COMPARISON OF THE GROWTH OF SHIGA TOXIN-PRODUCING \textit{ESCHERICHIA COLI} (STEC) ON DIFFERENT MEDIA

THESIS

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Abstract

This study was done to compare the growth of Shiga toxin-producing *Escherichia coli* (STEC) on 3 different culture media. Shiga toxin-producing *Escherichia coli* (STEC) are important and challenging human pathogen worldwide. This organism can cause zoonotic, foodborne and waterborne enteric illness in human association with diarrhea, hemolytic colitis and fatal hemolytic uremic syndrome (HUS). STEC consists of two main groups: O157 and non-O157 STEC. Human can acquire STEC infection by ingestion of contaminated food and water, animal-to-person and person-to-person contact. Cattle are identified as the main reservoir of STEC in United States. And cattle feces are considered the primary source of food supply and environment contamination. Cultural methods for isolation and detection of STEC, especially STEC O157:H7, are commonly and widely used. In past decades, more and more non-O157 STEC were reported in United States. CHROMagar STEC and enterohemolysin Agar were used to isolation and detection of STEC. However, there is no media that can differentiate all serigroups of STEC. Yet there is currently no comparison among different media for detection of non-O157 STEC. To fill this gap, three solid media (MAC, VTEC, and WSBA) were compared with the growth of STEC in this study. The individual prevalence of stx-positive colony growing on 3 media above is MAC (11.6%), VTEC (9.7%), and WSBA (7.8%). Statistical analysis revealed that there actually was no significant difference on the prevalence of stx-positiv colony among those 3 media.
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CHAPTER 1

LITERATURE REVIEW

1.1 *Escherichia coli*

*Escherichia coli* that are probably the most studied organism (Eckburg et al., 2005) is a common group of bacterium that colonize in the intestinal tract of human and warm-blood animals, and are shed in feces of warm-blood animals and humans. The bacterium was first described by Theodor Escherich in 1885 (Escherich, 1988; Escherich, 1989). In taxonomy, *Escherichia coli* belong to the enteric bacterial family, *Enterobacteriaceae*. The bacteria in this family share the same physiologic and biochemical characteristics: facultative anaerobes, non-sporeforming, gram-negative straight rods (0.3-1.8 μm). They can move by peritrichous flagella and ferment glucose to acquire energy. Most of those bacteria are catalase-positive and oxidase-negative. *Escherichia coli* can be categorized into two virotypes: non-pathogenic and pathogenic. Non-pathogenic *Escherichia coli* and its hosts can coexist in good health and with mutual benefits for each other (Kaper et al., 2004; Nataro and Kaper, 1998). Pathogenic *Escherichia coli* may cause diverse diseases in different host (Kaper et al., 2004; Nataro and Kaper, 1998). Some *Escherichia coli* strains are pathogens to human (Johnson et al., 2008; Kaper et al., 2004; Nataro and Kaper, 1998).

1.2 Pathogenic *Escherichia coli*

For many years, *Escherichia coli* were simply considered to be important members of the normal microflora (Eckburg et al., 2005; Gyles, 2007) of large intestine in human and
animals. However, some *Escherichia coli* strains can cause diseases under some conditions (Kaper et al., 2004). Clinical pathogenic *Escherichia coli* consist of two groups: extra-intestinal pathogens and intestinal pathogens. Extra-intestinal pathogens include strains that are responsible for urinary tract infections and neonatal meningitis (Kaper et al., 2004). Intestinal pathogens are the *Escherichia coli* strains that cause intestinal diseases including abdominal pain, watery or bloody diarrhea (Hunt, 2010; Kaper et al., 2004; Karmali, 2004). The diseases caused by particular strains of *Escherichia coli* depend on distribution and expression of an array of virulence determinants (Kaper et al., 2004), including adhesins, invasins, toxins, and abilities to withstand host defenses and the infection mechanism includes colonization of mucosal sites, evasion of host defense, multiplication, toxins release and host damage to infect their hosts. So far, six categories (Kaper et al., 2004) of *Escherichia coli* associated with diarrheal diseases are recognized: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC) and diffuse-adherent *E. coli* (DAEC). The unique characteristics of six categories of pathogenic *E. coli* in their interaction with eukaryotic cells are represented in Fig.1. 1.
Figure 1.1 Pathogenesis of diarrhagenic E. coli. (a) The pathogenesis of EPEC involves adherence to small bowel enterocytes, destroying the normal microvillar architecture and cytoskeletal derangements accompanied by inflammatory response and diarrhea. 1. Initial adhesion, 2. Protein translocation by type III secretion, 3. Pedestal formation. (b) EHEC can produce Shiga toxins that play an important role in the intense inflammatory response and also induce the attaching and effacing lesion in colon. (c) ETEC can colonize the GI tract by means of fimbrial adhesions that adhere to specific receptor on enterocytes and may secrete heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) resulting in diarrhea. (d) EAEC can adhere to small and large bowel epithelia in a thick biofilm and attach to tissue culture cells in aggregative manner. (e) EIEC can invade the colonic epithelia cell causing cell destruction, cell-to-cell spread and re-enter the baso-lateral plasma membrane. (f) DAEC elicit a
characteristic signal transduction effect in small bowel enterocytes that manifestations as the growth of a long finger-like cellular projections, which wrap around the bacteria. AAF, aggregative fimbriae; BEF, bundle-forming pilus; CFA, colonization factor antigen; DAF, decay-accelerating factor; EAST1, enteroaggregative \textit{E. coli}. Adapted from Kaper et al. 2004

1.3 Shiga toxin-producing \textit{Escherichia coli}

1.3.1 Emergence of Shiga toxin-producing \textit{Escherichia coli} as pathogen

Shiga toxin-producing \textit{Escherichia coli} (STEC) are a subtype of \textit{E. coli} that is characterized by production of Shiga toxins (Gyles, 2007). The name, Shiga toxin, was derived from similarity to a cytotoxin produced by \textit{Shegella dysenteriae} serotype 1 (O'Brien et al., 1982). The history of Shiga toxins may date back to late 1970s, when it was observed that some strains of \textit{E. coli} can produce cytotoxicity on Vero (African green monkey kidney) cells and the cytotoxin was termed verocytotoxin (Konowalchuk et al., 1977). Several years later, a study on \textit{E. coli} strains that were cytotoxic for Hela cells was published in 1980 and the cytotoxin was termed as Shiga-like toxin (O'Brien et al., 1980). Subsequent study showed that verocytotoxin and Shiga-like toxin were the same toxin(O'Brien et al., 1983a). STEC, VTEC, or SLTEC were used to represent the \textit{E. coli} that can produce Shiga toxins in publications. Those names can be used interchangeably, but the abbreviation of STEC is used in this thesis.

STEC identified as a distinct class of pathogenic \textit{E. coli} can be traced to the first reported STEC outbreak in US, which was associated with ingestion of contaminated hamburger at fast-food restaurant chain in Oregon and Michigan, 1982 (Riley et al., 1983). \textit{E. coli} O157:H7, the most popular STEC, was isolated from stool cultures from patients with
hemorrhagic colitis. At the same year, the association between hemolytic-uremic syndrome and STEC was established (Karmali et al., 1983). Those studies led to the establishment that STEC serve as important pathogen to cause intestinal and renal disease. In the past decades, STEC serotypes other than serotype *E. coli* O157:H7 (Hunt, 2010) were identified from cases and outbreaks of STEC infection worldwide and more than 100 non-O157 STEC strains have been recognized (Hunt, 2010; Konowalchuk et al., 1977). Like O157 STEC, non-O157 STEC can also produce Shiga toxins causing severe diseases. In North America, STEC serotypes O26, O45, O103, O111, O121, and O145 are most common (Brooks et al., 2005; Gyles, 2007). So far, it has been established that STEC are pathogens that can cause foodborne (Karmali, 1989) and waterborne (Swerdlow et al., 1992) illness ranging from mild intestinal disease (watery or bloody diarrhea) to severe kidney complications (hemolytic uremic syndrome HUS) (Kaper et al., 2004; Karmali, 2004; Nataro and Kaper, 1998). STEC infection has been a public health problem of serious concern (Karmali, 2004).

