Survival and Prevalence of Non-O157 Shiga Toxin Producing *Escherichia coli* (STEC) in Beef Cattle Preharvest and in the Environment

by

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Abstract

Cattle are primary reservoirs of shiga toxin producing *Escherichia coli* (STEC) and are considered "super-shedders" if they shed more than 10^4 CFU/g of *E. coli* O157:H7. In addition to *E. coli* O157:H7, six non-O157 STEC serogroups "big six" were added to the zero-tolerance adulterant list in June 2012. Limited information is available on the prevalence of non-O157 serogroups in beef cattle, especially during the pre-harvest stages.

Alabama has a large number of cow/calf operations. Most calves are birthed, weaned, and shipped from Alabama to commercial feedlots in the Midwest. Thus, the first purpose of this study was to determine the prevalence of non-O157 STEC among the beef cattle in Alabama. This was accomplished by acquiring fecal samples from cattle at 6 different Alabama Agricultural Experiment Station research centers and testing them for STEC. The second purpose was to identify shedding level changes in calves before and after weaning. For this study, 110 fecal samples were taken from calves located at E.V. Smith research center, both before and after weaning. These samples were tested for the presence of STEC. The third purpose was to isolate and serotype the STEC strains from "super-shedder" calves. The same E. V. Smith calf fecal samples as were used for purpose 2 were also used for purpose 3. Those fecal samples exhibiting a "super-shedder" load of STEC had their dominant STEC isolated and sent for serotyping. Based on findings, O26 proved to be the dominant "big six" member in both the research center and the calf study. STEC O26 isolates also displayed different virulence profiles in a multiplex PCR comparison, targeting three major virulence genes. For these reasons, the

fourth purpose was to investigate how O26's possession of different virulence profiles affected its survival in ground beef and different environmental samples. Overnight cultures of O26 strains containing two different virulence gene profiles were inoculated at various concentrations into separate samples of ground beef, cattle feces, bedding material, and trough water. STEC levels were observed over a 10 day period for the ground beef samples and a 30 day period for the environmental materials.

Results from these studies show that there were a greater number of positive STEC shedders in research centers sampled during the summer months and in herds containing calves. "Big six" STEC were isolated from the high shedders at 3 out of 6 research centers. STEC O103 was isolated from one research center and O26 was isolated from two others. The calf study shows that calves begin shedding STEC early in their lives and that weaning does generate an impact on STEC shedding levels. Nine out of 110 calves were found to be "super-shedders" both before and after weaning. "Big six" member O26 was also isolated from these "super-shedder" samples before and after weaning. Results show that virulence genes do not have an impact on STEC's ability to survive in ground beef and in the environment ($P > 0.05$), while the inoculation levels and sample types do ($P < 0.05$).

Further research should be conducted on control methods pertaining to the pre-harvest stage of cattle production. From this study's results, the gut microflora appear to be easily influenced since the dominant STEC before weaning was not the same as after. Also, more emphasis should be placed on studying and controlling STEC O26, as it was the dominant "big six" member in these studies.

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List of Abbreviations

CDC	Center for Disease Control
Ct	Cycle threshold
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *Escherichia coli*
FSIS	Food Safety and Inspection Service
HC	Hemorrhagic colitis
HUS	Hemolytic uremic syndrome
GFP	Green fluorescent protein
LB	Luria Bertani
OD	Optical density
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
RMAC	Ramnose-MacConkey agar
SDS	Sodium dodecyl sulfate
STEC	Shiga toxin Producing *Escherichia coli*
TBE	Tris borate EDTA
TE	Tris EDTA

TSA	Trypticase soy agar
TSB	Tryptic soy broth
USDA	United States Department of Agriculture
VTEC	Verotoxigenic *Escherichia coli*

Chapter 1. Introduction

1.1 Non-O157 STEC *Escherichia coli*

Escherichia coli is a very widely studied organism, but there is still a great deal unknown about the *Enterobacteriaceae* family. *E. coli* is a Gram negative, rod shaped, non-spore forming, facultative anaerobe, normally found in the lower gut of warm-blooded organisms. Most *E. coli* are part of the normal gut microflora and harmless to the human and animal population. One function of these is to inhibit other pathogenic bacteria from inhabiting the gut.

There are some which have gained more notoriety because of their association with human illness such as the Verocytotoxigenic *E. coli* (VTEC), also known as shiga toxin-producing *Escherichia coli* (STEC). These *E. coli* have gained virulence factors via plasmids, bacteriophages, transposons, etc. which make them harmful to humans (Lim *et al*, 2010). The names Verotoxin and Shiga toxin have been used interchangeably and are related to cellular cytotoxin production (Mathusa *et al*, 2010). Both shiga toxin and shiga-like toxin come from a group of protein toxins that will bind to the surface of the cell, gain entry, and once in the cytosol, will enzymatically inhibit protein synthesis or induce apoptosis.

1.1.1 Shiga toxin

Shiga toxin is made up of one catalytic A subunit and five B subunits which are capable of binding to receptors on a cell's surface. Once the B subunits have bound, the A subunit, an N-

glycosidase, will enter the cell using endocytosis and venture into the cytoplasm. Here it inactivates the RNA component of the ribosome, halts protein synthesis, and causes the death of the cell (Cary *et al*, 2000). Shiga toxin works to attack the blood vessels in the intestinal wall, causing hemorrhaging of the mucosa and bloody feces. In more severe cases, the shiga toxins will attack the endothelial cells of the kidneys. They are thought to gain access to the kidneys via the blood stream with their entry point being the intestinal wall. The shiga toxin is attracted to specific Globo series glycolipids like Globotriacylceramide (Gb3), which contain Galactose receptors. These are found in great quantities in the kidneys, specifically the glomerulus region, well known as the filtering system of the kidneys. The invasion and death of these endothelial cells can lead to clotting and renal failure in severe cases of infection (Cary *et al*, 2000).

The most common source of Shiga toxin is Shigella dysenteriae and a shiga-toxin producing *Escherichia coli* like O157:H7, O26, and other Enterohemorrhagic *E. coli* (EHEC). These EHEC are a subset of STEC which are known to cause bloody diarrhea, hemolytic uremic syndrome (HUS), hemorrhagic colitis (HC), fever, vomiting, and possible death in people who ingest them (Brooks *et al*, 2005; Mathusa *et al*, 2010). Symptoms of HUS are thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure, which can lead to death, especially in children (Etcheverria *et al*, 2013). According to Etcheverria *et al.* (2013), STEC usually affect children under 5, elderly, and people with immune systems which have been compromised.

There are two types of shiga toxin found in EHEC: shiga toxin 1(*stx*1) and shiga toxin 2 (*stx*2). Shiga toxin 1 is associated with less severe cases of illness and more closely related to the shiga toxin in *S. dysenteriae*, differing by only one amino acid. Serologically, they cannot be differentiated from one another, but *stx*2 is less closely related to the *S. dysenteriae* shiga toxin

and is known to cause more severe symptoms in the people it infects (Boerlin *et al,* 1999). It shares only 56% of the DNA sequence homology with *stx*1.

1.1.2 "Big six"

In past decades, studies on STEC have focused on *E. coli* O157:H7, simply due to its earlier discovery in human illness (Monaghan *et al,* 2011) and its involvement in the 1982 Michigan and Oregon outbreaks (Lim *et al,* 2010). Unfortunately, there is very little information on non-O157:H7 shiga toxin-producing *E. coli* (STEC), not because they lack involvement in human illness. More than 100 serotypes have been linked to cases of human illness, however, six have recently made a big impression. They are: *E. coli* O26, O111, O103, O121, O45, and O145 (Brooks *et al,* 2005). These six serotypes are now of greater importance than O157:H7 and have been placed on the zero tolerance adulterant list as of 2012 (USDA, 2011). According to the new regulation, any raw non-intact beef trimmings, both domestic and international, found to have non-O157 STEC must be considered adulterated and removed from the food chain (Stevens, 2012). For STEC, an infectious dose is thought to consist of less than 10 cells, so it takes only a few live pathogenic bacteria cells to make a human sick (Etcheverria *et al,* 2013; Tilden *et al,* 1996). Knowing that, the USDA has attempted to identify those STEC serogroups responsible for the most human illnesses and keep them from entering the food chain through systematic tests and recalls, starting with beef trimmings.

1.1.3 Outbreaks

Non-O157 STEC have been responsible for many illnesses and outbreaks all around the world. In 2006, STEC O103:H25 was involved in an outbreak which took place in Norway

(L'Abée-Lund *et al,* 2012). In 2011, there was a major outbreak involving STEC O104:H4, leaving 53 dead, 3,000 with acute gastroenteritis, and 600 with HUS (Hauswaldt *et al,* 2013). There was also a recent multistate outbreak of STEC O102 infections in the United States in March of 2013. The non-O157 STEC serogroups involved in these outbreaks cannot yet be rapidly identified and even though the number of clinical labs which test for them have increased, some still do not perform the required tests to identify these STEC. For these reasons, and according to an article on the Centers for Disease Control and Prevention website, many non-O157 STEC infections go unidentified and unrecorded (CDC, 2013). As of 2012, the CDC estimates that STEC cause 265,000 illnesses per year, 168,698 due to non-O157 STEC (CDC, 2012). Estimates of illnesses due to non-O157 STEC are quite possibly on the low side.

1.2 Virulence of STEC

Some people have noticed a problem with the STEC classification system and believe virulence should play a larger role in it (Mathusa *et al.* 2010). Instead of focusing on the virulence profiles to determine whether or not an organism is pathogenic, STEC, in the past, has been identified as pathogenic based solely on serotype (Mathusa *et al*, 2010). This is an inefficient system. For example, not all *E. coli* O103:H2, a serogroup on the zero tolerance list, are pathogenic. The public and laboratory workers only looking to identify serogroups will classify these STEC as harmful to the public when they may not be. More focus should be placed on the virulence of each strain rather than branding the whole serogroup as pathogenic.

There are several virulence factors involved in the pathogenicity of non-O157 STEC. The presence of *stx1*, *stx2*, or a combination of the genes is the most important indicator as to whether or not a bacterium is virulent (Etcheverria *et al,* 2013). Strains containing *stx*2 are

considered more virulent than those containing *stx1* and are more often associated with HUS (Etcheverria *et al,* 2013). The second most important virulence factor is the *eae* intimin gene, which is used by the bacteria to attach to epithelial cells, inducing an attaching and effacing (A/E) lesion on the surface of the target cell (Etcheverria *et al,* 2013). Even though the presence of virulence genes such as *eae* is a good indicator of pathogenicity, its absence does not necessarily mean it is not pathogenic (Beutin *et al,* 2006). The presence of intimin is associated with the more severe cases, but there have been less severe cases of sickness caused by intimin-negative STEC (Beutin et al, 2004).

The pathogenic non-O157 STEC were previously unrecognized as harmful in the food industry. This may be because they did not always have the virulence that they do today, or recently advanced methods of rapid detection now allow for the identification of these pathogenic bacteria that may not have necessarily been a concern in the past (Beutin *et al,* 2006). Research has been traditionally focused on O157:H7 (Beutin *et al*, 2006). Since 2000, researchers have become more aware of the existence of non-O157 STEC and testing has become more frequent, so it may only seem like these bacteria have become a greater problem these past few years (Mathusa *et al*, 2010).

1.3 Non-O157 STEC O26

Among the 6 STEC strains, a lot of the non-O157 STEC studies have been focused on non-O157 STEC O26 because it is notorious for having those virulence factors which can make people sick (Mathusa *et al*, 2010). One study in particular examines how the O26 virulence profile has changed over the years. The results from a study by Zhang *et al.* (2000) show that all O26 isolates collected between 1965 and 1994 expressed only *stx1* while most O26 isolates

collected between 1995 and 1999 had either *stx2* alone or a combination of *stx1* and *stx2*. This new virulence profile was found to have a "high pathogenic potential for humans" so more research is conducted on this STEC (Mathusa *et al,* 2010). Another study in France emphasizes the importance of researching the survival of non-O157, especially STEC O26, in the soil and environment (Fremaux *et al,* 2007). The researchers found that O26 has survivability similar to O157:H7 and its ability to survive for a very long time, up to and possibly more than 18 weeks in cow feces at 15°C (Fremaux *et al,* 2007).

1.4 Sources of STEC

Outbreaks are most often due to the consumption of beef, dairy, or vegetable products which have come in contact with cattle feces (Etcheverria *et al,* 2013). Ruminant animals like cattle are reservoirs for STEC (Mathusa *et al,* 2010). STEC which can cause illness in humans are not normally harmful to the adult cattle that harbor them. Some serogroups have been the cause of diarrhea in calves, but nothing more serious than this has been observed (Shaw *et al,* 2004). Beef carcass processing has been scrutinized as a major contamination control point (Mathusa *et al,* 2010). During the slaughter process, pathogens from the feces, hides, or environment can contaminate the carcasses (Midgley and Desmarchelier, 2001).

STEC contamination is not limited to cattle products, but there have been a higher frequency of cases involving beef or veal (Mathusa *et al,* 2010). Most illnesses associated with meat are due to the consumption of undercooked products (Soon *et al,* 2011). Even though these products are considered safe when prepared properly, such as cooking the meat to an internal temperature which would eliminate all possibility of illness, customers continue to demand a safer product. Even though there can be possible cross-contamination in the slaughter facility,

STEC do not originate there. Scientists can often link the STEC responsible for these illnesses back to the farm they originated from using a fingerprinting method call Pulse Field Gel Electrophoresis (Soon *et al*, 2011).

1.4.1 Super-shedders

According to Chase-Topping et al. (2008), the definition of super-shedding cattle are those which shed 10^4 CFU (colony forming unit)/g STEC O157:H7 or greater in their feces. This definition has also been applied to non-O157 STEC (Menrath *et al*, 2010). Super-shedder cattle are thought to be the major cause of higher transmission rates and cross-contamination within cattle producing environments (Arthur *et al*, 2013). Therefore, focusing research on reducing the number of STEC that these individual super-shedders put out will greatly reduce the contamination of the production environment (Arthur *et al*, 2013; Menrath *et al*, 2010). A study by Baines and Erb (2013) suggests that calves infected with *stx*-positive strains of STEC tend to shed significantly more STEC in their feces than those infected with *stx*-negative strains of STEC. The presence of the *stx* gene could possibly have an impact on the STEC survival in the cattle gut. More research needs to be done on methods for reducing the number of non-O157 STEC in these super-shedders because it could serve as a good control point before slaughter (Menrath *et al*, 2010).

1.4.2 Survival of STEC in the environment

Non-O157 STEC has seasonality as its numbers are highest between June and September and its peak has been observed in August (Brooks *et al*, 2005). Farmers and food industry workers should be aware of the greater chance of contamination during the summer months and

know that this is the time to take more precautionary measures. In several studies, the ability of *E. coli* O157:H7 and non-O157 STEC to survive long-term in feces has been observed. This being said, irrigating with a manure mixture or using the same field for another group of cattle could lead to contamination of that vegetable crop or its new occupants (Fremaux *et al*, 2007; Fukushima *et al*, 1999).

