Title: Evaluating the efficacy of Beef Slaughter Line Interventions by Quantifying the Six Major Non-O157 Shiga toxin Producing *Escherichia coli* Serogroups Using Real-Time Multiplex PCR

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Abstract

Six major Shiga toxin producing *Escherichia coli* (STEC) serogroups: O26, O103, O145, O111, O121, and O45 have been declared as adulterants in federally inspected raw beef in the USA effective June 4th, 2012 in addition to the routinely tested STEC O157: H7. This study uses a real-time multiplex PCR assay and pooling of the samples as a strategy to optimize the detection and quantification (prevalence and contamination) of six major non-O157 STEC, regardless of possessing Shiga toxins. Samples were collected from one large-scale slaughter plant (Plant LS) and one small-scale slaughter plant (Plant SS) located in the Mid-Western USA, in 2011, before the establishment of 2013 USDA laboratory protocols. Carcasses were sampled at consecutive intervention stations and beef trimmings were collected at the end of the fabrication process. Plant SS had marginally more contaminated samples than Plant LS (p-value 0.08). The post-hide removal wash, steam pasteurization, and lactic acid ($\leq 5\%$) spray used in Plant LS seemed to reduce the six serogroups effectively, compared to the hot-water wash and 7-day chilling at Plant SS. Compared to the culture isolation methods, quantification of the non-O157 STEC using real-time PCR may be an efficient way to monitor the efficacy of slaughter line interventions.

1. Introduction

Shiga toxin producing *Escherichia coli* (STEC, *syn.* VTEC) are foodborne pathogens that may cause bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in humans ([Karmali et al. 1983](#)). *E. coli* O157:H7 is the prominently discussed pathogenic serotype of the STEC. However, among 265,000 estimated annual STEC infections in the U.S. population, only 36% were due to STEC O157 while 64% were due to non-O157 STEC ([CDC 2012](#)). The most common foodborne pathogenic non-O157 STEC serogroups reported in the U.S. between 2000 and 2010 were O26 (26%), O103 (22%), O111 (19%), O121 (6%), O45 (5%), and O145 (4%) (Percentage of each serogroup compared to the total non-O157 STEC) ([Gould et al. 2013](#)). These six serogroups were recognized as the “major six non-O157 STEC serogroups” reported during 1983 – 2000 period as well ([Brooks et al. 2005](#)).

Raw meat, especially beef, is associated with foodborne STEC infections due to direct or cross contamination ([Erickson and Doyle 2007](#)). Ruminants harbor STEC in their gastrointestinal system and contamination of beef with STEC takes place at slaughter and processing plants ([Arthur et al. 2004](#)). In October 1994, the Food Safety and Inspection Service of the United States Department of Agriculture (USDA - FSIS) declared *E. coli* O157:H7 as an adulterant in the non-intact raw beef including ground beef and the federally inspected facilities regularly test their products for STEC O157:H7 ([U.S. Federal Register Vol 11, 1999](#)). USDA-FSIS declared the six major non-O157 STEC serogroups as adulterants in the non-intact raw beef including ground beef effective June 4th, 2012. Since then, federally inspected facilities routinely test beef trimmings for the presence of six major non-O157 STEC serogroups, in addition to STEC O157:H7 ([U.S. Federal Register Vol 77, 2012](#)).

U.S. beef industry is focused on the reduction of contamination, improvement of intervention methods, improvement of the detection methods, and optimization of the sample sizes to cost effectively test their production systems in order to mitigate the impact of pathogenic and adulterant STEC (Pihkala et al. 2012). Several publications introduced real-time multiplex PCR methods that can be used to quantify non-O157 STEC in slaughter line interventions and ground beef (Fratamico et al. 2011, Anklam et al. 2012, Singh and Mustapha 2015). There is no published study up-to-date which describe the use of real-time multiplex PCR as a tool to evaluate the efficacy of slaughter line interventions. The real-time PCR assays are suggested to be time and cost effective compared to conventional culture isolation methods (Singh and Mustapha 2015). Real-time multiplex PCR can be used as a first- or second order test that can be used to quantify the prevalence and the level of contamination of the six major non-O157 STEC (Singh and Mustapha 2015), therefore, can be used to evaluate the efficacy of beef slaughter line interventions. Depending on the real-time multiplex PCR results the necessity for further laboratory analysis such as culture and isolation or testing for Shiga toxins can be decided.

The objective of this study was to use a real-time multiplex PCR assay and pooling of the samples as a strategy to optimize the detection of six major non-O157 STEC, despite their possession of Shiga toxins. The real-time multiplex PCR & pooling method can be used as a first- or second order test to evaluate the efficacy of slaughter line interventions. The study targets the quantification of the six major non-O157 STEC at both the pool level and the individual carcass level. The contamination values of six major non-O157 STEC generated in this study may serve as an example for the potential use of real-time multiplex PCR and pooling of samples to assess the exposure levels of carcasses. This approach may contribute to the risk analysis and improvement of HACCP

protocols at the slaughter plants ([Wilhelm et al. 2011](#)). Moreover, this type of information may be beneficial when risk-based sampling for the slaughter plant intervention steps are being considered ([USDA-FSIS 1999](#), [U.S. Federal register Vol 80, 2015](#)).

2. Materials and Methods

2.1. Sampling locations

One large scale slaughter plant (Plant LS) which processes 250-300 carcasses per hour for 5 days a week was sampled during April- May 2011. Similarly, one small scale slaughter plant (Plant SS) which processes 30 - 40 carcasses per day once a week was sampled during July-August 2011. Both were located in the Midwestern United States.

2.2. Sample size calculation

Carcasses were sampled using a sponge at consecutive intervention stations and beef trimmings were collected in the end process. The sample size was calculated using AusVet Epitools pooled prevalence calculator based on a pilot study (data not shown here) (Sergeant, 2009). The expected prevalence of carcasses at the end process was assumed to be 0.04% with 95% sensitivity and specificity. The calculated pool size was 15 ($k=15$). Therefore, from each intervention step 15 sponge samples were pooled together. End process beef trimmings were collected according to USDA-FSIS guidelines ([USDA-FSIS 2008](#), [USDA-FSIS 2011](#)). The types of samples and sample sizes collected from the slaughter plants are summarized in Table 1.

2.3. Sampling Stations and Sampling Strategy

Sponge samples were collected according to the food safety monitoring protocols of the slaughter plants which were based on USDA-FSIS guidelines and the beef industry standards ([USDA-FSIS 1996](#), [Arthur et al. 2004](#)). **Sponge samples for each intervention step is a pool of samples collected from brisket, flank, or rump region.** Beef trimmings from Plant LS were collected

according to the N60 sampling method established by the USDA-FSIS for O157: H7 ([USDA-FSIS 2011](#)). A modified N60 method was used for the sampling of Plant SS ([USDA-FSIS 2008](#)). Plant SS received beef trimmings from external small scale slaughter plants. Therefore, meat trimmings of Plant SS should be considered as a combination of in-house and external meat trimming samples.

