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University of Arkansas, Fayetteville

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Shiga Toxin-Producing *Escherichia coli* (STEC) Detection Strategies with Formalin-Fixed
STEC Cells

Shiga Toxin-Producing *Escherichia coli* (STEC) Detection Strategies with Formalin-Fixed
STEC Cells

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Food Science

by

Christopher Adam Baker
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Dr. Steven C. Ricke
Thesis Director

Dr. Steven L. Foley
Committee Member

Dr. Jeffrey A. Lewis
Committee Member

Abstract

Certain pathogenic *Escherichia coli* known as Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are commensals in cattle, and typically cause bloody diarrhea in humans once the Stx toxin is secreted in invaded intestinal epithelial cells. Infections with STEC cells can lead to hemolytic uremic syndrome, which is commonly associated with kidney failure. Several STEC serogroups have been declared adulterants in raw, non-intact ground meat, and future regulations could potentially lead to a higher number of STEC serogroup detection strategies for these pathogenic microorganisms. Microbiological research laboratories may benefit from formalin-fixed STEC cells for periodic (daily, weekly, monthly, among others) instrument validation/calibration by serving as a working set of known cell concentration samples and internal standard i.e. positive control. These cell concentrations may be used across laboratories in different geographical locations, within an individual laboratory, and across a broad range of detection assays (molecular as well as immuno-based). This thesis consists of three research parts: a comprehensive literature review that covers STEC incidence in foods and molecular detection techniques (**chapter 1**), a literature review that covers immuno-based detection strategies (**chapter 2**), and a research manuscript that involves the development of an internal standard and positive control with formalin-fixed STEC cells that can be used for a broad range of molecular as well immuno-based detection assays for instrument calibration and validation purposes (**chapter 3**).

Table of Contents

| | | |
|------|------------------------------|-----|
| I. | Introduction | 1 |
| II. | Chapter 1 | 2 |
| III. | Chapter 2 | 56 |
| IV. | Chapter 3 | 124 |
| V. | Conclusion | 146 |
| VI. | Appendix | |
| | a. IBC Approval Letter | 148 |
| | b. Copyright Approval | 149 |

I. Introduction

This thesis focuses on molecular and immuno-based detection strategies for Shiga toxin-producing *Escherichia coli* (STEC). Although each article focuses on different facets of STEC, the common theme throughout these three chapters is the advantages and limitations of STEC detection methods, and future strategies for improvement of various detection methods. Commensal *Escherichia coli* are commonly utilized for investigating the genetic and biochemical requirements of microorganisms, and have served in a wide variety of applications. Certain pathogenic *Escherichia coli* known as STEC are a public health threat to the consumer, and are problematic for the food industry. Food products containing STEC are deemed unfit for human consumption, and STEC illnesses can cause hemolytic uremic syndrome (HUS), a disease affecting the kidneys in susceptible individuals. These pathogens are present in a wide range of environments, and have caused numerous foodborne outbreaks and recalls. These outbreaks and the increased awareness of STEC have led to certain STEC serotypes to be declared adulterants in non-intact raw meat. Various STEC detection methods have been investigated, and numerous cultural and molecular-based detection methods continue to be modified to meet regulatory requirements. However, STEC serotypes may possess certain characteristics that lead to bias in the likelihood of a certain serotype being detected in an assay. Understanding the components of these STEC serotypes will provide means for optimizing the detection platforms, and as a result limit foodborne illness and recalls caused by STEC due to enhanced cultural and molecular detection capabilities. Research aspects of this thesis focus on an internal standard i.e. positive control (formalin-fixed STEC cells) that can complement commercial detection assay kits for instrument calibration and validation.

II. Chapter 1

Shiga Toxin-Producing *Escherichia coli* in Food: Incidence, Ecology, and Detection

Strategies

Christopher A. Baker^{ab}, Peter M. Rubinelli^{ab}, Si Hong Park^{ab}, Franck Carbonero^b, and S. C. Ricke^{a*}

^a Center for Food Safety, 2650 N. Young Ave. University of Arkansas, Fayetteville, AR 72704

^b Department of Food Science, 2650 N. Young Ave. University of Arkansas, Fayetteville, AR 72704

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Address for correspondence: Dr. Steven C. Ricke, Department of Food Science, University of Arkansas, 2650 N. Young Ave., Fayetteville, AR 72704-5690. Tel: (479) 575-4678. Fax: (479) 575-6936. E-mail: sricke@uark.edu

Introduction

Escherichia coli isolates are characterized as Gram negative, chemoorganotrophic, oxidase negative commensal microorganisms that are 1.1 to 1.5 μm in diameter, and 2 to 6 μm in length (Brenner et al., 2005). As a member of the *Enterobacteriaceae* family, *E. coli* in general contain between 48.5 to 52.1 percent guanine-cytosine in their DNA (Brenner et al., 2005).

Escherichia coli can be isolated from humans and numerous animal hosts, and occur throughout most of the gastrointestinal tract. These microorganisms can grow within a pH range of 5 to 9 (Zilberstein et al., 1984), but can survive after a short exposure to a pH level as low as 2 (Small et al., 1994), which is required if they are to successfully pass through the acidic stomach environment prior to intestinal colonization.

Historically, commensal *E. coli* have been among some of the most thoroughly studied microorganisms in the biological sciences and have served as the basis for understanding many of the fundamental genetic and biochemical concepts (Koch and Schaechter, 1984; Sofos and Smith, 1993; Alteri and Mobley, 2012). Not surprisingly, *E. coli* offer tremendous utility for a wide range of applications due to their well-characterized genome (Souza et al., 2002; Kang et al., 2004; Baba et al., 2006; Lajoie et al., 2013). Since complex preformed organic molecules are generally not required by *E. coli* for growth (Koch and Schaechter, 1984), this biosynthetic capacity has been exploited for numerous genetic engineering applications. Consequently, *E. coli* strains have been employed as genetic engineering platforms for heterologous protein production (Richins et al., 1997; Terpe, 2006), biosensor development (Ryabova et al., 2008; Chalova et al., 2009, 2010; Potera, 2013), and indicator probes (Cha et al., 2000; Guido et al., 2006; Sanchez and Golding, 2013). More recently, progress has been made toward *E. coli* genome reconstruction to generate strains that can be used to monitor the intestinal tract by recognizing

metabolites characteristic of human disease, which could be applicable in diagnosis and therapy (Kotula et al., 2014). This in turn has implications for adjusting the molecular framework to regulate the gut microbiota that would potentially provide a more accurate model of the human gut-microorganism relationship (Koli et al., 2011).

Pathogenic *E. coli* have been identified as the leading cause of traveller's diarrhea (40 to 70% of reported cases), and as the name implies, most often occurs when humans consume contaminated food during their travels to less developed countries (Rowe et al., 1970; Cook, 1983; Sofos and Smith, 1993). The term Shiga toxin (Stx)-producing *E. coli* (STEC) was adopted due to the similarity of the toxin generated by the *stx1* gene to the Stx produced by *Shigella dysenteriae* (O'Brien et al., 1984; Lingwood, 1999). In addition to Stx1, STEC may possess the Stx2 toxin as well as various Stx1 and/or Stx2 variants (Oku et al., 1989; Willford et al., 2009; Skinner et al., 2013). STEC possess an *eae* gene that codes for intimin, a 94- to 97-kDa attachment and adhesion outer membrane protein, which enables the bacterium to invade the intestinal wall of a host (Nataro and Kaper, 1998; Spears et al., 2006). Upon invasion, Stxs are subsequently released and can cause life-threatening health complications. People that are immunocompromised, including infants and the elderly, are considered to be at the greatest risk to STEC (Boyce et al., 1995; Westerholt et al., 2003).

A major public health concern is the possibility of STEC infections becoming a more severe form of clinical disease classified as hemolytic uremic syndrome (HUS), which typically occurs in roughly 5% of the reported cases (Tuttle et al., 1999) 3 days after the onset of diarrhea (Karmali et al., 1985). The mortality rate of HUS illnesses is roughly 5% (Kaplan et al., 1998; Razzaq, 2006) although rates are higher in developing countries where most cases are not treated (Rivas et al., 2008; Heuvelink et al., 2002). This is likely due to the lack of resources. The

specific details of the onset, pathogenesis, and methods to treat HUS have been extensively reviewed elsewhere (Kaplan et al., 1998; Tarr et al., 2005; Welinger-Olsson and Kaijser 2005; Spears et al., 2006; Mayer et al., 2012). The objective of this review is to address the factors that impact the incidence of STEC as well as the procedures currently implemented for STEC detection in food.

STEC sources

Environmental – Animal reservoirs

Domestic and wild animals are a major source of STEC, which are often associated with cattle as well as corresponding environmental niche(s) contaminated with fecal matter. Animal reservoirs often lack clinical signs of disease, which makes the reduction and prevention of contamination a challenge. Animals originating from farms, slaughterhouses, county livestock fairs, and petting zoos are known to be sources of STEC (Varma et al., 2003). Petting zoos pose a particular problem (especially for children) due to close contact during the petting and feeding of possibly STEC-ridden animals that lack clinical signs of disease (Heuvelink et al., 2002). Atypical environments prone to fecal contamination are largely associated with cross contamination due to human error (Jones 1998; Fonesca and Ravishankar, 2007). Persons exposed to areas highly associated with STEC have been encouraged to be aware of possible risks in these types of environments (CDC, 2012).

A major contributor in animal-to-animal transmission is the high population levels of STEC that can be present in cattle feces. Cattle that shed feces highly concentrated in STEC (greater than 10^4 colony forming units (CFU)/g) have been termed super shedders (Arthur et al., 2009, 2010). Even in cattle feedlots with only a minimal amount of STEC carrying cattle, the

presence of just a few super shedders can result in widespread transmission and contamination. In one study, 96 % of all *E. coli* O157:H7 shed by a group of cattle were linked to only 9 % of the cattle (Omisakin et al., 2003). However, it is unknown whether this phenomenon only pertains to a portion of cattle or if all cattle transition into super shedders following STEC infection (Smith, 2014).

Although hides are considered a major contributor to carcass contamination (Schmidt et al., 2012), the actual role of hides in STEC contamination is a controversial topic as swabbing techniques and the location(s) on the hides that are swabbed can lead to inconsistent recoveries of STEC (Elder et al., 2000). This in turn can result in less than certain attributes for the role of hides in carcass contamination (Elder et al., 2000; Arthur et al., 2009). More incidences of STEC illnesses occur during the summer months (Banatvala et al., 2001) most likely due to the higher loads of pathogenic *E. coli* shed by cattle during this time, which leads to hide and eventual carcass contamination during processing (Elder et al., 2000).

The various cattle diets and management practices among feedlots should be considered when comparing data, and determining the efficiencies of management practices is problematic in that the cattle population density, season, and geographic locations in which the corresponding studies were conducted are all likely contributors to contrasting results and varying conclusions (Dargatz et al., 1997; Callaway et al., 2003). Various pre-harvest management practices to reduce STEC shedding and hide contamination are available (Callaway et al., 2004), but variable results on the efficacy of prevention measures make implementing the best management strategies a difficult task (Callaway et al., 2003).

Numerous investigations have supported the suggestion that grain-fed cattle typically shed higher levels of *E. coli* when compared to forage-fed cattle (Callaway et al., 2009). Diez-

Gonzalez et al., (1998) observed higher numbers of *E. coli* in the feces of cattle that were fed diets consisting of higher amounts of grain. Additionally, when cattle diets were switched from a 90% grain diet to a hay diet, these authors observed nearly 10^6 fold fewer *E. coli* after only five days from the diet alteration (Diez-Gonzalez et al., 1998). They also concluded that grain was more efficient with regard to cattle performance, and that strictly forage-based diets were unlikely to become commonplace in American cattle feed. However, they suggested that switching from a grain to forage diet prior to slaughter could potentially limit *E. coli* transmission from farm-to-slaughter (Diez-Gonzalez et al., 1998).

Following their research on the influence of diets and the prevalence of *E. coli* O157:H7 in sheep, Kudva et al., (1997) suggested that ruminants fed a grain based diet exhibited less *E. coli* O157:H7 shedding compared to ruminants fed a forage based diet. These authors also suggested that dietary changes could create a more favorable environment for *E. coli* O157:H7 in ruminants (Kudva et al., 1997), while Hovde et al., (1999) observed differences in the duration of STEC shedding by cattle fed grain and forage based diets. *E. coli* O157:H7 (10^{10} CFU) was administered into the rumen of cattle via a gastric tube, and these cattle were fed either grain or forage based diets. Fresh fecal samples were obtained every 3 to 4 days, and cultured for *E. coli* O157:H7. These authors were able to culture *E. coli* O157:H7 from forage fed cattle roughly 10 times longer than grain fed cattle – forage fed cattle contained *E. coli* O157:H7 for an average of 39 days (alfalfa hay) and an average of 42 days (grass hay), while grain fed cattle contained *E. coli* O157:H7 for an average of only 4 days (Hovde et al., 1999). Additionally, unlike Diez-Gonzalez et al., (1998) observations on increased recovery of acid-resistant *E. coli* from grain fed cattle, these authors found no differences in acid resistance of *E. coli* O157:H7 fed grain and forage based diets (Hovde et al., 1999). These contrasting results of *E. coli* and STEC levels in

grain or forage fed cattle could be due to varying sampling methods, the type of ruminant used for the research, and the geographic distribution of these ruminants.

The presence of litter material, hay, silage, fur, and feathers contributes to the prevalence of microorganisms in feedlots and animal housing, and it has been suggested that airborne transmission of STEC is possible (Al-Dagal and Fung, 1990; Zucker et al., 2000; Wilson et al., 2002; Pillai and Ricke, 2002; Varma et al., 2003; Lues et al., 2007; Patel and Nou, 2008; Martin et al., 2010). In addition to areas highly concentrated with livestock, a drier and warmer environment may also contribute to an increase in airborne transmission (Lutgring et al., 1997; Wilson et al., 2002). Likewise, the hide, skin, and carcass of slaughtered animals can be a major source of airborne microorganisms in processing plants (Rahkio and Korkeala, 1997; Ahmed and Sarangi, 2013). The direction of airflow influences airborne transmission (Rahkio and Korkeala, 1997; Cundith et al., 2002), and birds and insects can also act as vectors. In addition to distributing urine and feces, the aerosols they generate contain airborne microbial populations including pathogens (Hugh-Jones and Wright, 1970).

By considering the possibility of airborne transmission, it has been recommended that processing plants should be designed to consistently direct airflow from finished product(s) to raw materials (Lutgring et al., 1997). Additional prevention strategies include locating air filters as close as possible to the contamination source, using “clean room clothing” (head covering, masks, gloves, among others) (Al-Dagal and Fung, 1990), proper maintenance of ventilation systems (Lutgring et al., 1997), and implementing air cleaning systems (Cundith et al., 2002). The possibility of contamination by airborne microorganisms is often overlooked, and since a low infectious dose of STEC may lead to infection (it has been estimated an infectious dose of *E.*

coli O157:H7 ranges from 10 to 100 cells (Feng et al., 2011)), airborne transmission may be more of a concern for STEC compared to other less infectious pathogens.

Human-to-human transmission

Bacterial transmission occurs among humans, as well as from contaminated food and water (Keene et al., 1994; Islam et al., 2004; Fonesca and Ravishankar, 2007; Fischer et al., 2007; Craun et al., 2010). The original sources for STEC are likely to be cattle or human carriers (Wang and Doyle, 1998; Council for Agricultural Science and Technology, 2004). Nursing home outbreaks have been documented (Ryan et al., 1986; Carter et al., 1987) and nursing home safety awareness and regulatory measures are usually addressed (Rangel et al., 2005), but more needs to be done. For instance, improvements for infection control procedures are needed. This would include constant surveillance of the elderly and their dining facilities before, during, and after food consumption to reduce cross-contamination and illness, and any ill individuals should be isolated to prevent spread among nursing homes. Also, proper food preparation practices, eating habits, hygiene, and thoroughly cooking food are all considered effective means for preventing an outbreak in these types of environments (Ryan et al., 1986; Thoreau, 1986; Carter et al., 1987; Rangel et al., 2005).

Day-care facilities are also a high-risk environment for STEC infection. Greater infection rates are probably a consequence of children's behavioral patterns. Children more frequently place their hands in their mouth and are more likely to not wash their hands (Black et al., 2005). In addition, young children are less likely to be trained to use the restroom properly (Belongia et al., 1993). Occurrence of STEC illnesses have been linked to improperly chlorinated swimming pools (usually attributed to infants swimming in diapers) (Friedman et al., 1999), but identifying

a body of water as a source of contamination can be difficult. Water sources typically contain minimal or no nutrients, which can physiologically stress microorganisms and subsequently cause metabolic and morphological alterations.

Physiologically stressed STEC may be particularly problematic as cells in this state can be difficult to recover and detect. This is in part due to the fact that microorganisms that are exposed to a stressful environment can enter a survival state that has been defined as viable but nonculturable (VBNC) (Binsztein et al., 2004). Although cellular energy levels remain constant (Beumer et al., 1992), a reduction of nutrient transport, respiration, and macromolecular synthesis has been observed in VBNC cells (Oliver 2000, 2005). Also, Gram-negative bacteria can become reduced in size, and exhibit a more rounded morphology compared to cells in a more favorable growth environment (Rollins and Colwell 1986; Donelli et al., 1998; Signoretto et al., 2000).

Viable but nonculturable cells are troublesome in that they are not identifiable on conventional culture media, and can lead to false conclusions about the true population of cells in an environment. Also, *E. coli* that become VBNC pose a problem for assessing water quality since they are often the primary microorganisms used to indicate the fecal contamination level in water systems (Edberg et al., 2000; Oliver et al., 2005). The inability to culture VBNC cells leads to the possibility of underestimating, as well as not detecting these cells, and VBNC pathogenic microorganisms in food systems present a potential public health threat (Oliver et al., 2005).

Food sources

The public's lack of knowledge on proper food safety practices has been identified as a contributing factor to STEC outbreaks (Roseman and Kurzynske, 2006; Rivas et al., 2008). Consequently, it should be fairly well established from a public sector standpoint that any food has the potential to be unsafe due to contact with STEC, whether it occurs by human error or during production, processing, shipping, storage, or cooking. Food safety public awareness and control measures must be continually addressed to reduce and limit STEC. Proper hand washing, preventing cross-contamination while preparing food, storage of meat below 40 °F (4.4 °C), and using a thermometer to confirm an internal temperature of 160°F (71 °C) during cooking are considered necessary steps to take for preventing foodborne illnesses (Vogt and Dippold, 2005; FSIS, 2011).

As discussed previously, pathogen prevention measures (drastic pH changes, heat exposure, among others) during food processing can cause bacteria to become VBNC (Shen et al., 2009; Díaz et al., 2010). Stressed and damaged cells may not be identifiable when cultured, but may later resuscitate and subsequently proliferate (Hussong et al., 1987), which can result in an underestimation of the original levels of microorganisms present while being enumerated. The fact that microorganisms can adapt, survive, regrow, and disperse in various environments makes preventing contamination and illness a difficult task. Theoretically, even if quality assurance and control procedures are appropriately in place, illnesses and outbreaks can occur, and based on past experiences, an end to such occurrences does not seem likely.

Some specific instances of food preparation or lack thereof can also contribute to STEC occurrence. For example, raw milk consumption has recently become popular (Houser et al., 2008; Baylis, 2009; Claeys et al., 2013, 2014). It has been estimated that three percent of the U.S. population consumes raw milk each week (CDC, 2007a). Milk is rich in nutrients, and has a

neutral pH, which is favorable for bacterial survival and growth (Lejeune and Rajala-Schultz, 2009). Not surprisingly, raw milk has been identified as a source of STEC contamination, and the potential public health consequence(s) of consuming raw milk have been reviewed (Oliver et al., 2009). From 1973 to 1992, 1733 cases of infection were reported due to raw milk consumption. Of these cases, 6 were identified as being caused by *E. coli* O157:H7 and the source of 281 cases was declared unknown (Headrick et al., 1998). Conceivably, STEC contamination could have been the cause of many of the unknown cases. Milk contamination is likely due to cross-contamination of feces during or after the milking process (Heuvelink et al., 1998). It is becoming apparent that additional safety procedures should be addressed on dairy farms where raw milk is produced for retail to limit contamination. The CDC lists several ways in which pathogens can contaminate milk such as cattle feces, cattle udder infection (mastitis), processing equipment, insects and rodents, and humans from contaminated clothing and boots (CDC, 2014). With all of the numerous potential pathogen sources, it seems that appropriate safety procedures should include cross-contamination prevention measures, and continuous assessment of cattle udder health, equipment, milk collection areas, and human garment cleanliness.

