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Prevalence of Diarrhea-Associated Virulence Genes and Genetic Diversity in *Escherichia coli* Isolates from Fecal Material of Various Animal Hosts

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In order to assess the health risk associated with a given source of fecal contamination using bacterial source tracking (BST), it is important to know the occurrence of potential pathogens as a function of host. *Escherichia coli* isolates ($n = 593$) from the feces of diverse animals were screened for various virulence genes: stx_1 and stx_2 (Shiga toxin-producing *E. coli* [STEC]), *eae* and EAF (enteropathogenic *E. coli* [EPEC]), STh, STp, and LT (enterotoxigenic *E. coli* [ETEC]), and *ipaH* (enteroinvasive *E. coli* [EIEC]). Eleven hosts were positive for only the *eae* (10.11%) gene, representing atypical EPEC, while two hosts were positive for both *eae* and EAF (1.3%), representing typical EPEC. stx_1, stx_2, or both stx_1 and stx_2 were present in 1 (0.1%,) 10 (5.56%), and 2 (1.51%) hosts, respectively, and confirmed as non-O157 by using a *E. coli* O157 *rfb* (rfb_{O157}) TaqMan assay. STh and STp were carried by 2 hosts (2.33%) and 1 host (0.33%), respectively, while none of the hosts were positive for LT and *ipaH*. The repetitive element palindromic PCR (rep-PCR) fingerprint analysis identified 221 unique fingerprints with a Shannon diversity index of 2.67. Multivariate analysis of variance revealed that majority of the isolates clustered according to the year of sampling. The higher prevalence of atypical EPEC and non-O157 STEC observed in different animal hosts indicates that they can be a reservoir of these pathogens with the potential to contaminate surface water and impact human health. Therefore, we suggest that *E. coli* from these sources must be included while constructing known source fingerprint libraries for tracking purposes. However, the observed genetic diversity and temporal variation need to be considered since these factors can influence the accuracy of BST results.

Management of the microbiological quality and safety of source water used for drinking and recreation is imperative, since contamination of this system can present high risks to human health. Although *Escherichia coli* is considered a nonpathogenic commensal bacterium, several diarrheagenic pathotypes such as Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* have been recognized (1), and their role has been demonstrated in many waterborne outbreaks (2–4). Of these, STEC is more frequently associated with outbreaks in the developed world, especially serotype O157 (3, 4). In addition, STEC strains other than O157 (non-O157 STEC) are increasingly being recognized as a cause of water- and food-borne outbreaks worldwide (5, 6).

The ability of STEC to cause illness is due to the production of verotoxins or Shiga-like toxins, Stx1 and Stx2 (encoded by stx_1 and stx_2 genes), and related virulence factors. Although the key feature of EPEC pathogenesis is the production of "attaching and effacing" (A/E) lesions, which is characterized by the intimate adherence of the bacteria to the intestinal epithelium. The *eae* gene, which encodes intimin, an outer membrane protein product of the *eae* located in the pathogenicity island "locus of enterocyte effacement" (LEE), is responsible for the adhesion (1). EPEC strains are defined as intimin (*eae*)-containing diarrheagenic *E. coli* that possess the ability to form A/E lesions on intestinal cells but do not possess the *stx* gene (1). EPEC is further divided into two subtypes, typical (tEPEC) and atypical (aEPEC), depending on the presence or absence of the EPEC adherence factor (EAF) plasmid (1). Although EPEC is a significant cause of gastrointestinal disease in the developing world (1), outbreaks have also been reported in developed countries (2, 7); among them, tEPEC is more dominant in developing countries, while aEPEC seems to be more important in developed countries (8). ETEC produces heat-labile (LT) and/or heat-stable (ST; variants STh and STp) enterotoxins and is an important cause of diarrhea in infants and travelers. EIEC is associated with invasive, bloody diarrhea resembling that caused by *Shigella* sp. Invasion is mediated by genes encoding, for example, Ipa proteins, and their transcription regulator invE (1).

Cattle are considered the primary reservoir of O157 and non-O157 STEC (9). However, these strains have also been isolated from a wide variety of animal species, including sheep, goat, pig, water buffalo, and wild ruminant species (9, 10). Since animals can represent the reservoir of pathogenic *E. coli*, these organisms can be transmitted to humans through the ingestion of food or water contaminated with animal feces or through contact with infected animals or their environment (9). In Canada, microbial contamination of source water is a major environmental and health issue. Of the 288 waterborne outbreaks reported during 1974 to 2001, 45% were of unknown etiology and ca. 20% were of bacterial origin (11). Considering the public health risk posed by pathogenic bacteria originating from different animals sources, for example, cattle manure as the source of contamination in the

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TABLE 1 Month and year of fecal sample collections from different animals

Sources (no. of isolates)	Mo	Yr	Total no. of isolates
Black bear (5), buffalo (5), cow (26), coyote (20), deer (10), dog (18), donkey (20), horse (9), llama (5)	June and July	2004	118
Human (25), bison (6), black bear (43), mountain goat (20), cougar (20), cow (15), dog (10), elk (29), grizzly bear (20), horse (10), pony (19), rabbit (5), bighorn sheep (16)	April to October	2005	238
Bison (5), coyote (101), cow (8), deer (79), dog (20), donkey (5), groundhog (19)	October and December	2006	237

Walkerton outbreak (3), the protection of source water quality is a continuing public health priority.