As summarized in Table 2, sampling stations at Plant LS included 7 intervention steps and end process beef trimmings. Whereas, the sampling stations at Plant SS included 6 intervention steps, end- process beef trimmings, and meat samples from a waste bin located at the slaughtering line (the waste bin represents the highest possible environmental contamination). Carcasses were swabbed using sterile sponges at each intervention step (Sponges; Fisherbrand™ Cellulose sponge, Fisher Scientific, Pittsburg, PA). After swabbing, the sponges were immersed in 25 ml of Butterfield's Phosphate Buffered Diluent, pH 7.2 (BPBD; Remel™, Thermo Fisher Scientific, Inc., Waltham, MA) for Plant LS and in 25 ml of Phosphate Buffered Saline, pH 7.4 (PBS) (Sigma-Aldrich, Co., St. Louis, MO) for Plant SS, and transported to the laboratory under refrigeration ($\leq 4^{\circ}\text{C}$) ([USDA-FSIS 2011](#)). The term 'area' refers to the surface area of the carcass that was sponge-sampled. Carcass sampling locations are described using the industry terminology for commercial primal cuts of beef (Retail beef cuts, 2009). All the samples were kept at 4°C and processed within 24h of the collection.

2.4. Sample processing

Sponge samples were homogenized manually. Ten milliliters from 15 sponge samples collected from the same intervention step were pooled and were placed in three 50 ml conical tubes. This procedure was repeated for every sampling station. Conical tubes were centrifuged at $2880 \times g$ for 30 min at 27°C in an Eppendorf rotor -model 5810: A-4-44 centrifuge (Eppendorf North America,

Hauppauge, NY). The pellet was re-suspended into 3ml of the Modified *E. coli* broth (MEC) - (Thermo Fisher Scientific Remel™ products, Lenexa, KS) supplemented with 10mg/L cefsulodin (MP Biomedicals, LLC., Solon, OH).

Please note that at the time of the study (2011), the USDA processing standards for bacteriological cultures had not been published yet ([USDA-FSIS laboratory guidebook 2013](#)). Therefore, a real-time multiplex PCR protocol for the detection of the six major non-O157 STEC published by our research group was used ([Anklam et al. 2012](#)). The Prepman Ultra heat lysis protocol was used for the DNA extraction using a 1.5 ml aliquot of the pooled samples (Prepman®Ultra sample protocol by Applied Bio Systems, Carlsbad, CA). The remaining pool suspension was added to 5 ml of MEC-cefsulodin broth and enriched by incubating at 37°C for 12 h. A 1-ml aliquot of the enriched sample was subjected to DNA extraction. A DNA cleaning procedure was conducted using the PowerClean® DNA Clean-Up Kit which uses the patented Inhibitor Removal Technology® to remove possible inhibiting substances (Mo Bio Laboratories, Inc. Carlsbad, CA).

Ninety milliliters of MEC-cefsulodin broth was added to the beef trimming sample (375 g per sample) and homogenized manually for 5 minutes. From the homogenate, 1.5 ml aliquot was subjected to DNA extraction using the Prepman Ultra protocol. The remaining meat sample was enriched by incubating at 37° C for 12 h. A 1-ml aliquot of the enriched meat sample was subjected to DNA extraction and DNA cleaning procedures as described above. The same protocol was followed for the processing of waste meat bin samples of Plant SS.

2.5. Positive and negative controls

The positive controls were the pure cultures of non-O157 STEC inoculated onto Luria Bertani (LB) agar (Difco™ Luria- Bertani Broth, Miller, and Bacto™ Agar; Becton Dickinson and Company, Sparks, MD) and incubated at 37° C for 12 h. A 1.5 ml aliquot of MEC-cefsulodin broth was used as the negative control. Detection limits of the real-time multiplex PCR method used in this study ranged between 3 log CFU/ml– 4 log CFU/ml (detection limits for the pure cultures and artificially inoculated fecal samples) ([Anklam et al. 2012](#)).

2.6. Taqman real-time multiplex PCR screening of the samples

The real-time multiplex PCR assays described by Anklam *et al.* (2012) were used for the analysis of the samples ([Anklam et al. 2012](#)). The cutoff cycle threshold (Ct) value was 38. The Ct values for each serogroup resulted in the PCR assay were converted into a log CFU/ml using standard curves for STEC pure cultures described by Anklam *et al.* (2012) were used in the analysis ([Anklam et al. 2012](#)). These values represent the contamination levels. Regression equations for each serogroup derived from standard curves were as follows: O45: $\log \text{CFU/ml} = 14 - 0.322 * \text{Ct}$, O121: $\log \text{CFU/ml} = 13.9 - 0.313 * \text{Ct}$, O111: $\log \text{CFU/ml} = 14.9 - 0.311 * \text{Ct}$, O26: $\log \text{CFU/ml} = 15.8 - 0.343 * \text{Ct}$, O145: $\log \text{CFU/ml} = 15.7 - 0.312 * \text{Ct}$, and O103: $\log \text{CFU/ml} = 17.4 - 0.354 * \text{Ct}$ ([Anklam et al. 2012](#)).

2.7. Quantitative methods

Quantitative methods included two steps: 1) quantification of the prevalence and contamination level of six major non-O157 STEC in the pooled samples as well as in the individual carcasses and 2) statistical analysis of the prevalence and level of contamination data generated from

the first step. The statistical analysis was done to evaluate efficacy in reducing the six major non O-157 STEC serogroups at consecutive intervention steps along the process.

2.8. Quantification of the prevalence and level of contamination

Based on the PCR results, presumptive prevalence per pool (Prev_P) and presumptive prevalence per individual carcass (Prev_C) were calculated. Prev_P was defined as the number of positive pools per number of pools tested. Prev_P was estimated to have a beta distribution. The number of positive pools (a) and the total number of pools (b) were used to obtain the shape parameters alpha (α) and beta (β) of the beta distribution, as shown in Eq (1) and Eq (2).

$$\alpha = a-1 \text{ \#Eq (1)}$$

$$\beta = b-a+1 \text{ \#Eq (2)}$$

Using the parameters above, the distribution of the pooled prevalence in a given serogroup was simulated 1000 times (Vose, 2000). Descriptive statistics including median and 95% credibility interval were produced for the simulated Prev_P values for all the sponge and meat samples from Plant LS and Plant SS. Prev_C were derived from the Prev_P data using the Eq (3).

$$\text{Prev_C} = 1-(1-\text{Prev_P})^{1/k} \text{ \#Eq (3)}$$

The estimated average contamination level of a particular serogroup per each pool in “log CFU/pool” units (Contamination_P) was calculated using Eq (4). Where, x is the contamination level that was calculated using the regression equation for each serogroup (Unit: log CFU/ml) and k is the number of sponge samples pooled together. Since an aliquot of the sample was subjected to DNA extraction, the dilution factors were taken into consideration in order to calculate the contamination

in the original pool. The average contamination level of a unit area in the carcass (Contamination_C) with a particular serogroup was calculated using Eq (5). The unit of Contamination_C was in log CFU/cm². The assumption used in this calculation is that the contamination in the pool is a representation of the individual carcass contamination.

