Epidemiological studies on
Non-O157 Shiga toxin-producing Escherichia coli

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the
Graduate School of The Ohio State University

By

Anil Kenneth Persad DVM, MS

Graduate Program in Comparative and Veterinary Medicine

The Ohio State University

2016

Dissertation Committee:
Professor Jeffrey LeJeune (Advisor)
Dr. Gireesh Rajashekara
Dr. Qiuhong Wang
Dr. Joshua Daniels
Abstract

Shiga toxin-producing *Escherichia coli* (STEC), a diverse group of bacteria with over 400 serogroups, is estimated to cause over two million cases of human disease globally each year. STEC O157:H7 has been the pathotype most associated with human disease; however the number of human diseases associated with non–O157 serogroups has been increasing. STEC can be part of the normal gastrointestinal flora of animals and shed asymptotically. Humans are primarily infected via consumption of contaminated food or water, but can also be infected via direct-contact with animals and/or their environment and person to person transmission.

In chapter 2, we investigated the extent to which *E. coli* transfers from deer feces to soils where crops are cultivated in Ohio. Two experiments were performed assessing persistence and dissemination of *E. coli* in deer feces. In the first experiment, the total coliform and *E. coli* counts were determined in soil and surface debris samples collected from a vegetable production field naturally contaminated with deer feces and compared with samples collected away from any visible signs of fecal contamination. Samples were similarly assessed 60 days post-redmediation. In the second experiment, deer feces were deposited on soils in vegetable production fields and dissemination of *E. coli* in surrounding soils was determined at multiple distances over several time intervals. For produce grown in Ohio we can conclude: remediation of fields contaminated by deer
feces can minimize risk of transfer of microorganisms from feces to soil and employing no-harvest zones greater than 15cm around areas of fresh deer fecal contamination can minimize the risk of harvesting contaminated vegetables.

In chapter 3, our aim was to ascertain the minimum number of colony picks from a MacConkey agar plate required to recover at least one stx-positive colony from ruminant fecal enrichment samples testing PCR-positive for stx. There are over 200 STEC serotypes associated with disease and unfortunately, to date, highly selective and specific methods for the isolation of many of these serotypes are not available. Two studies using stx PCR-positive fecal samples obtained from cattle and small ruminants were performed. Enriched fecal samples were streaked onto MacConkey agar and suspect E. coli colonies screened for stx genes. Overall, at least one stx-positive colony was recovered from 79% of the PCR-positive fecal samples. Based upon the proportion of stx-positive E. coli present, selecting more than 20 suspect colonies per sample did not significantly increase in the probability of recovering a stx-positive colony.

In chapter 4, we investigated the prevalence of stx-positive colonies in healthy sheep and goats in Trinidad and Tobago. Based on PCR screening, goats had a higher stx prevalence than sheep. Upon culture, approximately two-thirds of all PCR-positive enrichments, regardless of species of origin, yielded colonies that only encoded stx1. The eae gene was detected in only three stx-positive isolates recovered from two different ovine enrichments. Possible risk factors for stx-positive carriage were also identified. From our results, we can conclude that stx-positive isolates shed by sheep and goats in Trinidad do not pose a significant risk to human health.
Acknowledgments

I am eternally grateful to my advisor Professor Jeffrey LeJeune for giving me the honor of being a member of his research team at The Ohio State University. I would like to sincerely thank him for all of his friendship, patience, guidance, support, and mentorship during both my Masters and PhD research. As a mentor he has not only fostered my development as a researcher, but also instilled in me the qualities of being a good supervisor, leader and mentor.

I would like to thank my committee members Dr. Gireesh Rajashekara, Dr. Qiuhong Wang and Dr. Joshua Daniels for their guidance and support.

To my parents, Seunarine Persad and Drupatee Persad, and sister Kavita Persad: I thank them for their love, support and encouragement, without them my academic and personal achievements would be impossible.

I wish to thank all my fellow lab members a.k.a. “My Wooster family”: Michael Kauffman, Pamela Schlegel, Jennifer Schrock, Nicholas Anderson, Ken Schenge, Michele Williams, Gayeon Won, Ana Castillo and Dr. Zhanqiang Su for their support, advice, friendship and encouragement during my five years in Ohio. I would also like to acknowledge all my Wooster friends especially Anand Kumar, Huang-chi Huang, Chung-Ming Lin and Ruby-Pina Mimbela, I hope we can have many for “pizza parties” in the future!
Finally I thank the major mentors in my life: Dr. Gustave Borde, for his guidance during my undergraduate studies and early Veterinary career; Dr. Jeffrey LeJeune for his mentorship during my graduate studies and introducing me to the sphere of Preharvest food safety; and my parents for being my life mentors. I thank you sincerely for all that you have taught me and for believing in me.
Vita

2002 – 2007 .................................. Doctor of Veterinary Medicine,
University of the West Indies, St. Augustine.

2007 – 2009 .................................. Teaching Assistant–Food Animal Medicine,
University of the West Indies, St. Augustine.

2009 – 2011 .................................. Research Assistant/Instructor/ Veterinarian
University of Trinidad and Tobago.

2011 – 2013 .................................. Graduate Research Fellow
M.S. Comparative and Veterinary Medicine
The Ohio State University.

2013 – 2016 .................................. Graduate Research Assistant,
PhD Comparative and Veterinary Medicine
The Ohio State University.
Publications


**Fields of Study**

Major Field: Comparative and Veterinary Medicine
# Table of Contents

Abstract ............................................................................................................................................ ii

Acknowledgments ................................................................................................................................. iv

Vita ......................................................................................................................................................... vi

Publications ........................................................................................................................................ vii

List of Tables ....................................................................................................................................... ix

List of Figures ..................................................................................................................................... x

Chapter 1: Literature Review: Shiga-toxin producing *Escherichia coli* ........................................ 1

Chapter 2: *Escherichia coli* in Ohio specialty-crop soils contaminated with deer feces. 133

Chapter 3: Isolation of non-O157 Shiga toxin – producing *Escherichia coli* from ruminant manure. ........................................................................................................................................... 156

Chapter 4: Shiga toxin (*stx*) encoding genes in sheep and goats reared in Trinidad and Tobago. ........................................................................................................................................... 179

Bibliography: ..................................................................................................................................... 214

List of Tables

Table 1.1  Categorization of STEC Sero-pathotypes associated with illness in humans................................................................. 83
Table 2.1  Rainfall and temperature data from sampling the different sampling blocks................................................................. 149
Table 3.1  Primers Sequences used in the PCR assays and the expected sizes of the products.............................................................. 173
Table 4.1  Multivariate effects logistic regression model of associations between farm management factors and prevalence of Stx in sheep and goat feces......................................................................................... 205
Table A.1  Comparison of results obtained from MALDI-TOF and conventional subtyping methods.............................................................. 292
List of Figures

Figure 2.1 Persistence of total coliform and *E. coli* in surface material and soil collected from clean and fecal contaminated (shaded) areas ........... 150

Figure 2.2 Comparison of total coliform (shaded) and *E. coli* (unshaded) counts at different distances from the site of fecal pat deposition on the different days ................................................................. 151

Figure 3.1 Comparison of probability rates for selecting one *stx* positive colony.. 171

Figure 3.2 Percentage of *stx*-positive isolates from each fecal sample screened in each study.......................................................... 172
Chapter 1: Literature Review: Shiga-toxin producing *Escherichia coli*
INTRODUCTION:

*Escherichia coli*, a gram negative rod shaped bacteria, is the predominant facultative anaerobic organism in the gastrointestinal flora of most vertebrate animals (H. C. Berg, 2008; Nataro & Kaper, 1998; Tenaillon, Skurnik, Picard, & Denamur, 2010). Theodore Escherich in 1885 was the first to identify *Escherichia coli* when he isolated it from the intestinal flora of infants. At that time he referred to it as *Bacterium coli commune*, but the name was subsequently changed to *Escherichia coli* in 1920, 9 years after his death (H. C. Berg, 2008).

*E. coli* isolates are primarily categorized based on serotyping of the somatic (O) and flagella (H) antigens (Nataro & Kaper, 1998). The somatic antigen, O, is determined by the polysaccharide segment of the cell wall lipopolysaccharide and the H antigen is based on flagella protein. There are 174 O antigens and 53 H antigens with multiple O and H combinations (Scheutz, Cheasty, Woodward, & Smith, 2004). Some *E. coli* isolates are non-motile since they lack a flagella and are consequently classified as NM (Gyles, 2007).

Intestinal colonization with *E. coli* can occur within hours of birth and many serotypes can exist as commensal organisms, rarely causing disease except in cases of breaches of intestinal integrity or immune-deficient hosts (Kaper, Nataro, & Mobley, 2004; Nataro & Kaper, 1998). All *E. coli* are not commensals, some have evolved to include virulence genes which allows them to cause intestinal and extra-intestinal disease (Gyles, 2007). This subset of *E. coli* which are able to cause disease are termed
‘Pathotypes’ (Kaper et al., 2004). Pathotypes which cause intestinal disease are further divided into 6 categories based on their disease causing mechanisms: (a) Enteropathogenic *Escherichia coli* (EPEC); (b) Enterohemorrhagic *Escherichia coli* (EHEC) / Shiga toxin-producing *Escherichia coli* (STEC); (c) Enterotoxigenic *Escherichia coli* (ETEC); (d) Entero-invasive *Escherichia coli* (EIEC); (e) Enteroaggregative *Escherichia coli* (EAEC) and (f) Diffusely Adherent *Escherichia coli* (DEAC). Those pathotypes which have evolved to cause extra-intestinal disease (e.g. meningitis or urinary tract infections) are termed Extra-intestinal Pathogenic *Escherichia coli* (ExPEC) or in the case of birds Avian Pathogenic *Escherichia coli* (Russo & Johnson, 2000).

In this next section we will briefly discuss the epidemiology of the *Escherichia coli* pathotypes associated with intestinal disease before proceeding to a more in depth discussion on Shiga toxin-producing *Escherichia coli*

**ESCHERICHIA COLI PATHOTYPES ASSOCIATED WITH INTESTINAL DISEASE:**

(i) **Enteropathogenic *Escherichia coli* (EPEC)**

EPEC was first associated with infantile diarrhea outbreaks in 1940s and continues to be a major cause of infantile diarrhea in developing countries resulting in over 1.3 million cases annually (Kenny et al., 1997; Lozano et al., 2012; Neter, 1955; Neter, Westphal, Luderitz, Gino, & Gorzynski, 1955). While EPEC doesn’t produce any toxins, it does produce attaching and effacing (A/E) lesions similar to STEC. However, unlike STEC, the target of EPEC cells is small intestine enterocytes and not the distal
colon (Kaper et al., 2004). The formation of these A/E lesions results in degeneration of the epithelial brush border microvilli and disruption of the architecture of microvilli leading to reduced absorption and consequently diarrhea (Kallonen & Boinett, 2016; Kenny et al., 1997). Infections with EPEC are most severe in children under two years of age and presents with acute onset diarrhea, vomiting, fever and dehydration (Kaper et al., 2004; Nataro & Kaper, 1998). Most cases of EPEC are self-limiting and can be treated with supportive therapy, but persistently infected persons may require the use of antimicrobials (Croxen et al., 2013). Many strains of EPEC however exhibit multidrug resistance especially to commonly used antibiotics (Subramanian et al., 2009).

(ii) Enterotoxigenic *Escherichia coli* (ETEC)

ETEC was first identified as a causative agent of human diarrhea in 1968 (Sack et al., 1971). All age groups are susceptible to infection with ETEC, but children are at greatest risk. This pathotype is one of the leading causes of Travelers’ diarrhea and estimated to cause almost 400 million cases of diarrhea annually in children less than five years resulting in almost 500,000 deaths (World Health Organization, 2006). ETEC is transmitted via the consumption of contaminated food and water and the infectious dose is approximately $10^8$ CFU (Nataro et al., 1995). ETEC as the name suggests produces two toxins, a heat stable (ST) and a heat labile toxin (LT), which mediate the occurrence of hypersecretory diarrhea. Briefly, ST causes diarrhea by stimulating intracellular cyclic guanosine 5’-monophosphate (cGMP) production. Increased cGMP inhibits intracellular fluid intake, resulting in increased intraluminal fluid volume (Evans Jr & Evans, 1996).
The mode of action of LT is different; it stimulates the activation of adenylate cyclase which produces cAMP which leads to hypersecretion of water and electrolytes into the lumen of the bowel (Evans Jr & Evans, 1996). ETEC usually presents as mild to profuse watery diarrhea and infected persons may also experience nausea, vomit, abdominal cramping, fever and headaches (Croxen et al., 2013; Qadri, Svennerholm, Faruque, & Sack, 2005). The duration of illness is usually three to five days, and the case fatality rate with proper treatment is less than one percent (Beatty et al., 2004; Dalton, Mintz, Wells, Bopp, & Tauxe, 1999; Qadri et al., 2005). Similar to EPEC, most cases of ETEC associated diarrhea are self-limiting with only supportive therapy required (Nataro & Kaper, 1998; Qadri et al., 2005). Anti-secretory drugs (e.g. loperamide) can be used in combination with antimicrobials (e.g. azithromycin) to reduce the associated diarrhea and duration of illness (Croxen et al., 2013). There are currently no vaccines available for preventing ETEC infections (Ahmed, Bhuiyan, Zaman, Sinclair, & Qadri, 2013).

(iii) Entero-invasive *Escherichia coli* (EIEC)

This pathotype was first identified as a cause of human diarrheal disease in 1971 (DuPont et al., 1971). EIEC is closely genetically related to *Shigella spp.*, and causes dysentery via the same mechanism as *Shigella* (van den Beld & Reubsaet, 2012). Despite their 80-90% nucleotide similarity, EIEC and *Shigella* can be easily differentiated based on their physiological and biochemical characteristics (Brenner, Fanning, Steigerwalt, Orskov, & Orskov, 1972; Brenner, Steigerwalt, Wathen, Gross, & Rowe, 1982; van den
Beld & Reubsaet, 2012). The infectious dose for EIEC and *Shigella* is estimated to be 10 CFU however the clinical signs of EIEC are usually less severe with most cases presenting with watery diarrhea (Kaper et al., 2004; Moreno, Gonçalves Ferreira, & Baquerizo Martinez, 2009). Diarrhea resulting from EIEC infection is not toxin mediated. The diarrhea results from the destruction of the colonic epithelium following invasion of the colon mucosa and intercellular movement by EIEC (Parsot, 2005). Invasion of the epithelial cells stimulate the release of inflammatory cytokines and chemokines resulting in the chemotactic movement of polymorphonuclear cells to the sites of mucosal and epithelial invasion resulting in inflammation and destruction of cellular architecture (Ud-Din & Wahid, 2014). The primary treatment method is oral rehydration since most cases are self-limiting. Additional treatment measures may include the antimicrobial drugs (e.g. azithromycin) especially in the cases of severe illness (Croxen et al., 2013).

**(iv) Enteroaggregative *Escherichia coli* (EAEC)**

EAEC was first associated with human disease in 1987 and has since been identified as an etiology of persistent diarrhea in children and a cause of Travelers’ diarrhea (Nataro et al., 1987; Okhuysen & Dupont, 2010). EAEC serotypes are heterogeneous and isolates carry a subset of virulence genes with no single factor being consistently associated with EAEC pathogenesis (Czeczulin, Whittam, Henderson, Navarro-Garcia, & Nataro, 1999; Navarro-Garcia, 2014; Okeke et al., 2010). EAEC adheres to epithelial cells of the small and large intestine forming a thick biofilm and
produce a combination of enterotoxins and cytotoxins which leads to tissue destruction (Nataro et al., 1987). Diarrhea can be watery to bloody and persistent in children (Wanke, Schorling, Barrett, Desouza, & Guerrant, 1991). One divergence from the classical EAEC infection model was the O104:H4 outbreak in Germany during 2011 (Rasko et al., 2011). This serotype in addition to having virulence genes associated with EAEC also had a prophage encoding for Shiga toxin 2 (Stx) production (Rohde et al., 2011). Stx encoding prophages are more commonly found in EHEC/STEC strains and not EAEC strains (Navarro-Garcia, 2014). The enhanced ability of EAEC to colonize intestinal epithelial cells, compared to STEC/EHEC strains, coupled with its ability to produce Shiga toxin made this EAEC hybrid more virulent than conventional STEC/EHEC and EAEC serotypes (Navarro-Garcia, 2014). Other incidences of EAEC acquiring the ability to produce Stx have also been described in Japan, France, northern Ireland and the Central African Republic (Croxen et al., 2013).

The treatment modality for EAEC infections is dependent on the presenting syndrome. For, travelers’ diarrhea the efficacy of antibiotics is dependent on geographical locations but antibiotics as ciprofloxacin, azithromycin and fluoroquinolones are usually indicated (Croxen et al., 2013). Anti-secretory drugs may be added to the treatment regime to reduce the severity of diarrhea. Unlike O157:H7, using antibiotics with Stx-producing EAEC actually reduces the Shiga toxin production (Steiner, 2014). For example, the German O104:H4 stereotype exhibited sensitivity to fluoroquinolones, carbapenems and azithromycin. The use of ciprofloxacin was shown to
reduce the progression to hemorrhagic uremic syndrome (HUS) and combination antibiotic therapy reduced the risk of patients developing HUS (Geerdes-Fenge et al., 2013; Menne et al., 2012)

(v) **Diffusely Adherent *Escherichia coli***

DAEC are a unique subset of diarrheagenic *E. coli* whose diffused attachment to the surface of epithelial cells is different to other *E. coli* adherence patterns (Croxen et al., 2013). DAEC has been recovered from the feces of both symptomatic and apparently healthy children and adults and the method of transmission is not conclusively known (Croxen et al., 2013; Mansan-Almeida, Pereira, & Giugliano, 2013). Once adhered to the epithelial cell surface, DAEC induces changes to the cellular cytoskeleton resulting in changes to microvilli architecture (Le Bouguenec & Servin, 2006; Servin, 2005). In young children infection with EAEC can present with watery diarrhea while it is speculated adults may be asymptotically infected which can progress to inflammatory intestinal diseases including Crohn’s Disease (Le Bouguenec & Servin, 2006). Treatment options are limited to supportive therapy and many strains exhibit multiple drug resistance (Croxen et al., 2013; Lopes et al., 2005).
(vi) Enterohemorrhagic *Escherichia coli (EHEC)* / Shiga toxin-producing *Escherichia coli* (STEC)

**Nomenclature:**

Shiga toxin-producing *Escherichia coli (STEC)* is also referred to as *Verocytotoxin-producing Escherichia coli (VTEC)*. Both STEC and VTEC are equivalent terms. The term VTEC is based on the cytotoxic effect observed in Vero cells (Konowalchuk, Speirs, & Stavric, 1977) while STEC reflects the fact that toxin produced is almost identical to Shiga toxin produced by *Shigella dysenteriae* (Nataro & Kaper, 1998). STEC serotypes associated with hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) are called Enterohemorrhagic *Escherichia coli (EHEC)* and these only represent of a subset of all STEC serotypes (Croxen et al., 2013).

**Introduction:**

Shiga toxin-producing *Escherichia coli (STEC)* is estimated to cause over 2 million cases of disease annually, with the laboratory confirmed incidence in 2014 the United States being 1,135 cases of illness with almost 25% requiring hospitalizations and 3 deaths (Caprioli, Morabito, Brugere, & Oswald, 2005; Crim et al., 2015). The actual incidence however is much higher due to under reporting and the annual incidence is estimated to be in excess of 265,000 cases of illness per year resulting in over 3,600 hospitalizations and at least 30 deaths annually (Scallan et al., 2011).
STEC was first identified as a foodborne pathogen 1982 when O157:H7 was associated with an outbreak of hemolytic uremic syndrome following consumption of contaminated food (Karmali, Steele, Petric, & Lim, 1983; Riley et al., 1983; J. G. Wells et al., 1983). STEC O157:H7 strains are believed to have evolved from the EPEC serotype O55:H7 via serial acquisition of virulence traits and a change in serotype (Zhang et al., 2007). The first step in the evolutionary process was lysogenization of the EPEC strain by a stx2-converting phage followed by acquisition of the O157 gnd-rfb locus which resulting in the serotype change from O55 to O157 (Tarr et al., 2000). Following serotype conversion, the pO157 plasmid was acquired and there was subsequent loss of sorbitol fermenting ability. The next step in the evolutionary process involved lysogenization by a stx1-converting phage and the finally the loss of β-glucuronidase activity (Feng, Lampel, Karch, & Whittam, 1998).

There are now over 400 known STEC serotypes (Farrokh et al., 2013), but most of these have not been associated with human disease (Bettelheim, 2007). The most pathogenic serotype for humans is O157:H7 and accounts for 75% of all STEC infections worldwide, but the incidence of human diseases associated with non-O157 STEC is increasing (Anonymous, 1998; Barkocy-Gallagher et al., 2005; Gould et al., 2013; Luna-Gierke et al., 2014; Scallan et al., 2011). For example, within the United States almost two thirds of human STEC cases during 2000 – 2008 were caused by non-O157 STEC serotypes (Scallan et al., 2011). In the United States, the main non-O157 STEC serogroups implicated in human disease are O26, O45, O111, O121, O103 and O145.
Globally, the serotype of importance may vary according to climatic and other environmental factors. In 2003, Karmali et al. proposed the categorization of STEC serotypes into 5 sero-pathotype categories based (A-E) based on their association with disease (Gyles, 2007; Karmali et al., 2003) (See Table 1.1)

STEC can be part of the normal gastrointestinal flora of many animals and these animals species can asymptptomatically shed the organism (Persad & LeJeune, 2014). Ruminant species including cattle are identified as a natural reservoir for STEC serogroups especially O157 but the predominant serotype can vary between animal species and geographic location (Gyles, 2007). Many other non-ruminant animal species including pigs, dogs, cats, rabbits, wildlife, fish and flies have been implicated as potential reservoirs, spill-over hosts or transient shedders (Ahmad, Nagaraja, & Zurek, 2007; Beutin, Geier, Steinruck, Zimmermann, & Scheutz, 1993; La Ragione, Best, Woodward, & Wales, 2009; Scaife, Cowan, Finney, Kinghorn-Perry, & Crook, 2006).
Main virulence factors:

(a) Shiga toxin.

The primary virulence factor associated with STEC infections is the production of Shiga toxin. Shiga toxin (Stx) was first identified in the 19th century when it was isolated from Shigella dysenteriae and was recovered from E. coli isolates almost 80 years later (Melton-Celsa, 2014). The Shiga toxin molecule is comprised of an A and B subunit. The A subunit inhibits protein synthesis by disrupting the 28S ribosomal subunit resulting in a “ribotoxic stress response” which is both pro-inflammatory and apoptotic (Jandhyala, Thorpe, & Magun, 2012). The B subunit has up to three binding sites for globotriaosylceramide-3 (Gb3) receptors which accounts for the high Stx specificity for Gb3 receptors (Ling et al., 1998; Nguyen & Sperandio, 2012).

There are 2 main types of Shiga toxins: Stx 1 and Stx 2. Both toxin types are encoded on prophages which are integrated into the E. coli chromosome (Croxen et al., 2013). Stx1 is almost identical to the toxin produced by S. dysenteriae, while Stx2 exhibits 55 – 60% similarity in amino sequence to stx1 but is genetically and immunologically distinct (Pennington, 2010). Stx1 is further divided in 3 subtypes (a, c, d) while Stx2 is divided into 7 subtypes (a – g), however Stx1a and Stx2a are the most toxin types associated with human disease (Croxen et al., 2013; Melton-Celsa, 2014). The disease in humans is more severe when the etiology is STEC serotypes which produce stx2 alone compared to STEC serotypes which produce stx1 alone. This is possibly due to the fact that stx2 is
over 1000 times more toxic to human renal endothelial cells compared to stx1 (Louise & Obrig, 1995)

(b) Locus of Enterocyte Effacement (LEE)

The LEE is a chromosomal pathogenicity island required for the formation of Attaching and Effacing (A/E) lesions (Gyles, 2007; Nguyen & Sperandio, 2012; Stevens & Frankel, 2014). A/E lesions represent the intimate attachment of STEC cells to the apical surface of enterocytes. These areas of attachment are usually pedestal like structures extending from the surface of enterocytes and almost enveloping the individual bacteria. The surrounding microvilli are usually destroyed.

The LEE consists of 41 genes and can be divided into five operons, LEE1 – LEE5. The first three operons are responsible for the Type III Secretion System, the fourth for a protein translocation system and the fifth is for the production of intimin and its receptor, Tir (translocated intimin receptor) (Gyles, 2007; Nguyen & Sperandio, 2012; Stevens & Frankel, 2014). Intimin is an outer membrane adhesin (94–97 kDA) produced by some STEC strains and is encoded for by the eae gene (Stevens & Frankel, 2014). There are over 17 different types of intimin proteins but one intimin type, γ1, has been commonly identified in highly pathogenic STEC serotypes (Blanco et al., 2005; Gyles, 2007; M. Rivas et al., 2006).

Following initial attachment of STEC to the intestinal epithelium, the Type III Secretion System ‘injects’ the protein Tir directly into cytoplasm of the epithelial cells.
Tir then becomes embedded in the apical plasma membrane of the epithelial cell in a hairpin-like structure with the central domain exposed at the epithelial cell surface, while the two terminal ends are embedded in the epithelial cell cytoplasm (Nguyen & Sperandio, 2012). The exact mechanism by which Tir embeds in the plasma membrane is unclear (Stevens & Frankel, 2014). Intimin located on the bacterial surface interacts with the exposed central portion of the Tir forming an intimate attachment between the STEC cell and the endothelial cell. The characteristic pedestal structure observed following attachment of STEC to intestinal epithelium is as a result the actions of Tir and the protein EspFu secreted by *E. coli*. Together they redirect actin formation and cytoskeletal structure resulting in actin accumulating below the area of STEC attachment eventually causing that area to become elevated and hence the pedestal lesion observed.

STEC isolates lacking the LEE pathogenicity island have also been implicated in HUS and HC in humans. STEC auto-agglutination adhesin (encoded by *saa* gene) has been identified in STEC isolates recovered from human HUS cases (Croxen et al., 2013; Jenkins et al., 2003; Paton, Srimanote, Woodrow, & Paton, 2001). Other proteins such sab, an auto-transporter associated with biofilm formation, and EibG, *E. coli* immunoglobulin binding protein G, have also been identified as contributing to attachment of LEE negative STEC isolates to the intestinal endothelial cells (Croxen et al., 2013).

(c) Additional Virulence factors.
Other putative virulence factors may be present but they are not essential for disease. These include enterohemolysin (ehlyA), long polar fimbriae (lpf), subtilase cytotoxin and outer membrane A (ompA) amongst others.
**STEC Transmission to Humans**

STEC transmission occurs via the fecal-oral route and most infections are a result of consumption of contaminated food products. Humans are infected with STEC via three methods: (a) **consumption of contaminated food or water**, (b) **direct contact with animals and/or their environment** or (c) **person to person transmission** (Gyles, 2007). The primary method of STEC transmission to humans is via consumption of contaminated food and water (Erickson & Doyle, 2007). Investigation of 90 confirmed STEC O157 outbreaks in the USA occurring between 1982 and 2006 revealed that food and water sources were identified as the origin for 64% of the infections, contact with animals and their environment accounted for 8% while person to person transmission accounted for 20% of outbreak cases (Snedeker, Shaw, Locking, & Prescott, 2009). Historically meat and animal products have been the source of most STEC diseases in humans (Painter JA, 2013; Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005), however due to increased abattoir sanitation, improved slaughter practices and increased consumer awareness the incidence of disease associated with animal products is decreasing.

While ground beef has been one of the main sources of STEC infection in the human population, quite interestingly, the second most common source of foodborne STEC outbreaks is not of animal origin but rather leafy vegetables (Painter JA, 2013). There were 502 reported foodborne disease outbreaks in the USA associated with leafy green consumption during the period 1973 to 2006 resulting over 18,000 cases of illness and at least 15 deaths (K. M. Herman, Ayers, T.L. & Lynch, M., 2008). Vegetable
consumption can be a major risk factor since most are eaten raw or with minimal cooking. Furthermore, once a bacterial contamination event occurs, in addition to surface contamination, bacterial contaminants can also be internalized within edible portions of plants rendering most post-harvest decontamination procedure ineffective (Mootian, Wu, & Matthews, 2009). The annual economic cost of STEC from plant sources is estimated to be over US 400 million dollars (Xicohtencatl-Cortes, Sanchez Chacon, Saldana, Freer, & Giron, 2009). In an attempt to reduce STEC transmission via vegetables, farmers are encouraged to adopt Good Agriculture Practices aimed at reducing the contamination of produce and also preventing contaminated produce from entering the food supply chain. Producers and regulatory agencies have also developed produce guidelines (e.g. Leafy Greens Marketing Agreement and Food Safety Modernization Act) aimed at ensuring the microbial safety of vegetable produce by reducing pre-harvest contamination (LGMA, 2013). In addition to food sources, both drinking and recreational water sources have also been implicated as the origin of STEC outbreaks in the human population (Rangel et al., 2005).

