Abstract

Thomas Matthew Taylor: Development of an Alternative Method to Concentrate *E. coli* O157:H7 From Meat Homogenates to Facilitate Detection by Cultural and Molecular Methods (Under Direction of Dr. MaryAnne Drake)

Escherichia coli O157:H7, first identified in 1982, is linked to approximately 73,500 cases of foodborne illness per year. Sensitive and efficient methods to detect this organism are crucial. Current bacterial concentration methods from foods, such as centrifugation and filtration, are problematic, often co-precipitating or becoming clogged with food particulates. Metal hydroxide concentration of bacterial cells has gained increased recognition as a new alternative method to non-specifically concentrate bacteria from foods (Lucore et al., 2000; Cullison and Jaykus, 2002). The objective of this research was to develop and optimize the application of a metal hydroxide concentration method for recovery of viable *E. coli* O157:H7 from ground beef with subsequent polymerase chain reaction (PCR) detection. Hypotheses that metal hydroxide concentration would lead to increased cell recoveries compared to controls and that subsequent detection by PCR would gain higher detection sensitivity were tested for *E. coli* O157:H7 in autoclaved and raw ground beef samples.

In the study, 10 g autoclaved and raw ground beef samples were artificially contaminated with *E. coli* O157:H7 cells at 10^2, 10^4, or 10^6 CFU/ml; autoclaved and raw ground beef samples were also seeded with a low level inoculum (1 CFU/g beef) and allowed to incubate to previously mentioned inoculum levels. Bacterial concentration consisted of two primary steps: centrifugation (primary concentration) and metal hydroxide immobilization (secondary concentration). PCR primers targeted a 254-bp portion of the *slt*-II gene. Southern hybridization was conducted

on nylon membranes to confirm presence of DNA amplicons and gain higher detection limits. Recoveries exceeded 70% for cooked beef samples without growth and 57% for cooked beef samples with growth. Further, recoveries for raw ground beef samples exceeded 66% for samples concentrated without growth and 55% for samples concentrated after growth in a selective medium. In all cases, loss to supernatant did not exceed 20%. Overall, there were no statistically significant differences in samples concentrated with the metal hydroxide and samples concentrated with a saline control. The PCR detection limit effectively was 10^2 CFU/ml for all samples except raw, no growth cell concentrates. Southern hybridization afforded confirmation and an increase in detection limits for all samples except from growth, raw beef cell concentrates.

Data indicate that model food systems may not always be accurate indicators of a bacterium's behavior in a food matrix. Future research is recommended to investigate the mechanisms by which bacterial cells are less readily recovered following growth in a food.

Development of an Alternative Method to Concentrate *E. coli* O157:H7 From Meat Homogenates to Facilitate Detection by Cultural and Molecular Methods

By

THOMAS MATTHEW TAYLOR

A thesis submitted to the Graduate Faculty of
North Carolina State University in partial fulfillment of the
requirements for the Degree of Master of Science

FOOD SCIENCE

Raleigh

2003

APPROVED BY:

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(Advisory Committee Chair)

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Biography

Thomas Matthew Taylor was born in Winston-Salem, NC on September 19, 1978. Upon graduation from Forbush High School in East Bend, NC, he entered NC State University, a student in Food Science and Sociology. He completed his B.S. degree in Food Science in May of 2000 and his B.A. degree in Sociology in December of 2000. He then began his M.S. degree in the Food Science Department in January of 2001 under the direction of Dr. MaryAnne Drake, Department of Food Science, NCSU. Upon completion of his M.S. degree he entered the University of Tennessee in Knoxville, TN, to begin working on a Ph.D. in Food Science.

Acknowledgements

The author sincerely wishes to express his gratitude to those who have significantly contributed to his completion both of the M.S. degree and this Thesis. To his family, thank you for your constant support and encouragement that allowed me to remain focused and determined to complete this work. To the many friends in the Food Science Department, in particular the members of the Drake and the Jaykus lab, the author wishes to truly express his thanks for their support and their guidance. Without their support and care, the author would not have finished this work. To his graduate committee, Drs. Drake, Jaykus, and Ward, thanks are due for the countless hours of instruction, guidance, encouragement given to him in order to help him attain this degree. Finally, the author wishes to thank the Lord God in Heaven, whose love and presence in his life has given him meaning and made this degree possible.

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Chapter 1: Comprehensive Literature Review on *E. coli* O157:H7 and Existing Methods for Concentration and Molecular Detection

Introduction

Escherichia coli O157:H7, a gram-negative bacterial pathogen, has emerged in recent years as a serious threat to the safety of the U.S. food supply. First identified as a human pathogen in 1982, it has been epidemiologically linked to a number of disease syndromes including diarrhea, hemmorhagic colitis, hemolytic-uremic syndrome, and thrombotic thrombocytopenia (Riley et al., 1983). While the exact prevalence of *E.* coil O157:H7 is not known precisely, Mead et al. (1999) estimated that approximately 73,500 cases of foodborne disease attributable to *E. coli* O157:H7 occur in the U.S. every year. Lindqvist et al. (1998), utilizing different molecular detection methods, estimated that approximately 4.0% of all imported and domestic beef was contaminated with EHEC species in Sweden.

There exists an established bovine reservoir for this environmentally ubiquitous organism and for this reason dairy and beef products have been shown to be at increased risk for transmission of this organism (Anon., 1994; Chapman et al., 1993; Griffin and Tauxe, 1991; Hancock et al., 1994). Researchers have demonstrated the long-term survival of EHEC strains in bovine feces and food samples at low temperatures, thus reinforcing the need for extreme care in the processing of bovine food products (Fukushima et al., 1999; Palumbo et al., 1997; Wang et al., 1997). Other foods have also been implicated in foodborne outbreaks involving *E. coli* O157:H7 including unpasteurized fruit juices, contaminated water, raw sprouts, semi-dry sausage, and other non-processed vegetables grown with fertilizer consisting of bovine feces (Wells et al., 1991; Cieslak et al., 1993; Anon., 1994; Anon., 1995; Chapman et al., 1993; Hancock et al., 1994; Paton et al., 1996;

Keene et al., 1997; Anon., 1999; Cody et al., 1999; Hara-Kudo et al., 1999; McDonough et al., 2000). Person-to-person transmission (fecal-oral transmission) has been established as a secondary transmission mode (Griffin and Tauxe, 1991; Reida et al., 1994). Further, several outbreaks from contaminated drinking and swimming water have been documented (Dev et al., 1991; Keene et al., 1994; Ackman et al., 1997). Researchers have also established the ability of *E. coli* O157:H7 to remain viable for long periods in cool waters and to possibly enter the viable but non-culturable state (VBNC) (Rigsbee et al., 1997; Wang and Doyle 1998).

E. coli O157:H7 belongs to a group of verotoxin-producing *E. coli* species deemed Enterohemorrhagic *E. coli* (EHEC), one of multiple groupings of *E. coli* species that are infectious to humans. Examples of non-*E. coli* O157:H7 EHEC strains isolated from humans and animals on the basis of the lipopolysaccharide (LPS) antigen (O) include O91 and O111 (Beutin et al., 1993; Beutin et al., 1995), O104:H21 (Feng et al., 2001), O113:H2 (Gyles et al., 1998), and O157:H^- (non-motile) (Karch and Bielaszewska, 2001). It has also been reported that exposure to non-O157 EHEC strains occurs more frequently than does exposure to *E. coli* O157:H7 (Tarr and Neill, 1996). Furthermore, it has been suggested that testing schemes that are more inclusive of various EHEC strains would improve the capability to effectively diagnose and treat diseased patients (Acheson, 2000).

EHEC Virulence and Pathogenesis

Shiga-toxin and Shiga-like toxin

There are multiple virulence and pathogenesis markers for EHEC strains, the primary one being the production of Shiga-like toxins 1 and 2 (SLT1; SLT2). Shiga-like toxin, a protein toxin, is highly similar to Shiga toxin (Stx) produced by *Shigella dysenteriae*, another gram-negative bacterial pathogen (Granum et al., 1995). Active portions on each toxin are highly homologous (Tesh and O'Brien, 1991). The holotoxin is phage encoded and consists of one A-subunit and a pentamer of B-subunits surrounding the A-subunit that aid in binding of the toxin to host cells (Scotland et al., 1983; Gruskin, 2000). The toxin is actively produced during the cell's vegetative state and is secreted throughout the ileum upon infection, and it is deemed heat-labile (Granum et al., 1995)

The toxin binds to the host cells, in many cases host endothelial cells, via glycosphingolipid receptors termed Gb3 receptors and it is thought that the toxin enters the cell through a receptor-mediated endocytosis process (Gruskin, 2000; Tesh and O'Brien, 1991). The toxin inhibits protein synthesis through cleaving a specific adenosine residue on the eukaryotic cell 28S rRNA (Law, 2000). In response to the toxin, inflammation mediators produced by the cell, Interleukin 1 (IL-1) and Tumor Necrosis Factor (TNF-α) are released. Interestingly, it has been demonstrated that the release of these mediators leads to an upregulation of the cellular Gb3 receptors, thus leading to an increase in toxin binding (Gruskin, 2000; Remuzzi, 2000). This cytotoxic response of host cells may constitute a possible explanation for the low infectious doses observed in many foodborne disease cases.

Despite our knowledge of the genetic sequence of the SLT's and their mechanism of action, there are still factors that limit our understanding (Calderwood

et al., 1987; Law, 2000). The most important of these limitations is the absence of a useful animal model for the study of EHEC genetics and pathogenesis (Li et al., 2000). Variants of Stx2 beyond SLT2 have been identified and scrutinized (Sung et al., 1990; Beutin et al., 1995; Paton and Paton, 1998). Currently, in situations where multiple variants of the toxin are involved, the individual contributions of each are just becoming known. Ludwig et al. (2001) reported a significantly higher percentage of HUS cases with detectable *Stx*II antibodies then with antibodies to *Stx*I. Furthermore, bacterial factors influencing the production of toxin have been demonstrated in an experiment using parent EHEC strains and *E. coli* K-12 cells with the toxin phage (Wagner et al., 1999). Lysogens of Shiga-toxin (Stx2) bacteriophage were created with K-12 strains and subsequent lysogens were placed in five distinct groups based on random fragment-length polymorphism (RFLP) analysis. These authors concluded that the genotype of the Shiga-toxin gene-carrying prophage might influence the degree of toxin expression; the prophage consequently exerts some influence over the corresponding EHEC strain.

Attachment and Effacement of EHEC

Another virulence marker of potentially equal significance is that of bacterial attachment/effacement (A/E). McDaniel and Kaper (1997) first termed the 35.6 kb chromosomal island necessary for the encoding of proteins used in the attachment of the bacterium to the host cell as the Locus of Enterocyte Effacement (LEE). Perna et al. (1998) sequenced the *E. coli* O157:H7 LEE and described it to be slightly larger than the LEE of enteropathogenic *E. coli* (EPEC) described by McDaniel and Kaper (1997). Based on molecular analyses of the LEE for EHEC

and EPEC strains, researchers have determined that the LEE was horizontally transferred from another bacterial species, although how and when remains unclear (Boerlin, 1999). The LEE encodes three main sets of products. The first is a set of proteins necessary for inducing signal transduction in host cells. The second set of genes encode proteins that constitute a Type III secretion system necessary for bacterial protein secretion, including products of the signal transduction proteins produced in the first region of the LEE. Finally, the third primary set of products of the LEE is intimin and Tir (Law, 2000). The *eae* gene encodes both and both function to provide intimate attachment capability to the EHEC cell. Tir (translocated intimin receptor) functions by entering the host cell, fixing itself to the outer membrane of the host cell, and binding to intimin on the bacterial membrane. The ability to intimately attach has been demonstrated in HEp-2 cells and has been strongly associated with virulence in both bovine and porcine models (McKee and O'Brien, 1996; Donnenberg et al., 1993; Paton and Paton, 1998). Gyles et al. (1998) demonstrated the significance of intimin by demonstrating that 92% of isolates defined as being commonly associated with foodborne disease expressed the *eae* gene versus 27% of isolates not commonly associated with human disease. Beutin et al. (1995) claimed that intimin was consistently less common in healthy animals versus diarrheagenic animals, demonstrating the significance of intimin in bacterial pathogenesis. Finally, researchers have demonstrated the presence of five intimin variants based on PCR and sequence analysis throughout EPEC and EHEC spp., including *E. coli* O157 (Oswald et al., 2000). These variants share a common function but differ at the 3' terminal end, which potentially leads to problems in

detection and characterization of pathogens by researchers. Furthermore, it is speculated that the presence of intimin variants allows attachment to a variety of host tissues, increasing the tropism of the bacterium (Tzipori et al., 1995; Oswald et al., 2000).

