EVALUATION OF ENRICHMENT, TRANSPORT, AND DETECTION METHODS RELATING TO SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC)

by

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are Gram negative rod-shaped bacteria that are causative agents of foodborne disease. While natural flora in the gastrointestinal tracts of bovines and other ruminants, certain enterohemorrhagic STEC strains cause serious or even fatal disease when ingested. In 2012, the USDA identified six STEC serotypes (O26, O45, O103, O111, O121, O145) as particularly dangerous (in addition to O157:H7; STEC-7) based upon data from the Centers for Disease Control and Prevention and designated them adulterants in raw ground beef, its component materials, and non-intact raw beef products. This research addressed three important components for detection of the recently declared STEC adulterant serotypes. Part one evaluated traditional *E. coli* O157 selective enrichment media, and additional media types and additive levels, for STEC-7 cultural amplification. Buffered peptone water (BPW), universal pre-enrichment broth (UPB), tryptic soy broth (TSB), TSB with 8 mg/L novobiocin, *Escherichia coli* broth (EC), and EC with 5, 8, and 20 mg/L novobiocin added were evaluated. EC broth performed equally well compared to non-selective media types, but addition of novobiocin supplement, typically used to suppress overgrowth by background flora, suppressed non-O157 STEC growth. Higher levels of novobiocin inhibited most serotypes. Part two tested the ability of transport media to maintain original STEC-7 concentrations as samples are transported to analytical laboratories. Transport media BPW, maximum recovery diluent (MRD), and Cary-Blair transport medium (CB) were inoculated with individual STEC serotypes and held at 4 or 10 °C for 72 h. Growth/survival curves indicated that CB maintained STEC-7 populations nearest to inoculation levels during storage at either temperature. Part three, part of a field study determining STEC-7 prevalence rates for cattle, hides and dressed carcasses, compared qualitative results generated by two molecular-based detection systems (BioControl Assurance GDS® and Neogen NeoSeek™), analyzing 576 selectively enriched beef carcass sponge samples collected from a commercial processing facility. The GDS and NeoSeek systems indicated 28.7 and 6.1 percent presumptive positive rates for STEC-7, respectively. It was speculated the higher GDS prevalence rate was due to false-positive determinations from the mixed culture enrichments, as viable STEC-7 cells were not recovered by immuno-magnetic bead culture isolation.

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Chapter 1 - **Introduction**

For as long as there has been consumption of food, there have been incidents of foodborne illness. It is estimated that each year 12,000,000 people get sick, 75,000 are hospitalized, and 2,268 die of foodborne diseases (Scallan et al., 2011). However, for the greater part of civilization these incidents have been often regarded as having mysterious or wrongly attributed origins. One of the earliest beliefs of disease origin was in the "Miasma Theory." Translated from Greek, this means "to pollute" and the conditions associated with it (i.e. swampland, fouled water, and spoiled food) were in actuality sources of what came to be known as germs. In 1546 Girolamo Fracastoro, an Italian intellectual, first postulated that disease was caused by agents that could be transferred between living organisms without direct contact between the two, thus laying the foundation of the Germ Theory of Disease. During the 1854 outbreak of cholera in London, it was John Snow who championed the cause of Germ Theory, and with the aid of the discovery and characterization of *Vibrio cholera*, helped progress the understanding of the causative agents of disease. Snow mapped the spread of the cholera epidemic and compared it to the system of wells that provided water for the city. His findings indicated that a source of the disease was a contaminated well known as the Broad Street pump. Paired with his "Grand Experiment", wherein water for a population from two sources was mapped, Snow showed the harmful effect of contaminated water in two nearly equivalent populations (Britannica 2014). One of the greatest contributors to Germ Theory, and the field of food science, was French chemist and microbiologist Louis Pasteur. Pasteur's work with the beer and wine industries in France led to new processes and understanding of the fermentation process and paved the way for development of pasteurization; a process in which a substance is heated so as to remove all pathogenic and most spoilage organisms, rendering it safe to consume for an extended period of time. Pasteur also utilized a "swan-neck" flask to debunk the pervading theory of spontaneous generation (i.e. life from nothingness). This simultaneous proof of bacteriology and disproving of spontaneous generation allowed for the general acceptance of sound scientific principles regarding microbiology as it is known today.

Food products are potential carriers of human pathogens, particularly those that are raw, only partially processed, or that have become recontaminated after processing. Foods therefore,

must be produced, processed and consumed in a manner that reduces human health risks as much as possible. Regulatory monitoring, protection of corporate brands, and buyer expectations all lead to a great number of microbiological analyses being conducted across the food system, many of which are directed towards detecting the presence of pathogens. In most cases, a qualitative (presence/absence) test is necessary since any level of the pathogen is considered unacceptable. However, in certain circumstances including the need for quantitative testing for research purposes, it is important that original pathogen population levels are maintained during sample collection and analysis so that an accurate estimate of pathogen levels in the original sample can be made. Shiga toxin-producing *Escherichia coli* are a group of pathogens that can be found in a wide variety of foods (raw and further processed meats, produce, juices, water, and various processed foods like cookie dough). Thus, an abundance of food product and ingredient testing occurs across the food industry each day to ensure public health against this group of pathogens or to identify the source of outbreaks attributed to STEC.

The objectives of this project were to *i*) establish how well the cultural methods traditionally used for enrichment of *E. coli* O157:H7 work to amplify serotypes O26, O45, O103, O111, O121, and O145 (the "Big Six" USDA-FSIS declared adulterants in raw non-intact beef) for detection assays; *ii*) assess three commonly used transport media for their ability to maintain population levels of STEC during simulated transportation and laboratory storage prior to analyses; and *iii*) conduct a qualitative STEC-7 screening of 576 commercial beef carcass sponge samples using two molecular based detection assays in support of a larger field study to ascertain STEC-7 prevalence in feedlot cattle feces and on their matched hides and resultant pre-washed carcasses at the abattoir.

Chapter 2 - **Literature Review**

The Organism *Escherichia coli*

Escherichia coli is in the family of bacteria known as Enterobacteriaceae, which includes other known foodborne pathogens such as *Salmonella* and *Yersinia* spp. These pathogens are facultative anaerobes with a Gram negative rod-shaped phenotype.

First discovered by Theodor Escherich in 1885, *Escherichia coli* has been established as a ubiquitous organism in the human gastrointestinal tract as well as that of other mammalian species. Since *E. coli* is primarily found in feces, it was put forth in 1892 that it could be used as an "indicator organism" for fecally contaminated water sources; the theory being that since *E. coli* was easy to isolate and identify through its lactose fermentation trait, it would only appear when fecal contamination of water had occurred. Although the concept of using *E. coli* as an indirect indicator of health risk was sound, it was complicated in practice due to the presence of other enteric bacteria like *Citrobacter*, *Klebsiella* and *Enterobacter* that can also ferment lactose and are similar to *E. coli* in phenotypic characteristics (Food and Drug Administration, 2011; BAM 8th Edition). Difficulty in distinguishing these bacteria led to the collective term "coliform" being used as a descriptor for these organisms. "Coliform" is not a taxonomic classification but rather a working definition used to describe a group of Gram-negative, facultative anaerobic rod-shaped bacteria that ferments lactose to produce acid and gas within 48 h at 35°C (Food and Drug Administration, 2011; BAM 8th Edition). Some coliforms can be found naturally in the environment, so the criteria for contamination was limited to fecal coliforms; coliforms that ferment lactose and grow at higher temperatures. While *Klebsiella* spp. are still considered to be a part of the fecal coliform collective, *E. coli* has been the primary indicator organism due to the increased ability of rapid tests to detect its presence. The role of *E. coli* has continued to change throughout history, because even though it was well known as an indicator organism, many strains were recognized to harbor various virulence characteristics causing pathogenicity findings towards humans and animals (Buchanan & Doyle 1997).

Illnesses attributed to this group of organisms are sometimes difficult to treat with conventional therapies due to their ability to resist antibiotics. *Klebsiella* species and *E. coli* are a

normal part of the human gut bacteria, and can become carbapenem-resistant (CDC, 2014). As carbapenems are currently our last good defense against multi-drug resistant Gram-negative bacteria (Wood et al., 2014), this is an issue when treating foodborne illness. Even with a number of severe foodborne illnesses being classified as infections (wherein the organism actively invades and grows inside the host causing illness), treatment with antibiotics is not always advised because mass cell death may lead to increased bacterial toxin release into the bloodstream and cause further damage to the host. The primary method of treating many foodborne illnesses is administration of fluids until the body is able to naturally pass the infectious organism (Feng et al., 2011).

Shiga-Toxigenic *E. coli*

While *E. coli* are predominantly non-pathogenic, they are opportunistic and, when ingested, are capable of causing gastrointestinal distress in humans (Food and Drug Administration, 2011; BAM 8th Edition). One group of pathogenic *E. coli*, known as enterohemorrhagic (EHEC) *E. coli*, produces a toxin that is similar to the one produced by the organism *Shigella*, the etiologic agent of dysentery. This Shiga toxin is also known as verotoxin (lethal to Vero cells in tissue culture) and has led to the organism being known as Shiga toxin-producing *E. coli* (STEC), verotoxin producing *E. coli* (VTEC), or the aforementioned EHEC. Not all STEC/VTEC are classified as EHEC.

Regardless of nomenclature (for the purpose of this document, organism references will be identified as STEC), infection with STEC can be accompanied by symptoms that include gastrointestinal distress, fever, nausea, and diarrhea. The majority of infections will be self-limiting and short lived; however, in some individuals (particularly immune-compromised such as the young or elderly) symptoms can progress to hemolytic uremic syndrome (HUS), a systemic form of infection that can cause kidney failure and even death. In most cases HUS occurs about 7 days after the first appearance of gastrointestinal distress. Characteristic symptoms are pallor, intravascular destruction of red blood cells (microangiopathic hemolytic anemia), depressed platelet counts (thrombocytopenia), lack of urine formation (oligo-anuria), swelling (edema), and acute renal failure (Buchanan and Doyle, 1997). While the most severe symptoms often manifest in children less than 10 years of age, for all cases of HUS, there is a 50% need for dialysis and a 3-5% mortality rate (Feng et al., 2011).

Such severe symptoms are due to the nature of Shiga toxin itself. The toxins that bind to cells effectively stop protein synthesis and are known to produce cytokines (a cell signaling protein) that signal cell death. Toxin production by itself is inadequate to cause illness. EHEC require the presence of other virulence markers to be fully pathogenic. The *eae* chromosomal gene (intimin) is ubiquitous among EHEC strains and encodes for an outer membrane protein associated with attachment (Buchanan and Doyle, 1997). This gene allows for greater attachment of the organism to the GI tract of the infected individual; thereby, preventing the organism from being passed while simultaneously increasing both the duration and severity of any illness associated with the infecting organism. Another gene that is believed to be vital to EHEC pathogenesis is hemolysin (*ehxA*), a portion carried on the EHEC plasmid. Serotypes that have been identified as the cause of 71% of non-O157 STEC disease are O26, O45, O103, O111, O121, and O145. Symptoms of non-O157 infection include mild diarrhea, HUS, or even death (Kalchayanand et al., 2012). Of these non-O157 serotypes, it has been observed that strains containing both genes, or merely stx2, induce more severe sickness than those caused by stx1 alone (Scheutz & Strockbrine, 2005).

STEC are part of the normal intestinal microflora of healthy cattle and other animals, particularly ruminants. Thus, they are shed in the animals' feces and can be found on the hides. During slaughter of food animals (particularly cattle) and application of various dressing procedures, this gut and hide STEC contamination can be spread to the surfaces of carcasses and ultimately find its way into the food supply (Rangel et al, 2005; Moxley and Acuff, 2014; Kalchayanand et al., 2012). More than 435 serotypes and 120 O serogroups of STEC have been shown to colonize the intestinal tract of cattle (Blanco et al., 2004; Gyles, 2007). More than 470 STEC serotypes have been isolated from humans and most have been isolated from cattle and/or beef (Brooks et al., 2005). The U.S. Centers for Disease Control and Prevention (CDC) reported 4,589 foodborne disease outbreaks for all food types involving 9.64 million cases between 1998 and 2008, of which beef caused 5% of the outbreaks. Of the outbreaks caused by STEC O157 and non-O157 STEC strains for all food commodities combined, 103 of 186 (55.3%) and 3 of 6 (50%), respectively, were attributable to beef (Moxley and Acuff, 2014). Cattle and sheep are considered primary reservoirs of STEC; however, several other animals have been found to carry infection. "Transport hosts", or vectors for transporting contamination from the reservoir to the food/water supply, include birds, rodents, and insects (i.e. flies and beetles) (Kaspar et al., 2010).

Human STEC infections have been attributed on frequent occasions to vegetables, raw milk, dairy products, and drinking water (Hussein, 2007). Other foods including salami, yogurt and apple juice/cider have been implicated in outbreaks (Reinders et al., 2001; Kaspar et al., 2010). According to a review of outbreak data by Kaspar et al. (2010), non-O157:H7 STEC outbreaks are more often traced back to person-to-person contact or to no known source compared to *E. coli* O157:H7, which is more often associated with meat and water contamination.

STEC Regulation of Beef Products

The O157:H7 strain of *E. coli* was first identified as a foodborne pathogen in 1982 during an outbreak at a fast food chain resulting in hemorrhagic colitis (Rangel et al., 2005). This outbreak was fairly limited in its impact, with approximately 47 individuals reported as being ill (Riley et al., 1983). However, it was after a 1993 outbreak involving multiple states in the Pacific Northwest that the USDA Food Safety and Inspection Service (FSIS) administrator Michael R. Taylor declared the implicated O157:H7 strain to be an adulterant in raw ground beef within the meaning of the Federal Meat Inspection Act in a speech given in September 1994. In this outbreak, 73 Jack-in-the-Box fast food restaurants served undercooked ground beef patties that sickened over 700 people and resulted in 4 deaths and 41 HUS cases. The FSIS justified this determination with the statement "These products are adulterated because they contain a poisonous or deleterious substance that may render them injurious to health" (Anonymous, 2011). Prior to this declaration, the requirement for determining a food to be adulterated had been restricted to things like harmful chemicals or foreign objects. For the first time, a microorganism naturally present in the gut of a cow would become an illegal substance in raw ground beef (Andrews, 2013). Now that *E. coli* O157:H7 was a nationally recognized organism of concern, testing increased and subsequently more outbreaks were identified and attributed for years after the declaration. The number of reported *E. coli* O157:H7 outbreaks began rising in 1993 and peaked in 2000 (Rangel et al., 2005). As the number of foodborne outbreaks increased, it was viewed by some to be indicative of a general increase in the number of outbreaks occurring. Others have attributed this so called "increase" to the increased ability of scientists to track the incidence of outbreaks due to improved sampling, detection and attribution technologies. PulseNet was formed in 1996 in order to better track and identify outbreaks after widespread criticism of the delay in identifying the 1993 Jack-in-the-Box outbreak. The CDC

and the Association of Public Health Laboratories developed the national PulseNet system so that foodborne disease outbreaks could be identified and stopped more quickly. This system works through public health labs that upload genetic "fingerprints" of human illness isolates (mostly from fecal sources) into the national database at the CDC. Utilizing pulsed-field gel electrophoresis, genetic sequences unique to different organisms can be identified, allowing PulseNet to establish whether separate incidents are part of a wider outbreak that can be epidemiologically investigated to identify a potential source of the contamination, often a food product (Anonymous, 2013).

As the number of cases attributed to *E. coli* O157:H7 in food has diminished, the number of cases caused by non-O157 STEC serotypes has increased. STEC as a total group are estimated to cause more than 265,000 illnesses each year in the United States, with more than 3,600 hospitalizations and 30 deaths. Accounting for under-diagnosis and under-reporting, an estimated 96,534 STEC O157 and 168,698 non-O157 STEC infections occur each year (CDC 2012). Of the non-O157 STEC food outbreak data reported by the CDC, six non-O157 *E. coli* serotypes (O26, O45, O103, O111, O121, and O145) cause 70-83 percent of confirmed STEC cases. With this information, FSIS further declared these additional six serotypes to be adulterants in raw, non-intact beef products (Anonymous, 2011). There has been some concern that the detection methods employed for STEC O157:H7 are inadequate or unproven for the task of detecting these new adulterants in variable food matrices.

STEC Control Measures by the Beef Industry

With the immense regulatory and customer pressure to ensure safe beef products, several antimicrobial intervention strategies/technologies are now commonly utilized by the beef industry to prevent the transmission of STEC into the food system. Although not the focus of this thesis, Moxley and Acuff (2014) recently provided a detailed summation of current technologies utilized across the beef processing industry. Good manufacturing practices for beef slaughter and fabrication are essential components of all pathogen control programs, and include not processing sick (downer) animals for human consumption, use of stringent sanitary dressing procedures, visual inspection of carcasses after hide removal to ensure absence of organic material, and proper chilling of dressed carcasses. Many beef processors use steam vacuums to sanitize defined surface areas of the carcass (i.e. areas where the hide is opened for subsequent

removal). The foundation of beef industry food safety programs is pre- and post-evisceration carcass treatments that primarily include steam or hot water treatment of whole carcasses or carcass sides, and spray treatments with various approved antimicrobial solutions. Additionally, various antimicrobial treatments are now being applied to chilled carcasses and wholesale cuts (primals and subprimals), along with beef trimmings destined for ground beef production. Application of 2% lactic acid and 2% acetic acid solutions have proven more effective than hot water washing alone in reducing bacterial levels on beef surface tissue when converted to ground beef and held at refrigeration temperatures (Dorsa et al., 1998). A 5% lactic acid solution was used to treat beef trim that had been artificially inoculated with rifampin-resistant strains of Big 6 STEC and O157:H7. Similar reductions were seen between Big 6 STEC and O157:H7 regardless of the lactic acid application temperature (25 and 55°C), achieving 0.5-0.9 and 1.0-1.4 log reductions at each temperature, respectively (Fouladkhah et al., 2012). This result is promising as it shows that methods already used widely throughout the beef industry may be capable of controlling the Big 6 STEC as well as O157:H7. Chilled beef carcass intervention strategies that have been evaluated are the use of 0.02% peroxyacetic, 0.16% sodium chloride, and 2 and 4% lactic acid solutions. At the given concentrations very little reduction in aerobic, coliform or *E. coli* counts occurred with peroxyacetic acid and sodium chloride, but lactic acid solutions were determined to be effective with particular regard to 4% which showed a reduction of ≥2 log units in some areas. However it was observed that there were variations in the meat that inhibited consistent application of antimicrobial solutions, which were magnified at chilled temperatures (Gill and Badoni, 2003). Various interventions have also been used in the treatment of beef hides as they have been identified as potential sources of contamination during processing. 10% solutions of lactic and acetic acid applied at 55^{o}C achieved at least a 2 log reductions in artificially introduced bacterial contamination levels on hide surfaces as well as removing visually apparent contamination (Carlson et al., 2008). Application of steam either to specific areas or whole hot beef carcasses has also proven effective at reducing overall bacterial load when used in conjunction with other carcass intervention strategies (Nutsch et al., 1997; Castillo et al., 1999). Use of steam alone with a vacuum system is also effective in reducing *E. coli* O157:H7 levels, however, this method is only suitable for spot treatment of beef carcasses (Dorsa et al., 1996).

While most research has focused on reduction of STEC O157 counts, one study indicated that in-plant antimicrobial interventions were able to reduce carcass prevalence for non-O157 STEC from 50% to 8.3% (Arthur et al., 2002). This indicates that the interventions used for control of STEC O157:H7 are likely effective in reducing non-O157 STEC numbers, but further research specifically targeting the recently added Big 6 STEC is needed. Microbiological screening of manufacturing beef trimmings prior to grinding, and finished ground beef products, is an additional component of verification of food safety programs across the beef industry. Currently, manufacturers test predominantly for *E. coli* O157:H7, while the USDA-FSIS collects and analyze products for both *E. coli* O157:H7 and the Big 6 STEC (Anonymous, 2013a).

STEC Detection in Beef Products

It was estimated by Strategic Consulting Inc. that 4.9 million tests for *E. coli* O157:H7 were performed in 2010, and that 3.9 million (or 80%) of these were conducted on red meat products; and they projected that STEC testing would equal this number by 2012 (Bryon, 2012). Although a vast community of knowledge exists for detecting *E. coli* O157:H7, significantly less understanding of effective detection methods exists for non-O157 STEC. Therefore, cultural protocols developed for *E. coli* O157:H7 have been applied in original or adapted formats in an attempt to isolate and identify other STEC. Wang et al. (2013) summarized inherent challenges for detecting pathogens in food, including that pathogens are often present in low levels amongst high levels of background microflora, they are often in an injured or stressed physiological condition, they almost always non-homogeneously distributed in the food, and many interfering factors within the food can complicate analyses. They further characterized difficulties in detecting non-O157 STEC to include the phenotypic ability of these strains to ferment sorbitol (thus, the absence of this trait cannot be used in differential plating as is done with serogroup O157), detecting multiple serogroups of highly pathogenic STEC (Big 6) requires a suite of assays for detection and confirmation, and that virtually no molecular detection assays can determine if all necessary virulence traits (O serogroup antigen, *stx* and *eae*) present in a mixed enrichment broth come from one cell (thus an adulterant) or from multiple cells (perhaps not an adulterant).

USDA-FSIS Official Methods

The USDA-FSIS considers raw, non-intact beef products or their components to be adulterated if *E. coli* O157:H7 or any of the "Big 6" STEC (O26, O45, O103, O111, O121 and O145) are detected, as defined in the Federal Register notice titled Shiga Toxin-Producing *Escherichia coli* in Certain Raw Beef Products (Anonymous, 2012). The FSIS confirmed in this announcement that it would implement routine verification testing for the Big 6 STEC and *E. coli* O157 in raw beef manufacturing trimmings beginning on June 4, 2012. The FSIS stated that it believes that steps taken within their regulatory STEC sampling and detection program to increase representative sample size and increase detection technology sensitivities will improve the public health outcome of the program (Anonymous, 2013a).

STEC detection methods, especially regarding non-O157:H7 serotypes, are complicated by a lack of common distinguishing biochemical characteristics compared to other *E. coli* (Kalchayanand et al., 2012). The current USDA method of analyzing beef and beef-associated samples for *E. coli* O157:H7 (USDA-FSIS MLG 5.08) became effective June 29, 2014 (Anonymous, 2013b) and includes placing samples into mTSB selective enrichment broth (1:4 dilution of product in TSB with added bile salts, vancomycin, cefixime, and cefsoludin [Fluka analytical No. 08069]) for 15-24 h at 42±1^{o}C followed by analysis. If fermented sausages are being analyzed, addition of 8 mg/L novobiocin to the mTSB with incubation at 42±1^{o}C for 15-22 h (1:10 dilution of product in broth; mTSB+n) is used. Detection, which is considered a qualitative screen, is accomplished using the BAX® System Real-Time PCR Assay (Qualicon #D14203648) (DuPont Qualicon, Wilmington, DE) for *E. coli* O157:H7 according to USDA-FSIS MLG 5A.04 (Anonymous, 2013c). If unavailable, the RapidChek® lateral flow device for O157:H7 (Romer Labs #7000160) (Strategic Diagnostics Inc., Newark DE) is a suitable alternative. Once a negative qualitative result for a sample has been obtained, samples may be declared negative. However, if a positive result is obtained, which is called a "potential positive", an isolation procedure follows to attempt organism recovery. Isolation begins with immunomagnetic separation using Dynal #710.04 *E. coli* O157:H7 paramagnetic beads (Dynal Inc., Lake Success, NY), which are added to the screen positive enrichment sample and allowed to attach. Once the separation procedure, which uses a recovery magnet to collect the paramagnetic beads, has been completed, 1:10 and 1:100 dilutions of bead suspensions are plated onto modified Rainbow Agar® (Biolog Inc., Hayward, CA) (Rainbow Agar with novobiocin, cefixime trihydrate, and potassium tellurite added) plates and incubated for 20-24 h at 35±2^{o}C.