Contact with animals and their environment have also been identified as a potential risk factor for STEC transmission to humans resulting in almost 16,000 disease cases each year (Hale et al., 2012; Henderson, 2008). STEC has been recovered from the feces of many asymptomatic ruminant, non-ruminant livestock, pets, birds, wildlife and even fish species (A. K. Persad & LeJeune, 2014). Direct transmission from animals to humans has been recorded to occur on numerous instances especially at petting zoos and
animal fairs. For example, sheep and goats which are two popular animal species at petting zoos have been demonstrated in multiple studies to be shedding STEC asymptomatically with carriage rates being up to 100% (La Ragione et al., 2009; Schilling et al., 2012). Durso et al (2008) also reported that for each day someone visited an animal fair the risk of contracting STEC increased by 51% (L. M. Durso, Reynolds, Bauer, & Keen, 2005). The risk of contracting STEC exists by just being in the animal’s environment even if there is no direct animal contact. This was exemplified by Henderson et al (2008) who reported that by just visiting a cattle farm some was ten times more likely to contract STEC than someone who had not (Henderson, 2008). Once shed, STEC serotypes can persist in the environment for extended periods with one study reporting STEC surviving for up to 266 days in manure amended soil (Franz, van Hoek, Bouw, & Aarts, 2011). Such long persistence in the environment means persons can be exposed to STEC long after any physical evidence of fecal contamination has dissipated.
Clinical considerations of STEC associated disease.

Despite being identified as a foodborne pathogen over 30 years ago, Shiga toxin – producing *Escherichia coli* (STEC) continues to be a cause of morbidity and mortality in both developed and undeveloped countries (Havelaar et al., 2015). Globally, this bacterial pathogen transcends both socio and economic boundaries causing over 2.5 million cases of human disease annually, however the actual incidence is unknown since many lesser developed countries lack the required surveillance and diagnostic capabilities (Caprioli et al., 2005; M. Rivas, Chinen, Miliwebsky, & Masana, 2014). The incidence of laboratory confirmed STEC is lower in Canada compared to the United States (1.9 vs 2.1 cases per 100,000 persons) (Government of Canada, 2015; M. Rivas et al., 2014). Compared to these two North American countries, the overall incidence of laboratory confirmed STEC cases in Europe is much lower and reported to be 0.96 cases per 100,000 persons. However within Europe there were clear differences in the incidence between countries. For example while 16 of 25 countries reported the incidence of STEC being less than 1 case per 100,000 persons, others such as Denmark, Sweden and Ireland reported incidences greater than 3.00 cases per 100,000 people. The highest reported of any Europeans country was Ireland where the incidence was 4.41 cases per 100,000 persons (European Centre for Disease Prevention and Control, 2013). In addition to virulence factors, the development of STEC associated disease is dependent several host factors including age, health status, medication use, genetic factors as well as environmental factors and behavioral practices may increase the predisposition to developing STEC.
associated disease (Karmali, 2004; M. Rivas et al., 2014; Tarr, Gordon, & Chandler, 2005).

(i) Host:

Age has been identified as a risk factor for developing STEC infections. The young and elderly are the most susceptible, but young children especially those less than 5 years are at most risk of contracting STEC associated disease and developing HUS (Byrne, Jenkins, Launders, Elson, & Adak, 2015; M. Rivas et al., 2014; Wong et al., 2012). Surprisingly gender has also been identified as another risk factor. Females are more likely to contract STEC than males; the underlying reason for this gender bias is unknown, however possible hypotheses include females having different consumption practices, or having higher exposure due to their increased role in kitchens, or increased exposure when tending to children shedding STEC (Byrne et al., 2015; Centers for Disease Control and Prevention, 2006; Vally et al., 2012). Another potential risk factor which has been identified is ethnicity. Persons of Caucasian ethnicity are more likely to develop HUS than non–Caucasian persons (Byrne et al., 2015; Karmali, 2004).

(ii) Medication:

Two of the main physical body defenses against foodborne disease are the gastric acidity of the stomach and the physical barrier of the mucosa. A gastric acid pH of 4.0 or lower will result in greater than 99% of ingested organisms being killed. Use of Proton Pump Inhibitors (PPI; e.g. Oemprazole) for the treatment of ulcers and gastro-esophageal reflux disease increases the gastric pH thus decreasing the effectiveness of this chemical
barrier in reducing the bacterial population which may be present in food consumed (Morgan, Chidi, & Owen, 2015). Prolonged antibiotic use causes the death of commensal organisms in gastrointestinal organisms. These commensal bacteria via production of organic acids and inhibitory substances, immunomodulation or competitive exclusion inhibit the colonization and subsequent infection with STEC (Corr, Hill, & Gahan, 2009). The use of antibiotics to treat suspected cases of STEC infections is contra-indicated since it may actually precipitate the development of HUS (Wong et al., 2012). Antibiotic usage results in the induction of the phage lytic cycle, bacteriophage replication and subsequently increased Stx production (Tarr et al., 2005). The use of anti-motility drugs and non-steroidal drugs are also contraindicated in the suspected STEC or HUS cases (Tarr et al., 2005).

(iii) Environmental factors and practices.

In England, two studies carried out thirty years apart, both identified persons living in rural areas were more likely to contract STEC compared to persons living in urban areas (Byrne et al., 2015; Trompeter et al., 1983). While no reason for this observation is suggested, it could be as a result of rural areas having more livestock farms thus persons in these areas may have greater exposure to ruminants and their manure (Byrne et al., 2015). Other factors identified are swimming pools, consumption of raw milk or undercooked meat, consumption of raw vegetables, drinking well water, poor hygiene practices following contact with animals and their environment, attending daycare and poor hygiene (Hadler et al., 2011; Jalava, Ollgren, Eklund, Siitonen, &
Kuusi, 2011; McPherson et al., 2009; M. Rivas et al., 2008; Rivero et al., 2011; Vaillant et al., 2009; Werber et al., 2007).
**Clinical Disease:**

Humans can become infected with after consuming as little as 100 STEC CFU (Karmali, 2004). Human infections with STEC can be asymptomatic or result in a wide spectrum of clinical signs ranging from mild to bloody diarrhea, with approximately 4% of adult cases and 15% of children (Tarr et al., 2005) progressing to more severe clinical syndromes such as hemolytic uremic syndrome (HUS). Mortality rate can be up to 3%, and 70% of cases recover fully within 5 years however, but the remainder can suffer lifelong neurologic and kidney function deficits (Helge Karch et al., 2015). Young children, elderly persons and immuno-compromised persons are at greatest risk of contracting STEC infections and developing complications.

The onset of clinical signs following of consumption of STEC ranges between 2 to twelve days but the average incubation time is 3.7 days (Bell et al., 1994; Riley et al., 1983). The first symptom observed is diarrhea which could be accompanied by abdominal cramping and vomiting. These signs persist for 2 – 3 days after which 10% of cases recover while the diseases progresses to bloody diarrhea for the other 90% of cases (Tarr et al., 2005). The colon is hemorrhagic and the lamina propria is edematous with areas of focal necrosis and leukocyte infiltration (Karmali, 2004). This disease condition is called hemorrhagic colitis based on the colon pathology and clinical signs observed. The hemorrhagic diarrhea may persist for up to 5 days with approximately 4% of adult cases and 15% of children progressing to more severe clinical syndromes such as hemolytic uremic syndrome (HUS) while the rest recover (Tarr et al., 2005). HUS is
defined by three conditions: thrombocytopenia, hemolytic anemia and acute renal failure (Croxen et al., 2013). HUS is number one cause of acute renal failure among children. Renal failure is as a result of circulating Shiga toxins binding to Gb3 receptors located on renal glomerular endothelial, mesangial and tubular epithelial cells (Boyd & Lingwood, 1989; Lingwood, 1994; Robinson, Hurley, Lingwood, & Matsell, 1995; Takeda et al., 1993). The mechanism of action of Stx on susceptible cells is discussed earlier under the virulence factors.
Animal Reservoirs of STEC*

STEC is frequently recovered from the gastrointestinal tract of many animal species. Within the gastrointestinal tract, STEC can either be resident flora or transient but differentiation of this status is difficult since perennial isolation of the organism could be the result of replication within the gastrointestinal tract or from recurrent re-exposure. Intestinal carriage of most STEC serotypes by these animals is usually of little or no clinical significance to either animal or human hosts since many animals lack Stx receptors and many of serotypes shed by these animals lack the virulence genes required to cause disease in humans (Etcheverria & Padola, 2013; Friedrich et al., 2002; Gyles, 2007). In addition to potentially serving as a source of infection to humans, animals can also serve as a source of inter and intra-species transmission of STEC. The prevalence and magnitude of STEC in animals is dependent upon a complex interaction of many factors including: age, immunity, housing, diet, climate, sanitation, frequency and dose of exposure, colonization susceptibility, and duration of shedding.

Animals can be categorized as reservoirs, spill-over hosts or dead-end hosts of STEC. Reservoirs are animals capable of maintaining STEC carriage in the absence of continuous exposure, or those that frequently are re-exposed to STEC from environmental sources. One of the most recognized reservoirs of STEC is cattle, but many other ruminant animal species can also act as reservoirs (Gyles, 2007). Spill-over hosts are animals that are susceptible to STEC colonization but are unable to maintain this carriage unless there is continuous exposure. Animal species such including birds,
dogs and horses can act as spill – over hosts. Finally, animals may be classified as dead – end hosts, these animals are unable to propagate the dissemination of STEC naturally. Examples of dead – end hosts include fin and shellfish which only transmit STEC if they are consumed (Bennani et al., 2011; Gourmelon et al., 2006a; Manna, Das, & Manna, 2008). Due to varying exotic diets and absence of strict hygienic slaughter practices, STEC in animals from lesser developed countries can potentially be a serious threat to food safety (Cunin et al., 1999; Effler et al., 2001).

(a) Cattle

Cattle like most other ruminant species are asymptomatic colonized with STEC and are recognized as a primary reservoir for STEC, especially the serogroup O157 (Gyles, 2007). Cattle are exposed to STEC via the consumption of contaminated food and water, or via direct contact with shedding animals, and comparable to humans, the infectious dose of only 300 CFU is low (Besser, Richards, Rice, & Hancock, 2001) (Vanaja, Springman, Besser, Whittam, & Manning, 2010).

The lymphoid follicle-dense mucosa of the Recto-Anal Junction (RAJ) has been identified as the main site of STEC colonization in cattle (Naylor et al., 2003). Pruimboom-Brees et al. (2000) reported that despite evidence of Stx binding and Gb3 receptor expression in renal tubules and brains of calves or adult cattle there were no evidence of pathologic lesions in any of these tissues suggesting that cattle of all ages are inherently resistant to Stx (Pruimboom-Brees et al., 2000).
The average duration of shedding of STEC by cattle is thirty days but animals can shed STEC in their feces for up to one year (Lim et al., 2007; Widiasih, Ido, Omoe, Sugii, & Shinagawa, 2004). Most cattle shed STEC at levels of 10–100 CFU/g but a subsection of the cattle population may shed STEC at rates greater than $10^4$ CFU/g and are termed “super-shedders” and are possibly responsible for most environmental contamination observed (Chase-Topping, Gally, Low, Matthews, & Woolhouse, 2008; Matthews et al., 2006; Omisakin, MacRae, Ogden, & Strachan, 2003). This elevated rate of STEC shedding may be representative of a stage of colonization and can be observed within a population at any given time as opposed to a specific animal predisposition (Williams, Pearl, Bishop, & Lejeune, 2013).

STEC can be found on almost all cattle farms with almost all animals shedding STEC at some point with unpredictable fluctuations in herd prevalence. Age-wise, calves tend to shed STEC at the lowest levels just prior to weaning, however the highest shedding is exhibited in period immediately post-weaning (Nielsen, Tegtmeier, Andersen, Gronbaek, & Andersen, 2002). STEC shedding also tends to be seasonal, with shedding highest in summer and lowest during winter (D. Hancock, Besser, Lejeune, Davis, & Rice, 2001).

While STEC is primarily shed in the feces, there are rare occasions where it has been attributed as a causative agent of mastitis (D. Hancock et al., 2001; Lira, Macedo, & Marin, 2004). Additional instances of STEC recovery from milk have been attributed to poor milk hygiene and not to inframammary infections. The STEC O157 prevalence
within cattle herds has been reported to range from 0% to 71% (Cerqueira, Guth, Joaquim, & Andrade, 1999) while herd infection rates has been reported to be up to 100% (Farrokh et al., 2013). Globally, prevalence of STEC O157 in dairy cattle has been reported to range from 0.2% to 48.8% and 0.2 to 27.8% for beef animals, while the non–O157 STEC in dairy and beef cattle ranges between from 0.4% to 74% (H. S. Hussein & Bollinger, 2005; H. S. Hussein & Sakuma, 2005).

Pen design and management factors may influence the prevalence of STEC in cattle. Contaminated feed, water troughs are potential sources of STEC infection to animals. Drinking water troughs may be contaminated with STEC by shedding animals allowing for dissemination of STEC within herds (J. T. LeJeune, Besser, Merrill, Rice, & Hancock, 2001). STEC survival in these water troughs has been estimated to be over six months (J. T. LeJeune, Besser, & Hancock, 2001). Manure removal from pens via flushing of alleyways, housing on sawdust and inter-farm movement of animals have all been identified as risk factors for increasing the prevalence of STEC on cattle farms (Cernicchiaro et al., 2009; Garber, Wells, Schroeder-Tucker, & Ferris, 1999; LeJeune & Kauffman, 2005).

(b) Small Ruminants

Small ruminants, particularly sheep and goats, are important reservoirs of STEC O157 (La Ragione et al., 2009). While there has been considerable focus on the role of sheep in the epidemiology of STEC the role of goats are less well described (La Ragione et al., 2009). Unlike the USA, in many countries small ruminants are identified as ‘hosts
of significance’ in the epidemiology of STEC and cited as reservoirs of over 100 serotypes including O157 and O26 (Brandal et al., 2012; Gyles, 2007; La Ragione et al., 2009).

Transmission of STEC to small ruminants occurs via the same pathway as cattle and similarly they tend to be asymptomatic shedders (Beutin et al., 1993; Cortes et al., 2005). Unlike cattle, exclusive tropism for RAJ has not been described for all small ruminants (La Ragione et al., 2009). Following exposure to STEC O157, some studies report few attaching and effacing lesions are visible on the intestinal mucosa and the entire distal intestine including the caecum, colon and rectum were colonized, and not only the RAJ (Grauke et al., 2002; La Ragione et al., 2009). Other studies have however shown exclusive RAJ colonization in small ruminants challenged with STEC O157:H7 strains of human origin (Woodward et al., 2003). Both orally and rectally inoculated sheep exhibit similar shedding patterns indicating the terminal rectum can be colonized but low numbers of A/E lesions at the RAJ compared to cattle may account for reduced shedding time (Best et al., 2009).

In addition to consumption of contaminated lamb, mutton or chevon, many cases of human disease are attributed to direct contact with sheep and goats at petting zoos and open farms (Heuvelink et al., 2002; LeJeune & Davis, 2004). The prevalence of STEC among sheep and goats at these farms has been reported to be up to 100% (Schilling et al., 2012). This high carriage coupled with the inquisitive behavior of these animals increases risk of transmission of STEC via direct contact to humans (La Ragione et al.,
The consumption of contaminated unpasteurized milk and cheese made from goat and sheep milk has also been associated with human outbreaks (Cortes et al., 2005; Espie et al., 2006). The risk of STEC transmission between humans and sheep is exemplified in Norway where almost 50% of the O26 isolates recovered from sheep had similar MLVA profiles to those recovered from human clinical cases (Brandal et al., 2012).

The importance of sheep and goats in the epidemiology of STEC is without question. Over 50% of Spanish and 90% of Australian sheep flocks sampled have been reported to have at least one animal shedding STEC (Djordjevic et al., 2001; Oporto, Esteban, Aduriz, Juste, & Hurtado, 2008). While overall herd prevalence can be high the actual animal prevalence is much lower. In Spain, the individual sheep O157 prevalence was reported to be 7.8%, while the prevalence in England and Holland was reportedly much lower at 1.0% and 4.0% (Heuvelink et al., 1998; Milnes et al., 2008; Oporto et al., 2008). The prevalence of STEC O157 and non – O157 in goats in the USA has been reported at 11.1% and 14.5% respectively (Jacob, Foster, Rogers, Balcomb, & Sanderson, 2013; Jacob, Foster, Rogers, Balcomb, Shi, et al., 2013). The prevalence in STEC O157 in goats sampled at slaughter in Bangladesh was a similar 10% (Islam et al., 2008). The goat prevalence in Vietnam was reported to be a much higher 65% and 100% of herds sampled had at least one animal shedding STEC (Vu-Khac & Cornick, 2008). Similar to cattle, the shedding of STEC has been reported to be age and seasonal dependent. Young sheep tend to have a lower prevalence of STEC than older animals, while shedding
tended to be higher in warmer months (Battisti et al., 2006; Cortes et al., 2005; Franco et al., 2009; Kudva, Hatfield, & Hovde, 1996; Orden et al., 2008)

(c) Water buffalo (*Bubalus bubalis*)

   The water buffalo (*Bubalus bubalis*) has also been identified as an important reservoir of STEC O157 in many countries (Galiero, Conedera, Alfano, & Caprioli, 2005). The water buffalo is reared in many lesser developed countries as a multipurpose animal, used for draught power, milk production and meat production. The animals are able to thrive on poor quality forages and yet produce milk with high butterfat content as well have good growth for meat production (Rastogi & Rastogi, 2004). Large buffalo herds are reared in Latin America, Asia and Europe for milk and meat. The prevalence of STEC O157 in water buffaloes was reported to be 38% in Bangladesh while the prevalence in Italy and Vietnam was reported to be 14.5% and 27% respectively (Galiero et al., 2005; Islam et al., 2008; Vu-Khac & Cornick, 2008).

(d) Deer

   The role of deer in the maintenance and dissemination of STEC is often overlooked. The demand to feed an ever increasing human population has necessitated conversion of natural forested and grasslands towards agricultural production (Tscharntke et al., 2012). As a result of loss of their natural habitat, feral deer often share pastures with cattle and are often found feeding in close proximity to livestock farms or intruding onto gardens and agriculture fields (Boase, 2014; Giclas & Wetherington, 2016; Sterba, 2012). The close association between deer and livestock can result in deer disseminating
STEC between cattle herds (Renter, Sargeant, Hygnstorm, Hoffman, & Gillespie, 2001; D. H. Rice, Hancock, & Besser, 2003). Deer have also been implicated in causing foodborne disease after defecating on produce fields (Laidler et al., 2013).

Deer in the United State were first identified as a reservoir of STEC O157 in 1999 (Sargeant, Hafer, Gillespie, Oberst, & Flood, 1999). Initial cross-sectional epidemiological studies reported the 2.4% of deer sampled were actively shedding STEC in their feces (Sargeant et al., 1999). Subsequent studies have reported the prevalence of STEC to range from 0.2% to 8.0% (Bardiau et al., 2010; Dunn, Keen, Moreland, & Alex, 2004; French, Rodriguez-Palacios, & LeJeune, 2010; Kistler, Mulugeta, & Mauro, 2011; Sanchez et al., 2009). The first STEC O157 case associated with the consumption of venison was reported in 1997 (Keene et al., 1997). Since then there have been multiple reports of STEC O157 and non – O157 associated disease in humans following consumption of venison or products contaminated with deer feces (Hilborn et al., 2000; Laidler et al., 2013; Rounds et al., 2012).

(e) Other Ruminant species

Similar to deer, elk (Cervus canadensis) have also been associated with numerous foodborne disease outbreaks (Franklin et al., 2013; Laidler et al., 2013). The prevalence of STEC in deer in Colorado and Idaho has been reported at 8 and 22 % respectively (Franklin et al., 2013; Gilbreath et al., 2009). In both of these studies the prevalence of STEC in elk was higher than deer which shared the same grazing fields in these regions.
The American Bison (*Bison bison*) and cattle have similar morphological RAJ characteristics and is also a potentially important reservoir of STEC (Kudva & Stasko, 2013). There has been increasing bison meat consumption in the United States and with this also increasing reports of foodborne disease outbreaks associated with STEC consumption (Heiman, Mody, Johnson, Griffin, & Gould, 2015; K. Johnson, 2011). The prevalence of STEC O157:H7 in bison in the United States has been reported to be 42% (Reinstein, Fox, Shi, Alam, & Nagaraja, 2007). STEC O157 has also been isolated from the carcass of slaughtered bison at a prevalence of 1.13% (Li, 2004).

In addition to the species discussed above, STEC O157 and non-O157 STEC has been isolated from numerous other captive and wild non-domesticated ruminant species including llamas, moose, alpacas, antelopes, and yaks, (Chandran & Mazumder, 2013; Cid et al., 2012; Gilbreath et al., 2009; Leotta et al., 2006; Mercado, Rodriguez, Elizondo, Marcoppido, & Parreno, 2004; Mohammed Hamzah, Mohammed Hussein, & Mahmoud Khalef, 2013).

(f) Equine

There is a paucity of published data on the epidemiology of STEC carriage in horses and additionally, despite an extensive literature search no published case reports describing the clinical features of STEC infection in horses were found. From the available published data, equids including horses and donkeys are not major reservoirs of STEC but may instead act as spill-over hosts (Chandran & Mazumder, 2013; D. D. Hancock et al., 1998; Larson, 2009; Lengacher, Kline, Harpster, Williams, & Lejeune,
2010; Momtaz, Farzan, Rahimi, Safarpoor Dehkordi, & Souod, 2012; Pichner, Sander, Steinruck, & Gareis, 2005; Pritchard, Smith, Ellis-Iversen, Cheasty, & Willshaw, 2009). The prevalence of STEC O157 in horses ranged from 0 to 2.6% (D. D. Hancock et al., 1998; Larson, 2009; Lengacher et al., 2010; Pichner et al., 2005). In two of these studies, the positive horses were from farms which reared ruminants also, and in the Ohio study both the horse and its caprine stall mate had indistinguishable MLVA patterns (Larson, 2009; Lengacher et al., 2010). Despite the low STEC prevalence in horses, there are reported human clinical cases associated with infection from horse contact (Chalmers et al., 1997), and as such one must be cognizant of this risk.

(g) Swine

Unlike the previous animal species reviewed above swine can exhibit severe clinical disease following exposure to STEC (Ferens & Hovde, 2011). When exposed to STEC with the toxin type Stx2e, swine can develop a highly fatal disease called edema disease (Pruimboom-Brees et al., 2000; Waddell, Coomber, & Gyles, 1998). Pigs can be colonized with various serotypes of STEC including O157 but the risk of transmission to humans is low (Fairbrother & Nadeau, 2006; Ferens & Hovde, 2011; Kaufmann et al., 2006). The prevalence of STEC O157:H7 in domestic pig has been reported to range from 0% to up to 10%, with the prevalence in the United States usually being less than 2% (Chapman, Siddons, Gerdan Malo, & Harkin, 1997; Fairbrother & Nadeau, 2006; Ferens & Hovde, 2011; Richards et al., 2006).
The pathogenesis for the disease observed in pigs following exposure to STEC is different from humans. In human STEC infections, the main toxins produced are stx1a and/or stx2a and they target Gb3 receptors, however in edema disease stx2e is the main toxin produced and this toxin type exhibits receptor affinity for Globotertaosylceramide (Gb4) receptors (Imberechts, De Greve, & Lintermans, 1992; Muthing et al., 2012). The stomach submucosa, colonic mesentery, periocular tissues, larynx and brain are the main tissues affected. Weaned pigs are most susceptible to edema disease and onset of clinical signs is peracute with animals exhibiting anorexia, periocular edema, subcutaneous and submucosal edema, ataxia, incoordination, stupor and recumbency (Imberechts et al., 1992; Meisen et al., 2013). The case fatality rate for affected animals is high and treatment is usually ineffective. Despite a low prevalence of pathogenic STEC serotypes (Gyles, 2007), the potential for human infection from consumption of pork still exists as reported in a 2011 Canadian outbreak of STEC O157:H7 associated with consumption of pork (Trotz-Williams, 2012)

(h) Feral swine

Similar to deer, feral swine is another wildlife species which has been associated with STEC disease in the human population. These animals can contaminate vegetable production fields and serve as vectors for STEC transmission between livestock farms. Feral swine was first identified as a reservoir for STEC O157:H7 in 2007 following a STEC O157 outbreak associated with spinach consumption California (Jay et al., 2007). STEC O157 has also been detected in the feces of feral swine in multiple countries with
reported prevalence ranging from 3.3 – 8% (Sanchez et al., 2010; Wacheck, Fredriksson-Ahomaa, Konig, Stolle, & Stephan, 2010). In the Californian study, the prevalence of STEC O157 in feral swine was 14.9% (Jay et al., 2007). The prevalence of non-O157 in Spanish feral swine was reported to be 5.2% (Sanchez et al., 2010).

(i) Birds

Wild birds were first identified as a spillover-over host for STEC in 1997 (Wallace, Cheasty, & Jones, 1997) and since then numerous other avian species including starlings, pigeons, sparrows have been implicated in the transmission of STEC (Pedersen, 2007; Wallace et al., 1997). Contamination of surface water and vegetable production fields with fecal bacteria have been attributed to water fowl, including geese and ducks (Ankney, 1996; Kirschner et al., 2004; Moriarty, Weaver, Sinton, & Gilpin, 2012; Pedersen, 2007). An individual goose can produce over 2 kilograms of feces per day and given they are usually found in flocks, their presence can result in mass environmental contamination (J. Bedard, 1986). These birds have also been identified as harboring multiple foodborne pathogens (Kullas, Coles, Rhyan, & Clark, 2002; Murinda et al., 2004; Rutledge et al., 2013; Siembieda et al., 2011).

The migratory pattern of birds and the fact they can traverse long distances in a single day means they are an excellent mechanism for STEC dissemination over large distances. For example, the presence starlings (*Sturnus vulgaris*) on dairy farms are positively correlated with incidence of O157:H7 in bovine fecal pats (Cernicchiaro et al., 2012). The role of starlings as disseminators of STEC was conclusively demonstrated by
William et al. (2011) who reported that cattle on geographically distinct farms had identical MLVA subtypes. Starlings which flew between these farms were found to have the same MLVA O157 subtype as the cattle (Williams, Pearl, & Lejeune, 2011).

Many other wild and domestic species of birds have been reported to shed STEC in their feces (Foster et al., 2006; Kobayashi, Kanazaki, Hata, & Kubo, 2009; LeJeune, Homan, Linz, Pearl, 2008). The STEC prevalence in the avian species is low with prevalences reported being approximately 2% (Foster et al., 2006; L. A. Hughes et al., 2009; H. Kobayashi et al., 2009; LeJeune, Homan, Linz, Pearl, 2008; Nielsen et al., 2004). Experimentally, birds have also been demonstrated to shed STEC O157 at levels greater than 100 CFU/gram feces for up to 13 days post-challenge (Kauffman & LeJeune, 2011). Peri-domestic birds such as pigeons and finches share a common environment with many migratory birds and can be colonized with STEC when they are exposed to STEC shed in the feces of migratory birds. The peri-domestic birds can propagate the transmission of STEC when they deposit STEC in their feces close to areas frequented by humans such as, parks and other recreational areas (Foster et al., 2006; Morabito et al., 2001).

