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Biofilm Formation, Virulence Gene Profiles, and Antimicrobial Resistance of Nine Serogroups of Non-O157 Shiga Toxin–Producing *Escherichia coli*

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Abstract

The objectives of this study were to characterize the phenotype and genotype of 36 non-O157 Shiga toxin–producing *Escherichia coli* (STEC) strains isolated from humans, ovines, or bovines, including the top 6 (O26, O45, O103, O111, O121, and O145) and three other serogroups implicated in serious illness (O91, O113, and O128). Biofilms were formed by all strains with intermediate to strong biofilm producers ($n=24$) more common at 22°C than at 37°C ($p<0.001$) and 48 and 72 h ($p<0.001$) than 24 h of incubation time. Biofilm-forming potential differed by serogroup and origin with O113 and human strains exhibiting the highest potential ($p<0.001$). Biofilm-associated genes, *csgA/csgD/crl/fimH* (100%), *flu* (94%), *rpoS* (92%), *ehaA*$^{\alpha}$ (89%), and *cah* (72%), were most prevalent, while *mlrA* (22%) and *ehaA*$^{\beta}$ (14%) were least prevalent, although there was no clear compliment of genes associated with strains exhibiting the greatest biofilm-forming capacity. Among 12 virulence genes screened, *iha* and *ehxA* were present in 92% of the strains. The occurrence of stx_1 in the top 6 serogroups (8/12, 67%) did not differ ($p=0.8$) from other serogroups (17/24, 71%), but stx_2 was less likely (confidence interval [CI] = 0.14–1.12; $p=0.04$) to be in the former (9/24, 38%) than the latter (9/12, 75%). Excluding serogroups, O91 and O121, at least one strain per serogroup was resistant to between three and six antimicrobials. Streptomycin (31%), sulfisoxazole (31%), and tetracycline (25%) resistance was most common and was 35–50% less likely ($p<0.05$) in human than animal strains. All non-O157 STEC strains were able to form biofilms on an abiotic surface, with some exhibiting resistance to multiple antimicrobials. Potential as a reservoir of antimicrobial resistance genes may be another hazard of biofilms in food-processing plants. As a result, future strategies to control these pathogens may include measures to prevent biofilms.

Introduction

Shiga toxin–producing *Escherichia coli* (STEC) are important foodborne pathogens worldwide (Etcheverria and Padola, 2013). *E. coli* O157:H7 is the predominant serotype associated with outbreaks of STEC infections, but a growing number of non-O157 serotypes have also been linked to human illness (Luna-Gierke *et al.*, 2014; Smith *et al.*, 2014). Severity of illnesses caused by non-O157 STEC may equal or even exceed that associated with STEC O157:H7 (Johnson *et al.*, 2006; Hermos *et al.*, 2011).

Non-O157 STEC outbreaks are typically associated with contaminated foods of bovine origin (Mathusa *et al.*, 2010; Robbins *et al.*, 2014) or vegetable products contaminated with bovine feces (Hussein and Bollinger, 2005; Smith *et al.*, 2014). Specifically, six serogroups, O26, O45, O103, O111, O121, and O145 (top 6), were responsible for ~70% of non-O157 infection from 1983 to 2002 in the United States (Brooks *et al.*, 2005) and were declared as adulterants in raw beef products in the United States (USDA, 2012). Although other non-O157 serogroups such as O91, O113, and O128 are less likely to be associated with outbreaks, they can cause severe illness (Johnson *et al.*, 2006; Bettelheim, 2007). The production of Shiga toxins (Stx) (Paton and Paton, 1998) and possession of mobile genetic elements (MGEs) (Tatsuno *et al.*, 2001; Imamovic *et al.*, 2010; Etcheverria and Padola,

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2013) are the main virulence features of STEC-associated pathogenesis. Moreover, genetic diversity in STEC virulence varies among strains (Anjum *et al.*, 2014; Tseng *et al.*, 2014) and geographical locations (Mellor *et al.*, 2013).

STEC may form biofilms on food or food contact surfaces (Silagyi *et al.*, 2009; Dourou *et al.*, 2011; Wang *et al.*, 2012). Decontaminating food-processing equipment of biofilms is particularly difficult as biofilms frequently slough off, releasing cells into food products (Vogeleer *et al.*, 2014). Biofilms can also form on food equipment surfaces, reducing the effectiveness of disinfectants (Vogeleer *et al.*, 2014). Most STEC biofilms studies have focused on O157:H7 (Dourou *et al.*, 2011; Uhlich *et al.*, 2013; Wang *et al.*, 2014) and few report biofilm formation by non-O157 STEC strains (Chen *et al.*, 2013; Uhlich *et al.*, 2014).

