondetection group	F-specific coliphage mean rank sum for virus detection group	F-specific coliphage mean rank sum for virus nondetection group	Positive association between virus and coliphage	*U* statistic of Mann-Whitney *U* test	Z adjusted	*P* value	Valid no. of virus detections	Valid no. of virus nondetections	Arithmetic mean of coliphage for virus detection group[a]	Arithmetic mean of coliphage for virus nondetection group[a]
Astrovirus	F-DNA	25,993	227,835	313	362	No	22,507	−2.59	0.010	83	629	2	8
Astrovirus	F-RNA	26,533	225,872	320	360	No	23,047	−2.03	0.042	83	627	58	72
Norovirus GI	F-RNA	37,478	609,475	658	564	Yes	25,735	2.63	0.009	57	1,080	447	28
Norovirus GII	F-RNA GI	36,225	610,728	636	565	Yes	26,988	2.21	0.027	57	1,080	440	24
Norovirus GIV	F-RNA GIII	26,134	320,394	443	414	Yes	21,243	2.26	0.024	59	773	0	1
Rotavirus	F-RNA	15,042	631,912	684	567	Yes	9,742	2.08	0.037	22	1,115	11	50
Human TTV	F-DNA	162,834	488,678	702	538	Yes	75,083	8.12	0.000	232	909	55	7
Human TTV	F-RNA	111,388	535,566	482	591	No	84,592	−5.66	0.000	231	906	5	61
Human TTV	F-RNA GI	113,684	533,270	492	589	No	86,888	−5.60	0.000	231	906	4	56
Human TTV	F-RNA GII	126,519	520,434	548	574	No	99,723	−2.09	0.036	231	906	1	4
Human TTV	F-RNA GIII	127,619	519,335	552	573	No	100,823	−2.35	0.019	231	906	0	1
Animal TTV	F-DNA	160,305	491,206	629	554	Yes	98,265	3.80	0.000	255	886	35	12
Animal TTV	F-RNA	136,154	510,800	540	577	No	104,276	−1.98	0.048	252	885	26	56
Adenovirus 40/41	F-DNA	11,246	508,444	402	513	No	10,840	−2.38	0.017	28	991	11	19
Adenovirus 40/41	F-RNA	11,682	503,938	417	511	No	11,276	−2.07	0.039	28	987	13	56
Any virus	F-DNA	367,617	283,895	587	551	Yes	151,025	2.20	0.028	626	515	26	7
Any virus	F-RNA	339,859	307,094	546	596	No	146,106	−3.21	0.001	622	515	54	45
Any virus	F-RNA GI	338,880	308,074	545	598	No	145,127	−3.84	0.000	622	515	49	41
Any TTV	F-DNA	235,088	416,423	641	538	Yes	116,498	5.89	0.000	367	774	37	8
Any TTV	F-RNA	191,163	455,791	527	589	No	125,097	−3.75	0.000	363	774	18	64
Any TTV	F-RNA GI	190,710	456,244	525	589	No	124,644	−4.31	0.000	363	774	12	61
Human virus	F-DNA	281,884	369,628	610	544	Yes	138,768	3.97	0.000	462	679	34	6
Human virus	F-RNA	245,238	401,716	532	594	No	138,747	−3.95	0.000	461	676	65	39
Human virus	F-RNA GI	246,422	400,531	535	593	No	139,931	−4.11	0.000	461	676	63	33
Human virus	F-RNA GII	255,196	391,758	554	580	No	148,705	−2.48	0.013	461	676	2	4
Animal virus	F-DNA	243,878	407,634	593	558	Yes	140,819	2.06	0.039	411	730	28	11

[a]Mean trends may differ from rank sum trends. Concentrations are in PFU · 100 ml^{-1}.

TABLE 4 Classification of virus detections on the basis of F-DNA coliphage densities using CART analyses

CART model	Dependent (target) variable	Classification criterion: F-DNA coliphage density (PFU · 100 ml^{-1})	No. of samples with:		% virus detection
			Virus detection	Virus nondetection	
1	Human virus	>87.5	41	6	87
	Human virus	≤87.5	421	673	39
2	Animal virus	>141.5	25	13	66
	Animal virus	≤141.5	386	717	35
3	Any virus	>141.5	36	2	95
	Any virus	≤141.5	590	513	54

Seasonality and environmental/land use associations with viruses and F-specific coliphage. Fig. 4 shows the seasonal detection of F-RNA and F-DNA coliphage and viruses. Levels of detection were greatest for human F-RNA coliphage, animal F-RNA coliphage, F-RNA and/or F-DNA coliphage, and F-DNA coliphage in fall (24%), fall (38%), summer (62%), and summer (47% samples), respectively. For the statistically significant seasonal differences, levels of detection of HAV, NoV (GI, GII, and GIV), human TTV, animal TTV, AdV 40/41, and general AdV were greatest in summer (47%), summer (3%), summer (6, 7, and 12%, respectively), summer (25%), fall (27%), spring (8%), and spring (4% of samples), respectively. NoV GIII detection was greatest in spring (16% of samples), a time period when livestock manure is applied to land. Seasonality was not significant for the detection of any TTV, animal virus, HEV, astrovirus, RV, or kobuvirus.