Bacterial adhesion is not afforded only through intimate attachment. The LPS O-antigen may play an indirect role in the adhesion of the bacterium (Law, 2000). Some researchers have shown that loss of the O-antigen resulted in increased adherence to the host cell through a possible upregulation of other outer membrane proteins (OMP's) (Bilge et al., 1996; Cockerill et al., 1996; Paton and Paton, 1998). Furthermore, an LPS toxin was claimed to produce an additive effect on the inhibition of host cell protein synthesis when introduced with SLT; an overall synergistic effect resulting in loss of host cell viability was also seen (Louise and Obrig, 1992).

There also exist outer membrane proteins and polysaccharide structures produced by EHEC strains. Zhao et al. (1996) used mutants lacking various OMP's to demonstrate a decreased ability to adhere to host cells. Other research has demonstrated that OMP-specific antibodies were effective at inhibiting the adherence of EHEC strains to host cells (Sherman and Shoni, 1988; Tarr et al., 1995). An exopolysaccharide (EPS) structure produced under anaerobic conditions by EHEC strains has been correlated with increased adhesion to human HEp-2 cells (James and Keevil, 1999). This EPS was composed partially of uronic acid and was deemed chemically similar to colanic acid. Furthermore, these researchers found that upon washing of cells in the chemostat environment, adhesion was significantly

decreased. This loss in adhesion was presumed to be reflective of the loss of the EPS layer.

α-Hemolysin

Many EHEC species produce hemolysis upon infection via hemolysins. Among these, α-hemolysin is likely the best characterized; the protein is encoded by four genes linked as cistrons (*hlyA, hlyB, hlyC, hlyD*) (Welch and Pellett, 1988). Secretion is mediated by proteins encoded on the *hlyB* and *hlyD* genes (Beutin, 1991). Furthermore, an OMP encoded by a gene outside this operon is also required for secretion of the hemolysin (Wandersman and Delepelaire, 1990). This hemolysin is structurally similar, yet not identical, to the enterohemolysin discussed later (Schmidt et al., 1995). In active form, the hemolysin is not stable and is influenced by pH, temperature, and media components (Beutin, 1991). Beyond the lysis of erythrocytes, α-hemolysin also retains cytotoxicity for leukocytes and fibroblasts (Cavalieri and Snyder, 1982a; Cavalieri et al., 1984). Cavalieri and Snyder (1982b) demonstrated that cells exposed to the hemolysin showed decreased ability to phagocytize particles and to perform chemotaxis. Other studies involving recombinant hemolysin-encoding plasmid insertion into non-hemolysin *E. coli* strains have confirmed that α-hemolysin constitutes a virulence factor for EHEC species: after insertion these cells became 10-1000x more virulent than parent strains (Welch, 1991). The protein is often seen in strains associated with extraintestinal infections in humans and urinary tract infections in dogs and humans (Cavalieri et al., 1984; Beutin, 1991). The protein can be produced under both aerobic and anaerobic conditions and is resistant to 1% formalin. Furthermore,

treatment with DNAse, RNAse, lecithinase, and lysozyme results in no loss of hemolytic activity (Cavalieri et al., 1984). However, lipases and urea have been shown to degrade activity of the hemolysin (Cavalieri et al., 1984).

Enterohemolysin & the pO157 Plasmid

Several EHEC species harbor a 60 MDa plasmid whose function is as of yet not wholly elucidated. While the plasmid was at one time believed to have some influence over attachment of the organism to host cells (Karch et al., 1987; Tzipori et al., 1987), it has since been demonstrated that this is not the case (Junkins and Doyle, 1989; Tzipori et al., 1989). These researchers demonstrated that EHEC strains without the plasmid were equally able to adhere and produce lesions on mucosal cells as those possessing the plasmid. The plasmid does contain an operon encoding an enterohemolysin, similar to the operon-encoded α-hemolysin found in EHEC species (Schmidt et al., 1995). Schmidt et al. (1995) sequenced this gene (*ehxA*) in EHEC species and observed the phenotype on blood agar plates containing washed, defibrinated sheep erythrocytes. These authors concluded that a possible synergism exists between SLT and enterohemolysin in the disturbance of host cell activities based upon immune response studies and the presence of enterohemolysin in SLT-producing *E. coli* species. Beutin et al. (1989) claimed that enterohemolysin activity can be observed only on blood agar using washed erythrocytes and only on stationary cells after 24 hours incubation (Beutin, 1991).

Physical Characteristics: Stress Response

Introduction to EHEC

EHEC species are extremely hardy organisms capable of withstanding a variety of processing parameters and environmental conditions. For example, it has been shown that EHEC species are capable of withstanding acid to the point of pH=2.5 (Lin et al., 1996). *E. coli* O157:H7 grows well at a temperature range of 30-42°C with short generation times (Doyle and Schoeni, 1984). The organism will not grow well at 45°C. It is quite able to withstand cold storage conditions in food products yet it is not extremely heat stable; proper cooking/pasteurization should ensure its inactivation (Padhye and Doyle, 1992).

Acid Stress Response

Besides virulence markers necessary for infection, EHEC strains also encode proteins necessary for bacterial survival in response to various environmental stresses. One of the most understood, and likely the most important for bacterial survival within the host, is the acid response. Protection from acid-induced stress is obviously vital for survival after ingestion and passage through the human small intestine to reach the colon, the desired colonization region. EHEC strains depend on three acid-response mechanisms: an acid response system in oxidatively metabolizing cells, a glutamate-dependent system, and an arginine-dependent system (Law, 2000). All are upregulated by the induction of the *rpoS* gene that encodes σ^S, a general stress response sigma factor. All will protect the *E. coli* cell at a pH=2.5 (Lin et al., 1996). However, only the glutamate and the arginine system are induced during anaerobic conditions and only in the presence of either arginine or glutamate (Lin et al., 1996). Lin et al. (1996) further demonstrated the inability of the cellular general response mechanism to effectively handle weak acids. In tests

with benzoic acid and a weak organic acid cocktail (acetic, butyric, and propionic), the general stress response was ineffective. Lin et al. (1996) also described the ability of EHEC cells to maintain acid resistance once placed in cold storage; this constitutes another example of how resistance mechanisms provide a blanket of protection for bacterial cells rather than protection against a single stress.

Acid resistance is afforded to the *E. coli* O157:H7 cell through the *rpoS* gene and RpoS sigma factor (σ^S). Sigma factors are small proteins that bind to RNA polymerase and then force the enzyme to bind at specific promoter sequences of the chromosome. The enzyme binds to the promoter and begins transcription (Abee and Wouters, 1999). The *rpoS* gene encodes over 30 general stress proteins including proteins protecting against environmental acid and giving increased resistance to other environmental stresses such as heat, osmotic pressure, and salt (Abee and Wouters, 1999). On the other hand, research indicates that the *rpoS* gene is quite mutable and the presence of alleles has been established with varying degrees of acid resistance being observed (Waterman and Small, 1996). This variance in acid resistance might also have implications for virulence in that cells with greater acid-sensitivity may have higher infectious doses than do more acid-resistant cells (Buncic and Avery, 1998; Waterman and Small, 1996).

Resistance of EHEC to various organic acids commonly used to preserve foods, notably lactic, acetic, and citric acid, has been investigated and demonstrated (Abdul-Raouf et al., 1993). Furthermore, other research has claimed that organic acids such as lactic acid may be more effective at increasing lag-phase growth and inactivation than inorganic acids such as HCl when at equal concentrations (Glass et

al., 1992). McKellar and Knight (1999) reported similar results when testing acetic acid and HCl at equal concentrations. Other researchers have claimed that processing hurdles such as NaCl, anaerobic conditions, and temperature can affect the time to development of acid resistance (Cheng and Kaspar, 1998). These researchers demonstrated that anaerobic conditions led to more rapid onset of acid resistance. Storage of cells at refrigeration temperatures in synthetic rumen fluid did not significantly affect survival or acid resistance, with acid resistance only being decreased after pre-incubation at 15ºC for 4 h (Cheng and Kaspar, 1998). On the other hand, Guraya et al. (1998) demonstrated decreased survival of *E. coli* O157:H7 cells following inoculation in various dairy products and storage at 4ºC and 12ºC at various pH, salt, and diacetyl concentrations. Together, these studies indicate that even with proper processing and storage of processed foods, it remains possible for *E. coli* O157:H7 to survive in various food products.

Heat Stress Response

Heat constitutes the second primary stress present in the human body against which the EHEC cell must protect itself. It has been shown that heat tolerance in an EHEC cell is raised after exposure to sub-lethal temperatures, due in part to the σ^{32} sigma factor, a heat-stress sigma factor (Abee and Wouters, 1999). Other researchers have shown that following heat shock, *E. coli* O157:H7 heat resistance is increased as shown by an increase in D-values compared to non-heat shocked cells (Williams and Ingham, 1997). The gene encoding this sigma factor encodes multiple heat-shock proteins (HSP's); these proteins perform primarily two functions. Chaperones assist the cell by maintaining the native folding of many

proteins subject to denaturation and thus help the protein maintain its functionality (Abee and Wouters, 1999; Law, 2000). Proteases recycle denatured proteins for other uses and for later reconstruction by other proteins after a return to non-stressful conditions (Abee and Wouters, 1999; Law, 2000).

Cold Stress Response

Cold stress is often incurred during post-processing storage of ground beef and other beef products. EHEC strains have also developed methods to maintain cell viability during refrigeration and freezing conditions. Cold shock proteins (CSP's) have been shown to increase the concentration of unsaturated fatty acids present in the cell membrane (Abee and Wouters, 1999). Further, some CSP's assist DNA in maintenance of supercoiling and thus help to regulate gene expression (Law, 2000). Studies have investigated the ability of bacterial cells, including EHEC, to maintain resistance to cold conditions after an initial exposure. Doyle and Schoeni (1984) demonstrated increased resistance to refrigeration/freezing after an initial sub-lethal exposure to cold conditions. However, despite increased resistance to cold conditions, it has been shown that cells remain in the lag phase longer and require extended enrichment conditions to culture and type (Uyttendaele et al., 1998).

Cold storage does not seem to negatively affect the expression of virulence factors: Buncic and Avery (1998) found that production of SLT at 37ºC was not significantly reduced in cells previously stored at 4ºC for four weeks in a nutritious medium. Furthermore, starved cells put in cold storage increased their production of SLT after incubation at 37ºC for 12 h. Similarly, previous storage at cold

temperatures can increase resistance to heat stress/shock (Ansay et al., 1999; Jackson et al., 1996). Ansay et al. (1999) reported that *E. coli* O157:H7 populations were not significantly reduced with storage at refrigeration and freezing temperatures for multiple weeks. Jackson et al. (1996) reported increased resistance of *E. coli* O157:H7 cells inoculated into ground beef patties to heat shock after storage at freezing temperatures.

Biochemical Typing Methods for *E. coli* O157:H7

Over the years, researchers have devised a number of methods to biochemically identify *E. coli* O157:H7 as well as other EHEC strains. These methods all share common advantages and disadvantages in relation to their use. Through their repeated use over the years, they have become refined and proven. In many cases, reagents used are relatively inexpensive. Furthermore, many of these methods include pre-enrichment and enrichment steps to gain the necessary concentration of cells to gain positive results as well as to allow injured organisms to resuscitate (Lantz et al., 1994a). However, these methods also have disadvantages associated with their use. The primary problem with many of these methods is that they often require in excess of five days to gain final results; in many foodborne disease cases, the infection has passed and the patient has recovered before medical personnel have identified the organism responsible. Many of these organisms are labor intensive to detect and require some degree of experience at interpreting results that are less than clear (Lantz et al., 1994a).

One of the primary media used to identify EHEC strains is Sorbitol MacKonkey Agar (SMAC): EHEC strains are widely unable to ferment sorbitol within

24 h. Again, pre-enrichment and enrichment are often necessary prior to the use of this medium to increase cell numbers and assure accurate testing. This media is not a foolproof method for detecting and differentiating EHEC; Gunzer et al. (1992) demonstrated that some *E. coli* O157 strains were able to ferment sorbitol within 24 hours (Paton and Paton, 1998).

E. coli O157:H7 reportedly does not encode the enzyme β-glucuronidase, capable of reacting with 4-methylumbelliferyl-D-glucuronide (MUG) and producing a fluorescent product. Yet again, multiple steps are required for this test as for other tests and *E. coli* O157 strains have been identified which do encode the enzyme of interest (Gunzer et al., 1992; Paton and Paton, 1998). Beutin et al. (1989) reported that many EHEC strains contained the gene encoding a hemolysin that produced small turbid zones on washed erythrocyte sheep blood agar after 24-hour incubation at 37°C.

