NOVEL APPROACHES TO DETECT AND DIFFERENTIATE PATHOGENIC *E. COLI*.

A University Thesis Presented to the Faculty

of

California State University, East Bay

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biological Science

By

Kristen Teuscher

December, 2016

NOVEL APPROACHES TO DETECT AND DIFFERENTIATE

PATHOGENIC *E. COLI.*

By

Kristen Teuscher

Approved: Date:

Kenneth Curr, PhD — OCT. 28, 2016

Maria Gallegos, PhD — 10/28/2016

Carol Lauzon, PhD — 10/28/2016

Table of Contents

List of Figures

Chapter One

Introduction

According to the Centers for Disease Control and Prevention (CDC), approximately 1 in 6 Americans contract a foodborne illness each year, and 128,000 of these patients are hospitalized. Three thousand of those patients will die (CDC, 2016a). Pathogens that cause foodborne illness get into food in a variety of ways. Sources include contaminated water used for irrigation, soil used to grow fruits and vegetables, animals hides that become contaminated during the slaughtering process, and through food handlers; this includes contaminated vessels used to transport food to manufacturing facilities, food not being kept at the proper storage temperatures, and people preparing food for manufacturing facilities or restaurants and other food establishments. If at any time food becomes contaminated and is not stored in proper refrigeration, or cooked to high temperatures, the amount of bacteria in the food can increase exponentially, increasing the risk of contracting a foodborne illness. Despite proper handling of contaminated food, toxins released by certain pathogens can remain and cause illness. In 2014, there were eight hundred and sixty four foodborne outbreaks caused by bacterial agents, and twenty-three of those were caused by Shiga toxin-producing *Escherichia coli* (STECs) (CDC, 2014).

Background

The CDC states there are 31 organisms that cause foodborne illnesses. Among these, some of the most common are in the genera *Campylobacter*, *Salmonella*, *Shigella*, *Listeria*, and *Escherichia* (CDC 2016a). Pathogens, such as those just mentioned, cause varying degrees of gastrointestinal symptoms, such as diarrhea, vomiting, nausea, and cramps. Some people display mild symptoms and they recover in a few days. People who are immunocompromised, young, and/or elderly are at especially high risk and usually suffer more severe consequences. It may be inaccurate to say that the incidence of these diseases has been on the rise over the past 20 years because methods and technologies used for pathogen detection have improved since. Many cases of foodborne illness, nevertheless, go unreported because they do not require hospitalization. Often hospitalization is warranted, however, because in severe cases for example, patients can develop hemolytic uremic syndrome (HUS) where the kidneys fail and the likeliness of death increases. Briefly, HUS is caused by the action of Shiga toxins released by STECs that permeate through the intestinal lining and into the bloodstream.

Epidemiology

Non-pathogenic *E. coli* is part of the normal microbiota of humans and certain other animals. Pathogenic strains, such as *E. coli* O157:H7 and other STECs are not part of most animal normal microbiota but are considered to be part of the normal gut microbiota of ruminants, particularly cattle, making them reservoirs. Once in the human gut, STECs release a toxin (Shiga toxin) that damages the integrity of the intestinal lining and causes severe diarrhea. The action of Shiga toxin will be described in more detail later.

Cattle account for the main source of contamination of STECs in ground beef (Arthur et al., 2010). When cows defecate, STECs are released into the soil and surrounding environment. Feedlots and pastures become contaminated and thus, other cows through normal foraging activities can acquire STECs. During the slaughtering process, the gut of the cow can be punctured and as a result, fecal contents containing STECS can contaminate the slaughtering machinery and other parts of the beef. Once the hide is contaminated, the rest of the cow can also become contaminated during further handling and butchering (Arthur et al., 2010).

There are 3 types of beef products: ground, non-intact and intact beef, and all products have different regulatory guidelines. Preparation of ground beef requires pooling meat from multiple cows that may have originated from various farms. If one

cow in the batch is contaminated, the entire batch of ground beef will become tainted. Grinding machinery subsequently becomes contaminated and any meat processed on the machinery could also get contaminated. Examples of raw ground beef products include meatballs, meat filling such as taco meat, soups such as chili, and sausages. The Food Safety and Inspection Service (FSIS) considers ground beef that is contaminated with *E. coli* O157:H7 as adulterated unless it is further processed in order to kill all pathogens (FSIS, 2002). Whole steaks may have *E. coli* O157:H7 on the surface and are not always considered adulterated because with proper cooking, the *E. coli* will be killed (FSIS, 2002).

Non-intact beef are cuts of beef that have been mechanically tenderized. This is achieved commonly by the insertion of many small needles into the beef. If *E. coli* is on the surface of non-intact meat, the insertion of needles can transfer pathogens to the interior of the meat. If the meat does not reach 140^{o}C in the interior during cooking, the pathogens will survive. If *E. coli* are found on non-intact beef, the beef is considered adulterated unless processed (cooked) for use in "Ready-to-Eat" products (FSIS, 2002).

According to FSIS, customary practices in the United States for consuming beef products by the general public include consumption of meat that is rare to medium rare. The Food Network is a popular television network that broadcasts meal preparation instructions and food related entertainment such as, highlighting certain establishments while traveling, to cooking competitions where contestants win cash and prizes. They also maintain a website in which users can access recipes and tips for food preparation in their homes. The website, foodnetwork.com, recommends cooking beef to 125^{o}F for rare,

130-135^{o}F for medium rare and 135-140^{o}F for medium (Meat and Poultry Temperature Guide: Food Network, 2016). However, FSIS does not recommend consuming beef products below 145^{o}F and also recommends a 3-minute rest between cooking and consumption. The Food Network classifies this as medium-well. Therefore, if ground and non-intact cuts are contaminated with STECs, there is a higher potential for the U.S. consumer to become ill if they are congruent with customary cooking practices. Contaminated intact beef is allowed for purchase because most pathogens are limited to the surface, which are easily killed during customary cooking. Some of these cuts may be irradiated before they are sold (FSIS, 2002). Irradiation techniques include infrared radiation, ultra-violet radiation, and ionizing radiation (FSIS, 1999).

FSIS uses one of two sampling programs for detection of STECS that are in accord with the intended use of each cut of meat. The Beef Manufacturing Trimmings Sampling Program (MT60) processes samples from beef trimmings that are slaughtered onsite. Cuts include trimmings from carcasses, primal cuts like sirloins and ribs intended for raw, non-intact use, and boneless beef in boxes or beef in vacuum shrink bags intended for raw, non-intact use. MT60 monitors contamination from the slaughtering and packaging process. The Bench Trim Sampling Program (MT55) is designed for cuts that are trimmed on a different site from where the cattle are slaughtered to ensure that the processing facility is not receiving contaminated product. MT60 and MT55 do not provide any guidelines for intact cuts of beef (USDA, 2013).

The incidence of foodborne illness has increased over the years for documented and a few likely, speculative reasons. For example, an increased portion of the population

may be becoming more sensitive to virulence factors of pathogenic *E. coli* due to an increase in the number of people in the elderly population. The average life expectancy in 1982 in the U.S. (when foodborne illnesses were first required to be reported,) was 74.5 years (Shrestha, 2006). In 2013, the average life expectancy rose to 78.8 years (CDC, 2016b). As people age, their ability to fight infection decreases because T-cells are not produced at the same rate, which affects the cell-mediated immune response. Elderly people may suffer from chronic diseases such as atherosclerosis, diabetes, cancer, or may be post-surgery, which compromise the immune system. Thus, they tend to have difficulty overcoming the symptoms and fighting infections. Some medications can suppress the immune system adding to a patient's vulnerability and decreasing their ability to fight infections (Strausbaugh, 2001). Since people are living longer and the elderly population has increased, this may contribute to the increase in reported foodborne illnesses.

In addition to the elderly, children under 5 years of age are also at high risk for acquiring an *E. coli* infection that develops in to HUS (Bell et al., 1997). An infant or child's immune system is similar to that of the elderly in their vulnerability in fighting infection. T cells in children are not well developed and are slower to proliferate, therefore less efficient in fighting pathogens (Holt & Jones, 2000). Bell et al., (1997); found an association between children with elevated leukocyte counts and vomiting, and the development of HUS post STEC infection. Nineteen percent of children who had vomiting within 3 days of an STEC infection, progressed to HUS and 38% of children with leukocyte levels greater than 13,000µl within 3 days of infection developed, HUS.

Gerber et al., (2002) speculated that Shiga toxin is transported throughout the body via leukocytes, which increases the spread of the toxin, and thus elevated leukocyte levels yielded a greater amount of toxin being distributed throughout the body. The number of children reported to have illness caused by *E. coli* may be misrepresented. When children are ill, they have a higher probability of acquiring medical care than older adults who let the illness pass without consulting a healthcare professional (FSIS, 2001).

According to the 2001 Food Safety and Inspection Service Public Health Risk Assessment, most ground beef consumption in the United States takes place outside of the home in retail food establishments. The total number of servings of hamburger patties and sandwiches consumed by all ages inside the home between 1994-1996 was 4,969,292,135 servings compared to 11,084,434,611 servings consumed outside the home. People between the ages 25-64 consumed the majority of servings. This age group also consumes a larger serving size versus people in other age groups. The CDC conducted a nation-wide case control study, and their findings suggest that the common dietary factor of individuals infected with *E. coli* O157:H7 was eating ground beef that is "pink in the middle" (Slutsker et al., 1998). Other common factors include consumption of lettuce, milk, apple cider, cheese curd, and fruit salad that became contaminated through cross contamination (Slutsker et al., 1998).

In 2009, 46 states reported a total of 2,215 O157 foodborne isolates yielding an overall isolation rate of 0.72 per 100,000 population. Thirty-five states reported a total of 893 non-O157 isolates from yielding an overall isolation rate of 0.29 per 100,000 population. Forty-five states submitted a total of 1,800 specimens to the National *E. coli*

Reference Laboratory (NECRL) for further characterization of serogroups and gene identification. Non-O157 serotypes were 90% stx-1 positive: O26, O103, O45, O118 and O69. 90% of isolates from O121 were stx-2 positive. 999 (94%) of the 1,066 presumptive non-O157 isolates were actually non-O157 STECs with the top 3 serotypes being O:103 at 203 isolates (20%), O:126 at 187 isolates (19%), and O:111 at 129 isolates (13%). NECRL is not intended to be a nationwide surveillance system and many states can determine serotypes on their own so they do not use the NECRL, therefore the NECRL likely does not represent all STEC cases (CDC, 2012a).

As mentioned earlier, the number of reported STEC infections in all populations may be an underrepresentation for a number of reasons. Also similar to those reported cases with children mentioned earlier, the common symptoms of diarrhea and vomiting may not be severe enough for people to feel the need for medical treatment (CDC, 1998). For those that do seek medical assessment, cases may be missed if the physician does not test for an *E. coli* infection, patients refuse to provide stool samples, labs results give false negatives, and a lack of reporting data to the public health department even though it is a reportable disease (Hedberg et al., 1997; CDC, 1997). While illness figures may be underestimated due to aforementioned factors, the number of illnesses associated with *E. coli* infection has increased over the past decades (FSIS, 2001). Improvements in detection methods, and monitoring and surveillance policies and programs are likely the reason behind this trend and are explained later.

The exact dose response of *E. coli* O157:H7 that causes human disease has not been determined. This is due to that fact that human experiments using STECS are unethical.

The dose response is instead estimated using animals or similar microorganisms and analyzing data from *E. coli* outbreaks. Dose response estimates use the number of cases of *E. coli* infection associated with ground beef consumption in order to produce a statistical distribution, which estimate the total number of cases per year. This distribution is used to determine the dose response using the beta-Poisson function (FSIS, 2001; Powell et al., 2000). Surrogate organisms are used to determine the upper and lower values of the dose response. The surrogate for the upper value is *Shigella dysenteriae*, which has similar virulence factors to *E. coli* O157:H7 but has a very low dose response (<10,000 organisms) (Levine et al., 1973). The lower value uses enteropathogenic *E. coli* because the dose response is high (100 million organisms) and is less pathogenic than *E. coli* O157:H7 (FSIS, 2001). The dose response can be seen in Figure 1.

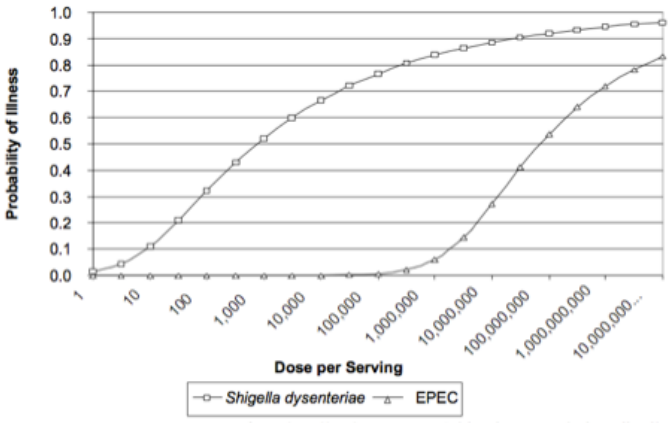

Figure 1. Dose Response Relationship. The dose-response relationship comparing *S. dysenteriae* and enteropathogenic *E. coli*. These organisms represent the upper and lower bound limits estimated with *E. coli* O157:H7 infection (FSIS, 2001).

S. dysenteriae is considered more pathogenic than *E. coli* and when the dose-response relationship was measured, 50% of the subjects became ill at a dose of 740 bacterial cells. When enteropathogenic *E. coli* was examined, the dose at which 50% of the people became ill was 68 million organisms. Therefore, the dose response for *E. coli* O157:H7 is expected to be between 740 and 68 million cells (FSIS, 2001).

Teunis et al. (2008) developed a method of determining dose response of *E. coli* O157 using data from human outbreaks. They estimated the dose from measuring colony-forming units (CFU) by estimating the amount of contaminated food consumed and the concentration of bacteria recovered in the contaminated product. They estimate a range from 3-93 CFU/gram but an exact dose could not be determined because factors like host susceptibility and amount consumed were varied. Serotypes of *E. coli* have varying degrees of virulence, which contributes to the large range in dose response. For example, enteropathogenic *E. coli* is one type of *E. coli* that is classified according to the sequellae of disease. This will be explained in more detail in subsequent chapters.

Outbreaks

Foodborne illnesses are most commonly monitored via outbreaks. According to the World Health Organization (WHO) (2013), an outbreak is defined as a disease occurring more often than what is normally expected in a community. The only time a single reported case is considered an outbreak is when the disease has not previously been seen in that community (WHO, 2013). There is a growing trend for large meat distribution centers across the country (Altekruse et al., 1997). This can lead to isolated incidents of illness that may not be linked as an outbreak. Notably, distribution companies may distribute different types of food, which can cause cross contamination if

transport vessels are not properly cleaned. International travelers have also caused outbreaks by bringing food home from other countries and getting themselves and family members sick. Cultural differences in food preparation also contribute to outbreak incidents (Altekruse et al., 1997).

Some notable *E. coli* O157:H7 outbreaks include the 1991 outbreak in Massachusetts involving raw apple cider, and the1993 outbreak that spanned multiple states and involved ground beef served at a fast-food chain. In the 1991 outbreak, the presence of *E. coli* was linked to contaminated apple cider from a specific apple farm in Massachusetts (Besser et al., 1993). This outbreak was abnormal because *E. coli* is not normally found on the skin of fruits or in fruit and vegetable products (Besser et al., 1993). The source of contamination was not determined, but after investigation, it was determined that the apples were not washed before they were pressed and the cider was not pasteurized (Besser et al., 1993). Laboratory tests also concluded that *E. coli* survived in samples of chilled cider for up to 20 days (Besser et al., 1993).

The 1993 outbreak involved contaminated hamburger patties sold at multistate Jack-in-the-Box locations sparked national attention. Contaminated patties were undercooked and served at 73 different locations causing over 600 people to become ill, many of them children (Bell et al., 1994). Jack-in-the-Box voluntarily recalled their hamburger meat and paid out millions of dollars in compensation to victims. As a result of the outbreak, the FDA raised the cooking temperature for hamburger from 140^{o}F to 155^{o}F and they implemented food labels that provide consumers with safe-food-handling recommendations on raw meat (Golan et al., 2004). More recently, in 2011 there was an

outbreak in Germany involving contaminated sprouts. In this case the causative agent was *E. coli* O104:H4. This organism was an enteroaggregative *E. coli* that had the added ability to secrete Shiga toxins (CDC, 2012b). The increased virulence resulted in 3,000 cases of illness, 18 deaths, and 855 cases of HUS (Burger, 2012).

