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Research article

Incidence of Top 6 shiga toxigenic *Escherichia coli* within two Ontario beef processing facilities: Challenges in screening and confirmation testing

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Abstract: The incidence of the Top 6 STEC serotypes was determined in two beef slaughter houses. In total, 328 samples were taken of hides, de-hided carcasses and the plant environment. Samples were enriched in Tryptic Soy Broth containing novobiocin then screened using RT-PCR GeneDisk® system that targeted *stx*, *eae* and *wzx* genes. It was found that 92.5% (172 of 186) of the hide samples. 72.5% (29 of 40) de-hided samples and 84.3% (86 of 102) of the environmental samples returned presumptive positive results. Serotypes O103, O45 and O121 were most commonly encountered although all the Top 6 serotypes were represented within individual samples. However, attempts to recover the Top 6 serotypes by culturing proved unsuccessful despite screening up to 20 colonies per CHROMAgar® plate of enriched sample. The reasons for the discrepancy between the RT-PCR and culture methods were found to be due to low levels of the target in enriched samples, presence of virulence factors in different cells and also the transient retention of *stx*. With regards the latter it was found that strains harboring a full set of virulence factors (*eae*, *stx*) were more common in grown cultures held post-incubation at 4 °C for 14 days. Moreover, no *stx* gene was recovered when isolates were sub-cultured on TSA but was present in the same strains grown on CHROMAgar®. In total 39 STEC isolates were recovered with the majority harboring stx_1, stx_2, *eae* and *hylA*. Only 3 of the isolates had stable complement of virulence factors and were identified as O172:H28, O76:H7 and O187:H52. Although no Top 6 STEC were isolated the presence of virulent strains on carcasses with the potential to cause Hemolytic Uremic Syndrome is of concern. The significance of those STEC

that transiently harbor virulence factors is unclear although clearly impacts on diagnostic performance robustness when screening for the Top 6 non-O157 STEC.

Keywords: STEC; Top 6; *Escherichia coli*; RT-PCR; cattle; beef; *stx*; *eae*

Abbreviations

aEPEC	Atypical Enteropathogenic *Escherichia coli*
C_T	Cycle threshold
eae	intimin encoding gene
EHEC	Enterohaemorrhagic *Escherichia coli*
HUS	Hemolytic Uremic Syndrome
RT-PCR	Real Time –Polymerase Chain Reaction
STEC	Shiga toxin producing *Escherichia coli*
stx	shiga toxin
TSA	Tryptone Soya Agar

1. Introduction

In recent years there has been a progressive increase in the number of reported cases of non-O157 Shiga Toxin producing *Escherichia coli* (STEC) infections [1]. It is estimated that there are 168, 000 cases of non-O157 STEC infections within the United States that compares with 96, 000 for the more studied O157:H7 serotype [2]. The incidence of non-O157 STEC is thought to be significantly underestimated due to lack of reporting, misdiagnosis and lack of detection using current clinical diagnostics [2,3,4].

The majority of non-O157 STEC infections are associated with lower virulence than O157:H7 yet some can still lead to hemolytic uremic syndrome (HUS) if the strain harbors the full complement of virulence factors. Specifically, genes encoding for initimin attachment factor (*eae*), shiga toxin (*stx*) and occasionally, hemolysin (*hylA*), are frequently correlated to virulence [5]. The *eae* gene forms part of the chromosome-located LEE island whereas *hylA* is plasmid-encoded [6]. The *stx* gene can be subdivided into stx_1 and stx_2 that also exists in several variants [7]. It is commonly accepted that stx_2 is the more potent form of the toxin compared to stx_1 that is typically associated with lower virulent strains [7]. The *stx* gene is encoded on a prophage that integrates into the chromosome and when expressed produces new phages along with releasing the shiga toxin before cell lysis [8]. Consequently, the expression of shiga toxin in most cases ultimately leads to the death of the *E. coli* producing cell [8].