For conventional milk production, pasteurization involves eliminating pathogens that may be present in raw milk by heat exposure for a short period of time (Oliver et al., 2009). The USDA recommends that the pasteurization process should be performed at one of three conditions: 63 °C for 30 minutes, 72 °C for 15 seconds, or 89 °C for 1 second (Rasooly and Do, 2010). Assessing the most safe milk pasteurization temperature(s) can be difficult; Stasiewicz et al., (2014) found that increasing the temperature from 72 °C to 82 °C for 25 seconds increased the total population of *Listeria monocytogenes* in milk. The authors hypothesized that the

inactivation of lactoperoxidase at 82 °C, an enzyme in milk that is bacteriostatic and bacteriocidal, was the cause of an increase in *L. monocytogenes* (Stasiewicz et al., 2014).

D'Aoust et al., (1988) investigated the survivability of *E. coli* O157:H7 in milk following pasteurization at temperature ranges of 60 °C to 72 °C for 16.2 seconds for each temperature tested. There was a 4-log reduction of *E. coli* O157:H7 at 63 °C for 16.2 seconds, but only temperatures of 64.5 °C and higher for 16.2 seconds were sufficient in deactivating all *E. coli* O157:H7 in milk (D'Aoust et al., 1988). Although this study identified which pasteurization temperatures would effectively inactivate *E. coli* O157:H7, direct plating techniques were used to determine the presence of *E. coli* O157:H7 in the pasteurized milk samples. Using a plating method may not have necessarily given a true indication of the present bacterial populations due to the possibility of VBNC cells that could have formed during the pasteurization process.

While *E. coli* O157:H7 was inactivated at temperatures exceeding 64.5 °C, milk containing high levels of STEC that produced large amounts of Stx toxin before pasteurization could still cause illness. Rasooly and Do (2010) investigated how Stx2 toxin survives the various USDA pasteurization recommendations. These authors justified investigating the effects of pasteurization on the Stx2 toxin rather than Stx1 because of a previous study where Tesh et al., (1993) had demonstrated that Stx2 was 400 times more virulent to mice than the Stx1 toxin (Tesh et al., 1993; Rasooly and Do, 2010). Rasooly and Do (2010) concluded that neither of the USDA recommended pasteurization conditions were sufficient to eliminate Stx2 toxin, which was only inactivated following pasteurization for 5 minutes at 100 °C. This study was useful in providing insight into the stability of Stx2 in conventional pasteurization temperatures, but the effect that these conditions have on STEC was not addressed. Unless a large number of STEC

(that have produced a substantial amount of Stx(s)) are present in the raw milk before pasteurization, eliminating STEC by pasteurization should be sufficient to ensure safe milk.

There will always be the threat of contamination of milk during the production process whether it be during raw milk production or after pasteurized milk has been heat treated (Oliver et al., 2009). Although heat treatment is still the best strategy to eliminate pathogens in milk, options for raw milk are much more limited. Therefore, control measures such as washing udders prior to milking would represent a possible means to limit contamination and therefore reduce illnesses within production parameters specified for raw milk (Baylis, 2009).

In the U.S., the most common food associated with STEC infections is improperly cooked ground beef (Boyce et al., 1995; Razzaq, 2006). Carcass contamination by feces during processing may be unavoidable, and STEC growth may occur following production. It has been suggested that the meat matrix (pH, percent sodium chloride, water activity among others), as well as the exposure and duration of temperature fluctuations during food processing, transportation, distribution, and storage (as well as a number of other factors) may contribute to STEC growth in ground beef (Walls and Scott, 1996; Cassin et al., 1998; Juneja et al., 1998; Ansay et al., 1999).

The sources and origins of ground beef partially accounts for the high risk of STEC contamination since ground beef is typically made from the trimmings of more than one carcass, thus increasing the likelihood of STEC contamination through multiple sources (Armstrong et al., 1996). Also, since ground beef is composed of many small meat particles, the product as a whole represents a large surface area, and is further troublesome in that the ground beef is more prone to STEC exposure due to contaminated surfaces, tools, and machinery (Surkiewicz et al., 1975; Pillai and Ricke, 1995; Schroeder et al., 2003). Additionally, the large surface area of

ground beef favors rapid bacterial growth by providing an aerobic environment for microorganisms to proliferate (Pohlman et al., 2002). The doubling time of *E. coli* is roughly 20 minutes under aerobic conditions, but in a study comparing the effect of media on growth rates, a doubling time as high as 16.8 minutes has been observed (Guerini et al., 2006). Theoretically, if a single STEC cell comes into contact with a food product at 8 am, and subsequently doubled in population every 20 minutes, there would be over 1000 STEC cells in the food product by 11:40 am (within 3 hours, 40 min). These circumstances are based on the assumption that the food product provided sufficient growth conditions (excluding refrigerated or frozen ground beef) comparable to broth-based culture conditions. With many animal sources, the potential for cross-contamination, and foods that are favorable to proliferation, STEC continues to be a major cause of foodborne illness and subsequent outbreaks (Hussein, 2007). Previous outbreaks associated with STEC were instrumental in the restructuring of how food safety analyses are conducted in the U.S. meat industry.

Epidemiology in the U.S.

The annual number of foodborne illnesses (Scallan et al., 2011), as well as the costs of these illnesses (Scharff, 2012) in the U.S. have been estimated (Table 1). Similar to many foodborne illnesses, the prevalence of STEC infection is likely underestimated for a variety of reasons (Wickham et al., 2006). For example, not all clinical laboratories have the means to screen fecal samples for STEC, resulting in fewer confirmed reported cases. Underestimation is also caused by incidences where individuals despite contracting STEC do not seek medical treatment due to mild symptoms and thus remain unreported.

It has been estimated that bacterial agents in beef products caused 482,199 illnesses; 2650 hospitalizations and 51 deaths annually in the U.S. from 1998 to 2008 (Painter et al., 2013).

Although foodborne illnesses occur more often with pathogens other than STEC, STEC are still considered a major risk to public health due to the very low doses required to cause an infection (Bell et al., 1994; Rowe et al., 1994; Ray and Bhunia, 2014); bacterial populations ranging from 10 to 100 *E. coli* O157:H7 cells are known to cause human illness (Feng et al., 2011).

STEC publicity and government action – A history of the U.S. experience

The first fully documented modern outbreak of STEC occurred in the early summer of 1982 (Riley et al., 1983). Illnesses and outbreaks continued throughout several countries for the next ten years (Karch et al., 1999; Rangel et al., 2005). It was not until the northwestern U.S. retail restaurant associated outbreak of 1992-1993 that public awareness and eventual government action spawned a general alarm and mobilized a comprehensive regulatory response to STEC (Schuller, 1998). During the course of this outbreak, foodborne illnesses caused by *E. coli* O157:H7 were reported in Washington, Idaho, Nevada, and California (Karch et al., 1999). Eighty-six percent of the cases were reported from the state of Washington and thus historically became identified as the “Washington experience” (Bell et al., 1994). This outbreak was traced to a single fast food chain that did not thoroughly cook hamburger patties, which led to 731 confirmed cases, 170 hospitalizations, 56 cases of HUS and the death of four children. Given the extent of the outbreak and the severity of the ensuing illness, it soon became apparent that the infectious agent involved had possessed considerable virulence potency that would be consistent with a low infectious dose. Consequently, not only did identifying the organism become important, but quantifying actual levels of the pathogen came to be considered equally critical.

Therefore, as part of the outbreak investigation, bacteria from frozen hamburger patty samples were enumerated to determine the amount of *E. coli* O157:H7 originating from individual patties.

This outbreak shed additional light on the lethal potential of STEC and the need for comprehensive and systematic control of this pathogen in ground beef in the U.S. in the mid-1990's. In response to national publicity and ensuing public concerns, regulatory action became a focal point for additional control measures to be taken, and publicity of the subsequent outbreak led to changes in the meat industry (Bottemiller, 2011). Following the U.S. Secretary of Agriculture's involvement in addressing the outbreak, more meat inspectors were hired and recall notification requirements were improved (Bottemiller, 2011). Also, in response to the well-publicized outcry, the U.S. Department of Agriculture (USDA)-Food Safety and Inspection Services (FSIS) adopted the zero-tolerance policy for visibly contaminated meat during processing (Kochevar et al., 1997).

Consequently, the USDA Deputy Commissioner for Policy at the time, led a strategic effort among agency legal counsel and research personnel to initiate a case for *E. coli* O157:H7 to be classified as an adulterant under the Federal Meat Inspection Act (FMIA) (Bottemiller, 2011). On September 29, 1994, Mike Taylor spoke at the American Meat Institute's annual convention and addressed the plan to consider *E. coli* O157:H7 as an adulterant (Bottemiller, 2011). This promptly spread controversy in the meat industry, and on December 13, 1994, several supermarket and meat industry organizations filed a legal suit against the USDA and stated the *E. coli* O157:H7 adulterant statute violated the Administrative Procedure Act and was outside the realm of the USDA's authority under the FMIA, but the Federal District Court upheld the USDA's official decision (Bottemiller, 2011).

Once adulterant status was established, the need for sensitive detection strategies became obvious and thus immediately impacted and accelerated the search for even faster and more sensitive detection systems. Several costly recalls followed (Table 2) (Schuller, 1998), and it became clear in the U.S. that detection capabilities would need to be vastly improved both in terms of speed and accuracy. One of the primary lessons that was learned from establishing the adulterant status was not just the need for sensitivity improvement but for more consistent and reliable detection technologies. Correctly identifying and classifying STEC each and every time as rapidly as possible was projected as a means to theoretically minimize illnesses during outbreaks as well as improve the treatment of infections and overall public health (Sobel et al., 2002). Aside from health costs directly due to illnesses, the issues of recalls, unused products, and possible lawsuits from undetected STEC continues to be an ongoing economic concern in the food industry. Contaminated products are not only a public health threat to the consumer but a potential economic disaster for the processor both in terms of loss of product and negative publicity –not to mention the potentially extensive litigation costs. For example, the Washington state experience in 1992-1993 led to several settlements in favor of the victims of the outbreak, and the largest settlement (\$15.6 million) went to a victim who spent 42 days in a coma (Schuller, 1998). Since then, STEC-associated foodborne outbreaks have been and continue to be a significant threat to public health and food industry economics.

Non-O157 STEC in the U.S.

Several pathogenic *E. coli* outbreaks not associated with *E. coli* O157 were documented following the Washington state experience (Brooks et al., 2005). Not surprisingly, as improved methods to identify non-O157 STEC surfaced, there was a four-fold increase in reports of non-

O157 illnesses from 2000 to 2006 (Windham et al., 2013) and awareness of these pathogenic serovars increased. On October 5, 2009, a citizens petition was filed to suggest non-O157 STEC also be considered adulterants (Clark, 2009), and in 2011 the FSIS declared six additional STEC (O26, O45, O103, O111, O121 and O145), alongside *E. coli* O157 as adulterants in raw, non-intact ground beef products (FSIS, 2011; Fratamico and Bagi, 2012).

Protocols for the detection of non-O157 STEC continue to be investigated (Hegde et al., 2012a, 2012b; Cowan-Lincoln, 2013) with the ultimate goal of devising and/or implementing new technologies and/or combining methods to optimize and exceed performance standards of the industry (Wang et al., 2013). Methods that rapidly produce the most reliable and consistent results represent the ultimate goal. Rapid detection has been defined as less than 12 hours (Guerini et al., 2006), and it has been suggested an 8 hour time period from initiation to reportable results would be optimal due to the fact that an 8-hour detection time would produce results on a set of food products before being shipped from a production facility (Cox, 1988; Gooding and Choudary, 1997, 1998; Guerini et al., 2006; Hagren et al., 2008; Wilkes et al., 2012). Much of this emphasis is due to the current requirement by USDA-FSIS for STEC test and hold of meat products prior to release in the commercial retail sector. Test and hold involves sampling ground beef or trim for adulterants. Trim is typically used to produce ground beef and is defined as the outer portions of the beef carcass that is removed from meat to make the prime cuts that are more commercially desirable (Smith, 2012). Acceptable products can be released into commerce distribution routes once the product sample test outcomes are confirmed negative for STEC (Guerini et al., 2006). In a two day time frame, product(s) can be confirmed as acceptable, and in four days product(s) can be confirmed as unacceptable following culture

confirmation (FSIS, 2013a). It should be noted that these time frames do not include the 12 to 24 hour minimum to freeze samples prior to transportation to a laboratory (FSIS, 2013a).

STEC growth enrichment approaches for detection

General concepts

For most foodborne pathogens, the frequency and total numbers of microorganisms on a particular food product are relatively low. This represents a challenge for detection since sampling protocols need to be designed to account for infrequent and non-uniform distributions of a pathogen and relatively low population numbers. Consequently, most detection methods include steps to increase (enrich) the initial number of the target pathogen to population levels for consistent and reliable detection. Thus, the first step for STEC detection is to enrich the sample to be analyzed. Several factors contribute to the type of enrichment procedure utilized: the sensitivity of the particular detection technology (Woo and Palavecino, 2013), level and complexity of background microflora (Vimont et al., 2006), and the presence of interfering substrates associated with the food matrix that would either inhibit STEC growth or interfere with the detection system output (Maciorowski et al., 2005). Enrichment media vary in composition but generally provide an environment appropriate to increase a bacterial cell population. Such constituents provide a supportive nutritional matrix for growth of microorganisms, or in the case of a selective enrichment medium, a particular segment of the bacterial population (Holt and Krieg, 1994).

To identify STEC, enrichment and culture media that are best suited to optimally support the growth physiology of all STEC requires an understanding of the metabolism of the organism. The specific biochemical growth limitations of STEC and generic *E. coli* have been described

(Atkinson et al., 2012), and efficient enrichment and culturing media containing various components to grow STEC have been reported in a series of publications over a number of years (Doyle and Schoeni, 1984; Wallace and Jone, 1996; McCarthy et al., 1998; Taormina et al., 1998; Vimont et al., 2006; Guerini et al., 2006) and have been reviewed previously (De Boer and Heuvelink, 2000). As would be expected, there are many factors that affect growth of STEC bacteria (Table 3). Although STEC can grow in various conditions, providing an environment with appropriate concentrations of required nutrients, a pH range of 6.5 to 7.5, and an adequate volume of enrichment culture are considered generally necessary to achieve optimal growth (Greasham and Herber, 1997).

In addition to decreasing detection time, analysis of media efficiency using smaller quantities and/or aliquots could reduce the cost, time, and required laboratory incubation space for the enrichment process (Guerini et al., 2006). When reducing the volume from 3.375 L to 1 L for the enrichment of artificially inoculated ground beef samples, Guerini et al., (2006) observed similar recovery rates of *E. coli* O157:H7, indicating a much lower volume is sufficient for enrichment purposes.

Other methods aimed at limiting the required volume(s) even more so involve using microtiter plates (a working volume of 100 to 300 μ L per well is common) to miniaturize growth-based assays for screening a large number of samples per analysis in μ L rather than L-based volumes (Hüser, 2006). While STEC isolates have not been examined directly, there is precedent for comparison of miniaturized growth conditions with standard incubation methods. Much of this work was done as a means to assess particular *E. coli* isolates as potential whole cell biosensors. Critical to this assessment was the impact on growth kinetics since growth response was the measurement being used. Accordingly, a series of studies were conducted with

nonpathogenic *E. coli* amino acid mutants to assess their growth responses to different quantities of the required amino acid and potential to serve as a bioavailability assay for that particular amino acid (Froelich Jr. et al., 2002b, 2005; Zabala-Díaz et al., 2004; Froelich Jr. and Ricke, 2005; Chalova et al., 2009, 2010). Froelich Jr. et al., (2002a, b) used growth kinetic estimates of these mutants after growth in minimal media based on Lineweaver-Burk plots of growth rate to assess response to incremental changes in amino acid concentration. By plotting the reciprocal exponential growth rate versus the initial concentration of methionine, the Lineweaver-Burk plot was used to calculate the affinity constant (substrate concentration at which the growth rate is at half-maximum) and maximum potential growth rate of microorganisms grown in media containing incremental increases of the essential amino acid (Froelich Jr. et al., 2002a, b).

In a follow up study, Zabala-Díaz et al., (2002) directly compared a classical tube assay with a microtiter plate format to determine the affinity and growth rate of an *E. coli* methionine auxotroph grown on the same incremental methionine concentrations (3.3 μM increments, 0 to 26.8 μM). While the affinity estimates between the two methods were similar, these authors observed the maximum growth rates to be significantly higher ($P < 0.05$) when grown in culture tubes versus microtiter plates. These differences suggest that bacterial growth responses are methodology dependent, and although microtiter plates are beneficial for reducing media, reagents, and labor, the differences in growth rates should be considered when employing growth based detection enrichment methods and most probable number types of isolation or estimation for enumeration when changing from culture tubes to microtiter plate assays (Zabala-Díaz et al., 2002).

More recently, Berghaus et al., (2013) miniaturized the most probable number assay to estimate the bacterial populations of *Campylobacter* and *Salmonella* spp. in farm and processing

plant samples from broiler chicken flocks. To estimate *Salmonella* populations, enrichment procedures as recommended by the USDA for *Salmonella* enrichment procedures were adapted for 2-mL deep-well microtiter plates (FSIS, 2014). Once samples were diluted across a microtiter plate containing tetrathionate media (0.8 mL), plates were incubated overnight, and 0.1 mL of culture from each microtiter plate well was transferred to corresponding microtiter plate wells containing Rappaport Vassiliadis media (0.9 mL). Following the secondary enrichment procedure, 1 μ L of culture from each microtiter plate well was transferred onto xylose lysine Tergitol 4 agar via a pin tool replicator, which negates the labor intensive practice of individually transferring large quantity liquid cultures to subsequent agar plates for enumeration (Berghaus et al., 2013). Similar methods that require evaluation of a large number of samples and implement smaller volumes of media, as well as limit the labor associated with conventional laboratory methods will likely be necessary to simplify, and reduce the time required to screen greater numbers of samples for microorganisms (Berghaus et al., 2013).

It has been suggested that an enrichment step may be unavoidable due to the requirement to detect single cells of STEC, and that it would be research time well spent to improve media for better growth of STEC (Wang et al., 2013). Among other aspects, additional studies remain to be conducted to determine growth variations between the relevant STEC serotypes. This is critical, as general assumptions cannot necessarily be made for other STEC serotypes based on results obtained from studies on only a few serotypes. When several strains of *Salmonella* serovars were grown in an enrichment culture, Gorski (2012) observed higher recovery rates of *Salmonella* serovars in serogroups C2 and E when compared to *Salmonella* serovars in serogroups B or C1. These recovery rate differences suggest that multiple enrichment protocols may be necessary to account for all of the microorganisms actually present in a sample (Gorski,

2012). In addition to adopting several enrichment protocols to reduce and potentially eliminate bias recovery of microorganisms, determining the media in which they grow most rapidly should reduce enrichment time and accelerate the overall food detection analytical process. The following subsection examines current media developed for use with STEC.

Current STEC culture growth procedures

The USDA-FSIS currently recommends the use of Modified Tryptone Soy Broth (mTSB) for enrichment of raw meat product samples (FSIS, 2013b). This medium is utilized for *E. coli* O157:H7, the “big six” STEC as well as *Salmonella* enrichment (FSIS, 2013c). The main component of the mTSB medium is pancreatic digest of casein (Holt and Krieg, 1994), which provides the carbon and nitrogen compounds essential for energy production, protein and nucleic acid synthesis, and growth (Roberts et al., 1955). Following an enrichment step in mTSB, samples are screened for the presence of virulence genes *stx* and *eae* with a polymerase chain reaction (PCR) assay, and positive samples are further tested for the “big six” with an additional PCR analysis (FSIS, 2011, 2013c). Following the “big six” PCR assay, immunomagnetic beads (specific for the serogroup(s)) are added to the respective potentially positive samples to recover the cells, and the immunomagnetic bead-cell complexes are plated on modified Rainbow Agar (mRBA) for culture confirmation and further analysis. Modified Rainbow Agar contains 0.15 mg/L of potassium tellurite, 5 mg/L of sodium novobiocin, and 0.05 mg/L of cefixime trihydrate (FSIS, 2013c). The antibiotics novobiocin and cefixime are added to the media to inhibit the growth of *Proteus* spp. and other microorganisms that are capable of reducing the tellurite, the selective compound in mRBA (Zadik et al., 1993). Traditionally, STEC serogroups have been considered difficult to distinguish on chromogenic agar, and although recent work suggests that

phenotypic distinctions may be possible, it is still recommended that independent confirmation assays should be used to complement the initial identification with chromogenic agar (Kalchayanand et al., 2013).