FSIS declared *E. coli* as a contaminant of raw beef and funding became available for CDC's PulseNet program (Golan et al., 2004). Part of this funding for PulseNet and other food safety programs came from the meat slaughtering and processing industries and companies who had a financial interest in food safety and outbreak prevention. A single point of contamination could mean a loss of millions of dollars for a company. USDA's Economic Research Service (ESR) conducted a survey of large and small meat companies. For example, Ollinger and Moore, (2004) examined the survey findings and discovered that one piece of meat that contaminated the equipment can lead to all meat processed with that equipment to become contaminated. If all this meat goes into 100 bins, then all 100 bins will be contaminated. This can be seriously detrimental to large companies who process large volumes of product. Smaller companies may not be as affected since they are processing smaller volumes of product and recalls can result in less product loss and/or easier remediation efforts, such as cleaning machinery. However, investing in expensive safety equipment and following safety procedures can increase production costs for all involved. Added to these costs are buyers' demands for higher quality, safer products (Ollinger and Moore, 2004).

The Pathogen Reduction and Hazard Analysis and Critical Control Point (PR/HACCP) is a system designed by FSIS to reduce the occurrence of pathogens in

meat and poultry products. The system requires that establishments have written standard operating procedures for sanitation, regular testing of slaughtering facilities and development standards that reduce the number of pathogens in the establishment. It also requires establishments to have guidelines for controls to prevent foodborne pathogens (Golan et al., 2004). This program allows each company to analyze their own protocols and develop methods of improvement allowing for each company to have customized safety procedures according to the demands of their individual operations (Ollinger and Moore, 2004). While measures have been taken to make food safer, and programs implemented to enforce sanitation regulations, the increased consumption of fruits and vegetables from 1980 to 2009 (United States Census Bureau, 2013), for example, challenges complete elimination of foodborne illness. If produce items are not washed properly, foodborne pathogens can remain on the food and cause illness. On June 4, 2012, FSIS began requiring routine testing for non-O157 STECs (O26, O45, O103, O111, O121, O145) in slaughtered cattle and raw beef products (FSIS, 2012).

In 1996, the CDC created PulseNet, a database that combines data from 87 laboratories across the United States to detect and investigate possible foodborne outbreaks. PulseNet utilizes Pulse-field Gel Electrophoresis (PFGE) to identify DNA "fingerprints" of different bacteria in order to link strains and connect them to a specific outbreak. In 1993, 700 people became sick and four people died due to an outbreak of *E. coli* O157:H7 found in contaminated beef patties. The DNA "fingerprints" were analyzed and it was determined that the same strain was found in the tainted beef patties and in the sick patients. It took over a month to identify the beef patties as the common source and

E. coli O157:H7 as the causative agent. PulseNet was created to give researchers access to documented "fingerprints" so that they can easily and quickly identify outbreaks caused by organisms with the same DNA "fingerprint."

The process for identifying an outbreak is as follows: A patient becomes ill and seeks treatment from a doctor. In most cases the doctor orders a stool sample and the causative agent is identified by the facility's laboratory. The laboratory informs the doctor of their findings and the doctor discusses treatment with the patient as well as notifies the state public health department of the illness. Samples are sent to the public health laboratory where they identify the species and they do PFGE to obtain the organism's DNA "fingerprint." The "fingerprint" pattern is uploaded to the PulseNet database, where microbiologists and epidemiologists from the public health department review it for abnormalities. Patients are interviewed in an attempt to identify the source of the organism. If patients are infected with a microorganism that contains the same PFGE pattern and are linked to the same source, an outbreak investigation is initiated.

The efficiency of PulseNet relies heavily on culture-independent laboratory techniques such as PFGE, and labs are progressing towards Next Generation Sequencing. Next Generation Sequencing allows laboratories to sequence the whole genome of microorganisms to provide specific genetic variances between microorganisms, and improve the accuracy of identifying outbreaks. Microorganisms must first be isolated and cultured, however; isolation must be achieved first by traditional media-based culture methods (CDC, 2013). Traditional culture methods can be problematic because one must first obtain a pure culture from a specimen that will likely have many different species of

microorganisms both pathogenic and non-pathogenic. Once microorganisms are isolated, they need to be grown on selective and/or differential media to further narrow down the possibility of the presence of pathogens. Incubation times of media can range from 24 hours to 16 days depending on the microorganism of interest (Lazcka, et al., 2007).

Gavin et al. (2004) claims that some laboratories only screen stool samples with visible blood due to financial reasons or because prevalence is low in that area. Selectively choosing stool samples for testing based on visible blood in the feces is not reliable because in some cases, blood is not visible and can be masked by the culture medium (Gavin, et al., 2004). The CDC now recommends that all stool samples from individuals suspected of possessing a foodborne pathogen be tested for *E. coli* O157 in addition to either Shiga toxin antigens or genes coding for Shiga toxin (Gould et al., 2009). Current methods of detection involve selective and/or differential media for *E. coli* O157 detection, however there is a lack of selective and/or differential media for detecting *E. coli* non-O157 isolates. Identification of non-O157 microorganisms relies on non-culture methods such as enzyme immunoasays. Enzyme assays are not completely reliable for

exclusive identification and detection for STECs due to the possibility of false positives, and traditional culture methods do not detect all strains (Kehl et al., 1997). A combination of culture-based and non-culture based methods are the most efficient methods for identifying non-O157 STECs (Cohen, 2002).

Consumers have become more vigilant in preventing foodborne illness. An example of this is the creation of the Safe Tables Our Priority (STOP) organization.

STOP is a consumer advocate group that lobbies lawmakers for stricter laws and enforcement of food safety. Participants involved with STOP notify the public of foodborne illnesses by making public service announcements, press releases, and make the media aware of personal stories of people who have battled a foodborne illness. They also provide a free helpline and peer mentoring for sufferers (Golan et al., 2004). Another example is the non-profit organization, Partnership for Food Safety Education. This organization was developed in 1997 in response to a report titled Putting the Food Handling Issue on the Table: The Pressing Need for Food Safety Education, which called for ways to educate people on food safety. They provide tools for health and food safety educators and are known for their Fight Bac campaign, which encourages proper food handling (Partnership for Food Safety Education, 2010).

Pathogenic *E. coli*

E. coli is a Gram-negative rod normally found in the gut of humans and cattle. As part of the normal microbiota, *E. coli* is usually nonpathogenic and does not cause disease (Janda and Abbott, 1998). There is growing concern, however, that different serotypes of *E. coli* that produce toxins cause severe gastrointestinal disease, in particular, Shiga toxin-producing *E. coli* (STEC). Kiyoshi Shiga (Fig. 2) discovered Shiga toxin (verotoxin, verocytotoxin, or Shiga-like toxin) during a *sekiri* or dysentery outbreak while he was attending medical school in Japan. At the time, the term "dysentery" was a vague term and referred to "red diarrhea" (Trofa et al., 1999). Thousands of people died as a result of the outbreak. Shiga studied 36 patients with dysentery and isolated a Gram-negative rod that ferments glucose, is indole negative and cannot ferment mannitol. Dogs were infected with the newly isolated microorganism and the dogs exhibited symptoms of dysentery. He determined that the bacteria had toxic properties, which later came to be known as Shiga toxins (Trofa et al., 1999).

Figure 2. Kiyoshi Shiga. Shiga discovered Shiga toxin during an outbreak of dysentery while in medical school

The major pathogenic serotype of concern is *E. coli* O157:H7 (Conedera et al., 2001). A serotype is a classification at the subspecies level. Serotypes are based on the

differences between O and H antigens on the cell surface. Cell walls of Gram-negative organisms contain an outer membrane and thin layer of peptidoglycan above the cell membrane. The outer membrane lines the outside surface of the cell and is exposed to the surrounding environment. Complex lipopolysaccharides (LPS) line the surface of the outer membrane (OM). The OM provides protection and permeability and contributes to the net negative charge of the cell (Wileyet al., 2008). Figure 3 shows the structure of a Gram-negative cell wall.

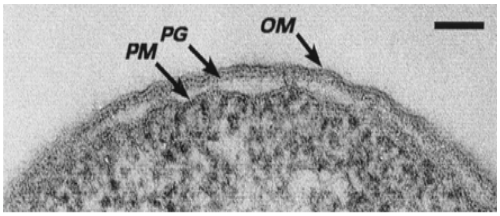

Figure 3. Structure of a Gram-Negative Cell Wall. Arrows indicate the plasma membrane (PM), peptidoglycan (PG), and outer membrane (OM) (Beveridge, 1999).

The LPS component of the OM is comprised of lipids and carbohydrates that are arranged in three parts: hydrophobic Lipid A, core polysaccharides, and the O antigen that extends out of the cell wall (Amor et al., 2000) (Fig. 4). Lipid A forms the top layer of the OM from which the polysaccharides extend outward. If the lipids are released from the OM, they can act as an endotoxin, which are toxic to the host (Talaro & Chess, 2008). When the LPS is released and acts as an endotoxin it triggers the release of host immune factors like interleukins and tumor necrosis factor causing macrophages to migrate to the area. A large influx of macrophages can stimulate an

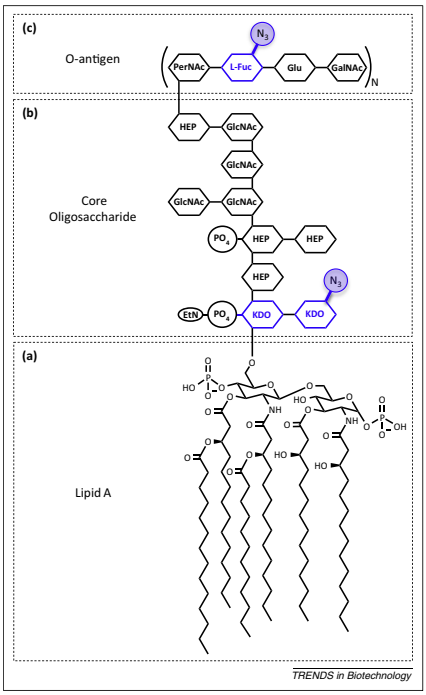

Figure 4. LPS Structure. (Gautam, et al., 2013)

increase in LPS release from degrading bacteria, thus increasing the amount of toxin in the bloodstream. This causes additional tissue damage to the host, eventually leading to septic shock. This is why antibiotics are not recommended to patients with STEC infections (Talaro & Chess, 2008).

Gram-negative bacteria can be further classified according to the components of the LPS. They are referred to as smooth or rough isolates (Pupo et al., 2013). Smooth isolates contain the Lipid A, core polysaccharides, and the O antigen, but rough isolates also have Lipid A, but in contrast to the smooth isolates, they have a shortened polysaccharide core and lack the O antigen (Amor et al., 2000). The core is highly conserved across different members of the *Enterobacteriaceae* family (Amor et al., 2000). There are 5 types of core structures found in *E. coli*: R1, R2, R3, R4 and K-12. The outer polysaccharide cores have the same structure but the order of the polysaccharides and the linkages of the side chain residues vary between serotypes (Amor et al., 2000).

O antigens play a role in pathogenicity. Host antibodies are specific to receptors on the ends of the polysaccharide chains of O antigens. If the polysaccharide chains are long, complement may be inhibited because the enzymes are deposited too far away from the bacterial cell (Lerogue & Vanderleyden, 2001). Some microorganisms can irreversibly lose their O antigens resulting in decreased pathogenicity. This can be due to extreme environmental stressors or in the absence of the host immune system where stressors are decreased and O antigens are not needed. By definition, when an isolate looses the O antigen it is called an S-R (smooth to rough) mutation. (Lukáčová, M. et al., 2008). Boyer et al. (2011), found that *E. coli* O157:H7 isolates that lost their O antigen

showed reduced attachment to lettuce leaves, making them less virulent. Bacteria can alter their O antigens through addition of acetyl or methyl groups, or the addition of fucosyl and glucosyl residues to the antigen (Lerogue & Vanderleyden, 2001). The structure of bacterial O antigens can vary within a species. Thus, serotyping is based on differences within the O antigen structure itself. Since host antibodies are antigen specific, immunity for one serotype does not constitute immunity for a different serotype (Todar, 2008). In addition to using O antigens for serotyping, H antigens are also identified.

H antigens are also called flagellar antigens and are found on the surface of bacterial flagella. Construction of flagella involves a Type III Secretion System (T3SS) located in the basal body. Flagellin proteins make up the H antigens and they are transported through the hollow core of the filament until they reach the filament cap. They bind together which allows the filament to grow from the tip rather than growing from the base (Wiley et al., 2008). Each strain of bacteria has a specific type of flagellin, which is determined by the loci of each flagellar protein within the bacterial chromosomes. Possible loci include *fliC*, *flkA*, *fllA* and *flmA*. The type of flagellin a bacterium displays is categorized as H1, H2, H3, for example (Ratiner et al., 2003).

Flagella protrude from the cell wall to aid in locomotion. They can be arranged in a variety of ways, a characteristic that also helps to identify bacteria. For example, *E. coli* have a peritrichous flagellar arrangement, which means they have many flagella that completely surround the cell. A flagellum (flagella, plural) is made of three parts: the flagellar filament, basal body, and flagellar hook. The flagellar filament is a long, cylindrical section made of the protein flagellin. The basal body is embedded in the cell

wall and the hook connects the filament to the basal body. Gram-negative organisms, such as *E. coli*, have four rings that make up the basal body in the cell wall.

The basal body is where flagellar movement is initiated. It can be broken down into two parts: a stationary part (stator) and a rotating part (rotator). A rod comes down the center of the basal body through the L ring in the LPS and the P ring in the peptidoglycan, and connects to the MS ring in the inner membrane. The MS ring is surrounded by the stator which is made up of two proteins: MotA and MotB. MotA and MotB create a proton channel that moves ions across the membrane. (Wiley et al., 2008; Blair, 2003). The flagellar structure within the bacterial membrane is seen in Figure 5.

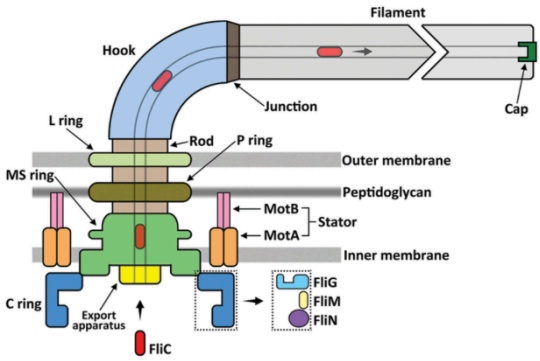

Figure 5. Protein Components of Flagella. (Xue et al., 2015).

Bacteria move by quick rotation of the flagella. Cells that are free to move about will move in the opposite direction as the flagella. If the flagellum rotates counter-clockwise then the cell moves clockwise. This propels the bacterium forward in a movement called a run. If the bacterium needs to change direction, the flagella will rotate the opposite way causing the cell to stop and tumble. When bacteria are attracted to or repelled by chemical signals this movement is called chemotaxis. If a bacterium is attracted to a substance, it will engage in longer runs, but, if a substance repels it, it will tumble in order to change its direction in an attempt to move away from the repellant (Willey et al., 2008). The ability for bacteria to move is considered a virulence factor

because a motile bacterium can evade phagocytosis or locate a site for attachment on the host membrane. While *E. coli* are motile, according to Girón et al., (2002), *E. coli* strains that lack flagella do exist and they show decreased pathogenicity.

One way in which *E. coli* is classified or grouped is based on its ability to cause harm, or virulence. Enteroaggregative *E. coli* (EAggEC) produce an enterotoxin that causes diarrhea that lasts more than 14 days. Enteroinvasive *E. coli* (EIEC) do not produce toxins but they can colonize epithelial cells in the intestine. This causes high volumes of diarrhea that may or may not contain blood. Enteropathogenic *E. coli* (EPEC) produce toxins that cause attachment-effacement (AE) lesions where the brush-border microvilli of the intestines are destroyed. Enterotoxogenic *E. coli* (ETEC) produce toxins once they are attached to the intestinal lining. They cause sudden diarrhea known as Travelers' Diarrhea. Enterohemorrhagic *E. coli* (EHEC) produce Shiga-like toxins that cause AE lesions and bloody diarrhea (Jay et al., 2005). This paper focuses on enterohemorrhagic *E. coli* and the production of Shiga toxin.

Shiga-like toxins released by STECs are similar to toxins released by *Shigella dysenteriae,* which causes shigellosis or dysentery. Shigellosis is characterized by watery diarrhea, abdominal cramps, tenesmus (constant feeling of the need to pass stools), fever, and blood and pus in the stool (Murray et al., 2009). STEC organisms can produce either Shiga-like toxin 1 (Stx1) and/or Shiga-like toxin 2 (Stx2). Stx1 more closely resembles Shiga toxins associated with *S. dysenteriae*. Genes for Stx are found in lambda phages that infect *E. coli*.