Since water quality assessment based on traditional fecal indicator bacteria does not provide information about the origin of fecal pollution, bacterial source tracking (BST) has emerged as a promising multibarrier approach as a means to protect and manage source water quality and also to assess the potential public health risk associated with fecal contamination from a particular host source. In recent years, various phenotypic and genotypic (library-dependent or library-independent) BST methods have been developed to identify the source of fecal pollution. Library-dependent methods require the construction of a comprehensive library of known source fingerprint profiles from nearby fecal pollution sources that are used for comparison with isolates collected from aquatic environment to determine the sources of contamination. However, the library-independent method relies on the direct detection of source-specific genetic markers (12, 13).

However, since the BST method does not provide information about pathogens, the assessment of the public health risk associated with a particular host source would not be accurate based on this method unless we know the distribution and occurrence of potential enteric pathogens as a function of host and the lack of such data would underestimate the actual risk from such sources. Therefore, it is essential to generate a prior knowledge about the prevalence of pathogens in the feces of different animal species that share the same watershed and their relative contributions of pathogens into the environment (watershed) where the BST is applied. Currently in Canada, little is known about the prevalence of different pathogenic *E. coli* in various animal host sources in addition to the extensively studied natural reservoir, such as cattle and other ruminants. Understanding their occurrence in various potential host sources of fecal contamination would help us to assess the health risk posed by such strains of *E. coli* from potential host sources by BST method and also help us to identify whether certain animal species may present a greater risk to human health than others. Regarding the BST method, high genetic diversity among *E. coli* populations has been reported to influence the accuracy of BST results (14); thus, more information is necessary on the genetic diversity of *E. coli* populations in a host source of interest for determining their applicability in DNA fingerprint-based source tracking studies. The objectives of the present investigation were thus to evaluate the prevalence of diarrheagenic virulence genes in *E. coli* isolated from the fecal samples of diverse animals, as well as to determine the genotypic diversity of these isolates by repetitive element palindromic PCR (rep-PCR) fingerprinting.

MATERIALS AND METHODS

***E. coli* sources and isolation.** A total of 593 *E. coli* strains were isolated from the fecal samples of the following animals during a 3-year period (2004 to 2006) from the Okanagan Lake (50.0000° N, 119.5000°W) watershed in British Columbia, Canada: bison, $n = 11$; black bear, $n = 48$; buffalo, $n = 5$; cow, $n = 49$; coyote, $n = 121$; deer, $n = 89$; dog, $n = 48$; horse, $n = 19$; donkey, $n = 25$; big-horned sheep, $n = 16$; llama, $n = 5$; pony, $n = 19$; rabbit, $n = 5$; mountain goat, $n = 20$; cougar, $n = 20$; elk, $n = 29$; grizzly bear, $n = 20$; and groundhog, $n = 19$. The fecal samples were collected and identified by the British Columbia Ministry of Forestry staff. The year and month of each sampling are given in Table 1. Human isolates ($n = 25$) were obtained from healthy volunteers at the University of Victoria. The isolates were confirmed as *E. coli* according to standard biochemical tests (15) and by PCR amplification of the gene encoding universal stress protein *uspA* (16).

Detection of virulence genes by real-time PCR assay. Bacterial cells were recovered from 1 ml of pure culture of *E. coli* grown for 18 h at 37°C and genomic DNA was extracted with InstaGene Matrix (Bio-Rad, Canada) according to manufacturer's protocol. Previously published primers and probes were used for the detection of the virulence genes of pathogenic *E. coli* (Table 2). A triplex TaqMan real-time PCR targeting stx_1, stx_2, and *eae* gene were used for the simultaneous detection of STEC and EPEC (17). Two separate singleplex reactions targeting *E. coli* O157 *rfb* (rfb_{O157}; which is responsible for the biosynthesis of the O antigen) and the EAF gene were used for the confirmation of *stx* positive *E. coli* as O157/non-O157 and EPEC as aEPEC/tEPEC, respectively (18, 19). The reaction mixtures were dispensed into 96-well, thin-wall PCR plates (MicroAmp; Applied Biosystems, USA). Each well contained (20 μl) 2× TaqMan Universal PCR master mix (Applied Biosystems) with optimized concentrations of primers, probes, and DNA template (Table 2). Plates were covered with optically clear sealing film (Applied Biosystems) and briefly centrifuged. After uracil *N*-glycosylase treatment at 50°C for 2 min to prevent amplification of carryover PCR products, followed by the activation of AmpliTaq Gold DNA polymerase at 95°C for 10 min, DNA was amplified for 40 cycles at 95°C for 15 s and 60°C for 1 min using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Two separate SYBR (duplex) green real-time PCR assays were also used for the detection of STh, LT, STp, and *ipaH* genes (17, 19). The reaction mix contained (20 μl) 2×Fast SYBR green master mix (Applied Biosystems) with optimized concentrations of primers and DNA template (Table 2). The DNA amplification (40 cycles) was carried out at 95°C for 1 min and 60°C for 20 s. After amplification, a melting-curve analysis of the amplified DNA was performed using the default temperature profile. The instrument software was used for data analysis (SDS, version 2.4). The following *E. coli* isolates were used as positive controls: ATCC 43887 (EPEC), EDL 933 *E. coli* O15:H7 (STEC), ATCC 43886, 43896 (ETEC), and ATCC 43893 (EIEC). Ultrapure water without DNA was used as a negative control.

rep-PCR genomic fingerprinting of *E. coli* isolates. The genomic DNA from each *E. coli* isolate was extracted with an InstaGene DNA extraction kit (Bio-Rad) according to the manufacturer's instructions. A BOX PCR was performed with the BOX A1R primer 5′-CTA CGG CAA GGC GAC GCT GAC G-3′ (Invitrogen). The reaction mix (25 μl) contained 5 μl (50 nM) DNA template, 1 μl (2 μM) primer, 10 μl of 2.5× 5 Prime Mastermix, 1 μl of $MgCl_2$ (1 mM), and ultrapure water. PCR amplifications were performed in a PxE0.2 thermal cycler (Thermo Electron Corp.) with an initial denaturation step (95°C, 2 min), followed by 30

