The Pennsylvania State University
The Graduate School
Department of Food Science

PRESENCE OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* O-GROUPS IN SMALL AND VERY SMALL BEEF PROCESSING PLANTS AND RESULTING BEEF PRODUCTS DETECTED BY A MULTIPLEX POLYMERASE CHAIN REACTION ASSAY

A Thesis in
Food Science
by
Amanda Lyn Svoboda

© 2012 Amanda Lyn Svoboda

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

August 2012
The thesis of Amanda L. Svoboda was reviewed and approved* by the following:

Catherine N. Cutter  
Associate Professor of Food Science  
Thesis Advisor

Edward W. Mills  
Associate Professor of Dairy and Animal Science

Edward Dudley  
Assistant Professor of Food Science

Chitrita DebRoy  
Director of E. coli Reference Center

John D. Floros  
Professor of Food Science  
Food Science Department Head

*Signatures are on file in the Graduate School.
ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) are pathogens attributed to numerous foodborne illnesses resulting in gastrointestinal disease of varying severity, including hemolytic uremic syndrome (HUS) in humans. Cattle, and consequently beef products, are considered a major source of STEC. *E. coli* O157:H7 has been regulated as an adulterant in ground beef since 1994. The USDA-Food Safety and Inspection Service (USDA-FSIS) has indicated that 6 additional STEC (O145, O121, O111, O103, O45, and O26) will be regulated as adulterants in raw beef trim and ground beef, beginning in June 2012.

However, the overall incidence of non-O157 STEC is difficult to estimate because routine screening of these serogroups is not performed, due to a lack of rapid molecular methods and/or standard culture methods. A standard detection protocol for non-O157 STEC is not currently established, largely due to the broad range of both phenotypic and genotypic characteristics this group of pathogens display. While some methods have been employed to detect STEC in large beef processing environments and/or products, little is known about the presence of STEC in small and very small beef processing plants or the resulting beef products.

The first objective of this study evaluated the effectiveness of three different enrichment media (tryptic soy broth (TSB) + novobiocin (TSBn); modified *E. coli* broth + novobiocin (mECn); and modified TSB (mTSB) containing 8 mg/L novobiocin, 16 mg/L vancomycin, 2 mg/L rifampicin, 1.5g/L bile salts, and 1 mg/L potassium tellurite; and described by Possé et al. (2008) to isolate a non-O157:H7 STEC. Carcass, environmental, fecal, or ground beef samples were artificially inoculated with approximately $10^2$ CFU/mL of *E. coli* O145 and enriched for 24 hr at 42°C with each of the selected enrichment media. After enrichments, O145 was confirmed using a multiplex PCR assay (DebRoy et al., 2011) which had been optimized for
the detection of STEC serogroups O157, O145, O121, O113, O111, O103, O45, and O26. All three enrichment media were effective for the detection of \textit{E. coli} O145. Interestingly, fewer background microorganisms were detected on agar plates when subjected to enrichment with mTSB.

The second objective of this study was to determine if small and very small beef processing plants are a potential source of STEC, utilizing the enrichment and detection methods optimized in the first objective. In this survey, environmental swabs, carcass swabs, hide swabs, fecal samples, and ground beef from small and very small beef processing plants were obtained to determine the presence of STEC. The previously optimized multiplex PCR assay was used to detect the presence of STEC O-groups and the presence of Shiga toxin (\textit{stx}) and intimin (\textit{eae}) genes were determined using PCR primers described by Paton and Paton (1998). Results demonstrated that 55.5\% (151/272) of the environmental samples, 36.9\% (75/203) of the carcass samples, 85.2\% (23/27) of the hide samples, 37.5\% (12/32) of the fecal, and 18.6\% (22/118) of the ground beef samples tested positive for one or more of the serogroups. However, only 7.7\% (21/272) of the environmental samples, 5.9\% (12/203) of the carcass samples, 0\% (0/27) of the hide samples, 0\% (0/32) of the fecal, and 0\% (0/118) ground beef samples tested positive for \textit{stx1} and/or \textit{stx2} genes. In addition, 13.6\% (37/272) of the environmental samples, 7.9\% (16/203) of the carcass samples, 0\% (0/27) of the hide samples, 0\% (0/32) of the fecal samples, and 0.8\% (1/118) of the ground beef samples tested positive for \textit{eae} gene.

A second survey was completed to evaluate the effectiveness of three different sampling methods (sponge swab, hide clipping, and a novel M-Vac sampling method (Microbial Vac Systems, Inc., Bluffdale, Utah) for detecting STEC on cattle, swine, and sheep hides at one
Samples were assayed for eight STEC serogroups, (O157, O145, O121, O113, O111, O103, O45, and O26) using a multiplex PCR assay (DebRoy et al., 2011). The presence of stx genes in samples were identified using an additional multiplex PCR assay (Paton and Paton, 1998). Results demonstrated that 92% (24/26) of cattle hides tested positive for one or more STEC O-groups using the sponge swab and hair clipping methods, while 88% (23/26) of cattle hides tested positive for one or more STEC O-groups using the M-Vac sampling method. Swine hides tested positive for one or more STEC O-group in 93% (28/30) of samples collected with the sponge swab method, as compared with 80% (24/30) of samples collected using the hair clipping method, while 97% (29/30) of samples collected were positive for the STEC O-group using the M-Vac sampling method. Sheep hides were positive for one or more STEC O-group in all (11/11) of the samples collected with the sponge swab and M-Vac methods, while 82% (9/11) of samples collected were positive with the hair clipping method. However, only 3.8% (1/26) of cattle hides, 40% (12/30) of swine hides, and 27.3% (3/11) of sheep hides tested positive for stx1 and/or stx2 genes, when all sampling methods were considered.

The data resulting from the beef processing plant survey may establish a baseline for the presence of non-O157 STEC in small and very small processing establishments in Pennsylvania and the resulting beef products. Results from the hide study may serve as an indication of the presence of STEC in small and very small beef slaughter facilities, which also may process multiple species. Collectively, these studies may be useful to regulatory officials, researchers, and public health personnel who are interested in determining the presence of these pathogens in the meat supply. Future studies focusing on specific locations in the beef processing environment or in beef trim, as well as resulting ground beef products, may result in a better understanding the path(s) that these pathogens take to reach the final product.
Understanding pathways of transmission in a beef processing environment may help researchers and processors determine interventions that can be employed to control these pathogens, resulting in a safer food supply. Funding for this project was provided by the USDA-NIFA grant 2009-03611.
# TABLE OF CONTENTS

LIST OF FIGURES .............................................................................................................. xi

LIST OF TABLES .................................................................................................................... xii

ACKNOWLEDGEMENTS ....................................................................................................... xiv

Chapter 1 LITERATURE REVIEW ...................................................................................... 1

*Escherichia coli* as a foodborne pathogen ..................................................................... 1
Description of *E. coli* ........................................................................................................... 2
*E. coli* O157:H7 .................................................................................................................. 6
Non-O157 STEC .................................................................................................................... 7
Incidence of STEC outbreaks ............................................................................................... 9
  Serogroup O157 .................................................................................................................... 10
  Serogroup O145 ................................................................................................................... 11
  Serogroup O121 ................................................................................................................... 12
  Serogroup O113 ................................................................................................................... 12
  Serogroup O111 ................................................................................................................... 13
  Serogroup O103 ................................................................................................................... 14
  Serogroup O45 .................................................................................................................... 14
  Serogroup O26 .................................................................................................................... 15
*E. coli* reservoirs .............................................................................................................. 16
STEC in livestock and resulting meat products .................................................................. 17
  STEC in cattle, beef processing, and resulting beef products ........................................... 17
  STEC in swine, pork processing, and resulting pork products .......................................... 19
  STEC in sheep, sheep processing, and resulting sheep products ....................................... 19
  Transfer of STEC between animals ................................................................................... 20
STEC detection in livestock processing environments ....................................................... 20
Vehicles of STEC transmission ........................................................................................... 21
Beef processing plants ........................................................................................................ 24
Regulations for *E. coli* in beef products .......................................................................... 25
  *E. coli* O157:H7 regulations .......................................................................................... 25
  Proposed non-O157 regulations ....................................................................................... 26
  Potential implications for beef processors ....................................................................... 26
Methods of STEC detection ............................................................................................... 27
  Selective/differential enrichments and media .................................................................. 27
  Agglutination .................................................................................................................... 29
  Immunomagnetic separation ............................................................................................ 29
  PCR, multiplex PCR, and real-time PCR ......................................................................... 30
Proposed methods of detection for non-O157 STEC ....................................................... 32
Purpose of survey ............................................................................................................... 35
Statement of objectives ...................................................................................................... 37
### Chapter 2  OPTIMIZATION OF SAMPLING TECHNIQUES TO DETECT, ISOLATE, AND CONFIRM STEC

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>51</td>
</tr>
<tr>
<td>Introduction</td>
<td>52</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>55</td>
</tr>
<tr>
<td>Bacterial strains and culture conditions</td>
<td>55</td>
</tr>
<tr>
<td>Optimization of multiplex PCR primers</td>
<td>58</td>
</tr>
<tr>
<td>Evaluation of enrichment media for non-O157 STEC experimentally inoculated in ground beef, fecal, environmental, and carcass samples</td>
<td>60</td>
</tr>
<tr>
<td>Ground beef sample collection</td>
<td>60</td>
</tr>
<tr>
<td>Fecal sample collection</td>
<td>60</td>
</tr>
<tr>
<td>Environmental sample collection</td>
<td>61</td>
</tr>
<tr>
<td>Beef carcass sample collection</td>
<td>62</td>
</tr>
<tr>
<td>Evaluation of background microflora from ground beef, fecal, environmental, and carcass samples</td>
<td>62</td>
</tr>
<tr>
<td>Isolation and identification of STEC in ground beef, fecal, environmental, and carcass samples</td>
<td>65</td>
</tr>
<tr>
<td>DNA isolation</td>
<td>65</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>66</td>
</tr>
<tr>
<td>Results</td>
<td>66</td>
</tr>
<tr>
<td>Discussion</td>
<td>69</td>
</tr>
<tr>
<td>References</td>
<td>71</td>
</tr>
</tbody>
</table>

### Chapter 3  SURVEY OF BEEF PROCESSING PLANTS AND RESULTING GROUND BEEF PRODUCTS FOR THE PRESENCE OF STEC

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>74</td>
</tr>
<tr>
<td>Introduction</td>
<td>75</td>
</tr>
<tr>
<td>Pathogenic <em>E. coli</em> (STEC)</td>
<td>76</td>
</tr>
<tr>
<td>Description of STEC</td>
<td>77</td>
</tr>
<tr>
<td>STEC in cattle, beef processing plants, and resulting beef products</td>
<td>78</td>
</tr>
<tr>
<td>Objective</td>
<td>81</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>82</td>
</tr>
<tr>
<td>Bacterial strains and culture conditions</td>
<td>82</td>
</tr>
<tr>
<td>Experimental design</td>
<td>84</td>
</tr>
<tr>
<td>Sample collection and enrichment</td>
<td>84</td>
</tr>
<tr>
<td>Environmental samples</td>
<td>84</td>
</tr>
<tr>
<td>Carcass samples</td>
<td>85</td>
</tr>
<tr>
<td>Hide samples</td>
<td>86</td>
</tr>
<tr>
<td>Fecal samples</td>
<td>86</td>
</tr>
<tr>
<td>Ground beef samples</td>
<td>86</td>
</tr>
<tr>
<td>Sample enrichment</td>
<td>87</td>
</tr>
</tbody>
</table>
Evaluation of background microflora from environmental, carcass, hide, and
ground beef samples ................................................................. 88
DNA isolation ........................................................................... 88
STEC analysis ........................................................................... 89
Immunomagnetic separation for STEC isolation .......................... 94
Statistical analysis .................................................................... 95
Results ....................................................................................... 95
Pathogen prevalence in environmental samples ....................... 95
Pathogen prevalence in environmental sample types ................ 96
Pathogen prevalence in carcass samples ................................... 96
Pathogen prevalence in hide samples ....................................... 97
Pathogen prevalence in ground beef samples ........................... 97
Pathogen prevalence in fecal samples ...................................... 97
Pathogen prevalence in all tested samples ............................... 98
Pathogen prevalence tested in individual beef processing plants . 98
STEC O-groups detected within sample types ........................... 99
Seasonal STEC variance in all sample types .............................. 99
APC and EC results ................................................................... 100
Discussion ................................................................................. 107
References ............................................................................... 110

Chapter 4  A SURVEY OF THE PRESENCE OF STEC ON CATTLE, HOG, AND SHEEP HIDES
USING THREE DIFFERENT SAMPLING METHODS .......................... 113

Abstract ..................................................................................... 113
Introduction ............................................................................... 114
*Escherichia coli* as a foodborne pathogen ................................ 114
*E. coli* reservoirs ...................................................................... 115
*E. coli* regulations ................................................................... 116
*E. coli* in cattle, beef processing, and resulting beef products . 117
*E. coli* in swine, pork processing, and resulting pork products . 118
*E. coli* in sheep, sheep processing, and resulting sheep products . 119
Transfer of STEC between animals ............................................ 120
Sampling methods ..................................................................... 120
Objectives .................................................................................. 122
Materials and methods ............................................................... 123
Bacterial strains and culture conditions ..................................... 123
Experimental design ................................................................... 125
Sample collection ...................................................................... 125
Sponge swab samples ............................................................... 125
Clipping samples ...................................................................... 126
M-Vac samples ......................................................................... 126
Sample enrichment ..................................................................... 127
STEC analysis ............................................................................ 127
Statistical analysis ..................................................................... 133
Results ....................................................................................... 133
Pathogen prevalence on cattle hides ................................................................. 133
Pathogen prevalence on pork hides ............................................................... 134
Pathogen prevalence on sheep hides ............................................................. 134
Discussion........................................................................................................ 138
References....................................................................................................... 140

Chapter 5  CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH........ 144

References .................................................................................................... 148

APPENDIX .................................................................................................... 149
LIST OF FIGURES

Figure 1-1: Number of non-O157 STEC outbreaks occurring in the United States 1990-2010. Adapted from USDA-FSIS, 2011 ................................................................. 9

Figure 2-1: Agarose gel of amplicons from the multiplex PCR assay of Shiga toxin-producing Escherichia coli (STEC) serogroups. ......................................................... 58

Figure 3-1: Agarose gel of amplicons from the multiplex PCR assay of Shiga toxin-producing Escherichia coli (STEC) serogroups. .............................................................. 90

Figure 3-2: Agarose gel of amplicons from the multiplex PCR assay for the virulence genes: stx1/stx2/eae in Escherichia coli (STEC). ................................................................. 92

Figure 4-1: Agarose gel of amplicons from the multiplex PCR assay of Shiga toxin-producing Escherichia coli (STEC) serogroups. .............................................................. 129

Figure 4-2: Agarose gel of amplicons from the multiplex PCR assay for the virulence genes: stx1/stx2/eae in Escherichia coli (STEC). ................................................................. 131
LIST OF TABLES

Table 2-1: Reference strains used as controls for the O-type determination in multiplex PCR assay.................................................................................................................. 56

Table 2-2: Bacterial strains used for multiplex PCR assay. ................................................. 57

Table 2-3: PCR primers used in this study........................................................................... 59

Table 2-4: Average aerobic plate counts (APC) and E. coli counts (EC) for ground beef, fecal, environmental, and carcass samples before artificial inoculation of STEC......... 64

Table 2-5: Frequency of detection of E. coli O145 in beef samples by agglutination and multiplex PCR. ...................................................................................................... 68

Table 3-1: Reference strains used as controls for O-type determination in culture and multiplex PCR assay........................................................................................................ 83

Table 3-2: O-Group PCR primers used in this study............................................................ 91

Table 3-3: stx/eae PCR primers used in this study.............................................................. 93

Table 3-4: Presence and percentages of positive samples for STEC O-groups found in beef processing environments and resulting ground beef products......................... 101

Table 3-5: Presence and percentages of positive samples for STEC virulence genes (stx1, stx2, and eae) found in small and very small beef processing environments and resulting ground beef products. ................................................................. 102

Table 3-6: Presence and percentages of positive samples for STEC O-groups found in small and very small beef processing plants ................................................ 103

Table 3-7: Presence and percentages of positive samples for STEC virulence genes (stx1, stx2, and eae) found in small and very small beef processing plants .................. 104

Table 3-8: Presence and percentages of positive samples for STEC O-groups and virulence genes (stx1, stx2, and eae) found in small and very small beef processing environments and resulting ground beef products................................. 105

Table 3-9: Average aerobic plate counts (APC) and E. coli (EC) for environmental, carcass, hide, and ground beef samples. ................................................................. 106

Table 4-1: Reference strains used as controls for O-type determination in multiplex PCR assay............................................................................................................. 124

Table 4-2: O-group PCR primers used in this study. .......................................................... 130
Table 4-3: stx/eaе PCR primers used in this study.................................................................132

Table 4-4: Presence of STEC O-groups in hide samples collected by different methods in beef, pork, and sheep. ........................................................................................................136

Table 4-5: Number of samples testing positive for one or more STEC in hide samples collected by different methods in beef, pork, and sheep.........................................................137

Table 4-6: Presence and percentages of samples testing positive for STEC O-group, and STEC virulence genes; stx1/stx2/eaе in beef, pork, and sheep hide samples. .........................137

Table A-1: Presence and percentages of positive samples for STEC O-groups found in the environment of small and very small beef processing plants. .................................149

Table A-2: Presence and percentages of positive samples for STEC O-groups found in the environment of small and very small beef processing plants. .................................150

Table A-3: Seasonal variation in the prevalence of STEC and virulence genes in cattle processing environments, on cattle carcasses and hides, and ground beef resulting from small and very small processing plants. .................................................................151

Table A-4: Seasonal variation in the prevalence of STEC and virulence genes in all cattle processing samples. .........................................................................................................................154

Table A-5: Culture positive isolates obtained from enriched beef processing plant and product samples, identified by O-group and presence of virulence genes (stx1, stx2, and eae). .........................................................................................................................155
ACKNOWLEDGEMENTS

First, I would like to thank my advising committee for their guidance through the last two years. My major professor, Dr. Catherine Cutter, has been not only an advisor, but a mentor to me throughout my time working with her. She continuously encouraged me when working through the design of my project with patience and understanding. Additionally, her contacts within the meat industry were essential to the success of my project. Dr. Cutter’s expertise and passion for food safety is expressed through her extension work with the food industry, and played a large role in my decision to continue pursuing my education in the area of food safety.

Dr. Edward Dudley is also deserving of my gratitude. As an essential member of my committee, Dr. Dudley provided knowledge in the molecular detection aspect of my project, which was a new and difficult topic for me. I also appreciate the use of laboratory equipment, which was graciously provided by the busy Dudley laboratory. Additionally, Dr. Dudley is an exceptional teacher, with a unique ability to instill the passion of learning in students.

Dr. Chitrita DebRoy was also a great help to me over the past two years, and is deserving of my many thanks. Her broad knowledge of the STEC was a great resource for me to get a good grasp on the importance of this research. Dr. DebRoy was always willing to provide me a good reference paper for a difficult topic, or to set down with me to discuss my project. Her kind words and reassurance were often just what I needed to get me back on track.

Dr. Edward Mills is an outstanding teacher with an evident passion for the meat industry. Dr. Mills provided a connection to local meat processors, and was always willing to contact industry members for my sampling purposes. Outside of my research project, I had the pleasure of working with Dr. Mills as his TA for his meat processing undergraduate course. During this time, Dr. Mills encouraged me to become involved by lecturing in lab, and working
closely with students on individual projects. Whether he knew it or not, Dr. Mills was pushing me to work through some of my greatest insecurities, and for that I will forever be grateful.

Though I can not reveal the identities of the small and very small establishments that I worked with to collect samples, I would like to say how very greatful I am for their participation. Each processor allowed me to interrupt their processing schedule to collect samples, without these privileges I would not have been able to earn this degree. Each and every processor was kind and inviting to me, showing genuine interest in the research. I hope that these strong industry connections can remain intact for many years to come here at Penn State.

Next, I thank my labmates, undergraduate research assistants, and fellow graduate students who have given me advice, helped me make media, wash dirty labware, and even just listened when I was having a rough day. Over the last two years, I have had the pleasure of working closely with Angela Richards, Wladir Valderrama, Joshua Scheinberg, Caroline Dartnell, Chris Gardner, Rebekah Miller, Rich Swartz, Robson Machado, Amanda Gipe, Audrey Draper, Samantha Bennett, and Alissa Allen. I have heard many times over that grad school is where you make life-long friends, and I truly hope this is so.

Lastly, I want to thank my family for their support through my program. My parents, who have encouraged me to stick it out when I was having hard times, provided financial support through my entire education, and even supplied numerous plane tickets when they knew I was especially homesick. My grandmother, Donna, who has always believed in me, and been my most important confidant at many points throughout my life. And, my late grandmother, Marcia, who displayed love and devotion to her family, and unbelievable strength throughout her life.
Chapter 1

Literature Review

*Escherichia coli* as a foodborne pathogen

It is estimated that 48 million people become ill as a result of foodborne pathogens annually in the United States (U. S. CDC, 2011b). Therefore, the microbial safety of the food supply in the United States is an important issue. Shiga toxin-producing *Escherichia coli* (STEC) are Gram-negative, rod-shaped bacteria that have been implicated in several foodborne illness outbreaks. Symptoms of STEC infection include watery diarrhea, severe stomach cramping, and dehydration. In some instances, the disease can progress to hemorrhagic colitis (HC), or bloody stools. HC is caused by the destruction of the red blood cells in the intestine, resulting from infection of *E. coli* and release of the Shiga toxin into the host’s system (Liu, 2010). Up to 22% of HC patients develop hemolytic uremic syndrome (HUS) resulting from *E. coli* infection (Frank et al., 2011). HUS symptoms include exaggerated HC symptoms in addition to vomiting, fever, weakness, decreased urine output, and kidney failure. In rare cases, neurological damage may occur (Su, 1995). The mortality rate of HUS is 2-10% (Johnson et al., 1996). Death occurs as a result of renal failure, severe hypertension, myocarditis, or neurological disease. There are no specific treatments for HUS and individuals contracting the disease often require prolonged clinical treatment, resulting in significant healthcare costs over time. Ten percent (10%) of HUS survivors suffer chronic renal failure, while 40% suffer renal insufficiency (Johnson et al., 1996). Individuals most susceptible to STEC infection include young children, the elderly, and those with weakened immune systems (Liu, 2010).
**Description of *E. coli***

Individual strains of pathogenic *E. coli* can be identified and classified based on both serotype and strain. Identification of *E. coli* is based on the O and H antigens present in the bacterium. O-antigens are found on the surface of the lipopolysaccharide layer of the outer membrane in *E. coli*. There are 174 known O-antigens in the *E. coli* classification system. H-antigens are found in the flagella of bacteria. Currently, fifty-three H-antigens are used to serotype *E. coli* strains. *E. coli* strains are then classified using a combination of O and H antigens (ex. *E. coli* O157:H7). However, not all pathogenic *E. coli* contain flagella; in which case, isolates would be serotyped based on the O-antigen only and identified as non-motile (NM) (ex. *E. coli* O157:NM) (Scheutz et al., 2004; Gyles, 2006). Non-O157 serotypes commonly implicated in human illness include O26:H11, O111:H- and O145:H-. Pathogenic *E. coli* also can be identified by serogroups, where the O-group antigens are used as a means of identification. For example, O157 is a serogroup in which serotype O157:H7 belongs (Liu, 2010).

Serotypes of pathogenic *E. coli* can be categorized into groups, based on characteristics, such as virulence. The group of most concern is enterohemorrhagic *E. coli* (EHEC), which are capable of causing diarrheagenic disease in humans. EHEC are recognized by their ability to attach to intestinal cells and cause diarrhea in humans. Shiga toxin-producing *E. coli* (STEC) are a subset of pathogens within this group that are recognized by their ability to produce Shiga-like toxins. As many as 400 STEC are known to exist but not all have been identified as causing human illness and not all cause human disease in the same severity (Johnson et al., 1996; Gyles, 2006; Liu, 2010). To better understand the classification of STEC and an individual strain’s potential for causing disease, a classification of seropathotypes was proposed in which strains would be grouped based on their virulence and severity of disease. Seropathotypes are labeled A-E and strains are placed into groups based on decreasing virulence. Seropathotype A consists
of O157:H7, which is considered to be the most virulent. Seropathotype B is similar to O157 in that organisms in this group exhibit similar disease symptoms, but outbreaks occur with less frequency. Seropathotype C has been implicated in sporadic cases of HUS, but not outbreaks. Seropathotype D has been implicated in sporadic cases of diarrhea, while seropathotype E has not been implicated in human illness (Karmali et al., 2003).

STEC are recognized by their ability to produce Shiga-like toxins (Stx). Stx is a potent cytotoxin, also identified as verotoxin, due to the cytotoxic effect on cultured Vero cells (Johnson et al., 1996; Clark, 2001). Therefore, VTEC (verotoxin producing Escherichia coli) and STEC are terms used interchangeably for E. coli possessing the Shiga toxin gene. Shiga toxins were first identified in Shigella dysenteriae, the organism responsible for shigellosis, and resulting in severe bloody diarrhea. Stx produced by STEC are very similar in structure, causing comparable disease in humans. Two types of Stx can be produced by pathogenic E. coli: Stx1 and Stx2. Variants of Stx1 include Stx1c, which has been identified in strains isolated from sheep, but has not been associated with severe forms of human disease. Stx2, however, has several variants which include Stx2c, Stx2d, Stx2e, and Stx2f. Stx2 and Stx2c are most commonly associated with severe forms of human illness (USDA-FSIS, 2010; Gyles, 2006). A survey in the U.S. demonstrated that non-O157 STEC isolates from human illness outbreaks were more likely to have Stx1 than E. coli O157:H7. However, this observation alone was not able to explain differences in severity of illness (Hedican et al., 2009).

Genes for Stx are located within the DNA of a bacteriophage. When E. coli become infected with the said bacteriophage, DNA becomes inserted into the genome of the host bacterium, allowing the production of Stx by E. coli (USDA-FSIS, 2010). Stx molecules consist of two subunits: A and B. The A subunit is responsible for enzymatic activity which allows for the destruction of the host cell’s 28s rRNA, therefore preventing protein synthesis and results in
apoptosis of the host cell. The B subunit allows for binding of the Stx molecule to glycolipid receptors called Gb3 and Gb4. Binding to these receptors initiates internalization of the toxin molecule into the host cell, allowing the action of the A subunit to occur (Gyles, 2006). Mature cattle lack the receptor in intestinal tissues, which allows them to be non-symptomatic carriers of pathogenic E. coli (Pruimboom-Brees et al., 2000).

Though Stx production is a critical virulence factor and indicative of pathogenicity, the ability to produce Shiga toxin alone does ensure a pathogenic strain of E. coli (Wickham et al., 2006). Extensive analysis of surveillance data and genetic research has demonstrated that several combinations of virulence factors may be responsible for human illness. Virulence genes that have been associated with HUS resulting from STEC infection include: the locus of enterocyte effacement (LEE), the intimin (eae) and intimin receptor (tir) genes, as well as the enterohemolysin (hly) gene.

Pathogenicity islands (PAI) are locations in the bacterial genome that contain groups of virulence genes. The locus of enterocyte effacement, or LEE, is a PAI which allows STEC to attach to enterocytes (differentiated cells on the surface of the small intestine). After attachment, the STEC cause effacement, which allows the bacterial cell to remain in the intestine long enough for the release of Stx into the host’s system. Many, but not all pathogenic strains of non-O157 STEC implicated in foodborne illness contain the LEE. Examples of LEE-negative STEC implicated in foodborne illness include O111:H2 and O113:H21. A strain of O111 was shown to be capable of forming adhesions to HEp-2 cells (a line of cells developed from human cancer cells used for in vitro studies) instead of the typical attaching and enfacing lesions on intestinal cells produced by STEC containing the LEE locus (Morabito et al., 1998). STEC of serotype O113 also may produce an autoagglutinating adhesion which is encoded by a locus other than LEE, termed saa.
This adhesion acts in a similar manner as LEE to allow colonization of the intestinal mucosa (Paton et al., 2001).

Intimin (eae) is a virulence gene located within LEE. This gene, along with the intimin receptor (tir), encode for bacterial proteins that mediate the adhesion of the bacterium to enterocytes (Lou et al., 2000). The action of eae and tir create attaching and effacing (AE) lesions, which result in structural changes in the epithelial cells, such as loss of microvilli and accumulation of cytoskeletal proteins, and direct attachment of the bacterium to the host cell follows (Gyles, 2006). Several distinct types of intimin have been identified. Intimin type ε, γ, and β are most commonly associated with STEC (Oswald et al., 2000).

Enterohemolysin (hly) is a cell-associated hemolysin released from bacteria which causes the destruction of red blood cells within the host (Taneike et al., 2002). Intimin (eae) and enterohemolysin (hly) have been found in over 90% of all STEC cases in the U. S. Between 1983 and 2002, the CDC reported that over 90% of illnesses resulting in bloody diarrhea caused by non-O157 STEC contained eae and/or hly genes (Brooks et al., 2005). One study suggested a synergistic effect of stx2 and eae that results in a more severe form of illness (Boerlin et al., 2005).

An additional PAI (PAI O#122) has been identified in some non-O157 STEC, as well as O157 STEC, that could contribute to the pathogenicity of the bacteria. Though not considered commonly associated with pathogenic E. coli infection, in vivo, this PAI has been shown to have involvement with the repression of host lymphocyte activation as well as adhesion to cultured cells. It has also been associated with the capability of colonizing the intestinal tract of cattle, causing diarrhea in young calves (Stevens et al., 2002).