Shiga toxins are proteins composed of one A subunit and five B subunits (Fig. 6). The A subunit acts as an enzyme that cleaves an adenine residue on the rRNA of the 60S ribosomal subunit to cease the elongation of the RNA thus, inhibit protein synthesis. The B subunits of the toxin bind to globotriaosyleramide (Gb3), a glycolipid on host endothelial cell membranes that line the intestines (Tesh & Brien, 1991; Fraser et al., 2004). Once attached to the host membrane, the toxin is endocytosed through the formation of a clathrin-coated pit that forms a sealed vesicle inside the cell. Some cells can fuse the vesicle with lysozymes to degrade the toxin but other cells transport the vesicle to the endoplasmic reticulum via the Golgi and then it translocates to the cytosol (Fig. 7). During this process, the A subunit is cleaved. The cleaved A subunit acts as an RNA N-glycosidase to cleave a N-glycosidic bond in the 28S rRNA which prevents binding of the acyl-tRNA to the 60S ribosomal subunit to inhibit peptide elongation during protein synthesis (Paton & Paton 1998).

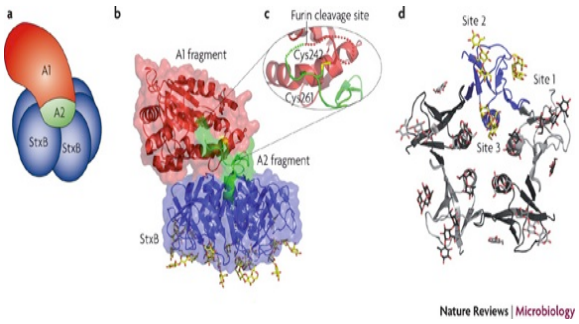

Figure 6. Shiga Toxin Protein Structures. (Johannes & Römer, 2010).

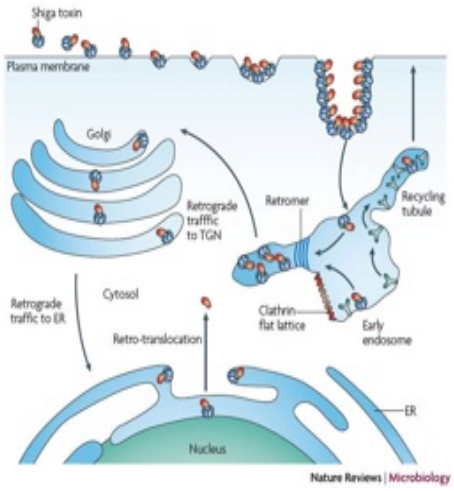

Figure 7. Transport of Shiga Toxin Through the Host Membrane. Toxin binds to the intestinal mucosa and enters the cell via clathrin-coated pit. It is subsequently transferred to the ER before it goes to the Golgi and nucleus (Johannes & Römer, 2010).

As mentioned earlier, Shiga toxin is associated with certain *Shigella* spp. and some *E. coli.* STEC infections with associated toxin production cause mild to severe outcomes, i.e., diarrhea to death of individuals with compromised immune systems (Gyles, 2007). STECs cause characteristic attaching and effacing (A/E) lesions. They colonize the intestine and attach to the microvilli of mucosal lining via bundle forming pili (Donnenberg et al., 1997). The bacteria release proteins that destroy the microvilli (effacement) and causes rearrangement of the actin of the enterocyte. The STEC attaches to the surface of the enterocyte via an outer membrane protein called intimin (Donnenberg et al., 1997). Further rearrangement of the actin produces a pedestal formation and causes alterations in the resting membrane potential of the enterocyte (Fig 8). Changes in resting membrane potential result in decreased intestinal absorption and diarrhea (Donnenberg et al., 1997). The genes necessary for the generation of A/E lesions are located on pathogenicity islands or locus for enterocyte effacement (LEE) (Wiley et al, 2008). Pathogenicity islands are a group of genes that encode proteins necessary for various virulence factors (Wiley et al., 2008). LEE includes a cluster of genes (*sepA* to *sepI*) that encode a T3SS and the eaeA gene that encodes intimin (Paton & Paton, 1998). LEE also encodes the receptor (Tir) for intimin.

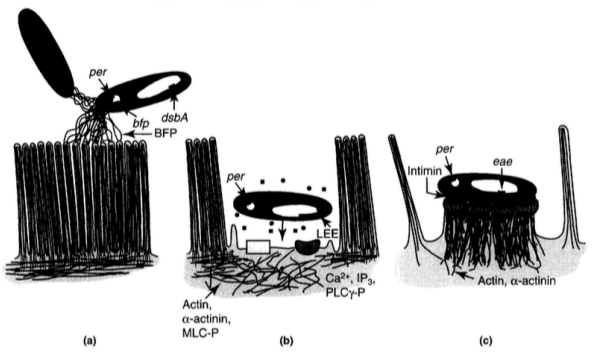

Figure 8. The Stages of AE Lesion Formation. Bacteria attach to the microvilli (a). Then it secretes proteins that facilitate attachment and rearrangement of actin (b) followed by pedestal formation (c) (Donnenberg et al., 1997).

Non-O157 strains do not produce A/E lesions therefore the attachment to the enterocyte is mediated by a fimbriae adhesion F107 (f*edA*) (Paton & Paton, 1998).

Shiga toxin is released by the STEC and penetrates the intestinal lining, inhibiting enterocyte protein synthesis, killing the cells. When multiple cells die, the lining of the intestine begins to lose integrity, and as a result, water is not absorbed from the stool, resulting in diarrhea and in some cases, bloody diarrhea. Once the intestinal lining has been infiltrated by STECs, the pathogens travel through the blood stream to the kidneys where the glomerulus is damaged to the point of kidney failure (Paton & Paton, 1998).

Individuals with kidney involvement suffer from what is known as hemolytic uremic syndrome (HUS). In HUS, the toxin binds to globotriaosylceramide (Gb3) receptors that line the epithelium of the glomerulus (Fig. 9). The toxin is transported to the ribosome where it cleaves an adenine form the host 28S rRNA to inhibit protein synthesis (Paton & Paton, 1998). This causes the glomerulus to detach and fibrin and platelets are deposited to the glomerular capillaries. Fibrin and platelets block blood flow to the kidneys (Paton & Paton, 1998). According to a review by Thorpe (2004), research shows that Shiga toxin induces the release of host cytokines that promote inflammation and apoptosis, which may increase the number of Shiga toxin receptors of host epithelium.

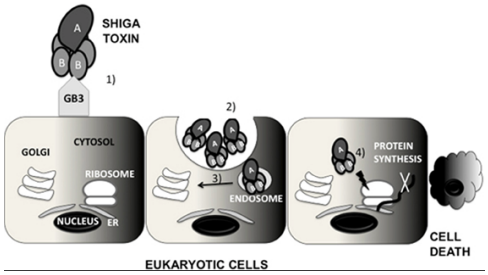

Figure 9. Binding of B Subunits to GB3 Receptors. Binding of host cells allows for endocytosis of the toxin leading to inhibition of protein synthesis (Pacheco & Sperandio, 2012)

While there are five different types of stx2 (genes), only one of them is found in O157 serotypes. However, pathogenicity of Stx1 and Stx2 (toxins) differ. Patients with *E. coli* O157:H7 that produce only Stx1 present with symptoms of bloody diarrhea. Patients with *E. coli* O157:H7 that produce Stx2 are more likely to develop HUS because Stx 2 toxins have a wider range of receptors that they can bind to (Fraser et al., 2004). Therefore, the varying degrees of pathogenicity are due to affinity of the toxins to receptors on host epithelium (Fraser et al., 2004).

Li et al., (2000) discovered certain proteins associated with *E. coli* virulence factors show promise toward creating vaccines against EHEC infection. Vilte et al., (2012) used bacterial ghosts (empty envelopes of Gram-negative cell walls with no cytoplasmic content) to elicit an immune response in cattle in an attempt to prevent *E. coli* O157:H7 colonization. They introduced the bacterial ghosts subcutaneously, then measured the shedding of *E. coli* O157:H7 in the feces and tested for immunoglobulins in the serum and saliva after challenging them with various doses of *E. coli* O157:H7. They found that vaccinated cattle had reduced shedding, or colonization, of *E. coli* O157:H7 and the shedding did not last as long as the non-vaccinated cattle (Vilte et al., 2012). This and multiple other studies (Babiuk et al., 2008; Mayr et al., 2005; Smith et al., 2008) show promise in using bacterial ghosts as potential vaccines in humans and cattle to reduce intestinal colonization of pathogenic *E. coli*.

Current Detection Methods

Detection of foodborne pathogens include sampling, microscopic examination, and traditional culture methods. Sampling methods include swabbing food with a cotton swab and streaking media, or direct contact with the food to the surface of an agar plate. Ultra-sonic devices are often used to help remove samples from swabs into a diluent. The agar syringe method can be used where a syringe is filled with agar and the agar is pushed past the syringe plunger. Agar that protrudes from the syringe makes contact with a surface and is cut off and put in a Petri plate. Other sampling methods include pouring tempered agar over a contaminated surface and letting the agar harden. The agar is then placed in a Petri plate for incubation (Jay et al., 2005).

Chemical detection methods involve testing for specific proteins or cell components that are specific to each pathogen. For example, *Limulus* amoebocyte lysate (LAL) is used to detect Gram–negative endotoxins (Jay et al., 2005). LAL contains hemolymph protein of the horseshoe crab, *Limulus polyphemous*, which is sensitive to the effects of Gram-negative endotoxins. Food samples are added to the lysate and the presence of endotoxins causes agglutination of the protein lysate. This method was first used to detect pathogens in ground beef (Jay et al., 2005). Since the discovery of LAL, newer methods for detecting pathogens in food aim to detect pathogen presence more rapidly and to increase pathogen specificity. For example, chromogenic substrates can allow for detection of a pathogen using chromogens, such as *o*-nitrophenyl-β-D-

galactopyranoside (ONPG), 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal) and indoxyl-β-D-glucuronide (IBDG) (Manafi et al., 1991). Bacterial enzymes cleave chromogens, and the product(s) cause a color reaction. Fluorescent products can also be visualized from bacterial enzymatic reactions such as the use of 4-methylumbelliferyl-β-D-glucuronide MUG where 4-methylumbelliferyl is hydrolyzed by β-glucuronidase resulting in the release of fluorogenic substances that can be detected under UV light (Power & Zimbro, 2003; Manafi et al., 1991).

Immunological methods, such as Enzyme Linked Immunosorbant Assay (ELISA) can also be used for pathogen detection. An ELISA can be used to test directly for the antigen or indirectly by testing for serum antibodies to the antigen. When the antigen and antibody bind to form a complex, a color appears to detect antigen or antibody presence. Antigens are added to a small, polystyrene coated well. Antibodies from the patient serum (or antibodies specific to the antigen being tested for in the indirect method) are added to the well. A second antibody is added. The second antibody is linked to an enzyme, usually horseradish peroxidase, and upon the addition of the colorless substrate, the enzyme reacts to produce a colored product (Jay et al., 2005; Nisonoff, 1984). The amount of color produced is measured using a spectrophotometer to determine the amount of antigen or antibody that is present (Nisonoff, 1984).

Polymerase Chain Reaction (PCR) can be also used for detection of specific pathogens or virulence genes (Jay, et al., 2005). PCR takes specific segments of DNA identified by a set of sequence specific primers and amplify the gene segments. The variety of genes that can be identified range from being able to identify a specific

taxonomic group of organisms or to identify the presence of genes responsible for certain virulence factors (Naravaneni & Jamil 2005). An advantage of using PCR is that a very small amount of DNA, sometimes from a single cell, is enough to produce a successful PCR application. PCR can be used in conjunction with biochemical media to identify different serogroups of STECs. Lin et al., (2011) used Real-time PCR (RT-PCR) to detect specific molecular structures of surface O antigens. The sensitivity of this assay allowed them to identify misclassified or unidentified STEC isolates. They recommended that RT-PCR be used in conjunction with screening for Shiga toxin genes since O antigen identification does not equate virulence and pathogenicity. Conrad et al., (2014) enhanced the work of Lin et al., (2011) by modifying the primer recognition sites in order to increase PCR sensitivity for genetically similar STECs such as O26, O45, O103, O111, O121, and O145.

When determining food quality and safety, researchers often test for indicator organisms rather than pathogens themselves. Typically, tests are specific for detection and isolation of *E. coli* because in most cases, *E. coli* indicates probable fecal contamination. Indicator organisms such as *E. coli*, can be detected using selective and differential media. Media such as MacConkey agar (Fig. 10) selects for Gram-negative enteric organisms by inhibiting Gram-positives and non-enterics using crystal violet and bile salts (Leboffe & Pierce, 2012).

Figure 10. *E. coli* on MAC Plates. Left: Non-lactose fermenting *E. coli*. Right: Lactose fermenting *E. coli*. Photos by

Eosin Methylene Blue Agar (EMB) is a selective and differential medium that helps to differentiate *E. coli* from *Enterobacter aerogenes,* two closely related coliform bacteria. EMB uses the dyes eosin Y and methylene blue to inhibit the growth of Gram-positive bacteria. Gram-negative organisms that grow on EMB are differentiated by their ability to ferment lactose in the media. If lactose is fermented by a bacterial species, colonies appear dark purple and if lactose is not fermented the colonies are light pink (Becton Dickinson, 2006) (Fig. 11). Eosin Y and methylene blue are also used as pH indicators to detect a significant decrease in pH as a result of *E. coli* lactose fermentation, and causes the metallic green sheen that is characteristic to *E. coli* (Leininger, et al., 2001). The use of media such as MacConkey agar and EMB only allow for isolation of specific of bacteria and further tests are usually necessary to identify specific species or serotypes. Thus, multiple steps of identification results in increased time needed to investigate the causative agent of an outbreak.

Figure 11. *E. coli* on EMB. Isolated colonies show characteristic metallic green sheen of colonies of white thicker growth shows purple color due to lactose fermentation. Photo taken by Kristen Teuscher

Federal agencies and researchers are pressed to find more accurate and faster detection methods for STECs. The USDA (2014) recommends *E. coli* O157:H7 are detected in a variety of methods and must be differentiated from non-O157:H7 serotypes. One way to do this is by using a sorbitol MacConkey agar (sMAC). *E. coli* O157:H7 serotypes are sorbitol negative and produce clear colonies on sMAC and non-O157:H7 serotypes are sorbitol positive with pink colonies on sMAC. However, other organisms

produce clear colonies on sMAC plates so this is not conclusive for *E. coli* O157:H7. The RIM® *E. coli* O157:H7 Latex Test Kit uses sorbitol negative colonies and differentiates *E. coli* O157:H7 from other sorbitol negative cultures. This test uses 3 latex reagents: one with anti-O157 antibodies, one with anti-H7 antibodies and one with rabbit globulin to serve as the negative control. Confirmation of *E. coli* O157:H7 occurs when agglutination occurs with mixing of samples with the anti-O157 antibody and anti-H7 antibody, with no agglutination in the negative control. Samples that are positive for agglutination are streaked on Sheep Blood Agar plates. Pure colonies are then identified using the biochemical identification assay VITEK®2. The VITEK®2 is an automated identification assay that uses inoculated cards with various metabolic substrates that are incubated in the machine and analyzed every 15 minutes for turbidity or color of the substrate. The reaction in each well of the card is compared to a database developed from a variety of clinical, industrial, and educational sources to identify each organism (Pincus, 2006).

Shiga toxin production is confirmed by the Meridian Primer® EHEC Kit or the BAX® System Real-time PCR Assay STEC Screening assay (Gould et al., 2009). The Meridian Primer® EHEC Kit tests for the presence of Shiga toxin in stool samples through an ELISA (enzyme linked immunosorbant assay) protocol. Prepared stool samples are added to a microwell pate. Then monoclonal and polyclonal anti-Shiga toxin antibodies are added to the wells. Addition of an enzyme and substrate catalyze a color change in the presence of Shiga toxins binding to the monoclonal antibodies (Meridian Bioscience, 2016). Real-time PCR is conducted selecting for *stx1, stx2* toxin genes and *uidA* genes for β-D-glucuronidase production. Real-time PCR is a sensitive PCR method

that allows the user to begin with a very small concentration of the sample nucleic acid. As the nucleic acid is replicated through the PCR process, the amount of nucleic acid is measured in real-time by fluorescent technology to determine the amount of nucleic acid present in the original sample (Fraga, et al., 2008). This allows for detection of small concentrations of toxin genes in a sample. Further testing for samples that are positive for the selected genes can be cultured on media that was described above such as, Sorbitol MacConkey Agar, Rainbow® Agar, EMB agar, and tested for latex agglutination and biochemically identified using the API20E or VITEK assays (Feng et al., 1998). Non-O157 STECs do not all share the same characteristics so their detection and differentiation can be difficult unless they are identified by toxin production and toxin characteristics (Fratamico, et al., 2014). My research aims to use simple methods utilizing agar media to quickly differentiate between general pathogenic strains of non-O157 *E. coli* and non-pathogenic *E. coli* strains and to develop a profile to differentiate between strains of non-O157 STECs.