A second plating of undiluted beads is conducted after an acid treatment to kill background microflora. Here, beads are exposed to 1N hydrochloric acid (bringing suspension pH down to 2.0-2.5) for 1 h at 18-30°C. Subsequently, beads are neutralized in buffer and plated on modified Rainbow Agar as described above. Colonies that display the appropriate black or gray coloration associated with *E. coli* O157 (O157 colonies may have a bluish hue if surrounded by non-toxigenic pink or magenta colonies) are then further tested using latex agglutination assays (RIM® *E. coli* O157:H7 Latex Test Kit, Remel, Lenexa, KS). At this point, samples demonstrating characteristic colony colors and agglutination with O157 antisera are called "presumptive positives". *E. coli* O157 latex positive colonies are further confirmed using biochemical testing, O and H latex agglutination assay, and the presence of Shiga toxins in culture isolates using a toxin assay. If Shiga toxins are not detected with the kit, PCR is used to determine the presence of toxin genes. A sample is "confirmed positive" if it is biochemically identified as *E. coli*, serologically or genetically determined to be of the serogroup O157, and is shown to produce Shiga toxin or possess at least one of the Shiga toxin genes (*stx*).

The current official USDA-FSIS detection and isolation method for non-O157 STEC from meat products, beef carcasses and environmental sponges (USDA-FSIS MLG 5B.05) became effective on June 29, 2014 (Anonymous, 2013c). In summary, this official method utilizes the BAX STEC suite to initially screen selectively enriched (1:4 dilution of product in mTSB incubated at 42°C for 15-24 h) samples, followed by cultural isolation from presumptively positive screens. The BAX screening assay targets the *stx* and *eae* gene markers initially. If positive, two additional BAX panels are used to identify genes within the O antigen gene cluster specific for each serogroup. Isolation of non-O157 cultures from presumptively positive STEC screens for the Big 6 (meaning having simultaneous presence of a Big 6 O antigen, *stx* and *eae* genes in the mixed culture enrichment broth) is done using immunomagnetic separation beads coated with sero-specific antibodies, and employing a post-separation acid treatment to the beads to reduce background microflora. These beads are recovered and plated onto modified Rainbow Agar, which contains 5.0 mg/L sodium novobiocin, 0.05 mg/L cefixime trihydrate, and 0.15 mg/L potassium tellurite. Suspect colonies appearing on modified Rainbow Agar are further screened using agglutination tests to indicate Big 6 serogroups, and any positive colonies by agglutination are streaked onto TSA containing 5% sheep blood and confirmed using BAX real-time PCR assays and other biochemical testing procedures.

Food Sample Enrichment for STEC Detection

Samples that have been received from the field often need to undergo an enrichment step to increase any target organisms of interest to detectable levels. Enrichment media may be non-selective, and enrich all organisms approximately equally, or selective and have additives to inhibit non-target organisms and suppress background flora. Tryptic soy broth (TSB) is a commonly used non-selective media containing pancreatic digest of casein, peptic digest of soybean meal, glucose, sodium chloride, and dipotassium hydrogen phosphate. Universal Pre-Enrichment Broth (UPB), which contains casein enzymic hydrolysate, proteose peptone, monopotassium phosphate, disodium phosphate, sodium chloride, dextrose, magnesium sulphate, ferric ammonium citrate, and sodium pyruvate, functions to resuscitate injured cells by forming a nutritious non-selective environment. UPB is primarily used for enrichment of samples possibly containing *Salmonella* or *Listeria*. Injured Enterobacteriaceae have also been enriched using non-selective buffered peptone water (BPW), which contains enzymatic digest of casein, sodium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate, to provide injured cells time to recover and replicate (Sadovski, 1977). Selective enrichment media includes *Escherichia coli* Broth (EC), which contains casein digest, lactose, dipotassium phosphate, potassium phosphate, sodium chloride, and bile salts. The bile salts inhibit background flora and enhance coliform growth (Perry and Hajna, 1944). Bile is a fluid produced in the digestive tract that contains various antimicrobial bile salts, which are stored in the gall bladder. Bile salts interact with bacterial cell membranes, and certain organisms including *E. coli* can expel bile salts from the cellular cytoplasm after passing through the cell membrane, providing resistance to bile salts (Merritt and Donaldson, 2009).

While each media type is useful for organism enrichment, the addition of antimicrobials allows greater selectivity during the enrichment process but may inadvertently suppress some target organisms. Kanki et al. (2009) found that UPB performed as well as, if not better, than EC broth containing novobiocin at 42°C for *E. coli* serotypes O157 and O26. Novobiocin is a natural coumarin type antibiotic that inhibits cell growth by blocking production of DNA gyrase, a vital component of DNA reproduction in prokaryotes, and is an additive in media for selective growth of Shiga-toxigenic *E. coli* (Nakada et al. 1994; USDA MLG 5.06). Novobiocin has been shown to inhibit Gram-positive bacteria (Meyer et al., 1995). Addition of novobiocin to select for *E. coli* O157:H7 has been used in USDA guidelines for sample analysis and is approved for use

when detecting non-O157 STEC (USDA MLG 5.06). This allowance differs from findings that showed that addition of novobiocin supplement may inhibit or slow the growth of non-O157 STEC (Vimont et al., 2006).

Tzschoppe et al. (2012) investigated cultural detection approaches for STEC, including O104:H4, in ready-to-eat salads and sprouted seeds. They found significant problems when trying to detect low (<10 cfu/25 g) STEC levels in the presence of high (10^5 cfu/g) background numbers of naturally occurring Enterobacteriaceae and *Pseudomonas*. At 37°C, the background flora saturation of enrichment broth was reached in 8-9 doubling periods, and they concluded that an enrichment period >6 h was disadvantageous for recovery of low EHEC levels in salads. They also found that enrichment medium composition greatly impacted growth and isolation of EHEC from food samples. They compared BPW and Brilliant Green Bile Lactose Broth (BRILA-broth; SIFIN, Berlin, Germany), both antibiotic free broths, and found BRILA-broth to be more effective in controlling background flora growth while allowing EHEC growth. Importantly, Tzschoppe et al. (2012) found that EHEC in salad stored at 6°C, in what they termed "stressed samples", were reduced in numbers to 14.6% (at 44°C) and 15.6% (at 37°C) compared to non-stressed cells, and three EHEC strains became undetectable after 72 storage using an initial inoculation level of 1-10 cfu/25 g.

A comprehensive evaluation of published literature over STEC enrichment methods from 1997-2006 found that primary enrichment media used in STEC research studies were BPW, TSB, and EC. Media often had antibiotics, primarily novobiocin, added with incubation occurring for 18-24 h at 35-37°C, but the methods and strains used for each study varied by researcher, limiting study comparisons (Vimont et al., 2005). Differing from Vimont's analysis, Gonthier et al. (2001) found that temperatures of 40.2 and 41.2°C were optimum enrichment temperatures for O157 and non-O157 serogroups, respectively. However, this study only looked at growth in Mueller-Hinton broth, a medium comprised of beef infusion solids, starch, and casein hydrolysate. It has been observed that for freeze-injured cells a 2-hour non-selective enrichment at 25°C prior to selective enrichment steps increases recovery of *E. coli* O157:H7 from frozen foods (Hara-Kudo et al., 2000). For enrichment in selective media, it was observed that *Escherichia coli* broth performed similarly to TSB for resuscitation of both freeze- and heat-injured *E. coli* O157:H7 cells at 37 and 42°C while suppressing growth of Gram positive bacteria (Restaino, 2001).

The use of novobiocin in selective enrichment broths to isolate *E. coli* O157:H7 is widespread, as it is effective in restricting background flora and Gram-positive organisms while allowing amplification of the target pathogen. However, research has shown that the use of novobiocin as an enrichment additive may repress the growth of non-O157 STEC strains and subsequently lead to false-negative results for detecting non-O157 STEC (Vimont et al., 2006). Novobiocin is often added to enrichment broth for *E. coli* O157:H7 detection at 20 μg/mL, but Vimont et al. (2007) showed that using a lower concentration (16 μg/mL) provided better efficacy in detecting non-O157 STEC. Numerous non-O157 STEC were used by Vimont, but the Big 6 STEC were not among the serotypes tested using novobiocin supplement.

Selective/Differential Plating Media for STEC

Escherichia coli O157:H7 has been the primary target for pathogen detection in beef since being deemed an adulterant in ground beef in 1993. Unlike other serotypes, O157:H7 is known for its inability to utilize sorbitol, which has allowed for the standard use of sorbitol containing MacConkey Agar for detection and isolation of the organism. MacConkey agar selects for the growth of Gram-negative rod-shaped bacteria and differentiates between lactose fermenters and nonfermenters. Many of the facultatively anaerobic bacteria in the intestines ferment lactose, including *E. coli* (ASM, 2014). March and Ratnam (1986) demonstrated that replacing lactose in MacConkey agar with sorbitol (SMAC agar) was effective in distinguishing *E. coli* O157 (which cannot ferment sorbitol) from fermenting competing microflora in stool samples. The relative frequency of non-sorbitol fermenting organisms other than *E. coli* O157:H7 appearing on SMAC agar was low at 10-20%. SMAC is comprised of peptone, D-sorbitol, bile salts, sodium chloride, neutral red, crystal violet and agar. SMAC replaces lactose with sorbitol as the fermented sugar, which results in colorless *E. coli* O157:H7 colonies that can be used for further identification (March and Ratnam, 1986).

However, the association of sorbitol-fermenting *E. coli* O157:H7 strains with HUS patients has been documented (Gunzer et al., 1992; Karch et al., 1993; Werber et al., 2011), and the recognition of other STEC that generally ferment sorbitol as significant contributors to disease, limits the use of sorbitol fermentation as a screening tool in detection/isolation of STEC (Gouali et al., 2013). Gouali et al. (2013) pointed out regarding STEC isolation from fecal samples that the low STEC density and potential inhibitors in stool specimens and the absence of culture characteristics common to all STEC make it very difficult to develop a "universal"

isolation medium. This characterization would hold true for STEC isolation from food matrices. This has more recently led to the development and marketing of various chromogenic media for STEC detection and differentiation that is not based upon sugar fermentation, but are based on presumptive identification from unique chromogenic substrate utilization characteristics of *E. coli* (Hirvonen et al., 2012). STEC selectivity of chromogenic media is often enhanced by the addition of compounds like cefixime, novobiocin and tellurite and by varying incubation temperatures. However, when using chromogenic agar, incubation temperature variations and use of plates that have been stored for an extended period may cause fluctuations in expected colony color (De Boer and Heuvelink, 2000).

Rainbow® Agar O157 (RBA) is a proprietary differential media (Biolog, Hayward, CA). RBA contains chromogenic substances specific for β-galactosidase and β-glucuronidase; chemicals that result in black/gray O157:H7 colonies, purple/violet/blue non-O157 pathogenic *E. coli*, or pink/magenta non-pathogenic *E. coli* (Rainbow® Agar, Biolog, 2006). Manafi and Kremsmaier (2001) compared Fluorocult HC (Merck, Darmstadt, Germany), RBA, and Biosynth Culture Medium O157:H7 (BCM O157; Biosynth, Staad, Switzerland) to SMAC (Merck), for detection of *E. coli* O157:H7 from raw ground beef and raw milk samples after enrichment in mEC+n and mTSB+n, respectively. In this comparison, RBA resulted in fewer false positives (2.1%) when analyzing enriched food samples, and had a sensitivity and specificity of 91.1 and 91.6%, respectively, using pure cultures (Manafi and Kremsmaier, 2001).

CHROMagar™ O157 (Becton Dickinson, Franklin Lakes, NJ) (chromopeptone, sodium chloride, chromogen mix, potassium tellurite, cefixime, cefsoludin, and agar) is designed to inhibit non-O157:H7 growth and simultaneously differentiate O157:H7 as mauve or pink colonies. While O157 has been shown to conform to these standards, other EHEC also stand out as blue-black, whereas O113 and some other EHEC strains were mauve, red or pink and indistinguishable from SLT-negative strains of *E. coli*. (Bettelheim, 1997).

In a clinical laboratory study in 2006 comparing the diagnostic efficacy of CHROMagar O157 (Becton Dickinson) to SMAC agar for direct plating of stool samples, Church et al. (2007) determined that CHROMagar O157 had a higher sensitivity (96.3%) and negative predictive value (100%) than SMAC, and a specificity and positive predictive value (100% each) that was the same as SMAC. However, they were able to make patient diagnostic determinations using 75% fewer colony picks and 43% fewer sero-agglutination tests with CHROMagar O157.

Further, the overall false-positive rate for colony picks was 65% and 20% for SMAC and CHROMagar O157, respectively.

CHROMagar STEC medium (CHROMagar Microbiology, Paris, France) was shown to be an effective medium for selectively propagating different STEC serotypes associated with human diarrhea (Hirvonen et al., 2012). They found high STEC detection sensitivities for serogroups O26 (90%), O111 (100%), O121 (100%), O145 (100%), and O157 (84.9%) and growth on CHROMagar STEC was highly associated with presence of the *terD* tellurite resistance gene. In an evaluation of the selective/differential agars CHROMagar STEC and TBX agar to detect low levels (1-10 cfu/25 g) of artificially inoculated STEC into ready-to-eat salad and sprouted seeds, Tzschoppe et al. (2012) found that plating from 6-h incubated enrichments onto both agar media with incubation at 44°C improved identification of EHEC. CHROMagar STEC was most suitable for the detection of the major EHEC strains, and growth was associated with tellurite resistance. Their study showed that TBX agar was better in isolating tellurite sensitive EHEC strains. A very important finding was that the presence of high levels (i.e. 10^5 cfu/g) of background flora in samples and their overgrowth during enrichments of longer than 6 h, or when using BPW as the enrichment broth, greatly reduced their ability to identify STEC on plates. This was mostly overcome by utilizing BRILA-broth for enrichment for 6 h followed by plating on CHROMagar STEC incubated at 44°C, as background flora appearing on plates was reduced by >1000 fold without having a deleterious effect on the growth and colony formation of the EHEC strains. Gouali et al. (2013) showed that CHROMagar STEC (CHROMagar Microbiology, Paris, France), after a 7 h enrichment step for stool samples from patients suspected of STEC infections, detected 15 STEC serotypes including non-sorbitol fermenting O157 and O104:H4 (when supplemented with cephalosporins). They concluded that CHROMagar STEC provided good performance, particularly relating to its high negative predictive value.

To detect STEC and differentiate between O157 and various non-O157 pathogenic *E. coli* serotypes, Posse et al. (2007) created a media containing chromogenic markers, pH indicators, and inhibitory compounds. This media was prepared from MacConkey agar base (BD Biosciences), sucrose, sorbose, bile salts No. 3, 5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal), isopropyl-b-D-thiogalactopyranoside (IPTG), novobiocin, and potassium tellurite. For O157 differential medium, sucrose and sorbose were replaced with

sorbitol (Posse et al., 2007). Due to the fermentation differences between serotypes, differences in colony colors associated with each serogroup allows for easier identification of organisms used in the study (O26, O103, O111, and O145). O26 appeared as bright red to dark purple, O103 and O111 were blue-purple, and O145 were identified by a green color. Non-coliforms were also recovered with colors ranging from pale yellow, to dark orange, to red. Color changes are due to the inclusion of IPTG to induce β-D-galactosidase activity, an enzyme that encourages cellular use of lactose through inhibition of the lactose inhibitor in a cell (Posse et al., 2007). When paired with X-Gal, IPTG will indicate β-D-galactosidase activity by allowing a dark center to be visible on fully grown colonies (Posse et al., 2007). The O157 media yielded high false positives for sorbitol-positive colonies and required subsequent confirmatory steps. Further research by Posse et al. (2008) found that the above mentioned media was suitable for use with feces and various food matrices when paired with further confirmatory steps such as more selective media and polymerase chain reaction sequencing. Others have found that colony color ambiguity between non-O157 serogroups can occur (Mathusa et al., 2010).

Lin et al. (2012) reported on an STEC detection agar called STEC heart infusion washed blood agar with mitomycin-C (SHIBAM agar), which is a modification of washed sheep blood agar that has mitomycin C added. This agar greatly improved non-O157 STEC recovery from inoculated produce (<10 cfu/25 g) by improving suspect colony selection based on a hemolysis zone of clearing (most STEC are hemolytic compared to background lettuce microflora).

Polymerase Chain Reaction for Detection/Confirmation of STEC

Polymerase chain reaction (PCR) is an analytical technique with a wide range of applications in microbiology. Attributed to Kary Mullis in the 1980's, PCR is a method of rapid DNA amplification in which target sequences are cleaved from the overall sequence and subjected to sessions of heating and cooling designed to denature strands of DNA and allow them to re-anneal to form exponential increases to the original amount of DNA present in a sample (Bartlett and Stirling, 2003). According to the National Center for Biotechnology Information (NCBI, 2014), PCR requires a DNA template, DNA polymerase, primers, and nucleotides in order to function. The template is comprised of the target sequence from the sample DNA to be replicated. DNA polymerase (commonly Taq polymerase from the organism *Thermis aquaticus*) is an enzyme that allows for complementary sequences to form to the cleaved target sequence. Primers are "short pieces of single-stranded DNA that are

complementary to the target sequence," (NCBI, 2014). These primers are carefully chosen by researchers to allow for only select DNA segments unique to the target segment to re-anneal. Nucleotides are the raw building blocks of DNA (guanine, adenine, thymine, and cytosine) that allow new segments of DNA to form for analysis. The process can be further refined for specificity by utilizing multiple primers that have small overlapping portions to detect longer unique sequences, this is known as multiplex PCR.

Genes that are indicative of the pathogenicity of STEC are *eae* (intimin) and *stx1/stx2* (genes responsible for shiga-toxin production). When paired with the location of the O antigen in the genetic sequence, multiplex PCR has the capacity to generate data with a high degree of specificity for the sample. This method has been proven to be able to simultaneously detect pathogenicity genes when present in samples of O157:H7 (Fratamico et al., 1995), and can distinguish other non-O157 STEC (Meng et al., 1996). Multiplex PCR has since been utilized to detect *E. coli* serotypes O26, O111, and O157 in stool samples where culture based methods were unable to recover pathogens (Louie et al., 1998). Another study used multiple (over 10) unique primers to detect genetic segments from the most commonly pathogenic strains to great effect (Perelle et al., 2003). PCR assays have also been developed to allow for all diarrheagenic *E. coli* categories to be detected at once, even allowing for virulence genes from a single stool sample to be differentiated based on the presence of shiga toxin-producing (containing *stx1* or *stx2* with *eae*), enteropathogenic (*eae* and *bfp*), enterotoxigenic (*st*II and lt), enteroinvasive (*vir*F and *ipa*H), enteroaggregative (*aaf*II), or diffuse adherent (*daa*E) genes (Vidal et al., 2005). The lower cost and increased speed with which results can be obtained is one of the primary benefits of PCR analysis. Combined with the specificity of properly designed primers, PCR analysis of samples is a vital tool in rapid pathogen detection. However, disadvantages include assays not being commercially available, inhibition by food matrix components, and the inability to determine live versus dead cells. These drawbacks can sometimes be minimized or eliminated by additional protocol steps such as the inclusion of internal amplification controls. Further, Wang et al. (2012) pointed out that a positive PCR determination from a mixed enrichment system does not always mean that a pathogenic STEC is present, as detection of gene targets from multiple strains could occur.

Immunomagnetic Separation

Immunomagnetic separation (IMS) is a cell isolation technique in which small beads of paramagnetic material (iron) are coated with antibodies specific to a desired target cell. Antibodies may bind directly to the bead, or used in conjunction with a secondary antibody that will bind to the cell, then attach to the antibody on the bead. These beads are added to a sample and bind to a specific cell type, which when subjected to a magnetic field, is separated from the sample. Remaining beads may be washed to remove non-target cells (Brooks and Schumacher, 2001). Developed for use with fluids like blood, the process has since been utilized for detecting bacteria, as the non-destructive nature of the separation method collects viable cells that may then be cultured for analysis. One of the benefits of this process is that a high concentration of cells can be obtained from a large sample (Olsvik et al., 1994). Work by Chapman et al. (1994) showed IMS to be a sensitive and simple technique for the isolation of *E. coli* O157 from bovine feces. Building on this, Karch et al. (1995) determined that IMS increased sensitivity in O157 detection compared to direct culturing on sorbitol MacConkey agar (SMAC) and cefixime tellurite SMAC, and that the IMS technique had the advantage of being less labor intensive and time-consuming than the molecular methods when direct culture, colony hybridization, and IMS were compared to analyze stool samples from children displaying hemolytic uremic syndrome (HUS). Results from the study also indicated that the IMS procedure was able to detect 10^2 colony forming units per gram (CFU/g) in the presence of 10^7 coliform background flora. Karch et al. (1999) stated that due to the rising numbers of non-O157 enterohemorrhagic *E. coli* cases, the use of additional methods besides IMS in the bacteriological diagnosis of HUS is necessary. Chapman and Siddons (1996) evaluated IMS for detection of *E. coli* O157 in fecal samples from asymptomatic human subjects, finding that it out-performed direct plating methods. IMS has also been proven effective in isolation of *E. coli* from untreated milk (Chapman et al., 1993). Research by Wright et. al. (1994) established that IMS was acceptable for use on multiple food matrices for isolation of *E. coli* O157. A comparison of methods during a milk-borne outbreak showed that detection rates were increased with use of IMS to levels comparable to that of PCR and that use of such techniques is recommended for laboratory analysis when attempting to isolate STEC (Cubbon et al., 1996).

Transportation of Field Samples to Laboratories

Sample analysis typically involves sample shipment from the collection location to a laboratory, and upon receipt at the laboratory, samples are generally stored for a short period of

time prior to analysis. During transport it is imperative that sample integrity is maintained, including maintaining viability of target organisms that may not compete well with native background microflora in the sample, or where target organisms may be in a stressed or injured state. Furthermore, when target organisms within a sample are to be quantified, media and extrinsic conditions during shipment should maintain microbial populations at levels near those at the time of sample collection. Extensive use of various transport media for clinical cultures is reported in the literature; however, fewer publications address the importance and use of transport media in food analysis.

Stuart et. al. (1954) devised a transport medium for the shipment of specimens to a central laboratory for the isolation of gonococci. Crookes and Stuart (1959) used this medium for a field study and found that 5% of the specimens collected were overgrown with *Escherichia coli*. To circumvent this, Aerosporin (polymixin B sulfate) was added to suppress coliform growth in the gonococci samples. Further research with Stuart's media by Cary and Blair (1964) showed that significant overgrowth of *Escherichia coli* still occurred in fecal samples after shipment from the collection location. The availability of nutrients combined with higher pH was identified as causative agents of bacterial growth in Stuart's medium. To reduce background flora growth while maintaining culture viability, criteria for an effective enteric transport media were established as (i) low nutrient content, (ii) low oxidation-reduction potential, and (iii) a high pH (Cary and Blair, 1964). Known as Cary-Blair Transport Medium (CB), this medium is comprised of disodium hydrogen phosphate, sodium thioglycollate, sodium chloride, calcium chloride, and agar. These researchers stated that the low nutrient content of the medium and utilization of phosphate as a buffering agent instead of sodium glycerophosphate prevents overgrowth by *Escherichia coli*, *Citrobacter freundii* and *Klebsiella aerogenes*. Replacement of glycerophosphate with phosphate is necessary to prevent dehydrogenation by *E. coli*.