Distinguishing *E. coli* O157:H7 from other *E. coli* strains with routine enteric media is difficult due to the fact that it requires testing for sorbitol fermentation (March and Ratnam, 1986). *E. coli* O157:H7 typically cannot ferment sorbitol (at least not in the first 24 hours) (Farmer III and Davis, 1985), and is distinguishable (appears as a colorless colony) from generic and non-O157 *E. coli* (Atkinson et al., 2012) when cultured on sorbitol-MacConkey agar (SMAC) (March and Ratnam, 1986), which is the primary medium used to culture *E. coli* O157:H7 (CDC, 2007b). However, there have been instances of *E. coli* O157:H7 fermenting sorbitol (Karch et al., 1993; Werber et al., 2011; Sallam et al., 2013), and an outbreak in 1999 due to sorbitol-fermenting *E. coli* O157:H7 exemplifies the limitations of SMAC (Ammon et al., 1999) and the need for more appropriate media.

Recent advances in microbiological methods may have overshadowed certain aspects of bacterial growth physiology during enrichment and there is a need for this to be reconsidered now that multiple serotypes are known to be involved (Chen et al., 1993; Brehm-Stecher et al., 2009). The argument that it is necessary to enrich samples for a substantial amount of time (16 hours or more) will likely always be raised, but research efforts on media improvements and increasing the ability to recover microorganisms following shorter enrichment periods will more precisely define the time needed for enrichment and the subsequent overall detection process. In order to achieve the fundamental objective of rapid detection (less than 12 hours), alternative approaches need to be explored to reduce the enrichment duration (Chen et al., 1993). Following the enrichment step, positive samples must be cultured and serotyped for epidemiology purposes

(Vallières et al., 2013). If reducing the background microflora is necessary due to the lack of suitable media for colony differentiation, then alternative approaches become obligatory. If there were an enrichment medium that could be consistently converted from a nonselective to a selective state after a given enrichment time, the issue of growing foodborne pathogens in a shorter amount of time, as well as being able to eventually reduce the background microflora for culturing purposes would be attainable. Compounds that inhibit non-relevant microbial populations could be either encapsulated or administered in some sort of initially inactive state or form to prevent initial inhibition, but allow eventual release into the medium at the proper time and inhibitory concentration(s). Additionally, devising a medium containing compounds that are not known to inhibit molecular detection assays may be appropriate.

With this in mind, there is also the likelihood that the growth response of STEC may vary in different food systems (Palumbo et al., 1997), thus several enrichment protocols may be needed to successfully enrich STEC serogroups that do not grow within the same response times in a single medium. If it is expected to produce rapid results, an enrichment period of 12 to 16 hours is unacceptable. Although the growth of unwanted background microbiota may outcompete the target microorganism(s) (Suo and Wang, 2013), a richer, less selective medium in some cases may still be applicable if the STEC in question can grow faster than the background microbiota. A longer duration of enrichment is not necessarily better, especially with techniques that enable pathogen detection by non-culture methodologies, such as molecular and immuno-based systems.

Bacteriophage and Immuno Assays

Bacteriophages have been implemented to reduce bacterial loads at the pre- and postharvest food processing stages and have been investigated for foodborne pathogen detection purposes (Ricke et al., 2012; Schmelcher and Loessner, 2014). Bacteriophages require bacteria as a host for replication and survival, and the understanding of the bacteriophage-bacteria relationship has provided alternatives to molecular methods and use of antibodies for detection. Bacteriophages have been used for magnetic separation of cells (phagomagnetic separation) (Liébana et al., 2013), use in an ELISA (Galikowska et al., 2011; Willford et al., 2011), and other detection purposes by direct fluorescent labeling (Goodridge et al., 1999; Willford et al., 2011). Compared to antibodies, bacteriophages are more stable, less expensive, easier to produce and isolate, have longer shelf lives, and do not require animals for production (Balasubramanian et al., 2007; Galikowska et al., 2011). Bacteriophages can be implemented in many detection assays, and a wide range of potential applications have been reviewed (Arugula and Simonian, 2014; Schmelcher and Loessner, 2014; Singh et al., 2013).

PCR Detection Assays

Molecular approaches involving the isolation, detection, and in some cases quantitation of either DNA or RNA were instrumental in the emergence of rapid detection systems for STEC. Consequently, extensive development and improvement on these techniques became possible for application to STEC. Once developed and optimized, commercialization and widespread adaptation by the food industry became commonplace. Much of this has been extensively reviewed elsewhere either as general concepts for all pathogens, or for specific organisms such as STEC and other pathogens (Higuchi et al., 1993; Malorny et al., 2003; Maciorowski et al., 2005; Girones et al., 2010; Elizaquível et al., 2014; Park et al., 2014). Therefore, in the current

review, only the highlights will be discussed for molecular detection of STEC since much of this has been covered in considerable detail elsewhere (DebRoy et al., 2011b; Gerritzen et al., 2011; Fratamico and Bagi 2012; Paddock et al., 2012; Svoboda et al., 2013).

Polymerase chain reaction-based methods can be used to identify the presence of microorganisms by targeting specific portions of the DNA or RNA that are characteristic of the target organism. These genomic sequences are typically amplified with sequence-specific primers and DNA polymerase. In addition to amplifying DNA or RNA, PCR can be applied to differentiate mutations and quantitate levels of gene expression (Park et al., 2014). Nucleic acids (nucleotides) are the structural components of DNA and RNA, and each organism possesses a unique sequence of these nucleotides. Many organisms contain similar DNA and RNA sequences, but there are also generally sequences among a genus, species, or serovar that are unique to that particular population, and can be targeted for identification. This is the principle upon which PCR is applied for detection and identification. Primers used for amplification are oligonucleotide sequences that are essentially identical to the nucleotide segment of an organism or group of organisms, and in theory, if the organism that the primers target is present, the nucleotide sequence(s) will be identifiable. A key component to the process of any PCR assay is to successfully isolate DNA from samples (Yeni et al., 2014), which can subsequently be detected with nucleotide specific primers. Primers have been developed to detect virulence genes such as *stx1*, *stx2* and *eae* (Bae et al., 2010; Bosilevac and Koohmaraie, 2012; Dargatz et al., 2013; Guy et al., 2014), and distinguish *E. coli* pathotypes (Nataro and Kaper, 1998), as well as common STEC serotypes (Paton and Paton, 1998; DebRoy et al., 2011b; Fratamico and Bagi, 2012; Paddock et al., 2012). In theory, with proper DNA extraction techniques and sufficient

DNA purity level, PCR methods can detect a single DNA molecule, which can be amplified to obtain a greater amount of DNA for further analysis (Hedman and Rådström, 2013).

Conventional single and multiplex PCR are used to amplify a specific DNA region to generate sufficient copies to be detectable such that the presence of the target organism(s) can be visibly determined by agarose gel electrophoresis. In a multiplex PCR assay, multiple primers, each specific for a certain serotype and/or virulence gene(s), can be included in a single PCR assay to detect several factors in a single reaction, but optimizing multiplex PCR conditions can be difficult (Maciorowski et al., 2005). Conventional single and multiplex PCR requires agarose gel electrophoresis for analysis, and certain parameters cannot be determined. Real-time PCR is advantageous in that it does not require gel electrophoresis for analysis – results as the name suggests can be analyzed in real-time (Yeni et al., 2014). Real-time quantitative PCR (qPCR) is a highly accurate and sensitive type of PCR that can be performed to quantitatively determine the phylogeny and function of genes in sample matrices (Smith and Osborn, 2009; Girones et al., 2010; Lin et al., 2011; Guy et al., 2014).

Real-time PCR is currently a key part of the screening process for pathogens in the food industry. One commercial method known as the BAX[®] System real-time PCR Assay is approved by the USDA-FSIS for STEC detection in the food industry (DuPoint, 2014). The BAX systems simplify a PCR assay by combining the necessary reagents into a single tablet (which is pre-packed into PCR tubes) to reduce the possibility of contamination and error during PCR preparation (Bailey, 1998; Shearer et al., 2001; Frausto et al., 2013).

Conclusions and Future Directions

As certain STEC serotypes emerged and were declared adulterants, there was an increase in detection methods. Determining the most appropriate assay will become difficult if additional serotypes are declared adulterants. It will be difficult to predict the most appropriate approaches in the future, and as more information on the characteristics of these serotypes is revealed, an increase in automated assays will likely be required. Additionally, procedures that concentrate large sample sizes (such as immunomagnetic separation) will more accurately determine the true bacterial population(s) in food matrices (Baker et al., 2015).

Although STEC serotypes may not proliferate uniformly in certain environments, implementing several enrichment protocols per sample would be a more time consuming and inconvenient exercise that will not likely be practiced by industry. However, if continued research proves the importance of several enrichment protocols, these practices may become commonplace for sample analyses. Revealing the physiological adaptation mechanisms of STEC has been shown to be a continuous and daunting task. As microorganisms continually change, researchers must optimize methods in accordance with regulations and ensure that they are adaptable to future regulations.

Whole genome sequencing of STEC has been performed as a means to investigate the different characteristics of STEC variants, and as whole genome sequencing continues to become more feasible, a large database will likely be available to determine why certain STEC serotypes are more prevalent in food products and associated with illness (Franz et al., 2014; Lindsey et al., 2014).

While molecular approaches have dramatically improved detection times and sensitivity, several issues remain. For example, attaining whole cells from samples would likely be useful for illness and outbreak investigations due to the fact that a pure culture is needed for serotyping

as well as for potential further characterization (Vallières et al., 2013). During outbreak investigations, stool samples acquired from ill persons could be lyophilized as whole cells following capture and separation with immunomagnetic beads, which would provide a stock of samples that could later be cultured and characterized to link illnesses. In the sense of having samples that could later be of use for serotyping, the use of immuno-based methods would be advantageous over cell destruction based methods such as molecular assays based on the extraction of DNA or RNA. For molecular techniques, DNA is generally extracted from cells for PCR analysis, and DNA samples are lyophilized and stored. These samples would be useful for additional analyses such as whole genome sequencing.

Stocks consisting exclusively of DNA may be suitable for whole genome sequencing, but preserving culture collections for future research may also be necessary, especially conventional systems that implement serotyping. The ability to metabolically characterize isolates could help better understand ecological niches, which may reveal why certain isolates suddenly become prevalent in certain environments. Maintaining culture collections provides the opportunity for subtyping in future laboratory procedures, which may help further characterize pathogens with various techniques. Additionally, as new detection and characterization platforms (advancements in whole genome sequencing technology) surface, being able to compare conventional assays with new technology may provide useful data to better understand and optimize the newly developed techniques. As always, confidently developing feasible and timely protocols will be beneficial to the food industry and public health.

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Table 1. Illness and Cost Estimates of *E. coli* and other common pathogens in the U. S. annually

| Pathogen | Illnesses [^] | Cost (millions)* |
|--|------------------------|------------------|
| Norovirus | 5,461,731 | 3,677 |
| <i>Salmonella</i> (nontyphoidal) | 1,027,561 | 11,391 |
| <i>Staphylococcus aureus</i> | 241,148 | 168 |
| STEC non-O157 | 112,752 | 154 |
| STEC O157 | 63,153 | 635 |
| Non shiga-toxin producing <i>E. coli</i> | 29,876 | 40 |

[^] Scallan et al., 2011

* Scharff, 2011, cost estimate model accounts for pain, suffering and disability

Table 2. Timeline of outbreaks, recalls, and government response with relation to STEC

| Year | Summary | Reference |
|------|---|------------------------------|
| 1977 | Cytotoxicity of <i>E. coli</i> is first observed | Konowalchuk and Speirs, 1977 |
| 1982 | Food-borne outbreaks led to the identification of <i>E. coli</i> O157:H7 | Riley et al., 1983 |
| 1993 | Washington state outbreak (including Idaho, Nevada, and California) | Bell et al., 1994 |
| 1994 | FSIS declares <i>E. coli</i> O157 to be an adulterant in raw non-intact beef | Schuller, 1998 |
| 1996 | <i>E. coli</i> O157:H7 outbreak in Sakai City, Japan - over 7,000 children linked to radish sprouts in school lunches | Sobel et al., 2002 |
| 1997 | Colorado recall of 25.6 million pounds. of frozen ground beef patties due to <i>E. coli</i> O157:H7 | CDC, 2007a |
| 2002 | Colorado recall of 18.6 million pounds of ground beef products due to <i>E. coli</i> O157:H7 | Vogt and Dippold, 2005 |
| 2006 | U. S. outbreak of <i>E. coli</i> O157:H7 linked to spinach | Grant et al., 2008 |
| 2011 | FSIS declares <i>E. coli</i> O26, O45, O103, O111, O121 & O145 to be adulterants in raw non-intact beef | FSIS, 2011 |

Table 3. Enrichment media and growth considerations

| Factor | Example | Reference |
|-------------------------|--|--|
| Temperature | 37 or 42°C | Guerini et al., 2006 |
| Sample | Type of carcass or product | Brehm-Stecher, Young et al., 2009 |
| Nutrient concentrations | Optimal for target microorganisms? | Greasham and Herber, 1997 |
| pH | 6.5-7.5 optimal for bacteria | Greasham and Herber, 1997; Grant, 2004 |
| Water | Type used for making media | Greasham and Herber, 1997 |
| Sterilization | No contamination | Greasham and Herber, 1997 |
| Labor | Difficulty/how time consuming? | Guerini et al., 2006 |
| Cost | | Guerini et al., 2006 |
| Volume | How much is efficient for growth? Static or Shaking | |
| Serotype | Variations among serotypes? | |

III. Chapter 2

Immuno-Based Detection of Shiga Toxin-Producing Pathogenic *Escherichia coli* in Food – A Review on Current Approaches and Potential Strategies for Optimization

Christopher A. Baker^a, Peter M. Rubinelli^a, Si Hong Park^a and Steven C. Ricke^{a*}

^a Center for Food Safety; Department of Food Science, 2650 N. Young Ave. University of Arkansas, Fayetteville, AR 72704

Baker CA, Rubinelli PR, Park SH, et al. (2015). Immuno-based detection of Shiga Toxin-Producing *Escherichia coli* in Food – A review on current approaches and potential strategies for optimization. Crit Rev Microbiol (Early Online, 1-20). DOI: 10.3109/1040841X.2015.1009824.

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Address for correspondence: Dr. Steven C. Ricke, Department of Food Science, University of Arkansas, 2650 N. Young Ave., Fayetteville, AR 72704-5690, USA. Tel: (479) 575-4678. Fax: (479) 575-6936. E-mail: sricke@uark.edu

Introduction

Although commensal *E. coli* are beneficial to fundamental biological research and are a part of the intestinal indigenous microflora population, certain serotypes of *E. coli* exist that are considered pathogenic and represent a high level risk to public health and the food industry. Any microorganism that can cause disease is defined as a pathogen. More than 400 serotypes of *E. coli* have been isolated, 100 of which have been associated with human illness thus far (Brenner et al., 2005). Pathogenic *E. coli* are divided into six pathotypes: enterotoxigenic, enteropathogenic, enterohemorrhagic, enteroaggregative, enteroinvasive, and diffuse-adhering *E. coli* (ETEC, EPEC, EHEC, EAEC, EIEC, DAEC, respectively) (Alteri and Mobley, 2012), and although there are similarities, each pathotype possesses unique pathogenic mechanisms (Nataro and Kaper, 1998). Enterohemorrhagic *E. coli* are especially problematic to human health, and these isolates exhibit unique virulence mechanisms. In 1977, cytotoxic effects from *E. coli* were first observed in a Vero cell (an African Green Monkey kidney cell line), and were described as verotoxigenic *E. coli* (VTEC) (Konowalchuk et al., 1977; Karmali et al., 1985). The VTEC strains are included in the EHEC pathotype and are better known as Shiga toxin (Stx)-producing *E. coli* (STEC).

The detection of STEC and Stx toxins in foods is important at the manufacturing, processing, and consumer settings. Immuno-based detection methods have been implemented for such purposes, and these non-molecular detection assays invoke a direct interaction(s) with intact STEC cells. This review has a particular emphasis on immunological approaches, and highlights potential future directions for developing detection systems and strategies for use in industrial settings. The objective of this review is to provide information on the immunological methods

that have emerged to as a means to detect and quantify pathogenic *E.coli* as well as concentrate and collect bacteria from food matrices.

Immuno-based detection assays

The immune response and the development of the immunoassay

Escherichia coli, like most bacteria and other organisms, possess structures on the outer surfaces that are well-defined antigens due to their ability to consistently trigger an immune response in the host. This response is characterized by specific host production of antibodies, which directly interact with these antigens in a consistent manner that lends to the ability to use this antigen-antibody matrix as a diagnostic tool.

Escherichia coli have many well defined antigens – the O antigen defines the serogroup, and the specific combination of O and H antigens classify the serotype (Edwards and Ewing, 1986; Stenutz et al., 2006; DebRoy et al., 2011). They possess an endotoxin known as the lipopolysaccharide (LPS) embedded in the outer membrane, which consists of three parts; the lipid A region, core region, and the O polysaccharide. The lipid A region, which is anchored in the hydrophobic portion of the Gram-negative bacterial outer membrane, contributes to the toxicity of the LPS, and is well conserved among Gram-negative bacteria (Stenutz et al., 2006; DebRoy et al., 2011). The core region, which consists of oligosaccharides, connects the lipid A region to the O polysaccharide, which is commonly referred to as the O antigen. *Escherichia coli* may also possess a flagellum or flagella, which is an outer membrane structure identified in serological reactions by the H antigen. While H antigen characterization is typically only necessary for outbreak investigation purposes, O antigen identification is the primary means of serotyping (Atkinson et al., 2012).

Antibody sources

General concepts

To understand the how antibodies interact with bacterial antigens, it is important to understand the function and sources of antibodies. Antibodies are a family of proteins of the adaptive immune system that defend the host by binding to antigens. Antibodies are produced by numerous unique B cells that have been stimulated by the antigen binding to the B cell's antigen-receptor to differentiate into antibody-producing cells (plasma cells) in the lymphoid organs (spleen, lymph nodes, among others). For instance, there are hundreds of millions of B cells in the human body, and each have a pre-determined specificity for a unique antigen that the immune system might potentially encounter (Greener, 2005). The antibody that is produced by the antigen-specific B cells is a soluble form of the B cell's antigen receptor.

All antibodies have a similar structure in common that determines the functionality of the antibody, and this portion of the antibody is known as the constant region. The variable regions, which contain the complementary determining sites for antibodies, are responsible for binding the respective antigens, and each antibody consists of two identical heavy chains and two light chains, thus providing at least two sites capable of binding an antigen (Abbas et al., 2012). Following exposure of an antigen to its specific B cell, clonal expansion occurs – B cells proliferate and differentiate into either effector cells (plasma cells) to combat the current antigen, or memory cells to address future antigen exposure. Affinity maturation occurs during this process - the genetic code for the variable region of the B cell rearranges, and antibodies that are unique for the antigen that originally contacted the B cell are subsequently produced (Abbas et al., 2012). The complementary determining regions of an antibody interact with an antigen at the

site known as an epitope, or determinant, which usually consist of 6 to 8 amino acids (Nelson et al., 2000). The strength at which a single antibody binds to an epitope is defined as the affinity, and the overall strength of the antibody-antigen interaction is defined as the avidity (Abbas et al., 2012).

Polyclonal antibodies

During an immune response, numerous clones of B cells with specificity for an epitope of a complex antigen will become activated. While each clone of B cells produces antibody with each cells' unique specificity, collectively the plasma cells originating from different B cell clones produce a heterogeneous mixture of antibodies, which are capable of binding several different antigen epitopes, and are known as polyclonal antibodies (Stills, 2012). Antibodies are typically generated by administering an antigen into an animal to produce an immune response, and rabbits are the most common animal model for polyclonal antibody production (Stills, 2012). The immunization process leads to the production of polyclonal antibodies, which respond to each of the specific different epitopes of a particular antigen.