In fields, fecal material has been shown to harbor *E. coli* for at least 150 days (Alexander *et al*, 2008). This also has implications for feedlots, transport trailers, and holding pens at slaughter facilities. At 15°C, cross contamination can easily happen 16-18 weeks after the cattle who shed the bacteria are gone (Fukushima *et al*, 1999). There have also been studies on clay and sandy soils containing non-O157 STEC and it took 40 days and 62 days for there to be a 1 log reduction of bacteria in clay and sandy soil respectively (Bolton *et al*, 2011). The clay had a faster reduction time because of its slightly acidic nature, which makes it a less favorable environment for STEC (Bolton *et al*, 2011). In another study, clay-ridden soils offered a more suitable environment for STEC because it is better able to hold bacteria and it creates a shield against microbial predators (Fremaux *et al*, 2007). These studies show that STEC are able to persist for an extended period of time in soils and this could be a detriment to human health.

Water troughs which have been contaminated with fecal material can also be a point of cross-contamination for cattle (Midgley and Desmarchelier, 2001). Many studies have shown that *E. coli* has an excellent survivability in the environment for an extended amount of time and that it is also a good source of antibiotic resistance, especially on a farm (Alexander *et al*, 2008). These antimicrobial resistant bacteria can contaminate any new residence of the field, transferring the resistance to the microbes in the gut of the next occupants. Because *E. coli* is a prominent bacterium in the cattle gut and is known to readily transfer resistance to other bacteria,

it can serve as a reservoir for antimicrobial resistance genes within the lower intestines of cattle (Alexander *et al.* 2008).

1.4.3 Survival of STEC in Food

Certain factors can influence the survival of STEC in foods such as temperature, pH, salt levels, and water availability (Mathusa *et al*, 2010). Studies have examined different food items for the presence of non-O157 STEC and have discovered them at various intensities in salads, milk, beef, pork, lamb, poultry, seafood, and cheeses (Mathusa *et al*, 2010). A study by Samadpour *et al.* (1994) searched for the *stx* genes in food samples and these researchers found that veal had the highest count (63%) and lamb followed with 48% of the samples being positive for *stx*. Some STEC are acid tolerant, surviving in low pH environments (Mand *et al*, 2012) and in foods like apple cider and fermented meats. A lot of preserved and processed foods have organic acids present, which some non-O157 STEC have the ability to adapt to, given the opportunity (Brudzinski and Harrison, 1998). By the mere fact that a low number of inoculants can make a person sick, they must have a resistance to the normal acid conditions of the human stomach (Cary *et al*, 2000). STEC strains are also very resistant to stressful conditions like high pH and salt concentrations, (Elhadidy and Muhammed, 2012) making it more difficult to find effective control measures in food industries, and improving the chances that they will come in contact with humans.

As for treatments on foods for the prevention of STEC growth, Gilbreth *et al*. (2009) looked into how O157:H7, O145, O111, and O26 were affected by antimicrobial treatments that would be sprayed on the surfaces of meat. After treating the contaminated meat with a solution of acidified sodium chloride, octanoic acid, and peracetic acid, the researchers concluded that

there was no large difference in sensitivity to the treatment (Gilbreth *et al*, 2009). They also concluded that those treatments previously used for O157:H7 control could be just as effective on non-O157 STEC (Gilbreth *et al*, 2009).

1.5 Antibiotic resistance of STEC

1.5.1 Antibiotic usage in animals

Antibiotics have been used since the 1950's to increase feed efficiency, for therapy, and to prevent disease in cattle during certain times when risk of disease is high (Alexander *et al*, 2008). The use of antibiotics like chlortetracycline on a regular basis as a growth promoter contributes to the rise in new antibiotic resistant *E. coli* pathogens (Alexander *et al*, 2008). For this reason, the European Union in 1999 banned the use of antimicrobial growth promoters (Casewell *et al*, 2003).

Oxytetracycline has been used to treat bacterial disease in cattle (Alexander *et al*, 2008). Many researchers are concerned about antibiotics used in both human and animal medicine such as tetracycline (Alexander *et al*, 2008). They fear that overusing these antibiotics in animals will result in bacterial resistance which could quite possibly transfer to humans and make human treatments with the same or similar antibiotics ineffective.

Tetracycline is known to have a broad ranging effect on both Gram-positive and Gram-negative bacteria, and different types of it are used as growth promoters, and to treat a whole host of bacterial infections (Alexander *et al*, 2013). Like tetracycline, both ampicillin and streptomycin have been used as a broad spectrum antibiotic against Gram positive and negative bacteria. Streptomycin has a history of being used to treat TB in humans and is also used in Veterinary medicine for large animals. Ampicillin has been used for many years to treat bacterial

infections in humans. It is cause for concern when studies like Srinivasan *et al*. (2007) are finding high numbers of multidrug resistant STEC coming from beef and dairy cattle in the United States.

Another antibiotic of concern is cephalosporin. Along with subsequent generations of this antibiotic, it has been used in human medicine as a broad spectrum treatment against both Gram positive and negative bacterial infections. There has been a noticeable increase in the number of cephalosporin resistant *E. coli* in the soil. Ceftiofur, a third generation cephalosporin, is used to treat foot, uterus, and respiratory infections in cattle. Due to its convenience and effectiveness, it is used a great deal by farmers. With its greater usage, scientists have been seeing an increase in enteric bacteria with ceftiofur resistance (Subbiah *et al*, 2012).

1.5.2 Antibiotic resistant bacteria formation

Bacteria can gain resistance naturally through random genetic mutation. Resistance can also be generated by the application of a stress on a population of bacteria (de Verdier *et al*, 2012). In this case, only those with the resistance gene can survive, multiply, and pass their genetic code onto subsequent generations and other bacteria. Bacteria can transfer the resistance to daughter cells by vertical gene transfer or to other bacteria in the vicinity through horizontal gene transfer involving a plasmid exchange (Price *et al*, 2008).

Antibiotics and antibiotic resistant bacteria can affect humans in a few different ways. When contaminated food is eaten, the resistant bacteria from the food may transfer resistance to other bacteria in the human gut (Sahoo *et al*, 2012). A study in Scotland reveals that the "release of free *stx*-encoding bacteriophages into the intestinal environment of cattle by STEC O157:H7 or other EHEC may permit horizontal transfer of virulence genes to *E. coli* O26," one of the six

zero tolerance *E. coli* (Chase-Topping *et al*, 2012). This emphasizes the possibility of transference within the intestines. Antibiotic residues in consumed food can also influence the human gut microflora by encouraging a natural selection of resistant bacteria (Sahoo *et al*, 2012). Gram negative bacteria such as *E. coli* are common inhabitants in the intestines of warm-blooded animals; these bacteria can often be carriers and conveyors of antimicrobial resistant genes, reflecting the intestine's past exposure to antimicrobials (de Verdier *et al*, 2012).

Another avenue of obtaining resistant bacteria is through its excretion and interaction with the environment (Sahoo *et al*, 2012). Resistant bacteria shed from the human or animal gut into the soil or water could interact with other *E. coli* in the environment (Sahoo *et al*, 2012). There is research by Yue *et al*, (2012) which says phage mediated gene transfer events can happen in the environment with the help of temperature and UV light. Feedlot sludge could also be a good environment for horizontal gene transfer, leading to the creation of new pathogenic bacteria if those genes contain the right virulence factors (Yue *et al*, 2012). According to Sáenz *et al*, (2004), normal microflora "play(s) a key role as an acceptor and donor of transmissible antimicrobial resistance mechanisms."

It is difficult to pinpoint exactly which antimicrobials most STEC are resistant to because resistance is different for every region, every animal, and every strain that is examined. It is also hard to compare different studies when they are not testing the same antimicrobials on STEC isolates. A few studies though, have noticed a high resistance to different forms of streptomycin, tetracycline, and ampicillin, among others (Mora et al, 2005 and Sasaki et al, 2012). Sasaki *et al*. (2012) noted, when comparing STEC O157:H7 isolates to O26 isolates in beef cattle, that the O26 strains had a significantly higher antimicrobial resistance rate. Several studies also mention an association between antimicrobial resistance and having the *eae* and *stx* virulence genes

(Mora *et al*. 2005; Sasaki *et al*. 2012). Mora *et al*. (2005) noticed a relationship between non-O157 STEC being resistant to multiple antimicrobials and also having the *eae* and *stx*1 virulence genes. Sasaki *et al*. (2012) had similar findings and suggests that the antimicrobial resistance has helped the O157:H7 containing both *stx*1 and *stx*2 genes to grow considerably greater in number.

1.6 Pre-Harvest Management and Control

1.6.1 Effects of stresses on STEC shedding

There are various stresses which can affect the normal physiological/psychological homeostasis of an animal. For cattle, some of these stresses are weaning, transportation, fasting, diet change, and handling (Rostagno and Marcos, 2009). Such stressors will affect the gastrointestinal tract, causing an increase in pH, intestinal motility, and passage of foodborne pathogens (Rostagno and Marcos, 2009). Weaning alone provides a number of stressors because farmers most often do it abruptly, unlike the natural, gradual process of weaning. This abrupt switch to a new diet, new environment, and new social situation provides great stress to the young animal. Although it is a very stressful event, it is necessary for cattle operations to do this abrupt weaning so that they can get their breeding herd pregnant again as soon as possible (Enriqeuz *et al*, 2011). Some producers like to transport their calves right after weaning to save on feed costs. Enriquez *et al*. (2011) suggests exposing the calves to certain things before weaning like the new feed, handling, and the new environment to reduce the amount of stress on the animal.

With an increase in stressors, the number of animals carrying foodborne pathogens will also increase because it suppresses their immune systems, making the GI tract more open for colonization (Salak-Johnson and McGlone, 2007). This could very well cause a shift in dominant

gut microflora (Edrington *et al*, 2011). Calves in particular are more susceptible to STEC infection and the possible development of chronic scours when stressed (Baines and Erb, 2013). While they are still young, their gut microflora is still being established and can easily be influenced. With an increase in shedding and an increase in the number of cattle open to colonization, more of the herd may be involved in cross-contamination. Also, with more fecal shedding and possible close contact with each other, more hides can become contaminated (Rostagno and Marcos, 2009).

1.6.2 Control methods

Due to the importance of STEC, researchers in the past have focused on finding control measures for reducing the *E. coli* contamination after slaughter with little headway recently (Etcheverria *et al*. 2013). Therefore, focus has switched to controlling pathogenic populations in and on the cattle before slaughter. If this can be done effectively, there will be minimal carcass contamination once the cattle reach the slaughter facility. This pre-harvest intervention, combined with controls already in place at the slaughter plant, should create an effective resistance against pathogenic non-O157 STEC.

Research has been done on controlling the number of *Escherichia coli* shed in the feces of cattle destined for slaughter by changing their diet and looking at how fasting affects the bacterial count. Cattle have shown a significantly lower concentration of *E. coli* in their feces when fed a high roughage diet for an extended period of time, but fasting can reverse the effects of the roughage diet, causing an increase in *E. coli* shedding only 24 hours after being implemented (Jordan *et al*, 1997). Also, although a study which fed cattle a high roughage diet 4 days before slaughter showed a significant decrease in fecal *E. coli* counts, it is neither practical

nor cost-effective for feedlot owners to switch to a diet that would reduce the rate of weight gain in their cattle (Jordan *et al*, 1997).

Some vaccines have been developed to reduce STEC shedding in cattle. One in particular is based on the type III secreted proteins; another uses the adherence proteins intimin, Tir, and EspA (Etcheverria *et al*, 2013). The type III secretion system is specific to Gram negative bacteria and their ability to secrete the toxins which make people sick.

Midgley and Desmarchelier. (2001) recommend some measures to be taken at the slaughter facility to reduce the spread of STEC. Providing clean feed and water to each new group of cattle, cleaning out fecal material in between groups, and holding high risk(calves) and low risk cattle(adults) in separate pens would greatly reduce cross contamination among the cattle (Midgley and Desmarchelier, 2001). Probiotics and prebiotics have also been implemented in an attempt to control STEC shedding. A study by Baines and Erb. (2013) investigated a prebiotic called Celmanax® on calves during the spring and summer months to see if it reduced STEC shedding. Celmanax® reduced the number of STEC shed and prevented the acquisition of new strains during the spring, but there was no difference seen in the summer when the calves were shedding significantly more STEC (Baines and Erb, 2013). The authors indicated that the stressful feedlot environment may have also had an influence on fecal shedding (Baines and Erb, 2013). An interesting study by Rund *et al*. (2013) looked at the probiotic effect of *E. coli* strain Nissle 1917 (EcN) on two O104:H4 isolates involved in the large Germany outbreak in 2011. In culture, it greatly reduced the adherence, growth, and *stx*2 production of the O104 isolates (2013). These promising results indicate that there is room for further advancement in the realm of probiotics to fight non-O157 STEC.

1.7 Rapid Detection Methods

Non-O157 STEC do not have distinct biochemical markers such as an inability by O157:H7 to ferment sorbitol or Beta-glucuronidase activity (Fratamico *et al*, 2009; Fremaux *et al*, 2007). This is what makes studying these bacteria difficult; such identifiers would help with screening for these pathogens.

There are multiple media types currently being used to isolate these STEC. One uses a base of MacConkey agar and this is supplemented with such things as sucrose, bile salts, sorbose, novobiocin, potassium tellurite, and a carbohydrate mixture (Mathusa *et al*, 2010). This has been used to isolate 4 specific serotypes: O26, O103, O111, and O145. They will show up as different colored colonies, which helps with identification. Another agar type being used is a chromogenic agar (Mathusa *et al*, 2010). This helps with isolating STEC in general, but when differing serotypes are plated on this agar, they cannot always be differentiated from one another and may appear differently based on incubation time and the number of colonies on the plate. Most colonies will appear mauve in color. A third agar being used is called Rainbow agar (Mathusa *et al*, 2010). STEC O26, O103, and O121 will appear pink; O45, O111, and O157 will show up as grey, light blue, or purple, and O145 may appear dark blue. An agar developed specifically for isolating *E. coli* O26 is called rhamnose-MacConkey agar (RMAC), based on O26's ability to ferment rhamnose, while other STEC cannot. STEC O111 has been effectively isolated on a chromocult agar mixed with cefixime, cefsulodin, and vancomysin (Mathusa *et al*, 2010). Colonies appear purple on this agar.