Many studies have focused on recovery of enteric *Shigella* and *Salmonella* from samples with few targeting *E. coli* specifically. Wells and Morris (1981) found that buffered glycerol saline (BGS) was superior to CB at room temperature, refrigeration (4°C) and freezing (-20°C) yielding better recovery of *Shigella spp.* Other studies agree and found that CB is suitable for storage as well as transport of *Shigella* and *Salmonella spp*. under refrigeration (4°C) and freezing (-70°C) conditions. Room temperature samples showed significantly greater loss in organism recovery than other temperatures (Wafsy et al., 1995). The CDC recommends CB for

fecal specimens with temperatures of 4°C if analyzed within 48 hours; otherwise freezing at -70°C is required to maintain sample viability (CDC, 1990).

Stern and Line (2009) compared sterile tap water, universal preenrichment broth and buffered peptone water for maintaining populations of *Campylobacter*, *Salmonella* and *E. coli* over 24 h at 4°C under simulated transport conditions of poultry carcass washes to a laboratory. Universal preenrichment medium provided lower recovery of *E. coli* compared to the other two transport media. Based on results from other studies, these parameters should be sufficient for maintaining sample viability during transport.

Chapter 3 - **Objectives**

The general goal of this project was to assess how well common cultural methods used for detection/isolation of *Escherichia coli* O157:H7 translate for the "Big 6" serotypes O26, O45, O103, O111, O121, and O145.

Specific goals of this series of studies included 1) the assessment of various media types for their efficacy as a transportation medium for STEC; with the aim of protecting the viability and maintaining initial target population concentrations during simulated shipment and laboratory storage representing proper, along with mildly abusive temperatures; and 2) a comparison of different non-selective and selective enrichment broths commonly used for isolation of *E. coli* O157 for their efficacy in amplifying the Big 6 STEC.

Lastly, in support of a larger field prevalence study, STEC O157 and non-O157 STEC (Big 6) prevalence was determined for matching hide and pre-intervention carcass sponge samples collected from fed cattle being harvested at a commercial processing plant. This comparison included qualitative PCR analysis of selectively enriched samples using the BioControl Assurance GDS® (BioControl Systems, Bellevue, WA) and the NeoSeek™ system (Neogen Inc., Lansing, MI).

Chapter 4 - **Growth of *Escherichia coli* O157:H7 and Six Non-O157 Shiga Toxin-Producing *E. coli* Serotypes in Various Enrichment Media**

Abstract

Detection of *Escherichia coli* O157:H7 has been a key focal point in food safety since the pathogen was identified as being foodborne in 1982, and subsequently being declared by the USDA-FSIS to be an adulterant in raw, non-intact beef in 1994. Analysis of food samples for *E. coli* O157:H7 requires enrichment to amplify populations to detectable levels. With the emergence of additional serogroups of Shiga toxin-producing *E. coli* (STEC) as significant foodborne pathogens, and with the USDA-FSIS declaration of an additional six serotypes (O26, O45, O103, O111, O121, and O145) as adulterants in 2012, the need to culture and detect the broader group of STEC has arisen. Methodologies to target non-O157 STEC have generally been modifications of O157 protocols; however, these methods may not always be applicable to other serotypes. This study sought to establish the efficacy of commonly used enrichment media for *E. coli* O157:H7 to amplify non-O157 STEC in pure culture. Broth media evaluated included buffered peptone water (BPW), universal pre-enrichment broth (UPB), tryptic soy broth (TSB), modified tryptic soy broth with 8 mg L^{-1} novobiocin (mTSB M), *Escherichia coli* broth (EC), and EC broth with 5, 8 and 20 mg L^{-1} novobiocin added. Samples were incubated at 37^{o}C and analyzed over a 24-h period to establish growth curves for each STEC serotype. The addition of novobiocin as a selective supplement to TSB or EC broth (at 5 mg L^{-1}) delayed rate of pure culture growth to reach peak population density and ultimately reduced the final microbial counts attained after 24 h of incubation, with growth in broth treatments using the EC media base being more restricted and variable. At medium (8 mg L^{-1}) and high (20 mg L^{-1}) concentrations in EC broth, novobiocin showed varying degrees of inhibition of STEC strains, and completely inhibited the growth of all six non-O157 STEC while allowing growth of O157:H7 at the high concentration. Due to non-O157 growth suppression in the presence of novobiocin, particularly in EC broth base, careful consideration should be given to its use in analysis of food samples for the presence of non-O157 STEC, understanding that the presence of competing background flora

in food and environmental samples can impact STEC recovery and must be suppressed during enrichment.

Introduction

The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) declared *E. coli* O157:H7 to be an adulterant in ground beef in 1994 after a multi-state outbreak linked to undercooked ground beef patties served by a fast food chain in 1993 (Wang et al., 2012). Shortly thereafter, USDA-FSIS began a sampling program for ground beef at federally inspected processing facilities and at retail. This sampling program was expanded in 1999 to include non-intact beef products (i.e. mechanically tenderized or injected beef cuts). After imposing this regulation, numerous methods have been developed over the years to specifically detect or control *E. coli* O157:H7 throughout the food industry due to its regulatory implications and the severity of illness that can occur when a susceptible individual becomes infected. The incidence of *E. coli* O157:H7 infections has declined according to CDC outbreak data (CDC, 2011), likely due to improvements in food process controls, greatly increased screening of foods for the organism, and due to numerous recalls of contaminated foods.

While improved control measures have prevented similar large-scale outbreaks from ground beef in subsequent years, the improved detection methods showed that the scope of STEC in the beef industry was still significant. The CDC estimates that Shiga toxin-producing *E. coli* are responsible for over 265,000 illnesses per year with non-O157 STEC contributing 168,698 cases annually (CDC, 2012). From 1983 to 2002, CDC data on STEC isolates identified six O groups comprising 13 serotypes as the cause of 71% on non-O157 STEC disease in the United States (Brooks et al., 2005). In response to growing numbers of illness being traced to these six STEC serotypes (O26, O45, O103, O111, O121, and O145), the USDA-FSIS expanded its list of adulterants in ground beef and other non-intact beef products (Anonymous, 2012).

Disease symptoms can include bloody diarrhea, gastrointestinal distress, fever, nausea, and in some cases can proceed to hemolytic uremic syndrome (HUS). Development of HUS can lead to renal failure and in certain cases may be fatal. Since STEC are most likely to cause serious injury to the young, elderly, or immune compromised the repercussions of an outbreak may be quite severe.

The USDA-FSIS has published preferred analytical protocols for detection of both O157:H7 and non-O157 STEC in its Microbiology Laboratory Guidebook (MLG). Generally, detection protocols prescribe real-time PCR screening of selectively enriched samples, followed by immunomagnetic separation and plating on chromogenic media for the PCR-positive samples (Moxley and Acuff, 2014). Since the methods in use for *E. coli* O157 have not been fully evaluated in relation to these new "Big Six" serotypes, there is a need to establish cultural detection procedures that are effective when applied to the new adulterants. Vimont et al. (2006) found that while several studies evaluating enrichment of non-O157 STEC have been reported, there was a distinct lack of consistency in the methods used. Specifically, the antibiotic novobiocin has been used as an additive to enrichment media when attempting to grow O157:H7. This is due to O157:H7 having a high resistance to novobiocin and the antibiotic's ability to suppress background flora; however, in previous studies it has been shown to inhibit non-O157 STEC (Vimont et al. 2006).

The goal of this study was to analyze the effects of various enrichment media on the growth patterns of the Big Six and O157:H7 STEC in order to ascertain which media is best suited for simultaneous enrichment of all serotypes listed.

Materials & Methods

STEC growth in three non-selective broth media types were evaluated; buffered peptone water [BPW; Sigma Aldrich, St. Louis MO: 10.0 g enzymatic digest of casein, 5.0 g sodium chloride, 3.5 g disodium hydrogen phosphate, 1.5 g potassium dihydrogen phosphate], universal pre-enrichment broth [Sigma Aldrich: 5.0 g casein enzymic hydrolysate, 5.0 g proteose peptone, 15.0 g monopotassium phosphate, 7.0 g disodium phosphate, 5.0 g sodium chloride, 0.5 g dextrose, 0.25 g magnesium sulphate, 0.10 g ferric ammonium citrate, 0.20 g sodium pyruvate], and tryptic soy broth [Becton Dickinson, Sparks, MD: 17.0 g tryptone, 3.0 g soytone, 2.5 g glucose, 5.0 g sodium chloride, 2.5 g dipotassium hydrogen phosphate]. Five selective enrichment broths were evaluated: TSB with 8 mg L^{-1} novobiocin (mTSB M), *Escherichia coli* Broth [Sigma-Aldrich: 20.0 g casein digest, 5.0 g lactose, 4.0 g dipotassium phosphate, 1.5 g sodium chloride, 1.5 g bile salts], EC with 5 mg L^{-1} novobiocin (mEC L), EC with 8 mg L^{-1} novobiocin (mEC M), and EC with 20 mg L^{-1} novobiocin (mEC H). These selective enrichment combinations were determined per enrichment methods for *E. coli* O157:H7 and non-O157

STEC defined by USDA-FSIS in their MLG, which allow for the use of either 8 or 20 mg L^{-1} novobiocin (Anonymous, 2011; Anonymous, 2012). A 5 mg L^{-1} addition of novobiocin was also evaluated to determine the point at which novobiocin begins to effect the target organisms, as well as, inclusion of EC broth with no additives to identify the base growth level for all seven serotypes. Novobiocin supplement was filter sterilized with 0.2 µm filters and added to the appropriate media after it had been autoclaved for 15 minutes at 121°C and allowed to cool to 50°C.

STEC serotypes O26:H11 (H30 Konowalchuk et al., 1977), O45:H2 (CDC 96-3285), O103:H2 (CDC 90-3128), O111:H- (JB1-95), O121:H19 (CDC 97-3068), O145:NM (83-75), and O157:H7 (ATCC 35150) were used to inoculate the listed media. Cultures were confirmed with API 20E biochemical kits (BioMerieux Inc., Hazelwood, MO) and latex agglutination (Wellcolex® O157; Remel Inc., Lenexa, KS and Prolex™ Non-O157 Identification Kit; Pro-Lab Diagnostics, Round Rock, TX) prior to use. Cultures were activated by streaking onto tryptic soy agar (Becton Dickinson Difco, Franklin Lakes, NJ) and incubated for 24 h at 37°C. Two colonies per serotype were picked from TSA plates and inoculated into 30 mL of pre-warmed (37°C) TSB in centrifuge tubes and incubated again for 24 h at 37°C. Tubes were centrifuged for 15 min at -4°C and 4960 x g and the supernatant decanted. Each bacterial pellet was reconstituted with 30 mL 0.1% peptone broth (PW; Difco) and re-centrifuged. Supernatants were then decanted and washed pellets were again reconstituted with 30 mL PW. Reconstituted STEC culture tubes were vortexed and serially diluted in PW to obtain a calculated target inoculum concentration of 100 cfu/mL. A 0.1 mL volume of this dilution was then added to 25 mL of each enrichment media (pre-warmed to 37°C) type to obtain a calculated test broth inoculation level of 10 cfu/mL (1 log cfu/mL). Inoculated enrichment broth tubes were then placed into an incubator at 37°C for the duration of the experiment. Aliquots (1 mL) of each enrichment tube were removed during enrichment at 4, 8, 12, 18, and 24 h and serial dilutions were plated on TSA in duplicate to enumerate population levels. Tryptic soy agar plates were then incubated at 37°C for 18-24 h prior to being removed and counted.

All combinations of media and serotype were replicated three times and the results averaged in the form of log colony forming units per milliliter (cfu/mL). Average counts were analyzed using the Proc Reg function of SAS with time set as a quadratic function to mimic logarithmic curves under ideal growth circumstances. Using $\alpha \leq 0.05$, each serotype and media

combination was evaluated on conformity to the ideal growth curve. Peak growth levels and time of occurrence were also recorded and compared with the generated growth curves to fully evaluate media efficacy.

Results

Although the calculated STEC serotype inoculation levels into test media were targeted to be 1 log cfu/mL (10 cfu/mL), the actual inoculation levels achieved ranged from 0.04-0.60 cfu/mL of test media. Growth curves of *E. coli* O157:H7 and the Big 6 STEC strains as pure cultures in BPW, UPB, TSB and mTSB M are shown in Figures 4.1-4.4. The growth profiles of all STEC cultures evaluated in three non-selective media and mTSB M were considered a good fit ($\alpha < 0.05$) as a quadratic function. This close fit indicates that the non-selective broths and mTSB M used for this experiment functioned well as enrichment media, in that STEC population amplification was effectively achieved. All seven STEC strains (representing seven serogroups) grew rapidly from approximately 0.05-0.2 cfu/mL (very low levels) to ≥8 log cfu/mL during the total 24 h of incubation at 37°C. By 4 h of incubation, all strains in BPW, UPB and TSB had increased to approximately 2 log cfu/mL, indicating a short lag period in these non-selective media. Logarithmic growth of all STEC strains was observed between 4 and 12 h of incubation. Serotypes O45 and O103 grew slightly more slowly in the three non-selective media, however, both serotypes achieved population densities of >7 log cfu/mL by 12 h of incubation.

All seven STEC serotypes had achieved their highest population densities by 12 h of incubation at 37°C in TSB (reaching 9-10 log cfu/mL levels). Slightly slower growth was seen in BPW and UPB, with serotypes O45, O157 and O103 requiring approximately 18 h to reach maximum population densities. These maximum cell densities were 1.5 and 1.0 log cfu/mL lower compared to TSB in BPW and UPB, respectively (Figs. 4.1-4.3).

When comparing growth of the seven STEC strains in TSB (Fig. 4.3) versus mTSB M (Fig. 4.4), the addition of 8 mg/L novobiocin to TSB negatively impacted both the growth rates and final population densities achieved over 24 h incubation at 37°C. All STEC strains were delayed in achieving maximum population density by approximately 6 hour (from 12 h in TSB reaching 9-10 log cfu/mL to 18 h in mTSB M reaching 7.5-9 log cfu/mL). Notable differences in the responses of the different STEC strains to 8 mg/L novobiocin were observed. *Escherichia coli* O157:H7 was the least affected by the antibiotic addition, reaching 9.7 log cfu/mL by 12 h

in TSB and 9.0 log cfu/mL by 18 h in mTSB M. Similarly, strain O26 grew well in mTSB M reaching an 8.5 log cfu/mL level by 24 h. The other five STEC strains (O45, O103, O111, O121 and O145) grew more slowly in mTSB M and reached maximum population densities of 7.7-7.9 log cfu/mL between 18-24 h of incubation. Even though all seven STEC strains were able to amplify in these four broth systems, serotypes O121, O103, O11 and O45 were the most restricted by 8 mg L^{-1} novobiocin in TSB, as determined by the slowest growth rates and/or lowest maximum population densities achieved during enrichment.

Figure 4-1: STEC-7 Growth in Enrichment Media Over 24 h (BPW)

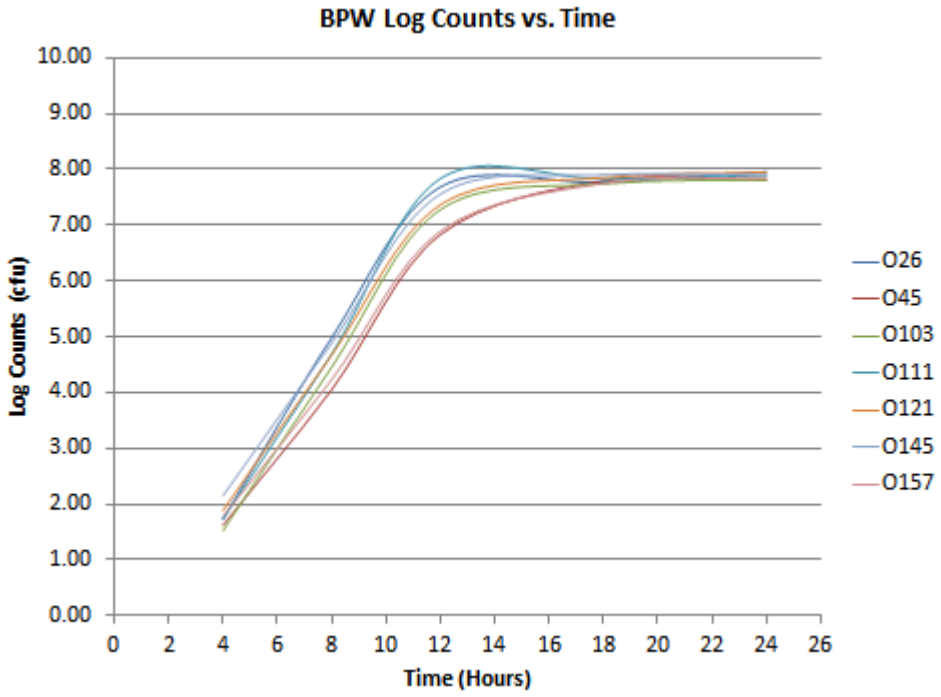


Figure 4-2: STEC-7 Growth in Enrichment Media Over 24 h (UPB)

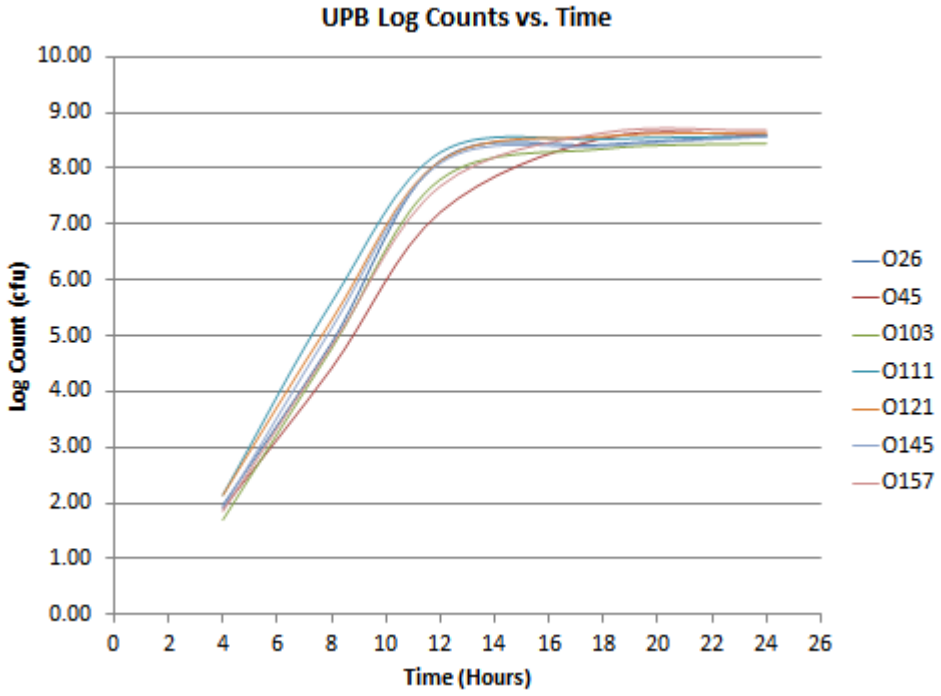


Figure 4-3: STEC-7 Growth in Enrichment Media Over 24 h (TSB)

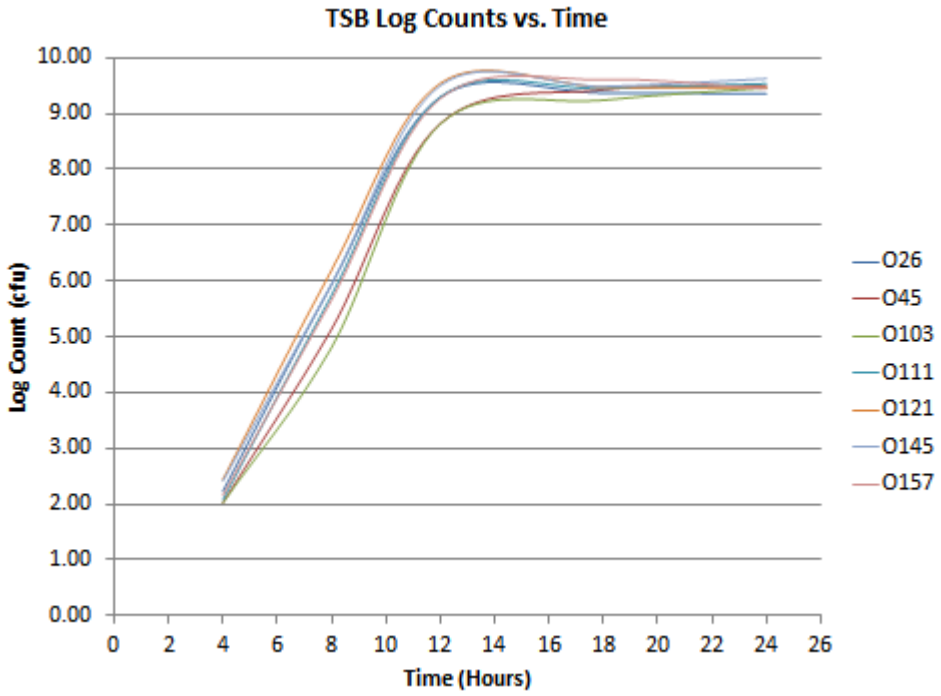


Figure 4-4: STEC-7 Growth in Enrichment Media Over 24 h (mTSB M)