1.3.2 Characteristics of STEC

STEC is a subtype of *E. coli* that has the ability to produce Shiga toxins. Like most *E. coli*, they can ferment glucose, lactose and sorbitol. And they are β-D-glucuronidase positive. However, serotype O157:H7 is an exception, which is β-D-glucuronidase negative and cannot ferment sorbitol. Some media are developed based on this exception to culture serotype O157:H7 from fecal and food samples such as sorbitol-MacConkey (SMAC) agar (March and Ratnam, 1986). Another exception is serogroup STEC O26 that
cannot ferment rhamnose. Rhamnose-MacConkey (RMAC) agar was developed to culture and isolate STEC O26 (Hiramatsu et al., 1999; Hiramatsu et al., 2002).

The most unique characteristic that distinguishes STEC from other *Escherichia coli* is that STEC have the ability to produce Shiga toxins. There are two Shiga toxins: Shiga toxin type 1 (Stx1) and Shiga toxin type 2 (Stx2) (Strockbine et al., 1986). Stx1 is just one amino acid difference from Shiga toxin produced by Shigella dysenteriae serotype 1 (Takao et al., 1988), whereas Stx2 only has approximately 60% amino acid similarity to Stx1 (Jackson et al., 1987). The gene coded for the production of Shiga toxin is located in STEC chromosome (Hayashi et al., 2001). The Figure 1.2 provides a graphic representation of the locations of gene coding for Stx1 and Stx2 in *E. coli* O157 chromosome. STEC can produce only Stx1, or Stx2, or both. Studies have suggested that STEC O157:H7 acquired its stx-gene when a bacteriophage transmitted horizontally toxin genetic code from Shigella to a formerly benign species of *E. coli*.
1.3.3 Typing of STEC

Typing STEC isolates can allow the comparison between isolates from human, animal, food and environment origin and provide information to aid on epidemiology, diagnosis and pathogenesis studies. Of those typing methods, classical serotyping is the most commonly used method to categorize *E. coli* strains (Blanco et al., 2004a; Blanco et al., 2004b; Prager et al., 2005).

There are 3 different surface antigens on the cell surface: O-, H- and K-antigen. Antigenic structure of *E. coli* is shown in Figure 1.3. Serotype of STEC is based on O and H antigen, which are various combinations of O and H antigens (Scheutz et al., 2004).
The O antigen is a polysaccharide composed of repeating monosaccharide trimmers in diverse combinations and sequences and anchored in the cell wall’s outer membrane by lipid moiety, Lipid A, by an oligosaccharide core (Hunt, 2010). The H antigen is flagellin. 

*E. coli* can move by means of flagella. The K antigens may be composed of proteinaceous organelles associated with colonization (e.g. CFA antigens), or made of polysaccharides, which serve as a virulence factor in extraintestinal colonization, urinary tract infections and invasive diseases (Hunt, 2010). So far, there are 174 O antigens (numbered 1 to 181, with numbers 31, 47, 67, 72, 93, 94, and 122 deleted) and 53 H antigens reported in the international serotype scheme.

Seropathotyping of STEC is another method that is proposed to classify STEC into 5 groups based on the association of the serotypes with disease of varying severity in human and with the frequency isolated from sporadic diseases and outbreaks (Karmali et al., 2003). Seropathotype A consists of O157:H7 and O157:NM. Those two serotypes are
the most virulent strains. Seropathotype B consists of serotypes O26:H11, O103:H2, O111:NM, O121:H19, and O145:NM. Those serotypes are similar to the O157 STEC in causing severe disease and outbreaks but occur at lower frequency. Seropathotype C includes serotypes O91:H21 and O113:H21 that is not associated with outbreak but can be related to potential sporadic HUS. Seropathotype D is composed of numerous serotypes that have been implicated in sporadic cases of diarrhea, and seropathotype E is composed of the many STEC serotypes that have not been implicated in disease in humans.

1.4 Survival of STEC in environment

STEC can survive in feces, soil and water (Duffy, 2003; Erickson and Doyle, 2007; Fremaux et al., 2008b; Maule, 2000). Survival of STEC in different conditions can be influenced by various factors such as temperature, moisture content, pH, and nutrition. 

*E.coli* O157:H7 can grow well at 10 to 45 °C in brain heart infusion and *E.coli* broth (Palumbo, 1995). In trypticase soy broth, *E. coli* O157:H7 can grow best at 37°C, but poorly at 44-45.5°C (Doyle and Schoeni, 1984).

Cattle is regarded as the major reservoir of STEC linked to human infection (Caprioli et al., 2005). Cattle can shed the STEC into environment by means of feces. Therefore, the studies on the survival of *E. coli*O157:H7 in cattle effluents (feces and slurry) were conducted. In untreated effluents, *E. coli*O157:H7 may persist for 49-126 days in bovine feces, and STEC O26:H11 and O111 can survive for 112 and 70 days in feces at 15°C.
respectively (Duffy, 2003; Fukushima et al., 1999). Furthermore, different studies revealed that *E. coli* O157:H7 and STEC O26:H11 could survive for up to 90 days in cattle slurry (Fremaux et al., 2007; McGee et al., 2001). Similarly, non-O157 STEC may survive *in vitro* for 90 days in untreated manure heaps at temperature of 15°C (Fremaux et al., 2007). Those studies found that temperature had negative effect on survival of *E. coli*. One study showed that the concentration of *E. coli* O157:H7 decreases by 4log$_{10}$ CFU/g within 3 hours at 55°C, versus within 15 minutes at 60°C, and 3 minutes at 65°C (Jiang et al., 2003). Considering cattle effluents treatment such as anaerobic digestion and feces composting can cause reduction of pathogenic microorganisms’ contamination by thermal destruction. Several studies found that *E. coli* O157:H7 survive longer in untreated manure than in treated manure (Fremaux et al., 2008a).

STEC can also survive in soil. Soil is contaminated due to direct shedding of STEC onto pasture land by animals, especially cattle and sheep. Other animals like pigeon (Morabito et al., 2001), deer (Asakura et al., 1998), and rabbit (Scaife et al., 2006) can also shed STEC into soil. *E. coli* O157:H7 can survive from 25-131 days (Fremaux et al., 2008b) at lab under experimental conditions. STEC O26:H11 may persist *in vitro* for more than one year in various manure-amend soils at both temperature of 4 and 20 °C (Fremaux et al., 2008a). Low temperature plays negative effects on the survival of *E. coli* O157:H7. It was demonstrated by study (Jiang et al., 2002) that O157:H7 can survive for up to 77, >226, and 231 days in manure-amended autoclaved soil held at 5, 15, and 21°C, respectively. Studies also revealed that moisture has little impact on survival of STEC.
*E. coli* O157:H7 can survive for a long time under very dry condition in soil (Berry and Miller, 2005; Jiang et al., 2002).

Contaminated water exposure is an important risk factor for STEC infection to human (Lee et al., 2002). Drinking or swimming in contaminated water are two major exposure to STEC. *E. coli* O157:H7 survival studies conducted at lab level revealed that the pathogen can survive for 14 days in farm water. The pathogen even can persist in marine water for 15 days and multiply in the medium containing 5% NaCl (Miyagi et al., 2001). Studies also demonstrated that STEC can live longer in cold water. Rice et al. (Rice et al., 1992) showed that the pathogen can survive in water for 40 days at 21°C and for more than 70 days at 5°C. Another finding is that *E. coli* O157:H7 can persist in commercial bottle water for 300 days at 22°C (Warburton et al., 1998). The possible explanation is the formation of biofilm on the bottle surface (Maule, 2000).

1.5 Epidemiology of STEC infection

STEC can be transmitted by eating uncooked or undercooked ground beef; consumption of contaminated vegetables, and unpasteurized milk; swimming in or drinking contaminated water; fecal-oral transmission, animal-to-person contact and person-to-person contact. The Fig. 4 briefly illustrates the transmission routes of STEC. Of all transmission routes, foods account for higher percentage of STEC infection. In the United States, foods are the most important vehicle of STEC infection. It was estimated that
around 52% of outbreaks of *E. coli* O157:H7 infection between 1982 and 2002 were associated with consumption of contaminated food (Rangel et al., 2005).