Differing evolutionarily from O157, non-O157 STEC are heterogeneous and information on their virulence, fitness, and stress responses is limited (Wang *et al.*, 2013). This lack of knowledge and strain heterogeneity increase complexity of developing strategies to reduce food and water contamination with these pathogens. Therefore, the aim of this study was to investigate molecular and phenotypic features of non-O157 STEC strains from nine major serogroups isolated from Canada. Strains were characterized for the presence of biofilm-forming- and virulence-associated genes, biofilm-forming ability, and antimicrobial resistance (AMR) profiles.

Materials and Methods

Bacterial strains and growth conditions

All strains used (Table 1 and Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/fpd) were of human ($n=22$), bovine ($n=12$), or ovine origin ($n=2$) and were obtained from the Public Health Agency of Canada (Guelph, ON). Four strains were evaluated of each top 6 non-O157 STEC serogroup (O26, O45, O103, O111, O121, and O145) and three other non-O157 STEC serogroups implicated in serious illness (O91, O113, and O128). All strains were streaked onto tryptic soy agar and incubated at 37°C for 18 h. An isolated colony was then inoculated into 10 mL of tryptic soy broth and incubated at 37°C overnight.

Biofilm formation assay

Biofilm formation was assessed in 96-well polystyrene microplates (Nunc, Edmonton, AB) using a modification of Uhlich *et al.* (2013). Briefly, LB broth with no salt (LB-NS) only (negative control, 200 μL) or LB-NS diluted overnight culture of each strain (200 μL) was dispensed into four replicate wells of a microplate. The plates were incubated at 22°C or 37°C for 24, 48, or 72 h. Following incubation, supernatants were removed and wells were washed thrice. Remaining attached bacteria were fixed with 250 μL of absolute methanol (Sigma-Aldrich, Okaville, ON) per well for 15 min. Plates with biofilms were then emptied, air-dried, and stained with 1% (w/v) crystal violet (Sigma-Aldrich) solution for 15 min, followed by three water washes and air-drying. The dye bound to the biofilm was then dissolved with 33% glacial acetic acid (200 μL; Sigma-Aldrich) per well and OD_{590nm} values were measured.

Based on the OD_{590nm} produced by biofilms, strains were classified as no biofilm, weak, intermediate, or strong biofilm producers, as previously described (Stepanovic *et al.*, 2000). Briefly, cutoff optical density value (ODc) of 0.071 was three standard deviations above mean OD of negative controls. Strains were classified as $OD \leq ODc$, no biofilm producer; $ODc < OD \leq 2 \times ODc$, weak biofilm producers; $2 \times ODc < OD \leq 4 \times ODc$, intermediate biofilm producers; and $4 \times ODc < OD$, strong biofilm producers.

Polymerase chain reaction assay

All strains were screened by polymerase chain reaction (PCR) for the presence of major genes associated with biofilm formation and virulence (Tables 1 and 2). Bacterial DNA was isolated from 18-h cultures using the NucleoSpin® Tissue Kit (Macherey-Nagel, Bethlehem, PA). All PCR assays excepting bacteriophage insertions in *mlrA* were conducted individually using HotStar Plus MasterMix (Qiagen, Mississauga, ON) and 0.2 μM of each primer at annealing temperatures as indicated (Supplementary Table S2). The bacteriophage insertions in *mlrA* (*yehV*) were investigated using multiplex PCR and primers (Supplementary Table S2) complementary to regions flanking bacteriophage insertion site in *mlrA* (primers A and B) and to ends of predominant bacteriophage occupying *mlrA* insertion site (primers E and F) (Shaikh and Tarr, 2003). Multiplex PCR assays used QuantiFast Multiplex Master Mix (Qiagen) and 0.5 μM of each primer at annealing temperature of 57°C. Each PCR contained positive and negative controls. Samples were then electrophoresed on 2% agarose gels (w/v) stained with ethidium bromide and visualized with a UV transilluminator (Alpha Innotech, San Leandro, CA).

