

Comparison of the Prevalences and Diversities of *Listeria* Species and *Listeria monocytogenes* in an Urban and a Rural Agricultural Watershed

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Foods and related processing environments are commonly contaminated with the pathogenic *Listeria monocytogenes*. To investigate potential environmental reservoirs of *Listeria* spp. and *L. monocytogenes*, surface water and point source pollution samples from an urban and a rural municipal water supply watershed in Nova Scotia, Canada, were examined over 18 months. Presumptive *Listeria* spp. were cultured from 72 and 35% of rural and urban water samples, respectively, with 24% of the positive samples containing two or three different *Listeria* spp. The *L. innocua* (56%) and *L. welshimeri* (43%) groups were predominant in the rural and urban watersheds, respectively. Analysis by the TaqMan assay showed a significantly ($P < 0.05$) higher prevalence of *L. monocytogenes* of 62% versus 17% by the culture-based method. Both methods revealed higher prevalences in the rural watershed and during the fall and winter seasons. Elevated *Escherichia coli* (≥ 100 CFU/100 ml) levels were not associated with the pathogen regardless of the detection method. Isolation of *Listeria* spp. were associated with 70 times higher odds of isolating *L. monocytogenes* (odds ratio = 70; $P < 0.001$). Serogroup IIa was predominant (67.7%) among the 285 *L. monocytogenes* isolates, followed by IVb (16.1%), IIb (15.8%), and IIc (0.4%). *L. monocytogenes* was detected in cow feces and raw sewage but not in septic tank samples. Pulsotyping of representative water ($n = 54$) and local human ($n = 19$) isolates suggested genetic similarities among some environmental and human *L. monocytogenes* isolates. In conclusion, temperate surface waters contain a diverse *Listeria* species population and could be a potential reservoir for *L. monocytogenes*, especially in rural agricultural watersheds.

Listeria monocytogenes is a Gram-positive bacterium that is able to tolerate high salt concentrations, cold temperatures, and acid stress (1). Pathogenic *Listeria* species include *L. monocytogenes* and *L. ivanovii* (2), while 13 nonpathogenic species exist: *L. innocua*, *L. welshimeri*, *L. grayi*, *L. seeligeri*, *L. rocourtia*, *L. marthii*, *L. weihenstephanensis*, *L. fleischmannii*, *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. grandensis*, and *L. riparia* (3, 4).

Listeria spp. are considered ubiquitous in the natural environment, and representatives of the genus, including *L. monocytogenes*, have been isolated from soil, surface waters, animal feeds, animal feces, sewage, food processing plants, and farm environments (5–11). In one New York study, *L. seeligeri* and *L. welshimeri* were dominant species in water and other environmental samples from pristine environments, while *L. innocua* and *L. monocytogenes* were associated with urban environments (8). Focusing on *Listeria* spp. diversity, Chapin et al. (12) reported finding *Listeria* spp. (excluding *L. monocytogenes*) in 22 and 51% of the surface water samples from pristine and produce growing areas, respectively, in New York. Linke et al. (11) detected *Listeria* spp. at similar levels (26%) in surface water samples from the pristine natural environment in different parts of Austria. As for the pathogenic *L. monocytogenes* in surface waters, recent culture-based studies have reported prevalence of 10% in Ontario, Canada (13), and 28 to 43% in various watersheds in New York and California (14–17).

Several methods of characterizing *L. monocytogenes* isolates in water- and food-borne outbreaks and ecological studies are available to elucidate the transmission of the pathogen through the environment and into our food and drinking water supply. A multiplex PCR method divides *L. monocytogenes* isolates into five serogroups (IIa [1/2a and 3a serovars], IIb [1/2b, 3b, and 7 serovars],

IIc [1/2c and 3c serovars], IVa [4c serovar], and IVb [4b, 4d, and 4e serovars]) (18, 19). The serogroups represent evolutionary complexes and separate the four serovars (1/2a, 1/2c, 1/2b, and 4b) that cause >98% of human listeriosis cases (20, 21). For studies of strains at a more detailed level, genotyping methods such as ribotyping, pulsotyping using pulsed-field gel electrophoresis (PFGE), multiple-locus variable-number tandem-repeat analysis (MLVA), and multilocus-sequence based typing are useful (7, 22–24), and application of these methods has shown there to be a great biodiversity of *L. monocytogenes* in surface waters (11, 13, 14, 17).

Many communities rely on surface waters as a source of raw drinking water, as well as for recreational and agricultural uses. Protection of the source water is an important step in a proactive multibarrier approach to safeguard the safety of drinking and ir-

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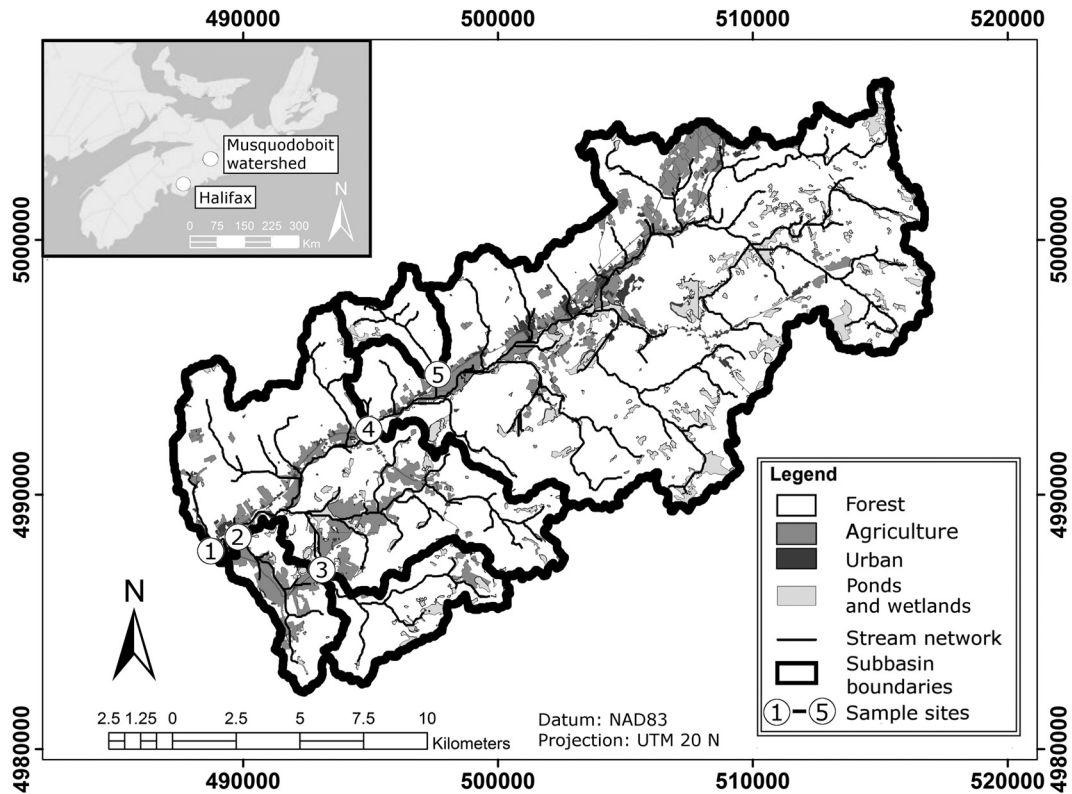


FIG 1 Middle Musquodoboit watershed in Canada and sampling locations (MR1 to MR5). (Map created using ArcGIS v10.2.)

rigation water (25). The quality of source water is commonly assessed by monitoring levels of fecal indicator bacteria (FIB) such as *Escherichia coli*. Although *E. coli* levels have been shown to predict the presence of zoonotic pathogens such as *Salmonella* spp. (26, 27), it is less certain that this approach can be used to predict the presence of an indigenous pathogen such as *L. monocytogenes*, which may or may not originate from fecal sources.