#Eq (4)

#Eq (5)

In equation 5, N is the pool size and A is the sampled area. Samples that resulted positive before the enrichment were used to represent contamination levels. The pool size for sponge samples was 15, and it is assumed that each N-60 beef trimming sample represents at least 3 carcasses. Any sample that was positive after the enrichment was assigned a contamination level 2 log CFU/ml. The Contamination_C values less than 1 log CFU/cm² were reported as <1 log CFU/cm². Please note that throughout the text ‘prevalence’ is used as a presumptive prevalence and ‘contamination level’ is an estimated contamination level based on the real-time PCR results.

2.9. Statistical analysis of the prevalence

In order to locate the intervention steps with a significant reduction of STEC contamination, a logistic Linear Mixed-Effect Regression (LMER) model was used. Only the sponge sample results were included in LMER models. The R-package “lme4” and the R statistical program version 3.0.2 were used for the analysis ([Bates et al. 2012](#), [R Core Team 2013](#)). The outcome or the dependent variable (y) which represents the presence (“1”) or absence (“0”) of the particular STEC serogroup is considered to have a Binomial distribution with the probability of presence p (i.e. $y \sim \text{Bin}(p)$). Units of analysis in the LMER models were pools. The variables “Sampling station” and the “STEC

serogroup” were set as the fixed effects, and the “Pool identification” (i.e. the identification number of a particular sponge sample pool) was set as a random effect for the model (De Boeck et al. 2011). The logistic LMER model is shown in Eq (6).

$$\text{logit}(p) = \beta_0 + \beta_{\text{Sampling station}} + \gamma_{\text{Serogroup}} + \eta_r \quad \# \text{Eq (6)}$$

Where, p is the probability of the presence; a vector of dependent variable which represents the presence or absence of the particular STEC serogroup (i.e. $p(y=1)$). Therefore, $p/(1-p)$ is the odds of presence and $\text{logit}(p) = \log(p/(1-p))$ is the log odds of presence. The β_0 is the log odds for the reference serogroup at the first sampling station. The $\beta_{\text{Sampling station}}$ is the vector of the fixed effects of Sampling station. The $\gamma_{\text{Serogroup}}$ is the vector of the fixed effects of “Serogroup” and η_r is the vector of the random effects of “Pool identification” where r is the pool identification number.

The LMER models were used to predict the odds of being positive for any serogroup in the consecutive sampling stations (De Boeck et al. 2011). The model compares the significance of consecutive sampling stations from the Hide-on station at both slaughter plants (Hide-on was the reference station). On the other hand, the model also compares the significance of the other five non O-157 STEC serogroups compared to the reference serogroup, STEC O103. The OR and CI for the estimates were calculated according to the Eq (7), Eq (8), and Eq (9).

$$\text{Point estimate of OR} = e^{\beta} \quad \# \text{Eq (7)}$$

$$\text{Lower OR limit} = e^{\beta - 1.96 \cdot \text{SE}} \quad \# \text{Eq (8)}$$

$$\text{Upper OR limit} = e^{\beta + 1.96 \cdot \text{SE}} \quad \# \text{Eq (9)}$$

To compare between Plant LS and Plant SS performance in reducing the prevalence of six major non-O157 STEC, results of the sponge samples from both Plant LS and Plant SS were analyzed with a logistic LMER (Eq 10). The “Slaughter plant” and the “Serogroup” were the fixed effects and the “Pool identification” was the random effect.

$$\text{logit}(y) = \beta_0 + \beta_{\text{Slaughter plant}} + \gamma_{\text{Serogroup}} + \eta_z \quad \text{\#Eq (10)}$$

Where, y is the vector of dependent variable which represents the presence or absence of the particular STEC serogroup, β_0 is the log odds the reference serogroup O103. The reference slaughter plant was Plant LS. The log odds of prevalence are calculated as $\text{logit}(y) = \log(y / (1-y))$. $\beta_{\text{Slaughter plant}}$ is the vector of fixed effect for the “Slaughter plant” and $\gamma_{\text{Serogroup}}$ is the vector of fixed effect for the “Serogroup”. The η_z is the vector of the random effect for the “Pool” where z is the pool identification number (the pool identification numbers were assigned differently for each slaughter plant). In confirmation of the LMER model results, a Tukey’s test was performed for all the pairwise comparisons among the six major STEC serogroups (i.e. a contrast analysis). The p-values resulted using the pairwise comparisons were considered more accurate. Therefore, when summarizing the results for LMER models, p-values from the Tukey’s test were used.

The 87 beef trimmings collected at Plant LS in 2011 and the 75 beef trimmings collected at Plant SS in 2011 were subjected to Chi-square tests to determine if the prevalence of each serogroup as well as all six major non-O157 STEC as a group differed among the two plants.

2.10. Statistical analysis of the contamination levels

A paired T-test was used to compare between contamination levels at consecutive stations. Statistical significance in reducing the contamination level of each serogroup as well as all the six major non-O157 STEC serogroups was tested for all the consecutive sampling stations.

3. Results

3.1. Summary of statistical analysis of the prevalence data

As summarized in Table 3, results of the logistic LMER model for Plant LS indicate that when compared to the prevalence at Hide-on station (LS1) the Hide-off (LS2) is not statistically different. The following station, which is the Pre Evisceration Wash (LS3) shows a trend in reduction when compared to the Hide-on station (LS1) at a p-value of < 0.1 . All subsequent sampling stations after Pre Evisceration Wash (LS3) showed a significantly lower prevalence compared to the Hide-on station (LS1). According to the contrast analysis, Pre- HWSP (LS4) had significantly higher odds of prevalence compared to the Post-HWSP (LS5). Comparisons among the other consecutive sampling stations were not significantly different. In comparison of the prevalence of serogroups at plant LS, serogroup O103 had significantly higher odds of prevalence compared to serogroups O111 and O26 ($p < 0.05$). According to the contrast analysis, serogroup O45 had significantly higher odds of prevalence compared to the serogroups O111 and O26 ($p < 0.05$). Serogroup O103 showed marginally significant odds to be more prevalent than O145 ($p < 0.1$). All other serogroup odds were not significantly different from each other.

As summarized in Table 4, results of the logistic LMER model for Plant SS indicates that when compared to the Hide-on station (SS1) the prevalence of six STEC at the Hide-off (SS2) and Post Evisceration wash (SS3) was not statistically different. However, prevalence of STEC serogroups at the Post Hot wash (SS4) station was significantly reduced ($p < 0.05$) when compared to the Hide-on (SS1). All the subsequent stations were significantly different from the Hide-on station (SS1) ($p < 0.05$) with reduced prevalence of STEC serogroups at each station. According to the contrast analysis, Exit cooler (SS5) station had a significantly lower prevalence compared to the

preceding Post-Hot Wash (SS4) station ($p < 0.05$). However, the Cutting board (SS6) indicated a trend of increased prevalence when compared to the preceding station: Exit cooler (SS5) ($p < 0.1$). The difference between the Post-Hot Wash (SS4) and Exit cooler (SS5) showed a decreasing prevalence with estimate of log odds ratio at -4.36. Whereas the difference between Exit cooler (SS5) and Cutting board (SS6) showed an increasing prevalence (estimate of the log odds ratio at 2.86). None of the other consecutive sampling stations were significantly different. In comparison of the prevalence of serogroups at plant SS, serogroup O103 had significantly higher odds of prevalence compared to O111 and O145 ($p < 0.05$). At Plant SS, serogroups O121, O145, O26, and O45 ($p < 0.05$) had significant odds of having higher prevalence compared to the serogroup O111.