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TABLE 2 Primers and probes used in the present study

E. coli pathotype	Gene[a]	Primer or probe	Sequence (5′–3′)	Final concn (nmol/liter)	Amplicon size (kb)
STEC	stx_1*	Forward primer	ACTTCTCGACTGCAAAGACGTATG	200	132
		Reverse primer	ACAAATTATCCCCTGAGCCACTATC	200	
		TaqMan probe	VIC-CTCTGCAATAGGTACTCCA-MGB	100	
	stx_2*	Forward primer	CCACATCGGTGTCTGTTATTAACC	500	93
		Reverse primer	GGTCAAAACGCGCCTGATAG	500	
		TaqMan probe	NED-TTGCTGTGGATATACGAGG-MGB	200	
	rfb_{O157}‡	Forward primer	ATG CTG CCC ACA AAA ATA ATG TAAA	600	89
		Reverse primer	TTC CAT AAT CGG TTG GTG TGC TAA	600	
		TaqMan probe	NED-AAC TGC TTT TCC TCG GTT CGT CGT GTA T-MGB	200	
EPEC	*eae**	Forward primer	CCGATTCCTCTGGTGACGA	200	105
		Reverse primer	CCACGGTTTATCAAACTGATAACG	200	
		TaqMan probe	FAM-CGTCATGGTACGGGTAA-MGB	100	
	EAF‡	Forward primer	GTT CTT GGC GAA CAG GCT TGT C	600	107
		Reverse primer	TTA AGC CAG CTA CCA TCC ACC C	600	
		TaqMan probe	TET-AGT ACT GAC GTG CAG GTC GCC TGT TCG-MGB	200	
ETEC	STh†	Forward primer	CCTTTCGCTCAGGATGCTAAAC	500	128
		Reverse primer	CAGTAATTGCTACTATTCATGCTTTCAG	500	
	LT†	Forward primer	TTCCCACCGGATCACCAA	100	62
		Reverse primer	CAACCTTGTGGTGCATGATGA	100	
	STp†	Forward primer	CTTTCCCCTCTTTTAGTCAGTCAACT	300	137
		Reverse primer	GCAGTAAAATGTGTTGTTCATATTTTCTG	300	
EIEC	*ipaH*†	Forward primer	GAA CTC AAA TCT TGC ACC ATT CA	600	107
		Reverse primer	CGT CCG TCC GAG AAC AAT TAA G	600	

[a] *, triplex reaction; †, duplex reaction; ‡, singleplex reaction.

cycles of denaturation (94°C for 3 s and 92°C for 30 s), annealing (50°C for 1 min), and extension (65°C for 8 min), and then a single final extension step (65°C for 8 min). A negative control (molecular-biology-grade water; Fermentas, Canada) and a positive control (genomic DNA of *E. coli* K-12 strain W3110) were included in the PCR assays. The amplification products were separated by horizontal electrophoresis on a 1.8% agarose gel after mixing with gel loading dye (Fermentas) at 100 V for 3 h. GeneRuler 1-kb Plus molecular weight marker (Fermentas) was loaded into a well for every eight samples as an external reference standard. The gel was then stained with DNA gel stain (SYBR Safe; Life Technologies, Canada) in 1× TAE buffer. The banding patterns of the gels were captured and processed using a Gel Doc XR+, molecular imager, and Image Lab 4.0 image acquisition and analysis software (Bio-Rad).

Computer-assisted fingerprint data analysis. BioNumerics version 7 (Applied Maths, Belgium) was used to analyze the rep-PCR DNA fingerprints of *E. coli* isolates. Each gel was normalized using 1-kb Plus DNA ladders (Fermentas), in the range of 75 to 2,000 bp as an external reference standard. DNA fingerprint similarities were calculated using the curve-based Pearson coefficient with 1% optimization. A dendrogram was generated using the unweighted-pair-group method using arithmetic averages (UPGMA). A similarity score of 92% was used as the cutoff value for the same strain types. Isolates with similarity scores below this value were considered unique. Clustering of the isolates were accomplished by multivariate analysis of variance (MANOVA), a form of discriminant analysis accounting for variance (20). A binary band-matching character table generated by using the BOX-derived rep-PCR DNA fingerprint data was used for discriminant analysis and MANOVA. The Shannon diversity index (H') was used to calculate the genetic diversity of the *E. coli* isolates (21). The diversity index was calculated as follows:

$$H' = -\sum_{i=1}^{s} pi \ln pi,$$

where S is the number of unique genotypes, and p_i is the number of isolates sharing the same genotype i over the total number of isolates.

RESULTS

Prevalence of virulence genes. In the present study, we analyzed the presence of major virulence genes associated with diarrheagenic *E. coli* in the fecal samples collected from human and different animal hosts in order to determine whether they could be a significant source of these pathogens. The results indicated that *eae* was the most frequently detected gene compared to the others and was carried by 60 isolates (10.11%) out of the 593 isolates analyzed (Table 3). Among the 11 host sources found positive for *eae* gene, the highest prevalence was observed in horses (50%), followed by dogs (41.66%), ponies (26.31%), groundhogs (15.78%), coyotes (11.57%), and humans (8%). In other hosts, relatively lower prevalences were noticed (≥5%). In addition, 8 isolates (1.3%) were found to be positive for both the *eae* and the EAF genes, 7 carried by coyotes (5.78%) and 1 carried by dogs (2.08%).