The prevalence of STEC O157 in domestic chicken is similar to wild avian species ranging from 0% to 1.5% (Chapman et al., 1997; Doane et al., 2007; Doyle & Schoeni, 1987; Ferens & Hovde, 2011). The STEC prevalence in turkeys was higher than that of chickens, with up to 7.5% of fecal samples testing positive (Doane et al., 2007; Ferens & Hovde, 2011). Experimentally inoculated chickens have been reported to shed
STEC O157 in their feces in excess of eleven months (Schoeni & Doyle, 1994). Other avian species such including canaries (Serinus canaria domestica) have also been identified as being positive for shedding STEC (Gholami-Ahangaran & Zia-Jahromi, 2012)

(j) Fish and shellfish

Fish and shellfish are not recognized as STEC reservoirs but instead classified as dead – end hosts. These animals are exposed to STEC when their aquatic habitat is contaminated with fecal matter from animals shedding STEC and STEC has been recovered from finfishes living close to livestock facilities (Tuyet et al., 2006). Shellfish, for example oysters, are filter feeders and able to concentrate and retain pathogens from their environment thus presenting a significant risk to humans health when these fish are consumed (Bennani et al., 2011; Guyon et al., 2000). Both STEC O157 and non – O157 STEC serotypes have been recovered from the carcass and intestinal contents of finfish and shell fish sold at markets (Gourmelon et al., 2006b; Gupta, Ghatak, & Gill, 2013; Sanath Kumar, Otta, Karunasagar, & Karunasagar, 2001; Surendraraj, Thampuran, & Joseph, 2010; Thampuran, Surendraraj, & Surendran, 2005)

(k) Rodents

Another animal species identified as being capable of harboring STEC within their gastrointestinal tract is rodents (Blanco Crivelli, Rumi, Carfagnini, Degregorio, & Bentancor, 2012; Cizek et al., 1999; Kilonzo et al., 2013; Nielsen et al., 2004). Both O157 and non – O157 STEC serotypes have been recovered from Rattus spp. residing on
farms and within houses in urban areas (Blanco Crivelli et al., 2012; Cizek et al., 1999). Experimentally, *Rattus spp.* have been demonstrated to not be long-term reservoirs of STEC but are still capable of shedding STEC at high concentrations for up to 16 days post inoculation and viable STEC can be recovered from rat feces up to nine months post defecation (Cizek, Literak, & Scheer, 2000). Their role in the dissemination of STEC on farms is without question as molecularly identical STEC serotypes have been recovered from both cattle and rat feces on farms (Nielsen et al., 2004). Although rats are not recognized as major reservoirs of STEC O157, they still play an important part in the epidemiology of STEC (D. D. Hancock et al., 1998; Nkogwe, Raletobana, Stewart-Johnson, Suepaul, & Adesiyun, 2011; D. H. Rice et al., 2003). There is a dearth of published information on the prevalence of STEC in naturally infected mice; however these animals have been used for many years as animal models for studying the pathogenesis of STEC (Krystle L. Mohawk, & Brien, 2011; Wadolkowski, Burris, & O'Brien, 1990).

(I) Raccoons (*Procyon lotor*)

Raccoons reside in a wide range of habitats including agricultural, forested and urban areas and can potentially serve as an excellent disseminator of pathogens. While multiple reports of raccoons being reservoirs for *Salmonella*, *Leptospira*, and *Campylobacter*, despite an extensive literature search, only one published report of STEC in raccoons could be found. (Beltrán-Beck, García, & Gortázar, 2012; Compton et al., 2008; M. Jay-Russell et al., 2010; K. Lee et al., 2011; Renter, Sargeant, Oberst, &
Samadpour, 2003). This animal was living in the hay bards of a dairy farm and could be a spill-over host as opposed to a reservoir (Shere, Bartlett, & Kaspar, 1998).

(m) Insects

STEC O157 has been recovered from numerous insect species including houseflies (Musca domestica), dump flies (Hydrotaea aenescens) and dung beetles (Catharsius molossus) (Alam & Zurek, 2004; Keen, Wittum, Dunn, Bono, & Durso, 2006; Szalanski, Owens, McKay, & Steelman, 2004; Xu et al., 2003). The transmission potential of houseflies was demonstrated when previously naïve calves were exposed to STEC inoculated houseflies and within 24 hours all calves were shedding STEC in their feces (Ahmad et al., 2007). In addition to acting as a vector for dissemination of STEC to animals, houseflies can also contaminate vegetable produce with STEC (Wasala, Talley, Desilva, Fletcher, & Wayadande, 2013). Houseflies are potentially spill over hosts and not just mechanical vectors, since STEC could be recovered from their gastrointestinal tracts up to three days post inoculation (M. Kobayashi et al., 1999).

(n) Pets

Pets have a close interaction with their human owners, which can result in an exchange of microbiota resulting in the possible transmission of STEC between species. Common animal pets including dogs, cats, rabbits and birds have all been identified as being either reservoirs or spill – over hosts of STEC (Beutin, 1999; Gholami-Ahangaran & Zia-Jahromi, 2012; Hogg et al., 2009; LeJeune & Hancock, 2001; Panda et al., 2010; Roopnarine, Ammons, Rampersad, & Adesiyun, 2007). Dogs (Canis lupus familiaris) and
cats (*Felis catus*) are capable of shedding a diverse range of STEC serotypes in their feces (Bentancor et al., 2012; Beutin, 1999; Beutin et al., 1993; Hogg et al., 2009; LeJeune & Hancock, 2001; Roopnarine et al., 2007). While both O157 and non–O157 serotypes have been recovered from dogs, only non–O157 serotypes have been reported in cats. Cats have also implicated as possible vectors in transmission of the highly a pathogenic 145:NM serotype to humans in both Argentina and Germany (Busch et al., 2007; Rumi, Irino, Deza, Huguet, & Bentancor, 2012). Human infections following exposure canids have also been reported (Anonymous, 2011).

(o) Animal models

Animal models provide valuable insight into STEC intestinal colonization, pathogenesis, immune responses and treatment efficacy (Mayer, Leibowitz, Kurosawa, & Stearns-Kurosawa, 2012; K. L. Mohawk, Melton-Celsa, Zangari, Carroll, & O'Brien, 2010). Many animal species including mice, rats, chickens, rabbits, cows, greyhounds, baboons, and macaques have been used animal models of STEC infection (Garcia et al., 2006; Kang et al., 2001; K. L. Mohawk & O'Brien, 2011; Sueyoshi & Nakazawa, 1994; Taylor et al., 1999; Tzipori et al., 1986; Zotta, Lago, Ochoa, Repetto, & Ibarra, 2008). The mouse models are preferred for in vivo STEC for a number of reasons including their availability, size, cost and easy husbandry (K. L. Mohawk & O'Brien, 2011). Four of the more popular mouse models are (i) streptomycin treated model, (ii) streptomycin and mitomycin C/ ciprofloxacin treated, (iii) intragastric fed but not streptomycin treated and (iv) the malnourished mouse models (Melton-Celsa & O'Brien, 2003). The response of
mouse models to STEC inoculation is dependent on the method of inoculation, the strain of STEC and mouse model used (Melton-Celsa & O'Brien, 2003)

Detection and Isolation of STEC

STEC O157:H7, the serotype first associated with human disease, has been the focus of multitudinous research projects aimed at improving isolation of this pathogen (Wang et al., 2013). Consequently, a number of culture based methods have been developed to aid in the isolation of O157:H7 (Beutin & Fach, 2014). For the isolation of STEC O157, the commonly employed methods include colony hybridization, immunomagnetic separation, and chromogenic media have been developed to aid in the detection, differentiation and isolation of O157:H7 colonies. These assortments of tests do not exist for all non-O157 serotypes and attempts have made to extrapolate O157:H7 techniques for non-O157 serotypes but have not had the same success (Bettelheim, 1998a; Beutin & Fach, 2014; Cooley et al., 2013; Gill, Huszczynski, Gauthier, & Blais, 2014; Wylie et al., 2013).

Although multiple molecular tests have been developed to detect the presence STEC, culture based methods are still regarded as the standard for pathogen detection (Buchan et al., 2013; Wang et al., 2013). Furthermore isolation of a colony is required for additional confirmatory biochemical, serological and molecular analysis as well as for epidemiological investigations (Baker, Rubinelli, Park, Carbonero, & Ricke, 2016; Wang et al., 2013).

Enrichment of Samples:

STEC when present in feces, are usually present in such lows numbers that it can be almost impossible to isolate via direct plating (Chase-Topping et al., 2008; Delbeke et
al., 2015; Jeffrey T LeJeune, Dale D Hancock, & Thomas E Besser, 2006). A number of various protocols are used to enhance the sensitivity of detection and recovery of STEC isolates from samples, but the efficacy of many of these methods have not been validated or only validated for use for a finite number of serotypes in limited matrices (Noll et al., 2015). While the composition of the enrichment media may vary, the essential goal is to increase the population of the target population by providing a supportive nutritional base for its growth (Baker et al., 2016). Additives such as bile salts, antibiotics (e.g. Novobiocin, Vancomycin) and other ionic salts (e.g. Potassium tellurite) are sometimes included in these enrichment formulations to make them more selective for growth of the target population by reducing the growth of back ground flora (Beutin & Fach, 2014).

Enrichment of samples in a non-selective media provides the benefit for allowing recovery of chemically or thermally stressed STEC cells which may not grow in a highly selective media (Rocelle, Clavero, & Beuchat, 1995; Bavo Verhaegen, De Reu, Heyndrickx, Van Damme, & De Zutter, 2015). Increasing the sensitivity of STEC detection can be achieved by non-selective enrichment at high temperatures (35 – 41.5 ± 1°C) which tends to retard the growth of background fecal flora but still allow proliferation of STEC populations in fecal samples (Barkocy-Gallagher et al., 2005; L. M. Durso, 2013; Jeffrey T LeJeune et al., 2006). Additionally, it should be noted that while the use of highly selective broth may enhance the growth of certain STEC serotypes, it can also inhibit the growth of others and caution must be exercised when adding these selective agents to avoid false-negative results (Baylis, 2008; Gill et al.,
Following enrichment, samples are usually screened for the presence of Shiga toxins genes (stx) after which positive samples are subjected to colony isolation procedures (Beutin & Fach, 2014; Bryan, Youngster, & McAdam, 2015; Lisa M Durso, 2013; Wang et al., 2013). In the following section we briefly discuss methods for screening of enriched samples for STEC and discuss methods of isolating STEC colonies including four media types commonly used.

**STEC screening**

The methods employed for the detection of STEC can be classified as being either molecular or phenotypic:

(i) *Phenotypic assays* - Beutin and Fach (2014) identifies the use of tissue culture cytotoxicity assays as the ‘gold standard’ for Shiga toxin detection (Beutin & Fach, 2014). In these assays Vero or HeLa cell lines are used because of their high concentration of Gb3 and Gb4 receptors (L. Rivas, Mellor, Gobius, & Fegan, 2015a). Cell monolayers are inoculated with sterile filtrates of the enriched sample and observed for cytotoxicity. The degree of cytotoxicity observed can vary with the Stx variant (Dorothea Orth et al., 2007). Differentiation of toxin type requires the addition of Stx-specific antisera to neutralize the effects of the toxin in mono-cell layer. Additionally neutralizing antisera is required to ensure the cytotoxicity observed is as a result of Shiga-toxins and not due to the effect of virulence factors which may be present in background flora. While tissue culture cytotoxicity assays are reportedly more sensitive than
immunological assays, this method is not commonly used in epidemiological studies because it is time consuming, requires specialized equipment and usually requires 72 hours before results can be interpreted (Beutin & Fach, 2014; J. C. Paton & A. W. Paton, 1998; L. Rivas et al., 2015a).

Immunological based tests are also used for the detection of STEC in various matrices (Beutin & Fach, 2014). The basis of these tests is the interactions between polyclonal or monoclonal bodies and target surface antigens on specific STEC serogroups or toxins. Although these tests offer quick results (<4 hours) with high sensitivity and specificity there is always the risk for false positive or false negative test results especially since Stx toxin variants can be missed or cross reaction between serogroups (Wang et al., 2013). Stx detection can be enhanced by adding Mitomycin C to the enrichment broth (Johnson, Thorpe, & Sears, 2006). Another disadvantage of this method is that organisms are not isolated and subsequent isolation and identification of the STEC serotype can be challenging (Rivas et al., 2015a).

(ii) DNA based detection – Molecular testing has better sensitivity and higher throughput than phenotypic assays and are independent of culture facilities and the need for antisera (Beutin & Fach, 2014; Bryan et al., 2015; Noll et al., 2015). Theoretically, with highly purified and properly extracted DNA, PCR methods should be able to identify as little as one molecule of DNA in a sample (Hedman & Radstrom, 2013). Conventional and RT PCR assays have been developed for the detection of certain O serogroups, H antigens, Intimin and stx and its variants. These PCR assays can be run as single PCR reactions
with only one set of primers targeting one specific gene or it can be multiplex targeting with multiple primer sets targeting multiple genes (Baker et al., 2016). Real-time PCR detection allows for very low detection limits is included as part of the STEC screening process recommended by USDA-FSIS (Bryan et al., 2015; Chui et al., 2011; Gerritzen, Wittke, & Wolff, 2011; Wang et al., 2013). The interpretation of PCR results can be confusing at times since multiple STEC colonies may be present in the sample and one cannot identify the origin of the virulence genes without isolation and subsequent re-screening of \( \text{stx} \)-positive colonies. Additional drawbacks to the use of PCR assays include the presence of PCR inhibitors in the sample which can prevent amplification of DNA (Bessetti, 2007) and there can also be amplification of DNA from dead cells. Current European and American regulatory protocols recommend the screening of samples for the Shiga toxins encoded by \( \text{stx1} \) and \( \text{stx2} \) genes and Intimin encoded for by \( \text{eae} \) gene (Beutin & Fach, 2014). Possible outcome classification of samples when screened with PCR assays for \( \text{stx} \) and \( \text{eae} \) are:

<table>
<thead>
<tr>
<th>( \text{stx} )</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Eae} ) Positive</td>
<td>Potentially</td>
<td>Possible EPEC Strain or STEC Strain that has lost ( \text{stx} )</td>
</tr>
<tr>
<td>Pathogenic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{Negative} )</td>
<td>Low risk</td>
<td>No STEC present</td>
</tr>
</tbody>
</table>

Following PCR screening, there is still need for isolation of STEC isolates and confirmation of the presence of virulence genes.
**Metagenomics:** Metagenomics is a culture independent method for analysis of the all the genetic material within a given environment (Culligan, Sleator, Marchesi, & Hill, 2014). While conventional 16S rRNA sequencing allows for taxonomic classification up to the genus or possibly species level, shotgun metagenomic sequencing can be used to identify the possible contaminating organism at the species or subspecies level and can also identify the virulence genes (Leonard, Mammel, Lacher, & Elkins, 2015). Leonard et al. (2015) demonstrated that *E. coli* O157 could be only detected from a 100g of spinach spiked at concentration of 10,000,000 CFU/100g. The authors also reported *E. coli* O157:H7 and the virulence genes present could be detected if samples inoculated at 10 CFU/100g were incubated in an enrichment broth at for 24hrs following inoculation (Leonard et al., 2015). Similar to conventional PCR assays metagenomics analysis can result in the detection of DNA from dead cells and as such samples can be classified as being positive. This can be problematic especially in cases where food samples have been subjected to pasteurization (Bergholz, Moreno Switt, & Wiedmann, 2014).

**Isolation of STEC colonies:**

(i) *Colony hybridization* offers a highly sensitive and specific method for the identification of STEC colonies from an agar plate. This method is standard STEC isolation technique used in many diagnostic laboratories. Oligonucleotide probes were developed based on highly conserved regions of the toxin types allowing identification of all Shiga toxin types, and under stringent washing conditions probes could be used to differentiate between Stx1 and Stx2 (James C Paton & Adrienne W Paton, 1998). In
epidemiological studies where large numbers of samples are processed, this method is unsuitable due to its laborious and time consuming nature (Beutin & Fach, 2014).

(ii) Another method used to recover and isolate STEC isolates from samples is *immuno–magnetic separation assays (IMS)*. Microscopic paramagnetic beads coated with specific anti O-antibodies are mixed with an aliquot of the sample. Target STEC isolates bind to these beads as a result of surface O antigen – antibody affinity. The beads with the bound STEC isolates are then recovered from the application of a magnetic field and undergoes multiple washing to remove any inhibitors (James C Paton & Adrienne W Paton, 1998). The washed beads are then plated on non-selective agar plates or indicator media supplemented with chemicals or antibiotics to improve their selectivity. The efficiency of detection and recovery of STEC by IMS is dependent on a number of factors including the matrix the organism is in, the target organism and the interaction between the antigen and antibody coated paramagnetic beads (Cooley et al., 2013; Dwivedi & Jaykus, 2011). Additionally, it should be noted that there can be non-specific binding between beads and non-target STEC serotypes (Cooley et al., 2013). The use of IMS is further limited since assays have not been developed for all STEC serogroups (Beutin & Fach, 2014; Wang et al., 2013).

(iii) *Culture media*

The ideal selective culture media should be one that is supportive of the growth of STEC serotypes, but inhibit the growth of other background organisms. The large number of STEC serotypes coupled with the fact that (1) at this time there are no identified
common traits that differentiates them from other bacteria and (2) serotypes possess varying physiological and biochemical properties and antibiotic sensitivities makes development of the perfect selective media for all serotypes almost impossible.

**MacConkey agar** – MacConkey agar (MAC) is a minimally selective media used for the isolation of gram-negative enteric bacteria. Bile Salts and crystal violet additives inhibit the growth of gram positive bacteria while allowing enteric gram-negative organisms to proliferate. Enteric gram-negative bacteria ferment lactose resulting in drop in the pH around the bacteria colonies and there is a color change in the neutral red pH indicator and hence the pinkish - purple color of the colonies. For recovery of STEC, MAC is supportive of the growth of most STEC strains but offers limited specificity since growth of other enteric lactose fermenters are not inhibited (Gill et al., 2014).

**CHROMagar® STEC** – CHROMagar® STEC is a chromogenic media which allows phenotypic differentiation of STEC colonies from background flora. STEC colonies are mauve in color while non –STEC colonies are blue, colorless or inhibited. While CHROMagar®STEC offers high STEC specificity, the sensitivity is reduced as reported by Verhaegen et al. and others (Church, Emshey, Semeniuk, Lloyd, & Pitout, 2007; B. Verhaegen, De Reu, Heyndrickx, & De Zutter, 2015; Wylie et al., 2013). Gill et al. (2014) evaluated CHROMagar® STEC against 7 other agar formulations for the growth of 96 strains of STEC and reported that this media was the most inhibitory to STEC growth (Gill et al., 2014). Growth of STEC on CHROMagar® STEC was found to be strongly correlated with the presence of the *Ter* gene complex. This gene complex which
encodes for tellurite resistance, is comprised for four essential genes (*TerB, TerC, TerD* and *TerE*) and bacteria lacking this gene are unable to grow on CHROMagar® STEC agar (B. Verhaegen et al., 2015). Most STEC serotypes outside of the ‘big 6’ and O157:H7 lack these *Ter* genes and as such will not grow on CHROMagar® STEC (Orth, Grif, Dierich, & Wurzner, 2007; Tzschoppe, Martin, & Beutin, 2012).

**Rainbow Agar** – Developed originally for detecting and isolating O157, Rainbow agar (RA) is another chromogenic media which allows phenotypic differentiation of STEC colonies. The media tests for the presence of two enzymes β-galactosidase and β-glucoronidase. STEC O157:H7 is usually β-galactosidase negative and as such the colonies are charcoal grey to black in color (Bettelheim, 1998b). Non-O157 colonies may appear a variety of colors including pink, mauve, white or blue grey (Bryan et al., 2015; Gill et al., 2014). Antimicrobial supplements including Novobiocin, Potassium tellurite and cefixime trihydrate are added to RBA to enhance its selectivity against non-STEC cells but these agents have also shown to reduce the sensitivity of the media (Gill et al., 2014).

**Enterohemolysin agar** – Enterohemolysin agar is a non-selective indicator media comprised of nutrient source (e.g. Tryptose Blood agar base) supplemented with washed defibrinated sheep blood (Beutin & Fach, 2014). The media function on the premise that the virulence factor Enterohemolysin, encoded by the genes *ehxA/ehlyA*, is present and will produce narrow turbid zones of hemolysis around the colonies. While the majority of O157 strains possess this virulence factor and will be identified using this media, for non
– O157 STEC less than 50% have these genes and will consequently not be detected (Beutin & Fach, 2014). While over 85% of non-STEC E. coli strains evaluated did not possess this virulence factor, there are exceptions such as EPEC strains O26:H11 which contain the *ehxA/ehlyA* genes and will appear identical to STEC isolates *(Beutin & Fach, 2014; Lin et al., 2012; Wang et al., 2013)*

**Possé Media** – This media was first described by B. Possé et al. (2008) and was designed to allow for color based separation of STEC serogroups O26, O103, O111 and O145 (Posse, De Zutter, Heyndrickx, & Herman, 2008). The media is comprised of a MacConkey agar base supplemented with sucrose, bile salts, novobiocin, potassium tellurite, 5-Bromo-4-chloro-3-indolyl-β-galactopyranoside and isopropyl-β-D-thiogalactopyranoside. While this media offers good specificity, it also inhibits the growth of STEC strains and is reported to have a comparable sensitivity to CHROMagar®STEC (B. Verhaegen et al., 2015).

From the literature cited above, it is evident that there is no media type which provided high sensitivity and specificity for STEC growth. Additionally it is discernible that addition of selective agents to increase culture specificity has the negative effect of hindering growth of certain STEC serotypes. Recovery of STEC serotypes from agar plates may possibly accomplished by screening an appropriate number of colonies from a minimally selective media.

**Serotyping of STEC**
(i) Agglutination reactions – STEC serotypes are identified based on the observation for agglutination reactions between somatic and flagellar antigens and various antisera (Ewing, 1986). There are in excess of 400 different serotypes of STEC which makes serotype identification via agglutination reactions time consuming, laborious and requiring a wealth of reagents. Furthermore, strains of STEC may spontaneously agglutinate or cross react with antisera or in some cases no reactions are observed (Beutin & Fach, 2014). Serotyping of STEC via this method is usually limited to the ‘Big 6’ and O157 serogroups in many laboratories with only few specialized reference laboratories using agglutination for identification of all STEC serotypes ("European Centre for Disease Prevention and Control. Fourth external quality assessment scheme for typing of verocytotoxin-producing E. coli (VTEC), Stockholm, ECDC," 2014).

(ii) Molecular based – Numerous PCR assays for identification of the STEC ‘big 6’ and O157 isolates recovered from culture agar have been developed (Wang et al., 2013). Most PCR assays designed for serogrouping of STEC target the \(wzx\) and \(wzy\) genes however other genes such as the \(gnd\) gene have also been used for serogrouping of STEC (Beutin & Fach, 2014). In most serogroups, genes encoding for O antigen flippase (\(wzx\)) and O antigen polymerase (\(wzy\)) are sufficiently unique to allow differentiation of serogroups (DebRoy, Roberts, & Fratamico, 2011). Other than the 7 main STEC serogroups associated with human disease, there are at minimum DNA sequences for 88 other O-antigen clusters deposited in GeneBank. Currently there are primer sequences
available for O antigen identification and differentiation of at least 58 STEC serogroups (DebRoy et al., 2011). Multiplex PCR assays have also been developed to rapidly screen if isolates belong to the 7 main serogroups of STEC and characterize the main virulence genes (stx1, stx2, eae, ehlyA) present (Bai, Shi, & Nagaraja, 2010; Z. Paddock, Shi, Bai, & Nagaraja, 2012). Primers have also available to the typing of 47 H antigens based on variations on the fliC gene (Beutin & Fach, 2014).

(iii) Matrix Laser Desorption/Ionization Time of Flight (MALDI – TOF) -
MALDI – TOF is a library-based approach to bacterial identification, offering a rapid, reproducible method with a high sensitivity and specificity and at minimal cost (Christner et al., 2014; Kliem & Sauer, 2012). MALDI-TOF uses the mass-to-charge ratio profile of bacterial microbial proteins and peptides for bacterial identification (Sandrin, Goldstein, & Schumaker, 2013). This mass-to-charge ratio profile or mass spectral profile analysis is usually confined to the 2 – 20 kDA which is representative of ribosomal proteins since these proteins are less influenced to by variability in cultivation conditions (Dingle & Butler-Wu, 2013). Bacterial isolates can be characterized at the genus and species level via the identification of a unique biomarker ion peak(s) or by matching the mass spectral profile or “fingerprint” of query bacteria with the spectral profiles of known bacterial species within the established MALDI-TOF library using pattern recognition algorithms (Carbonnelle et al., 2011; Dieckmann & Malorny, 2011; Karger et al., 2011; Kliem & Sauer, 2012). Currently the designers of MALDI –TOF analytical systems do not offer standardized proprietary spectral libraries for differentiation between Shigella spp. and E.
coli serotypes. Users wishing to attempt to identify E. coli serotypes via MALDI-TOF are required to create their own E. coli serotype reference libraries. There is a paucity of published literature describing use of MALDI-TOF to serotype E. coli but recent research has shown promise for the development of MALDI-TOF identification protocols. Clarke et al. (2013) reported that they were able to correctly classify 136 E. coli isolates based on pathotypes (Clark et al., 2013). Later, Christner et al. (2014) reported success in being able design reference spectra that correctly classified 292/293 E. coli isolates obtained from the German O104:H4 outbreak and elsewhere (Christner et al., 2014).

Commonly used STEC Subtyping methods:

(i) Pulse Field Gel Electrophoresis (PFGE) – PFGE was first identified in 1984 as method of separating large fragments of DNA (J. Besser, 2015). The first use of PFGE in O157:H7 outbreak surveillance investigations was in 1994 in Minnesota when four O157:H7 outbreaks were only identified based on clustering of molecular subtypes (Bender et al., 1997). Since then it has become the ‘gold standard’ method used in many laboratories for the subtyping of O157:H7 serotypes due to its high discriminatory power and has been extensively used for subtyping of O157:H7 in many epidemiological studies (Beutin & Fach, 2014; Karama & Gyles, 2010).

Briefly, bacterial cells are suspended in agarose plugs and cells treated with cell lysis/proteinase K which lyses bacterial cell walls and inactivates any endogenous nuclease. The plugs are then repeatedly washed to remove any cellular debris after which the purified DNA is treated with restriction enzymes (e.g. XbaI). The restriction
enzyme cleaves DNA into fragments ranging from 20 Kb to 1Mb or greater in size. DNA fragments are then separated via gel electrophoresis and fragments visualized under U.V light. PFGE patterns are interpreted using imaging software following which dendrograms to illustrate similarities can be developed (L. Rivas, Mellor, Gobius, & Fegan, 2015b). This method of STEC molecular subtyping can be laborious, technically demanding and requires standardized protocols to be religiously followed if inter-lab comparisons of PFGE patterns are to be done (Dallman et al., 2015; Gerner-Smidt et al., 2006; Karama & Gyles, 2010).

(ii) **Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA)** – MLVA is a rapid method of molecular subtyping of STEC with the similar discriminatory power as PFGE but offers higher throughput, portability and robustness (Hyytia-Trees, Cooper, Ribot, & Gerner-Smidt, 2007). Prior to the recent introduction of whole genome sequencing, PFGE and MLVA were the only methods employed by PulseNet for the surveillance of STEC O157 (L. Rivas et al., 2015b).

Variable Number Tandem Repeat (VNTR) loci are amplified via a multiplex PCR assay using fluorescently labelled primers located in highly conserved areas which flank the VNTR loci (L. Rivas et al., 2015b). The size of each amplicon is determined using high resolution capillary electrophoresis and the copy number determined. Using this copy number, the number of VNTR at each locus can be calculated and a numeric MLVA profile is generated. The MLVA profile is then compared to other profiles to type the bacteria. While a common MLVA protocol for O157 is well established there is no
established non-O157 MLVA protocol since and each STEC serotype may have different set of VNTR loci (L. Rivas et al., 2015b). Some researchers have suggested increasing the number of VNTR loci from 7 which is sufficient to discriminate between O157:H7 subtypes to 10 VNTR loci for subtyping of non-O157 serotypes (González, Sanso, Lucchesi, & Bustamante, 2014; Løbersli, Haugum, & Lindstedt, 2012). In addition to deciding on the number of VNTR loci to be used for subtyping non-O157 STEC, consensus also has to be arrived on use of common PCR reagents and primers and electrophoresis platforms all of which can affect the reproducibility of MLVA data (L. Rivas et al., 2015b)

(iii) Whole Genome Sequencing (WGS) – WGS allows for high throughput molecular subtyping of bacterial isolates with higher discriminatory resolution than existing methods (Bergholz et al., 2014; Chattaway et al., 2016). Theoretically WGS can differentiate between isolates whose genomes differ by a single nucleotide (Salipante et al., 2015). Another tangible benefit of WGS is faster detection of foodborne disease outbreaks and easy sharing of data between laboratories (Bergholz et al., 2014). Chattaway et al. (2016) reported that WGS offered higher resolution for serotyping of non-O157 serotypes compared to traditional agglutination methods (Chattaway et al., 2016). In that study as 27% (38/140) of the isolates tested could not be assigned a serogroup based on agglutination reactions, but almost all of these isolates (37/38) were serogrouped using WGS. This study also compared PCR screening of virulence genes to WGS, and once again WGS performed better. Another study conducted by Joensen et al.
(2014) compared WGS with traditional typing methods (PFGE) for STEC isolates recovered from human patients (Joensen et al., 2014). In this study WGS provided results not only faster and lower cost than traditional typing methods but was also able to accurately discriminate between outbreak and sporadic isolates. While WGS offers fast results with high discriminatory resolution, there are still many drawbacks to this method including high cost of equipment and the need for bioinformatics expertise (Singhal, Kumar, Kanaujia, & Virdi, 2015).
PREHARVEST MEASURES TO REDUCE THE PREVALENCE OF STEC

Effective control of STEC encompasses all stages of food production. Preharvest food safety can be defined as the sum of on farm interventional strategies implemented to reduce the rate of foodborne pathogen contamination of animal and vegetable produce and in turn reduce human exposure (T. E. Besser, Schmidt, Shah, & Shringi, 2014; LeJeune & Wetzel, 2007). Supplemental to reducing contamination of food products, on-farm mitigation strategies will also reduce environmental contamination and incidence of disease associated with direct animal contact (Karmali, Gannon, & Sargeant, 2010).