Antimicrobial resistance

AMR was determined against 12 antimicrobials using the disc diffusion method (CLSI, 2014). Antimicrobial discs (BD, Mississauga, ON) were used: ampicillin (AMP; 10 μg), amoxicillin–clavulanate (AMC; 20/10 μg), ceftazidime (CAZ; 30 μg), tetracycline (TET; 30 μg), kanamycin (KAN; 30 μg), nalidixic acid (NAL; 30 μg), streptomycin (STR; 10 μg), chloramphenicol (CHL; 30 μg), neomycin (NEO; 30 μg), enrofloxacin (ENR; 5 μg), trimethoprim–sulfamethoxazole (TMS; 1.25/23.75 μg), and sulfisoxazole (SUL; 250 μg). Inoculum of each strain was streaked on Mueller–Hinton agar (Dalynn Biologicals, Calgary, AB), and the appropriate drug-impregnated discs were placed on the agar surface. Plates were inverted and incubated (37°C, 18 h), and zones of inhibition measured.

Statistical analysis

Results from biofilm formation were compiled from two independent experiments. No biofilm and weak biofilm formation were scored as negative (designated as low biofilm-forming potential) and intermediate or strong biofilm formation (designated as high biofilm-forming potential) was scored as positive. Influence of origin (animal vs. human), serogroups, incubation temperature, and time on biofilm-forming potential was analyzed using GLIMMIX with random measures. Odds ratios were calculated for the

Table 1. Presence or Absence of Genes Associated with Biofilm Formation in Non-STEC O157 that Differ in Biofilm-Forming Ability

					Presence of biofilm-forming-associated genes						
Strains	*Reference ID*	*Serotype*	*Origin*[a]	*Biofilm-forming ability*[b]	csgA/ csgD/ crl/fimH	rpoS	flu	cah	ehaA$^{\alpha}$	ehaA$^{\beta}$	*Prophage* mrlA *insertion*[c]
EC19930517	R1	O26:H11	B	+	+	+	+	+	+	—	Intact
EC19960464	R2	O26:H11	B	+	+	+	+	+	+	—	Intact
EC19970119	R3	O26:H11	H	+++	+	+	+	+	+	—	Intact
EC19990859	R4	O26:H11	H	+	+	+	+	+	+	—	Intact
EC19940040	R5	O45:H2	B	+++	+	+	+	+	+	—	Intact
EC19970074	R6	O45:H2	H	++	+	+	+	+	+	—	Intact
EC19970086	R7	O45:H2	H	++	+	+	+	-	+	—	Intact
EC19970358	R8	O45:H2	H	++	+	+	+	+	+	—	Intact
EC19970327	R9	O103:H2	B	+++	+	+	+	+	+	—	Intact
EC19970345	R10	O103:H2	H	+++	+	+	+	+	+	—	Intact
EC20010670	R11	O103:H2	B	+	+	+	+	+	+	—	Intact
EC20020219	R12	O103:H2	H	+++	+	+	+	+	+	—	Intact
EC19930467	R13	O111:H8	B	++	+	+	+	+	+	—	Intact
EC20000612	R14	O111:H8	B	++	+	+	+	+	+	—	Intact
EC20000927	R15	O111:NM	H	+	+	+	+	+	+	—	Variant-L
EC20030053	R16	O111:NM	B	++	+	+	+	+	+	—	Intact
EC19960807	R17	O121:H19	H	+++	+	+	+	+	+	+	Intact
EC19990161	R18	O121:H19	H	+++	+	+	+	—	—	+	Intact
EC20020234	R19	O121:H19	H	+++	+	+	+	+	+	+	Intact
EC20040083	R20	O121:H19	B	+	+	+	+	—	+	+	Intact
EC19970355	R21	O145:NM	H	+	+	+	—	+	+	—	Intact
EC19990166	R22	O145:H25	H	+++	+	+	+	+	+	—	Intact
EC19990324	R23	O145:NM	H	+	+	+	+	+	+	—	Intact
EC20020231	R24	O145:NM	H	+	+	+	+	+	+	—	Variant-L
EC19950329	R25	O91:NM	O	+	+	+	+	—	+	—	Variant-R
EC19950340	R26	O91:NM	O	++	+	+	+	—	+	—	Variant-R
EC20010076	R27	O91:H21	H	+++	+	+	+	—	+	—	Intact
EC20020030	R28	O91:H21	B	++	+	+	—	—	+	—	Variant-R
EC19960371	R29	O113:H4	B	+++	+	—	+	+	—	—	Variant-R
EC19960434	R30	O113:H4	B	+++	+	—	+	+	—	—	Variant-R
EC19970352	R31	O113:H21	H	+++	+	+	+	+	+	—	Variant-R
EC20020170	R32	O113:H21	H	+++	+	+	+	—	+	—	Intact
EC19960949	R33	O128:NM	H	+++	+	+	+	—	+	—	Intact
EC19990162	R34	O128:H2	H	++	+	+	+	—	+	+	Intact
EC20000914	R35	O128:H10	H	+	+	-	+	+	—	—	Intact
EC20100049	R36	O128:NM	H	+	+	+	+	+	+	—	Intact
Total No. of Strains (%)	36				36 (100)	33 (92)	34 (94)	26 (72)	32 (89)	5 (14)	8 (22)