In this study, we set out to characterize the prevalence and diversity of *Listeria* spp., in particular *L. monocytogenes*, in an urban and a rural municipal source water system to understand the ecology and occurrence of this pathogen in the environment. Our specific objectives were to (i) determine the diversity of *Listeria* spp. in the urban and rural watersheds during an 18-month study period to cover all seasons, (ii) characterize the prevalence and diversity of *L. monocytogenes* in the surface waters, (iii) determine whether *E. coli* density, *Listeria* presence, or other water quality characteristics could predict *L. monocytogenes* presence in surface water, and (iv) compare PFGE patterns obtained for representative source water isolates to patterns from clinical *L. monocytogenes* strains representing all human clinical cases that occurred in Nova Scotia, Canada, during the study period.

MATERIALS AND METHODS

Collection of water and point source pollution samples. Surface water samples were collected in 33 separate sampling events from five sites in the rural and agricultural Musquodoboit River (MR) watershed (Fig. 1) and five sites in the urban/suburban Lake Fletcher (LF) watershed (Fig. 2) in Canada over the study period from January 2012 to August 2013 for a total of 329 water samples (one urban site was inaccessible in January 2012). The MR watershed is located upstream of the intake (site 1 in Fig. 1) to the

community of Middle Musquodoboit's drinking water treatment plant. The watershed covers a drainage area of approximately 135 km² and is home to several small dairy and beef cattle operations (approximately 500 animals), some of which are located along smaller tributaries flowing into the river (sites 2 and 3 in Fig. 1). The primary inflow to Lake Fletcher (site 1 in Fig. 2) drains a large (128 km²) urban catchment that originates in the city of Dartmouth, while additional smaller tributaries drain areas of suburban development (sites 3, 5, and 6 in Fig. 2).

Water samples were collected monthly during the months of November to March and biweekly for the remainder of the year. The sample sites were selected for the purpose of monitoring the effects of key agricultural and residential land uses on source water quality. On each of the sampling events, four liters of surface water was collected aseptically into 1-liter sample bottles, which had been washed and rinsed in 70% (vol/vol) ethanol.

Cow fecal samples ($n = 24$ [9 in 2012 and 15 in 2013]) were obtained from one farm in the rural watershed. In addition, septic tank samples ($n = 3$) were collected from a home located in the urban watershed (in 2012). Incoming raw sewage samples ($n = 3$) were also collected on two occasions (in 2012 and 2013) from the wastewater treatment plant located within the urban watershed (site 5 area in Fig. 2). All samples were transported back to the laboratory in coolers (maximum temperature, 5°C), and the analyses were begun immediately upon arrival (i.e., a maximum of 5 h after being sampled).

Enrichment and isolation of *Listeria* spp. from water and point source pollution samples. Aliquots of 500 ml of sample water were filtered through 0.45- μ m-pore-size membrane filters (Millipore, Etobicoke, Ontario, Canada). These filters were then placed in *Listeria* enrichment broth (LEB; Oxoid, Nepean, Ontario, Canada) and incubated for 24 h at 37°C. Next, 1 ml from the LEB culture was transferred to 9 ml of Fraser broth (Oxoid), followed by incubation for another 24 h at 37°C.

Fecal samples (250 mg) were also enriched in LEB, followed by Fraser

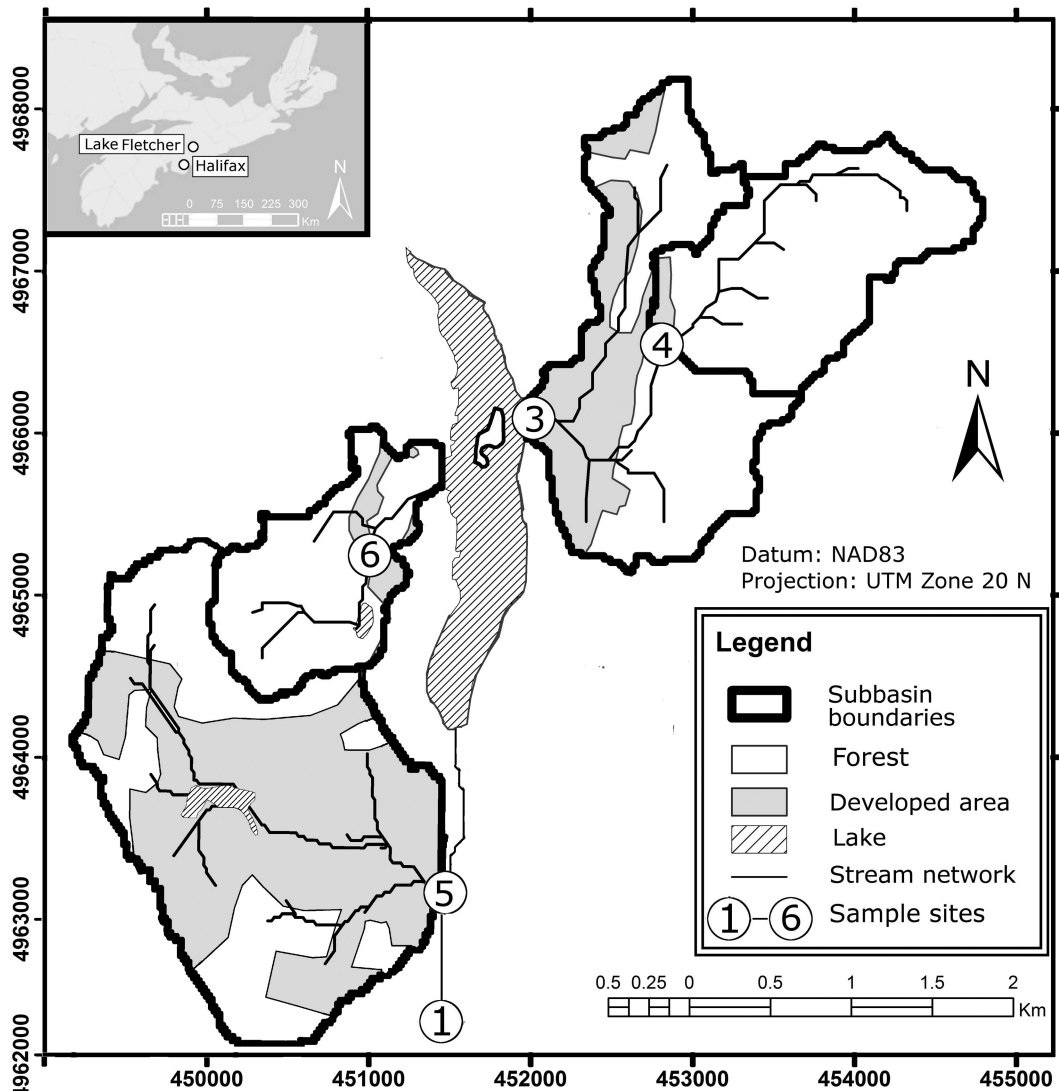


FIG 2 Lake Fletcher/Collin's Park watershed system and sampling locations (LF1 to LF6). (Map created using ArcGIS v10.2.)

broth, according to the protocol described above. Septic tank and raw sewage samples (500 ml) were mixed well prior to transferring 10 ml into a 15-ml Falcon tube for centrifugation at $3,200 \times g$ for 10 min. The pellets (ca. 250 mg) were then spiked into LEB and enriched for *Listeria* spp. as described above.