Karch and Bielaszewska (2001), in a review of *E. coli* O157:H^- strains, further described a set of Shiga-toxin producing strains capable of sorbitol fermentation within 24 hours and exhibiting β-glucuronidase activity. These strains constituted a unique subset of EHEC strains due to proposed differences in reservoirs and transmission vehicles. These non-motile strains could not be typed by their flagellar antigen, but they did contain the necessary *fliC* gene as demonstrated by RFLP typing (Bielaszewska et al., 2000). Of strains typed, all but one contained *stx*2 as the sole SLT producing gene. Other virulence markers such as the *eae* gene were shared with motile EHEC strains. However, these strains constitute not only a new potential disease threat but also a serious obstacle to correct diagnosis.

In the past few years, researchers have sought to update these conventional methods and decrease the time necessary for positive results. An overnight plating method using HC media (Tryptone, bile salts, NaCl, Sorbitol, MUG, and Bromocresol Purple) has been developed with a mean 96.5% recovery possible after incubation at 44.5°C (Szabo et al., 1986). However, as previously discussed, *E. coli* O157:H7 isolates that are sorbitol fermenters and that produce β-glucuronidase have since been identified, thus reducing the effectiveness of this medium to correctly estimate bacterial loads. Weagant et al. (1995) developed a rapid biochemical method devised from several existing biochemical tests with results obtainable in 30 hours or less. However, this method suffers a loss of sensitivity when testing for the presence of cold-stressed organisms, likely due to the time increase in lag-growth phase. Hudson et al. (2000) developed an EHEC agar that exhibited high sensitivity in milk products but decreased sensitivity in fermented meat products.

Antigen/Antibody Binding Detection Assays

Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) constitutes a heterogeneous immunoassay in that unbound antibody in the reaction well must first be removed before the enzyme/substrate reaction for confirmation of the antigen's presence can be performed (Barbour and Tice, 1997). There are multiple ELISA formats currently available commercially. Simply, antibodies and antigen are mixed and depending on the reaction format, the presence or absence of an enzyme/substrate product may be interpreted as a positive result indicating the antigen's presence. ELISA methods feature a number of positive aspects including their ease of use, improved sensitivity

over biochemical (phenotypic) methods, decreased time for detection, variety of formats, and the use of enzymes versus radioactive isotopes (Hornbeck et al., 1991). However, ELISA reactions have been reported to be inhibited by food matrices and other cross-reactants (Vernozy-Rozand, 1997; Weeratna and Doyle, 1991). Multiple ELISA polyclonal antibody assays have been developed for the detection of Shiga-like toxin from *E. coli* (Basta et al., 1989; Downes et al., 1989; Acheson et al., 1990). However, these methods have been replaced by more sensitive ELISA methods that require smaller sample volumes and have improved detection limits with reduction in the occurrence of false positive results (Milley and Sekla, 1993; Novicki et al., 2000).

Nucleic Acid Based Detection/Hybridization

DNA Hybridization

DNA hybridization methods make use of the relatively unchanging nature of bacterial DNA versus other methods that rely on gene expression. Target cells are first lyzed to release the DNA and RNA. After splitting into two single strands, a probe designed to be complementary to a target sequence is introduced into the reaction well. DNA, mRNA, or rRNA may act as a target in hybridization reactions. The target sequence is bound to an enzyme-labeled probe and is then removed by one of several methods. The mixture is washed to remove unbound DNA or RNA and the researcher proceeds to immobilize following a separation step (Southern blot or colony dot blot) (Barbour and Tice, 1997). The solution is then mixed with the enzyme/substrate to confirm the presence of the target nucleic acid sequence (Barbour and Tice, 1997). Advantages to hybridization methods include their use of

nucleic acids versus phenotypic markers and their increased specificity over other methods. However, they require increased time over other methods for enrichment procedures to gain the necessarily high numbers (10^5-10^7 CFU) for a clear signal (Karch et al., 1996; Barbour and Tice, 1997). Hybridization methods have been developed for the immobilization and detection of EHEC virulence factors including the Shiga-like toxins and intimin (Jerse and Kaper, 1991; Willshaw et al., 1992; Willshaw et al., 1994; Huck et al., 1995), with varying degrees of success. In tests using a probe designed for O157 EHEC species, cross-reactivity with 5 non-*E. coli* O157 species was also reported by Huck et al. (1995). These authors were only able to correct this problem with the addition of an overnight hybridization, thus increasing the time to detect. Other researchers have claimed that food matrices may reduce the test specificity, thus requiring culture methods for confirmation of all positive results (Mozola, 1997).

The Polymerase Chain Reaction (PCR)

From these methods has risen the polymerase chain reaction (PCR). These reactions utilize primers to amplify targeted portions of the DNA. Amplification occurs exponentially from a very small sample size, thus effectively allowing detection based on specificity of nucleic acid sequence. Like DNA hybridization methods, PCR relies on the relative stability of the DNA sequence versus the biochemical products of gene expression. PCR was first introduced in 1985 by researchers at Cetus and was first used to amplify portions of human DNA using the Klenow fragment of *E. coli* DNA polymerase I (Erlich et al., 1991). PCR was first utilized as a diagnostic tool in the detection of sickle cell anemia via amplification of

different β-globin sequences (Saiki et al., 1985). Researchers performed hybridization with labeled probes or restriction site analysis to visualize amplicons and differentiate between normal and mutant cells (Saiki et al., 1985; Mullis et al., 1987).

PCR constitutes an in vitro method for amplifying specific portions of the bacterial DNA using the sample nucleic acid, reaction buffer, target primers, Mg^{++}, deoxyribonucleoside triphosphates (dNTP's), and a thermostable DNA polymerase isolated from an extreme thermophile (Coen, 1994). The sample is heated to denature the DNA and allow the primers to anneal to the complementary sequence of interest. Upon annealing, the enzyme uses dNTP's to replicate new DNA. This sequence of events is cycled approximately 30 times to allow exponential amplification of the target sequence and is controlled by the concentration of reagents, reaction time, and environmental temperature (Kramer and Coen, 1999). Reaction products are then visualized using gel electrophoresis with ethidium bromide stain on various gel types such as agarose or polyacrylamide (Kramer and Coen, 1999).

One benefit of PCR is the short time necessary for results: after extraction of bacterial DNA, a PCR cycle can be completed in a matter of hours. Further, the method successfully amplifies a small sample of DNA and it is theoretically possible to get detection from a single strand of target DNA (Lantz et al., 1994a). Thus the reaction is extremely sensitive and can be engineered to be highly specific as well based on the selection of target and primer sequences (Scheu et al., 1998). This sensitivity can be invaluable to the food microbiologist working with food samples in

which there are only a few organisms present. PCR reactions can also be engineered to amplify multiple target sequences (multiplex) to assist in species confirmation or to identify multiple organisms. Furthermore, while in the past PCR reactions have required operators to have a fair degree of technical training, newer methods are being developed to make PCR a more user-friendly research tool. Shearer et al. (2001) reported on BAX, a commercially available PCR kit in which all reagents and enzymes come in tablet form premixed and ready to use, including negative and positive control samples.

Multiplex-PCR

Henegariu et al. (1997) defined multiplex PCR as the simultaneous amplification of two or more target sequences. They described reaction protocol changes that require modification of the uniplex PCR design due to the nature of the reaction including the extension temperature and time, annealing time/temperature, primer concentration, and dNTP concentration (Henegariu et al., 1997). Multiplex PCR must therefore be optimized from standard PCR conditions to give the best chances for amplification of multiple targets and clear product visualization. Furthermore, the chance that false positive results will appear increases in a multiplex PCR due to the increased use of primer pairs. This increase in primers can produce false positive results if primer pairs share homology. This problem must be counteracted by design of primer pairs that do not amplify sequences that are homologous to a high extent to retain specificity (Chamberlain and Chamberlain, 1994).

Multiplex PCR methods for EHEC strains are numerous and make use of a variety of virulence and pathogenesis markers. Pollard et al. (1990) utilized four primers based on the sequences of SLT-1 and SLT-2 and tested them on 40 SLT-producing *E. coli* strains along with 43 other isolates. All but two strains that produced a SLT-II variant that were scored as positive for SLT production by cytotoxicity assay gave matching positive results with multiplex-PCR. Gannon et al. (1992) described a method for the detection of SLT genes in *E. coli* obtaining a final sensitivity of 1 CFU/g. Cebula et al. (1995) used primers for the *uidA* gene, encoding β-glucruonidase, and the SLT's in a mismatch multiplex PCR. Multiplex methods for the detection of the *eaeA* gene, a portion of the 60 MDa-plasmid, and conserved regions of the SLT's have also been devised to control against false positive results that may occur with the use of any one primer pair (Fratamico et al., 1995; Deng and Fratamico, 1996; Reischl et al., 2002). In an analysis of 151 strains, Meng et al. (1997) demonstrated a multiplex-PCR for which *E. coli* O157:H7 could easily be differentiated from *E. coli* O55 strains. Gannon et al. (1997) amplified a portion of the flagellar (*fliC*) gene along with SLT and *eaeA*. Venkateswaran et al. (1997) used a filtration technique to remove PCR-inhibitory food particles prior to a multiplex-PCR for EHEC, and a plasmid-encoded hemolysin gene was amplified with the flagellar gene by Fratamico et al. (2000).

PCR Inhibitors

PCR reactions do have disadvantages associated with their use. There are multiple compounds and conditions that may inhibit the amplification reactions, giving false negative results. Rossen et al. (1992), using *L. monocytogenes* as a

source of template DNA plus various culture media and food samples, demonstrated that different food components exerted differing degrees of inhibition on PCR reactions. In the case of enterohemmorhagic *E. coli*, components of ground beef, bean sprouts, and oyster flesh have all been documented as inhibitory (Andersen and Omiecinski, 1992; Jinneman et al., 1995; Witham et al., 1996). Brian et al. (1992) reported that components in feces might inhibit PCR reactions. It has been reported that polyphenolic compounds in various fruit juices and plant products may also be inhibitory and should be considered when testing these types of food products (De Boer et al., 1995; Rafii and Lunsford, 1997; Ogunjimi et al., 1999). Koonjul et al. (1999) reported these compounds can form complexes with nucleic acids and successfully inhibited formation of these complexes with polyvinylpyrrolidone (PVP). Drake et al. (1996) and McKillip et al. (2000) reported PCR inhibitory compounds present in cheeses.

Stewart et al. (1998) reported that boiling of fecal samples prior to PCR constituted an efficient method of sample purification. Sample dilution, while potentially reducing reaction sensitivity, can also effectively dilute inhibitors to the extent necessary to gain evidence of amplification (Wilson, 1997). Furrer et al. (1991) reported that centrifugation represented a viable option for the removal of PCR inhibitors found in milk. Sub-culturing of food samples on selective agars and then using plated colonies for PCR reactions can reduce inhibition by food particulates (Golsteyn Thomas et al., 1991). Methods to remove the bacteria from the food sample, such as immuno-magnetic separation (IMS), effectively reduce the

inhibition of PCR reactions by food components and environmental sample components.

Components in food do not constitute the sole source of PCR inhibition: other non-food compounds have been shown to reduce the sensitivity of PCR. Detergents used in the lysis of bacteria to induce the release of DNA have also been demonstrated to be inhibitory to PCR (Weyant et al., 1990). Tryptic Soy Broth (TSB), a common enrichment medium for many species including EHEC, was found to be inhibitory to PCR reactions involving *Y. enterocolitica* (Lantz et al., 1998). Lantz et al. (1998) also reported that background micro-flora inhibited the detection of a desired strain when inoculated into TSB. Weaver and Rowe (1997) reported similar findings with respect to experiments testing the sensitivity of PCR to detect SLT-producing *E. coli* among large concentrations (~10^9 CFU/ml) of a non-pathogenic *E. coli* strain. These researchers hypothesized that a large amount of non-target DNA represented a physical barrier to the effective amplification of target DNA. Even laboratory equipment, such as gloves with powder, has been shown to be inhibitory to PCR reaction; plastic-ware and cellulose have also been implicated as inhibitors (Wilson, 1997).

Reverse Transcription PCR (RT-PCR)

Probably the primary problem with PCR is that it amplifies DNA directly only and it has been demonstrated that DNA can persist in a system or on a food sample long after the organism has lost viability (Allmann et al., 1995). Solutions to this problem have been devised. One method to eliminate this problem is to perform an enrichment step prior to the PCR reaction to differentiate viable cells from non-viable

cells. This has been shown to increase sensitivity by decreasing false positive results due to intact but dead cells (Lantz et al., 1994a; Scheu et al., 1998).