1.7.1 Polymerase chain reaction (PCR)

PCR has been used to test for the presence of the shiga toxin 1(*stx*1) and shiga toxin 2(*stx*2) genes, which would indicate the presence of STEC with the potential to be pathogenic. The polymerase chain reaction amplifies a certain length of DNA millions of times in a very short amount of time. The thermo-cycler will cycle through various temperatures to create the right environment for amplification. First, the machine raises the temperature to 94-96°C, which denatures or breaks up the DNA strands into single-stranded DNA chains. Next, the temperature is lowered to 50-65°C so that the forward and reverse primers can attach or anneal to the complimentary length of base pairs on the DNA strands. After the annealing step, the temperature is raised to 72°C for 1 minute or more, allowing *Taq* polymerase to bind to each priming site and extend/synthesize a new DNA strand. The thermo-cycler then raises the temperature once again to 94-96°C to denature the DNA and start the cycle over again. This goes on for about 30 cycles. If the annealing temperature is too high or too low, the primers will either not bind at all or lose specificity, binding to other sites of similar sequence. Specificity is the propensity for a primer to bind to a specific target region of the DNA strand and not to other non-specific target areas (Bartlett and Stirling, 2003). This can be difficult when working with multiplex PCR, as there will be multiple primers with different melting temperatures.

In preparing the DNA for PCR, the DNA must first be extracted from the cells. A simple cell suspension in water and boiling for 10 minutes will work, but it does not give the best results. Suspending the cells in the PrepMan Ultra reagent produces a purer sample of DNA. It also puts out a higher concentration of DNA so that less template needs to be used to produce the same results (Wasilenko *et al*, 2012) Using a TE buffer method of DNA extraction does not give as pure, or as highly concentrated a sample as PrepMan, but it does give a lower cycle threshold (Ct) value when used in real time PCR. Lower Ct values indicate strong positive reactions, so in

the interest of saving money, it is acceptable to use TE buffer for real time PCR preparation or the simple water boiling method for regular PCR DNA extraction.

Research studies have been devoted to finding a reliable rapid detection method for identifying STEC strains in meat products and feces. PCR has become a common practice in microbiology/food safety labs, using the amplification of certain known virulence genes to test for the presence of certain pathogens. Within the same serogroup such as O26, there can be *stx*-positive and *stx*-negative versions of the bacteria (Fratamico *et al*. 2009). Most of the time, researchers are concerned with those containing the *stx* genes because they contain the means to make the toxin which makes us sick. Some researchers also use *wxz* and *wxy*, which are o-antigen gene clusters specific to the antigen and valuable for identifying individual O serogroups (Wang *et al*. 2010). If the goal is to identify a specific, already known serogroup within a material, the best method would be to use the wxz and wxy gene primers. This is not an effective method if the serogroup(s) is/are unknown.

1.7.2 Pulse Field Gel Electrophoresis (PFGE)

In addition to PCR, another technology has been widely used to better identify the contamination sources, Pulse Field Gel Electrophoresis (PFGE). PFGE is used for separating large pieces of DNA in a gel, similar to a regular electrophoresis technique. PFGE periodically switches voltage paths in three different directions, but the DNA continues to run along one central axis. This helps pull the larger DNA fragments apart. In preparing the DNA, the protocol calls for the lysis of the organism imbedded in agarose and the usage of restriction enzymes which cut the DNA at certain restriction sites, resulting in 10 to 20 fragments. These fragments will be loaded in an agarose gel and pulled apart in the electrophoresis machine over a period of

approximately 19 hours. Larger DNA fragments will be located towards the top of the gel and smaller ones will be located towards the bottom. PFGE is often used to compare strains of the same serogroup. If the strains are exactly the same, the enzymes will cut the DNA strands at the same points, resulting in fragments of the same size, which will appear at the same points along the electrophoresis gel.

Researchers like Miko *et al*. (2013) use PFGE to compare the genetic similarity of pathogenic outbreak strains to other outbreak strains or other farm strains. This way, strains that make humans sick can be traced back to the farm they originated from. After identifying the source of contamination and investigating the operation's regular practices, more control measures may be needed to prevent any future pathogens from entering the food chain at that particular facility. If necessary, PFGE can provide the evidence needed for the recall of a contaminated product.

1.8 Summary

E. coli are natural inhabitants of the gastrointestinal tracts of warm-blooded animals such as cattle, sheep, humans, etc., but there are a few specific strains in the EHEC subgroup which have been known to make people sick. Sickness can vary in severity depending on what virulence genes the strain contains and the state of the infected person's immune system. STEC strains containing the *stx*2 gene have been associated with more severe cases of sickness, as these are more likely to have HUS. The first STEC to be investigated and identified as harmful was *E. coli* O157:H7, but recently, 6 non-O157 STEC have become a more important public health concern and have been placed on the zero tolerance list.

There is no well-established method for testing for these STEC, but some rapid detection methods like PCR and PFGE are currently being implemented in combination with different selective and differential medias like Rainbow, Chromogenic or a MacConkey-based agar. Many scientists are presently researching new methods for isolation and identification of the non-O157 STEC, especially the "big six." The classification of pathogenic STEC must also be taken into consideration when trying to identify these STEC. Identifying them as virulent based on their serotype may not be the best course of action when individual strains have been shown to vary in their virulence profiles.

Another major topic of concern, related to these STEC, is the control of antimicrobial resistant bacteria. *E. coli* is known to readily transfer resistance to other bacteria and since it resides naturally in the gut, *E. coli* in general is a good reservoir for antimicrobial resistant genes.

It has been suggested that the best course of action for controlling these pathogens is to use a combination of pre-harvest and post-harvest control measures. Using vaccinations, probiotics, prebiotics, proper herd management, and studying "super-shedding" cattle are all being researched for improving pre-harvest control. Post-harvest control measures, such as acid washes used for O157:H7 control, have been fairly effective against non-O157 STEC.

More research needs to be conducted on the pre-harvest management side to find the best combination of control methods. Advancement in identifying and isolating these non-O157 STEC is also a necessary endeavor.

Chapter 2. Materials and Methods

2.1 Collection and processing of samples from the research centers

Six Alabama Agricultural Experiment Station research centers were sampled including Wiregrass, Sand Mountain, Gulf Coast, Tennessee Valley, Upper Coastal Plain, and Black Belt research centers. Approximately 30 fecal samples were taken rectally from cattle at each of these research centers and shipped back to the lab in coolers. These samples were processed immediately upon arrival. Twenty five grams of each sample was measured out into a Whirl-pak® bag (Nasco, Fort Atkinson, WI) and 100ml of 0.1% peptone water was added to this. Samples were then stomached for 2 minutes in a Smasher™ lab blender (AES, Hamilton, NJ). Two 100µl samples of the slurry were then plated in duplicate on two plates of CHROMagar™ STEC (CHROMagar, Paris, France). Plates were incubated overnight at 37°C and enumerated the following day. Positive STEC colonies on the agar were expected to display a mauve or pale purple color and negative colonies were expected to be colorless, blue, or inhibited.

2.2 Sample collection and processing procedures for calf testing

Fecal samples were taken from 110 beef calves at both 6 months (before weaning) and 9.5 months of age (after weaning).

At the E.V. Smith research center, fecal samples were taken rectally from the cattle and put in 100ml sterile specimen containers (VWR® Radnor, PA). ID numbers were written down in a notebook and on each sample cup. These numbers were located on the cattle's ear tags.

Samples were placed in a cooler on ice, and taken back to the food microbiology lab in Upchurch hall for further processing. After arriving at Upchurch Hall, 25g samples of feces were removed from each cup using a clean spatula and place in a labeled, filtered Whirl-pak® bag (Nasco, Fort Atkinson, WI). 112.5 ml of 0.1% peptone water was added to the filtered Whirl-pak® bag (Nasco, Fort Atkinson, WI) and then the mixture was placed in a smasher for 2 minutes at regular speed. All fecal samples were processed as described above.

Two CHROMagar™ STEC (CHROMagar, Paris, France) plates, two CHROMagar™ O157 (CHROMagar, Paris, France) plates, and one MacConkey plate was plated for each sample. The plates were labeled with the sample number and then 100µl of each sample was pipetted from the Whirl-pak® bags (Nasco, Fort Atkinson, WI) bag onto each plate and spread evenly. Plates were allowed to sit for 10 minutes before being inverted and placed in a 37˚C incubator for 24 hours. Lastly, 111.5ml of the 2xlactose broth was added to each Whirl-pak® bag (Nasco, Fort Atkinson, WI) containing a fecal sample. These were enriched for 24 hours at 35˚C.

After 24 hours, the positive colonies were counted. For any samples that were completely negative for STEC or O157, samples were taken from the enriched, incubated Whirl-pak® bags (Nasco, Fort Atkinson, WI) and streaked onto the appropriate agar to determine if the samples were positive for STEC or O157:H7. These plates were put in the incubator at 37˚C for 24 hours.

STEC plates from super-shedder cattle were saved. Three positive, well-isolated colonies were picked from each plate and streaked on CHROMagar™ O157 (CHROMagar, Paris, France) plates to make sure they were not O157:H7. Once those colonies were confirmed to be non-O157 STEC, one was transferred to trypticase soy agar (TSA) slants for further analysis.

2.3 DNA extraction (Simple boiling method)

Purified non-O157 isolates were then tested for the presence of shiga toxin 1(*stx*1), shiga toxin 2 (*stx*2), and intimin (*eae*A) genes. Overnight fresh broth cultures were obtained for each isolate and 1ml of each culture was washed and resuspended in 100µl of ultrapure water. The resuspended cultures were boiled for 10 minutes and centrifuged for 2 minutes at 12000rpm. The supernatant containing DNA was then transferred to new tubes and stored at -20°C or used right away for PCR.

2.4 DNA extraction (Prepman® boiling method)

Procedures were the same as the simple boiling method except for one part. Instead of resuspending the pellet the second time with 100µl of ultrapure water, 100µl of Applied Biosystems® Prepman® Ultra (Life Technologies, Carisbad, CA) was used. The Prepman® Ultra was used for testing the "big six" non-O157 STEC cultures obtained from the Thomas S Whittam STEC Center at Michigan State University and also the STEC O26 used in the ground beef and environmental survival tests. Any other DNA isolates were extracted using the simple boiling method.

2.5 Polymerase chain reaction (PCR)

A multiplex PCR assay targeting three different virulence genes determined the virulence profiles for all six O26 isolates. Target virulence genes included the *eae*A intimin gene, (*stx*1) and (*stx*2). AccuStart™ II PCR Supermix (2x) (Quanta BioSciences, Gaithersburg, MD) was used for the multiplex PCR. A 1:10 dilution of the DNA samples with ultrapure water was used as a PCR template. Primers were synthesized by Integrated DNA Technologies (IDT®)

(Coralville, Iowa) and those primers were resuspended in a 1x TE buffer solution. The concentration of each primer stock solution was 100µM and these primers were stored at -20°C before being diluted for PCR usage. The working solution for each primer was 10µM. In each 0.1ml PCR tube, the following reagents were mixed together:

Table 2.1: Volumes of Reagents and Primers for PCR

Reagent/Primer	Volume (µl)	Concentration
PCR Template	5.0	Varies with sample
*stx*1 Forward Primer	0.25	10µM
*stx*1 Reverse Primer	0.25	10µM
*stx*2 Forward Primer	0.25	10µM
*stx*2 Reverse Primer	0.25	10µM
*eae*A Forward Primer	0.25	10µM
*eae*A Reverse Primer	0.25	10µM
PCR Super Mix	12.5	1x
Ultrapure Water	6.0	
Total Volume	25 per sample	

The template was replaced with TE buffer or water as a negative control. The PCR reaction was carried out in Applied Biosystems® Veriti® 96-Well Fast Thermal Cycler (Life Technologies™, Carlsbad, CA). 30 cycles of 30 sec. 94°C denaturation, 30 sec. 58°C annealing, and 1 minute 72°C extension were carried out following the initial 3 minute 94°C denaturation. Samples were then held at 4°C for 30min before being taken out of the thermocycler. After the run, PCR products were checked by running the samples on a 2% agarose gel at 120 volts for 42 minutes.

Table 2.2: PCR Primer sequences

PCR Primers	5'-3'	Bp	Tm	Ta	Reference
***stx*1 Forward**	ATAAATCGCCATTCGTTGACTAC	**180**	**58.4**°C	**52.4**	**(Paton and Paton, 1998)**
***stx*1 Reverse**	AGAACGCCCACTGAGATCATC		**58.9**°C		
***stx*2 Forward**	GGCACTGTCTGAAACTGCTCC	**255**	**59.6**°C	**55.0**	
***stx*2 Reverse**	TCGCCAGTTATCTGACATTCTG		**58.0**°C		
***eae*A Forward**	GACCCGGCACAAGCATAAGC	**384**	**62.9**°C	**51.8**	
***eae*A Reverse**	CCACCTGCAGCAACAAGAGG		**61.3**°C		

2.6 Pulse field gel electrophoresis (PFGE)

A PFGE was completed for the farm and clinical-isolated strains of O26 to identify any similarities.

A well isolated colony was selected and streaked from test cultures onto Trypticase Soy Agar (TSA) plates for confluent growth. These cultures were incubated at 37°C for 14-18 hours. A 1% agarose mixture was made and divided into glass tubes for future use. TE buffer, cell suspension buffer, and cell lysis buffer were made according to the following procedures:

-TE buffer was made using10 ml of 1 M Tris (pH 8.0) and 2 ml of 0.5 M EDTA (pH 8.0). This mixture was diluted to 1000 ml with ultrapure water.

-Cell Suspension Buffer was made by mixing100 ml of 1 M Tris (pH 8.0) and 200 ml of 0.5 M EDTA (pH 8.0). This was diluted to 1000 ml using ultrapure water.

-Cell Lysis Buffer was made using 25 ml of 1 M Tris (pH 8.0), 50 ml of 0.5 M EDTA (pH 8.0), and 50 ml of 10% Sarcosyl (N-lauroylsarcosin, sodium salt). This mixture was diluted to 500 ml with ultrapure water.

The next day, an Eppendorf™ Thermomixer™ R (Fisher Scientific, Pittsburgh, PA) was set to 55°C and a regular water bath was also set to 55°C. One ml of 20% SDS was preheated to 55°C in the water bath along with 3 liters of TE buffer and 2 liters of ultrapure water. The premade agarose was reheated in the small glass tube until completely melted. Five hundred microliters of 20% pre-heated SDS per 9.5 ml agarose was added and mixed well. The final

concentration of SDS was 1%. The agarose tube was recapped and returned to the 55°C water bath.

Fifteen milliliter centrifuge tubes were labeled with culture numbers and 5 ml of Cell Suspension Buffer was added to the tubes. A sterile loop was used to remove some of the growth from each agar plate. Cells were suspended in the cell suspension buffer by spinning the loop gently to evenly disperse them. The concentration (OD_{600}) of cell suspensions was measured by the use of an Ultrospec 10 Cell Density Meter (GE Healthcare, Piscataway, NJ). The OD value of each sample was adjusted to 1.3-1.4 by the addition of more cells or cell suspension buffer. Along with the other samples, the *Salmonella* ser. Braenderup H9812 standard was prepared. It was used as a control in this experiment.