To isolate *Listeria* spp., an aliquot from the Fraser broth culture was streaked onto Palcam agar (Oxoid) and incubated for 48 h at 35°C. Up to eight black, presumptive *Listeria* colonies were picked from each positive plate, grown in brain heart infusion broth (Oxoid) for 48 h at 37°C, followed by the addition of glycerol (15% [vol/vol]; Sigma, Oakville, Ontario, Canada) and storage at -80°C until further analysis.

Diversity of *Listeria* species. Frozen cultures of the presumptive *Listeria* isolates were streaked directly on RAPID[®] *L. Mono* chromogenic agar (Bio-Rad Laboratories, Hercules, CA) which separates *Listeria* species based on their phosphoinositide phospholipase C activity and xylose metabolism. The inoculated plates were incubated aerobically at 37°C for 30 h. This method allowed us to divide the presumptive *Listeria* isolates into four groups: *L. monocytogenes*, *L. ivanovii*, the *L. innocua* group (*L. seeligeri*, *L. marthii*, and *L. grayi*), and the *L. welshimeri* group, which includes *L. rocourtiæ*, *L. fleischmannii*, *L. weihenstephanensis*, *L. floridensis*, *L. aquatic*,

L. cornellensis, *L. grandensis*, and *L. riparia* (see Table S1 in the supplemental material).

Serogrouping of *L. monocytogenes* isolates. A multiplex PCR method was performed to confirm the identity (*prfA* positive) and determine the serogroup of each *L. monocytogenes* isolate (Table 1). The method was based on the work by Doumith et al. (18) and K erouanton et al. (19) and was performed in a Biometra T-Gradient thermocycler (Biometra, G ttingen, Germany) using the following PCR program: initial denaturation for 3 min at 94°C, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 53°C for 45 s, and extension at 72°C for 1 min 15 s, and then a final extension for 7 min at 72°C. Each colony PCR (25 μl) consisted of ~ 0.5 μl of colony mass, 1 U of *Taq* DNA polymerase (IDTaq *Taq* polymerase kit; IDTaq, London, Ontario, Canada), $1 \times \text{MgCl}_2$ -free buffer, 3 mM MgCl_2 , 1 mM concentrations of deoxynucleoside triphosphates, 0.4 μM concentrations of each of the *lmo737F/R*, *lmo1118F/R*, *orf2819F/R*, and *orf2110F/R* primer sets, and a 0.2 μM concentration of the *LIP1/LIP2a* (*prfA*-F/R) primer set and diethyl pyrocarbonate-water.

The amplified PCR products were transferred to the wells of a 2% (wt/vol) agarose (Fisher Scientific, Oakville, Ontario, Canada) gel, containing 0.016% (vol/vol) GelRed (Biotium, Hayward, CA) for visualiza-

TABLE 1 Primers and probes used for the TaqMan assay and multiplex PCR experiments

Assay and primer	Sequence (5'–3') ^a	Annealing temp (°C)	Amplicon size (bp)	Reference
TaqMan assay for detection of <i>L. monocytogenes</i>				
HlyQF	CATGGCACCACCAGCATCT	56	64	28
HlyQR	ATCCGCGTGTTCCTTTTCGA			
HlyQP	FAM-CGCCTGCAAGTCCTAAGACGCCA-TAMRA			
Serogrouping of <i>L. monocytogenes</i> (by PCR)				
Lmo737F	AGGGCTTCAAGGACTTACCC	53	691	18
Lmo737R	ACGATTTCTGCTTGCCATTC			
Lmo1118F	AGGGGTCTTAAATCCTGGAA	53	906	
Lmo1118R	CGGCTTGTTCCGCATACTTA			
Orf2819F	AGCAAATGCCAAAACCTCGT	53	471	
Orf2819R	CATCACTAAAGCCTCCCATTTG			
Orf2110F	AGTGGACAATTGATTGGTGAA	53	597	
Orf2110R	CATCCATCCCCTTACTTTGGAC			
LIP1 (<i>prfAF</i>)	GATACAGAAAACATCGGTTGGC	53	274	19
LIP2a (<i>prfAR</i>)	GTGTAATCTTGATGCCATCAGG			

^a TaqMan probe labels: FAM, fluorescein; TAMRA, 5(6)-carboxytetramethylrhodamine.

tion, in 1 × Tris-borate-EDTA buffer (Sigma) and resolved by electrophoresis (80 min, 85 V) in a Hoefer submarine HE33 (GE Health Care Life Sciences, Baie d'Urfe, Quebec, Canada). The resulting gel band patterns were then used to confirm the identity and assign the *L. monocytogenes* isolates to their respective serogroups.

L. monocytogenes presence or absence testing using a TaqMan assay.

For TaqMan real-time PCR (TaqMan assay) detection of *L. monocytogenes* in the water samples, 2 ml of the Fraser enrichment broth was centrifuged at 3,200 × g for 10 min. The supernatant was removed, and 250 µl of resuspended pellet was subjected to DNA extraction using the PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions.

DNA was directly extracted from the bovine feces samples (250 mg; $n = 24$) by using a PowerSoil DNA extraction kit (MoBio Laboratories). Septic tank and raw sewage samples (500 ml) were mixed well prior to transferring 10 ml into 15-ml Falcon tubes for centrifugation (3,200 × g, 10 min). The supernatant was decanted, and 250 mg of resuspended pellet was used for DNA extraction using a PowerSoil DNA extraction kit (MoBio). Along with the samples, DNA was also extracted from a negative extraction control (250 µl of sterile water) and other negative controls (enrichment media). All DNA samples were stored at –20°C until further analysis.

Each TaqMan assay reaction mixture (25 µl) consisted of the following: DNase-free water (7.7 µl; Fisher Scientific, Ottawa, Ontario, Canada), master mix (12.5 µl; Applied Biosystems Fast Advanced 2×; Applied Biosystems, Burlington, Ontario, Canada), 0.3 µl each of 10 µM concentrations of forward and reverse primers, 0.2 µl of 10 µM concentrations of TaqMan probes, and 4 µl of sample DNA. Primers and probes (Sigma, Oakville, Ontario, Canada) for *L. monocytogenes* targeted the *hly* gene as outlined by Rodríguez-Lázaro et al. (28) (Table 1). Amplification and detection were done using a StepOne Plus system (Applied Biosystems) and the following thermocycler program: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min.

The test results were scored as *L. monocytogenes* being present or absent in 500 ml of water, 10 ml of wastewater, or 250 mg of bovine feces. DNA extracted from *L. monocytogenes* 568 (serogroup IIa) was used as the positive control in each TaqMan assay along with negative or no-template controls. The TaqMan assay PCR efficiencies ranged from 88 to 105% (within the standard operating procedures for pathogen detection), as determined using a 10-fold dilution series of the positive-control sample. The limit of detection (LOD) for the (enriched) water samples was 1 CFU in 500 ml (enriched to minimum levels of 50 CFU/ml in the Fraser en-

richment broth), while LODs for wastewater and bovine feces samples were 10 and 100 CFU/g, respectively. The absence of PCR inhibitors in each sample type was confirmed in spiking assays with the positive-control strain (data not shown).

PFGE on a subset of *L. monocytogenes* isolates. Results from the serogrouping multiplex PCR showed that all six to eight *L. monocytogenes* isolates from a culture-positive sample belonged to the same serogroup. Therefore, one *L. monocytogenes* isolate was selected from each culture-positive water sample, for a total of 54 *L. monocytogenes* strains, to be subjected to the PFGE analysis (see Table 3; 2 of the 56 positive samples were inadvertently omitted from the analyses).