Surveillance system data have demonstrated that a combination of these virulence genes is most commonly associated with human illness. STEC most often isolated from
individuals with disease and containing some combination of these genes, include the serogroups O26, O45, O103, O111, O121, O145, and O157 (Brooks et al., 2005; Hedican et al., 2009; Thompson et al., 2005). Studies have analyzed the association of one or more virulence genes with the ability to cause disease of varying severity, as well as the variability of the presence of these genes among various STEC serotypes (Brandt et al., 2011).

For example, in an outbreak of STEC O111, \textit{stx1}, \textit{stx2}, \textit{eae}, and \textit{hly} were identified in the pathogen. Another outbreak due to STEC O103 was found to harbor two virulence genes: \textit{stx2} and \textit{eae}. In another case, O111 harbored only \textit{stx1} and \textit{stx2}. In another outbreak, STEC O26 harbored \textit{stx1}, \textit{eae}, and \textit{hly}, but caused only 20 cases of illness, no HUS cases, and no deaths. These cases suggest that, while virulence factors play a major role in the severity of disease caused by STEC, the genetic makeup that results in a pathogenic strain of STEC capable of causing severe human disease is not understood completely (USDA-FSIS, 2010).

\textit{Escherichia coli O157:H7}

\textit{E. coli} O157:H7 is the most commonly isolated pathogenic strain resulting in infection in the United States (CDC, 2011a). \textit{E. coli} O157:H7 has been recognized as a foodborne pathogen since 1982 (Riley et al., 1983) when it was isolated from individuals in several states and traced back to the consumption of undercooked ground beef. Since that time, the pathogen has been linked to several foodborne illness outbreaks, including a large outbreak in 1993 following the consumption of undercooked hamburgers, resulting in 477 illnesses and four deaths (CDC, 1993). In 1994, the U. S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) declared \textit{E. coli} O157:H7 an adulterant in ground beef processed under federal inspection (USDA-FSIS, 1999). Between 1982 and 2002, the United States Center for Disease Control and
Prevention (CDC) reported a total of 350 outbreaks due to *E. coli* O157:H7 with 8,598 illnesses, 1,493 hospitalizations, 354 cases of HUS, and 40 deaths (Rangel et al., 2005).

**Non-O157 STEC**

*E. coli* O157:H7 is the STEC most commonly associated with severe forms of disease, but it is not the only STEC known to cause disease in humans. At least 60 strains of STEC have been linked to human illness worldwide (Bettelheim, 2003). Non-O157 STEC are of major concern in many areas of the world. Some European countries report that over one half of confirmed STEC infections are caused by non-O157 STEC (Monaghan et al., 2011; Arthur et al., 2002; Brooks et al., 2005). It has been estimated that *E. coli* O157:H7 strains cause two-thirds of all *E. coli* human infection cases in the U. S., while non-O157 strains are responsible for the remaining cases (Mead et al., 1999). The geographic differences in reported STEC frequencies can be attributed to differences in diagnostic approaches. Many diagnostic laboratories are equipped to screen only for O157:H7 in patients with bloody diarrhea. Different food preferences, animal species, production and distribution practices may also contribute to varying frequencies of STEC detected in different areas of the world (Johnson et al., 1996).

When compared to *E. coli* O157:H7 and other enteric pathogens, non-O157 STEC are infrequently isolated and implicated in foodborne illness outbreaks. It is believed that this group of pathogens is largely under-accounted for, presumably due to ineffective laboratory screening and culturing methods (Possé et al., 2008). Thus, non-O157 STEC are not newly emerging pathogens. They have been implicated in clinical cases of human disease and have been of increasing public health concern since the early 1990’s (USDA-FSIS, 2010). The first non-O157 outbreak reported in the U. S. occurred in Montana in 1994 in which 11 cases of HC were reported as a result of *E. coli* O104:H21 contamination associated with a pasteurized dairy
product (CDC, 1995). This pathogen was isolated from fecal samples of patients using the same methods for *E. coli* O157:H7 detection, since it possessed the similar growth characteristics on selective growth media.

A survey completed in the U.S. between the years of 1982-2002, reported a total of 940 non-O157 STEC infection cases resulting in illness (Liu, 2010). It was not until 2000 that infections caused by non-O157 STEC were acknowledged as a nationally notifiable disease (Brooks et al., 2005). The number of reported cases of illness caused by non-O157 STEC to the CDC has steadily increased since that time. In addition, isolation of non-O157 STEC strains from clinical cases, as well as environmental and food sources has been increasing (Possé et al., 2008), likely due to a better awareness of the pathogens, as well as improving cultural and epidemiological techniques. Between 2001 and 2003, the CDC reported a 284% increase of laboratory-confirmed infections due to non-O157 STEC, while a 27% decrease in laboratory-confirmed infections with O157 STEC occurred (USDA-FSIS, 2011).

Non-O157 STEC are believed to cause diarrhea at rates similar to other enteric pathogens and are capable of causing disease resulting in HUS (Brooks et al., 2005). *E. coli* O157:H7 is known to cause illness in individuals who ingest as few as 10 cells (Tilden et al., 1996). In comparison, an outbreak of STEC O26 in fermented beef sausage resulted from an infectious dose of approximately 100 cells (Ethelberg et al., 2009). In another case, an outbreak of STEC O111 associated with beef sausage resulted from an infectious dose of 1 to 10 cells (Paton et al., 1996). Finally, an outbreak of STEC O145 in ice cream resulted from an infectious dose of approximately 400 cells (Buvens et al., 2011).

A study completed by the CDC between 1983 and 2003 demonstrated as many as 14 different serogroups were implicated in human disease resulting from *E. coli* infection, in addition to illnesses that resulted in undetermined serotypes (Brooks et al., 2005). However, the
same study demonstrated that approximately 70% of the infections caused by non-O157 STEC infections, that could be serotyped, were attributed to only 6 serotypes: O145, O121, O111, O103, O45, and O26, which have been identified by the CDC and USDA-FSIS as the “Big 6” non-O157 STEC. Of the “Big 6” serogroups, O45 has been reported to cause HC, while O26, O103, O111, O121, and O145 have been reported to cause disease which progresses to HUS (USDA-FSIS 2011). World-wide, other serogroups including O91, O128, and O55 have been implicated in human illnesses (Bell, 2002; Ito et al., 1990; Perelle et al., 2004).

**Incidence of STEC outbreaks**

Since 1990, the CDC has implicated the following serogroups in foodborne illness outbreaks: O111, O104, O121 O145, O103, O26, O45, O165, O84, and O141, with serogroups O26, O45, O111, O121, O103 and O145 implicated the most often. Data collected through the CDC also indicates that 75-80% of all reported and serogrouped non-O157 STEC isolates are attributed to these six serogroups. The data demonstrate that the remaining isolates (approximately 15%) are attributed to 53 different serogroups (USDA-FSIS, 2011).

![Figure 1: Number of non-O157 STEC outbreaks occurring in the United States 1990-2010. Adapted from USDA-FSIS, 2011.](image-url)
Vehicles of transmission in these outbreaks have included ill workers, dairy products, lettuce, water, animal contact, apple cider, and berries. While only one non-O157 outbreak has been linked to a beef product in the U. S., several outbreaks linked to ground meat have occurred world-wide. The first reported outbreak of non-O157 STEC linked to ground beef occurred in Italy in 1992. In this outbreak, serotype O111 was the causative agent, resulting in 9 illnesses that progressed to HUS with one death occurring. Additional outbreaks recorded and linked to non-O157 STEC in beef products include the serotypes O111 in Australia, O26 in Germany, O148 in France, O103 in Norway, O26 in Denmark, and O123 in France (USDA-FSIS, 2011).

Serogroup O157

From a total of 3,704 laboratory confirmed cases, it is estimated that *E. coli* O157:H7 causes approximately 96,500 cases of illness and 31 deaths each year in the U. S. (Scallan et al, 2011). As such, pathogenic *E. coli* belonging to serotype O157:H7 have been recognized as a major foodborne pathogen by the CDC since the early 1990’s. O157 is considered an important serogroup in many parts of the world including the United Kingdom, Canada, and Japan (Erickson and Doyle, 2007). Serotypes most commonly associated with human illness include O157:H7 and O157:NM.

(Söderström et al., 2005). This outbreak was linked to the consumption of iceberg lettuce. In 2000, an outbreak of O157 occurred at a camp in Scotland, resulting in 20 illnesses and one case of HUS, in which environmental exposure was deemed the causative agent (Howie et al., 2003).

**Serogroup O145**

Pathogenic *E. coli* belonging to serogroup O145 have been isolated from patients with bloody and nonbloody diarrhea, HC, and HUS worldwide (Fratamico et al., 2009; Feng et al., 2005). Serotypes most commonly associated with human illness include O145:NM, O145:H-, O145:H8, O145:H16, O145:H25, and O145:H28 (Fratamico et al., 2009). STEC O145 was ranked among the top six non-O157 serogroups isolated from individuals with sporadic illness reported to the CDC between 1983 and 2002 (Brooks et al., 2005). Outbreaks associated with O145 in the U. S. have been linked to a daycare center (Minnesota, 1999), drinking water (Oregon, 2005), and lettuce (multi-state outbreak, 2010) (USDA-FSIS, 2011).

According to Enter-net data, between the years 2000-2004, O145 was the fourth most common serogroup linked to human illness in mainland Europe, when the United Kingdom and Ireland are not considered (Kraigher et al., 2005). In 2005, *E. coli* O145 caused a case of bloody diarrhea which lead to HUS and the death of a 22 month old girl in Slovenia (Kraigher et al., 2005). Minced meat bought at a local butcher was thought to be the most likely source of infection. In 2007, an outbreak of *E. coli* O145 and O26 occurred in Belgium, resulting in 12 illnesses and 5 cases of HUS (De Schrijver et al., 2008). The source of infection was ice cream produced at a local farm. Isolates from patients, the ice cream, and the farm where the ice cream was produced and sold, were indistinguishable. Cattle have been identified as the major reservoir of STEC O145, but they have also been isolated from other food and companion animals (Fratamico et al., 2009).
**Serogroup O121**

Pathogenic *E. coli* belonging to serogroup O121 have been isolated from patients with HC and HUS worldwide (Fratamico et al., 2003). The serotype most commonly associated with human illness is O121:H19. STEC O121 also was ranked among the top six non-O157 serogroups isolated from individuals with sporadic illness reported to the CDC between 1983 and 2002 (Brooks et al., 2005). Outbreaks associated with O121 in the U. S. have been linked to a camp (Montana, 1998), lake water (Connecticut, 1999), lettuce (Utah, 2006), a daycare (Nebraska, 2006), a correctional facility (Colorado, 2007), and raw milk (Washington, 2009) (USDA-FSIS, 2011). In 2004, an outbreak of *E. coli* O121 occurred in school children in Japan after exposure to infected cattle on a ranch used for public education on dairy farming (Akiba et al., 2005). In this outbreak, 63 children developed symptoms of *E. coli* infection and serotype O121:H19 was isolated from patients and cattle on the ranch. As such, cattle are considered the major reservoir of STEC O121 (Akiba et al., 2005).

**Serogroup O113**

Pathogenic *E. coli* belonging to serogroup O113 were among the first STEC to be associated with HUS (Karmali et al., 1985). Serotypes most commonly associated with human illness include O113:H-, O113:H4, and O113:H21, with the latter comprising approximately half of all reports in this serogroup (Bettelheim, 2007). In 1998, an outbreak of *E. coli* O113 occurred in Southern Australia, where 3 children developed HUS (Paton et al., 1999). This group of organisms can produce intimin (*eae*), and have been shown to produce a subtilase cytotoxin, as well as the autoagglutinating adhesion (*saa*) (Paton et al., 1999). While cattle are considered the major reservoir of O113, the organism has also been isolated from venison, a horse in Germany, and sheep in Switzerland (Bettelheim, 2007).
Serogroup O111

Pathogenic *E. coli* belonging to serogroup O111 are recognized as significant human pathogens (Erickson and Doyle, 2007; Bettelheim, 2007). Serotypes most commonly associated with human illness include O111:H- and O111:H8 (Bettelheim, 2007). STEC O111 also was ranked among the top six non-O157 serogroups isolated from individuals with sporadic illness and reported to the CDC between 1983 and 2002 (Brooks et al., 2005). Outbreaks associated with O111 in the U. S. have been linked to a salad bar and ice (Texas, 1999), contact with calves (Minnesota, 2000 and 2001), irrigation water (Utah, 2000), a daycare (South Dakota, 2001 and 2009, Maine, 2007, Minnesota, 2008), unpasteurized apple cider (New York, 2004), a private home (North Dakota, 2007), and an elementary school (North Dakota, 2007). In addition, the largest STEC O111 outbreak in the U. S. occurred in Oklahoma where 340 people became ill, 72 were hospitalized, and 1 person died (USDA-FSIS, 2011). This outbreak was linked to a restaurant, but the vehicle of transmission was not identified.

In 1992, an outbreak of *E. coli* O111:NM occurred in Italy, resulting in nine children developing HUS, and one death (Caprioli et al., 1994). Cases of illness were widespread, and no source was identified. An outbreak of *E. coli* O111 occurred in France in 1992, which resulted in 10 school-age children developing HUS (Boudailliez et al., 1996). Person-to-person contact was determined to be the mode of transmission in this outbreak. In 1995, an outbreak of *E. coli* O111:H- occurred in Australia which resulted in 161 cases of illness, 23 cases of HUS, and one death (CDC, 1995). Uncooked fermented beef sausage was deemed the vehicle of transmission in this outbreak. Cattle are considered the major reservoir for STEC O111, yet the organism has rarely been isolated from the animals. It is also important to note that STEC O111 have been isolated from diseased chicken in Canada and wild deer in Japan (Bettelheim, 2007).
Serogroup O103

Pathogenic *E. coli* belonging to serogroup O103 are recognized as significant human pathogens (Erickson and Doyle, 2007; Bettelheim, 2007). Serotypes most commonly associated with human illness include O103:H- and O103:H2. Combined, these two serotypes make up 82% of this STEC serogroup (Bettelheim, 2007). STEC O103 was ranked among the top six non-O157 serogroups isolated from individuals with sporadic illness reported to the CDC between 1983 and 2002 (Brooks et al., 2005). One outbreak associated with O103 has been reported in the U. S., linked to water-based punch (Washington, 2000) (USDA-FSIS, 2011).

In 2006, an outbreak of *E. coli* O103:H25 occurred in Norway, resulting in 17 cases of illness, 9 cases of HUS, and one death (Sekse et al., 2009). Fermented sausage made from lamb meat was deemed to be the vehicle of transmission in the outbreak. An outbreak of *E. coli* O103:H2 occurred in Japan at a nursery, resulting in eight illnesses, three of which were children (Muraoka et al., 2007). The vehicle of transmission was not determined in this outbreak. Like other STEC, cattle are considered the main reservoir of this serogroup, yet are rarely isolated from these animals (Bettelheim, 2007).

Serogroup O45

Pathogenic *E. coli* belonging to serogroup O45 have been isolated from sick animals and humans, and have been classified as both enterotoxigenic *E. coli* (ETEC) and STEC (DebRoy et al., 2005). Serotypes most commonly associated with human illness include O45:H2 and O45:NM (USDA-FSIS, 2011). STEC O45 was ranked among the top six non-O157 serogroups isolated from individuals with sporadic illness and reported to the CDC between 1983 and 2002 (Brooks et al., 2005). Outbreaks associated with O45 in the U. S. have been linked to food workers (New York, 2005) and contact with goats (North Carolina, 2006) (USDA-FSIS, 2011). STEC O45 has been
associated with human illness resulting in HC, but has never been reported as progressing to cases of HUS or death. Cattle are recognized as a major reservoir of this serogroup. *E. coli* O45 strains have been associated with both diarrheic dairy calves and postweaning pigs. This serogroup has also been associated with feral pigeons. (DebRoy et al., 2005).

**Serogroup O26**

Pathogenic *E. coli* belonging to serogroup O26 have been linked to both human and animal illness for more than 25 years (Valadez, 2010). STEC O26 were the first STEC to be described in the literature and were essential to the early research on verocytotoxin identification (Smith and Linggood, 1971). Serogroup O26 has been increasingly associated with diarrheal disease and is frequently linked to outbreaks and cases of HUS (Jenkins et al., 2007). Interestingly, STEC O26 possesses many of the similar virulence factors as O157, but has the additional ability to infect animals (Jenkins et al., 2007). While incidence of O26 is considered low in the U. S., in countries such as Germany and the United Kingdom, this serogroup is believed to cause as much disease as O157 does in the U. S. (Caprioli et al., 1997). Serotypes most commonly associated with human illness include O26:H- and O26:H11 (Bettelheim, 2007). Serogroup O26 appears to survive in the bovine intestinal tract, rather than that of other food animals (Bettelheim, 2007).

STEC O26 was ranked among the top six non-O157 serogroups isolated from individuals with sporadic illness reported to the CDC between 1983 and 2002 (Brooks et al., 2005). Outbreaks associated with O26 in the U.S. have been linked to lake water (Minnesota, 2001), a daycare (Nevada, 2005, Iowa, 2007), blueberries and strawberries (Massachusetts, 2006), raw milk (Washington, 2010), and ground beef (multistate outbreak, 2010) (USDA-FSIS, 2011).
In 1997, an outbreak of O26 occurred in a nursery in Japan, resulting in 32 children developing diarrhea (Hiruta et al., 2001). Contaminated food samples (mixed vegetables with bean sprouts and spinach, and sliced watermelon) were deemed the likely source of transmission in this outbreak. In 2000, an outbreak of O26:H11 occurred in Germany, which resulted in 11 cases of diarrhea (Weber et al., 2002). A 2-year old child was hospitalized due to dehydration, but no cases of HC or HUS were reported. The vehicle of transmission was not confirmed, but epidemiological data suggested that a beef product called seemerrolle was a possible source. In 2007, an outbreak of O26:H11 occurred in Denmark, where 20 individuals with a median age of 2 years developed symptoms (Ethelberg et al., 2009). Credit card information was used to determine the source of infection to be an organic fermented beef sausage.

**E. coli reservoirs**

*E. coli* exists as a commensal organism in the gastrointestinal tract of animals, as well as humans. Many strains of *E. coli* are harmless to both humans and animals, but a subset (STEC, as discussed previously) is capable of causing serious illness in humans. While Shiga toxin can be pathogenic to humans, it has no negative effects on ruminant carriers of the pathogen, due to the lack of vascular receptors for the toxin (Pruimboom-Brees, 2000). Therefore, ruminants, and cattle in particular, are major reservoirs of STEC. Surveys have demonstrated that cattle may harbor more than 100 STEC serotypes, 60% of which have been associated with HC and HUS in humans (Cleary, 1992; Johnson et al., 1996). An earlier study from Japan detected STEC in 39.4-78.9% of fecal samples from cattle ranging in age from less than 2 months to adult (Shinagawa et al., 2000). These studies indicated that the highest rates of fecal shedding of STEC occurred in cattle immediately following weaning, and decreased with age. However, it also has been
demonstrated that the prevalence of STEC in cattle depends on the season, with the highest rates of fecal shedding occurring during the warmer months. Studies also indicate that O157 STEC are shed in loads varying from 2 to 5 $\log_{10}$ CFU/gram of fecal matter, depending on the season (Shere et al., 1998; Zhao et al., 1995).

Other ruminant species associated with pathogenic STEC include sheep, goats, and wild animals, such as deer and water buffalo. Sheep are the second most common animal reservoir for STEC, with serogroups such as O91, O128, and O146 having been isolated from the intestinal tract of the species. Goat milk also has been associated with an outbreak of *E. coli* O157 infection. *E. coli* O157 has been isolated from wild deer in several cases, and consumption of deer venison has been linked to human illness (Keene et al., 1997). In 2010, an outbreak of *E. coli* linked to O103 and O145 occurred in a school and was traced back to the consumption of deer venison (Rounds et al., 2012). STEC also have been isolated from non-ruminants such as horses, dogs, rabbits, pigs, and wild birds. However, it is likely that these animals are vectors for the pathogen, rather than reservoirs (Caprioli et al., 2005).

**STEC in livestock and resulting meat products**

**STEC in cattle, beef processing, and resulting beef products**

Extensive research has demonstrated that cattle are the major reservoir of Shiga toxin-producing *E. coli*. Cattle harbor STEC in the gut and can shed the bacteria in their feces. Shinagawa et al. (2000) demonstrated that cattle can shed STEC in their feces throughout their lifespan. One study indicated that there appears to be a correlation between the presence of STEC in feces and carcass contamination (Elder et al., 2000).

Fecal shedding of pathogens acts as a vehicle of transmission from one animal to another (Ransom et al., 2002). This observation is due primarily to animal coats and hooves.
easily being contaminated with fecal material (Small et al., 2002). As a result, cattle hides have been recognized as a source of microbial contamination on carcasses. Barkocy-Gallagher et al. (2003) suggested that cattle hides may be a greater source of STEC than feces. In fact, it has been reported that incidence of *E. coli* O157:H7 can be as much as ten times higher on cattle hides than in the feces of cattle (Ransom et al., 2002). Additionally, different areas of the hide have been reported to have varying incidence of O157 STEC. A study by Reid et al. (2002) indicated that O157 was present on the brisket of cattle hides most often (22.2% of samples), but less so on the rump and flank areas of the hide (3.3% and 4.4%, respectively). Incidence of O157 STEC on cattle hides have been reported, including 10.7% (Elder et al., 2000), 18% (Ransom et al., 2002), 28.8% (Small et al., 2002), and 60.6% (Barkocy-Gallagher et al., 2003). Incidence of non-O157 STEC has been reported as high as 77.7% on cattle hides in the fall months (Barkocy-Gallagher et al., 2003).

Hide to carcass transfer of microbial contamination can occur during slaughter and is more likely to occur at several points during de-hiding, such as making initial cuts through the skin (particularly in the brisket area), when alternate use of the same hand occurs for handling the hide and the carcass, and during roll-back of the hide. Ransom et al. (2002) reported 30-100% of carcasses from animals with hides contaminated with O157 also tested positive for O157 after de-hiding. Elder et al. (2000) also demonstrated a significant positive correlation between the prevalence of O157 in feces and hides, and prevalence of carcass contamination with O157.

Contamination on carcasses that follows through processing can lead to contaminated beef products, with ground beef being of particular concern. A survey of ground beef products demonstrated a prevalence of O157:H7 to be 3.7% (Doyle and Schoeni, 1987), and prevalence of
all STEC demonstrated presence to be between 12.1-40.8% (Read et al., 1990; Samadpour et al., 1994; Brooks et al., 2001).

**STEC in swine, pork processing, and resulting pork products**

Limited studies detecting the presence of STEC in swine and pork products have been completed. Feder et al. (2003) reported an O157:H7 prevalence of 2.0% in pig fecal samples. An additional study in central Mexico demonstrated an *E. coli* O157:H7 prevalence rate of 2.1% in swine feces collected on farms (Callaway et al., 2004). To the best of our knowledge, no studies have been conducted to determine the prevalence of STEC on pork hides or on pork carcasses. Surveys of ground pork products have demonstrated a prevalence of O157:H7 to be 1.5% (Doyle and Schoeni, 1987) and prevalence of all STEC to be between 4.0-18.0% (Read et al., 1990; Samadpour et al., 1994; Brooks et al., 2001).

**STEC in sheep, sheep processing, and resulting sheep products**

Limited studies of sheep and lamb products have demonstrated that sheep are reservoirs of STEC. Fegan and Desmarchelier, (1999) detected STEC in 45% of sheep feces and 36% of lamb feces. A survey of healthy lambs in Spain detected O157:H7 in 0.4% of fecal samples, and non-O157 STEC in 36% of fecal samples (Blanco et al., 2003). Sumner et al. (2003) surveyed lamb carcasses at abattoirs and very small plants, detecting *E. coli* on 61.5% of carcasses at the larger abattoirs and on 18.5% of carcasses at very small plants. Surveys of lamb and mutton products have demonstrated a prevalence of O157:H7 to be 2.0% (Doyle and Schoeni., 1987) and prevalence of all STEC to be between 17.1-48.0% (Samadpour et al., 1994; Brooks et al., 2001).
Transfer of STEC between animals

Contamination of animal hides with pathogens can come from soil, feces, other animals, and the environment in which animals are housed. It has been suggested that animal hide contamination can come from the farm, during transport to abattoirs, during unloading into holding pens, and during the lairage of animals at abattoirs prior to slaughter (Barkocy-Gallagher et al., 2003; Small et al., 2002). Routes of contamination transmission onto hides can include animal-animal contact, animal-environment contact, and environment-animal contact (Small et al., 2002). Animals held in lairage can pick up contamination on their hides by mingling with other animals or via contact with walls, floors, or water troughs in the lairage environment. Small et al. (2002) demonstrated that *E. coli* O157 contamination in lairage areas can be carried over from one day to the next, in spite of routine cleaning. Additionally, studies have demonstrated extended lairage of swine increases the contamination of slaughtered pigs with salmonella, because of the high prevalence of the pathogen in the lairage environment (Hurd et al., 2001). Given these factors, the possibility exists that animals coming from one farm that are not contaminated with a pathogen can become contaminated with pathogens when introduced into the lairage environment by previously contaminated animals. This observation becomes an even greater issue for small processors, who often house and slaughter multiple species in the same area, and on the same day.

STEC detection in livestock processing environments

Sampling techniques for animal hides utilize a gauze or sponge swab pad to collect the sample from the surface (Elder et al., 2000; Reid et al., 2002; O’Brien et al., 2005). Ransom et al. (2002) compared methods for collecting fecal, hide, and carcass samples using a variety of methods, including three-site sponge swabbing, hair clippings, hide excision, rinsing, and gauze
swabbing beef hides. Results of the study indicated that for *E. coli* O157:H7 detection, three-site sponge swab sampling and hair clippings appear to be the most effective on cattle hides, though these methods were not statistically different from the other sampling methods investigated. However, for the detection of *Salmonella* on beef hides, rinsing of the hide was statistically more effective (P < 0.05) than hair clippings and hide excision, but not more effective than sponge or gauze swabbing. To the best of our knowledge, a study has not been completed to compare hide sampling techniques among various species. As such, it may be possible that effective techniques for beef hides are not sufficient for pork or sheep hides.

A novel sampling method, known as the Microbial Vacuum (M-Vac; Bluffdale, UT) system, was designed to sample a large surface area, thereby recovering more microbial contamination (according to the manufacturer). The M-Vac system uses a vacuum and sterile solution to create agitation, detaching bacteria from the surface and transferring the mixture into a sterile collection bottle. The system aims to replace traditional sampling methods by sampling a larger surface area, increasing collection off the surface, and recovering more from sampling devices. Previous studies have demonstrated that the M-Vac system is effective for pathogen (*Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes*) recovery on various surfaces such as plastic, steel, and tile, and on beef flank and pork sides (Ngadze and Cutter, 2009; unpublished data). In some cases, the M-Vac system recovered more pathogens than sponge swabs on these surfaces.

**Vehicles of STEC transmission**

Vehicles of transmission for STEC include contaminated water, direct contact with infected humans or animals, and contaminated food products such as produce. In some instances, irrigation water can become contaminated with fecal matter and when used on fresh
produce, can lead to contamination. A multistate outbreak of *E. coli* O157:H7 associated with bagged spinach occurred in 2006. This outbreak was linked to irrigation water, which was contaminated by cattle feces. In this case, cattle were grazing in pastures and the surface water used for animal consumption also was used for irrigation of the spinach fields (CDC, 2006). In addition, feral pigs have been linked to water contamination, which led to an outbreak of *E. coli* O157:H7 in bagged spinach grown in central California in 2006 (Jay et al., 2007). Direct contact with infected humans or animals is another form of transmission for STEC infections. Several STEC outbreaks have occurred in a number of other settings: daycare centers where infants and toddlers are more susceptible to STEC infection; ill food workers contaminating prepared products; or human contact with infected animals at a petting zoo (USDA-FSIS, 2011). However, the most common vehicle of transmission for STECO157:H7 is contaminated foods, with contaminated beef products being the major source of infection.

Past foodborne illness outbreaks associated with meat consumption have led to extensive research efforts focusing on meat products as a source of pathogenic *E. coli*. Beef is the second most consumed meat product in the U.S., second only to chicken (U.S. Census Bureau, 2011). This statistic, combined with the number and severity of foodborne illness outbreaks traced to beef products, has led to considerable knowledge in the area of foodborne pathogens associated with beef. Since the major outbreak of *E. coli* O157:H7 in 1993 associated with ground beef, extensive research has focused on beef cattle as reservoirs of STEC. It has been demonstrated that the most common source of meat contamination with STEC is contamination of carcasses and plant environments with fecal material during slaughter (Johnson et al., 1996).