[a]Non-O157 STEC strains isolated from a bovine (B) and human (H).
[b]Biofilm-forming ability was scored based on biofilm formation under optimal experimental conditions: +, weak; ++, intermediate; +++, strong.
[c]Intact indicates detection of amplicon A/B only; Variant-L indicates detection of amplicons A/B and A/E; Variant-R indicates detection of amplicons A/B and B/F.
STEC, Shiga toxin–producing *Escherichia coli*.

percentage of high biofilm-forming potential with cohorts of animal origin, serogroup O26, at 37°C and 24 h showing a low percentage of high biofilm-forming ability as referent. Within each serogroup, OD_{590nm} values were transformed and then analyzed using MIXED and least-squares differentiated means ($p<0.05$). Correlations between biofilm-forming potential and biofilm-forming genes and between serogroup or origin and virulence-associated genes were assessed using tetrachoric correlation and Cochran–Mantel–Haenszel statistics of FREQ. All analyses were conducted with SAS (version 9.3; SAS Institute, Cary, NC).

Results

Biofilm formation

Generally, biofilm-forming potential differed by serogroup and depended on incubation temperature and time ($p<0.001$) with human strains nearly twice as likely ($p<0.001$) to form strong biofilms than animal strains (Table 3). Among serogroups, O113 exhibited the overall highest biofilm-forming potential ($p<0.001$), followed by O91 ($p<0.01$), O103 and O121 ($p<0.05$), and O45 and O128 ($p<0.05$), respectively. Strains of O111, O145, and O26 formed the least biofilms. Across all serogroups, intermediate to strong

Table 2. Prevalence of Virulence Genes in Non-O157 STEC Strains

	Serogroup	No. of strains	% Strains positive by PCR assay												
			stx_1	stx_2	eae	iha	ehxA	saa	toxB	adfO	sodCF	tccp	ureA	efa1-5′	efa1-75′
Top 6	O26	4	100	0	100	100	100	0	75	100	0	0	75	100	100
	O45	4	100	0	100	100	75	0	75	100	100	0	100	100	100
	O103	4	100	25	100	100	100	0	50	100	100	0	100	100	75
	O111	4	100	0	100	100	100	0	50	100	100	0	100	100	100
	O121	4	0	100	100	75	100	0	100	100	100	75	100	100	100
	O145	4	25	100	100	75	100	0	100	100	100	0	100	100	75
Others	O91	4	75	100	0	100	50	50	0	0	0	0	25	0	25
	O113	4	50	100	0	100	100	50	0	50	0	0	75	25	0
	O128	4	75	25	25	75	100	25	0	50	25	0	50	25	25
% Gene presence	All strains	36	70	50	70	92	92	14	50	78	58	8	81	72	67
	Top 6	24	71	38	100	92	96	0	75	100	83	13	96	100	92
	Others	12	67	75	8	92	83	42	0	33	8	0	50	17	17

PCR, polymerase chain reaction; STEC, Shiga toxin–producing *E. coli*.

biofilms were more likely at 22°C than 37°C ($p < 0.001$) and with 48 and 72 h ($p < 0.001$) than 24-h incubation times. Bofilms developed at 37°C, but became less established with longer incubation times in some O45, O91, O113, and O128 strains.

Although the ability to form biofilms varied among strains within serogroup ($p < 0.001$; Fig. 1), all strains produced biofilms under some conditions with 24/36 strains showing intermediate or strong biofilm (Table 1).

Table 3. Odds Ratios Representing the Likelihood of Biofilm-Forming Potential of Different Serogroups ($N = 4$) and Origin with Different Incubation Temperatures and Time

Factors	% of high biofilm-forming potential	Odds ratio[a]	95% CI	p
Serogroups				
O26	8			
O45	26	3.95	2.15–7.28	<0.001
O103	37	6.99	3.85–12.66	<0.001
O111	15	1.91	0.99–3.67	0.05
O121	32	5.46	3.00–9.95	<0.001
O145	9	1.14	0.56–2.31	0.7
O91	52	13.5	7.47–24.39	<0.001
O113	83	65.44	34.34–124.69	<0.001
O128	18	2.43	1.29–4.58	0.006
Temperature (°C)				
37	26			
22	36	1.86	1.46–2.37	<0.001
Time (h)				
24	22			
48	32	1.92	1.41–2.60	<0.001
72	39	3.00	2.21–4.07	<0.001
Origin				
Animal	28			
Human	32	1.94	1.45–2.59	<0.001

[a]Odds ratio for serogroups, temperature, time, and origin were generated, respectively, with cohorts of serogroup O26, at 37°C, 24 h, and animal origin as the referent.