Plug making: Wells of the disposable plug molds were labeled. Four hundred microliters of adjusted cell suspensions were transferred to labeled 1.5 ml centrifuge tubes. Twenty microliters of Proteinase K (20 mg/ml) was added to each tube and mixed gently by pipetting up and down 5-6 times. Four hundred microliters of the melted agarose/SDS mixture were added. The mixture was quickly but also gently mixed together, avoiding the formation of bubbles, and dispersed into wells of plug mold. Two plugs per strain were made. Plugs were allowed to solidify at 4°C for 5 min. The agarose tubes were kept in a beaker of warm water (55°C) to keep it from cooling too quickly.

Lysis of cells in agarose plugs: Two milliliter centrifuge tubes were labeled with culture number and lysis reagents were measured out into the test tubes. One and a half milliliters of cell lysis buffer and 25 µl Proteinase K (20 mg/ml) was added to each 2 ml tube. After they

solidified, plugs were transferred from mold to the prepared tubes (one tube per strain). Tubes were placed in a rack and incubated in the water bath at 55°C for 1 hour with constant and vigorous agitation (150-175 rpm).

Washing of agarose plugs after cell lysis: The temperature of the shaking water bath was lowered to 50°C and the tubes were removed. The sterile green wash column was placed under the hood and column caps were placed wide-end-side down. A clean spatula was used to remove plugs from tubes and place them on narrow end of green lid. Caps were labeled with a marker and screwed onto the next cap. This was repeated for each sample. The green wash column was then placed inside a plastic cylinder and 750 ml of sterile water, preheated from 50°C water bath, was poured in. The lid was screwed on and the cylinder was placed securely in the gyratory water bath at 50°C for 15 min (75 rpm). The water was poured off from the cylinder and the last step was repeated once more. The water was again poured off and pre-heated (50°C) sterile TE buffer was added. The cylinder was shaken in the 50°C water bath for 15 min. TE buffer was poured off and the wash step was repeated with the pre-heated TE 3 more times. A total of 2 water washes and 4 TE washes were completed. Using a clean spatula, the plugs were transferred to 1.5 ml tubes with 1 ml TE buffer in them and stored at 4°C for Day 3.

Restriction digestion of DNA in agarose plugs with *Xba*I: On the third day, 1.5 ml micro centrifuge tubes were labeled with culture/sample numbers. Plugs were removed one at a time from their tubes and placed on a large, clean glass slide. Excess buffer was wiped away with a Kimtech Science™ Kimwipe™ (Kimberly-Clark, Roswell, GA). Two pieces of 2.5mm×3mm bands were cut and transferred to the labeled 1.5 ml centrifuge tubes. A master mix was made in

a 2 ml tube using 87µl of water, 10µl H buffer, and 3µl of the enzyme (XbaI 10U/µl). The restriction enzyme and H buffer were kept on ice at all times.

One hundred microliters of master mix was added to each tube along with the cut plugs. Tubes were closed and mixed by gentle tapping. Plugs in the enzyme mixture were incubated in the 37°C water bath for 3 hrs with constant agitation. To stop the digestion, the enzyme was removed and replaced with 200 µl TE buffer. Plugs were dried a little, using a Kimwipe™ (Kimberly-Clark, Roswell, GA), before they were added to the TE buffer.

Casting the agarose gel and running PFGE: Two liters of 0.5X TBE buffer were added to the PFGE machine, the power switch was turned on, and the pump was set to a level at which the buffer would run at a smooth, consistent rate through the system. At the same time, the temperature of the cooling unit was set to 14°C. The water bath was turned on and set to 55°C. 1.2% SeaKem® Gold Agarose (SKG) (Lonza, Allendale, NJ) in 0.5X TBE was made by weighing out 1.2g of SKG into 500 ml screw-cap flask and adding 120 ml of 0.5X TBE or TE. The mixture was swirled gently to disperse the agarose and microwaved for 60 seconds. The flask was removed, swirled gently, and microwaved in 15 second intervals until the agarose was completely dissolved. After being completely dissolved, the flask was placed in a 55°C water bath. Next, the gel mold was assembled. The comb was placed on top of the mold and the gel plugs were loaded on the bottom of the comb teeth. Each plug was dried before being placed on the comb. The control (H9810) was placed on the first tooth and loaded again for every 5 samples. These were allowed to dry for about 1 minute, making sure there was no visible buffer on or surrounding the plugs. The comb was positioned in the gel mode and the plugs were confirmed to have an even alignment on the bottom of the teeth. 100 ml of warm agarose, cooled

to 55°C, was carefully poured into the mold. The gel was allowed to solidify for 30 min and then the comb was removed. The wells were sealed with left over warm agarose and the gel was loaded into the machine. Electrophoresis conditions were programmed into the machine and the run was started. Run time took 19 hours or less depending on the samples.

Electrophoresis conditions: The Bio-Rad CHEF Mapper® XA Pulsed Field Electrophoresis System (Hercules, CA) was set up with the following conditions: Temperature 14°C, Angel 120, 30 kb-low MW, 600 kb-high MW, Run time=19.5 hour, Initial switch time= 2.16S, final switch time= 54.17 for *E. coli*).

Gel staining and imaging: After the run completed the next day, the gel was stained with GelRed™ (Biotium, Hayward, CA) for 20 minutes. After 20 minutes, the gel was de-stained in 500 ml ultrapure water for 20 min. The gel was then washed once more with fresh 500 ml ulrapure water. During this time, the buffer was drained from the PFGE machine tubing and was washed with ultrapure water several times. Lastly, the gel was placed in the GelDoc-It$^{®2}$ Imager (UVP, Upland, CA) so that a photo could be taken.

2.7 Transformation of *Escherichia coli* cells:

In preparation for the ground beef and environmental survival experiments, the farm and clinical-isolated *E. coli* O26 cells needed to be transformed. By doing this, the bacteria inoculated into the sample can more easily be differentiated from the other bacteria that show up on the growth media. They were transformed using a green fluorescent protein (GFP) plasmid from the bioluminescent jellyfish *Aequorea victoria*.

Making competent cells: Bacteria to be transformed were first streak plated onto regular Luria Bertani (LB) plates. These plates were grown overnight at 37°C.

A Bio-Rad pGLO™ Transformation Kit (Bio-Rad Laboratories, Herculues, CA) was used to help in transforming the cells. A single colony was inoculated into 25ml LB in a 250ml bottle. The bottle was shaken at 37°C for 4-6hrs. Cells were put on ice for 10min and kept cold for the rest of the procedure. The shaken and incubated samples were then centrifuged at 3800rpm for 15min at 4°C. The supernatant was discarded and the pellet was re-suspended in 10ml cold 0.1M $CaCl_2$. The new suspension was next placed in ice for 20min. Samples were centrifuged again at 3800rpm once again for 15min. Supernatant was discarded and the pellet was resuspended in 5ml cold 0.1 $CaCl_2$. To increase the efficacy of transformation four to six-fold, cells were stored at 4°C in $CaCl_2$ solution for 24-48 hrs. This helped in gaining more competent cells.

Transformation: Two hundred and fifty micro liters of the competent cells were placed in a small microcentrifuge tube. A sterile loop was dipped into the plasmid DNA solution, withdrawn, and mixed into the cell suspension. If this did not work, 5µl was extracted and mixed into the cell mixture. Tubes were incubated on ice for 10min and then transferred to a 42°C water bath for exactly 50 seconds. Tubes were then placed back on ice for 2 minutes. This exchange was made as rapidly as possible because the cells need to be shocked. Tubes were then placed on the bench top and 250µl of LB nutrient broth was added. Tubes were incubated for 10min at room temperature. One hundred microliters was pipetted onto LB plates supplemented with 0.1mg/ml ampicillin and 50mg/ml arabinose, spread evenly, and incubated at 37°C overnight.

2.8 Antimicrobial tests:

The antimicrobial susceptibility of the farm and clinical-isolated O26 strains was tested against 13 different antimicrobials using the disk diffusion method.

Bacteria to be tested were spread on TSA plates and incubated overnight at 37°C. Mueller Hinton Agar was prepared and antimicrobial disks were kept refrigerated at 8°C or below.

Sealed packages containing the discs were removed from refrigeration one to two hours before use so that they could acclimate to room temperature before use. After opening the package, they were placed in an autoclaved container for further storage in the refrigerator or freezer. One well-isolated colony of the same morphological type was selected from each TSA plate and transferred to tubes of tryptic soy broth (TSB). These were incubated overnight at 37°C. The overnight culture was taken and diluted until an OD_{600} value of 1.0 was obtained. One hundred microliters of the cell suspension was added to the Mueller Hinton plates and spread evenly over the surface. The plates were divided into four parts and the appropriate drug-filled disks were placed carefully on the surface of the inoculated plates. Using sterile forceps, the disks were pressed down gently. Disks were not placed any closer than 24mm from the center of one disk to another. The disks were also kept away from the edge of the plates. The plates were then inverted and incubated at 37°C for 16-18hrs.

The diameters of the zones of inhibition were examined and measured in millimeters. The organisms were reported as susceptible, intermediate, or resistant to each antimicrobial agent.

Table 2.3: Antimicrobials used

Class	***Antibody***	***Disk concentration***
Penicillin	Amoxicillin-clavulanic acid	20µg

Penicillin	Ampicillin	10 µg
Cephalosporin	Ceftriaxone	30µg
Cephalosporin	Cephalothin	30 µg
Phenicol	Chloramphenicol	30 µg
Quinolone & Fluoroquinolones	Ciprofloxacin	5 µg
Cephalosporin	Cefoxitin	30 µg
Cephalosporin	Ceftiofur	30 µg
Aminoglycoside	Gentamicin	10 µg
Quinolone & Fluoroquinolones	Naladixic acid	30 µg
Sulfonamide	Sulfamethoxazole	----
Sulfonamide	Sulfamethoxazole/Trimethoprim	23.75/1.25 µg
Tetracycline	Tetracyline	30 µg

2.9 Ground beef 10 day trials

Ground Beef trials were done on STEC O26 containing one virulence gene (*eae*A) and O26 containing two virulence genes (*eae*A and *stx*1) to find out if the presence of multiple genes influenced survival. The ground beef trials were done for 10 days, samples were held at normal refrigeration temperature, and three different inoculation levels were examined: (10^2, 10^6, and 10^8 log CFU's/g).

Ground beef was purchased from a local store. Samples used in this experiment were 85% lean and 15% fat. Before inoculation, the ground beef was confirmed to be STEC-free by taking a 25g sample and enriching it with 225ml TSB. This was incubated at 37˚C overnight. Enriched beef samples were plated on CHROMagar™ STEC (CHROMagar, Paris, France) and incubated again overnight at 37°C. Positive, mauve colored colonies were further tested using PCR to determine if they contain the *stx* or *eae*A intimin genes. LB plates supplemented with ampicillin and arabinose were made two days in advance to allow the plates time to thoroughly dry before use.

Previously transformed farm and clinical-isolated strains were streaked to isolation on the supplemented LB plates. These were incubated overnight at 37°C. A 0.1% buffered peptone

water solution, TSB, and 9ml dilution tubes were made. Measuring tools for weighing out the beef were autoclaved.

For each strain, a single, well isolated colony showing fluorescence was chosen and 10ml TSB tubes were inoculated. TSB tubes were incubated overnight at 37°C. This gave approximately 10^9 CFU's/ml for inoculation the next day.

Bacteria cells were washed by centrifugation and resuspended in autoclaved ultrapure water. In order to reach target inoculations of 10^2, 10^6 and 10^8 log CFU/g, dilutions of 10^3, 10^7 and 10^9 log CFU/g were made by taking 1ml samples from the TSB overnight cultures and diluting them accordingly. A cocktail of bacteria containing the gene *eae*A was made. The same was done with bacteria containing both *eae*A and *stx*1 genes.

Twenty five gram beef samples were then weighed out into filtered Whirl-pak® bags (Nasco, Fort Atkinson, WI). Bags were labeled according to the strain, temperature, and dilution. Each bag was inoculated with 1ml of the chosen *E. coli* cocktail.

Every day for ten days, 6 bags containing the various inoculation levels and cocktails were removed from the 4°C refrigerator. One hundred mililiters of 0.1% buffered peptone water was added to each bag and put through the Smasher™ lab blender (AES, Hamilton, NJ) for 2 minutes. Bacteria were plated by spreading 100µl of the smashed contents on a STEC plate and incubated at 37°C overnight. This plating was done in duplicated for each bag. If required, samples were diluted to reach a countable range (between 25 and 200 colonies) on the plates. If plates showed zero colonies, bags were enriched with 100ml of lactose broth, incubated overnight at 37˚C, and re-plated the following day.

2.10 Environmental 30 day trials

Environmental trials were conducted for 30 days, and samples were held at room temperature. Only 2 different target inoculation levels were examined in these experiments (10^6 and 10^4 log CFU/g) and three different environmental sample types were tested: bovine feces, bedding, and trough water. Bedding in this case was dirt that the cattle had been milling around and laying in.

The same bacteria used in the ground beef trials were streaked onto supplemented LB plates. Inoculation cocktails were prepared in the same manner as those in the beef trials, except the target inoculations here were 10^6 and 10^4 log CFU/g.

About 800ml water, 1800g fecal material, and 1800g bedding were retrieved from E.V. Smith Research Center and brought back to the lab on ice. Twenty five grams of fecal or bedding samples were measured out into Whirl-pak® bags (Nasco, Fort Atkinson, WI). Bags were labeled according to the day, gene(s), and target inoculation. Each bag was inoculated with 1ml of the chosen *E. coli* cocktail. The farm water was separated so that there were four cups containing 100ml of the farm water. One milliliter of chosen *E. coli* cocktail was added and containers were labeled. On days 0, 5, 10, 15, 20, 25, and 30, 4 water cups and 8 bags of the various cocktails and inoculation levels were taken out of room temperature storage. One hundred mililiters of 0.1% peptone water was then added to each bag of fecal or bedding material and these were shaken for 2 minutes. Water cups were also shaken for 2 minutes. One hundred microliter samples were taken from these bags and cups and spread onto supplemented LB plates. If necessary, samples were diluted to reach a countable range on the agar plates. If the results from the previous sampling day gave a count below 25, then 1ml of the sample was divided and spread onto 4 LB plates to give a more accurate and lower limit of detection. All plates were incubated at 37°C and all fluorescent colonies were counted and recorded.

2.11 Statistical Analysis

In examining the prevalence of STEC in the calf trial, before and after weaning, a Chi square test was completed along with a paired Student t-test. Statistics for the ground beef and environmental study included a one-way proc ANOVA which was completed using SAS. Inoculation levels, gene profiles, materials, and days were all examined to find out if there was a statistical difference. If *P* was less than 0.05, the data were considered different.

Chapter 3. Results and Discussion

3.1 Research Center studies

Six research centers all around Alabama were sampled during the summer and fall months of 2013. Their locations are marked in (Figure 3.1). The Tennessee Valley research center, along with the Sand Mountain and Upper Coastal Plain research centers are located in north Alabama. The Gulf Coast, Black Belt, and Wiregrass research centers are located in south Alabama. Upper Coastal Plain has more of a northwestern location while Gulf Coast and Black Belt have a southwestern position. Sand Mountain and Wiregrass have more of a northeast and southeastern orientation respectively. About 30 fecal samples were taken from each research center and processed within 24 hours of being received in the lab.