One of the earliest underpinnings of modern preharvest food safety programs in the United States was the successful bovine tuberculosis eradication program. Authorities from 1917 to 1942 were able to reduce the incidence of tuberculosis in both humans and cattle by instituting a *Mycobacterium bovis* eradication program in the cattle population (Smith, Novotnaj, & Smith, 2010). Preharvest programs have also been instituted to reduce STEC carriage in livestock and to prevent contamination of vegetable produce. Reducing STEC carriage in animals has been shown to reduce the STEC contamination of carcasses. This was demonstrated by Arthur et al. (2009) who reported that if the prevalence of STEC O157:H7 was less than 20% in cattle pens then carcass contamination post evisceration was 0%; conversely if STEC prevalence in pens was greater than 20% then carcass contamination post evisceration was 0.7% (Arthur et al., 2009). STEC contamination of vegetable produce is also of particular concern, since vegetable produce is often eaten raw and most post-harvest intervention strategies for
decontamination are ineffective; contamination prevention thus paramount to reduce human exposure to STEC (Herman, Hall, & Gould, 2015; Solomon, Yaron, & Matthews, 2002). In this section we will briefly discuss some on-farm strategies to mitigate the risk of STEC contamination of livestock products.

**Diet:**

There a myriad of studies discussing the effect diet of on animal carriage of STEC serotypes especially O157: H7. The vast majority of these studies have focused on bovine carriage of STEC O157:H7 and the general consensus is both the constituent of diet as well its hygiene influences the prevalence (J. Berg et al., 2004; T. R. Callaway, Carr, Edrington, Anderson, & Nisbet, 2009; Jacob, Callaway, & Nagaraja, 2009; Jacob & Nagaraja, 2012; LeJeune & Wetzel, 2007).

(i) Feed Composition: Manipulating the overall grain and forage composition of the beef cattle diet as a potential method of reducing generic *E. coli* shedding in cattle was first reported in 1967 by Brownie and Grau (Brownlie & Grau, 1967). Later based on research findings of Diez-Gonzalez et al. (1998), it was recommended that beef cattle be switched to all hay diets five days prior to slaughter to reduce generic *E. coli* contamination of abattoirs (Diez-Gonzalez, Callaway, Kizoulis, & Russell, 1998). Such interventions, while improving carcass hygiene were found no have no effect on the overall carcass weight, dressing percentage and carcass grades (T. R. Callaway et al., 2009). The impact of such drastic shift in diet composition on STEC O157:H7 in beef cattle was first described by Keene et al. (1999) who reported that beef cattle whose diet
was switched from grain to hay had a significantly lower prevalence of STEC compared to those who continued to receive all grain diets (18% vs 52%) (T. R. Callaway et al., 2009). Conversely, while short term drastic shifts in feeding to all forage diets prior to slaughter may reduce O157 shedding, it should be noted that long term feeding of all forage diets does not have that same effect on O157 shedding and may actually increase shedding in some cases.

One plausible explanation for this difference in generic E. coli and STEC O157:H7 prevalence observed with drastic shifts to hay based diets is there increased undigested matter reaching the large intestine which undergoes fermentation producing L-lactate which is known to have antimicrobial effects against E. coli O157 and non-O157 E. coli isolates (McWilliam Leitch & Stewart, 2002; Shelef, 1994). Another possibility is that forages used to produce hay may contain phenolic compounds which can inhibit growth of E. coli and O157:H7 (Berard et al., 2009; J. Wells, Berry, & Varel, 2005). One theory postulated by Callaway et al (2009) is that due to the diet change, the digestive products reaching the colon are suitable for growth of other bacterial species and thus via exclusion prevent STEC colonization (Callaway et al., 2009). This change in microbial population either as a result of antimicrobial effects of L-Lactate and/ or phenols or change in substrate matter, is supported by Rice et al. (2012) who reported significant changes at the phyla and genera level in the fecal microbiome of animals fed wet distillers’ grain versus animals in the control group (Rice, Galyean, Cox, Dowd, & Cole, 2012). Another theory postulated by Callaway et al. (2009) is that undigested
material reaching the large intestine mechanically removes STEC O157:H7 by a scraping action at the Recto-anal junction which is its site of colonization (Callaway et al., 2009).

Another factor that influences the prevalence of STEC is the constituents that make up the diet (Berg et al., 2004). Animals fed corn based diets were found to have a shed generic *E. coli* in their feces at higher levels compared to animals fed barley but conversely had lower STEC O157:H7 prevalence and shed STEC O157:H7 at lower levels (J. Berg et al., 2004). The effect of distillers’ grain on the prevalence of STEC O157 is debatable. There are numerous research articles but reporting that inclusion of distillers’ grain increases shedding of STEC while others report that its inclusion has no effect on prevalence of O157:H7 (Durso et al., 2012; Fink et al., 2013; Hallewell, Barbieri, Thomas, Stanford, & McAllister, 2013; Jacob, Paddock, Renter, Lechtenberg, & Nagaraja, 2010; Paddock et al., 2013; Wells et al., 2009). These differences may be due to a combination of effects including differences in the diet formulation, inter-animal variations, management factors and variability in strains of O157:H7 used in the experiments. Supplementation of feed with antibiotics such as tylosin and neomycin have also been explored as mechanisms to reduce STEC carriage in livestock and while they have shown some effectiveness in trials, their use is not recommended due possibility of antimicrobial resistance development (Callaway et al., 2009).

The method of diet preparation can also affect O157:H7 prevalence, for example animals fed a dry rolled grain diet had lower O157:H7 prevalence than animals fed steam flaked grain diets (Fox, Depenbusch, Drouillard, & Nagaraja, 2007). Steam flaking of
grain increases the ruminal digestibility of grain in the rumen of beef cattle compared to dry roll processing (Owens, 2005). Consequently there is the possibility of increased post ruminal digestion and fermentation of undigested material when dry rolled grain is fed compared to steam flaked and this may account for the decrease in STEC O157:H7 for reasons discussed above.

(ii) Direct-fed Microbials (DFM) – Also called Probiotics, are defined by the United States Food and Drug Administration as ‘products that contain live organisms’ (U.S. Food and Drug Administration CPG Sec. 689.100 Direct-Fed Microbial Products).

These microorganisms when fed in sufficient quantities can elicit some beneficial health effect on the host (Jacob & Nagaraja, 2012; LeJeune & Wetzel, 2007; Soon, Chadd, & Baines, 2011). The concept of consuming microorganisms to improve health was first suggested by Elie Metchnikoff at the beginning of the 20th century; he postulated that enriching the gut microflora with lactic acid producing bacteria could have beneficial health effects since these organisms prevented the putrefaction of fermented milk (Tannock, 1997).

DFMs have been fed to cattle for many years primarily to improve growth rate, milk production and overall production efficiency but its use as a method of reducing carriage of foodborne pathogens is more recent development (Callaway et al., 2009; LeJeune & Wetzel, 2007). The main DFMs investigated to reduce STEC O157:H7 prevalence in cattle include Streptococcus bovis, Sacchromyces cerevisiae, Escherichia coli, Propionibacterium freudenreichii but most intervention studies have focused on
**Lactobacillus spp.** especially the strain *Lactobacillus acidophilus* (NP51) (Jacob & Nagaraja, 2012; Wisener, Sargeant, O'Connor, Faires, & Glass-Kastra, 2015). There are three proposed modes of action by which these DFMs exert their effects against STEC O157:H7: (a) production of antibacterial properties, (b) competitive exclusion and (c) Immunomodulation. While these mechanisms have been demonstrated to be practical in small animal models and *in vitro* studies, the mode(s) of action of DFMs in live cattle warrants further investigation (Jacob & Nagaraja, 2012). Overall, a meta-analysis of 20 research papers conducted by L.V. Wisener et al. (2015) concluded that feeding DFMs to cattle was a method of significantly reducing fecal prevalence of STEC O157:H7 (Wisener et al., 2015). The authors however concluded that since most of these studies were conducted at research farms, these results may not be applicable to commercial feedlots due to differences in cattle stocking density, geographic conditions and other factors which may affect pathogen transmission.

(iii) **Prebiotics** – These are organic compounds that cannot be digested by the host animal but are available for utilization by the microbial population within the gastrointestinal system (Callaway, Edrington, Harvey, Anderson, & Nisbet, 2012; LeJeune & Wetzel, 2007). The addition of prebiotics to animal diets has been demonstrated to increase microbial diversity of the gastrointestinal tract and herein may be one mechanisms of reducing STEC colonization (Callaway et al., 2012). Prebiotics such oligosaccharides reach the large intestine undegraded and are utilized by colonic bacteria resulting in amplification of colonic microbial populations, which may prevent
STEC colonization. In vitro studies have also demonstrated the possibility of prebiotics reducing the adhesion of STEC O157:H7 to colonic mucosa (Baines et al., 2011). Although prebiotics have been used in humans and multiple other livestock species, its use in ruminants have been limited due to (a) the large size of the rumen (b) degradation by rumen microbes and (c) cost (Callaway et al., 2012; Callaway, Edrington, Loneragan, Carr, & Nisbet, 2013; Soon et al., 2011). In calves however, the use of prebiotics have been associated with increased body weight, feed conversion efficiency and decreased incidence of diarrhea (Nagpal, Shrivastava, Kumar, Dhewa, & Sahay, 2015).

**Bacteriophages:**

Bacteria are susceptible to infection with viruses called Bacteriophages. These viruses have a narrow target spectrum and can be found in the environment and within the gastrointestinal tracts of many animals (Callaway et al., 2013; LeJeune & Wetzel, 2007). Once infected, the bacteriophage directs the host bacteria to produce copies of the bacteriophage and stimulate lysis of the cell thereby releasing multiple copies of the bacteriophage into the surrounding environment (Callaway et al., 2013). The bacteriophage progeny can then infect other target cells and the replication procedure is repeated as before. Bacteriophage therapy is most effective when a cocktail of different bacteriophages are used and also helps to reduce incidence of bacterial resistance to phages (Callaway et al., 2013; Soon et al., 2011). Bacteriophage cocktails which target
both O157 and non O157 STEC, quickly initiate production of progeny and stimulate cell lysis are desired since rapid destruction of cells reduces the opportunities for target cells integrate phage DNA into their genome (Joerger, 2003). Bacteriophages will persist as long as the target Besserbacteria are present. The use of both rectally and orally administered bacteriophage cocktails in cattle has been demonstrated to reduce but not eliminate carriage of STEC O157:H7 (Rozema et al., 2009; Sheng, Knecht, Kudva, & Hovde, 2006). Ruminants naturally infected with bacteriophages have also been demonstrated to be more resistant to colonization with STEC O157:H7 (Raya et al., 2006). Soon et al. (2011) suggested that bacteriophage therapy is most effective if O157:H7 populations are greater than $10^4$ CFU/g (Soon et al., 2011). While the use of bacteriophages has been shown to be effective under experimental conditions, the production of a product which can be used on a large scale has not been as successful (Callaway et al., 2013; LeJeune & Wetzel, 2007).

**Feed and Water Hygiene:**

Feed and water can be vehicles for the entry and dissemination of O157 and non – O157 STEC with animal populations. This was confirmed by Joris et al. (2013) who recovered molecularly identical O157 and non-O157 STEC serotypes from cattle, feed and water toughs (Joris, Verstraete, De Reu, & De Zutter, 2013). STEC is transmitted via the fecal – oral route, thus maintaining proper hygiene of feed and water is potentially one critical step in reducing its transmission on the farm. Contamination of feed and
water troughs may occur by both domestic and wild animals, including birds defecating in these troughs or in the case of ruminants via rumination action resulting in the oral cavity being contaminated with STEC which is then transmitted via saliva when these animals eat or drink (Besser et al., 2014; LeJeune, Homan, Linz, Pearl, 2008; Soon et al., 2011).

LeJeune et al. (2001) identified water troughs as a potential long term reservoir for STEC O157 and a source of infection for cattle (LeJeune, Besser, & Hancock, 2001). In that study, O157:H7 was able to persist for more than 6 months in contaminated water troughs. Other studies have reported the water trough contamination with O157 to range between 1.3 to 35% (Joris et al., 2013; Lammers et al., 2015; LeJeune, Besser, Merrill, et al., 2001; Van Donkersgoed et al., 2001). Water in water troughs could be contaminated by animals shedding STEC in the pen or contaminated at its source. The potential contamination of drinking water at its source was demonstrated by Won et al. (2013) who reported that up to 4% of wells in dairy intensive areas were contaminated with *E. coli* O157 (Won, Gill, & Lejeune, 2013). The STEC contamination of water troughs has also been demonstrated to be seasonal and parallels that seen in cattle, with the STEC prevalence in water troughs being highest during summer and lowest in winter (Besser et al., 2014; Van Donkersgoed et al., 2001). Intervention strategies to improve water hygiene may include regular cleaning and removal of sediment from water troughs (Ahmadi et al., 2007; LeJeune, Besser, Merrill, et al., 2001). The use of water chlorination as a method of reducing transmission is debatable, while it may be effective
for treating water contaminated at its source it is not effective in reducing STEC in contaminated water troughs (LeJeune, Besser, & Hancock, 2001; J. LeJeune et al., 2004). Other methods suggested for reducing STEC in water troughs include electrolyzed oxidation of water or addition of compounds such as cinnamaldehyde and sodium caprylate, but the effectiveness of these measures when applied to farm scenarios and their effects on water palatability need further investigation (Callaway, 2010).

In addition to the type of feed given to animals have affecting the prevalence of STEC, the feed can also be a source of STEC contamination (Soon et al., 2011). Feed can be contaminated at its source or when placed in feed troughs with up 17% of feed troughs and 4% of feed components before being placed in troughs being contaminated with STEC (Doane et al., 2007; Joris et al., 2013). STEC has been demonstrated to persist on soils for over four months, thus grazing animals can be exposed to the STEC via the feces of STEC shedding wild or domestic animals or by untreated manure spread onto pastures (Jones, 1999).

**Non – Livestock Animals:**

Many domestic and wild animals living on or in close proximity to livestock production farms can act as vectors of both O157 and non – O157 STEC. The potential role of insects and animals as reservoirs, spillover hosts or dead end hosts of STEC were discussed in the previous section under animal reservoirs of STEC. From the literature cited in that section, these animals play a significant role in the transmission and
dissemination of STEC on farms and their exclusion from access to farm livestock can potentially result in reduction of STEC prevalence on farms.

**Vaccination**

There are two commercial STEC O157 vaccines approved for use in bovid: (1) a Type III Secretion System (TTSS) protein based vaccine and (2) a siderophore receptor and porin (SIR) based vaccine. Both vaccines have been evaluated extensively in field studies and have their use has been found to be protective against STEC. For example, a meta-analysis of data from 8 research manuscripts on the TTSS based vaccine and 4 manuscripts on the SIR based conducted by Snedeker et al. (2012) concluded that odds ratio for recovering STEC from cattle vaccinated with the TTSS based vaccine was 0.38 and 0.42 for cattle receiving the SIR based vaccine (Snedeker, Campbell, & Sargeant, 2012). Due to serotype specificity, these vaccines do not offer good cross protection against STEC serotypes other than O157 (Asper, Sekirov, Finlay, Rogan, & Potter, 2007). These two approved vaccines have two different modes of action:

(a) Type III Secretion System protein based vaccine. This vaccine stimulates the production of mucosal antibodies preventing adherence and eventual colonization of bovine intestinal mucosa with STEC O157 (Besser et al., 2014). The efficacy of this vaccine is improved by using multiple doses with three doses reported to reduce STEC colonization by up to 98% and decrease shedding of STEC by 73% (Peterson, Klopfenstein, Moxley, Erickson, Hinkley, Bretschneider, et al., 2007; Peterson, Klopfenstein, Moxley, Erickson, Hinkley, Rogan, et al., 2007).
(b) Siderophore receptor and porin (SIR) based vaccine. This vaccine works by stimulating production of antibodies which bind to SIR on the outer membrane of the cell thereby blocking iron transport into the bacteria. This action results in the STEC being able to amplify less efficiently than other intestinal microflora thereby reducing its potential colonization of the intestinal mucosa (Smith, 2014). Similar to the results observed with the TTSS vaccine, administration of multiple doses elicited the greatest efficacy with a three dose vaccine regimen reducing STEC recovery by 85% compared to control animals, and reduced the prevalence of ‘super shedders’ by almost 73% (Cull et al., 2012; Thomson et al., 2009). Overall, given that these two vaccines function via separate mechanisms, the possibility exist that vaccine efficacy can be increased via synergistic effects from combination vaccination therapy (Besser et al., 2014). Further investigation is however needed to determine the duration immunity of these vaccines.

**Summary and Conclusion:**

From the literature cited in this section it is apparent that there exist numerous potential preharvest measures which if applied correctly either individually or in combination can reduce the STEC prevalence especially serogroup O157 in the bovine population. However with increasing incidences of non–O157 infections in the human population, research needs to be directed to interventional on farm strategies which can be applied to not only reduce non–O157 carriage in cattle but other livestock and non-livestock species.
PREHARVEST FOOD SAFETY RESEARCH IN TRINIDAD AND TOBAGO

Globally, there are an estimated 600 million cases of foodborne disease resulting in over five hundred thousand deaths annually (Havelaar et al., 2015). Foodborne disease, defined as diseases which occur as a result of ingestion of foodstuffs contaminated with microorganisms or chemicals, is one of the major causes of diarrheal diseases and poses a major health and economic burden to many Latin American and Caribbean countries (Pires, Vieira, Perez, Lo Fo Wong, & Hald, 2012). Trinidad and Tobago (T&T), located in the Southern Caribbean is one such country where foodborne disease affects almost 10% of the human population annually with an economic burden conservatively estimated to be in excess of US$135 million dollars (Lakhan, Badrie, Ramsubhag, Sundaraneedi, & Indar, 2013). The actual incidence and etiology of foodborne disease in T&T and most Caribbean countries is however unknown due to the absence of proper surveillance or diagnostic programs necessary for detecting the occurrence of foodborne disease and/or identifying the responsible pathogen (Pires et al., 2012).

Foodborne disease can be attributed to enteric bacterial, viral or parasitic pathogens, chemical contaminants or biotoxins (World Health Organization, 2008). Within Latin America and the Caribbean, bacterial pathogens were responsible for almost 70% of the recorded outbreak cases during the period 1993 – 2010 (Savio, 1999). Meat and dairy products were the origin for 30% of the outbreaks, 10.6% originated from water and vegetable sources accounted for almost 9% (Pires et al., 2012). Many of these bacterial pathogens (e.g. Shiga toxin-producing Escherichia coli, Salmonella,
Campylobacter and Listeria spp.) can be part of the resident enteric flora of animals and shed in animal feces.

Humans are infected either via consumption of food and water contaminated with the feces of these animals; via direct contact with the animal or its environment; or via person to person transmission. The infectious dose is dependent on the pathogen, but can be as little as 10 cells (Kothary & Babu, 2001). Onset of clinical signs is variable, and infections can be asymptomatic or manifest with a spectra of clinical signs including, mild to bloody diarrhea, fever, vomiting and systemic disease. Infections can be self-limiting or become systemic resulting in chronic disease and/or potentially life threatening complications. The young, elderly and immune-compromised are usually at greatest risk of infection and developing complications.

Historically most bacterial foodborne disease has been mainly associated with the consumption of meat and animal products, however the incidence of foodborne disease associated with vegetable consumption, especially leafy greens has been increasing (K. M. Herman et al., 2015). Fecal contamination of vegetables is of particular concern, since many vegetables are eaten raw or with minimal cooking and this coupled with the fact that post-harvest intervention strategies for decontamination are ineffective makes contamination prevention of paramount (K. M. Herman et al., 2015; Solomon et al., 2002). In an effort to decrease the incidence of foodborne illness many producers have adopted programs such as the ‘farm to fork’ approach which encourages the implementation of pre-harvest practices (Good Agriculture Practices (GAPs)) and post-
harvest measures such as Hazard Analysis Critical Control Plans (HACCP) aimed at minimizing the risk of pathogen contaminated produce entering the food chain (Food & Administration, 2010; Health & Services, 1998; Newell et al., 2010; Olaimat & Holley, 2012).

Although bacterial foodborne disease affects a large section of the Caribbean population annually, there is a dearth of published literature focusing on pre-harvest food safety in T&T and the wider Caribbean. In this section, we will focus on two main bacterial causes of bacterial foodborne disease and the research gaps which exist in understanding the epidemiology of STEC and Salmonella in T&T.

**Shiga toxin-producing *Escherichia coli***:

Shiga toxin-producing *Escherichia coli* is estimated to cause over 2 million cases of disease globally each year yet there are no reported cases of human disease in T&T (Caprioli et al., 2005). Interestingly, despite this apparent absence of human illness attributable to STEC, evidence of STEC in humans was reported by R. Roopnarine et al (2007) who reported that approximately 1.4% of dairy farmers feces sampled were positive for *stx genes* (Roopnarine et al., 2007). The virulence properties of these isolates were however not reported; and despite an exhaustive literature search there were no other published reports of STEC surveillance in the human population.

STEC in T&T was first reported by A.A. Adesiyun (1993) in beef and pork samples collected from fresh meat markets (AA Adesiyun, 1993). The first report of STEC in livestock was made in 1994, when 14% of dairy cattle, 11% of pigs and 23% of
sheep sampled were positive for STEC (Abiodun A Adesiyun & Kaminjolo, 1994). In that study however, the serogroups present were not identified nor were virulence profiles evaluated. STEC including serogroup O157 has since been detected in raw bovine milk samples collected from milk churns and bulk milk tanks (Adesiyun, Stoute, & David, 2007; Adesiyun, Webb, Romain, & Kaminjolo, 1997). STEC is rarely associated with mastitis and its presence in raw milk is more indicative of poor milking hygiene protocols (Oliver, Jayarao, & Almeida, 2005). This particularly disconcerting since it is estimated that up to 35% of dairy farmers in T&T consume raw milk (A. Adesiyun et al., 1997). Pet dogs including dogs reared on bovine farms in T&T have also been demonstrated to shed STEC (Adesiyun, Campbell, & Kaminjolo, 1997; Roopnarine et al., 2007). Approximately 9% of fecal samples from farm dogs residing on bovine farms tested positive for stx genes, however whether this was transient shedding or as a result of dogs being colonized was not determined. Neither the serogroups recovered from dogs nor the genetic relatedness of the isolates to those recovered from cows were reported (Roopnarine et al., 2007). E. coli O157 has also been isolated from oysters sold by roadside vendors (Rampersad et al., 1999). Mammalian wildlife species, rats and diarrheic foals have also been screened for STEC in subsequent studies, but none tested positive (Adesiyun, 1999; Harris et al., 2012; Nkogwe et al., 2011).

There is dearth of published literature describing the role of non-animal sources in the transmission and propagation of STEC. An extensive literature search revealed only two published papers evaluating the microbial contamination of drinking water, one
evaluating vegetable contamination and no reports on the microbial quality of irrigation water. One of the papers on microbial quality of drinking water focused on urban drinking water and the other on rural drinking water. Quite disconcerting is that 67% of rural and 33% of urban water samples were contaminated with *E. coli* (Agard et al., 2002; Welch et al., 2000). Even more startling is that 15% of the *E. coli* colonies recovered from the rural drinking water samples were *stx* positive and 2% were identified as being O157 colonies. None of the *E. coli* isolates recovered from the urban drinking water samples were reported as being *stx* positive or of the O157 serogroup. As for microbial contamination of vegetables only one published report was found and this evaluated ready to eat vegetable samples collected at supermarkets. Of the 71 samples screened, one sample was positive for *E. coli* but the *Stx* profile and or virulence profile was not investigated (Hosein, Muñoz, Sawh, & Adesiyun, 2008).

*Salmonella enterica subspecies enterica*:

*Salmonella enterica* are gram negative facultative anaerobic bacteria which can be harbored as part of the enteric flora of many animals and shed in the feces of these animals. The duration of animal *Salmonella* shedding can be variable, for example the median shedding time for cattle is reported to be 50 days and a maximum shedding period for up to 391 days (Cummings et al., 2009). Once in the environment, *Salmonella* has also been demonstrated to persist for up to two years (Jay-Russell et al., 2013; Toth, Aceto, Rankin, & Dou, 2011; You et al., 2006). The survival of *Salmonella* in the environment can differ between serovars and can be dependent on many factors including
the chemical characteristics, pH, and moisture content of soil and fecal material. 

*Salmonella* environmental persistence can also be dependent on interactions with other microorganisms in the environment. (Kirchner et al., 2012).

Serovars of *Salmonella enterica* have been associated with foodborne disease globally for over 100 years (Lee, Runyon, Herrman, Phillips, & Hsieh, 2015). The global incidence of *Salmonella* is estimated to be almost 100 million cases (Majowicz et al., 2010). Within T&T the annual incidence is estimated to be over 5 cases per 100,000 persons, and 1.7% of childhood diarrhea cases screened between 1998 – 2000 were *Salmonella* positive (Berger, 2015; Khan-Mohammed, Adesiyun, Swanston, & Chadee, 2005). The largest outbreak of non-typhoidal human salmonellosis in T&T occurred in 1973 when over 3000 persons became ill as a result of consumption of contaminated powdered milk (Weissman, Deen, Williams, Swanston, & Ali, 1977). Other sporadic outbreaks have since occurred linked to consumption of contaminated food and water (Hyatali, Quamina, Mohess, & Foster, 1992; Koplan, Deen, Swanston, & Tota, 1978). Most human *Salmonella* cases are self-limiting; however young children under five years, the elderly and immunocompromised are at most risk of becoming infected and also developing complications (Acheson & Hohmann, 2001; Baird-Parker, 1990).

The majority of published *Salmonella* research in T&T has focused on the role of poultry and table eggs as source of infection to the human population (Adesiyun et al., 2005; Adesiyun et al., 2007; Dookeran, Baccus-Taylor, Akingbala, Tameru, & Lammerding, 2012; Indar, Baccus-Taylor, Commissiong, Prabhakar, & Reid, 1998).
Indar et al. (1998) reported that 1.2% of the internal contents and 4.7% of egg shells were contaminated with *Salmonella* (Indar et al., 1998). Another survey in 2005, testing eggs sold at market, revealed that 2.9% of egg shells and 8% of egg contents were positive for *Salmonella*. This same study reported that 18% of egg shells and 3.6% of egg contents were positive for *E. coli* (A Adesiyun et al., 2005). Another more recent study reported *Salmonella* was recovered from 60% of layer farms, and 12.5% of egg shells sampled. The egg contents were however all negative for *Salmonella* (A. Adesiyun et al., 2014).

Historically *Salmonella* Enteritidis has been the serovar associated with poultry and egg contamination but recent research has postulated the emergence of new serovars (A. Adesiyun et al., 2014). The risk of *Salmonella* transmission to humans from poultry is quite profound as one study evaluating the prevalence of *Salmonella* in chicken carcasses sold at retail reported 80% of carcasses were positive for *Salmonella* (Dookeran et al., 2012; Thomas, Lallo, & Badrie, 2006). This high retail *Salmonella* prevalence could be as a result of the combination of two factors: (i) a high on-farm *Salmonella* prevalence in live broiler birds and (ii) poor slaughter and processing practices that allows for cross contamination during processing (Dookeran et al., 2012; World Health Organization, 2002). The prevalence in live Muscovy ducks reared for human consumption was reported to be as high as 40% based on PCR screening (J. Rampersad, Johnson, Brown, Samlal, & Ammons, 2008).