TABLE 5 Significant Fisher's exact test results among virus targets, and associated Phi coefficients

Virus vs virus	Phi coefficient	Fisher's exact test *P* value
Hepatitis E virus vs norovirus GI	0.07	0.05
Hepatitis E virus vs norovirus GIII	0.24	0.00
Hepatitis E virus vs norovirus GIV	0.15	0.00
Hepatitis E virus vs adenovirus 40/41	0.16	0.00
Hepatitis E virus vs kobuvirus	0.33	0.02
Hepatitis E virus vs any virus	0.12	0.00
Hepatitis A virus vs astrovirus	0.15	0.00
Hepatitis A virus vs any virus	0.12	0.00
Astrovirus vs norovirus GIII	0.10	0.02
Astrovirus vs any virus	0.41	0.00
Norovirus GI vs norovirus GII	0.11	0.00
Norovirus GI vs norovirus GIII	0.12	0.00
Norovirus GI vs norovirus GIV	0.12	0.00
Norovirus GI vs human TTV	−0.09	0.00
Norovirus GI vs any virus	0.19	0.00
Norovirus GII vs norovirus GIII	0.10	0.01
Norovirus GII vs animal TTV	−0.08	0.01
Norovirus GII vs any virus	0.21	0.00
Norovirus GIII vs norovirus GIV	0.27	0.00
Norovirus GIII vs any virus	0.40	0.00
Norovirus GIV vs animal TTV	−0.07	0.02
Norovirus GIV vs any virus	0.32	0.00
Rotavirus vs any virus	0.13	0.00
Human TTV vs animal TTV	0.35	0.00
Human TTV vs any virus	0.46	0.00
Animal TTV vs any virus	0.49	0.00
Adenovirus 40/41 vs general adenovirus	0.31	0.00
Adenovirus 40/41 vs any virus	0.14	0.00
General adenovirus vs any virus	0.13	0.00
Kobuvirus vs any virus	0.51	0.00

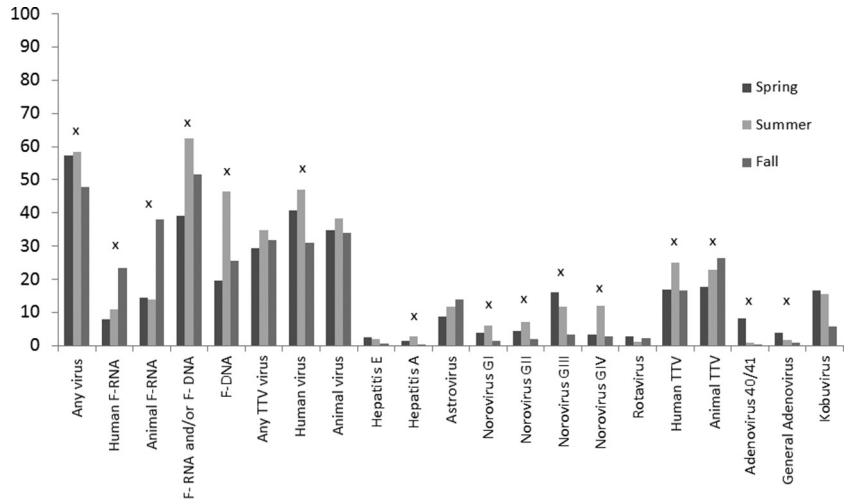

FIG 4 The percentage of various virus and F-specific coliphage detection and nondetection in samples grouped by season. Significant seasonality was determined via Fisher's exact test (3 seasons by presence/absence). x = $P \leq 0.05$.

Table 6 documents exploratory data mining results for a suite of virus and F-specific coliphage targets. The independent variables used in this analysis are given in Table S1 and are exploratory variables that have been utilized consistently in other works for pathogen association (see, e.g., reference 13). The approach defines optimal independent variable conditions that classify dependent variables (virus and F-specific coliphage detection and nondetection) on the basis of independent variable criteria. Some noteworthy results are as follows: for F-DNA coliphage, most detections were associated with daily mean air temperature of >4.25°C and total rainfall on the day of sampling of ≤4.1 mm. The majority of the F-RNA coliphage as well as animal F-RNA coliphage detections occurred during fall, in relation to the other seasons. Human F-RNA coliphage were most strongly associated with a daily mean air temperature of ≤9.25°C and developed land upstream (5 km) of >1%; the developed land variable split condition suggests there was a positive association between human F-RNA coliphage and degree of urban/rural development for daily mean air temperatures of ≤9.25°C. Regarding human virus, a majority of the detections were associated with mean daily river discharge of ≤1.64 $m^3 \cdot s^{-1}$ and developed land upstream (2 km) of >0.2%. The positive relationship between detection and human development for the discharge condition highlights positive-occurrence links between lower-discharge conditions and human land development. For animal virus, two independent variable criteria defined conditions associated with higher relative levels of virus detection; these were mean daily river discharge of >0.39 $m^3 \cdot s^{-1}$ and total rainfall on the day of sampling of >0.30 mm, and a mean daily river discharge of ≤0.39 $m^3 \cdot s^{-1}$. These are two contrasting situations, the first being higher relative discharge and rainfall inducing detection, and the second being lower-discharge conditions exclusively (animal virus presence is basically bimodal in the context of discharge). Some other notable virus-specific results include those for HEV, where greater HEV detection occurred when the total rainfall on the day of sampling was >0.60 mm and the electrical conductivity of water was >0.60 $mS \cdot cm^{-1}$ (higher salt content is usually linked to fertilizer-based nutrients in water in this area [13] under rainfall conditions). For NoV, the associations were different for each genogroup, where the detection of most NoV GI was associated with a daily mean air temperature of >5.90°C and a total rainfall on the day of sampling of ≤5.30 mm; proportionally higher levels of NoV GII detection occurred when the daily mean air temperature was >23.40°C. NoV GIII was associated with an oxidation-reduction potential of ≤145.10 mV or a combination of an oxidation-reduction potential of >145.10 mV and daily mean air temperature of ≤21.15°C, while NoV GIV detection was strongly