CI, confidence interval.

Detection of biofilm-associated genes

Biofilm-associated genes, *csgA/csgD/crl/fimH* (100%), *flu* (94%), *rpoS* (92%), $ehaA^{\alpha}$ (89%), and *cah* (72%), were most prevalent, while *mlrA* (22%) and $ehaA^{\beta}$ (14%) were least prevalent (Table 1), although no correlation ($p > 0.1$) was found between biofilm-forming ability and the presence of these genes. Most strains (33/36, 92%) carried the *rpoS* gene, but this gene was lacking in R29, R30, and R35. Of 36 strains, *mlrA*-intact (amplicon A/B only) was detected in 28 (78%), indicating that these strains did not carry the prophage stx_1 in *mlrA*.

Detection of virulence-associated genes

Prevalence of virulence genes in the non-O157 STEC strains ranked as *iha/ehxA* (92%), *ureA* (81%), *adfO* (78%), *efa1-5′* (72%), stx_1/*eae* (70%), *efa1-3′* (67%), *sodCF* (58%), stx_2/*toxB* (50%), *saa* (14%), and *tccP* (8%; Table 2). Of the strains, 18 contained only stx_1, 11 were only positive for stx_2, and 7 carried both *stx* genes. Prevalence of stx_1 from the top 6 serogroups (8/12, 67%) did not differ ($p = 0.8$) from that of other serogroups (17/24, 71%), but detection of stx_2 was 40% less likely (confidence interval [CI] = 0.14–1.12; $p = 0.04$) in the top 6 (9/24, 38%) than in the latter (9/12, 75%).

Other genes associated with virulence, including *eae*, *adfO*, *efa1-5′*, *efa1-3′*, *toxB*, *ureA*, and *sodCF*, were more ($p < 0.05$) common in top 6 strains than other serogroups. All strains from the top 6 were positive for *eae*, *adfO*, and *efa1-5′*, whereas these genes were only present in 8–33% of the 12 strains from O91, O113, and O128. In contrast to the top 6 strains, O91, O113, and O128 were negative for both *toxB* and *tccP*. Additionally, most O91, O112, and O128 strains lacked an intact *efa1* gene, even though four strains possessed either *efa1-5′ or efa1-3′*. A lower prevalence of *ureA* (50%) was also observed in O91, O113, and O128 serogroups than in the top 6 serogroups (96%). No correlation ($p > 0.1$) was found between any virulence genes and strain origin.

Antimicrobial susceptibility

Excluding serogroups, O91 and O121, 12 strains from other serogroups were resistant to three to six antimicrobials