PFGE was performed following the standard CDC PulseNet Protocol (29) on a Bio-Rad Chef Mapper (Bio-Rad, Hercules, CA). Isolates were grown overnight at 37°C in tryptic soy broth (Oxoid). Plugs were created using 1% (wt/vol) Megabase agarose (Bio-Rad) and digested separately with the restriction enzymes AscI (New England BioLabs [NEB], Whitby, Ontario, Canada) and ApaI (NEB) for 4.5 h at 37°C (AscI) and 25°C (ApaI). *Salmonella enterica* serotype Braenderup (H9812) was digested for 4.5 h with the XbaI enzyme (NEB) and used as a reference standard (30). Run conditions on the Chef Mapper were as follows: 18 h at 6 V/cm with switch times of 4 s to 40.01 s. PFGE images were captured with a Bio-Rad Versa Doc. Patterns from PFGE images were analyzed by using BioNumerics software version 7 (Applied Maths, Saint-Martine, Belgium). Included in the analyses were the PFGE patterns from the 19 human clinical listeriosis cases that occurred in Nova Scotia, Canada, from 2011 to 2013, which were generously provided by the PulseNet Canada Team at the National Microbiology Laboratory (NML), Public Health Agency of Canada (Winnipeg, Canada) and R. Davidson (Departments of Microbiology and Immunology/Medicine, Dalhousie University). The human *L. monocytogenes* strains were originally isolated from blood, cerebrospinal, or peritoneal fluids from affected individuals at the Anchor Laboratories of the Provincial Public Health Laboratory Network, Nova Scotia and subsequently pulsed at NML using the CDC PulseNet Protocol.

Water quality testing and enumeration of *E. coli* and total coliforms.

The turbidity in all water samples was determined using a 2100AN IS laboratory turbidimeter (Hach, Mississauga, Ontario, Canada). Using a handheld 600R Sonde device (YSI, Yellow Springs, OH), water temperature (°C) was measured during each sampling event.

E. coli and total coliforms were enumerated using the m-ColiBlue medium (Millipore) according to the manufacturer's instructions. Briefly, sample (undiluted or diluted 10-fold in phosphate-buffered saline) volumes of 100 ml were filtered through a 0.45-µm-pore-size mem-

TABLE 2 Prevalence and diversity of *Listeria* spp. in water samples from the rural (MR) and urban (LF) source watersheds^a

Site	<i>Listeria</i> spp. (%) ^b	No. (%) of water samples						
		Single species ^c				Mixed species ^d		
		<i>L. innocua</i>	<i>L. welshimeri</i>	<i>L. ivanovii</i>	<i>L. monocytogenes</i>	Two spp.	Three spp.	Mixed
Rural	72.1	45 (37.8)	23 (19.3)	2 (1.7)	18 (15.1)	24 (20.2)	7 (5.9)	18 (15.1)
MR1 WTP ^e intake/river	84.8	12	8	0	1	7	0	4
MR2 tributary A	75.8	13	2	1	2	4	3	4
MR3 tributary B	78.8	14	4	0	4	3	1	3
MR4 river (upstream)	69.7	4	3	1	7	6	2	4
MR5 tributary C	51.5	2	6	0	4	4	1	3
Urban	35.4	13 (22.4)	18 (31.0)	3 (5.2)	13 (22.4)	11 (19.0)	0 (0)	7 (12.1)
LF1 Lake Fletcher run/inlet	18.2	3	0	1	0	2	0	0
LF3 tributary A (outlet to lake)	27.3	5	2	0	1	1	0	1
LF4 tributary A (headwater)	48.5	1	4	2	5	4	0	2
LF5 tributary B	60.9	4	10	0	3	3	0	3
LF6 tributary C	21.9	0	2	0	4	1	0	1
Total	53.8	58 (32.8)	41 (23.2)	5 (2.8)	31 (17.5)	35 (19.8)	7 (4.0)	25 (14.1)

^a *Listeria* spp. were detected after enrichment and plating on Palcam agar, followed by determination of the species for up to eight *Listeria* isolates per water sample on RAPID[®] L. Mono agar to yield *Listeria* species diversity.

^b Percent positive samples (33 samples per site [32 for LF6] for a total of 165 and 164 for the rural and urban watersheds, respectively).

^c Number of water samples (% of all *Listeria* spp.-containing samples) where all *Listeria* spp. isolates belonged to the same species. The *L. innocua* group includes *L. seeligeri*, *L. marthii*, and *L. grayi*; the *L. welshimeri* group includes *L. rocourtae*, *L. fleischmannii*, *L. weihenstephanensis*, *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. grandensis*, and *L. riparia*.

^d Number of water samples that contained two or three different *Listeria* spp. "Mixed" refers to water samples in which *L. monocytogenes* cooccurred with one ($n = 20$) or two ($n = 5$) other *Listeria* spp.

^e WTP was the sampling site located immediately adjacent to the intake of raw source water from the river to the local drinking water treatment plant.

brane (Millipore). Filters were transferred aseptically to petri dishes (37 mm in diameter; Millipore) containing absorbent pads with 2 ml of m-Coliblu broth. The plates were incubated at 37°C for 24 h and enumerated.

Statistical analysis. The normality of the data was evaluated by using Shapiro-Wilk normality tests in SYSTAT software (Chicago, IL). Binary logistic regression analyses were carried out in SYSTAT to determine the odds ratios (ORs) for the ability of the *L. monocytogenes* TaqMan assay results to predict the presence of culturable *Listeria* spp. and *L. monocytogenes* in water samples (ORs with 95% confidence intervals [CI]). Other OR calculations included evaluating whether the detection of *Listeria* spp. predicted the presence of culturable *L. monocytogenes* and explored possible associations between elevated *E. coli* (≥ 100 CFU/100 ml) and turbidity (nephelometric turbidity units [NTU] ≥ 1.0) levels and presence of *Listeria* spp. and *L. monocytogenes* (as detected by the molecular and culture-based methods). Multidimensional scaling (MDS) and cluster analysis using the Dice coefficient and unweighted pair group matching algorithm (UPGMA) with a tolerance of 1.5% of PFGE pattern data were completed in BioNumerics 7 (Applied Maths). Simpson's index of discrimination (D) for *Listeria* spp. and *L. monocytogenes* pulsed-field diversity in the watersheds was calculated as outlined by Hunter and Gaston (31).

RESULTS

Prevalence and diversity of presumptive *Listeria* spp. in water and point source pollution samples. Presumptive *Listeria* spp. were isolated from 177 (53.8%) of the 329 water samples, which were collected over the course of the study, with the detection rates being higher in the rural watershed (72.1%) than in the urban watershed (35.4%) (Table 2). Consequently, more *Listeria* isolates were obtained from rural watersheds ($n = 860$) than the urban watersheds ($n = 424$) for a total of 1,284 isolates.