TABLE 6 CART classification tree results targeting detected F-specific coliphage and viruses using independent variables listed in Table 2

Dependent (target) variable	CART classification criteria	No. of detections	No. of nondetections	Total samples	% positive	% negative	CART nodal class designation[a]	Classification accuracy (%)	Weighted classification accuracy of CART model (%)
F-DNA coliphage	RUS_MEANTEMP ≤4.25°C	19	125	144	13	87	−	87	52
F-DNA coliphage	RUS_MEANTEMP >4.25°C AND RUS_TOTALRAIN ≤4.10 mm	344	519	863	40	60	+	40	
F-DNA coliphage	RUS_MEANTEMP >4.25°C AND RUS_TOTALRAIN >4.10 mm	20	142	162	12	88	−	88	
F-RNA coliphage	SEASON = FALL	157	190	347	45	55	+	45	67
F-RNA coliphage	SEASON = SPRING, SUMMER AND RUS_MEANTEMP ≤22.90°C	143	595	738	19	81	−	81	
F-RNA coliphage	SEASON = SPRING, SUMMER AND RUS_MEANTEMP >22.90°C	30	50	80	38	63	+	38	
Human F-RNA	RUS_MEANTEMP >9.25°C	75	716	791	9	91	−	91	76
Human F-RNA	RUS_MEANTEMP ≤9.25°C AND DEVELP_5K ≤1%	2	85	87	2	98	−	98	
Human F-RNA	RUS_MEANTEMP ≤9.25°C AND DEVELP_5K >1%	84	203	287	29	71	+	29	
Animal F-RNA	SEASON = FALL	132	215	347	38	62	+	38	69
Animal F-RNA	SEASON = SPRING, SUMMER AND RUS_MEANTEMP ≤22.90°C	91	647	738	12	88	−	88	
Animal F-RNA	SEASON = SPRING, SUMMER AND RUS_MEANTEMP >22.90°C	25	55	80	31	69	+	31	
Any virus	DIS_RUS ≤0.39 $m^3 \cdot s^{-1}$	56	0	56	100	0	+	100	62
Any virus	DIS_RUS >0.39 $m^3 \cdot s^{-1}$ AND TEMP ≤12.89°C	146	230	376	39	61	−	61	
Any virus	DIS_RUS >0.39 $m^3 \cdot s^{-1}$ AND TEMP >12.89°C	432	291	723	60	40	+	60	
Human virus	DIS_RUS ≤1.64 $m^3 \cdot s^{-1}$ AND DEVELP_2K ≤0.2%	14	67	81	17	83	−	83	65
Human virus	DIS_RUS ≤1.64 $m^3 \cdot s^{-1}$ AND DEVELP_2K >0.2%	276	215	491	56	44	+	56	
Human virus	DIS_RUS >1.64 $m^3 \cdot s^{-1}$	178	405	583	31	69	−	69	
Animal virus	DIS_RUS ≤0.39 $m^3 \cdot s^{-1}$	55	1	56	98	2	+	98	60
Animal virus	DIS_RUS >0.39 $m^3 \cdot s^{-1}$ AND RUS_TOTALRAIN ≤0.30 mm	94	371	465	20	80	−	80	
Animal virus	DIS_RUS >0.39 $m^3 \cdot s^{-1}$ AND RUS_TOTALRAIN >0.30 mm	268	366	634	42	58	+	42	
Hepatitis E virus	RUS_TOTALRAIN ≤0.60 mm	2	525	527	0	100	−	100	68
Hepatitis E virus	RUS_TOTALRAIN >0.60 mm AND CONDUCTIVITY ≤0.60 mS $\cdot$ cm^{-1}	0	246	246	0	100	−	100	
Hepatitis E virus	RUS_TOTALRAIN >0.60 mm AND CONDUCTIVITY >0.60 mS $\cdot$ cm^{-1}	18	364	382	5	95	+	5	
Hepatitis A virus	RUS_MEANTEMP ≤11.50°C	2	379	381	1	99	−	99	72
Hepatitis A virus	RUS_MEANTEMP >11.50°C AND TOTPHO ≤0.08 mg $\cdot$ $liter^{-1}$	15	287	302	5	95	+	5	
Hepatitis A virus	RUS_MEANTEMP >11.50°C AND TOTPHO >0.08 mg $\cdot$ $liter^{-1}$	1	348	349	0	100	−	100	
Astrovirus	ORP ≤174.35 mV	31	407	438	7	93	−	93	81
Astrovirus	ORP >174.35 mV AND TEMP ≤11.13°C	13	139	152	9	91	−	91	
Astrovirus	ORP >174.35 mV AND TEMP >11.13°C	39	92	131	30	70	+	30	
Norovirus GI	RUS_MEANTEMP ≤5.90°C	1	226	227	0	100	−	100	35
Norovirus GI	RUS_MEANTEMP >5.90°C AND RUS_TOTALRAIN ≤5.30 mm	46	750	796	6	94	+	6	
Norovirus GI	RUS_MEANTEMP >5.90°C AND RUS_TOTALRAIN >5.30 mm	1	131	132	1	99	−	99	
Norovirus GII	RUS_MEANTEMP >23.40°C	13	50	63	21	79	+	79	78
Norovirus GII	RUS_MEANTEMP ≤23.40°C AND DIS_RUS ≤6.33 $m^3 \cdot s^{-1}$	44	851	895	5	95	−	95	
Norovirus GII	RUS_MEANTEMP ≤23.40°C AND DIS_RUS >6.33 $m^3 \cdot s^{-1}$	0	197	197	0	100	−	0	
Norovirus GIII	ORP ≤145.10 mV	45	209	254	18	82	+	18	61
Norovirus GIII	ORP >145.10 mV AND RUS_MEANTEMP ≤21.15°C	21	451	472	4	96	−	96	
Norovirus GIII	ORP >145.10 mV AND RUS_MEANTEMP >21.15°C	23	95	118	19	81	+	19	
Norovirus GIV	DIS_RUS ≤1.56 $m^3 \cdot s^{-1}$ AND PASFOR_10K ≤35%	51	222	273	19	81	+	19	73
Norovirus GIV	DIS_RUS ≤1.56 $m^3 \cdot s^{-1}$ AND PASFOR_10K >35%	1	102	103	1	99	−	99	
Norovirus GIV	DIS_RUS >1.56 $m^3 \cdot s^{-1}$	7	461	468	1	99	−	99	