Many antigens are too small to trigger an immune response alone, and require a carrier protein to which the antigen (hapten) is bound making the small antigen immunogenic. A hapten is composed of small functional groups (such as glucose) (Berzofsky and Berkower, 1999a) and any alteration in these small structures can potentially change the function of an epitope. To limit conformational changes of epitopes throughout the immunization process, microorganisms are typically purified or fixed with formaldehyde before being injected into a host. Formaldehyde reacts with the amino acids (primarily lysine) to form cross-links between proteins, and stabilize the epitope structure (Hopwood, 2002). Purification and fixation procedures ensure the antigen

retains its structural conformation throughout the immunization process, which increases the likelihood that B cells will only produce antibodies specific for the immunogen. If cells are not fixed prior to immunization, the protein or sugar structure of the antigen can change conformation while inside the host, and B cells that recognize these altered antigen structures will produce unwanted antibodies. Additionally, fixation ensures the following injections into a host help B cells eventually secrete more specific antibodies due to repeated exposure to an identical antigen, and fixed antigens can result in greater immunogenicity when compared to unfixed antigens, which are more susceptible to degradation (Van Epps, 2006).

The time at which the B cells are extracted from a host is critical – if not gathered during an active immune response to the administered antigen, there will likely be too many memory B cells of different specificities to sort through since there are millions of B cells (each with unique specificity) in a host at any given time (Zeliadt, 2014). Following the initial immunization, several booster injections can be administered, which is necessary to continue exposure of antigens to B cells. Once the antigen in a host is neutralized, a small number of activated B cells will differentiate into memory cells, and most will die by apoptosis (Schijns et al., 2008). Additionally, booster injections typically result in a higher concentration of antigen-specific antibodies. For example, Potter et al., (2004) observed a 45 fold increase in specific antibody titer following a booster vaccination 21 days after the initial immunization of *E. coli* O157:H7 type III secreted proteins in eight calves (Potter et al., 2004).

Polyclonal antibodies are often acquired from a host via blood sampling, which involves collection from the host every two weeks (Shimizu et al., 1988), and upon acquiring the blood, isolating antibodies is a relatively simple procedure. Blood can be stored at 4 °C to initiate clotting, and centrifugation will separate the clots from the serum that consists of the polyclonal

antibodies (Layton et al., 2011). Once the blood clots are removed, sodium azide can be used to preserve the sample, which can be stored at 4 °C until the antibodies are analyzed.

Chicken laying hen eggs have been utilized as an alternative method to recover polyclonal antibodies. Polyclonal antibodies are present in the yolk of hen eggs (commonly referred to as egg yolk antibodies (IgY)), and are a source of passive immunity to newly hatched chicks, which have an immune system that is initially incapable of fighting infection (Malkinson, 1965). By immunizing a hen with an antigen, high concentrations of egg yolk antibodies (IgY) (typically higher than in mammalian hosts) can be recovered from hen eggs on a daily basis during an elevated immune response that produces the antigen-specific polyclonal antibodies (Ricke et al., 1988; Ricke and Schaefer, 1990; Biswas et al., 2010; Herrera et al., 2013). Additionally, unlike mammalian antibodies, IgY does not inadvertently activate the mammalian complement system, which results in an undesired immune response (Akita and Nakai, 1993).

Limited work has been done with egg yolk antibodies produced against STEC, and most of this has focused on their use as a therapeutic agent in animals susceptible to STEC. Sunwoo et al., (2002) isolated egg yolk antibodies from chickens that were immunized with *E. coli* O157:H7 whole cells. *E. coli* O157:H7-specific egg yolk antibodies were sufficient in inhibiting *E. coli* O157:H7 growth at concentrations higher than 1.17 mg/mL, and following visual analyses of immunoelectron micrographs, these authors suggested the specific egg yolk antibodies were capable of binding to the bacterial surface of *E. coli* O157:H7, which altered the bacterial surface structure(s) and inhibited growth (Sunwoo et al., 2002; Schade et al., 2005).

In efforts to produce *E. coli* specific IgY antibodies as a food ingredient, Shimizu et al., (1988) injected formalin-fixed *E. coli* and an adjuvant into White Leghorn hens to elicit the production of anti-*E. coli* Immunoglobulin Y (IgY). An enzyme-linked immunosorbant assay

(ELISA) was used to determine IgY yield by adding the anti-*E. coli* IgY to microtiter wells containing predetermined concentrations of heat-killed *E. coli* lipopolysaccharide (Shimizu et al., 1988). Similarly, Akita and Nakai, (1993) compared four methods to purify anti-*E. coli* IgY from egg yolk, which typically contains a substantial amount of lipids. These authors experienced a higher yield, purity, and ease of use with a method known as the water dilution method when compared to three alternative methods involving polyethelene glycol, dextran sulphate, and xanthan gum (Akita and Nakai, 1993).

Bacterial and viral diseases in calves and piglets have been treated with IgY antibodies to neutralize and/or eradicate the bacterial or viral antigen(s) (Schade et al., 2005). Yokoyama et al., (1992) reduced diarrheal disease in neonatal piglets by orally administering spray-dried egg yolk antibodies (specific for ETEC). Following ELISA trials that revealed egg yolk specificity for each ETEC strain, a scanning electron microscope was used *in vitro* to observe the adhesion ability of these ETEC strains in the small intestine of piglets, with and without orally administered egg yolk antibodies. These authors observed successful adhesion of ETEC strains to the duodenal and ileal epithelial cells of untreated piglets, and due to the reduced adhesion of ETEC strains in treated piglets, suggested egg yolk antibodies exhibited specific anti-fimbrial mechanisms (Yokoyama et al., 1992).

Egg yolk antibodies involve relatively simple procedures, as well as the opportunity to frequently isolate high concentrations of antibodies (it has been suggested approximately 17 to 35 g of total IgY can be acquired from a single hen annually) (Schade et al., 2005). Also, hen eggs provide a feasible source of antibodies compared to procedures involving other animals (the cost to maintain hens is generally less when compared to rabbits) (Schade et al., 2005).

Monoclonal antibodies

While polyclonal antibodies are specific for multiple epitopes of an antigen, monoclonal antibodies have a unique specificity and have revolutionized antibody-based therapeutics, but production requires a more labor intensive and time-consuming *in vitro* process for development. The basis and methods of monoclonal antibody production have been extensively described elsewhere (Strockbine et al., 1985; Nelson et al., 2000; Skinner et al., 2013), and will only be briefly discussed here.

Once B cells are activated, they typically have a short life span following the neutralization of an antigen in a host. The fact that an activated B cell (plasma cell) secretes antibodies of a single specificity provides an opportunity to join a plasma cell with an immortal tissue cell culture, and create unique cells that become a large source of antigen-specific antibodies due to their continued proliferation. Köhler and Milstein, (1975) first described efforts to produce monoclonal antibodies by fusing plasma cells to myeloma tissue culture cells to produce antibody-producing permanent tissue culture cell lines (Köhler and Milstein, 1975).

The primary method of monoclonal antibody production is via hybridoma technology, which involves the production of an immortal plasma cell *in vitro* (Nelson et al., 2000; Byrne et al., 2009). Plasma cells from the bone marrow, primary lymph nodes, and spleen of an immunized host can be recovered and fused with immortal myeloma cells, but murine-derived splenocytes are used for the vast majority of hybridoma fusions. Hybridoma technology is used to develop immortalized plasma cells (hybridoma cells), each of which produce identical monoclonal antibodies of unique specificity for an epitope of an antigen (Köhler and Milstein, 1975; Leenaars et al., 1999; Byrne et al., 2009).

Polyethylene glycol is generally used as the hybridoma cell fusing agent, and causes extensive cross-linking between a myeloma and B cell (Clark, 1991). A common problem with hybridoma cell culture production is the presence of unfused myeloma cells that can ultimately outgrow successfully fused hybridoma cells. The fusion process as well as myeloma-cell elimination procedures that follow are based on nucleotide biosynthesis concepts that are characteristic of all cells. Nucleotide synthesis is essential for survival of cells, and cells produce nucleotides by either the *de novo* or the salvage pathway. Only cells that contain an enzyme known as hypoxanthine guanine phosphoribosyltransferase (HGPRT) can utilize the salvage pathway. To eventually eliminate unfused myeloma cells, HGPRT-deficient myeloma cells are used in the fusion process, and are limited to the *de novo* nucleotide biosynthesis pathway.

Following the fusion process, cell cultures are incubated in HAT selective medium, which ultimately eliminates any unfused myeloma cells (Nelson et al., 2000). The HAT selective medium contains hypoxanthine, aminopterin, and thymidine, and aminopterin blocks the *de novo* pathway, which is required for HGPRT negative cells, thus eliminating any myeloma cells that did not fuse with a B cell. Since hybridoma cells consist of B cells, these cells can utilize the salvage pathway for nucleotide synthesis and survive.

Once cells are grown in the HAT selective medium, each microtiter well containing antibodies that react with an administered purified antigen can be transferred to a separate microtiter plate. Cells are diluted throughout the microtiter plate with the intention to isolate one hybridoma cell per microtiter well, thus acquiring a source of antibodies with a unique specificity (Clark, 1991). This ensures that a particular population of antibodies are the source of a single hybridoma cell (Clark, 1991; Nelson et al., 2000; Huang et al., 2013; Zeliadt, 2014).

Current monoclonal antibody production methods are time consuming and complicated, and require screening procedures to obtain a culture containing a single hybridoma cell.

Alternative monoclonal antibody production methods are surfacing due to the fact that animal-derived antibodies can trigger an undesired immune response in humans. In a somewhat similar fashion to hybridoma technology, human memory B cells can be isolated from a purified blood sample and infected with Epstein-Barr virus to obtain immortal cell cultures that secrete an antibody of a desired specificity (Zeliadt, 2014).

Monoclonal antibodies are important in Western blots, which are commonly used for protein analyses. Once proteins are separated by gel electrophoresis, labeled monoclonal antibodies can be applied to detect various proteins, revealing the different types of proteins (antigens) from a sample. A primary motivation for monoclonal antibody production is due to the potential for therapeutic applications in humans and animals (Nelson et al., 2000; Greener, 2005; DeKosky et al., 2013). Monoclonal antibodies are a useful diagnostic tool, and can be used to measure hormone levels, as well as the presence of toxins in biological fluids (Berzofsky et al., 1999b). In addition to many other applications, monoclonal antibodies can be used to detect proteins and toxins in food systems.

He et al., (2013) recently developed five Stx2-specific monoclonal antibodies to detect the Stx2 toxin in milk with immuno-based detection assays. Following the production of the five Stx2-specific monoclonal antibodies, Western blots were used to determine toxin binding and specificity. These authors observed no cross reactivity with the Stx1 toxin, and each distinct monoclonal antibody displayed different levels of affinity for A and/or B subunits of the Stx2 toxin. Two of the produced monoclonal antibodies were specific for Stx2 subunits A and B and both exhibited lower disassociation constants than that of the three antibodies specific for only

subunit A (He et al., 2013). Each of the monoclonal antibodies (except Stx2-2) bound to all four of the Stx2 variants tested in the Stx2 variant binding experiments (He et al., 2013).

The type of antibody most appropriate for immunoassays is dependent on several factors such as the format of an assay in which the antibodies will be used, as well as the other advantages and limitations often times associated with monoclonal and/or polyclonal antibodies (Table 1). Although either monoclonal or polyclonal antibodies are most appropriate in certain circumstances, some immunoassays involve both types. The general concepts of these immunoassays will be discussed in the following sections.

Immunoassay

General concepts

Once the realization that antibodies could be used to discern certain antigens, it became intuitively apparent that antibody-antigen reactions could be used to classify, detect, and ultimately quantify microorganisms. Not surprisingly, numerous strategies were conceived and developed to detect this reaction as a function of an immunoassay. Numerous immunoassays have emerged over the years for targeting pathogens in clinical stool samples or industrial food products. In the food industry, immunoassays have been applied to detect and quantify numerous food components (proteins, enzymes, vitamins, among others) and contaminants (microorganisms, toxins, pesticides, hormones, among others) (Fukal and Káš, 1989).

Immunoassays can be designed for detection due to the understanding of the immunochemical antigen-antibody complex that is formed during an immunological reaction between the analyte (antigen) and the reagent (antibody) (Káš et al., 1986). An immunoassay can be used to assess the presence and/or concentration of an antigen. Early work focused on

visualization of the agglutination and subsequent visible clumping of extensive numbers of cells and antibodies that formed large lattice type structures, which could be viewed on a microscope and scored accordingly (Burnet, 1934; Pauling, 1940).

Further refinement occurred with the development of radial immunodiffusion assays that were first described in 1949 (Ouchterlony, 1949), and are commonly referred to as the double disk diffusion assay or Ouchterlony assays. Radial immunodiffusion assays were based on the principle that similarly-structured molecules will diffuse at similar rates through an agarose gel that allows free movement of the molecules (Lam and Mutharia, 1994). The double disk diffusion assay can be used to determine the lowest concentration of antigen needed for precipitation of an antibody-antigen complex; the lowest antigen concentration to form a precipitin line can be used in additional assays to conserve antigen stock(s).

In a follow-up to the early development research, Ouchterlony, (1968) published a methodology oriented handbook which describes the conventional, as well as alternative formats, techniques, and modifications that were available at that time to perform a double disk diffusion assay. As described at the time, the center portion of the agarose gel typically consisted of an antibody in a well (disk), and surrounding wells containing various antigens could be positioned of equal distance around the center antibody. Following diffusion of the antigen and antibodies through the agarose gel, a streak or band-like precipitate (precipitin line) would form between wells of the antibody and antigens, which indicated a corresponding binding of the antibody and antigen. Although the antibody was typically positioned in the center well to be surrounded by peripheral wells containing different types of antigens, the antibody and antigen arrangement was interchangeable, and a known antigen could be used to distinguish binding characteristics among antibodies.

The double disk diffusion assay has been used to identify various components associated with *E. coli* such as enzymes (Lee and Englesberg, 1962), surface proteins (Guinee et al., 1976; Isaacson, 1977), enterotoxins (Honda et al., 1981; Klipstein and Engert, 1985; Tsuji et al., 1985), and Stxs (Oku et al., 1989). Oku et al., (1989) used a double disk diffusion assay to differentiate a Stx2 variant. These authors purified a cytotoxin that was present in an *E. coli* isolate, which was obtained from a patient that had developed HUS. The toxin, along with purified Stx2, was analyzed in a double disk diffusion assay for reactivity against anti-Stx2, as well as against anti-purified toxin. These authors observed a spur formation in both Ouchterlony assays, which they interpreted as a partial identity between the two antigens analyzed.

Likewise, Klipstein and Engert (1985) compared the immunological similarities of *E. coli*, *Campylobacter*, and cholera enterotoxins with a double disk diffusion assay. Following the purification of the toxins, a B subunit antibody was arranged central to the circular placement of *E. coli*, *Campylobacter*, and cholera toxins in a gel, and partial identity between enterotoxins was determined upon observation of spur formations.

Fluorescent labeling of antibodies and bacterial cells

In addition to double disk diffusion assays, immunofluorescence techniques provide researchers with the ability to visibly detect antibody-antigen associations. Fluorescent molecules emit the light they absorb at a certain wavelength range that is at a greater wavelength than the wavelength that was originally absorbed, which can be analyzed in several ways. It is common to fluorescently label antibodies, which can be performed by covalently linking fluorescent molecules to antibodies. The presence of functional groups (carboxylic acids, aromatic or aliphatic amines, hydroxyl groups, among others) on the antibodies makes

fluorescent labeling possible, and often requires conjugation of fluorescent molecules with reactive intermediates (esters, acid anhydrides, diazonium salts, isothiocyanates, among others) to achieve the covalent interactions with antibodies (Hemmiliä, 1985). Upon incubation of these fluorescently labeled antibodies with samples potentially containing a target antigen, a researcher can visually determine antigen presence, whether it is with a light microscope or flow cytometry. Fluorescent antibody tests can detect microorganisms that cannot be grown on culture media, but does not distinguish between dead and living cells (Thomason et al., 1959).

However, there are several ways that fluorescence can be used to detect living cells, such as color formation due to the presence of substrate, as well as DNA labeling techniques. Microtiter plates can be used in prevalence assays to detect microorganisms that produce certain components, which react with a substrate to produce a visibly fluorescent reaction. A fluorogenic compound (4-methylumbelliferone glucuronide (MUG)) has been used to detect *E. coli* based on the research of Kilian and Bulow (1976) that approximately 97% of *E. coli* produce beta-glucuronidase, which cleaves MUG to produce a fluorogenic substrate visible under long-wave UV light (Kilian and Bulow, 1976; Feng and Hartman, 1982).

Also, labeling dyes have been used to stain cellular components such as DNA (Bernander et al., 1998), as well as antibodies specific for certain antigens. There are several ways to enumerate viable cells. Only viable cells possess a membrane potential, and a variety of dyes are available to determine cell viability based on this concept. The membrane of a viable cell will either take up or resist dyes depending on the structure of a dye.

Solid phase-based immunoassays

General concepts

Immunoassays progressed as it was realized that due to the hydrophobic nature of the interaction between an antibody and antigen, either component could be attached to hydrophobic surfaces to quantitate the particular antigen-antibody relationship. Antibodies (or antigens) could be bound to surfaces such as plastic as a preliminary step in several revolutionizing immunoassay formats that involved determining antigen or antibody presence and/or concentrations by way of antibody labeling.

Immunoassays that are formatted in microtiter plate wells (96 well plates are common) enabled the simultaneous screening of many samples, a capability that was not available in earlier developed immunoassays. In addition to determining the presence of antigen(s) in many samples, quantifying antigen(s) in samples became possible by developing a standard curve in which predetermined concentrations of an antigen were applied to microtiter plates. By creating a standard curve, the concentration of samples could be used to approximate the quantity of an antigen in a sample. Once the ability to bind antibodies to solid phase surfaces was developed, it was realized that such antibodies could be quantified if some sort of label was attached to the antibody that could be detected by external instrumentation. The following sections discuss these approaches.

Radioimmunoassay

The first immunoassay to incorporate labels was developed in 1960 in the form of the radioimmunoassay (Yalow and Berson, 1960; Lequin, 2005), and the labels are most commonly the radioisotopes ^{125}I and ^3H (Greenberg et al., 1977; Frantz and Robertson, 1981; Soergel et al., 1982; De Boever et al., 1983). Once antibodies were labeled and the unbound radioactivity was removed, analysis of the antigen-antibody reaction(s) could be performed with a liquid

scintillator counter, which ultimately quantified the particles that were emitted by radioactive decay (Chase, 1980; Yalow, 1980).

Several concerns were associated with the radioimmunoassay – labeled reagents were subject to radiolysis, the radioisotopes possessed a short half life, proper radioactive waste disposal was an issue, and the possibility of radioactive exposure was less than ideal (De Boever et al., 1983). Shortly after the development of the radioimmunoassay, there was a growing focus on the potential use of nonradioactive labels such as enzyme-based chromophore systems as a means to indirectly generate a detectable label in an immunoassay reaction (Lequin, 2005).

Enzyme-linked immunosorbant assay

In 1971, both Engvall and Perlmann, (1971) and Van Weeman and Schuurs, (1971) successfully implemented an enzyme label for detection purposes, and this ultimately led to a variety of enzyme-associated immunoassay formats (Lequin, 2005). A primary motivation for the development of the ELISA was to improve detection methods in a clinical and public health setting that at the time used cell culture or radioimmunoassays (Downes et al., 1989). In addition to being able to design an ELISA for various antigens, various formats evolved that could be employed for antigen detection and quantification.

One of the most common immunoassay formats that emerged was the enzyme-linked immunosorbant assay (ELISA) (Fukal and Káš, 1989). The ELISA is a multistep process commonly used to determine the amount of an antigen in samples (McCarthy et al., 2001). In a sandwich ELISA, capture antibodies are bound to a solid surface (typically a 96-well microtiter plate) followed by a wash step to remove unbound antibodies. Sample dilutions containing the putative antigen are subsequently added to interact with the plate-bound capture antibodies.