Although poultry and eggs have been identified as possibly the main source of *Salmonella*, the role of livestock is without doubt just as important. Cazabon et al. (1978)
first reported pork as possible vehicle for Salmonella transmission in T&T, in that study 18% of swine carcasses sampled at the abattoir were positive for Salmonella (Cazabon, Berment, & Supersad, 1978). A more extensive abattoir study conducted in 1993 detected Salmonella in 3% of goat meat samples but all pork, beef, mutton and chicken samples screened were negative (Adesiyun, 1993). Despite goat meat being a delicacy in T&T and having been identified as a vehicle for the transmission of Salmonella, there is only one published study investigating Salmonella epidemiology in goats. In that study, a very small sample size of ten goats was tested and all tested negative for Salmonella (Adesiyun et al., 2001). Another cross-sectional study conducted by Adesiyun et al. (1994) reported the Salmonella prevalence in cattle, pig and sheep feces was 5%, 4% 3% respectively (Abiodun A Adesiyun & Kaminjolo, 1994). Among pets, Salmonella has also been detected in dogs (3.6%), cats (2.1%), pet birds (0.9%) and aquarium fish (0.4%) (Seepersadsingh, Adesiyun, & Seebaransingh, 2004; Seepersadsingh & Adesiyun, 2003; Seepersadsingh, Adesiyun, & Seebaransingh, 2005).

Similar to STEC, the role of vegetable produce in the epidemiology of Salmonella epidemiology is a neglected sphere of Salmonella research. Vegetable produce contamination can occur in the same manner as STEC contamination (Brackett, 1999) however there is no published literature in T&T investigating non-animal sources of Salmonella. There are also no published reports investigating Salmonella contamination of surface or ground water used for irrigation or drinking. Given the previous detection of E.coli in both urban and rural drinking water, it is plausible that these water sources may
also be contaminated with *Salmonella*, (Agard et al., 2002; Welch et al., 2000). Adding credence to water sources being contaminated with *Salmonella* is the fact that *Salmonella* has been recovered from ponds where commercial fish are reared. *Salmonella* has also been recovered from free-living aquatic shellfish such as oysters harvested from mangrove swamps (Newaj-Fyzul, Adesiyun, & Mutani, 2006; Rampersad et al., 1999). These swamps are usually the terminal point of many rivers and streams from which irrigation water is sometimes collected raising the possibility that irrigation water may also be contaminated with *Salmonella*.

**Knowledge gaps in STEC and *Salmonella* epidemiology.**

From the literature cited above it is indisputable there are many knowledge gaps in the epidemiology of STEC and *Salmonella* in T&T which must be filled before one can fully comprehend the epidemiology pathogens:

Firstly, the role of vegetable produce in the epidemiology of both of these pathogens remains a largely unexplored sphere in T&T. Vegetable contamination on the farm can occur via number of routes including wind dispersal of pathogens from nearby livestock farms; animals defecating in the production field; use of improperly treated manure or biosolids; poor worker hygiene; use of contaminated irrigation water; flooding and via contaminated equipment (Brackett, 1999). The potential for indirect contamination of vegetable fields and produce from human, livestock farms is without question. In a 1998 study conducted on water quality by the Environmental Management Authority of Trinidad and Tobago, all major ground and surface waters sources were

79
reportedly contaminated or at risk of contamination by effluents from housing and livestock farms (Anonymous, 1998). Additionally many vegetation plots in T&T are at risk of flooding on an annual basis which can lead to further contamination of produce and produce fields from these contaminated surface water sources (Persad, Ramnarine, Wilson., 2007).

While the role of vegetables in foodborne disease is largely unexplored in T&T, the importance of vegetables in the epidemiology of the foodborne disease pathogens is demonstrated in the United States where over 50% of the outbreak foodborne disease cases from 1998 – 2008 were attributed to plant commodities (Painter JA, 2013). During this period, leafy vegetables alone were responsible for almost 2.2 million disease cases, the most of any food commodity. This was a drastic increase from 1973 – 2006 data reported by Herman et al (Herman, Ayers, & Lynch., 2008) who found that only 6.5% (18,242) of disease cases were attributable to leafy greens. In the USA, from 1998 – 2008, plant commodities were also responsible for an estimated 24,000 hospitalizations and over 300 deaths (Painter JA, 2013). From 2009 to 2010, there over 1,527 foodborne disease outbreaks in the United States, with *Salmonella* and STEC being the leading etiologies of outbreak related hospitalizations (Centers for Disease & Prevention, 2013). In T&T a survey of dietary consumption patterns revealed that 38% of the participants regularly ate mixed raw vegetable salad and a further 22% ate reported eating raw lettuce or tomatoes thus the risk of consumption of contaminated produce is very apparent (Ramdath, Hilaire, Cheong, & Sharma, 2011). This high consumption rate coupled with
low compliance of farmers to GAPs makes vegetables a high risk factor foodborne disease (Ganpat et al., 2014).

Another critical void is the absence of recent research focusing on livestock species used for meat consumption. There are an estimated 35,000 pigs, 20,000 cattle, 11,000 sheep, 4,000 goats and 2,000 buffalo reared in T&T and with the exception of cattle and water buffalo the rest are reared solely for meat production (Central Statistical Office - Number of Small Ruminants by Region (Trinidad), 2012; Singh, 2006). Other than poultry, there is a dearth of recent research focusing on these livestock species. There have been many changes to animal genetics, nutrition, management and production systems and emergence of new diseases. One would expect that with these changes there would be concurrent changes to exposure potential and susceptibility to certain pathogens.

For example, while cattle and buffalo are slaughtered at properly constructed abattoir facilities, other smaller sized ruminants and sometimes pigs are slaughtered using backyard / road side facilities in low input slaughter facilities usually devoid of running water and proper sanitation protocols. Despite this appreciable risk there is a paucity of STEC and Salmonella research focused on these animals especially sheep and goats.

Another important observation from the review of the existing published literature is that diagnostic methods for STEC did not include the critical step of sample enrichment. Sample enrichment increases the sensitivity of diagnostics tests and is necessary since STEC when present in feces, are usually present in such lows numbers
that it can be almost impossible to isolate via direct plating (Chase-Topping et al., 2008; Delbeke et al., 2015; Jeffrey T LeJeune et al., 2006). Another critical epidemiological factor missing in existing STEC literature is the absence of virulence profiling. Although some studies report the stx profile, most do not evaluate other virulence factors such Intimin or Enterohemolysin (Etcheverria & Padola, 2013; Friedrich et al., 2002; Gyles, 2007).

Even with limited detection and surveillance programs, the incidence of foodborne disease in T&T continues to increase annually (Isaac, 2015). The reduction in foodborne disease can only be achieved with multifaceted projects which incorporate research, application and education programs. From the literature cited, it is indisputable that there are many critical knowledge gaps in the epidemiology of foodborne pathogens in T&T. The goal of future research endeavors must not only be aimed at filling these gaps but also include identifying and evaluating appropriate preharvest intervention mechanisms to reduce transmission of foodborne pathogens. There is also need for education programs which inculcate the importance for good agriculture practices as well as safe post-harvest handling and preparation of food.
Table 1.1 Categorization of STEC Sero-pathotypes associated with illness in humans

(Farrokh et al., 2013; Gyles, 2007; Karmali et al., 2003).

<table>
<thead>
<tr>
<th>Sero-pathotypes</th>
<th>Serotype Examples</th>
<th>Incidence of disease</th>
<th>Association with Outbreaks</th>
<th>Implicated in HUC / HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>O157:H7, O157:NM</td>
<td>High</td>
<td>Common</td>
<td>Yes</td>
</tr>
<tr>
<td>B</td>
<td>O26:H11, O103:H2, O111:NM, 121:H19, O145:NM</td>
<td>Moderate</td>
<td>Uncommon</td>
<td>Yes</td>
</tr>
<tr>
<td>C</td>
<td>O91:H21, O104:H21, O113:H21 etc.</td>
<td>Low</td>
<td>Rare</td>
<td>Yes</td>
</tr>
<tr>
<td>D</td>
<td>O7:H4, O69:H11, O103:H25 etc.</td>
<td>Low</td>
<td>Rare</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>O6:H35, O8:H19, O39:H49, O76:H7 etc.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
References:


Chandran, A., & Mazumder, A. (2013). Prevalence of diarrhea-associated virulence genes and genetic diversity in Escherichia coli isolates from fecal material of


Cull, C. A., Paddock, Z. D., Nagaraja, T. G., Bello, N. M., Babcock, A. H., & Renter, D. G. (2012). Efficacy of a vaccine and a direct-fed microbial against fecal shedding
of Escherichia coli O157:H7 in a randomized pen-level field trial of commercial feedlot cattle. *Vaccine, 30*(43), 6210-6215. doi: 10.1016/j.vaccine.2012.05.080


95


108


followed by immunomagnetic separation and direct plating methodologies. 
Journal of clinical microbiology, 44(3), 872-875.

Longitudinal study of fecal shedding of Escherichia coli O157: H7 in feedlot 
cattle: predominance and persistence of specific clonal types despite massive 

prevalence of Escherichia coli O157:H7 in horses in Ohio, USA. J Food Prot, 
73(11), 2089-2092.

metagenomic sequencing to food safety: detection of Shiga Toxin-producing 

Leotta, G. A., Deza, N., Origlia, J., Toma, C., Chinen, I., Miliwebsky, E., . . . Rivas, M. 
(2006). Detection and characterization of Shiga toxin-producing Escherichia coli 
in captive non-domestic mammals. Vet Microbiol, 118(1-2), 151-157. doi: 
10.1016/j.vetmic.2006.07.006

LGMA. (2013). Commodity Specific Food Safety Guidelines for the production and 
harvest of lettuce and leafy greens., 2015, from http://www.lgma.ca.gov/wp-

Li, Q; Sherwood J; Logue, C. (2004). The prevalence of Listeria, Salmonella, Escherichia 
coli and E. coli O157:H7 on bison carcasses during processing. Food Microbiol, 
21(6), 791-799. doi: http://dx.doi.org/10.1016/j.fm.2003.12.006,

coli O157:H7 colonization at the rectoanal junction of long-duration culture-
positive cattle. Appl Environ Microbiol, 73(4), 1380-1382. doi: 
10.1128/AEM.02242-06

Isolation of Shiga toxin-producing Escherichia coli from fresh produce using 
STEC heart infusion washed blood agar with mitomycin-C. J Food Prot, 75(11), 
2028-2030. doi: 10.4315/0362-028X.JFP-12-157


Savio, María. (1999). Sistema de vigilancia epidemiológica de las enfermedades transmitidas por alimentos *Sistema de vigilancia epidemiológica de las enfermedades transmitidas por alimentos*.


128


131
Chapter 2: *Escherichia coli* in Ohio specialty-crop soils contaminated with deer feces.
Research Note

Running title: *E. coli* in Soils

*Escherichia coli* in Ohio specialty-crop soils contaminated with deer feces.

Anil K. Persad, Michael D. Kauffman, Terrence Kline, Jeffrey T. LeJeune*

Food Animal Health Research Program, The Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Ave, Wooster, Ohio 44691

*Escherichia coli*, vegetables, contamination, soil, dispersion,

* Corresponding author: Jeffrey T. LeJeune
Ph: (330) 263-3739 Fx: (330) 263-3677
Email: lejeune.3@osu.edu

134
Abstract

Deer excrete numerous foodborne pathogens in their feces; therefore, their intrusion into fields where fruits and vegetables are grown can result in food safety risks. The purpose of this study was to determine the extent to which *E. coli* transfers from deer feces to soils where crops are grown in Ohio. Total coliform and *E. coli* counts were assessed in soil and surface debris samples collected from areas of a vegetable production field naturally contaminated with deer feces and compared with counts present in soil and debris collected from the same field away from any visible signs of fecal contamination. Samples were collected and assessed similarly again 60 days after mitigation (feces removal and field fencing). In a separate experiment, fresh deer feces was deposited on soils in vegetable production fields and *E. coli* counts present in surrounding soils was determined at multiple distances away from the inoculum at several time intervals. Under naturally contaminated conditions, surface material from the visually contaminated areas initially had a higher generic *E. coli* counts than samples collected from uncontaminated areas (*P* < 0.02). Sixty days later there was no difference in *E. coli* counts between the surface material and soils in the previously contaminated and uncontaminated areas (*P* = 0.35; *P* = 0.97). Under experimental conditions, generic *E. coli* counts in soils decreased sharply from the point of inoculation, with counts being significantly lower at 15cm compared to the site of inoculation (*P* < 0.05). *E. coli* counts in soils greater than 15cm away from the point of fecal deposition did not differ
significantly from counts 5 m away. Remediation of fields contaminated by deer can minimize risk of transfer of microorganisms from feces to soil. Moreover, employing no-harvest zones greater than 15 cm around areas of fresh deer fecal contamination minimize the risk of harvesting vegetables from soils contaminated with fecally-transmitted bacteria.

Introduction

The demand to feed an ever increasing human population has necessitated conversion of natural forested and grasslands towards agricultural production (Tscharntke et al., 2012). This coupled with loss of habitat due to urbanization has resulted in reports of intrusion of native wildlife species such as deer, onto gardens and agriculture fields (Boase, 2014; Giclas & Wetherington, 2016; Sterba, 2012). In recent years, numerous foodborne disease outbreaks have been linked to wildlife incursion and subsequent contamination of agriculture produce (Gardner et al., 2011; Jay et al., 2007; Laidler et al., 2013).

Deer can asymptptomatically harbor a diverse population of pathogenic bacteria including *Salmonella*, Shiga toxin-producing *Escherichia coli* and *Listeria* spp. within their gastrointestinal tract (French et al., 2010). Wild deer are known to share pastures with cattle and can also be found in close proximity to many dairy farms. The interaction between deer and livestock allows deer to serve as reservoir hosts and disseminate foodborne pathogens between and within livestock herds (LeJeune & Pearl, 2014; Renter et al., 2001). Deer can also serve as a vehicle for the dissemination of pathogens onto
produce fields: For example, in 2011, 15 people were sickened and two people died following the consumption of strawberries from a field contaminated with deer feces (Laidler et al., 2013).

Historically most bacterial foodborne disease has been mainly associated with the consumption of meat and animal products, however the incidence of foodborne disease associated with vegetable consumption, especially leafy green has been increasing (K. M. Herman et al., 2015). Fecal contamination of vegetables is of particular concern, since many vegetables are eaten raw or with minimal cooking and most post-harvest intervention strategies for decontamination are ineffective; contamination prevention is paramount to reduce human exposure to these foodborne pathogens from produce (K. M. Herman et al., 2015; Solomon et al., 2002). In an effort to decrease the incidence of foodborne illness associated with vegetable consumption, producers and regulators have sought to develop and implement pre-harvest practices aimed at minimizing the risk of pathogen contaminated produce entering the food chain (Food & Administration, 2010; Health & Services, 1998; Olaimat & Holley, 2012).

The extent of fecal contamination is commonly implied by determining the concentration of generic *Escherichia coli* on produce, in soil and irrigation water. *Escherichia coli* is a normal inhabitant of the gastrointestinal tract of warm blooded animals and its presence has been traditionally interpreted as evidence of fecal contamination and possible presence of other pathogens (Dufour, 1977). These pre-harvest guidelines aim to minimize the risk of human exposure to these foodborne
pathogens by regulating the location of produce fields, the use of manure, the establishment of buffer zones around animal fecal deposits found in the field and up to and including condemnation of entire produce blocks if there is evidence of widespread wildlife intrusion (LGMA, 2013)

Despite the strong scientific rationale for these measures, empirical data to support the exact details (e.g. distance for exclusion, waiting intervals to harvest etc.) are not widely available. Since bacterial persistence in the environment is dependent on a number of factors including climatic factors, the soil and animal fecal physical and chemical properties and soil microbial diversity (Coroller, Leguerinel, Mettler, Savy, & Mafart, 2006; Couvert, Gaillard, Savy, Mafart, & Leguerinel, 2005; Ravva, Sarreal, & Mandrell, 2010; Xiong, Xie, Edmondson, & Sheard, 1999), this study seeks to partially fill this gap in knowledge by (1) Determining the *E. coli* counts in soils at various distances from deer fecal deposits in the fields and (2) Evaluating the effectiveness of a field remediation strategy to reduce risks of contamination following deer fecal deposits in produce fields in Ohio.

**Materials and Methods**

Persistence of Coliforms and *Escherichia coli*:

During April 2014, evidence of deer intrusion onto a commercial produce field in NE Ohio was noted. These animals had defecated at various locations throughout the field. The areas contaminated with deer feces were marked with flags, and an electric perimeter fence established around the field to prevent further intrusion and field contamination.
Five contaminated and five uncontaminated sites, at least 10m away from visible fecal material, were identified and overlying vegetation, surface debris and soil samples were collected. Surface samples (straw, and overlying vegetation) were collected aseptically from an area 9cm x 9cm. The underlying soil was also sampled using a spade which had been disinfected using 10% bleach solution. Soil samples were collected to a depth of three centimeters and placed in separate Ziploc ® Bags. This procedure was repeated at each of the five sampling sites and all tools disinfected between sites. Samples were transported to the laboratory and processed within two hours of collection.

At the laboratory, the bags containing the surface debris were weighed on an electronic balance (CS 200 OHAUS Corporation, USA) and sterile phosphate buffered saline (AMRESCO, OH) was added to each bag at a volume equivalent to twice the weight of the surface debris. The contents were manually agitated for two minutes. One milliliter aliquots from each of the suspensions were removed and ten-fold dilutions of each sample were made in phosphate buffered saline solution and spread plated using sterile glass beads on Tryptone Bile X- Glucoronide (TBX) agar (Accumedia, MI). Plates were incubated for 32 – 36 hours at 37°C and the number of presumptive E. coli and total coliform on each plate recorded.

The soil samples in each bag were thoroughly mixed. Twenty five grams of soil was removed and added to 50ml of sterile phosphate buffered saline in 24oz Whirlpak® bag (Nasco, WI). Each bag was mixed thoroughly for two minutes via manual agitation. Similarly, as with the surface debris samples, one milliliter aliquots from each of the
suspensions were then sampled and ten-fold dilutions of each sample were made in phosphate buffered saline solution and spread plated using sterile glass beads on TBX agar. Plates were incubated for 32 – 36 hours at 37°C and the number of presumptive *E. coli* and total coliforms recorded.

Field remediation involved workers identifying fecal contaminated areas and manually removing any feces. Sixty days post remediation; samples were once again collected from another five previously contaminated areas and five uncontaminated areas. Samples were processed as described previously.

Dissemination distance:

An experiment to determine the concentration of *E. coli* in soils surrounding deer fecal deposits was conducted in duplicate at two different locations near Wooster, OH during the months of August to October. At each location there were three replicates of the study. Temperature and rainfall data for each location is presented in Table 2.1. For each replicate, freshly deposited deer fecal samples were collected and pooled and placed at a location in the field in a container with a mesh base. Surface and soil samples were collected from underneath the fecal sample by lifting the mesh bottom container, and at distances of 15, 30, 100, 150, 300 and 500 centimeters from the deposited deer feces daily for four days. A randomly generated number between 0 and 360 provided the directional coordinates from where the specimens were collected at each distance. One sample was collected at each distance per day for four days.
Samples were collected using sterile tongue depressors. At each sampling location, soil and surface samples of approximately 4cm radius and 2cm depth were collected aseptically and placed into 4 oz. Whirlpak® bags (Nasco, Fort Atkinson, WI) and transported to the laboratory for processing within two hours of sample collection. At the laboratory, the contents of the individual bags were thoroughly mixed and a 10 gram aliquot mixed with 90 milliliters of sterile phosphate buffered saline (AMRESCO, OH). One milliliter aliquots from each of the suspensions were then sampled and ten-fold dilutions of each sample were made in phosphate buffered saline solution and spread plated on Tryptone Bile X- Glucoronide (TBX) agar (Accumedia, MI). Plates were incubated for 32 – 36 hours at 37°C and the number of presumptive *E. coli* and total coliforms recorded.

**Statistical Analysis**

Dilutions resulting in plate counts between 30 – 300 colonies were used for analysis. Count data was log\(_{10}\) transformed prior to analysis. Statistical analyses were done using Minitab 15.1® statistical software (Minitab Inc., State College, Pa., USA.). For the natural contamination study, differences between contaminated and non-contaminated areas evaluated using a 2 sample t-test. For the dissemination distance experiment, a Repeated Measures ANOVA model for total coliforms and *E. coli* were developed, with day and distance, and their interaction included as predictor variables for both bacterial counts. For multiple comparison analysis, Dunnett’s test was to determine if there were any differences between the respective colony counts at 500 cm
(uncontaminated) and the counts at the different distances. For both studies we evaluated the Spearman Rank correlation to evaluate if total coliform counts could be used as a predictor of generic $E.\ coli$ counts. Differences were considered to be statistically significant at $P <0.05$.

**Results**

Persistence of Coliforms and *Escherichia coli*:

Surface material: Initially the surface material from the visually contaminated areas had a higher total coliform counts ($6.59 \log_{10} \text{CFU/g}$ versus $5.54 \log_{10} \text{CFU/g}$) ($P <0.01$) and generic $E.\ coli$ ($3.88 \log_{10} \text{CFU/g}$ versus $0.46 \log_{10} \text{CFU/g}$) ($P <0.02$) than the uncontaminated areas (Figure 1). Sixty days later the coliform counts for both surface material matrices from previously contaminated areas ($5.90 \log_{10} \text{CFU/g}$) and uncontaminated areas ($5.87 \log_{10} \text{CFU/g}$) were similar ($P =0.94$). $E.\ coli$ counts decreased in both the contaminated ($0.86 \log_{10} \text{CFU/g}$) and uncontaminated areas (undetectable) in relation to the initial sampling; this difference in $E.\ coli$ counts between the contaminated and uncontaminated areas was not significant ($P = 0.35$).

Soil: In contrast to the results obtained for the surface material, at the time of initial sampling, there was no statistical difference ($P = 0.23$) between the coliform counts for soil samples obtained from the contaminated areas ($5.28 \log_{10} \text{CFU/g}$) and the uncontaminated areas ($5.10 \log_{10} \text{CFU/g}$). Initial generic $E.\ coli$ counts obtained from the contaminated areas ($0.97 \log_{10} \text{CFU/g}$) and the uncontaminated areas ($1.0 \log_{10} \text{CFU/g}$) were similar ($P = 0.97$).
When sampled sixty days later, the soil from the previously contaminated areas actually had a significantly lower total coliform count (4.52 log$_{10}$ CFU/g) than the soil collected from the uncontaminated areas (5.08 log$_{10}$ CFU/g) ($P = 0.02$). Sixty days after remediation, there was no statistical difference ($P = 0.32$) between the generic $E.\ coli$ counts obtained from the contaminated areas (undetectable) and the uncontaminated areas (0.32 log$_{10}$ CFU/g).

Dissemination distance:

The generic $E.\ coli$ count was highest at the point of deposition (0.27 log$_{10}$ CFU/g) and decreased to 0.02 log$_{10}$ CFU/g at 15cm. No generic $E.\ coli$ were detected at distances 30, 100, and 150 cm for the duration of the experiment (Figure 2). Generic $E.\ coli$ counts were detected at 300 and 500cm on day 3 from one of six sampling blocks. No generic $E.\ coli$ was recovered from any of the other sampling distances at this farm. Additionally it should be noted, there was no rainfall at this site on the day prior to collection and less than 0.03cm of rainfall on that day positive samples were recorded at 300 and 500cm. No generic $E.\ coli$, were recovered from the other sampling block, which also received similar rainfall levels. When evaluating, the generic $E.\ coli$ count, an interaction between day and distance was identified ($P = 0.001$), therefore the analysis was conducted for each day. Only distance measured from fecal deposit was found to be significant ($P < 0.001$). Similarly, sampling block had no effect on generic $E.\ coli$ counts ($P = 0.11$). When the generic $E.\ coli$ counts at 500cm were compared to the counts at the
other sampling points, only the counts at the site of fecal deposition was significantly different ($P < 0.05$).

Sampling distance ($P = 0.038$), but not sampling day ($P = 0.11$), nor distance by day interaction interactions ($P = 0.12$) were found to affect total coliform counts. Total coliform counts however varied significantly between sampling locations ($P < 0.01$).

Coliform counts and *E. coli* counts (from soil and debris) were correlated in in the natural contamination study ($r = 0.31; P = 0.05$), but this association was weaker in the dissemination study ($0.11; P = 0.07$)

**Discussion**

One main goal achieved by these experiments is the provision of metric guidelines for reducing the risk of pathogen contaminated produce grown in Ohio from entering the food system. The first experiment demonstrates that removal of visible fecal contamination and prevention of recontamination resulted in indicators of fecal contamination falling to levels equivalent to non-contaminated areas within 60 days.

Secondly, given the limited dissemination of bacteria from deer fecal pats observed under these experimental conditions support the use of no-harvest zones greater than 15 cm.

As frequently observed in water quality testing the correlation between both total coliforms and generic *E. coli* counts in matrices evaluated (soil and surface material) in both the natural contamination event and the experimental study was weak (Elmund, Allen, & Rice, 1999; Evans, 1996; Steele & Odumeru, 2004). This indicates that the total coliform count was a poor estimator for *E. coli* contamination of soil. Total coliform
counts can include many harmless bacteria ubiquitous in the environment and thus is not a good indicator of fecal contamination (Tallon, Magajna, Lofranco, & Leung, 2005). Coliform counts should not be used as a measure of soil microbial safety.

Although there was a decrease in *E. coli* counts in areas contaminated with deer feces, *E. coli* persisted in the both contaminated and uncontaminated areas of the field. Of interest also, is the observation that the total coliform and *E. coli* counts in soil samples did not differ between fecal contaminated and uncontaminated areas. One possible reason for this observation is that the layer of surface material had insulated the underlying soil from contamination. Another reason could be weather conditions from the time of fecal deposition to the time of sampling did not facilitate leaching of *E. coli* colonies from the pellet to the soil below.

The survival of bacteria in the environment can be described as being biphasic with the population being comprised of a stress sensitive and a stress resistant subpopulation (Coroller et al., 2006; Couvert et al., 2005; Xiong et al., 1999). This stress resistant subpopulation may persist for prolonged periods in the environment. This persistence of *E. coli* in the environment has also been described by Ogden et al (2001) who reported that *E. coli* present in manure slurry exhibited two distinct rates of die off, with one sub-population having a half-life of 4 days while the other sub-population had a half-life 17.8 days. This sub-population persisted in the environment for the duration of the experiment (Ogden, Fenlon, Vinten, & Lewis, 2001). A similar decrease was observed with fields contaminated *E. coli* O157:H7 where within 20 days, the detection
of *E. coli* O157:H7 in deer fecal pellets had decreased from 20% to less than 2% (Laidler et al., 2013). While certain guidelines propose up to one year, no harvest intervals following fecal contamination of produce fields, our research has demonstrated that with remediation, after 60 days there was no statistical difference in generic *E. coli* counts between contaminated and uncontaminated areas. Extending no harvest intervals beyond this time period may not further reduce contamination risk due to the environmental persistence of the stress resistant *E. coli* sub-population.

Since post-harvest decontamination of produce eaten fresh or minimally processed is not currently possible, good agricultural practices should be employed to limit the possibility of contamination. Harvesting of produce in direct contact with fecal material is clearly unacceptable; however, there is a paucity of information about how far away is far enough to minimize risk of contamination. Rain splash is a known method for pathogen dispersal (Gregory, Guthrie, & Bunce, 1959) however dispersal distances on lands with flat topography have been demonstrated to not extend beyond 5-9 cm from the fecal source (Boyer, 2008; Cevallos-Cevallos, Danyluk, Gu, Vallad, & van Bruggen, 2012).

Current guidelines, such as the LGMA, recommends 5 feet (152cm) radius buffer around fecal deposits (LGMA, 2013). Our research demonstrates that in Ohio, for deer feces, buffer distances much smaller than those recommended by LGMA may provide a similar reduction in contamination risks.
Food security not only involves production of adequate quantities of nutritious food, but also food that is safe for human consumption (Pinstrup-Andersen, 2009). While guidelines and regulations are imperative for production of safe food, one must be careful with the formulation and implementation of these guidelines to minimize any unnecessary adverse economic effects on producers (Ribera & Knutson, 2011). Our study, in addition to being the first to provide metrics for the establishment of Ohio specific guidelines, also demonstrates that guidelines formulated in one region may not be applicable on a national basis. Guidelines should therefore be tailored to reflect varying climatic conditions, regional agricultural practices and contamination scenarios.

**Limitations**

There are several limitations with this data, and as such caution must be exercised when attempting to extrapolate these findings. Firstly, we did not measure produce contamination. We measured contamination of soils and surface material and although the presence of *E. coli* in soil may indicate the possibility of produce contamination, it is not definite and cannot provide an estimate for produce contamination (Mootian et al., 2009). Secondly, we were not able to identify the exact time of field contamination nor could we determine if the fecal contamination had been as a result of a single contamination event or as a result of multiple contamination events. Additionally, the quantity of *E. coli* present may differ between fecal samples (Guber, Fry, Ives, & Rose, 2015). Another limitation is that although *E. coli* is a suitable indicator for fecal contamination, it is not reflective of the presence of other pathogenic organisms or their
concentrations (Miskimin et al., 1976). Finally, this proposed buffer distance and remediation/no harvest interval may not be applicable in other locations where there are varying climatic, environmental, and production conditions.