As summarized in Table 5, when compared between Plant LS and Plant SS sponge sampling stations, Plant SS showed a marginal significance with higher prevalence of the six major non-O157 STEC compared to Plant LS ($p < 0.1$). Regardless of the sampling station, serogroup O103 was more common in all the sponge samples collected at both slaughter plants compared to the serogroups O111, O145 and O26 ($p\text{-value} < 0.05$). Contrast analysis indicated that serogroup O111 had significantly less prevalence compared to O121 and O45 ($p < 0.05$). All other serogroup odds were not significantly different from each other.

As shown in Table 6, the Chi-square tests results indicate that, the STEC serogroups O103 and O45 were more prevalent in Plant SS beef trimmings compared to that of Plant LS ($p < 0.05$). Plant LS beef trimmings had a significantly higher prevalence of O145 compared to Plant SS beef trimmings ($p < 0.05$). Serogroup O111 indicates a marginally significant trend of being prevalent at Plant LS beef trimmings compared to that of Plant SS ($p < 0.1$). The overall prevalence of all six

major non-O157 STEC in beef trimmings was significantly higher in Plant SS compared to Plant LS ($p < 0.05$).

3.2. Prevalence and contamination level of major six non O-157 STEC in the pools and individual samples

The quantified prevalence and contamination level of six major non O-157 STEC serogroups in the pooled samples and individual carcasses are summarized in the Figures 1 and 2. Each figure contains two sub figures. The figures 1.A and 2.A show the prevalence and contamination level in the pooled samples at Plant LS and Plant SS respectively, whereas, figures 1.B and 2.B illustrate the prevalence and level of contamination at the carcasses. In both figure 1 and 2, the graphs on top illustrates the prevalence and the graphs on bottom illustrates the level of contamination (Unit: log CFU/cm²). The statistical procedures leading to the significance values were the LMER models and the Tukey's tests described under section 2.9. Prevalence of the six serogroups ranged between 43.3% and 90.3% in the pooled hide-on samples collected from both Plant LS and Plant SS. At the end process, Plant LS showed prevalence of six serogroups ranged between 9.3% and 50.7% (Pre fabrication i.e. LS7 station), whereas, prevalence of the six serogroups at the end process of Plant SS ranged between 9.3% and 23.5% (Exit cooler i.e. SS5 station).

The level of contamination of six major non-O157 STEC in positive beef trimming samples ranged from 1.3 - 3.59 log CFU/g in Plant LS and 0.5 - 3.21 log CFU/g in Plant SS. The waste meat bin sampling station showed level of contamination between 1.2 - 4.28 log CFU/g which is the highest level of contamination detected in all the meat samples tested (Plant SS only). The level of

contamination and the pooled prevalence of the waste meat bin samples from the Plant SS were as follows: O26, 2.9 log CFU/g (68.5%); O145, 3.57 log CFU/g (24.4%); O103, 4.28 log CFU/g (91.6%); O111, 3.79 (11.6%); O121, 2.03 log CFU/g (27.7%); and O45, 2 log CFU/g (59.6%). Both prevalence and level of contamination at the carcass level mirrors the pattern of pooled sample outputs.

4. Discussion

This study provides an example of using real-time multiplex PCR as a tool to evaluate the efficacy of beef slaughter line interventions in reducing the non-O157 STEC contamination. The real-time multiplex PCR method described by [Anklam et al. \(2012\)](#) was used to quantify the six major non-O157 STEC serogroups at consecutive slaughter line intervention steps, at two large and small scale slaughter plants. In addition to the use of real-time PCR assays, this study approach suggests laboratory testing of limited number of samples by pooling the samples. It is noteworthy that due to the higher sensitivity of the real-time PCR techniques, the prevalence and level of contamination values for the six major non-O157 STEC generated in the study are higher values compared to the traditional culture – isolation techniques. The technique is capable of detecting the changes in contamination levels throughout the slaughter process. This study exhibits the potential use of real-time multiplex PCR assays as a first- or second order test that can be used to quantify the prevalence and the level of contamination of the six major non-O157 STEC.

The quantified prevalence and contamination levels suggest that Plant LS intervention steps are more effective in reduction of six major non O-157 STEC when compared to Plant SS. However, this finding can be attributed to several factors, including the following: 1) differences in antimicrobial interventions used in the two facilities, 2) differences in sources of cattle received by each facility, 3) difference between sampling periods, and 4) difference in the origin of Plant SS beef trimmings. Regarding the first factor, Plant LS underwent three antimicrobial interventions: steam vacuum and lactic acid spray, pasteurization cabinet, and chilling. Whereas, Plant SS used two antimicrobial interventions: hot wash and cold storage for 7 days. The steam pasteurization and hot water pasteurization are considered similarly effective in reducing generic *E.coli*, and lactic acid

washes further reduce contamination levels ([Greig et al. 2012](#)). Therefore, the comparatively effective outcomes at Plant LS can be explained as a result of combined antimicrobial intervention steps. Regarding the second factor, Plant LS received cattle from comparatively larger herds from multiple Midwestern locations, whereas, Plant SS received cattle from a rather confined area around the facility. Regarding the third factor, the seasonality of STEC shedding may have contributed to the high STEC contamination at Plant SS, since Plant SS was sampled during summer (July-August) when compared to Plant LS, which was sampled during spring (April – May) ([Barkocy-Gallagher et al. 2003](#)). Simultaneous sampling of both locations was not possible in this study due to logistic difficulties. In relation to the fourth factor, the significantly high contamination of beef trimmings at Plant SS could be due to the mixing of in-house and external small scale slaughter plant beef trimmings. The trimmings were mixed in a final container and processed using the same equipment; therefore, the separation of in-house and external trimmings was not attempted.

Based on the results researchers proposed two potential intervention steps in order to improve the performance at Plant SS; incorporation of a pre evisceration wash and incorporation of an antimicrobial intervention technique for the Cutting board (SS6). The first suggestion to incorporate a pre evisceration wash was based on the fact that, contamination of six major non O157 STEC at the Plant SS was not significantly different for three consecutive steps: Hide-on (SS1), Hide-off (SS2), and Post Evisceration Wash (SS3). Continued high contamination of non O-157 STEC at SS3 indicates the probability of passing the organisms to consecutive stations. The second suggestion was a result of the finding that prevalence and level of contamination was comparatively higher at the Cutting board (SS6) when compared to the preceding Exit cooler station (SS5). This evidence of increased contamination suggests that the cutting board is a critical control point where potential re-

contamination and/or cross-contamination may occur. Therefore, the reduced detection of the six major non O-157 STEC at the preceding Exit-cooler station (SS5) at Plant SS could be attributable to the fact that carcass surfaces became dry and desiccated after the seven-day cold storage. The sponges could not be moistened using PBS during sampling, since the carcasses were ready for final commercial packing. As mentioned in Table 2, the practice of seven-day cold storage at Plant SS was an antimicrobial measure, as well as an aging (i.e. ripening) measure. The seven-day cold storage is anticipated to **increase** the tenderness of meat and develop a peculiar taste through proteolytic activity ([FAO 2016](#)).