![Figure 1.4 The possible transmission routes of Shiga toxin-producing *Escherichia coli* (C. L Gyles, 2007)](image)

The most common time of year for STEC infection is summer and autumn season. The possible reason may link to food and environmental exposure such as water and farm animals. Children and elder are high risk groups for STEC infection. Paton JC and Paton AW reported that O157 and O111 STEC have low infectious dose (< 100 organisms) (Paton and Paton, 1998). The infectious dose of other serogroups is not known (Gould et al., 2009). The incubation period ranges from 1 to 8 days, though typically it is 3 to 5 days. Typical symptoms of STEC infection include severe abdominal cramping, sudden onset of watery or bloody diarrhea, and vomiting. However, fever is not common in STEC infection. Most of the illness is mild and self-limited, generally lasting 1-3 days. However, serious complications such as hemorrhagic colitis, Hemolytic Uremic Syndrome (HUS) can occur in up to 10% of cases. The highest infection rates are
reported in children under age 5. Elderly patients also account for a large number of cases.
Diagnostic methods include STEC strain recovery from fecal or foods samples and Shiga
toxin detection assay. The treatment of STEC infection consists of fluid replacement,
supportive care, and careful monitoring of kidney function, without antibiotic use.
Antibiotic therapy can aggravate the STEC infection. A possible explanation is that
antibiotic use may increase the production of Shiga toxin (Grif et al., 1998). Figure 1.5
provides an overview of STEC infection.

Figure 1.5. Overview of disease in humans due to enterohemorrhagic Escherichia coli (EHEC) (C. L
Gyles, 2007)

1.6 Reservoir of STEC
STEC has been identified in
cattle, goats, sheep, cats, chickens, dogs, horses, pigs, deer, rabbits, pigeons, and rats (Caprioli et
al., 2005). In North America, cattle are the most significant reservoir of STEC (Gyles,
2007). In countries such as Australia, sheep are of greater significance (Gyles, 2007). It
has been recognized that ruminants are the major reservoir of STEC. Human illnesses due to STEC infection have been traced in most cases to cattle, their manure, or their edible products. Therefore, determining the prevalence of serotypes of STEC in cattle is a public health concern. Although numerous investigations were performed to determine the prevalence of STEC in cattle, it is still difficult to make comparisons among those studies due to different sampling and methodology applied such as sample collection time and sites and investigation protocols. In general, higher prevalence of STEC is commonly observed in feedlots and grazing cattle. The high prevalence could be explained by horizontal transmission through the close cattle-to-cattle contact during grazing and the continuous contamination of the pasture with feces from infected cattle. The use of irrigation in some cow-calf operations also contribute to enhanced STEC dissemination or maintenance. Additionally, it is reported that prevalence of STEC in cattle significantly changes over time (Dunn et al., 2004). Study on testing beef cattle over 1 year in US reported that the higher and lower prevalence of STEC were observed in the fall and winter, respectively (Cobbold et al., 2004; Hussein, 2007). Another study on fecal testing of grazing beef heifers over 1 year revealed the highest (15%) prevalence rate to occur in winter and the lowest (4%) to occur in the spring and fall (Thran et al., 2001). Many previous studies focused on *E. coli* O157:H7 due to its importance in human disease. Prevalence of *E. coli* O157 has been reported in cattle ranging from 0.3 to 19.7% in the feedlot, from 0.7 to 27.3% on pasture (Hussein, 2007; Hussein and Bollinger, 2005b). Furthermore, previous studies have revealed that more and more non-O157 STEC were identified in cattle due to more specific and sensitive detection technologies.
applied in studies (Beutin et al., 1993; Blanco et al., 2004b). Non-O157 STEC infection has already drawn public health attention. However, of those serotypes, *E. coli* O157:H7 and O157:NM are the predominant STEC serotype that are known to cause major outbreaks and sporadic cases of human illnesses, including HC, HUS, and TTP. Serogroups O26, O45, O103, O111, O121, O145 are the top six non-O157 STEC in the US (Gyles, 2007).

1.7 STEC in foods

In 1982, the first recorded STEC outbreak in the United States was associated with O157:H7 contaminated hamburger. Since then, a large number of reported outbreaks and sporadic case with STEC infection occurred. And contaminated foods were identified as the principal vehicle for transmission of STEC to human. The high risk food includes undercooked ground beef, unpasteurized juice, raw milk, and raw produce (lettuce, spinach, and alfalfa sprouts). Additionally, drinking contaminated water is also an important transmission route for STEC infection. In the US, 52% of outbreaks of *E. coli* O157:H7 infection between 1982 and 2002 were associated with consumption of contaminated food (Rangel et al., 2005). A study about *E. coli* O157 outbreaks conducted in Scotland from 1994 to 2003 showed that approximately 40% of the outbreaks were foodborne (Strachan et al., 2006).

Cattle are considered to be the major reservoir for STEC in the US and can pose significant health risks to humans. Beef products, raw milk and dairy products were
reported in association with STEC infection in previous studies. Hussein and Sakuma’s study revealed that prevalence of *E. coli* O157 ranges from 0.1 to 54.2% in ground beef, 0.1 to 4.4% in sausage, 1.1 to 36.0% in unspecified retail cuts, and 0.01 to 43.4% in whole carcasses (Hussein and Bollinger, 2005a). They also reported that more non-O157 than O157 STEC was isolated from foods, with prevalence rates ranging from 2.4 to 30.0% in ground beef, 17.0 to 49.2% in sausage, 11.4 to 49.6% in unspecified retail cuts, and 1.7 to 58.0% in whole carcasses (Hussein and Bollinger, 2005a). The prevalence of STEC in raw milk was also reviewed in 2005, with the rates ranging from 0.75 to 16.2%. In previous STEC outbreak, ground beef was reported to be the most common risk food. The U.S. Department of Agriculture has announced that six additional serogroups (O26, O103, O45, O111, O121 and O145) of pathogenic *E. coli* have been declared adulterants in non-intact raw beef. If these organisms are found in raw ground beef or tenderized steaks, those products will be considered adulterated and will be prohibited from entering market. Additionally, fresh produce is another highly risk food including leafy greens (lettuce and spinach), herbs and fruits (cantaloupe). Furthermore, apple cider, yogurt, juice, cheese, mushroom, sprout and seafood were also reported to be transmission vehicle for STEC infection to human.

1.8 STEC in human

STEC infection usually occurs by the ingestion of contaminated food or water, or by person-to-person transmission, or by animal-to-person contact. The foods include beef and beef products, raw milk and dairy products, vegetables and fruits, juice and so on.
STEC infection is typically common in summer and fall season, and mainly affects young children less than 5-year-old. The elder are also at risk of infection. STEC infections are largely sporadic, and the majority of reported outbreaks and HUS cases in human are caused by *E. coli* O157:H7. However, non-O157 STEC (e.g. O26, O103, O111, and O145) are increasingly being associated with outbreaks and HUS (Gyles, 2007).

Figure 1.6 briefly illustrates the typical course of *E. coli* O157 infection. The incubation period is 1-3 days. The symptoms include watery diarrhea, abdominal pain, sometimes vomiting. Fever is less common. Typical *E. coli* O157:H7 infection is 1–3 days of non-bloody diarrhea after which the diarrhea becomes bloody. There are presently no specific measures for treating STEC infection. Regular treatments include fluid replacement, pain control and supportive cares. Patients with STEC infection should not be given antibiotics therapy because the evidences suggest that antibiotics increase the risk of HUS. A possible mechanism for this observation is that antibiotics might lead to bacterial lysis, which increases the availability of stx for systemic absorption. Also, bacteriophages that contain the stx genes might be stimulated by the antibiotics, leading to increased stx generation.
Figure 1.6 Progression of E. coli O157:H7 infections in children. Adapted from Tarr et al. 2005

About 15% STEC infection may develop HUS (Tarr et al., 2005). HUS is the abbreviation of hemolytic uremic syndrome, which was first described in 1955 (Gasser et al., 1955). Five children were reported to die with small-vessel renal thrombi, thrombocytopenia and Coombs-negative hemolytic anemia. In 1983, association of childhood HUS with a cytotoxin produced by E. coli in stools was observed (Karmali et al., 1983). In the same year, two outbreaks in adults with painful bloody diarrhea were reported and E. coli O157:H7 was identified from the patient stools in both of two outbreaks (Riley et al., 1983). It was still at the same year that O’Brien et al. established that E. coli O157:H7 produced a toxin similar to that produced by Shigella dysenteriae serotype 1, which can lead to HUS in human (O’Brien et al., 1983b).