A major challenge in surveillance of non-O157 STEC is the wide distribution and diversity of the *E. coli* subgroup. The majority (>70%) of non-O157 STEC encountered have low virulence due to restricted *stx* expression and lack of a full complement of virulence factors such as *eae* [9]. However, those STEC that express shiga toxin along with attachment factors can result in HUS development within a susceptible host and hence are classed as EHEC. Given that EHEC only makes up a small proportion of STEC encountered in animals, foods and the environment, there was a focus

on those that represent the main risk to public health. To this end, it was noted that 70% of clinically confirmed non-O157 STEC could be attributed to 6 serotypes; O26, O45, O103, O111, O121 and O145 that became known as the Top or Big 6 [6].

It was assumed from the outset that the Top 6 STEC followed the same dissemination routes as O157:H7 in that cattle were the main source of the pathogens, along with beef being the main food vehicle for transmission. Consequently, legislation was passed in 2011 to designate the Top 6 non-O157 STEC as adulterants in beef trim then later in ground beef [10]. This in turn led to the development of diagnostics (primarily methods based on RT-PCR) to screen for the Top 6 non-O157 STEC in cattle, the environment and beef. The methods follow a similar format of enriching samples in TSB with novobiocin followed by screening with multiplex RT-PCR to detect *stx* and *eae* followed by a second panel that screens for the *wzx* gene that encodes LPS associated with the Top 6 serotypes. There have been several diagnostic platforms developed for screening for the Top 6 non-O157 STEC with the GeneDisk® from the PALL Corporation and BAX from Dupont being two examples [5,11]. A common feature of surveillance studies, either performed on beef or cattle, is that a high prevalence (0.4–74%) and diversity of Top 6 non-O157 STEC are encountered [12–15]. However, attempting to recover isolates from presumptive positive samples remains a challenge. Specifically, the extent of recovery of culture positive can be <1% of those samples testing positive by RT-PCR [16–21]. The underlying reasons for the low culture positive samples has been proposed to be due to the target being at low levels, virulence and LPS genes being present in different cells [22]. It has also been proposed that the lack of distinguishing phenotypes between non-O157 STEC and non-pathogenic *E. coli* can result in high background interference thereby making isolation of the former problematic during culturing [11,23].

The following reports on the incidence of the Top 6 non-O157 STEC associated with cattle and processing environment within Ontario beef processing operations. An RT-PCR platform was applied and problems with isolating culture positive samples addressed through extensive screening of colonies derived from presumptive positive samples. Specifically, whereas other studies select 2–5 colonies per plate the current study screened up to 20 colonies to increase the probability of isolating a Top 6 non-O157 STEC target.

2. Materials and Method

2.1. Experimental design

Samples were obtained from two different beef processing facilities in South-Western Ontario over 4 visits within a 5 month period. Hide, carcass, fecal and environmental samples were collected from individual animals for detection of *E. coli* non-O157:H7. Specifically, ten incoming cattle were selected at random and a saline pre-moistened sterile sponge (Nasco, Fort Atkinson, WI) was used to take a composite sample of the neck, flank and round. The same carcass was sampled following the de-hiding process. In addition, samples (10 cm^2 areas) were also collected using sponges from the holding pens and also the stun floor onto which the cattle rolled onto following stunning. All the samples were transferred to a cold box and processed within 24 h.

2.2. Top 6 STEC screening

The sponge samples were enriched in 100 mL TSB containing 20 mg/L novobiocin overnight at 42 °C. The enriched samples were screened using RT-PCR GeneDisk system (GeneDisc® Cycler, Pall Corporation, Port Washington, NY, USA) in accordance to the manufacturer's instructions. Here, 50 µL of enriched sample was heated at 100 °C for 10 min to extract the DNA. Aliquots (18 µL) of the DNA template were added along with an equal volume of quantification master mix (QMM) containing primers targeting *stx*$_1$, *stx*$_2$, and *eae* genes. The PCR routine was performed over 45 cycles and reaction monitored through the increase in fluorescent signal. Samples testing positive for *eae* and *stx* were taken forward for a second round of screening for presence of genes for the Top 6 O-antigens, as per the manufacturer's instructions.