Another solution to the problem of amplification of DNA from non-viable cells is reverse-transcription PCR (RT-PCR). This variation of PCR allows a better estimation of the viability of a cell by amplifying bacterial RNA that has a significantly shorter half-life then DNA (Kushner, 1996). RT-PCR amplification methods have been developed for both rRNA and mRNA (McKillip et al., 1998; McKillip et al., 1999; Klein and Juneja, 1997; Sheridan et al., 1999). rRNA boasts high copy numbers in the cells and is better understood at the molecular level, thus making it easier to work with then mRNA (McKillip et al., 1998). Furthermore, while problems with the extraction of detectable amounts of mRNA have been reported, there is at least theoretically a greater range of possibilities for designing primers with a mRNA template then with rRNA (Sheridan et al., 1998; Sheridan et al., 1999). However, it has been shown that the physiological state of the cells can have effects on the sensitivity of a RT-PCR reaction.

Samples for RT-PCR amplification are prepared in a manner similar to PCR reactions. RNA is separated from DNA to prevent crossover contamination and the potential for inhibition. Researchers have utilized DNAse enzymes to digest DNA after precipitation of RNA (McKillip et al., 1998; McKillip et al., 1999). Klein and Juneja (1997), due to the instability of mRNA, included a stabilization step to preserve extracted *L. monocytogenes* mRNA prior to RT-PCR. Primers may be chosen on the basis of desired targets just as in PCR. RT-PCR reactions utilize dNTP's, chelating ions (Mg, Mn), sample RNA, a reverse transcriptase for the

formation of DNA from template RNA, and a thermostable polymerase. RT-PCR can be carried out as either a two-step or a one-step process. Sheridan et al. (1998) performed both procedures in the detection of *E. coli* rRNA and mRNA targets. In both cases, the procedure was comprised of an initial RNA-synthesis step and is followed by PCR amplification. One-step reactions were seen as beneficial in reducing the opportunity for cross-contamination and allowing all reverse-transcribed RNA to be subsequently available for amplification (Sheridan et al., 1998).

Despite the advantages of RT-PCR versus the conventional PCR reaction, there are inhibiting compounds that may lower the sensitivity of the reaction. The first of these may be DNA itself; DNA present in an mRNA sample may also be amplified during the reaction, thus potentially giving a false positive result (Simpkins et al., 2000), especially if one goal of the RT-PCR procedure may be to estimate cell viability. Further, while a few papers have been published describing RT-PCR inhibiting substances, there has been no focused effort at studying inhibitors specific to RT-PCR amplification. It has been shown that proteases (specifically proteinase K) are inhibitory to RT-PCR and PCR reactions (Engstrand et al., 1992). Scwab et al. (2000) developed an RT-PCR procedure for Norwalk-like Viruses and found that most inhibiting substances such as lipids in food samples were effectively rendered nil by dilution. Others have found that PCR enhancement agents could be used to combat the effects of background nucleic acid and other inhibitors (Jaykus et al., 1996). Another method to control for the effects of inhibitors is to add an internal control consisting of species RNA to amplification reactions to test for the sensitivity

of the primers and the presence of inhibitors (Kaucner and Stinear, 1998; Schwab et al., 2001).

Southern Blotting

Introduced by Southern (1975), Southern blotting involves the transfer of fragmentary DNA obtained from an electrophoresis gel onto a solid support as a precursor to hybridization. In the course of the blotting procedure, the DNA fragments are denatured, transferred to the solid support (most often either a nitrocellulose or nylon membrane), and ultimately immobilized using alkaline solutions, baking, or UV radiation (Brown, 1993).

The parameters of greatest interest include the amount of target DNA estimated to be present, the membrane, the transfer buffer and duration of transfer, and the method of transfer (Brown, 1993). The volume of target DNA present has a direct influence on the amount of DNA that must be loaded into the system. Nylon membranes, despite problems with high background noise, have been shown to be superior to the nitrocellulose membranes first used in the procedure. This superiority stems from the higher tensile strength of the nylon as well as the ability to covalently bind the DNA to the membrane resulting in stronger attachment and the ability to probe the DNA multiple times (Sambrook and Russell, 2001). Nylon membranes, versus nitrocellulose, are able to effectively bind DNA in the presence of buffers of varying acidity and ionic strength (Brown, 1993). The duration of transfer can be directly manipulated by the transfer method chosen. An ascending capillary transfer may need to run for 18 hours, while a descending capillary transfer may be completed within one to two hours (Lichtenstein et al., 1990). Vacuum

blotting, while requiring careful supervision of system pressure, can be completed in as little as 30 minutes (Brown, 1993).

Concentration/Removal Methods

Introduction

For the successful application of many of these rapid detection methods to food products, an effective bacteria concentration and removal step seems necessary. It has been repeatedly suggested that such a step would aid in the purification of target bacteria as well as prevent contamination of samples with reaction inhibitors (Swaminathan and Feng, 1994; de Boer and Beumer, 1999). Methods for the separation of bacteria from food products are rare and none are highly efficient and simple. A method that could effectively concentrate viable organisms from a food sample would have a significant advantage over conventional methods. Any method that has increased ability to remove bacterial cells from such a complex medium such as a food sample is superior to other methods. Furthermore, the ability to recover viable cells for later use would be beneficial to researchers who wish to serotype the organisms recovered and use them for further work. Methods such as centrifugation, filtration, immuno-magnetic separation (IMS), and bacterial immobilization have all been developed and tested in food products. These methods will be reviewed here.

Centrifugation

Centrifugation operates on the principal that rotational (centrifugal) forces are able to move large particles through a medium; this movement is dependent on particle size and density (Catsimpoolas, 1976). Differential centrifugation and

density gradient centrifugation are two methods commonly applied to food and environmental samples for the removal of bacterial cells (Cullison, 2000). Differential centrifugation functions primarily to separate cells on the basis of their size. Rodrigues-Szulc et al. (1996) applied this method to beef homogenates. Differential centrifugation has also been used in the removal of cells from soil samples (Jacobsen and Rasmussen, 1992). Density gradient centrifugation uses a liquid medium with an increasing density gradient. The sample is loaded on top of this medium and spun. Cells of similar size and density clump together tightly (Jacobsen and Rasmussen, 1992).

Other researchers have investigated the use of high-speed centrifugation to sediment bacterial cells. Fliss et al. (1991) concentrated *Lactobacillus* and *Lactococcus* species from pure culture at 10,000 x g with a high reported yield of DNA. Other researchers have concentrated cells out of blood specimens and growth media (Tjhie et al., 1994; Darby et al., 1970). However, this method has a tendency to sediment compounds that may be inhibitory to other detection assays such as IMS or PCR. Neiderhauser et al. (1992) increased the sensitivity of a PCR assay by 3 log cycles after first spinning a meat sample at a low rate to remove meat particles and then spinning at a higher rate to sediment the bacteria. Meyer et al. (1991) used this technique in a similar fashion to concentrate *E. coli* from soft cheese.

Centrifugation does have its problems: density gradient columns may not always be compatible with food samples that possess their own density gradient profile (Payne and Kroll, 1991). Jaykus (1993) observed that inhibitors are likely to

be transferred to the PCR reaction if centrifugation is the sole concentration method used. Foods that have large particles, such as fat globules, may trap bacteria during the spin and thus lead to erroneous estimates of cell concentration in a sample (Lantz et al., 1994b).

Filtration

Filtration methods are designed to separate cells based on solubility differences and size. In general, filtration methods have not been widely studied and developed for foods. Filtration methods have been paired with other detection/capture methods to remove and identify cells from a food sample. Wang et al. (1992) filtered cells from meat samples and then heated the cells to induce lysis for subsequent PCR amplification. These researchers claimed a detection of less than 10 CFU/g *L. monocytogenes* from artificially contaminated meat products, yet they could not get any detection of cells from inoculated soft cheese samples. Kirk and Rowe (1994) utilized polycarbonate membrane filters to remove *Campylobacter jejuni* and *Campylobacter coli* from water samples with a detection limit of 10 CFU/ml following enrichment and PCR. Venkateswaran et al. (1997) reported a sensitivity of 1 CFU/g of *E. coli* O157:H7 in beef slurry following a two-step filtration technique and PCR amplification. However, on the whole, filtration methods are largely unacceptable. Compounds inhibitory to PCR are sometimes filtered out along with the target bacteria; some filters have been shown to possess PCR inhibition properties; and filters may become clogged with large food particles thus rendering them useless (Oyofo and Rollins, 1993). In the case of the two-step

filtration technique, an 18-h enrichment was necessary to gain amplification at low inocula, thus increasing time to detection (Venkateswaran et al., 1997).

Immuno-Magnetic Separation (IMS)

Immuno-magnetic separation (IMS) functions on the principle of antibody-antigen binding. Antibodies specific to an antigen characteristic of an organism are chosen for assays. IMS protocols can utilize either monoclonal or polyclonal antibodies. Numerous IMS methods have been developed for the detection of foodborne pathogens from a variety of media, including pure culture, various food matrices, environmental samples, and clinical samples (Patchett et al., 1991; Fratamico et al., 1992; Fluit et al., 1993; Mitchell et al., 1995; Luk and Lindberg, 1991).

According to Ogden et al. (2000), the benefits of using IMS are two fold: the method has enhanced recovery of organisms from samples with a greater sensitivity when compared to alternative methods, and the method is relatively simple requiring little technical training to perform. A prototype method for the isolation of an organism would begin with dilution of the sample in a buffer solution and subsequent enrichment. This enrichment step has been shown to dramatically increase the test sensitivity, but it also increases the time to detection (Karch et al., 1996; Ogden et al., 2000). After enrichment, antibody coated beads (antigen specific) are added to the enriched culture and allowed to mix. The beads are then removed with a magnet and washed. The remaining supernatant is usually discarded (Chapman et al., 1994; Karch et al., 1996). Finally, the beads can be re-suspended in a buffer

and plated for identification (Chapman et al., 1994; Pyle et al., 1999), or used in PCRs for detection (Tortorello et al., 1998).

While IMS is the preferred method of detection and recovery for the research community today, it has its shortcomings. Reactions often require an enrichment step to increase cell numbers to a detectable level, thus adding to the time to detect a pathogen from a food or clinical sample. This enrichment increases the bacterial load to 10^3-10^4 CFU/ml often necessary for detection, a concentration of cells not often found in naturally contaminated food samples. Further, it is known that cross-reactivity may occur, thus producing false positive results (Karch et al., 1996). Pyle et al. (1999) reported that cells could be injured during the IMS process, but that was specific to the suspension medium. Furthermore, Ogden et al. (2000) demonstrated that large volumes of antibody-coated beads are often necessary for efficient capture and removal of cells; beads are commercially available but they are not cheaply priced. Moreover, these beads are only commercially available for a few pathogens today. Therefore, while IMS remains the concentration method of choice for researchers today, newer methods are necessary to overcome the inherent problems and improve assay sensitivity without sacrificing cost efficiency and ease of use.

Metal Hydroxide Immobilization and Concentration

Kennedy et al. (1976) first reported the use of metal hydroxides via a chelation process for the purpose of concentrating viable bacterial cells. These researchers hypothesized the immobilization process as the loss of hydroxyl groups on the metal hydroxide in favor of cell or enzyme ligands with the formation of

covalent bonds. Furthermore, these researchers demonstrated the strength of the attachment of the cells to the hydroxide using *E. coli* and *S. marcescens* (Kennedy et al., 1976).

Ibrahim et al. (1985a) developed both a radioimmunometric and an enzyme-immunometric method for the detection of *Salmonella* spp. in food samples following enrichment. Using titanous hydroxide as the solid phase in these assays, these researchers reported excellent specificity with no false-positive results observed. These researchers demonstrated an increase in sensitivity with respect to minimum detectable bacterial population of 100- to 160-fold over microtiter plates. Ibrahim et al. (1985b) extended this work to include three hydroxide solutions: titanous hydroxide, titanic hydroxide, and zirconium hydroxide. All three hydroxides recovered ≥90% of the initial inoculum of viable cells. These researchers also studied the effect of several variables on the ability of the hydroxide to immobilize viable cells. It was observed that an increase in the volume of hydroxide increased binding efficiency, possibly due to the larger concentration of potential hydroxide binding sites. The highest percentages of recovered cells were observed between pH=5.0 and pH=7.25 (Ibrahim et al., 1985b). Shaking of samples for 10 min. was observed to be the maximum time necessary for efficient binding of hydroxide to cell. Finally, in testing efficiency of binding to non-target organisms, immobilization percentages ranged from ~89% cells bound to ~99% cells bound.