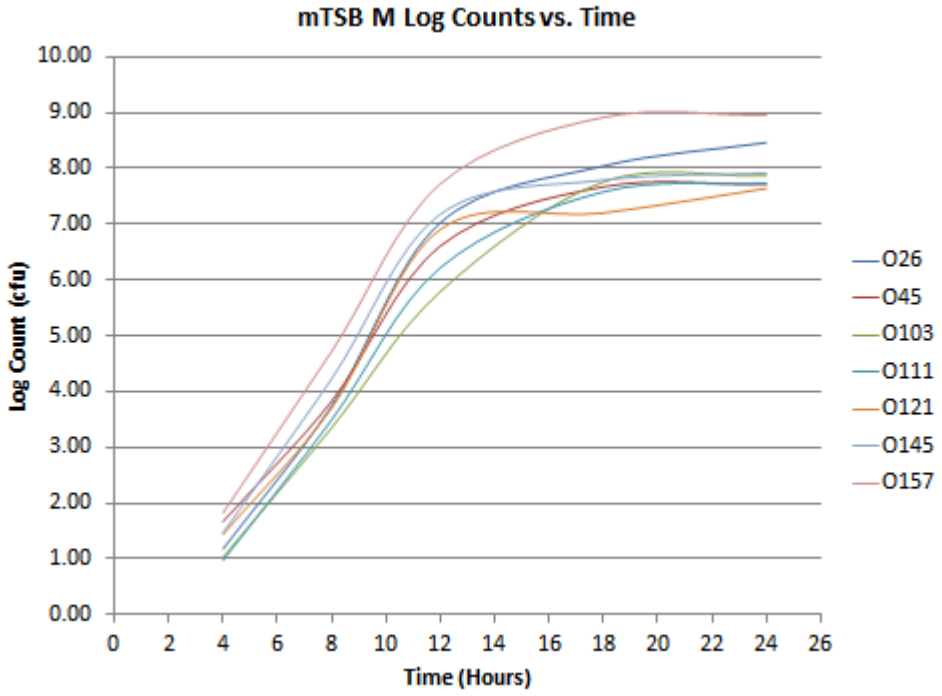
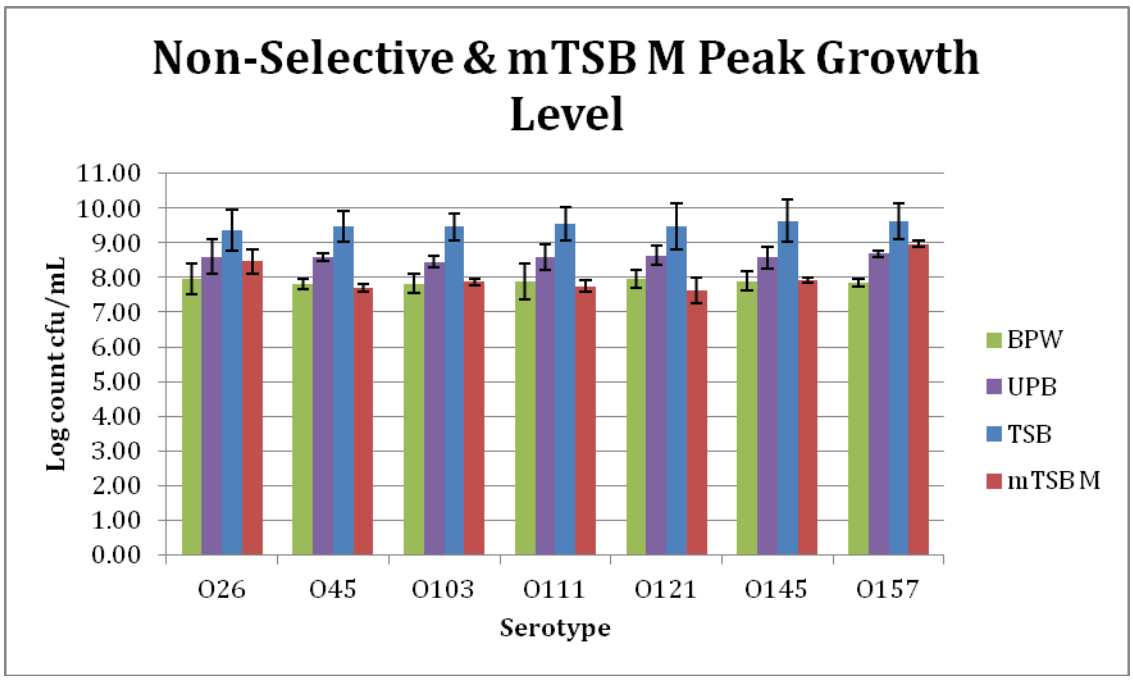


Figure 4.5 summarizes the comparison of maximum growth densities achieved by the seven STEC strains (representing seven serotypes) across BPW, UPB, TSB and mTSB M enrichment media. The highly nutritive TSB provided the greatest level of growth for all strains, with densities reaching >9 log cfu/mL. UPB also consistently supported growth of the seven STEC strains, with population densities reaching approximately 8.5 log cfu/mL. The third non-selective medium, BPW, provided slightly less support for population amplification at 37°C but levels of all seven STEC strains approached maximum densities of 8.0 log cfu/mL. Even though the addition of 8 mg L^{-1} of novobiocin to TSB somewhat restricted the growth of all seven strains compared to their growth in TSB alone, population amplification still occurred over the 24 h of incubation to ultimately achieve >7.5 log cfu/mL levels. Growth to maximum population density in mTSB M was similar to growth in the non-selective BPW (a common STEC enrichment medium) reaching slightly greater than 7.5 log cfu/mL, except for serotypes O26 and O157, which showed greater growth in mTSB M compared to BPW. Error bars indicate the standard deviation associated with the growth of each serotype in that media.

Figure 4-5: Non-Selective Media & mTSB M Peak Cell Population Levels



The growth characteristics of the seven STEC strains were compared in EC media supplemented with different levels of novobiocin (Figs. 4.6-4.9). *Escherichia coli* broth in this study, a medium designed to inhibit Gram positive organisms and support growth of enteric coliforms, provided for variable STEC growth across serotypes at 37°C.

EC broth with no novobiocin showed a good quadratic fit for growth of all serotypes ($\alpha \leq 0.05$), with the exception of O26 (no growth recovered at 4 h) and O103 (Fig. 4.6). Despite a good fit for the model, the peak growth levels were delayed until the 18 h incubation mark. For the six STEC strains that demonstrated an ability to grow in EC broth, strains O157, O45, O11 and O145 reached a maximum population density of approximately 9.5 log cfu/mL, while strains O103 and O121 only reached densities of 8.0 log cfu/mL. It was also noted that longer lag periods (2-3 h) were observed for all strains in EC (base broth with no antibiotic supplementation) versus TSB and mTSB M. This lag period was extended to approximately 8 h in EC broth for strain O103. Beginning with very low inoculation levels (0.05-0.2 cfu/mL), STEC populations required 4-5 h at 37°C to reach counts of 1 log cfu/mL, with O103 requiring >8 h and O26 not growing at all over the 24 h period.

With 5 mg L^{-1} novobiocin addition (mEC L; Fig. 4.7), a good quadratic fit was only achieved with serotypes O45, O145 and O157, which showed maximum population densities being reached at 18 h, but at approximately 1 log cycle lower than in non-supplemented EC broth (9.5 versus 8.5 log cfu/mL). The maximum population densities reached by O103 and O121 in mEC L were approximately 0.7 log cycles lower than in EC broth. In addition to O26, which did not grow in EC broth, strain O111 was not recovered in mEC L by 4 h.

STEC growth became more notably restricted in the mEC M broth system (8 mg L^{-1} of novobiocin) (Fig. 4.8). Serotypes O45 and O157 exhibited a good quadratic model fit; however, strains O26, O103, O121, and O145 were completely inhibited by 8 mg L^{-1} novobiocin supplementation. In addition to *E. coli* O157, serotype O45 grew slowly in mEC M, with its lag period extended to 12 h and a population density of 5.5 log cfu/mL attained after 24 h of 37°C incubation. It is important to note relative to Fig. 4.8 that strain O111 was recovered from mEC M broth, but this only occurred during one of the three experimental replications. In this one replication, the lag phase was 12 h and it grew to approximately 4 log cfu/mL over 24 h. Addition of 20 mg L^{-1} (mEC H; Fig. 4.9), novobiocin was completely inhibitory for all STEC serotypes, with the exception of O157, which still exhibited good model fit ($\alpha \leq 0.05$). Growth of *E. coli* O157:H7 in EC broth did not seem to be impacted at any novobiocin level, and maximum population density for *E. coli* O157:H7 was approximately 8.5 log cfu/mL.

Figure 4-6: STEC-7 Growth in Enrichment Media Over 24 h (EC)

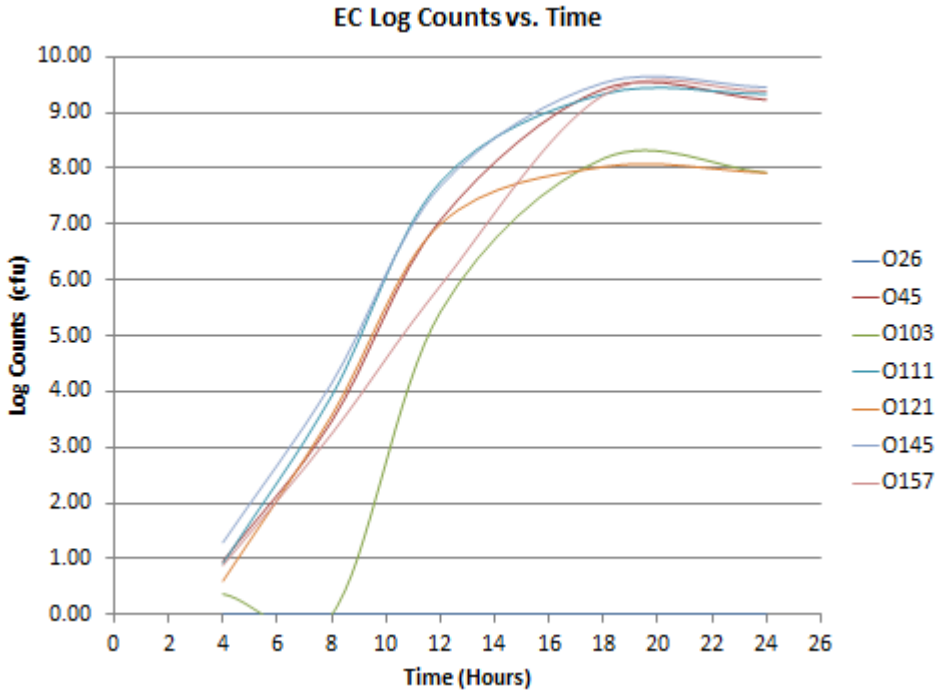


Figure 4-7: STEC-7 Growth in Enrichment Media Over 24 h (mEC L)

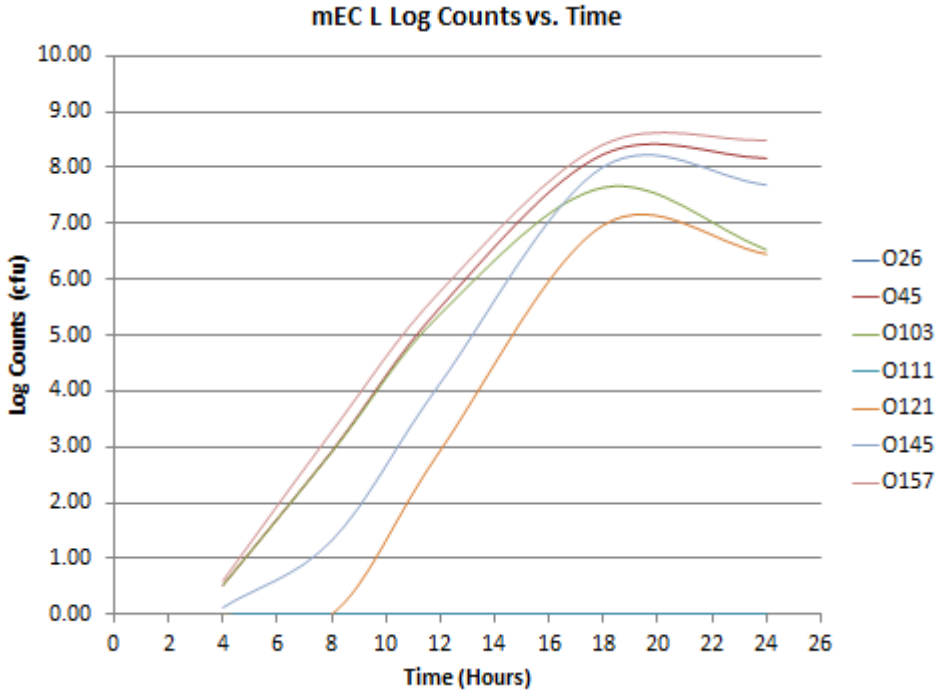


Figure 4-8: STEC-7 Growth in Enrichment Media Over 24 h (mEC M)

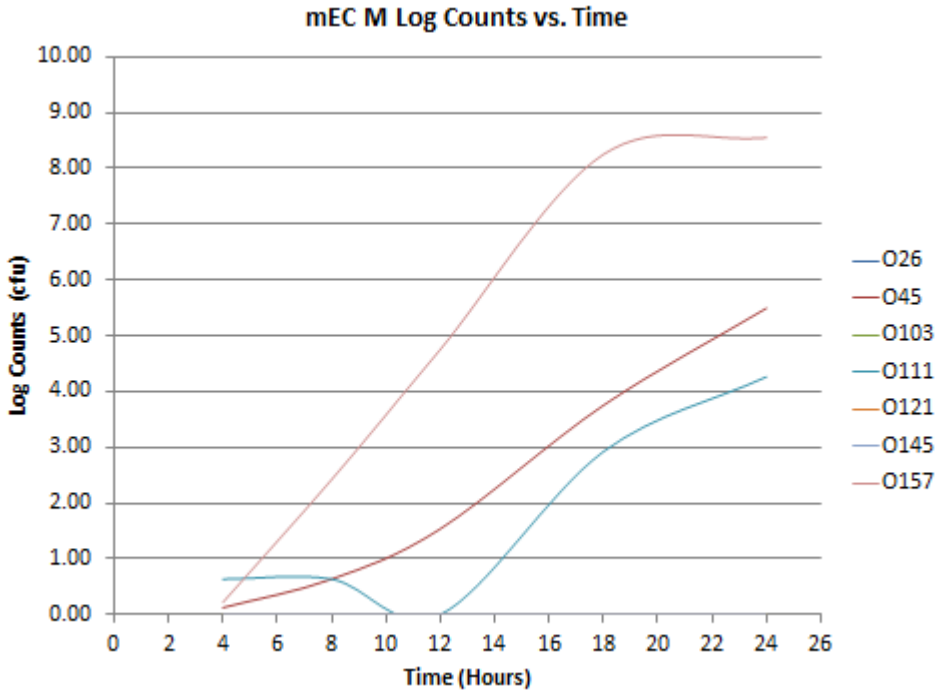


Figure 4-9: STEC-7 Growth in Enrichment Media Over 24 h (mEC H)

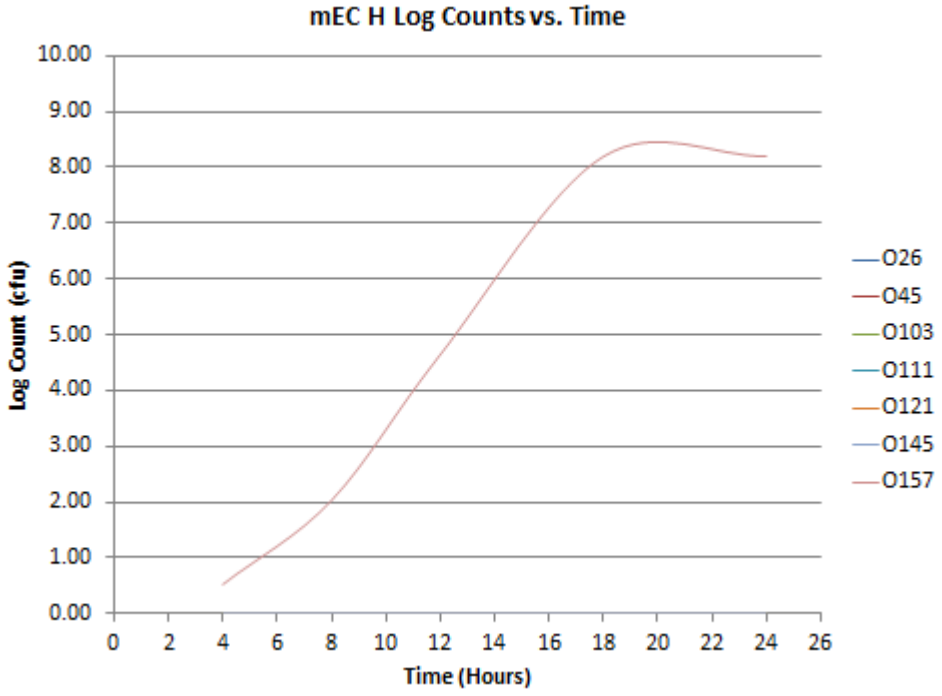
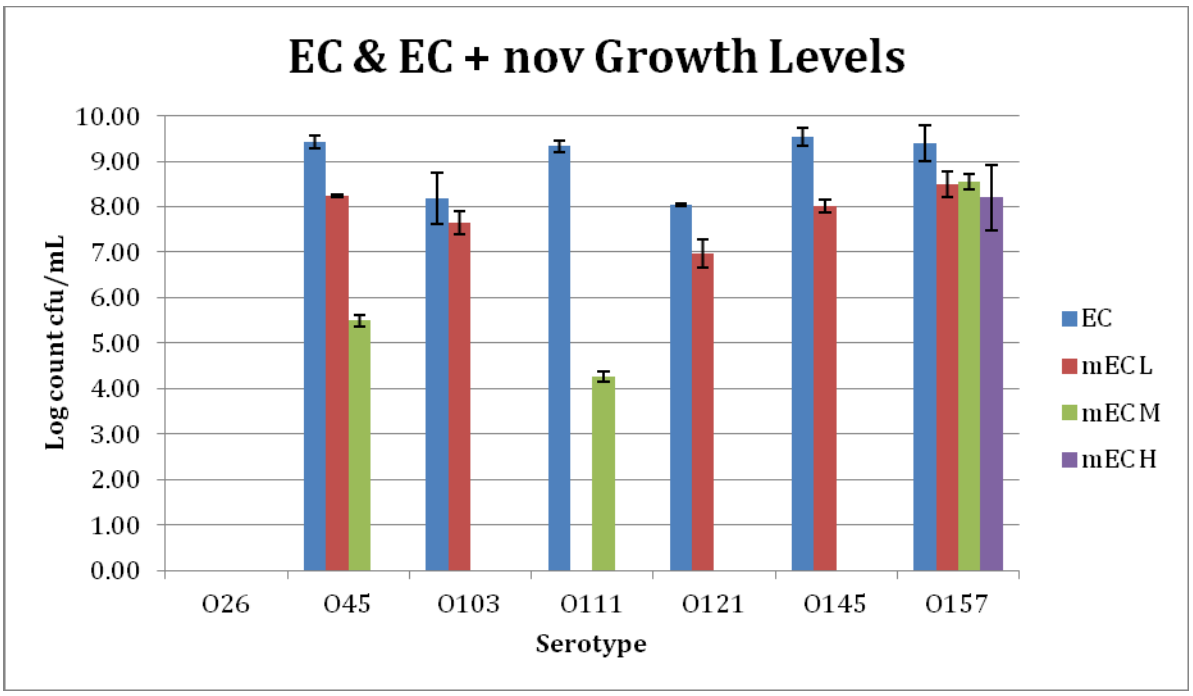


Figure 4.10 summarizes maximum population densities reached by the seven STEC strains during growth in EC broth supplemented with novobiocin at varying levels (0, 5, 8 and 20 mg L^{-1}) when incubated at 37°C for 24 h, beginning with a low inoculation level of 0.05 to 0.20 cfu/mL. Strain O26 was not detected at any time post-inoculation in any of the four broth systems. All other strains reached >8 log cfu/mL in unsupplemented EC broth. STEC non-O157 serotypes O45, O103, O121 and O145 grew well in the mEC L (5 mg L^{-1} novobiocin) reaching at least 7 log cfu/mL. In mEC M (8 mg L^{-1} novobiocin), the only non-O157 STEC serotypes that grew were O45 and O111 (which grew in only one of three replications), and these two strains only achieved a final population density in 24 h of 5.5 and 4.2 log cfu/mL, respectively. *E. coli* O157 was the only serotype that grew well in EC broth supplemented with novobiocin at all three levels, reaching greater than 8 log cfu/mL in mEC H (20 mg L^{-1} novobiocin). Error bars indicate the standard deviation in serotype growth across three replications.

Figure 4-10: *Escherichia coli* Broth & modified EC Broth



Discussion

The variance in STEC growth between BPW and TSB is similar to that seen by Rocha and Piazza (2007) during which two strains of STEC (one with *stx1*, one with *stx2* genes) were compared in different media types for growth characteristics and Shiga toxin production. While it has been suggested that UPB is a superior enrichment at 42°C for *E. coli* (Kanki et al., 2009; Kanki et al., 2011), when compared with non-selective media types at 37°C, EC broth performed at a comparable level in terms of reaching maximum population density. The exception was serotype O26 used in this experiment, which showed no growth in any EC broth based medium (with or without novobiocin supplementation). Subsequent work with serotype O26 in our laboratory showed that growth in EC broth occurred when higher initial inoculation levels (compared to this study's inoculation levels of 0.05 to 0.20 cfu/mL). Overall growth levels correlate with previous research that indicates addition of novobiocin supplements can have an adverse effect on the growth of non-O157 STEC (Vimont et al., 2006). Such inhibition of non-O157 STEC by novobiocin was also seen by Catarame et al. (2003) for serotypes O26 and O111 when enriching minced beef at a level of 40 mg L^{-1}, although this level is double the amount recommended for enrichment by the USDA. This experiment indicated, however, that novobiocin alone was not responsible for decreased cell growth rates and density levels attained, as high levels of STEC growth of all strains evaluated occurred when novobiocin supplement was added to TSB at 8 mg L^{-1}. The presence of bile salts in the EC broth also appears to have contributed to the inhibitory effect of the novobiocin supplement. However, it is the presence of these bile salts that is desirable in EC broth, as they work to suppress Gram positive organisms and other background flora in enrichment samples.

Based on these results generated by evaluating pure cultures, use of modified tryptic soy broth (mTSB) supplemented with 8 mg L^{-1} novobiocin would appear to offer the best opportunity for detection of very low levels of non-O157 STEC, while providing some level of selectivity against background microflora that can overgrow the target STEC. Further research should confirm this suggestion using actual STEC-spiked field samples. Additionally, a level of enhanced enrichment selectivity should be evaluated by applying a higher (i.e. 42°C) enrichment temperature. Finally, due to the likely stressed or sublethally injured STEC cells that would likely comprise field samples, it is possible that using one of the three non-selective enrichment

broths with a short pre-enrichment resuscitation period (i.e. 2 h) at a lower incubation temperature (room temperature or 37 °C), followed by enrichment in mTSB M at 42 °C would provide optimal detection capability. These scenarios should be investigated in conjunction with the effect of transport medium use, as discussed in Chapter 5.

Chapter 5 - **Evaluation of Three Transport Media for Maintaining Shiga Toxin-Producing *Escherichia coli* Populations During Simulated Sample Transport**

Abstract

Food and environmental samples must often be shipped from the field (i.e. processing facility) to laboratories for analysis to determine qualitative presence of target pathogens. Due to the typically low concentrations of pathogens in food matrices, and accounting for various physiological stresses and sublethal injury that may have occurred, conditions during this shipping and pre-analysis sample storage can impact pathogen detection outcomes. Additionally, when quantitative determinations of target microbial populations are desired, maintaining microbial levels similar to those at the time of sampling is important. Transport media are designed to maintain viability of target microbial cells in samples during chilled shipment and short laboratory storage periods. A comparison of three transport media was conducted using Shiga toxin-producing *Escherichia coli* (STEC) strains O26, O45, O103, O111, O121, O145, and O157:H7 at 4 and 10°C storage temperatures. Transport media evaluated were buffered peptone water (BPW), maximum recovery diluent (MRD), and Cary-Blair (CB) transport medium. Each media was individually inoculated with the seven serotypes and stored at either 4 or 10°C. Sets were sampled at 0, 24, 48, and 72 h and enumerated for comparison. CB medium showed the best ability to maintain cell counts at original inoculation levels for both temperatures. BPW showed slight STEC population loss at 4°C and growth at 10°C. MRD allowed cell growth throughout the sampling period at both temperatures. It is advised that Cary-Blair Transport Medium be utilized when sample shipment times are involved and/or when temperature fluctuations might occur prior to sample analysis.