When compared the survival capacity of six major non O-157 STEC, serogroup O103 was the most frequently detected non O-157 STEC in the sponge samples from both processing plants. Serogroup O103 was prominent in the waste bin meat samples in Plant SS as well. According to the literature, a similar study conducted in Ireland reported frequent detection of serogroup O103 in slaughter plant interventions ([Thomas et al. 2012](#)). Another experimental study, conducted in the Midwestern U.S., reported higher survival of serogroup O103 in pepperoni production ([Glass et al. 2012](#)). These findings may support the hypothesis that STEC serogroup O103 has a higher survival capacity compared to the other non O-157 STEC. However, the most prevalent serogroups in beef trimmings at Plant LS contained: 17.9% of O121, 8.6% of O45, and 8.6% of O111, while the beef trimmings from Plant SS contained: 31% of O103, 24.6% of O121, and 21.8% of O45 (**Table 6**).

This study was conducted in 2011, when neither the USDA laboratory protocols nor the regulations regarding the six major non-O157 STEC were established. Therefore, it is possible that a similar study conducted under current USDA guidelines would differ from our results. The real-time multiplex PCR detection and mathematical quantification were used in the study rather than

enumeration of viable STEC on bacteriological cultures. The assumptions made in the process of calculations were based on the literature and expert opinions available as of 2011. Results from the study are not an indication of the viable non O-157 STEC.

Real-time PCR is commonly used for the quantification of STEC. For example, a study by Luedtke and Bosilevac (2015) described real-time PCR assays as being able to enumerate enterohemorrhagic *E. coli* in cattle hide within a range of 34 to 91,412 CFUs/100 cm² (95% CI 4-113,460 CFUs/100 cm²) (Luedtke and Bosilevac 2015). The contamination levels reported at “Hide-on” stations in this study are within this range (Figure 1 and 2, level of contamination per carcass). Real-time PCR methods are highly sensitive and capable of detecting as low as 50 CFU per PCR from artificially inoculated ground beef samples (Fratamico et al. 2011). Pure culture based detection limit for the real-time multiplex PCR used in this study ranged between 3 log CFU/ml– 4 log CFU/ml. Therefore, the use of pure culture based real-time multiplex PCR standard curves for the quantification of STEC was considered informative and compatible with other methods available at the time of the study. However, due to high sensitivity, PCR based methods results in higher STEC contamination levels when compared to culture based methods (Noll et al. 2015). For example, Arthur et al. (2004) reported 180 non-O157 STEC isolated from 334 samples (53.9%) at pre-evisceration and 27 non-O157 STEC isolated from 326 samples (8.3%) at post-processing (Arthur et al. 2004). The corresponding prevalence generated in this study are relatively higher compared to Arthur et al. (2004) (Table 3 and 4, column “positive %”).

This study only quantified the prevalence and contamination level of the six major non-O157 STEC serogroups, not their virulence factors. Although the virulence genes are commonly associated with pathogenicity, not all STEC serogroups are equally pathogenic (Bosilevac and Koohmaraie

2012). On the other hand, presence of virulence genes in non-O157 STEC is rare (Dewsbury et al. 2015). Admittedly, the mere recovery of the STEC serogroups in beef trimmings does not directly imply the risk of pathogenicity. However, the quantified prevalence and contamination levels of each serogroup at slaughter plant interventions, would provide background reference values in the implementation of targeted interventions. Furthermore, quantification of O157:H7 alongside the six non-O157 STEC would have given a complete visualization of the seven pathogenic and adulterant STEC contaminations in the beef slaughter plant process; however, this was not possible due to industry concerns.

There are a few suggestions outlined by the study. One of them is, use of real-time multiplex PCR as a tool to evaluate the efficacy of slaughter line interventions and pooling the samples in effort to limit the number of samples tested. Combination of the real-time PCR and pooling would be both time and potentially cost effective approach (Singh and Mustapha 2015). Using the pooled sample results, it is possible to estimate the prevalence and bacterial concentrations of six major non O-157 STEC, at both the pool level and the individual carcass level. The second suggestion is considering the prevalence and level of contamination values generated by this study as a basis when exposure assessments are conducted in a risk analysis, in order to improve of HACCP protocols at the slaughter plants (USDA-FSIS 1999). The information generated in this study may also be valuable if the beef industry is considering risk-based sampling of slaughter plant intervention steps. The third suggestion is that criteria be established for the contamination tolerance for different antimicrobial intervention steps and sampling frequencies be adjusted for different slaughter plant facilities. This is because, the intervention techniques may vary between large and small scale facilities which leads to varying contamination levels in retail beef products (Cates et al. 2008).

5. Conclusions

In conclusion, this study elucidates an example of the use of real-time multiplex PCR as a tool to evaluate the efficacy of slaughter line interventions in reducing the six major non-O157 STEC; serogroups O26, O103, O111, O121, O45 and O145. Because these six STEC serogroups have been declared as adulterants in ground beef in the USA since June 4th 2012, the beef industry is focused on the reduction of contamination, detection methods, and optimization of the sample sizes, and risk-based sampling procedures applicable for surveillance. Quantified contamination levels of the six major non-O157 STEC along the beef processing lines in addition to the enumeration of the generic *E. coli*, may be useful as a first or second order test that would measure the efficacy of commonly used antimicrobial intervention techniques and may benefit improvement of current HACCP protocols used in slaughter plants. Our results could potentially address some of the knowledge gaps. The prevalence and level of contamination values for six major non-O157 STEC generated in the study using the real-time multiplex PCR assays are higher values compared to the culture – isolation techniques. Therefore, when extracting information from this study, consideration on the higher sensitivity of real-time PCR is recommended.

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References

1. Anklam K.S., Kanankege K.S.T., Gonzales T.K., Kaspar C.W., Dopfer D., 2012. Rapid and reliable detection of Shiga toxin-producing *Escherichia coli* by real-time multiplex PCR. *J. Food Prot.* 75, 643-650.
2. Arthur T.M., Barkocy-Gallagher G.A., Rivera-Betancourt M., Koohmaraie M., 2004. Prevalence and characterization of non-O157 Shiga toxin producing *Escherichia coli* on carcasses in commercial beef cattle processing plants. *Appl. Environ. Microbiol.* 68, 4847-4852.
3. Barkocy-Gallagher G.A., Arthur, T.M., Rivera-Betancourt, M., Nou, X.W., Shackelford, S.D., Wheeler, T.L., Koohmaraie, M., 2003. Seasonal prevalence of Shiga toxin-producing *Escherichia coli*, including O157: H7 and non-O157 serotypes, and *Salmonella* in commercial beef processing plants. *J. Food Prot.* 66, 1978-1986.
4. Bates D., Maechler M., Bolker B., 2012. Package ‘lme4’: Linear mixed-effects models using S4 classes. In *R: A language and environment for statistical computing*, <lme4-authors@R-forge.wu-wien.ac.at>, ed. (CRAN). Fit linear and generalized linear mixed-effects models.
5. Bosilevac, J.M., Koohmaraie, M., 2012. Predicting the presence of non-O157 toxin producing *Escherichia coli* in ground beef by using molecular tests for Shiga toxins, intimin, and O serogroups. *Appl. Environ. Microbiol.* 78, 19, 7152-7155.
6. Brooks, J.T., Sowers E.G., Wells, J.G., Greene, K.D., Griffin, P.M., Hoekstra, R.M., Strockbine, N.A., 2005. Non-O157 Shiga toxin producing *Escherichia coli* infections in the United States, 1983-2002. *J. Infect. Dis.* 192, 1422-1429.
7. Cates, S.C., Viator, C.L., Karns, S.A., Muth, M.K., 2008. Food safety practices of meat slaughter plants: findings from a national survey. *Food Prot. Trends.* 28, 26-36.