Following incubation, a wash step is performed to remove unbound antigen. Lastly, a detection antibody is added to interact with an available epitope of the bound antigen. It is common practice for the detection antibody to be bound to an enzyme. Van Weeman et al., (1971) successfully conjugated the enzyme horseradish peroxidase (HRP) to the human chorionic gonadotropin (HGC) antigen (Van Weemen and Schuurs, 1971). The HRP enzyme is still commonly used in ELISA's, and is popular in part due to its high turnover number and its small molecular size (Gosling, 1990). Alkaline phosphatase, and beta-D-galactosidase are also common labeling enzymes that can be used in ELISA assays, and numerous other available immunoassay labels have been reviewed extensively elsewhere (Gosling, 1990; Kricka, 1994). Following incubation and a wash step to remove unbound antibody-enzyme complexes, an enzyme-specific chromogenic soluble substrate is applied. For example, 4-nitrophenyl phosphate can be used as a substrate to conjugate alkaline phosphatase. This enzyme-substrate hydrolysis reaction results in a color change (peak absorbance of 400 nm) of the solution (Voller et al., 1976), and is in direct correlation with antigen presence (Hegde et al., 2012a).

Although ELISA's have been implemented to detect certain STEC serogroups, it became common to design an assay to detect Stx as a screen for all STEC (Kehl et al., 1997). Skinner et al., (2013) developed a sandwich assay for an *E. coli* Stx2 subtype known as Stx2f (Skinner et al., 2013). These authors noted that very few assays were capable of detecting the Stx2 subtype Stx2f toxin protein, which is one of the seven known Stx2 subtypes (Skinner et al., 2013). The Stx2f toxin is not known to be associated with serious human disease (Melton-Celsa, 2014), but the assay could be useful to indicate the presence of uncharacteristic phages and plasmids in isolates of *E. coli* (Skinner et al., 2013). Before an immunoassay for the Stx2f toxin was developed, monoclonal antibodies were generated by immunizing mice with a Stx2f A subunit,

and the splenocytes from the mice were fused with myeloma cells. Following fusion of hybridoma cells and screening antibodies for reactivity with Stx2f, four Stx2f specific antibodies (designated Stx2f-1, Stx2f-2, Stx2f-3, and Stx2f-4) were found to be specific for the Stx2f toxin. The optimal sandwich ELISA format was determined to be with Stx2f-1 as the capture antibody, and Stx2f-4 as the detection antibody, which exhibited a detection limit of 0.123 ng/mL of purified toxin. These authors suggest that although catch-all assays are useful for monitoring food systems, subtype-specific assays would be valuable for tracking Stx subtypes and *stx* encoding phages among microorganisms (Skinner et al., 2013).

The Premier EHEC Assay (Meridian Diagnostics Inc., Cincinnati, Ohio) is a commercially available alternative to conventional culture that has been promoted for detecting the presence of Stxs in samples. In 1997, the Premier EHEC Assay was used to screen for the presence of Stx(s) in 974 stool specimens in an ELISA format (Kehl et al., 1997). Stool specimens were enriched overnight, and 50 μ L of the overnight cultures were added to wells containing 200 μ L of diluent for enzyme immunoassay testing. Following the reaction of stool specimen culture and diluent, samples were considered positive if the optical density (450 nm) was equal to or greater than 0.150. In comparison to conventional culture, the Premier EHEC Assay was determined to be more sensitive (100%) than conventional culture on sorbitol-MacConkey agar (60 %). This sensitivity was calculated by dividing the number of samples detected as positive by the total number of samples that contained Stx (Kehl et al., 1997).

Downes et al., (1989) developed an ELISA to distinguish between Stx1 and Stx2 for evaluating culture and stool specimens. First, monoclonal antibodies (each specific for Stx1 and Stx2 toxin proteins) were bound to microtiter plate wells as the primary antibody to capture Stx1 and/or Stx2. Next, a blocking step was performed to remove unbound monoclonal antibodies,

and culture or stool samples were distributed among wells to interact with the monoclonal antibodies for a one-hour incubation period. A wash step was performed to ensure that unbound Stx(s) were removed from the wells (ideally, only bound antigen would interact with a secondary antibody as unbound antigen(s) could compete with the administered secondary antibody if it were removed by washing). Following the washing step, two types of polyclonal antibodies (each specific for Stx1 or Stx2) were added to the corresponding wells to bind either Stx1 or Stx2. An additional washing step was performed to remove unbound polyclonal antibodies, and alkaline phosphatase-labeled anti-rabbit antiserum was added to each well, followed by a washing step. After an incubation period to allow an enzyme-substrate interaction, a stop solution was added to inhibit additional color development, and the optical density was measured to determine the presence of Stx1 and Stx2. Both ELISA's exhibited 100% specificity, and it was suggested that the detection limit for the Stx1 ELISA was 200 pg of purified Stx1, and the 75 pg of purified Stx2 for the Stx2 ELISA. These authors noted that the Stx2 ELISA was the first to specifically target Stx2, and that detecting both Stx1 and Stx2 was considered necessary to confirm the presence of STEC (Downes et al., 1989).

A more recent study suggests certain types of Stx proteins may be undetectable with ELISA's that were designed to specifically target Stxs (Willford et al., 2009). The performances of three commercially available ELISA kits (Premier EHEC test (Meridian Bioscience Inc., Cincinnati, OH), the ProSpecT Shiga toxin *E. coli* (STEC) Microplate Assay (Remel Inc., Lenexa, KS), and the Ridascreen Verotoxin Enzyme Immunoassay (r-Biopharm AG, Darmstadt, Germany) were evaluated for the ability to detect Stx variants (Willford et al., 2009). Each ELISA kit was conducted as a sandwich format, and positive results were recorded by visual observation or by an optical density measurement. The type of Stx(s) in each sample was

determined by PCR. The detection limit of the Premier EHEC test and the Ridascreen Verotoxin Enzyme Immunoassay was established as 10^6 CFU/mL, and 10^7 CFU/mL for the Ridascreen Verotoxin Enzyme Immunoassay, respectively. Two Stx variants that were PCR-confirmed were shown to be undetectable by all three evaluated ELISA kits. The authors suggested the STEC *stx* genes might not have been detected due to mutations within the opening reading frames of these genes, which would result in a non-functional gene protein product that was structurally altered enough to no longer be recognized by the antibody generated against the original toxin protein structure (Willford et al., 2009).

There are other instances that also suggest an ELISA assay may be inefficient in detecting all STEC – in 2013, of the 632 stool specimens that were tested for the presence of STEC, twenty one samples were positive. While all of the twenty one positive stool samples were detected by PCR, only six were detected by ELISA (Vallières et al., 2013). The results indicated that the presence of STEC by PCR, that resulted in false negatives with ELISA, were likely due to the fact that PCR has shown to exhibit a sensitivity that is 10^4 CFU/mL greater than ELISA based assays (Vallières et al., 2013).

To improve ELISA techniques, modifications and variations have been developed. For example, Galikowska et al., (2011) utilized bacteriophages to serve as the initial capture components for various strains of *Salmonella enterica* and *E. coli* in a variation of an ELISA format (Galikowska et al., 2011). The ELISA sandwich format was performed as usual, except that instead of initially coating wells with capture antibodies; phages (10^8 /well) were adsorbed to the polystyrene surface of ELISA plate wells. Following the conventional incubation, blocking, and washing steps with the subsequent addition of antigen, capture antibody, and labeled enzyme, the sensitivity of the bacteriophage-based ELISA assay was determined to be 10^6

cells/mL, which is similar to other ELISA assays that involve coating primary antibodies to a solid phase (Galikowska et al., 2011).

Bacteriophages are attractive capture components due to the multivalent interaction(s) with host cells, and Petrenko and Vodyanoy, (2003) suggested that there are thousands of binding sites/particles on the bacteria that are associated with a phage (Petrenko and Vodyanoy, 2003). Bacteriophages can be implemented in various detection assays, and a wide range of potential applications have been reviewed (Singh et al., 2013; Arugula and Simonian, 2014; Schmelcher and Loessner, 2014).

Regardless of the capturing components utilized in an ELISA, this may not be the best available method for determining the presence of STEC. Also, the ELISA is a laborious technique (requires several wash and incubation steps), takes several hours (Teel et al., 2007), and is not likely to be implemented for pathogen detection in the food industry, especially as a stand-alone assay.

Immunoassays that can be analyzed visually are also available, and since the assays are generally easy to perform, they are useful for clinical laboratories to determine further detection approaches and treatment. Additionally, immunomagnetic separation beads can be used on the front end of an assay to extract microorganisms from samples, which helps reduce the sample volume and concentrate target cells. Immunoassays that can help visually identify, as well as concentrate target cells will be discussed in later sections of this review.

Additionally, lateral flow (dipstick) immunoassays are simple and cost effective immunoassays that are available for prompt analysis of a sample for Stx(s), as well as STEC(s) (Aldus et al., 2003; Willford et al., 2009; Noguera et al., 2010), and these have been extensively reviewed (Posthuma-Trumpie et al., 2009; Sajid et al., 2014; Zhu et al., 2014).

In addition to being at the forefront for implementation in food systems, immunoassays and immuno-based sensors are also being investigated for biomedical applications (Wu et al., 2007). Certainly, immuno-based sensors have the potential to be incorporated in food detection systems. These techniques have been discussed and described extensively (Ricci et al., 2007; Yang and Bashir, 2008; Tokarsky and Marshall, 2008; Clotilde et al., 2011; Zhu et al., 2014) and therefore will not be discussed further in this review.

Latex agglutination

Antibody specificity (Hegde et al., 2012b), as well as the presence of pathogens in mixed microbial populations can be determined by coating latex particles with target antibodies in a process known as latex agglutination, which is the most common clinical laboratory method used for O antigen identification (Atkinson et al., 2012). Latex-bound antibodies form a complex with any antigen present in a sample to form visually identifiable precipitates (De Boer and Heuvelink, 2000).

A microtiter plate format has been utilized with a latex agglutination assay to detect the Stx1 and Stx2 toxin proteins (Karmali and Petric, 1999). Briefly, polyclonal antibodies (specific for Stx1 and Stx2) were bound to latex particles, and applied to wells containing human isolates that potentially contained STEC. Following a 24-hour incubation period, Stx presence and the antibody titer was determined (Karmali and Petric, 1999). Titer is often determined by observing the most dilute sample that results in a positive reaction. Such an assay could provide useful for epidemiological (Karmali and Petric, 1999), as well as clinical information due to the fact that Stx2 has been shown to be more virulent than Stx1 (Tesh et al., 1993; Vallières et al., 2013).

Determining the type of Stx isolated from a patient may lead to a different treatment (possibly closer monitoring for HUS for patients with Stx2-containing stool).

Additionally, a method known as the Optical Immunoassay (OIS) is available for visual identification of microorganisms. The BioStar OIA SHIGATOX kit (Inverness Medical Professional Diagnostics, Inc.) can be used to visually identify the presence of Stx1 and Stx2, but this method does not distinguish between the two toxins (Teel et al., 2007). A mixture of anti-Stx antibodies (specific for either Stx1 or Stx2) is coated to a silicon wafer. After addition of a sample, secondary anti-Stx1 and anti-Stx2 antibodies are added to the sample. The light reflection from the silicon wafer typically appears gold, but in samples containing Stx, the thickness of the wafer increases, which causes an altered light reflection that appears purple (Teel et al., 2007). The OIS can be analyzed in less than 20 minutes, and can be visually determined positive by observing color development. The performance of the OIA SHIGATOX assay was similar to that of the Premier EHEC assay when screening frozen and broth-enriched samples. However, the sensitivity of the OIA SHIGATOX test (96.8%) was superior to the Premier EHEC assay (83.9%) when screening fresh direct stool samples (Teel et al., 2007).

Multifunctional immunoassays for separation and detection

General concepts and the issue of sampling

Knowing the true population or presence of a cell type is difficult due to the fact that the sampling strategy presents a problem for pathogen detection. In general, only a small portion of a product is sampled for microorganisms, and conclusions about the total product have to be made based on results from this small sample size. Techniques that condense a cell population from a sample enable the possibility that a larger sample per product can be tested to achieve a better

cell population estimate. Filtration and centrifugation procedures are common approaches that can be used to concentrate cells in a large sample volume, while also removing unwanted debris and components in food and environmental samples. Although optimizing cell concentration methods seems to be appropriate, one must also consider that food producers will not likely test larger sample quantities of each product to circumvent product loss due to sampling. Aside from such considerations, methods that are proven to condense or concentrate cell populations in samples will aid the sampling process – concentrating cells will result in a reduced volume size that is generally easier to work with during sampling procedures.

Designing a representative collection protocol is mostly a statistical exercise based on estimating the likelihood of isolating the pathogen within the logistical constraints of sampling. However, developing high-throughput sampling systems that can accurately reflect the desired statistical goal is a technological challenge. Certainly a number of molecular methods can be used for the final detection steps, but the true challenge is collecting enough representative samples to have confidence in the outcome.

The practice of quantifying antigens with immunoassays such as ELISA is useful for many applications, but the availability of antibodies and their well characterized affinity properties have led to their use not just for detection, but initial capture from sample matrices. This concept has been employed as the basis for emergence of an entire line of technological developments for utilizing antibodies to capture or sequester target antigens from complex matrices. This has in turn led to new approaches for sampling.

This has fostered advancements in antibody construction to generate antibody complexes with iron for ease of separation due to magnetic employment separation. Immunomagnetic separation can be used to limit the duration of enrichment, concentrate low cell populations

dispersed in a large sample volume, and ultimately reduce a sample volume to a more workable form that can be analyzed employing advanced detection systems. More complex approaches have ushered in an array of specialized cell separation equipment that does not rely on magnetic properties of specifically constructed antibody complexes, but are capable of recognizing individual antibody-antigen complexes. These are discussed in the following sections.

Magnetic immuno-beads

Immunomagnetic separation is used to recover target microorganisms from samples by applying magnetic beads bound to antibodies (Veal et al., 2000). Samples are mixed with antibody-bound magnetic beads for a short period of time (approximately 10 minutes). A separator that produces a magnetic field is used to recover the bacteria that are bound to the antibody-bead complex (Auvray et al., 2007). Recovered beads are washed, enriched, and spread on plates for follow-up incubation and eventual enumeration (Seo et al., 1998; Varela-Hernández et al., 2007; Çadırcı et al., 2010). Immunomagnetic separation is gaining considerable attention as a necessary step in the pathogen detection process (Potter, 2014), and as a result, automated IMS methods have been developed (Chen et al., 2014).

A potential application for the food industry would be to apply a capture antibody or antibodies that binds all *E. coli* in a sample. Such a step would reduce sample volume as well as background noise associated with food matrices. Following separation, these cells could be further analyzed with an instrument capable of measuring individual cells. A capture antibody would need to target live and intact whole cells given that only live cells will lead to human illness. Whole cell detection would be applicable by targeting an exposed target such as a universally expressed surface protein. Consequently, antibodies would only bind to cells with an

intact membrane, which is more likely representative of living cells (Riedy et al., 1991). This would also eliminate false positives that can occur with the inclusion of intracellular detection targets. Intracellular components could still be bound once a cell is lysed, which would not be the case for an epitope present on an intact membrane. Finding an extracellular commonality of all *E. coli* would be considered the first step in the development of an antibody that could bind to all *E. coli*. The efficiency of such an assay would depend on the consistent accessibility of the universal epitope, continuous expression of the protein under all growth conditions, along with the antibody sensitivity and specificity to the epitope. This would likely involve analysis of binding efficiencies for each type of samples (different food matrices) in which the assay would be applied.

Flow cytometry

Basic concepts

Flow cytometry has been applied to a wide range of scientific fields since the idea was first described in 1934 (Moldavan, 1934), and there are a variety of flow cytometers currently available (Table 2). A flow cytometer is used to analyze a population of cells by determining the percentage of cells in a population that are of a certain size, composition, or that express a particular antigen of interest. In addition to identifying cells of certain types, flow cytometers can be programmed to separate cells that exhibit distinct characteristics. It is common to fluorescently label antibodies that are specific for an antigen/surface protein of interest, and such approaches for labeling were discussed in a previous section. The first successful cell separation technique was performed in 1965 (Fulwyler, 1965) when multiple scientific disciplines were combined to adapt the droplet sorting technology used in ink jet printers (Davey and Kell, 1996).

This technique was made possible due to the ability of the cell sorter to recognize and sort target cells based on similar features such as size, density, membrane potential, adherence ability, and fluorescence (Herzenberg et al., 1976). The technology was essentially based on droplets of cells becoming electrostatically charged after being passed through two high voltage plates, and cells with different characteristics subsequently being deflected into their respective receptacles (Holmes et al., 2014).

Herzenberg and Sweet, (1976) first described fluorescence-activated cell sorting (FACS) nearly 40 years ago where they discussed the advances such a tool would bring to molecular biology due to the ability to sort cells by phenotypic similarities. Employment of a FACS approach allows for retrieval of homologous microbial cell populations from samples containing heterogeneous organisms. If fluorescently labeled antibodies specific for an antigen were applied to a sample, in theory, any cells containing the target antigen could be detected in the sample, and subsequently separated into a uniform population of cells. FACS can be a useful tool for obtaining homogeneous populations when the objective of studying a particular organism and/or phenotype is desired.

Instrumentation

Flow cytometers rapidly analyze individual cells (i.e. particles in a suspension), one cell at a time, and samples can be analyzed within seconds. The degree at which light interacts with these particles reveals detailed output on the population of cells in a suspension. Cells are passed by a laser in single-file fashion and are intersected by a laser for a period of approximately 0.2 to 4 μ s. Individual cells scatter light depending on size, internal complexity, and fluorescence emission. Lenses collect and focus the light on to light detectors; diffracted light in the same

direction as the laser (forward scatter) represents cell size, and refracted and reflected light at a 90° angle (side scatter) represents internal complexity (Bernander et al., 1998; Givan, 2004). An event is considered any type of an observed particle (Wood, 2001; Givan, 2004). There is a relationship between flow rate and the probability that more than one particle is identified as an event, and with more concentrated samples there is a greater likelihood that events will be missed and therefore underestimated (Givan, 2004). The process of acquiring the proper events for analysis is known as gating, and a detailed description of this complex process is available (Wood, 2001). Proper gating assures that formulated data from a sample are a product of viable target organism cells (Herzenberg et al., 1976). Since events that are not representative of a target organism will likely be counted, gates can be set for multiple factors such as forward versus side scatter and green versus red fluorescence. Gates consist of thresholds that although detected, will exclude the irrelevant events, and the events inside the gate will only be considered in analyses. Although it may take several trials to determine the proper gating parameters for a particular protocol, once formulated, these gates can assure final analyses will account for only the target organism.

Along with analyzing forward and side scatter parameters, multi-dimensional flow cytometers can analyze parameters with the use of fluorescent labels that emit light when exposed to certain wavelengths. The laser most commonly used in flow cytometers is the 488 nm argon laser (Givan, 2004), and more than one laser is available in advanced flow cytometers for multifluorescent analysis (Herzenberg et al., 2002). The contrasting fluorescence emitted by fluorochromes enables characterization of multiple cellular components (parameters) within an analysis (Givan, 2004). Bacteria cannot always be identified with forward and side scatter parameters due to their small size (Bernander et al., 1998), and fluorescently-labeled antibodies

represent a more reliable approach (Wood, 2001). Technical improvements are still being made for prokaryote research with fluorescently labeled antibodies and nucleic acid binding dyes (Wood, 2001; Müller and Nebe-von-Caron, 2010).

Fluorescent sources

Propidium iodide is a common dye used to stain dead bacterial cells (Subires et al., 2014), and Syto 9 can be applied to label viable bacteria prior to analysis in a flow cytometer (Duncan et al., 2006). In theory, cells with a compromised plasma membrane will take up propidium iodide, and be identifiable in a flow cytometer as dead cells (Díaz et al., 2010). By labeling the dead cells with propidium iodide, the number of viable cells can be calculated by subtracting the dead cell events (outside a predetermined gating parameter) by the total events. Although propidium iodide can give indication of cell viability, it is often applied in conjunction with other labeling dyes, and there have been instances of live cells being mislabeled (Díaz et al., 2010; Buzatu et al., 2013). Syto 9 dye has been used with propidium iodide, and Syto 9 labels all the cells in a population as green, while cells consisting of Syto 9 and propidium iodide emit a red fluorescence (Rudi et al., 2005).