Introduction

With the recent addition of six new strains of Shiga (vero) toxin-producing *Escherichia coli* (STEC) to the list of adulterants in ground beef, it can be expected that there will be greater

need for sample analysis to determine the safety of the food supply. Generally accepted methods allow for the refrigeration or freezing of samples collected in the field to ensure accuracy of analysis. However, samples collected in the field or processing facility are often shipped overnight to an off-site laboratory for analysis, and upon receipt at the laboratory, samples may be held for up to a day before being analyzed. Ensuring proper chilled shipment and storage temperature (4-5°C) of the samples is of utmost importance; although, it is a frequent observation that fluctuations in sample temperature can occur when samples are shipped via commercial carriers. Scenarios whereby sample shipments become delayed, or are shipped during periods of warm weather, an increase in sample temperatures may result.

The type of media used to contain samples during transport is vital to maintaining microbial viability. Stuart (1946) developed an early transport media for use with samples potentially containing *Neisseria gonorrhoeae*, which was made using sodium glycerophosphate as a buffering agent that also served to reduce the oxidation effects of the media. One issue associated with this medium was that coliform presence interfered with *N. gonorrhoeae* isolation. To combat this it was found that addition of aerosporin (polymyxin B sulfate) inhibited the growth of coliforms and while allowing survival of the target organism (Crookes and Stuart, 1959). Despite this, overgrowth of fecal coliforms still occurred when using Stuart transport medium. Cary and Blair (1964) developed a new transport medium containing sodium thioglycolate (to inhibit oxidation), calcium chloride (to reduce available moisture), sodium chloride, disodium phosphate to bind moisture and raise pH, and agar (Becton Dickinson Difco, Franklin Lakes, NJ). All ingredients aside from calcium chloride were mixed and heated until clear. After cooling to 50^{o}C, calcium chloride was added and the pH of the media was raised to 8.4. The high pH further serves to reduce microbial activity and cellular processes. This new Cary-Blair medium proved to be effective for stable transport of *Shigella*, *Salmonella* and *Escherichia coli* and has since been adopted by the CDC as a designated transport medium for fecal samples (Cary and Blair, 1964; Centers for Disease Control and Prevention, 1990).

Maximum recovery diluent may be used in place of 0.1% peptone buffer for dilution of samples and has proven effective for resuscitation of injured cells (Jordan et al., 1999). This property makes it a logical candidate for use as a transport medium as it may increase recovery of stressed or injured cells.

The current USDA Meat Animal Research Center (Clay Center, NE) carcass sampling protocol calls for sponges to be hydrated with buffered peptone water prior to use, then refrigerated and shipped for analysis, with the sample to be analyzed within 24 h. Buffered peptone water is known for its function as a pre-enrichment medium that can reduce organism stress. Despite any precautions taken, there exists a possibility of temperature abuse when shipping samples for analysis. Therefore, another desirable characteristic of a media used during sample transport is the ability to maintain static microbial population levels for short to moderate time periods when slight temperature rises occur. In most cases, this is accomplished by using a medium that contains minimal nutritive components necessary for cell growth. This study was conducted to establish suitability of various media for transport of samples that may contain STEC using simulated good and moderately abusive shipment temperatures.

Materials & Methods

Three types of media were selected for evaluation for their ability to maintain STEC-inoculated samples in a state of stasis; Cary Blair transport media (Oxoid CB: 1.1 g disodium hydrogen phosphate, 1.5 g sodium thioglycollate, 5.0 g sodium chloride, 0.09 g calcium chloride, 5.6 g agar), maximum recovery diluent (Difco MRD: 1.0 g peptone, 8.5 g sodium chloride), and buffered peptone water (Oxoid BPW: 10.0 g peptone, 5.0 g sodium chloride, 3.5 g disodium phosphate, 1.5 g potassium dihydrogen phosphate). Two temperatures were selected to simulate both a good (4°C) and moderately abusive (10°C) temperature for shipment of samples.

STEC strains O26:H11 (H30; Konowalchuk et al., 1977), O45:H2 (CDC 96-3285), O103:H2 (CDC 97-3068), O111:H- (JBI-95), O121:H19 (CDC 97-3068), O145:NM (83-75), and O157:H7 (ATCC 35150) were confirmed prior to use by latex agglutination (Wellcolex® O157; Remel Inc., Lenexa, KS and Prolex™ Non-O157 Identification Kit; Pro-Lab Diagnostics, Round Rock, TX) and BioMerieux (Durham, NC) aerobic profile index 20E strips.

Confirmed cultures were plated on tryptic soy agar (TSA; Becton Dickinson, Franklin Lakes, NJ) and incubated at 37°C for 24 h. One colony was then selected for each serotype and used to inoculate centrifuge tubes containing 30 mL tryptic soy broth. Inoculated tubes were incubated for 24 h at 37°C. After 24 h, the inoculated tubes were centrifuged at -4°C and 4960 x g for 15 minutes. The TSB was decanted and the pellet reconstituted with 30 mL 0.1% peptone, vortexed, and centrifuged a second time under the same conditions. After the second

centrifugation, the 0.1% peptone was decanted and the remaining pellet was again reconstituted with 0.1% peptone and vortexed. The bacterial inoculum solution was serially diluted to 10^7 cfu/mL, and 10 µL was inoculated into 10 mL of each media/temperature combination contained in 30 mL screw-cap tubes to target an initial concentration of approximately 10^4 cfu/mL of each STEC strain. Test tubes containing the inoculated media were placed in refrigerators at either 4 or 10°C. From these tubes, 1 mL aliquots were removed at the 0, 12, 24, 48, and 72 h marks. Aliquots were plated onto a modified form of the chromogenic media described by Posse et al. (2008) (40 g Difco MacConkey agar base, 6 g sucrose, 6 g sorbose, 3.5 g bile salts #3, 0.05 g X-gal, 0.05 g IPTG, 5 mg novobiocin, 0.5 mg potassium tellurite; prepared as described by Posse et al. 2008) and incubated at 37°C for 18-24 h prior to enumeration.

All STEC strain/media/storage temperature combinations were replicated 3 times and microbial counts were analyzed using the Proc Mixed function in SAS. Plates were counted and average counts were log transformed prior to analysis. Sampling times were analyzed in comparison to the media/serotype/temperature interaction. The average logarithmic variations from inoculation levels are shown based on the different levels of bacteria recovered at the given sampling time.

Results

Figures 5.1-5.6 show the population levels of each of the seven STEC strains over a 72 h storage period at either 4 or 10°C temperatures. Values were analyzed as a quadratic function to determine how stable bacterial levels remained throughout the duration of the experiment. At $P < 0.05$, values that were lower than this indicated that the levels of bacteria recovered were a statistical fit for a quadratic model. Therefore a higher P value is indicative of a linear growth curve, i.e. no change. This goodness of fit served to identify whether a given media could maintain cellular stasis under the given conditions.

Using BPW as the transport medium (Figs. 5.1 and 5.2), only serotype O26 showed significant change at 4^oC. At 10^oC, however, all serotypes except O111 experienced significant change in population levels across sampling times. This indicates that under good chilled temperature BPW adequately maintains STEC population levels (<1 log cfu/mL loss of counts). If sample temperatures increase to 10^oC, BPW allowed STEC counts to increase by up to 2 log

cycles in 72 h. In most cases laboratories will analyze samples within 24 h of collection. In this case, BPW counts at 10°C were similar to T0 counts and would be appropriate. Additionally, if qualitative (+/-) pathogen detection is the goal, as opposed to quantitative enumeration, a slight increase in STEC level would not be problematic.

Figure 5-1: Cell Levels in Buffered Peptone Water at 4°C

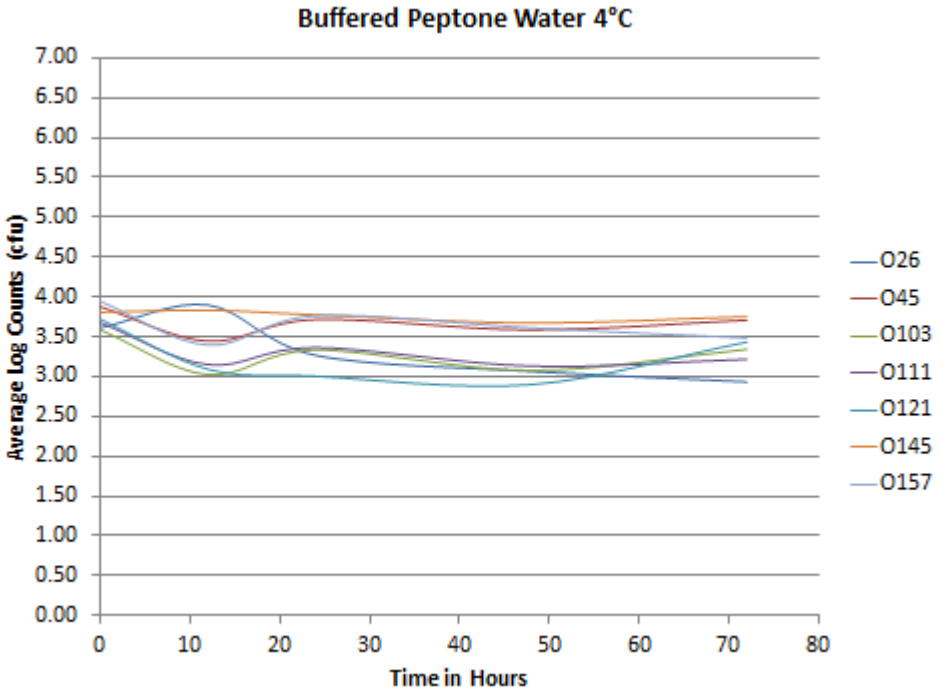
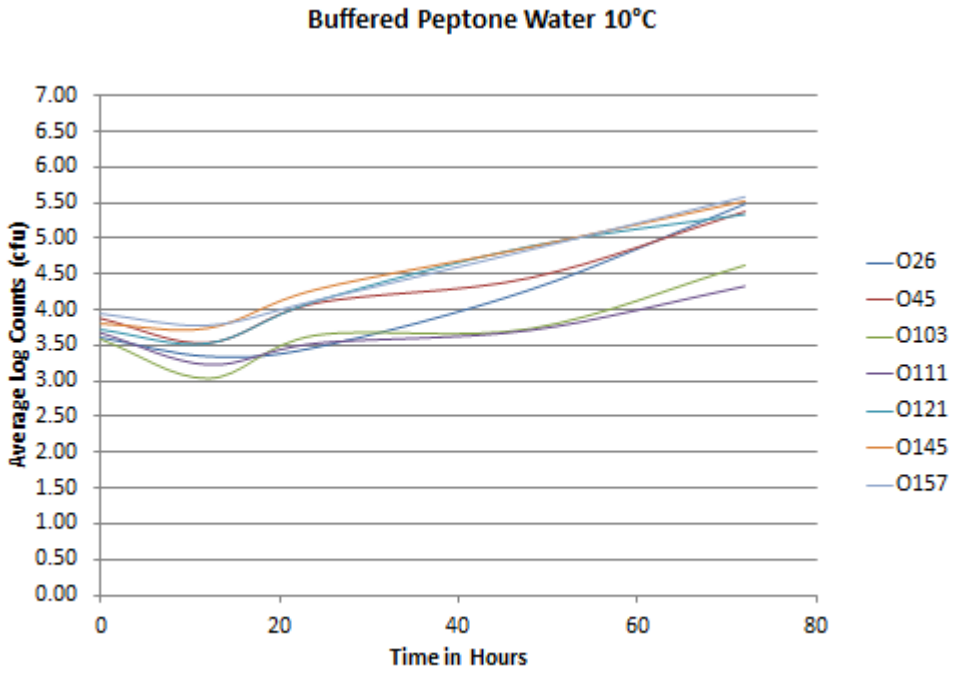


Figure 5-2: Cell Levels in Buffered Peptone Water at 10°C



STEC population profiles for storage in MRD are provided in Figures 5.3 and 5.4. Interestingly, counts recovered at T0 from the inoculated medium showed a substantial degree of variation across STEC strains compared to T0 counts observed in BPW and CB transport media. In MRD at 4°C, populations of serotypes O26, O121, and O145 experienced significant change over the 72 h storage; however, under the simulated abuse temperature of 10°C, all seven serotypes experienced a significant change (growth by 2-3 log cycles) in bacterial levels recovered over the course of the experiment. This inability to prevent significant change within the samples in the event of mishandling lessens the desirability of MRD as a transport medium. At 4°C, STEC O103 and O111 seemed to not thrive well, as indicated by a steady decline in numbers over the first 48 h (in the case of O103) or a reduced T0 recovery followed by slow recovery over 72 h (in the case of O111).

Figure 5-3: Cell Levels in Maximum Recovery Diluent at 4°C

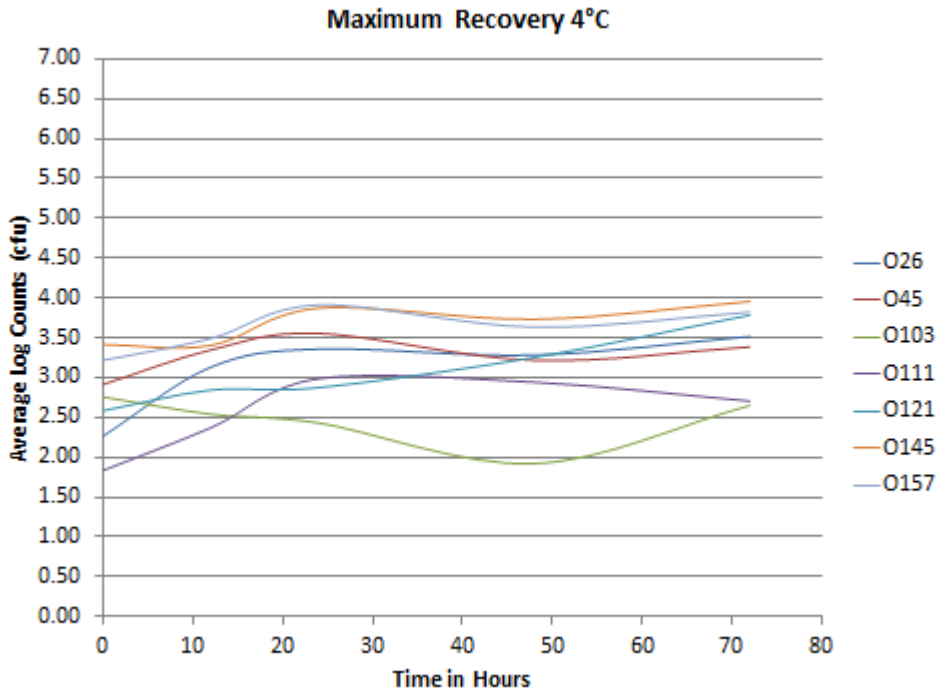
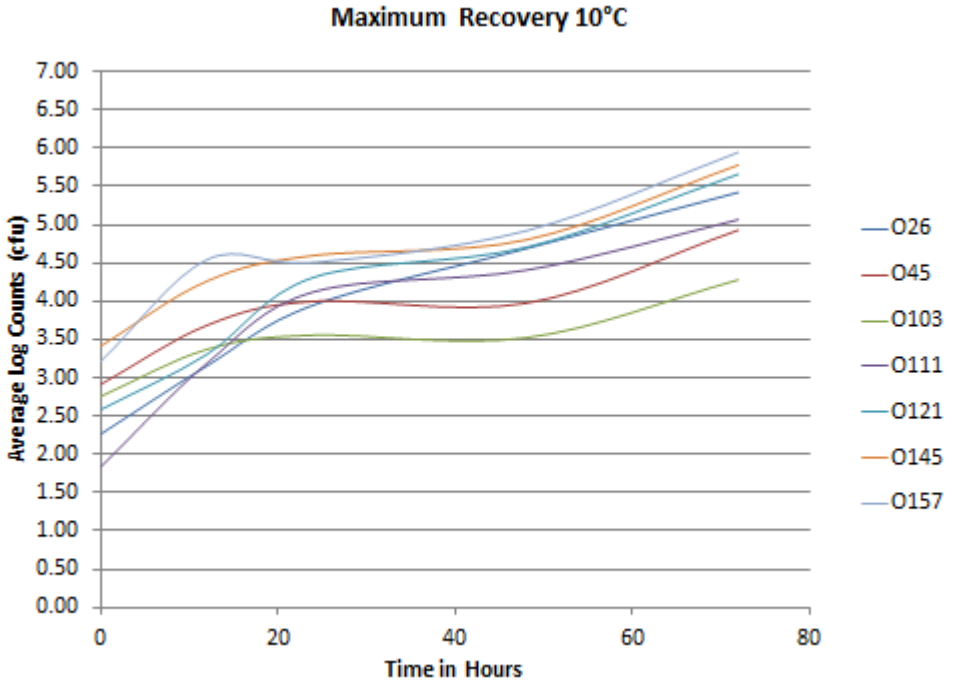


Figure 5-4: Cell Levels in Maximum Recovery Diluent at 10°C



STEC population profiles for CB medium are shown in Figs. 5.5 and 5.6. Statistically, Cary-Blair transport medium showed significant ($P \leq 0.05$) change at 4°C for O45, O103, and O157; however, at 10°C only a change in population of STEC O26 was deemed significant. Significance applies across the entire sampling period and is based on the calculated standard error from three replications. It must be noted, however, that this statistically significant change detected was primarily due to very small standard errors for each organism at each sampling time. CB provided the most stable population levels for all STEC strains at both storage temperatures over the 72 h time period. At both 4°C and 10°C, STEC population levels varied across the 72 h of storage by <0.5 log cycles. This resistance to change in STEC population levels is a desirable characteristic where there exists a higher likelihood of either temperature abuse, or prolonged shipping time.

Figure 5-5: Cell Levels in Cary-Blair Transport Medium at 4°C

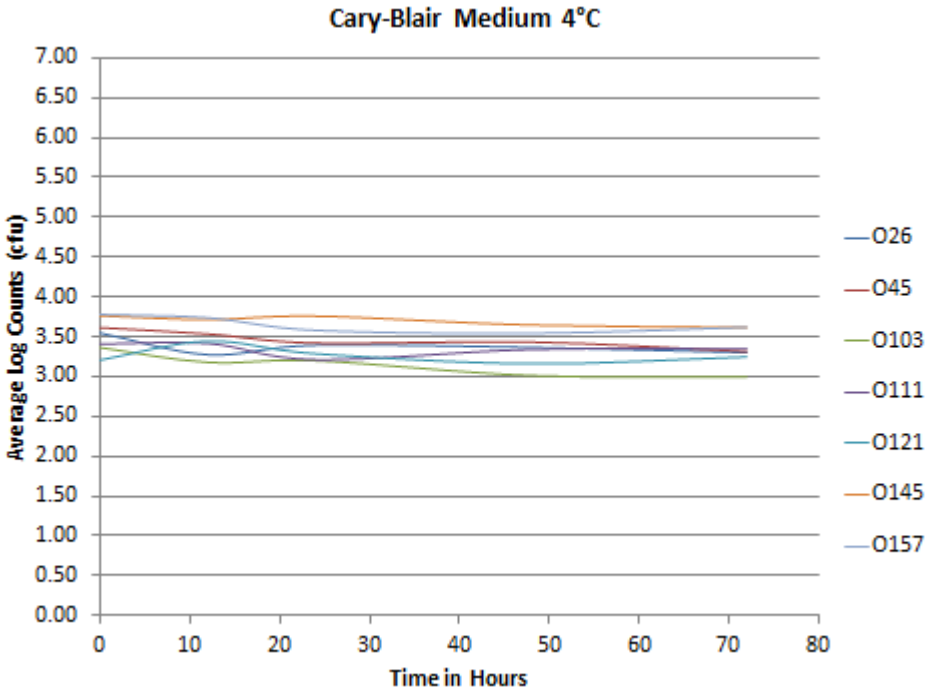
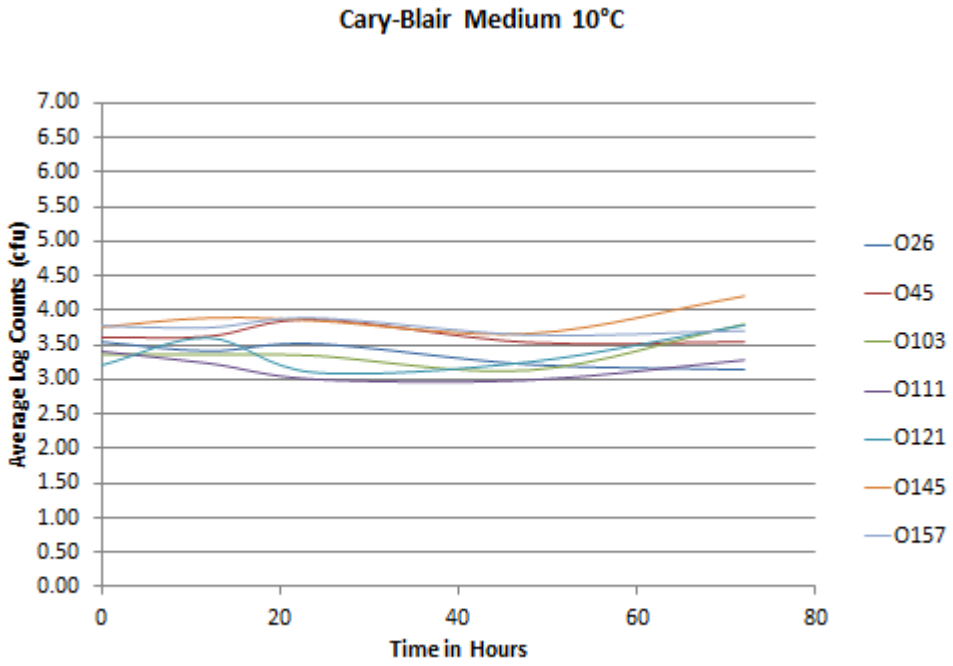


Figure 5-6: Cell Levels in Cary-Blair Transport Medium at 10°C



Figures 5.7 through 5.14 depict the levels of bacterial change that were observed at each time and temperature as they relate to the organism levels at time 0. Error bars were compiled from the SAS output based on growth variation specific to organism-temperature interaction within a given media. Overall trends show that organisms at 10°C displayed higher amounts of positive change relative to the starting microbial concentration over the duration of the sampling period. Meanwhile at 4°C, there appeared to be an overall reduction amongst bacterial levels with the exception of cultures in MRD, which exhibited positive growth over the entire sampling period with the exception of serotype O111.