(Continued on following page)

TABLE 6 (Continued)

Dependent (target) variable	CART classification criteria	No. of detections	No. of nondetections	Total samples	% positive	% negative	CART nodal class designation[a]	Classification accuracy (%)	Weighted classification accuracy of CART model (%)
Rotavirus	TOTKN ≤0.88 mg · liter^{-1} AND RUS_MEANTEMP ≤13.65°C	11	343	354	3	97	+	3	50
Rotavirus	TOTKN ≤0.88 mg · liter^{-1} AND RUS_MEANTEMP >13.65°C	7	228	235	3	97	+	3	
Rotavirus	TOTKN >0.88 mg · liter^{-1}	5	561	566	1	99	−	99	
Human TTV	RUS_MEANTEMP ≤20.40°C AND TEMP ≤16.06°C	74	477	551	13	87	−	87	72
Human TTV	RUS_MEANTEMP ≤20.40°C AND TEMP >16.06°C	158	246	404	39	61	+	39	
Human TTV	RUS_MEANTEMP >20.40°C	4	196	200	2	98	−	98	
Animal TTV	DIS_RUS ≤0.39 m^3 · s^{-1}	55	1	56	98	2	+	98	56
Animal TTV	DIS_RUS >0.39 m^3 · s^{-1} AND ORP ≤174.35 mV	54	444	498	11	89	−	89	
Animal TTV	DIS_RUS >0.39 m^3 · s^{-1} AND ORP >174.35 mV	151	450	601	25	75	+	25	
Any TTV	DIS_RUS ≤0.49 m^3 · s^{-1}	82	18	100	82	18	+	82	61
Any TTV	DIS_RUS >0.49 m^3 · s^{-1} AND ORP ≤171.85 mV	66	398	464	14	86	−	86	
Any TTV	DIS_RUS >0.49 m^3 · s^{-1} AND ORP >171.85 mV	226	365	591	38	62	+	38	
Adenovirus 40/41	SEASON = SUMMER, FALL	5	750	755	1	99	−	99	89
Adenovirus 40/41	SEASON = SPRING AND DIS_RUS ≤2.29 m^3 · s^{-1}	22	103	125	18	82	+	18	
Adenovirus 40/41	SEASON = SPRING AND DIS_RUS >2.29 m^3 · s^{-1}	1	151	152	1	99	−	99	
Adenovirus	RUS_MEANTEMP ≤12.65°C	4	413	417	1	99	−	99	50
Adenovirus	RUS_MEANTEMP >12.65°C AND ORP ≤105.15 mV	0	84	84	0	100	−	100	
Adenovirus	RUS_MEANTEMP >12.65°C AND ORP >105.15 mV	18	513	531	3	97	+	3	
Kobuvirus	NITRATE ≤1.42 mg · liter^{-1}	7	35	42	17	83	+	17	50
Kobuvirus	NITRATE >1.42 mg · liter^{-1} AND TEMP ≤13.20°C	1	46	47	2	98	−	98	
Kobuvirus	NITRATE >1.42 mg · liter^{-1} AND TEMP >13.20°C	8	26	34	24	76	+	24	

[a]+, classed as a detection group; −, classed as a nondetection group.

associated with mean daily river discharge of ≤1.56 $m^3 \cdot s^{-1}$ and pasture forage land upstream (10 km) of ≤35%.

DISCUSSION

Detection and densities of F-specific coliphage and viruses in water and environmental/land use associations. The detection of viruses in surface water varies greatly in the literature (54, 57). Payment and Locas summarize a large 20-year assessment of somatic and F-specific RNA coliphage and a suite of enteric viruses primarily in groundwater in Canada (58). Other studies focusing on surface water, such as those by Baggi et al. (59), Hot et al. (60), Skraber et al. (18), Westrell et al. (61), Espinosa et al. (62), Lodder et al. (63), and Viau et al. (64), examined collocated enteric virus and somatic and/or F-specific coliphage occurrence in fresh surface waters within a variety of impacted and lower-impacted regions throughout the world. But, to our knowledge, studies evaluating this broad suite of viral and F-specific coliphage genogroup targets over multiple years and surface waters from multiple (sub)watersheds have not been conducted for mixed-use predominantly agricultural regions. Untangling sources of fecal pollution in agro-ecosystems impacted by mixes of wildlife, livestock, and human fecal pollution is a rapidly emerging source water protection issue, and in parallel with new advances in molecular detection methods in combination with geospatial and statistical/data mining techniques, this work helps provide a Canadian baseline for future surveillance initiatives within an agricultural context.