Species determination on RAPID[®] L. Mono agar of up to eight isolates obtained from each *Listeria*-positive sample showed that two or three *Listeria* spp. were present in 42 (23.8%) of the sam-

ples (Table 2). Of the 56 samples that tested positive for *L. monocytogenes* by the culture method, the pathogen was found to co-occur with other *Listeria* species in 25 (44.6%) samples. The diversity of *Listeria* spp. in the rural watershed was dominated by the *L. innocua* group, which was found in a total of 56.3% of single and mixed species *Listeria* genus-positive samples, followed by the *L. welshimeri* group (37%), *L. monocytogenes* (30.3%), and *L. ivanovii* (8.4%). Members of the *L. welshimeri* group (43.1%) were more common in water samples from the urban watershed, followed by the *L. innocua* group and *L. monocytogenes* (both 34.5%) and *L. ivanovii* (6.9%). Only 5 of the 177 *Listeria*-positive samples from the watersheds contained *L. ivanovii* alone with another 9 samples being positive for *L. ivanovii*, together with other *Listeria* spp., including *L. monocytogenes*, indicating that *L. ivanovii* occurred in 7.9% of the *Listeria*-positive samples and in 4.3% of all samples. The *Listeria* diversity was slightly higher for the urban watershed listerial population ($D = 0.687$) compared to the rural watershed population ($D = 0.622$).

The culture-based method yielded no *L. monocytogenes* isolates from the point source samples (cow feces [$n = 15$; only samples from 2013 were tested], sewage [$n = 3$], and septic tanks [$n = 3$]). Other *Listeria* species were isolated, including the *L. innocua* group and *L. ivanovii* from two cow fecal samples and *L. ivanovii* from one of the raw municipal sewage samples.

***L. monocytogenes* detection in samples from the rural and urban watersheds.** Analyses by the TaqMan assay revealed detection rates of *L. monocytogenes* ranging from 57.6 to 75.8% (average, 67.3%) and 48.5 to 60.6% (average, 56.1%) at the rural and urban watershed sampling sites, respectively (Table 3). Of the sampling sites, MR2 (MR tributary A in close vicinity of cattle farms), LF4 (undeveloped forested area, headwaters of LF tributary A), and LF5 (LF tributary B, close to urban development)

TABLE 3 Prevalence of *L. monocytogenes* as determined using TaqMan assay and culture methods in surface water samples from a rural and urban watershed in Nova Scotia, Canada, during an 18-month monitoring period from 2012 to 2013

Watershed	Type	Detection (% positive) ^a		<i>L. monocytogenes</i> serotype diversity (%) ^b			
		TaqMan assay	Culture based	IIa	IIb	IIc	IVb
Musquodoboit River	Rural	67.3 (111/165)	21.8 (36/165)	76.8	12.5	0.6	10.1
MR1	WTP intake/river	66.7 (22/33)	15.2 (5/33)	34.8	65.2	0	0
MR2	Tributary A	75.8 (25/33)	18.2 (6/33)	66.7	33.3	0	0
MR3	Tributary B	57.6 (22/33)	21.2 (7/33)	96.7	0	3.3	0
MR4	River (upstream)	72.7 (24/33)	33.3 (11/33)	71.7	0	0	28.8
MR5	Tributary C	72.7 (24/33)	21.2 (7/33)	100	0	0	0
Lake Fletcher	Urban	56.1 (92/164)	12.2 (20/164)	54.7	20.5	0	24.8
LF1	Lake Fletcher run/inlet	51.5 (17/33)	0	0	0	0	0
LF3	Tributary A (outlet to lake)	48.5 (16/33)	6.1 (2/33)	0	100	0	0
LF4	Tributary A (headwater)	60.6 (20/33)	21.2 (7/33)	76.6	0	0	23.4
LF5	Tributary B	60.6 (20/33)	18.2 (6/33)	71.0	25.8	0	54.8
LF6	Tributary C	59.4 (19/32)	15.6 (5/32)	54.7	20.5	0	24.8
Total		61.7 (203/329)	17.0 (56/329)	67.7	15.8	0.4	16.1

^a Values are expressed as the percentage of *L. monocytogenes*-positive samples (number of positive results/total number of samples).

^b Serogroup diversity among *L. monocytogenes* isolates ($n = 285$).

showed the highest levels of *L. monocytogenes* detection of 75.8 and 60.6% in their respective watersheds. In contrast, the culture-based detection gave fewer positives for *L. monocytogenes*, with detection rates ranging from 15.2 to 33.3% (average, 21.8%) and 0 to 21.2% (average, 12.2%) for the sampling sites in the rural and urban watersheds, respectively (Table 3). It should be noted that the TaqMan assay was performed on DNA extracted from the enrichment cultures, making it less likely that the positive amplification results were due to amplification of DNA from dead cells.

Both detection methods pointed to *L. monocytogenes* being more prevalent in the rural watershed than in the urban watershed. Detection of *L. monocytogenes* by the TaqMan assay was associated with a 4.7 times higher risk of obtaining *L. monocytogenes* isolates from the same sample (OR = 4.7, CI = 2.1 to 10.3, $P < 0.001$, $\rho^2 = 0.064$). A positive TaqMan assay result predicted a 2.3-fold higher risk of *Listeria* spp. being cultured from the same water samples (OR = 2.3, CI = 1.4 to 3.6, $P < 0.001$, $\rho^2 = 0.030$). Importantly, detection of *Listeria* spp. was associated with a 70-fold higher risk of obtaining a culture-based positive *L. monocytogenes* result (OR = 69.9, CI = 9.5 to 512.2, $P < 0.001$, $\rho^2 = 0.232$).

In 2012, the majority of dairy cows (randomly selected across all age groups) tested positive for *L. monocytogenes* (88.8%, or 8/9) by the TaqMan assay, while the opposite occurred in 2013, where only 13% (2/15) of the bovine feces contained *L. monocytogenes*. All raw municipal sewage samples tested positive for *L. monocytogenes* (3/3) in the TaqMan assay, while the septic tank samples tested negative (0/3).

PCR-based serogrouping of the 285 *L. monocytogenes* isolates showed that serogroup IIa strains dominated at most watershed sampling sites, with the exception of MR1 (main river, adjacent to the intake to the water treatment plant) and LF3 (bottom of tributary A, outlet to lake) where serogroup IIb strains prevailed, and LF6 (tributary C in developed urban area) where serogroup IVb strains formed the majority (Table 3). Serogroup IIc accounted for only 0.4% of the *L. monocytogenes* isolates, followed by IIb (15.8%), IVb (16.1%), and IIa (67.7%).

***E. coli* and turbidity as predictors of the presence of *Listeria* spp. and *L. monocytogenes* in surface waters.** *E. coli* levels in the water samples ranged from below the detection limit of 1 to 2,080 CFU/100 ml, with the concentrations of *E. coli* being significantly ($P < 0.05$) lower in the urban watershed (median, 2 CFU/100 ml) than in the rural watershed (median, 22 CFU/100 ml). *E. coli* concentrations of ≥ 100 CFU/100 ml occurred in 12 and 35 water samples from the urban and rural watersheds, respectively, and were associated with a 5.2-fold increased likelihood of the simultaneous presence of *Listeria* spp. (OR = 5.2, CI 2.3 to 11.4, $P < 0.001$, $\rho^2 = 0.046$) but not *L. monocytogenes* ($P > 0.05$) regardless of the detection method.

Turbidity (NTU) is regularly measured by water utilities as a source water quality indicator. NTU levels of ≥ 1.0 came with a 17.3-fold higher risk (OR = 17.3, CI 4.1 to 73.1, $P < 0.001$, $\rho^2 = 0.130$) of detecting *E. coli* levels at or above 100 CFU/100 ml and a 2.1-fold (OR = 2.1, CI 1.3 to 3.4, $P < 0.01$, $\rho^2 = 0.023$) higher likelihood of detecting *Listeria* spp. in the samples. However, there was no significant ($P > 0.05$) relationship between NTU levels of ≥ 1.0 and the detection of *L. monocytogenes*.