Surveys addressing the prevalence of STEC in cattle have indicated rates as high as 71% (Cerqueira et al., 1999). However, these data were collected with a major emphasis on O157:H7
identification, due to the importance placed on the specific pathogen at the time. A more broad survey of non-O157 STEC may have indicated even higher prevalence rates. Limited studies available for non-O157 STEC indicate a prevalence of 19.3% in feces, 57% on hides, 58.2% on pre-eviscerated carcasses, and 9% on post-intervention carcasses (Barkocy-Gallagher et al., 2003). This study indicated comparable prevalence rates of E. coli O157:H7 as well, which included 6% in feces, 61% on hides, 27% on pre-eviscerated carcasses, and 1.3% on post-intervention carcasses (Barkocy-Gallagher et al., 2003). Other reports have demonstrated that dairy cattle also are reservoirs for non-O157 STEC, which is important to the beef industry since cull dairy cows make up approximately 17% of ground beef produced in the U.S. (Troutt, 1997). Microbial surveys of retail ground beef and other retail meat products found 23-25% of samples to be positive for STEC (Acheson et al., 1996; Samadpour et al., 1994). A survey completed by the USDA-Agricultural Research Service (USDA-ARS) found similar results, with 24.3% (1,006/4,133) of ground beef samples from the U.S. testing positive for one or more STEC (Bosileveć and Koohmarraie, 2011.) Another survey indicated an STEC presence of 16.8% in retail ground beef and 18.4% in fecal samples (Samadpour et al., 2002). Similar results were obtained in the United Kingdom, where 17% of beef samples tested positive for STEC (Willshaw et al., 1993).

Of the non-O157 STEC outbreaks reported in the U.S., vehicles of transmission include dairy products, vegetables, water, apple cider, berries, and animal contact. However, no report of human illness from a non-O157 STEC infection has been reported from a beef product in the U.S. until 2010. In this outbreak, a Pennsylvania beef processing plant recalled approximately 8,500 pounds of ground beef following illnesses resulting from an E. coli O26 infection that was traced back to a ground beef product (USDA, 2010b). Three individuals reported illness, though no reports of HUS resulted.
Beef processing plants

It is the responsibility of beef processors to ensure the product that they put into commerce is safe for consumer consumption. Processors and their employees must be aware of how their actions on the processing floor and during fabrication will impact the food safety of products they are supplying to consumers. Every processing plant under federal inspection is required to develop a Hazard Analysis and Critical Control Point (HACCP) plan specialized to their plant and processing systems and to follow this plan throughout their daily operations. HACCP systems are designed to help control chemical, physical, and biological hazards throughout a process to ensure the safety of all meat products. Generally, biological hazards require considerably more attention, due to the significant impact on public health. In addition to following a HACCP plan, each federally inspected plant must test carcasses for generic E. coli to ensure critical control points are being met (USDA-FSIS, 1996).

The USDA classifies plants under federal regulations into size categories, based on the number of employees and the annual sales of a plant. Large plants are described as employing more than 500 workers, small plants as employing between 10 and 500 workers, and very small plants as employing 10 or fewer workers, or having annual sales of less than $2.5 million (USDA-FSIS, 1996). There are approximately 6000 federally inspected plants in the U. S. In the Northeast U.S. (Pennsylvania, New York, New England), there are 2 large plants in operation and approximately 280 small and very small, federally inspected plants (Pennsylvania State University, 2004). It is estimated by the USDA-FSIS that the four largest federally inspected slaughter establishments account for approximately 85% of slaughter production in the U. S., resulting in the majority of the nation’s fresh beef supply (P&SP, 2012). In addition, 81% of all slaughterhouses are classified as small and very small federally inspected establishments, and,
combined, make up the remaining of the total national production (USDA-FSIS, 1996). All plants under federal inspection are expected to comply with regulatory requirements, regardless of income or plant size. Given the size and income classifications of plants, it is clear that small and very small plants work with restricted labor and finances to meet the same regulations that large plants meet, which includes mandatory testing for biological hazards, such as *E. coli* O157:H7.

**Regulations for pathogenic *E. coli* in beef products**

*E. coli* O157:H7 regulations

In 1994, and in response to several large outbreaks, USDA-FSIS announced that *E. coli* O157:H7 would be considered an adulterant in raw ground beef. By implementing the Pathogen Reduction, HACCP Final Rule in 1996 (USDA-FSIS, 1996), USDA-FSIS also acknowledged that several pathogens including, *Salmonella*, *E. coli* O157:H7, *Campylobacter*, and *Listeria monocytogenes* were significant food safety hazards associated with meat and poultry products, resulting in over 4,000 deaths and 5 million hospitalizations annually. As part of the Pathogen Reduction act, USDA-FSIS proposed three measures to be applied in establishments during the introduction of HACCP: the implementation of sanitation standard operating procedures (SSOP’s), the requirement of at least one effective antimicrobial treatment in slaughter establishments, and standards for cooling red meat carcasses to prevent the growth of harmful bacteria (USDA-FSIS, 1996).

In addition to this rule, USDA-FSIS established performance standards for pathogen prevalence on raw products and determined that testing for generic *E. coli* is an effective means of verifying that a slaughter process is under control. Generic *E. coli* was established as a way to measure the process control for enteric pathogens because of the strong association between *E.
coli and the presence of fecal contamination. Thus, generic *E. coli* is considered an indicator of enteric pathogens, thereby permitting more rapid and more frequent adjustments of process control than can be done with pathogens (USDA-FSIS, 1996). Cattle slaughter establishments which fail to meet the generic *E. coli* testing standards and produce ground beef, are required to test their ground product according to the *E. coli* O157:H7 ground beef testing program implemented by the USDA-FSIS (1996).

**Proposed non-O157 regulations**

In September 2011, the USDA-FSIS announced that six other serogroups of STEC, including O26, O45, O103, O111, O121, and O145, would be considered adulterants in ground beef and non-intact raw beef products, as outlined in the Federal Meat Inspection Act (FMIA). This rule will require beef processing establishments to identify interventions to control (i.e., treatment of beef trim with antimicrobials), as well as perform additional verification procedures (i.e., testing beef trim and other raw ground beef products) for the presence of STEC. This rule followed a petition filed by food safety advocate groups who argued that all pathogenic STEC posed threats equal to *E. coli* O157:H7. Some have argued that the rule was made without sufficient scientific research to determine the extent and prevalence of these pathogens in the nation’s beef supply. Interestingly, USDA-FSIS plans to perform a nationwide microbiological baseline survey on beef carcasses prior to, or at the time of implementation of the proposed rule (USDA-FSIS, 2011).

**Potential implications for beef processors**

Given the recent developments by USDA-FSIS, it is estimated that a significantly larger number of beef samples (4-10%) will test “potentially positive” for the “big 6” STEC, as
compared to O157:H7-tested samples (Marsden, 2011), especially when one considers that the potential positive rate is 2% for *E. coli* O157:H7 testing (FSIS, 2011). Once beef is found presumptive positive, product will be held for further testing and confirmation, meaning that fresh product will have a shorter shelf-life once it reaches consumers, or that ground beef will be sold frozen.

It is important to note that screening for the additional STEC requires more advanced laboratory techniques than testing for *E. coli* O157:H7. At this point, cost effective assays for non-O157 STEC are not available commercially. The lack of commercially available assays for the beef industry will make testing difficult, resulting in longer times for “test and hold” procedures. This situation, combined with the much higher percentage of product expected to be held for further testing due to “presumptive positives”, may put a significant financial strain on small and very small processing establishments.

**Methods of STEC detection**

**Selective/differential enrichments and media**

Effective methods for isolation and identification of *E. coli* O157:H7 have been used since the early 1990’s, when regulation of the pathogen occurred. Isolation of this pathogen has been facilitated by its inability to ferment sorbitol. MacConkey Sorbitol Agar (SMAC) is a selective medium for the isolation of enterohemorrhagic *E. coli* that utilizes D-sorbitol in the formulation. Since *E. coli* O157:H7 cannot ferment this carbohydrate, the organism grows as a colorless colony (Difco Laboratories, 1998). However, other strains of STEC ferment sorbitol and grow as pink colonies on SMAC, making them indistinguishable from each other. The addition of antimicrobials, such as cefixime and potassium tellurite to SMAC (yielding CT-SMAC), results in a more selective media for the identification of *E. coli* O157:H7. Cefixime inhibits the growth of
Proteus spp., which can grow as a non-Sorbitol fermenting colony on SMAC. Potassium tellurite inhibits Providencia and Aeromonas species, as well as inhibiting the growth of some non-O157 STEC (TOKU-E, 2012). This phenomenon was demonstrated in a study using Dutch veal calves in which CT-SMAC effectively inhibited 67% of non-O157 STEC (Heuvelink et al., 1996).

Cultural methods designed for the identification of specific non-O157 STEC are available, but because non-O157 STEC lack distinguishable characteristics, these methods do not identify a broad range of serogroups. Posse et al. (2008), developed a selective, differential, chromogenic medium for the isolation of STEC serotypes O26, O103, O111, and O145, utilizing a mixture of carbohydrate sources, β-D-galactose activity, and selective components to separate the serotypes based on color. Rainbow® Agar O157 is another selective chromogenic media for the detection and identification of Shiga toxin-producing strains of Escherichia coli, in particular serotype O157:H7. This media utilizes two chromogenic substrates specific to E. coli associated enzymes, β-galactosidase and β-glucuronidase to identify serotypes based on color. Since E. coli O157:H7 is considered glucuronidase-negative, the colonies appear black or grey on the media. Non-O157 STEC generally over-produce β-galactosidase in comparison to β-glucuronidase, resulting in the growth of purple, violet, or blue colonies. Non-pathogenic strains of E. coli are considered glucuronidase-positive, which causes the growth of pink or magenta colonies. Potassium tellurite and/or novobiocin can be added to Rainbow® Agar to increase selectivity. While the manufacturer of Rainbow® Agar describes the medium as being differential for STEC O157, O26, O48, O111, and non-pathogenic E. coli (Biolog, 2003), USDA-FSIS has utilized this growth media to identify typical phenotypes of serogroups O26, O45, O103, O111, O121, and O145 (USDA, 2010a).
**Agglutination**

Latex agglutination is a rapid method for detection of *E. coli* O157 that became commercially available in the 1980s. This detection method utilizes latex particles coated in antibodies against *E. coli* cell-surface antigens to detect the bacterium (March and Ratnam, 1989). In the presence of the specific *E. coli* cell wall antigens, the latex particles will agglutinate, or clump together. This assay is intended to provide a fast and simple screening tool for *E. coli*, taking 5-10 minutes to perform. March and Ratnam (1989) described the *Escherichia coli* O157 latex agglutination test as a simple, highly efficient, and reliable test for detection of the pathogen with 100% sensitivity and specificity. In addition, agglutination kits have been developed to detect serogroups other than O157, including but not limited to O26, O45, O103, O111, O121, and O145 (Medina et al, 2012).

**Immunomagnetic separation**

Immunomagnetic separation (IMS) became commercially available in the 1980s, following the discovery of spherical polystyrene beads of exactly the same size (Dynal Life Technologies, 2012). This method was originally designed for the isolation of cells from blood, but was later applied to the microbiological field. IMS utilizes superparamagnetic, spherical polystyrene particles coated with monoclonal antibodies to bind to specific antigens (Dynal Life Technologies, 2012). IMS beads are combined with a pre-enriched sample and incubated for a period of time with gentle agitation, at which point the antibodies will bind to the matching cell surface antigen. The beads are then concentrated using a magnet particle concentrator (MPC; Sarian, 2005). Washing the incubated beads with phosphate buffered saline containing Tween-20 helps to reduce non-specific binding (Wright et al., 1994). IMS beads are then spread on a selective and/or differential plating medium for the growth of the desired bacteria. Pre-
enrichment of a sample followed by IMS has been shown to yield results that were 100 times more sensitive in detection than using a direct sample for \textit{E. coli} detection (Chapman et al., 1994; Wright et al., 1994). IMS anti-O157, O145, O111, O103, and O26 are commercially available (Dynal Life Technologies, 2012).

\textbf{PCR, multiplex PCR, and real-time PCR}

Molecular methods are utilized widely for the identification and characterization of many foodborne pathogens, including STEC. A conventional polymerase chain reaction (PCR) assay was first described in 1986 and has become an established lab technique that is used for the rapid screening and identification of foodborne pathogens (Liu, 2010). This technique is used for the amplification of double or single stranded DNA sequences \textit{in vitro} (Liu, 2010). PCR utilizes primers targeting specific DNA sequences chosen by the user, deoxyribonucleoside triphosphates (dNTPs), a thermostable DNA polymerase, magnesium chloride, and a DNA template (DNA from the tested sample; Liu, 2010). In a series of heat and cool steps, the primers anneal to the exposed DNA fragment and extend the sequence, essentially amplifying the desired DNA fragment. Following amplification, DNA can then be visualized using gel electrophoresis. Conventional PCR is one of the most popular molecular detection techniques, due to its relatively low cost, in comparison to other molecular methods, its ease of use, and the rapid process of detection (DNA is amplified to detectable limits ranging in time from minutes to a few hours; Liu, 2010). PCR has been utilized for the detection of the O-antigen gene cluster of several STEC, including but not limited to O157, O145, O121, O113, O111, O103, O91, O45, and O26 (DebRoy et al., 2011; Valadez et al., 2011; Fratamico et al., 2011). PCR detection limits have been reported to be as low as 2 cells for \textit{E. coli} O157:H7 (Liu, 2010).
Multiplex PCR (mPCR) follows the same concept of conventional PCR, but combines primers for the detection of several different DNA fragments into one reaction, allowing the identification of several genes at once. This technique has been utilized largely for the detection of foodborne pathogens. For example, Hill et al. (2011) developed a protocol for the simultaneous detection of *Salmonella* and enterohemorrhagic *E. coli* by combining primers for the detection of two *Salmonella* genes and stx1 and stx2 genes. Li et al. (2005) developed a mPCR protocol for the simultaneous detection of *E. coli* O157:H7, *Salmonella* spp., and *Shigella* spp. in raw and ready-to-eat meat products. In addition, mPCR protocols have been developed to detect specific STEC serotypes by combining primers targeting O-antigen gene clusters and primers targeting the Shiga toxin genes (Fratamico et al., 2009). DebRoy et al. (2011) developed a mPCR protocol for the simultaneous detection of STEC serogroups O157, O145, O121, O113, O111, O103, O45, and O26.

Real-time PCR also follows the same concept of conventional PCR, but has the capability of quantifying pathogen-specific gene sequences (Perelle et al., 2004). This approach allows one to determine the presence/absence of a gene sequence as the reaction is occurring, cutting out the extra step of running generated DNA through gel electrophoresis for detection. Real-time PCR is considered a more rapid method for molecular detection, with the ability to run thousands of samples per day (Liu, 2010). This method is not as widely used as conventional PCR, since it is a newer technology, as well as the higher costs of equipment and reagents. Several real-time PCR protocols have been developed for the detection of STEC utilizing primers targeting short sequences in the Shiga toxin genes and serogroup specific O-antigen gene sequences (O’Hanlon et al., 2004; Beutin et al., 2009; Fratamico et al., 2011).
Proposed methods of detection for non-O157 STEC

Following increased public concern over the non-O157 STEC serogroups most commonly implicated in foodborne STEC outbreaks, the USDA-FSIS developed a method to detect and isolate the six major non-O157 serogroups (O26, O45, O103, O111, O121, and O145) from ground beef products (USDA, 2010a). This method utilizes selective enrichment, multiplex real-time PCR assays, and selective and differential cultural techniques, as described below.

To screen ground beef samples for the presence of the six non-O157 STEC, USDA-FSIS suggests the following steps. A 325 gram sample will be aseptically collected and combined in a 1:4 dilution with modified TSB + casamino acids and novobiocin. The test sample will be incubated at 42 °C for 22-24 hours. Following enrichment, a 1.5 ml aliquot of the test sample will be collected and DNA extraction performed. The test sample DNA will be subjected to a multiplex real-time PCR assay to screen for the presence of stx and the eae intimin gene. If the test sample tests positive in this initial real-time PCR screen, it will be subjected to three additional multiplex real-time PCR assays which will identify the serogroup (O26, O45, O103, O111, O121, or O145). Serogroups O26 and O111 are screened in one real-time PCR reaction, serogroups O45 and O121 in another, and serogroups O103 and O145 in the third reaction. Following serogroup identification, a 2-5 ml aliquot of the 24 hour enrichment will be subjected to immunomagnetic separation (IMS beads) for the suspect serogroup. For example, if the real-time PCR assays indicate the presence of STEC O26, anti-O26 IMS beads will be utilized. Once IMS has been completed on the test sample, it will be spread-plated onto Rainbow ® Agar and incubated at 35 °C for 20-24 hours. Following incubation, plates will be examined for colonies exhibiting phenotypes of the suspect serogroup. Colonies expressing the correct phenotype will be collected and confirmed using the stx and eae multiplex real-time PCR assay; and the serogroup-specific multiplex real-time PCR assay. Up to 15 colonies will be collected from the
original Rainbow spread-plate. Initially, five suspect colonies will be subjected to the real-time PCR assays. The additional ten colonies will be plated onto a non-selective media, such as TSB +5% sheeps blood. If the first five colonies screen negative, the remaining colonies will be utilized for further screening (USDA-FSIS, 2010a).

This protocol requires sophisticated laboratory techniques not available to most meat processors. Real-time PCR assays require expensive equipment and reagents. Thermocyclers, clean-air hoods, and micro-pipettors would need to be available to enable laboratories to screen for the non-O157 STEC using the protocol provided by USDA-FSIS. In addition, PCR reagents are costly, ranging in price of approximately $130 to $170 for 20 real-time PCR reactions. Using the USDA-FSIS protocol, at least one real-time PCR reaction would be necessary to identify a negative sample and a minimum of 14 real-time PCR reactions would be required to detect and isolate a pathogen from a positive sample. This approach could result in as much as $100 for PCR reagents alone, for each sample tested.

Additional costs would include enrichment media, plating supplies, the cost of labor, and the income lost to processors when their product is being held for an extended period of time while awaiting test results. When considering that small and very small processing plants would need to develop laboratories equipped to handle this screening or pay for testing of their products using contract laboratories, the costs for screening non-O157 STEC become expensive. In addition, screening products for non-O157 STEC takes considerably more time than screening for O157:H7. To identify negative samples, it would take approximately 36 hours to complete the above-mentioned protocol. A positive sample would take an additional 44 to 72 hours, resulting in as much as 4-5 days to identify and isolate STEC from a positive sample. For many small processors that lack the distribution capabilities of larger companies, this approach would require fresh ground beef to be sold frozen to increase the shelf-life of the product.
Additionally, immunomagnetic separation is a crucial step in the isolation of non-O157 STEC, given that the USDA-FSIS protocol depends on this method to detect the suspect serogroup. While immunomagnetic separation beads containing antibodies for serogroups O157, O26, O103, O111, and O145 are commercially available, antibodies for O45 and O121 are not yet commercially available for use in private laboratories.
Purpose of survey

Non-O157 STEC have been linked to numerous outbreaks world-wide since the 1980’s. Yet, few laboratories perform regular screening for these pathogens. This limitation is likely due to the lack of reliable detection methods available to screen for non-O157 STEC and the low incidence in food products. However, with improvements in detection and isolation methods, these pathogens have become of larger concern in the food industry in recent years. While scientists currently lack reliable methods for characterizing and isolating individual STEC strains, rigorous immunological and molecular methods have paved the way to a better understanding of the importance of these pathogens in our food supply.

As such, the USDA-FSIS has determined that six strains of non-O157 STEC are of significant importance in the beef industry and have deemed them to be adulterants in raw ground beef and trim. Past research has demonstrated that cattle are a major reservoir of STEC and appear to be responsible for further contamination of beef products during slaughter and further processing. Focus has been placed on the presence of STEC in large processing facilities due to the higher number of cattle going through and the amount of beef produced by such plants. However, it is not known what role, if any, STEC play when beef is processed in small and very small processing plants, or the impact that these new regulations will have on these establishments. Small and very small processors are much more prevalent in the northeastern United States, Pennsylvania in particular. Testing for the non-O157:H7 STEC has the potential to create a financial burden on these smaller businesses. Therefore, a better understanding of the presence of the non-O157 STEC in small and very small beef processing plants will help researchers and regulatory personnel determine how best to regulate these facilities. By having data from these establishments, researchers and regulatory officials also can determine best
practices or adequate interventions to prevent STEC contamination of the beef supply originating from small and very small processors.
Statement of objectives

The overall objective of this project is to determine the prevalence of non-O157:H7 and O157:H7 STEC from carcasses, environment, and resulting ground beef produced in small and very small beef processing establishments throughout Pennsylvania. Along these lines, this project proposes to:

1. sample carcass, environmental, and ground beef samples for non-O157:H7 and O157:H7 STEC from small and very small beef processing establishments throughout Pennsylvania;

2. utilize a multiplex PCR assay previously developed at Penn State University (DebRoy et al., 2011), in combination with additional primers, to detect O-groups, genes for Shiga toxins 1 & 2, and intimin for the detection of pathogenic STEC in samples obtained in objective 1;

3. establish a baseline for the presence of non-O157 STEC in small and very small processing establishments and resulting beef products located in Pennsylvania;

4. evaluate the efficacy of various methods of sampling beef, pork, and sheep hides under slaughterhouse conditions for detecting STEC; and

5. establish a baseline for the presence of non-O157 STEC on beef, pork, and sheep hides in a very small processing establishment.
References


Chapter 2

Optimization of Sampling Techniques to Detect, Isolate, and Confirm STEC

Abstract

Non-O157 Shiga toxin-producing *Escherichia coli* (STEC) have been linked to a number of foodborne illness outbreaks since the early 1980’s. However, the overall incidence of these STEC is difficult to estimate, because routine screening of these serogroups is not performed, due to a lack of screening tools. Currently, a standard detection protocol for non-O157 STEC has not been established, largely due to the broad range of both phenotypic and genotypic characteristics this group of pathogens displays. This study evaluated the effectiveness of three different enrichment media (TSB + novobiocin (TSBn); modified *E. coli* broth + novobiocin (mECn); and modified TSB (mTSB) containing 8 mg/L novobiocin, 16 mg/L vancomycin, 2 mg/L rifampicin, 1.5g/L bile salts, and 1 mg/L potassium tellurite; described by Possé et al. (2008) to isolate a non-O157:H7 STEC. Carcass, environmental, fecal, or ground beef samples were artificially inoculated with approximately $10^2$ CFU/mL of STEC O145 and enriched for 24 hours at 42°C with each of the selected enrichment media. After enrichments, O145 was confirmed using a multiplex PCR assay (DebRoy et al., 2011), which had been optimized for the detection of STEC serogroups O157, O145, O121, O113, O111, O103, O45, and O26. All three enrichment media were effective for the detection of STEC O145. Interestingly, fewer background microorganisms were detected on agar plates when subjected to enrichment with mTSB. This combined method of mTSB enrichment and multiplex PCR assay will be utilized for future surveys of beef processing plants and resulting ground beef products for the detection of non-O157 STEC.
Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are Gram-negative, rod-shaped bacteria that have been implicated in several foodborne illness outbreaks. Symptoms of STEC infection include watery diarrhea, severe stomach cramping, and dehydration progressing to hemorrhagic colitis (HC) or bloody stools (Liu, 2010). At least 60 of the more than two hundred known strains of STEC have been linked to human illness worldwide (Bettelheim, 2003). The Centre for Disease Control and Prevention (CDC) has estimated approximately 265,000 STEC infections occur in the United States annually. The most well-known STEC, O157:H7, is believed to cause about 64% of these infections, with the remaining illnesses believed to be caused by non-O157 STEC (CDC, 2011).

However, the overall incidence of non-O157 STEC is difficult to estimate because routine screening of these serogroups is not performed (Bettelheim, 2007). The lack of screening tools is mainly due to the absence of a specified International Organization for Standardization (ISO) method of non-O157 STEC detection. A standard detection protocol for non-O157 STEC is difficult to establish because this group of pathogens displays a broad range of both genotypic and phenotypic characteristics. Individual strains of the non-O157 STEC most often lack characteristics distinguishing them from other strains (Possé et al., 2008a). This is not the case with the well-known pathogen, *E. coli* O157:H7, which possesses a unique phenotypic characteristic—the inability to ferment sorbitol (Difco Laboratories, 1998). Since non-O157 STEC lack distinguishable characteristics, detection of these pathogens has focused on non-cultural methods including enzyme immunoassays, biosensors, and molecular detection methods, such as DNA probe assays and polymerase chain reactions (PCR) (Nataro and Kaper, 1998).
Cattle are considered the primary reservoir of both O157 and non-O157 STEC (Bettelheim, 2000). These pathogens can be transferred to beef products throughout the production process (Barkocy-Gallagher, 2002). During processing, fecal contamination can occur during evisceration or de-hiding, which facilitates the transmission of STEC onto the beef carcass surface (Arthur et al., 2002). To achieve a better understanding of STEC transmission through the beef supply, surveys determined the major sources of STEC are cattle feces (Barkocy-Gallagher et al., 2003; Samadpour et al., 2002), beef carcass surfaces (Barkocy-Gallagher et al., 2003; Arthur et al., 2002), beef hides (Barkocy-Gallagher et al., 2003), and ground beef products (Bosilevac et al., 2011; Samadpour et al., 2002).

Since standardized methods of non-O157 STEC detection are not available, current studies rely on detecting genes for Shiga toxins 1 and 2 (stx) utilizing PCR. However, enrichment media used prior to PCR detection has varied, including but not limited to tryptic soy broth (TSB) (Bosilevac et al., 2011; Barkocy-Gallagher et al., 2003), brilliant green bile broth 2% (Arthur et al., 2002), and a modified TSB supplemented with bile salts no. 3, dibasic sodium phosphate, and novobiocin (Samadpour et al., 2002). Barkocy-Gallagher et al. (2003) also utilized Escherichia coli broth, or EC broth, a commercially available selective enrichment media approved for the growth of coliforms to isolate non-O157 STEC from food and environmental samples. EC broth contains the selective agent bile salts no. 3 which is known to inhibit the growth of Gram-positive bacteria. Additionally, a selective enrichment method was described by Possé et al. (2008b) that includes a pre-enrichment of samples in TSB containing 8 mg/L novobiocin and 16 mg/L vancomycin for 6 hours at 37°C, followed by the addition of 2 mg/L rifampicin, 1.5g/L bile salts, and 1 mg/L potassium tellurite, with incubation at 42°C for an additional 18 hours. After enrichment and isolation, STEC have been confirmed further using biochemical assays and/or API 20E strips and serotyped using DNA probes or PCR.
Additional research to develop a multiplex PCR assay to identify seven STEC serogroups (O103, O91, O113, O145, O111, O157, and O26) in artificially inoculated beef carcass swabs, beef trim, and ground beef, utilized different enrichments for each product, including TSB, modified TSB + novobiocin (TSBn), and a modified E. coli medium + novobiocin (mECn), respectively (Valadez, 2010). It is the goal of this project to sample carcass, environmental, fecal, and ground beef samples for the presence of non-O157 and O157 STEC from small and very small beef processing establishments throughout Pennsylvania. To simplify the enrichment process, it would be beneficial to utilize one enrichment medium for all sample types. To determine if one enrichment medium can be used effectively for the recovery of non-O157 STEC in carcass, environmental, fecal, and ground beef samples, each sample type was artificially inoculated with a non-O157 STEC. Samples were enriched with three different enrichment media: TSB + novobiocin (TSBn; Difco Laboratories, Detroit, MI), modified E. coli broth + novobiocin (mECn; Difco laboratories, Detroit, MI), and modified TSB (mTSB) containing 8 mg/L novobiocin and 16 mg/L vancomycin for pre-enrichment, followed by the addition of 2 mg/L rifampicin, 1.5g/L bile salts, and 1 mg/L potassium tellurite (Possé et al., 2008b). Presence of a non-O157 STEC were confirmed utilizing latex agglutination and primers for a multiplex PCR assay previously described (DebRoy et al., 2011).
Materials and methods

**Bacterial strains and culture conditions**

*E. coli* reference strains belonging to serogroups O157, O145, O121, O113, O111, O103, O45, and O26 were used as positive controls, and *E. coli* DH5α as the negative control in this study. In addition, representative strains from each serogroup were used for multi-STEC detection utilizing the multiplex PCR assay described by DebRoy et al. (2011). All strains were obtained from the *E. coli* Reference Center in the Department of Veterinary and Biomedical Sciences at the Pennsylvania State University (University Park, PA) and are listed in Tables 1 and 2.