The performance of the RT-PCR GeneDisc® system was verified by inoculating homogenates of environmental, hide and carcass samples with a cocktail of Top 6 non-O157 STEC serotypes provided by the Public Health Agency of Canada (Guelph, ON, Canada). The individual isolates were cultivated 50 mL of TSB overnight at 37 °C. The culture was used to inoculate (final cell density of *ca.* 3 log cfu/mL) TSB containing 20 mg/L novobiocin from a homogenate derived from hide, carcass or environment. The culture was enriched overnight at 42 °C before removing an aliquot for screening using the GeneDisc® system.

For enriched samples testing positive for virulence factors and Top 6 O-antigens were put through a confirmatory second screening using the BAX RT-PCR STEC assay. Here, aliquots (20 µL) of enriched samples were transferred to reaction tubes containing 200 µL of lysis reagent that was then heated for 20 minutes at 37^{o}C. The temperature was then increased to 95 °C and held for 10 min and 30 µL of the DNA template mixed with reaction mix within PCR tubes containing primers targeted towards s*tx* and *eae* genes. Samples testing positive were taken forward for a secondary screening for the O antigen gene (*wzx)* of the Top 6 STEC using two separate panels for O26, O111, and O121 (first panel) and O45, O103, O145 (second panel).

2.3. Culture confirmation testing

Enriched samples testing positive for *eae*, *stx* and Top 6 *wzx* were taken forward for culture confirmation testing. Here, the enriched samples were plated onto CHROMAgar® (BioMed Diagnostics, Oregon, USA) and incubated for 24 h at 37 °C. Ten presumptive STEC isolates (mauve colonies) were picked per plate and transferred to a new CHROMAgar® plate (subsequently incubated at 37 °C for 24 h) and the remainder of the inoculm to 500 µL molecular grade water (Invitrogen Life Technologies, Mississauga, Ontario, Canada). All 10 selects colonies were pooled into the tube containing 500 µL water to make the composite sample and placed into a boiling water bath to extract the DNA. PCR protocol, primers, and thermal cycling conditions (MyCycler® Thermal cycler, BIO-RAD, California, US) for *stx*, *eae* and *hylA* screening were followed as previously described by Paton & Paton [17]. DreamTaq Green PCR master mix (ThermoFisher, NY, USA) was used in the PCR reactions with nuclease-free water and 2 µM primer concentrations combined with 1 µL DNA template (Table 1). The PCR amplification products were separated on a 1.5% w/v agarose gel containing ethidium bromide. The gel bands were visualized under UV using a Bio-Rad Gel Doc EZ System (Bio-Rad, Hercules, CA).

In the event that composite samples tested positive for *eae* and *stx* the ten individual colonies that comprised the presumptive positive composite samples were individually screened by the same PCR technique to identify those that contributed to the positive result. Isolates harboring *eae* and *stx* were confirmed as *E. coli* by using the VITEK® MS IND system (BioMerieux, Laval, QC, Canada).

Table 1. Primers used for amplification of virulence genes *stx1, stx2, eae, hly* and ERIC PCR.

Target	Fragment Size (bp)	Primer	Sequence	Source
stx_1	180	stx1F	ATAAATCGCCATTCGTTGACTAC	[17]
		stx1R	AGAACGCCCACTGAGATCATC	
stx_2	255	stx2F	GGCACTGTCTGAAACTGCTCC	
		stx2R	TCGCCAGTTATCTGACATTCTG	
eae	384	eaeF	GACCCGGCACAAGCATAAGC	
		eaeR	CCACCTGCAGCAACAAGAGG	
hlyA	534	hlyF	GCATCATCAAGCGTACGTTCC	
		hlyR	AATGAGCCAAGCTGGTTAAGCT	
ERIC	various	ERIC1	ATGTAAGCTCCTGGGGATTCAC	[24]
		ERIC2	AAGTAAGTGACTGGGGTGAGCG	

2.4. Serotyping of culture positive Top 6 STEC isolates

Culture positive *E. coli* isolates harboring *eae* and *stx* were sub-cultured onto TSA and incubated for 24 h at 37 °C. The Prolex® *E. coli* non-O157 Identification latex agglutination test (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada) was used to screen for the Top 6 non-O157 STEC serotypes. Selected isolates were also submitted to the Public Health Agency of Canada (Guelph, Ontario) for confirmatory serotyping.