3.1.1 Shedding and super-shedding prevalence

Cows and calves were sampled at only two of the research centers: Wiregrass and Sand Mountain. As observed in (Table 3.1), these two research centers have the highest STEC shedding prevalence (100% and 85%). For the other four research centers, only cows were sampled; no calf samples were collected. They exhibited a relatively lower number of positive STEC shedding samples as compared to the first two research centers, but still high: (76.5%, 55.6%, 38.7%, and 68.5% positive for STEC). The cow-only herds contained fewer high and "super-shedder" cattle as compared to the two research centers that had only calves in their herd at the time. High-shedders were defined by the author as: containing around a 3.0 log CFU/g STEC prevalence in their fecal samples. As for the prevalence of "super-shedding" cattle,

Wiregrass contained only one “super-shedder,” Sand Mountain had 11 “super-shedders,” and Black Belt had 1 (Table 3.1).

Results would be better interpreted if samples were received from each research center at the same time of the year and from the same herd type, either cows or a herd with cows and calves. The high positive STEC counts at Wiregrass and Sand Mountain may not only be due to calves being in the sampling herd, but could also be due to the samples being obtained during the warmer months of the year.

3.1.2 PCR examination and serotyping results of isolates from high-/super-shedder cattle

Three isolated colonies were taken from any high- or “super-shedder” research center fecal samples, DNA was isolated, and they were tested by PCR for three target virulence genes. Those containing at least one target gene were sent out for serotyping. Isolates from all but the Gulf Coast area were sent for serotyping (Table 3.2). The high shedders from the Gulf Coast center did not exhibit any of the target virulence genes, so serotyping was not warranted. Two STEC isolates from Wiregrass, 5 from Sand Mountain, 1 from Black Belt, 2 from Tennessee Valley, and 3 from Upper Coastal Plain were sent for serotyping. The PCR results for these isolates are shown in Figure 3.2. All of those sent out exhibited the *eae*A intimin gene. Seven contained the *eae*A and the *stx*2 genes, while three contained all 3 genes. STEC O26 was isolated from the high shedders at both Black Belt and Upper Coastal Plain while STEC O103 was isolated from the Sand Mountain research center (Table 3.2).

3.2 Calf Trials

Fecal samples were directly grabbed from 110 calves at E.V. Smith research center while they were still with the dam at 6 months old and just after weaning at 9.5 months of age. All fecal samples were processed, plated on CHROMagar™ STEC (CHROMagar, Paris, France), and incubated. The next day they were enumerated and results were recorded.

3.2.1 Shedding level and prevalence changes during weaning

All of the shedding results from the calf trial, before and after weaning, are presented in Table 3.3. Any plates showing over 250 colonies were considered too numerous to count, so the upper limit of detection for these tests was 4.14 log CFU/g. No change was seen in the shedding levels before and after weaning for 26% of the calves. This means that if the calf was a high-shedder before weaning, it was high after. If the calf was a low-shedder before weaning, then it was low after, and so on. Two calves changed from high STEC shedders to non-shedders and one calf changed from being a non-shedder to being a high shedder. Out of the rest, 34 increased in their shedding level and 45 decreased. Overall, the shedding level of 74% of the calves changed.

As seen in Table 3.4, the number of calves positive for STEC went from 95.5% to 96.5%, staying about the same before and after weaning. Statistics here included a Chi square test ($P > 0.05$). Shedding level, on the other hand, changed from an average of 3.00±1.42 before weaning to 2.32±1.62 log CFU/g feces after. By conducting a paired Student t-test, the decrease in shedding levels between the two sampling times was discovered to be different ($P < 0.05$).

3.2.2 Super-shedders

Colonies were picked from the consistently “super-shedding” samples. These isolates were purified and their DNA was extracted for PCR analysis. A total of 18 isolates, 9 before and

9 after weaning, were tested in a multiplex PCR assay targeting the three virulence genes and then sent out for serotyping.

3.2.3 PCR results of super-shedder isolates

The 9 consistently high "super-shedders" in this study are highlighted in Table 3.5, which also outlines all of the high-shedders in the group of calves. Eighteen samples from the 9 calves, before and after weaning, were tested by multiplex PCR to see if they contained any target virulence genes: *eae*A, *stx*1, and/or *stx*2. PCR results showed that 10 out of 18 were positive for at least one target gene. Also, 11 out of the 18 isolates contained *eae*A and 2 out of 18 had the *stx*1 gene. The number of isolates discovered per serogroup and their virulence profiles are shown in Table 3.6. The PCR image of the amplified target genes for the 18 super-shedder isolates is displayed in Figure 3.4.

3.2.4 Serotyping results

The serotyping results for these 18 isolates are displayed in Table 3.7, which shows the O-type of the isolates found before and after weaning. Since these dominant isolates shown in the table are the result of only a few randomly picked colonies out of 250 or more colonies on the plates, statistics could not be completed to confirm that these are the true dominant O-types for these samples. Of the "big six," *E. coli* O26 turned up most frequently, twice before and once after weaning. All three O26 isolates contained the *eae*A virulence profile. One isolated before weaning contained the *eae*A and *stx*1 virulence profile. Just from observing the potentially dominant isolates from Table 3.7, none of the calves possessed the same dominant *stx*-containing O-group before as they did after weaning.

3.3 Michigan State reference strain evaluation

Two of each “big six” non-O157 STEC strains were obtained from the STEC Center at Michigan State University (Table 3.8) and their virulence genes were examined by multiplex PCR. As see in Figure 3.5, four of the six “big six” did not display a different virulence profile than that which was displayed by the serogroup partner. Both O26 and O111 showed a different number of virulence genes within their individual serogroups. One O26 isolate held all three target genes while the other contained only *eae*A and *stx*1. The same was seen with O111. One isolate had three while the other only held two of the target genes. This shows that even among the individual serogroups on the recall list, there are various pathogenic gene compositions present.

3.4 Comparison of O26 isolates

Because O26 was discovered in both the E.V. Smith calf trials and the Alabama research centers, further tests were conducted to examine its survival in ground beef and the farm environment. The two clinical-isolated strains of O26 from Michigan State University were compared to the four farm-isolated O26 strains from the E.V. Smith calf testing. The multiplex PCR comparison of these six O26 can be seen in Figure 3.6. As determined by PCR, three farm O26 strains contained the *eae*A intimin gene only. The fourth farm strain, along with one clinical strain (TWO7814), contained the *eae*A and *stx*1 virulence genes. The last clinical isolate (TWO8031) contained all three.

3.4.1 PFGE comparison of O26 isolates

All four farm isolates from the calf trials and one clinical isolate (TWO7814) were used in the survival tests. The PFGE of these O26 isolates showed that the farm strains were more closely related to each other than they were to the clinical strain (Figure 3.7-3.8). The farm strain

containing *stx*1 was more closely related to the clinical strain than the other three farm strains were.

3.4.2 Antimicrobial test results

Antimicrobial tests were also done on the farm and clinical strains. All isolates had a similar susceptibility to the antimicrobials tested, except for one of the clinical isolates (Table 3.9). It was completely resistant to three antibiotics: Ampicillin, Sulfamethoxazole, and Tetracycline. The clinical isolate had a human source and most likely developed a resistance when the person went through an antibiotic treatment.

3.5 Survival studies

The GFP transferred farm-isolated and clinical-isolated cells were added to ground beef at three different inoculation levels and to the environmental samples at two different inoculation levels. For every sampling day, previously inoculated samples were taken and plated on LB agar supplemented with ampicillin and arabinose. Examples of what the fluorescent colonies on the LB supplemented plates look like with a lot of background microflora and with very little are shown in Figure 3.9. The figure also shows what the plates looked like under normal fluorescent light, compared to UV light. As the bedding pictures show, there were oftentimes other colonies on the supplemented LB plates that looked very similar in appearance. By transforming the colonies, the inoculants could easily be distinguished from the background microflora and counted without too much trouble.

3.5.1 Ground beef study

Every day for 10 days, the previously inoculated ground beef samples were taken out of the 4°C storage and plated on supplemented LB agar. Any fluorescent colonies on the plates

were enumerated the next day. For the 10 day ground beef tests, the counts did not vary much from the initial inoculation level (Figure 3.10). The two higher inoculations: 10^8 and 10^6 log CFU/g showed about 0.5 log reduction over time whereas the 10^2 log CFU/g inoculation showed about 1 log reduction over the 10 day period. From the two higher inoculations, there was no difference seen when comparing the different virulence gene profiles ($P > 0.05$). Even though there seems to be more variation in the lower inoculation level (10^2 log CFU/g), the standard deviation for that inoculation shows that the count from the two different gene profiles still did not deviate enough to be considered different from one another. There was, however, a difference in the inoculation levels ($P < 0.05$).

3.5.2 Environmental study

The same inoculation and plating process used in the ground beef study was repeated for the environmental samples, except samples were plated every 5 days for 30 days total. Materials inoculated include: feces, bedding, and water. Cattle feed was not used in this study because preliminary tests showed that mold and other bacteria dominated the plates and made it very difficult to count the GFP transformed colonies. An accurate count would not be possible with this material unless an effective mold suppressing supplement were added to the media.

The fecal test results can be observed in Figure 3.11. The graph shows a 1 log CFU/g increase in bacteria count during the first 5 days for all inoculation levels and genes profiles except the eaeA/stx1 profile at target inoculation level 10^4 log CFU/g. This profile also saw an increase in the first 5 days, just not as great an increase as the others. They then showed variable results over the following 25 days. Looking at the overall trend, the count did not change from the initial inoculation level ($P > 0.05$).

For the bedding test seen in Figure 3.12, the bacteria count declined steadily over the whole 30 day period. In this case, the bacterial count at the end of the 30 days was different from the initial inoculation level (P <0.05).

For the farm water test seen in Figure 3.13, there was a steep decline in the bacteria count over the 30 day trial period. Similar to the bedding sample results, the bacteria count on day 30 for these water samples were different from the initial inoculation levels (P > 0.05). Most counts were 0 or close to 0 by day 30, but enriching the samples and plating showed that the STEC O26 inoculants were still present in all samples.

Statistics for the ground beef and environmental study included a one-way ANOVA which was completed using SAS. Inoculation levels, gene profiles, materials, and days were all examined to find out if there was a statistical difference. If P was less than 0.05, the data were considered different.

Over all the trials, there were differences seen in the types of materials that were inoculated and also in initial inoculation levels (P <0.05). The limit of detection for these trials was 0.6 log CFU/g. Looking at all of the materials, the inoculants survived best in the refrigerated ground beef samples. This is most likely due to the temperature preventing other microbes from growing and influencing the inoculants. The temperature also slows the metabolism of the O26 inoculants, so rapid growth or death was not likely to happen. The ground beef would also be the material with the least amount of background microflora, so it would see the least amount of microbial competition. The fecal samples saw the most variation as they held the nutrients to support the growth and survival of STEC, yet they also held other microflora which could offer some competition. The inoculants did not survive as well in the bedding and water samples as they did in the beef and fecal samples. There were fewer nutrients in the

bedding and water. For the bedding samples in particular, a greater amount of background microflora was observed on the plates. The mold and other bacteria seemed to flourish at room temperature. This may have had some influence on the results for this material.

Chapter 4. Conclusion

4.1 Research center studies

Alabama research centers were sampled to determine the non-O157 STEC prevalence in beef cattle all around the state of Alabama. In each herd with cows only, there was only 1 super-shedder cow. The presence of only one or two super-shedder cattle per herd is supported by research (Aurthur *et al*, 2013). Only about 2% of the cattle displayed the "super-shedder" status. Results indicate that the time of year and/or herd type have an impact on the number of positive STEC shedders. The presence of calves in the herd seems to have an impact on the number of high-shedders in the herd, as seen at the Sand Mountain research center, where there were 11 "super-shedders."

4.2 Calf trial

The purpose of the E.V. Smith calf sampling, before and after weaning, was to observe the impact weaning may have on calf STEC shedding. The calves at E.V. Smith experienced a decrease in their shedding level ($P < 0.05$), but not in the number of calves positive for STEC ($P > 0.05$). Because the consistently "super-shedder" calves did not have the same dominant STEC before weaning as they did after (Table 3.7), it suggests that their gut microflora is easily influenced while they are still young. Of course, only a few colonies were picked randomly from the plated "super shedder" samples and multiple tests could give varying results on the dominant microflora present in the sample. There are quite possibly multiple dominant STEC in each fecal

sample, so it must be said that there are limitations of currently available testing protocols. As seen by the numbers in Table 3.4, weaning does have an impact on shedding level. There was a decline the amount of STEC shed by the calves on average ($P < 0.05$). So, it would be a good idea to look at weaning as a possible control point for reducing the amount of STEC shedding even further, as it already occurs naturally.

4.3 O26 isolate evaluation

As seen in both the clinical O26 isolates obtained from the STEC center at Michigan State University and with the O26 farm isolates from the calf study, STEC can have multiple virulence profiles within the same serogroup. Therefore, the virulence profiles should be more important for determining virulence rather than looking at specific serogroups.

4.4 Survival studies

Non-O157 STEC O26 has been discovered to be the dominant "big six" member in these studies, as it showed up in both the research center study and in the E.V. Smith calf study. Because this STEC also displayed a propensity to have different virulence profiles, as discovered in the multiplex PCR analysis of the different clinical and farm isolates, it was examined further in the ground beef and environmental survival tests. Based on the results from the survival tests, it has the ability to survive for more than 30 days at different temperatures and in different samples. Having the *stx*1 gene, or the lack thereof, does not seem to influence its survival ($P > 0.05$).

4.5 CHROMagar™ STEC

CHROMagar™ STEC (CHROMagar, Paris, France) agar was the best media to fit the needs of this study. Research shows that this agar has a 98.9% specificity; only 3 out of 264 non-STEC bacteria strains could produce mauve colonies when plated (Hirovonen *et al*. 2012). Growth on this media is highly associated with the tellurite resistance genes (TerZABCDEF). Not all of the STEC isolated by the agar contained the *stx* gene, but the existence of the gene could easily be discovered by running them through a PCR assay amplifying the specific gene sequences. This agar was very effective in obtaining a total STEC count in our fecal samples and also isolating those with the *stx* genes.

4.6 Overall Conclusions

Calves start shedding STEC early in their lives and in greater quantities than their dams. Calves are more likely to be "super-shedders" than adult cattle. The results of this study show that weaning does influence the calf gut. This could be a good pre-harvest control point for reducing the number of STEC in the calf gut before they are shipped to a feedlot or to slaughter. More research should be done on possible methods for control, especially on the "super-shedding" cattle. If the count in these "super-shedding" cattle can be reduced, it will more than likely be effective for the whole herd.