The overall prevalence of *stx* genes (stx_1, 0.1%; stx_2, 5.56%; stx_1 plus stx_2, 1.51%) detected from different animals was 7.25% (43/593) (Table 3). Among these, stx_1 was detected only in mountain goats (5%), whereas stx_2 was detected in humans (24%), bison (45.45%), buffalo (20%), cows (16.32), coyote s(4.13%), dogs (2.08%), donkeys (20%), mountain goats (5%), and groundhogs (21%). However, only humans (6/25; 24%) and rabbits (3/5, 60%) harbored *E. coli* carrying both stx_1 and stx_2 together. Further characterization of *stx*-positive *E. coli* with an O157 (rfb_{O157})-specific TaqMan assay revealed that all of the STEC isolates were non-O157 since none of them were positive for this gene. The prevalence of STh and STp genes was relatively lower (0.33%, 2/593),

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TABLE 3 Prevalence of virulence genes in *E. coli* isolated from the feces of humans and various animals

Source[a] (no. of isolates)	No. of positive isolates (%)[b]						
	stx$_1$	*stx*$_2$	*stx*$_1$ plus *stx*$_2$	*eae*	*eae* plus EAF	STh	STp
Human (25)		2 (8)	6 (24)	2 (8)			
Bison (11)*		5 (45.45)					
Black bear (48)							
Buffalo (5)*		1 (20)					
Cow (49)*		8 (16.32)					
Coyote (121)		5 (4.13)		14 (11.57)	7 (5.78)		
Deer (89)*				1 (1.12)		1 (1.112)	
Dog (48)		1 (2.08)		20 (41.66)	1 (2.08)		2 (4.16)
Horse (19)				10 (50)			
Donkey (25)		5 (20)		1 (4)			
Bighorn sheep (16)*							
Llama (5)							
Pony (19)				5 (26.31)		1 (5.26)	
Rabbit (5)		1 (20)	3 (60)				
Mountain goat (20)*	1 (5)	1 (5)		1 (5)			
Cougar (20)							
Elk (29)*				2 (7)			
Grizzly bear (20)				1 (5)			
Ground hog (19)		4 (21.05)		3 (15.78)			
Total (n = 593)	1 (0.1)	33 (5.56)	9 (1.5177)	60 (10.11)	8 (1.349)	2 (2.337)	2 (0.337)

[a] *, ruminants (all other animal sources are nonruminants).
[b] No isolates were positive for LT or *ipaH*.

whereas no isolates were positive for the LT and *ipaH* gene. Nevertheless, STh was observed in deer (1/89; 1.12%) and ponies (1/19; 5.26%), and STp was observed only in dogs (2/48; 4.16%). Although detected separately in different isolates, *E. coli* carrying three or more genes was present in humans (*stx*$_1$, *stx*$_2$, and *eae*), dogs (*stx*$_2$, *eae*, *eae* plus EAF, and STp), and mountain goats (*stx*$_1$, *stx*$_2$, and *eae*).

Genetic diversity of *E. coli*. In the present study, rep-PCR DNA fingerprint analysis indicated that *E. coli* isolates obtained from different animals displayed high degree of genetic variability since we obtained 221 unique fingerprints (<92% similarity) out of the 593 isolates analyzed, with an overall Shannon diversity index of 2.67 (Table 4). Although the fingerprint patterns of the remaining isolates (n = 372) were >92% similar, most of the isolates obtained from the same animal species were not identical. The H' index calculated for *E. coli* obtained from each host sources were ranged from 0.4 to 3.1. Among them, the highest diversity was observed in coyotes (3.1), followed by bighorn sheep (2.68), cows (2.42), bison (1.89), deer (1.84), cougars (1.84), grizzly bears (1.67), and black bears (1.56). The H' index was <1.5 for all other hosts.

In order to study the diversity without clonality, only unique fingerprints (n = 221) were used for dendrogram analysis. When the dendrogram was collapsed at 60% similarity cutoff value, it generated 25 clusters each containing at least two isolates (Fig. 1). Of the 221 unique fingerprints examined, 211 (96%) fell into 25 clusters, whereas 10 fingerprints (4%) had individual lineages with DNA fingerprint similarities ranging from 5 to 55%. Among different clusters, cluster 4 contained the largest number of isolates (40) that originated from 12 animals with DNA fingerprint similarities ranging from 61 to 91%, followed by cluster 9 (22 isolates, 62 to 91% similarity), cluster 13 (21 isolates, 64 to 91% similarity), cluster 11 (18 isolates, 64 to 88% similarity), cluster 20 (15 isolates, 65 to 92% similarity), cluster 5 (15 isolates; 61 to 88% similarity), cluster 6 (13 isolates; 63 to 91% similarity), and cluster 21 (10 isolates; 64 to 87% similarity). The remaining 17 clusters comprised fewer than 10 isolates, with fingerprint similarities ranging from 60 to 83%. These results demonstrate that there is a high degree of genetic diversity in *E. coli* populations isolated from different host sources. In several instances, genotypes were shared among different hosts, indicating the circulation of isolates in dif-