For my thesis research, I analyzed three different possible capabilities of certain STECs to determine if any or all of these capabilities might be used for improved detection protocols. All of these capabilities can be easily visualized on bacteriological media rather quickly, i.e, within 24 h. The first capability involved the potential susceptibility of certain STECs to a bacteriophage. I refer to this examination as a "biological detection method. " Bacteriophages are viruses that infect bacteria. I tested the ability of a bacteriophage to infect certain STECs known to infect non-pathogenic *E. coli*. I want to see if STECs have a defense mechanism against predators such as a

bacteriophage, which may add to their virulence or if they can be destroyed in a phage assay. I also surveyed certain STECs for the ability to produce products associated with biochemical pathways, such as siderophore and quorum sensing compound production and to see if these abilities are correlated with toxin production. Siderophores are iron-chelating compounds that sequester iron from the environment and bring them into the bacterial cell (Garénaux et al., 2011). Iron is used in bacterial cells for functions such as cell growth, enzyme production (peroxidase and superoxide dismutase), and metabolism (toxins, vitamins, tricarboxylic acid cycle, and electron transport) (Messenger & Barclay, 1983). I aim to determine if siderophore detection could be used to differentiate stains of STEC from each other O157:H7 *E. coli.* Finally, I tested for the production of quorum sensing compounds. Gram-negative bacteria communicate with each other through quorum sensing compounds, particularly acyl-homoserine lactones (Shaw et al., 1997). Studies have shown that quorum sensing compounds influence the expression of virulence factors (Natrah et al., 2011; Le Berre et al., 2008) including toxin production. My research similarly aimed to determine if certain STECs produce quorum-sensing compounds and if their production can be used to differentiate strains of STEC from each other and from O157:H7 *E. coli*.

Chapter Two

Bacteriophage

Bacteriophage (phage) are viruses that infect bacteria. They are composed of nucleic acids within their core, and a protein coat, or capsid, in the shape of a hexagon. The single units that make up the capsid are called capsomeres. A tail extends from the head and it is the tail that contains a contractile sheath (reviewed by Bradley, 1967). When a phage infects a bacterial cell, it must first adsorb to the cell wall of the bacterium. Once the phage attaches to the host, the tail fiber binds to the surface of the host and the tail sheath shortens. The phage releases lysosomes so the central tube can penetrate the host peptidoglycan and transfer its nucleic acid into the cytoplasm of the cell. Host RNA polymerase makes early mRNA from the phage DNA, which is responsible for proteins and enzymes necessary for phage generation. Host DNA is degraded and subsequently, nucleotides can be used for phage components. Once all phage DNA is created, proteins bind together to build the tail, sheath and capsid and phage DNA are packaged inside the capsid. Once all virions are assembled, the phage releases lysozyme to degrade the peptidoglycan and create holes in the cell wall to fully destroy the cell. The viral particles are released from the cell and attach to neighboring cells where the infection process occurs again with a new host cell (Weinbauer, 2004).

Depending on the type of phage, either virulent or temperate, one of two things can happen to the nucleic acid. Virulent phage undergo the lytic cycle where host DNA replication ceases and only viral proteins are synthesized. Phage nucleic acid is replicated, proteins assemble and the host cell lyses to release the virions. Temperate phage undergo the lysogenic cycle where phage nucleic acid can be incorporated into the host genome and remain dormant, creating a prophage. The host uses its replication machinery to replicate both genomes and all of the host progeny contain both types of nucleic acid. Usually an environmental stressor, such as UV light, triggers the prophage nucleic acid to be excised from the host chromosome and the phage switches from a lysogenic cycle to a lytic cycle (Wiley et al., 2008). Host DNA replication ceases, phage nucleic acid is replicated and assembled before the host cell lyses and virion particles are released (Singh et al., 2009).

PhiX174 belongs to the family *Microviridae* and genus *Microvirus* and has an icosahedral capsid with a circular single stranded DNA core (Roux et al., 2012). PhiX174 is used in detecting fecal contamination in water because it targets specific receptors found on 3% of *E. coli* strains (Michel et al., 2010). Michel et al. (2010) reviewed the sensitivity of 783 different *E. coli* strains to PhiX174 by analyzing genotypic and phenotypic traits of the O antigen. The strains that were sensitive to PhiX174 are rough with no O antigen bound in their core LPS. A Triplex PCR phylotyping method was used to determine the A_1, A_0, B1 and $B2_3$ subgroups of *E. coli* strains that were tested. The reference strain used in the study was sensitive to PhiX174 and belonged to serogroup A_0, which implies that all sensitive strains have a rough characteristic. This likely means

that entry into the host is the limiting factor in determining phage infection. Smooth strains were manipulated to become rough strains and found that after they became rough they became sensitive to infection. PCR was conducted to determine the LPS core types of all *E. coli* and they found the *E. coli* that were sensitive to infection had core types R1, R2 or R3. From the combined tests, they concluded that phage sensitivity is not dependent on specific components of the LPS but rather depended on the overall makeup of the LPS. Also, PhiX174 demonstrated some flexibility to host attachment and entry, and other bacterial species with similar LPS structures have the potential to be sensitive to infection (Michel et al., 2010). A plaque assay can be used to test bacteriophage activity and host susceptibility. Agar plates are prepared, and dilutions of lytic phage are prepared in tubes of tempered agar. The tempered phage dilutions are mixed with host bacteria then poured over the hardened agar plates. As the bacteria grow to stationary phase, if they are susceptible, they are infected by the phage. Phage progeny infect neighboring bacteria to create a clearing called a plaque. The opaque growth around the plaques is where uninfected bacteria are present. Plaques are counted to determine the concentration of phage in the original sample (Anderson et al., 2011).

Methods

Biochemical Profiles

All cultures: *E. coli* O45:S107, *E. coli* O103:S7, *E. coli* O111:E3, *E. coli* O121:S19, *E. coli* JZ Green, *E. coli* Lac-, *E. coli* O157:H7 ATCC 43894, *E. coli* O157:OC-1, *Shigella boydii* ATCC 20249, *Shigella dysenteriae* CDC-85-3131, *Shigella flexneri* ATCC 20239, *Shigella sonnei* ATCC 9290, *E. coli* ATCC 25922, *E. coli* CN-13 ATCC 700609, were grown first in Tryptic Soy Broth (TSB) (Becton, Dickinson and Company, Sparks, MD) prepared following manufacturer's directions. *Shigella* spp. was used as positive controls due to their known ability to secrete Shiga toxins. Biochemical tests were performed on all strains as a part of routine identification and differentiation for these pathogens.

Lactose broth: tubes of Phenol Red Broth (PRB) (Becton, Dickinson and Company, Sparks, MD) were made according to manufacturer instructions. Seven and one-half grams of PRB and 5 grams of lactose (Becton, Dickinson and Company, Sparks, MD) was added to 500 milliliters of distilled water and mixed well. Five milliliters of the combination was aliquoted into test tubes and Durham tubes were added individually to each tube. The broths were autoclaved at 121°C at 15 psi for 15 minutes. *Methyl Red/Vogues-Proskauer* (MRVP) broth: eight and one-half grams of MRVP (Becton,

Dickinson and Company, Sparks, MD) was mixed with 500 milliliters of distilled water and 4 milliliters of the broth was aliquoted into individual test tubes and autoclaved as described earlier. *Tryptone* broth: fifteen grams of tryptone (EM Science, Gibbstown, NJ) was added to 500 milliliters of distilled water and 5 milliliters of broth was aliquoted into individual test tubes and autoclaved as described earlier. *Citrate* slants: citrate (Becton, Dickinson and Company, Sparks, MD) medium was prepared by adding 250 milliliters of distilled water to 6.05 grams of citrate medium to and dissolved by heating. Four milliliters was aliquoted into individual test tubes and autoclaved as described earlier. Tubes were cooled on their side to create a slant. *Motility Test Medium (MTM) with Triphenyltetrazolium Chloride (TTC)*: five and one-half grams of MTM (Becton, Dickinson and Company, Sparks, MD) was mixed with 250 milliliters distilled water and 0.05g of TTC was added. Four milliliters of the media was aliquoted into tubes and autoclaved. *Triple Sugar Iron (TSI)* slants: thirty two and one-half grams of TSI (Becton, Dickinson and Company, Sparks, MD) was added to 500 milliliters of distilled water and 6 milliliters was aliquoted into tubes and autoclaved. The tubes were cooled on their side to create a slant. *Nitrate*: four and one-half grams of nitrate (Becton, Dickinson and Company, Sparks, MD) was added to 500 milliliters of distilled water and 5 milliliters was aliquoted into tubes. A Durham tube was added to each tube and then autoclaved. *Gelatin*: thirty-two grams of gelatin (Difco, Detroit, MI) was added to 250 milliliters of distilled water and 4 milliliters was aliquoted into tubes and autoclaved.

Bacteriophage Assay

E. coli O45:S107, *E. coli* O103:S7, *E. coli* O111:E3, *E. coli* O121:S19, *E. coli* JZ Green, *E. coli* Lac-, *E. coli* O157:H7 ATCC 43894, *E. coli* O157:OC-1, *Shigella boydii* ATCC 20249, *Shigella dysenteriae* CDC-85-3131, *Shigella flexneri* ATCC 20239, *Shigella sonnei* ATCC 9290, *E. coli* ATCC 25922, *E. coli* CN-13 ATCC 700609 were subcultured in Tryptic Soy Broth (TSB) (Becton, Dickinson and Company, Sparks, MD) and incubated overnight at 35^{o}C. *E. coli* CN-13 ATCC 700609 was used at the positive control because it is the typical host for PhiX174 and has created plaques on the agar plates when used in previous experiments. One milliliters of each sample was put aseptically into 30 milliliters of TSB and incubated at 35^{o}C for 4 hours. A dilution series was set up using the bacteriophage PhiX174 (ATCC# 13706 B1). One hundred microliters of phage was put in 0.9 milliliters TSB and mixed by pipetting. One hundred microliters was removed and diluted in 0.9 milliliters TSB and mixed well. Serial dilutions were carried out through 10^{-6}. One milliliters of each STEC and *Shigella* spp. culture was put in 2.5 milliliters of tempered 1.5% Tryptic Soy Agar (TSA) overlay tubes and mixed. Then, each phage dilution was added to an overlay tube, which was then poured onto a prepared TSA plate. Plates were left to harden and then put in an incubator at 35^{o}C. All dilutions were performed in triplicate.

Results

Biochemical Profiles

Figures 12 and 13 show the results of the biochemical tests. All of the organisms showed the same reactions for MRVP, indole, citrate, and gelatin. The TSI results were congruent with the results of lactose fermentation on MAC plates. STECS that were able to ferment lactose exhibited an A/A reaction with the presence of gas, indicating the production of CO_2 upon lactose and glucose fermentation. *S. boydii*, *S. sonnei*, and *S. dysenteriae* showed A/A with visible gas production in the TSI tube, however, the PRB tubes were pink indicating no lactose fermentation. Since no lactose was fermented, these bacterial species must have fermented glucose and sucrose in the TSI. *E. coli* Lac-, *E. coli* O121:S19, and *S. flexneri*, showed a K/A reaction in the TSI with no lactose fermentation in the PRB tubes. This indicates that these organisms are only capable of fermenting glucose.

Biochemical Tests

	MR	VP	Indole	Citrate	MTM	Nitrate	TSI	Gelatin	Lac on MAC	Lac in PRB	Lac in PRB after 5 days
E. coli O45 S107	+	-	+	-	+/-	NO_2	A/AG	-	+	AG	AG
E. coli O121 S19	+	-	+	-	+	NO	K/A	-	-	-	AG
E. coli O111 E3	+	-	+	-	+	NO	A/AG	-	+	AG	AG
E. coli O103 S7	+	-	+	-	+	NO	A/AG	-	+	AG	AG
E. coli O145 S23	+	-	+	-	+	NO	A/AG	-	+	AG	AG
E. coli O157 43894	+	-	+	-	+	NO_2	A/AG	-	+	AG	AG
E. coli O157 OC-1	+	-	+	-	+	NO	A/AG	-	+	AG	AG
E. coli 25922	+	-	+	-	+	NO	A/AG	-	+	AG	AG
E. coli CN-13 700609	+	-	+	-	+	NO	A/AG	-	+	AG	AG
E. coli Lac-	+	-	+	-	+	NO	K/A	-	-	-	AG
E. coli JZ Green	+	-	+	-	+	NO/NO/-	A/AG	-	+	AG	AG
S. dysenteriae CDC 85-3131	+	-	+	-	+	NO_2	A/AG	-	+	-	-
S. boydii 20249	+	-	+	-	+	NO_2	A/AG	-	+	-	-
S. sonnei 9290	+	-	+	-	+	NO_2	A/AG	-	+	-	-
S. flexneri	+	-	+	-	-	NO	A/AG	-	+	AG	AG
P. agglomerans	+	-	++-	-	-	NO_2	NC	-	+	-	-

Figure 12. Summary of Biochemical Results. Biochemical tests for all samples. (+) indicates a positive result. (-) indicates a negative result. NO or NO_2 indicates reduction to nitric oxide or nitrite. A indicates acid, AG indicates acid with CO_2 gas, and K indicates basic.

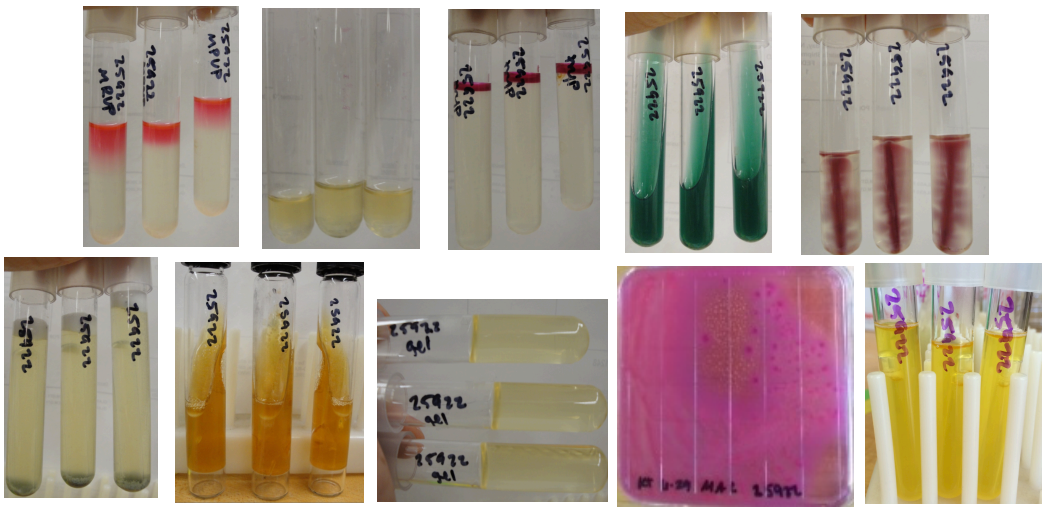

Figure 13. Biochemical reactions for *E. coli* 25922. A. methyl red test, positive for acidic byproducts of glucose fermentation **B.** Vogues-Proskauer test, negative for acetoin production as a result of glucose fermentation **C.** Tryptone water, positive for indole production **D.** Citrate tubes, negative for citrase production **E.** MTM tubes, positive for motility **F.** Nitrate test with addition of sulfanilic acid (reagent A) and naphthylamine (reagent B) and Zinc powder, positive for nitrate reduction **G.** TSI, positive for lactose and glucose fermentation and CO_2 production, negative for H_2S production **H.** Gelatin test, negative for gelatinase production **I.** MAC plate, positive for lactose fermentation **J.** Lactose tubes, positive for lactose fermentation with CO_2

Bacteriophage

No plaques were observed for any of the STEC preparations. As expected, plaques were visible on the *E. coli* CN-13 for the 10^{-3}, 10^{-4}, 10^{-5} dilutions (Figs. 14 and 15).