More recently, researchers investigated the efficiency of metal hydroxide immobilization in dairy products (Lucore et al., 2000). These researchers reported consistent recovery rates of approximately 97-98% for *L. monocytogenes* and *S.*

enteriditis from pure culture. Direct plating of precipitated pellets produced recoveries ranging from 25-143% (titanous hydroxide) and 58-143% (zirconium hydroxide) from non-fat dry milk (reconstituted). These authors claimed recoveries of 83-122% for whole milk samples and 65-161% for ice cream samples with all but 10 of 48 samples being confirmed by PCR. These researchers concluded that metal hydroxides were a rapid and efficient means of recovering viable cells from dairy products. McKillip et al. (2000) studied the effectiveness of bacterial concentration and a solvent extraction technique (Drake et al., 1996) for recovery of bacterial DNA from five different dairy products. In a comparison of the solvent extraction method and immobilization by titanous hydroxide, these authors reported overall greater sensitivity of the solvent extraction method as indicated by PCR results and overall increased DNA yield from solvent extraction versus immobilization/concentration (McKillip et al., 2000). Nonetheless, these researchers reported a higher degree of purity of DNA yield following concentration versus solvent extraction. They concluded that the choice of method for DNA recovery is dependent on desired time to detect, desirable PCR sensitivity, and other research goals. Also, they noted that extraction techniques, regardless of methodology, must be optimized for each food product.

Conclusions

When viewed as a whole, there seems to be no one superior method of recovering bacterial cells and nucleic acids for pathogen identification from foods. The need for a recovery/identification method that can also estimate concentration of EHEC cells from a sample without the limitations of extensive enrichment steps or

costly reagents is still a reality. It is the goal of this research to develop such a methodology. Specific goals included: (1) the optimization of the metal hydroxide immobilization method for recovery of an EHEC species in autoclaved and raw ground beef homogenates; (2) the optimization of nucleic acid extraction from these samples; (3) the detection/confirmation of an EHEC spp. with PCR/Southern blots designed to target the sequence responsible for encoding the *slt*-II (Shiga-like toxin β-subunit) virulence factor common to infectious EHEC species. Finally, the method will be applied to beef homogenates in which EHEC cells are both inoculated directly (no growth) and homogenates in which EHEC cells are inoculated at a low inoculum (1 CFU/g beef) and allowed to incubate to a desired concentration prior to metal hydroxide concentration and detection. Recovery efficiency and PCR detection for both raw versus autoclaved beef and no growth versus growth will be evaluated.

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Chapter II

Metal Hydroxide Concentration of *E. coli* O157:H7 From Ground Beef With Cultural and Molecular Detection

Metal Hydroxide Concentration of *E. coli* O157:H7 From Ground Beef With Cultural and Molecular Detection

Abstract

This study examined the ability of double strength titanous hydroxide (2x TiOH) to effectively immobilize and concentrate *E. coli* O157:H7 cells from ground beef. *E. coli* O157:H7 cells were homogenized with 99 ml broth (TSB or mEC+n) and ten gram samples of autoclaved or raw beef at 10^3, 10^5, and 10^7 CFU/10 g beef. Further, experiments determining the ability of 2x TiOH to concentrate cells directly inoculated (no growth) versus cells inoculated at a low level (1 CFU/g beef) and allowed to incubate (from growth) were undertaken. Concentrated pellets were resuspended and plated to determine cell recovery efficiency and to provide DNA template for subsequent PCR detection and Southern hybridization. Cell loss to supernatant was always less than 30% although cell recovery varied according to treatment, with the highest recovery from autoclaved ground beef samples without growth (~71%). PCR detection limits were 10^2 CFU/ml for all treatments except for samples concentrated from raw ground beef with no growth where PCR detection was 10^4 CFU/ml. DNA hybridization was able to increase the detection limit for samples concentrated from autoclaved ground beef. With growth of in autoclaved and raw ground beef (versus direct inoculation in autoclaved and raw ground beef), bacterial cells were less readily recovered. [Data indicate that the use of a model food system, such as autoclaved ground beef, may not always provide an accurate description of real world conditions.] Future research is needed to investigate the mechanisms by which bacterial cells are less readily recovered and their DNA less readily amplified following growth in a food.

Key Words: Bacterial concentration, metal hydroxides, PCR detection, ground beef, *E. coli* O157:H7

Introduction

It is estimated that approximately 73,500 cases of foodborne disease attributable to *Escherichia coli* O157:H7 occur every year in the United States (Mead et al., 1999). While its exact prevalence is not known at this point, the organism has emerged as a serious threat to the integrity of the food supply. First identified as a human pathogen in 1982 (Riley et al., 1983), *E. coli* O157:H7 has been epidemiologically related to a number of disease syndromes including diarrhea, hemorrhagic colitis, hemolytic-uremic syndrome, and thrombotic thrombocytopenia (Riley et al., 1983). A bovine reservoir for the organism has been identified; thus beef and dairy products are at increased risk for contamination and transmission of *E. coli* O157:H7 (Chapman et al., 1993; Griffin and Tauxe, 1991; Hancock et al., 1994). Furthermore, products such as contaminated drinking water and unpasteurized fruit juices have been identified as transmission vehicles for the organism (Swerdlow et al., 1992; Besser et al., 1993). Person-to-person transmission (fecal-oral transmission) has been established as a secondary transmission mode (Griffin and Tauxe, 1991; Reida et al., 1994).

E. coli O157:H7 belongs to a group of Shiga-toxin-producing *E. coli* species deemed Enterohemorrhagic *E. coli* (EHEC), one of multiple groupings of *E. coli* species that are infectious to humans. While *E. coli* O157:H7 appears to be the EHEC species most often reported, examples of non-O157:H7 EHEC strains isolated from humans and animals on the basis of the lipopolysaccharide (LPS) antigen (O) include the O91 and O111 (Beutin et al., 1993; Beutin et al., 1995), O104:H21 (Feng et al., 2001), O113:H2 (Gyles et al., 1998), and O157:H^- (non-

motile) strains (Karch and Bielaszewska, 2001). *E. coli* O157:H7 and other EHEC species are hardy pathogens capable of withstanding a myriad of processing and environmental conditions. Defense systems have been identified that aid in the resistance of the bacterium to various environmental stresses, the most significant of which is its ability to withstand acidic conditions (Law, 2000; Lin et al., 1996; Abee and Wouters, 1999; Doyle and Schoeni, 1984). Furthermore, due to a characteristic low infectious dose (Doyle et al., 1997), detection methods that are reliable and sensitive are a clear necessity.

It has been repeatedly suggested that effective bacterial concentration methods would aid in the purification of target bacteria for detection as well as in the removal of matrix-associated inhibitory substances (Swaminathan and Feng, 1994; de Boer and Beumer, 1999). Current methods for the separation of bacteria from food products are far from ideal and none are highly efficient or simple. Centrifugation, filtration, immuno-magnetic separation (IMS), and bacterial immobilization using metal hydroxides have all been developed and tested in food products (Rodrigues-Szulc et al., 1996; Wang et al., 1992; Karch et al., 1996; Lucore et al., 2000). Centrifugation works well to sediment bacteria, yet it is also capable of sedimenting and concentrating unwanted compounds and food particulates. Filtration techniques are useful at removing large food particulates, but they may contain materials inhibitory to DNA amplification procedures or they may become clogged and inefficient (Thomas, 1998). Immuno-magnetic separation represents a great advance in the ability of researchers to selectively remove bacteria from a food or environmental sample, but it can be problematic in that a relatively high inoculum

(10^4 CFU/ml) is often required to gain the necessary degree of sensitivity (Karch et al., 1996; Pyle et al., 1999). Alternatively, an enrichment period may be required prior to the procedure to achieve the minimum concentration of cells.

Another bacterial concentration method that has shown promise utilizes metal hydroxides to immobilize and concentrate bacteria from a liquid medium. Kennedy et al. (1976) first reported the use of metal hydroxides via a chelation process for the purpose of concentrating viable bacterial cells. *E. coli* and *Serratia marsescens* cells were used to demonstrate the ability of the hydroxide to bind to cell membranes tightly. Ibrahim et al. (1985a; 1985b) experimented with different metal hydroxides and different experimental protocols to investigate the effectiveness of the metal hydroxides at recovering viable cells from a food sample as well as testing immobilization capability of the hydroxides after altering reaction conditions. These researchers reported >90% recovery for target spp. More recently, researchers have applied metal hydroxides for bacterial concentration from dairy products like ice cream, reconstituted non-fat dry milk, cheddar cheese, and yogurt (Lucore et al., 2000; McKillip et al., 2000; Cullison and Jaykus, 2002).

Here we report the application of double strength (2x) titanous hydroxide (2x TiOH) solution to concentrate *E. coli* O157:H7 from ground beef homogenates followed by PCR and Southern blot detection/confirmation. The impact of growth of the pathogen in ground beef on subsequent concentration and detection was also compared to direct ground beef inoculation (no growth). Finally, we compare bacterial concentration efficiency and detection between a model food matrix (autoclaved ground beef) and a “natural” food matrix (raw ground beef).

Materials and Methods

Bacterial Cultures and Media

Escherichia coli O157:H7 (ATCC 43895) cells were maintained by daily transfers in tryptic soy broth (TSB) (Accumedia, Baltimore, MD) and incubation at 37°C. Bacterial enumeration was conducted using tryptic soy agar (TSA) (Accumedia) using pour plates following serial dilutions in 0.1% peptone water (Fisher, Fair Lawn, NJ). Plates were incubated aerobically at 37°C for 24 hours.

For experiments utilizing raw ground beef, *E. coli* O157:H7 (ATCC 43895) was transformed with a plasmid encoding the Green Fluorescent Protein (EC-GFP) and containing an antibiotic-resistance marker (ampicillin, 100 µg/ml). The EC-GFP was utilized to allow differentiation between target cells and background microflora for enumeration. This strain was transferred daily in TSB containing 100 µg/ml filter-sterilized ampicillin (Sigma, St. Louis, MO). Green fluorescent protein is a 238 amino acid protein (Prasher et al., 1992) forming a chromophore within its primary structure (Prasher, 1995) that maximally fluoresces green upon exposure to 509 nm light (Suarez et al., 1997). Enumeration was conducted using TSA plus filter-sterilized ampicillin (100 mg/l) using pour plates following serial dilutions in 0.1% peptone water (Fisher). Plates were incubated at 37°C aerobically for 24 hours. Periodically, both cultures were streaked onto Sorbitol MacKonkey agar to confirm inability to ferment sorbitol within 24 hrs. and culture purity (Novicki et al., 2000). The presence of the *stx* (McIngvale et al., 2002), *eae* (Gannon et al., 1993), and *hly* (Meng et al., 1998) genes were also confirmed by PCR.

Transformation of E. coli O157:H7 with GFP

Competent cells were prepared according to a method of Sambrook and Russell (2001). Overnight culture of *E. coli* O157:H7 was diluted and inoculated into 100 ml TSB to 10^6 CFU/ml and then allowed to incubate at 37°C with agitation (150 rpm) in an Orbit Environ Shaker (Labline, Melrose Park, IL). Bacteria were then harvested during exponential growth (OD_{600nm}=~0.3, ~10^7 CFU/ml) via centrifugation at 7600 x g for 10 min. at 4°C. The bacterial pellet was washed in cold, sterile 0.9% saline (100 ml); this spin/wash was repeated once with 100 ml of saline and then repeated with 50 ml saline. Cells were then washed in two ml glycerol (10%) and re-suspended in 10% glycerol (250 µl) prior to transferring 50 µl aliquots into micro-centrifuge tubes. Tubes were then frozen quickly using liquid N_2 and stored at -80°C until needed.

E. coli O157:H7 competent cells were thawed on ice and mixed with 1 µl of pGFP DNA (200 ng), generously provided by R.W. Worobo (Cornell University, Geneva, NY). The solution was then pipetted into an electroporation cuvette (BTX, San Diego, CA) and transformation was carried out using a GenePulser apparatus with a voltage pulse of 2.5 kV and a time constant of 4.5 msec. (BioRad Laboratories, Richmond, CA). Cells were then quickly rinsed with one ml Luria Broth (Becton Dickinson, Sparks, MD), transferred to 9 ml Luria Broth, and incubated at 37°C with agitation (150 rpm) for one hour. The resulting cell suspension was plated on to TSA spread plates supplemented with 100 µg/ml Ampicillin (Sigma). Transformants, those colonies displaying ampicillin resistance and fluorescence at

366 nm with a Mineralight UVGL-58 lamp (UVP, Upland, CA), were then transferred to individual broth tubes and incubated at 37°C for 18 hours prior to use.