Acknowledgements

This material is based, in part, upon research supported by the National Institute of Food and Agriculture, Specialty Crop Research Initiative, Award No. 2011-51181-30767. The authors would like to acknowledge the assistance of Jennifer Schrock and Pam Schlegel in conducting this study and also thank the farmers for their co-operation and participation.
Table 2.1 Rainfall and temperature data from sampling the different sampling blocks.

<table>
<thead>
<tr>
<th>Sampling block</th>
<th>Sampling Day</th>
<th>Rainfall (cm)</th>
<th>Minimum Temperature (Fahrenheit)</th>
<th>Maximum Temperature (Fahrenheit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A+B</td>
<td>0</td>
<td>0.97</td>
<td>55.9</td>
<td>60.5</td>
</tr>
<tr>
<td>A+B</td>
<td>1</td>
<td>0.15</td>
<td>48.3</td>
<td>56.6</td>
</tr>
<tr>
<td>A+B</td>
<td>2</td>
<td>0</td>
<td>35.4</td>
<td>52.7</td>
</tr>
<tr>
<td>A+B</td>
<td>3</td>
<td>0.03</td>
<td>30.6</td>
<td>60.3</td>
</tr>
<tr>
<td>C+D</td>
<td>0</td>
<td>0</td>
<td>35.2</td>
<td>54.3</td>
</tr>
<tr>
<td>C+D</td>
<td>1</td>
<td>0</td>
<td>34.5</td>
<td>51.8</td>
</tr>
<tr>
<td>C+D</td>
<td>2</td>
<td>0.08</td>
<td>32.3</td>
<td>41.9</td>
</tr>
<tr>
<td>C+D</td>
<td>3</td>
<td>0.66</td>
<td>38.3</td>
<td>42.6</td>
</tr>
<tr>
<td>E+F</td>
<td>0</td>
<td>0</td>
<td>56.9</td>
<td>69.5</td>
</tr>
<tr>
<td>E+F</td>
<td>1</td>
<td>0</td>
<td>54.3</td>
<td>77.78</td>
</tr>
<tr>
<td>E+F</td>
<td>2</td>
<td>0</td>
<td>52.3</td>
<td>82.7</td>
</tr>
<tr>
<td>E+F</td>
<td>3</td>
<td>0</td>
<td>58.2</td>
<td>81.6</td>
</tr>
</tbody>
</table>
Figure 2.1: Persistence of total coliform and *E. coli* in surface material and soil collected from clean and fecal contaminated (shaded) areas.

Comparison of total coliform counts in surface material (A) and soil (B) samples and generic *E. coli* counts in surface material (C) and soil (D) samples. Values are expressed
as the mean $\log_{10}$ CFU/g of minimum of five samples ± standard error. Statistically significant differences are labelled “a”

Figure 2.2: Comparison of total coliform (shaded) and *E. coli* (unshaded) counts at different distances from the site of fecal pat deposition on the different days.

Values are expressed as the mean $\log_{10}$ CFU/g of minimum of four samples ± standard error. Horizontal line is the median (the second quartile), boxes are the first to third quartile. Whiskers represent the range of data from the first and third quartiles to their respective extremes and outliers (o).
References


Miskimin, DK, Berkowitz, KA, Solberg, M, Riha, WE, Franke, WC, Buchanan, RL, & O'leary, V. (1976). Relationships between indicator organisms and specific


Chapter 3: Isolation of non-O157 Shiga toxin – producing *Escherichia coli* from ruminant manure.
Isolation of non-O157 Shiga toxin – producing *Escherichia coli* from ruminant manure.

Anil K. Persad, Gaochan Wang, Jeffrey T. LeJeune*

Food Animal Health Research Program, The Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Ave, Wooster, Ohio 44691

*Escherichia coli*, shiga toxin, MacConkey, minimum number

* Corresponding author: Jeffrey T. LeJeune
Ph: (330) 263-3739 Fx: (330) 263-3677
Email: lejeune.3@osu.edu
Abstract

There are over 200 serotypes of Shiga toxin – producing Escherichia coli (STEC) associated with human disease, some of which may colonize the ruminant gastrointestinal tract and/or contaminate foods. Unfortunately, to date, highly selective and specific diagnostic methods for the isolation of many of these serotypes are not available. The objective for this study was to ascertain the minimum number of colony picks from a MacConkey agar plate required to recover at least one stx-positive colony from ruminant fecal enrichment samples testing PCR-positive for stx. Two studies using stx PCR-positive fecal samples obtained from cattle (n=28) and small ruminants (n=116) were performed. Enriched fecal samples were streaked onto MacConkey agar and suspect individual E. coli colonies selected and screened for stx genes. Overall, at least one stx-positive colony was recovered from 79% of the PCR-positive fecal samples screened. The proportion of stx-positive colonies identified from a single PCR-positive enrichment ranged from 0% to 100%. Based upon the proportion of suspect stx-positive E. coli present in these enrichment, selecting greater than 20 colonies per sample did not significantly increase the probability of recovering at least one stx-positive cell. This information can be used to optimize the culture methods for STEC from ruminant fecal enrichments.
Introduction

Although identified as a source of foodborne disease over thirty years ago, Shiga toxin-producing *Escherichia coli* (STEC) remains a major cause of foodborne disease globally (Karmali et al., 1983; Riley et al., 1983). There are over 400 serotypes of STEC with approximately 200 of these serotypes having been associated with human disease (Caprioli et al., 2005). Annually, the global incidence of STEC associated human disease exceeds two million cases (Majowicz et al., 2014) with an estimated 250,000 cases in the United States (Scallan et al., 2011) and over 6000 confirmed cases in Europe (B. Verhaegen et al., 2016). Historically, STEC O157:H7 has been the serotype most associated with human disease in the USA, however almost two thirds of human cases are now associated with non-O157 serotypes with O26, O45, O105, O111, O145 and O121 being responsible for most cases (Barkocy-Gallagher et al., 2005; Gould et al., 2013; Luna-Gierke et al., 2014; Scallan et al., 2011). Globally, the serotype of importance may vary in different geographic, climatic and cultural conditions (Beutin, Krause, Zimmermann, Kaulfuss, & Gleier, 2004; J. M. Hughes, Wilson, Johnson, Thorpe, & Sears, 2006).

STEC O157:H7, the serotype first associated with human disease, has been the focus of multitudinous research projects aimed at improving detection and isolation of this pathogen (Wang et al., 2013). Consequently, a number of culture based methods employing the use of phenotypic characteristics, hybridization, immuno-magnetic separation, and chromogenic media have been developed to aid in the detection,
differentiation and isolation of O157:H7 colonies (Beutin & Fach, 2014). However, this array of isolation techniques do not exist for all non-O157 serotypes (Cooley et al., 2013). Attempts have been made to extrapolate O157:H7 isolation techniques to non-O157 serotypes, but these methods have had their inherent faults including being labor intensive, time consuming, and poor sensitivity thus resulting in low recovery rates of non-O157 isolates from experimental microcosms (Bettelheim, 1998a; Beutin & Fach, 2014; Gill et al., 2014; Wylie et al., 2013).

Although multiple molecular tests have been developed to detect STEC, culture based methods are still regarded as the standard for pathogen detection (Buchan et al., 2013; Wang et al., 2013). Furthermore isolation of a colony is required for additional confirmatory biochemical, serological and molecular analysis as well as for epidemiological investigations (Baker et al., 2016; Wang et al., 2013). Multiple studies have investigated the use of various selective media for the recovery of both O157 and non-O157 STEC, however the general consensus is that under experimental conditions, STEC isolates can grow on MacConkey agar but grow less efficiently on other selective media (Gill et al., 2014). While MacConkey agar is supportive of the growth of most STEC, when used to recover STEC isolates from fecal samples, we face the challenge of differentiating STEC colonies from phenotypically similar background generic E. coli and other enteric lactose fermenters. For this reason, we conducted this study to evaluate the minimum of number of suspect E. coli colonies which has to be selected from a
MacConkey agar plate to recover at least one STEC isolate from a Shiga toxin-positive fecal enrichment.

**Methodology**

Preliminary study: A preliminary study was carried out using 28 enriched Shiga toxin-positive bovine fecal samples. These bovine fecal samples were originally collected in 2008 and enriched in Buffered Peptone Water (BPW; Acumedia, East Lansing, Mi) at 42°C for 18 – 24 hours. Following enrichment, fecal samples were screened for the presence of stx genes as described by LeJeune et al (2006) (LeJeune, Hancock, Wasteson, Skjerve, & Urdahl, 2006). One milliliter aliquots of the positive enriched samples were banked with 300μl of buffered glycerol and stored at -80°C for STEC isolation at a later date.

Sample recovery: 100μl of each of the previously banked enriched bovine fecal samples, were added to 1.7ml microcentrifuge tubes containing one milliliter of BPW and incubated for 18 – 24 hours at 37°C. The next day, serial dilutions ranging from 10^-5 to 10^-7 were prepared with phosphate buffered saline and 100μl of each dilution spread plated onto MacConkey (MAC; Acumedia, MI) agar plates and incubated for 18 – 24 hours at 37°C.

The next day, individual suspect *E. coli* colonies, up to 288 were selected from the MAC plates and transferred to sterile 96 well plates containing EC media (Acumedia, Neogen Corporation, Lansing, MI, USA) supplemented with 10 mg ml^-1 4-methylumbelliferyl-β-d-glucuronide (Biosynth Ag, Staad, Switzerland) (EC-MUG).
These 96 well plates were then incubated overnight at 37 °C. The following day, individual colonies from 8 wells were picked and pooled together in a 1.7ml microcentrifuge tubes containing 100µl of sterile DNAase-free water. The tubes were then placed in a water bath at 100°C for 10 minutes. The cell lysates were then used as the DNA template for PCR screening for the detection of stx as described by Karch and Meyer (1989) (H. Karch & Meyer, 1989). Briefly, amplifications were performed in a 50µl reaction mixture containing 25µl GoTaq Green Mastermix (Promega, United States), 5µl MK1 (0.6 pmole/µl), 5µl MK2 (0.6 pmole/µl), 10µl H2O, and 5µl DNA template. The samples were heated to 94°C for 5 minutes, then 39 cycles of amplification at 94°C for 1.5 minutes, 47°C for 2 minutes, 72°C for 3 minutes, and final extension at 72°C for 7 minutes. E. coli O157 strain EDL933 was employed as positive control. The PCR products were separated on 2% agarose gels, stained with ethidium bromide and visualized using UV light. The individual colonies of each pool were then subjected to PCR screening if the pool tested positive, using the same amplification conditions as stated above.

Supplementary study: The supplementary study was conducted using 116 enriched Shiga toxin-positive small ruminant fecal samples obtained from a cross sectional study conducted in 2015. The fecal samples were collected and enriched in BPW at 42 °C for 18 – 24 hours and screened for the presence of stx genes using a method adapted from LeJeune et al (2006) and Hu et al (1999) (Hu, Zhang, & Meitzler, 1999; LeJeune, D. Hancock, et al., 2006).
Sample recovery: 100μl of each of the previously banked enriched small ruminant fecal samples, were added to 1.7ml microcentrifuge tubes containing one milliliter of BPW and incubated for 18 – 24 hours at 42°C. Following incubation, 100μl of this enrichment was then streaked onto MAC agar plates and incubated for 18 – 24 hours at 42°C. The next day, individual suspect E. coli colonies, up to 30, were selected from the MAC plates and with portions of each colony being inoculated into individual wells of sterile 96 well plates containing Brain-Heart infusion broth (BHI, Acumedia, East Lansing, Mi). The 96 well plates were incubated for 18 – 24 hours at 42°C and the inoculated BHI wells mixed with buffered glycerol and stored at -80°C. The other portion each colony was transferred to Whatman FTA Cards (GE Healthcare, Piscataway, NJ) and the cards shipped to Food Animal Health Program (FAHRP), OARDC, Wooster, Ohio for stx screening.

At the FAHRP laboratory, a 6mm sterilized paper punch was used to excise portions of the FTA card which contained the colony smears. These portions of the card were transferred to clean 1.7ml microcentrifuge tubes containing 400μl of Millipore water and washed by vortexing three times for twenty seconds. The rinsate was then aspirated and 150μl of Millipore water added to each microcentrifuge tube. DNA elution was completed by placing the tubes in a water bath at 95°C for thirty minutes. The DNA extract was transferred to clean microcentrifuge tubes and stored at -20°C. Following DNA extraction, 10μl of DNA extract from 5 colonies were pooled together in a 1.7ml microcentrifuge tube and subjected to PCR screening for detection of stx genes as
described by Hu et al (1999) (Hu et al., 1999). Briefly, amplifications were performed in a 100μl reaction mixture containing 50μl GoTaq® Green Master Mix, 3.0 μl of each primer (SLT 1F, SLT 1R, SLT11F, SLT 11R), 2.5 μL MgCL$_2$, 30.5 μl sterile water and 5μl DNA template. The reaction mixture was heated to 94°C for three minutes, followed by 30 cycles of amplification at of 94°C for 30 seconds, 59°C for one minute and one minute at 72°C and final extension was done at 72°C for 10 minutes. Similarly as before, the PCR products were separated on 2% agarose gels stained with ethidium bromide and visualized using UV light. The individual colonies of each pool were then subjected to PCR screening if the pool tested positive, using the same amplification conditions as stated above.

**Statistical Analysis**

The number of individual colonies testing positive or negative for $stx$ was tabulated, and the prevalence of STEC colonies in each fecal sample determined. Using this data, for the preliminary study, the probability of picking a STEC colony if 1 to 50 colonies were randomly selected from a MAC plate was calculated. In the second study, using the cumulative probability, we determined the probability of recovering a $stx$ positive cell from a $stx$ positive fecal sample, given the number of positive individual PCR-positive colonies present in each sample. Samples were classified as culture positive if we were able to recover at minimum one $stx$ positive isolate. Statistical analyses were done using Minitab 16.2 statistical software (Minitab Inc., State College, Pa., USA.). The Friedman test, a non-parametric repeated measures analysis test, was used to determine if
the probability of choosing a STEC positive cell varied with the number of colonies picked. Post Hoc comparisons were done using Tukey’s test to identify the minimum number of suspected *E. coli* colonies which could be picked from a MAC agar to recover at least one *stx*-positive cell from a *stx* positive fecal sample. Using the data from both experiments, a bootstrapping resampling method with 1000 iterations was applied to the data to obtain an estimate for the mean probability and confidence interval estimate if the sampling had been done from a hypothetical larger pool. Differences were considered to be statistically significant at $P < 0.05$.

**Results**

Overall we recovered at least on *stx* positive isolate from 79% of the fecal samples screened. A total of 4640 (first study) and 3480 (second study) suspected *E. coli* colonies were picked from MAC agar plates and screened for *stx* genes. For the first study, at least one *stx* positive sample was recovered from 89% (25 of 28) of the fecal samples. With the second study however, at least one *stx* positive colony was recovered from only 77% (89 of 116) of the fecal samples. In both studies, the probability of recovering a *stx* positive cell was dependent on the number of colonies picked ($P < 0.001$). Although the recovery rate was higher in the first study, for both studies, there was no statistical difference in the probability of recovering a *stx*-positive cell if 20 or greater suspected *E. coli* cells were picked from a MAC agar plate ($P=0.2$).
Bootstrapping of the means revealed no difference between the means obtained in these experiments and hypothetical experiments with 1000 iterations (Figure 1).

In the initial study, no stx-positive cells were recovered from 3 bovine fecal samples (11%). Overall 371 of the 580 pools screened had at least one stx-positive isolate. For the majority of fecal samples (23/28) less than 20% of cells screened were stx-positive. The highest stx cell prevalence recorded for any sample was 24%.

In the supplementary study, we failed to recover any stx-positive isolates from 27 (23%) of the PCR positive fecal samples (Figure 2). Similarly as the first study, less than 20% of the cells screened were stx-positive for the majority of samples (76/116). However, 13% of fecal samples had a stx cell prevalence of 50% or greater.

Discussion

Herein we provide a scientific justification for the optimal number of colonies to screen from enrichments of bovine manure to maximize the likelihood of recovering STEC colonies while at the same time conserving valuable resources and expense: Selecting 20 suspected E. coli colonies from a MacConkey agar plate will provide the same probability of recovering at least one stx-positive isolate as screening greater than 20 colonies. In both studies, using a minimally selective media we demonstrated that by picking 20 suspected E. coli colonies from a MAC plate we were able to recover a STEC isolate in 77% and 72% of the PCR positive fecal samples screened in both studies. For culture positive fecal samples, screening 20 suspected E. coli cells results in the probability of at least one stx cell selected being 86% and 90% respectively. For both
PCR positive and culture positive samples, increasing the number of colonies screened on a MAC plate will increase the probability of detection of a STEC isolate however these increases are not statistically significant.

STEC when present in feces, are usually present in such low numbers that it can be almost impossible to isolate via direct plating (Chase-Topping et al., 2008; Delbeke et al., 2015; LeJeune, Hancock, & Besser, 2006). Increasing the sensitivity of STEC detection can be achieved by non-selective enrichment at high temperatures which tends to retard the growth of background fecal flora but still allow proliferation of STEC populations in fecal samples (Barkocy-Gallagher et al., 2005; Durso, 2013; LeJeune et al., 2006).

The ideal selective culture media should be one that is supportive of the growth of STEC serotypes, but inhibit the growth of other background organisms. STEC isolation strategies include the use of colony hybridization, immune-magnetic separation assays, selective media and nonselective indicator media. While colony hybridization is the preferred method in some diagnostic laboratories, this procedure can be time consuming and laborious and may not be suitable for epidemiological studies where large numbers of samples are processed (Beutin & Fach, 2014). The use of immuno-magnetic separation (IMS) is another method employed for the detection and isolation of STEC serogroups. The efficiency of detection and recovery is however dependent on a number of factors including the matrix the organism is in, the target organism and the interaction between the antigen and antibody coated paramagnetic beads (Cooley et al., 2013; Dwivedi &
The use of IMS is further limited since assays have not been developed for all STEC serogroups (Beutin & Fach, 2014; Wang et al., 2013). There are a number of different selective media available for isolation STEC isolates. These media utilize differential bacterial physiology characteristics such as, carbohydrate fermentation and antimicrobial susceptibility to allow for identification of STEC isolates via phenotypic characteristics such as colony morphology and color. However, these highly selective media also inhibit the growth of certain STEC serogroups. STEC serogroups can exhibit varying susceptibilities to antibiotics such as tellurite and novobiocin (D. Orth et al., 2007; Vimont, Delignette-Muller, & Vernozy-Rozand, 2007) which are commonly incorporated into selective media and consequently their growth may be inhibited (Gill et al., 2014).

As can be inferred from the discussion above, there are currently no selective culture media available for the detection of all STEC serotypes. MacConkey agar is a minimally selective media commonly used for the isolation of gram-negative enteric bacteria including *Escherichia coli*. The problem faced with the use of MacConkey is while it exhibits good sensitivity, it lacks STEC specificity, hence the need to screen multiple colonies. Herein lies the benefit of our research; we were able to demonstrate that screening more than 20 colonies does not significantly increase the probability of recovering a STEC isolate.

The disparity in the number of positive PCR positive samples and the number of culture positive samples could be due to a number of reasons. One possibility is that there
were no viable stx-producing cells in the fecal samples, and amplification of DNA from dead cells or viable but non-culturable cells occurred (Noll et al., 2015). Additionally there are a vast number of STEC serotypes and many have differing optimum growth conditions (Baker et al., 2016; Ferenc, Oliver, Witkowski, McLandsborough, & Levin, 2000; Gill et al., 2014; Vimont, Vernozy-Rozand, et al., 2007). Consequently, the enrichment conditions used may not have facilitated the growth of the stx serotype present in the feces. Another possibility is the presence of background/competitive flora may have prevented the proliferation of the STEC population (Hara-Kudo, Ikedo, Komatsu, Yamamoto, & Kumagai, 2002) thus reducing the sensitivity of our detection method.

There are several limitations to this study, and caution should be exercised when attempting to extrapolate these findings. Firstly the results obtained are specific for the growth and enrichment conditions used in this experiment. The type of enrichment broth and incubation conditions can affect the growth of STEC serotypes and thus affect its recovery from the MAC agar plate (De Boer & Heuvelink, 2000). Another limitation, one must be cognizant of, is samples may contain more than one STEC serotype and using this method does not guarantee recovery all of possible serotypes (Arthur, Barkocy-Gallagher, Rivera-Betancourt, & Koohmarai, 2002). Finally this method is not meant to be used as a stand-alone method for detection of STEC in fecal material. This method can however be used in conjunction with PCR screening for the isolation of STEC isolates from fecal samples.
Conclusion

Under these experimental conditions, selecting 20 suspected *E.coli* colonies will offer the same probability of recovering a STEC isolate as selecting greater number of colonies. The method described here, provides a simple yet efficient method for recovery of STEC isolates which can be employed in laboratories where more expansive methods are unavailable. Recovery of STEC isolates can be dependent on a number of factors, including STEC serotype, the matrix it is in, enrichment conditions, isolation technique and plating media. Further research into the physiological characteristics of the various STEC serotypes will possibly enable the identification of a common distinguishing trait and thus development of an appropriate isolation technique universal for all STEC isolates.

Acknowledgements

This material is based, in part, upon research supported by Caribbean Public Health Agency and state and federal funds allocated to the Ohio Agricultural Research and Development Center. The authors would like to acknowledge the assistance of Michael Kauffmann, Jennifer Schrock and Pam Schlegel in conducting this study.
Figure 3.1 Comparison of probability rates for selecting one *stx* positive colony from a PCR positive fecal sample for studies 1(●) and 2(▲) and the difference between the mean obtained. Values are expressed as the mean probabilities for ± 95% Confidence Interval.
Figure 3.2 Percentage of stx-positive isolates from each fecal sample screened in each study.
Table 3.1 Primers Sequences used in the PCR assays and the expected sizes of the products

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide Sequence (5’ to 3’)</th>
<th>Product Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK1</td>
<td>TTTACGATAGACTTCTCGAC</td>
<td>≈230</td>
<td>(H. Karch &amp; Meyer, 1989)</td>
</tr>
<tr>
<td>MK2</td>
<td>CACATATAAAATTATTTTCGCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLT 1 – F</td>
<td>TGTAACCTGAAAGGTGGAGTATAAC</td>
<td>210</td>
<td>(Hu et al., 1999)</td>
</tr>
<tr>
<td>SLT 1 – R</td>
<td>GCTATTCTGAGTCAACGAAAAATAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLT 11 – F</td>
<td>GTTTTTCTTCGATCCTATTCCG</td>
<td>484</td>
<td>(Hu et al., 1999)</td>
</tr>
<tr>
<td>SLT 11 – R</td>
<td>GATGCACTCTCTGGTCATTGTATTAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
References:


Chapter 4: Shiga toxin (stx) encoding genes in sheep and goats reared in Trinidad and Tobago.
Shiga toxin (stx) encoding genes in sheep and goats reared in Trinidad and Tobago.

Anil K. Persad¹, A.A. Adesiyun², Jeffrey T. LeJeune¹*

¹ Food Animal Health Research Program, Ohio Agriculture Research and Development Center, The Ohio State University, Wooster, Ohio, 44691

² School of Veterinary Medicine, The University of the West Indies, St. Augustine, Trinidad and Tobago

Running title: Stx in sheep and goats

Key words: Escherichia coli, Shiga toxin, sheep, goats, Trinidad, Caribbean

* Corresponding author: Jeffrey T. LeJeune

Ph: (330) 263-3739 Fx: (330) 263-3677

Email: lejeune.3@osu.edu
Abstract

Ruminants including sheep and goat have been identified as potential reservoirs for Shiga toxin-producing *Escherichia coli* (STEC). Trinidad and Tobago is one of the largest livestock producers and consumer of sheep and goat meat in the Caribbean. The epidemiology of STEC in these animals, though of utmost importance, has not been previously described. The prevalence of stx was investigated in healthy sheep (n=204) and goats (n=105) in Trinidad. Based on PCR screening, goats had a higher stx prevalence than sheep (46% vs 35%, P = 0.06). Upon culture, approximately two-thirds of all PCR-positive enrichments, regardless of species of origin, yielded colonies that only encoded stx1; however, isolates that were both stx1 and stx2 positive were more likely to be recovered from sheep compared to goats (24% vs 17%, P = 0.34). The eae gene was detected in only three stx-positive isolates recovered from two different ovine enrichments. None of the recovered isolates were of the O157 serogroup.

In both species, the prevalence of stx was higher in animals less than 3 months old versus older animals (sheep: 55% vs 30%, P = 0.003; goats: 56% vs 39%, P = 0.11). Sheep reared on deep litter flooring (43%) had a higher prevalence than sheep reared on slatted (27%) or concrete floorings (21%, P = 0.007), while goats reared on slatted flooring were 8.2 (95% CI: 1.45 – 45.84; P = 0.02) times as likely to shed stx than goats on deep litter. The presence of cows on the same premise was not an associated predictor for stx in sheep (P = 0.61) or goats (P = 0.09).
This study demonstrates that although sheep and goats in Trinidad are reservoirs for non-O157 stx-positive isolates, they do not play a significant role in the epidemiology of O157 STEC. Furthermore, it appears that non-O157 stx-positive isolates harbored by these animals do not pose a significant threat to human health

**Introduction.**

The annual foodborne disease burden in Trinidad and Tobago is estimated to be over 100,000 cases, or almost one tenth of the population, but the actual incidence and etiology of most cases is unknown since many cases are not reported (Lakhan et al., 2013). Globally, Shiga toxin-producing *Escherichia coli* (STEC), a diverse group of bacteria with over 400 serogroups, is estimated to cause over two million cases of human disease annually (Caprioli et al., 2005). Historically, STEC O157:H7 has been the pathotype most reported with human disease, however due to improved awareness and diagnostics capabilities, the number of human diseases associated with non–O157 serogroups has been increasingly recognized (Barkocy-Gallagher et al., 2005; Gould et al., 2013; Luna-Gierke et al., 2014; Scallan et al., 2011). Humans are primarily infected via consumption of contaminated food or water, but they can also be infected via direct contact with animals and/or their environment and via person to person transmission (Gyles, 2007). The median infectious dose (ID$_{50}$) for humans is estimated to be less than 100 cells (Karmali, 2004). Infections in humans can be asymptomatic, or present with a spectrum of clinical signs including mild to bloody diarrhea. Most infections are self-limiting, but approximately five percent of cases progress to hemorrhagic uremic
syndrome, thrombotic thrombocytopenic purpura and possibly death. Elderly persons, young children and immuno-compromised persons are at greatest risk of developing these complications.

Shiga toxin-producing *Escherichia coli* (STEC) can be harbored within the gastrointestinal tract of many animals and ruminants, including sheep and goats. These small ruminants have also been identified as potential reservoirs of STEC O157:H7 and non-O157 serogroups (Persad & Lejeune, 2015). The role of small ruminants in the epidemiology of STEC is exemplified by sheep being identified as the STEC reservoir of significance in Australia (Gyles, 2007). Similar to their bovine counterpart, these small ruminants tend to be asymptomatic shedders of STEC (Beutin et al., 1993; Cortes et al., 2005). Sheep and goats have been cited as reservoirs for over 100 other serotypes of STEC including O26, O115, O128 and O130 (Brandal et al., 2012; La Ragione et al., 2009). The predominant STEC serotype shed may vary with geographic location and *stx* prevalence in animals may vary due to a number of factors including age of animals, diet, climate and sanitation (Fraser, MacRae, Ogden, Forbes, & Strachan, 2013; Gyles, 2007; Persad & Lejeune, 2015). For example, in Switzerland the main STEC serotypes shed by sheep are O91:H- and O128:H2 while the main serotypes harbored by sheep in Brazil are O76:H19 and O65:H- (Martins, Guth, Piazza, Leão, Ludovico, Ludovico, Dahbi, Marzoa, Mora, Blanco, et al., 2015; Zweifel, Blanco, Blanco, Blanco, & Stephan, 2004). In Spain the 72% of isolates recovered from sheep belonged to one of these 12 STEC serogroups: O5:H- , O6:H10, O91:H-, O117:H-, O128:H-, O128:H2, O136:H20,
O146:H8, O146:H21, O156:H−, O166:H28, and ONT:H21 (Blanco et al., 2003). The influence of diet on sheep STEC O157 prevalences was reported by Fraser et al. (2013) who reported sheep fed brassica diets were found to have lower prevalence than animals fed conventional diets (Fraser et al., 2013), while other authors have reported that abruptly switching sheep to low quality forages increases the shedding of STEC O157 (Callaway et al., 2009). Although O157 shedding has been demonstrated to be seasonal, with higher prevalences in summer than winter (Fraser et al., 2013; Kudva et al., 1996), no such shedding pattern has been reported for non-O157 STEC with prevalence rates remaining almost constant irrespective of season (Hussein, Thran, & Glimp, 2003; Sánchez et al., 2010).