Influences of season and storm events on prevalence of *L. monocytogenes*. *L. monocytogenes* was detected year round in both watersheds, with TaqMan assay-positive detection in 76.9, 59.1, 55.4, and 72.0% of the water samples obtained during the winter (December to February, $n = 39$, average water temperature of 1.2°C), spring (March to May, $n = 110$, average water temperature of 8.1°C), summer (June to August, $n = 130$, average water temperature of 17.8°C), and fall (September to November, $n = 50$, average water temperature of 13.0°C) periods, respectively.

Six and three storm events (>20 -mm rainfall) were captured in the rural and urban watershed, respectively, during the study. Storm event-associated detection rates of *L. monocytogenes* in the TaqMan assay tended to be higher than base-flow detection rates in the rural watershed with 73.3% of the 30 storm samples in the rural watershed testing positive versus 65.8% of the 135 base-flow samples. The opposite was observed in the urban watershed, where the *L. monocytogenes* TaqMan assay yielded lower detection rates of 40% (6 of the 15 samples) during storm events compared

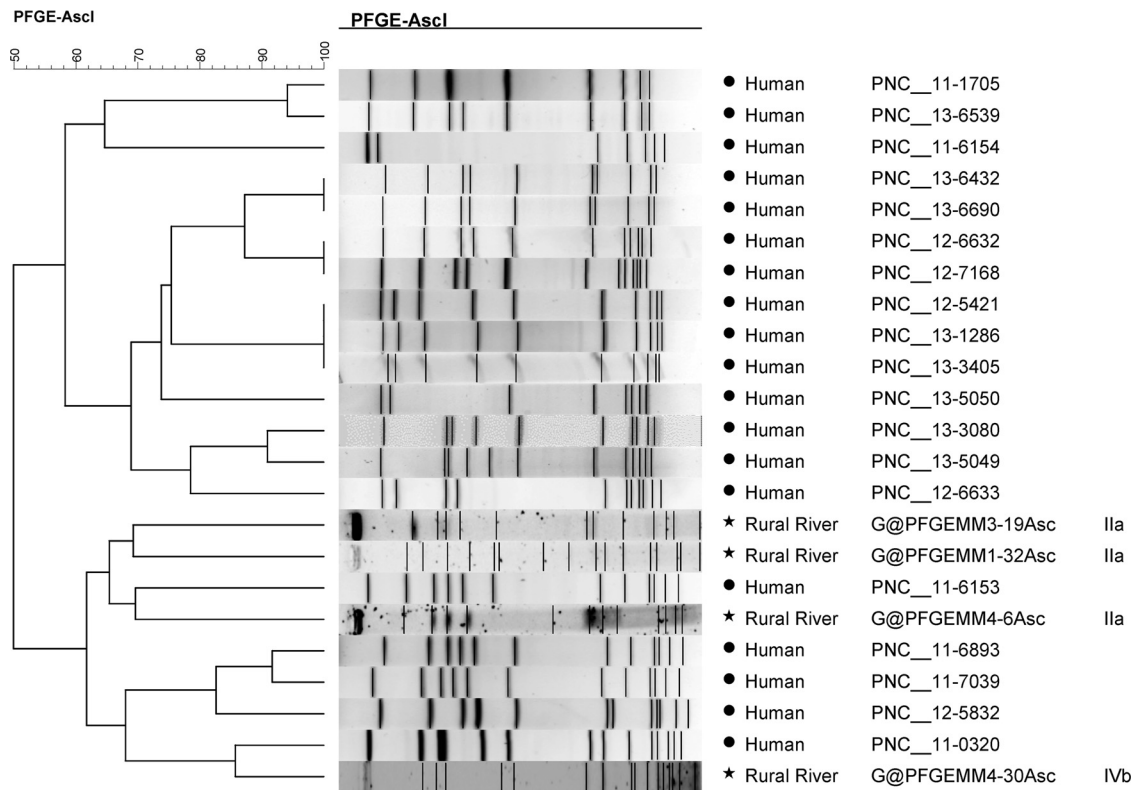


FIG 3 UPGMA clustering analysis of human *L. monocytogenes* isolate AscI PFGE patterns and selected *L. monocytogenes* watershed isolates using the Dice coefficient with a tolerance of 1.5%. *L. monocytogenes* NS human food-borne outbreak isolates are labeled “human,” MR *L. monocytogenes* isolates are labeled “rural river,” and LF isolates are labeled “urban stream.”

to the 57.7% testing positive found in 149 base-flow water samples.

Pulsotype comparison of representative *L. monocytogenes* surface water and NS human isolates. A large diversity of pulsotypes ($n = 52$) was found among isolates collected from water samples ($n = 54$) (see Fig. S1 and S2 in the supplemental material), leading to a Simpson’s index of discrimination (D) of 0.99 for both watersheds. One strain (LF6-5) did not produce a pattern. Only two isolates collected during the same sampling run (MR2-4 and MR3-4, collected ~8 km apart in the rural watershed) showed matching AscI/ApaI combination patterns (>80% similarity). Fourteen PFGE types were found among the human clinical isolates and several (9/19) showed identical AscI/ApaI patterns with >95% similarity (Fig. 3 and 4).

When AscI and ApaI patterns were analyzed separately (Fig. 3 and 4), the AscI pattern of human isolate 11-0320 showed 85% similarity with the rural river water isolate MR4-30. Similarly, the human isolate 12-5832 shared 83% similarity with the urban water strain LF5-10 using ApaI patterns, while human isolates 13-3080 and 13-5049 both shared 80% similarity with urban watershed strains LF5-17 and LF3-9.

The pulsotypes did not cluster by serogroup (see Fig. S1 and S2 in the supplemental material). Isolates from water samples that were highly similar to human isolates using AscI patterns were not similar to the same human isolates using ApaI patterns. Furthermore, water isolates that were highly similar in regard to their AscI patterns were not grouped together using ApaI patterns and vice versa, with the exception of MR2-4 and MR3-4 and human isolates noted above.

Results from cluster analysis and multidimensional scaling (MDS) of AscI and ApaI patterns from the pulsed *L. monocytogenes* isolates ($n = 53$ of the original 285 isolates) and 2011-3 Nova Scotia human isolates ($n = 19$; total $n = 72$) indicated a higher level of similarity among the rural and urban watershed isolates. The MDS analysis (Fig. 5) did not separate strains based on their origin, indicating overlaps in genetic traits among clinical and environmental isolates from the rural and urban watershed. However, some but not all human isolates formed a distinct cluster MDS plot based on the ApaI patterns (Fig. 5).