Figure 5-7: Bacterial Level of Change at 12hr 4°C

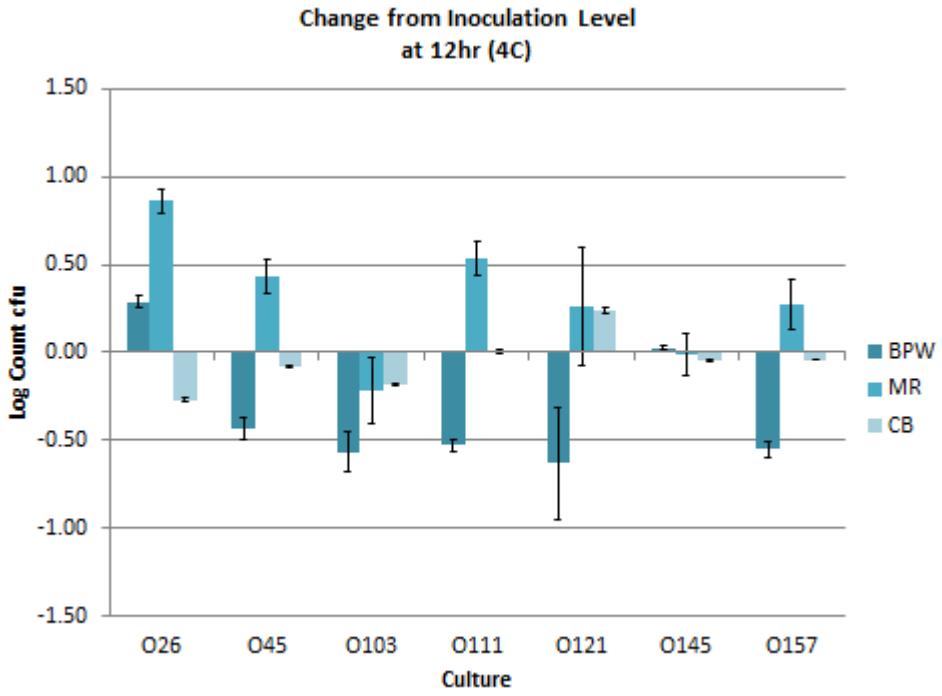


Figure 5-8: Bacterial Level of Change at 12hr 10°C

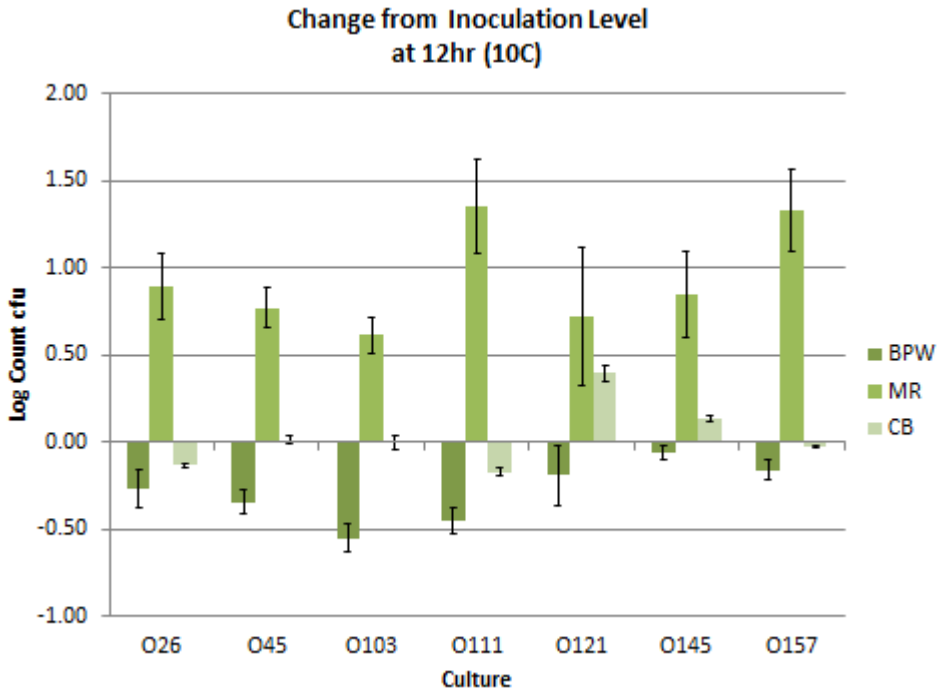


Figure 5-9: Bacterial Level of Change at 24hr 4°C

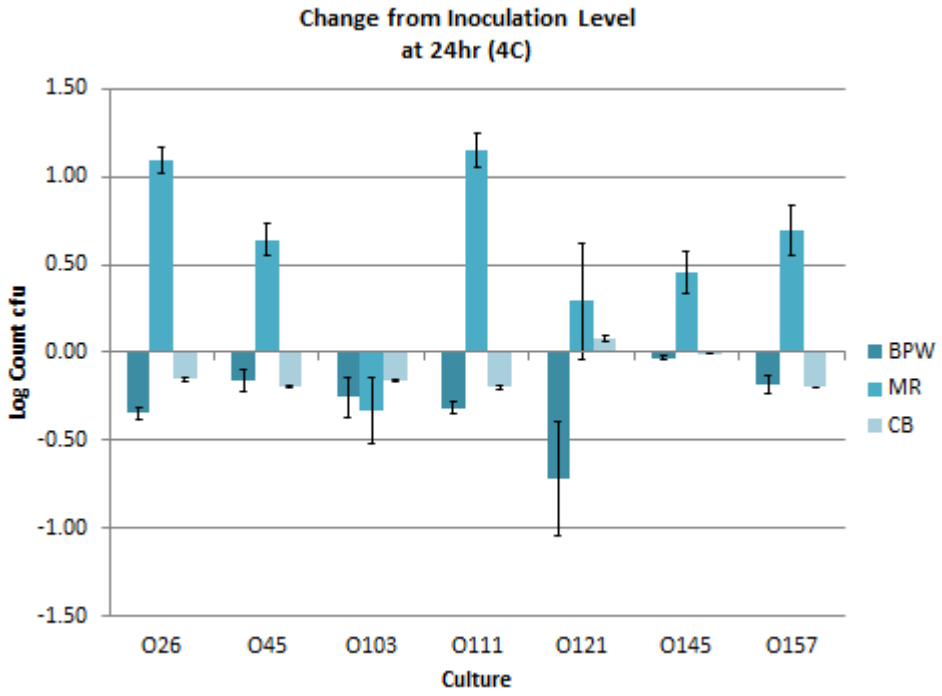


Figure 5-10: Bacterial Level of Change at 24hr 10°C

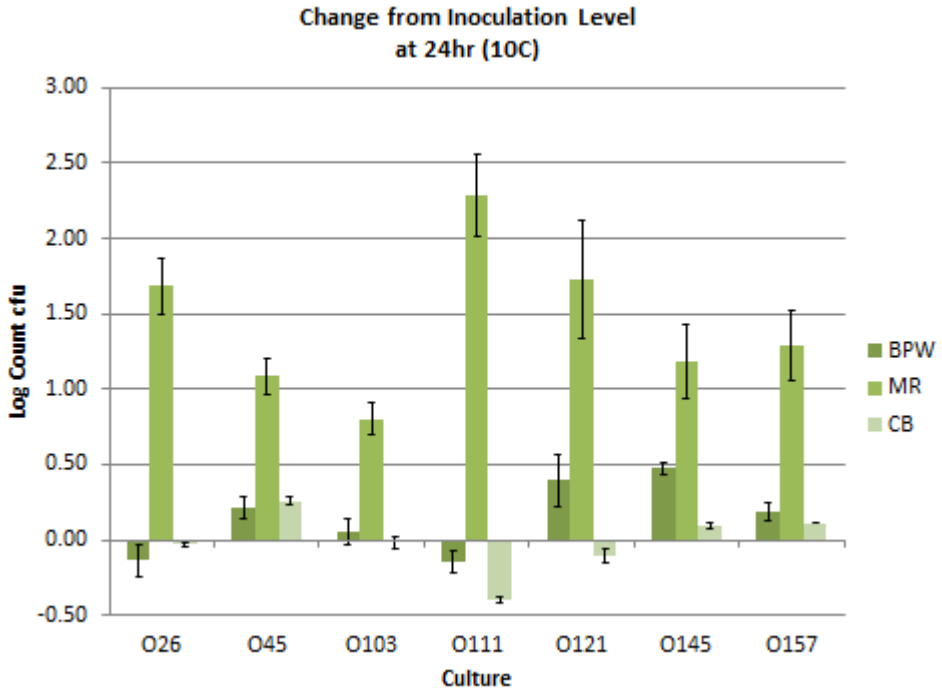


Figure 5-11: Bacterial Level of Change at 48hr 4°C

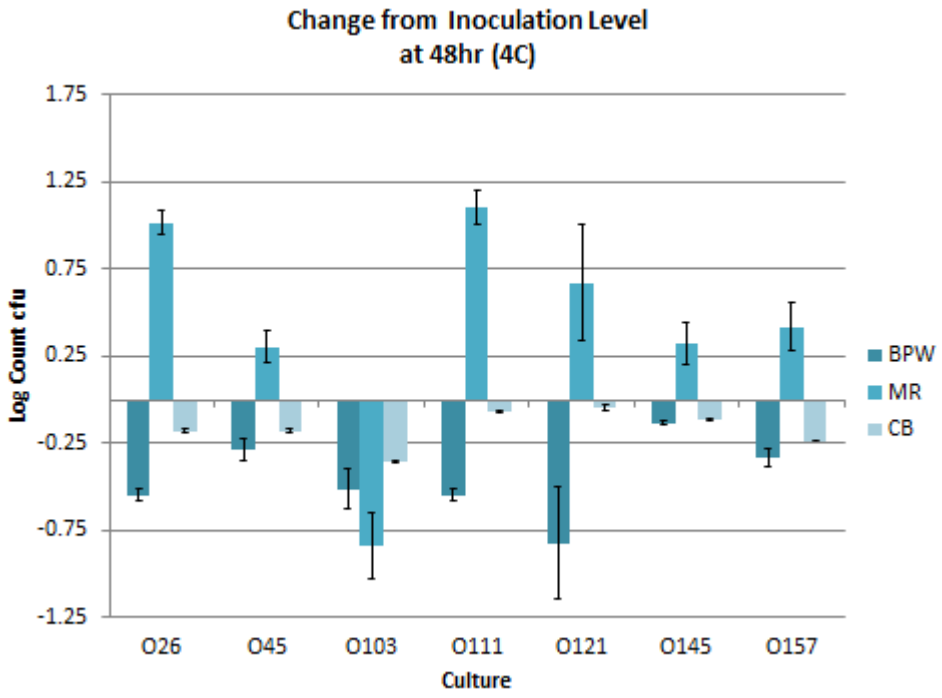


Figure 5-12: Bacterial Level of Change at 48hr 10°C

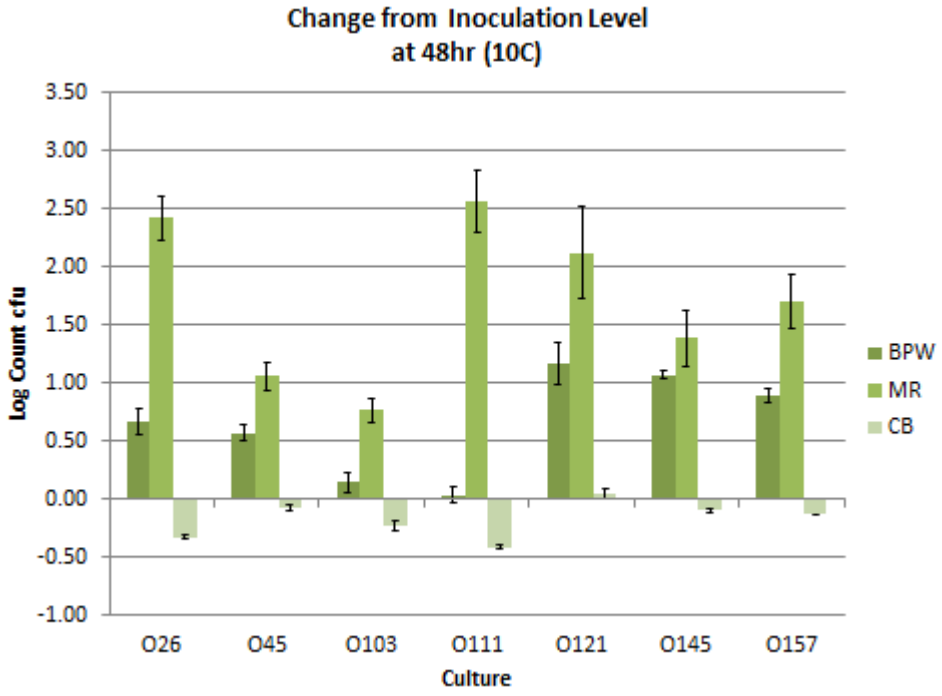


Figure 5-13: Bacterial Level of Change at 72hr 4°C

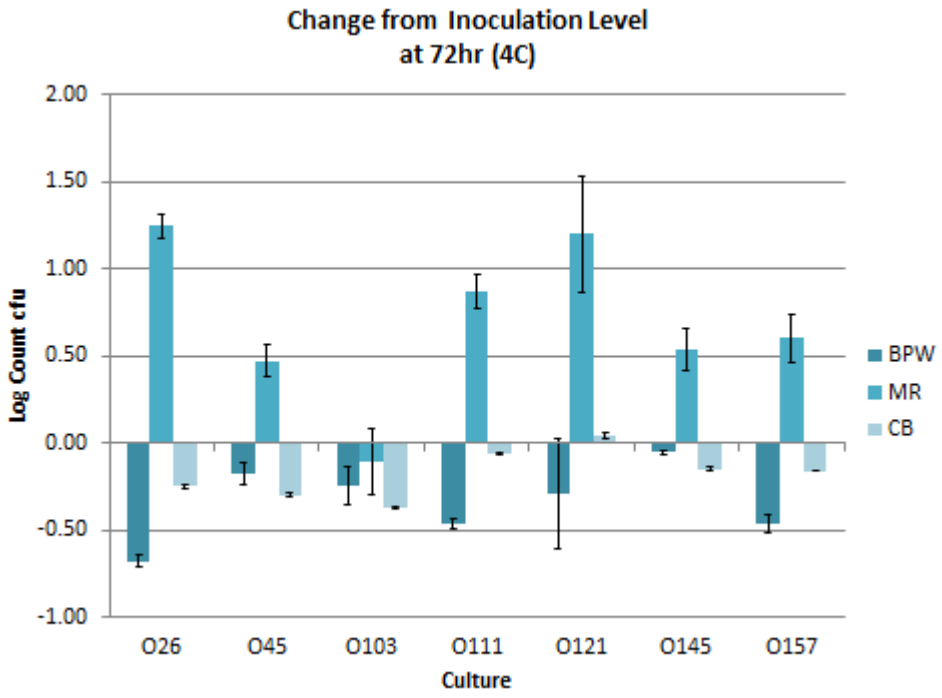
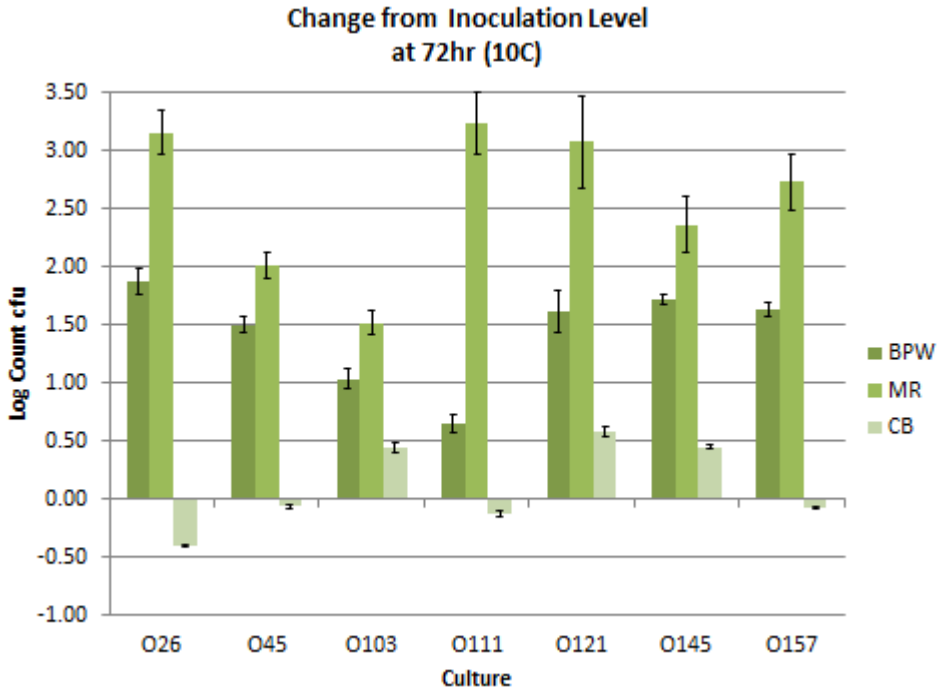


Figure 5-14: Bacterial Level of Change at 72hr 10°C



Discussion

Transport media have two critical roles to play in sample analysis for pathogens; they must protect the viability of the target pathogen even if it is stressed or injured, and they should maintain the level of the target microorganism at near the level of sampling while preventing natural background microflora overgrowth in the medium. If a transported sample will be qualitatively analyzed for a target pathogen, increases in that pathogen's numbers during transport is not an issue, unless concurrent increases in background microflora inhibit or mask the target pathogen's detection.

During sample shipping, delivery and pre-analysis storage, slight to moderate fluctuations in temperature frequently occur. It is important that the transport medium be able to maintain stable microbial population numbers, particularly if a quantitative type of analysis is to be conducted. In this study, consideration must be given to the fact that the seven STEC serotype making up the inoculum were laboratory cultured (therefore, not stressed or injured), they were inoculated into the transport in pure culture, and there were no food matrix components to influence microbial growth and/or stability.

Observation of population profiles of all STEC strains in these three potential transport media clearly established Cary-Blair medium as superior at maintaining stable populations over the entire storage time at both storage temperatures. BPW would serve as an acceptable transport medium, particularly if proper chilled transport temperatures were maintained and samples were analyzed within 48 h of collection. However, at 10^{o}C STEC counts rapidly increased. It is likely that competing background microflora would likewise increase and potentially interfere with STEC growth and detection. Of the three media tested, MRD was the least preferred as a transport medium due to variability in recovery of STEC at T0 and because all strains grew rapidly when slightly abusive temperatures were encountered.

Results from this study agree with previous findings that indicate at 4°C Cary-Blair is suitable for recovery of *E. coli* from samples (Dan et al., 1989). Other research indicates that CB is equally effective in recovering both *Shigella* and *Salmonella* species from stored samples, thereby broadening its applications (Wafsy et al., 1995). CDC protocol recommends that samples that cannot to be analyzed within 3 days should be frozen at -70°C to promote recovery of

organisms. CB has been shown to be effective as a storage solution at -70^{o}C for enteropathogenic *E. coli*, as well as *Salmonella typhimurium*, *Shigella flexneri*, *Shigella, sonnei*, *Campylobacter jejuni*, and *Yersinia enterocolitica* (Dan et al., 1989).

Sample collectors (researchers, food safety or quality managers, etc.) in the field may not always possess the ability to analyze samples on-site or nearby. Samples that are shipped to outside locations may face delays due to weather or other unforeseen circumstances. In these instances, it is best err on the side of caution and utilize a media that can maintain cell stasis should such events occur. Use of Cary-Blair medium for collection of field specimens is advised for instances where knowledge of organism levels is desired.

Chapter 6 - **Prevalence of STEC in Feedlots**

Abstract

With the recently added serotypes of *E. coli* to the list of adulterants there is greater pressure on the beef industry to test for, and prevent, their presence in the food supply. To aid in this, rapid methods of detection with greater sensitivity and specificity are being increasingly relied on detect the target organisms. This study utilized two PCR systems to attempt to identify the prevalence of STEC in the beef industry by analyzing 576 carcass samples from a processing facility. Samples were enriched and analyzed with either BioControl's Gene Detection System, or with Neogen's GeneSeek system. Positive samples from the GDS were then subjected to Abraxis immunomagnetic separation and plated on modified Posse agar. Colonies with an appearance similar to presumed typical *E. coli* were then subjected to further confirmation methods. GDS and NeoGen indicated a prevalence of 28.65 and 6.1 percent respectively in the samples collected. Attempts to isolate STEC from the GDS presumptive positive samples were unsuccessful.

Introduction

It is estimated that 9.4 million cases of foodborne illness, 55,961 related hospitalizations, and 1,351 related deaths annually in the United States, are caused by 31 identified causes (Scallan et al., 2011). Of these illnesses 63,153 are attributed to O157 and 112,752 to non-O157 Shiga toxin-producing *E. coli* (STEC). This represents a difficulty in testing for STEC as testing for O157 only began in earnest in 1994 following a massive outbreak in restaurants (Mead et al., 1999) with methods primarily targeting that organism, while in 2012 the United States Department of Agriculture added six new serotypes (O26, O45, O103, O111, O121, and O145) to the list of adulterants due to increased incidences of foodborne illness caused by these non-O157 STEC. Standard culture methods require several steps using selective media in conjunction with other methods to prevent false readings. These methods are time consuming and can be quite costly especially to an industry that faces constant high demand for a fresh and safe product.

One method popular for its accuracy and short time is polymerase chain reaction (PCR). Discovered in the 1980's, this method of DNA amplification revolutionized the microbiology world by allowing relatively small amounts of genetic material to be utilized for scientific analysis. In early trials polymerase, from *E. coli* was utilized but required careful attention and repetitive addition of polymerase. Discovery of the organism *Thermophilus aquaticus* (Taq) and the ability of its polymerase to resist degradation at high temperatures that allowed for greater use of this technology (Bartlett and Stirling, 2003). PCR works by cleaving the DNA sequence at set points to target gene sequences unique to a specific organism. After adding nucleotides and primers, the sample is then combined with Taq polymerase to allow sequences of heating and cooling to cleave the DNA, then re-anneal to exponentially increase the amount of DNA in a sample. Known as multiplex PCR, the number of primers can be increased to form overlapping sequences that improve specificity and reduce the incidence of false positives. Genes that may be targeted are the specific O groups of *E. coli* and genes indicative of pathogens such as the *stx1/stx2* (shiga-toxin producing) and *aggR* (enteroaggregative) genes. Multiplex PCR has already been proven to be effective in detecting the main genes identified as pathogenicity markers in *E. coli* from a single sample (Toma et al., 2003). PCR may be utilized in conjunction with immuno-magnetic separation (IMS) to further increase detection abilities. IMS involves coating very small paramagnetic beads with antibodies that are keyed to lock with certain microbes (Olsvik et al., 1994). Beads are inoculated into a medium that contains the target organism remaining sample material then removed. The beads are washed to remove excess flora, and then either inoculated into a resuspension media or plated. Cultures may be analyzed further to confirm identity. One study found that use of IMS for isolation of O157:H7 was able to recover 72.6% of the total isolates recovered from inoculated samples while direct plating recovered only 27.4% (Chapman et al., 1994). Findings by Sanderson et al. (1995) agreed with Chapman's work and confirmed that IMS is more sensitive than direct plating methods for recovery of O157:H7. A study by Elder et al. (2000) found that 43% of pre-evisceration carcasses sampled in a processing facility were positive for enterohemorrhagic *E. coli* O157 while this number declined with further processing; 18% and 2% for post-evisceration pre-intervention, and after entering the cooler, respectively. Another study found that 54% of pre-evisceration carcasses were positive for non-O157 STEC, with only 8% positive post-intervention. These results indicate that while there is a high frequency of STEC on incoming

carcasses, current intervention methods appear effective at reducing bacterial loads on beef carcasses (Arthur et al., 2002). This study was conducted as a part of a larger study to compare STEC counts on feedlot cattle at different points prior to and during processing. For this study, two commercial PCR-based detection systems were compared for sensitivity and accuracy in conjunction with IMS.