F-DNA and F-RNA coliphages in this study were detected in 33 and 28% of the water samples, respectively. Wolf et al. found that 100% of river water samples likely impacted by both human and animal contamination were positive for F-RNA coliphage, with maximum numbers similar to those found herein (65). In this study, F-RNA coliphage GI was the most frequent and most abundant genogroup detected, which was expected, given that the samples were predominantly from agriculturally and wildlife-impacted surface waters (12). Moreover, increased rates of survival for F-RNA coliphage GI compared to those of other F-RNA coliphage subgroups at different temperatures may contribute to a greater prevalence or persistence of F-RNA coliphage GI in surface water (17, 30, 33, 66, 67). The stream systems studied had varied depth and substrates, which will contribute to there being a range in surface water temperatures affecting both persistence and viability. The lack of detection of F-RNA coliphage GIV in surface water found here is also in agreement with other studies (17, 65, 66).

Human and animal F-RNA coliphage detection levels were relatively low at site 22, an agricultural stream at the end of a cattle pasture area with a protected riparian buffer (55). Interestingly, this location has historically received highly transient fecal pollution inputs from leaking septic systems from homes immediately upstream, and from a relative framework, enhanced inputs of wildlife fecal matter resulting from riparian zone protection (e.g., Canada goose *Bacteroidales* markers [68]). Animal F-RNA was detected in relatively high quantities while human F-RNA was detected in relatively low quantities at site 24, a forested catchment area with no known upstream impact by humans or agriculture. This site did have some of the highest levels of detection of human and Canada goose *Bacteroidales* markers; however, as observed by Marti et al., the reasons for these observations are not fully clear (68). Frey et al. observed the presence of *Campylobacter*, *Salmonella*, and ruminant/bovine *Bacteroidales* markers in runoff from sediments in the area of study, especially around site 10 (impacted by pasturing cattle and direct cattle access to water) (56). In this study, at site 10, we observed some of the highest levels of detection of animal F-RNA coliphage, which is consistent with the Frey et al. ruminant/bovine marker observations. Site 12, located on a tributary with mixed land uses, had the greatest any virus detection, with associated detection levels of ~20% human F-RNA and ~78% animal F-RNA. For this site, few human, no pig, ~20% ruminant, ~7% muskrat, and no goose *Bacteroidales* markers were observed (68). The results of the site virus, F-specific coliphage, and source detections discussed here indicate that sources of viruses/coliphages are complex and

vary in both time and space transiently; moreover, they are not always coherent and consistent with detections associated with other biomarkers and pathogens.

The detection results obtained in this study show that 55% of the samples analyzed had at least one virus detected, suggesting that there is a need to carry out more-refined microbial risk assessments (10). Human and animal TTV viruses were most frequently detected in 20% and 23% of the surface water samples, respectively. This is not surprising, since the TTV virus is highly prevalent in humans, yet its link with disease is not well defined. Some authors have reported that TTV could be part of the normal flora for both humans and animals (making it a potential source tracking marker, which would help explain its high prevalence in certain populations and in water observed in this study [69, 70]). Some recent studies have suggested that human TTV virus could be involved in cases of gastroenteritis and that TTVs could be responsible for pathologies in swine as well, particularly in coinfection with circovirus type 2 (50, 71–73). The presence of human TTV in water has been frequently observed by others. A recent study reported that human TTV was found in 38 to 100% of wastewaters (74).

Levels of detection of the main human-pathogenic viruses in the surface waters from this study varied from 2% for HAV and HEV up to 12% for astrovirus. Astrovirus is responsible for a considerable proportion of gastroenteritis cases in young children, the elderly, and the immunosuppressed (75–77). NoV GII, which is considered to be an etiologic agent frequently associated with cases of gastroenteritis, was found in only 5% of water samples, perhaps reflecting the agricultural impact in the watershed (as reflected also in the somewhat higher level of detection of NoV GIII, at ~11%). Overall, the persistence of NoV in the environment, particularly in water, has often been observed, but there is in tandem high variability in terms of both prevalence and concentrations in surface and waste waters (78–80).

In this study, there were seasonal associations with the detection of F-DNA coliphages, where detection was highest in summer (when animal activity in the basin is greatest). Animal and human F-RNA coliphages were associated with the fall season. Cole et al. reported that the prevalence of F-DNA coliphages was significantly higher during the warmer months and early fall (17). A seasonal effect was reported for the survival of F-RNA coliphage in surface waters in California (67). F-DNA and F-RNA coliphages can die off faster at temperatures of 20°C or warmer than at 10°C or lower (27, 67). The presence or absence of a seasonal effect will be influenced by the climatic region in which the sample was taken (as well as the water body in terms of substrate and depth; see reference 81), as systemically higher maximum water temperatures in warmer climates may not be typical or sustained in a more northern climate. While overall, F-specific colipages were found in this study to be most strongly linked to seasonal attributes, the greatest number of human F-RNA coliphage detections were found via CART data mining analyses to be associated with both lower relative mean air temperature (i.e., cooler seasons) and higher relative degrees of developed land. This was the only CART-based F-specific coliphage finding to classify data on the basis of an urban/rural land development variable, with a 76% weighted classification accuracy. The CART result appears to be coherent with potential septic leakages that can occur in the area, as well as wastewater treatment plant effluents, which are typically discharged under higher-river-flow conditions in the cooler spring and fall seasons (6). Somewhat like human F-RNA coliphage, human virus associations with environment/land use indicated that most detections occurred where urban/rural development was relatively higher.