Other methods of cell labeling methods are available. For example, apple juice, milk, and food samples containing non Stx-producing *E. coli* O157 and *E. coli* K-12 have been analyzed with an EPICS ELITE flow cytometer by double-staining cells with 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) and fluorescein isothiocyanate (FITC). A CTC approach enables analysis of the respiratory activity in bacteria, and FITC is a fluorochrome used for antibody labeling. CTC and the FITC-labeled antibody are detectable due to the characteristic red and green fluorescent signals emitted by each component, respectively. To compare the efficiency of

the method, 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) was utilized to counterstain CTC and FITC stained samples, which were enumerated with an epifluorescent microscope (Yamaguchi et al., 2003). These authors reported that the optimal conditions for CTC staining was a 30 minute sample incubation at 37 °C in a CTC concentration of 2 mM. The efficiency and application of similar dyes have been reviewed previously (Müller and Nebe-von-Caron, 2010; Elizaquível et al., 2014).

Clinical and industrial flow cytometry applications

The breadth of research with flow cytometers is extensive; research includes the study of eukaryotes, drug development, enumerating bacteria (Clarke and Pinder, 1998; Comas-Riu and Rius, 2009), determining cell size (Skarstad et al., 1983), shape, density, and cell cycle kinetics (Steen et al., 1982) among others (Jaroszeski and Radcliff, 1999; Müller and Nebe-von-Caron, 2010). Flow cytometry has also created an avenue for characterizing the morphology of cells, a practice extremely difficult to do microscopically. More recently, the effect of certain enzymes on the morphology of *E. coli* cells has been investigated (Meberg et al., 2004; Laubacher et al., 2013).

Flow cytometry has also been used to determine the effect of diet on host susceptibility to Stxs by detecting the presence of Stx receptors (Gb3) on the surface of human colonic epithelial cells (Zumbrun et al., 2013). Human colonic epithelial cells that were exposed to sodium butyrate (characteristic of a high-fiber diet (HFD)) exhibited a ten-fold increase in Gb3 levels compared to cells with no treatment. Follow up experimentation included comparing the number of Gb3 receptors in HFD mice to low fiber diet (LFD) mice, which revealed that the HFD mice possessed significantly more Gb3 receptors. Lastly, HFD and LFD mice were infected with *E.*

coli O157 and HFD mice exhibited a greater level of infection as well as lesions (Zumbrun et al., 2013). This work demonstrates that flow cytometry could be used to provide strong support for results that were acquired with different methods such as immunofluorescence tissue and cell staining, cytotoxicity assays, and gas chromatography due to the ability of analysis at the cellular level with flow cytometry (Zumbrun et al., 2013).

Hegde et al., (2012b) determined the required duration time for enrichment (6, 8, or 12 hours) to detect STEC in artificially spiked ground beef samples via flow cytometry. Ground beef samples were spiked with each of the “big six” STEC serogroups, enriched, and analyzed by both aerobic plate enumeration and flow cytometry. Prior to analysis of the spiked samples with a flow cytometer, polyclonal antibodies were raised in rabbits against heat-killed whole cells for each of the “big six” STEC serotypes. These polyclonal antibodies were subsequently fluorescently labeled with the Alexa Fluor 488 label from the Zenon rabbit IgG labeling kit (Molecular Probes), and mixed with 200 μ L of bacterial cells, and incubated for 1 h at room temperature to allow the fluorescently-labeled antibodies to bind the respective STEC cells. Following 3 washes with phosphate buffer saline, samples were analyzed in a Beckman Coulter FC500 flow cytometer (Hegde et al., 2012b). These authors concluded that the flow cytometry assays could detect spiked samples after 8 h of enrichment, except for serotypes O45 and O103, which could be detected after 6 h of enrichment due to an accelerated growth rate. The relationship between the percentage of detected cells via flow cytometer and cell number based on aerobic plate count exhibited a regression coefficient of 0.9809, and these authors reported that the detection limit of the flow cytometry protocol ranged from 1 to 10 CFU (Hegde et al., 2012b).

Wilkes et al., (2012) and Buzatu et al., (2013) utilized the recently developed RAPID-B model 9013 flow cytometer (LITMUS RAPID-B, LLC, Little Rock, AR) flow cytometer to detect *E. coli* O157:H7 in several food matrices. Wilkes et al., (2012) determined the detection efficiency and levels of background noise associated with fifteen food systems, and Buzatu et al., (2013) investigated ways to limit the autofluorescence caused by food particles in fresh spinach. These authors optimized RAPID-B protocols for various food systems to meet the goal of attaining sample results within an 8 hour production shift (Wilkes et al., 2012; Buzatu et al., 2013). Wilkes et al., (2012) investigated several options for background noise reduction; optimizing growth conditions for *E. coli* O157:H7, filtration, centrifugation, oxygen bleaching, and gating optimization (Wilkes et al., 2012). These authors determined the most appropriate background noise reduction procedures for each food sample. For example, 500 000 false positive counts were observed in ground beef samples prior to background noise reduction procedures. However, following two cycles of centrifugation, decanting the supernatant, suspension with PBS, and diluting with PBS, only one false positive count was observed. In addition to reducing background noise, high speed centrifugation and subsequent sample dilutions enabled target bacteria to become more concentrated, and increased the sensitivity of the protocol (Wilkes et al., 2012).

Buzatu et al., (2013) determined that phyloxine B was a sufficient photobleaching agent to reduce background noise associated with spinach, and that the limit-of-detection for the optimized RAPID-B assay to detect *E. coli* O157:H7 in spinach was 1 viable cell per 25 g of raw spinach (Buzatu et al., 2013). More recently, Buzatu et al., (2014) further optimized a pathogen-specific detection system with the RAPID-B model 9013 flow cytometer (Vivione Biosciences, LLC, Pine Bluff, AR). With this assay, live and dead cells can be enumerated, and 15 to 20

samples could be analyzed per hour (Buzatu et al., 2014). These authors compared the cell counts of *E. coli* O157 between conventional plate counts and RAPID-B flow cytometry analysis. Subsamples were analyzed in duplicate for each method, and results indicated 10 to 50 percent greater counts were observed with the RAPID-B assay compared to plate count methods. In addition to greater cell recovery, these authors emphasized that the RAPID-B system could be performed over an 8 hour time period, while the conventional plating method would have required a time span of 48 to 56 hours (Buzatu et al., 2014).

Comparison between flow cytometers can be difficult due to differences in configuration, operating conditions, lasers, and setting variations (Bernander et al., 1998). The lack of standardization is problematic, and until a system is validated, there will be a need to optimize research protocols in accordance with the flow cytometer system, antibodies, and labeling reagents on hand to target microorganisms. Moreover, performance tests to optimize flow cytometry protocols need to be conducted by inoculating foods such as ground beef with known quantities of STEC versus natural contamination. To further support bacterial counts as determined by flow cytometry, additional procedures to compare bacterial counts are often times appropriate. For example, in addition to analysis of samples via flow cytometry, a fluorescent microscope can be used to visually analyze bacterial populations. Fluorescently labeled polystyrene beads can be applied to samples to interact with target cells. Once target cells are bound, these cells can then be enumerated in a hemacytometer via fluorescence microscopy. Upon enumeration of target cell populations in samples by flow cytometry, the ratio of flow cytometer counts to fluorescent microscopy counts can subsequently be calculated to determine a regression coefficient among cell populations, and provide a more supportive enumeration of cells (Tortorello et al., 1997).

The need for low cell detection and high sensitivity in STEC protocols makes reduction of background noise an important hurdle to address. Before analyzing samples in a flow cytometer, samples are typically filtered to remove as much debris as possible (Hegde et al., 2012a, 2012b; Wilkes et al., 2012) due to the possibility of large particles clogging a flow cytometer. Background noise can result in a dead time due to excessive and undesired signals, and methods to reduce background noise have been reviewed (Stevens and Jaykus, 2004). Additionally, problems can arise when analyzing plant-based samples in conjunction with markers that emit green fluorescence when excited by lasers. These foods contain components capable of fluorescently emitting light and reducing detection sensitivity. Sodium bicarbonate has been applied to limit background noise from light emitting molecules (Buzatu et al., 2013), but its corresponding use is application dependent. Sodium bicarbonate can compromise cell membranes, making propidium iodide no longer applicable for distinguishing viable from nonviable cells (Wilkes et al., 2012). Storage time has proven to influence the detection ability with flow cytometry, especially of injured cells (Subires et al., 2014).

If flow cytometry protocols that are designed for STEC become reliable and validated, a flow cytometer could become a staple for quality and assurance in the food industry. An individual sample can be analyzed within seconds, which suggests that flow cytometry could be an instrument for analyzing large numbers of samples in a work shift following sample preparation (incubation, washing, target labeling, among others). Also, once a protocol becomes repeatable, and meets regulatory standards, there is potential for automation of the instrumentation, which creates an avenue for even more high-throughput technology. Since there are potentially several benefits including cost savings in personnel time if flow cytometers are to be more routinely employed in commercial settings such as food testing laboratories, it will be

necessary to address issues that can arise due to pathogen adaptation(s), as well as how to promptly detect emerging pathogens, which will be discussed in the following sections.

Future directions and developments for immunoassays and *E. coli*

General issues

Considering the reactionary-based history of the regulatory adaptation processes for food testing procedures in the USA, it is likely that as new serovars continue to emerge and once again ignite public health concerns, this will in turn lead to a longer and longer list of serovars that eventually become defined as adulterants. If such instances reoccur, more readily adaptable assays that can be implemented for new serovars will become critical, and understanding the nature of antibodies, and the practical means that they can be used to detect emerging serovars will aid in the process of adapting and eventually implementing an assay. Similar to currently available immuno-based methods, several issues will remain to be addressed due to the antibody-antigen interaction variations in different environments (food matrices, stool samples, among others).

Optimizing the antigen-antibody relationship

Assays performed in different food systems may not necessarily have similar levels of efficiency. It is difficult to compare assays due to varying consistencies (powder, paste, or liquid) and processing conditions among food systems (Fukal and Káš, 1989). In addition, the use of different sources of antibodies makes immunoassays difficult to compare due to the differing affinities of antibodies for antigens. One cannot state that one assay is better than another unless

identical antibodies and antigens are used in both assays. For this reason, antibody performance must be systematically evaluated for a variety of assays and food systems.

As previously described, the variable region of an antibody is responsible for binding an antigen. A sequence of roughly fifteen amino acids in the variable region will physically associate with an epitope (Frank, 2002). There are potentially numerous combinations of the fifteen amino acid sequences that can form a binding site, many of which overlap one another, thus providing the potential for interacting with many epitopes in a variable region (Frank, 2002).

These potential sequences bind an antigen at different affinity levels, which can result in cross reactivity and false positives (Frank, 2002). Antibodies produced against one antigen may interact with a structurally related epitope of a different antigen and is commonly referred to as cross reactivity, which is the interaction of an antibody with a different antigen(s) than the antibody was intended (Frank, 2002; Abbas et al., 2012). The inclusivity of a detection method is defined as the percentage of target organisms that are recognized in an assay, while exclusivity is defined as the percentage of non-target organisms that are falsely recognized by an assay. Target organisms are defined based on relatedness (such as taxonomic, immunological, or genetic composition) (FDA, 2011). For example, by applying an antibody that targets antigen A, a desired assay will be 100% inclusive (all antigen A in a sample will be recognized), and 100% exclusive (all antigens besides antigen A will not be recognized).

Theoretically, any antibody molecule can cross react and bind a different antigen. Such circumstances are a troublesome reality for general antibody use for detection and quantitation due to the misleading conclusions (false positives). Therefore, it is necessary to determine the cross reactivity of antibodies among antigens to determine the likelihood of implementing an

antibody (or set of antibodies) in a detection application. Immunoassays such as latex agglutination and ELISA can be used to estimate cross reactivity (Medina et al., 2012). For example, certain antibodies that target *E. coli* O26 are known to also interact with strains of *E. coli* O103 (Medina et al., 2012).

The availability of an epitope for antibody binding can be dependent on the state of the molecule that possesses the antigenic determinant. One type of antigen epitope (conformational epitope) becomes inaccessible following protein denaturation (Abbas et al., 2012). Quality control steps during food processing can alter microorganisms without complete destruction, which can lead to protein denaturation and the loss of epitope availability. One way to overcome an inaccessible antigenic determinant would be to apply an additional antibody that binds to a site accessible after protein denaturation (linear determinant). Applying more than one antibody would in theory increase the sensitivity of an assay, regardless of the structure of the target protein.

Deciding on the antibody to use can be complicated, and the ultimate decision comes down to how the antibody will be applied (Lipman et al., 2005). Optimizing an antibody and immunoassay for pathogen detection would likely be dependent on the accuracy, sensitivity, repeatability, and feasibility of an antibody and assay. With these characteristics in mind, determining the most appropriate immunoassay would likely involve analyzing the optimal antibody sensitivity in different immunoassays. Consequently, understanding of the antibody-antigen interaction, as well as establishing the target epitope of an antigen would be considered necessary. Although most researchers take advantage of readily available commercial antibodies, there are tools available to determine epitope uniqueness of target organisms.

Identifying and optimizing unique antigen targets

Depending on the target organism (or target antigen) for an assay, an optimal antibody may not be available or in existence. By sorting through the genome sequences of many organisms, a researcher can locate an antigen sequence unique to the target organism that could potentially be used to produce an antigen-specific antibody that was not previously available. The Basic Local Alignment Search Tool (BLAST) is a program available to researchers on the National Center for Biotechnology (NCBI) website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). BLAST can be used to identify nucleotides and proteins that are unique to the target organism. The genomes of a wide range of organisms have been sequenced and are available as a library on the NCBI website. A BLAST search can be used to make genetic comparisons between organisms, whether it is DNA similarities based on nucleic acid sequences or protein similarities based on amino acid sequences. During a search, all known genomes with similar protein or nucleotide sequence(s) are typically scanned to provide a comprehensive data set on which organisms also possess a certain cellular component, and to what extent.

There are different ways to conduct a BLAST search. It is common practice to search with BLAST to develop PCR primers that are useful in identifying specific genes or organisms of interest. An example would be adding the nucleic acid sequence of a bacteria and searching all known organisms with similar DNA, thus possibly delineating any uniqueness that could be used in primer development. Primer development with BLAST is particularly useful for conventional multiplex PCR to distinguish closely related species of microorganism. Since protein sequences can also be compared among organisms, a BLAST search could easily serve as a starting point for antibody development for whole cell detection by determining the amino acid sequence of an extracellular epitope either shared by all *E. coli* or epitopes highly specific to one serotype.

Extracellular protein data is readily available in the literature and can include accession numbers of the organism(s) possessing such proteins. By inserting the accession number and protein name into the NCBI search bar, information on the organism, protein sequence, and other organism protein sequences becomes available. Also, by inserting a protein sequence into a BLAST search (protein blast), all known genomes can be searched to provide information on other organisms that entirely or partially contain the protein sequence. A search is typically conducted within a relatively short period time period (typically several minutes), and the search page will subsequently reload and provide the “time since input.” Not every known genome has to be included in a search, and a search can be performed on a single organism. BLAST search results provide a list of organisms in ascending order based on the degree of sequence similarities.

As of 2011, the genetic clusters of 96 O antigens had been sequenced, and at that time 78 additional O groups were still being pursued (DebRoy et al., 2011). Determining the genetic sequences of O antigens will not only help determine how these genes impact virulence mechanisms, but will provide information on any universal structural components between O antigens, and will likely be useful for antibody production (DebRoy et al., 2011).

Alternative antibody sources

Requirements for antibody sources

With regard to immuno-based systems, deciding on antibodies for a particular application involves complex obstacles. Determining the best target location of an antigen needs to be carefully considered; specificity, selectivity (Table 3), and reproducibility are important criteria to consider when selecting antibodies for assay development (Bordeaux et al., 2010). Antibody

development by laboratories is often limited due to high costs, difficulty, excessive labor, and time; hence the popularity of commercial antibody sources.

There are many factors that contribute to the variation of antibody recognition of an epitope (assay format, protein conformation, nonspecific binding, among others) (Perkel, 2014). To ensure that the properties of antibodies are accurately portrayed, peer reviewed publication submission requirements have been laid out to combat publication of poor data from immuno-based research (Perkel, 2014). When purchasing commercially generated antibodies, possessing certain fundamental information about the type of antibody is essential (Saper, 2005). Knowing specifically its source, how it has been characterized, the controls used for development, and the antigen (and specific sequence) that the respective antibody was raised against are considered necessary background information that should be, but are not always, provided by a supplier (Saper, 2005). Another driving force for requiring technical antibody information in published research articles is due to the difficulty of exactly reproducing similar data, especially if reagents and antibody information is not fully provided (Perkel, 2014). There are over 300 suppliers of antibodies worldwide (Bird, 2012). In a survey conducted by Frost and Sullivan of which 1155 subscribers to *The Scientist* magazine (LabX Media Group, Midland, Ontario, Canada) participated, 88% of subscribers claimed antibodies were currently used in their laboratories (Bird, 2012), however, antibody sources were not identified.

Issues with current antibody sources

Although necessary, maintaining a proper environment for animals to remain healthy over the duration of their use for research purposes can be represent a tremendous investment in labor. For example, designer rodents are more vulnerable to infectious agents, and continuous

monitoring and husbandry of animals can require extensive efforts (Cork et al., 1997). Along with continuously monitoring the well being of animals, and although animal pain and suffering during immunization is typically minimal, there are those individuals and corresponding organizations who are universally opposed to the use of animals for research purposes (Hurnik, 1990; Conn and Parker, 2008), and in response to these concerns, animal welfare has become more heavily regulated (Cork et al., 1997; Matheny and Leahy, 2007; Rushen et al., 2011). Any incidents that violate the Animal Welfare Act will likely lead to more stringent regulations for animal-based antibody suppliers to recognize and prevent any incidents of animal neglect. Resistance to heightened regulations could drive animal-based antibody producers out of the commercial market following fines, lawsuits, or a perceived reputation for inhumane treatment of animals, and it has been speculated that as new technologies emerge, the use of animal-based research will likely require even more rigorous justification (Dresser, 1992; Cork et al., 1997; Conn, 2009; Rushen et al., 2011; Cossins, 2012).

In addition to more justification, to avoid confrontation in the animal welfare controversy, antibody users will more likely consider employing alternative sources of antibodies. Antibody development without animals is possible, and due to the ensuing recent publicity of animal abuse with some commercial antibody suppliers (Grant, 2014), alternatives would eliminate the growth of an unsettling consumer conscience with regard to the source of antibodies. These alternative sources will be discussed in the following sections.

Novel clone libraries and systems specific for antibody production

Alternatives of antibody sources include modified monoclonal antibody production, cloning antibody variants into yeast and bacterial cultures, as well as creating multi-specific

antibodies in transgenic plant systems (Plückthun, 1991; Hayden et al., 1997; Brekke and Løset, 2003; Kipriyanov and Gall, 2004; Berghman et al., 2005; Zeliadt, 2014). An additional process of monoclonal antibody production involves isolating B cells by flow cytometry, distributing the B cells in 384 well plates, and culturing the B cells in an environment supportive of growth and antibody secretion. After two weeks of culture, the secreted antibodies are screened to determine their ability to neutralize an antigen. The heavy and light chain(s) of antibodies that show neutralizing activity are cloned into expression vectors and are expressed by transfection to produce recombinant antibodies (Huang et al., 2013). This method is advantageous in that it can be performed in much less time (about 3 weeks) than the typical monoclonal antibody production time (3 to 6 months) (Leenaars et al., 1999; Lipman et al., 2005). However, it is believed to be more applicable for laboratories with an automated liquid-handling device due to the large number of wells involved.

An even more rapid technique (within a week) of monoclonal antibody production can be performed with flow cytometry, PCR, and next generation sequencing. Following B cell isolation via flow cytometry, single B cells can be isolated in microwells embedded on a slide, and PCR primers (each specific for heavy and light chain genes) are used to construct a single DNA fragment that consists of both heavy and light chain genes. Recovered DNA strains are then analyzed with next generation sequencing to identify unique heavy and light chain pairs, and sequences that are most appropriate to develop a recombinant antibody are determined. Although this method can be performed in a week, the microwell slides are not commercially available, and data analysis typically requires extensive bioinformatics knowledge (DeKosky et al., 2013; Zeliadt, 2014).