2.5. Enterobacterial Repetitive Intergenic Consensus (ERIC) sequence PCR

The isolated to be typed was cultivated on Luria-Bertani (LB) agar at 37 °C for 24 h and a single colony suspended in 500 µL water. The sample was placed in a boiling water bath for 10 min followed by centrifugation at 13,000 g for 2 min to remove cell debris. Aliquots (1 µL) were mixed with 24 µL of DreamTaq Green PCR master mix (ThermoFisher, NY, USA) and 2 µM primers (Table 1). The PCR reaction was performed in a MyCycler® Thermal cycler (Bio-Rad, California, US) with an initial cycle for 3 min at 94 °C, then 35 cycles consisting of 30 s at 94 °C, 1 min at 52 °C, and 4 min at 65 °C. The last cycle was for 8 min at 65 °C [24]. The PCR amplification products were separated on a 1.5% w/v agarose gel and band patterns visualized using a Bio-Rad Gel Doc EZ System (Bio-Rad, Hercules, CA).

3. Results

3.1. Sampling and RT-PCR screening

Hide (n = 186), de-hided carcasses (n = 40) and environmental (n = 62 holding pen n = 40 stun floor) samples were collected from two participating beef processors over a 5 month period. The samples were enriched in TSB containing novobiocin then screened for the Top 6 STEC using RT-PCR GeneDisk® system. It was found that positive samples (i.e. harboring *stx*, *eae* and *wzx* for Top 6 STEC serotypes) were returned for 92.5% (n = 172) of the hide samples, 72.5% (n = 29) de-hided samples and 84.3% (n = 86) of the environmental samples screened. A secondary RT-PCR assay using BAX System confirmed the presence of *stx* and *eae* in enriched samples along with the occurrence of the LPS genes for the Top 6 STEC. The two RT-PCR platforms were in agreement with respect to *stx* and *eae*, along with the occurrence of the Top 6 STEC LPS encoding genes. With respect to the latter, a common feature in all the samples was the high diversity of the Top 6 serotypes encountered within the individual samples. The most commonly encountered serotypes were O103, O45 and O121 although all the Top 6 STEC serotypes were encountered to varying degrees (Figure 1). A further observation was the high diversity of Top 6 non-O157 STEC within the individual samples that ranged from three to all six serotypes.

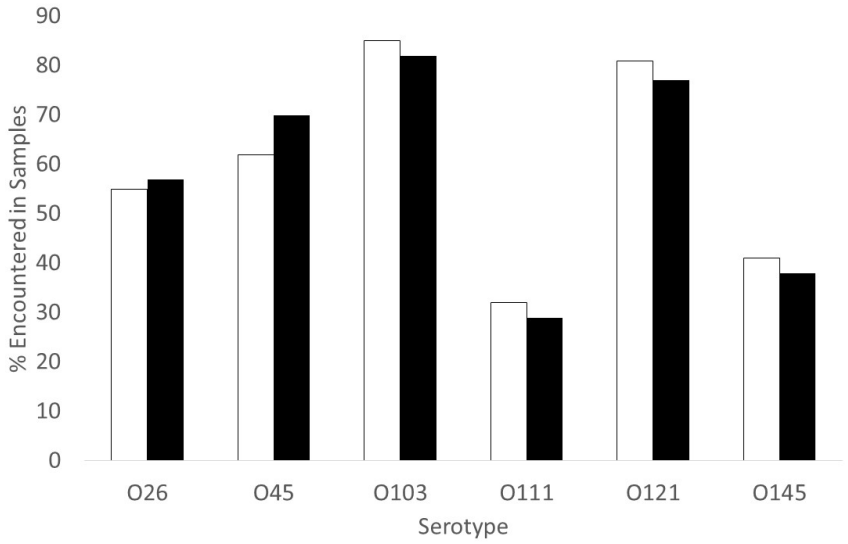

Figure 1. Distribution of Top 6 non-O157 STEC serotypes within RT-PCR presumptive positive samples recovered from hide, carcasses and environmental samples collected from beef slaughter houses. Open bars are serotypes determined by GeneDisk® system with closed bars being detected using BAX system.