Materials & Methods

Two sponge swabs were hydrated with 15 mL of 0.1% peptone water and used to sample various locations on a post hide removal, pre-evisceration beef carcass at a processing facility in Nebraska. Sponges were refrigerated and sent overnight to be analyzed the following morning. It was observed that some sponges contained foreign material such as blood, hair, or dirt. This information was recorded for comparison with presumptive positives. Samples had 1 mL of diluent removed and frozen at -80°C, after which 80mL of *Escherichia coli* broth (EC; Sigma-Aldrich: 20.0 g casein digest, 5.0 g lactose, 4.0 g dipotassium phosphate, 1.5 g potassium phosphate, 5.0 g sodium chloride, 1.5g bile salts) was added and the sample allowed to rest at room temperature (22-24°C) for 2 h. After resting, samples were placed in an incubator at 41°C for 8hr. Upon removal, two 1-mL aliquots from each enriched sample were placed at -80C. One mL aliquots from the enriched sample bag were then used to run the commercially available PCR system from BioControl, the BioControl Assurance Gene Detection System (sample remainders were stored at 4°C until further needed), a system that combines immunomagnetic separation with polymerase chain reaction assays to determine the presence of both Big Six Shiga Toxigenic *E. coli* and the O157:H7 serotype. Post-enrichment samples were also taken from -80°C storage and sent to Neogen (Lincoln, NE) for analysis using their NeoSeek PCR system. BioControl GDS analysis was conducted following system protocol provided by the manufacturer. Briefly, 0.2µL of immunomagnetic separation Big Six STEC beads were added to covered sample wells, available from BioControl, containing 1 mL of sample and rocked for 10 minutes to allow adhesion. A magnetic tipped PickPen (BioControl, Bellevue, WA) covered in a thin latex sheath (BioControl) was submerged in the solution and gently used to stir the mixture for 30 seconds to collect the beads and transfer them to a second well containing 1 mL wash solution from the MPX Top 7 STEC GDS kit. Beads were detached from the pick-pen and allowed to rest in the wash solution for 30 seconds so that trapped non-target organisms could

disperse. After exchanging the latex sheath on the magnetic pen, it was submerged in the wash solution and the sample stirred for 30 seconds prior to moving the pen to another well containing 1 mL wash solution. Beads were not detached from the pen at this time and the pen was used to gently stir the solution for 30 seconds prior to moving the pen to a resuspension plate containing 0.45 µL of resuspension solution and detaching the beads, then stirring the beads for an additional 30 seconds. The resuspension solution functioned to lyse the cells and prevent degradation of the DNA prior to analysis. 0.3 µL of this was then transferred to amplification tubes containing the polymerase and nucleotides, and then shaken gently to ensure mixing of the solution. Once this process was completed for all samples, the amplification tubes were placed into the BioControl GDS unit for analysis. Results from the GDS indicate the presence of *stx1, stx2, eae*, or O157 genes in the sample being analyzed and determine presence of possible Big Six STEC based on these results.

Samples that were presumptive positive by GDS for either Big Six or O157 STEC were analyzed using Abraxis Big Six IMS (Abraxis BioScience, Warminster, PA) and Dynal O157 IMS beads (Thermo Fisher Scientific, Waltham, MA). Beads were inoculated separately into 1 mL aliquots of the post-enrichment sample (Bead O26 in 1 mL, O45 in a separate mL, etc.) and allowed to rest for 20 minutes with gentle rocking. Tubes were then placed into a magnetic separator (Abraxis 6 separator) for 3 minutes to allow beads to be separated from the suspension. The remaining solution was then removed with care taken not to disturb the beads on the side of the tube. One mL of phosphate buffered saline was then added to the beads and the tube was then vortexed and returned to the magnetic separator for 3 additional minutes. The wash steps were repeated twice and, after the final rehydration and vortexing, 0.1 mL of the washed solution and a 1:10 dilution of the washed beads in 0.1% peptone were plated on modified Posse media (40 g Difco MacConkey agar base, 6 g sucrose, 6 g sorbose, 3.5 g bile salts #3, 0.05 g X-gal, 0.05 g IPTG, 5 mg novobiocin, 0.5 mg potassium tellurite; prepared as described by Posse et al., 2008). Plates were incubated for 18-24 h at 37°C. Significant growth of multiple colors (blue, mauve, green, purple, milky white, clear) was observed on plates for all bead types. To obtain isolated colonies, a 1 µL loop was drawn across the 1:10 dilution plate and streaked for isolation on modified Posse media, then incubated for 18-24 h at 37°C. Colonies displaying typical *E. coli* color patterns (blue, mauve, purple, green) were harvested and analyzed by latex agglutination (Remel: Wellcolex *E. coli* O157:H7, Prolex: Pro-Lab Diagnostics *E. coli* non-O157). Colonies

that were analyzed by latex agglutination were also pooled and analyzed with the GDS system to attempt organism isolation.

Results

Figure 6-1: GDS vs GeneSeek Total Count Positives

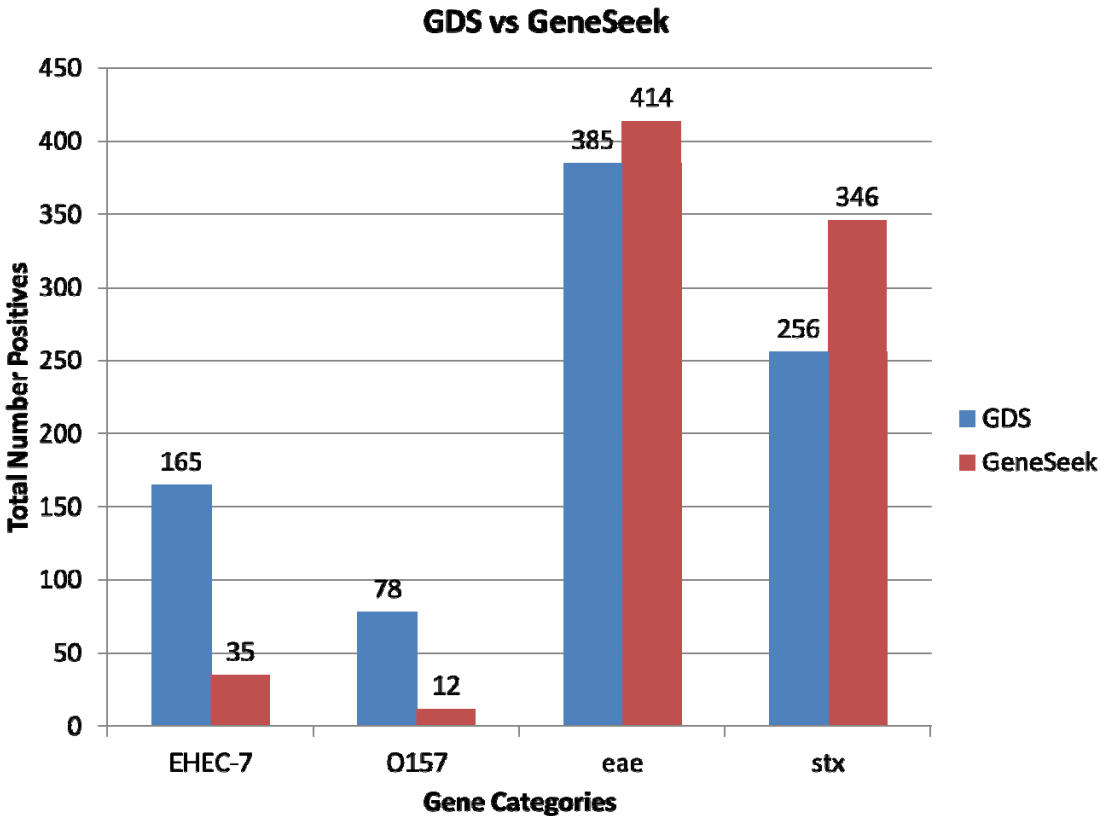
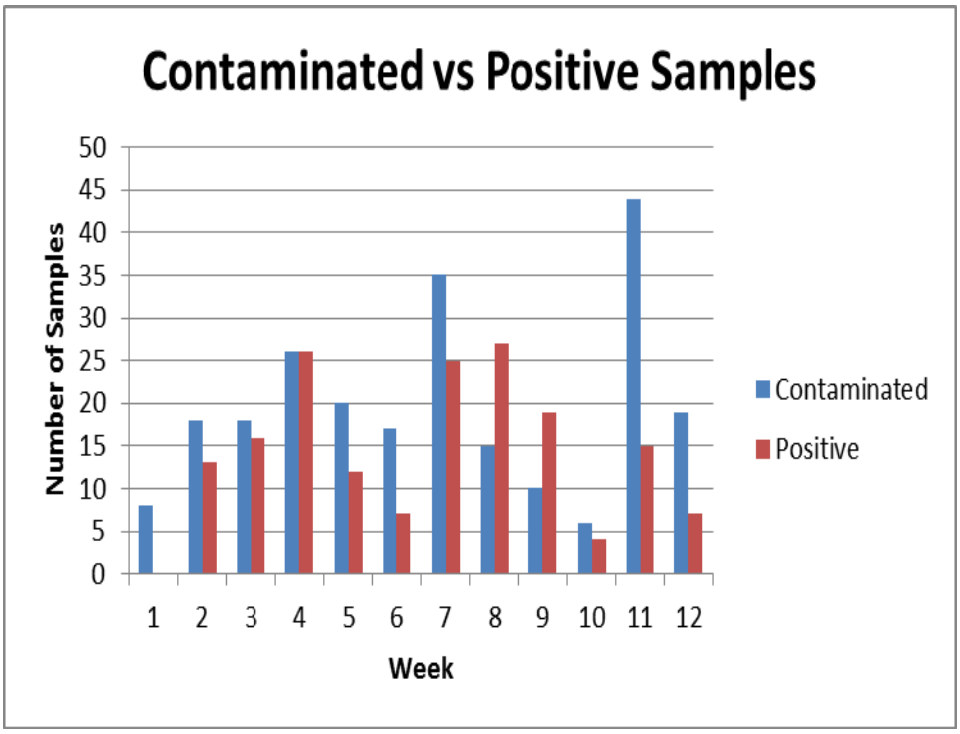


Table 6-1: Comparison of Total Percentage of Presumptive Positives; GDS vs GeneSeek

% Positives				
	EHEC-7	O157	eae	stx
GDS	28.65	14.93	66.84	44.44
GeneSeek	6.1	2.1	71.9	60.1

Figure 6-2: Sponges with Foreign Material vs GDS Positive Sample Amount per Week



Discussion

Results collected from the GDS indicate that there was a combined O157 and non-O157 STEC prevalence rate of 43.58% in the samples collected. Similar counts were obtained by Arthur et. al. (2002) during a study of United States beef processing plants with 54% of carcasses sampled pre-evisceration positive for non-O157 STEC. Data from NeoGen indicated the prevalence at a lower amount of only 6.1% STEC positive samples. Of the samples that contained foreign material (229 out of 576 total samples), 37.12% and 57.14% of presumptive positive samples were identified by GDS and GeneSeek, respectively. There did not appear to be any correlation between the amount of foreign material on sponge samples and the amount of presumptive positives recovered, evaluation was purely subjective based on visual inspection of samples and observed number of GDS presumptive positive samples.

A possible explanation for the discrepancy between systems is the methods employed by each system for determining whether or not a sample is positive. The GDS system has the capability of identifying the stx1, stx2, and eae genes simultaneously with markers for the O157 serotype in a sample, but relies on the use of IMS for reduction of false positives during separation of the Big Six STEC. As such when determining presence of Big Six STEC, any

detection of eae and one of the stx genes in a single sample will be declared presumptive positive for Big Six STEC by the GDS system even if the genes are from separate organisms (Anonymous, 2014). In contrast, NeoGen claims to use over 70 markers to be able to link targeted genes to a single organism even in mixed culture, thereby preventing false positives (Hosking and Petrik, 2013). Their results showed a high prevalence of non-pathogenic E. coli serotypes, eae, and stx genes in the samples recovered. The high levels of shiga toxin genes and intimin detected by both GDS and NeoSEEK systems (66.8 % intimin and 44.4 % shiga toxin: 71.9 % intimin and 60.1 % shiga toxin respectively), combined with the relatively low levels of STEC reported (28.6 % and 6.1 % respectively); indicate that a large proportion of the virulence traits needed for identification of a potential STEC may be present in other non-STEC organisms. For a system such as the GDS that relies on use of IMS as a pre-screen and lacks the ability to link target markers to a single organism there exists increased chances for false positive results to be obtained.

Presumptive positive samples from the GDS were analyzed using Abraxis IMS beads, latex agglutination, multiplex PCR, and additional analysis with the GDS. . Of the total samples analyzed with these methods no STEC cultures were successfully isolated from the enriched samples.

While useful as a measure to prevent contaminated product from entering the food supply, the use of the GDS may result in a high number of false positives and loss of product for the industry thus increasing product costs. The ability of the NeoGen system to distinguish between pathogenic and non-pathogenic serotypes lends itself towards reducing the number of false positives obtained and thereby reducing product loss. However it should be noted that there is a high difference between price per sample for the two systems used. Based on component evaluation and company pricing, the average cost per sample is 10 $ for GDS and 27 $ for NeoSEEK. Even when paired with selective media, the use of IMS beads for analysis does not guarantee isolation of the target organism. Furthermore, the use of modified Posse agar for STEC does not appear to increase effectiveness of cultural isolation methods as the colony colors may not be indicative of the actual organisms present, particularly in samples that may contain high levels of background flora.

Chapter 7 - **Conclusions & Implications**

Enrichment

Of the enrichment medias used Buffered Peptone water and Universal Preenrichment broth generated lower growth levels than either Tryptic soy broth or *Escherichia coli* broth, which both achieved growth of over 9 log cfu/mL. With the exception of O157, the addition of 8 mg novobiocin to TSB elicited nearly a full log reduction in STEC growth. This pattern was also observed with *E. coli* broth when increased amounts of novobiocin were added. Addition of novobiocin to EC broth (5, 8, and 20 mg) even at currently recommended levels for STEC enrichment resulted in greatly reduced organism recovery for all target serotypes with the exception of O157. Serotype O26 was inhibited completely at all levels in EC broth, however subsequent evaluation of the strain used showed that growth did occur at levels greater than those used in the study. There exists the possibility that during dilution of the master inoculum for the study that due to the low levels of organism desired at the start (10 cfu/mL) the organism was not transferred to the enrichment broth tube. This would also explain the incidence of O111 being unrecoverable at 5 mg novobiocin but showing decent growth at 8 mg. EC broth has a greater capacity than TSB to reduce background flora and generated growth as effectively, further indicating that for STEC enrichment it would be more advantageous to use EC broth with no novobiocin.

Transport

The USDA dictates that for carcass sampling sponges are to be hydrated with buffered peptone water and either analyzed immediately or refrigerated and analyzed within 24h. Results from this study showed that organisms placed in BPW at 4°C experienced some decline in bacterial levels over the course of 24 h. Such sample degradation could be detrimental to analysis if low levels of organisms are already present in the sample and levels decreased further. If temperature abuse occurred (i.e. raised temperature), enumeration of bacterial load would be compromised as some serotypes used experienced an increase of up 0.5 log cfu during the first 24 h sampling period in BPW.

Maximum recover diluent experienced increased cell counts under refrigeration and abuse temperatures at all sampling times with the exception of serotype O103, which had decreased counts for all times at 4°C. The ability of MR to increase cell counts could be beneficial as a replacement for sponge hydration during prevalence studies in which detection without enumeration of the sample is the goal. However use of MR as a primary transport medium for enumeration studies is ill-advised due the capacity for increasing the bacterial load inherent to the media.

Bacterial counts in Cary-Blair medium fluctuated the least at both temperatures over the sampling period for the medias tested. At both standard refrigeration and abuse temperatures the ability of CB to maintain cell stasis was superior over the 72 h sampling period. Due to the consistency of the medium it would be impractical for use hydrating sponges or samples. However the properties of CB that allow it to maintain cells levels at initial levels for both 4 and 10°C make it ideal to store other sampling implements in CB for the duration of transport if there exists the possibility of extended shipment times or temperature abuse.

Detection

As the amount of organisms that samples are tested for continues to increase the ability of detection methods will be ever more important. The methods used in this study were evaluated for their ability to perform as intended and how the results obtained from them could impact the industry.

The 576 sponge samples from Nebraska were analyzed with both the BioControl GDS system and NeoGen's GeneSeek system. These two systems use multiplex PCR to determine whether or not samples are positive for STEC by detecting target gene sequences. BioControl's system relies on immunomagnetic separation prior to PCR analysis to eliminate non-O157 STEC and identifies presumptive positives based on the presence of *eae* and *stx* genes. It does check for the O157 serotype but requires the presence of the other two pathogenicity genes to declare a presumptive positive. NeoGen claims to utilize over 70 genetic markers to ensure the accuracy of its results. Results from NeoGen claimed to be able to distinguish between different O groups while detecting pathogenicity genes and, that genes must come from one organism in order to be identified as STEC. As such the GDS system identified a significantly higher percentage of samples received as STEC than the NeoGen system; 28.65% and 6.1% respectively. This

difference could be due to the presence of other organisms containing the pathogenicity genes being detected by the GDS while only individual organisms were identified by GeneSeek. Having a significant number of false positives is undesirable as it can lead to increased production costs. Further research comparing the two systems is needed before a definitively superior option can be declared for sample analysis.

Abraxis and Dynal immunomagnetic beads were also used in conjunction with modified Posse agar to attempt to isolate STEC from the received samples. While both the beads and the agar are identified as being selective for specific serotypes a large number of colonies were obtained when IMS samples were plated on Posse. From these colonies it was determined that while many were in fact *E. coli*, but there were also a high number of non-target organisms collected. These colonies were further analyzed with latex agglutination, aerobic profile index (BioMerieux API 20E), and some were analyzed with the GDS system. All colonies tested, regardless of appearance, tested negative for either the presence of pathogenicity genes on the GDS or for the O antigen group with the latex agglutination assay. Based on these results it is surmised that the bead mechanisms for identification were overwhelmed by the high concentration of non-target organisms in enriched samples. Furthermore, modified Posse agar does not appear to possess the ability to selectively grow and differentiate non-O157 STEC as many of the plates in question were observed to have both high colony counts and multiple morphologies present regardless of the IMS bead used prior to plating. Using the results of this study it is inadvisable to rely solely on modified Posse or IMS to identify STEC. These methods should be used with other confirmatory steps to ensure accuracy.

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Appendix A - SAS

Enrichment Media Code

```
proc sort data=set1; by media organism time day;run;
proc means mean noprint;
by media organism time;
var cfu;
output out=new mean=mcfu;
data new; set new; time2=time**2;
proc print data=new;
proc reg data=new;
by media organism;
model mcfu=time;
model mcfu=time time2;
run;
```

Enrichment Media Output

Data was analyzed as a quadratic function as that most closely mimicked the natural growth curve of bacteria in an ideal environment. Using $\alpha < 0.05$ the given P values in the table indicate whether or not the data is described by the function.

Table A-1: Enrichment Media Anova Data- Non- Selective Media

Media	Organism	MSE	P value	Rsq Adj
BPW	O26	0.436	0.029	0.942
	O45	0.165	0.011	0.978
	O103	0.277	0.018	0.963
	O111	0.521	0.034	0.931
	O121	0.255	0.018	0.964
	O145	0.289	0.021	0.957
	O157	0.118	0.008	0.983
UPB	O26	0.500	0.029	0.943
	O45	0.110	0.007	0.987
	O103	0.165	0.009	0.981
	O111	0.373	0.023	0.954
	O121	0.274	0.017	0.966
	O145	0.315	0.019	0.962
	O157	0.095	0.005	0.989
TSB	O26	0.599	0.031	0.939
	O45	0.437	0.021	0.959

	O103	0.379	0.017	0.965
	O111	0.475	0.023	0.954
	O121	0.658	0.036	0.929
	O145	0.902	0.033	0.934
	O157	0.524	0.026	0.948
mTSB	O26	0.347	0.019	0.961
	O45	0.115	0.008	0.984
	O103	0.104	0.006	0.988
	O111	0.168	0.011	0.979
	O121	0.370	0.025	0.950
	O145	0.071	0.005	0.991
	O157	0.092	0.005	0.991

Table A-2: Enrichment Media Anova Data- Selective Media

Media	Organism	MSE	P value	Rsq Adj
EC	O26	0.000	0.000	0.000
	O45	0.134	0.013	0.974
	O103	0.558	0.146	0.708
	O111	0.124	0.011	0.979
	O121	0.029	0.012	0.975
	O145	0.192	0.008	0.984
	O157	0.394	0.015	0.970
mEC L	O26	0.000	0.000	0.000
	O45	0.023	0.009	0.982
	O103	0.259	0.149	0.702
	O111	0.000	0.000	0.000
	O121	0.316	0.110	0.780
	O145	0.139	0.046	0.908
	O157	0.290	0.014	0.972
mEC M	O26	0.000	0.000	0.000
	O45	0.136	0.027	0.947
	O103	0.000	0.000	0.000
	O111	0.119	0.141	0.719
	O121	0.000	0.000	0.000
	O145	0.000	0.000	0.000
	O157	0.177	0.015	0.970
mEC H	O26	0.000	0.000	0.000
	O45	0.000	0.000	0.000
	O103	0.000	0.000	0.000
	O111	0.000	0.000	0.000
	O121	0.000	0.000	0.000
	O145	0.000	0.000	0.000

O157	0.728	0.037	0.926

Transport Media Code

```
proc sort data=set3; by organism media temp time day;run;
proc mixed covtest cl data=set3;
by organism media temp;
class day;
model log10cfu=time/solution cl output=new1;
random day;
run;
proc univariate normal plot data=new1;
var resid;
run;
data set3;
     set set3;
     time2 = time**2;
run;
proc mixed covtest cl data=set3;
by organism media temp;
class day;
model log10cfu=time time2/solution cl output=new2;
random day;
run;
proc univariate normal plot data=new2;
var resid;
run;
```

Transport Media Output

This section of research was attempting to establish the best media to maintain stasis for the organisms used. To determine this, the data was analyzed as a quadratic function and the effect of time measured. At $\alpha < 0.05$ any P values that are significant are indicative that the media does not support stasis under the prescribed conditions for the given organism.

Table A-3: Transport Media Output

Buffered Peptone Water														
				4 C							10 C			
Culture	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**	**O157**	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**	**O157**
Std Er	0.0343	0.0629	0.1110	0.0310	0.3198	0.0131	0.0486	0.1094	0.0686	0.0854	0.0745	0.1748	0.0412	0.0585
P value	0.0063	0.9367	0.9487	0.1130	0.8663	0.6617	0.3710	0.0011	0.0010	0.0121	0.0647	0.0020	0.0001	0.0001

Maximum Recovery Diluent														
				4 C							10 C			
Culture	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**	**O157**	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**	**O157**
Std Er	0.0694	0.0935	0.1893	0.0963	0.3348	0.1210	0.1388	0.1891	0.1179	0.1060	0.2669	0.3953	0.2463	0.2360
P value	0.0125	0.4644	0.5088	0.1076	0.0428	0.0496	0.1078	0.0004	0.0020	0.0036	0.0015	0.0009	0.0033	0.0021

Cary-Blair Transport Medium														
				4 C							10 C			
Culture	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**	**O157**	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**	**O157**
Std Er	0.0125	0.0096	0.0062	0.0108	0.0174	0.0073	0.0044	0.0138	0.0227	0.0403	0.0204	0.0466	0.0176	0.0049
P value	0.2515	0.0382	0.0022	0.8284	0.7966	0.2129	0.0356	0.0195	0.2839	0.9657	0.2973	0.1095	0.2864	0.1853

Appendix B - Raw Data

Enrichment Media Data

Shown below is the raw data used for the enrichment portion of the presented research. Counts were averaged and then transformed by adding "1" to each averaged value to allow for transformation into log counts for analysis.