The genetic regions that are responsible for the complementary determining regions of an antibody can be rearranged to become more specific for a particular antigen to create recombinant antibodies. Following genetic alterations, the antibodies that are most specific for the antigen can be selected for and isolated. These complementary determining regions can be paired with the constant portion of an antibody, thus providing an antibody with a specific target and functionality (Waltz, 2006).

Regardless of the antibody source, after collecting a pool of polyclonal antibodies, a researcher is often left with antibodies that may or may not be sufficient for a particular application. Developing methods to sort out and identify the interaction each polyclonal antibody has with the respective antigen is a critical step in taking advantage of a source of antibodies. Protein microarray systems have been investigated as means for screening a large number of antigens to determine which antigen(s) an antibody will best recognize (most sensitive and specific) (Lee et al., 2012; Yu et al., 2013). Also, a protein microarray can be used to determine the affinity of an antigen against a library of antibodies (Rivas et al., 2008; Ferreira and Finlay, 2012). These methods will potentially be useful in vaccine and cancer research (Yu et al., 2013), as well as in other areas where the affinity of a large number of antigens or antibodies needs to be characterized.

For example, a method known as *in vivo*-induced antigen technology (IVIAT) has been used to detect antigens in the human gastrointestinal tract. The IVIAT approach was performed by John et al., (2005) to reveal the virulence of *E. coli* O157 by determining which proteins of *E. coli* O157 are expressed during human infection (John et al., 2005). These authors identified two hundred and twenty three proteins that were expressed during human infection of *E. coli* O157, some of which were not accounted for upon analysis of a mid-exponential phase *E. coli* O157

culture. These authors suggest these *in vivo*-induced (ivi) proteins could provide insight to human infection caused by *E. coli* O157, and be appropriate targets for detecting *E. coli* O157 in stool specimens. As protein detection systems become more sophisticated, virulence mechanisms, as well as the most relevant antigens will likely be revealed.

Since protein microarrays are useful for determining the antigen that an antibody is most compatible for, an antibody can be screened against a library of antigens (or vice versa), and depending on the amount of antibodies/antigens that are available, highly sensitive and specific antibodies can be isolated and used in other immuno-based detection assays. Antibodies of non-animal origin or from hen eggs could be used to screen numerous antigens of interest, and eventually collect a set of useful antibodies.

Progress toward alternative antibody production could lead to a supply of more reliable and feasible antibodies. If such alternatives were proven to be equivalently reliable as previous methods, novel antibody production methods could become the preferred method of antibody production. As new antibody development techniques surface, researchers can implement such techniques for creating an antibody most fit for the application in mind (Berghman et al., 2005).

Compared to conventional methods, alternative methods may provide more rapid antibody production. In addition to more readily available antibody sources, these methods and BLAST will ultimately result in a library and unlimited supply of antibodies that can be used to target the nested epitope sequences of relevant pathogens. As technology progresses, developing alternative methods will likely provide much greater flexibility in the antibody construction process.

Advances in capture antibody systems

A generic capture antibody could initially be used to detect and recover all *E. coli* with an available sorting system such as FACS. The antibody would need to target only one site of an *E. coli* extracellular protein – an antibody of single specificity will be necessary to ensure two events are not counted where only one *E. coli* actually exists in a sample. In a similar theory to the BAX system, antibodies congruent with unique fluorescence that are specific for each serogroup could be applied to determine which serogroups were present in the sample. Multifluorescent analysis is becoming more advanced (Baumgarth and Roederer, 2000; Herzenberg et al., 2002; De Rosa et al., 2003; Perfetto et al., 2004), and with an available generic antibody, such methods could be integrated into detection. Upon the initial sorting and acquisition of a generic *E. coli* population, one could detect the “big six” via flow cytometry with six available fluorescent markers that aligned with each serogroup. This procedure would only be applicable with flow cytometers that contained more than one laser. If six distinguishable markers were not readily available, two markers (each specific for three of the “big six”) could be used and would also make sorting capabilities less complex. Following the secondary flow cytometry analysis and sorting process, IMS could be applied to samples that were positive for respective serogroups. Afterward, culture and identification could be performed as currently practiced. Cross-reactivity of antibodies and protocol accuracy would need to be investigated and addressed prior to becoming implemented in the food industry. If deemed as being acceptable, optimized flow cytometry protocol(s) could reduce the duration of the test and hold procedure and accelerate food distribution without compromising product safety.

Conclusions

There will likely always be a strong push to create and optimize highly sensitive detection methods for use in food systems associated with low infectious dose pathogens such as STEC (Palumbo et al., 1997). Correctly and consistently identifying these harmful bacteria is important due to significant health and economic implications that can result from illnesses. More efficient assays (shorter time-to-results, increased accuracy, and minimal preparation and/or labor) would accurately guide outbreak investigations and provide the food industry confidence that products are safe.

Detection methods were developed for *E. coli* O157:H7 once it was considered an adulterant, and with the emergence of regulations for the “big six” such methods only targeting *E. coli* O157:H7 are no longer applicable. With increased diversity in serotype prevalence, continued public reaction, and corresponding government action, current protocols considered efficient for regulatory purposes will need to be altered more frequently to account for new serotypes, leading to the need for new approaches or adjustments to satisfy new food safety standards. The USDA-FSIS has provided considerations and suggestions for assay validation to become equal in the eyes of current regulations and requirements (FSIS, 2010). When developing protocols it should be kept in mind that emerging pathogens and the possibility for them to interfere with food safety regulations can occur and requires strategic efforts to create assays adaptable to new serotypes. Also, declaring samples as presumptive positives only if both *stx* and *eae* genes are present may need to be reconsidered. There have been instances of STEC causing human illness that do not possess *eae* (Paton et al., 1999; Eklund et al., 2001; Willford et al., 2009). If PCR were the only method used for testing, STEC lacking *eae* could potentially lead to false negative results and undetected risk coming through the food supply system. An increase in outbreaks due to *eae*-negative STEC would likely lead to restructuring of current

detection requirements and initiate the edict that samples positive for the *stx* or *eae* genes would need to be considered as presumptive positives. As more STEC become associated with illness, development of PCR primers specific for these STEC will be in demand (Auvray et al., 2007; Fratamico and Bagi, 2012).

In addition to the need to develop assays adaptable to new serotypes, such assays should have a broad application to many food systems that have been and could potentially become associated with STEC and foodborne pathogens in general. Unforeseen obstacles have risen and are likely to continue in the future for detection systems due to the physiological elusiveness of STEC and their ability to adapt and either not code the usual virulence gene(s) or implement similar virulence factors with different genes and cause illness.

For example, a serotype never before known to cause infection in humans resulted in a 2011 outbreak in Northern Germany. *E. coli* O104:H4 was the causative agent, and the isolate had acquired both a phage encoding the *stx* gene and a plasmid containing EAEC virulence genes. The outbreak was unfortunate in that an uncharacteristically high percentage (25%) (Zhao et al., 2011) of illnesses progressed to HUS, more so than usual (5%) (Tuttle et al., 1999). It has been hypothesized such an increase in HUS cases was due to the acquired EAEC virulence factors that aided in the uptake of the Stx (Muniesa et al., 2012). The ability of *E. coli* O104:H4 to form such uncharacteristic modes of virulence is a troublesome reality in epidemiology, and there are concerns that comparable adaptations will occur and result in similar outbreaks (Chattaway et al., 2011). Such instances of gene transfer via plasmids (Ogura et al., 2009) and phages (Muniesa et al., 2003, 2004) to *E. coli* contribute to the virulence of pathogenic *E. coli*. Such situations are a major threat to public health due to the possibility that the current detection methodologies will not be applicable for emerging serotypes (Wang et al., 2013).

The evolution of methods with increased sensitivity will likely continue as it has in the past due to consumer demand, as well as threats of bioterrorism (Sobel et al., 2002). Although certain methods may be highly accurate, their chance of being implemented into clinics and the food industry is hindered by the difficulty to perform these methods by clinicians and laboratory technicians. Creation of protocols that can be performed with minimal training will be an asset to the food detection systems. The cost to train lab technicians on specific protocols also needs to be considered (Hoorfar, 2011).

Implementing a new pathogen detection method in the food industry is a complex issue for several reasons. The efficiency of an assay can vary depending on the type of food and its components, so protocols need to be specific for food types and be validated when applied to untried food systems (Hoorfar, 2011). It is also possible that assay efficiencies will differ in similar foods due to the ingredient type and even quality. The age and concentration of organic molecules or electrolytes can vary in meat products and in turn alter the function of an assay (Fu et al., 2005). To our knowledge, the quality of food ingredients and the effects on detection capabilities has not been investigated in immuno-based and flow cytometry protocols. It might be worthwhile to investigate how the grade and quality of a food affects detection sensitivity while detection protocols are being developed for food systems. It is possible that a highly efficient assay not known to produce false negatives may not apply to food systems that extensively vary in quality and composition. If a new system were to be introduced to the food industry, knowing any quality limitations would be important, especially in production facilities characteristic of product diversity. One can gauge the efficiency of an assay for new products based on the assay performance in a similar food system, but a confirmation analysis will be necessary.

When discussing detection methods several terms are commonly used with regard to analyzing results. Readers should take caution when using these terms and acknowledge the author's reference point. Accurate, efficient, rapid, specific, sensitive, quick, and simple are terms that are frequently encountered descriptions of flow cytometry and immunoassay methods when discussed in the published literature. When it is stated that an assay is "rapid" or "simple", one must realize that this may refer to a specific portion of the protocol, and does not necessarily account for the steps to prepare for the assay. For example, incubation and wash steps during an ELISA assay, the practice of follow-up or conformation PCR assays, and sample preparations such as DNA extraction for PCR or removing concentrated food constituents should be considered. Immunoassays will continue to be applicable as antibodies are further characterized (Skinner et al., 2013). As STEC evolve and serotypes become associated with human infection and food products, the need for antibodies in these detection systems will benefit human health and the food industry. If available tools such as organism search engines (BLAST) and antibody production alternatives (*in vitro*, IgY production from hen eggs, among others) continue to emerge and become applicable, researchers can potentially keep pace with what is needed for detecting new serotypes by immuno-based methods.

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IV. References

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Table 1. Monoclonal and Polyclonal Advantages and Limitations

| | Monoclonal | Polyclonal |
|-------------|--|--|
| Advantages | Unique/single epitope specificity Large quantities can be acquired Uniformity among batches | Multiple epitopes for an antigen Epitope rearrangements/mutations less likely a problem due to multiple epitope targets ^{^#} Relatively short production period (4 to 8 weeks) More stable and less sensitive to pH and salt concentration fluctuations [#] |
| Limitations | Only applicable for detecting one epitope Possible false negatives from epitope rearrangement/mutation* Time consuming (3 to 6 months) ^{^#} Rarely precipitate antigen (one binding site limits cross-linking abilities) | Wider antigen specificity (similar protein and sugar antigens among nontarget bacteria can result in false positives [@] Batch-to-batch variation [^] |

Lipman et al., 2005

^ Leenaars et al., 1999

* Herzenberg, Sweet, & Herzenberg, 1976

@ Fukal & Káš, 1989

Table 2. Flow Cytometers

| Instrument | Laser (nm) | Reference |
|--|-------------------|--|
| Beckman Coulter FC500 | 488, 633 | Hegde et al., 2012b |
| BD Bryte HS Instrument (BioRad Laboratories) | 470-490 | Tortorello et al., 1997 |
| FACSCalibur (BD, Sydney, Australia) | 488 | Gunasekera et al., 2003 |
| FACSSStar + (BD, San Jose, CA) | * | Bernander et al., 1998 |
| EPICS Elite (Beckman Coulter, Fullerton, CA) | 488 | Yamaguchi, 2003; Tortorello et al., 1997 |
| LSR Flow Cytometer (BD) | * | Zumbrun et al., 2013 |
| Argus 100 (Skatron Lier, Norway) | * | Bernander et al., 1998 |
| RAPID-B model 9013 (LITMUS RAPID-B) | 488 | Wilkes et al., 2012 |
| BD FACScan | 488 | Riedy et al., 1991 |

* Multiple lasers

BD - Becton Dickinson

Table 3. Terminology

| | |
|-------------|---|
| Sensitivity | probability that a positive sample will be screened as negative (positive samples that are determined "positive" are known as false negatives)* |
| Specificity | probability that a negative sample will be screened as negative (negative samples that are determined "positive" are known as false positives)* |

* FSIS, 2010

IV. Chapter 3

Formalin-Fixed Cells As An Internal Standard Approach for the Detection and Quantitative Assessment of Shiga Toxin-Producing *Escherichia coli* (STEC)

Christopher A. Baker^a, Si Hong Park^a, Peter M. Rubinelli^a, Stephanie M. Roto^a, Shawn Ramsaroop^b, Melinda Miller^b, and Steven C. Ricke^a

Center for Food Safety; Department of Food Science, 2650 N. Young Ave. University of Arkansas, Fayetteville, AR 72703^a

Vivione Biosciences, LLC, Pine Bluff, AR 71602^b

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*Corresponding author: Steven C. Ricke, Center for Food Safety; Department of Food Science, University of Arkansas, Fayetteville, AR 72704. Phone: 479-575-4678, Fax: 479-575-6936
Email: sricke@uark.edu

Introduction

Although there are forms of *Escherichia coli* which reside in the intestinal tract of humans as commensal microorganisms, a certain subtype (enterohemorrhagic *E. coli* (EHEC)) of pathogenic *E. coli* known as Shiga toxin-producing *E. coli* (STEC) produce shiga toxin(s). Shiga toxin-producing *E. coli* are considered to be a high-risk to humans due to the low infectious dose that can result in foodborne illness. Additionally, ill persons infected with EHEC pathogens can develop a disease known as hemolytic uremic syndrome (HUS), which can be fatal, especially in children and the elderly (Tarr et al., 2005; Mayer et al., 2012).

Major concerns have resulted from the emergence of the *E. coli* O157:H7 serotype (Armstrong et al., 2006), and more recently the non-O157 serogroups (commonly referred to as the “big six” (serogroups O26, O45, O103, O111, O121, and O145)), which are all considered adulterants in raw non-intact ground meat (Fratamico and Bagi, 2012; FSIS, 2014a,b). Outbreaks and recalls have been frequently associated with these STEC throughout the past decades, resulting in commercial economic loss, illness, and possibly death (CDC, 2014a, b). More sensitive and specific STEC detection assays continue to be investigated, and optimization of these assays will be advantageous to the food industry and consumers to limit recalls and foodborne illnesses, respectively.

The USDA recommends the practice of PCR (BAX[®]) for detecting O26, O45, O103, O111, O121, and O145 serogroups (FSIS, 2014a, b), which is typically preceded by a lengthy and necessary enrichment step. Quantitative PCR (qPCR) assays are useful for enumerating cell populations in a sample (Smith and Osborne, 2009), and this method has previously been utilized for STEC detection (Debroy et al., 2004, 2005; Fratamico et al., 2005, 2011, 2012). Quantitative

PCR assays can be advantageous since analyses provide an approximate enumeration of the target organism(s) in samples, and yields a more in-depth analysis (quantification) opposed to conventional PCR platforms that simply provide a presence/absence analysis.

In addition to PCR assays, future commercial detection procedures may integrate immuno-based detection systems (Baker et al., 2015). Flow cytometry continues to be optimized for detection strategies, and these platforms are beneficial due to the potential for automation as well as a shortened time to results (Hegde et al., 2012a, b; Buzatu et al., 2014; Wilkes et al., 2012; Beers et al., 2015). For instance, a flow cytometry assay (RAPID-B[®]) was recently compared to the BAX[®] system, and in addition to showing parallel results, this method was performed in a mere 8 h (Beers et al., 2015), which provides the opportunity for large scale sample analysis, especially if several instruments were utilized simultaneously.

Commercial laboratories may utilize a variety of detection assays, and it is difficult to directly compare different nonculture-based rapid detection systems (flow cytometry versus gene amplification) in food matrices since these systems focus on distinct microbial cell components (protein antigen versus molecular, respectively). Regardless of the assay(s) that becomes (or continues to be) commonplace in large-scale commercial pathogen detection procedures, having stocks of absolute and known quantities of cells could be useful as an internal standard positive control for periodic instrument and assay validation. Utilizing versatile and universally accurate internal standards that complement a broad range of multiple and different assays, whether molecular or immuno-based, would be beneficial for pathogen detection laboratories and large-scale commercial operations where routine detection assays are conducted.

Bacterial cell fixation provides a means to obtain a stabilized bacterial cell population that could be advantageous as an internal standard – fixatives help maintain cell structure,

permeabilize cells (which enhances accessibility of antibodies with their target(s)), and terminate the biological mechanisms of cells. This provides a means to revisit samples at a later date and determine their properties at the time of fixation (Bongaerts et al., 2002). An internal standard is commonly used to adjust and calibrate instruments in analytical chemistry, and provides appropriate measurements in conjunction with samples to obtain a more reliable data set (de Oliveira, 2010). Formalin-fixed cells would provide a calibration technique similar to an internal standard by yielding a positive control that can be used to quantitatively assess and compare with food or environmental subsamples. These procedures may also save time and increase laboratory safety measures (Bongaerts et al., 2002) by eliminating the need to repeatedly enrich/culture pathogens, extract DNA, and perform serial dilutions for calibration. Additionally, these procedures may reduce labor requirements and further support automation for foodborne pathogen detection.

In this study we present an alternative method that utilizes formalin-fixed STEC cells for standard curve development for different quantitative assays that can potentially be incorporated into commercial operations. Several detection instruments can be calibrated with the same set of fixed cells, which will provide a baseline and more uniform positive control for detection assays. The objective of this study was to compare fixation methods (8% versus 10% formalin-fixative) with BAX[®] PCR, qPCR, and flow cytometry platforms, as well as determine the recovery rate of spiked cells in ground beef for STEC serotypes O26:H11, O45:H2, O103:H3, O111:H-, O121:H19, and O145:H18 with quantitative PCR and flow cytometry assays.

Materials and methods

Escherichia coli strains, culturing conditions, and cell preparation

Strains used in this study are listed in Table 1. Isolated colonies from each STEC serotypes were inoculated in 10 mL of Luria-Bertani (LB) Broth and incubated at 37°C for 24 h to obtain overnight cultures. All overnight cultures were centrifuged (5 min, 8,000 x g) with an Avanti J026 XP centrifuge in a JA-17 rotor (Beckman Coulter, Inc. Brea, CA, USA). Following centrifugation, the supernatant was discarded, and the cell pellet was stored until fixation.

Cell fixation

An overnight culture of each STEC serotype (O26:H11, O45:H2, O103:H3, O111:H-, O121:H19, and O145:H18) was obtained as previously described, and the cells were subjected to 10 mL of fixative. Cell pellets were resuspended in 10 mL of fixative, and the cells were incubated at room temperature for 30 minutes with periodic vortexing. Fixed cells were centrifuged (5 min, 8,000 x g) and washed twice with PBS (pH 7.4) before being resuspended with PBS. A previously published fixative procedure (8% formal-saline) (22-24) as well as a commercial fixative (10% formalin-fixative) (Sigma-Aldrich® Formalin Solution 10%, Neutral Buffer, St. Louis, MO) was evaluated for each STEC serotype.

Approximation of STEC serotype cell concentrations – Phase Contrast Microscopy

Cells of each respective STEC serotype (O26:H11, O45:H2, O103:H3, O111:H-, O121:H19, and O145:H18) were enriched, fixed, and washed as previously described, and the concentration of each STEC serotype (for each fixation method) was approximated in a Petroff-Hausser Bacteria Counter (1/400 Sq. mm, 1/50 mm deep, C. A. Hausser & Son, Phila., PA, USA) under oil immersion (1000 X magnification) with a Nikon Phase Contrast microscope

(Nikon Inc., Tokyo, Japan). For each cell suspension, sixteen squares in total were counted and averaged, and the following equation was used to approximate each cell concentration:

$$\text{cells/mL} = (\text{average cells/square}) / 5 \times 10^{-8}$$

DNA extraction

Prior to all PCR reactions, DNA was extracted from samples (1 mL) with a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the instructions of the manufacturers.