1.9 Virulence factors

The Shiga toxin-producing Escherichia coli were first recognized in an outbreak of hemorrhagic colitis in 1982, North America (Law, 2000; Riley et al., 1983). So far, more
than 200 STEC serotypes have been identified (Boerlin, 1999). However, only a few STEC strains were responsible for outbreak and cases in human (Griffin and Tauxe, 1991). Based on in vitro and animal model studies, several virulence factors have been described in STEC (Gyles, 1992). Of those virulence factors, Shiga toxins (Stx1 and Stx2) are the defining virulence factors (Gyles, 1992; Melton-Celsa et al., 1998). In addition to Shiga toxin production, a protein called intimin (Donnenberg et al., 1997; Nataro and Kaper, 1998) encoded by eae gene is responsible for intimate attachment of STEC to the intestinal epithelial cells, causing attaching and effacing (AE) lesions in the intestinal mucosa. The third virulent factor that links to STEC is the enterohemolysin (Ehly) that is encoded by ehxA gene (Schmidt et al., 1995; Schmidt and Karch, 1996). STEC strains with gene for intimin (eae) and/or enterohemolysin (ehxA) are more commonly recovered from the feces of human with hemolytic colitis and HUS than STEC serotypes that do not harbor those virulence factors. Furthermore, two putative virulence factors, a protease (EspP) (Brunder et al., 1997) and a catalase peroxidase (Brunder et al., 1996), have also been reported previously. However, due to lack of experimental proof, the roles of two putative virulence factors above in the virulence of STEC still have not been well understood.

1.9.1 Shiga toxins

Shiga toxin-producing *Escherichia coli* (STEC) are named by their ability to produce Shiga toxins. It is therefore that Shiga toxins are the most characteristic and unique virulence factor in STEC infection. O’Brien in 1980s recognized that some *E. coli*
isolates are capable of producing a toxin that was in close association with Shiga toxin and named these *E. coli* strains Shiga-like toxin-producing *E. coli* (O'Brien et al., 1982).

In 1982, Shiga toxin produced by *E. coli* O157:H7 was first identified from two outbreak of hemorrhagic colitis related to consumption of undercooked ground beef in US (Riley et al., 1983). In 1983, STEC was recognized to be associated with HUS. Since then, intensive studies on STEC revealed its secret veil.

Shiga toxins are encoded by the stx (stx1 and stx2) gene reside in STEC chromosome. Stx1 encodes for Shiga toxin 1 (Stx1) and stx2 is responsible for the production of Shiga toxin 2 (Stx2). Fig. 3 indicates the location of stx1 and stx2 gene in O157 chromosome. Stx1 has just one amino acid different from Shiga toxin produced by *Shigella dysenteriae* serotype 1, whereas Stx2 has only 60% identicalness at amino acid sequence level to Stx1. Stx1 is highly homogenous with only one variant (Stx1c). However, Stx2 has several variants (Stx2c, Stx2d, Stx2e, and Stx2f) that are 84-99% homogenous to Stx2. Table 1 presents a brief summary of Shiga toxin variants.

The Stx (70 kDa) molecule has an A\(_1\)B\(_5\) structure, which consists of one A subunit (32 kDa) and five B subunits (7.7 kDa each) (Gyles, 2007). The A subunit are capable of entering the cytosol and inhibit the protein synthesis in target cells and eventually cause cell death (Gyles, 2007). The B subunit is responsible for binding of the toxins to cell surface receptors (Johannes and Romer, 2010). The most common receptor at the cell surface is globotriaosylceramide (Gb3). However, Stx2e can bind to
globotetrosylceramide (Gb4). Glomerular, colonic, and cerebral endothelial cells are rich in Gb3 receptors, as are renal mesangial and tubular epithelial cells. This might account for the localization of tissue damage in HUS. Previous intensive studies concluded that most of STEC O157:H7 harbor the stx2 gene. And STEC O157 strains producing Stx1 only or both Stx1 and Stx2 are less virulent than those only generating Stx2. John T. Brooks et al performed an analysis on the virulence gene profiles of non-O157 STEC infections in the United States from 1983 through 2002 (Brooks et al., 2005). The virulence gene profiles were as follow: 61% stx1 but not stx2; 22% stx2 but not stx1; 17% both stx1 and stx2; 84% intimin (eae); and 86% enterohemolysin (ehx). The study (Brooks et al., 2005) also revealed that the most common serogroups were O26 (22%), O111 (16%), O103 (12%), O121 (8%), O45 (7%), and O145 (5%). Strains that produce Shiga toxin 2 are much more likely to cause HUS than those that produce Shiga toxin 1 alone.
<table>
<thead>
<tr>
<th>Toxin types</th>
<th>Sequence similarity to Shiga toxin</th>
<th>Characteristics</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A subunit</td>
<td>B subunit</td>
<td></td>
</tr>
<tr>
<td>Stx1</td>
<td>97%</td>
<td>98%</td>
<td>Highly identical to Stx produced by <em>Shigella dysenteriae</em> serotype 1</td>
</tr>
<tr>
<td>Stx1c</td>
<td>97%</td>
<td>98%</td>
<td>Found in some eae-negative STEC; associated with no symptoms or mild diarrhea in humans</td>
</tr>
<tr>
<td>Stx2</td>
<td>53%</td>
<td>64%</td>
<td>Associated with severe disease in humans</td>
</tr>
<tr>
<td>Stx2c</td>
<td>53%</td>
<td>61%</td>
<td>Associated with diarrhea and HUS in humans; common in ovine STEC</td>
</tr>
<tr>
<td>Stx2d</td>
<td>54%</td>
<td>61%</td>
<td>Associated with eae-negative STEC and mild disease in humans</td>
</tr>
<tr>
<td>Stx2e</td>
<td>53%</td>
<td>61%</td>
<td>Associated with the piglet edema disease</td>
</tr>
<tr>
<td>Stx2f</td>
<td>54%</td>
<td>60%</td>
<td>Frequently isolated from pigeon droppings; rare in human disease</td>
</tr>
</tbody>
</table>

Table 1.1 Summary of Shiga toxins and their variants. Adapted from Johannes and Romer, 2010.

1.9.2 Intimin

Intimin, a 94-kDa outer membrane protein, is another virulence factor associated with STEC infection. It has been well established that intimin plays an important role for some STEC strains to tightly attach to epithelial cell of intestine to cause intestinal epithelial cells profound structural modifications named attaching and effacing (A/E) lesions.

Boerlin P et al. reported a multivariate analysis about interaction between the *eae* and
stx2 genes supported the hypothesis of the synergism between the intimin and Shiga toxin2. Intimin production in STEC is encoded by the eae gene that resides in a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) that is a 35-kbp region in chromosome. Figure 1.2 provides the graphic representation of the location of LEE in O157 chromosome. The LEE can be divided into several regions. At one end are esp genes, encoding secreted proteins for signal transduction and A/E lesions. At the other end are the genes encoding a type III secretion system. Type III secretion system is associated with secretion and translocation of virulent determinants and proteins. In the middle of LEE lie the eae gene encoding for intimin and Tir encoding the protein Tir (translocated intimin receptor). The protein Tir acts as the receptor for intimin.

It has been demonstrated that intimin is necessary for intimate attachment of the bacterium to colonic epithelial cells in vivo and in vitro. The patterns of attachment and interaction between STEC and epithelial cells are significantly different between eae-positive and eae-negative STEC. Eae-positive STEC harbors LEE. The A/E lesion is a complex process including several stage: (1) STEC strains initial attachment to epithelial cells; (2) Tir proteins produced by Tir III protein system and other proteins bind to the cell surface act as receptor for intimin, (3) intimate bacterial attachment via intimin binding to Tir receptors, (4) accumulation of tightly attached bacteria. Little is known about eae-negative STEC attaching to epithelial cells.