O26 was found to be the dominant "big six" member in both the research center and calf studies, begging the question: does O26 have some better means of surviving in the environment? Survival studies comparing different virulence profiles revealed that the existence of the *stx*1 gene in STEC does not influence O26 survival in refrigerated ground beef or other environmental samples. More research should look into the *stx*2 gene and how it influences

survival, especially since it is more often associated with HUS, more severe sicknesses, and outbreaks.

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Tables

Table 3.1: Research Center Results

	Research Center	***% cattle positive for STEC***	***Super-shedders***	***Type of cattle***	***Month Sampled***
1	Wiregrass	100%	1	cow/calves	May
2	Sand Mountain	85.70%	11	cow/calves	June
3	Gulf Coast	76.50%	0	cows	June
4	Black Belt	55.60%	1	cows	July
5	Tennesse Valley	38.70%	0	cows	October
6	Upper Coastal Plain	64.50%	0	cows	November

Table 3.2: Research center serotyping results

	Research Center	***# Sent for Serotyping***	***Dominant STEC***
1	Wiregrass	2	O4
2	Sand Mountain	5	O103/O49
3	Gulf Coast	0	-----
4	Black Belt	1	O26
5	Tennessee Valley	2	O177/O46
6	Upper Coastal Plain	3	O26/O10/O182

Table 3.3: Enumeration of STEC from calf trial

Cattle Ear Tag No.	*6-Month-Old log CFU/g (Before weaning)*	*9.5-Month-Old log CFU/g (Right after weaning)*
1001	2.089848	(e)>4.14
1002	4.051599	3.790978
1003	(e)>4.14	3.248879
1004	(e)>4.14	2.129438
1005	(e)<1.74	2.518514
1007	negative	2.041393
1008	positive	(e)>4.14
1009	(e)>4.14	(e)>4.14
1010	3.183608	3.786055
1011	(e)>3.83	2.915487
1012	(e)>4.14	1.890878
1013	1.985277	3.085461
1014	(e)>3.73	(e)>4.14
1015	positive	2.328408
1016	3.775566	3.202502
1017	(e)>4.14	2.390878
1018	(e)>4.14	4.071647
1019	2.779953	2.761059
1020	(e)>4.14	(e)>4.14
1021	(e)>4.14	2.430468
1022	2.73818	3.946526
1023	3.687244	3.550951
1024	positive	2.566969
1025	(e)>4.14	(e)>4.14
1026	(e)>4.14	3.435034
1027	3.373831	3.226001
1028	negative	2.279953
1029	4.166679	negative
1030	(e)>4.36	(e)>4.14
1032	positive	(e)>4.14
1033	3.740145	3.258078
1034	3.228079	2.551987
1035	(e)>4.14	2.313427
1036	3.482868	(e)<1.74
1037	3.322836	3.735199
1038	3.740363	3.573434
1039	3.889636	(e)>4.14
1040	positive	3.003532

1041	3.314355	(e)>4.14
1042	positive	(e)>4.14
1043	negative	2.518514
1044	(e)<2.04	3.051987
1045	3.394645	2.492938
1046	2.089848	3.284431
1047	3.75293	4.184228
1048	(e)>4.14	3.855715
1049	3.40054	2.041393
1050	3.542205	2.818031
1051	2.719883	2.717484
1052	(e)>4.14	(e)<2.04
1054	2.492938	4.315106
1055	3.61368	(e)>4.14
1056	3.144806	3.786372
1057	positive	(e)>4.14
1058	(e)>4.14	3.470059
1059	2.478923	positive
1060	3.460062	3.30777
1062	(e)>4.14	3.142773
1063	2.298656	4.254201
1064	(e)>4.14	3.850744
1065	(e)>4.14	(e)>4.14
1067	4.256986	2.342423
1068	2.834123	3.282631
1069	(e)<2.22	4.133291
1071	4.22582	2.731498
1072	2.089848	3.336925
1073	4.118977	(e)>4.14
1074	negative	4.241672
1076	(e)>4.14	negative
1077	3.711019	3.228079
1078	2.041393	1.740363
1079	1.978923	(e)>4.14
1080	(e)>4.14	(e)<2.34
1081	(e)>4.14	2.217484
1082	3.757557	3.202502
1083	3.931479	3.071269
1084	3.23195	positive
1086	(e)4.14	1.890878
1088	3.89216	(e)>4.14
1089	4.382664	2.191908
1090	(e)4.14	2.957647

1091	2.739705	1.890878
1092	1.978923	positive
1094	positive	3.013634
1095	positive	negative
1097	(e)4.47	(e)>4.14
1098	(e)4.14	3.257075
1099	positive	2.867999
1100	(e)4.62	2.640033
1101	negative	2.240363
1103	3.812681	(e)>4.14
1104	3.60069	3.539649
1105	(e)>4.14	2.580983
1106	2.037811	2.861882
1107	(e)>4.14	3.964762
1108	4.044529	3.146819
1109	3.451165	3.631169
1111	3.784253	3.370875
1112	(e)>4.14	3.211367
1113	3.653173	2.669029
1114	3.991098	2.328408
1115	(e)<1.74	2.129438
1116	(e)4.31	2.439333
1117	(e)>4.14	3.071742
1118	3.998035	3.010772
1119	(e)>4.14	2.901472
1120	(e)>4.14	4.018514
1121	2.390878	2.662912
1122	2.943633	4.000691

Table 3.4: Shedding levels and STEC prevalence in calves at 6 and 9.5 months of age

	STEC shedding level (log CFU/g feces)	*STEC prevalence (% Positive)*
6-Month Old	3.00± 1.42 [a]	95.5%
9.5 Month Old	2.32 ± 1.62 [b]	96.5%

Table 3.5: Enumeration of STEC from "super-shedders" in calf trial

Cattle Ear Tag No.	*6-Month-Old log CFU/g (Before weaning)*	*9.5-Month-Old log CFU/g (Right after weaning)*
1002	4.05 ± 0.03	3.79 ± 0.07
1003	>4.14	3.25 ± 0.03
1004	>4.14	2.13 ± 0.12
1009	>4.14	>4.14
1012	>4.14	1.89 ± 0.21
1017	>4.14	2.39 ± 0.07
1018	>4.14	4.07 ± 0.02
1020	>4.14	>4.14
1021	>4.14	2.43 ± 0.55
1025	>4.14	>4.14
1026	>4.14	3.44 ± 0.09
1029	4.17 ± 0.02	Negative
1030	>4.36	>4.14
1035	>4.14	2.31 ± 0.38
1048	>4.14	3.86 ± 0.02
1052	>4.14	<2.04
1058	>4.14	3.47 ± 0.07
1062	>4.14	3.18 ± 0.08
1064	>4.14	3.85 ± 0.02
1065	>4.14	>4.14
1067	4.26 ± 0.11	2.34 ± 0.43
1071	4.23 ± 0.04	2.73 ± 0.12
1073	4.12	>4.14
1076	>4.14	Negative
1080	>4.14	<2.34
1081	>4.14	2.22 ± 0.01
1086	4.12 ± 0.02	1.89 ± 0.21
1089	4.38 ± 0.04	2.19 ± 0.21
1090	>4.14	2.96 ± 0.02
1097	4.47 ± 0.09	>4.14
1098	>4.14	3.26 ± 0.06
1100	4.62 ± 0.14	2.64 ± 0.08
1105	>4.14	2.58 ± 0.09
1107	>4.14	3.96 ± 0.36
1108	4.04 ± 0.01	3.15 ± 0.01
1112	>4.14	3.21 ± 0.10
1116	4.31 ± 0.01	2.44 ± 0.01
1117	>4.14	3.07 ± 0.04
1118	4.00 ± 0.09	3.01 ± 0.13
1119	>4.14	2.90 ± 0.02
1120	>4.14	4.02 ± 0.10

Table 3.6: Serotyping and PCR results for 9 consistently high "super-shedder" calves

Isolates	*# of Isolates Before Weaning*	*# of Isolates After Weaning*	*PCR results*
O26	2	1	*eae*A, (*eae*A, *stx*1)
O49	2	0	*eae*A
O120	2	4	---
O152	1	0	---
O172	1	0	*eae*A
O98	1	1	*eae*A, (*eae*A *stx*1)
O177	1	1	*eae*A
O159	0	1	---
O109	0	1	*eae*A

Table 3.7: Serotyping results for consistently high "super-shedder" calves

Tag #	*Before Weaning*	*After Weaning*
1009	O26	O109
1018	O152	O159
1020	O98/O172	O26
1025	O49	O120
1030	O49	O120
1065	O120	O177
1073	O26	O120
1097	O120	O98
1120	O177	O120

Table 3.8: Isolates from the STEC Center at Michigan State University

STEC	*Accession number*	*Isolate*	*Sources (Human)*
O26	TW07814	97-3250	(Female 2yr) – USA(Idaho)
O26	TW08031	MT#10	USA(Mont.)
O45	TW09183	M103-19	(Male 45yr) – USA(Mich.)
O45	TW14003	MI05-14	(Male 12yr) – USA(Mich.)
O103	TW08101	MT#80	USA(Mont.)
O103	TW07971	TB154A	USA(Wash.)
O111	TW07926	3215-99	(Female 18yr) - USA(Tex.)
O111	TW014960	0201 9611	USA(Conn.)
O121	TW07614	MDCH-4	(Female 51yr) – USA(Mich.)
O121	TW08039	MT#18	USA(Mont.)
O145	TW09356	4865/96	Germany
O145	TW09153	IH 16	Uruguay

Table 3.9: Antimicrobial resistance results

Antimicrobials	*Clinical(mm)*		*Isolated from Farm (mm)*			
	TWO8031	**TWO7814**	**22B**	**41C**	**38H**	**31-1**
Amox-clavulanic acid	**7.00** ±0.00	**6.50** ±1.22	**8.75** ±0.65	**8.13** ±0.85	**7.88** ±0.75	**8.13** ±0.75
Ampicillin	**7.00** ±1.15	**0.00** ±0.00	**8.25** ±1.44	**7.50** ±0.58	**7.75** ±1.19	**7.88** ±0.63
Ceftriaxone	**13.88** ±0.75	**14.38** ±0.48	**13.88** ±0.75	**13.63** ±0.25	**12.50** ±0.58	**14.63** ±0.48
Cephalothin	**6.25** ±0.65	**5.63** ±1.03	**6.63** ±0.48	**6.25** ±0.29	**6.88** ±0.25	**7.25** ±0.29
Chloramphenicol	**9.38** ±0.75	**9.50** ±0.41	**9.88** ±0.25	**9.00** ±0.58	**8.50** ±0.58	**9.88** ±0.25
Ciprofloxacin	**14.50** ±0.41	**15.00** ±1.2	**13.50** ±0.71	**13.63** ±0.95	**14.38** ±1.03	**15.88** ±1.93
Cefoxitin	**9.63** ±0.75	**9.75** ±0.29	**9.50** ±0.41	**9.88** ±0.48	**10.25** ±1.44	**10.38** ±0.48
Ceftiofur	**11.50** ±0.41	**11.75** ±0.87	**11.00** ±0.00	**12.00** ±0.00	**11.63** ±0.75	**12.00** ±0.00
Gentamicin	**8.63** ±0.48	**8.25** ±0.29	**8.25** ±0.29	**8.25** ±0.29	**8.00** ±0.00	**8.88** ±0.25
Naladixic acid	**9.75** ±0.29	**11.5** ±0.58	**10.38** ±0.25	**10.00** ±0.71	**9.75** ±0.29	**11.00** ±0.00
Sulfamethoxazole	**8.13** ±0.25	**0.00** ±8.13	**6.88** ±0.48	**8.25** ±0.29	**7.25** ±0.29	**6.88** ±0.48
Sulfameth/Trimeth	**11.75** ±0.65	**8.13** ±0.48	**11.63** ±0.25	**12.00** ±0.71	**11.25** ±0.87	**11.13** ±0.48
Tetracylcine	**9.00** ±0.00	**0.00** ±0.00	**8.63** ±0.48	**8.50** ±0.41	**8.00** ±0.41	**8.63** ±0.25