TABLE 4 Shannon diversity index of each host analyzed

Source	No. of isolates	No. of unique genotypes	Shannon diversity index
Human	25	3	0.4987
Bison	11	7	1.8937
Black bear	48	16	1.5607
Buffalo	5	3	1.3321
Cow	49	26	2.4200
Coyote	121	70	3.1385
Deer	89	31	1.8425
Dog	48	9	0.8945
Horse	19	7	1.3750
Bighorn sheep	16	14	2.6859
Pony	19	2	0.4094
Cougar	20	10	1.8444
Elk	29	9	1.3012
Grizzly bear	20	9	1.6768
Ground hog	19	5	0.9998
Llama	5	0	0
Rabbit	5	0	0
Donkey	25	0	0
Mountain goat	20	0	0
Total	593	221	2.67

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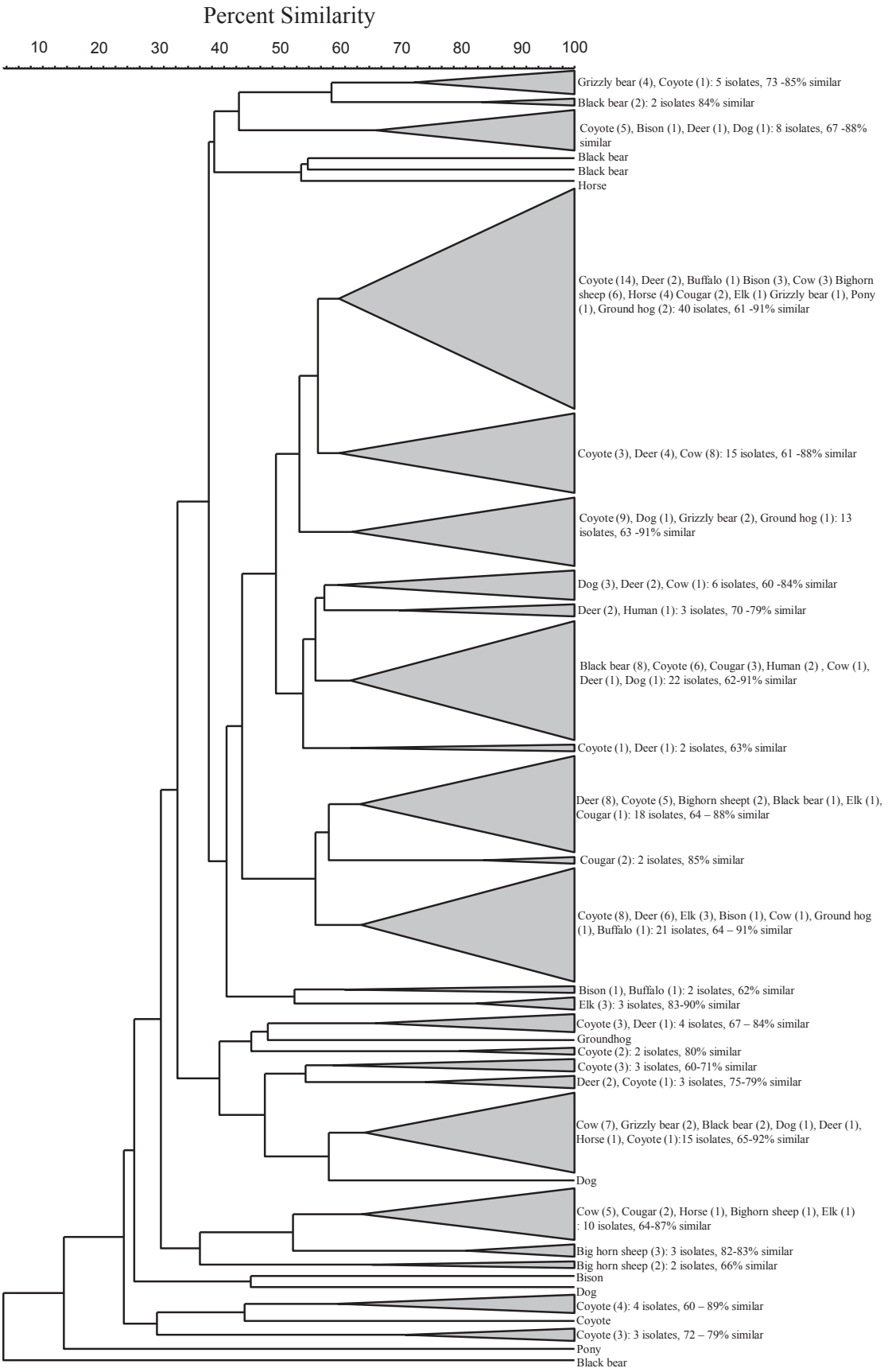

FIG 1 Dendrogram showing the relatedness of *E. coli* strains isolated from different animal hosts as determined by rep-PCR fingerprint analysis using BOX A1R primer. Because of the large population size ($n = 221$), a condensed dendrogram (using a 60% cutoff value) has been presented to show 25 major clusters. DNA fingerprint similarities were calculated by the curve-based Pearson coefficient, and dendrograms were generated by UPGMA. The source, number of isolates, total number of isolates in each cluster, and percent similarity is indicated next to each cluster.