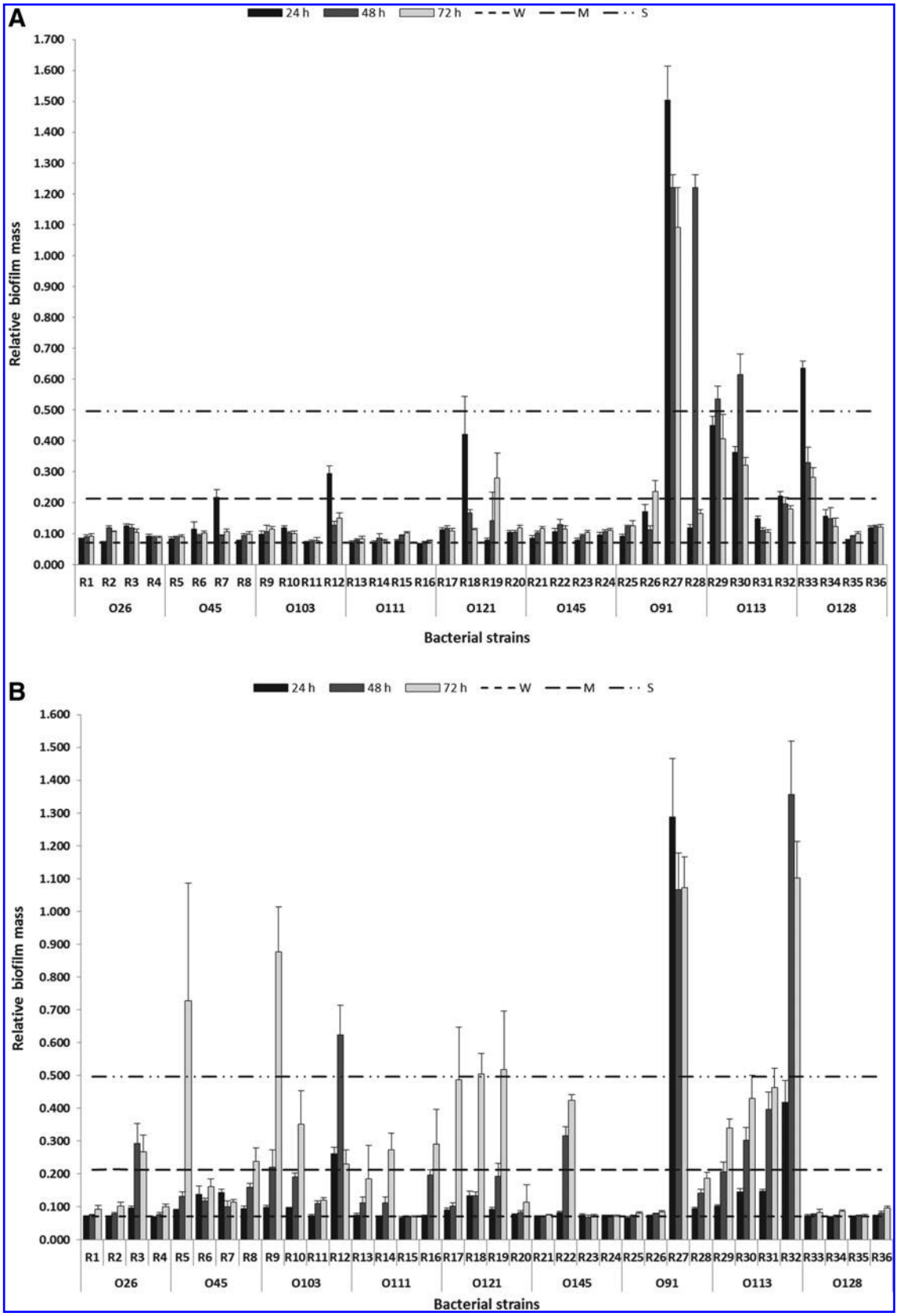

FIG. 1. Biofilm formation of non-O157 STEC on polystyrene surface at 37°C **(A)** and 22°C **(B)**. The vertical axis represents the median OD of at least eight replicates of each strain, determined at 590 nm. Horizontal lines represent the cutoff values between weak, intermediate, and strong biofilm producers. The ODc is defined as three standard deviations above the mean OD of the negative control. Strains were classified as follows: OD ≤ ODc, no biofilm producer; ODc < OD ≤2 × ODc, weak biofilm producer; 2 × ODc < OD ≤4 × ODc, intermediate biofilm producer; and 4 × ODc < OD, strong biofilm producer. OD, optical density; ODc, cutoff OD value; STEC, Shiga toxin–producing *E. coli*.

Table 4. Characterization of Multiple Drug-Resistant Non-O157 STEC Strains

Resistance pattern (No. of antimicrobials)	*No. of strains*	*Reference ID*	*Serotype*	*Origin*[a]	*Biofilm-forming ability*[b]	*Shiga toxin genes*
AMP-STR-SUL (3)	1	R31	O128:H10	H	+	stx_1
AMP-STR-TET (3)	1	R2	O26:H11	B	+	stx_1
STR-SUL-TET (3)	3	R5	O45:H2	B	+++	stx_1
		R13	O111:H8	B	++	stx_1
		R16	O111:NM	B	++	stx_1
AMP-STR-SUL-TET (4)	2	R29	O113:H4	B	+++	stx_1+stx_2
		R30	O113:H4	B	+++	stx_1+stx_2
STR-SUL-TET-TMS (4)	1	R11	O103:H2	B	+	stx_1
AMP-KAN-NEO-SUL (4)	1	R21	O145:NM	H	+	stx_2
AMP-KAN-NEO-SUL-STR (5)	1	R7	O45:H2	H	++	stx_1
AMP-KAN-NEO-SUL-STR-TET (6)	2	R3	O26:H11	H	+++	stx_1
		R14	O111:H8	B	++	stx_1