Environmental occurrences of and infections by NoV, AdV, RV, and astrovirus have been associated with cooler seasons (45, 82–84). In this work, we observed increased levels of detection of NoV GI, GII, and GIV in summer, while GIII was highest in spring, which could be related to agricultural practices, such as bovine manure applications on fields. Rotavirus was rarely observed, and astrovirus detections showed no seasonality.

The highest levels of animal virus detection were associated with higher relative water flow conditions and relatively higher daily rainfall, suggesting more spatially

uniform hydrologically driven inputs (e.g., surface runoff, tile drainage, and in-stream mobilization of antecedent waters/sediment/stream detritus [55, 85]). HEV detection was also linked to relatively higher daily rainfalls, as well as higher relative water electrical conductivity values (i.e., salts). Wilkes et al. documented livestock *Cryptosporidium* and higher odds of bacterial pathogen occurrence in fall when stream/river discharge and nitrate (a salt that will increase electrical conductivity in water) concentrations in water were relatively higher (12). TTV was bimodal in detection with respect to relative river discharge (which might be suggestive of systemic hydrologically driven inputs from land to stream) and intrinsic inputs from wildlife/urban under nonflushing conditions (i.e., low-flow conditions where contaminants can accumulate).

Overall, seasonality, expressed through air and water temperature and season variables, was extremely important for delineating the detection of viruses and F-specific coliphage in water. Seasonality in pathogen/indicator prevalence in the study region is linked to seasonal application of livestock manures to land (spring and fall) and subsequent movement of fecal pollution to stream, wastewater treatment plant (WWTP) lagoon dumping (spring and fall) in the South Nation River proper, wildlife fecal inputs into water courses, and wetter fall conditions promoting water course fecal pollution (13, 86).

F-specific coliphage–virus relationships. Associations between F-specific coliphage and viruses vary from modestly strong to negative (e.g., see references 21, 54, and 87). In this study, significant positive associations were found between the presence of F-DNA coliphages and human TTV, animal TTV, and any TTV. As TTV was the most frequently detected virus in the any virus group, the positive associations of F-DNA coliphages with the combined virus grouping were significantly influenced by TTV. In contrast, the presence of AdV 40/41, kobuvirus, astrovirus, human TTV, and any TTV (which includes human TTV as a subset) were associated with the absence of F-specific coliphage (F-RNA and F-DNA combined). The significance and number of the negative associations among the detection of viable F-specific coliphage and molecularly detected viruses were not entirely unexpected. Negative correlations might indicate that the viruses and F-specific coliphages had different fecal pollution sources and environmental reservoirs (54). Another factor could be that F-specific coliphages were detected by plaque assay and the viruses detected by molecular methods. Enteric viral genomes protected by a protein viral capsid can persist longer than some other infectious particles (42, 53). This also has bearing regarding differences in environmental persistence of the two microbial targets. Wu et al. reported that correlations between indicators and pathogens were strongest for conventional detection methods (odds ratio [OR], 2.36, positive association) and weakest for molecular detection methods (OR, 0.40) (21). In addition to the differences in the detection of infectious particles versus genomes, the absence of detection of F-specific coliphages and presence of other viruses may also be due to relative differences in viral and coliphage loads detected in the water samples. Fecal contamination from a single animal may also not be easily detected, since not all animals in a herd, for example, will carry and shed F-specific coliphages at equal levels; hence, inputs may be overwhelmingly diluted once entering the broader surface water environment (22, 88).

In our study, relatively small volumes of water were concentrated for the detection of viruses, while in many other surface water studies, larger volumes of water, up to 10 to 1,000 liters, are not uncommon (22, 65, 89). In the absence of significant fecal pollution inputs, the levels of F-specific coliphage may be well below the limit of detection. In such cases, perhaps an enrichment method for the detection of F-specific coliphages would provide more valuable information than direct enumeration (87). When Ballester et al. retested archived seawater sample concentrates prepared from large volumes (>100 liters of water) for the presence of coliphages, the rate of detection increased from 8% to 58% using a two-step enrichment method (87). However, with respect to sampling and laboratory management, processing larger volumes of water can be logistically cumbersome and would preclude, potentially, the

identification of viral contamination in surface waters captured via the smaller-volume approaches defined in the current study, where over 1,150 samples from 24 surface water sampling sites were processed and analyzed. Such virus molecular detection approaches can be viewed as a preliminary screening for identifying where and when more-intensive monitoring or microbial risk assessments (e.g., viability) need to be performed, and potentially where coliphage could be used as an indicator of viral pollution in water.

Virus versus virus associations. In this study, modestly strong associations were observed between the detection of astroviruses versus any virus, norovirus GIII versus any virus, animal and human TTV and any virus, as well as kobuvirus versus any virus. These virus groups were the most frequently detected viruses in the water samples. NoV GIII is associated with bovines, while kobuviruses are found in animals and humans (90, 91). Associations were not observed between TTV and kobuvirus. Information on correlations between TTV, kobuvirus, or NoV GIII and other pathogenic viruses is scarce (43). Diniz-Mendes et al. reported a lack of correlation between TTV and HAV in streams in a setting where both viruses were endemic (45).

TTV is being considered a suitable indicator of viral contamination in surface and drinking water broadly because it chronically infects humans; some studies have shown it is relatively abundant throughout the seasons, relative to human NoV and human AdV (for example, see references 45, 46, and 92–94). In this study, TTV was found to be positively associated with F-DNA coliphage and relatively abundant seasonally compared to other viruses.