Garrido et al. (Garrido et al., 2006) employed PCR scheme and the oligonucleotide microarray to demonstrate that there are 16 existing variants (alpha1, alpha2, beta1, beta2,
gamma1, gamma2/theta, delta/kappa, epsilon, zeta, iota, lambda, mu, nu, xi, omicron) of the eae genes, 4 existing variants (alpha1, beta1, gamma1, gamma2/theta) of the Tir genes. Intimin is present in most of STEC O157 isolates. Guth et al. reported that all 22 STEC O157 strains isolated from animals and food in Argentina (n=44) and Brazil (n=20) carried eae and ehx genes in 2003(Guth et al., 2003). M. Blanco et al. analysis of 384 Ovine STEC strains demonstrated the eae gene was found in serotype O26:H11, O156:H11, OX177:H11, O49:H−, O52:H12, O156:H−, and O156:H25 in 2003 (Blanco et al., 2003). Islam MA et al. reported that more than 93% of the STEC O157 isolated from buffalo, cows, and goats in Dhaka, Bangladesh, were positive for the stx2, eae and ehx genes (Islam et al., 2008). Unlike STEC O157, most of the non-O157 STEC isolates (78.9%) were positive for stx1. Only 7.0% of the isolates were positive for ehx, and none were positive for eae. These studies reveal that the eae gene is more common in STEC O157, and less frequently found in non-O157 STEC.

1.9.3 Enterohemolysin
Beutin et al. first described a novel hemolysin termed enterohemolysin (Ehx) in association with verotoxin-producing *Escherichia coli* strains in 1989 (Beutin et al., 1989). Unlike the best characterized alpha-hemolysin produced by Uropathogenic *Escherichia coli* (UPEC), which produces large clear zones of hemolysis expression after 4h incubation on blood agar with washed or unwashed erythrocytes, Enterohemolysin (ehx) is defined by the production of a small turbid zone of hemolysis after 18-24h incubation on blood agar only containing defibrinated washed erythrocytes. The
The enterohemolysin gene is located on a large plasmid, encoding for the synthesis of enterohemolysin. Schmidt et al. demonstrated that the enterohemolysin is encoded by a large plasmid of *E. coli* O157:H7, termed pO157 and the *ehx* gene is located from position kb 7.83 to position kb 15 in the physical map of plasmid pO157. Four genes, *ehxCABD*, constitute an operon that is responsible for the synthesis, activation, and transport the protein out of the bacterial cell. The enterohemolysin is encoded by *ehxA* gene. It was reported that enterohemolysin participates in the inflammatory process of epithelial cell or amplified effects of LPS and Shiga toxins. It was also suggested that enterohemolysin acts synergistically with Shiga toxins to disrupt important cell function. Djordjevic et al. reported that STEC strains possessing *ehxA* gene and *eae* gene are more commonly recovered from feces of humans with hemolytic colitis and HUS than STEC strains that do not harbor those two virulence genes (Djordjevic et al., 2004).

The previous studies have demonstrated that the *ehxA* gene is present in all *E. coli* O157, most of non-O157 EHEC strains and most *eae*-positive STEC isolates from animals. The STEC serotypes include O157:H7, O26:H11, and O103:H2, O5:H*, and O84:H2/ H*.

Those serotypes are very commonly associated with STEC infection. It is therefore that *ehxA* gene now has been used as a marker for identification of STEC as well as a virulence factor. Djordjevic SP et al. reported that a total of 78 of 90 STEC strains isolated from 1623 ovine fecal samples from healthy slaughter-age sheep in Australian expressed Shiga toxin in Vero cell culture and 75 of 84 *ehxA*-positive isolates expressed enterohemolysin on washed sheep blood agar (Djordjevic et al., 2004). Blanco Met al.
developed a PCR method to detect ehxA gene in 384 ovine STEC strains. The results showed that 106 (28%) of the STEC strains possess the ehxA gene (Blanco et al., 2003). Mora et al. reported that enterohemolysin (ehxA) and intimin (eae) virulence genes were detected in 43 (45%) and in 25 (26%) of the 95 STEC isolates in minced beef in Lugo (Spain) from 1995 through 2003, respectively (Mora et al., 2007).

1.10 Detection of STEC
Timely and accurate diagnosis of STEC isolates is extremely important on behalf of public health and disease management. In the outbreak setting, rapid determination of pathogen is of significant importance to carry out effective epidemiologic intervention. It is observed that the number of STEC in feces is large at the early stage of infection in human. With the infection progress, the STEC number in feces drops dramatically. Therefore, sensitivity, specificity, and time are essential criteria to evaluate detection methods. The method for detection of STEC is based on detection of Shiga toxins (Stx) from samples (food, feces, and water), or isolation of STEC strains from samples, or detection of stx genes in organisms cultured from samples. The methods differ in complexity, speed, sensitivity, specificity, and cost.

1.10.1 Detection of Shiga toxins: Tissue Culture Cytotoxicity Assays
The Vero (African green monkey kidney) and HeLa cell lines are very sensitive to Shiga toxins, because they are rich in Gb3 and Gb4, the receptors for all Shiga toxin variants. Cytotoxicity assay currently is the gold standard for detection of Stx in fecal samples with high sensitivity. Cytopathic effect can be confirmed by neutralization using anti-Stx antibodies. The test usually takes 48-72h. However, this method is not routinely used because this method is labor-intensive, time-consuming, cumbersome, and require tissue culture technique and specific antibodies. Therefore, immunological and DNA-based methods have replaced the Vero cell assay for confirmation of Stx production.

1.10.2 Detection of Shiga toxins: Shiga toxin immunoassays

The immunological methods utilize the stx-specific poly- or monoclonal antibodies. The methods can be applied to pure or mixture culture (enrichment cultures of food and feces). When positive results detected, the broth can be subcultured onto isolation medium and pure or pooled colonies can be further examined. Immunological methods are usually reliable and most assays are easy to implement at lab and do not require expensive equipment. Several enzyme immunoassays (EIA) have been employed to direct detection of Shiga toxin and have commercial kit available. The advantages of Shiga toxin immunoassay are time-saving, high sensitivity and specificity. The test needs less than 24h. The commercial kit can give result in one hour. The Food and Drug Administration (FDA) has approved several immunoassays for the detection of Shiga toxin in human specimens. Due to low amount of free fecal Shiga toxin in stools, EIA testing of
enrichment broth cultures incubated overnight (16—24h at 37°C) has higher sensitivity and specificity than direct testing of stool specimens

<table>
<thead>
<tr>
<th>Immunoassay Kit</th>
<th>Company</th>
<th>Target toxin</th>
<th>Time</th>
<th>Sensitivity (%)</th>
<th>specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premier EHEC</td>
<td>Meridian Diagnostics, Inc. (Cincinnati, Ohio)</td>
<td>Shiga toxins; cannot differentiate between 1 and 2</td>
<td>3.5 hrs</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>ProSpecT Shiga Toxin E. coli Microplate Assay</td>
<td>Remel (Lenexa, Kansas)</td>
<td>Shiga toxins; cannot differentiate between 1 and 2</td>
<td>3hrs</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Immunocard STAT! EHEC</td>
<td>Meridian Diagnostics, Inc. (Cincinnati, Ohio)</td>
<td>Shiga toxins; can differentiate between 1 and 2</td>
<td>20min</td>
<td>92</td>
<td>100</td>
</tr>
<tr>
<td>DuopathVerotoxins Gold Labeled Immunosorbent Assay</td>
<td>Merck (Germany)</td>
<td>Shiga toxins; can differentiate between 1 and 2</td>
<td>20min</td>
<td>100 (Shiga toxin 1) 99 (Shiga toxine 2)</td>
<td>98 (Shiga toxin 1) 97 (Shiga toxin 2)</td>
</tr>
</tbody>
</table>

Table 1.2 Summary of four EIA test Kit approved in US by FDA
1.10.3 Detection of stx genes: DNA-based methods

DNA-based methods are considered as a good alternative to culture or immunological methods due to low detection limits. DNA-based methods directly detect the presence of stx genes. Of those methods, PCR is the most widely used technology for detecting stx gene in STEC studies. The sequence of stx1, stx2, and their variants can be used to develop stx-specific primer in PCR. Single colony, mixed culture broth, and direct DNA extraction from feces, food samples and other sources can be used as templates for PCR. PCR products can be detected by ethidium bromide staining after separation of the reaction mix by agarose gel electrophoresis. Usually, PCR is designed to test isolated colonies from plated media after 18-24hrs incubation at 37°C. The time required to obtain results ranges from 3 hours to 24-36hrs. Previous studies demonstrated that only several serogroup of STEC account for the STEC infection (Brooks et al., 2005; Wang et al., 2012). Therefore, in addition to stx genes, the specific primers based on O/H antigen and virulence factors (intimin and enterohemolysin) have also been developed. Although those assays do not target for stx genes, the test results can provide supportive evidence for subtyping STEC strains and virulence profile. The DNA-based methods are able to detect the DNA from viable but non-cultural cells in samples.