Generation of Growth Curves for E. coli O157:H7 in TSB

Growth curves of *E. coli* O157:H7 were conducted in 99 ml TSB with 10 g sterile ground beef at 37°C with shaking at 200 rpm in an Orbit Environ Shaker (Labline). Beef homogenate packs were initially inoculated with at ca. 1 CFU/g (confirmed by MPN). *E. coli* O157:H7 growth packs were stomached for 2 min. at 230 rpm in a Stomacher 400 (Seward, U.K.) and allowed to incubate. It was determined over triplicate experiments that 10^2 CFU/ml, 10^4 CFU/ml, and 10^6 CFU/ml were achieved at 4.5 hrs., 7.0 hrs., and 9.0 hrs. incubation, respectively (Appendix 1).

Generation of Growth Curves for EC-GFP in mEC+n

Growth curves were generated in Bacto modified *E. coli* broth (Becton Dickinson, Sparks, MD) with Bacto novobiocin antimicrobic supplement at 20 mg/l media (Okrend and Rose, 1989; Okrend and Rose, 1990) (mEC+n). Growth packs containing 99 ml mEC+n broth and 10 g raw ground beef were artificially contaminated at ca. 1 CFU/g (confirmed by MPN), stomached for 2 min. at 230 rpm in a Stomacher 400 (Seward) and allowed to incubate at 37°C. Final inoculum levels of 10^2, 10^4, and 10^6 CFU/ml were obtained at 6, 8.5, and 11 hrs. incubation, respectively (Appendix 2). Cell numbers were confirmed via plating with TSA-Ampicillin.

Preparation of Metal Hydroxides

Titanous Hydroxide (TiOH) and Zirconium Hydroxide (ZrOH) were prepared according to protocols previously reported (Lucore et al., 2000). For 1x TiOH, 136.6 µl Titanium Chloride (Sigma) was added to 200 ml distilled water and then the pH was adjusted to pH=7.0 +/- 0.2 by dropwise addition of 2 M ammonium hydroxide with constant stirring. The suspension was then washed three times with 5 ml sterile saline (0.9%). After mixing, the suspension was allowed to settle for 10 minutes and the clear top phase decanted. The final volume measured was autoclaved (121ºC, 15 min.), and stored in the dark. Single strength ZrOH was prepared in a similar manner: 2 g Zirconium Chloride (Sigma) was added to 40 ml distilled water and pH adjusted to pH=7.0 +/-0.2 by dropwise addition. The suspension was then washed three times with 150 ml sterile saline (0.9%), mixed gently, and then allowed to settle for 10 min. After each washing, the clear top phase was decanted and the final volume measured approximately 200 ml. This solution was also autoclaved (121ºC, 15 min.) and stored in the dark. For experiments requiring double-strength hydroxides, the initial volume or mass of the metal chloride was doubled.

Experimental Overview

The concentration method employed here has been previously reported (Cullison and Jaykus, 2002; Lucore et al., 2000), although past work has focused on the removal of bacterial pathogens from dairy products. Simply, metal hydroxide concentration consists of two steps: primary and secondary concentration. In this work, viable *E. coli* O157:H7 cells were inoculated into ground beef/broth homogenate. A sample was pipetted off and centrifuged (1° concentration) to sediment bacterial cells. Following the centrifugation, cell pellets were resuspended

in 0.9% saline, the metal hydroxide was added, and the resulting suspension was mixed gently for ten minutes at room temperature (2° concentration). The resulting mixture was then centrifuged at a low speed to re-pelletize bacterial cells. The supernatant was poured off and cells were resuspended in sterile 0.9% saline prior to enumeration and/or DNA extraction (Appendix 4).

Experimentation began with sample clarification and the optimization of the primary concentration (centrifugation) method. We then moved on to the optimization of the secondary concentration (bacterial immobilization) method, testing different metal hydroxides and concentration enhancement agents. Once the entire method was optimized, we optimized the PCR detection of extracted DNA from concentrated bacterial pellets. Following optimization of PCR, the concentration and detection methods were paired together and DNA amplicons were confirmed via Southern hybridization.

Experimental Controls

Throughout all experimentation, two centrifuge tubes containing stomached TSB and beef, but no bacterial cells, were processed in the same manner simultaneously with experimental samples. These served as negative control samples to test for contamination. These negative controls also served to monitor the specificity of PCR reactions.

Mathematical Determination: Supernatant Loss and Recovered Pellet

Pour plating or Most Probable Number (MPN) technique prior to experimentation confirmed initial experimental inocula. Throughout all experiments, percent losses to supernatant and pellet recovery were evaluated after both the

primary and secondary concentration steps via direct plating and comparison to plate counts from the initial inocula. Percent recovery of the precipitated pellet at both the primary and secondary concentration steps was calculated as reported by Lucore et al. (2000): % Recovery= (Total population in pellet after concentration)*100%/(Total population in sample before concentration). Similarly, loss to supernatant was calculated using the following equation: % Loss= (Total population in supernatant after concentration)*100%/(Total population in sample before concentration).

Sample Clarification

In order to clarify the samples before beginning primary concentration, sterile cheesecloth was employed to strain large beef particulates, similar to methods reported by Lucore et al. (2000) and Cullison and Jaykus (2002). Cheesecloth was autoclaved at 121°C for 15 min. Following stomaching in the filter bag, sample was pipetted through cheesecloth and centrifuged for 10 min. at 7600 x g, 0°C. Samples were then removed and the cheesecloth was discarded.

Primary Concentration Optimization

Prior to beginning work with metal hydroxides, experiments using only high-speed centrifugation were performed to optimize the primary concentration step. Autoclaved ground beef was used in these experiments in an effort to increase experimental control due to high levels of indigenous microflora (Klein & Juneja, 1997; McIngvale et al., 2002). For all primary concentration experiments, ground beef (~93% lean/7% fat) was purchased at a local grocery store. Immediately following purchase, beef was aliquoted into 10 g samples, wrapped in aluminum foil,

and stored at -20°C until needed. Immediately prior to use, a 10 g ground beef sample was autoclaved (121°C, 15 min.), cooled to room temperature, mixed with 99 ml TSB in a filter stomacher bag (Nasco, Ft. Atkinson, WI), seeded with *Escherichia coli* O157:H7 (10^2, 10^4, or 10^6 CFU/ml), and stomached in a Stomacher 400 (Seward) at 230 rpm for two minutes. Following this step, 20 ml of filtrate was pipetted into a 50 ml centrifuge tube (VWR, Suwanee, GA) and 1 ml was plated out to determine initial inocula. The 20 ml filtrate (representing 20% of the total sample) was centrifuged in a Sorvall RC-5B (Dupont, Wilmington, DE) centrifuge for 10 min. at 7600 x *g* at 0°C. Upon completion, one ml of the resulting supernatant was plated out to determine bacterial loss to supernatant. The total volume of supernatant was recorded and the supernatant was discarded. The remaining pellet was resuspended in 5 ml 0.9% saline, the total volume of resuspended pellet was recorded (~5.5-5.7 ml), and the pellet was plated to determine the bacterial recovery after primary concentration.

Secondary Concentration Optimization

Following primary concentration, the remainder of the resuspended pellet was subjected to secondary concentration using 2x TiOH or 2x ZrOH in a 2:1 ratio of hydroxide:sample. The solution was mixed gently for 10 min. on the lowest setting on a vortexer. After mixing, the tubes were removed and centrifuged at 1900 x *g* for 6 min. at 0°C. The tubes were removed and one ml of the supernatant was withdrawn and plated for determination of loss to supernatant. Total supernatant volume was recorded and the remaining supernatant was discarded. The precipitated pellet was weighed using a tared empty centrifuge tube and then

resuspended in 5 ml 0.9% saline and plated to determine bacterial recovery after secondary concentration.

Optimization of Metal Hydroxide Concentration to Ground Beef

We began by testing, in triplicate, the recovery of *E. coli* O157:H7 using either TiOH or ZrOH, based on data previously reported by Lucore et al. (2000), in order to determine if one metal hydroxide outperformed the other. We then tested double strength (2x) TiOH and ZrOH to determine whether the 2x solution provided better recovery of cells versus the 1x solution. Experiments were conducted using an inoculum of 10^6 CFU/ml and were performed as described previously.

To explore the possibility of bacterial clumping in the final reconstituted pellet following secondary concentration, experiments utilizing suspension buffer containing Tween 20 (Sigma) were conducted. Reconstitution buffer supplemented with 1%, or 5% Tween 20 were used. Secondary pellets were mixed in the Tween 20/0.9% saline solution as previously described, then plated to determine percent recovery.

A separate set of experiments were conducted using polyethylene glycol (PEG) 8000 (4%) (Fisher) added to the stomacher bag at the outset of the experiment. The use of PEG 8000 to increase the precipitation of protein was attempted based on findings that PEG 8000 aided in the recovery and detection of viable bacteria from other food matrices (Cullison, 2000).

Viability Studies

To confirm that *E. coli* O157:H7 cells remained viable when mixed with TiOH and 2xTiOH over an extended time period, *E. coli* was suspended in sterile saline

(0.9% NaCl), adjusted to ~10^8 CFU/ml, and mixed with either TiOH or 2x TiOH in a 1:2 volume ratio (sample: metal hydroxide) (Cullison and Jaykus, 2002). A control was also performed using the same ratio, except that the metal hydroxide was replaced with sterile 0.9% NaCl. Samples were collected at 0, 2, 6, 12, 24, and 48 hours. Samples were serially diluted in 0.1% peptone water, plated on to TSA, and incubated at 37°C for 24 hours. Previous research has confirmed that neither ZrOH nor 2x ZrOH damaged *E. coli* or other food-borne pathogens (Kennedy et al., 1976; Lucore et al., 2000).

Optimized Concentration Method for Autoclaved Ground Beef Slurries For No Growth and Growth Experiments

An autoclaved (121°C, 15 min.) 10 g ground beef was mixed with 99 ml TSB in a filter stomacher bag (Nasco), seeded with *Escherichia coli* O157:H7 (10^2, 10^4, or 10^6 CFU/ml), and stomached in a Stomacher 400 (Seward) at 230 rpm for two minutes (no growth). For homogenates requiring incubation (growth), packs were inoculated at 1 CFU/g beef and placed at 37°C and shaken at 200 rpm (Labline) to gain the desired 10^2, 10^4, or 10^6 CFU/ml. Following this step, 20 ml of filtrate was pipetted into a 50 ml centrifuge tube (VWR) and 1 ml was plated out to determine initial inocula. The 20 ml filtrate was centrifuged in a Sorvall RC-5B (Dupont) centrifuge for 10 min. at 7600 x *g* at 0°C. Upon completion, 1 ml of the resulting supernatant was plated to determine loss to supernatant. The total volume of supernatant was recorded and the supernatant was discarded. The remaining pellet was resuspended in 5 ml 0.9% saline, the total volume of resuspended pellet was

recorded, and the pellet was plated to determine percent bacterial recovery in the pellet following primary concentration.

After primary concentration, the reconstituted pellet was subjected to secondary concentration using 2x TiOH in a 2:1 ratio of hydroxide:sample. The solution was mixed gently for 10 min. on the lowest setting on a vortexer. After mixing, the tubes were removed and centrifuged at 1900 x *g* for 6 min. at 0°C. The tubes were removed and 1 ml of the supernatant was withdrawn and plated for loss to supernatant following secondary concentration. Total supernatant volume was recorded and the supernatant was discarded. The precipitated pellet was weighed using a tared empty centrifuge tube and then resuspended in 5 ml 0.9% saline and plated for determination of recovery following secondary concentration.

Concentration of E. coli O157:H7 from Raw Ground Beef Homogenates

Upon completion of all optimization and concentration trials using autoclaved ground beef, we proceeded to more fully test the ability of 2x TiOH to concentrate *E. coli* O157:H7 by experimenting with raw ground beef and mEC+n in place of TSB. In order that we might distinguish the target bacterium from background microflora that might be present in the beef sample, we utilized the transformed EC-GFP strain described previously. Concentration experiments were conducted identically to those described above at concentrations of 10^2, 10^4, and 10^6 CFU/ml from no growth and growth scenarios.