8. CDC, Centers for Disease Control and Prevention, 2012. National Enteric Disease Surveillance: STEC Surveillance Overview. U.S. Department of Health and Human Services. Atlanta, Georgia, USA.
9. De Boeck, P., Bakkar, M., Zwitser, R., Nivard, M., Hofman, A., Tuerlinckx, F., Partchev, I., 2011. The estimation of item response models with the lmer function from the lme4 package in R. *J. Stat Software.* 39, 1-28.
10. Dewsbury, D.M., Renter, D.G., Shridhar, P.B., Noll, L.W., Shi, X.R., Nagaraja, T.G., Cernicchiaro, N., 2015. Summer and Winter Prevalence of Shiga Toxin-Producing *Escherichia coli* (STEC) O26, O45, O103, O111, O121, O145, and O157 in Feces of Feedlot Cattle. *Foodborne Pathog. Dis.* 12, 8, 762-732.
11. Erickson, M.C., Doyle, M.P., 2007. Food as a vehicle for transmission of Shiga toxin-producing *Escherichia coli*. *J. Food Prot.* 70,10, 2426-2449.
12. FAO, Food and Agriculture Organization, 2016. FAO corporate document repository. Manual on meat cold store operation and management. Produced by agriculture and consumer protection. Available from: <http://www.fao.org/docrep/004/t0098e/T0098E01.htm#TopOfPage>. Accessed 2016 January 23.
13. Fratamico, P.M., Bagi, L.K., Cray, W.C., Narang, N., Yan, X.H., Medina, M., Liu, Y.H., 2011. Detection by multiplex real-time polymerase chain reaction assays and isolation of Shiga toxin producing *Escherichia coli* Serogroups O26, O45, O103, O111, O121, and O145 in Ground Beef. *Foodborne Pathog. Dis.* 8, 601-607.
14. Glass, K.A., Kaspar, C.W., Sindelar, J.J., Milkowski, A.L., Lotz, B.M., Kang, J.H., Faith, N.G., Enache, E., Kataoka, A., Henry, C., 2012. Validation of pepperoni process for control of Shiga toxin producing *Escherichia coli*. *J. Food Prot.* 75, 838-846.

15. Gould, L.H., Mody, R.K., Ong, K.L., Clogher, P., Cronquist, A.B., Garman, K.N., Larthrop, S., Medus, C., Spina, N.L., *et al.* of Emerging Infections Program of FoodNet, 2013. Increased Recognition of Non-O157 Shiga Toxin-Producing *Escherichia coli* Infections in the United States During 2000-2010: Epidemiologic Features and Comparison with *E. coli* O157 Infections. *Foodborne Pathog. Dis.* 10, 453-460.
16. Greig, J.D., Waddell, L., Wilhelm, B., Wilkins, W., Bucher, O., Parker, S., Rajic, A., 2012. The efficacy of interventions applied during primary processing on contamination of beef carcasses with *Escherichia coli*: A systematic review-meta-analysis of the published research. *Food Control.* 27, 385-397.
17. Karmali, M.A., Petric, M., Lim, C., Fleming, P.C., Steele, B.T., 1983. *Escherichia coli* cytotoxin, Hemolytic Uremic syndrome, and hemorrhagic colitis. *Lancet.* 2, 1299-1300.
18. Luedtke, B.E., Bosilevac, J.M., 2015. Comparison methods for the enumeration of enterohemorrhagic *Escherichia coli* from veal hides and carcasses. *Frontiers in Microbiol.* 6, 1062. DOI:10.3389/fmicb.2015.01062.
19. Noll, L.W., Shridhar, P.B., Dewsbury, D.M., Shi, X.R., Cernicchiaro, N., Renter, D.G., Nagaraja, T.G., 2015. A comparison of culture- and PCR –based methods to detect six major non-O157 serogroups of Shiga toxin-producing *Escherichia coli* in cattle feces. *PlosOne.* 10, 8, e0135446. DOI: 10.1371/journal.pone.0135446.
20. Pihkala, N., Bauer, N., Eblen, D., Evans, P., Johnson, R., Webb, J., Williams, C., and the FSIS non-O157 working group, 2012. Risk profile for pathogenic non-O157 Shiga toxin producing *Escherichia coli* (non-O157 STEC). Office of Public Health Science, Office of Policy and Program Development, USDA-FSIS. Available from: <http://www.fsis.usda.gov/wps/wcm/connect/92de038d-c30e-4037-85a6-65c3a709435/>

- Non_O157_STEC_Risk_Profile_May2012.pdf?MOD=AJPERES. Accessed 2015 January 23.
21. R Core Team, 2013. Version 3.0.2. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org>.
 22. Retail beef cuts and recommended cooking methods, 2009. Beef made easy. Cattlemen's beef board and national cattlemen's beef association. Available from: <http://nationaladmin.beef.org/CMDocs/BeefRetail/efcdisplaytools/BeefMadeEasyCutChart.pdf>. Accessed 2015 January 12.
 23. Sergeant, E.S.G., 2009. EpiTools epidemiological calculators: Pooled prevalence calculator (AusVet Animal Health Services and Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease).
 24. Singh, P., Mustapha, A., 2015. Multiplex real-time PCR assays for detection of eight Shiga toxin-producing *Escherichia coli* in food samples by melting curve analysis. *Int. J. Food Microbiol.* 215, 101-108.
 25. Thomas, K.M., McCann, M.S., Collery, M.M., Logan, A., Whyte, P., McDowell, D.A., Duffy, G., 2012. Tracking verocytotoxigenic *Escherichia coli* O157, O26, O111, O103 and O145 in Irish cattle. *Int. J. Food Microbiol.* 153, 13, 288-296.
 26. U.S. Federal Register. Vol 80 (No. 82), 2015. Risk-based sampling of beef manufacturing trimmings for *Escherichia coli* (E.coli) O157:H7 and plans for beef baseline (April 28, 2015). 23761 – 23765.
 27. U.S. Federal Register Vol 11. 1999. Beef products contaminated with *Escherichia Coli* O157:H7, Action: Policy on beef products contaminated with E. coli O157:H7. U.S. Federal Register 11 (January 19, 1999). 2803-2805.