Although the C_t values for the O antigens were low (<20 cycles), those associated with the virulence factors had values ranging from 10–39 cycles (Figure 2). Control samples derived from enriched cultures derived from those inoculated with top 6 STEC strains returned positive results indicating no inhibitors were present in the different matrices (hide, carcass and manure).

The C_t values for *eae* and *stx* for each sample were within 5 cycles for 81% of samples, and within one cycle for 22% of cycles, suggesting that these targets were present in relatively equivalent amounts. Yet, the high C_t values would suggest the virulence genes were at relatively low concentration within the enriched samples (Figure 2).

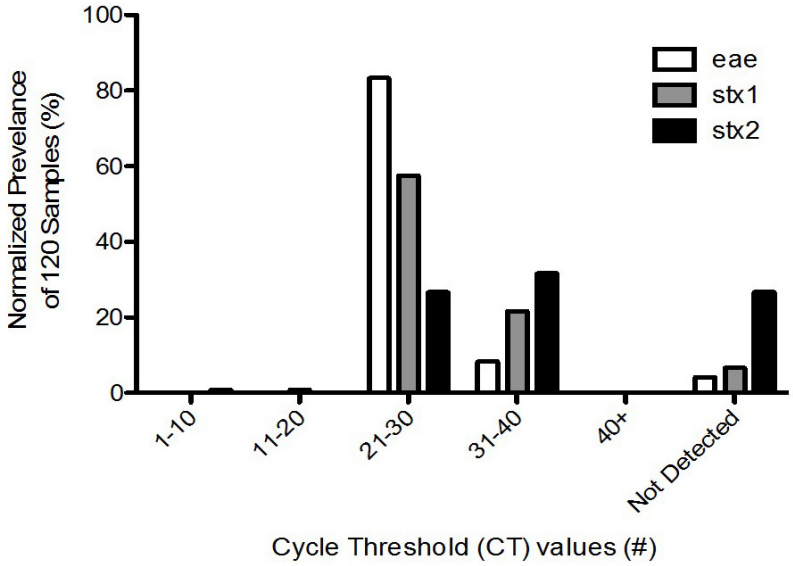

Figure 2. C_T values for *stx* and *eae* detection of RT-PCR presumptive positive samples as determined by the GeneDisk® system.

3.2. Culture confirmation of presumptive positive samples

A sub-set of 120 presumptive positive enrichment samples were individually plated onto CHROMAgar® that was subsequently incubated at 37 °C for 24h. Mauve colonies (n = 10) from individual plates were sub-cultured onto a new CHROMAgar® plate and also combined to form a composite sample. The composite was taken forward to screen for the presence of stx_1, stx_2, *eae* and *hlyA* by conventional multiplex PCR. With composite samples testing positive for *eae* and *stx* the individual colonies making up the composite were screened to identify those contributing to the PCR positive result. After the first round of colony screening, 4 of 120 samples screened yielded composite sample pools containing virulence factors. When the individual colonies making up the sample composites were screened, at least one individual isolate harboring a full complement of virulent factors (*eae* & *stx*) was recovered from two of the samples, providing a rate of recovery of 1.7%. In the other two composite samples testing positive, the virulence factors were identified but in different isolates (Figure 3).

Figure 3. Virulence fingerprint of a composite sample containing ten colonies derived from isolates derived from enriched sample L1-35 (lane 4). Lanes 5-14 shows the virulence fingerprint of the individual isolates making up the composite. Lane 2 contains a positive control for virulence genes *stx*$_1$, *stx*$_2$, *eae,* and *hly*, and lanes 3 and 15 contain a negative control. 1kb DNA ladder shown in Lanes 1 and 16.