Several variations of the standard PCR have appeared and assisted in producing more sensitive detection methods, such as real-time quantitative PCR and multiplex PCR, were proving to be the most popular. Real-time PCR allows reactions to be characterized by the time when amplification of the PCR product is first detected by use of a fluorescent probe. Multiplex PCR allows several targets to be co-amplified in one PCR reaction mix.
when multiple primers applied. There are many studies on detection of STEC by employing real-time PCR and multiple PCR. Perelle et al. reported to identify the specific genes of O-antigen cluster from Shiga toxin-producing *Escherichia coli* O103 and performed a specific real-time PCR test for rapid detection of *E. coli* O103 (Perelle et al., 2005). This approach has been widely applied to specific detection of microorganisms present at low concentration in environmental samples, and is likely to be further developed in the future. Hu et al. described a multiplex PCR using five sets of primers which amplified regions of *eaeA, slt1, slt2, fliC* and *rfbE* genes (Hu et al., 1999). By analyzing 82 *E. coli* strains, it was found that the assay could successfully distinguish O157:H7 from other serotypes.

1.11 Isolation and enrichment of STEC

1.11.1 Isolation of O157 STEC

Unlike the majority of *E. coli*, O157:H7 does not ferment sorbitol in 48 hours at 35 to 37°C, and does not produce β-D-glucuronidase that can be detected by the enzyme substrate 4-methylumbelliferyl-β-D-glucuronide (MUG). Based on biochemical characteristics above, some modified media are developed to screen for O157 STEC. Sorbitol-MacConkey agar (SMAC) is the modified agar prepared by replacement of lactose with sorbitol in MacConkey agar. SMAC plates are inoculated and incubated at 37°C overnight for presence of colorless, sorbitol-negative colonies. Individual colony can be tested by latex agglutination with O157- and H7-specific antisera. Since not all O157 *E. coli* produce Shiga toxins, STEC O157 can be confirmed by PCR and other
methods above. In order to improve the sensitivity of isolating O157, selective reagents such as Cefixime that can inhibit *Proteus* and Potassium Tellurite that can inhibit other non-sorbitol fermenting organisms such as *Aeromonas* are added into SMAC to develop a modified SMAC agar named SMAC-CT. Rainbow agar O157 is another example of selective and differential medium for isolating STEC O157. Incorporation of selective agent for *E. coli* and chromogenic substrates for β-D-glucuronidase and β-galactosidase, Glucuronidase-negative and Galactosidase-positive O157 appears black or gray colony on plate. One study demonstrated that the Rainbow agar O157 is superior to SMAC (Novicki et al., 2000). CHROMagar O157 (Paris, France) can also be used for screening O157, based on the fact that O157 colonies are mauve and other bacteria are blue or colorless. Recent study showed that CHROMagar O157 have a higher sensitivity (96.3%) and negative predictive value (100%) and a better diagnostic efficiency than SMAC agar for isolation of O157 STEC from human stools (Church et al., 2007). Conventional cultural methods are both time-consuming and laborious. Therefore, Immunomagnetic separation (IMS) is introduced to increase the sensitivity of culture STEC O157. In samples such as human stool, there is a large amount of background microflora except STEC. IMS application is able to concentrate the STEC cells in specimen. IMS procedure involves immunomagnetic beads that are coated with antibodies to O157 antisera. The target organisms (STEC O157) bind to immunomagnetic beads, which are separated from other organisms in a magnetic field. After extensive washing to remove the nonspecific organisms bound, the beads are directly plated onto selective solid medium for STEC O157.
1.11.2 Isolation of non-O157 STEC

More and more non-O157 STEC has been isolated from bovine feces, human and animals (Brooks et al., 2005; Gyles, 2007). There are no international accepted standard methods for the isolation of non-O157 STEC. Due to lack of media for screening for non-O157 STEC, it is believed that the prevalence of O157 STEC may be over-estimated depending only on methods for isolation of O157 STEC. A solid medium that can detect non-O157 STEC is washed sheep blood agar supplemented with calcium (WSBA-Ca) (Beutin et al., 1996). This medium is based on the fact that most of non-O157 STEC has a diagnostically important phenotypic characteristic, which is enterohemolysin production (Beutin et al., 1989). STEC O157:H7 possess a plasmid-encoded gene that is responsible for production of enterohemolysin, which can produce a small turbid hemolytic zone on blood agar containing washed sheep erythrocyte supplemented with Ca²⁺ after 18-24h incubation at 37°C. The medium correctly identified 97.6% of the isolates tested and the use of EHEC agar for detecting STEC in stool samples has been reported by Beutin et al (Beutin et al., 1996). Bettelheim also concluded that Shiga toxin production and enterohemolysin production were closely associated, and that Shiga toxin production and alpha-hemolysis appeared to be mutually exclusive (Bettelheim, 1995). However, some questions have arisen about whether the test for enterohaemolysin production is sufficiently comprehensive. A study tested 36 STEC O111:H-isolates and found that only 20 of them were positive on EHEC agar, and only 22 of the isolates possessed the haemolysin gene (Schmidt and Karch, 1996). *Proteus* and *Pseudomonas*
can grow on sheep blood agar, which can be very annoying. A modified WSBA (BVCCA) developed by incorporation of Cefixime-Vancomycin-Cefsulodin (*Proteus* and *Pseudomonas* inhibitor) can improve the observation of enterohemolysis. Some non-O157 STEC grow poorly on BVCCA and BVCCA do not select for eaeA and ehxanegative STEC (Hornitzky et al., 2001). However, enterohemolysin detection should be combined with stx detection in positive colonies. And most of sorbitol-fermenting O157:H- strains that do not produce enterohemolysin can be overlooked on this medium.

CHROMagar STEC (Paris, France) is another medium developed for isolation of non-STEC and O157 STEC. On CHROMagar STEC, O157 STEC colony is mauve and non-fluorescent under UV lamp (365nm), whereas non-O157 STEC colonies are mauve and with/without fluorescence under 365nm UV lamp. PCR and latex agglutination can be used to determine the serogroups and serotypes. A recent study reported the use of CHROMagar STEC in detection and isolation of enterohaemorrhagic *Escherichia coli* (EHEC) serogroups O26, O103, O111, O118, O121 and O157 strains and the aggregative EHEC O104:H4 strain from ready-to-eat vegetables. The study showed that thirteen (18.3%) of the stx-negative strains formed mauve and fluorescent colonies on CHROMagarSTEC that was found most suitable for the selection of strains belonging to major EHEC groups (Fratamico et al., 2011). The limitation of CHROMagar STEC medium is that some STEC strains grow poorly, even do not grow on media.
There are still some selective media for isolation of non-O157 STEC. CHROMagar STEC-O104 supplement medium for isolating STEC O104:H4 whose colony is mauve. Other STEC strains and bacteria appear colorless or blue, and others are inhibited. However, there is currently no evaluation about this medium. Another selective medium is Rhamnose-MacConkey (RMAC) for isolation of STEC O26 serogroup. *E. coli* O26 cannot ferment rhamnose. Therefore, STEC O26 colony is colorless on RAMC. A modification is the incorporation of cefixime and tellurite (CT-RMAC). In one study, CT-RMAC was demonstrated to be better than tryptone bile X-glucuronide (TBX) agars for isolation of STEC O26 from feces samples (Evans et al., 2008). Like isolation media for STEC O157, it should be highlighted that the final confirmation of STEC should be done by slide or latex agglutination, or other methods.