28. U.S. Federal Register Vol 77. 2012. Shiga toxin producing *Escherichia coli* in certain raw beef products. U.S. Federal Register Vol 77 (May 31, 2012) 105, 31975-31981.
29. USDA laboratory guide book, 2013. Detection and Isolation of non-O157 Shiga toxin Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges. Revised. MLG 5B. 04.
30. USDA-FSIS, Food Safety and Inspection Service of the United States Department of Agriculture, 1996. Guidelines for *Escherichia coli* Testing for Process Control Verification in Cattle and Swine Slaughter Establishments. Available from: http://www.fsis.usda.gov/wps/wcm/connect/cebac8d0-954b-4cc2-abcf-205ad4f4ddcd/Guideline_for_Ecoli_Testing_Cattle_Swine_Estab.pdf?MOD=AJPERES. Accessed 2012 January 05.
31. USDA-FSIS, Food Safety and Inspection Service of the United States Department of Agriculture, 2008. Guidance for small and very small establishment on sampling beef products for *E. coli* O157:H7. Draft for stakeholder comment. August 12, 2008.
32. USDA-FSIS, Food Safety and Inspection Service of the United States Department of Agriculture, 2011. Sampling protocol for testing beef trim for *E. coli* O157:H7. Office of Inspector general. Audit report 24601-9-kc. Feb 2011. 5-6. Verification activities for *Escherichia coli* O157:H7 in raw beef products. FSIS Directive 10,010.1 Rev. 3.
33. Vose, D.J., 2000. Risk Analysis - A quantitative guide, 2nd edition. Edition. John Wiley and Sons Ltd., Chichester, England.
34. Wilhelm B, Rajic A, Greig JD, Waddell L, Harris J. 2011. The effect of hazard analysis critical control point programs on microbial contamination of carcasses in abattoirs: A systematic review of published data. *Foodborne Pathog Dis* 8: 949-960.

Table 1. Summary of the total samples collected from both large scale slaughter plant (Plant LS) and small scale slaughter plant (Plant SS)

Slaughter plant	S a m p l i n g period	Type of the sample	Description	Sample size per station
Plant LS	2011 (April-May)	Sponge samples	15 samples from 7 stations were collected for 6 weeks*	(6 * 15) = 90 carcasses per station
		Beef trimmings	375g per each sample	87 samples ^{##}
Plant SS	2011 (July-August)	Sponge samples	15 samples from stations 1, 2, 3, and 4 were collected for 7 weeks*	(7*15)=105 carcasses per station for stations 1, 2, 3, and 4
			15 samples from stations 5 and 6 were collected for only 6 weeks*	(6 * 15) = 90 carcasses per station for stations 5 and 6
		Beef trimmings	375g per each sample	75 samples
		Beef trim from the waste bin	375g per each sample	30 samples

^{##}the meat sample size of Plant LS is higher than the calculated number due to an additional week of sampling provided by the slaughter plant.

*One sampling session was conducted each week. Therefore, the number of pools per each station is the same as number of weeks.

Table 2. Sampling stations at each slaughter plant

Sampling stations at plant LS*			Sampling stations at plant SS [#]		
LS1	Hide-on	A 100 cm ² area of the 'Round' of carcass after the stunning but before hide [‡] removal	SS 1	Hide- on	An area 100 cm ² of the 'Round' after the stunning and head removal but before the removal of hide
LS2	Hide-off	An area of 4000 cm ² of the 'Round' after removal of the hide but before steam vacuum and organic acid spray	SS 2	Hide-off	An area of 4000 cm ² of the 'Round' area after removal of hides but before evisceration
LS3	Pre - Evisceration Wash	An area of 4000 cm ² of the 'Round' area of the carcass after the steam vacuum and organic acid spray (at 43°-54°C, ≤5% lactic acid) but before the evisceration	SS 3	Post-Evisceration. Wash	An area of 4000 cm ² of the 'brisket/ plate area' after the splitting and removal of viscera and the brief hot-water wash
LS4	Pre-Hot Wash or Steam Pasteurization at >74 °C (HWSP)	An area of 4000 cm ² of the 'brisket or plate area' at post evisceration and splitting before the pasteurization cabinet	SS 4	Post-Hot Wash	An area of 4000 cm ² of the 'brisket/ plate area' after the inspection followed by a high pressure hot-water wash at ≥74 °C but before the cooler
LS5	Post-HWSP prior to final pasteurization	An area of 4000 cm ² of the 'brisket or plate area' at the post-pasteurization cabinet and the lactic acid spray	SS 5	Exit Cooler	An area of 4000 cm ² of the 'brisket/ plate area' after the carcasses were held for 7 days from slaughter in the cooler. Seven-day period in cooler is intended to reduce microbial activity as well as meat aging (i.e. ripening) step
LS6	Pre-Hot box	An area of 4000 cm ² of the 'brisket or plate area' immediately prior to chilling i.e. the cooling chamber which is commonly referred as the "Hot box" where carcasses of cows stored for 24h and carcasses of steers stored for 24-48h	SS 6	Cutting Board	A cutting board where all the carcasses were processed. An area of 4000 cm ² was sampled
LS7	Pre-Fabrication	An area of 4000 cm ² of the 'brisket/ plate area' post chilling prior to the fabrication process	SS 7	Beef trimmings	Collected according to a modified N60 sampling method for small scale slaughter plants of the USDA-FSIS (USDA-FSIS 2008)
LS8	Beef trimmings	Collected according to N60 sampling protocol of the USDA-FSIS (USDA-FSIS 2011)		Waste bin meat samples	A waste bin placed in the processing area. Represents the highest possible environmental contamination

*LS = Large scale slaughter plant and #SS = Small scale slaughter plant . ‡hide = hide here refers to the skin with hair

Table 3. Statistical analysis of the results from Plant LS

Variable	Class	Number of pools ^{##}	Positive %	OR [#]	95% CI	p-value	Significance of the contrasts
Sampling Station	Hide on (LS1)	36	86.11%		Ref		
	Hide off (LS2)	36	69.44%	0.31	0.09-1.11	0.27	S1 vs S2 n.s.
	Pre evisceration wash (LS3)	36	58.33%	0.17	0.05-0.61	0.03	S2 vs S3 n.s.
	Pre HWSP* (LS4)	36	41.66%	0.08	0.02-0.27	< 0.01	S3 vs S4 n.s.
	Post HWSP* (LS5)	36	8.33%	0.01	0.00-0.04	< 0.01	S4 vs S5 sig. (<0.05)
	Pre hot box** (LS6)	36	13.88%	0.01	0.00-0.06	< 0.01	S5 vs S6 n.s.
	Pre Fabrication (LS7)	36	16.66%	0.01	0.00-0.06	< 0.01	S6 vs S7 n.s.
Total		252					
Serogroup	O103	42	57.14%		Ref		
	O111	42	28.57%	0.13	0.04-0.44	0.01	O111 vs. O45 sig. (<0.05)
	O121	42	50%	0.52	0.17-1.61	0.86	
	O145	42	33.33%	0.19	0.06-0.61	0.06	
	O26	42	28.57%	0.13	0.04-0.44	0.01	O26 vs. O45 sig. (<0.05)
	O45	42	54.76%	0.85	0.28-2.61	1	
	Total	252					

*HWSP = Hot Wash or Steam Pasteurization at >74 °C and ** Pre hot box = The cooling chamber in which carcasses of cows stored for 24h and carcasses of steers stored for 24-48h.