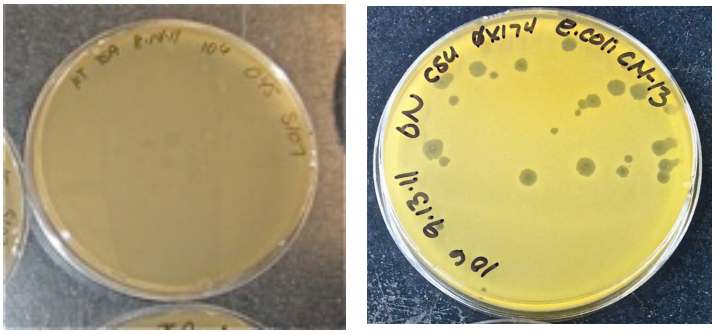

Figure 14. Plaques on *E. coli* plates. Left, PhiX174 plaques on lawn of *E. coli* CN-13. Right, absence of PhiX174 plaques on a lawn containing an STEC

Ability of PhiX175 to infect STECs

Non-O157 STECs		Serotype O157:H7		*Shigella* spp		Common *E. coli* (usually non-pathogenic)	
E. coli O45:S107	-	*E. coli* O157:H7 ATCC 43894	-	*Shigella boydii* 20249	-	*E. coli* 25922	-
E. coli O103:S7	-	*E. coli* O157 OC-1	-	*Shigella dysenteriae* CDC-85-131	-	*E. coli* CN-13 ATCC 700609	+
E. coli O111:E3	-			*Shigella* 20239	-		
E. coli O121:S19	-			*Shigella sonnei* 9290	-		

Figure 15. Summary of Plaque Formation. Bacteriophage plaques on TSA plates. (–) indicates no plaque formation. (+) indicates plaque formation.

Discussion

Biochemical Profiles

Bacteria typically display a biochemical profile specific to their species (Abbott et al., 1994). There may be slight variations within a species due to the acquisition of genes through horizontal gene transfer. *E. coli* usually show the following results:

***E. coli* biochemical profile**

MR	VP	Indole	Citrate	MTM	Nitrate	TSI	Gelatin	Lac on MAC	Lac in PRB
+	-	+	-	+	NO_2	A/AG	-	+	+

All of the *E. coli* that I tested showed biochemical reactions consistent with the expected profiles except *E. coli* O121:S19, which showed lactose fermentation in the phenol red broth after 5 days of incubation. *E. coli* Lac- is of unknown origin but was previously tested resulting in lactose negative fermentation. The results of the Mac plate, PRB tubes and TSI all show that *E. coli* Lac- is not a lactose fermenter. The expected biochemical profile for *Shigella* spp. is as follows:

***Shigella* spp. biochemical profile**

MR	VP	Indole	Citrate	MTM	Nitrate	TSI	Gelatin	Lac on MAC	Lac in PRB
+	-	+	-	+	NO_2	A/AG	-	-	-

The strains of *Shigella* I used followed this biochemical pattern except for *S. flexneri*. That strain of *S. flexneri* displayed no motility and it was able to ferment lactose.

Bacteriophage Plaque Assay

Phi X174 bacteriophage cannot be used to differentiate the strains of STECs used in my study from O157:H7 *E. coli*. Research previously described by Michel et al. (2010), showed that species of bacteria with a similar LPS composition can be infected by a phage that is host-specific because it is the overall make up of the LPS that allows for phage attachment versus a single receptor. My results lead me to conclude that *E. coli* CN-13, which is the host for PhiX174, may have a different LPS composition from the STECs that I tested because it was susceptible to phage infection and the others were not.

It is possible that other phage that are less host-specific have the potential to be used to differentiate STECS from O157:H7 *E. coli*. Phage typing has been utilized in identification of *Staphylococcus* and *Salmonella* species. Islam et al., (2012) used a double agar method similar to the method I used but added mitocycin C (MMC) and Ca^{2+} to the agar. They believed that MMC helped prevent lysogeny and induced the lytic cycle to foster host cell infection. They used a Stx2 phage, which can transduce virulence factors to *E. coli*. Stx2 phage gene sequences have been found in EHEC chromosomes, which may yield the propagation of an Stx2 phage with an increased broad range host specificity.

Chapter Three

Siderophores

Most all bacteria, including STECs, require iron as a cofactor for enzyme activity. Iron is used for some metabolic processes, such as the electron transport chain, and is a component of some proteins (Miethke & Marahiel, 2007). The ferric state of iron is insoluble and therefore not bioavailable to most organisms (Andrews et al., 2003), and in addition, some forms of iron can be highly toxic. This makes it difficult for many organisms, such as bacteria, to obtain iron from the environment and maintain its levels for biological processes (Andrews et al., 2003). In the presence of oxygen and water, iron is usually found in the form FeIII, which bacteria cannot uptake very easily, and thus, in most environments the amount of soluble iron is too low to meet their needs (Miethke & Marahiel 2007). In mammals, iron is protected from bacterial uptake because it is bound to proteins called hemoglobin. When mammals transfer iron from the hemoglobin to tissues it does so by using transport proteins, transferrin and lactoferrin, which help protect the iron. With environmental iron being insoluble and mammalian iron protected by proteins, bacteria have evolved ways to draw iron into their cells (Miethke & Marahiel 2007).

One way that bacteria can acquire iron is through the use transferrin or lactoferrin receptors. These receptors bind the transferrin or lactoferrin transport proteins and allow for the iron to pass through their membrane and into the cytoplasm. Transferrin and lactoferrin can bind two FeIII molecules limiting iron access to microorganisms (Miethke & Marahiel 2007). Bacterial transferrin and lactoferrin receptors are very specific and will bind transfer proteins only from specific sources. If a different source is available then the receptors cannot bind the proteins. For example, some bacteria produce hemophores that bind iron from hemoglobin in the blood and cannot access iron from any other sources. (Miethke & Marahiel, 2007).

Another method of iron uptake by bacteria involves the use of iron-chelating compounds known as siderophores. Siderophores are composed of proteins called non-ribosomal peptide synthases (NRPS), which act like a magnet to aid in the binding and uptake of iron (Andrews et al., 2003). Siderophores have a high affinity to ferric iron and are released when the environment has low levels of iron available (Andrews et al., 2003). Siderophores, unlike transferrin receptors, are not source specific (Miethke & Marahiel, 2007) and can bind iron from the environment or from hemoglobin. They are produced when the iron concentration goes below 10^{-6} moles per liter and can chelate either FeII or FeIII. If FeIII is available it is either taken directly into the cell through transport proteins in the cell membrane, or they are first reduced to FeII before being brought into the cell. Some bacteria can produce xenosiderophores that uptake siderophores produced by other bacteria. This allows them to conserve their energy and not waste energy on unnecessary proteins (Miethke & Marahiel, 2007). The gain of iron for the bacterium comes at a cost

to both the host and the environment. In humans, siderophore action can result in anemia and interfere with the immune response. Thus, siderophore production is considered to be a virulence factor (Andrews et al., 2003).

One siderophore released by *E. coli* is enterobactin. Enterobactin (Fig. 16) is a catecholate released through the cell membrane via ABC transport proteins (Neildans, 1995). The chelating mechanism of enterobactin causes an ionic change in the iron compound converting the compounds into ferric enterobactin (FeEnt). FeEnt binds to a specific outer membrane protein, FepA, allowing transfer of iron across the membrane using the TonB system (Thulasiraman et al., 1998). The FepA receptors are β–barrel transmembrane receptors that allow for the ferric iron siderophore complex to go directly to the periplasm (Fig. 17). The presence of the membrane receptors is triggered by the lack of iron in the environment. A bacterium usually possesses multiple ferric iron siderophore receptors each with different specificities to particular siderophores (Andrews et al., 2003).

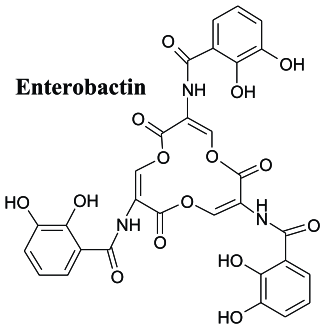

Figure 16. Structure of Enterobactin. (Andrews et al., 2003).

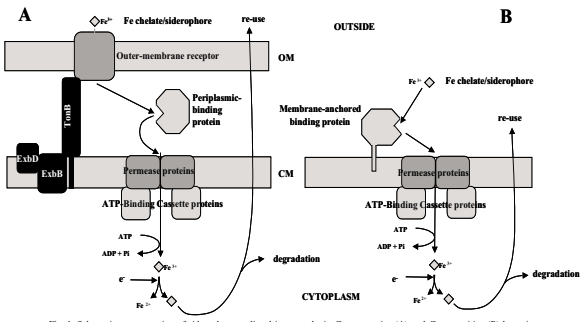

Figure 17. Cell Wall Proteins and Fe-siderophore Complex. Siderophore transfer in Gram-negatives (A) and Gram-positives (B) (Andrews et al., 2003).

The transport of siderophores across the membrane requires energy derived from an electrochemical potential across the cytoplasmic membrane and transmitted to the outer membrane through the Ton system (Braun et al., 1996). Ton system is composed of a series of proteins (TonB, ExbB and ExbD) (Fig. 18) in the cytoplasmic membrane (Braun et al., 1996). TonB spans from the cytoplasm, through the cytoplasmic membrane and contacts the FepA receptor that reacts with enterobactin. The electrochemical gradient in the cytoplasmic membrane causes ExbB and ExbD to induce a conformational change in TonB which induces a conformational change in FepA, which allows the ferric iron-siderophore complex to enter the periplasmic space (Andrews et al., 2003). Once the siderophore enters the periplasmic space, TonB goes back to its original conformation.

Once inside the cytoplasm, the ferric iron needs to be dissociated from the siderophore so that it can be utilized for biological processes. This involves an esterase, which breaks the ester bonds to create a weak siderophore. It also acts as a ferric iron reductase. The combination of the ferric iron being reduced and the weakened affinity of the siderophore to the ferric iron cause the dissociation of the complex. The ferrous iron can be transported through many different transport systems and can be utilized for different processes inside the cell and also can be reused to chelate extracellular iron (Andrews et al., 2003).

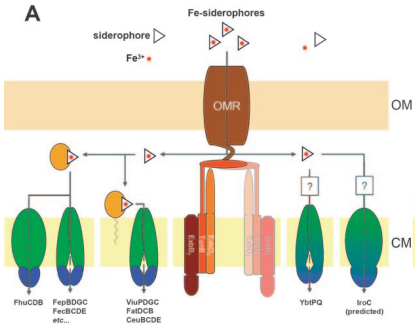

Figure 18. Siderophore transport. Transport through cell membrane and the Ton system (Miethke & Marahiel, 2007).

Siderophore production is regulated by the ferric uptake regulation (*fur*) gene (Neilands, 1995) and is induced according to the availability of iron in the environment. Fur genes are responsible for biosynthesis of the Fur protein, which forms a complex with FeII to repress transcription of the *fur* gene (Andrews et al., 2003). If FeII is available in excess it binds to Fur to inhibit transcription of siderophore proteins. If FeII is only available in small amounts it does not bind to Fur and transcription of *fur* genes occurs, synthesizing siderophore proteins (Andrews et al., 2003).

Siderophores can be detected by using Chrom Azural S medium (CAS). The medium is blue when FeIII binds to a chromagen. When siderophores are produced they bind to FeIII, mobilize it, and the iron-siderophore complex releases the chromagen and the medium turns orange.

Quorum Sensing

Quorum sensing is a process that bacteria use to communicate with each other via chemical signals. According to a review by Whitehead et. al. (2001) quorum sensing is regulated by a set of genes in an operon. These genes include the *lux* genes and were first discovered in *Vibrio fischeri* (Visick & McFall-Ngai, 2000) and are responsible for bioluminescence in certain *Vibrio* species. *Vibrio spp.* have evolved a symbiotic

relationship with certain cephalopods where bacteria are captured in the seawater and stored in the mouth of the cephalopod (Visick & McFall-Ngai, 2000). Bacteria secrete quorum-sensing signals that induce neighboring *Vibrio* to bioluminesce. Bioluminescence inside the mouth of the cephalopod attracts prey directly into the cephalopod's mouth (Visick & McFall-Ngai, 2000). In return for the bacteria aiding in the capture of food for the cephalopod, bacteria are provided food and shelter inside the mouth. In the morning when the sunlight penetrates the ocean water, the cephalopod expels the bacteria into the water. This prevents bacteria from building up in the cephalopod and causing disease (Visick & McFall-Ngai, 2000).

Genes of the *lux* Operon

lux Gene	Function
A	luciferase subunits
B	luciferase subunits
C	substrate used by luciferase
D	substrate used by luciferase
E	substrate used by luciferase
G	flavin reductase
I	produces autoinducer to regulate bioluminescence
R	produces autoinducer to regulate bioluminescence

Figure 19. Genes of the *lux* Operon. Adapted from Whitehead et al., 2001.

The ability of the bacteria to bioluminescence is highly controlled by the *lux* operon. Research has been done on quorum sensing in other species of bacteria and it has been found that other bacteria have operons, such as the Lac operon, that are homologous to the *lux* genes found in *Vibrio*. The *lux* operon is composed of eight genes (*lux*A-E, *lux*G, *lux*I, and *lux*R) (Engebrecht et al., 1983). Each gene function is listed in Figure 19.

In the case of *V. fischeri,* when cell density is low, there is little if any transcription of the *lux* genes. When the population of *V. fischeri* increases and reaches a threshold, *lux*I releases the autoinducer in the form of an acyl-homoserine lactone (AHL). The AHL binds to a transcription activator protein LuxR, which changes conformation and the complex binds to the *lux* box. The *lux* box is located upstream from the transcription start site. Transcription of the *lux* genes is induced. This causes a positive

feedback loop, which increases the amount of bioluminescence and AHL production. Once certain levels of AHLs are synthesized, the LuxR transcription activator represses transcription of *lux*R (Bassler, 1999).

The feedback mechanism of the *lux* operon in *V. fischeri* can be applied to virulence in *E. coli* via homologous genes. SdiA is homologous to LuxR, which acts to bind to the autoinducer (AHL) but it also acts as a transcription activator for proteins of cell division and as a negative regulator for other virulence factors such as cell attachment (Kanamaru et al., 2000). When the *E. coli* population is dense, AHLs are released and bind to SdiA which inhibits expression of EspD and intimin (both needed for host cell pore formation and attachment respectively) and the bacterial cells can no longer attach to the host epithelium so they are shed in the feces, thus promoting spreading to other hosts (Kanamaru et al., 2000; Anand & Griffiths, 2003).

Quorum sensing can be detected by utilizing the Lac operon as an indicator gene. The Lac operon is a set of genes in a bacterial chromosome that are activated and allows for transcription of those genes to produce enzymes that assist the organism in catabolizing lactose molecules. The enzyme β-galactosidase cleaves lactose into individual glucose and galactose molecules, which can be transported into the cell. The genes that code for β-galactosidase are inducible so they are activated only when lactose is present near the cell.

Agrobacertium tumefaciens produces β -galactosidase in the presence of AHL's (Kawaguchi et. al., 2008). The addition of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) acts to mimic the presence of lactose in the environment. The

Lac operon is only transcribed in the presence of lactose, so when X-Gal (normally colorless) is mixed into the media this triggers the cell to make β-galactosidase, which cleaves X-Gal into galactose and 5,5'-dibromo-4,4'-dichloro-indigo. 5,5'-dibromo-4,4'-dichloro-indigo is a blue color, which changes the color of the growth medium to blue. Since *A. tumefaciens* only produces β-galactosidase in the presence of AHL, the presence of a blue color indicates AHL production. Strains of *A. tumefaciens* used for these assays do not produce their own autoinducers, which prevents stimulation of the Lac operon to produce β-galactosidase (Kawaguchi et. al., 2008).

Recent research suggests detection of AHL's may not be the best way to screen for quorum sensing compounds because quorum sensing is not limited to AHL molecules. For example, Tommonaro et al., 2011 researched signaling molecules in archaea and found that the AHL bioreporters were activated in the absence of AHL molecules in the sample. Rajput et al., 2015 compiled literature and organized quorum sensing molecules into categories like signaling system, signaling molecule, synthase and recipient genes, organism, and strain and developed an online database (SigMol) consisting of 1382 entries and 182 molecules involved in quorum sensing. They encompass quorum sensing molecules such as diketpiperazines (DPK's), 4-hydroxy-2-alkylquinolines (HAQ's), diffusible signal factors (DSF's), and autoinducer-2 (AI-2).