Figures

Figure 3.1: Alabama research centers tested

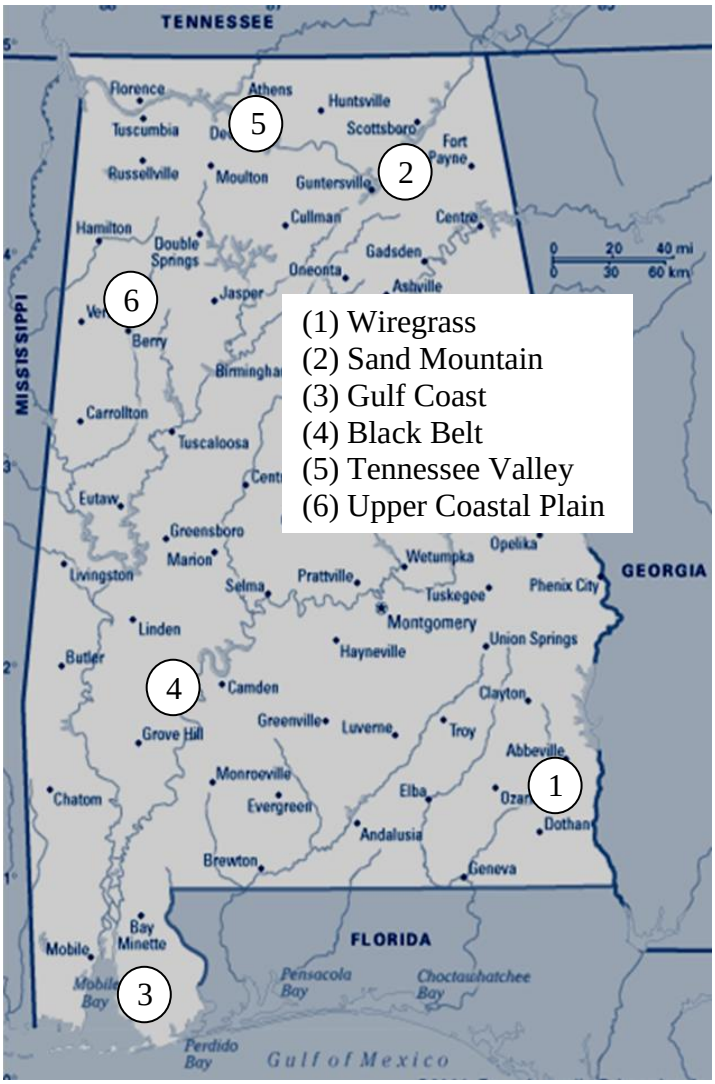


Figure 3.2: PCR results for research center samples sent for serotyping

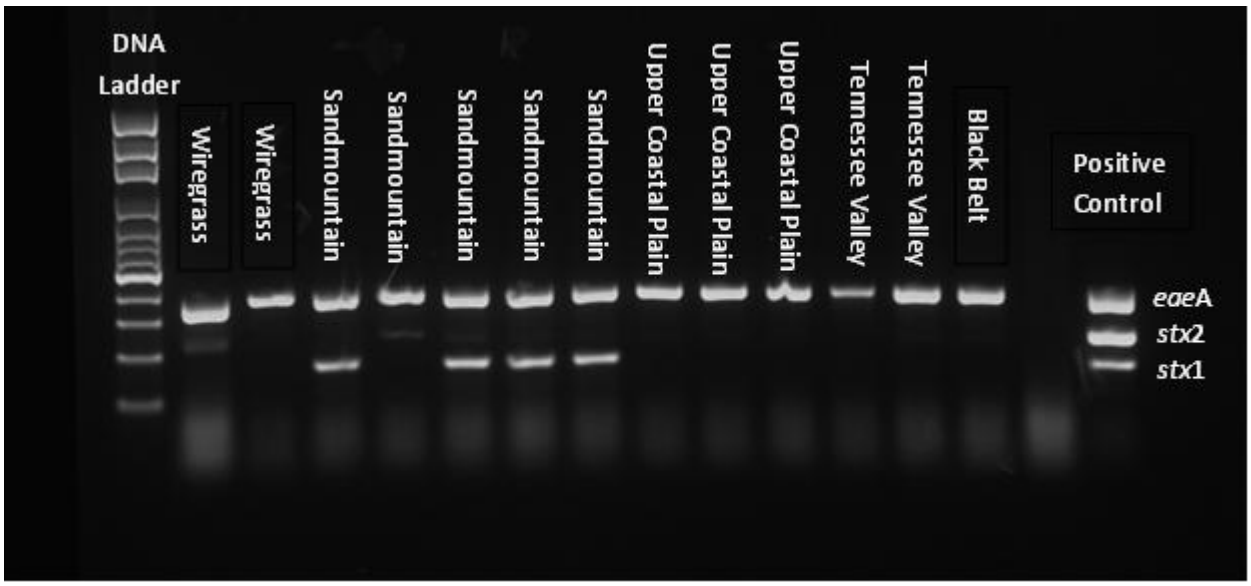


Figure 3.3: Shedding level for calf trial

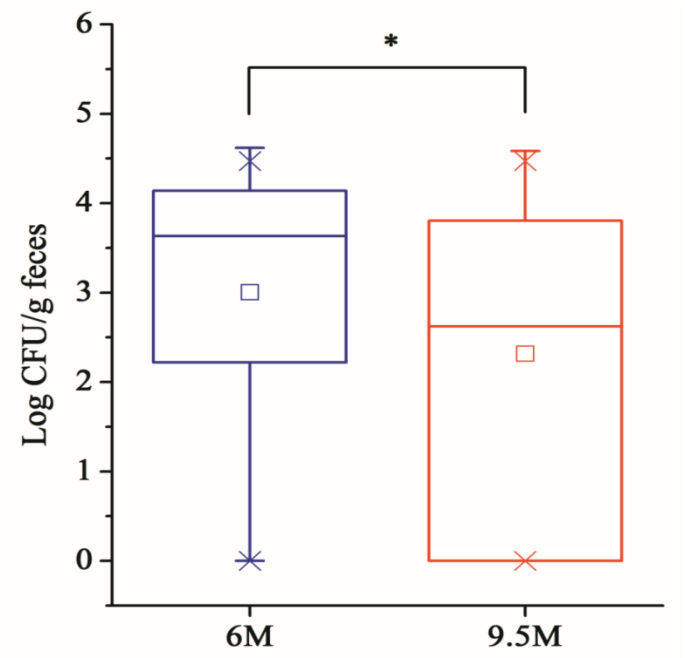


Figure 3.4: PCR of the 9 "super-shedder" isolates from the calf trial, sent for serotyping

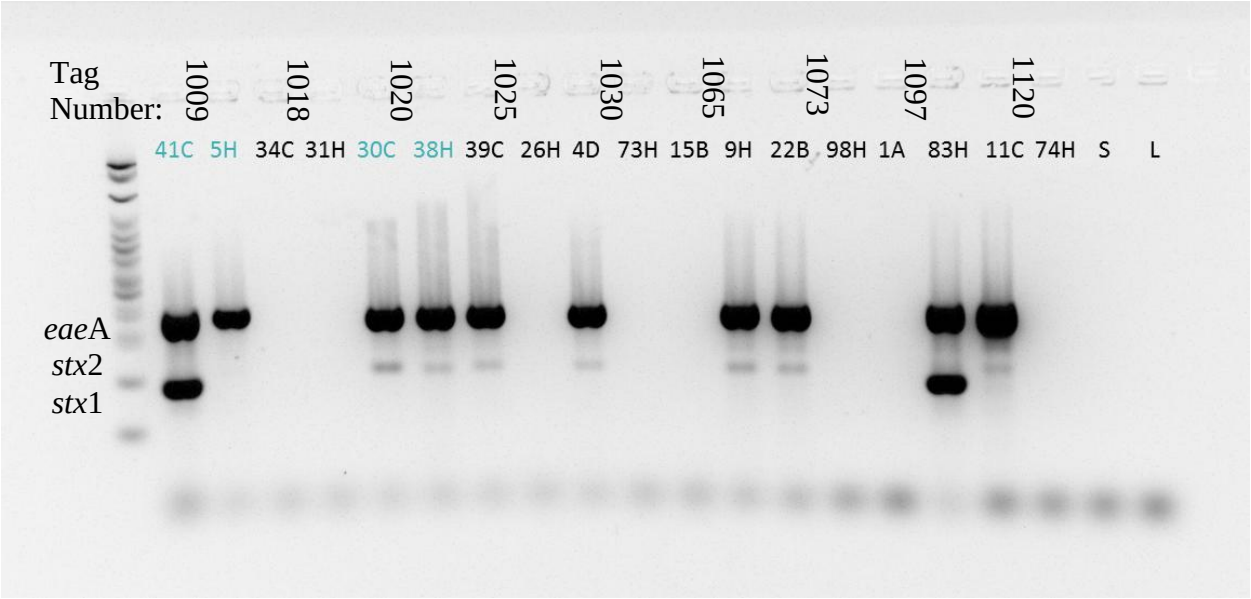


Figure 3.5: PCR of "big six" STEC clinical isolates from Michigan State University STEC center

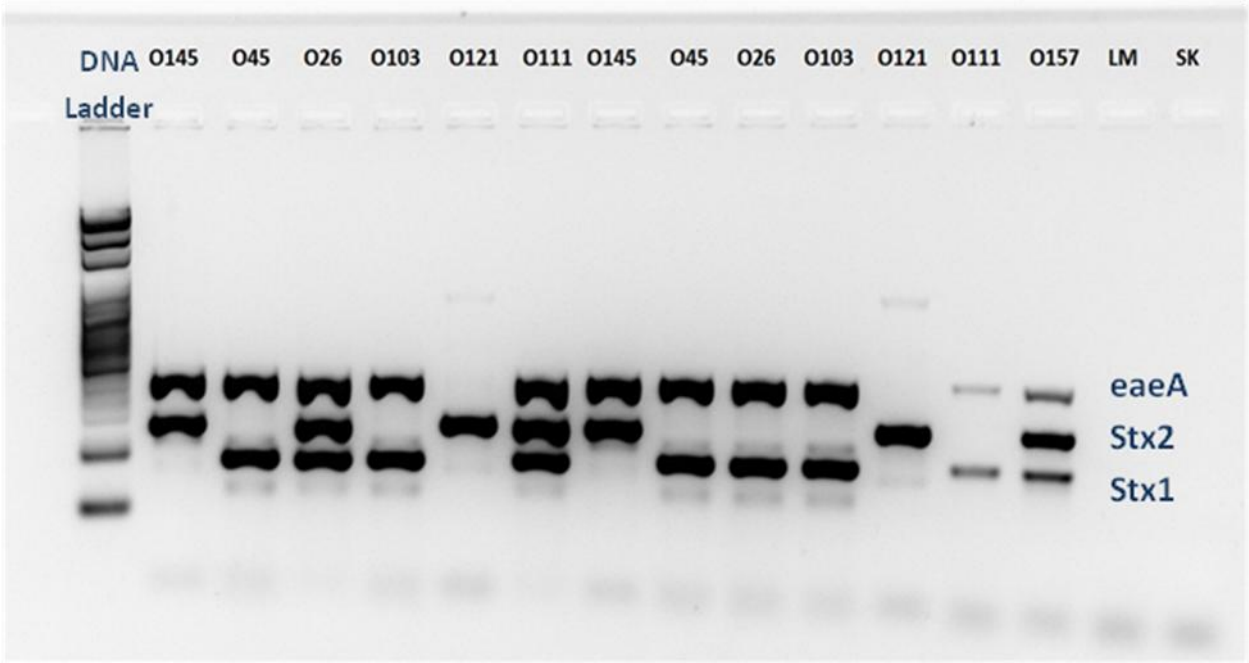


Figure 3.6: PCR of 4 farm-isolated O26 strains and 2 clinical isolated O26 strains