OR[#]= Odds ratio. ^{##} Number of pools per station is calculated as 6 pools (i.e. one pool per week) * 6 serotypes = 36.

p-value = p-value for the comparison of a given station or a given serogroup compared to the reference category. Reference category for the comparison between stations was the LS1 (i.e. Hide-on) and the reference serogroup was O103.

Last column represents contrast analysis (i.e. Tukey's correction). Only the significantly different intervention stations or serogroups are indicated.

n. s. = the contrasts were not significant ($p \text{ value} \geq 0.05$) and sig. = contrasts were significant ($p \text{ value} < 0.05$)

Table 4. Statistical analysis of the results from Plant SS

Variable	Class	Number of pools ^{##}	Positive %	OR [#]	95% CI	p-value	significance of contrasts
Station	Hide on (SS1)	42	90.48%		Ref		
	Hide off (SS2)	42	78.57%	0.2	0.04-0.93	0.13	S1 vs S2 n. s.
	Post evisceration wash (SS3)	42	61.90%	0.07	0.01-0.32	0.30	S2 vs S3 n. s.
	Post-hot wash (SS4)	42	61.90%	0.00	0.00-0.01	< 0.01	S3 vs S4 n. s.
	Exit cooler (SS5)	36	2.77%	0.01	0.00-0.04	< 0.01	S4 vs S5 sig. (< 0.01)
	Cutting board (SS6)	36	25%	0.01	0.00-0.06	< 0.01	S5 vs S6 trend (0.06)
Total		240					
Serogroup	O103	40	72.5%		Ref		
	O111	40	22.5%	0.01	0.00-0.06	< 0.01	O111 vs. O121, O145, O26, and O45 sig. (<0.05)
	O121	40	62.5%	0.24	0.06-0.98	0.35	
	O145	40	50%	0.11	0.03-0.45	0.02	
	O26	40	47.5%	0.24	0.06-0.98	0.35	
	O45	40	65%	0.37	0.09-1.52	0.74	
Total		240					

OR[#]= Odds ratio. ^{##} Number of pools per station for stations was calculated SS1:SS4 as 7 pools (i.e. one pool per week) * 6 serotypes = 42 and for stations SS5:SS6 as 6 pools (i.e. one pool per week) * 6 serotypes = 36.

p-value = p-value for the comparison of a given station or a given serogroup compared to the reference category. Reference category for the comparison between stations was the SS1 (i.e. Hide-on) and the reference serogroup was O103.

Last column represents contrast analysis (i.e. Tukey's correction). Only the significantly different intervention stations or serogroups are indicated.

n. s. = the contrasts were not significant ($p \text{ value} \geq 0.05$) and sig. = contrasts were significant ($p \text{ value} < 0.05$)

Table 5. Results of statistical analysis for the comparison of the prevalence of the six major non-STEC serogroups in Plant LS and Plant SS

Variable	Class	Number of observations (Number of pools)	Positive %	OR#	95% CI	p-value	significance of contrasts
Slaughter plant	Plant LS	252	106/252 (42.06%)		Ref		
	Plant SS	240	128/240 (53.33%)	1.7	0.94-3.08	0.08	Plant SS vs Plant LS marginally significant (0.08)
	Total	492					
Serogroup	O103	82	53/82 (64.63%)		Ref		
	O111	82	21/82 (25.61%)	0.16	0.08-0.32	< 0.01	O111 vs. O121 and O45 sig. (<0.05)
	O121	82	46/82 (56.09%)	0.59	0.31-1.11	0.57	
	O145	82	34/82 (41.46%)	0.35	0.18-0.66	0.02	
	O26	82	31/82 (37.80%)	0.39	0.20-0.74	0.04	
	O45	82	49/82 (59.76%)	0.76	0.40-1.45	0.96	
	Total	492					

OR# = Odds ratio

p-value = p-value for the comparison between prevalence at small scale slaughter plant compared to the large scale slaughter plant or significance of a given serogroup compared to the reference serogroup O103.

Last column represents contrast analysis (i.e. Tukey's correction). Only the significantly different serogroups are indicated.

n. s. = the contrasts were not significant i.e. with $p\text{-value} \geq 0.05$ and sig. = contrasts were significant

Table 6. Summary of Chi-square tests for the comparison of non-O157 STEC prevalence in the beef trimmings at the end of slaughter-fabrication process from Plant LS and Plant SS

Serogroup	Percentage of positive beef trimmings in Plant LS 2011	Percentage of positive beef trimmings in Plant SS 2011	Chi-square test result (P-value)
O26	3/87 (3.45%)	8/75 (10.67%)	0.131
O145	7/87 (8.04%)	1/75 (1.33%)	0.019
O103	7/87 (8.04%)	23/75 (30.66%)	< 0.01
O111	8/87 (9.19%)	1/75 (1.33%)	0.06
O121	15/87 (17.24%)	17/75 (22.67%)	0.508
O45	7/87 (8.04%)	16/75 (21.33%)	0.028
All 6 serogroups	47/87 (54.02%)	65/75 (86.67%)	<< 0.01

< 0.01 represents the p-values resulted less than 0.01 and << 0.01 represents the p values resulted with less than 0.001

Figure 1. A. Plant LS sampling results for the pools

(A) The pooled prevalence of the six major non-O157 STEC in the large scale slaughter plant (Plant LS). (B) The level of contamination of the pools of sponge samples collected from sampling stations LS1:LS7 (unit: log CFU/ml), beef trimmings (unit: log CFU/g). (C) The level of contamination of the beef trimmings (SS7) ***

Figure 1. B. Plant LS sampling results for the individual carcasses

(A) The prevalence of the six major non O-157 STEC in beef carcasses of the large scale slaughter plant (Plant LS). (B) The level of contamination of the carcasses at each sampling station, LS1:LS8 (unit: log CFU/ml), beef trimmings (unit: log CFU/g). (C) The level of contamination of the beef trimmings (SS8) ***

Figure 2. A. Plant SS sampling results for the pools

(A) The pooled prevalence of the six major non-O157 STEC in the small-scale slaughter plant (Plant SS). (B) The level of contamination of the pools of sponge samples collected from sampling stations SS1:SS6 (unit: log CFU/ml), beef trimmings (unit: log CFU/g). (C) The level of contamination of the beef trimmings (SS7) ***

Figure 2. B. Plant SS sampling results for the individual carcasses

(A) The prevalence of the six major non O-157 STEC in beef carcasses of the small-scale slaughter plant (Plant SS). (B) The level of contamination of the carcasses at each sampling station, SS1:SS6 (unit: log CFU/ml), beef trimmings (unit: log CFU/g). (C) The level of contamination of the beef trimmings (SS7) ***