Bacteria were suspended in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) containing 10% glycerol and stored at -80°C. Prior to experiments, cultures were propagated twice in 10 mL TSB at 37°C for 18-24 hours and maintained on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI). Prior to the experiments, each culture was grown overnight on TSA at 37°C, and a single colony was selected and inoculated into 10 mL TSB under static conditions. Using this method, the starting concentration of each of the strains was approximately $10^9$ CFU/mL. Serial dilutions of the culture were made in buffered peptone water (BPW; Difco Laboratories, Detroit, MI) and the actual CFU/mL was determined by plating serial dilutions on TSA and incubating for 24 hours at 37°C.
Table 1. Reference strains used as controls for O-type determination in multiplex PCR assay.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Culture ID</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>ATCC 43895</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O145:K-:H-</td>
<td>E1385(3)</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O121</td>
<td>39W</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O113:H21</td>
<td>6182-50</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O111:H-</td>
<td>Stoke W</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O103:K+:H8</td>
<td>H515b</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O45</td>
<td>K61</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O26:H-</td>
<td>H311b</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>Negative Control</td>
<td>Invitrogen (Carlsbad, California)</td>
</tr>
</tbody>
</table>
Table 2. Bacterial strains used for multiplex PCR assay.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>ECRC#**</th>
<th>Source</th>
<th>Location</th>
<th>stx1</th>
<th>stx2</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli O157:H7</td>
<td>6.1693</td>
<td>WHO*</td>
<td>Denmark</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td>7.1495</td>
<td>Ground Beef</td>
<td>DE, USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli O145:H2</td>
<td>4.0968</td>
<td>Rabbit</td>
<td>MA, USA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>E. coli O145:H+</td>
<td>6.1598</td>
<td>WHO</td>
<td>Denmark</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>E. coli O113:H4</td>
<td>9.0109</td>
<td>WHO</td>
<td>Denmark</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli O113:H21</td>
<td>9.0532</td>
<td>Unknown</td>
<td>OH, USA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>E. coli O111:?</td>
<td>4.0522</td>
<td>Cow</td>
<td>PA, USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli O111:H8</td>
<td>7.1639</td>
<td>WHO</td>
<td>Denmark</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli O103:H2</td>
<td>9.0108</td>
<td>WHO</td>
<td>Denmark</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E. coli O103:H2</td>
<td>3.2608</td>
<td>Horse</td>
<td>CO, USA</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E. coli O26:H11</td>
<td>7.3964</td>
<td>Unknown</td>
<td>OH, USA</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E. coli O26:H30</td>
<td>8.0176</td>
<td>Unknown</td>
<td>OH, USA</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*World Health Organization  
** E. coli Reference Center, Department of Veterinary and Biomedical Sciences; Pennsylvania State University, University Park, PA  
Stx: Shiga toxin 1/2, virulence genes of pathogenic E. coli  
- indicates the organism is negative for the Stx1 or Stx2 gene  
+ indicates the organism is positive for the Stx1 or Stx2 gene
Optimization of multiplex PCR primers

Primers were empirically tested and optimized to produce a visible band using a combination of all 8 control DNA templates and to decrease primer-dimer accumulation in the PCR reaction (Brownie et al., 1997) (Figure 1). The PCR assay was performed using a Mastercycler pro thermocycler (Eppendorf, Westbury, NY). Thermocycler conditions are as follows: Initial denaturation of 95°C for 15 minutes, followed by 30 cycles of 94°C for 30 seconds, 61°C for 1.5 minutes, and 72°C for 1.5 minutes, followed by a final extension of 72°C for 10 minutes. Primer concentrations used in the multiplex PCR assay are provided in Table 3.

Figure 1. Agarose gel of amplicons from the multiplex PCR assay of Shiga toxin-producing Escherichia coli (STEC) serogroups. Lane 1: Molecular weight ladder, 100 bp. Lane 2: No template control. Lane 3: 2-strain control. Lane 4: 4-strain control. Lane 5: 6-strain control. Lane 6: 8-strain control. Lane 7: STEC O157. Lane 8: STEC O145. Lane 9: STEC O121. Lane 10: STEC O113. Lane 11: STEC O111. Lane 12: STEC O103. Lane 13: STEC O45. Lane 14: STEC O26.
Table 3. O-group PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Primer Sequence (5’-3’)</th>
<th>Target Gene</th>
<th>Primer Concentration (µM)</th>
<th>Amplicon Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>O157</td>
<td>TCG AGG TAC CTG AAT CTT TCC TTC TGT ACC AGT CTT GGT GCT GCT CTG ACA</td>
<td>wzx F</td>
<td>0.015</td>
<td>894</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td>O145</td>
<td>TTC ATT GTT TTG CTT GCT CG GGC AAG CTT TGG AAA TGA AA</td>
<td>wzx F, wzx R</td>
<td>0.03</td>
<td>750</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td>O121</td>
<td>TCC AAC AAT TGG TCG TGA AA AG AAG TGT GAA ATG CCC GT</td>
<td>wzx F, wzx R</td>
<td>0.029</td>
<td>628</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td>O113</td>
<td>TGC CAT AAT TCA GAG GGT GAC AAC AAA GCT AAT TGT GGC CG</td>
<td>wzx F, wzx R</td>
<td>0.029</td>
<td>514</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td>O111</td>
<td>TGT TTC TGC GAT GTT GCG AG GCA AGG GAC ATA AGA AGC CA</td>
<td>wzx F, wzx R</td>
<td>0.029</td>
<td>438</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td>O103</td>
<td>TTG GAG CGT TAA CTG GAC CT GCT CCC GAG CAC GTA TAA AG</td>
<td>wzx F, wzx R</td>
<td>0.03</td>
<td>321</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td>O45</td>
<td>TGC AGT AAC CTG CAC GGG CG AGC AGG CAC AAC AGC CAC TAC T</td>
<td>wzx F, wzx R</td>
<td>0.063</td>
<td>238</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td>O26</td>
<td>CAA TGG GCG GAA ATT TTA GA ATA ATT TTC TCT GCC GTC GC</td>
<td>wzx F, wzx R</td>
<td>0.17</td>
<td>155</td>
<td>DebRoy et al., 2011</td>
</tr>
</tbody>
</table>
Evaluation of enrichment media for non-O157 STEC experimentally inoculated in ground beef, fecal, environmental, and carcass samples

Ground beef sample collection

Non-irradiated ground beef (80:20; lean:fat) was purchased from a local retailer (n=10). The ground beef was held at 4°C until sampling. Ground beef packages were aseptically opened, 25 gram aliquots were transferred aseptically to a filtered stomacher bag (Interscience, Rockland, MA), and artificially inoculated with 1 mL of approximately $10^2$ CFU/mL E. coli O145. Inoculated samples were mixed gently and left for at least 15 minutes to allow the bacteria to adhere to the meat surface (Brichta-Harhay et al., 2007; Pepperell et al., 2005). The inoculated beef was diluted 1:10 in 225 mL of BPW (Difco Laboratories, Detroit, MI) and stomached for 2 minutes at 230 rpm (Stomacher® 400 Circulator, Steward®, United Kingdom). One mL of the sample was then combined with 9 mL of each medium (mTSB, TSBn, or mECn) and incubated as follows. TSBn and mECn enrichments were incubated for 24 hours at 42°C. Samples enriched in mTSB were incubated for 6 hours at 37°C. Following the 6 hour pre-enrichment, additional antimicrobials were added, samples were then incubated for 18 hours at 42°C. After enrichments, samples were streaked for isolation on CT-SMAC and Rainbow® agar. Agar plates were incubated for 18-24 hours at 37°C.

Fecal sample collection

Fecal samples (n=10) were collected from the Penn State Dairy Barns in sterile Whirl-Pak® bags (Nasco®, Fort Atkinson, WI) and held at 4°C until sampling. Four grams of the fecal sample were aseptically transferred into a Stomacher® 400 Circulator Standard Bag (Steward®, United Kingdom), diluted 1:9 in 36 mL of BPW, and stomached for 30 seconds at 230 rpm. Nine
mL of the stomached sample was inoculated with 1 mL of approximately $10^2$ CFU/mL E. coli O145. One mL of the sample was then combined with 9 mL of each medium (mTSB, TSBn, or mECn), and incubated and plated as described as above.

**Environmental sample collection**

Beef processing environmental samples (n=10) were collected from the PSU Meats Lab abattoir. All samples were collected using a Spongesicle® swab (Biotrace International; Seattle, WA) moistened with 25 mL of sterile BPW. Environmental sampling was performed according to USDA-FSIS guidelines (USDA-FSIS, 2009). Briefly, sponges were wrung out and aseptically removed from the bag. Sampling was performed aseptically using sterile latex gloves changed between samples. For each environmental sample, one square foot was swabbed. Swab samples consisted of 10 passes vertically, 10 passes horizontally, and 10 passes diagonally. The swab was flipped midway through taking the sample. Environmental samples collected included a drain, the chute floor, a knife, a walk-in cooler handle, and a skinning cradle. The samples were stored in the collection bag at 4°C until processing was possible. Upon arrival at the lab, samples were processed immediately. Swabs were stomached at 230 rpm for 2 minutes, swabs were wrung out, and sample solution collected aseptically. Nine mL of the stomached sample was inoculated with 1 mL of approximately $10^2$ CFU/mL E. coli O145. One mL of the sample was then combined with 9 mL of each medium (mTSB, TSBn, or mECn) and incubated and plated as described previously.
Beef carcass sample collection

Beef carcass samples (n=5) were collected from the PSU Meats Lab abattoir. All samples were collected using a dry-sponge (3M™, St. Paul, MN) moistened with 25 mL of sterile BPW. Carcass samples were collected according to USDA-FSIS guidelines (USDA-FSIS, 1996). Briefly, sponges were wrung out, aseptically removed from the bag, and used to sample one half of the carcass using a sterile 100 cm² template. Sampling was performed aseptically using sterile latex gloves and changed between samples. For each carcass sample, 100 cm² was swabbed at three sites: flank, brisket, and rump, in the respective order. Swab samples consisted of 10 passes vertically and 10 passes horizontally. The swab was flipped midway through taking the sample. The samples were stored in the collection bag at 4°C until processing was possible. Upon arrival at the lab, samples were processed immediately. Swabs were stomached at 230 rpm for 2 minutes, swabs were wrung out, and sample solution collected aseptically. Nine mL of the stomached sample was inoculated with 1 mL of approximately 10² CFU/mL E. coli O145. One mL of the sample was then combined with 9 mL of each medium (mTSB, TSBn, or mECn) and incubated and plated as described previously.

Evaluation of background microflora from ground beef, fecal, environmental and carcass samples

Levels of aerobic plate counts (APC) and generic E. coli (EC) were evaluated in all samples. Ground beef (25 g), fecal (4 g), environmental (25 mL), and carcass (25 mL) samples were aseptically transferred to a stomacher bag. Ground beef and fecal samples were diluted 1:10 and 1:9, respectively in sterile BPW. The samples were stomached for 2 minutes (ground beef, environmental, and carcass samples) or 30 seconds (fecal samples) at 230 rpm. Following
stomaching, 1 mL of each sample was serially diluted 1:10 in 9 mL of sterile BPW. APC and EC of the samples were measured by plating a 1 mL aliquot of the serial dilutions onto APC and EC Petrifilm (3M™ Healthcare, St Paul, MN). The Petrifilm was incubated at 37°C for 24 hours, manually counted, and resulting populations were recorded (Table 4).
Table 4. Average aerobic plate counts (APC) and *E. coli* counts (EC) for ground beef, fecal, environmental, and carcass samples before artificial inoculation of STEC.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sample Size</th>
<th>APC (log_{10} CFU)</th>
<th>EC (log_{10} CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Beef (80:20)</td>
<td>25 g</td>
<td>4.93 x 10^5</td>
<td>6.5 x 10^1</td>
</tr>
<tr>
<td>Fecal</td>
<td>4 g</td>
<td>6.01 x 10^{10}</td>
<td>1.18 x 10^7</td>
</tr>
<tr>
<td>Environmental Swab</td>
<td>1 ft^2</td>
<td>4.10 x 10^8</td>
<td>4.35 x 10^5</td>
</tr>
<tr>
<td>Carcass Swab</td>
<td>100 cm^2</td>
<td>1.64 x 10^4</td>
<td>1.87 x 10^1</td>
</tr>
</tbody>
</table>
Isolation and identification of STEC in ground beef, fecal, environmental, and carcass samples

Following incubation as described above for each of the enrichment media, presumptive isolates were selected and picked for agglutination using Dryspot E. coli Seroscreen (Oxoid Microbiology, Hampshire, UK). Isolates positive for agglutination testing were selected and placed in TSB. Following an 18-24 hour incubation at 37°C, DNA isolation was performed (see below), and isolates were confirmed as E. coli O145.

DNA isolation

For DNA isolation, all bacterial DNA was extracted using Epicentre Biotechnologies’ MasterPure™ DNA Purification Kit (Madison, WI) according to manufacturer’s instructions with minor modifications. Briefly, 1 mL of enrichment broth was concentrated by centrifugation for 5 minutes at 7,000 x g in a microcentrifuge (Galaxy 16, VWR International, Radnor, PA) and supernatant was discarded. The cell pellet was resuspended in 300 µl of tissue and cell lysis solution containing 1 µl of 50 µg/µl proteinase K. The cells were lysed by incubating at 65°C for 15 minutes with agitation every 5 minutes using a vortex (VWR International, Radnor, PA). Samples were cooled to 37°C for 10 minutes, 1 µl of 5 µg/µl RNase A added to the sample, mixed thoroughly, and incubated at 37°C for 30 minutes. Samples were then placed on ice for 5 minutes. MPC Protein Precipitation Reagent (175 µl) was added to the lysed sample and vortexed vigorously for 10 seconds. The cell debris was pelleted by centrifugation for 10 minutes at 10,000 x g in a microcentrifuge. The supernatant was transferred to a clean microcentrifuge tube, 500 µl of isopropanol was added, and the tube inverted 30-40 times. The total nucleic acid was pelleted by centrifugation for 10 minutes in a microcentrifuge and the supernatant was carefully poured out without dislodging the pellet. The pellet was then rinsed twice with 70%
ethanol and allowed to dry aseptically for 15-20 minutes. The pellet was then resuspended in 100 µl of sterile deionized water. All DNA preparations were stored at -20°C until use.

**Statistical analysis**

A Chi-Square Goodness of Fit test (SAS program; Cary, NC) was conducted on the enrichment media data for each sample type to determine if the enrichment media type influenced the detection of non-O157 STEC (P ≤ 0.05).

**Results**

Results for the detection of *E. coli* O145 from ground beef, fecal, environmental swabs, and carcass swabs in 3 different enrichment mediums (TSBn, mECn, or mTSB) are summarized in Table 5. All samples were analyzed before artificial inoculation for the presence of *E. coli* O145 and none were found to be positive for the pathogen (data not shown). After experimental inoculation of samples, *E. coli* O145 was detected in all sample types, using all enrichment medium types. In ground beef samples, O145 was detected in 10/10 of the samples, regardless of medium. In fecal samples, O145 was detected in 9/10 of the samples enriched in TSBn, in 10/10 of the samples enriched in mECn, and in 8/10 of the samples enriched in mTSB. In environmental samples, O145 was detected in 9/10 of the samples enriched in TSBn, in 9/10 of the samples enriched in mECn, and in 2/10 of the samples enriched in mTSB. In carcass samples, O145 was detected in 5/5 of the samples enriched in TSBn and mECn, and in 2/5 of the samples enriched in mTSB. For each sample type, there was no significant difference (P > 0.05) in the number of samples in which O145 was detected between media. These data suggest that any of
the media evaluated could be used in subsequent experiments for detection of *E. coli* O145 in a variety of samples.
Table 5. Frequency of detection of STEC O145 in beef samples by agglutination and multiplex PCR.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Enrichment Media Type</th>
<th>TSBn % Positive</th>
<th>mECn % Positive</th>
<th>mTSB % Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Beef</td>
<td>10/10</td>
<td>100 A *</td>
<td>100 A</td>
<td>100 A</td>
</tr>
<tr>
<td>Fecal</td>
<td>9/10</td>
<td>90 A</td>
<td>10/10</td>
<td>8/10</td>
</tr>
<tr>
<td>Environmental Swab</td>
<td>9/10</td>
<td>90 A</td>
<td>9/10</td>
<td>2/10</td>
</tr>
<tr>
<td>Carcass Swab</td>
<td>5/5</td>
<td>100 A</td>
<td>5/5</td>
<td>2/5</td>
</tr>
</tbody>
</table>

* Means within each sample type bearing a common letter are not significantly different (P > 0.05).
Discussion

Isolation of foodborne pathogens is vital to the confirmation of foodborne illness outbreaks. Pathogens are traced through the food chain; including pre-harvest, processing environment, and product sampling. Isolation of these pathogens can prove difficult, due to the large number of background microflora present in many samples. Conventional methods for the detection of *E. coli* O157:H7 include isolation and confirmation of the bacterium using serotyping methods. Due to the lack of distinguishing physical characteristics of non-O157 STEC, this task is difficult and time consuming. Therefore, several studies have utilized PCR methods to detect and serotype STEC based on O-antigens (Perelle et al., 2004; DebRoy et al., 2001; Fratamico et al., 2011), as well as the presence of virulence genes including *stx1*, *stx2*, *eae*, and *hylA* (Barkocy-Gallagher et al., 2003).

Limited studies have demonstrated the presence of *E. coli* O157:H7 and non-O157 STEC in large beef plants and beef products. Barkocy-Gallagher et al. (2003) indicated an STEC prevalence of 19.3% in feces, 57% on hides, 58.2% on pre-eviscerated carcasses, and 9% on post-intervention carcasses. This study utilized PCR detection for STEC virulence genes *stx1*, *stx2*, *eae*, and *hylA*. Another survey found 24.3% (1,006/4,133) of ground beef samples from the U. S. to be positive for one or more STEC (Bosilevec and Koohmaraie, 2011). This survey utilized a combination of PCR, conventional plating, and biochemical assays to identify and characterize STEC.

The current study utilizes previous PCR methodologies (DebRoy et al., 2011), in combination with conventional enrichment and plating techniques, to detect non-O157 STEC (O145) in ground beef samples, fecal samples, beef processing environmental swabs, and beef
carcass swabs. Three different enrichment media were utilized to detect STEC O145: TBS + novobiocin (TSBn), modified E. coli broth + novobiocin (mECn), or modified TSB (mTSB) containing 8 mg/L novobiocin and 16 mg/L vancomycin for a pre-enrichment period, followed by the addition of 2 mg/L rifampicin, 1.5g/L bile salts, and 1 mg/L potassium tellurite (Possé et al., 2008b). Results demonstrated that all 3 medium types were equally effective for the detection of non-O157 STEC in different sample types. Additionally, plated samples demonstrated fewer background microorganisms when enriched in mTSB. Therefore, it was decided that mTSB enrichment medium would be utilized for further experiments. A multiplex PCR assay utilizing primers previously described by DebRoy et al. (2011) was effective at detecting non-O157 STEC (O145) from all sample types. Results from this study will be used for a future survey of STEC in beef processing facilities and resulting products. Additionally, this work, in combination with studies to improve cultural methods (Posse et al., 2008a; 2008b) may benefit the current work being conducted to create a standard method for the detection of non-O157 STEC in real-world samples.
References


Chapter 3

Survey of Beef Processing Plants and Resulting Ground Beef Products for the Presence of STEC

Abstract

Shiga toxin-producing Escherichia coli (STEC) are pathogens attributed to numerous foodborne illnesses resulting in gastrointestinal disease of varying severity, including hemolytic uremic syndrome (HUS) in humans. Cattle and consequently, beef products are considered a major source of STEC. E. coli O157:H7 has been regulated as an adulterant in ground beef since 1994. The USDA-Food Safety and Inspection Service has indicated that 6 additional STEC (O145, O121, O111, O103, O45, and O26) will be regulated as adulterants in beef trim and raw ground beef, beginning in June 2012. Little is known about the presence of STEC in small and very small beef processing plants. It is the goal of this research to determine if small and very small beef processing plants are a potential source of STEC. In this study, environmental swabs, carcass swabs, hide swabs, fecal samples, and ground beef from small and very small beef processing plants were obtained from October 2010 to December 2011 to determine the presence of STEC. A multiplex polymerase chain reaction assay was used to determine the presence of STEC O157, O145, O121, O113, O111, O103, O45, and O26 in the samples (DebRoy et al., 2011). Results demonstrated that 55.5% (151/272) of the environmental samples, 36.9% (75/203) of the carcass samples, 85.2% (23/27) of the hide samples, 37.5% (12/32) of the fecal, and 18.6% (22/118) of the ground beef samples tested positive for one or more of the serogroups.
However, only 7.7% (21/272) of the environmental samples, 5.9% (12/203) of the carcass samples, 0% (0/27) of the hide samples, 0% (0/32) of the fecal, and 0% (0/118) ground beef samples tested positive for one or more of the Shiga toxin genes. Based on this survey, small and very small beef processors may be a source of the 6 non-O157:H7 STEC. The information from this study may be of interest to regulatory officials, researchers, public health personnel, and the beef industry that are interested in the presence of these pathogens in the beef supply.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are Gram-negative, rod-shaped bacteria that have been implicated in several foodborne illness outbreaks. Symptoms of STEC infection include watery diarrhea, severe stomach cramping, and dehydration. In some instances, the disease can progress to hemorrhagic colitis (HC) or bloody stools. HC is caused by the destruction of the red blood cells in the intestine, resulting from infection of *E. coli* and release of the Shiga toxin into the host’s system (Liu, 2010). Up to 22% of HC patients develop hemolytic uremic syndrome (HUS) resulting from *E. coli* infection (Frank et al., 2011). HUS symptoms include exaggerated HC symptoms, in addition to vomiting, fever, weakness, decreased urine output, and kidney failure. In rare cases, neurological damage has occurred (Su, 1995). The mortality rate of HUS is 2-10% (Johnson et al., 1996). Ten percent (10%) of HUS survivors suffer chronic renal failure, while 40% suffer renal insufficiency (Johnson et al., 1996). Individuals most susceptible to STEC infection include young children, the elderly, and those with weakened immune systems (Liu, 2010).
Pathogenic *E. coli* (STEC)

At least 60 strains of STEC have been linked to human illness worldwide (Bettelheim, 2003). *E. coli* O157:H7 is the most commonly isolated pathogenic strain resulting in infection in the United States (CDC, 2011). *E. coli* O157:H7 has been recognized as a foodborne pathogen since 1982 (Riley et al., 1983) when it was isolated from individuals in several states and traced back to the consumption of undercooked ground beef. Since that time, the pathogen has been linked to several foodborne illness outbreaks, including a large outbreak in 1993 following the consumption of undercooked hamburgers, resulting in 477 illnesses and four deaths (CDC, 1993). In 1996, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) declared *E. coli* O157:H7 as an adulterant in ground beef under the Federal Meat Inspection Act (FMIA) (FSIS, 1999).

When compared to *E. coli* O157:H7 and other enteric pathogens, non-O157 STEC are not as frequently isolated and implicated in foodborne illness outbreaks. It is believed that this group of pathogens is largely under-accounted for, presumably due to the lack of effective laboratory screening and culture methods (Possé et al., 2008). However, these pathogens have been implicated in clinical cases of human disease and have been of increasing public health concern since the early 1990’s (USDA-FSIS, 2010). It has been estimated that *E. coli* O157:H7 strains cause two-thirds of all *E. coli* human infection cases in the United States, while non-O157 strains are responsible for the remaining cases (USDA-FSIS, 2011). A survey completed in the U.S. between the years of 1982-2002, reported a total of 940 non-O157 STEC infection cases resulting in illness (Liu, 2010). Between 2001 and 2003, the CDC reported a 284% increase of laboratory-confirmed infections due to non-O157 STEC, while a 27% decrease in laboratory-confirmed infections with O157 STEC occurred (USDA-FSIS, 2011).
A study completed by the CDC between 1983 and 2003 demonstrated as many as 14 different serogroups were implicated in human disease resulting from pathogenic *E. coli*, in addition to illnesses that resulted in undetermined serotypes (Brooks et al., 2005). However, the same study demonstrated that approximately 70% of the infections caused by non-O157 STEC infections, which could be serotyped, were attributed to only 6 serotypes: O145, O121, O111, O103, O45, and O26. These STEC have been identified by the CDC as the “Big 6” non-O157 STEC. Non-O157 STEC are believed to cause diarrhea at rates similar to other enteric pathogens and are capable of causing disease resulting in HUS (Brooks, 2005). The combination of these findings have prompted the USDA-FSIS to announce that six additional serogroups of STEC including O26, O45, O103, O111, O121, and O145 will be considered adulterants in ground beef and non-intact raw beef products, as outlined in the Federal Meat Inspection Act (FMIA), beginning in June of 2012.

**Description of STEC**

As indicated above, individual strains of pathogenic *E. coli* can be identified and classified based on serotype. Serotypes of pathogenic *E. coli* can be categorized into groups, based on characteristics, such as virulence. The group of most concern is enterohemorrhagic *E. coli* (EHEC), which are capable of causing diarrheagenic disease in humans. EHEC are recognized by their ability to attach to intestinal cells and cause diarrhea in humans. Shiga toxin-producing *E. coli* (STEC) are a subset of pathogens within this group that are recognized by their ability to produce Shiga-like toxins. Pathogenic *E. coli* also can be identified by serogroups, where the O-group antigens are used as a means of identification. For example, O157 is a serogroup in which serotype O157:H7 belongs (Liu, 2010). Serogroups O157, O145, O121, O111, O103, O45, and O26 are all identified as STEC.
STEC are recognized by their ability to produce Shiga-like toxins (Stx). Stx is a potent cytotoxin, also identified as verotoxin, due to the cytotoxic effect on cultured Vero cells (Johnson et al., 1996; Clark, 2001). Two types of Stx can be produced by *E. coli*: Stx1 and Stx2. Genes for Stx are located within the DNA of bacteriophage. When *E. coli* become infected with the bacteriophage, DNA becomes inserted into the genome of the host bacterium, allowing the production of Stx by *E. coli* (USDA-FSIS, 2010). Stx molecules consist of two subunits: A and B. The A subunit is responsible for enzymatic activity which allows for the destruction of the host cell’s rRNA, therefore preventing protein synthesis, resulting in apoptosis of the host cell. The B subunit allows for binding of the Stx molecule to a glycolipid receptor called Gb3. Binding to this receptor initiates internalization of the toxin molecule into the host cell, allowing the action of the A subunit to occur (Gyles, 2006).

Though Stx production is a critical virulence factor and indicative of pathogenicity, the ability to produce Shiga toxin alone does not ensure a pathogenic strain of *E. coli* (Wickham et al., 2006). Extensive analysis of surveillance data and genetic research has demonstrated that several combinations of virulence factors may be responsible for severe human illness. Virulence genes that have been associated with HUS resulting from STEC infection include; the locus of enterocyte effacement (LEE) gene, the intimin (*eae*) and intimin receptor (*tir*) genes, as well as the enterohemolysin (*hly*) gene.

**STEC in cattle, beef processing plants, and resulting beef products**

*E. coli* exists as a commensal organism in the gastrointestinal tract of animals, as well as humans. Many strains of *E. coli* are harmless to both humans and animals, but a subset (STEC, as discussed previously) is capable of causing serious illness in humans. While Shiga toxin can be pathogenic to humans, it has no negative effects on ruminant carriers of the pathogen, due to
the lack of vascular receptors for the toxin (Pruimboom-Brees, 2000). Therefore, ruminants and cattle in particular, are major reservoirs of STEC. Surveys have shown that the cattle may harbor more than 100 STEC serotypes, 60% of which have been associated with HC and HUS in humans (Johnson et al., 1996). Earlier studies detected STEC in 39.4-78.9% of fecal samples from cattle ranging in age from less than 2 months to adult (Shinagawa et al., 2000). This study also demonstrated that the prevalence of STEC in cattle depends on the season, with the highest rates of fecal shedding occurring during the warmer months. Studies also indicate that O157 STEC are shed in loads varying from 2 to 5 log_{10} CFU/gram of fecal matter, depending on the season (Shere et al., 1998; Zhao et al., 1995).

Since the first outbreak of *E. coli* O157:H7 in 1982 associated with ground beef, extensive research has focused on beef cattle as reservoirs of STEC. It has been shown that the most common source of meat contamination with STEC is soiling of carcasses and plant environments with fecal material during slaughter (Johnson et al., 1996). Surveys addressing the prevalence of STEC in cattle have indicated rates as high as 71% (Cerqueira et al., 1999). However, these data were collected with a major emphasis on O157:H7 identification, due to the importance placed on the specific pathogen at the time. A more broad survey of non-O157 STEC may have indicated even higher prevalence rates. More recent, but limited studies available for non-O157 STEC indicate a prevalence of 19.3% in feces, 57% on hides, 58.2% on pre-eviscerated carcasses, and 9% on post-intervention carcasses (Barkocy-Gallagher et al., 2003). This study indicated comparable prevalence rates of *E. coli* O157:H7 as well, which included 6% in feces, 61% on hides, 27% on pre-eviscerated carcasses, and 1.3% on post-intervention carcasses (Barkocy-Gallagher et al., 2003). This survey was completed at three large beef processing plants located in the Midwest United States.
To the best of our knowledge, a survey has not been completed to determine the presence of STEC in small and very small processing plants. However, 81% of all federally inspected establishments are classified as small and very small, though combined, they make up only approximately 15% of total national beef production (P&SP, 2012). The USDA classifies plants under federal regulations into size categories, based on the number of employees and the annual sales of a plant. Large plants are described as employing more than 500 workers, small plants as employing between 10 and 500 workers, and very small plants as employing 10 or fewer workers, or having annual sales of less than $2.5 million (USDA-FSIS, 1996).

Small and very small processors are much more prevalent in the northeastern United States, Pennsylvania in particular. It is not known what role, if any, STEC play when beef is processed in small and very small processing plants, or the impact that these new regulations will have on these establishments. All plants under federal inspection are expected to comply with regulatory requirements regardless of income or plant size, including the newly proposed regulations on non-O157 STEC. Given the size and income classifications of plants, it is clear that small and very small plants work with restricted labor and finances to meet the same regulations that large plants meet. Testing for non-O157 STEC has the potential to create a financial burden on these smaller businesses. A better understanding of the presence of the non-O157 STEC in small and very small beef processing plants will help researchers and regulatory personnel determine how best to regulate these facilities. By having data from these establishments, researchers and regulatory officials also can determine best practices or adequate interventions to prevent STEC contamination of the beef supply originating from small and very small processors.
Objective

The overall objective of this survey is to establish a baseline for the presence of non-O157 STEC in small and very small processing establishments and resulting beef products located in Pennsylvania by collecting and analyzing environmental, carcass, hide, fecal, and ground beef samples from these processors.
Materials and methods

Bacterial strains and culture conditions

*E. coli* reference strains belonging to serogroups O157, O145, O121, O113, O111, O103, O45, and O26 were used as positive controls, and *E. coli* DH5α as the negative control in this study. All strains were obtained from the *E. coli* Reference Center in the Department of Veterinary and Biomedical Sciences at the Pennsylvania State University (University Park, PA), listed in Table 1.