DISCUSSION

Prevalence and diversity of *Listeria* spp. in surface water and point source pollution samples. The present study discovered a diverse *Listeria* population in 35.4 and 72.1% of the urban and rural watershed samples, respectively, indicating differences among the watersheds. Other studies also report widespread but variable occurrence of *Listeria* spp. in the environment. Sauders et al. (8) reported an overall *Listeria* prevalence of 16 and 33% in surface waters from urban and natural environments, respectively, in New York State (NYS). A survey of five produce farms and five natural sites in NYS over a 2-year period yielded 51% culture-positive water samples ($n = 264$), which was higher than the prevalence in soil (17%), drag swab (21%), and fecal samples (16%) (12). Interestingly, we found a similar culture-based prevalence of just 14% in our fecal point source samples (cow feces, raw sewage, and septic tank). In Austria, Linke et al. (11) isolated *Listeria* from 26.5% of the water samples, while Frances et al. (32)

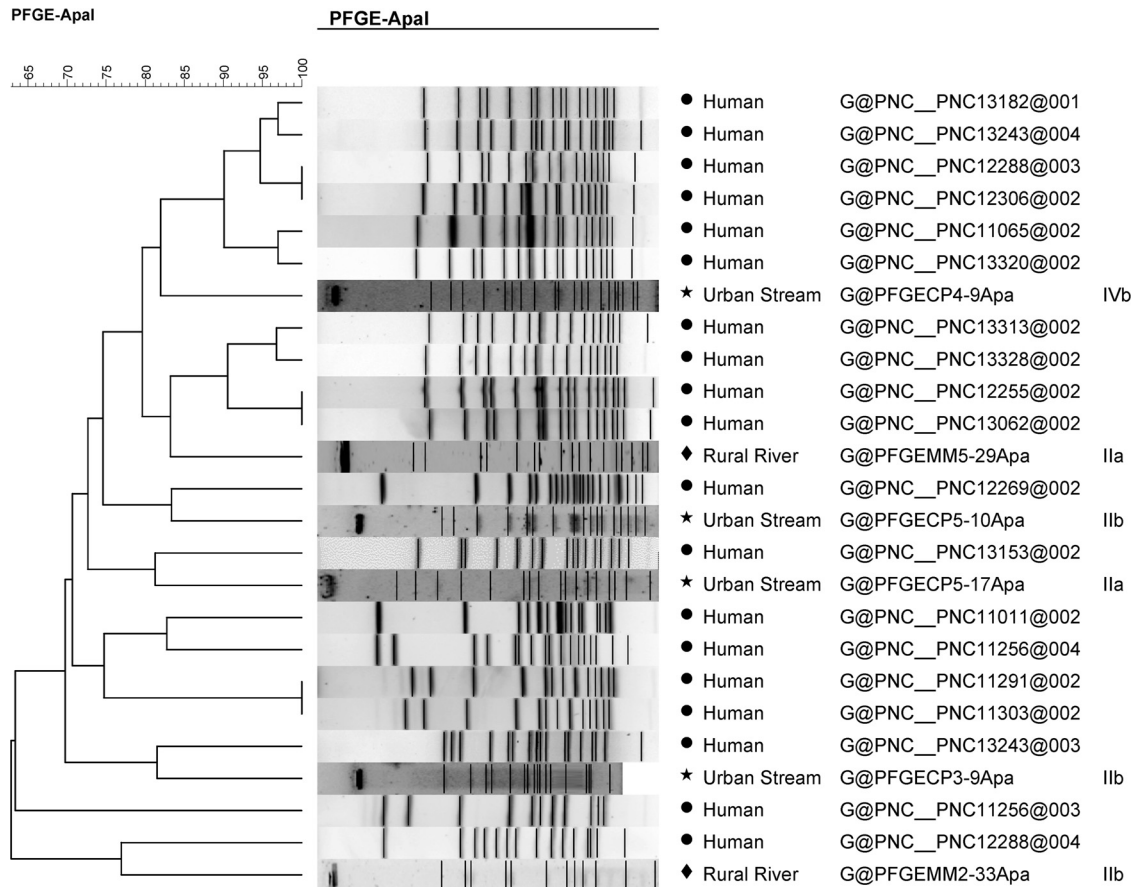


FIG 4 UPGMA clustering analysis of human *L. monocytogenes* isolate ApaI PFGE patterns and selected *L. monocytogenes* watershed isolates using the Dice coefficient with a tolerance of 1.5%. *L. monocytogenes* NS human food-borne outbreak isolates are labeled “human,” MR *L. monocytogenes* isolates are labeled “rural river,” and LF isolates are labeled “urban stream.”

in a small culture-based study of surface waters ($n = 21$) in the United Kingdom reported finding *Listeria* in 27% of samples.

Isolation of up to eight strains from each *Listeria*-positive sample (average of 7.25 strains/sample) showed that 23.8% of the samples contained two or three *Listeria* spp., which is higher than the 6 to 8% previously reported in studies where three or four isolates per sample were characterized (7, 11). The diversity in the rural watersheds was slightly lower ($D = 0.622$) and dominated by the *L. innocua* group (including *L. seeligeri*) compared to urban samples ($D = 0.687$), which were dominated by the *L. welshimeri* group. The diversity of *Listeria* spp. in surface water samples appear to vary among regions as studies in NYS reported high levels of *L. seeligeri* and *L. welshimeri* in pristine environments and *L. innocua* in produce farming and urban environments (7, 12). The Austrian study similarly showed significant geographical variations in the dominant *Listeria* populations (11). Taken together, this indicates considerable geographical variation in listerial biodiversity, which likely is linked to farming practices and other anthropogenic impacts.

Our culture-based analysis of the cow feces and raw municipal sewage samples yielded only *L. innocua* group and *L. ivanovii* isolates. A molecular-detection-based survey of urban wastewater treatment effluent in France indicated the presence of *Listeria* spp. in 84% of samples with *L. monocytogenes* and *L. innocua* in 75 and 63% of the samples, respectively, indicating co-occurrence (5).

***L. monocytogenes* in surface water and point source pollution samples.** Higher detection rates of *L. monocytogenes* occurred when the water and point source pollution samples were analyzed by the TaqMan assay (61.7%) compared to the culture-based method (17%). This discrepancy may be due to *L. innocua*, and possibly other *Listeria* spp., outcompeting *L. monocytogenes* during the selective enrichment process (33–36). Oravcová et al. (37) reported that culture-based detection of *L. monocytogenes* in the presence of *L. innocua* was consistently lower than when the same samples were analyzed by PCR, indicating that the TaqMan assay results in our study may more accurately reflect the prevalence of the pathogen. Also, the use of Palcam and RAPID[®] *L. Mono* agar may result in lower recoveries of less virulent strains (38), which might have been detected in our TaqMan assay. Interestingly, a recent European validation study found >90% agreement between plating methods using Palcam and quantitative PCR (qPCR) detection (39). Lastly, our culture-based approach characterized eight isolates per sample, meaning that *L. monocytogenes* populations constituting <12.5% of the *Listeria* population in a given sample might have been missed and possibly contributing to the discrepancy between culture-based and molecular methods.

Detection of *L. monocytogenes* was a common occurrence in both natural and anthropogenically impacted sampling sites (Table 3), pointing to watershed as an important reservoir. The reported culture-based prevalence of *L. monocytogenes* in different