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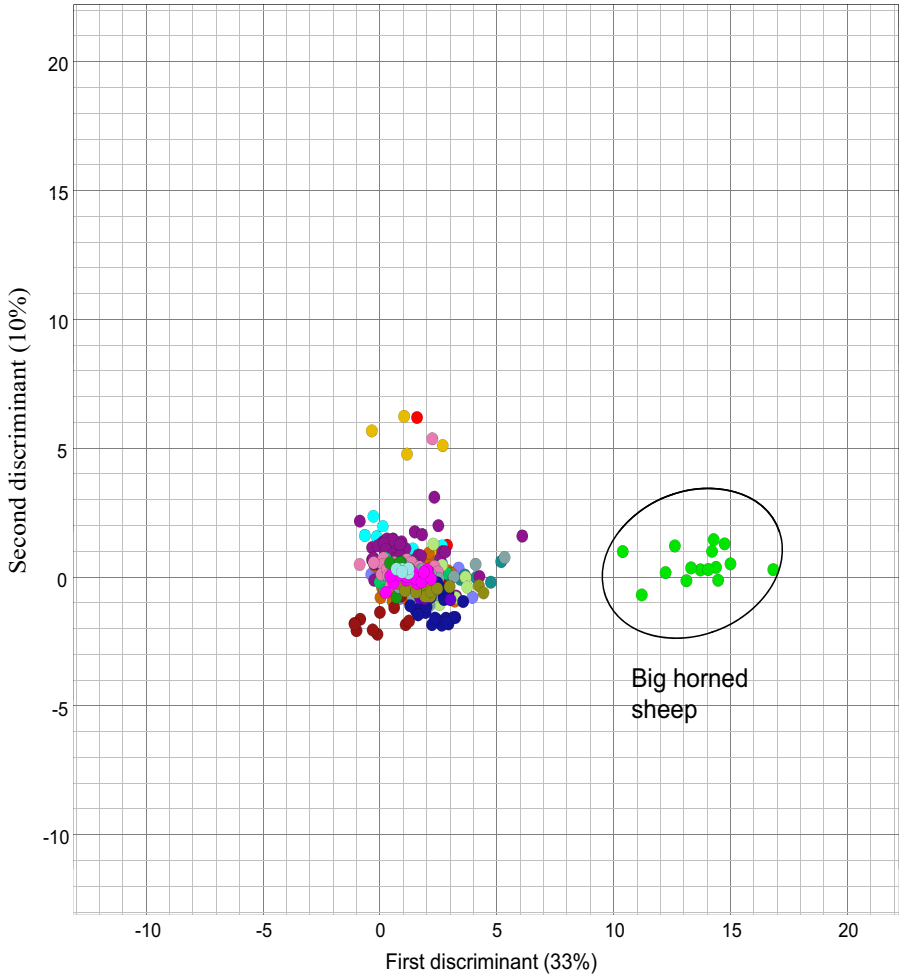

FIG 2 MANOVA plot of rep-PCR DNA fingerprints from *E. coli* ($n = 593$) isolated from different hosts. A binary band-matching character table was analyzed by discriminant analysis. Only the first two discriminants are presented in this graph, as shown by the distances along the *x* and *y* axes.

ferent host. Source-wise MANOVA of *E. coli* fingerprints ($n = 593$) revealed that isolates from big horned sheep formed a separate cluster distinct from other isolates representing a unique group (Fig. 2). The first and second discriminants accounted for 33 and 10% of the variation, respectively. We also performed MANOVA on the fingerprint data of *E. coli* isolates ($n = 593$) obtained in 2004 ($n = 118$), 2005 ($n = 238$), and 2006 ($n = 237$) to determine whether *E. coli* diversity correlated with the sampling year. A vast majority of the isolates showed a tendency to cluster according to the year (Fig. 3). The first and second discriminants accounted for 100% of the canonical variation (73 and 27%, respectively). The result indicated that there was a significant temporal variation in the occurrence of *E. coli* isolates in different hosts, suggesting that they exhibit complex population structures and dynamics.

DISCUSSION

Prevalence of virulence gene. The hazards to human health represented by zoonotic pathogens in animal excreta present special challenges for maintaining the quality of surface waters used for recreation and as a source of drinking water. Thus, understanding the distribution of pathogens in the host source of fecal contamination is important for evaluating their relative contribution to overall pathogen loads into the environment and assessing subsequent health risks from specific host sources. Caution is needed when comparing prevalence between studies due to variations in methods, particularly in molecular detection, isolation, and characterization (22). The majority of the EPEC isolates (60/593, 10.11%) encountered in the present study were aEPEC, since the EAF gene was not detected in these isolates. However, the isolates obtained from coyotes (7 isolates) and dogs (1 isolate) in which EAF was detected were tEPEC (1). This is in full agreement with the observation that humans are the only living reservoir of tEPEC (1), with the exception of a few isolates from dogs (23). Compared to our results, a similar study conducted in Minnesota and western Wisconsin (*E. coli*, $n = 1,531$) reported a lower prevalence of *eae* and EAF genes in humans, cats, dogs, deer, cows, and horses (24). In contrast to that result, we did not find the *eae* gene in samples from cows and buffalos; a similar observation was made by Rogerie et al. (25). However, Koyabashi et al. (26) found that 16.7% of *eae*-positive isolates from cattle were aEPEC. There are very few published reports available on the occurrence of EPEC in coyotes, donkeys, ponies, mountain goats, elk, grizzly bears, or groundhogs, and hence the detection of EPEC in these hosts indi-