DNA Extraction

Following concentration, DNA was extracted from one ml of the reconstituted pellet obtained after secondary concentration using a guanidinium isothiocyanate

(GITC)-based method reported by McKillip et al. (2000). Four one-ml samples were prepared from the reconstituted bacterial pellet for DNA extraction. Cells were sedimented by centrifuging at ~9,000 x *g* for five minutes at room temperature in a Micro 240-A microcentrifuge (Spintron Inc., Metuchen, NJ). Bacterial pellets were reconstituted in 600 µl of a solution containing 4 M GITC (ICN Biomedicals, Aurora, OH), 20 mM Sodium Acetate (Sigma), 0.1 mM dithiothreitol (DTT) (Sigma), and 0.5% sodium dodecyl sulfate (SDS) (Fisher). To this, 100 µl of a 2% Triton X-100 (Sigma) solution was added and the tubes were gently agitated for 10 min. on a vortexer set at the lowest setting. After agitation, DNA was extracted with 700 µl phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen, Carlsbad, CA). DNA was precipitated by the addition of one volume isopropanol (Sigma), 0.1 volume 2.5 M ammonium acetate (Sigma), and 5 µl glycogen (Fisher) (10 µg/ml); tubes were then centrifuged for 30 min. at ~18,000 x *g*. Supernatants were discarded and pellets were dried at 37°C for 15 minutes. The precipitated DNA was reconstituted in 50 µl molecular grade water (Geno Tech, St. Louis, MO) and the four reconstituted pellets were pooled together to reduce variance between samples. In order to quantify the volume of extracted DNA, optical density (260/280 nm) of the pooled pellets was determined using 10 µl extracted DNA plus 990 µl water using a Beckman DU-530 spectrophotometer (Fullerton, CA).

Primers

The 254 bp portion of the *stx*-II gene (B-subunit) previously utilized by McIngvale et al. (2002) was chosen for PCR detection based on reported primer specificity. While Shiga-like toxins (*stx*) are phage-encoded and may be unstable at

low cell inocula (Wagner et al., 1999), early optimization work indicated that greater specificity was possible with *stx*-based primers and stability was not affected by concentration. The primer sequences for the forward and reverse primers, respectively, were as follows: TXBF=(5'-TGTTTATGGCGGTTTTATTTG-3') and TXBR=(5'-ATTATTAAACTGCACTTCAG -3'). All primers were obtained from Invitrogen (Carlsbad, CA).

Primers were also tested for specificity with PCR using various *Enterobacteriacae* spp. Test bacteria included enterohemhorragic *E. coli* (EHEC) (5 strains) and enteropathogenic *E. coli* (EPEC) (2 strains), non-pathogenic *E. coli* strains (2 strains), an enteroinvasive *E. coli* strain, and other gram-negative and gram-positive pathogens (*S. flexneri, L. monocytogenes, S. typhimurium, E. aerogenes, A. hydrophila, V. vulnificus*). A Basic Local Alignment Search Tool (BLAST) sequence check (National Center for Biotechnology Information, Bethesda, MD; [www.ncbi.nih.gov]) was also conducted to confirm primer specificity.

PCR Reagents and Cycling Conditions

PCR Master Mix (41 µl/reaction tube) (Promega, Madison, WI) consisted of *Taq* DNA polymerase (50 u/ml), 3 mM magnesium chloride ($MgCl_2$), and 400 µM each of dATP, dGTP, dCTP, and dTTP. To this was added 4 µl extracted DNA and 2 µl [20 pmol] forward and reverse primers. Samples were placed in an MJ Research PTC 200 Thermal Cycler (Watertown, MA) and run for 36 cycles, following a 2 min. 94°C denaturation step, according to the following protocol: 94°C for 1 min., 54°C for 1.5 min., and 72°C for 2 min. with a final 4°C hold step. PCR amplicons

were subsequently visualized via electrophoresis using a 0.5% agarose gel stained with ethidium bromide.

PCR Optimization

PCR optimizations utilizing differing reagent combinations and annealing temperatures were conducted. Initial trials focused on the volume of primers added to the reaction tube. Four µl DNA concentrated from 10^4 CFU/ml and 10^6 CFU/ml bacterial cells was added to reaction tubes (Fisher Scientific) containing 41 µl PCR mix (Promega) and either 2, 4, or 10 µl primers. Detection of 1/10 serial dilutions of DNA and robustness of bands was used to judge the optimal volume of primers and resuspended DNA for experimentation. Various other reagents known to assist PCR including one µl 25 mM magnesium chloride ($MgCl_2$) (Sigma), 3% dimethyl sulfoxide (DMSO) (Sigma) (Filichkin and Gelvin, 1992), 0.5 mM DTT (Sigma), 2.5% PEG 8000 (Fisher) (Traore et al., 1998), 8% Glycerol (Sigma) (Wilson, 1997), and additional *Taq* DNA polymerase (Promega) (1 µl, 5 u/µl) were also evaluated on DNA extracted from cells concentrated from autoclaved beef without growth. PCR reactions were also prepared as previously described using a temperature gradient range of 46°C-56°C containing DNA extracted from 10^4 CFU/ml cells to determine the optimum annealing temperature.

Southern Transfer/Blotting

Southern hybridizations were performed on representative PCR amplicons. Genescreen™ nylon membranes (Perkin Elmer, Boston, MA) for hybridizations were prepared according to the method of Southern (Sambrook and Russell, 2001), specifically using an ascending transfer setup. Gels were soaked in a denaturing

solution (0.5 M NaOH, 1.5 M NaCl) for 30 min. and subsequently soaked in a neutralizing buffer (0.5 M Tris-Cl, 1 M NaCl), also for 30 min. Membrane transfer was allowed to proceed overnight. Membranes were cross-linked under a UV lamp (254 nm) for three minutes to bind DNA to the membrane.

Biotinylated probes were created with the Random Primer Biotin Labeling Kit with Streptavidin-AP (NEN Life Science Products, Boston, MA) according to manufacturer's instructions following the method of McIngvale et al. (2002). Briefly, a tube of PCR product (~45 µl) using DNA obtained from pure culture in nutrient broth was denatured in boiling water for 3-5 minutes. After denaturation, the DNA was immediately chilled on ice and mixed with random primers, labeled nucleotides, and Klenow fragment to begin synthesis of the double stranded probe. The probe was incubated at 37°C for one hour. The probe synthesis was then terminated with the addition of 0.1 M EDTA and stored at –20°C until ready for use.

DNA hybridization was carried out according to manufacturer's instructions (NEN Life Science Products). Following the transfer, the membrane was subjected to a prehybridization-buffer wash for one hour at 65°C in a Hybaid oven (Hybaid, Vangard Intl., Neptune, NJ). The prehybridization buffer containing 2x SSC, 0.5% Blocking Reagent, 5% Dextran Sulphate, and 0.1% SDS, was mixed with 50 µl of carrier DNA (Salmon Sperm DNA (Invitrogen)). Following prehybridization, the DNA probe hybridization solution was thawed, boiled for approximately four minutes, and added to the hybridization tube with 10 ml of fresh prehybridization buffer/50 µl Salmon Sperm DNA mix after discarding old prehybridization solution. Hybridization was allowed to continue overnight at 65°C.

Following hybridization, the membrane was subjected to a series of washes to remove unbound probe. The washing procedure began with a 15 min. wash of the membrane in 5 ml 20x SSC, 500 µl 10% SDS, and 44.5 ml water at 65°C. Immediately after this first stringency wash, the membrane was subjected to a second, less-stringent solution containing 500 µl 20x SSC, 50 µl 10% SDS, and 49.45 ml water for 15 min. at 65°C. The membrane was then rinsed for 5 min. at room temperature in a 50 ml solution (Wash 1) containing 0.8% NaCl (Sigma), 0.22% sodium phosphate (dibasic) (Fisher), 0.02% KCl (Fisher), 0.1% SDS (Sigma), 0.024% potassium phosphate (monobasic) (Fisher) and 0.05% Tween 20 (Sigma). The membrane was then blocked in 10 ml of solution Wash 1 supplemented with 0.5 g blocking agent (NEN Life Science Products) for one hour at room temperature.

After washing away non-specifically bound probe, membranes were soaked in streptavidin-alkaline phosphatase conjugate buffer (NEN Life Science Products) for one hour at 27°C. Chemiluminescent detection utilizing CDP-Star reagent (NEN Life Science Products) was conducted and blots were visualized using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Data Analysis

All experiments were performed in triplicate. Data analysis for bacterial concentration was conducted using Instat v2.05 statistical analysis software (Graphpad, San Diego, CA). PCR and Southern blot results were determined by consistent visualization of bands upon triplicate replications.

Results

E. coli Viability Study

The TiOH (1x and 2x) reagent did not significantly inactivate the *E. coli* O157:H7 cells through a 48 hr. exposure period. Cell counts from both the saline control and the two TiOH solutions increased by approximately 1.0 $\log_{10}$ from time zero hrs. to time 24 hrs.; thereafter, the counts remained stable (Figure 2.1).

Primary Concentration of Cells From Autoclaved Ground Beef Samples

The primary concentration protocol was performed on autoclaved meat homogenates at three different levels of bacteria (10^2, 10^4, 10^6 CFU/ml). Meat samples were seeded by direct inoculation (no growth) or inoculation and growth to target (from growth). Bacterial loss to supernatant was consistently low (<5%) across all inocula for both treatments (Table 2.1). Statistical analysis across inoculum levels and between treatments (no growth vs. growth) revealed no significant differences ($p \leq 0.05$). Although some variability was evident, we concluded that primary high-speed centrifugation effectively concentrated the bacterial cells from the food matrix.

Concentration Optimization: Tween 20 & PEG 8000

Despite early indications of the occurrence of bacterial clumping in our samples, the addition of 1% and 5% Tween 20 to the final reconstitution buffer (post-2° concentration) did not result in any significant increase in the recovery of bacterial cells from the meat homogenates compared with the control (0% Tween 20) (Appendix 3). Furthermore, optimizations with 4% PEG 8000 in order to precipitate protein did not result in increased cell recoveries (Appendix 3). Finally, analysis of

the data revealed no statistically significant differences between any of the treatments (1% Tween 20, 5% Tween 20, 4% PEG 8000) with respect to both supernatant loss and pellet recovery.

Concentration Experiments: TiOH vs. ZrOH

Use of the 2x TiOH presented an advantage, although not statistically significant, over that of 1x TiOH and 1x ZrOH (Table 2.2). Furthermore, 2x TiOH was superior to 2x ZrOH for bacterial concentration of *E. coli* O157:H7 in terms of final percent recovery (Table 2.2). Large 2x ZrOH/bacteria pellets were very difficult to effectively reconstitute fully in the prescribed volume, even after extended mixing. Henceforth, all experimentation utilized 2x TiOH in subsequent work.

Concentration of "No Growth" Meat Homogenates With Autoclaved Beef

No statistically significant differences were observed between 2x TiOH and 0.9% NaCl (Control) in terms of ability to concentrate viable *E. coli* O157:H7 cells from the ground beef homogenates within each inoculum with the exception of 10^2 CFU/ml (Table 2.3). Further, after testing between inoculum levels within a treatment, no significant differences were observed. Data presented demonstrate a high degree of similarity both in percentage of recovered cells as well as the degree of variation between experimental replications. The loss to supernatants were very low for both the treatment (2x TiOH) and the control (Table 2.3), indicating that discrepancies between expected pellet recoveries based on loss to supernatant and actual recoveries based on direct plating of the pellet might be due to inherent variation associated with enumeration of microorganisms, a phenomenon observed by others (Cullison and Jaykus, 2002; Jarvis, 1989; Hsu et al., 2002).

Concentration of "From Growth" Meat Homogenates With Autoclaved Beef

Over triplicate replications, a higher loss to supernatant was observed with bacteria concentrated from autoclaved ground beef homogenates using the 0.9% NaCl control versus 2x TiOH ($p<0.05$) (Table 2.4). Across all inocula the percent of cells lost to supernatant was higher in saline-concentrated samples versus 2x TiOH-concentrated cells (Table 2.4).

Conversely, there existed no statistically significant differences with respect to the recovery of cells in the final sample pellet; there were no statistical significant differences between the various inocula for either treatment. Across all inocula the recovery of bacteria in the final pellet was consistent with recoveries ranging 55.42%-60.73% for cells concentrated with 0.9% NaCl and 42.36%-57.74% for cells concentrated using 2x TiOH (Table 2.4).

Concentration of "No Growth" Meat Homogenates With Raw Ground Beef Using the GFP-Mutant

Percent cell recovery from no growth raw beef homogenates roughly mirrored that of percent recovery of cells concentrated from no growth autoclaved beef samples. There were no differences with respect to loss to secondary supernatant between controls and 2x TiOH, regardless of inoculum (Table 2.5). With respect to bacterial recovery based on direct plating of precipitated pellets, only the saline control at inoculum level of 10^2 CFU/ml showed statistically significant increased recovery compared to saline recoveries at 10^4 and 10^6 CFU/ml. No significant differences were observed between saline recoveries compared to 2x TiOH ($P<0.05$) (Table 2.5).

Concentration of "From Growth" Meat Homogenates With Raw Ground Beef Using the GFP-Mutant

When bacterial concentration was applied to raw ground meat homogenates in which bacterial cells were allowed to grow, secondary supernatant losses were lower as compared with supernatant losses recorded from no growth concentration experiments using raw ground beef (Tables 2.5, 2.6). Losses to supernatant ranged from 5.76%-11.39% for samples concentrated using 0.9% NaCl and from 6.00%-9.57% for samples concentrated with 2x TiOH. Statistical analysis of percent loss to supernatant indicated no significant difference between treatments or between inocula within a treatment (Table 2.6).