Human infections have also been linked to the consumption of lamb, mutton and goat meat (Bekal et al., 2014; Momtaz, Dehkordi, Rahimi, Ezadi, & Arab, 2013) as well as consumption of unpasteurized milk and cheese made from contaminated caprine and ovine milk (Cortes et al., 2005; Espie et al., 2006). The risk of transmission of STEC from small ruminants to humans is demonstrated by Brandal et al., who reported that almost 50% of the sheep STEC O26 isolates recovered in Norway had similar MLVA profiles to that found in human clinical cases (Brandal et al., 2012). Schimmer et al. also identified human STEC O103:H25 infections in Norway as having originated in sheep products. (Schimmer et al., 2008). Other reports have also implicated sheep as potential sources of human infection. (Blanco et al., 2003; La Ragione et al., 2009; Soderlund, Hedenstrom, Nilsson, Eriksson, & Aspan, 2012). Many direct contact human infections
are attributed to contact with sheep and goats at petting zoos and open farms (Heuvelink et al., 2002; LeJeune & Davis, 2004). Small ruminants, especially goats, generally exhibit inquisitive behavior, thus may have greater contact with humans increasing the potential for transmission to humans (La Ragione et al., 2009). Environmental contamination with STEC shed by sheep could also be a source of human infection, as was demonstrated in Scotland when 20 scouts were infected with *E.coli* O157 after camping on lands which were previously grazed by sheep. The *E. coli* O157:H7 isolates recovered from both scouts and deposited sheep feces were molecularly indistinguishable confirming the transmission from sheep to humans (Howie, Mukerjee, Cowden, Leith, & Reid, 2003).

Sheep and goat meat is considered a delicacy in Caribbean islands. Trinidad and Tobago, located in the southern Caribbean, is one of the leading consumers of sheep and goat meat per capita in the world with over 2kg of lamb and goat meat consumed annually per capita (Singh, 2006). Livestock husbandry practices in Trinidad mirrors that of the other Caribbean islands; most farms are semi-intensive and it is not uncommon to find more than one ruminant species on a farm. Most sheep and goat are primarily reared for meat production. In fact, Trinidad and Tobago is also one of the largest livestock producers in the Caribbean with an estimated 40,000 ruminants reared including 4,000 goats and 11,000 sheep annually (Central Statistical Office - Number of Small Ruminants by Region (Trinidad), 2012). Animals are frequently slaughtered under low-input, non-commercial, backyard or roadside locations in crudely constructed facilities usually devoid of running water and proper sanitation protocols. The high consumption of sheep
and goat meat, coupled with poor slaughter practices, increases the risk of persons in Trinidad and Tobago contracting foodborne STEC infections from these animals.

Although being a main cause of foodborne disease globally, there is a limited published literature on STEC epidemiology in Trinidad and Tobago. Literature searches of computer indexed databases yielded no recent publications describing the epidemiology of STEC carriage in live sheep and goats reared in this part of the world. Our literature search did however yield two publications from the early 1990s, with one study comparing the STEC prevalence in diarrheic and non-diarrheic sheep (Abiodun A Adesiyun & Kaminjolo, 1994) and another reporting the prevalence in goat meat sold at market (AA Adesiyun, 1993). Neither study reported the serotype nor the virulence profile of the STEC isolated.

While there are defined methods for the isolation of STEC O157, this is not the case for non-O157 serotypes (Beutin & Fach, 2014). For this study we used detection of Shiga toxin genes (stx), the main virulence factor of STEC, as an indicator for STEC presence. Stx is rarely reported in micro-organisms other than E. coli. The prototypic Stx-encoding organism is Shigella spp. but ruminants are not susceptible to colonization by Shigella spp. (LeJeune, D. Hancock, et al., 2006). Given the critical void in information on STEC epidemiology in Trinidad, we sought to partially fill this gap in knowledge by (1) Determining prevalence of stx in the feces of sheep and goats and identify any management risk factors, (2) Evaluate the virulence profile of stx-positive isolates
obtained and (3) Determine the genetic relatedness of caprine and ovine stx-positive isolates and human isolates obtained from human diarrheic fecal samples.

**Methodology**

During the months of January and February 2015, a cross sectional study was carried out to determine the prevalence of stx in healthy sheep and goats, and diarrheagenic human fecal samples. A total of 204 sheep and 105 goat fecal samples were collected from 10 sheep and 7 goat farms in northern and central Trinidad. The overall number of samples collected would have given us the estimated prevalence stx for the entire sheep and goat population in Trinidad at a 95% Confidence level and 5% confidence interval. No data was available for the overall number of sheep and goat farms in Trinidad and their herd sizes. Farms were selected, based primarily on the farmers’ willingness to participate in the study. At each farm visit we collected a minimum of 20 sheep fecal samples and/or 10 goat fecal samples. Using previously published prevalence estimate data for sheep and goats this sampling number per farm would have given us a greater than 95% of detecting a STEC positive animals on the farm (Abiodun A Adesiyun & Kaminjolo, 1994; Jacob, Foster, Rogers, Balcomb, & Sanderson, 2013; Jacob, Foster, Rogers, Balcomb, Shi, et al., 2013). Animals with a recent history of diarrhea, other illness, or receiving antibiotics were excluded from this study. Selected farm management practices were assessed using a questionnaire and discussion with the farmers during farm visits. The key areas of focus were age of
animals, housing, management system, and proximity to cattle. De – identified human diarrheal fecal samples were kindly provided by the North-Central Regional Health Authority Adult Hospital, Mt. Hope, Trinidad and Tobago. No Epidemiological information was provided for the human fecal samples. All tests were completed in compliance with institutional (OSU) guidelines for the Care and Use of Animals and human subjects.

Collection of small ruminant fecal samples: Fecal samples were collected from sheep and goats housed on ten sheep and seven goat farms. During each visit, individual fecal samples were collected aseptically per rectum from these small ruminants using sterile gloves. These samples were placed into individually labeled Whirl-pak® bags (Nasco, Fort Atkinson, WI) and transported on ice in a cooler to the laboratory and processed within 24 hours of collection.

Processing of fecal samples: At the laboratory, fecal samples were screened for the presence of stx genes using a method adapted from LeJeune et al (2006) and Hu et al (1999) (Hu et al., 1999; LeJeune, D. Hancock, et al., 2006). Briefly, individual fecal samples were crushed and mixed to attain a homogenized sample. A five gram aliquot was inoculated into 45 milliliters of Buffered Peptone Water (BPW) (Acumedia, East Lansing, MI). This suspension was then thoroughly mixed via manual agitation for two minutes and incubated overnight at 42°C. Following enrichment, 100 µl was removed for
detection of Shiga toxin genes (stx$_1$ and stx$_2$) and 200 µl of the enrichment mixed with Buffered Glycerol and frozen at -80°C for further analysis at a later date.

Screening for stx genes: 100 µl of the BPW enrichment added to a 1.7ml microcentrifuge tube containing 900µl of BPW. The contents of the tube were then mixed using a vortex and the cells pelleted via centrifugation at 18,000 x g for three minutes. The supernatant was decanted and pellet resuspended in 1 ml of BPW. This procedure was repeated three times. After the third washing procedure, the enriched fecal suspensions were boiled at 100°C for 15 minutes. The suspensions were then allowed to cool to room temperature and 4 µl RNase (0.5 mg/ml) was added to each sample and incubated for 30 minutes at 37°C. The cell lysates were then used as the DNA template for PCR screening for the detection of stx$_1$ (210 bp) and stx$_2$ (484 bp) genes as described by Hu et al (1999) (Hu et al., 1999). Amplifications were performed in a 100µl reaction mixture containing 50µl GoTaq® Green Master Mix (Promega, Madison, WI), 3.0 µl of each primer, SLT IF (5’ TGTAA CTGGAAAGGTGGAGTATAC 3’), SLT IR (5’GCTATTTCTGAGTCAACGAAAAATA AC3’), SLT IIF (5’GTTTTTCTTCGGGTATCCTATCCCG3’), SLT IIR (5’GATGCATC TCTGGTCATTGTATTAC3’), 2.5 µL MgCl$_2$, 30.5 µl sterile water and 5µl DNA template. The reaction mixture was heated to 94°C for three minutes, followed by 30 cycles of amplification at of 94°C for 30 seconds, 59°C for one minute, one minute at 72°C, and final extension was done at 72°C for 10 minutes. The PCR products were separated on 2% agarose gels stained with GelRed (Biotium, Cambridge Bioscience Ltd, 189
UK) and visualized using UV light. Samples testing positive for stx genes were subjected to further testing to identify the stx profile and virulence genes of individual colonies. Screening of individual colonies: 100μl of each of the previously frozen enriched small ruminant fecal samples, were added to 1.7ml microcentrifuge tubes containing one milliliter of BPW and incubated for 18 – 24 hours at 42°C. Following incubation, 75μl of this enrichment was then streaked for colony isolation onto MacConkey (MAC; Acumedia, East Lansing, MI) agar plates and incubated for 18 – 24 hours at 42°C. The next day, up to 30 individual lactose-fermenting, suspected E. coli colonies were selected from the MAC plates. These colonies were 1) transferred to Whatman FTA Cards (GE Healthcare, Piscataway, NJ) and the cards shipped to Food Animal Health Program (FAHRP), OARDC in Wooster, Ohio for stx screening; and 2) inoculated into Brain-Heart infusion broth (BHI, Acumedia, East Lansing, MI) in 96-well plates. These 96-well plates were incubated for 18 – 24 hours at 42°C. Subsequently buffered glycerol added to each well and stored at -80°C.

At the FAHRP laboratory, a 6mm sterilized paper punch was used to excise portions of the FTA card that contained the colony smears. These portions of the card were transferred to 1.7ml microcentrifuge tubes containing 400μl of Millipore water. Tubes were vortexed three times for twenty seconds to dislodge attached cellular debris. The rinsate was then aspirated and 150μl of Millipore water added to each microcentrifuge tube. DNA elution was completed by placing the tubes in a water bath at 95°C for thirty minutes. The DNA extract was transferred to clean microcentrifuge tubes
and stored at -20°C. Following DNA extraction, 10μl of DNA extract from 5 colonies were pooled together and subjected to pooled PCR screening for detection of stx genes as described previously. The PCR products were separated on 2% agarose gels stained with ethidium bromide and visualized using UV light. From positive pools, the individual colonies comprising each pool were also subjected to PCR screening using the same amplification conditions as stated above. Extracted DNA from stx-positive colonies were screened for the presence of eae (368 bp), rfb (292 bp), and flic (625 bp) genes described by Hu et al (1999) (Hu et al., 1999). Amplifications were performed in a 50μl reaction mixture containing 25μl GoTaq® Green Master Mix (Promega, Madison, WI), 1.0 μl of each of forward and reverse primers for flic (F:5’GACTGTCGATGCATCAGGCAAAG3’; R:5’CAACGCTG ACTTATCGCCATTCC3’) and eae (F:5’GACTGTCGATGCATCAGGCAAAG3’; R: 5’TGGAGTATTAACATTAACCCCAGG3’) and 1.5 μl of forward and reverse primers from rfb (F:5’GTGTCCATTATACGGACATCCATG CCAAAG3’; R:5’ CCTATAACGTCATG CCAATATTGCC3’), 2.5μL MgCL₂, 0.5 μl BSA, 10.0 μl sterile water and 5μl DNA template. The reaction mixture was heated to 94°C for three minutes, followed by 30 cycles of amplification at of 94°C for 30 seconds, 59°C for one minute, one minute at 72°C, and final extension was done at 72°C for 10 minutes. The PCR products were separated on 2% agarose gels stained with ethidium bromide and visualized using UV light.

191
**Statistical Analysis.**

Animal and herd prevalence values for *stx* were tabulated. Statistical analyses were done using Minitab 16.0® statistical software (Minitab Inc., State College, Pa., USA.). Univariate differences in the frequency of specific on farm management practices were compared using Fisher Exact Test. Multivariate logistic regression modelling was used to identify potential risk factors for the presence of one or more *stx*-positive sheep or goat on Trinidad farms. Potential predictor variables were assessed for collinearity using Spearman rank correlation. If the correlation value between two independent values was greater than |0.7| (*P*<0.05) then, based on biological plausibility, only one of the variables would be selected for inclusion in model (Cernicchiaro et al., 2009). Differences were considered to be statistically significant at *P* <0.05.

**Results**

We tested 204 sheep, 105 goat and 45 human fecal samples. We classified animals less than three months as being young and those greater than three months as being adult. Animals were housed either under fully intensive (zero grazed) or semi-intensive production systems. Those under semi-intensive productions systems were usually allowed to graze during the day and confined to pens at night. Due to the risk of praedial larceny, most farms we visited confined the goats to pens and were not allowed to graze. The diets of all animals were supplemented with commercially produced pellet rations. All farms provided chlorinated water for drinking to the animals. Pen flooring differed between farms, with animals being reared on either wooden slatted flooring,
solid concrete, or on deep litter. Cattle were also reared on some of these farms and we investigated this as a possible risk factor for stx in sheep and goats.

Overall the stx prevalence in goats was 45% (48/105; 95% C.I: 36-55%) while the prevalence in sheep was 35% (71/204; 95% C.I: 28-41%; P = 0.06). No human samples tested positive for stx. All sheep farms (100%) and six of seven goat farms (88%) sampled had at least one animal shedding stx in its feces. On farms which reared both sheep and goats, the prevalence in goats was higher than sheep (48% vs 32%; P = 0.029).

The average within-farm prevalence for goats was 46% (95% CI 25 – 67%). When stratified according to age, the stx prevalence in fecal samples from young goats was higher compared to adult goats (56% (95% CI: 41-72%) vs 39% (95% CI: 27-51%; P = 0.11). The stx prevalence in goats which were allowed to graze was slightly lower than goats that were allowed to graze intermittently (45% (95% CI: 31-59%) vs 46% (95% CI: 33-59%); P = 0.99). Goats which were reared on farms with cows had a stx prevalence of compared to goats reared on farms without cows (50% (95% CI: 35-65%) vs 43% (95% CI: 31-55%); P = 0.55). The stx prevalence in goats reared on slatted floors was higher than goats reared on deep litter bedding (52% (95% CI: 40-64%) vs 34% (95% CI: 19-49%); P = 0.10).

The average within-farm prevalence for stx in sheep fecal samples 35% (95% CI: 28-41%). When stratified according to age, young sheep had higher stx prevalence than adult sheep (55% (CI: 39-71%) vs 30% (CI: 23-37%); P = 0.004). Unlike goats, sheep that were confined to pens had a higher prevalence compared animals that were allowed
to graze (36% (95% CI: 28-43%) vs 32% (95% CI: 20-43%) P = 0.62). The prevalence of stx in sheep reared on farms with cows was 52% (95% CI: 39-65%) compared to farms without cows (27% CI: 20-35%; P = 0.007). With Bonferroni’s correction, pairwise analyses for flooring type demonstrated that animals housed on deep litter had a higher stx prevalence of 43% (95% CI: 34-52%; P = 0.007) compared to animals on slatted flooring (27% CI: 13-40%) and animals on solid concrete flooring (22% CI: 9-33%).

Based on PCR screening of fecal samples, stx$_1$ alone was detected in the majority (68% vs 69%, P = 0.90) of PCR-positive enrichments from sheep and goat fecal samples. A higher proportion of goat PCR positive fecal enrichments were stx$_2$ positive alone compared to sheep samples (14% vs 8%; P = 0.55), but a higher proportion of sheep fecal samples contained isolated both stx$_1$ and stx$_2$ genes compared to goat fecal samples (24% vs 17%, P = 0.34).

We recovered at least one stx positive cell from 77% of the PCR positive fecal samples and all the isolates from 74% of the fecal samples had a single stx type, 18% had isolates with two stx different profiles, and 9% of the fecal samples had isolates with 3 distinct stx profiles (stx$_1$, stx$_2$, stx$_{1+2}$). Although PCR positive, we did not recover any stx-positive cells from 28% of goat samples and 20% of sheep samples (P = 0.37). The majority of both sheep and goat fecal samples had isolates one stx profile (53% vs 60%, P = 0.57) while a higher proportion of sheep fecal samples had isolates with two different stx profiles compared to goat fecal samples (17% vs 9%; P = 0.27). In only 4% of goat
and 9% of sheep PCR positive samples did we recover \textit{stx}-positive isolates which were either \textit{stx}_1, \textit{stx}_2 or \textit{stx}_{1+2}-positive \((P = 0.47)\).

Overall we recovered 685 \textit{stx}-positive isolates; 453 from sheep and 232 from goats. Of this, 74% were \textit{stx}_1 alone, 14% were \textit{stx}_2 alone, and 12% were both \textit{stx}_1 and \textit{stx}_2-positive. A higher proportion of the isolates recovered from goats (79%) were \textit{stx}_1-positive alone compared to 71% for sheep \((P = 0.035)\). Similarly, as \textit{stx}_1-positive alone isolates, a greater proportion of goat isolates were \textit{stx}_2-positive alone compared to sheep (18% vs 11%, \(P = 0.024\)). However unlike the other two other \textit{stx} profiles, a higher proportion of sheep isolates were \textit{stx}_{1+2}- positive compared to goat isolates (18% vs 8%; \(P <0.001\)). Only three \textit{stx}-positive isolates tested positive for \textit{eae} gene and none tested positive for \textit{rfb}_{O157}. All three \textit{eae}-positive isolates came from sheep and were \textit{stx}_1 (alone) positive. Of these three isolates, two came from the same animal and both of these isolates were also \textit{flic} negative, while the isolate from the other animal was \textit{flic} positive. The two positive animals, one a young male and the other an adult female came from different farms; however, it should be noted that one of these farms supplies breeding stock to other farms.

Risk factor analysis.

Variables which were significantly associated with the presence of \textit{stx} in sheep fecal samples were age of animals and grazing. For goats, the predictor variables included age and the flooring type. The statistically significant variables in this model, with the exception of grazing for sheep, were all associated with increased odds for the
animal fecal sample testing positive. Allowing sheep to graze had a protective effect and reduced the odds of testing positive for stx. The odds ratio and P values for the significant predictors are presented in table 1.

Discussion

This is the first study documenting the stx prevalence in goats in not only Trinidad but the Caribbean. In our study, 45% of goats and 36% of sheep tested positive for shedding STEC in their feces. However despite this high prevalence, none of the recovered isolates belonged to the O157 serogroup and only three isolates possessed intimin. These results indicate that while sheep and goats are reservoirs for non-O157 STEC, they do not play a significant role in the epidemiology of the more pathogenic O157 STEC serogroup. The severity of diseases associated with non-O157 STEC infections is usually milder than O157 infections (Johnson et al., 2006), and this coupled with the low percentage of eae-positive isolates indicate that stx-positive isolates harbored by sheep and goats in Trinidad do not pose a significant threat to human health.

The prevalence of stx in sheep obtained in this study is higher than the one previously published report of 22% STEC prevalence in sheep reared in Trinidad (P = 0.05) (Adesiyun & Kaminjolo, 1994). The prevalence of stx in sheep and goats in this study is also higher than previously published data for other animal species in Trinidad (Adesiyun, 1999; Harris et al., 2012; Roopnarine et al., 2007). One possible reason for this difference is because our method for stx detection is more sensitive since we included an enrichment step in our detection and recovery of stx-positive isolates which was
absent from other Trinidad STEC studies. STEC when present in animal feces is usually present at such low numbers that it may be missed via direct plating, thus cultural enrichments are required for detectible limits to be reached (Beutin & Fach, 2014).

Another hypothesis for the higher stx prevalence compared to other species is stx-positive serotypes found in Trinidad preferentially colonizes sheep and goats as opposed to other animal species. A similar phenomenon is seen in Australia where sheep are considered the host of significance for STEC (Gyles, 2007). The plausibility of this species–specificity hypothesis needs to be further investigated.

The high prevalence of non-O157 stx-positive isolates in both sheep and goats is consistent with previously published literature from other geographical regions (Blanco et al., 2003; Cortes et al., 2005; Orden et al., 2008; Vu-Khac & Cornick, 2008). In Spain, Blanco et al. (2003) reported that 36% of lambs sampled from 63 flocks shed non-O157 STEC while the prevalence in sheep reared in New Zealand is reported at 66% (Blanco et al., 2003; Cookson, Taylor, & Attwood, 2006). Other studies in Spain and Vietnam focusing on goats, reported a STEC prevalence in goats similar to what we report herein: In Spain, 48% of dairy goats sampled from 12 farms were positive for non-O157 STEC while the prevalence of STEC in goats in Vietnam was 39% (Cortes et al., 2005; Vu-Khac & Cornick, 2008). Within the United States, the prevalence of non-O157 STEC in goats was reported to be 14% which is lower than our study, however this U.S. study only focused on serogroups O26, O45, O103, O111 and O145 and samples were only considered positive if one of these serotypes plus stx1 or stx2 were present (Jacob, Foster,
Rogers, Balcomb, Shi, et al., 2013). In our study, and others cited above, samples were classified as positive if stx\(_1\) and/or stx\(_2\) positive isolates were present and no serogroup presence criterion was included for classification samples. This difference in classification criteria may be the reason for the lower prevalence reported in the US.

The majority of fecal samples harbored stx-positive isolates with only one stx profile. Isolates with multiple stx profiles were recovered from 27% of the fecal samples screened. The varying stx profiles could indicate the animals were shedding multiple serotypes of STEC or there were serotypes with different stx profiles.

The preponderance of stx\(_1\) isolates recovered from small ruminant animals has also been recorded in other epidemiological studies (Cortes et al., 2005; Martins, Guth, Piazza, Leão, Ludovico, Ludovico, Dahbi, Marzoa, Mora, & Blanco, 2015; Orden et al., 2003). This high proportion of stx\(_1\)-positive isolates could be as a result of these isolates being better adapted to colonization of small ruminants than stx\(_2\) or stx\(_{1+2}\)-positive isolates. Another reason is that stx-positive isolates in small ruminants were resistant to lysogenization by stx\(_2\) bacteriophages. The exact reason for this observation needs to be further investigated.

In our study none of the stx-positive 685 colonies screened belonged to the O157 serogroup and only three were intimin positive. Similar results have been reported in New Zealand, where Cookson et al. (2006) screened 442 stx-positive sheep isolates and failed to recover any O157 positive isolates. In that study, only three 0.7% (3/442) versus our 0.4% (3/685) of the stx-positive isolates were also eae-positive (Cookson et al.,
Shilling et al. (2012) also failed to recover any O157 serogroups from 193 stx positive isolates obtained from sheep and goats and only 0.52% of stx-positive isolates screened were eae-positive (Schilling et al., 2012). The absence or low prevalence of O157 serogroups and eae-positive isolates in stx positive small ruminant samples was also confirmed by Cortes et al. (2005) who screened 105 different STEC serotypes recovered from goat feces, and reported the serogroups neither belonged to the O157 serogroup nor were they eae-positive (Cortes et al., 2005). Studies which have successfully identified O157 serogroup in sheep or goat feces report a prevalence of between 1.8 – 11.7% (Chapman et al., 1997; Franco et al., 2009; Hoar et al., 2013; Soderlund et al., 2012; Solecki, MacRae, Strachan, Lindstedt, & Ogden, 2009). The absence or low prevalence of O157 STEC from sheep and goat feces indicates that sheep and goats are an unlikely source of human O157 infection.

For both sheep and goats, age of the animal was found to significantly affect the odds of an animal testing positive for stx. For goats, young animals were almost five times as likely to test positive as an adult goat and similarly, young sheep were three times as likely to test positive as an adult sheep. Adult animals have a more diverse gastrointestinal microbial flora than young animals and such either via competitive exclusion STEC isolates may not be able to attach and colonize to the intestinal epithelium (Zhao et al., 2013). Another possibility is that younger animals may have been colonized with different STEC serogroups which are from the adults. Other plausible explanations for this difference in prevalence include differences in diet, immune status.
and stress which may have made younger animals more susceptible to STEC colonization (Djordjevic et al., 2004). Young animals (less than 6 months) are not slaughtered in Trinidad and thus although being more likely to harbor stx-positive compared to adults, they would not be a food safety risk. Although they do not enter the food chain, young animals can serve as maintenance hosts and can amplify and shed stx-positive colonies resulting in environmental contamination and transmission to other animals. Young lamb and kids are popular at open farms and petting zoos especially with little children, and as such they can be a source of infection to humans via direct contact.

Grazing reduced odds of a sheep shedding stx. Animals which were allowed to graze were almost five times less likely to shed stx than sheep not allowed to graze. One possible reason for the higher prevalence in non-grazing animals is that they are confined to pens and there is of greater contact between animals and the feces from animals shedding stx-positive colonies resulting in new animals being colonized or recolonization of animals (Urdahl, Beutin, Skjerve, Zimmermann, & Wasteson, 2003). The effect of grazing as a predictor of STEC prevalence in goats could not be reliably investigated since most of the goats sampled (97/105) were reared in confined pens.

The prevalence of stx in sheep and goats was not affected by the presence of cows on the farm. This is consistent with research findings of Urdahl et al. (2003) who also reported that there was no difference in the distribution of stx in sheep reared on farms with/without cattle (Urdahl et al., 2003). The fact that our study demonstrates that presence of cattle on a farm is not a risk factor for stx carriage in sheep and goats lends
further support to the hypothesis that STEC serogroups exhibit an animal-host relationship and STEC serogroups will preferentially colonize certain animal species. Previous epidemiological studies have also postulated the existence of such a relationship since there was a high degree of disparity in the STEC serogroups recovered from different animal species reared on the same farm (Beutin et al., 1997; Urdahl et al., 2003). There are over 400 different STEC serotypes and while cattle has been identified as a main reservoir for O157, it is clear there may multiple other animal reservoirs for non-O157 STEC. For example Djorodjevic et al. (2004), reported that despite cattle and sheep grazing together on the same pasture, 15 non-O157 STEC serotypes, 3 of which were also eae-positive were only recovered from sheep and not from cattle (Djordjevic et al., 2004). Accordingly, if the incidence of STEC-associated human disease is to be controlled, research should be directed towards identifying the various animal or environmental reservoirs of non-O157 STEC and intervention programs tailored appropriately and not just towards cattle.

Another factor identified as a possible predictor for stx prevalence was the flooring type. Sheep housed on deep litter bedding were four times more likely to test positive for stx compared to sheep housed on slatted or solid concrete flooring. One possible reason for this is deep litter bedding allowed for greater accumulation of feces in the environment compared to solid concrete flooring which were cleaned daily and slatted flooring which allowed for feces to drop below the pen and not remain in the direct environment of the animal. The overall better pen hygiene may with pens that are slatted
flooring or bare concrete may account for the lower prevalence observed compared to sheep reared on deep litter. Another possibility is that although sheep do not typically consume the litter, bacterial contaminants in the litter may become aerosolized and contaminate feed and water troughs or attach to the animal hide. Additionally these aerosolized particles may become trapped in the upper airway and consequently be swallowed (Farrokh et al., 2013; LeJeune & Kauffman, 2005). Interestingly the opposite was observed with goats. Goats on slatted flooring were eight times as likely to test positive for \textit{stx} compared to goats on deep litter flooring. Goats tend to prefer diets with higher roughage content compared to sheep (Wahed & Owen, 1986), and given their browsing and inquisitive nature they are more likely to consume bedding in their pen. Consumption of high roughage bedding can result in increased undigested matter reaching the large intestine which undergoes fermentation and L-lactate is produced which is known to have antimicrobial effects against \textit{E. coli} O157 and non- O157 \textit{E. coli} isolates (Leitch & Stewart, 2002; Shelef, 1994). Another possibility is that forage based bedding may contain phenolic compounds which can inhibit growth of \textit{E. coli} and O157:H7 (Berard et al., 2009; Wells et al., 2005). The bedding may also harbor a diverse bacterial population and when consumed, may reduce intestinal colonization by STEC via competitive exclusion (Callaway et al., 2009). These factors may account for differences in \textit{stx} prevalence but further research into the exact mechanism for the discrepancy in \textit{stx} prevalence between sheep and goats is warranted.
The small size (n=45), could have accounted for the absence of any human stx positive fecal samples since the diarrhea may have been due to multiple other etiologies. An expanded study targeting the human population is required to fully elucidate the STEC epidemiology in humans and possibly identify the factors which can account for the apparent absence of stx in human population.