Bacteria were stored in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) containing 10% glycerol at -80°C. Prior to experiments, cultures were propagated twice in 10 mL TSB at 37°C for 18-24 hours and maintained on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI). For each survey, cultures of each control were streaked for isolation on Rainbow® Agar (Biolog, Hayward CA) modified with 10 mg/L novobiocin (USDA-FSIS, 2010) to use as a control for visual comparison to samples.
Table 1. Reference strains used as controls for O-type determination in culture and multiplex PCR assay.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Culture ID</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>ATCC 43895</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O145:K-:H-</td>
<td>E1385(3)</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O121</td>
<td>39W</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O113:H21</td>
<td>6182-50</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O111:H-</td>
<td>Stoke W</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O103:K+:H8</td>
<td>H515b</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O45</td>
<td>K61</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O26:H-</td>
<td>H311b</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>Negative Control</td>
<td>Invitrogen (Carlsbad, California)</td>
</tr>
</tbody>
</table>
**Experimental design**

Samples were collected from seven different small and very small beef processors throughout the state of Pennsylvania and one plant located in New Jersey, over a 14 month period, from October 2010 to December 2011. Samples collected at each processing plant included environmental swabs and carcass swabs. Representative environmental samples were collected at each processing plant including: lairage, chute/slaughter area, de-hiding machinery/cradles, hide knives, split saws, sinks, drains, viscera collection sites, head racks, and processing tables. Due to the small number of animals processed at the very small processing facilities, carcass samples were collected from each animal slaughtered on every day of collection. When possible, hide samples also were collected. Fecal samples were collected at one site only. Additionally, ground beef samples were collected from retail stores of some processors or from farmers’ markets throughout the state. Purchased ground beef samples were traced to ensure a small or very small processor origin, using USDA establishment numbers. Combined, a total of 652 samples were collected, including: 272 environmental swabs, 203 carcass swabs, 27 hide samples, 32 fecal samples, and 118 ground beef samples.

**Sample collection and enrichment**

**Environmental samples**

Representative beef processing environmental samples were collected from each processor in this study. All samples were collected using a Spongesicle® sampling swab (Biotrace International; Seattle, WA) moistened with 25 mL of sterile BPW, performed according to USDA-FSIS guidelines (USDA-FSIS, 2009). Briefly, sponges were wrung out and aseptically removed from the bag. Sampling was performed aseptically using sterile latex gloves changed between samples. For each environmental sample, approximately one square foot was swabbed. Swab
samples consisted of 10 passes vertically, 10 passes horizontally, and 10 passes diagonally. The swab was flipped midway through taking the sample. The samples were stored in the collection bag at 4°C until processing was possible. Upon arrival at the lab—within 6-8 hours of collection, samples were processed within 24 hours of collection. Swabs were stomached at 230 rpm for 2 minutes in the sample collection bag (Stomacher® 400 Circulator, Steward®, United Kingdom), wrung out, and the sample solution collected aseptically in 50 mL test tubes (VWR International, Radnor, PA). An aliquot of the sample (2 mL) was used for background aerobic and generic E. coli analysis.

Carcass samples

Beef carcass samples were collected from each processor in this study. All samples were collected using a 3M™ dry-sponge (3M™, St. Paul, MN) moistened with 25 mL of BPW, according to USDA-FSIS guidelines (USDA-FSIS, 1996). Briefly, sponges were wrung out, aseptically removed from the bag, and used to sample one half of the carcass using a sterile 100 cm² template (3M™, St. Paul, MN). Sampling was performed aseptically using sterile latex gloves changed between samples. For each carcass sample, 100 cm² was swabbed at three sites: flank, brisket, and rump. Swab samples consisted of 10 passes vertically and 10 passes horizontally. The swab was flipped midway through taking the sample. The samples were stored in the collection bag at 4°C until processing. Samples were processed within 24 hours of collection. Swabs were stomached at 230 rpm for 2 minutes in the sample collection bag, wrung out, and the sample solution collected aseptically in 50 mL test tubes. An aliquot of the sample (2 mL) was used for background aerobic and generic E. coli analysis.
**Hide samples**

Beef hide samples were collected from selected processors aseptically using sterile latex gloves changed between samples. All samples were collected with a 3M™ dry-sponge moistened with 25 mL of BPW, using the 3-site sampling method for carcass sample collection (USDA-FSIS, 1996). Briefly, sponges were wrung out, aseptically removed from the bag, and used to sample the animal after rendering it unconscious. A sterile 100 cm² template was used to swab three sites: flank, brisket and rump. Swab samples consisted of 10 passes vertically and 10 passes horizontally. The swab was flipped midway through taking the sample. The samples were stored in the collection bag at 4°C until processing. Samples were processed within 24 hours of collection. Swabs were stomached at 230 rpm for 2 minutes in the sample collection bag, wrung out, and the sample solution collected aseptically in 50 mL test tubes. An aliquot of the sample (2 mL) was used for background aerobic and generic *E. coli* analysis.

**Fecal samples**

Fecal samples were collected from another study completed at the Penn State Dairy Barns and frozen until processing. Samples were collected from cattle in a feeding study on the farm site, and not at the time of slaughter of the cattle. To process, ten grams of the thawed (4°C for 24 hrs) fecal sample were aseptically transferred into a Stomacher® 400 Circulator Standard Bag (Steward®, United Kingdom), diluted 1:9 in 90 mL of BPW, and stomached for 30 seconds at 230 rpm. The sample solution was then collected aseptically in 50 mL test tubes.

**Ground beef samples**

Ground beef samples were collected at the processing plant, retail store, or purchased at farmers’ markets throughout the state of Pennsylvania and held at 4°C until sampling. All
samples were processed within 24 hours of purchasing. Ground beef was sampled according to USDA guidelines for detection and isolation of non-O157 STEC from meat products (USDA-FSIS, 2010), with minor modifications. Briefly, ground beef packages were aseptically opened, 325 grams of ground beef were transferred aseptically to a filtered stomacher bag (Interscience, Rockland, MA), diluted 1:3 in 975 mL modified TSB (mTSB) containing 8 mg/L novobiocin and 16 mg/L vancomycin, and stomached for 2 minutes at 230 rpm. An aliquot of the sample was collected to perform background aerobic and generic E. coli analysis. The samples were then pre-enriched at 37°C for 6 hours. Following pre-enrichment, 2 mg/L rifampicin, 1.5g/L bile salts, and 1 mg/L potassium tellurite were added, and samples were enriched for an additional 18 hours at 42°C. Enriched samples were collected (10 mL) and stored at 4°C for up to 24 hours before STEC analysis was performed.

**Sample enrichment**

Environmental, carcass, hide, and fecal samples were enriched as follows. The collected stomachate of each sample was combined 1:4 with mTSB containing 8 mg/L novobiocin and 16 mg/L vancomycin, and pre-enriched at 37°C for 6 hours. Following pre-enrichment, 2 mg/L rifampicin, 1.5g/L bile salts, and 1 mg/L potassium tellurite were added, and samples were enriched for an additional 18 hours at 42°C. Enriched samples were collected (3 mL) and stored at 4°C for up to 24 hours before STEC analysis was performed.

**Evaluation of background microflora from environmental, carcass, hide, and ground beef samples**

Levels of aerobic plate counts (APC) and generic E. coli (EC) were evaluated in environmental, carcass, hide, and ground beef samples. Following stomaching as described
above, 1 mL of each sample was serially diluted 1:10 in 9 mL of sterile BPW. APC and EC of the samples were measured by plating a 1 mL aliquot of the serial dilutions onto APC and EC Petrifilm (3M™ Healthcare, St Paul, MN). The Petrifilm was incubated at 37°C for 24 hours, manually counted, and resulting populations were recorded. Resulting counts are shown in Table 9.

**DNA isolation**

DNA isolation of enrichments was performed within 24 hours of collection. For DNA isolation, all bacterial DNA was extracted using Epicentre Biotechnologies' MasterPure™ DNA Purification Kit (Madison, WI), according to manufacturer's instructions with minor modifications. Briefly, 1 mL of enrichment broth was concentrated by centrifugation for 5 minutes at 7,000 x g in a microcentrifuge (Galaxy 16, VWR International, Radnor, PA), and supernatant was discarded. The cell pellet was resuspended in 300 µl of tissue and cell lysis solution containing 1 µl of 50 µg/µl proteinase K. The cells were lysed by incubating at 65°C for 15 minutes with agitation every 5 minutes using a vortex (VWR International, Radnor, PA). Samples were cooled to 37°C for 10 minutes, 1 µl of 5 µg/µl RNase A added to the sample, mixed thoroughly, and incubated at 37°C for 30 minutes. Samples were then placed on ice for 5 minutes. MPC Protein Precipitation Reagent (175 µl) was added to the lysed sample and vortexed vigorously for 10 seconds. The cell debris were pelleted by centrifugation for 10 minutes at 10,000 x g in a microcentrifuge. The supernatant was transferred to a sterile microcentrifuge tube, 500 µl of isopropanol was added, and the tube inverted 30-40 times. The total nucleic acid was pelleted by centrifugation for 10 minutes in a microcentrifuge and the supernatant was carefully poured out without dislodging the pellet. The pellet was then rinsed twice with 70% ethanol and allowed to dry aseptically for 15-20 minutes. The pellet was then
resuspended in 100 µl of sterile deionized water. All DNA preparations were stored at -20°C until use.

**STEC analysis**

Following DNA isolation, samples were tested for the presence of 8 STEC O-groups (O157, O145, O121, O113, O111, O103, O45, and O26) using a multiplex PCR assay previously described by DebRoy et al. (2011) and shown in Figure 1. The PCR assay was performed using a Mastercycler pro thermocycler (Eppendorf, Westbury, NY). Thermocycler conditions are as follows: Initial denaturation of 95°C for 15 minutes, followed by 30 cycles of 94°C for 30 seconds, 61°C for 1.5 minutes, and 72°C for 1.5 minutes, followed by a final extension of 72°C for 10 minutes. Primer sequences and concentrations of this O-group multiplex PCR assay are provided in Table 2.

In addition, samples were tested for the presence of stx1, stx2, and eae genes, utilizing an additional multiplex PCR assay as described by Paton and Paton (2008), as shown in Figure 2. The PCR assay was performed using the Mastercycler pro thermocycler with the following conditions: Initial denaturation of 94°C for 4 minutes, followed by 30 cycles of 90°C for 45 seconds, 55°C for 30 seconds, and 72°C for 50 seconds, followed by a final extension at 72°C for 8 minutes. Primer sequences and concentrations are provided in Table 3.
Figure 1. Agarose gel of amplicons from the multiplex PCR assay of Shiga toxin-producing *Escherichia coli* (STEC) serogroups. Lane 1: Molecular weight ladder, 100 bp. Lane 2: No template control. Lane 3: 2-strain control. Lane 4: 4-strain control. Lane 5: 6-strain control. Lane 6: 8-strain control. Lane 7: STEC O157. Lane 8: STEC O145. Lane 9: STEC O121. Lane 10: STEC O113. Lane 11: STEC O111. Lane 12: STEC O103. Lane 13: STEC O45. Lane 14: STEC O26.
Table 2. O-group PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Primer Sequence (5'→3')</th>
<th>Target Gene</th>
<th>Primer Concentration (µM)</th>
<th>Amplicon Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>O157</td>
<td>TCG AGG TAC CTG AAT CTT TCC TTC TGT ACC AGT CTT GGT GCT GCT CTG ACA</td>
<td>wzx F</td>
<td>0.015</td>
<td>894</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O145</td>
<td>TTC ATT GTT TTG CTT GCT CG GGC AAG CTT TGG AAA TGA AA</td>
<td>wzx F</td>
<td>0.03</td>
<td>750</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O121</td>
<td>TCC AAC AAT TGG TCG TGA AA AG AAG TGT GAA ATG CCC GT</td>
<td>wzx F</td>
<td>0.029</td>
<td>628</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O113</td>
<td>TGC CAT AAT TCA GAG GGT GAC AAC AAA GCT AAT TGT GGC CG</td>
<td>wzx F</td>
<td>0.029</td>
<td>514</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O111</td>
<td>TGT TTC TTC GAT GTT GCG AG GCA AGG GAC ATA AGA AGC CA</td>
<td>wzx F</td>
<td>0.029</td>
<td>438</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O103</td>
<td>TTG GAG CGT TAA CTG GAC CT GCT CCC GAG CAC GTA TAA AG</td>
<td>wzx F</td>
<td>0.03</td>
<td>321</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O45</td>
<td>TGC AGT AAC CTG CAC GGG CG AGC AGG CAC AAC AGC CAC TAC T</td>
<td>wzx F</td>
<td>0.063</td>
<td>238</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O26</td>
<td>CAA TGG GCG GAA ATT TTA GA ATA ATT TTC TCT GCC GTC GC</td>
<td>wzx F</td>
<td>0.17</td>
<td>155</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Agarose gel of amplicons from the multiplex PCR assay for the virulence genes: stx1/stx2/eaе in Escherichia coli (STEC). Lane 1: Molecular weight ladder, 100 bp. Lane 2: No template control. Lane 3: eaе control (890bp). Lane 4: stx1 control (600 bp). Lane 5: stx2 control (255bp). Lane 6: 3-gene combination (eaе, stx1, stx2).
<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Primer Sequence (5'-3')</th>
<th>Primer Concentration (uM)</th>
<th>Amplicon Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx1</td>
<td>ACA CTG GAT GAT CTC AGT GG CTG AAT CCC CCT CCA TTA TG</td>
<td>0.5</td>
<td>600</td>
<td>Paton and Paton, 1998</td>
</tr>
<tr>
<td>stx2</td>
<td>GGC ACT GTC TG AAC TGC TCC TCG CCA GTT ATC TGA CAT TCT</td>
<td>0.5</td>
<td>255</td>
<td>Paton and Paton, 1998</td>
</tr>
<tr>
<td>eae</td>
<td>GTG GCG AAT CT GGC GAG ACT CCC CAT TCT TTT TCA CCG TCG</td>
<td>0.5</td>
<td>890</td>
<td>Paton and Paton, 1998</td>
</tr>
</tbody>
</table>
**Immunomagnetic separation for STEC isolation**

Immediately following identification of STEC O-group and virulence gene presence, any stored samples that tested positive for an O-group in the PCR reaction were subjected to immunomagnetic separation (IMS) to isolate STEC from the enrichment. The Dynabeads® Anti-\textit{E. coli} (Invitrogen, Grand Island, NY) protocol for all commercially available anti-serum available, including O157, O145, O111, O103, and O26 was used, with minor modifications. Briefly, 20 µl of the resuspended anti-\textit{E. coli} specific serogroup was transferred aseptically to a sterile 1.5 mL microcentrifuge tube (VWR International, Radnor, PA). A 1 mL aliquot of the enriched sample was aseptically added to the microcentrifuge tube and the tube inverted several times. The sample/anti-\textit{E. coli} suspension was incubated at room temperature for 10 minutes with gentle continuous agitation using the Dynal sample mixer (Dynal Biotech INC, Lake Success, NY). Microcentrifuge tubes were then transferred to a magnetic particle concentrator (Dynal Biotech INC, Lake Success, NY) and subjected to a magnetic strip. The rack of tubes was then inverted several times to concentrate the beads into a pellet on the side of the tube and allowed to stand for 3 minutes for maximum recovery of Dynabeads. The sample supernatant was aspirated off, while being careful not to disturb the beads collected along the side of the tube. The magnetic strip was removed from the rack and 1 mL of wash buffer (PBS Tween; VWR International, Radnor, PA) was added to the beads. The tube was inverted several times to resuspend the beads and the magnetic strip replaced. The rack of tubes was inverted to collect the beads along the side and remained undisturbed for 3 minutes for maximum recovery. This rinsing was repeated 2 more times and the remaining Dynabeads were resuspended in 100 µl of the wash buffer. Following a rinse, the 100 µl suspension was spread on Rainbow® Agar modified with 10 mg/L novobiocin (USDA-FSIS, 2010), and incubated for 18-24 hours at 37°C. Following incubation, 5-15 isolates were collected from each plate, grown in TSB for 18-24 hours, and -
analyzed for STEC O-group confirmation using the multiplex PCR assays. Results of STEC isolation are shown in Table A-5.

**Statistical analysis**

Data from STEC analyses were reported as percentages of samples testing positive for each of the tested pathogens and virulence genes, divided by the total samples taken. Differences of percentage positive samples for different O-groups within each sample type and plant identified were determined using a Proc GLM test with a Tukey separation of means (SAS Program, Cary, NC). Differences of percentage positive samples for each O-group between all sample types were determined using a Proc Mixed test with a LSMeans separation.

**Results**

**STEC prevalence in environmental samples**

A total of 272 environmental samples were collected and were representative of each plant including lairage, chute/slaughter area, de-hiding machinery/cradles, hide knives, split saws, sinks, drains, viscera collection sites, head racks, and processing tables. Overall, 151 (55.5%) of the 272 samples tested positive for one or more STEC O-groups. However, only 21 (7.7%) tested positive for stx1 and/or stx2, suggesting the presence of an STEC strain (Table 5). The STEC O-groups most commonly found in environmental samples include O157, O121, and O45. O-groups O145 and O111 were found in significantly fewer samples than the rest of the O-groups (Table 4).
STEC prevalence in environmental sample types

Results for STEC prevalence, from environmental samples are demonstrated in table A-1. Of the environmental samples taken at the small and very small processing plants, hose, saw, and ladder samples tested positive most often for O-group O157 (40%, 47%, and 47%, respectively). Samples from sink (8.3%), eviscera area (7.1%), hose (6.7%), drain (6.3%), and lairage (3.3%) tested positive for O-group O145 most often. Samples from saw (33%), drain (31%), ladder (27%), and dehider (26%) samples tested positive for O-group O121 most often. STEC O-group O113 was not found in a particular sample more often than any other. STEC O-group O111 was found in knife (6.7%), drain (6.3%), and lairage (3.3%) samples most often. STEC O-group O103 was not found in a particular sample more often than any other. STEC O-group O45 was found most often in dehider (37%) samples. Drain samples (31%) also tested positive for STEC O26 most often. The stx1 gene was not detected in a particular sample more often than any other. The stx2 and eae genes (20% and 33%, respectively) were detected most often in hose samples.

Results for STEC prevalence within each sample type are demonstrated in table A-2. In the sink, drain, offal barrel, lairage, eviscera area, hook, floor/wall, and knife samples, no STEC O-group was detected more often than any other. In chute samples, STEC O-groups O45 (29%) and O157 (26%) were detected most often. In dehider samples, STEC O-group O45 (37%) was detected most often. In ladder, saw, and hose samples, STEC O-group O157 (40%, 47%, and 47%, respectively) was detected most often.

STEC prevalence in carcass samples

A total of 203 carcass samples were collected. Overall, 75 (36.9%) of the 203 samples tested positive for one or more STEC O-groups. However, only 8 (3.9%) tested positive for stx1
and/or stx2 (Table 5). The STEC O-groups most commonly found in carcass samples included O157, O121, O103, and O45. O-groups O145, O113, and O111 were found in significantly fewer samples than the rest of the O-groups (Table 4).

STEC prevalence in hide samples

A total of 27 hide samples were collected. Overall, 23 (85.2%) of the 27 samples tested positive for one or more STEC O-groups. However, no samples tested positive for stx1 and/or stx2 (Table 5). The STEC O-groups most commonly found in hide samples included O121, O103, O45, and O26. O-groups O145, O113, and O111 were found in significantly fewer samples than the rest of the O-groups (Table 4).

STEC prevalence in ground beef samples

A total of 118 ground beef samples were collected. Overall, 22 (18.6%) of the 118 samples tested positive for one or more STEC O-groups. However, no samples tested positive for stx1 and/or stx2 (Table 5). The STEC O-groups most commonly found in ground beef samples included O157 and O103, though they were not significantly different than any other O-groups (Table 4).

STEC prevalence in fecal samples

A total of 32 fecal samples were collected. Overall, 12 (37.5%) of the 32 samples tested positive for one or more STEC O-groups. However, no samples tested positive for stx1 and/or stx2 (Table 5). The STEC O-groups most commonly found in fecal samples included O157, O103, and O26 (Table 4). It is important to note, tested fecal samples had been frozen for an extended
period of time prior to analysis, and no culture positive isolates were obtained from these samples.

**STEC prevalence in all tested samples**

Overall, 283 (43.4%) of the total 652 samples tested positive for one or more STEC O-group. Only 32 (4.9%) of the total samples tested positive for stx1 and/or stx2, indicating potentially pathogenic strains of *E. coli* (Table 5). The STEC O-groups most commonly found in beef processing plant and product samples include O157 and O45. O121 and O103 were the second most prevalent STEC O-groups found in the samples, followed by O26. STEC O145, O113, and O111 were found in significantly fewer samples than the rest of the O-groups (Table 4).

**STEC prevalence tested in individual beef processing plants**

A total of 8 different processing plants were sampled. In Plant 1, 62 (41.4%) of 151 samples tested positive for one or more STEC O-group, with 4 (2.6%) of the samples testing positive for stx2 (Table 7). STEC O-groups O157 and O121 were the most prevalent serogroups in Plant 1 (Table 6). In Plant 2, 35 (39.8%) of 88 samples tested positive for one or more STEC O-group, with 1 (1.1%) of the samples testing positive for stx2 (Table 7). STEC O-groups O103, O45, and O26 were the most prevalent serogroups in Plant 2 (Table 6). In Plant 3, 9 (23.1%) of 39 samples tested positive for one or more STEC O-group, with 2 (5.1%) of the samples testing positive for stx2 (Table 7). STEC O-group O157 was the most prevalent serogroup in Plant 3 (Table 6). In Plant 4, 37 (46.8%) of 79 samples tested positive for one or more STEC O-group, with 8 (10.1%) of the samples testing positive for stx2 (Table 7). STEC O-groups O157, O45, and O103 were the most prevalent serogroups in Plant 4 (Table 6). In Plant 5, 17 (68.0%) of 25 samples tested positive for one or more STEC O-group, with 7 (28.0%) of the samples testing
positive for stx1 and/or stx2 (Table 7). STEC O-groups O45 and O26 were the most prevalent serogroups in Plant 5 (Table 6). In Plant 6, 38 (74.5%) of 51 samples tested positive for one or more STEC O-group, with 4 (7.8%) of the samples testing positive for stx2 (Table 7). STEC O-group O121 was the most prevalent serogroup in Plant 6 (Table 6). In Plant 7, 22 (88.0%) of 25 samples tested positive for one or more STEC O-group, with 1 (4.0%) of the samples testing positive for stx2 (Table 7). STEC O-group O103 was the most prevalent serogroup in Plant 7 (Table 6). In Plant 8, 29 (65.9%) of 44 samples tested positive for one or more STEC O-group, with 5 (11.4%) of the samples testing positive for stx1 (Table 7). STEC O-group O157 was the most prevalent serogroup in Plant 8 (Table 6).

**STEC O-groups detected within sample types**

Statistically, STEC O-groups O157 and O113 were found more commonly in environmental samples, while STEC O-groups O121, O103, O45, and O26 were found primarily in hide samples (Table 8). STEC O145 and O111 were found in significantly fewer samples and were not found in a specific sample type. The stx2 gene was found more commonly than stx1 and was most commonly detected in carcass and environmental samples (Table 5).

**Seasonal STEC variance in all sample types**

Results for seasonal variance of STEC in environmental samples are provided in table A-3. All STEC O-groups were detected most often in the fall season. However, samples testing positive for stx2 and eae genes were collected most often in the summer season. In carcass samples, all STEC O-groups were detected most often in the fall and summer months. No differences were seen in seasonal variance of virulence genes in carcass samples. Hide samples were only collected in the summer and fall seasons. STEC O-groups O157, O121, O103, and O45
were found most often in the fall season, while O113 was found most often in the summer season. No differences were seen in seasonal variance of virulence genes in hide samples.

Ground beef samples were collected in the summer, fall, and winter seasons only. No variations in STEC O-groups or virulence genes were observed in ground beef samples, except STEC O103, which was detected in the fall months most often.

Overall, seasonal variance was not observed in STEC O-groups O145, O113, and O111. STEC O157 was detected most often in the summer and fall seasons, while O121, O103, O45, and O26 were detected most often in the fall season. No seasonal variance was observed in the detection of the stx1 virulence gene, while stx2 and eae were detected most often in the summer season. Results are shown in Table A-4.

**APC and EC results**

Environmental and hide swabs had the highest average aerobic plate counts (APC), at approximately $7 \log_{10}$ CFU. Both carcass swabs and ground beef samples had average APC levels detected at approximately $5 \log_{10}$ CFU. Additionally, environmental and hide swabs also had the highest average generic *E. coli* (EC) counts, at approximately $4 \log_{10}$ CFU. Carcass swabs had average EC counts detected at $2 \log_{10}$ CFU per mL, and ground beef samples were undetectable on Petrifilm.
Table 4. Presence and percentages of positive samples for STEC O-groups found in beef processing environments and resulting ground beef products.

<table>
<thead>
<tr>
<th>Sample type</th>
<th># Samples</th>
<th>O157</th>
<th>O145</th>
<th>O121</th>
<th>O113</th>
<th>O111</th>
<th>O103</th>
<th>O45</th>
<th>O26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental</td>
<td>272</td>
<td>66</td>
<td>6</td>
<td>49</td>
<td>17</td>
<td>3</td>
<td>41</td>
<td>64</td>
<td>37</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>24^A</td>
<td>2.2^B</td>
<td>18^AB</td>
<td>6.3^CD</td>
<td>1.1^D</td>
<td>15^B</td>
<td>24^A</td>
<td>14^BC</td>
</tr>
<tr>
<td>Carcass</td>
<td>203</td>
<td>36</td>
<td>3</td>
<td>22</td>
<td>4</td>
<td>0</td>
<td>24</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>18^A</td>
<td>1.5^C</td>
<td>11^AB</td>
<td>2.0^C</td>
<td>0.0^C</td>
<td>12^AB</td>
<td>14^A</td>
<td>4.9^BC</td>
</tr>
<tr>
<td>Hide</td>
<td>27</td>
<td>6</td>
<td>0</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>10</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>22^BC</td>
<td>0.0^A</td>
<td>44^AB</td>
<td>3.7^C</td>
<td>0.0^C</td>
<td>37^AB</td>
<td>56^A</td>
<td>37^AB</td>
</tr>
<tr>
<td>Ground Beef</td>
<td>118</td>
<td>6</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>5.1^A</td>
<td>0.0^A</td>
<td>3.4^A</td>
<td>3.4^A</td>
<td>0.0^A</td>
<td>4.2^A</td>
<td>3.4^A</td>
<td>1.7^A</td>
</tr>
<tr>
<td>Fecal</td>
<td>32</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>22^A</td>
<td>0.0^B</td>
<td>0.0^B</td>
<td>3.1^B</td>
<td>0.0^B</td>
<td>13^AB</td>
<td>3.1^B</td>
<td>6.3^AB</td>
</tr>
<tr>
<td>Total</td>
<td>652</td>
<td>123</td>
<td>9</td>
<td>87</td>
<td>26</td>
<td>3</td>
<td>84</td>
<td>114</td>
<td>60</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>19^A</td>
<td>1.4^B</td>
<td>13^BC</td>
<td>4.0^D</td>
<td>0.5^D</td>
<td>13^BC</td>
<td>18^AB</td>
<td>9.2^C</td>
</tr>
</tbody>
</table>

*Percentages within each sample type bearing a common letter are not significantly different (P >0.05).*
Table 5. Presence and percentages of positive samples for STEC virulence genes (stx1, stx2, and eae) found in small and very small beef processing environments and resulting ground beef products.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th># Samples</th>
<th># Samples Pos. for at least 1 O-Group</th>
<th>stx 1</th>
<th>stx 2</th>
<th>eae⁴</th>
<th>1/eae⁴</th>
<th>2/eae⁵</th>
<th>1 &amp; 2⁶</th>
<th>1/2/eae⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental</td>
<td>272</td>
<td>151</td>
<td>4</td>
<td>18</td>
<td>37</td>
<td>2</td>
<td>9</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>% Positive</td>
<td>56</td>
<td>1.5⁵</td>
<td>6.6⁵</td>
<td>14⁵</td>
<td>0.7⁵</td>
<td>3.3⁵</td>
<td>0.4⁵</td>
<td>0.4⁵</td>
<td></td>
</tr>
<tr>
<td>Carcass</td>
<td>203</td>
<td>75</td>
<td>0</td>
<td>8</td>
<td>16</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>% Positive</td>
<td>37⁴</td>
<td>0.0⁸</td>
<td>3.9⁸</td>
<td>7.9⁸</td>
<td>0.0⁸</td>
<td>1.5⁸</td>
<td>0.5⁸</td>
<td>0.5⁸</td>
<td></td>
</tr>
<tr>
<td>Hide</td>
<td>27</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td>85⁴</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td></td>
</tr>
<tr>
<td>Ground Beef</td>
<td>118</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td>19⁴</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.8⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td></td>
</tr>
<tr>
<td>Fecal</td>
<td>32</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td>38⁴</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td>0.0⁸</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>652</td>
<td>283</td>
<td>8</td>
<td>26</td>
<td>54</td>
<td>2</td>
<td>12</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>% Positive</td>
<td>43⁴</td>
<td>1.2⁸</td>
<td>4.0⁸</td>
<td>8.3⁸</td>
<td>0.3⁸</td>
<td>1.8⁸</td>
<td>0.3⁸</td>
<td>0.3⁸</td>
<td></td>
</tr>
</tbody>
</table>

*Percentages within each sample type bearing a common letter are not significantly different (P >0.05).