3.3. Effect of extended holding period at 4°C on apparent STEC prevalence

In an attempt to recover more isolates with a full complement of virulence factors, further colonies were recovered from the original plates that had been stored at 4 °C for 14 days. Here, 10 further (different) colonies were taken to prepare the composite samples. Interestingly, on this occasion, the number of composite samples harboring the full compliment of virulence factors was 17 compared to only 2 in the first round. Isolates containing a full complement of virulence factors (i.e. *eae* and *stx*) were recovered from 16 of the composite samples (representing a recovery rate of 13%) with one sample having the virulence factors distributed between different cells.

In total, 39 individual isolates were recovered harboring full complement of virulence genes (Table 2). All the isolates were confirmed as *E. coli* with growth at 37 °C but not 4 °C. The most prominent strains harbored *stx*$_1$, *stx*$_2$, *eae*, *hylA* with *stx*$_2$ being encountered more so than the less potent *stx*$_1$ (Table 2). The majority of isolates were recovered from cattle hides but also the de-hided carcass but less from the environment of the slaughterhouses (Table 2).

As part of the culture maintenance the 39 isolates were sub-cultured onto TSA agar. It was found that the *stx* gene in all but 3 of the isolates was absent. Yet, when sub-cultured onto CHROMAgar® the *stx* gene was retained. This was illustrated in isolate L1-17 that when cultured on CHROMAgar® harbored *eae*, *stx*$_2$ and *hylA* but were devoid of the latter two virulence factors when grown on TSA agar (Figure 4). By using ERIC-PCR it was found that both isolates recovered from CHROMAgar® and TSA had the same fingerprint indicating they belonged to the same strain (Figure 5).

Table 2. Occurrence of *stx*, *eae* and *hlyA* genes amongst the isolates recovered from RT-PCR presumptive positive samples.

Sample ID	Slaughter House	Sample Source	Virulence Genes
L1-3	1	Hide	*stx_1, stx_2, eae*
L1-3	1	Hide	*stx_1, stx_2, eae*
L1-3	1	Hide	*stx_1, stx_2, eae*
L1-12	1	Hide	*stx_1, stx_2, eae, hly*
L1-14	1	Hide	*stx1, stx2, eae,*
L1-14	1	Hide	*stx_1, stx_2, eae*
L1-14	1	Hide	*stx_1, stx_2, eae*
L1-14	1	Hide	*stx_1, stx_2, eae*
L1-14	1	Hide	*stx_1, stx_2, eae*
L1-17	1	Hide	*stx_1, stx_2, eae*
L1-17	1	Hide	*stx_1, stx_2, eae, hly*
L1-17	1	Hide	*stx_1, stx_2, eae, hly*
L1-24	1	Hide	*stx_1, stx_2, eae*
L1-24	1	Hide	*stx_1, stx_2, eae*
L1-24	1	Hide	*stx_1, stx_2, eae*
L1-27	1	Hide	*stx_1, stx_2, eae*
L1-32	1	Carcass	*stx_1, stx_2, eae*
L1-32	1	Carcass	*stx_1, stx_2, eae*
L1-32	1	Carcass	*stx_1, stx_2, eae*
L1-32	1	Carcass	*stx_1, stx_2, eae*
L1-32	1	Carcass	*stx_1, stx_2, eae*
L1-32	1	Carcass	*stx_1, stx_2, eae*
L1-38	1	Carcass	*stx_1, stx_2, eae, hly*
L1-38	1	Carcass	*stx_1, stx_2, eae hly*
L2-1	2	Hide	*stx_2, eae, hly*
L2-5	2	Hide	*stx_1, stx_2, eae, hly*
L2-7	2	Hide	*stx_2, eae, hly*
L2-7	2	Hide	*stx_2, eae*
L2-8	2	Hide	*stx_2, eae*
L2-8	2	Hide	*stx_2, eae, hly*
L2-10	2	Hide	*stx_2, eae, hly*
L2-10	2	Hide	*stx_2, eae, hly*
L2-10	2	Hide	*stx_2, eae, hly*
L2-13	2	Hide	*stx_2, eae*
L2-36	2	Carcass	*stx_1, eae, hly*
L2-44	2	Floor	*stx_1, stx_2, eae, hly*
L2-46	2	Floor	*stx_1, eae, hly*
L2-46	2	Floor	*stx_2, eae*
L2-49	2	Floor	*stx_2, eae*