From data attained in this study, we can conclude that sheep and goats in Trinidad are important reservoirs of stx-positive non-O157 isolates but not O157. The absence of eae-positive strains in goats indicate that these strains are less pathogenic to humans since adherence of STEC to enterocytes, mainly dependent on the expression of eae gene, is highly correlated with pathogenicity (Johnson et al., 2006). Although the risk is low, these strains can nevertheless potentially result in disease since there are reports of eae-negative STEC isolates infections causing hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) in humans (Bekal et al., 2014). Other virulence factors including STEC auto-agglutination adhesin (encoded by saa gene), sab, an auto-transporter associated with biofilm formation, and EibG, E. coli immunoglobulin binding protein G, have also been identified as contributing to attachment of eae-negative STEC isolates to the intestinal endothelial cells (Croxen et al., 2013; Jenkins et al., 2003; Paton et al., 2001).

On the other hand, a low percentage (1%) of sheep fecal samples had stx-positive isolates which were also eae-positive. All of these isolates were stx₁-postive alone. Isolates with stx₂ or stx₁ and stx₂ are more likely associated with development of HUS and
subsequent STEC associated complications. The risk of human disease from these \textit{stx}_1-positive isolates recovered from sheep though usually not as severe as \textit{stx}_2+\textit{eae}-positive isolates should not be ignored; since there are case reports of severe human infections being caused by \textit{stx}_1-positive only STEC isolates (Sadjia Bekal et al., 2014).

Additionally, although there was a low prevalence of highly pathogenic STEC recovered from sheep and goats one must be cognizant of the possibility of \textit{stx} phages from STEC isolates being lysogenized into an Enteroaggregative \textit{Escherichia coli} strain as was the case in German 2011 O104:H4 outbreak (Frank et al., 2011).

In conclusion, we have identified age, flooring type and absence of grazing as risk factors which affect the prevalence of STEC in sheep and goats. Despite the high prevalence of \textit{stx}-positive isolates shed by sheep and goats, these results indicate that the sheep and goats do not play a significant role in the epidemiology of O157 STEC. Furthermore the majority of isolates were \textit{eae}-negative (99%) indicating they were not likely to be able to effectively colonize the human intestinal epithelium. We can therefore conclude that \textit{stx}-positive isolates shed by sheep and goats in Trinidad do not pose a significant risk to human health

\textbf{Acknowledgements}

This material is based, in part, upon research supported by Caribbean Public Health Agency and state and federal funds allocated to Jeffrey LeJeune and the Ohio Agricultural Research and Development Center. The authors would like to acknowledge
the assistance of Aphzal Mohammed, Narendra Siew, Hasani Stewart and Amar Soonar of The University of Trinidad and Tobago for their assistance in identification of farms and collection of fecal samples. The authors would also like to acknowledge the assistance of Michael Kauffman, Jennifer Schrock, Nicholas Anderson and Hannah Preston in conducting this study. Finally we wish to thank the co-operating farmers.

Table 4.1. Multivariate effects logistic regression model of associations between farm management factors and prevalence of Stx in sheep and goat feces

<table>
<thead>
<tr>
<th>Species</th>
<th>Model Predictor</th>
<th>Odds Risk (OR)</th>
<th>OR (95% CI)</th>
<th>P – value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>Age (Young)</td>
<td>2.89</td>
<td>1.28 – 6.54</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Grazing</td>
<td>0.27</td>
<td>0.09 – 0.81</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Flooring (Deep Litter)</td>
<td>4.38</td>
<td>1.40 – 13.75</td>
<td>0.01</td>
</tr>
<tr>
<td>Goat</td>
<td>Age (Young)</td>
<td>4.95</td>
<td>1.73 – 14.21</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Flooring (Slatted Flooring)</td>
<td>8.16</td>
<td>1.45 – 45.84</td>
<td>0.02</td>
</tr>
</tbody>
</table>
References:


Bibliography:


Dan Ramdath, D., Hilaire, Debbie G, Cheong, Kimlyn D, & Sharma, Sangita. (2011). Dietary intake among adults in Trinidad and Tobago and development of a
quantitative food frequency questionnaire to highlight nutritional needs for lifestyle interventions. *International journal of food sciences and nutrition*, 62(6), 636-641.


experimentally infected with enterohemorrhagic Escherichia coli. *J Infect Dis, 193*(8), 1125-1134. doi: 10.1086/501364


Jacob, Megan E, Callaway, Todd R, & Nagaraja, TG. (2009). Dietary interactions and interventions affecting Escherichia coli O157 colonization and shedding in cattle. *Foodborne Pathogens and Disease, 6*(7), 785-792.


237


238


Persad, Anil K., & Lejeune, Jeffrey T. (2015). Animal Reservoirs of Shiga Toxin-Producing *Escherichia coli* *Enterohemorrhagic Escherichia coli* and Other Shiga Toxin-Producing *E. coli*: American Society of Microbiology.


257


Savio, María. (1999). Sistema de vigilancia epidemiológica de las enfermedades transmitidas por alimentos *Sistema de vigilancia epidemiológica de las enfermedades transmitidas por alimentos.*


261


Tzschoppe, M., Martin, A., & Beutin, L. (2012). A rapid procedure for the detection and isolation of enterohaemorrhagic Escherichia coli (EHEC) serogroup O26, O103,


Appendix A: Identification and subtyping of *Salmonella* isolates using Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF)
Identification and subtyping of *Salmonella* isolates using Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF)

Anil K. Persad, Hanan Fahmy, Jing Cui, Jeffrey T. LeJeune*

Food Animal Health Research Program, The Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691 (Persad, LeJeune); Animal Disease Diagnostic Laboratory, Ohio Department of Agriculture, 8995 East Main St, Building 6, Reynoldsburg, OH 43068 (Cui);

* Corresponding author: Jeffrey T. LeJeune, Food Animal Health Research Program, The Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691; Email: lejeune.3@osu.edu

Short title: Identification of Salmonella serovars using MALDI-TOF
Abstract

Subtyping of bacterial isolates of the same genus and species is an important tool in epidemiological investigations. A number of phenotypic and genotypic subtyping methods are available; however, most of these methods are labor-intensive, time-consuming, require considerable operator skill, and a wealth of reagents. Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF), an alternative to conventional subtyping methods, offers a rapid, reproducible method for bacterial identification with a high sensitivity and specificity and at minimal cost. The purpose of this study was to determine the feasibility using MALDI-TOF to differentiate between six *Salmonella* serovars recovered from experimental microcosms inoculated with known strains of *Salmonella*. Following the establishment of a MALDI-TOF reference library for this project, the identity of 965 *Salmonella* isolates recovered from experimental microcosms was assessed using both MALDI-TOF and conventional methods (serotyping /PCR). All 965 isolates were identified as being *Salmonella* species. Overall, 937/965 (97%) of these isolates were identified similarly using the two different methods. Positive percent agreement at the serovar level ranged from 82 -100% and negative percent agreement for all serovars was greater than 99%. There was no statistical difference between results obtained using MALDI-TOF and conventional serotyping methods. Cohen’s kappa ranged from 0.82 to 0.99 for different the serovars. This study demonstrates that MALDI-TOF is a viable alternative for the rapid identification and differentiation of *Salmonella* serovars.

272
**Keywords:** Mass spectrometry, Matrix-Assisted Laser Desorption-Ionization, *Salmonella*, subtyping,

**Introduction**

*Salmonella enterica* serovars have been reported with foodborne disease globally for over 100 years (Lee, Runyon, Herrman, Phillips, & Hsieh, 2015). While the global incidence is unknown, within the United States the disease burden is estimated to be over one million cases annually, with 16,000 hospitalizations and almost 500 deaths (Scallan et al., 2011). Most human cases are self-limiting; however young children under five years, the elderly and immunocompromised are at most risk of becoming infected and also developing complications (Baird-Parker, 1990). Humans can be infected from the consumption of contaminated food and water, direct contact with infected animals and their environment or via person to person transmission. One necessity in the successful treatment, prevention and control of foodborne disease outbreaks is the rapid and accurate identification of the offending pathogen (Sabat et al., 2013).

Bacterial subtyping of isolates of the same genus and species is an important tool in disease surveillance, outbreak investigations and epidemiological research. A number of phenotypic and genotypic subtyping methods are available (Carbonnelle et al., 2011); however, for foodborne pathogens, such as *Salmonella enterica* subsp *enterica* serotyping is often among the most widely used initial characterizations performed on isolates.
Salmonella serotyping, is a phenotypic subtyping method which has been in existence for over eighty years and is still the primary screening method in many laboratories (Wattiau, Boland, & Bertrand, 2011). The basis of this method of subtyping is observation for agglutination reactions occurring between specific antisera and somatic (O) and flagellar (H) antigens of the Salmonella isolate. The Salmonella isolate is then classified using the Kauffman-White scheme (Grimont & Weill, 2007). However given there are over 2500 serovars of Salmonella enterica, with 46 O antigens and 114 H variations (McQuiston, Fields, Tauxe, & Logsdon Jr, 2008), serotyping can be quite exhausting, time consuming and require a vast number of antisera (Dieckmann & Malorny, 2011). Additionally, the possibility exists for inaccurate classification as a result of observer error, nonspecific agglutination, auto-agglutination or loss of antigenic expression (Schrader, Fernandez-Castro, Cheung, Crandall, & Abbott, 2008; Wattiau et al., 2011).

In comparison, Matrix Laser Desorption/Ionization Time of Flight (MALDI–TOF), a library-based approach to bacterial identification, offers a rapid, reproducible method for bacterial identification with a high sensitivity and specificity and at minimal cost (Christner et al., 2014; Kliem & Sauer, 2012). MALDI-TOF uses the mass-to-charge ratio profile of bacterial microbial proteins and peptides for bacterial identification (Sandrin, Goldstein, & Schumaker, 2013). This mass-to-charge ratio profile or mass spectral profile analysis is usually confined to the 2 – 20 kDa since the majority of peaks in this range are representative of ribosomal proteins which are less influenced to by variability in cultivation conditions (Dingle & Butler-Wu, 2013). Bacterial isolates can be

274
characterized at the genus and species level via the identification of a unique biomarker ion peak(s) or by matching the mass spectral profile or “fingerprint” of query bacteria with the spectral profiles of known bacterial species within the established MALDI-TOF library using pattern recognition algorithms (Carbonnelle et al., 2011; Dieckmann & Malorny, 2011; Karger et al., 2011; Kliem & Sauer, 2012). Identification at the subspecies level can however be more difficult due to the lack of unique ion peaks between serotypes and also due to poor differentiation of mass spectral profiles between the serotypes (Sandrin et al., 2013). Although MALDI-TOF use has been well validated for bacterial identification at the species level, differentiation at the subspecies level is less well described.

The purpose of this study was to determine the feasibility of using MALDI-TOF technology to differentiate six known Salmonella serovars by comparing MALDI-TOF subtyping with conventional subtyping of Salmonella isolates recovered from experimental microcosms inoculated with these same strains of Salmonella enterica serovars.

Materials and Methods.

Bacterial strains: The Salmonella enterica serovars used in this study were S. Anatum (K2669 CDC clinical isolate), S. Braenderup (04E61556), S. Javiana (ATCC® BAA-1593TM), S. Montevideo (Human- tomato linked), S. Newport (Environmental isolate) and S. Typhimurium (ATCC® 700720TM). The serovars and their epidemiological history
were kindly provided by Dr. Michelle Danyluk, Citrus Research and Education Center, University of Florida.

**Experimental microcosm:** The six *Salmonella* serovars were transformed to exhibit Rifampicin resistance at 80µg/ml via daily serial passage in increasing concentrations of Rifampicin \(^a\). These rifampicin resistant *Salmonella* isolates were then used in a fecal survival study conducted at four different laboratories in California, Delaware, Florida and Ohio. Briefly, the rifampicin resistant serovars were cultured separately overnight at 37°C in Buffered Peptone Water (BPW) \(^b\). Following incubation, 45 ml samples of each *Salmonella* broth culture were placed in 50 ml centrifuge tubes and centrifuged at 4600 xg for 20 minutes. The supernatant was decanted and the bacterial pellet resuspended in 45 ml of 1x phosphate buffered saline \(^c\). This ‘washing’ procedure was repeated two more times to ensure removal of any nutrient content or antibiotic selective pressure. After the third washing and re-suspension of the bacterial pellet, the optical densities of the resuspended solutions were measured to attain an inoculation dose predicted to be between $10^4$ to $10^5$ CFU/gram feces. The population of *Salmonella* in each fecal sample was enumerated at days 1, 3, 5, 7, 14 and monthly by surface plating 1ml dilutions ($10^{-1} - 10^{-6}$) of each fecal sample on LB agar, Lennox (LB)\(^b\) plates containing 80 µg/ml rifampicin and 50 µg/ml cycloheximide \(^d\). Up to ten (if present) *Salmonella* colonies recovered from each fecal sample on every sampling day were transferred from the LB agar plates to 2.0 ml centrifuge tubes containing 1.0ml brain heart infusion (BHI)\(^b\) and
cultured overnight at 37°C. The next day 300µl of buffered glycerol were added to each tube and the contents mixed. These cultures were then stored at -80°C until identification.

**Construction of Reference Spectra:** The reference *Salmonella* strains were streaked onto XLT-4 agar plates and incubated at 37°C for 18 – 24 hours. Following incubation one colony from each plate was selected and streaked onto LB agar plates supplemented with rifampicin (80µg/ml) and incubated at 37°C for 18-24 hours. Following incubation, 15 colonies were selected from each serotype and subjected to protein extraction procedures as described by the manufacturer (Schumann & Maier, 2014). Briefly, each colony was transferred from the agar plate to a 1.7ml microcentrifuge tube containing 300µl of HPLC grade water. The contents of the tube were mixed thoroughly by vortexing for approximately one minute. Following thorough mixing, 900µl of HPLC grade Ethanol was added to each tube and the contents mixed via vortexing for one minute. Each tube was then centrifuged at 18,000 x g for two minutes at 4°C. After centrifugation, the supernatant was carefully decanted and the tubes centrifuged again at 18,000 x g for two minutes at 4°C. Following this second centrifugation procedure, the residual supernatant was carefully removed via pipetting ensuring the bacterial pellet was not disturbed. The tubes containing the bacterial pellet were then left uncovered and allowed to air dry at room temperature.

Following air drying, 10µl of 70% of Formic Acid was added to each tube and the contents agitated via vortexing for one minute to ensure the re-suspension of the
bacterial pellet. The suspensions were then left to stand for five minutes after which, 10\(\mu\)l of HPLC grade Acetonitrile \(^a\) was added to each tube and mixed thoroughly via vortexing. The tubes were then centrifuged at 18,000 x g for two minutes at 4°C.

After centrifugation, 1\(\mu\)l of the supernatant was pipetted without disturbing the pellet and transferred to one well on the MSP 96 target polished steel BC (target)\(^j\). This was done in triplicate for each sample. The 1\(\mu\)l aliquots of supernatant on the target were allowed to air dry at room temperature following which each well was overlayed with 1\(\mu\)l of Bruker Matrix HCCA (\(\alpha\)-Cyano-4-hydroxycinnamic acid), portioned (HCCA matrix)\(^j\) and allowed to air dry. The mass spectrum of each protein extract was then assessed using Bruker Microflex LT\(^j\) and reference Mass Spectral Profile (MSP) created as described below.

**Mass Spectral Profile Creation:** Prior to analyzing the mass spectra of protein extracts, each target was calibrated to ensure accuracy of measurements. Calibration was achieved by placing 1\(\mu\)l of Bacterial Test Standard (BTS)\(^j\) on one empty well of the target containing the protein extract samples. Once dried, this well was then overlayed with HCCA matrix as described above and the auto-calibration option in the flexControl software\(^j\) was used to facilitate calibration. Once calibrated, spectra were acquired using the AutoXecute function within flexControl. Three spectra were recorded from each sample well using recommended setting for bacterial species identification (linear
positive mode, 20-Hz laser frequency, 20-kV acceleration voltage, 18.5 kV IS2 voltage, 250 ns extraction delay).

Following acquisition, all spectra for each serotype were then imported into flexAnalysis. Spectra were subjected to baseline subtraction and mass spectrum smoothing for evaluation of their uniformity and detection of abnormal peaks, flatlines or other anomalies. The identity of any discrepant spectra was recorded and these spectra removed from further analysis. All spectra passing the previous quality control screening were then imported into MALDI Biotyper 3.0 and the new MSP for each serotype created. The new MSP was then screened against the constituting spectra to ensure a biotyper score of 2.7 or greater was attained. Additionally, the peak list was evaluated to ensure there were a minimum of 70 peaks per MSP and these peaks were in 90% or greater of the constituting spectra. All six MSPs were then added to create a new reference library which the unknown Salmonella isolates recovered from the survival study were compared against as described below.

**Isolate Identification:** The unknown Salmonella isolates recovered from the survival study were subjected to Intact Cell Mass Spectrometry (ICMS). One loopful of the previously frozen culture broth was streaked for colony isolation onto LB agar plates supplemented with rifampicin (80µg/ml) and incubated at 37°C overnight. The following day, one individual colony was selected and smeared onto two separate wells of the MALDI-TOF target. The wells were then overlaid with 1µl of HCCA matrix allowed to
air dry at room temperature analyzed using Bruker Microflex LT\textsuperscript{3} using the instrument settings described earlier. Identification of samples was done automatically using Bruker Realtime Classification \textsuperscript{4} software.

The identities of the isolates were confirmed via a combination of serotyping of somatic antigens and a PCR based assay. The unknown isolates were streaked for colony isolation onto LB agar plates supplemented with rifampicin (80µg/ml) and incubated at 37°C overnight. The next day, one individual colony was selected from each plate and mixed with 10µl of Salmonella antisera \textsuperscript{k} for somatic groups B, C1, C2, D and E1 and observed for agglutination. Two of the serotypes, S. Montevideo and S. Braenderup, belong to Group C1 and agglutinated equally when mixed with C1 antisera. Differentiation of these two serotypes was accomplished via a PCR based assay adapted from J. Jean-Gilles Beaubrun et al. (2012) (Jean-Gilles Beaubrun et al., 2012). Briefly, individual colonies from samples agglutinating with C1 were placed in 1.7ml microcentrifuge tubes containing 100µl of sterile DNAase- free water. The tubes were then placed in a water bath at 100°C for 10 minutes. The cell lysates were then used as the DNA template for PCR screening for detection of \textit{stm1350} (171 bp) and \textit{sty0346} and \textit{sty0347} (262 bp)(Jean-Gilles Beaubrun et al., 2012). The primer sequences used were \textit{stm1350F}: 5’ TCAAAATTACGGGCCGCA 3’; \textit{stm1350R}: 5’TTTTAAGACTACATA CGCGCATGAA3’; \textit{STY0346}: 5’GGCTGGAGCAGCCTTACAAAA3’; and \textit{sty0347} 5’AAGAGTTTGCTGGCTGGTAAAA3’. Amplifications were performed in a 50µl reaction mixture containing 25µl GoTaq Green Mastermix \textsuperscript{1}, 2µl \textit{sty0346} (5µM/µl), 2µl
*sty0347* (5μM/μl), 2μl *stm1350* F (5μM/μl), 2μl *stm1350* R (5μM/μl), 14μl H2O and 3μl DNA template. The reaction mixture was heated to initial denaturation at 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 52°C for 1 minute, 72°C for 1 minutes and final extension at 72°C for 5 minutes. The PCR products were separated on 2% agarose gels stained with ethidium bromide and visualized using UV light.

Results were tabulated using 2x2 contingency tables for each serotype to reflect the agreement between the results obtained from MALDI-TOF and serotyping/PCR. The overall agreement, positive percent agreement, negative percent agreement and Cohen’s kappa were calculated to evaluate the agreement between methods. Cohen’s Kappa provides a measure of the degree to which the two methods concur in their respective classification of the different serovars (Landis & Koch, 1977). McNemar’s test was used to identify if there were any statistical differences in the subtyping results obtained between MALDI-TOF and conventional methods (α = 0.05). Statistical calculations were done using VassarStats m.

**Results**

Spectra Validation: To validate the accuracy of the MSPs created, 40 samples of each serotype whose identity was known was run against newly created MSP library. All correctly identified reference isolates had an overall mean score for all serotypes of 2.54 (95% CI: 2.52 – 2.56). For identification and subtyping of isolates we conservatively used the lower limit of 2.52 as our cut-off. If an unknown isolate achieved a biotyper
score of less than 2.52 for the best match organism when analyzed, the isolates were restreaked and fresh colonies reanalyzed.

Salmonella subtyping: MALDI-TOF subtyped 97% (938/965) of Salmonella isolates the same as the conventional serotyping and PCR combination. The majority of isolates recovered, 455, belonged to the serovar S. Javiana, while the least number of recovered isolates, 29, belonged to the S. Newport serovar (Table 1). The positive percent agreement which is a measure of MALDI-TOF ability to identify a serovar compared to the conventional methods sample was lowest for S. Newport (82.8%) and highest for S. Typhimurium where 100% of isolates were correctly matched. The negative percent agreement which is a measure of MALDI-TOF ability to correctly classify a sample as not being a particular serotype was above 99% for all serotypes.

We also calculated Cohen’s Kappa as another measure to evaluate the agreement between the subtyping results obtained for MALDI-TOF and the conventional serotyping/PCR method. The subtyping of S. Newport received the lowest Cohen’s Kappa score (0.82) while subtyping of S. Typhimurium received the highest score (0.99) (Table 1). Overall there was no statistical difference between results obtained from MALDI-TOF and the conventional serotyping/PCR method for any of the serovars (Table 1).
Discussion

Herein we report that MALDI-TOF is capable of subtyping *Salmonella* using mass spectral profile analysis equally as well as conventional methods (serotyping + PCR), while at the same time offering rapid identification at reduced cost: We can thus propose that MALDI-TOF can be used to an alternative method for the rapid identification of *Salmonella* serovars used in epidemiological studies.

Surprisingly, although MALDI-TOF technology has been in existence for over twenty years, there is a paucity of published literature describing its ability and use to identify *Salmonella* serovars. At the species level, *Salmonella enterica* subspecies enterica was first differentiated from other *Salmonella enterica* subspecies using mass spectrometry in 2008 (Dieckmann, Helmuth, Erhard, & Malorny, 2008). Serovar differentiation capability was also first reported by Dieckmann et al. in 2011 when he reported successful ICMS differentiation of *S. Enteriditis*, *S. Typhimurium*, *S. Virchow*, *S. Infantis* and *S. Hadar* (Dieckmann & Malorny, 2011). The authors further postulated the existence of potential discriminatory biomarker ions in *Salmonella enterica* serotypes but these have not been extensively evaluated. Discrimination at this phylogenic level was later demonstrated by Kuhns et. al (2012) who was able to differentiate *Salmonella Typhi* from other serovars, but not able to differentiate between the other eleven serovars of *Salmonella enterica* subspecies *enterica* tested (Kuhns et al., 2012).
MALDI-TOF biotyper scores are assigned based on the similarity of the organism’s mass spectra to the reference spectra (Dingle & Butler-Wu, 2013). The quality of spectra can be dependent on a number of factors including age of culture, sample preparation, thickness of colony smear on target and matrix used (Ford & Burnham, 2013; Giebel et al., 2010; Holland et al., 2014). Ford et al. (2013) reported that rate of identification dropped as the culture age increased, and Veelo et al. (2014) further reported that thickness of the smear on the target can affect identification of the organism (Ford & Burnham, 2013; Veelo, Elgersma, Friedrich, Nagy, & van Winkelhoff, 2014). It is for these reasons, if isolates obtained low scores below our cut-off values, we repeated the culture and smear preparation and reanalyzed the isolates with reference strains to validate preparation procedures.

Biotyper scores are logarithmic ranging between 0 and 3, and high values in this score represent a high similarity with the database entry (Richter et al., 2012). Currently MALDI-TOF scores of greater 2.3 are suggested by the manufacturer to indicate a high probability of species identification. However, for this experiment a higher discriminatory power for serovar identification is required and no MALDI-TOF scores are suggested for this level of discrimination. Consequently since we required an increased similarity match for serovars versus that normally required for species identification we increased our serovar classification score above the 2.3 suggested for species identification and used the a score of based on screening of the reference spectra against the newly created Salmonella library as our cut-off. Adjustment to biotyper cut-
off scores is not unique, other authors have also proposed adjustments to biotyper classification cut-off scores to optimize classification of organisms, but these studies were confined to improving genus and species classification (Barberis et al., 2014; Schmitt, Cunningham, Dailey, Gustafson, & Patel, 2012; Schulthess, Bloemberg, Zbinden, Böttger, & Hombach, 2014) and not serovar classification as we did.

Although the initial cost of MALDI-TOF machines can be high, money saved from the cost of diagnostic reagents and decreased labor requirements, coupled with the rapid identification time makes MALDI-TOF identification of bacteria a suitable alternative to conventional organism identification methods (Dingle & Butler-Wu, 2013). These benefits are most pronounced in epidemiological studies like this one where large numbers of samples are processed. For example, the average cost of consumables used for serotyping a Salmonella isolate is estimated to be US$8.00 (Association of Public Health Laboratories - Salmonella Serotyping in US Public Health Laboratories - White Paper. Available at: http://www.aphl.org/AboutAPHL/publications/Documents/FS_SalmonellaSustainabilityWhitePaper_Nov2014.pdf. Accessed 3/13/2016), while consumables used in MALDI-TOF costs an estimated US$0.50 (Dingle & Butler-Wu, 2013; Singhal, Kumar, Kanaujia, & Virdi, 2015). The difference in processing time was also very apparent since identification of a Salmonella isolate using the combination of serotyping and PCR took in excess of three hours for some isolates, while MALDI-TOF identification took as little
as three minutes per isolate, inclusive of time taken for sample preparation and MALDI-TOF analysis.

In conclusion, the MALDI-TOF method proposed here as a rapid, cost-effective method for identification of these *Salmonella* serovars was proven to have equal diagnostic capabilities as conventional subtyping methods. Consequently, this method can be used to complement conventional methods of serovar identification but if definitive of serovar identification and strain discrimination is required as in the case of outbreak scenarios, identification of isolates will still rely on molecular based confirmatory tests.

**Acknowledgements:**

The authors would like to acknowledge the assistance of Dr. Fei Wang, University of Maryland-College Park, College Park, MD for serotyping of some isolates. The authors would also like to thank Dr. Michelle Danyluk (University of Florida), Dr. Michele Jay Russel (University of California, Davis), and Dr. Kali Kneil (University of Delaware) for providing unidentified *Salmonella* isolates recovered from *Salmonella* survival studies conducted in their laboratories. Finally we would like to thank Michael Kauffman, Jennifer Schrock, Dr. Dominika Jurkovic and Mary-Beth Weisner for their assistance in conducting this study.
Sources and Manufacturers.

a  Fischer Scientific, Fair lawn, NJ
b  Acumedia, East Lansing, MI
c  AMRESCO, Salon, OH
d  Sigma – Aldrich, St. Louis, MO
e  VWR International, Radnor, PA
f  EMD Chemicals – Gibbstown, NJ
g  ACROS Organics, Fischer Scientific, Fair Lawn, NJ
h  Microfuge 2, Beckman Coulter, CA
i  Fluka, Sigma Aldrich, St. Louis, MO
j  Bruker Daltonics, Billerica, MA
k  Difco, BD Diagnostics, Sparks, MD
l  Promega, Madison, WI
m  Vassarstat - http://vassarstats.net/

Funding:

This material is based, in part, upon research supported by the National Institute of Food and Agriculture, Specialty Crop Research Initiative, Award No. 2011-51181-30767.
References:


289


Table A.1. Comparison of results obtained from MALDI-TOF and conventional subtyping methods

<table>
<thead>
<tr>
<th>Salmonella Serotype*</th>
<th>Number of isolates</th>
<th>MALDI – TOF</th>
<th>PPA †</th>
<th>NPA ‡</th>
<th>Cohen’s Kappa #</th>
<th>McNemar’s Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S.T</td>
<td>S.M</td>
<td>S.A</td>
<td>S.J</td>
<td>S.B</td>
</tr>
<tr>
<td>S.T</td>
<td>69</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.M</td>
<td>44</td>
<td>41</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>S.A</td>
<td>199</td>
<td>198</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>S.J</td>
<td>455</td>
<td>1</td>
<td>1</td>
<td>449</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>S.B</td>
<td>169</td>
<td>1</td>
<td>7</td>
<td>3</td>
<td>1</td>
<td>156</td>
</tr>
<tr>
<td>S.N</td>
<td>29</td>
<td>2</td>
<td></td>
<td>3</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

* S.T – *Salmonella* Typhimurium, S.M – *Salmonella* Montevideo, S.A – *Salmonella* Anatum, S.J – *Salmonella* Javiana, S.B – *Salmonella* Braenderup, S.N – *Salmonella* Newport; † PPA – Positive Percent Agreement; ‡ NPA – Negative Percent Agreement; # Kappa scores greater than 0.81 are considered to indicate almost perfect agreement between testing methods (Landis & Koch, 1977)