Methods

Siderophores

A detection medium was developed using a combination of methods from Alexander & Zuberer (1991) and Louden et al. (2011) and consisted of 3 parts: nutrients, a buffer, and an indicator. All glassware was rinsed with 6 moles per liter HCL prior to use. A sugar solution was made by combining 2 grams glucose, 2 grams mannitol, 493 grams MgSO4, 1 milligram $CaCl_2$ (calcium chloride), 1.17 milligrams MnSO4 , 1.4 milligrams H_3BO_3 0.04 milligrams CuSO4 1.2 milligrams ZnSO4, and 1 milligrams Na_2MoO_4 in 70 milliliters deionized water. The buffer solution as made by combining 0.3g KH_2PO_4 0.5 grams NaCl, and 1 gram NH_4CL in 750 milliliters deionized water. Fifteen grams of piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES) (pH range 6.1-7.5) buffer and 15 grams 3-(*N*-morpholino) propanesulfonic acid (MOPS) (pH range 6.5-7.9) buffer was added to the salt solution. PIPES does not dissolve in solution in a pH less than 5 therefore 50% KOH was used to adjust the pH to 6.8. Distilled water was added to bring the final volume to 800 milliliters. Fifteen grams of agar was added and the solution was boiled to dissolve the agar. The indicator solution was made by combining 3 different solutions: Solution 1 consisted of 0.06 grams chrome azural S agar (CAS) in 50 milliliters deionized water; Solution 2 consisted of 0.0027 grams $FeCl_3$ in 10 millimoles

HCL; and Solution 3 consisted of 0.073 grams HDTMA in 40 milliliters deionized water. Solution 1 was mixed with 9 milliliters of Solution 2. The combined solutions were poured into Solution 3 and a blue color developed. This solution was the indicator solution. The sugar solution, buffer solution and indicator solutions were autoclaved at 121^{o}C for 15 minutes and cooled to 50^{o}C. The salt solution and the sugar solutions were subsequently combined. A solution of casamino acids was prepared by combining 3 grams of casamino acids with 27 milliliters of deionized water. This solution was mixed with 50 milligrams per liter of 3% 8-hydroxyquinoline and then filter sterilized through a 0.45 microliter filter (Corning, New York). The casamino acid solution was poured into the sugar-buffer solution while stirring slowly. Then the indicator solution was added and the medium was poured into sterile Petri plates. Twenty-four hour cultures in Nutrient broth (Becton, Dickinson and Company, Sparks, MD) were used to streak CAS plates and incubated at 37^{o}C overnight. A broth version of CAS media was made the same way except the agar was omitted and PIPES was not used. Instead, 30.24 grams of MOPS was used. Three milliliters of the broth was aliquoted into tubes that had been rinsed previously with 6 molar HCL and autoclaved. Twenty-four hour cultures in Nutrient broth were used to inoculate the tubes, with 500 microliters of each culture and performed in triplicate.

Quorum Sensing

A culture of *Agrobacterium tumefaciens* was grown in 50 millilters of Luria Bertani broth (LB) (Becton, Dickinson and Company, Sparks, MD) overnight at 35^{o}C. STEC cultures and *Pantoea agglomerans* containing the fluorescent marker, Ds red was grown in 3 milliliters of LB and incubated overnight at 35^{o}C. *P. agglomerans* is used as the positive control for this experiment. Eighteen grams of LB was mixed in 600 milliliters water with 10.8 grams agar and heated until boiling. The agar was autoclaved and tempered. One milliliter of 60 milligrams per milliliter X-gal in dimethyl sulfoxide (DMSO) was mixed with *A. tumefaciens* then poured into the tempered agar. The agar/*A. tumefaciens* solution was poured onto prepared plates of LB agar and cooled. Plates were streaked with STECs and *P. agglomerans* and incubated at 35^{o}C overnight. The same process was done for negative controls for each STEC and *P. agglomerans*, however, *A. tumefaciens* was not added to the tempered agar. All microorganisms were tested in triplicate.

Results

Siderophores

All samples grew on the CAS plates except for *E. coli* O45:S107, *S. sonnei*, and *S. dysenteriae* (Figs. 20 and 21). The remaining samples presented as orange growth. Positive and negative results were more difficult to determine in the broth. Growth was apparent from visible turbidity of the tubes but the color intensity was not as apparent as it was on the plates. After 24 hours of incubation *E. coli* O45:S107, *S. sonnei*, *S. dysenteriae*, *E. coli* JZ green, *S. flexneri*, *S. boydii* did not grow. The tubes were incubated for another 24 hours and checked again. *E. coli* JZ green, *S. flexneri,* and *S. boydii* had growth after another 24 hours, however, *E. coli* O45:S107, *S. sonnei*, and *S. dysenteriae*, still did not grow. This growth pattern reflected the same growth pattern that was displayed on the solid media.

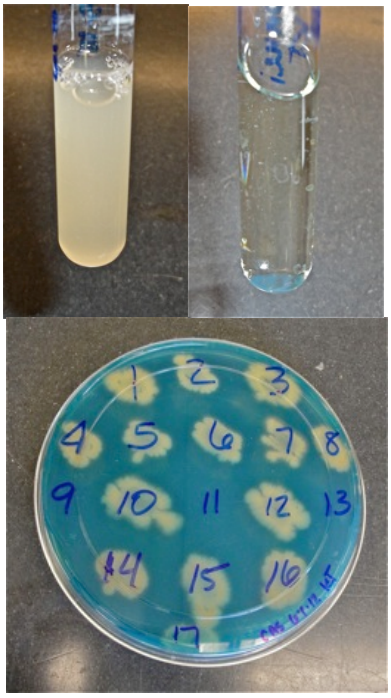

Figure 20. Growth on CAS Media. A. Liquid CAS media negative for siderophore production due to the lack of orange pigmentation. **B.** Liquid CAS media positive for siderophore due to the orange pigmentation. **C.** Solid CAS media with 17 STEC samples. All are positive except for samples 9, 11, and 13.

Siderophore production on CAS media

		Siderophore Production (on agar)	Siderophore Production (in broth)
Non-O157 STECs	*E. coli* O45 S107	+	-
	E. coli O103 S7	+	+
	E. coli O111 E3	+	+
	E. coli O121 S 19	+	+
	E. coli JZ green	+	+
	E. coli Lac-	+	+
Serotypes O157:H7	*E. coli* O157 H7 ATCC 43894	+	+
	E. coli O157 OC-1	+	+
***Shigella* spp.**	*Shigella boydii* 20249	+	+
	Shigella dysenteriae CDC-85-3131	+	-
	Shigella flexneri 20239	+	+
	Shigella sonnei 9290	+	-
Common *E. coli* (usually nonpathogenic)	*E. coli* 25922	+	+
	E. coli CN-13 ATCC 700609	+	+

Figure 21. Results of siderophore production on CAS media.

Quorum Sensing

A summary of quorum sensing results are presented in Figure 22. Controls included LB plates with an overlay of sterile LB that lacked *A. tumefaciens* (negative control) and LB plates with an overlay of LB mixed with *A. tumefaciens* (positive control for growth of *A. tumefaciens*). The negative control plates displayed no growth and the positive controls possessed a lawn of bacterial growth. None of the control plates turned blue (Fig. 23). All plates were observed after 24 hours of incubation.

Figure 22. Summary of Quorum Sensing Results. Growth of STECs on agar plates in pure culture versus in co-culture with *A. tumefaciens*. Color of colony is indicated as yellow or blue growth. Light blue growth indicates a less intense pigment without a ring around the colony. NG indicates no growth.

		Quorum sensing					
		AHL production with A. tumefaciens			AHL production without A. tumefaciens		
Non-O157 STECs	*E. coli* O45 S107	yellow	yellow	light blue	blue	blue	blue
	E. coli O103 S7	light blue	blue	light blue	blue	light blue	blue
	E. coli O111 E3	yellow	yellow	light blue	blue	blue	blue
	E. coli O121 S 19	yellow	blue	light blue	yellow	yellow	yellow
	E. coli O145 S23	yellow	yellow	yellow	light blue	light blue	light blue
	E. coli JZ green	light blue	blue	light blue	blue	blue	blue
	E. coli Lac-	light blue	light blue	blue	blue	blue	blue
Serotypes O157:H7	*E. coli* O157 H7 ATCC 43894	yellow	yellow	light blue	blue	blue	blue
	E. coli O157 OC-1	yellow	blue	light blue	blue	blue	blue
***Shigella* spp.**	*Shigella boydii* 20249	yellow	yellow	light blue	blue	light blue	light blue
	Shigella dysenteriae CDC-85-3131	NG	NG	NG	yellow	yellow	yellow
	Shigella flexneri 20239	light blue	light blue	light blue	blue	blue	blue
	Shigella sonnei 9290	yellow	yellow	yellow	blue	light blue	light blue
Common *E. coli* (usually nonpathogenic)	*E. coli* 25922	yellow	blue	light blue	blue	blue	blue
	E. coli CN-13 ATCC 700609	light blue	light blue	blue	blue	blue	blue
Control	*P. agglomerans*	yellow	little growth	little growth	blue	blue	blue

Figure 23. Plates With AHL Production. The plates on the left show growth without the presence of *A. tumefaciens*. The plates on the right show growth in the presence of *A. tumefaciens.*

Discussion

Siderophores

The use of CAS media was efficient and fast in determining siderophore production for the isolates tested, however, there was no general difference in siderophore production between STEC and O157:H7 *E. coli*. However, siderophore production did differ between *E. coli* O45:S107, *S. sonnei*, and *S. dysenteriae*, and the

rest of the samples. Unlike the other STECs, these organisms did not grow on solid media, so this method may be useful in distinguishing these organisms from the other pathogens. Keihan et al., 2011, developed a multiplex PCR that differentiates between species of Shigella organisms. These findings along with the methods of Keihan et al., 2011 could be used to assist with source tracking.

Perez-Miranda et al., (2007) propose doing an overlay of CAS medium using methods of Schwyn & Neilands (1987) for siderophore detection. They tested *Aspergillus niger*, *Rhizopus oligosporus*, and *Bacillus cereus* for siderophore production with the CAS overlay and were able to distinguish between types of siderophores produced according to the color change of the media *A. niger* produces a catechol which turns the media purple, *R. oligosporus* (a carboxylate producer) which turns the media light yellow, and *B. cereus* (a hydroxamate producer) which turns the media orange. Enterobacteriaceae produce Enterobactin (Ferreira et al., 2016), which is a catecholate so a purple color change would be expected assuming methods of a CAS overlay. Verma et al., 2012, used CAS media for siderophore detection in *Rhizobium meliloti* organisms that produce nodules in plants. They characterized a positive result as growth with an orange halo and a negative result as no growth without a halo. Multiple researchers state that fastidious organisms are difficult to grow on CAS media due to the presence of HDTMA (Verma et al., 2012; Louden et al., 2011; Schwyn & Neilands, 1987). Therefore, Verma et al., acknowledge the growth of fastidious organisms as growth with small halo formations.

The medium has its drawbacks in determining negative results. A negative result was designated as no growth on the agar. Pigment detection was easier on agar than in a broth and some organisms grew better on the agar than the broth.

Quorum Sensing

It was expected that there would be no blue pigment on the plates that lacked *A. tumefaciens* in the overlay. All but two (*E. coli* 121:S19 and *S. dysenteriae*) of the organisms had blue growth on the plates without *A. tumefaciens* but the blue pigment varied in intensity for each organism. If the blue color was present, then it was darker around the edge of the growth. The plates that had *A. tumefaciens* had more variation in pigment production. Most of these plates had either yellow or light blue pigment indicating a stronger reaction in the absence of *A. tumefaciens*. *E. coli* O121:S19 showed blue pigment on 2 of the 3 replicates on the plates that had *A. tumefaciens*. *E. coli* O121:S19 is a good control microorganism for this assay because it showed negative results on the plate without *A. tumefaciens* and positive results on the plates that had *A. tumefaciens*. *S. dysenteriae* had no growth on the plates with *A. tumefaciens* but growth on the plates without *A. tumefaciens* without AHL production. *S. sonnei* produced results contrary to what was expected by only yielding blue pigment on the plates that contained

A. tumefaciens making this microorganism a good candidate for use as a control organism for this assay. *P. agglomerans* was the organism used as the control for this experiment, however it showed results inconsistent with what was expected. It was assumed that *P. agglomerans* produced AHLs from previous studies, however, but they did not produce them in this assay. This leads to the conclusion that the strain of *P. agglomerans* used may not be the ideal microorganism to use as a control.

This method may be useful in differentiating a few of the microorganisms used in this study. For instance, *S. dysenteriae* showed no pigment on either of the overlay plates but it was lactose positive. This can be differentiated from *S. boydii*, *S. sonnei*, and *S. flexneri* because the former 3 organisms all turned blue on the plates with *A. tumefaciens* in the overlay. *S. sonnei* can be differentiated from *S. boydii* and *S. flexneri* because it did not turn blue on the plate that has *A. tumefaciens* when both *S. boydii* and *S. flexneri* did. *E. coli* O121:S19 was the only isolate that was negative for lactose fermentation and showed no blue on the plate without *A. tumefaciens*. *E. coli* O145:S23 was the only *E. coli* that was yellow on the plates with *A. tumefaciens* and blue on the plates without *A. tumefaciens*. Most of the organisms displayed reactions that contradict the theory that AHL production can be determined by blue color on plates with the biosensor, *A. tumefaciens*, and no color on plates that lack *A. tumefaciens*. This leads to further questioning regarding what other compounds are present that can cause this reaction. A summary of CAS and quorum sensing results are seen in Figure 24.

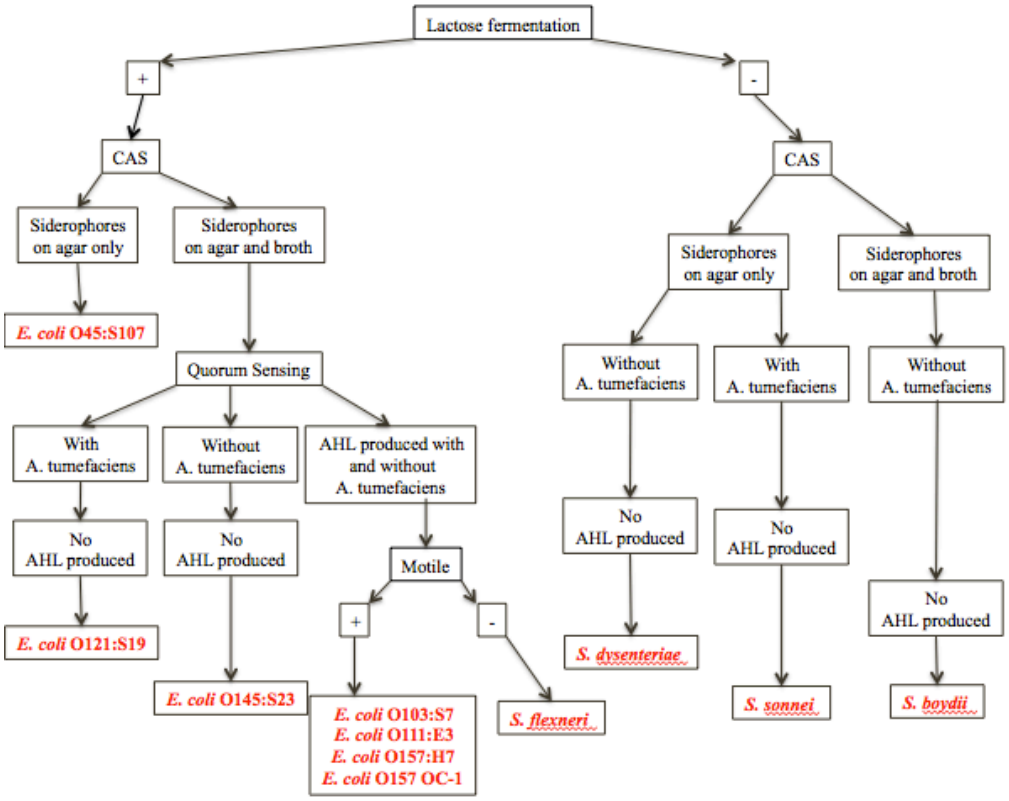

Figure 24. Schematic for Differentiation. Differentiation of STECs and *Shigella* spp. from each other using different types of media.

Holden et al. (1999), describes a group of molecules that are similar to AHLs that were isolated from cell-free supernatants of a *Pseudomonas aeruginosa* culture. They identify these molecules as diketopiperazines (DKPs). DKPs have shown to activate or inhibit *lux*-based AHL biosensors, including *A. tumefaciens*. This could lead to false positives and thus, when trying to identify AHL production using a biosensor, MS and NMR used in conjunction with biological assays is recommended.

Zhu et al., (2003) addresses the narrow specificity that indicator organisms like *A. tumefaciens* typically have when detecting AHL's. For example, *Chromobacterium violaceum* cannot detect 3-hydroxy AHL derivatives and some organisms may only be able to detect the 3-oxo forms and not the 3-hydroxy forms. They used a T7 bacteriophage to induce overexpression of TraR to create a new strain of *A. tumefaciens* that was capable of detecting lower concentrations of AHL's as well as new AHL forms that the original strain was not able to detect.