[a]Non-O157 STEC strains isolated from a bovine (B) and human (H).
[b]Biofilm-forming ability was scored based on biofilm formation under optimal experimental condition: +, weak; ++, intermediate; +++, strong.
AMP, ampicillin; KAN, kanamycin; NEO, neomycin; STEC, Shiga toxin–producing *E. coli*; STR, streptomycin; SUL, sulfisoxazole; TET, tetracycline; TMS, trimethoprim–sulfamethoxazole.

and concurrent STR-SUL-TET resistance was most common (8/12, 67%; Table 4). Moreover, STR (31%), SUL (31%), and TET (25%) resistance was 35–50% less likely ($p < 0.05$) to be detected from strains with human origin than those from bovine origin. Of the 12 multidrug-resistant (MDR) strains, 8 possessed an intermediate or strong ability to form a biofilm, and all strains but one (R21, O145:NM) possessed stx_1 or both *stx* genes (Table 4). In addition, all strains tested were susceptible to AMC, CAZ, CHL, ENR, and NAL.

Discussion

This study was the first to evaluate potential molecular and biological hazards of STEC strains representing nine important non-O157 serogroups from Canada.

Biofilm formation and its associated genes

All non-O157 STEC strains were able to form biofilms with the majority showing high biofilm-forming potential, although there was variation in biofilm formation among serogroups. This variation may be attributed to the relatively limited number of strains studied, but biofilm formation of STEC O157, O26, O91, O103, O111, and O113 on polystyrene surfaces has been recognized to be strain dependent (Biscola *et al.*, 2011; Wang *et al.*, 2012). In the present study, all strains of serogroup O113 readily formed biofilms. Across all serogroups and times, biofilm formation on polystyrene surfaces occurred most readily at room temperature. This agrees with the observation that 19 non-O157 STEC strains from O26, O45, O103, O111, O113, O121, and O145 formed more extensive biofilms at 25°C than at 37°C (Uhlich *et al.*, 2014). Similar results were also obtained by Nesse *et al.* (2014) as strains of O103:H2 produced less biofilm at 37°C than at 20°C. Additionally, biofilm mass was generally increased with exposure time, confirming the results of Fouladkhah *et al.* (2013) where biofilm formation was more extensive after 7 days compared with on day 0 at 15°C and 25°C. No reports, to our knowledge, have compared the biofilm-forming ability between human and animal strains of STEC, but Vijay *et al.* (2015) found that human isolates of Enteroaggregative *E. coli* produce comparatively more biofilm than did animal isolates.

To identify biofilm-associated genes, a panel of adhesin genes and autotransporter protein-associated genes was screened by PCR. For STEC, biofilm formation in most strains depended on the expression of curli fimbriae (Biscola *et al.*, 2011; Wang *et al.*, 2012). In the present study, type 1 fimbriae-encoding gene *fimH*, curli gene *csgA*, and *crl*, as well as central biofilm-regulating gene *csgD*, were present in all strains examined. However, biofilm formation potential of these strains was highly variable, likely reflecting divergence in the expression of these genes.

Previous studies have shown that variations in *rpoS* and prophage insertions in *mlrA* limited CsgD-dependent biofilm formation in O157:H7 and non-O157 strains (Chen *et al.*, 2013; Uhlich *et al.*, 2013). Even though mutations of the *rpos* were not assessed in the present study, two of three non-O157 strains lacked the *rpoS*, but surprisingly, still exhibited high biofilm-forming capacity. To date, frequency of RpoS in STEC strains is unknown, but Franz *et al.* (2015) reported that two strains from O128:H2 and O107:H2 were *rpoS* negative. Presumably, another sigma factor gene may play an *rpoS*-like role in regulating the transcription of *csgD* in response to environmental stress. In addition, fewer non-O157 strains studied harbored prophage stx1 in *mlrA*, consistent with the previous findings that *mlrA* prophage insertions in strains of non-O157 serogroups are not as common as in O157:H7 strains (Shaikh and Tarr, 2003; Chen *et al.*, 2013). Interestingly, stx_1 was detected in 25 strains, suggesting that stx_1-encoding phages may have a different chromosomal insertion site in some non-O157 STEC strains (Shaikh and Tarr, 2003; Kondo *et al.*, 2010).

Autotransporters are other important factors for biofilm formation of non-O157 STEC (Jaglic *et al.*, 2014). In the present study, autotransporter protein-associated genes, *cah* and *flu*, were detected in most non-O157 STEC strains. It is interesting that among the strains lacking *cah* in the present study, 60% exhibited reduced biofilm formation with increasing incubation time at 37°C. As the presence of none of the genes was statistically related to biofilm formation,

further investigation of expression of these genes is required to determine their relative importance in biofilm formation.