Nevertheless, TTV results vary geographically, with reported human carriage rates as low as 10% to as high as 96% depending on the region/country (43, 47, 95, 96). In some cases, low concentrations of TTV DNA in sample water suggest that TTV may not necessarily be suitable as a single indicator of viral contamination of surface water (47). The limitations of TTV as a potential indicator virus were also raised by Verani et al. due to its low prevalence (97).

NoV GIII is associated with bovines, and very little information is currently available about its prevalence in Canadian cattle herds. However, the virus does seem to have the ability to transport and persist in the environment (98, 99), and in the South Nation River basin, where dairy livestock operations predominate, NoV GIII was observed in many surface water samples. NoV GIII had many significant positive associations with other viruses and virus groups. Hence, its potential as a virus indicator is promising. To our knowledge, the studies by Wilkes et al. (4, 39) are some of the first to report NoV GIII in an agriculture-dominated watershed setting.

Conclusion. This study was one of the first surveillance attempts to assess associations and detections among viable F-DNA and F-RNA coliphage and a suite of molecularly detected viruses in surface waters of a mixed-use but predominantly agricultural region in Canada. Viable F-DNA coliphage was found to be a reasonable indicator of molecularly detected TTV. Stronger positive associations and codetection were found between F-DNA coliphage and TTV than between F-RNA coliphage and TTV. This has not been previously reported, to our knowledge. F-RNA coliphage, unlike F-DNA coliphage, was a reasonable predictor of NoV and RV, however. There was a large number of F-specific coliphage-versus-virus relationships that were negative, which suppresses the broad utility of F-specific coliphage as an indicator of virus occurrences in raw surface water, as documented in this study. Water sample size was relatively small to accommodate logistical constraints of this broader surveillance initiative, and therefore, we may have underestimated the presence of viruses in some circumstances where abundance may have been naturally lower. F-specific coliphages were also present at low levels in surface water samples, suggesting that alternate testing methods, such as a presence/absence approach using an enrichment method, may improve the frequency of detection. Notwithstanding, molecular methods for the detection of viruses in water surveillance initiatives, such as this, are desirable from a logistical and cost perspective; moreover, they provide for the detection of a vast array

of viral targets to define fecal pollution sources and pollution hot spots. It can be seen in this way as a functional first-screening approach in terms of where to focus surface water sampling for future assessments of viability and for quantitative microbial risk assessments (10).

It was identified that F-DNA coliphage densities of >142 PFU · 100 ml^{-1} delineated conditions when ~95% of the water samples contained some type of detected virus. This F-DNA coliphage density could be tested as a threshold value for virus detection in other surface waters to evaluate linkages with, for example, recreational virus exposure risks (10).

Generally speaking, the most important variables associated with virus and F-specific coliphage occurrence in surface water were season/temperature and surface water discharge. These factors are direct and indirect drivers of fecally derived pollution in this river basin. Seasonality is potentially linked to factors, such as wildlife activity, municipal wastewater lagoon discharge, septic leakages, land application of manure, and soil and hydrological conditions that promote off-field and instream transport processes. However, it was found that where human intervention was relatively lower, levels of detection of human viruses and human F-RNA coliphage were relatively lower (e.g., forest stream and small agricultural drainage systems hydrologically disconnected from human fecal pollution sources), and where human intervention was relatively more intensive, levels of detections of human viruses and human F-RNA coliphage were relatively higher.

MATERIALS AND METHODS

Study site and sample collection. Water samples were collected in the South Nation River basin, in eastern Ontario, Canada, from spring 2008 to fall 2010 and spring 2013 to fall 2014 (Fig. 1). General basin land use is given in Table 2, where agriculture (dairy-based activities, including livestock cropping and land applications of manure in spring and fall), urban, forest, and wetlands occupied, respectively, 0 to 90, 0 to 5, 9 to 100, and 0 to 4% of the upstream contributing areas of water sample sites (using 2013 data). See two studies by Wilkes et al. for further details on the site characteristics and land use (13, 86). The maximum and minimum temperatures observed during the study period were 35°C (26 May 2010) and −35°C (17 December 2013). Total annual rainfall for the study period years ranged between 680 mm (2009) and 804 mm (2014) (climate data for Agriculture and Agri-Food Canada's WEBs station measured with Hobo weather station [Onset Computer Corporation, Bourne, MA] [Fig. 1]).

Water sampling occurred on a biweekly basis at sites 1 to 24 (Fig. 1 and Table 2). A total of 1,155 site visits were made during this period, where a distinct 1 liter of sample water was collected for the molecular detection of viruses, and a separate 1 liter of sample water was collected for quantification of viable F-specific coliphages. Water samples were shipped overnight on ice to Agriculture and Agri-Food Canada's (AAFC's) research center in Lacombe, Alberta, Canada, for characterization of F-RNA and F-DNA coliphage, and to AAFC's research center in Saint-Hyacinthe, Quebec, Canada, for molecular detection of enteric viruses.