Table B-1: Number of colony forming units counted during Set 1, Rep 1 of the Enrichment Study

Media	Culture	4 Count (cfu)	8 Count (cfu)	12 Count (cfu)	18 Count (cfu)	24 Count (cfu)
BPW	O26	25	17200	13100000	83000000	83000000
	O45	66	16700	5700000	70000000	86000000
	O103	29	11000	7800000	58000000	67000000
	O104	133	510000	69000000	195000000	106000000
	O111	90	91000	41000000	85000000	90000000
	O121	144	84000	38000000	92000000	95000000
	O145	300	119000	37000000	91000000	99000000
	O157	109	18500	7400000	81000000	81000000
UPB	O26	159	120000	105000000	395000000	520000000
	O45	125	6900	4900000	444000000	520000000
	O103	11	7200	2600000	232000000	340000000
	O104	211	340000	159000000	282000000	360000000
	O111	67	490000	143000000	322000000	500000000
	O121	250	250000	99000000	488000000	630000000
	O145	69	101000	43000000	339000000	570000000
	O157	91	45000	14500000	522000000	480000000
mTSB M	O26	16	0	600	76000	340000000
	O45	104	670	76000	8800000	57000000
	O103	1	100	90000	330000	97000000
	O104	64	28000	29000000	280000000	370000000
	O111	1	0	900	9400	7400000
	O121	61	5300	690000	15100000	30000000
	O145	23	2600	119000	55000000	72000000
	O157	106	55000	16300000	788000000	1110000000
mEC H	O26	0	0	0	0	0

	O45	0	0	0	0	0
	O103	0	0	0	0	0
	O104	0	0	0	0	0
	O111	0	0	0	0	0
	O121	0	0	0	0	0
	O145	0	0	0	0	0
	O157	4	40	100	14000	250000

Table B-2: Number of colony forming units counted during Set 1, Rep 2 of the Enrichment Study

		4	8	12	18	24
Media	**Culture**	**Count (cfu)**	**Count (cfu)**	**Count (cfu)**	**Count (cfu)**	**Count (cfu)**
BPW	O26	31	31000	85000000	40000000	132000000
	O45	21	7900	7000000	41000000	35000000
	O103	32	64000	35000000	56000000	74000000
	O104	77	77000	45000000	47000000	40000000
	O111	30	26000	129000000	44000000	71000000
	O121	37	18200	12700000	38000000	65000000
	O145	87	67000	28000000	53000000	58000000
	O157	24	6700	1350000	21400000	58000000
UPB	O26	14	52000	58000000	70000000	320000000
	O45	56	36000	11500000	150000000	250000000
	O103	47	94000	136000000	229000000	220000000
	O104	14	11600	11000000	83000000	520000000
	O111	41	55000	58000000	203000000	280000000
	O121	27	41000	15700000	75000000	250000000
	O145	37	58000	31000000	113000000	196000000
	O157	33	37000	9400000	135000000	400000000
mTSB M	O26	9	14900	31000000	19500000	58000000
	O45	8	5000	5000000	55000000	34000000
	O103	8	1740	460000	66000000	34000000
	O104	6	5100	3400000	5600000	2800000
	O111	7	1120	78000	56000000	97000000
	O121	12	6600	13000000	7500000	45000000
	O145	31	26000	29000000	60000000	90000000
	O157	9	6400	8600000	540000000	640000000
mEC H	O26	0	0	0	0	0
	O45	0	0	0	0	0
	O103	0	0	0	0	0
	O104	1	10	200	4600	970000
	O111	1	0	0	0	0

	O121	1	0	0	0	0
	O145	0	0	0	0	0
	O157	3	280	31000	96000000	171000000

Table B-3: Number of colony forming units recovered during Set 1, Rep 3 of the Enrichment Study

		4	8	12	18	24
Media	**Culture**	**Count (cfu)**	**Count (cfu)**	**Count (cfu)**	**Count (cfu)**	**Count (cfu)**
BPW	O26	101	240000	47000000	52000000	57000000
	O45	35	9200	7600000	76000000	72000000
	O103	36	10600	14300000	51000000	55000000
	O104	97	138000	65000000	63000000	90000000
	O111	40	30000	35000000	72000000	72000000
	O121	47	39000	18600000	84000000	107000000
	O145	32	45000	43000000	95000000	82000000
	O157	45	26000	14600000	66000000	75000000
UPB	O26	99	52000	250000000	330000000	320000000
	O45	55	36000	33000000	450000000	430000000
	O103	89	76000	51000000	208000000	280000000
	O104	95	146000	270000000	460000000	410000000
	O111	306	650000	380000000	470000000	350000000
	O121	137	280000	290000000	560000000	440000000
	O145	133	250000	300000000	300000000	350000000
	O157	88	116000	120000000	640000000	560000000
mTSB M	O26	17	1640	810000	310000000	470000000
	O45	25	14500	7100000	76000000	64000000
	O103	20	4900	1300000	105000000	89000000
	O104	188	440000	41000000	51000000	60000000
	O111	17	8200	4900000	58000000	59000000
	O121	6	3300	10500000	25000000	56000000
	O145	31	22200	16000000	70000000	85000000
	O157	82	95000	133000000	1130000000	960000000
mEC H	O26	0	0	0	0	0
	O45	0	0	0	0	0
	O103	0	0	0	0	0
	O104	0	0	0	0	0
	O111	0	0	0	0	0
	O121	0	0	0	0	0
	O145	0	0	0	0	0
	O157	0	0	101000	372000000	300000000

Table B-4: Number of colony forming units recovered during Set 2, Rep 1 of the Enrichment Study

Media	Culture	4 Count (cfu)	8 Count (cfu)	12 Count (cfu)	18 Count (cfu)	24 Count (cfu)
EC	O26	0	0	0	0	0
	O45	4	970	650000	620000000	550000000
	O103	4	0	800000	450000000	250000000
	O104	25	30000	37000000	560000000	680000000
	O111	9	14600	25000000	540000000	550000000
	O121	9	11100	30000000	320000000	250000000
	O145	4	5500	6200000	700000000	680000000
	O157	4	1050	420000	500000000	680000000
mEC L	O26	0	0	0	0	0
	O45	0	0	0	0	0
	O103	0	0	0	0	0
	O104	16	13400	30000000	23000000	6000000
	O111	0	0	0	0	0
	O121	0	0	2600	28000000	8500000
	O145	1	60	42000	310000000	147000000
	O157	5	4200	1030000	280000000	480000000
mEC M	O26	0	0	0	0	0
	O45	0	0	100	5300	260000
	O103	0	0	0	0	0
	O104	0	50	2500	6100000	135000000
	O111	10	10	0	2500	55000
	O121	0	0	0	0	0
	O145	0	0	0	0	0
	O157	1	300	40000	130000000	560000000
TSB	O26	370	1340000	432000000	500000000	570000000
	O45	260	300000	295000000	750000000	550000000
	O103	250	131000	243000000	720000000	430000000
	O104	890	6900000	494000000	850000000	560000000
	O111	250	1260000	296000000	880000000	620000000
	O121	400	2500000	724000000	630000000	1030000000
	O145	360	1010000	298000000	730000000	710000000
	O157	217	650000	534000000	860000000	900000000

Table B-5: Number of colony forming units recovered during Set 2, Rep 2 of the Enrichment Study

Media	Culture	4 Count (cfu)	8 Count (cfu)	12 Count (cfu)	18 Count (cfu)	24 Count (cfu)
EC	O26	0	0	0	0	0
	O45	5	2900	29000000	6300000000	4200000000
	O103	0	0	0	0	0
	O104	26	34000	660000000	4000000000	5300000000
	O111	13	10400	145000000	5900000000	5900000000
	O121	0	0	0	0	0
	O145	32	10700	85000000	8700000000	7300000000
	O157	9	1670	1190000	4900000000	6100000000
mEC L	O26	0	0	0	0	0
	O45	0	0	0	0	0
	O103	0	0	0	0	0
	O104	25	16400	290000000	1140000000	950000000
	O111	0	0	0	0	0
	O121	0	0	0	0	0
	O145	0	0	0	0	0
	O157	3	0	5000	70000	4900000
mEC M	O26	0	0	0	0	0
	O45	1	10	0	11600	670000
	O103	0	0	0	0	0
	O104	0	0	0	0	0
	O111	0	0	0	0	0
	O121	0	0	0	0	0
	O145	0	0	0	0	0
	O157	1	490	130000	404000000	500000000
TSB	O26	111	1200000	5200000000	6000000000	5000000000
	O45	20	72000	1610000000	6800000000	7800000000
	O103	57	58000	1700000000	4100000000	7500000000
	O104	300	8400000	7800000000	6700000000	7800000000
	O111	39	159000	5300000000	6700000000	9000000000
	O121	350	1990000	9300000000	7700000000	6600000000
	O145	194	1080000	8500000000	8000000000	10900000000
	O157	112	420000	4400000000	10400000000	6200000000

Table B-6: Number of colony forming units recovered during Set 2, Rep 3 of the Enrichment Study

Media	Culture	4 Count (cfu)	8 Count (cfu)	12 Count (cfu)	18 Count (cfu)	24 Count (cfu)

EC	O26	0	0	0	0	0
	O45	15	4900	5500000	960000000	320000000
	O103	0	0	0	0	0
	O104	0	0	0	0	0
	O111	0	0	0	0	0
	O121	0	0	0	0	0
	O145	20	26000	51000000	790000000	550000000
	O157	7	2500	740000	770000000	510000000
mEC L	O26	0	0	0	0	0
	O45	7	2500	950000	520000000	440000000
	O103	7	2380	720000	132000000	10100000
	O104	0	0	0	0	0
	O111	0	0	0	0	0
	O121	0	0	0	0	0
	O145	0	0	0	0	0
	O157	1	1400	800000	500000000	440000000
mEC M	O26	0	0	0	0	0
	O45	0	0	0	0	0
	O103	0	0	0	0	0
	O104	0	0	0	0	0
	O111	0	0	0	0	0
	O121	0	0	0	0	0
	O145	0	0	0	0	0
	O157	0	0	0	0	0
TSB	O26	24	76000	280000000	490000000	1220000000
	O45	37	43000	86000000	460000000	790000000
	O103	4	5000	5400000	390000000	710000000
	O104	113	820000	900000000	740000000	800000000
	O111	67	260000	330000000	860000000	680000000
	O121	73	250000	470000000	590000000	1180000000
	O145	220	570000	750000000	480000000	1050000000
	O157	99	320000	670000000	990000000	1230000000

Transport Media Data

The data below was used for the transportation media segment of the presented research. Counts were averaged and the transformed into log counts for analysis.

Table B-7: Base Counts for Transport Media rep 1

Media	Temp	Culture	Time 0	Time 1	Time 2	Time 3	Time 4
BPW	**4 C**	**O26**	7150	1250	1185	975	540
		O45	8450	1285	2200	1465	2450
		O103	4350	720	1995	945	2550
		O111	6000	740	2295	1250	1420
		O121	11200	405	52	7	585
		O145	6150	11600	5100	5950	6100
		O157	8450	1140	3150	1180	2850
	10 C	**O26**	7150	3500	4860	16150	91900
		O45	8450	2200	5850	15100	70000
		O103	4350	1400	3890	3240	49600
		O111	6000	2500	4750	7100	12900
		O121	11200	8450	20100	101500	81200
		O145	6150	10000	15650	81000	281800
		O157	8450	9050	10200	60000	118400
MR	**4 C**	**O26**	280	1410	2715	2755	2650
		O45	2075	2200	2105	1210	315
		O103	1490	210	83	79.5	108
		O111	157	365	2220	2490	625
		O121	16	1800	355	515	69
		O145	4140	2175	2800	3540	5850
		O157	2900	5050	4980	2760	2600
	10 C	**O26**	280	3550	4000	22050	62800
		O45	2075	6500	2940	2750	17600
		O103	1490	3900	1475	3540	36150
		O111	157	2600	11250	8200	14050
		O121	16	3250	11650	8600	33600
		O145	4140	27500	33600	82500	172500
		O157	2900	80000	35800	27000	54800
CB	**4 C**	**O26**	5100	1665	1635	1510	1070
		O45	4900	1810	3200	1545	1740
		O103	2700	1070	1880	1330	760
		O111	3950	2650	1540	1870	1840
		O121	1470	5100	1640	1650	1235
		O145	8400	5300	7250	2900	3545
		O157	4950	5550	3850	3100	3450
	10 C	**O26**	5100	3450	7300	1315	1660

	O45	4900	4200	11550	1270	1505
	O103	2700	4750	4300	1120	17300
	O111	3950	2950	930	1265	3640
	O121	1470	9100	1805	3050	7950
	O145	8400	11550	9100	5000	37150
	O157	4950	6750	9200	4200	6000

Table B-8: Base Counts for Transport Media rep 2

Media	Temp	Culture	Time 0	Time 1	Time 2	Time 3	Time 4
BPW	**4 C**	**O26**	3100	20800	2500	1455	1105
		O45	5900	345	5800	4700	6250
		O103	3400	35	2650	2050	2245
		O111	5400	1230	2500	2405	2145
		O121	610	55	1560	1885	4250
		O145	6000	1755	7150	4550	5950
		O157	7650	705	7050	4850	2800
	10 C	**O26**	3100	820	1400	36500	810000
		O45	5900	1690	15300	58000	620000
		O103	3400	245	6150	9250	73000
		O111	5400	370	2900	7000	48500
		O121	610	305	8300	131000	490000
		O145	6000	1320	26000	111000	638000
		O157	7650	1150	18000	126000	940000
MR	**4 C**	**O26**	65	2080	2250	2350	6050
		O45	120	1495	3300	2900	4300
		O103	70	0	35	77	2
		O111	25	20	90	65	41
		O121	1130	45	625	3980	14350
		O145	3620	2250	13700	8100	15100
		O157	2000	2000	13400	7450	12250
	10 C	**O26**	65	730	22300	123500	730000
		O45	120	1835	15100	20500	216500
		O103	70	630	6250	4200	18800
		O111	25	1150	21350	67500	320500
		O121	1130	170	32000	113000	1264000
		O145	3620	15200	54500	106500	1400000
		O157	2000	11600	43000	200000	2444000
CB	**4 C**	**O26**	1700	1710	3350	3250	3100
		O45	2900	2585	1995	2795	2745
		O103	1630	1540	1145	890	1470
		O111	1330	1255	1720	2615	3300
		O121	815	825	1950	785	2175

		O145	3150	3200	5000	5300	5150
		O157	6800	4350	4350	4600	5250
	10 C	**O26**	1700	1530	795	1690	1160
		O45	2900	3300	4200	3650	3850
		O103	1630	900	1390	2240	925
		O111	1330	1330	875	790	1505
		O121	815	580	860	1350	8700
		O145	3150	5150	5550	4600	6650
		O157	6800	3800	8000	5100	5450

Table B-9: Base Counts for Transport Media rep3

Media	Temp	Culture	Time 0	Time 1	Time 2	Time 3	Time 4
BPW	**4 C**	**O26**	1990	1645	1855	1045	930
		O45	8300	6700	7600	5400	6600
		O103	3850	2400	1815	575	1805
		O111	2850	2260	2125	395	1375
		O121	4000	3250	1410	485	3300
		O145	7050	7050	5650	3600	4900
		O157	10350	5600	7200	6200	3500
	10 C	**O26**	1990	2350	2660	4740	11000
		O45	8300	6400	15900	11100	28500
		O103	3850	1630	3150	3780	3840
		O111	2850	2225	2505	1415	2950
		O121	4000	1430	11050	1220	83500
		O145	7050	5300	15750	34600	82000
		O157	10350	8000	12150	23600	94500
MR	**4 C**	**O26**	205	545	1870	645	1120
		O45	260	2955	5340	815	2650
		O103	150	825	685	90	1225
		O111	20	315	585	65	850
		O121	0	240	1260	870	3820
		O145	30	3160	5800	4550	6100
		O157	25	2205	6050	2650	5000
	10 C	**O26**	205	15	400	395	1045
		O45	260	6200	11850	5000	19900
		O103	150	2525	3080	2225	2045
		O111	20	890	6950	570	14000
		O121	0	2650	17650	29500	69000
		O145	30	12300	30500	665	228500
		O157	25	12850	17450	22000	149500
CB	**4 C**	**O26**	3750	2270	2440	2195	1825
		O45	4500	5850	2700	3780	1690

	O103	2590	1910	1710	860	735
	O111	2400	3900	1620	2075	1545
	O121	2550	2400	2225	1910	1870
	O145	5800	7050	4950	5100	3650
	O157	6250	6500	3200	2745	3800
10 C	**O26**	3750	2800	1805	1960	1360
	O45	4500	5200	6550	5400	5250
	O103	2590	1280	955	755	865
	O111	2400	890	1230	905	615
	O121	2550	2400	1135	1015	1785
	O145	5800	6750	6750	4350	4850
	O157	6250	6450	6150	4000	3900

Detection & Prevalence Data

Table below shows the data obtained using the BioControl GDS Assurance system and from NeoGen's GeneSeek system. A value of "1" indicates a positive reading from the system.

Table B-10: GDS vs NeoGen Prevalence

	GDS Results					NeoGen Results		
Sample	**Top STEC**	**O157**	**eae**	**stx2**	**stx1**	**EHEC-7**	**Eae**	**Stx**
1	0	0	0	0	0	0	1	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	0	0	1	0	0	0	0	0
9	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
11	0	0	1	0	0	0	1	0
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	1	0	0	0	0	0
16	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0
25	0	0	1	0	0	0	1	0
26	0	0	0	0	0	0	0	0
27	0	0	1	0	0	0	1	0
28	0	0	0	0	0	0	1	0
29	0	0	1	0	0	0	1	0
30	0	0	1	0	0	0	1	0

31	0	0	1	0	0	0	1	0
32	0	0	1	0	0	0	0	0
33	0	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0	0
35	0	0	1	0	0	0	1	0
36	0	0	1	0	0	0	1	0
37	0	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0	0
39	0	0	1	0	0	0	1	0
40	0	0	0	0	0	0	0	0
41	0	0	1	0	0	0	1	0
42	0	0	0	0	0	0	0	0
43	0	0	1	0	0	0	1	0
44	0	0	1	0	0	0	1	0
45	0	0	0	0	0	0	0	0
46	0	0	1	0	0	0	1	0
47	0	0	1	0	0	0	0	0
48	0	0	0	0	0	0	0	0
49	0	0	1	0	0	0	1	0
50	1	1	1	1	0	1	1	1
51	1	1	1	1	0	0	1	1
52	0	0	1	0	0	0	1	1
53	0	0	1	0	0	0	1	1
54	0	0	1	0	0	0	1	1
55	0	0	1	0	0	0	1	0
56	0	0	1	0	0	0	1	0
57	0	0	1	0	0	0	1	0
58	0	0	1	0	0	0	1	0
59	0	0	0	0	0	0	0	0
60	0	0	1	0	0	0	1	0
61	0	0	1	0	0	0	1	1
62	0	0	1	0	0	0	1	1
63	0	0	1	0	0	0	1	1
64	0	0	1	0	0	0	1	1
65	1	0	1	0	1	0	1	1
66	1	0	1	0	1	0	1	0
67	0	0	1	0	0	0	1	0
68	1	1	1	1	1	1	1	1
69	0	0	1	0	0	0	1	0
70	1	0	1	1	0	0	1	1
71	1	0	1	0	1	0	1	1
72	0	0	1	0	0	0	1	0
73	1	0	1	0	1	0	1	1

74	0	0	1	0	0	0	1	1
75	1	0	1	1	1	0	1	1
76	0	0	0	0	0	0	0	1
77	0	0	1	0	0	0	1	0
78	0	0	1	0	0	0	1	0
79	1	0	1	1	0	0	1	1
80	0	0	0	0	0	0	0	0
81	0	0	1	0	0	0	1	0
82	1	0	1	1	0	0	0	1
83	0	0	1	0	0	0	1	0
84	0	0	1	0	0	0	1	1
85	0	0	1	0	0	0	1	1
86	0	0	1	0	0	0	0	0
87	0	0	1	0	0	0	0	0
88	0	0	1	0	0	0	1	0
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516	0	0	1	0	0	0	0	0
517	0	0	1	0	0	0	0	0
518	0	0	1	0	0	0	1	0
519	0	0	1	0	0	0	1	0
520	1	1	1	1	1	1	1	1
521	0	0	1	0	0	0	0	0
522	0	0	1	0	0	0	1	0
523	1	1	1	0	1	0	1	1
524	1	1	1	1	1	1	1	1
525	1	0	1	0	1	0	1	1
526	1	1	1	1	1	0	0	1
527	1	1	1	1	1	0	0	0
528	1	0	1	0	1	0	1	1
529	1	1	1	1	0	0	1	0
530	1	0	1	0	1	0	1	1
531	0	0	1	0	0	0	1	1
532	0	0	1	0	0	0	1	0
533	0	0	1	0	0	0	0	0
534	1	0	1	1	0	0	0	0
535	0	0	1	0	0	0	0	0
536	0	0	1	0	0	0	0	0
537	0	0	0	0	0	0	0	0
538	0	0	1	0	0	0	0	0
539	0	0	1	0	0	0	0	0
540	0	0	1	0	0	0	0	0
541	0	0	0	0	0	0	0	0
542	0	0	0	0	0	0	0	1
543	1	0	1	1	0	0	0	0
544	0	0	1	0	0	0	0	0
545	0	0	0	0	0	0	0	0
546	0	0	0	0	0	0	0	0

547	1	1	1	0	1	0	0	0
548	0	0	1	0	0	0	0	0
549	0	0	0	0	0	0	0	0
550	0	0	1	0	0	0	0	0
551	0	0	0	0	0	0	0	0
552	0	0	0	0	0	0	1	0
553	0	0	1	0	0	0	0	0
554	0	0	1	0	0	0	0	0
555	0	0	1	0	0	0	0	0
556	0	0	1	0	0	0	0	0
557	0	0	1	0	0	0	0	0
558	1	1	1	1	1	0	0	0
559	0	0	1	0	0	0	0	0
560	0	0	0	0	0	0	0	0
561	0	0	0	0	0	0	0	0
562	0	0	0	0	0	0	0	0
563	0	0	0	0	0	0	0	0
564	0	0	0	1	1	0	0	1
565	0	0	0	0	0	0	0	0
566	0	0	1	0	0	0	0	0
567	0	0	1	0	0	0	0	0
568	0	0	1	0	0	0	1	0
569	0	0	0	0	0	0	0	0
570	0	0	0	0	0	0	0	0
571	0	0	1	0	0	0	0	0
572	0	0	1	0	0	0	0	0
573	0	0	1	0	0	0	1	0
574	1	1	1	1	1	0	1	1
575	0	0	1	0	0	0	0	0
576	0	0	0	0	0	0	0	0
Total	165	86	385	138	118	35	414	346
% Positive	28.65	14.93	66.84	23.96	20.49	6.1	71.9	60.1
	Top STEC	**O157**	**eae**	**stx2**	**stx1**	**EHEC-7**	**Eae**	**Stx**
	GDS Results					**NeoGen Results**		