1 Samples positive for Shiga toxin 1 gene
2 Samples positive for Shiga toxin 2 gene
3 Samples positive for intimin (eae) gene
4 Samples positive for Shiga toxin 1 and eae genes
5 Samples positive for Shiga toxin 2 and eae genes
6 Samples positive for Shiga toxins 1 and 2 genes
7 Samples positive for Shiga toxins 1, 2, and eae genes
Table 6. Presence and percentages of positive samples for STEC O-groups found in small and very small beef processing plants.

<table>
<thead>
<tr>
<th>Plant ID</th>
<th># Samples</th>
<th>O157</th>
<th>O145</th>
<th>O121</th>
<th>O113</th>
<th>O111</th>
<th>O103</th>
<th>O45</th>
<th>O26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant 1</td>
<td>151</td>
<td>40</td>
<td>0</td>
<td>33</td>
<td>8</td>
<td>0</td>
<td>4</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>27\textsuperscript{A}</td>
<td>0.0\textsuperscript{c}</td>
<td>22\textsuperscript{A}</td>
<td>5.3\textsuperscript{bc}</td>
<td>0.0\textsuperscript{c}</td>
<td>2.6\textsuperscript{bc}</td>
<td>6.6\textsuperscript{bc}</td>
<td>9.9\textsuperscript{b}</td>
</tr>
<tr>
<td>Plant 2</td>
<td>88</td>
<td>10</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>18</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>11\textsuperscript{Ab}</td>
<td>2.3\textsuperscript{b}</td>
<td>3.4\textsuperscript{b}</td>
<td>3.4\textsuperscript{b}</td>
<td>0.0\textsuperscript{c}</td>
<td>21\textsuperscript{A}</td>
<td>24\textsuperscript{A}</td>
<td>22\textsuperscript{A}</td>
</tr>
<tr>
<td>Plant 3</td>
<td>39</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>15\textsuperscript{A}</td>
<td>5.1\textsuperscript{ab}</td>
<td>2.6\textsuperscript{ab}</td>
<td>2.6\textsuperscript{ab}</td>
<td>0.0\textsuperscript{b}</td>
<td>0.0\textsuperscript{c}</td>
<td>5.1\textsuperscript{ab}</td>
<td>5.1\textsuperscript{ab}</td>
</tr>
<tr>
<td>Plant 4</td>
<td>79</td>
<td>24</td>
<td>0</td>
<td>6</td>
<td>3</td>
<td>0</td>
<td>13</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>30\textsuperscript{A}</td>
<td>0.0\textsuperscript{c}</td>
<td>7.6\textsuperscript{bc}</td>
<td>3.8\textsuperscript{bc}</td>
<td>0.0\textsuperscript{c}</td>
<td>17\textsuperscript{ab}</td>
<td>32\textsuperscript{A}</td>
<td>1.3\textsuperscript{c}</td>
</tr>
<tr>
<td>Plant 5</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>0.0\textsuperscript{c}</td>
<td>0.0\textsuperscript{c}</td>
<td>20\textsuperscript{bc}</td>
<td>4.0\textsuperscript{c}</td>
<td>0.0\textsuperscript{c}</td>
<td>8.0\textsuperscript{c}</td>
<td>60\textsuperscript{a}</td>
<td>40\textsuperscript{ab}</td>
</tr>
<tr>
<td>Plant 6</td>
<td>51</td>
<td>5</td>
<td>0</td>
<td>32</td>
<td>4</td>
<td>0</td>
<td>7</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>9.8\textsuperscript{c}</td>
<td>0.0\textsuperscript{c}</td>
<td>63\textsuperscript{a}</td>
<td>7.8\textsuperscript{c}</td>
<td>0.0\textsuperscript{c}</td>
<td>14\textsuperscript{c}</td>
<td>39\textsuperscript{b}</td>
<td>9.8\textsuperscript{c}</td>
</tr>
<tr>
<td>Plant 7</td>
<td>25</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>19</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>0.0\textsuperscript{c}</td>
<td>4.0\textsuperscript{c}</td>
<td>4.0\textsuperscript{c}</td>
<td>4.0\textsuperscript{c}</td>
<td>76\textsuperscript{a}</td>
<td>44\textsuperscript{b}</td>
<td>0.0\textsuperscript{c}</td>
<td></td>
</tr>
<tr>
<td>Plant 8</td>
<td>44</td>
<td>26</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>12</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>59\textsuperscript{A}</td>
<td>9.1\textsuperscript{bc}</td>
<td>2.3\textsuperscript{c}</td>
<td>0.0\textsuperscript{c}</td>
<td>4.5\textsuperscript{c}</td>
<td>27\textsuperscript{b}</td>
<td>14\textsuperscript{bc}</td>
<td>9.1\textsuperscript{bc}</td>
</tr>
<tr>
<td>Total</td>
<td>502</td>
<td>111</td>
<td>9</td>
<td>82</td>
<td>21</td>
<td>3</td>
<td>75</td>
<td>110</td>
<td>56</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>22\textsuperscript{A}</td>
<td>1.8\textsuperscript{c}</td>
<td>16\textsuperscript{ab}</td>
<td>4.2\textsuperscript{c}</td>
<td>0.6\textsuperscript{c}</td>
<td>15\textsuperscript{c}</td>
<td>22\textsuperscript{A}</td>
<td>11\textsuperscript{b}</td>
</tr>
</tbody>
</table>

*Percentages within each plant identification bearing a common letter are not significantly different (P >0.05).*
Table 7. Presence and percentages of positive samples for STEC virulence genes (stx1, stx2, and eae) found in small and very small beef processing plants.

<table>
<thead>
<tr>
<th>Plant ID</th>
<th># Samples</th>
<th># Samples Pos. for at least 1 O-Group</th>
<th>stx1</th>
<th>stx2</th>
<th>eae</th>
<th>1/eae</th>
<th>2/eae</th>
<th>1 &amp; 2</th>
<th>1/2/eae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant 1</td>
<td>151</td>
<td>62</td>
<td>0</td>
<td>4</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Positive</td>
<td>41(^A)</td>
<td>0.0(^c)</td>
<td>2.6(^BC)</td>
<td>7.9(^b)</td>
<td>0.0(^c)</td>
<td>1.3(^BC)</td>
<td>0.0(^c)</td>
<td>0.0(^c)</td>
<td>0.0(^c)</td>
</tr>
<tr>
<td>Plant 2</td>
<td>88</td>
<td>35</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Positive</td>
<td>40(^A)</td>
<td>0.0(^b)</td>
<td>1.1(^b)</td>
<td>5.7(^b)</td>
<td>0.0(^b)</td>
<td>0.0(^b)</td>
<td>0.0(^b)</td>
<td>0.0(^b)</td>
<td></td>
</tr>
<tr>
<td>Plant 3</td>
<td>39</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Positive</td>
<td>23(^A)</td>
<td>0.0(^b)</td>
<td>5.1(^b)</td>
<td>10(^A)</td>
<td>0.0(^b)</td>
<td>5.1(^b)</td>
<td>0.0(^b)</td>
<td>0.0(^b)</td>
<td></td>
</tr>
<tr>
<td>Plant 4</td>
<td>79</td>
<td>37</td>
<td>0</td>
<td>8</td>
<td>13</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Positive</td>
<td>47(^A)</td>
<td>0.0(^c)</td>
<td>10(^BC)</td>
<td>17(^B)</td>
<td>0.0(^c)</td>
<td>8.9(^BC)</td>
<td>0.0(^c)</td>
<td>0.0(^c)</td>
<td></td>
</tr>
<tr>
<td>Plant 5</td>
<td>25</td>
<td>17</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>% Positive</td>
<td>68(^A)</td>
<td>12(^b)</td>
<td>24(^b)</td>
<td>24(^b)</td>
<td>8.0(^b)</td>
<td>4.0(^b)</td>
<td>8.0(^b)</td>
<td>8.0(^b)</td>
<td></td>
</tr>
<tr>
<td>Plant 6</td>
<td>51</td>
<td>38</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Positive</td>
<td>75(^A)</td>
<td>0.0(^b)</td>
<td>7.8(^b)</td>
<td>0.0(^b)</td>
<td>0.0(^b)</td>
<td>0.0(^b)</td>
<td>0.0(^b)</td>
<td>0.0(^b)</td>
<td></td>
</tr>
<tr>
<td>Plant 7</td>
<td>25</td>
<td>22</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Positive</td>
<td>88(^A)</td>
<td>0.0(^c)</td>
<td>4.0(^RC)</td>
<td>20(^B)</td>
<td>0.0(^c)</td>
<td>0.0(^c)</td>
<td>0.0(^c)</td>
<td>0.0(^c)</td>
<td></td>
</tr>
<tr>
<td>Plant 8</td>
<td>44</td>
<td>29</td>
<td>5</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Positive</td>
<td>66(^A)</td>
<td>11(^b)</td>
<td>0.0(^c)</td>
<td>18(^b)</td>
<td>4.5(^RC)</td>
<td>0.0(^c)</td>
<td>0.0(^c)</td>
<td>0.0(^c)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>502</td>
<td>249</td>
<td>8</td>
<td>26</td>
<td>53</td>
<td>2</td>
<td>12</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>% Positive</td>
<td>50(^A)</td>
<td>1.6(^CD)</td>
<td>5.2(^b)</td>
<td>11(^b)</td>
<td>0.4(^CD)</td>
<td>2.4(^CD)</td>
<td>0.4(^b)</td>
<td>0.4(^CD)</td>
<td></td>
</tr>
</tbody>
</table>

*Percentages within each plant identification bearing a common letter are not significantly different (P >0.05).
Table 8. Presence and percentages of positive samples for STEC O-groups and virulence genes (*stx*1, *stx*2, and *eae*) found in small and very small beef processing environments and resulting ground beef products.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th># Samples</th>
<th>O157</th>
<th>O145</th>
<th>O121</th>
<th>O113</th>
<th>O111</th>
<th>O103</th>
<th>O45</th>
<th>O26</th>
<th>stx 1</th>
<th>stx 2</th>
<th>eae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental</td>
<td>272</td>
<td>68</td>
<td>6</td>
<td>49</td>
<td>17</td>
<td>3</td>
<td>41</td>
<td>65</td>
<td>37</td>
<td>4</td>
<td>18</td>
<td>37</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>25^A</td>
<td>2.2^A</td>
<td>18^A</td>
<td>6.3^A</td>
<td>1.1^A</td>
<td>15^A</td>
<td>24^A</td>
<td>14^A</td>
<td>14^A</td>
<td>14^A</td>
<td>6.2^A</td>
</tr>
<tr>
<td>Carcass</td>
<td>203</td>
<td>36</td>
<td>3</td>
<td>22</td>
<td>4</td>
<td>0</td>
<td>24</td>
<td>28</td>
<td>10</td>
<td>4</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>18^B</td>
<td>1.5^A</td>
<td>11^C</td>
<td>2.0^B</td>
<td>0.0^A</td>
<td>12^B</td>
<td>14^A</td>
<td>4.9^C</td>
<td>14^A</td>
<td>29^A</td>
<td>57^A</td>
</tr>
<tr>
<td>Hide</td>
<td>27</td>
<td>6</td>
<td>0</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>10</td>
<td>15</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>22^AB</td>
<td>0.0^A</td>
<td>44^A</td>
<td>3.7^AB</td>
<td>0.0^A</td>
<td>37^A</td>
<td>56^A</td>
<td>37^A</td>
<td>0.0^A</td>
<td>0.0^B</td>
<td>0.0^C</td>
</tr>
<tr>
<td>Ground Beef</td>
<td>118</td>
<td>6</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>5.1^C</td>
<td>0.0^A</td>
<td>3.4^C</td>
<td>3.4^AB</td>
<td>0.0^A</td>
<td>4.2^C</td>
<td>3.4^D</td>
<td>1.7^C</td>
<td>0.0^A</td>
<td>25^AB</td>
<td></td>
</tr>
<tr>
<td>Fecal</td>
<td>32</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>22^AB</td>
<td>0.0^A</td>
<td>0.0^—</td>
<td>3.1^AB</td>
<td>0.0^A</td>
<td>13^UL</td>
<td>3.1^UL</td>
<td>6.3^UL</td>
<td>0.0^A</td>
<td>0.0^B</td>
<td>0.0^C</td>
</tr>
</tbody>
</table>

*Percentages within each O-group or virulence gene identification bearing a common letter are not significantly different (P >0.05).*
Table 9. Average aerobic plate counts (APC) and *E. coli* counts (EC) for Environmental, carcass, hide, and ground beef samples.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sample Size</th>
<th>APC (log$_{10}$ CFU)</th>
<th>E. coli (log$_{10}$ CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental swab</td>
<td>1 ft$^2$/25 mL</td>
<td>7.1</td>
<td>4.52</td>
</tr>
<tr>
<td>Carcass swab</td>
<td>100 cm$^2$/25 mL</td>
<td>5.41</td>
<td>2.28</td>
</tr>
<tr>
<td>Hide swab</td>
<td>100 cm$^2$/25 mL</td>
<td>7.48</td>
<td>4.31</td>
</tr>
<tr>
<td>Ground beef</td>
<td>325 g</td>
<td>5.53</td>
<td>&lt; 1*</td>
</tr>
</tbody>
</table>

* < 1 is below the detection limit of the Petrifilm.
Discussion

Foodborne illnesses have been attributed to STEC (O157 and non-O157) since the early 1980s. Non-O157 STEC have been under-reported due to the inability of laboratories to identify and characterize these pathogens (Bettelheim, 2007). However, recent improved detection methods have indicated a prevalence of non-O157 STEC to be similar to that of O157 STEC (Barkocy-Gallahger et al., 2003; Brooks et al., 2001; Samadpour et al., 1994). An increasing concern over non-O157 STEC contamination of food, beef products in particular, has led to proposed regulations for 6 non-O157 STEC in raw, ground and non-intact beef products beginning in June of 2012. A study completed in large Midwestern beef processing plants indicated non-O157 STEC are prevalent in the processing plant environment (Barkocy-Gallahger et al., 2003). To the best of our knowledge, no studies have been conducted to indicate the presence of non-O157 STEC in small or very small processing plant environments. The current study utilized previously developed molecular detection methods to detect the presence of non-O157 STEC in small and very small beef processing plants in the state of Pennsylvania.

In this study, all sample types (environmental, carcass, hide, fecal, and ground beef) tested positive for STEC O-groups and in all plants surveyed. Of all sample types, environmental and hide samples tested positive more frequently and supports previous research in this area (Ransom et al., 2002; Barkocy-Gallahger et al., 2003). Sample sizes between sample types were not equal, with fecal and hide sample sizes considerably smaller than environmental, carcass and ground beef sample sizes. A more even sample size of each sample type may have indicated fecal samples to be similarly contaminated with STEC O-groups as environmental and hide samples, had samples been collected to represent seasonal variations (Barkocy-Gallahger et al., 2003; Shere et al., 1998; Zhao et al., 1995). When considering all samples, STEC O-groups O157,
O45, O121, and O103 were most prevalent and at similar rates. While STEC O-groups were prevalent in beef processing environments and resulting beef products, samples testing positive for a Shiga toxin gene were significantly lower, indicating that, while *E. coli* O-groups designated as adulterants may be present in small and very small beef processing environments, STEC strains are less so.

Within individual small and very small beef processing plants surveyed, some STEC O-groups were more prevalent in some plants than in others. This observation indicates that some processing plants may have specific pathogens within their environments, which has been reported previously (Small et al., 2002). Additionally, STEC O-groups most prevalent in some processing plants were non-O157 STEC, indicating that these 6 additional non-O157 STEC may be present within these small and very small processing environments. Due to availability and location of some plants, sample sizes among processing plants are not equal, which may have resulted in a more broad seasonal survey of some processing plants than others. Two of the eight processing plants were sampled only once, so conclusions cannot be drawn with regard to seasonality within these plants.

Further surveys of non-O157 STEC within small and very small beef processing plants that focus on more specific areas in the beef processing environment may result in a better understanding of the path that these pathogens take to reach the final product and may help researchers determine effective methods of controlling these pathogens through interventions. Additionally, survey work looking more specifically at beef trim used to process ground beef, with a larger sample size, may indicate the prevalence of these STEC in raw ground beef products processed and sold from small and very small beef processing plants. Overall, results from this study could help researchers and regulatory personnel determine how best to regulate these small and very small facilities. By having data from these plants, researchers and
regulatory officials also have an established baseline for the presence of non-O157 STEC in small and very small processing establishments and resulting beef products located in Pennsylvania, which can be a starting point to help determine best practices or adequate interventions to prevent STEC contamination of the beef supply originating from them.
References


Chapter 4

A Survey of the Presence of STEC on Cattle, Hog, and Sheep Hides Using Three Different Sampling Methods

Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are well-known foodborne pathogens that exist as commensal organisms in the gastrointestinal tract of animals, cattle in particular. STEC are known to be shed in fecal matter of ruminant animals, and as a result, can contaminate animal hides in feedlots, during transport and in lairage areas at slaughter facilities. Small and very small processing plants often house and process several animal species in the same areas, which can facilitate the transfer of foodborne pathogens, such as STEC, onto different species’ hides. This study evaluated the effectiveness of three different sampling methods (sponge swab, hide clipping, and a novel M-Vac sampling method (Microbial Vac Systems, Inc., Bluffdale, Utah)) for detecting STEC on cattle, swine, and sheep hides at one abattoir. Samples were assayed for eight STEC serogroups, (O157, O145, O121, O113, O111, O103, O45, and O26) using a multiplex PCR assay (DebRoy et al., 2011). The presence of Shiga toxin genes in samples were identified using an additional multiplex PCR assay (Paton and Paton, 1998). Results demonstrated that 92% (24/26) of cattle hides tested positive for one or more STEC O-groups using the sponge swab and hair clipping methods, while 88% (23/26) of cattle hides tested positive for one or more STEC O-groups using the M-Vac sampling method. Swine hides tested positive for one or more STEC O-group in 93% (28/30) of samples collected with the sponge swab method, as compared with 80% (24/30) of samples collected using the hair clipping method, while 97% (29/30) of samples collected were positive for the STEC O-group using the M-Vac sampling method. Sheep hides were positive for one or more STEC O-group in all (11/11) of the samples
collected with the sponge swab and M-Vac methods, while 82% (9/11) of samples collected were positive with the hair clipping method. However, only 3.8% (1/26) of cattle hides, 40% (12/30) of swine hides, and 27.3% (3/11) of sheep hides tested positive for \textit{stx1} and/or \textit{stx2} genes, when all sampling methods were considered. Results from this study may serve as an indication of the presence of STEC in small slaughter facilities that process multiple species and may be useful to regulatory officials, researchers, and public health personnel who are interested in the presence of these pathogens in the meat supply.

\textbf{Introduction}

\textit{Escherichia coli} as a foodborne pathogen

Shiga toxin-producing \textit{Escherichia coli} (STEC) are Gram-negative, rod-shaped bacteria that have been implicated in several foodborne illness outbreaks. Symptoms of STEC infection include watery diarrhea, severe stomach cramping, and dehydration progressing to hemorrhagic colitis (HC), (bloody stools; Liu, 2010). Up to 22% of HC patients develop hemolytic uremic syndrome (HUS) resulting from \textit{E. coli} infection (Frank et al., 2011). HUS symptoms include exaggerated HC symptoms in addition to vomiting, fever, weakness, decreased urine output, and kidney failure. In rare cases, neurological damage has occurred (Su, 1995). The mortality rate of HUS is 2-10\% (Johnson et al., 1996). Individuals most susceptible to STEC infection include young children, the elderly, and those with weakened immune systems (Liu, 2010).

STEC are recognized by their ability to produce Shiga-like toxins (Stx). Stx is a potent cytotoxin, also identified as verotoxin, as evidenced by the cytotoxic effect on cultured Vero cells (Johnson et al., 1996; Clarke, 2001). HC is caused by the destruction of the red blood cells in the intestine, resulting from infection of \textit{E. coli} and release of the Stx into the host’s system (Liu,
Though Stx production is a critical virulence factor and indicative of pathogenicity, the ability to produce Stx alone does not ensure a pathogenic strain of *E. coli* (Wickham et al., 2006). Extensive analysis of surveillance data and genetic research has demonstrated that several combinations of virulence factors may be responsible for human illness. Virulence genes that have been associated with HUS resulting from STEC infection include: the locus of enterocyte effacement (LEE) gene, the intimin (*eae*) and intimin receptor (*tir*) genes, as well as the enterohemolysin (*hly*) gene.

At least sixty of the more than two hundred known strains of STEC have been linked to human illness worldwide (Bettelheim, 2003). The Centre for Disease Control and Prevention (CDC) has estimated approximately 265,000 STEC infections occur in the United States annually. The most well-known STEC, O157:H7, is believed to cause about 64% of these infections, with the remaining illnesses believed to be caused by non-O157 STEC (USDA-FSIS, 2011). However, the overall incidence of non-O157 STEC is difficult to estimate, because routine screening of these serogroups has not been performed (Bettelheim, 2007).

*E. coli* reservoirs

*E. coli* exists as a commensal organism in the gastrointestinal tract of animals, as well as humans. Many strains of *E. coli* are harmless to both humans and animals, but the aforementioned subset (STEC) is capable of causing serious illness in humans. Ruminants, cattle in particular, are major reservoirs of STEC. Surveys have demonstrated that cattle may harbor more than 100 STEC serotypes, 60% of which have been associated with HC and HUS in humans (Johnson et al., 1996). Other ruminant species associated with pathogenic STEC include sheep, goats, and wild animals, such as deer and water buffalo. Sheep are the second most common animal reservoir for STEC, with serogroups such as O91, O128, and O146 having been isolated
from the intestinal tract of the species. Goat milk also has been associated with an outbreak of
*E. coli* O157 infection. *E. coli* O157 has been isolated from wild deer in several cases, and the
consumption of deer venison has been linked to human illness (Keene et al., 1997). In 2010, an
outbreak of *E. coli* O103 and O145 occurred in a school and was traced back to the consumption
of undercooked venison (Rounds et al., 2012). STEC also have been isolated from non-ruminants
such as horses, dogs, rabbits, pigs, and wild birds. However, it is likely that these animals are
vectors for the pathogen, rather than reservoirs (Caprioli et al., 2005).

*E. coli* regulations

In 1994, the United Sates Department of Agriculture Food Safety Inspection Service
(USDA-FSIS) announced that *E. coli* O157:H7 would be considered an adulterant in raw ground
beef in response to several large outbreaks in beef products (FSIS, 1996). This rule was later
expanded to include all non-intact raw beef products, as outlined in the Federal Meat Inspection
Act (FMIA). In September 2011, the USDA-FSIS announced that six other serogroups of STEC
including O26, O45, O103, O111, O121, and O145 would be considered adulterants in ground
beef and non-intact raw beef products, beginning in June 2012. This rule will require beef
processing establishments to identify interventions to control (i.e., treatment of beef trim with
antimicrobials), as well as perform additional verification procedures (i.e., testing beef trim and
other raw ground beef products) for the presence of these STEC. This rule followed a petition
filed by food safety advocate groups who argued that all pathogenic STEC posed threats equal to
*E. coli* O157:H7. Some have argued that the rule was made without sufficient scientific research
to determine the extent and prevalence of these pathogens in the nation’s beef supply.
Interestingly, USDA-FSIS plans to perform a nationwide microbiological baseline survey on beef
carcasses prior to, or at the time of, implementation of the proposed rule (USDA-FSIS, 2011).
E. coli in cattle, beef processing, and resulting beef products

Extensive research has demonstrated that cattle are the major reservoir of pathogenic E. coli. Cattle harbor STEC in the gut and can shed the bacteria in their feces. Shinagawa et al. (2000) detected STEC in 39.4-78.9% of fecal samples from cattle ranging in age from less than 2 months to adults. This study demonstrated that the prevalence of STEC in cattle depends on the season, with the highest rates of fecal shedding occurring during the warmer months. Studies have indicated that there appears to be a correlation between the presence of STEC in feces and carcass contamination (Elder et al., 2000).

Fecal shedding of pathogens acts as a vehicle of transmission from one animal to another (Ransom et al., 2002). This observation is due primarily to animal coats and hooves easily being contaminated with fecal material (Small et al., 2002). As a result, cattle hides have been recognized as a source of microbial contamination on carcasses. Barkocy-Gallagher et al. (2003) suggested that cattle hides may be a greater source of STEC than feces. In fact, it has been reported that incidence of E. coli O157:H7 can be as much as ten times higher on cattle hides than in the feces of cattle (Ransom et al., 2002). Additionally, different areas of the hide have been reported to have varying incidence of O157 STEC. A study by Reid et al. (2002) indicated that O157 was present on the brisket of cattle hides most often (22.2% of samples), but less so on the rump and flank areas of the hide (3.3% and 4.4%, respectively). Incidence of O157 STEC on cattle hides have been reported, including; 10.7% (Elder et al., 2000), 18% (Ransom et al., 2002), 28.8% (Small et al., 2002), and 60.6% (Barkocy-Gallagher et al., 2003). Incidence of non-O157 STEC has been reported as high as 77.7% on cattle hides in the fall months (Barkocy-Gallagher et al., 2003).
Hide-to-carcass transfer of microbial contamination can occur during slaughter and is more likely to occur at several points during de-hiding, such as making initial cuts through the skin (particularly in the brisket area), when alternate use of the same hand occurs for handling the hide and the carcass, and during roll-back of the hide. Ransom et al. (2002) reported 30-100% of carcasses from animals with hides contaminated with O157 also tested positive for O157 after de-hiding. Elder et al. (2000) also demonstrated a significant positive correlation between the prevalence of O157 in feces and hides, and prevalence of carcass contamination with O157.

Contamination on carcasses that follows through processing can lead to contaminated beef products, with ground beef being of particular concern. A survey of ground beef products have demonstrated a prevalence of O157:H7 to be 3.7% (Doyle and Schoeni, 1987), and prevalence of all STEC to be between 12.1-40.8% (Read et al., 1990; Samadpour et al., 1994; Brooks et al., 2001).

**E. coli in swine, pork processing, and resulting pork products**

Limited studies detecting the presence of STEC in swine and pork products have been completed. Feder et al. (2003) reported an O157:H7 prevalence of 2.0% in pig fecal samples. An additional study in central Mexico demonstrated an E. coli O157:H7 prevalence of 2.1% in swine feces collected on farms (Callaway et al., 2004). To the best of our knowledge, no studies have been conducted to determine the prevalence of STEC on pork hides or on pork carcasses. Surveys of ground pork products have demonstrated a prevalence of O157:H7 to be 1.5% (Doyle and Schoeni, 1987), and prevalence of all STEC to be between 4.0-18.0% (Read et al., 1990; Samadpour et al., 1994; Brooks et al., 2001).
**E. coli in sheep, sheep processing, and resulting sheep products**

Limited studies of sheep and lamb products have demonstrated that sheep are reservoirs of STEC. Fegan and Desmarchelier, (1999) detected STEC in 45% of sheep feces and 36% of lamb feces. A survey of healthy lambs in Spain detected O157:H7 in 0.4% of fecal samples, and non-O157 STEC in 36% of fecal samples (Blanco et al., 2003). Sumner et al. (2003) surveyed lamb carcasses at abattoirs and very small plants, detecting *E. coli* on 61.5% of carcasses at the larger abattoirs and on 18.5% of carcasses at very small plants. Surveys of lamb and mutton products have demonstrated a prevalence of O157:H7 to be 2.0% (Doyle and Schoeni., 1987), and prevalence of all STEC to be between 17.1-48.0% (Samadpour et al., 1994; Brooks et al., 2001).

**Transfer of STEC between animals**

Contamination of animal hides with pathogens can come from soil, feces, other animals, and the environment in which animals are housed in. It has been suggested that animal hide contamination can come from the farm, during transport to abattoirs, during unloading into holding pens, and during the lairage of animals at abattoirs prior to slaughter (Barkocy-Gallagher et al., 2003; Small et al., 2002). Routes of contamination transmission onto hides can include animal-animal contact, animal-environment contact, and environment-animal contact (Small et al., 2002). Animals held in lairage can pick up contamination on their hides by mingling with other animals or via contact with walls, floors, or water troughs in the lairage environment. Small et al. (2002) demonstrated that *E. coli* O157 contamination in lairage areas can be carried over from one day to the next, in spite of routine cleaning. Additionally, studies have demonstrated extended lairage of swine increases the contamination of slaughtered pigs with *Salmonella* spp., because of the high prevalence of the pathogen in the lairage environment.
(Hurd et al., 2001). Given these factors, the possibility exists that animals coming from pathogen-free farms can become contaminated with pathogens when introduced into the lairage environment by previously contaminated animals. This observation becomes an even greater issue for very small processors, who often house and slaughter multiple species in the same area, and on the same day.

**Sampling methods**

Sampling techniques for animal hides utilize a gauze or sponge swab pad to collect the sample from the surface (Elder et al., 2000; Reid et al., 2002; O’Brien et al., 2005). Ransom et al. (2002) compared methods for collecting fecal, hide, and carcass samples using a variety of methods, including three-site sponge swabbing, hair clippings, hide excision, rinsing, and gauze swabbing beef hides. Results of the study indicated that for *E. coli* O157:H7 detection, three-site sponge swab sampling and hair clippings appear to be the most effective on cattle hides, though these methods were not statistically significant from the other sampling methods investigated. However, for the detection of *Salmonella* on beef hides, rinsing of the hide was statistically more effective (P < 0.05) than hair clippings and hide excision, but not more effective than sponge or gauze swabbing. To the best of our knowledge, a study has not been completed to compare hide sampling techniques between various animal species. As such, it may be possible that effective techniques for beef hides are not sufficient for pork or sheep hides.