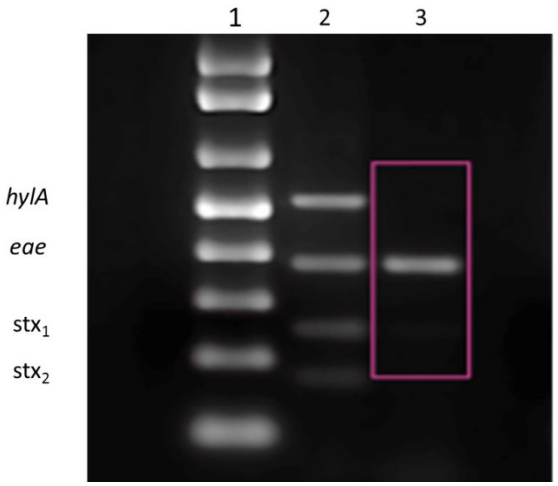

Figure 4. Loss of *stx* and *hylA* from L1-17 STEC isolate when cultivated on TSA agar. The isolate was cultivated on CHROMAgar® (lane 2) then sub-cultured onto TSA (lane 3). Lane 1 shows the 1kb ladder.

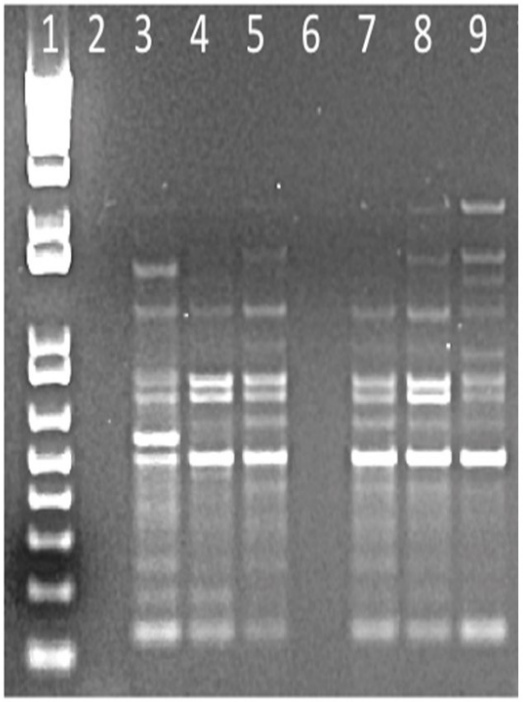

Figure 5. ERIC-PCR profile of L1-17 STEC isolate from TSA agar (Lanes 3-5) or CHROMAgar® (Lanes 7-9). Lane 1 contained illustrates a 1 kb DNA ladder.

The three isolates that exhibited stable virulence factor composition were identified as serotypes O172:H28, O76:H7 and O187:H52, all of which harbored *stx*$_1$, *stx*$_2$, *eae* and *hylA*. The other isolates screened did not belong to the Top 6 STEC as determined by latex agglutination assay.