BAX[®] PCR

A single colony of each respective STEC serotype (O26:H11, O45:H2, O103:H3, O111:H-, O121:H19, and O145:H18) was inoculated into 5 mL TSB broth, and incubated at 37°C for 18 to 24 h. Following enrichment, cells were fixed and washed as previously described. Fixed cells were subsequently diluted, and various concentrations of fixed cells (8 and 10% formalin fixatives) for STEC serotype were analyzed by BAX[®] PCR according to the instructions of the manufacturers (DuPont, 2013) to compare fixatives, as well as determine the limit of detection for both assays.

Quantitative PCR standard curve generation and conditions

The standard curve was generated for each STEC serotype by determining the genome size and copy number of the respective STEC serotypes. Standard curve dilutions were performed with nuclease free water (Integrated DNA Technologies, Coralville, IA, USA). All qPCR experiments (standard curves and samples) were performed in triplicate, as well as in conjunction with melting curve analysis. The qPCR reaction mixtures consisted of 5 µL of DNA,

500 nM of each primer (Table 2), 10 μ L of SYBR[®] Premix Ex Taq[™] II (Takara Bio, Inc., Shiga, Japan) and nuclease free water to bring the final reaction volume of 20 μ L. Quantitative PCR reactions were performed as follows: 95°C for 2 min, and 40 cycles of 95°C for 15 s, annealing at 60°C for 15 s, extension at 68°C, followed by a melting curve analysis consisting of an increasing temperature of 0.5°C per min for 20 min from 60°C to 95°C. All analyses were performed with a standard curve simultaneously in triplicate.

Spiking ground beef samples

Following fixation and approximation of STEC serotype cells, the cells were diluted ten-fold, and the various cell concentrations were spiked in ground beef samples. One mL of each fixed cell dilution for each STEC serotype was inoculated in 25 g \pm 1 g of commercially irradiated ground beef in a stomacher bag, and incubated at room temperature for 10 min. Following incubation, 225 mL of mTSB + novobiocin (8mg/L) (Modified Tryptone Soya Broth, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was added to the spiked ground beef and stomached for 2 min after which subsamples (1 mL) were obtained for DNA extraction and qPCR.

Flow cytometry analysis of fixed cells

The fixed cells that were spiked into ground beef samples and evaluated by qPCR were analyzed via flow cytometry with the RAPID-B[®] model 9013 flow cytometer (Vivione Bioscience, LLC, Pine Bluff, AR). One mL of sample was filtered (5.0 μ M), and 100 μ L of filtered sample was incubated with PBS, Reagent B, and antibodies (total volume 1 mL) from the

Non-O157 STEC Test Kit (Vivione Biosciences, LLC) for 25 min, and samples were evaluated on the RAPID-B[®] platform according to the instructions of the manufacturers.

Statistical analysis

The coefficient of variation for STEC serotypes was determined for the quantitative PCR cell recovery of ground beef spiked samples. All statistical analyses were performed on log cells/mL inoculated in ground beef, which was evaluated with a single factor one-way ANOVA test ($P \leq 0.05$) in Microsoft Excel[®] (Microsoft Corporation, Redmond, WA).

Results

BAX[®] PCR, fixed cell comparison, and limit of detection

Cells were subjected to each fixative and subsequently analyzed by PCR (BAX[®] Real Time (Dupont, 2013)) as recommended by USDA (FSIS, 2014a, b). The average limit of detection among 10% and 8% formalin-fixed cells was determined to be 488 and 3,821 cells/mL, respectively.

Comparison of 8 and 10% formalin fixative methods by qPCR

The cell concentration for each sample analyzed (excluding serotypes O45:H2 and O145:H18) was higher for 8 versus 10% fixed cells (Table 3), yet the cycle threshold value for 8% formalin-fixed cells was higher among all serotypes tested.

Spiked ground beef cell recovery and limit of detection

The average percent cell recovery exceeded 100% (an overestimation of copy number) for ground beef samples spiked with serotypes O26:H11 ($107 \pm 11\%$), O45:H2 ($101 \pm 5\%$), O103:H3 ($108 \pm 6\%$), and O145:H18 ($107 \pm 3\%$), and serotypes O111:H- and O121:H19 exhibited a recovery level lower than 100% ($97 \pm 6\%$ and $95 \pm 16\%$, respectively) (Table 4). The average limit of detection for the STEC fixed cells was determined to be 272 ± 342 cells/g (average \pm standard error) of ground beef.

Flow cytometry detection and analysis of fixed cells

Spiked ground beef samples containing fixed cells were analyzed with the RAPID-B[®]. Assays designed to enumerate STEC serogroups in ground beef following an enrichment step have been utilized (Buzatu et al., 2014). The samples containing STEC fixed cells without enrichment were subjected to the antibodies specific for STEC serogroups O26, O45, O103, O111, O121, and O145. Total events for trial 1 samples were roughly a log lower for each serotype when compared to the cells/mL approximation with phase contrast microscopy as well as the quantitative PCR evaluations (Table 5). In general, trial 2 samples exhibited similar (within a log cells/mL) cell number analyses in comparison with the phase contrast cell approximation and quantitative PCR evaluations (Table 5).

Comparison of different approximation and quantitation techniques

A single factor one-way ANOVA analysis ($P \leq 0.05$) was performed to compare the approximation of log cells/mL by phase contrast microscopy with the quantitation of log cells/mL by flow cytometry and quantitative PCR methods, and the results were not considered statistically significant.

Discussion

Flow cytometry can provide a means of rapidly analyzing a large amount of samples (100 to 1000 samples per day) with limited user assistance or automation, and commercial detection kits (antibodies, reagents, among others) may incorporate various stock concentrations of fixed cells to complement a wide range of assays. This will provide internal positive control sample volumes to calibrate as well as validate protocols. To our knowledge, fixed cells have not been incorporated into a qPCR assay, and similar stock concentrations of fixed cells would supplement qPCR by negating the enrichment of isolated colonies for standard curves used in the assay.

For the quantitative PCR assays, there were several instances of underestimation of cell number, which could be problematic if the goal of a qPCR assay was to enumerate absolute counts of a STEC serotype in a sample, but since certain STECs are adulterants (Fratamico and Bagi, 2012), the qPCR methods described here would be sufficient for a reliable presence/absence assay. Decreased sensitivity is an issue in high fat or protein food matrices, which can affect amplification efficiency and primer binding to the target template (Yeni et al., 2014).

Overestimation of cells is likely due to the fact that the standard curve was based on copy number and genome size of each serogroup. It is generally assumed that one copy (a single gene product) is equivalent to one cell, but it has been suggested that the genome size can vary among a population of cells (Bignell et al., 2004; Li and Olivier, 2012). If a cell population were fixed in the process of continued growth, it is possible that a single fixed cell could constitute multiple partial chromosomes (Li and Olivier, 2012), in which gene copy number would possibly not

correlate with absolute cell number. Detection capabilities of molecular methods such as qPCR are food and microorganism dependent (Yeni et al., 2014) – optimizing these assays for many different food matrices can be laborious and time consuming, and a beneficial standard stock of fixed cells may provide a means to optimize detection assays in a more timely and feasible fashion. Additionally, obtaining a more precise copy number from fixed cells may be possible with a continuous culture system, which during steady state at a set cell division would provide synchronized cell populations that could be used over a long duration of time (Yaginuma et al., 2014).

The flow cytometry assay used in this research was modified from the manufacturers procedures in that 225 mL of broth was used to simulate enrichment (75 mL of broth is recommended in the protocol). There were several instances of underestimation of cell number for the assay performed, which can result from inhibitory effects of fats present in the ground beef samples.

Developing a reliable internal standard with fixation methods will provide a means of reliably calibrating pathogen detection instruments periodically. For food quality control personnel, conducting assays on a variety of platforms as well as having a reliable internal standard would be beneficial for sample testing when compared to other methods that go through steps of isolation, enrichment, and DNA extraction prior to the development of a standard curve template. Of particular importance is the availability of stable intact bacterial cells that can be used for diverse detection methods that involve identifying intact cells with consistent antigen presentation in immuno-based methods, or as an extraction source of DNA for molecular techniques. Fixed cell standards could be used for on-site applications for transportable assays,

and the same set of fixed cell standards could be used throughout different laboratories for a more uniform and homogenous calibration protocol.

It is possible that STEC fixed cells bind to various components in ground beef or be non-randomly distributed, which could also play a role in not recovering 100% of cells. The extent to which fixed cells interact with these components warrants further investigation. Fixative procedures have been implemented in several instances. Chao and Zhang (2011) investigated fixation methods to study cell morphology and structure of *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas putida* by atomic force microscopy. These authors concluded that the most effective fixative for these microorganisms was 2.5% glutaraldehyde. The effects of fixation on bacterial structure and outer membrane integrity have been investigated, but it seems very few questions have been addressed with regard to how fixation procedures effect detection of microorganisms. Kamiya et al., (2007) investigated fixed marine bacteria and how storage time compromised quantitative assays via flow cytometry. These authors determined that their fixation procedure did not affect bacterial counts but that an increased storage time did have an effect on enumeration procedures via flow cytometry (Kamiya et al., 2007).

Storing samples for an extended amount of time could potentially impact detection efficacy. Gasol and Del Giorgio (2000) suggested that fixatives could be implemented prior to flow cytometry assays to preserve planktonic bacteria that cannot be evaluated immediately once samples are freshly processed. Similar methods could be used to evaluate foodborne pathogens in food or for on-site sampling.

Initial fixation with formaldehyde limits autolysis of cells (Kiernan, 2000), and reacts with proteins, lipids, and nucleic acids (French and Edsall, 1945; Hopwood, 1969). The protein-formaldehyde interaction provides the opportunity for additional protein(s) to interact with the

end group of the formaldehyde-bound protein and if these two reactive end groups are within close enough proximity to one another, a cross-link (methylene bridge) may form (Kiernan, 2000; Dapson, 2007). Formaldehyde fixatives interact with biological molecules to form what has been referred to as addition products, which are reactive and have the potential to crosslink the end groups of other molecules (Dapson, 2007). Several fixatives have been utilized for a wide range of purposes, and the most appropriate fixative is application and objective-dependent (Hopwood, 1969; Fox et al., 1985).

A primary advantage of fixing cell concentrations for a positive control is to avoid fluctuations in cell populations, which can occur over time in populated cells. It has been suggested that fixation can influence cell structure and morphology (Chao and Zhang, 2011), and although this may lead to changes that negatively impact antibody-based detection strategies, many of the detection analyses may not require absolute cell concentration analysis, and a rough estimate of cell number in a sample may suffice. An internal standard detection method may limit overall detection time by eliminating the need to enrich cells and DNA extraction for creating genomic DNA-based standard curves. Additionally, these methods may limit risk and result in a safer working environment by limiting hands-on applications with live STEC cells.

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Table 1. Shiga toxin-producing *Escherichia coli* strains used in this study

| Serotype | ID | Source | Reference |
|-----------------|-----------|-------------------------------|----------------------------------|
| O26:H11 | 05-6544 | human | National Microbiology Laboratory |
| O45:H2 | 05-6545 | human | Nova Scotia Public Health Lab |
| O103:H3 | E67 | cattle | Francisco Diez-Gonzalez |
| O111:H- | JB1-95 | Mettwurst (fermented sausage) | Australia outbreak, 1995 |
| O121:H19 | 03-2832 | human | Alberta Public Health Lab |
| O145:H18 | 03-4699 | human | Alberta Public Health Lab |

Table 2. Primers used in quantitative PCR assays

| Serogroup | Sequence | Gene | Product | Reference |
|------------------|--|-------------|----------------|------------------|
| O26 | TAAATTGCGGGGAAAGAATG GACTTCATGGGTACCGCCTA | wzy | 276 | (10) |
| O45 | TATGACAGGCACATGGATCTGTGG TTGAGACGAGCCTGGCTTTGATAC | wzx | 255 | (11) |
| O103 | TTGGAGCGTAACTGGACCT GCTCCCGAGCACGTATAAAG | wzx | 321 | (12) |
| O111 | TGTTCCAGGTGGTAGGATTCG TCACGATGTTGATCATCTGGG | wzx | 237 | (13) |
| O121 | AGGCGCTGTTTGGTCTCTTAGA GAACCGAAATGATGGGTGCT | wzx | 189 | (13) |
| O145 | AAACTGGGATTGGACGTGG CCCAAACTTCTAGGCCCG | wzx | 135 | (13) |

Table 3. Quantitative PCR Ct value of 8% and 10% formalin fixed cell for STEC serotypes O26:H11, O45:H2, O103:H3, O111:H-, O121:H19, O145:H18

| Cells/mL | 10 ⁶ | | 10 ⁵ | | 10 ⁴ | |
|----------|---------------------------|-----------------|-----------------|--------------|-----------------|----------------|
| | 10% | 8% | 10% | 8% | 10% | 8% |
| O26:H11 | 20.90 ± 0.09 ^a | 22.26 ± 0.12 | 24.30 ± 0.16 | 25.56 ± 0.14 | 27.44 ± 0.19 | 29.12 ± 0.36 |
| O45:H2 | 17.07 ± 0.09 | 19.20 ± 0.08 | 21.24 ± 0.16 | 23.19 ± 0.07 | 24.54 ± 0.07 | 27.22 ± 0.09 |
| O103:H3 | 20.51 ± 0.05 | 24.27 ± 0.27 | 24.11 ± 0.11 | 27.12 ± 0.07 | 27.39 ± 0.23 | 30.53 ± 0.42 |
| O111:H- | 22.89 ± 0.16 | 22.75 ± 0.14 | 26.69 ± 0.09 | 25.88 ± 0.40 | 29.50 ± 0.11 | 29.86 ± 0.73 |
| O121:H19 | 19.74 ± 0.12 | 23.42 ± 0.12 | 23.18 ± 0.15 | 26.67 ± 0.07 | 26.12 ± 0.29 | 30.43 ± 0.14 |
| O145:H18 | 20.24 ± 0.09 | 25.92 ± 0.10 | 23.81 ± 0.07 | 30.22 ± 0.11 | 27.12 ± 0.20 | 33.40 ± 0.34 |
| | 10 ³ | | 10 ² | | Efficiency (%) | R ² |
| | 10% | 8% | 10% | 8% | | |
| O26:H11 | 31.53 ± 1.31 | 32.04 ± 0.30 | ND | 36.39 | 96 | 0.993 |
| O45:H2 | 29.38 ± 0.25 | 30.48 ± 0.55 | 32.30 ± 0.75 | 35.38 ± 2.15 | 91 | 0.987 |
| O103:H3 | 30.42 ± 0.22 | 32.85 ± 0.23 | 33.78 ± 0.28 | 34.23 | 89 | 0.997 |
| O111:H- | 32.23 | 31.5 ± 0.91 | 35.62 ± 0.37 | 35.39 ± 0.19 | 96 | 0.997 |
| O121:H19 | 29.85 ± 0.59 | 34.82 ± 0.51 | 32.27 ± 0.81 | 35.93 ± 2.41 | 92 | 0.993 |
| O145:H18 | 30.46 ± 0.15 | ND ^b | 32.84 ± 0.60 | ND | 97 | 0.99 |

^avalues represent Ct value ± SD

^bND denotes not detected

Table 4. Quantitative PCR recovery of STEC fixed cell

| Serotype | Average cell recovery (%) | CV [#] (%) |
|----------|---------------------------|---------------------|
| O26:H11 | 107 ± 11 | 10.36 |
| O45:H2 | 101 ± 5 | 5.24 |
| O103:H3 | 108 ± 6 | 5.97 |
| O111:H- | 97 ± 6 | 5.93 |
| O121:H19 | 95 ± 16 | 16.82 |
| O145:H18 | 107 ± 3 | 3.16 |

[#]CV denotes the coefficient of variation (CV = standard deviation: average x 100)

*Limit of detection among all serotypes 272 ± 342 cells/g

Table 5. Log cells/mL evaluation with phase contrast microscopy, flow cytometry, and quantitative PCR

| Serotype | Log cells/mL | | | | | |
|----------|--------------|------|------|---------|------|------|
| | Trial 1 | | | Trial 2 | | |
| | PC | FC | qPCR | PC | FC | qPCR |
| O26:H11 | 5.60 | 4.51 | 5.04 | 6.43 | 6.25 | 6.41 |
| | 4.60 | 3.48 | 4.85 | 5.43 | 5.16 | 5.18 |
| | 3.60 | 2.62 | 3.97 | 4.43 | ND | 4.51 |
| O45:H2 | 6.49 | 5.77 | 6.24 | 6.20 | 6.70 | 6.49 |
| | 5.49 | 4.65 | 5.55 | 5.20 | 5.38 | 5.31 |
| | 4.49 | 4.88 | 4.40 | 4.20 | 4.40 | 4.22 |
| O103:H3 | 5.90 | 4.84 | 5.38 | 6.11 | 5.91 | 6.49 |
| | 4.90 | 3.85 | 4.50 | 5.11 | 4.88 | 5.39 |
| | 3.90 | 2.99 | 5.61 | 4.11 | 3.78 | 4.56 |
| O111:H- | 6.58 | 5.67 | 6.44 | 6.34 | 6.29 | 6.12 |
| | 5.58 | 4.75 | 5.39 | 5.34 | 5.20 | 5.21 |
| | 4.58 | 3.73 | 4.79 | 4.34 | 4.23 | 4.13 |
| O121:H19 | 6.62 | 4.73 | 4.91 | 6.18 | 6.54 | 6.63 |
| | 4.62 | 3.60 | 3.97 | 5.18 | 5.16 | 5.37 |
| | 3.62 | 3.26 | 3.04 | 4.18 | 4.40 | 4.42 |
| O145:H18 | 6.28 | 5.48 | 6.32 | 6.23 | 6.50 | 6.30 |
| | 5.28 | 4.52 | 5.31 | 5.23 | 5.27 | 5.25 |
| | 4.28 | 3.54 | 4.39 | 4.23 | 4.11 | 4.21 |

*Three ten-fold dilutions for each serotype were evaluated for each detection method

PC - Phase contrast microscopy for cell approximation

FC - Flow cytometry (RAPID-B[®])

qPCR - quantitative PCR

ND – not detected

IV. Conclusion

Pathogenic *E. coli* known as Shiga toxin-producing *E. coli* (STEC) have been and continue to be associated with various food products including ground beef. The regulatory requirements and the adulterant status of several STEC serogroups should be considered for assay development and improvements, and as certain STEC serogroups become more prevalent these will also likely become declared adulterants. Molecular assays continue to be the primary means of STEC detection, and immuno-based detection methods have also gained considerable attention for future developments. Optimizing detection methods in foods have been focused on more prompt and accurate analysis. Immunoassay antibody capture systems and flow cytometry platforms have been implemented into several food-based detection systems. By applying antibodies that will interact with target microorganisms, immunoassays can be used to directly detect and quantify pathogens. Immuno-based protocols could potentially be further implemented into the food industry, limit the duration of the detection process, and increase accuracy. Several STEC are considered adulterants in raw, non-intact ground meat, and several detection assays for these microorganisms have been investigated. The objective of the research in this thesis was to determine the efficacy of formalin-fixed STEC cells as a potential means to calibrate various detection assays. Eight and ten percent formalin-fixed cells were compared with quantitative PCR (qPCR), and the results suggest that the ten percent formalin fixed cells were a more sufficient means to quantitative cell populations. These results were based on the approximation of cells/mL by means of phase contrast microscopy. Fixed cells were spiked into ground beef samples, and the recovery rate for each STEC serotype was determined by qPCR. Additionally, the log cells/mL of spiked samples were determined by qPCR and flow cytometry. The log cells/mL evaluated for each assay were determined to not be statistically significant,

which suggests that formalin fixed cells could be used across a wide range of detection platforms (immuno-based versus molecular). Commercial detection kits could potentially implement formalin-fixed cells as a more standardized positive control to calibrate detection platforms simultaneously as well as among laboratories.

V. a. Appendix



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Office of Research Compliance

July 11, 2013

MEMORANDUM

TO: Dr. Steven Ricke

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 14002

Protocol Title: "Comparison of shiga-toxin Escherichia coli (STEC) detection limit between multiplex PCR and flow cytometry"

Approved Project Period: Start Date: July 11, 2013
Expiration Date: July 10, 2016

The Institutional Biosafety Committee (IBC) has approved Protocol 14002, "Comparison of shiga-toxin Escherichia coli (STEC) detection limit between multiplex PCR and flow cytometry" You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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Thank you.
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May 1

Dean, Clare <Clare.Dean@tandf.co.uk>

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