A novel sampling method known as the Microbial Vacuum (M-Vac; Bluffdale, UT) system was designed to sample a large surface area, thereby recovering more microbial contamination (according to the manufacturer). The M-Vac system uses a vacuum and sterile solution to create agitation, detaching bacteria from the surface and transferring the mixture into a sterile collection bottle. The system aims to replace traditional sampling methods by sampling a larger
surface area, increasing collection off the surface, and recovering more from sampling devices. Previous studies have demonstrated that the M-Vac system is effective for pathogen
(Escherichia coli O157:H7, Salmonella Typhimurium and Listeria monocytogenes) recovery on various surfaces such as plastic, steel, and tile, and on beef flank and pork sides (Ngadze and Cutter, 2009; unpublished data). In some cases, the M-Vac system recovered more pathogens than sponge swabs on these surfaces. However, the M-Vac system has not been utilized for microbial recovery on animal hides. A comparison of the M-Vac system with traditional hide sampling techniques could be beneficial to researchers, regulatory officials, and industry members who are interested in utilizing additional sampling techniques in slaughter and processing establishments.
Objectives

The objectives of this study are to:

1. Sample beef, pork, and sheep hides for the presence of non-O157 and O157 STEC;
2. Evaluate the efficacy of various methods of sampling beef, pork, and sheep hides under slaughterhouse conditions for detecting STEC; and
3. Establish a baseline for the presence of non-O157 STEC on beef, pork, and sheep hides in a very small processing establishment.
Materials and methods

Bacterial strains and culture conditions

*E. coli* reference strains belonging to serogroups O157, O145, O121, O113, O111, O103, O45, and O26 were used as positive controls, and *E. coli* DH5α as the negative control in this study. All strains were obtained from the *E. coli* Reference Center in the Department of Veterinary and Biomedical Sciences at the Pennsylvania State University (University Park, PA) and listed in Table 1.

Bacteria were stored in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) containing 10% glycerol at -80°C. Prior to experiments, cultures were propagated twice in 10 mL TSB at 37°C for 18-24 hours and maintained on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI). For each survey, cultures of each control were streaked for isolation on Rainbow® Agar (Biolog, Hayward CA) modified with 10 mg/L novobiocin (USDA-FSIS, 2010) to use as a control for visual comparison to samples.
Table 1. Reference strains used as controls for O-type determination in multiplex PCR assay.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Culture ID</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>ATCC 43895</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O145:K-:H-</td>
<td>E1385(3)</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O121</td>
<td>39W</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O113:H21</td>
<td>6182-50</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O111:H-</td>
<td>Stoke W</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O103:K+:H8</td>
<td>H515b</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O45</td>
<td>K61</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> O26:H-</td>
<td>H311b</td>
<td>Orskov et al., 1977</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>Negative Control</td>
<td>Invitrogen (Carlsbad, California)</td>
</tr>
</tbody>
</table>
Experimental design

Samples were collected from the Penn State Meats Lab where multiple species are processed weekly. Species slaughtered include beef, sheep and pork, in the respective order, on the same day. Samples were collected from all species. Due to the small number of animals processed on site, samples were collected from each animal slaughtered on every day of collection. Hide samples were collected from each carcass immediately following exsanguination, prior to an antimicrobial intervention. This study was conducted over a 15 week period from September to December 2011. A total of 67 samples were collected, including 26 beef hide, 30 pork hide, and 11 sheep hide samples.

Sample collection

Sponge swab samples

Swab samples were collected using a 3M™ dry-sponge (3M™, St. Paul, MN) moistened with 25 mL of BPW. Hide samples were collected according to USDA-FSIS guidelines, following the 3-site sampling method for carcass sampling (USDA-FSIS, 1996). Briefly, a moistened sponge swab was wrung out, aseptically removed from the bag, and used to sample one half of the beef carcass before de-hiding. Sampling was performed aseptically using sterile latex gloves, changed between samples. For each carcass sample, 100 cm² was swabbed at each of the three anatomical sites (flank, brisket, and rump) using a sterile template (Cattle template, 3M™, St. Paul, MN). Swab samples consisted of 10 passes vertically and 10 passes horizontally. The moistened sponge swab was flipped midway through taking the sample. The samples were stored in the collection bag at 4°C until processing. Upon arrival at the lab, samples were processed immediately. Sponge swabs were stomached at 230 rpm for 2 minutes (Stomacher® 400 Circulator, Steward®, United Kingdom) in the sample collection bag, swabs were wrung out,
and sample solution collected aseptically by transferring approximately 20 ml to a sterile capped tube (VWR International, Radnor, PA).

**Clipping samples**

Hide clipping samples were collected using cordless clippers (Oster, Niles, IL). A 100 cm² area of the exterior of the hide was shaved with the cordless clippers, using a sterile template placed along the midline and anterior to the naval. The clipped hair was collected in a sterile Whirl Pak bag (Nasco®, Fort Atkinson, WI) as it fell from the hide. Clipper blades were sanitized between samples by submerging in hot water (80°C) on the kill floor for 10 seconds. Clippings were stored in the collection bag at 4°C until processing was performed. Upon arrival at the lab, clipping samples were combined with 25 mL BPW, stomached at 230 rpm for 2 minutes in a sterile Stomacher® 400 Circulator Standard Bag (Steward®, United Kingdom), and collected aseptically for enrichment by transferring approximately 20 ml to a sterile capped tube.

**M-Vac samples**

All samples were collected with the microbial vacuum system (M-Vac; Microbial Vacuum System, Bluffdale, Utah), using the 3-site sampling method described previously by the USDA-FSIS (2006). The flank, brisket, and rump were rinsed with BPW using the M-Vac system and 25 mL of the solution was collected from approximately 1 ft² at each site. The M-Vac sampling head was sanitized before each sample by submerging in hot water (80°C) for 10 seconds. The rinse solution was collected aseptically in a collection bottle and stored at 4°C until processing. Upon arrival at the lab, samples were processed immediately for enrichment.
**Sample enrichment**

Following collection and processing, samples were combined 1:4 with modified TSB (mTSB) containing 8 mg/L novobiocin (VWR International, Radnor, PA) and 16 mg/L vancomycin (Remel, Lenexa, KS) for a pre-enrichment at 37°C for 6 hours, followed by the addition of 2 mg/L rifampicin (Sigma-Aldrich, St. Louis, MO), 1.5g/L bile salts #3 (Difco Laboratories, Detroit, MI), and 1 mg/L potassium tellurite (Sigma-Aldrich, St. Louis, MO) and incubation at 42°C for an additional 18 hours (Possé et al., 2008b). An aliquot of each enrichment was collected in 1.5 mL microcentrifuge tubes (VWR International, Radnor, PA) and frozen for further analysis.

**STEC analysis**

Prior to STEC analysis, DNA isolation was performed on each enriched sample, using Epicentre Biotechnologies’ MasterPure™ DNA Purification Kit (Madison, WI), according to manufacturer’s instructions with minor modifications. Briefly, 1 mL of enrichment broth was concentrated by centrifugation for 5 minutes at 7,000 x g in a microcentrifuge (Galaxy 16, VWR International, Radnor, PA), and supernatant was discarded. The cell pellet was resuspended in 300 µl of tissue and cell lysis solution containing 1 µl of 50 µg/µl proteinase K. The cells were lysed by incubating at 65°C for 15 minutes with agitation every 5 minutes using a vortex (VWR International, Radnor, PA). Samples were cooled to 37°C for 10 minutes, 1 µl of 5 µg/µl RNase A added to the sample, mixed thoroughly, and incubated at 37°C for 30 minutes. Samples were then placed on ice for 5 minutes. MPC Protein Precipitation Reagent (175 µl) was added to the lysed sample and vortexed vigorously for 10 seconds. The cell debris were pelleted by centrifugation for 10 minutes at 10,000 x g in a microcentrifuge. The supernatant was transferred to a clean microcentrifuge tube, 500 µl of isopropanol was added, and the tube inverted 30-40 times. The total nucleic acid was pelleted by centrifugation for 10 minutes in a
The supernatant was carefully poured out without dislodging the pellet. The pellet was then rinsed twice with 70% ethanol (VWR International, Radnor, PA) and allowed to dry aseptically for 15-20 minutes. The pellet was then resuspended in 100 µl of sterile deionized water. All DNA preparations were stored at -20°C until use.

Following DNA isolation, samples were tested for the presence of 8 STEC (O157, O145, O121, O113, O111, O103, O45, and O26) using a multiplex PCR assay previously described by DebRoy et al. (2011) and shown in Figure 1. The PCR assay was performed using a Mastercycler pro thermocycler (Eppendorf, Westbury, NY). Thermocycler conditions are as follows: Initial denaturation of 95°C for 15 minutes, followed by 30 cycles of 94°C for 30 seconds, 61°C for 1.5 minutes, and 72°C for 1.5 minutes, followed by a final extension of 72°C for 10 minutes. Primer sequences and concentrations of this O-group multiplex are provided in Table 2.

Samples were also tested for the presence of three virulence genes: stx1, stx2, and eae utilizing additional PCR primers, as described by Paton and Paton (2008), shown in Figure 2. The PCR assay was performed using the Mastercycler pro thermocycler with the following conditions: initial denaturation of 94°C for 4 minutes, followed by 30 cycles of 90°C for 45 seconds, 55°C for 30 seconds, and 72°C for 50 seconds, followed by a final extension of 72°C for 8 minutes. Primer sequences and concentrations are provided in Table 3.
Figure 1. Agarose gel of amplicons from the multiplex PCR assay of Shiga toxin-producing *Escherichia coli* (STEC) serogroups. Lane 1: Molecular weight ladder, 100 bp. Lane 2: No template control. Lane 3: 2-strain control. Lane 4: 4-strain control. Lane 5: 6-strain control. Lane 6: 8-strain control. Lane 7: STEC O157. Lane 8: STEC O145. Lane 9: STEC O121. Lane 10: STEC O113. Lane 11: STEC O111. Lane 12: STEC O103. Lane 13: STEC O45. Lane 14: STEC O26.
<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Primer Sequence (5'-3')</th>
<th>Target Gene</th>
<th>Primer Concentration (µM)</th>
<th>Amplicon Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>O157</td>
<td>TCG AGG TAC CTG AAT CTT TTC TGT ACC AGT CTT GGT GCT GCT CTG ACA</td>
<td>wzx F</td>
<td>0.015</td>
<td>894</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O145</td>
<td>TTC ATT GTT TTG CTT GCT CG GGC AAG CTT TGG AAA TGA AA</td>
<td>wzx F</td>
<td>0.03</td>
<td>750</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O121</td>
<td>TCC AAC AAT TGG TCG TGA AA AG AAG TGT GAA ATG CCC GT</td>
<td>wzx F</td>
<td>0.029</td>
<td>628</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O113</td>
<td>TGC CAT AAT TCA GAG GGT GAC AAC AAA GCT AAT TGT GGC CG</td>
<td>wzx F</td>
<td>0.029</td>
<td>514</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O111</td>
<td>TGT TTC TTC GAT GTT GCG AG GCA AAG GAC ATA AGA AGC CA</td>
<td>wzx F</td>
<td>0.029</td>
<td>438</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O103</td>
<td>TTG GAG CGT TAA CTG GAC CT GCT CCC GAG CAC GTA TAA AG</td>
<td>wzx F</td>
<td>0.03</td>
<td>321</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O45</td>
<td>TGC AGT AAC CTG CAC GGG CG AGC AGG CAC AAC AGC CAC TAC T</td>
<td>wzx F</td>
<td>0.063</td>
<td>238</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O26</td>
<td>CAA TGG GCG GAA ATT TTA GA ATA ATT TTC TCT GCC GTC GC</td>
<td>wzx F</td>
<td>0.17</td>
<td>155</td>
<td>DebRoy et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wzx R</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Agarose gel of amplicons from the multiplex PCR assay for the virulence genes: 

*stx1/stx2/eae* in *Escherichia coli* (STEC). Lane 1: Molecular weight ladder, 100 bp. Lane 2: 
No template control. Lane 3: *eae* control (890bp). Lane 4: *stx1* control (600 bp). Lane 5: 
*stx2* control (255bp). Lane 6: 3-gene combination (*eae, stx1, stx2*).
Table 3. *stx/eaе* PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Primer Sequence (5’-3’)</th>
<th>Primer Concentration (uM)</th>
<th>Amplicon Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx1</td>
<td>ACA CTG GAT GAT CTC AGT GG CTG AAT CCC CCT CCA TTA TG</td>
<td>0.5</td>
<td>600</td>
<td>Paton and Paton, 1998</td>
</tr>
<tr>
<td>stx2</td>
<td>GGC ACT GTC TG AAC TGC TCC TCG CCA GTT ATC TGA CAT TCT</td>
<td>0.5</td>
<td>255</td>
<td>Paton and Paton, 1998</td>
</tr>
<tr>
<td>eae</td>
<td>GTG GCG AAT CT GGC GAG ACT CCC CAT TCT TTT TCA CCG TCG</td>
<td>0.5</td>
<td>890</td>
<td>Paton and Paton, 1998</td>
</tr>
</tbody>
</table>
**Statistical analysis**

Data from STEC analyses were reported as percentages of samples testing positive for each of the tested pathogens, divided by the total samples taken. Differences of percentage positive samples between sampling methods were determined using a proc GLM test with a Tukey separation of means (SAS Program, Cary, NC) within each species and O-group type. Percentages of overall STEC presence in each species and between sampling methods were also determined using a proc GLM test with a Tukey separation of means.

**Results**

**STEC prevalence on cattle hides**

A total of 26 cattle hides were sampled. Overall, 24 out of the 26 samples tested positive for one or more STEC O-group using the sponge swab and hair clippings sampling methods, and 23 out of the 26 samples tested positive for one or more STEC O-group using the M-Vac sampling method (Table 5). While 92% of beef hide samples tested positive for at least one O-group, only 3.8% (1/26) tested positive for a stx gene (Table 6). The STEC O-groups most commonly found in beef samples include O157, O121, O113, O45, and O26 (Table 4). Detection of different O-groups was similar for each sampling method on beef hides. Most O-groups did not have statistically different positive percentages between the sponge swab, hair clipping, and M-Vac sampling methods (Table 4). However, for O157 and O113, the M-Vac sampling method detected significantly fewer positives than the sponge swab sampling method, but was not significantly different than the hair clippings sampling method. Detection of one or more O-groups in each sample was not significantly different in any of the sampling methods (Table 5).
STEC prevalence on pork hides

A total of 30 pork hides were sampled. Overall, 29 out of the 30 samples tested positive for one or more STEC O-group using the M-Vac sampling method; 28 out of the 30 samples tested positive for one or more STEC O-group using the sponge swab sampling method; and 24 out of the 30 samples tested positive for one or more STEC O-group using the hair clipping sampling method (Table 5). While 97% of pork samples tested positive for at least one O-group, only 40.0% (12/30) tested positive for a stx gene (Table 6). The STEC O-groups most commonly found in hog hide samples included O157, O145, O121, and O103 (Table 4). Detection of different O-groups was similar for each sampling method on hog hides. Most O-groups did not have statistically different positive percentages between the sponge swab, hair clipping, and M-Vac sampling methods (Table 4). However, for O113, the sponge swab sampling method detected significantly more positives than the hair clipping sampling methods, but was not significantly different from the M-Vac sampling method. Detection of one or more O-groups in each sample was significantly greater in the M-Vac sampling method than the hair clipping sampling method, but was not significantly different than the sponge swab sampling method (Table 5).

STEC prevalence on sheep hides

A total of 11 sheep hides were sampled. Overall, 100% (11/11) of samples tested positive for one or more STEC O-group using the sponge swab and M-Vac sampling methods. Nine of the 11 samples tested positive for one or more STEC O-group using the hair clipping sampling method (Table 5). While 100% of sheep samples tested positive for at least one O-group, only 27.3% (3/11) tested positive for a stx gene (Table 6). The STEC O-groups most commonly found in sheep samples included O157, O121, and O45 (Table 4). Detection of
different O-groups was similar for each sampling method on sheep hides. Most O-groups did not have statistically different positive percentages between the sponge swab, hair clipping, and M-Vac sampling methods (Table 4). However, for O157, the M-Vac sampling method detected significantly more positives than the hair clipping method, but was not significantly different from the sponge swab sampling method. For O45, the sponge swab method detected significantly more positives than the M-Vac sampling method, but was not significantly different from the hair clipping method. Detection of one or more O-groups in each sample was not significantly different for any of the sampling methods (Table 5).
Table 4. Presence of STEC O-Groups in hide samples collected by different methods in beef, pork, and sheep.

<table>
<thead>
<tr>
<th>Species</th>
<th>O157</th>
<th>O145</th>
<th>O121</th>
<th>O113</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Swab</td>
<td>Clippings</td>
<td>M-Vac</td>
<td>Swab</td>
</tr>
<tr>
<td>Beef</td>
<td>16/26</td>
<td>11/26</td>
<td>7/26</td>
<td>1/26</td>
</tr>
<tr>
<td>% Positive</td>
<td>61.5(^{A})</td>
<td>42.3(^{AB})</td>
<td>26.9(^{A})</td>
<td>3.9(^{A})</td>
</tr>
<tr>
<td>Pork</td>
<td>14/30</td>
<td>13/30</td>
<td>16/30</td>
<td>22/30</td>
</tr>
<tr>
<td>% Positive</td>
<td>46.7(^{A})</td>
<td>43.3(^{A})</td>
<td>53.3(^{A})</td>
<td>73.3(^{A})</td>
</tr>
<tr>
<td>% Positive</td>
<td>90.9(^{AB})</td>
<td>63.6(^{A})</td>
<td>100(^{A})</td>
<td>9.1(^{A})</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>O111</th>
<th>O103</th>
<th>O45</th>
<th>O26</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Swab</td>
<td>Clippings</td>
<td>M-Vac</td>
<td>Swab</td>
</tr>
<tr>
<td>Beef</td>
<td>2/26</td>
<td>2/26</td>
<td>1/26</td>
<td>12/26</td>
</tr>
<tr>
<td>% Positive</td>
<td>7.7(^{A})</td>
<td>7.7(^{A})</td>
<td>3.9(^{A})</td>
<td>46.2(^{A})</td>
</tr>
<tr>
<td>Pork</td>
<td>0/30</td>
<td>0/30</td>
<td>0/30</td>
<td>12/30</td>
</tr>
<tr>
<td>% Positive</td>
<td>0.0(^{A})</td>
<td>0.0(^{A})</td>
<td>0.0(^{A})</td>
<td>40.0(^{A})</td>
</tr>
<tr>
<td>Sheep</td>
<td>0/11</td>
<td>1/11</td>
<td>0/11</td>
<td>3/11</td>
</tr>
<tr>
<td>% Positive</td>
<td>0.0(^{A})</td>
<td>9.1(^{A})</td>
<td>0.0(^{A})</td>
<td>27.3(^{A})</td>
</tr>
</tbody>
</table>

*Percentages within each species and O-group type bearing a common letter are not significantly different (P >0.05).
### Table 5. Number of samples testing positive for one or more STEC in hide samples collected by different methods in beef, pork, and sheep.

<table>
<thead>
<tr>
<th>Species</th>
<th>One or more STEC O-groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Swab</td>
</tr>
<tr>
<td>Beef</td>
<td>24/26</td>
</tr>
<tr>
<td>% Positive</td>
<td>92⁰A</td>
</tr>
<tr>
<td>Pork</td>
<td>28/30</td>
</tr>
<tr>
<td>% Positive</td>
<td>93⁰AB</td>
</tr>
<tr>
<td>Sheep</td>
<td>11/11</td>
</tr>
<tr>
<td>% Positive</td>
<td>100⁰A</td>
</tr>
</tbody>
</table>

*Percentages within each species bearing a common letter are not significantly different (P >0.05).

### Table 6. Presence and percentages of samples testing positive for STEC O-group, and STEC virulence genes; stx1/stx2/eae in beef, pork, and sheep hide samples.

<table>
<thead>
<tr>
<th>Species</th>
<th># Samples Pos. for at least 1 O-group</th>
<th>stx1</th>
<th>stx2</th>
<th>eae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>24/26</td>
<td>1/26</td>
<td>1/26</td>
<td>5/26</td>
</tr>
<tr>
<td>% Positive</td>
<td>92⁰A</td>
<td>3.8⁰B</td>
<td>3.8⁰B</td>
<td>19⁰B</td>
</tr>
<tr>
<td>Pork</td>
<td>29/30</td>
<td>0/30</td>
<td>12/30</td>
<td>2/30</td>
</tr>
<tr>
<td>% Positive</td>
<td>97⁰A</td>
<td>0⁰C</td>
<td>40⁰B</td>
<td>6.7⁰C</td>
</tr>
<tr>
<td>Sheep</td>
<td>11/11</td>
<td>0/11</td>
<td>3/11</td>
<td>0/11</td>
</tr>
<tr>
<td>% Positive</td>
<td>100⁰A</td>
<td>0⁰C</td>
<td>27⁰B</td>
<td>0⁰C</td>
</tr>
</tbody>
</table>

*Percentages within each species bearing a common letter are not significantly different (P >0.05).
Discussion

All hide sampling methods detected STEC in all tested species. No statistical differences existed between any of the sampling methods within each species, indicating that all sampling methods were effective for the detection of STEC in multiple species, especially after an enrichment step. However, non-statistical trends were seen within individual STEC O-groups detected in pork hide samples, which suggested that hair clippings may not be as effective at recovering pathogens from pork hides. A larger sample size may have indicated a statistical difference between sampling methods on pork hides and would be supported by the difference in hair type of pork hides, when compared to the more dense cattle and sheep hides.

Observations of statistical differences indicated that the M-Vac is a possible new tool for sampling animals in the processing plant and could be of interest to researchers and processors. Currently, the 3-site sponge swab sampling method is used by industry and regulatory personnel. This study demonstrates that the M-Vac is as effective as the 3-site sponge swab method, yet is unlikely to be adapted primarily due to the large initial cost to obtain and maintain the M-Vac, as well as the maneuverability of the machine in small or tight places on the slaughter floor. However, the M-Vac system may be more adaptable for researchers and possibly large slaughter establishments.

No differences between STEC O-groups in each species tested were evident. However, STEC O111 was the least detected STEC in all species. This finding could be indicative of the organism, which has been isolated rarely from animals, or it could be representative of the environment of this specific processing facility. It is important to note that STEC O-groups were detected in at least one sample from each species. However, STEC O157 and O121 were the only O-groups present in all species.
Additionally, on sample collection days when multiple species were slaughtered, no trends were noted with regard to the presence of specific O-groups of different species (data not shown). Therefore, no conclusions can be drawn on the effect of holding different species in lairage prior to slaughter with the presence of STEC on animal hides in this facility. However, multiple species were slaughtered on only two of the sample collection days. In addition, due to the close proximity of the university farm sites, this particular slaughter facility does not hold animals for an extended lairage period. Results from this study may serve as an indication of the presence of STEC in small slaughter facilities that process multiple species. This study can be expanded upon in the future by taking a more extensive survey, including more samples from each species, and by surveying additional processing facilities to achieve a representative population of processors that either process more animals of different species, or that hold incoming animals for an extended lairage period.
References


Chapter 5

Conclusions and Suggestions for Future Research

Shiga toxin-producing *Escherichia coli* (STEC) are pathogens attributed to numerous foodborne illnesses resulting in gastrointestinal disease of varying severity, including hemolytic uremic syndrome (HUS) in humans. Cattle and consequently, beef products are considered a major source of STEC. *E. coli* O157:H7 has been regulated as an adulterant in ground beef since 1994. The USDA-Food Safety and Inspection Service has indicated that 6 additional STEC (O145, O121, O111, O103, O45, and O26; termed the “big-6” non-O157 STEC) will be regulated as adulterants in raw ground beef, beginning in June 2012.

It is believed that non-O157 STEC are largely under-reported, due to the broad range of both phenotypic and genotypic characteristics this group of pathogens display and the lack of cultural and diagnostic methods available to identify non-O157 STEC. A study of three large beef processing plants in the Midwest United States indicated an STEC prevalence of 19.3% in feces, 57% on hides, 58.2% on pre-eviscerated carcasses, and 9% on post-intervention carcasses (Barkocy-Gallagher et al., 2003). This study indicated prevalence rates of *E. coli* O157:H7 as well, which were comparable to rates of all STEC. However, little is known about the presence of STEC in small and very small beef processing plants. It was the goal of this research to use a combination of cultural and molecular detection methods to identify and isolate STEC from small and very small beef processing plants and resulting beef products in Pennsylvania.

First, an effective method of culturing and identifying non-O157 STEC was determined. Three different enrichment media (TSBn, mECn, and mTSB) were evaluated. Carcass, environmental, fecal, or ground beef samples were artificially inoculated with approximately $10^2$
CFU/mL of STEC O145 and enriched for 24 hr at 42°C with each of the selected enrichment media. Samples plated on Rainbow® agar demonstrated that all three enrichment media were effective for the enrichment of non-O157 STEC in multiple sample types and that samples enriched in mTSB (Possé et al., 2008) contained less background microflora growth. Therefore, mTSB was used for the following beef plant surveys. A multiplex PCR assay (DebRoy et al., 2011) was optimized for the detection of STEC serogroups O157, O145, O121, O113, O111, O103, O45, and O26. This multiplex PCR assay was used for initial identification of positive samples and confirmation of isolates was made from said samples. An additional multiplex PCR assay described by Paton and Paton (2005) was used to identify the presence of STEC virulence genes; Shiga toxin 1, Shiga toxin 2, and intimin (eae).

Small and very small beef processing environments and resulting ground beef were surveyed to determine if these establishments could be a potential source of STEC in the beef supply. Results demonstrated that 55.5% (151/272) of environmental, 36.9% (75/203) of carcass samples, 85.2% (23/27) of hide samples, 37.5% (12/32) of fecal, and 18.6% (22/118) of ground beef samples tested positive for one or more of the tested STEC serogroups. However, only 7.7% (21/272) of the environmental samples, 5.9% (12/203) of the carcass samples, 0% (0/27) of the hide samples, 0% (0/32) of the fecal, and 0% (0/118) ground beef samples tested positive for one or more of the Shiga toxin (stx) genes. Significantly fewer (P < 0.05) samples tested positive for the Shiga toxin gene than those testing positive for an STEC O-group. In some, but not all beef processing plants sampled, one or more STEC O-groups were more prevalent (P < 0.05) than others within the individual processing plant. Overall, STEC O157 and O45 were the most prevalent STEC (P < 0.05) in the beef processing samples collected in this study.

Additionally, this research evaluated the effectiveness of three different sampling methods (sponge swab, hide clipping, and a novel M-Vac sampling method) for detecting STEC
on cattle, swine, and sheep hides at one abattoir, which processes all species in a single day.

Results demonstrated that 92% (24/26) of cattle hides tested positive for one or more STEC O-groups using the sponge swab and hair clipping methods, but only 88% (23/26) of cattle hides tested positive for one or more STEC O-groups using the M-Vac sampling method. Swine hides tested positive for one or more STEC O-group in 93% (28/30) of samples collected with the sponge swab method, as compared with 80% (24/30) of samples collected using the hair clipping method, while 97% (29/30) of samples collected were positive for the STEC O-group using the M-Vac sampling method. Sheep hides were positive for one or more STEC O-group in all (11/11) of the samples collected with the sponge swab and M-Vac methods, while 82% (9/11) of samples collected were positive with the hair clipping method. However, only 3.8% (1/26) of cattle hides, 40% (12/30) of swine hides, and 27.3% (3/11) of sheep hides tested positive for stx1 and/or stx2 genes, when all sampling methods were considered. Overall, significantly fewer samples (P < 0.05) from all species tested positive for the Shiga toxin genes than those which tested positive for one or more STEC O-group.

Future surveys looking more specifically at individual small or very small processors over a more extended time period could give researchers a better idea of the seasonal variance of non-O157 STEC in these plants. In addition, more extensive surveys of these small processors, which focus on specific areas of the processing facility environments, may result in a better understanding of the path that these pathogens take to reach the final product, thereby helping researchers determine effective methods of controlling these pathogens.

Research should also investigate the transfer of STEC onto hides and resulting meat products of multiple species housed in the same processing facility, by performing a more extensive survey of these small and very small processors. By surveying a larger sample size of beef trim and ground beef samples from more small and very small processors, a more
comprehensive survey of the presence of non-O157 STEC in ground beef products from these processors can be attained.

Future research should determine if primers for STEC virulence genes such as Shiga toxins 1 and 2, and intimin (eae) could be incorporated into the current multiplex PCR assay. This step could improve the ability to screen for STEC by decreasing the time and costs required to do so, thereby benefitting researchers, beef processors, and regulatory officials.

Finally, this assay could be used to detect STEC presence in specialty or game meats. For example, venison, which is not regulated by the USDA-FSIS, has been linked to foodborne illness outbreaks of non-O157 STEC (Rounds et al., 2012). Additionally, it is recommended that researchers evaluate veal products, which are not often investigated (Flowers, 2002), or in beef specialty products, such as organ meats, for the presence of these STEC.
References


### Appendix

Table A-1. Presence and percentages of positive samples for STEC O-groups found in the environment of small and very small beef processing plants.