Based on the successful application of IMS for isolation of O157 STEC, IMS has been introduced to isolate several non-O157 STEC serogroups of O26, O103, O111, and O145 whose immunomagnetic beads are commercially available. The IMS protocol for non-O157 STEC is similar to that for O157 detection. Recently, IMS has been widely used as a concentration step for detection of STEC. Fratamico et al. reported the use of IMS for isolation and detection of Shiga toxin-producing *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 in ground beef by employing multiplex real-time polymerase chain reaction assays (Fratamico et al., 2011).
1.11.3 Enrichment of STEC

Fecal samples, food and environmental samples usually contain low numbers of STEC together with abundant background flora. Before directly plating clinical specimens onto media for isolation of STEC, enrichment is a necessary and required step. The main enrichment protocol factors are the enrichment broth (basal medium), the addition of selective ingredients, the temperature and the duration of incubation period (Vimont et al., 2007). Enrichment broth is categorized into two groups: selective and non-selective. A study (Vimont et al., 2006) reported that the most commonly used enrichment broths are trypticase soy broth (TSB), E. coli broth (EC) and buffered peptone water (BPW). Selective enrichment broth is supplemented with selective agents, such as novobiocin, bile salts, vancomycin, cefsulodin and cefixime (Catarame et al., 2003). The enrichment process is usually 16-24 hours at 37ºC. Vimont et al. reported that the use of EC broth was more appropriate for detection of non-O157 STEC from bovine samples, which can help avoid false negative results that may lead to underestimation of the STEC prevalence in cattle (Vimont et al., 2007).

1.12 Study objective

Disease caused by STEC ranges from self-limiting diarrhea to hemorrhagic colitis and hemolytic uremic syndrome (HUS). STEC O157:H7 is a well-recognized pathogen. However, a total of 940 human non-O157 STEC infection cases were confirmed between 1983 and 2002 in United States (Brooks et al., 2005). Non-O157 STEC infection now is an important concern of public health. For STEC O157, selective culture media have
been employed and demonstrated to isolate and detect the pathogen with high specificity. Currently, there are several media for isolation of non-O157 STEC. However, there is no accepted medium for isolation of non-O157 STEC, and there is no comparison among those media used for detection of non-O157 STEC. In this study, the objective is to compare the growth of STEC including O157 and non-O157 on several media developed for isolation and detection of STEC. We also want to answer the question that how many colonies should be picked to ensure at least one stx-positive colony.
CHAPTER 2

MATERIALS AND METHODS

2.1 Fecal samples

The fecal samples were collected at beef feedlot in Washington State, 2008. Fecal samples were transported on ice to lab. When the samples arrived, 10g of fecal samples was weighed into sterile WhirlPac bags, and 90 ml Buffered Peptone Water (BPW) was added, and stomached for 120 seconds, then incubated 42°C for 18hr. 1ml of enrichment was mixed with 300µl of buffered glycerol by inversion and stored at -70°C freezer as bank. Shiga toxin-positive frozen bank proved by PCR in previous study was selected in this study.

2.2 Media

The media used in this study are MacConkey agar (MAC), CHROMagar STEC, VTEC agar, Washed Sheep Blood Agar (WSBA), EC-MUG and buffered peptone water (BPW). All of the media were prepared at lab.

MAC was made by suspending 50g of the Medium base (Neogen Corporation, Michigan US) in 1 liter distilled water, heated with frequent agitation and boiledto completely dissolve the medium base. After autoclaveat 121°C for 15minutes, the medium was cooled to 50 °C in water bathe, and 25ml was poured into Petri Dish plate. The plates were cooled to room temperature and stored at 4 °C for use. CHROMagar STEC, WSBA, and VTEC media were made in the same procedure under instructions.
EC-MUG medium was made by dissolving 37g EC base (Neogen Corporation, Michigan US) into 1 liter distilled water, and heated with frequent agitation and boiled to completely dissolve the medium base. After autoclave at 121ºC for 15 minutes, the medium was cooled to 50 ºC, and 0.1g 4-Methylumbelliferyl-β-D-Glucuronide (MUG) was added into medium solution. Pour 200 µl medium into each well on 96-well plate by pipette. The EC-MUG plates were cooled to room temperature and stored at 4 ºC for use.

Buffered peptone water (BPW) was made by dissolving 20 g of the medium base in 1 liter purified water, mixed thoroughly and autoclaved at 121ºC for 15 minutes. After autoclave, the broth was cooled to room temperature and stored at 4 ºC for use.

Phosphate Buffered Saline (PBS) was made by diluting 20x PBS to 1x PBS. 1x PBS was autoclaved at 121ºC for 15 minutes, cooled at room temperature and stored at 4ºC for use.

2.3 Bacterial DNA preparation
Stx-positive frozen bank stored at -70 ºC was selected for bacterial DNA preparation. 100 µl frozen bank enrichment was added to 1ml BPW, incubated at 37 ºC overnight to recover the microorganisms in bank. Serial dilutions ranging from 10^{-1} to 10^{-7} of the enrichment broth were prepared with PBS and plated 100µl of the 10^{-5}, 10^{-6} and 10^{-7} dilutions onto MAC, WSBA, CHROMagar STEC, and VTEC Agar in duplicate and incubate at 37 ºC 18-24hrs. Individual colony was picked and transferred to 96-well EC-
MUG plate and incubate at 37 °C overnight. Eight wells from 96-well EC-MUG were pooled together and pooled colonies were boiled to extract DNA for PCR use.

2.4 PCR and analysis of PCR products

PCR was performed to detect the stx-gene by using the primers termed as MK1-MK2 (Karch and Meyer, 1989). The primer MK1 (5’-TTTACGATAGACTTCTCGAC-3’) and MK2 (5’-CACATATAAAATTATTTCGCTC-3’) were prepared as previously described by Karch and Meyer (Karch and Meyer, 1989). Amplifications were performed in 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 5µlDNA template. The sample was heated to 94°C for 5 minutes, then 39 cycles of amplification at 94°C for 1.5 minutes to denature the DNA, 47°C for 2 minutes to anneal the primers, 72°C for 3 min for extension, and at 72°C for 7 min for final extension. *E. coli* O157 strain EDL933 was employed as positive control. The PCR product was an approximately 230 bp fragment that was visualized in 2% agarose gel with ethidium bromide staining.

2.5 Pool testing

Pooled testing, also named group testing, is a procedure that can significantly reduce cost, labor, and time by combining individual sample into pooled samples for a binary response (positive or negative) (Bilder and Tebbs, 2012; Saraniti, 2006). Pools that test negative mean that all the individuals within pools are negative. Pools test positive mean that at least one individual in the pools is positive. Retesting each individual in pools can
differentiate positive individuals from negative individuals. Pooled testing can significantly reduce the number of tests (cost) when an optimal N that represents the number of individual sample containing in a pool is employed. Pooled testing was widely used in human medicine (Bilder and Tebbs, 2012; Kline et al., 1989; Tu et al., 1994). In veterinary medicine, two examples that pooled testing was used were detection of trichinellosis in pork and *Salmonella Enteritidis* in shell egg (Cowling et al., 1999).

Since basic pooled testing was first introduced to detect syphilis in 1943 by Dorfman (Bilder and Tebbs, 2012; Saraniti, 2006), researchers have extended the basic model on two ways to further reduce cost: sorting and multistage testing (Saraniti, 2006). Scientists also developed some statistical methods to estimate the individual prevalence based on the results of pooled tests. Sorting model is to sort individuals into high and low risk group. For the lower (higher) risk group, more (less) individual specimen were included in a pool. Multistage model is to subdivide the positive pools into smaller pools and re-test the smaller pools. Those two optimizations can result in additional improvement over basic model under certain conditions. Furthermore, when individual prevalence is lower, and the goal of study is to estimate the individual prevalence, calculators were developed and applied to estimate the individual prevalence based on pooled testing instead of re-testing the individuals in positive pools, or multistage testing, and the precision of estimated prevalence obtained from pooled testing was comparable to individual test. In this case, the assumption that sensitivity and specificity of pooled test are approximately the same as individual test should be highlighted.
In this study, we employed an estimator developed by Hauck and Kline et al. to calculate the prevalence of individual stx-positive \textit{E. coli} based on pooled PCR (Cowling et al., 1999; Hauck, 1991; Kline et al., 1989). This method assumes 100\% test sensitivity and specificity and fixed pool size. The improvement of this method is that the exact confidence limits (CL) are calculated based on binomial theory.