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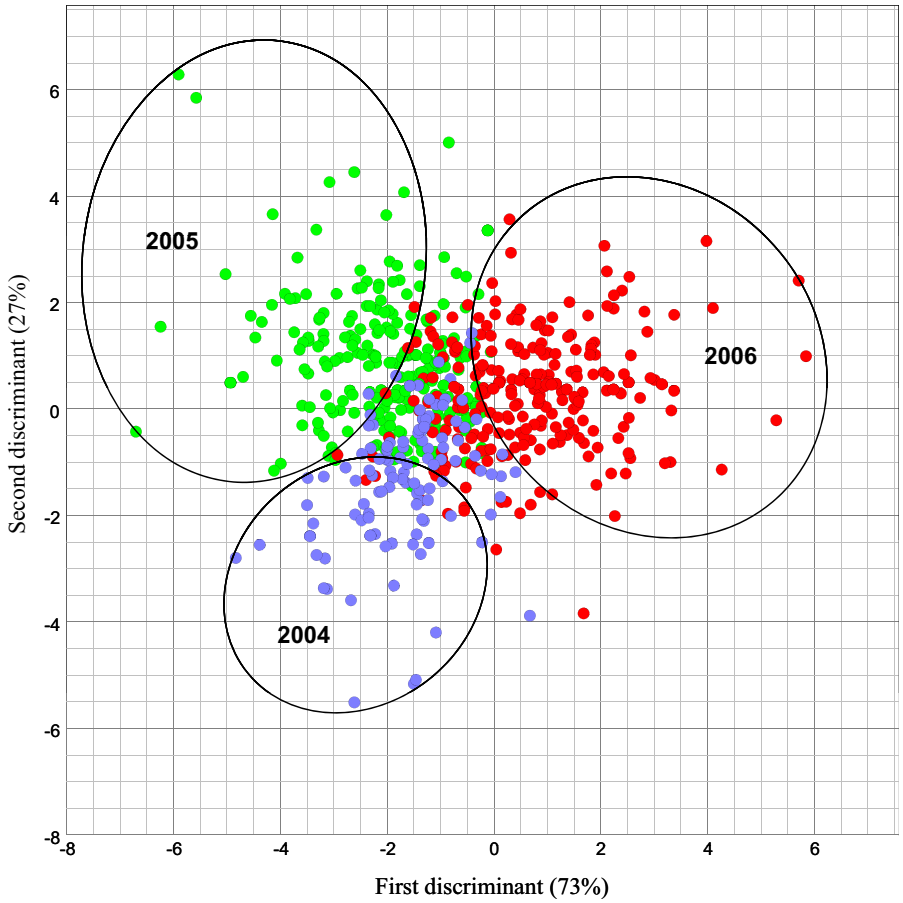

FIG 3 MANOVA plot of rep-PCR DNA fingerprints from *E. coli* ($n = 593$) isolated from human and various animals collected during different years. Binary band-matching character table was analyzed by discriminant analysis. Only the first two discriminants are presented in this graph, as shown by the distances along the *x* and *y* axes.

cates that they can be a significant source of this pathogen. To our knowledge, this is the first report of the presence of aEPEC in these hosts and the presence of tEPEC in coyotes. It has been reported that EPEC is a significant cause of gastrointestinal disease in the developing world (1). However, outbreaks have also been reported in developed countries, mainly outbreaks due to aEPEC (2, 7), indicating its role as an important human pathogen, and like STEC, such isolates appear to be emerging pathogens (8). Although there is no evidence of direct transmission from animals to humans, some aEPEC strains isolated from animals have been implicated in human diseases, suggesting that animals may represent important reservoirs of aEPEC, which can be transmitted to humans (27).

In general, cattle and other ruminants are considered to be the primary reservoirs of STEC, especially serotype O157 (9). However, we found here a greater occurrence of non-O157 STEC in nonruminant hosts (27 isolates) compared to ruminant (16 isolates) hosts (Table 3). Except for one isolate obtained from mountain goat (stx_1 positive), all other isolates recovered from ruminant hosts harbored only stx_2. In agreement with our results, Rogerie et al. (25) observed that all STEC isolates obtained from healthy cattle in France were non-O157, with a prevalence of 30 to 53% of stx_2, which is higher than what we observed in bison, buffalos, and cows (16 to 45%). In contrast, Oliveira et al. (28) detected stx_1 at prevalence levels of 15 to 27% in cattle and 25% in goats. However, the prevalence of stx_2 in goats (34.7%) was higher than our results (5%). Similar to our findings, these researchers also demonstrated a higher prevalence of the stx_2 gene compared to stx_1, substantiating other, similar observations from healthy cattle (22) and other ruminants (29). None of the isolates from deer and bighorn sheep were positive for either stx_1 or stx_2. In contrast to our results, non-O157 STEC was detected from deer and wild sheep carrying different combinations of stx_1 and stx_2 (24, 29).

Among the nonruminant hosts, STEC was more dominant in humans (32%, 8/25), with 8% positive for stx_2 genes and 24% positive for both stx_1 and stx_2 genes. It has been reported that humans can act as healthy asymptomatic carriers of non-O157 STEC (30). In addition to humans, rabbits also harbored STEC carrying both stx_1 and stx_2 genes. Rabbits have been identified as a new reservoir host of EHEC and are able to cause a zoonotic risk for humans (31). In that study, Garcia and Fox found 25% (7/28) of the isolates were non-O157 EHEC harboring both stx_1 and the *eae* gene. In contrast, the isolates in our sample were not non-O157 EHEC, since we did not detect the presence of the *eae* gene in any of these isolates, and stx_2 was lacking in their samples compared to our results. These results indicate that rabbits can be a significant source of O157 and non-O157 STEC with different

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combinations of virulence genes. Relatively higher occurrences of non-O157 STEC were reported in dogs (32), while Ritchie et al. (33) found stx_1-positive non-O157 isolates in coyotes. The STEC prevalences were 21% in groundhogs and 20% in donkeys, animals not previously reported as non-O157 STEC carriers. Therefore, this is the first report the occurrence of non-O157 STEC in these animals in Canada. Since cattle and other ruminants are the reservoir of *E. coli* O157 and are associated with outbreaks, most of the studies have focused on the detection of *E. coli* O157, and hence there is limited knowledge of the prevalence of non-O157 STEC isolates in other animals. Overall, the higher occurrence of non-O157 *E. coli* in nonruminant hosts suggests that they can be a potential reservoir for these pathogenic *E. coli*.