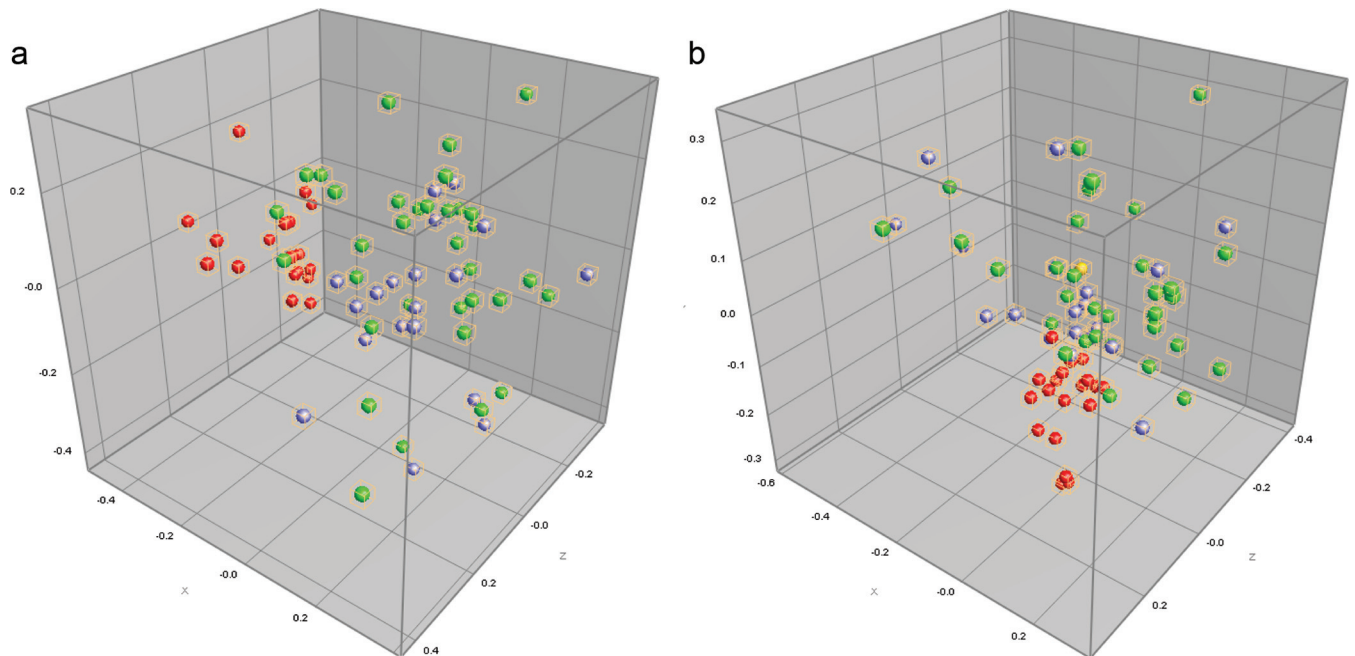


FIG 5 AscI multidimensional scaling in three dimensions in the coordinate space (a), followed by ApaI multidimensional scaling in three dimensions (b). Samples from the rural watershed (MR) are green/yellow, while samples from the urban watershed (LF) are represented by purple symbols. NS human food-borne outbreak isolates are indicated in red. The proportion of the data variance (r^2) accounted for by MDS was 0.72.

geographical regions ranges from 50% in South African rivers (40), 43% in five Californian watersheds (14), 30% in water samples from a produce-producing region in NYS (15), 30% in river, lake and stream samples ($n = 206$) from the Central California Coast (17), 12.8% of spring and river samples ($n = 148$) from Switzerland (41), 10% of water samples ($n = 314$) from a mixed-use watershed in Ontario, Canada (13), 1.7 and 10.9% of natural and urban water samples ($n = 486$), respectively, in NYS (8), and 3.9% in surface water samples ($n = 128$) in Northern Greece (42) to none in water samples ($n = 30$) obtained in Cheshire and Northern Wales (32) and from the natural environment ($n = 68$) in Austria (11).

Although we detected more *L. monocytogenes*-positive samples in the rural agricultural watershed, Sauders et al. (8) reported a significant association between culturable *L. monocytogenes* (and *L. innocua*) and urban watersheds. This is likely due to differences in local farming practices, urban development, drainage, wastewater management, etc.

Most of the cattle tested positive for *L. monocytogenes* (by the TaqMan assay) in 2012; however, only 13% tested positive in 2013, indicating variability in carriage among farm animals. *L. monocytogenes* has previously been cultured from animal feeds and feces (43–46) and *L. monocytogenes* was among the most abundant pathogens found in feedlot cattle manure in Australia (47).

In our study, serogroup IIa (1/2a and 3a serovars, 68%) were predominant among the *L. monocytogenes* isolates from the watersheds. These results are similar those reported in another Canadian study, where serogroups IIa and IVb constituted 50 and 32% of the isolates, respectively, in a mixed-use watershed (13) but different from the findings of predominantly serogroup IVb (>80%) isolates in water ways on the Central Coast of California

(14, 17), suggesting that *L. monocytogenes* populations are diverse in surface waters and the natural environment.

***E. coli*, turbidity, and *Listeria* spp. as predictors of *L. monocytogenes* in surface water.** Elevated *E. coli* levels (≥ 100 CFU/100 ml) were associated with 5.2-fold-higher likelihood of detecting *Listeria* spp. but were not related to the presence of *L. monocytogenes* as determined using a culture-based or molecular detection method. Other studies have shown absence of relationships between *E. coli*/FIB and specific pathogens (48, 49), which may be due to differences in physiological factors between the pathogen and indicator organisms (50). However, in the case of *L. monocytogenes* it is most likely due to it being naturalized in the environment, as also indicated by our detection of the pathogen at the pristine headwater of tributary A in the urban watershed (LF4, Table 3). Turbidity levels of ≥ 1 NTU were associated with higher risks of detecting elevated *E. coli* levels and *Listeria* spp. but not *L. monocytogenes*, indicating the limitations of this widely used source water quality indicator. Culture-based detection of *Listeria* spp. came with a 70-fold-higher risk of also isolating *L. monocytogenes* from the sample, supporting the suggestion of Chapin et al. (12) that *Listeria* spp. can be used as index organisms for the presence of *L. monocytogenes*.

Seasonality and storm-related detection of *L. monocytogenes*. In contrast to the findings of detection of *L. monocytogenes* being associated with storms and flooding in Austria (11), the prevalence of *L. monocytogenes* was not related to storm events in the present study. Although *L. monocytogenes* was detected year round in water samples, detection rates tended to be higher in the temperate to colder months (September to February), possibly due to less competition from the autochthonous microflora and adaptation to colder temperatures (1, 51). Seasonal differences in *L. monocytogenes* prevalence were also found in Ontario (Canada),

California, and NYS, where abundance tended to be higher during cooler periods (14, 16, 52).

Comparison of the pulsotypes of representative *L. monocytogenes* surface water and NS human isolates. The finding of 52 pulsotypes among 53 representative *L. monocytogenes* isolates revealed large biodiversity ($D = 0.99$) in both watersheds. The presence of host organisms such as wild animals, cattle in the rural watershed, and humans in both watersheds may have contributed to this diversity. Twenty-one pulsotypes were discerned among 32 *L. monocytogenes*-positive samples from a watershed in Ontario, leading to $D = 0.885$ (13). Similarly, a large diversity of MLVA types were found in a study of watersheds on the Central California Coast (17) and in *sigB* allelic types in NYS surface water samples (16). Taken together, these studies point toward a large biodiversity of *L. monocytogenes* in surface waters.

The two catchments in the present study may potentially serve as a reservoir for pathogenic *L. monocytogenes*, as shown by genetic similarity among some of the human and water *L. monocytogenes* isolates and the lack of distinct origin-based clusters in the MDS plots (Fig. 5). Although certain genotypes have been associated with specific sources such as humans and farms, some *L. monocytogenes* subtypes are widely distributed, including in the natural environment (53). Other studies have indicated that *L. monocytogenes* subtype populations found in farming environments overlap with human listeriosis strains (13, 44). In the former study, the majority of the pulsotypes of fecal *L. monocytogenes* isolates from livestock, wildlife, and humans in Ontario, Canada, matched clinical isolate patterns in the PulseNet Canada National *Listeria* Database (13). Such virulent *L. monocytogenes* can possibly be transferred to soil and water ways via nonsanitized sludge being spread onto agricultural fields (54) or from failing on-site septic systems. The risk of transmission of *L. monocytogenes* from different point sources into surface waters warrants further study, including the possible connectivity to *L. monocytogenes* subtypes in food plant environments and potential to infect humans and animals.

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