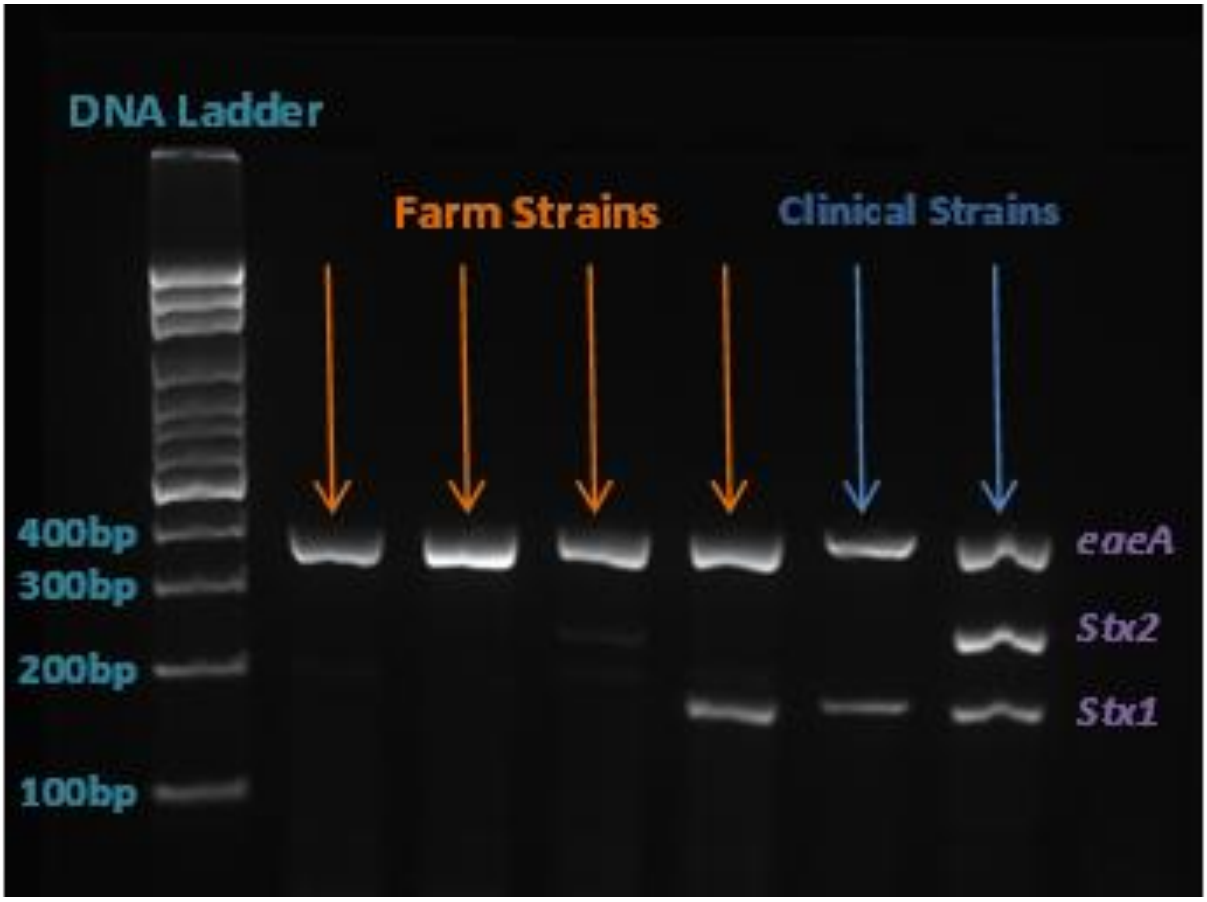


Figure 3.7: PFGE results from 4 farm and 1 clinical O26 strain

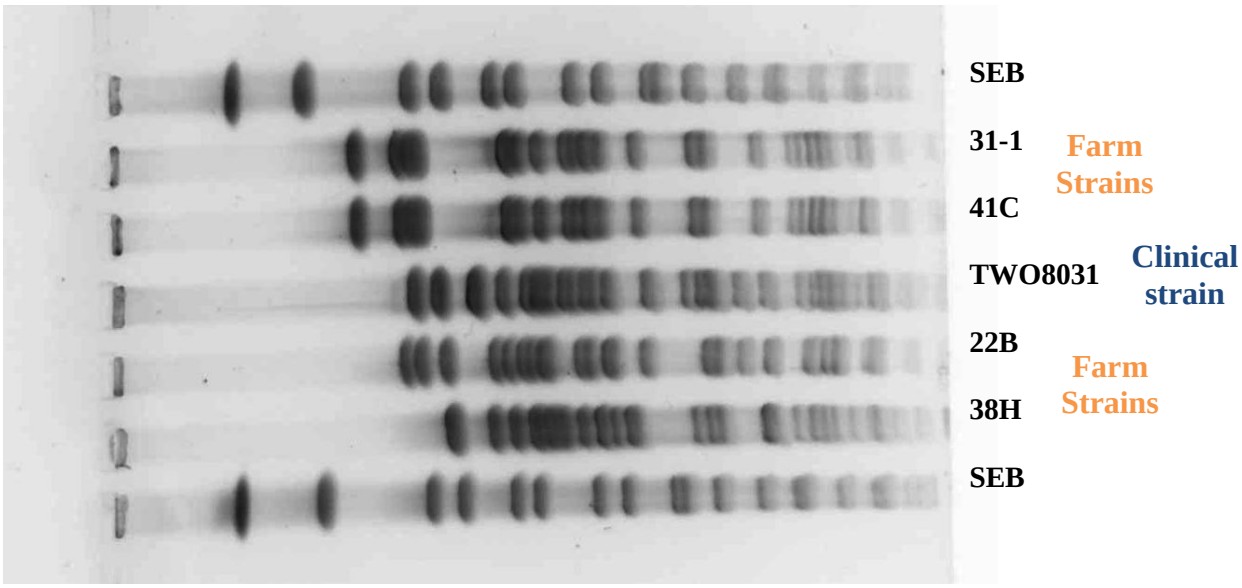


Figure 3.8: Dendrogram of PFGE fingerprinting profile

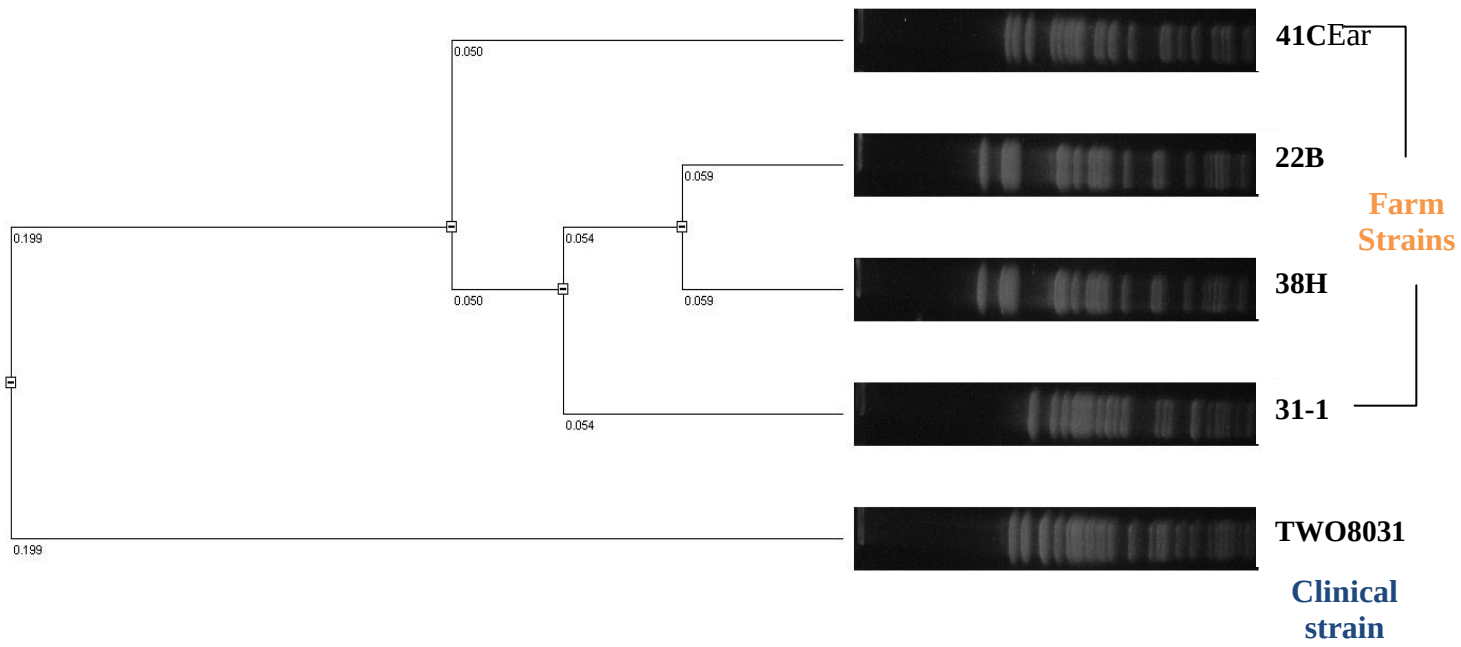


Figure 3.9: GFP transferred bacteria on supplemented LB agar

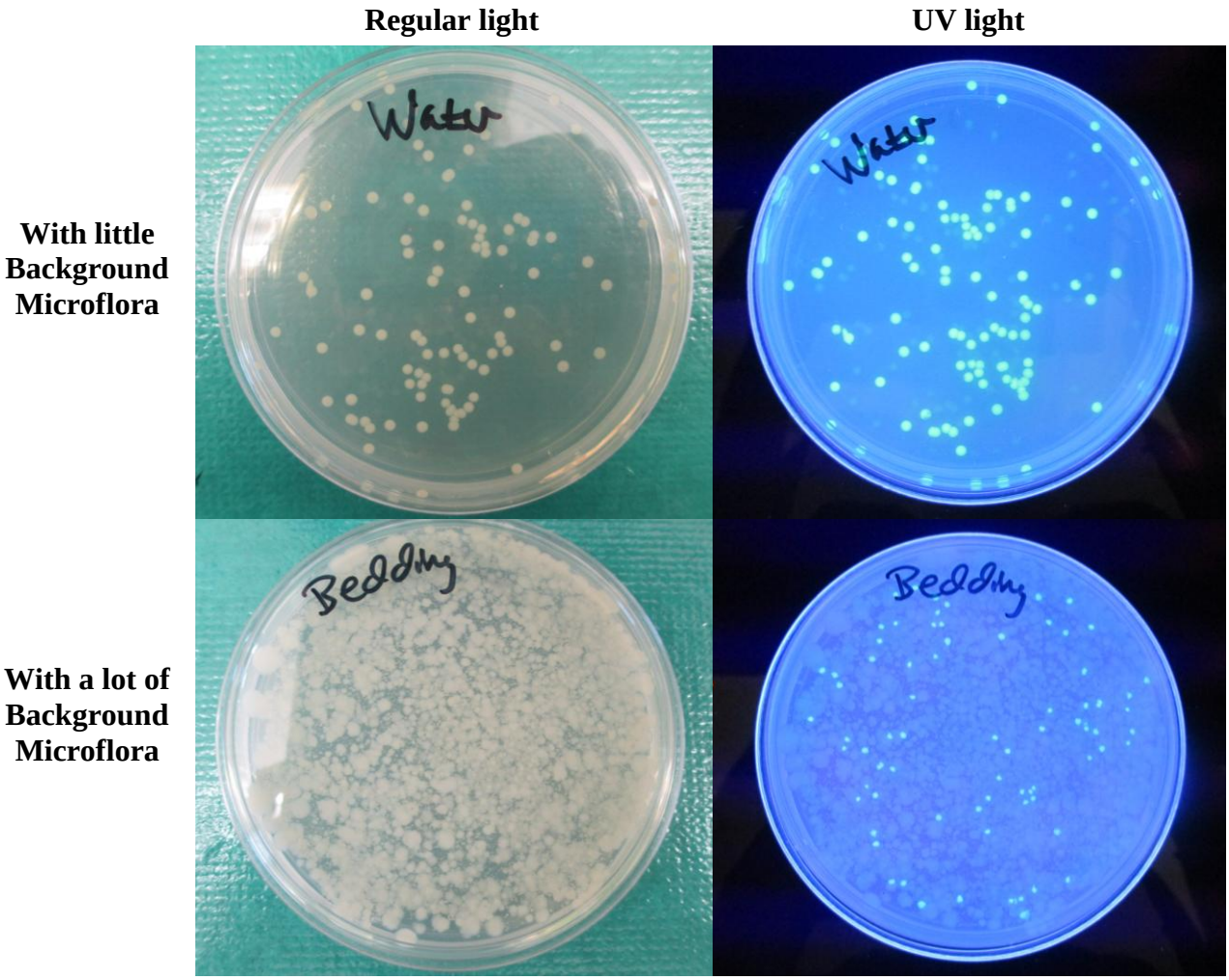
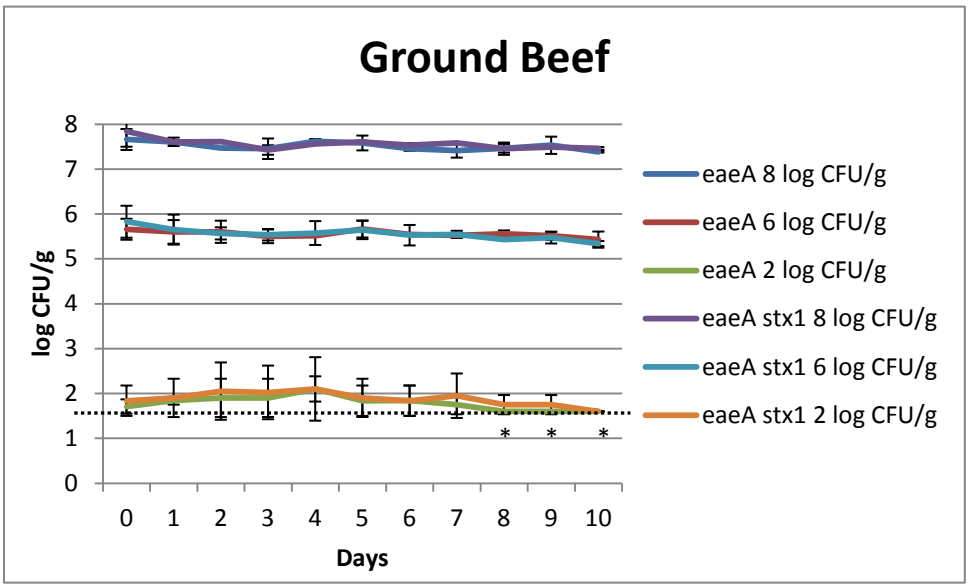


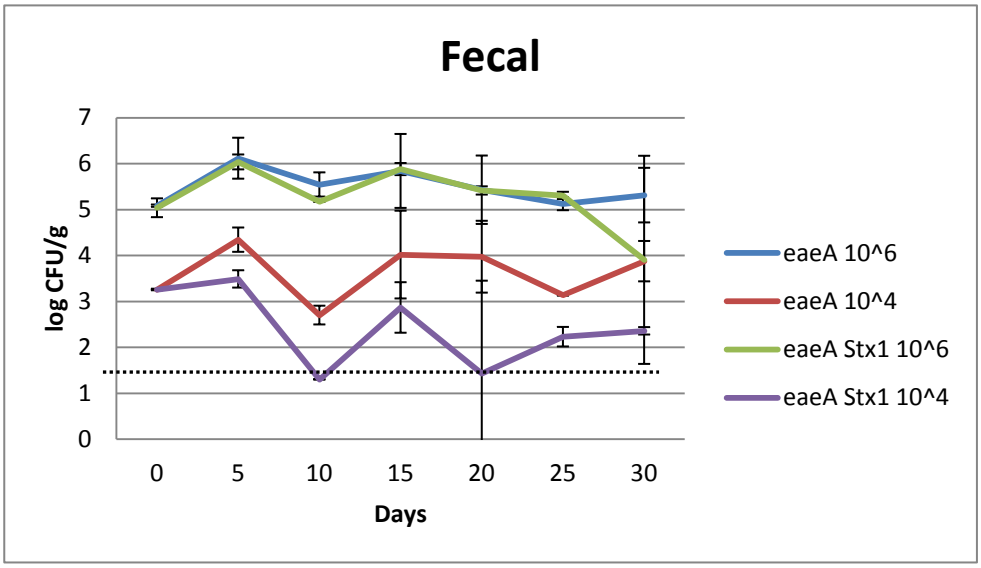
Figure 3.10: Ground beef trial



····· Limit of detection (1.6 log CFU/g)

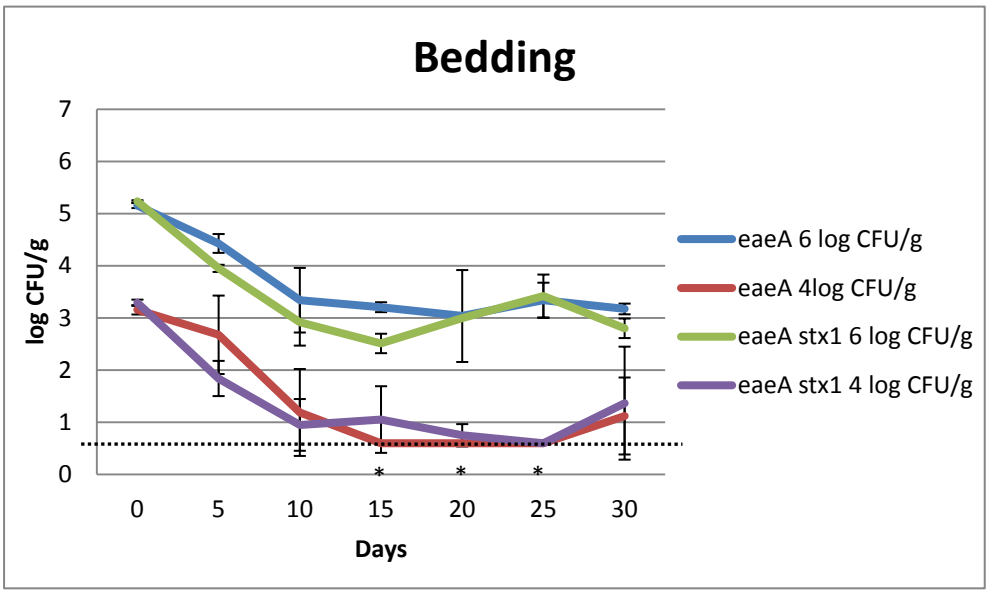
*Enriched and re-plated

Figure 3.11: Fecal environmental trial



Limit of detection (0.6 log CFU/g)
*Enriched and re-plated

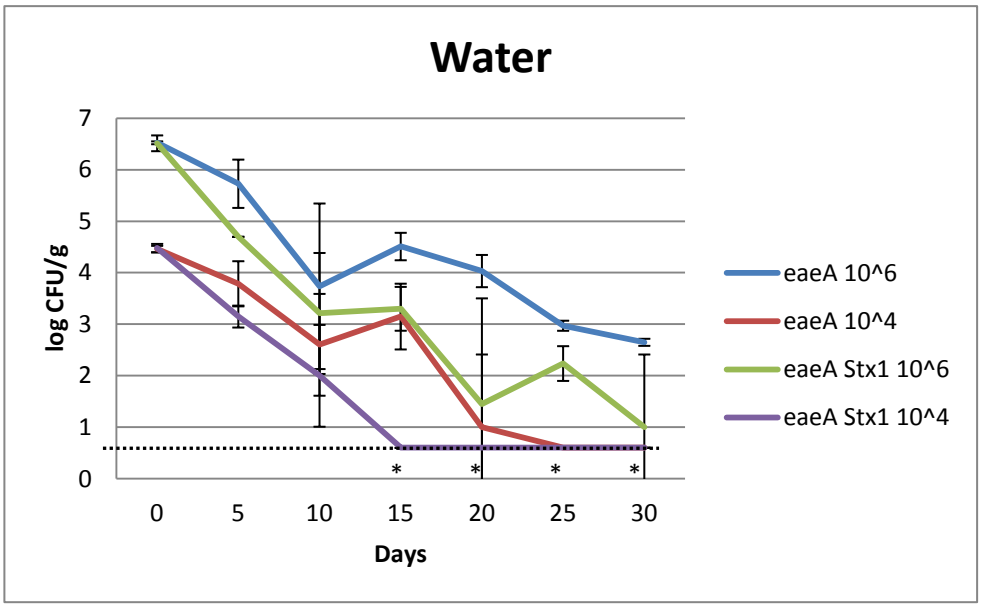
Figure 3.12: Bedding environmental trials



····· Limit of detection (0.6 log CFU/g)

*Enriched and re-plated

Figure 3.13: Water environmental trials



····· Limit of detection (0.6 log CFU/g)
*Enriched and re-plated