Although *eae* is an important virulence determinant in STEC infection, none of the *E. coli* isolates were found to harbor *eae* gene in association with *stx* gene. This was similar to the findings of Ishii et al. (24), who observed no *eae* gene in association with the *stx* gene in ruminant hosts, while it was opposed to the findings of Sandhu et al. (34), who observed the presence of the *eae* gene in STEC isolates obtained from cattle. As observed in the present study, the presence of *eae*-negative STEC identified in various animal hosts has particular health significance in view of the disease outbreaks of bloody diarrhea and hemolytic-uremic syndrome (HUS) caused by non-O157 STEC lacking *eae* (10, 35), suggesting that the primary virulence trait responsible for causing HUS is the production of Shiga toxin. Comparatively, we detected a higher incidence of stx_2 genes than of stx_1 genes. It has been documented that the stx_2 gene was the most commonly detected virulence gene and was found to be more strongly correlated with severe human disease than was stx_1 (36, 37), indicating the importance of stx_2 in human infection. The surveillance of Shiga toxin in stool specimens from diarrheal patients in Canada has revealed that a substantial portion of STEC illness is due to non-O157 STEC (38, 39). In response to the high prevalence of non-O157 STEC in the United States, the Centers for Disease Control has recently begun surveillance for non-O157 STEC through its FoodNet program. Thus, it appears that STEC serogroups other than O157 may be responsible for up to 50% of STEC-related illness in both Canada and the United States (40).

The prevalences of virulence genes associated with ETEC (LT, STh, and STp) and EIEC (*ipaH*) were relatively lower compared to *stx* and *eae* genes. It has been reported that both STh (STIb) and STp (STIa) may be produced by animal isolates (41). However, Carlos et al. (42) did not find ETEC- and EIEC-related virulence genes in human and ruminant animals compared to *stx* and *eae* genes. Therefore, from our results it can be concluded that the virulence genes *stx* and *eae* are more prevalent in humans and animals studied in Canada compared to other genes. This suggests that the identified host source may pose public health risk from non-O157 STEC and EPEC infection than from ETEC and EIEC.

In most of the outbreaks related to pathogenic *E. coli*—including non-O157 STEC and EPEC—water has been identified as the leading mode of transmission (11, 43, 44, 45), indicating the widespread occurrence of these organisms in the environment and especially in water. Several studies have quantified the relative impacts of animal derived fluxes of microbial pollution in water (46, 47) and their association with disease (48). This indicates that pathogens from animal feces may enter waterways by direct deposition or as a result of overland runoff containing fecal material deposited in the watershed. Thus, water bodies with substantial animal inputs can result in potential human health risks. Hence, the broad distribution of aEPEC and non-O157 STEC observed in diverse animal host sources has a considerable public health significance in terms of their ability to act as a potential reservoir and the potential to contaminate surface water.

Generally, the pathogens that could be expected to occur in contaminated waters are dependent on the host source reservoir from which they are derived. Therefore, the identification of major animal sources of fecal contamination is extremely important to properly assess the potential public health risk associated with fecal contamination from various host sources based on BST methods. In considering the BST efforts to identify fecal sources based on library-dependent (*E. coli* fingerprints) methods, it is essential that the library be large enough and contain a sufficiently diverse set of profiles to be representative of all of the potential animal sources in a particular watershed (14). However, most of the libraries that are currently in use do not have representative fingerprints from the all of the potential animal sources. Thus, library-dependent (fingerprint based) source tracking efforts can greatly reduce the accuracy and would significantly underestimate the actual health risk from potential host sources that carry pathogens. Thus, based on our results, we suggest that the hosts in which pathogens are detected should be considered as a potential risk source for contamination, and *E. coli* isolates from these host sources must be included when constructing known source fingerprint libraries for source tracking purposes in areas where these animals shares a common watershed.

Genetic diversity of *E. coli*. rep-PCR fingerprinting has been extensively used to study genetic variability in *E. coli* populations from diverse habitats and also to generate fingerprint libraries for microbial source tracking studies (20). Overall, rep-PCR DNA fingerprint analysis revealed extensive genetic diversity among *E. coli* strains isolated from different host sources. Shannon diversity indices obtained in the present study were higher than the H' index reported by Carlos et al. (42) for cows and sheep but lower than for humans and goats. However, the numbers were almost similar to the H' index previously reported based on ribotypes of *E. coli* isolated from the feces of humans and horses (49) but much higher for isolates from cows. Similarly, Johnson et al. (14) observed a high genetic diversity among *E. coli* isolated from humans and various animal sources. As observed here, several studies have also reported the occurrence of diverse *E. coli* (pathogenic and nonpathogenic) populations in several hosts and environments (14, 21, 29, 50).

Temporal variation in the composition of *E. coli* populations in different hosts and environments has been previously reported and is mainly attributed to diet (21, 51). It has been reported that temporal changes in the genetic composition of *E. coli* populations occur at scales beyond that of an individual host (51). Complex population dynamics of *E. coli*, such as high genetic diversity, differences in diversity among hosts, and temporal variability have all been reported to influence the accuracy of BST results (49, 52). It has also been reported that a strong selection takes place following excretion into the environment and that certain *E. coli* types can form stable populations in the environment, which can also influence the source tracking results (53). Hence, based on our results we suggest that the genetic diversity and temporal variation of *E. coli* observed in the present study should be considered while con-

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structing a DNA fingerprint library using an *E. coli* population from these host sources for source tracking purposes.

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