Correlation among virulence determinants and non-O157 STEC strains

Karmali *et al.* (2003) proposed grouping STEC strains into five seropathotypes (SPTs), from A through E, according to their reported frequencies in outbreaks, human illness, and the presence of MGEs such as locus of enterocyte effacement (LEE) and OI-122. In our study, all top 6 strains were classified as SPT-B (associated with outbreaks and Hemolytic uremic syndrom (HUS), but less commonly than serotype O157:H7) with strains from O91, O113, and O128 classified as SPT-C (associated with sporadic HUS, but not typically with outbreaks). Previous studies demonstrated a relationship between the presence of stx_2 and the severity of human disease, including the development of HUS and bloody diarrhea with O26, O103, O111, and O145 (Boerlin *et al.*, 1999; Friedrich *et al.*, 2002). However, in the present study, we found that stx_2 genes were more prevalent in the SPT-C. A low prevalence of stx_2 in top 6 strains in the present study aligned with previous findings where a higher percentage of stx_1 than stx_2 was observed in strains from serogroups, O26, O103, and O111 (Tayzar *et al.*, 2013; Anjum *et al.*, 2014).

The *eae* in the LEE is also thought to be a significant determinant of STEC virulence (Etcheverria and Padola, 2013). In this study, *eae* was detected in all strains from top 6 serogroups and in only one O128:NM strain, an observation that agrees with previous reports (Girardeau *et al.*, 2005; Kobayashi *et al.*, 2013).

Other virulence genes, including *toxB*, *adfO*, *sodCF*, *ureA*, and *efa1*, were primarily associated with top 6 strains. Anjum *et al.* (2014) showed that the top 6 STEC were likely to harbor *adfO*, *sodCF*, *ureA*, and *efa1*. All of these virulence-associated genes are located on MGEs in STEC, including OI-57 (*adfO*) (Imamovic *et al.*, 2010), lambdoid prophage (*sodCF*) (D'Orazio *et al.*, 2008), OI-43 or OI-48 (*urea*) (Yin *et al.*, 2009), and OI-122 (*efa1*) (Karmali *et al.*, 2003). Overall, these data suggest that the MGEs substantially contribute to the virulence of STEC strains.

MDR of non-O157 STEC

AMR among foodborne bacteria has been rising since the early 1990s (Walsh and Fanning, 2008). One-third of non-O157 STEC strains evaluated were MDR, with the highest resistance rates observed for STR, SUL, and TET. Other studies from United States (Ju *et al.*, 2012), Spain (Cabal *et al.*, 2013), Belgium (Buvens *et al.*, 2010), and India (Rajkhowa and Sarma, 2014) have made similar observations. Interestingly, STR-SUL-TET resistance was more likely to be detected from animal than human strains. The high frequency of STR-SUL-TET resistance reported among non-O157 strains may, in part, reflect the common use of these antibiotics for growth promotion and disease prevention in food animals (McEwen and Fedorka-Cray, 2002; USDA, 2009)

In the present study, MDR was found in 12 non-O157 strains with 67% possessing a strong or intermediate ability to form biofilms. Previously, Ito *et al.* (2009) indicated that generic *E. coli* in mature biofilms were highly resistant to antimicrobial agents, an outcome that could complicate the disinfection of food contact surfaces (Giaouris *et al.*, 2014). STEC strains forming biofilms with resistance to antimicrobials highlight current challenges of antimicrobial-based sanitation measures for biofilm removal. This is especially true as strains exhibiting AMR often exhibit cross-resistance to many common disinfectants (Ryu *et al.*, 2004; Fouladkhah *et al.*, 2013).

Conclusions

All non-O157 STEC strains evaluated were able to form biofilms, with the majority exhibiting high biofilm-forming potential. Biofilm formation varied with serogroup and origin was generally enhanced at room temperature with prolonged incubation times. Of the genes screened for biofilm formation, no specific combination appeared to be associated with enhanced biofilm formation. In addition, *eae*, *toxB*, *adfO*, *sodCF*, *ureA*, and *efa1* were the dominant virulence profile in top 6 strains. However, a high percentage of strains from serogroups, O91, O113, and O128, carried stx_2, highlighting that serogroups other than top 6 can carry genes with implications for human health. Furthermore, MDR was found in non-O157 STEC strains and most displayed intermediate or strong ability to produce biofilms. Consequently, future strategies to control non-O157 STEC may include measures that effectively control biofilms.

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Disclosure Statement

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