<table>
<thead>
<tr>
<th>Sample Location</th>
<th># Samples</th>
<th>O157</th>
<th>O145</th>
<th>O121</th>
<th>O113</th>
<th>O111</th>
<th>O103</th>
<th>O45</th>
<th>O26</th>
<th>stx1</th>
<th>stx2</th>
<th>eae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sink</td>
<td>24</td>
<td>12*</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>25*</td>
<td>8.3</td>
<td>1.7</td>
<td>0.0</td>
<td>0.0</td>
<td>4.2</td>
<td>4.2</td>
<td>8.3</td>
<td>4.2</td>
<td>8.3</td>
<td>21</td>
</tr>
<tr>
<td>Chute</td>
<td>42</td>
<td>11</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>8</td>
<td>12</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>26*</td>
<td>0.0</td>
<td>14</td>
<td>4.8</td>
<td>0.0</td>
<td>19</td>
<td>29</td>
<td>29</td>
<td>2.4</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Dehider</td>
<td>27</td>
<td>4</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>10</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>15*</td>
<td>0.0</td>
<td>26</td>
<td>11</td>
<td>0.0</td>
<td>15</td>
<td>37</td>
<td>26</td>
<td>3.7</td>
<td>3.7</td>
<td>11</td>
</tr>
<tr>
<td>Drain</td>
<td>16</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>31*</td>
<td>6.3</td>
<td>13</td>
<td>13</td>
<td>6.3</td>
<td>13</td>
<td>31</td>
<td>31</td>
<td>0.0</td>
<td>6.3</td>
<td>25</td>
</tr>
<tr>
<td>Offal Barrel</td>
<td>26</td>
<td>6</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>23*</td>
<td>0.0</td>
<td>15</td>
<td>3.8</td>
<td>0.0</td>
<td>12</td>
<td>15</td>
<td>7.7</td>
<td>0.0</td>
<td>3.9</td>
<td>12</td>
</tr>
<tr>
<td>Lardage</td>
<td>30</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>7</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>27*</td>
<td>3.3</td>
<td>23</td>
<td>6.7</td>
<td>3.3</td>
<td>23</td>
<td>27</td>
<td>13</td>
<td>3.3</td>
<td>3.3</td>
<td>13</td>
</tr>
<tr>
<td>Evisceria Area</td>
<td>14</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>7.1</td>
<td>7.1</td>
<td>0.0</td>
<td>14</td>
<td>0.0</td>
<td>7.1</td>
<td>14</td>
<td>0.0</td>
<td>0.0</td>
<td>14</td>
<td>0.0</td>
</tr>
<tr>
<td>Ladder</td>
<td>15</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>40</td>
<td>0.0</td>
<td>27</td>
<td>13</td>
<td>0.0</td>
<td>6.7</td>
<td>20</td>
<td>13</td>
<td>0.0</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Hook</td>
<td>17</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>18*</td>
<td>0.0</td>
<td>24</td>
<td>5.9</td>
<td>0.0</td>
<td>18</td>
<td>24</td>
<td>12</td>
<td>0.0</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>Saw</td>
<td>15</td>
<td>7</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>47</td>
<td>0.0</td>
<td>33</td>
<td>6.7</td>
<td>0.0</td>
<td>20</td>
<td>27</td>
<td>20</td>
<td>0.0</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Hose</td>
<td>15</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>47</td>
<td>6.7</td>
<td>6.7</td>
<td>0.0</td>
<td>0.0</td>
<td>27</td>
<td>33</td>
<td>6.7</td>
<td>0.0</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>Floor/Wall</td>
<td>12</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>17*</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>17</td>
<td>17</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>8.3</td>
</tr>
<tr>
<td>Knife</td>
<td>15</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>13*</td>
<td>0.0</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
<td>20</td>
<td>6.7</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Percentages within each O-group bearing a common letter are not significantly different (P >0.05).
Table A-2. Presence and percentages of positive samples for STEC O-groups found in the environment of small and very small beef processing plants.

<table>
<thead>
<tr>
<th>Sample Location</th>
<th># Samples</th>
<th>O157</th>
<th>O145</th>
<th>O121</th>
<th>O113</th>
<th>O111</th>
<th>O103</th>
<th>O45</th>
<th>O26</th>
<th>stx1</th>
<th>stx2</th>
<th>eae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sink</td>
<td>24</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>25(^{A})</td>
<td>8.3(^{A})</td>
<td>1.7(^{A})</td>
<td>0.0(^{A})</td>
<td>0.0(^{A})</td>
<td>4.2(^{A})</td>
<td>17(^{A})</td>
<td>8.3(^{A})</td>
<td>4.2(^{A})</td>
<td>8.3(^{A})</td>
<td>21(^{A})</td>
</tr>
<tr>
<td>Chute</td>
<td>42</td>
<td>11</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>8</td>
<td>12</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>26(^{A})</td>
<td>0.0(^{B})</td>
<td>14(^{AB})</td>
<td>4.8(^{B})</td>
<td>0.0(^{B})</td>
<td>19(^{AB})</td>
<td>29(^{A})</td>
<td>12(^{AB})</td>
<td>0.0(^{B})</td>
<td>2.4(^{B})</td>
<td>4.8(^{B})</td>
</tr>
<tr>
<td>Dehider</td>
<td>27</td>
<td>4</td>
<td>0</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>10</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>15(^{AB})</td>
<td>0.0(^{B})</td>
<td>26(^{AB})</td>
<td>11(^{AB})</td>
<td>0.0(^{B})</td>
<td>15(^{AB})</td>
<td>37(^{A})</td>
<td>26(^{AB})</td>
<td>3.7(^{B})</td>
<td>3.7(^{B})</td>
<td>11(^{AB})</td>
</tr>
<tr>
<td>Drain</td>
<td>16</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>31(^{A})</td>
<td>6.3(^{A})</td>
<td>31(^{A})</td>
<td>13(^{A})</td>
<td>6.3(^{A})</td>
<td>13(^{A})</td>
<td>31(^{A})</td>
<td>31(^{A})</td>
<td>0.0(^{A})</td>
<td>6.3(^{A})</td>
<td>25(^{A})</td>
</tr>
<tr>
<td>Offal Barrel</td>
<td>26</td>
<td>6</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>23(^{A})</td>
<td>0.0(^{A})</td>
<td>15(^{A})</td>
<td>3.8(^{A})</td>
<td>0.0(^{A})</td>
<td>12(^{A})</td>
<td>15(^{A})</td>
<td>7.7(^{A})</td>
<td>0.0(^{A})</td>
<td>3.9(^{A})</td>
<td>12(^{A})</td>
</tr>
<tr>
<td>Lairage</td>
<td>30</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>7</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>27(^{A})</td>
<td>3.3(^{A})</td>
<td>23(^{A})</td>
<td>6.7(^{A})</td>
<td>3.3(^{A})</td>
<td>23(^{A})</td>
<td>27(^{A})</td>
<td>13(^{A})</td>
<td>3.3(^{A})</td>
<td>3.3(^{A})</td>
<td>13(^{A})</td>
</tr>
<tr>
<td>Eviscera Area</td>
<td>14</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>7.1(^{A})</td>
<td>7.1(^{A})</td>
<td>0.0(^{A})</td>
<td>14(^{A})</td>
<td>0.0(^{A})</td>
<td>7.1(^{A})</td>
<td>14(^{A})</td>
<td>0.0(^{A})</td>
<td>0.0(^{A})</td>
<td>14(^{A})</td>
<td>0.0(^{A})</td>
</tr>
<tr>
<td>Ladder</td>
<td>15</td>
<td>6</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>40(^{A})</td>
<td>0.0(^{B})</td>
<td>27(^{AB})</td>
<td>13(^{AB})</td>
<td>0.0(^{B})</td>
<td>6.7(^{AB})</td>
<td>20(^{AB})</td>
<td>13(^{AB})</td>
<td>0.0(^{B})</td>
<td>13(^{AB})</td>
<td>13(^{AB})</td>
</tr>
<tr>
<td>Hook</td>
<td>17</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>18(^{A})</td>
<td>0.0(^{A})</td>
<td>24(^{A})</td>
<td>5.9(^{A})</td>
<td>0.0(^{A})</td>
<td>18(^{A})</td>
<td>24(^{A})</td>
<td>12(^{A})</td>
<td>0.0(^{A})</td>
<td>12(^{A})</td>
<td>18(^{A})</td>
</tr>
<tr>
<td>Saw</td>
<td>15</td>
<td>7</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>47(^{A})</td>
<td>0.0(^{B})</td>
<td>33(^{AB})</td>
<td>6.7(^{AB})</td>
<td>0.0(^{B})</td>
<td>20(^{AB})</td>
<td>27(^{A})</td>
<td>20(^{AB})</td>
<td>0.0(^{B})</td>
<td>13(^{AB})</td>
<td>13(^{AB})</td>
</tr>
<tr>
<td>Hose</td>
<td>15</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>47(^{A})</td>
<td>6.7(^{AB})</td>
<td>6.7(^{AB})</td>
<td>0.0(^{B})</td>
<td>0.0(^{B})</td>
<td>27(^{AB})</td>
<td>33(^{AB})</td>
<td>6.7(^{AB})</td>
<td>0.0(^{B})</td>
<td>20(^{AB})</td>
<td>33(^{AB})</td>
</tr>
<tr>
<td>Floor/Wall</td>
<td>12</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>17(^{A})</td>
<td>0.0(^{A})</td>
<td>0.0(^{A})</td>
<td>0.0(^{A})</td>
<td>17(^{A})</td>
<td>17(^{A})</td>
<td>0.0(^{A})</td>
<td>0.0(^{A})</td>
<td>0.0(^{A})</td>
<td>8.3(^{A})</td>
<td></td>
</tr>
<tr>
<td>Knife</td>
<td>15</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>% Positive</td>
<td></td>
<td>13(^{A})</td>
<td>0.0(^{A})</td>
<td>6.7(^{A})</td>
<td>6.7(^{A})</td>
<td>6.7(^{A})</td>
<td>6.7(^{A})</td>
<td>6.7(^{A})</td>
<td>20(^{A})</td>
<td>6.7(^{A})</td>
<td>0.0(^{A})</td>
<td>13(^{A})</td>
</tr>
</tbody>
</table>

*Percentages within each sample type bearing a common letter are not significantly different (P >0.05).*
Table A-3. Seasonal variation in the prevalence of STEC and virulence genes in cattle processing environments, on cattle carcasses and hides, and ground beef resulting from small and very small processing plants.

<table>
<thead>
<tr>
<th>Season</th>
<th>O157</th>
<th>O145</th>
<th>O121</th>
<th>O113</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Positive</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Environmental</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>45</td>
<td>3</td>
<td>6.7\textsuperscript{B}</td>
<td>45</td>
</tr>
<tr>
<td>Summer</td>
<td>47</td>
<td>15</td>
<td>32\textsuperscript{A}</td>
<td>47</td>
</tr>
<tr>
<td>Fall</td>
<td>159</td>
<td>50</td>
<td>31\textsuperscript{A}</td>
<td>159</td>
</tr>
<tr>
<td>Winter</td>
<td>21</td>
<td>0</td>
<td>0.0\textsuperscript{B}</td>
<td>21</td>
</tr>
<tr>
<td>Carcass</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>17</td>
<td>0</td>
<td>0.0\textsuperscript{B}</td>
<td>17</td>
</tr>
<tr>
<td>Summer</td>
<td>18</td>
<td>3</td>
<td>17\textsuperscript{AB}</td>
<td>18</td>
</tr>
<tr>
<td>Fall</td>
<td>156</td>
<td>33</td>
<td>21\textsuperscript{A}</td>
<td>156</td>
</tr>
<tr>
<td>Winter</td>
<td>16</td>
<td>0</td>
<td>0.0\textsuperscript{B}</td>
<td>16</td>
</tr>
<tr>
<td>Hide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Summer</td>
<td>5</td>
<td>0</td>
<td>0.0\textsuperscript{A}</td>
<td>5</td>
</tr>
<tr>
<td>Fall</td>
<td>22</td>
<td>6</td>
<td>27\textsuperscript{A}</td>
<td>22</td>
</tr>
<tr>
<td>Winter</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Ground Beef</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Summer</td>
<td>42</td>
<td>3</td>
<td>7.1\textsuperscript{A}</td>
<td>42</td>
</tr>
<tr>
<td>Fall</td>
<td>40</td>
<td>3</td>
<td>7.5\textsuperscript{A}</td>
<td>40</td>
</tr>
<tr>
<td>Winter</td>
<td>36</td>
<td>0</td>
<td>0.0\textsuperscript{A}</td>
<td>36</td>
</tr>
</tbody>
</table>

*Percentages within each O-group and sample type bearing a common letter are not significantly different (P >0.05).
Table A-3. Seasonal variation in the prevalence of STEC and virulence genes in cattle processing environments, on cattle carcasses and hides, and ground beef resulting from small and very small processing plants.

<table>
<thead>
<tr>
<th>Season</th>
<th><strong>O111</strong></th>
<th></th>
<th><strong>O103</strong></th>
<th></th>
<th><strong>O45</strong></th>
<th></th>
<th><strong>O26</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#</td>
<td>Positive</td>
<td>#</td>
<td>Positive</td>
<td>#</td>
<td>Positive</td>
<td>#</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td></td>
<td>n</td>
<td></td>
<td>n</td>
<td></td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>Environmental</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>45</td>
<td>0</td>
<td>45</td>
<td>2</td>
<td>45</td>
<td>1</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>4.4(^B)</td>
<td></td>
<td>2.2(^C)</td>
<td></td>
<td>0.0(^B)</td>
</tr>
<tr>
<td>Summer</td>
<td>47</td>
<td>0</td>
<td>47</td>
<td>8</td>
<td>47</td>
<td>10</td>
<td>47</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>17(^{AB})</td>
<td></td>
<td>21(^{AB})</td>
<td></td>
<td>11(^{AB})</td>
</tr>
<tr>
<td>Fall</td>
<td>159</td>
<td>3</td>
<td>159</td>
<td>31</td>
<td>159</td>
<td>53</td>
<td>159</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.9(^A)</td>
<td></td>
<td>20(^A)</td>
<td></td>
<td>33(^A)</td>
<td></td>
<td>20(^A)</td>
</tr>
<tr>
<td>Winter</td>
<td>21</td>
<td>0</td>
<td>21</td>
<td>0</td>
<td>21</td>
<td>1</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>0.0(^B)</td>
<td></td>
<td>4.8(^{BC})</td>
<td></td>
<td>0.0(^B)</td>
</tr>
<tr>
<td>Carcass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>17</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>0.0(^A)</td>
</tr>
<tr>
<td>Summer</td>
<td>18</td>
<td>0</td>
<td>18</td>
<td>2</td>
<td>18</td>
<td>4</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>11(^A)</td>
<td></td>
<td>22(^A)</td>
<td></td>
<td>0.0(^A)</td>
</tr>
<tr>
<td>Fall</td>
<td>156</td>
<td>0</td>
<td>156</td>
<td>21</td>
<td>156</td>
<td>23</td>
<td>156</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>14(^A)</td>
<td></td>
<td>15(^A)</td>
<td></td>
<td>6.4(^A)</td>
</tr>
<tr>
<td>Winter</td>
<td>16</td>
<td>0</td>
<td>16</td>
<td>1</td>
<td>16</td>
<td>1</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>6.3(^A)</td>
<td></td>
<td>6.3(^A)</td>
<td></td>
<td>0.0(^A)</td>
</tr>
<tr>
<td>Hide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Summer</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>0.0(^B)</td>
<td></td>
<td>0.0(^B)</td>
<td></td>
<td>20(^A)</td>
</tr>
<tr>
<td>Fall</td>
<td>22</td>
<td>0</td>
<td>22</td>
<td>10</td>
<td>22</td>
<td>15</td>
<td>22</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>46(^A)</td>
<td></td>
<td>68(^A)</td>
<td></td>
<td>41(^A)</td>
</tr>
<tr>
<td>Winter</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Ground Beef</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Summer</td>
<td>42</td>
<td>0</td>
<td>42</td>
<td>0</td>
<td>42</td>
<td>1</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>0.0(^B)</td>
<td></td>
<td>2.4(^A)</td>
<td></td>
<td>0.0(^A)</td>
</tr>
<tr>
<td>Fall</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>5</td>
<td>40</td>
<td>2</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>13(^A)</td>
<td></td>
<td>5.0(^A)</td>
<td></td>
<td>5.0(^A)</td>
</tr>
<tr>
<td>Winter</td>
<td>36</td>
<td>0</td>
<td>36</td>
<td>0</td>
<td>36</td>
<td>1</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0(^A)</td>
<td></td>
<td>0.0(^B)</td>
<td></td>
<td>2.8(^A)</td>
<td></td>
<td>0.0(^A)</td>
</tr>
</tbody>
</table>

*Percentages within each O-group and sample type bearing a common letter are not significantly different (P >0.05).
Table A-3. Seasonal variation in the prevalence of STEC and virulence genes in cattle processing environments, on cattle carcasses and hides, and ground beef resulting from small and very small processing plants.

<table>
<thead>
<tr>
<th>Season</th>
<th>stx1 n</th>
<th># Positive</th>
<th>% Positive</th>
<th>stx2 n</th>
<th># Positive</th>
<th>% Positive</th>
<th>eae n</th>
<th># Positive</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Environmental</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>45</td>
<td>0</td>
<td>0.0^A</td>
<td>45</td>
<td>0</td>
<td>0.0^B</td>
<td>45</td>
<td>2</td>
<td>4.4^B</td>
</tr>
<tr>
<td>Summer</td>
<td>47</td>
<td>0</td>
<td>0.0^A</td>
<td>47</td>
<td>8</td>
<td>17^A</td>
<td>47</td>
<td>14</td>
<td>30^A</td>
</tr>
<tr>
<td>Fall</td>
<td>159</td>
<td>4</td>
<td>2.5^A</td>
<td>159</td>
<td>10</td>
<td>6.3^B</td>
<td>159</td>
<td>21</td>
<td>13^B</td>
</tr>
<tr>
<td>Winter</td>
<td>21</td>
<td>0</td>
<td>0.0^A</td>
<td>21</td>
<td>0</td>
<td>0.0^B</td>
<td>21</td>
<td>0</td>
<td>0.0^B</td>
</tr>
<tr>
<td><strong>Carcass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>17</td>
<td>0</td>
<td>0.0^A</td>
<td>17</td>
<td>0</td>
<td>0.0^A</td>
<td>17</td>
<td>0</td>
<td>0.0^A</td>
</tr>
<tr>
<td>Summer</td>
<td>18</td>
<td>0</td>
<td>0.0^A</td>
<td>18</td>
<td>2</td>
<td>11^A</td>
<td>18</td>
<td>3</td>
<td>17^A</td>
</tr>
<tr>
<td>Fall</td>
<td>156</td>
<td>4</td>
<td>2.6^A</td>
<td>156</td>
<td>6</td>
<td>3.8^A</td>
<td>156</td>
<td>13</td>
<td>8.3^A</td>
</tr>
<tr>
<td>Winter</td>
<td>16</td>
<td>0</td>
<td>0.0^A</td>
<td>16</td>
<td>0</td>
<td>0.0^A</td>
<td>16</td>
<td>0</td>
<td>0.0^A</td>
</tr>
<tr>
<td><strong>Hide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Summer</td>
<td>5</td>
<td>0</td>
<td>0.0^A</td>
<td>5</td>
<td>0</td>
<td>0.0^A</td>
<td>5</td>
<td>0</td>
<td>0.0^A</td>
</tr>
<tr>
<td>Fall</td>
<td>22</td>
<td>0</td>
<td>0.0^A</td>
<td>22</td>
<td>0</td>
<td>0.0^A</td>
<td>22</td>
<td>0</td>
<td>0.0^A</td>
</tr>
<tr>
<td>Winter</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Ground Beef</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Summer</td>
<td>42</td>
<td>0</td>
<td>0.0^A</td>
<td>42</td>
<td>0</td>
<td>0.0^A</td>
<td>42</td>
<td>0</td>
<td>0.0^A</td>
</tr>
<tr>
<td>Fall</td>
<td>40</td>
<td>0</td>
<td>0.0^A</td>
<td>40</td>
<td>0</td>
<td>0.0^A</td>
<td>40</td>
<td>1</td>
<td>2.5^A</td>
</tr>
<tr>
<td>Winter</td>
<td>36</td>
<td>0</td>
<td>0.0^A</td>
<td>36</td>
<td>0</td>
<td>0.0^A</td>
<td>36</td>
<td>0</td>
<td>0.0^A</td>
</tr>
</tbody>
</table>

*Percentages within each O-group and sample type bearing a common letter are not significantly different (P >0.05).
Table A-4. Seasonal variation in the prevalence of STEC and virulence genes in all cattle processing samples.

<table>
<thead>
<tr>
<th>Season</th>
<th>O157</th>
<th>O145</th>
<th>O121</th>
<th>O113</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td># Positive</td>
<td>% Positive</td>
<td>n</td>
</tr>
<tr>
<td>Spring</td>
<td>62</td>
<td>3</td>
<td>4.8&lt;sup&gt;A&lt;/sup&gt;</td>
<td>62</td>
</tr>
<tr>
<td>Summer</td>
<td>112</td>
<td>21</td>
<td>19&lt;sup&gt;B&lt;/sup&gt;</td>
<td>112</td>
</tr>
<tr>
<td>Fall</td>
<td>377</td>
<td>92</td>
<td>24&lt;sup&gt;A&lt;/sup&gt;</td>
<td>377</td>
</tr>
<tr>
<td>Winter</td>
<td>73</td>
<td>0</td>
<td>0.0&lt;sup&gt;B&lt;/sup&gt;</td>
<td>73</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Season</th>
<th>O111</th>
<th>O103</th>
<th>O45</th>
<th>O26</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td># Positive</td>
<td>% Positive</td>
<td>n</td>
</tr>
<tr>
<td>Spring</td>
<td>62</td>
<td>0</td>
<td>0.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>62</td>
</tr>
<tr>
<td>Summer</td>
<td>112</td>
<td>0</td>
<td>0.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>112</td>
</tr>
<tr>
<td>Fall</td>
<td>377</td>
<td>3</td>
<td>0.8&lt;sup&gt;A&lt;/sup&gt;</td>
<td>377</td>
</tr>
<tr>
<td>Winter</td>
<td>73</td>
<td>0</td>
<td>0.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>73</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Season</th>
<th>stx1</th>
<th>stx2</th>
<th>eae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td># Positive</td>
<td>% Positive</td>
</tr>
<tr>
<td>Spring</td>
<td>62</td>
<td>0</td>
<td>0.0&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Summer</td>
<td>112</td>
<td>0</td>
<td>0.0&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fall</td>
<td>377</td>
<td>8</td>
<td>2.1&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Winter</td>
<td>73</td>
<td>0</td>
<td>0.0&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Percentages within each O-group bearing a common letter are not significantly different (P >0.05).
Table A-5. Culture positive isolates obtained from enriched beef processing plant and product samples, identified by O-group and presence of virulence genes (stx1, stx2, and eae).

<table>
<thead>
<tr>
<th>Isolate Identification</th>
<th>Date Collected</th>
<th>O-group</th>
<th>stx1</th>
<th>stx2</th>
<th>eae</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6/27/2011</td>
<td>O157</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>2</td>
<td>6/27/2011</td>
<td>O157</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>3</td>
<td>7/12/2011</td>
<td>O157</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>4</td>
<td>7/12/2011</td>
<td>O157</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>5</td>
<td>8/17/2011</td>
<td>O103</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>6</td>
<td>8/17/2011</td>
<td>O103</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>7</td>
<td>8/17/2011</td>
<td>O26</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>8</td>
<td>9/12/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>9</td>
<td>9/12/2011</td>
<td>O26</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>10</td>
<td>9/12/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>11</td>
<td>9/12/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>12</td>
<td>9/12/2011</td>
<td>O26</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>13</td>
<td>9/12/2011</td>
<td>O26</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>14</td>
<td>9/12/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>15</td>
<td>9/12/2011</td>
<td>O26</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>16</td>
<td>9/12/2011</td>
<td>O103</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>17</td>
<td>9/12/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>18</td>
<td>9/12/2011</td>
<td>O26</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>19</td>
<td>9/12/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>20</td>
<td>9/12/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>21</td>
<td>9/12/2011</td>
<td>O26</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>22</td>
<td>9/13/2011</td>
<td>O121</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>23</td>
<td>9/13/2011</td>
<td>O121</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>24</td>
<td>9/13/2011</td>
<td>O121</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>25</td>
<td>9/13/2011</td>
<td>O157</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>26</td>
<td>9/13/2011</td>
<td>O121</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>27</td>
<td>9/13/2011</td>
<td>O121</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>28</td>
<td>9/13/2011</td>
<td>O121</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>29</td>
<td>9/13/2011</td>
<td>O121</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>30</td>
<td>9/13/2011</td>
<td>O121</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>31</td>
<td>9/20/2011</td>
<td>O121</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>32</td>
<td>9/20/2011</td>
<td>O26</td>
<td>(+)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>33</td>
<td>9/30/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>34</td>
<td>9/30/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>35</td>
<td>9/30/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>36</td>
<td>9/30/2011</td>
<td>O103</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>37</td>
<td>9/30/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>38</td>
<td>9/30/2011</td>
<td>O103</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>39</td>
<td>9/30/2011</td>
<td>O145</td>
<td>(+)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>40</td>
<td>9/30/2011</td>
<td>O103</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>41</td>
<td>9/30/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>Isolate Identification</td>
<td>Date Collected</td>
<td>O-group</td>
<td>stx1</td>
<td>stx2</td>
<td>eae</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------</td>
<td>---------</td>
<td>------</td>
<td>------</td>
<td>-----</td>
</tr>
<tr>
<td>42</td>
<td>9/30/2011</td>
<td>O45</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>43</td>
<td>9/30/2011</td>
<td>O145</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>44</td>
<td>9/30/2011</td>
<td>O103</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>45</td>
<td>9/30/2011</td>
<td>O103</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>46</td>
<td>9/30/2011</td>
<td>O103</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>47</td>
<td>9/30/2011</td>
<td>O103</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>9/30/2011</td>
<td>O45</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>49</td>
<td>9/30/2011</td>
<td>O45</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>9/30/2011</td>
<td>O45</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>51</td>
<td>9/30/2011</td>
<td>O103</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>52</td>
<td>10/6/2011</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>53</td>
<td>10/6/2011</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>54</td>
<td>10/6/2011</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>10/6/2011</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>56</td>
<td>10/6/2011</td>
<td>O157</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>57</td>
<td>10/6/2011</td>
<td>O26</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>58</td>
<td>10/6/2011</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>59</td>
<td>10/11/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>10/11/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>61</td>
<td>10/11/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>62</td>
<td>10/11/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63</td>
<td>10/11/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>64</td>
<td>10/11/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>65</td>
<td>10/11/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>66</td>
<td>10/11/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>67</td>
<td>10/25/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>68</td>
<td>10/25/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>69</td>
<td>10/25/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>70</td>
<td>10/25/2011</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>71</td>
<td>10/25/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>72</td>
<td>10/25/2011</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>73</td>
<td>10/25/2011</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>74</td>
<td>10/25/2011</td>
<td>O121</td>
<td>(-)</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>75</td>
<td>10/25/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>76</td>
<td>10/25/2011</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>77</td>
<td>10/25/2011</td>
<td>O121</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>78</td>
<td>10/28/2011</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>79</td>
<td>10/28/2011</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>80</td>
<td>10/28/2011</td>
<td>O103</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>81</td>
<td>10/28/2011</td>
<td>O103</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>82</td>
<td>10/28/2011</td>
<td>O103</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>83</td>
<td>10/28/2011</td>
<td>O157</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>84</td>
<td>11/10/2011</td>
<td>O103</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>85</td>
<td>11/10/2011</td>
<td>O45</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Isolate Identification</td>
<td>Date Collected</td>
<td>O-group</td>
<td>stx1</td>
<td>stx2</td>
<td>eae</td>
</tr>
<tr>
<td>------------------------</td>
<td>----------------</td>
<td>---------</td>
<td>------</td>
<td>------</td>
<td>-----</td>
</tr>
<tr>
<td>86</td>
<td>11/10/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>87</td>
<td>11/10/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>88</td>
<td>11/10/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>89</td>
<td>11/10/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>90</td>
<td>11/10/2011</td>
<td>O103 (+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>91</td>
<td>11/10/2011</td>
<td>O103 (+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>92</td>
<td>11/10/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>93</td>
<td>11/10/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>94</td>
<td>11/10/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>95</td>
<td>11/10/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>96</td>
<td>11/14/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>97</td>
<td>11/14/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>98</td>
<td>11/14/2011</td>
<td>O157 (+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>99</td>
<td>11/14/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>100</td>
<td>11/14/2011</td>
<td>O157 (+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>101</td>
<td>11/14/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>102</td>
<td>11/14/2011</td>
<td>O157 (+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>103</td>
<td>11/14/2011</td>
<td>O157 (+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>104</td>
<td>11/14/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>105</td>
<td>11/14/2011</td>
<td>O157 (+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>106</td>
<td>11/14/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>107</td>
<td>11/14/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>108</td>
<td>11/14/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>109</td>
<td>11/14/2011</td>
<td>O157 (+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>110</td>
<td>11/14/2011</td>
<td>O157 (+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>111</td>
<td>11/14/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>112</td>
<td>11/31/2011</td>
<td>O26</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>113</td>
<td>11/31/2011</td>
<td>O111</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>114</td>
<td>11/31/2011</td>
<td>O26</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>115</td>
<td>11/31/2011</td>
<td>O145 (+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>116</td>
<td>11/31/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>117</td>
<td>11/31/2011</td>
<td>O45</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
</tbody>
</table>