Chapter Four

Assays such as phage infection, quorum sensing, and siderophore production have potential for differentiating between pathogenic and non-pathogenic bacteria that cause foodborne illness. The phage assay was not effective using PhiX174 however more research and tests should be done on using other types of phage in an attempt to find one that infects STECs and O157:H7 *E. coli* organisms differently. A preliminary screening based on CAS media and siderophore production also has potential to differentiate *E. coli* O45:S107 and *Shigella spp*. from other non-STECs particularly when combined with methods such as multiplex PCR. Quorum sensing has the most potential in determining pathogenic from non-pathogenic organisms. It is likely that these organisms secrete different compounds that can be identified through assays like mass spectrometry and nuclear magnetic resonance spectroscopy. Combining this with the quorum sensing assay performed in this paper, along with PCR, it is possible that there will be differences in the types of quorum sensing compounds making them easy to differentiate from each other.

Foodborne illnesses are seen worldwide and kill thousands of people each year. Food products are shipped to and from local farms and facilities and are distributed across the globe to reach millions of people. With such a widespread distribution, it is crucial to have the ability to perform fast, simple analysis of food quality.

References

Abbott, S. L., Hanson, D. F., Felland, T. D., Connell, S., Shum, A. H., & Janda, M. (1994) *Escherichia coli* O157:H7 generates a unique biochemical profile on MircoScan conventional Gram-negative identification panels. *Journal of Clinical Microbiology*, 32(3), 823-824.

Alexander, D. B., & Zuberer, D. A. (1991). Use of chrome azural S reagents to evaluate siderophore production by rhizosphere bacteria. *Biology and Fertility of Soils, 12*, 39-45. Retrieved from http://link.springer.com/article/10.1007/BF00369386

Altekruse, S. F., Cohen, M. L., & Swerdlow, D. L. (1997). Emerging Foodborne Diseases. *Emerging Infectious Diseases, 3*(3), 285-293. doi:10.3201/eid0303.970304

Amor, K., Heinrichs, D. E., Frirdich, E., Ziebell, K., Johnson, R. P., & Whitfield, C. (2000). Distribution of core oligosaccharide types in lipopolysaccharides from *Escherichia coli*. *Infection and Immunity, 68*(3), 1116-1124. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC97256/

Anand, S. K., & Griffiths, M. W. (2003). Quorum sensing and expression of virulence in *Escherichia coli* O157:H7. *International Journal of Food Microbiology, 85*, 1-9. doi:10.1016/S0168-1605(02)00482-8

Anderson, B., Rashid, M. H., Carter, C., Pasternack, G., Rajanna, C., Revazishvili, T., Dean, T., Senecal, A., & Sulakvelidze, A. (2011). Enumeration of bacteriophage particles: Comparative analysis of the traditional plaque assay and real-time QPCR- and nanosight-based assays. *Bacteriophage*, *1*(2), 86–93. http://dx.doi.org/10.4161/bact.1.2.15456

Andrews, S. C., Robinson, A. K., & Rodriguez-Quinones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiology Reviews, 27*, 215-237. http://dx.doi.org/10.1016/S0168-6445(03)00055-X

Arthur, T. M., Brichta-Harhay, D. M., Bosilevac, J. M., Kalchayanand, N., Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (2010). Super shedding of *Escherichia coli* O157:H7 by cattle and the impact on beef carcass contamination. *Meat Science, 86*, 32-37. doi:10.1016/j.meatsci.2010.04.019

Babiuk, S., Asper, D. J., Rogan, D., Mutwiri, G. K., & Potter, A. A. (2008). Subcutaneous and intranasal immunization with type III secreted proteins can prevent colonization and shedding of *Escherichia coli* O157:H7 in mice. *Microbial Pathogenesis, 45*(1), 7-11. doi:10.1016/j.micpath.2008.01.005

Bassler, B. (1999). How bacteria talk to each other: Regulation of gene expression by quorum sensing. *Current Opinion in Microbiology, 2*(6), 582-587. http://dx.doi.org/10.1016/S1369-5274(99)00025-9

Becton Dickinson. (2006). BBL™ Levine EMB Agar. Quality Control Procedures. Retrieved from https://www.bd.com/ds/technicalCenter/inserts/L007387(10).pdf

Bell, B. P., Griffin, P. M., Lozano, P., Christie, D. L., Kobayashi, J. M., & Tarr, P. I. (1997). Predictors of hemolytic uremic syndrome in children during a large outbreak of *Escherichia coli* O157:H7 infections. *Pediatrics, 100*(1). doi:10.1542/peds.100.1.e12

Besser, R. E., Weber, J. T., Doyle, M. P., Barrett, T. J., Wells, J. G., & Griffin, P. M. (1993). An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh pressed apple cider. *Journal of the American Medical Association, 5*(269), 17th ser., 2217-2220. doi:10.1001/jama.1993.03500170047032

Beveridge, T. J. (1999). Structures of gram-negative cell walls and their derived membrane vesicles. *Journal of Bacteriology*, *181*(16), 4725–4733. Retrieved from http://jb.asm.org/content/181/16/4725.full.pdf+html

Blair, D. F. (2003). Flagellar movement driven by proton translocation. *FEBS Letters, 545*, 86-95. doi:10.1016/S0014-5793(03)00397-1

Boyer, R., Summer, S. S., Williams, R. C., Kniel, K., & McKinney, J. M. (2011). Role of O-antigen on the *Escherichia coli* O157:H7 cells hydrophobicity, charge and ability to attach to lettuce. *International Journal of Food Microbiology, 147*(3), 228-232. doi:10.1016/j.ijfoodmicro.2011.04.016

Bradley, D. E. (1967). Ultrastructure of bacteriophage and bacteriocins. *Bacteriological Reviews*, *31*(4), 230–314. Retrieved from http://mmbr.asm.org/content/31/4/230.full.pdf

Braun, V., Gaisser, S., Herrmann, C., Kampfenkel, K., Killmann, H., & Traub, I. (1996). Energy-coupled transport across the outer membrane of Escherichia coli: ExbB Binds ExbD and TonB in vitro, and leucine 132 in the periplasmic region and aspartate 25 in the transmembrane region are important for ExbD activity. *Journal of Bacteriology, 178*(10), 2836-2845. doi:10.1128/jb.178.10.2836-2845.1996

Burger, R. (2012). EHEC O104:H4 in Germany 2011: Large outbreak of bloody diarrhea and haemolytic uraemic syndrome by Shiga toxin producing *E. coli* via contaminated food. *Improving Food Safety Through a One Health Approach: Workshop Summary,* 115-130. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK114499/

Centers for Disease Control and Prevention. (1997). *MMWR Weekly: Foodborne Diseases Active Surveillance Network*. Retrieved from https://www.cdc.gov/mmwr/preview/mmwrhtml/00046981.htm

Centers for Disease Control and Prevention (CDC). (1998) FoodNet Surveillance Report for 1997. Final Report. Retrieved from http://www.cdc.gov/foodnet.

Centers for Disease Control and Prevention (CDC). (2012a) National Shiga toxin-producing *Escherichia coli* (STEC) Surveillance Annual Summary, 2009. Atlanta, Georgia: US Department of Health and Human Services. Retrieved from http://www.cdc.gov/ncezid/dfwed/PDFs/national-stec-surv-summ-2009-508c.pdf

Centers for Disease Control and Prevention (CDC). (2012b) Questions about the 2011 *E. coli* outbreak in Germany. Retrieved from http://www.cdc.gov/ecoli/general/germany.html

Centers for Disease Control and Prevention (CDC). PulseNet. (2013) PulseNet Frequently Asked Questions. Retrieved from http://www.cdc.gov/pulsenet/about/faq.html.

Centers for Disease Control and Prevention (CDC). (2014) Surveillance for Foodborne Disease Outbreaks United States. 2014 Annual Report. The National Outbreak Reporting System (NORS). Retrieved November 28, 2016, from http://www.cdc.gov/foodsafety/pdfs/foodborne-outbreaks-annual-report-2014-508.pdf

Centers for Disease Control and Prevention (CDC). (2016a) Estimates of Foodborne Illness in the United States. Retrieved from http://www.cdc.gov/foodborneburden/pdfs/factsheet_a_findings_updated4-13.pdf.

Centers for Disease Control and Prevention (CDC). (2016b) Life expectancy. Retrieved from http://www.cdc.gov/nchs/fastats/life-expectancy.htm

Cohen, M. B. (2002). Shiga toxin-producing *E coli*: Two tests are better than one. *Journal of Pediatrics, 141,* 155–156.

Conedera, G., Chapman, P. A., Marangon, S., Tisato, E., Dalvit, P., & Zuin, A. (2001). A field survey of *Escherichia coli* O157 ecology on a cattle farm in Italy. *International Journal of Food Microbiology, 66*, 85-93. doi:10.1016/S0168-1605(00)00489-X

Conrad, C. C., Stanford, K., McAllister, T. A., Thomas, J., & Reuter, T. (2014). Further development of sample preparation and detection methods for O157 and the top 6 non-O157 STEC serogroups in cattle feces. *Journal of Microbiological Methods, 105*, 22-30. doi:10.1016/j.mimet.2014.06.020 0167-7012

Donnenberg, M. S., Kaper, J. B., & Finlay, B. B. (1997). Interactions between enteropathogenic *Escherichia coli* and host epithelial cells. *Trends in Microbiology, 5*(3), 109-114. doi:10.1016/S0966-842X(97)01000-7

Engebrecht, J., Nealson, K., & Silverman, M. (1983). Bacterial bioluminescence: Isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell, 32*(3), 773-781. http://dx.doi.org/10.1016/0092-8674(83)90063-6

Feng, P., Weagant, S. D., & Jinneman, K. (1998). Chapter 4A Diarrheagenic *Escherichia coli*. In *Bacteriological Analytical Manual* (8th ed.). Silver Spring, MD: U.S. Food and Drug Administration.

Ferreira, D., Seca, A. M., GAD, C., & Silva, A. M. (2016). Targeting human pathogenic bacteria by siderophores: A proteomics review. *Journal of Proteomics, 145*, 153-166. http://dx.doi.org/10.1016/j.jprot.2016.04.006

Food Safety and Inspection Service (FSIS). (1999) USDA Issues Final Rule on Meat and Poultry Irradiation. United States Department of Agriculture (USDA).

Food Safety and Inspection Service (FSIS). (2001). Risk assessment of the public health impact of *Escherichia coli* O157:H7 in ground beef. United States Department of Agriculture (USDA).

Food Safety and Inspection Service (FSIS). (2002). Comparative Risk Assessment of Intact (Non-Tenderized) and Non-Intact (Tenderized) Beef: Technical Report. Office of Public Health Risk Assessment Division.

Federal Safety and Inspection Service (FSIS). (2012) Shiga toxin-producing *Escherichia coli* in certain raw beef products. *Federal Register, 77*(105).

Fraga, D., Meulia, T., & Fenster, S. (2008). Real-Time PCR. *Current Protocols Essential Laboratory techniques*. Retrieved from https://www.researchgate.net/publication/277699881_Current_Protocols_Essential_Laboratory_Techniques

Fraser, M. E., Kujinaga, M., Cherney, M. M., Melton-Celsa, A. R., Twiddy, E. M., O'Brien, A. D., & James, M. N. (2004). Structure of Shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. *Journal of Biological Chemistry, 279*(26), 27511-27517. doi:10.1074/jbc.M401939200

Fratamico, P. M., Wasilenko, J. L., Garman, B., DeMarco, D. R., Varkey, S., Jensen, M., Rhoden, K., & Tice, G. (2014). Evaluation of a multiplex real-time PCR method for detecting Shiga toxin-producing *Escherichia coli* in beef and comparison to the U.S. Department of Agriculture Food Safety and Inspection Service Microbiology Laboratory Guidebook Method. *Journal of Food Protection, 2*, 180-344. doi:10.4315/0362-028X.JFP-13-248

Garenaux, A., Caza, M., & Doznois, C. M. (2011). The ins and outs of siderophore mediated iron uptake by extra-intestinal pathogenic *Escherichia coli*. *Veterinary Microbiology*. doi:10.1016/j.vetmic.2011.05.023

Gautam, S., Gniadek, T. J., Kim, T., & Spiegel, D. A. (2013). Exterior design: Strategies for redecorating the bacterial surface with small molecules. *Trends in Biotechnology, 31*(4), 258-267. doi:10.1016/j.tibtech.2013.01.012

Gavin, P. J., Peterson, L. R., Pasquariello, A. C., Blackburn, J., Hamming, M. G., Kuo, K. K., & Thomson, R. B., Jr. (2004). Evaluation of performance and potential clinical impact of ProSpecT Shiga toxin *Escherichia coli* microplate assay for detection of Shiga toxin-producing *E. coli* in stool specimens. *Journal of Clinical Microbiology, 42*(4), 1652-1656. doi:10.1128/JCM.42.4.1652-1656.2004

Gerber, A., Karch, H., Allerberger, F., Verweyen, H. M., & Zimmerhackl, L. B. (2002). Clinical course and the role of Shiga toxin-producing *Escherichia coli* infection in the hemolytic uremic syndrome in pediatric patients, 1997-2000, in Germany and Austria: A prospective study. *Journal of Infectious Diseases, 186*, 493-500. Retrieved from https://jid.oxfordjournals.org/content/186/4/493.full.pdf

Giron, J. A., Torres, A. G., Freer, E., & Kaper, J. B. (2002). The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. *Molecular Microbiology, 44*(2). 361-379. doi:10.1046/j.1365-2958.2002.02899.x

Golan, E., Roberts, T., Salay, E., Caswell, J., Ollinger, M., & Moore, D. (2004). *Food Safety Innovation in the United States: Evidence from the Meat Industry* (United States, Department of Agriculture).

Gould, L. H., Bopp, C., Strockbine, N., Atkinson, R., Baselski, V., Body, B., Carey, R., Crandall, C., Hurd, S., Kaplan, R., Shea, S., Somsel, P., Tobin-D'Angelo, M., Griffin, P.M., & Gerner-Smidt, P. (2009). *MMWR: Recommendations for Diagnosis of Shiga Toxin-Producing Escherichia Coli Infections by Clinical Laboratories*. Retrieved from https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5812a1.htm

Gyles, C. L. (2007). Shiga toxin-producing *Escherichia coli*: An overview. *Journal of Animal Science, 85*(13), 45-62. doi:10.2527/jas.2006-508

Hedberg, C., F. Angulo, J. Townes, J. Hadler, D. Vugia, & J. Garley. (1997). Differences in *Escherichia coli* O157:H7 annual incidence among FoodNet active surveillance sites. 5th International VTEC Producing *Escherichia coli* Meeting. Baltimore, MD.

Holden, M. T., Chhabra, S. R., DeNys, R., Stead, P., Bainton, N. J., Hill, P. J., Manefield, M., Kumar, N., Labatte, M., England, D., Rice, S., Givskov, M., Salmond, G.P.C., Stewart, G.S.A.B., Bycroft, B.W., Kjelleberg, S., & Williams, P. (1999). Quorum sensing cross talk: Isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other Gram-negative bacteria. *Molecular Microbiology, 33*(6), 1254-1266. doi:10.1046/j.1365-2958.1999.01577.x

Holt, P. G., & Jones, C. A. (2000). The development of the immune system during pregnancy and early life. *Allergy, 55*, 688-697. doi:10.1034/j.1398-9995.2000.00118.x

Islam, M. R., Ogura, Y., Asadulghani, M., Ooka, T., Murase, K., Gotoh, Y., & Hayashi, T. (2012). A sensitive and simple plaque formation method for the Stx2 phage of *Escherichia coli* O157:H7, which does not form plaques in the standard plating procedure. *Plasmid, 67*, 227-235. doi:10.1016/j.plasmid.2011.12001

Janda, J. M., & Abbott, S. (1998). *The Enterobacteria*. Philadelphia, PA: Lippincott-Raven.

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). *Modern Food Microbiology* (7th ed.). New York, NY: Springer.