F-RNA and F-DNA coliphages. Isolates were selected as described in reference 55. The RNA from confirmed F-RNA coliphage isolates was obtained from F-RNA coliphage suspensions that were thawed and diluted 1:50 in DNase/RNase-free water, boiled for 5 min, held on ice for 2 min, and then centrifuged at 14,000 × *g* for 10 s. The RNA extracts of F-RNA coliphages were genotyped into genogroups I through IV by real-time reverse transcription-PCR (RT-PCR). Real-time RT-PCRs were carried out with a QuantiTect multiplex no-ROX RT-PCR kit (Qiagen, Inc., Mississauga, Ontario, Canada) on a Stratagene MX3005P quantitative PCR (qPCR) thermocycler (Agilent), using conditions as described by Jones et al. (100). Genogroups I and IV were detected by a duplex assay using LV1 and GIV primers and probes, as described by Jones et al. (100), and genogroups II and III were detected in individual reactions using the primers and probes as described in Wolf et al. (65). Each 25-μl reaction mixture contained 200 nM each forward and reverse primers and probe, 0.25 μl of QuantiTect multiplex RT mixture, 0.03 μM of ROX reference dye, and 2.5 μl of RNA extract in 1× QuantiTect multiplex RT-PCR mastermix. RNA extracts that were positive for GI or GIV were assigned to F-RNA coliphage of animal origin, and those that were positive for GII or GIII were assigned to F-RNA coliphage of human origin for each water sample (taking into consideration that source definitions may not be absolute). The detection limits for viable phages were 5 liter^{-1}, and the genome copies by RT-PCR were 120 liter^{-1}.

Viruses. Water samples were processed for the detection of HAV, HEV, astrovirus, NoV GI, GII, GIII, and GIV, RV, human TTV, animal TTV, AdV 40/41, general AdV, and kobuvirus. The samples were processed as described in reference 6. Feline calicivirus was added to each sample of water before any manipulation as an internal process control to monitor the concentration and extraction processes and to evaluate the presence of inhibitors; negative controls were also employed (6). For each detected virus, standard curves were made to estimate the amount of viral genomes, as described by Wilkes et al. (6).

For kobuvirus, which we describe in detail here (not described in the previous methods), the cDNA synthesis was carried out using SuperScript III (Invitrogen), according to the manufacturer's recommendations. Briefly, a 20-μl final volume containing 10 μl of RNA extract, 10 mM deoxynucleotide triphos-

phate (dNTP), 4 μl of 5× first-strand buffer, 10 pmol of the antisense primer, 40 U of RNaseOUT, and 200 U of SuperScript III. The reverse transcription was performed at 55°C for 1 h. PCRs were performed in a total volume of 50 μl using the HotStar *Taq* Plus master mix 2× kit (Qiagen), according to the manufacturer's recommendations, in an Mastercycler gradient PCR system (Eppendorf, Mississauga, Ontario, Canada). cDNA (10 μl) was used as the template. The PCR primers Univ-KOBU-F and Univ-KOBU-R were used at a final concentration of 500 nM each (90). They were designed for the conserved RNA-dependent RNA polymerase (RdRp) gene of kobuviruses and amplify a 216-bp-long PCR fragment. The PCR was conducted under the following conditions: 1 cycle at 95°C for 15 min and 40 cycles of 95°C for 30 s, 51°C for 90 s, and 72°C for 45 s, followed by a final elongation step of 72°C for 10 min. The amplified products were separated on a 2% agarose gel with amplicons visualized with ethidium bromide staining.

For this study, broad groupings of viruses were created based on their generalized host association (again taking into consideration that assignations may not be absolute) for microbial source tracking purposes (4). For defining animal virus, the grouped viruses were norovirus GII, norovirus GIII, animal TTV, kobuvirus, RV, and HEV. For human virus, the grouped viruses were HAV, astrovirus, norovirus GI, norovirus GII, norovirus GIV, RV, human TTV, AdV 40/41, and general AdV.

Statistical treatment. Fisher's exact tests were used to examine the significance of contingency among the detection/nondetection of F-specific coliphage and detection nondetection of singular viruses (groups) (2 by 2 table) (R version 3.2.2; The Foundation for Statistical Computing, Vienna, Austria). For these same comparisons, Phi coefficients were calculated in R to discriminate the strength of the association between the detection/nondetection of viruses and the detection/nondetection of F-specific coliphage. A Phi coefficient of 1 is indicative of a strong positive correlation between categorical data, a Phi coefficient of −0.3 to 0.3 is indicative of no effective correlation, and a Phi coefficient of −1 is indicative of a strong negative correlation between categorical data. Fisher's exact tests were also used to test for seasonal significance among the singular virus (virus groups) and F-specific coliphage using a 3 by 2 (3 season and detection/nondetection) contingency table approach (R version 3.2.2, fisher.test function of stats package [101, 102]). To determine if quantitative F-specific coliphage distributions were significantly different among detection and nondetection groups of viruses, nonparametric Mann-Whitney *U* rank sum tests, using continuity corrections, were employed (Statistica version 12; StatSoft, Inc., Tulsa, OK). For all of the previous statistical treatments of the data, significance was defined as a P value of ≤ 0.05.

Data mining was conducted to explore associations among the detection/nondetection of microbiological targets (dependent variables) and a suite of independent land use (proportions of developed land, pasture and forage land, and agriculture land), physiographic (Strahler stream order [104]), and hydrological variables (e.g., stream flow) (Table S1). Classification and regression tree (CART) (Salford Predictive Modeler version 7.0 64-bit; Salford Systems, San Diego, CA) data mining was employed in classification tree mode, according to approaches outlined in reference 12. Also, we used CART in classification mode to evaluate threshold F-DNA densities associated with the detection and nondetection of viral targets. For CART analyses, we only present cross-validated tree models generated to a maximum of two levels (maximum of four classification groups) (4, 13).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02763-16.

TEXT S1, PDF file, 0.2 MB.

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