4. Discussion

The original objective of the study was to determine the incidence of the Top 6 STEC within two Ontario beef processing facilities. In agreement with other studies, it was found that samples associated with cattle had a high prevalence and diversity of the Top 6 serotypes when initially screened using RT-PCR. Similar to other studies, serotype O103 was most frequently recovered, suggesting host related associations with cattle [14,19,25]. Yet, despite the apparent high prevalence of the Top 6 non-O157 STEC recorded using RT-PCR no culture positive isolates of the serotypes were recovered. The high prevalence of the Top 6 O serotypes could not be assigned to false positives given the results were confirmed using a different RT-PCR platform. Instead, it is more likely that genes encoding for the O antigens were present but within *E. coli* cells not harboring virulence factors. This represents a key limitation of screening for the Top 6 *E. coli* serotypes given that there is a poor correlation between O antigen and virulence factors unlike the more well characterized O157:H7 [26]. Yet, additional factors were also evident to explain the low recovery on plates from samples returning high prevalence according to RT-PCR. Specifically, the high C_T values for *eae* and *stx* would suggest that the target genes were in low abundance in the enriched cultures. Cooley et al. [22] suggested that only samples with C_T values >27 cycles the probability of isolating culture positives would be <1% [22]. Other researchers have encountered the same low recoveries of Top 6 STEC when C_T values were high [14,19]. In the current study, the C_T values were >20 suggesting that the Top 6 non-O157 STEC could have been in the sample but the high background made isolation problematic.

An additional factor that contributed to the apparent high presumptive positive RT-PCR results was the presence of virulence factors in different cells. This was indeed found to be the case with virulence factors being present in composite samples taken from colonies from presumptive positive enrichment samples. The result is in agreement with other researchers that have also isolated virulence factors within different cells that constituted a presumptive PCR positive sample [27,28,29]. In the current study, the presence of virulence factors in different cells likely contributed to a minor extent given that the occurrence of *stx* and *eae* by RT-PCR was similar. If the virulence factors were in different cells then it would be expected that one of the factors would be disproportionate relative to the other. In addition, only two of the composites samples containing pooled isolates was demonstrated to have virulence factors in different cells.

A further factor that contributed to the apparent high prevalence of STEC by RT-PCR was the mobility of virulence factors (*stx* and to some extent *hylA*). Although *E. coli* did not grow at 4 °C it was evident that *stx* genes (prophages) were exchanged between cells making up the colony. The acquisition of *stx* by aEPEC is well documented although the heterogeneous distribution of *stx* within the same colony has not been reported to any great extent [30]. The extent of *stx* exchange was found to be influenced by the growth medium with TSA being less likely to harbor *stx* and *hylA* compared to CHROMAgar® . The heterogeneous distribution of *stx* within a population and influence of sub-culturing has been previously observed. Specifically, Bielaszewska et al. [31] demonstrated how Top 6 non-O157 STEC convert from EHEC to aEPEC by losing *stx* during cell division. The researchers observed loss of stx_2 in up to 14% of EHEC colonies tested following sub-culturing. Conversely, aEPEC were also found to acquire *stx* when incubated in the presence of shiga toxin phages [32]. The transient recovery of *stx* in *E. coli* was also observed from an isolate from a patient diagnosed with HUS [33]. The loss of *stx* has also been reported for STEC when sequentially sub-cultured [34].

However, there have been no reports on medium composition having an influence on the distribution of *stx* or *hylA* within *E. coli* populations. CHROMAgar® selectivity is primarily based on cefixime and tellurite with differentiation by hydrolysis of chromogenic constituents. Therefore, it is possible that the mobility of *stx* prophage and/or *hylA* encoding plasmid was enhanced due to imposed stress by selective agents that were not present in TSA. In evolutionary terms, there is a strong incentive for *E. coli* to resist infection by *stx* prophage given expression ultimately means death of the producing cells.

Even though the study did not recover any Top 6 non-O157 samples there were three isolates that carried virulence factors that could potentially cause HUS. The serotypes recovered have not been previously implicated in non-O157 STEC infections, so their clinical significance is unclear. Yet, the research does underline the need for better diagnostics to identify STEC strains of importance to food safety above that of screening for serotypes.

5. Conclusion

The study has further highlighted the challenges in screening for the Top 6 non-O157 STEC in samples derived from cattle and beef processing environments. Although RT-PCR platforms provide a high throughput and convenient screening technique the results are only useful if an isolate is recovered in the end. The study illustrated that the disconnect between RT-PCR and culture based methods is due to a combination of targets being present at low levels, virulence factors being distributed between different cells and the transient acquisition of *stx*. In the broader sense, the study has further underlined the need for improved diagnostics to identify those STEC of significance to public health.

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Conflict of Interest

There is no conflict of interest relating to the research.

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