Johannes, L., & Romer, W. (2010). Sdia toxins - from cell biology to biomedical applications. *Nature Reviews Microbiology, 8*, 105-116. doi:10.1038/nrmicro2279

Kanamaru, K., Kanamaru, K., Tatsuno, I., & Tobe, T. (2000). SdiA, an *Escherichia coli* homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7. *Molecular Biology, 38*(4), 805-816. doi:10.1046/j.1365-2958.2000.02171.x

Kawaguchi, T., Chen, Y. P., Norman, R. S., & Decho, A. W. (2008, June). Rapid screening of quorum-sensing signal N-acyl homoserine lactones by an in vitro cell-free assay. *Applied and Environmental Microbiology, 74*(12), 3667-3671. doi:10.1128/AEM.02869-07

Kehl, K. S., Havens, P., Behnke, C. E., & Acheson, D. W. (1997). Evaluation of the Premier EHEC assay for detection of Shiga toxin-producing *Escherichia coli*. *Journal of Clinical Microbiology, 35,* 2015–2054. Retrieved from http://jcm.asm.org/content/35/8/2051.long

Keihan, A. H., Kamal, Z., Ghaffarisharaf, M., & Kamali, M. (2011). Differentiation and identification of *Shigella dysenteriae*, *Shigella flexneri* and *Shigella sonnei* by multiplex PCR assay. *Clinical Biochemistry, 44*(13). doi:10.1016/j.clinbiochem.2011.08.997 /react-text react-text: 59 /react-text react-text: 60

Lazcka, O., Del Campo, F. J., & Muños, F. X. (2007). Pathogen Detection: A perspective of traditional methods and biosensors. *Biosensors and Bioelectronics, 22(*7), 1205-1217. doi:10.1016/j.bios.2006.06.036

Le Berre, R., Nguyen, S., Nowak, E., Kipnis, E., Pierre, M., Ader, F., Courcol, R., Guery, B. P., & Faure, K. (2008). Quorum-sensing activity and related virulence factor expression in clinically pathogenic isolates of *Pseudomonas aeruginosa*. *Clinical Microbiology and Infectious Diseases, 14*(4), doi:10.1111/j.1469-0691.2007.01925

Leboffe, M. J., & Pierce, B. E. (2012). *Microbiology: Laboratory Theory and Application*. United States: Morton Publishing Company. pp. 413.

Leininger, D. J., Robertson, J. R., & Elvinger, F. (2001). Use of eosin methylene blue agar to differentiate *Escherichia coli* from other gram-negative mastitis pathogens. *Journal of Veterinary Diagnostic Investigation, 13*(3), 273-275. doi:10.1177/104063870101300319

Lerouge, I., & Vanderleyden, J. (2001). O-antigen structural variation: Mechanisms and possible roles in animal/plant-microbe interactions. *FEMS Microbiology Reviews, 26*, 17-47. http://dx.doi.org/10.1111/j.1574-6976.2002.tb00597.x

Levine, M., DuPont, H., Formal, S., Hornick, R., Takeuchi, A., Gangarosa, E., Snyder, M., & Libonati, J. (1973). Pathogenesis of *Shigella dysenteriae* 1 (Shiga) Dysentery. *The Journal of Infectious Diseases, 127*(3), 261-270. Retrieved from http://www.jstor.org/stable/30108820

Lin, A., Sultan, O., Lau, H. K., Wong, E., Hartman, G., & Lauzon, C. R. (2011). O serogroup specific real time PCR assays for the detection and identification of nine clinically relevant non-O157 STECs. *Food Microbiology, 28*(3), 478-483. doi:10.1016/j.fm.2010.10.007

Li, Y., Frey, E., Mackenzie, A. M., & Finlay, B. B. (2000). Human response to *Escherichia coli* O157:H7 infection: Antibodies to secreted virulence factors. *Infection and Immunity, 68*(9), 5090-5095. doi:10.1128/IAI.68.9.5090-5095.2000.

Louden, B. C., Haarmann, D., & Lynne, A. M. (2011). Use of blue agar CAS assay for siderophore detection. *Journal of Microbiology & Biology Education : JMBE*, *12*(1), 51–53. doi:10.1128/jmbe.v12i1.249

Lukáčová, M., Barák, I., & Kazár, J. (2008). Role of structural variations of polysaccharide antigens in the pathogenicity of Gram-negative bacteria. *Clinical Microbiology and Infection, 14*(3), 200-206. http://dx.doi.org/10.1111/j.1469-0691.2007.01876.x

Manafi, M., Kneifel, W., & Bascomb, S. (1991). Fluorogenic and chromogenic substrates used in bacterial diagnostics. *Microbiological Reviews*, *55*(3), 335–348. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC372823/pdf/microrev00034-0011.pdf

Mayr, U.B., Haller, C., Haidinger, W., Atrasheuskaya, A., Bukin, E., Lubitz, W., & Ignatyev, G. (2005). Bacterial ghosts as an oral vaccine: a single dose of *Escherichia coli* O157:H7 bacterial ghosts protect mice against lethal challenge. *Infectious Immunology*, *73*(8), 4810–4817. doi:10.1128/IAI.73.8.4810-4817.2005

Meat and Poultry Temperature Guide: Food Network. (n.d.). Retrieved from http://www.foodnetwork.com/recipes/articles/meat-and-poultry-temperature-guide.html

Meridian Bioscience. (n.d.). Premier EHEC Product Details. Retrieved from http://www.meridianbioscience.com/diagnostic-products/foodborne/premier/premier-ehec.aspx

Messenger, A. J. M., & Barclay, R. (1983). Bacteria, iron, and pathogenicity. *Biochemical Education*, 11(2), 54-63. Retrieved from http://onlinelibrary.wiley.com/store/10.1016/0307-4412(83)90043-2/asset/5690110203_ftp.pdf?v=1&t=iw3338n9&s=d3291423cb24990b0518488f1eacb2c5c9e6281d

Michel, A., Clermont, O., Denamur, E., & Tenaillon, O. (2010). Bacteriophage PhiX174's ecological niche and the flexibility of its *Escherichia coli* lipopolysaccharide receptor. *Applied and Environmental Microbiology, 76*(21), 7310-7313. doi:10.1128/AEM.02721-09

Miethke, M., & Marahiel, M. A. (2007). Sideropore-based iron acquisition and pathogen control. *Microbiology and Molecular Biology Reviews, 71*(3), 413-451. doi:10.1128/MMBR.00012-07

Murray, P. R., Rosenthal, K. S., & Pfaller, M. A. (2009). *Medical Microbiology* (6th ed.). Philadelphia, PA: Mosby Elsevier.

Naravanei, R., & Jamil, K. (2005). Rapid detection of food-borne pathogens by using molecular techniques. *Journal of Medical Microbiology, 54,* 51-54. doi:10.1099/jmm.0.45687-0

Natrah, F. M., Ruwandeepika, H. A., Pawar, S., Karunasagar, I., Sorgeloos, P., Bossier, P., & Defoirdt, T. (2011). Regulation of virulence factors by quorum sensing in *Vibrio harveyi*. *Veterinary Microbiology,154*(1-2), 124-129. doi:10.1016/j.vetmic.2011.06.024

Neilands, J. B. (1995). Siderophores: Structure and function of microbial iron transport compounds. *The Journal of Biological Chemistry, 270*(45), 26723-26726. doi:10.1074/jbc.270.45.26723

Nisonoff, A. (1984). *Introduction to Molecular Immunology Second Edition*. United States: Sinauer Associates Inc.

Ollinger, M., & Moore, D. (2004). Food safety investments in the meat and poultry industries survey results. Food Safety Innovation in the United States/AER-831. *Economic Research Service*.

Pacheco, A. R., & Sperandio, V. (2012). Shiga toxin in enterohemorrhagic *E.coli*: regulation and novel anti-virulence strategies. *Frontiers in Cellular and Infection Microbiology*, *2*, 81. doi:10.3389/fcimb.2012.00081

Partnership for Food Safety Education. (n.d.). Retrieved from http://www.fightbac.org/about-us/

Paton, J. C., & Paton, A. W. (1998). Pathogens and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clinical Microbiology Reviews, 11*(3), 450-479. Retrieved from http://cmr.asm.org/content/11/3/450.long

Perez-Miranda, S., Cabirol, N., George-Tellez, R., Zamudio-Rivera, L.S., & Fernandez, F.J. (2007). O-CAS, a fast and universal method for siderophore detection. *Journal of Microbiological Methods, 70*(1), 127-131. doi:10.1016/j.mimet.2007.03.023

Pincus, D. H. (2006). Microbial identification using the bioMérieux Vitek 2 system. *Encyclopedia of Rapid Microbiological Methods.* Bethesda, MD. (Parenteral Drug Association).

Powell, M. R., Ebel, E., Schlosser, W., Walderhaug, M., & Kause, J. (2000). Dose-response envelope for *Escherichia coli* O157:H7. *Quantitative Microbiology, 2*(2), 141-163. doi:10.1023/A:1011557402204

Power, D. A., & Zimbro, M. J. (2003). EC medium with MUG. *Difco & BBL Manual: Manual of Microbiological Culture Media*. Becton Dickinson. Retrieved from http://www.mercantillab.com.pe/wp-content/uploads/2015/11/Difco™-EC-Medium-with-MUG.pdf

Pupo, E., Lindner, B., Brade, H., & Schromm, A. B. (2013). Intact rough- and smooth-form lipopolysaccharides from *Escherichia coli* separated by preparative gel electrophoresis exhibit differential biologic activity in human macrophages. *FEBS Journal, 280*(4), 1095-1111. doi:10.1111/febs.12104

Rajput, A., Kaur, K., & Kumar, M. (2015). SigMol: repertoire of quorum sensing signaling molecules in prokaryotes. *Nucleic Acid Research*. doi:10.1093/nar/gkv1076

Ratiner, Y. A., Salmenlinna, S., Eklund, M., Keskimäki, M., & Siitonen, A. (2003). Serology and Genetics of the Flagellar Antigen of *Escherichia coli* O157:H7a,7c. *Journal of Clinical Microbiology*, *41*(3), 1033–1040. doi:10.1128/JCM.41.3.1033-1040.2003

Roux, S., Krupovic, M., Poulet, A., Debroas, D., & Enault, F. (2012). Evolution and Diversity of the *Microviridae* Viral Family through a Collection of 81 New Complete Genomes Assembled from Virome Reads. *PLoS ONE, 7*(7), doi:10.1371/journal.pone.0040418

Schwyn, B., & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry, 160*(1), 47-56. Retrieved from https://www.researchgate.net/profile/Bernhard_Schwyn/publication/20204289_Universal_CAS_assay_for_the_detection_and_determination_of_siderophores/links/53d24ded0cf228d363e9315f.pdf

Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, J. E., Jr., Rinehart, K. L., & Farrand, S. K. (1997, June). Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proceedings of the National Academy of Sciences of the United States, 94*, 6036-6041. Retrieved from http://www.pnas.org/content/94/12/6036.full.pdf

Shrestha, L. B. (2006). Life expectancy in the United States. CRS Report for Congress. *The Library of Congress*. Retrieved from http://www.aging.senate.gov/crs/aging1.pdf.

Singh, A., Glass, N., Tolba, M., Brovko, L., Griffiths, M., & Evoy, S. (2009). Immobilization of bacteriophages on gold surfaces for the specific capture of pathogens. *Biosensors and Bioelectronics, 24,* 3645-3651. Retrieved from http://www.academia.edu/18562147/Immobilization_of_bacteriophages_on_gold_surfaces_for_the_specific_capture_of_pathogens

Slutsker, L., Ries, A. A., Maloney, K., Wells, J. G., Greene, K. D., & Griffin, P. M. (1998). A nationwide case-control study of *Escherichia coli* O157:H7 infection in the United States. *Journal of Infectious Diseases, 177*(4), 962-966. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9534969

Smith, D. R., Moxley, R. A., Peterson, R. E., Klopfenstein, T. J., Erickson, G. E., & Clowser. S. L. (2008). A two-dose regimen of a vaccine against *Escherichia coli* O157:H7 type III secreted proteins reduced environmental transmission of the agent in a large-scale commercial beef feedlot clinical trial. *Foodborne Pathogens and Disease, 5,* 589–598. http://dx.doi.org/10.1089/fpd.2008.0136

Strausbaugh, L. J. (2001). Emerging health care-associated infections in the geriatric population. *Emerging Infectious Diseases*, *7*(2), 268–271. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2631705/pdf/11294721.pdf

Talaro, K., & Chess, B. (2008). *Foundations in Microbiology* (8th ed.). New York, NY: McGraw-Hill.

Tesh, V. L., & O'Brien, A. D. (1991). The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins. *Molecular Microbiology, 5*(8), 1817-1822. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC281015/pdf/iai00020-0300.pdf

Teunis, P. F. M., Ogden, I. D., & Strachan, N. J. C. (2008). Hierarchical dose response of *E. coli* O157:H7 from human outbreaks incorporating heterogeneity in exposure. *Epidemiology and Infection*, *136*(6), 761–770. doi:10.1017/S0950268807008771

Thorpe, C. M. (2004). ShigaToxin-Producing *Escherichia coli* Infection. *Clinical Infectious Diseases, 38*(9), 1298-1303. doi:10.1086/383473

Thulasiraman, P., Newton, S. M. C., Xu, J., Raymond, K. N., Mai, C., Hall, A., Montague, M. A., & Klebba, P. E. (1998). Selectivity of Ferric Enterobactin Binding and Cooperativity of Transport in Gram-Negative Bacteria. *Journal of Bacteriology*, *180*(24), 6689–6696. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC107775/pdf/jb006689.pdf

Todar, K. (2008). Todar's Textbook of Bacteriology. Retrieved from http://textbookofbacteriology.net/endotoxin.html

Tommonaro, G., Abbamondi, G. R., Iodice, C., Tait, K., & De Rosa, S. (2011). Diketopiperazines produced by the halophilic archaeon, *Haloterrigena hispanica*, activate AHL bioreporters. *Microbial Ecology, 63*(3), 490-495. doi:10.1007/s00248-011-9980-y

Trofa, A. F., Ueno-Olsen, H., Oiwa, R., & Yoshikawa, M. (1999). Dr. Kiyoshi Shiga: Discoverer of the dysentery bacillus. *Clinical Infectious Diseases, 29*, 1303-1306. doi:10.1086/313437

United States Census Bureau. Department of Commerce. (2013). Per capita utilization of selected commercially produced fruits and vegetables: 1980-2009. http://census.gov/compendia/statab/2012/tables/12s0218.pdf

United States Department of Agriculture (USDA) (2013). Clarification and expansion of sampling eligibility criteria for the routine beef manufacturing trimmings (MT60) and bench trim (MT55) sampling programs. (n.d.). Retrieved from http://www.fsis.usda.gov/wps/wcm/connect/2a7c08da-727f-4784-a2cb-c1b3353c62d2/PolicyPoint-39-14.pdf?MOD=AJPERES

United States Department of Agriculture (USDA) (2014). Detection and isolation of non-O157 Shiga toxin-producing Escherichia coli (STEC) from meat products and carcass and environmental sponges. (2013, September 30). Retrieved from http://www.fsis.usda.gov/wps/wcm/connect/51507fdb-dded-47f7-862d-ad80c3ee1738/MLG-5.pdf?MOD=AJPERES

Verma, V., Joshi, K., & Mazumdar, B. (2012). Study of siderophore formation in nodule-forming bacterial species. *Research Journal of Chemical Sciences, 2*(11), 26-29. Retrieved from http://www.isca.in/rjcs/Archives/v2/i11/5.ISCA-RJCS-2012-166.pdf

Vilte, D. A., Larzabal, M., Mayr, U. B., Garbaccio, S., Gammella, M., Rabinovits, B. C., Delgado, F., Meikle, R. J. C., Lubitz, P., Lubitz, W., Cataldi, A., & Mercado, E. C. (2012) Systemic vaccine based on *Escherichia coli* O157:H7 bacterial ghosts (BGs) reduces the excretion of *E. coli* O157:H7 in calves. *Veterinary Immunology and Immunopathology, 146*(2), 169-176. doi:10.1016/j.vetimm.2012.03.002

Visick, K., & McFall-Ngai, M. (2000). An exclusive contract: specificity in the *Vibrio fischeri-Euprymna scolopes* partnership. *Journal of Bacteriology, 182*(7), 1779-1787. doi:10.1128/JB.182.7.1779-1787.2000

Weinbauer, M. G. (2004) Ecology of prokaryotic viruses. *FEMS Microbiology Reviews, 28*(2), 127-181. http://dx.doi.org/10.1016/j.femsre.2003.08.001

Whitehead, N. A., Barnard, A. M., Slater, H., Simpson, N. J., & Salmond, G. P. (2001). Quorum sensing in gram-negative bacteria. *FEMS Microbiology Reviews, 25*, 365-404. http://dx.doi.org/10.1111/j.1574-6976.2001.tb00583.x

World Health Organization (WHO). (2013). Disease outbreaks. Retrieved from http://www.who.int/topics/disease_outbreaks/en/

Willey, J., Sherwood, L., & Woolverton, C. (2008). *Microbiology* (7th ed.). New York, NY: McGraw Hill. pp. 59-60.

Xue, R., Ma, Q., Baker, M. A. B., & Bai, F. (2015). A delicate nanoscale motor made by nature-the bacterial flagellar motor. *Advanced Science, 2*. doi:10.1002/advs.201500129

Zhu, J., Chai, Y., Zhong, Z., Li, S., & Winans, S. (2003). *Agrobacterium* bioassay strain for ultrasensitive detection of N-acylhomoserine lactone-type quorum-sensing molecules: detection of autoinducers in *Mesorhizobium huakuii*. *Applied and Environmental Microbiology, 69*(11), 6949-6953. doi:10.1128/AEM.69.11.6949-6953.2003