Equation:
\[
\Omega = 1 - (1 - x/m)^{1/k}
\]

Confidence limits are calculated by
\[
CL_L = 1 - (1 - P_L)^{1/k} \\
CL_U = 1 - (1 - P_U)^{1/k}
\]

Where:
\[
\Omega = \text{estimated individual prevalence} \\
k = \text{pool size} \\
x = \text{the number of positive pools} \\
m = \text{the number of pools tested} \\
P = \text{prevalence of positive pools (x/m)} \\
L = \text{lower} \\
U = \text{upper}
\]

This is a free online service that provides calculator particularly designed for estimating prevalence where samples are tested in groups (pools) or where one or more tests are used with imperfect sensitivity and/or specificity. The site was developed by AusVet Animal Health Services, with funding from the Australian Biosecurity Cooperative Research Centre. The advantages of this online service are free, convenient, and fast, just entering the value of k, x, and m (Fig. 1). The limitation is that the calculator do not work when all of the pools are positive (x=m) or all of the tested pools are negative (x=0). When x=0, Ω=0; x=m, Ω cannot be determined; 0<x<m, Ω can be calculated by the equation above.

2.7 Statistic analysis

Statistical analysis was conducted by using Minitab software version15.
**Pooled prevalence for fixed**

**Input Values**

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</thead>
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<td>Method 1</td>
</tr>
<tr>
<td>Method 2</td>
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**Test results:**

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**Set confidence limits for output**

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**Method 1**

This method assumes input values. Input k value

**Method 2**

This method assumes input values. Input m value

**Input values**

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**Outputs**

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<th>Outputs for these confidence limits if</th>
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</table>

**Please Note:**

For both methods, input value, default 0.975

Input value, default 0.025

---

Figure 2.1 EpiTools screenshot for inputting value.
CHAPTER 3

RESULTS and DISCUSSIONS

3.1 Descriptive results

3.1.1 Fecal samples

28 beef fecal samples that were demonstrated to be stx-positive were used in this study. Up to 288 colonies were picked up from each sample and medium. 8 colonies were put together to form a pool except sample 15107 and 15108 that there were 12 colonies in one pool. Table 3.1 summarizes the number of pools formed from each sample and medium.

3.1.2 Pooled PCR

A total of 2242 colony pools were collected in this study from June to September, 2011. The fecal samples were plated onto MAC, VTEC and WSBA media at the same time; the samples were plated onto CHROMagar STEC medium at a different time. Of 28 beef fecal samples selected, 2 samples (15113 and 15132) were missing from WSBA (no colonies growing under plating dilutions), 1 sample (15106) was missing from CHROMagar STEC due to none colonies growing. For MAC, there were 28 samples tested, but 26 samples were positive for stx; the other two samples were negative to stx. For VTEC and WSBA, all samples tested were positive for stx. For CHROMagar STEC, only 15 samples of 27 tested were positive for stx. Regarding of sample 15145, all of the pools from 4 media were positive for stx.
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<td>96</td>
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</table>

Table 3.1 Summary of descriptive results

3.1.3 Individual stx-positive colony prevalence based on pool testing

The individual prevalence of stx-positive colony was calculated based on the method above. The mean prevalence is MAC (11.6%), VTEC (9.7%), WSBA (7.8%) and CHROMagar STEC (3.7%).

![Average prevalence %](image)

Figure 3.1 The average prevalence of individual stx-positive colony.
3.2 Statistical analysis

Due to plating samples onto CHROMagar STEC later than that onto other three media, the data collected from CHROMagar STEC was not included into statistical analysis. The detailed reason will be discussed in next section.

3.2.1 Nonparametric ANOVA analysis

Statistical analysis was performed by Minitab software version 15. Kruskal-Wallis Test was employed to compare whether there is significant difference among different medium.

Null hypothesis \((H_0)\): the medians of individual stx prevalence from three media are all equal

Alternative hypothesis \((H_1)\): the medians are not all equal

Figure 3.2 Z value of Kruskal-Wallis Test (alpha=0.05)
Kruskal-Wallis test instead of one-way ANOVA was employed to analyze the data. As shown in Figure 3.2, we noticed that the test statistic had a $p$-value of 0.155 (unadjusted) and 0.154 (adjusted), indicating that the null hypothesis cannot be rejected at alpha=0.05. In other words, there is no significant difference among these media on growth of STEC from fecal samples.

3.2.2 Tukey’s test

Tukey’s test was employed to test all pairwise comparison among level of different medium types. Confidence intervals with 98.08% individual confidence levels to obtain a 95% joint confidence level

![Figure 3.3 Tukey’s test output.](image)

Multiple comparison tests (Tukey’s test) were used to assess all pair-wise comparisons varying in the approach to estimating family-wise error rates. As shown in Figure 3.3, Tukey’s test output 2 sets multiple comparisons and 95% CI was estimated. In the first set of multiple comparisons, mean of MAC medium was subtracted from that of
VTEC/WSBA, zero was included in 95% CI, which means that all of the means are not statistically different. In the second set of comparisons, mean of VTEC was subtracted from that of VTEC/WSBA, zero was included in 95% CI, which means that none of means are statistically different.

3.3 Discussions

3.3.1 Data set

The data set was built up consisting of sample ID, Medium type, pool number, colonies in one pool, positive pool number, and estimated prevalence. Of those factors, estimated prevalence is response factor; others are variant factors that may affect the response factor. We assume that the medium that we made and PCR that can detect stx gene would not change through the experimental period. So, the time factor was not included in data set.

3.3.2 Freeze injured STEC

In this study, the fecal samples were collected in 2008 and the fecal enrichments were stored at -70°C. Previous study revealed that the optimal temperature for O157 and non-O157 STEC were 40.3°C and 41.3°C, and stopped grow when the temperature below 4.9°C and 11.2°C (Rosso et al., 1993). Also, STEC can survive well in chilled and frozen foods. In this study, several sample were missing due to none colonies growing under plating dilutions, and some samples that were proved to be stx-positive were negative to
stx genes in this study. The reason was not clear. However, the possible explanation is that freeze temperature killed or injured the STEC that cannot be recovered by BPW.

3.3.3 Recovery of freeze injured STEC
The main enrichment protocol factors are the enrichment broth (basal medium), the addition of selective ingredients, the temperature and the duration of incubation period. The enrichment step is difficult to control, because background microflora may influence the growth of STEC. Little is known about the interaction between STEC and background microflora. However, one study has demonstrated a simple competition interaction between non-O157 STEC and the prevailing background microflora during the enrichment of fecal samples (Vimont et al., 2007). STEC growth stops as soon as BM reaches its maximal level $8 \log_{10} (\text{CFU ml}^{-1})$, no matter what protocol was employed (Vimont et al., 2007). The incubation temperature is $37^\circ C$ or $42^\circ C$, and the incubation time $16-24$ hrs. The optimal temperatures for O157 and non-O157 STEC were $40.2^\circ C$ and $41.2^\circ C$ (Gonthier et al., 2001), respectively. Study showed that the best approach for the recovery of stressed STEC O15:H7 cells is a nonselective pre-enrichment for at least 18-24 hours (Stephens and Joynson, 1998). In this study, BPW, a non-selective enrichment broth, served as enrichment broth for STEC. It was reported that the use of EC broth was more appropriate for detection of non-O157 STEC from bovine samples, which can help avoid false negative results that may lead to underestimation of the STEC prevalence in cattle (Vimont et al., 2007). Buffered Peptone water (BPW) was used in this study, $37^\circ C$ overnight. The efficiency of BPW was not clear.
3.3.4 Nonparametric ANOVA test

In this study, we employed Kruskal-Wallis test to analyze the data instead of one-way ANOVA. The reason was that the data set was incomplete. The individual prevalence was calculated based on pool testing. When all of the pools are positive, the prevalence cannot be calculated by the method above. Take the sample 15145 for example; all of the pools from 4 media were test positive. The Equation cannot be used to calculate the prevalence. Therefore, in this data set, some data were blank. Furthermore, there are several outliers in the data set. In order to perform one-way ANOVA analysis, the data set must be complete and does not violate the normality assumption, randomness assumption and homogeneity of variance assumption. We cannot remove those samples with incomplete data and outliers. Otherwise, the statistic conclusion was questionable. In this situation, nonparametric test (Kruskal-Wallis test) was a good alternative method to perform statistical analysis.

3.4 Conclusion

Although high prevalence of stx colony was detected on MAC (11.6%), there is no significant difference among MAC, VTEC, and WSBA based on statistical analysis. Further work is needed to compare the four media on isolation of STEC.
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Appendix A: Data
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Data set of pooled PCR and individual prevalence estimation