Bacterial recoveries from pellets obtained in growth experiments from raw ground beef were lower and more variable than for any of the other treatments (autoclaved beef and raw beef, no growth and growth). Recoveries in the secondary pellet ranged from 15.88%-74.98% for samples concentrated using 0.9% NaCl and from 35.48%-55.57% for samples concentrated using 2x TiOH. Statistical analysis revealed more statistically significant differences between treatments and between inocula within treatments (Table 2.6). Of particular interest is the 15.88% recovery for samples concentrated from 10^2 CFU/ml using 0.9% NaCl (Table 2.6). Primary concentration consistently achieved 90% or greater recovery of the inoculum for these experiments. In addition, a low mean loss to supernatant suggests effective concentration despite low final cell count recovery. In summary, that the performance of bacterial concentration, on growth samples from raw ground beef,

when evaluated through plating of precipitated pellets, is more variable and frequently less efficient than that obtained from the no-growth samples.

2x TiOH vs. 2x ZrOH Coupled with PCR

Multiple experiments were conducted to determine if one metal hydroxide was superior at providing PCR-amplifiable DNA. Though it has already been stated that 2x ZrOH did not provide efficient bacterial concentration, it was hypothesized that zirconium might have a positive effect on PCR detection by way of decreased co-precipitation of PCR inhibiting food components. Thus, trials were conducted to determine if one of the bacterial concentrating reagents, 2x TiOH or 2x ZrOH, was superior at providing PCR detection. Four DNA samples tubes were obtained from 10^6 CFU/ml inoculum level following concentration and serially diluted. DNA yields for cells concentrated with 2x TiOH were approximately twice those obtained by concentrating with 2x ZrOH (2x TiOH=1.31 µg/µl$\pm$0.71 µg/µl; 2x ZrOH=0.67 µg/µl$\pm$0.33 µg/µl). Also PCR amplicons from 2x TiOH-concentrated cells were clear and robust while banding was not observed from samples concentrated with 2x ZrOH (Appendix 6). Thus, it was determined that a 2x TiOH-GITC concentration-extraction protocol would be adopted for subsequent work.

PCR Optimizations

Triplicate PCR reactions at all levels of inocula revealed that 4 µl of primers should be added to PCR reaction tubes to allow the most consistent amplification of template DNA. Duplicate PCR tests run at different annealing conditions concluded that a 55°C annealing step provided more consistent and robust banding then did annealing at 52°C. PCR experiments showed no significant beneficial effect of any

of the following PCR enhancement agents: additional $MgCl_2$, DTT, DMSO, Glycerol, and PEG 8000 (Appendix 7). However, an additional 1 µl of *Taq* DNA Polymerase to PCR reactions did allow more robust banding with a lower detection limit based on PCR with serially diluted DNA.

Results of Primer Specificity Tests

After completing PCR amplification of various EHEC, EPEC, and other *Enterobacteriacae* spp., gel electrophoresis and Southern blots confirmed the absence of non-specific binding of the primers designed for this study. Three other EHEC strains (O55:H7; O157:H-; O157:H7) and one *Shigella flexneri* (ATCC 12022) did yield amplicons of identical molecular weight as that of the positive control (Appendix 5). These results were not unexpected, as McIngvale et al. (2002) gained amplicons from various EHEC spp. and *Shigella* spp. using the same primers to amplify the same portion of the *stx*-II gene. Other bacteria tested did not yield amplicons.

Detection and Confirmation of No Growth Meat Homogenates With Cooked Ground Beef

PCR detection of *E. coli* O157:H7 DNA extracted from ~10^6 CFU/ml and 10^4 CFU/ml was consistent across three replications for both the 2x TiOH and 0.9% NaCl control. Furthermore, detection at the lower level inoculum (10^2 CFU/ml) was present across all replications of both treatment and control (Fig. 2.2). Negative beef controls did not produce amplicons. DNA hybridization experiments confirmed the presence of the *stx* target amplicon. Detection limits for 0.9% NaCl and 2x TiOH were identical with the only noticeable difference existing in the intensity of the blots.

The detection limit for either saline or 2x TiOH was the 1/10 dilution of DNA extracted from 10^2 CFU/ml *E. coli* O157:H7.

Detection and Confirmation of From Growth Meat Homogenates With Cooked Ground Beef

When compared to PCR detection from samples concentrated from a cooked ground beef "no growth" homogenate, detection for samples concentrated from a "from growth" homogenate increased (Fig. 2.3). Gels were again consistent with respect to detection limits and band intensity between DNA from both treatment and control. However, detection limits of DNA hybridization did not increase versus previous hybridization results from "no growth" cooked ground beef regime. More intense bands at the 1/10 dilution were observed for both sample sets versus PCRs of "no growth" samples. Experiments to confirm the presence of the target DNA via hybridization did not demonstrate any significant differences in the ability to amplify concentrated DNA between samples concentrated with 0.9% NaCl and 2x TiOH. Detection sensitivity was identical for both sets of samples over duplicate blots, with sample detection ending at a 1:10 dilution of a DNA sample concentrated from 10^2 CFU/ml (Fig. 2.3).

Detection and Confirmation of No Growth Meat Homogenates With Raw Ground Beef

PCR detection of *E. coli* O157:H7 GFP from raw ground beef decreased compared to previous data from experiments with cooked ground beef. PCR amplicons were observed from 10^4 CFU/ml and 10^6 CFU/ml, but not from 10^2 CFU/ml (Fig. 2.4). Bands were robust and for the samples concentrated with 2x

TiOH, PCR detection was also obtained at the 1:10 dilution of DNA extracted from recovered cells at both concentrations (Fig. 2.4). In contrast, amplicons from 1/10 dilutions were not visible from saline-concentrated cells. DNA hybridization provided detection at the 10^2 CFU/ml inoculum in both sample sets (0.9% NaCl- and 2x TiOH-concentrated) and higher serial dilutions from 10^4 CFU/ml and 10^6 CFU/ml (Fig. 2.4).

Detection and Confirmation From Growth Meat Enrichments With Raw Ground Beef

For cells grown in raw ground beef prior to concentration, PCR detection was achieved at every level of inoculum (10^6, 10^4, and 10^2 CFU/ml) and DNA hybridization confirmed the presence of target DNA at these concentrations. However, detection was not achieved for any of the serially diluted DNA samples, with the exception of the 1/10 dilution of the 10^6 CFU/ml sample for both saline-concentrated and 2x TiOH-concentrated samples (Fig. 2.5).

Discussion

Methods for the concentration of bacterial pathogens from a food matrix have been tested before by several researchers (Kennedy et al., 1976; Ibrahim et al., 1985a; Lucore et al., 2000; Cullison and Jaykus, 2002). This study represents the first investigation into the feasibility of metal hydroxides to concentrate *E. coli* O157:H7 from ground beef homogenates. Furthermore, due to the existence of a bovine reservoir for *E. coli* O157:H7 (Chapman et al., 1993; Hancock et al., 1994) and the lack of efficient, reliable methods for the concentration of bacterial cells from a food matrix, the need for a method that could quickly and effectively immobilize

and concentrate viable *E. coli* O157:H7 cells from ground beef still would be beneficial.

Throughout the initial phases of experimentation, modifications were made to the experimental design in order to optimize cellular recovery. We tested the addition of the surfactant Tween 20 at various concentrations (1%, and 5%) to our final resuspension buffer of 0.9% saline in order to alleviate an apparent microbial clumping phenomenon we believed might be hindering the optimization process. We also tested the addition of 4% PEG 8000 to the homogenate in the initial stomacher bag in order to aid in precipitating protein, based on work by Cullison (2000), who reported the use of PEG 8000 as beneficial in the removal of heavy food components from bacterial pellets. We believed this might facilitate with subsequent PCR detection by removing potential PCR inhibitors. We observed no apparent impact between samples with PEG 8000 added and samples without the addition of PEG 8000.

Although a number of trials were done with various agents demonstrated to reduce PCR inhibition, only additional *Taq* DNA Polymerase enzyme enhanced PCR detection. This may be attributable to protein in extracted DNA samples that may have hindered enzyme binding to the template DNA or potentially due to the inactivation of the enzyme. Other researchers have noted that the addition of more *Taq* DNA polymerase increased PCR detection limits from ground beef (McIngvale et al., 2002; Li and Drake, 2003).

Our data do not suggest that 2x TiOH represents a more effective tool for the concentration of *E. coli* O157:H7 from ground beef compared to saline. Metal

hydroxides have demonstrated increased efficiency compared to saline at recovering other food-borne pathogens from other food matrices, primarily dairy products (Cullison and Jaykus, 2002; Lucore et al., 2000). Guan and Levin (2002), following simple inoculation and immediate centrifugation of raw ground beef samples artificially contaminated with *E. coli* O157:H7 (~10^4 CFU/ml), reported approximately 55% recovery of cells for subsequent PCR detection. This reported percent cell recovery is comparable to our secondary concentration (final) percent cell recoveries. Lucore et al. (2000) reported approximately 95% recovery of *L. monocytogenes* and *S. enterica* serovar Enteriditis from cheese and non-fat dry milk based on loss to supernatant with TiOH, but less than 80% recovery when a saline control was used in lieu of the metal hydroxide. Berry and Siragusa (1997), though working with hydroxyapatite versus metal hydroxides, reported only ~50% recovery of *E. coli* O157:H7 with considerable variability in recovery from different meat and environmental matrices. In contrast, they also reported recoveries approximating 98% for *L. monocytogenes*, *S. typhimurium*, *S. aureus*, as well as other bacterial spp. (Berry and Siragusa, 1997). Taken as a whole, *E. coli* O157:H7 may present special challenges in terms of the ability to effectively recover cells from a food sample. McKillip et al. (2002) also concluded that techniques should be optimized for each food and bacterium as each food matrix/bacterium combination may represent unique challenges.

One difference observed throughout experimentation was between “no growth” and “growth” experiments with respect to both cell recovery and PCR detection. Data indicated a decrease in overall cell recovery across inoculum levels

when one shifts from a simple inoculation (no growth) to an incubation (from growth) regime, both in the case of cooked ground beef and raw ground beef, similar to data reported by McKillip et al. (2002) for autoclaved skim milk. These researchers reported the effects of growth of *E. coli* O157:H7 in a food medium, specifically autoclaved skim milk, both in terms of DNA extraction and PCR detection efficiency. DNA extracted from cells grown in skim milk versus that from cells directly inoculated and immediately concentrated from skim milk was less pure based on optical density ratios; PCR detection of DNA extracts from cells grown in skim milk also suffered both in band intensity and overall detection limit (McKillip et al., 2002). McKillip et al. (2002) speculated the decrease in DNA purity and PCR detection is either the result of a function of close associations formed between the bacteria and the food matrix or is the result of a shielding effect provided by the food matrix, allowing for decreased DNA extraction efficiency (McKillip et al., 2002). While our work is not directly comparable to these researchers' findings due to differences in food matrix, the similarity in findings suggests a discrepancy between detection of cells grown in the matrix as opposed to those simply inoculated into the matrix (Berry and Siragusa, 1997; Uyttendaele et al., 1999; Lucore et al., 2000; Cullison and Jaykus, 2002). The use of direct inoculation followed by experimentation is a common practice. Our results indicate that this practice may lead to results that are not identical to situations where bacteria are allowed to grow in food.

It is not evident why PCR detection limits suffered with the use of raw ground beef versus cooked ground beef. Many studies have reported food (specifically ground beef) as an inhibitor of PCR and the existence of practical hindrances to

detecting DNA derived from a small initial concentration (Andersen and Omiecinski, 1992; Oliveira et al., 2002). Uyttendaele et al. (1999) reported ground beef specifically to be inhibitory to PCR amplification of *E. coli* O157:H7 DNA, potentially due to the presence of nucleases or the binding of bacterial DNA to the matrix. We were able to gain detection at the lower limit inoculum (10^2 CFU/ml) with "from growth" raw ground beef homogenates on both an agarose gel and nylon membrane compared to direct inoculation (Fig. 5). This phenomenon is not totally surprising based on data reported by Oberst et al., (1998); these researchers reported a 5' nuclease (Taqman) PCR detection protocol for *E. coli* O157:H7 and ground beef that was artificially spiked. They reported PCR detection from ground beef samples was improved from $\geq 10^4$ CFU/ml to $\geq 10^2$ CFU/ml following an IMS and pre-enrichment procedure, reporting PCR detection limits comparable to those obtained in this study (Fig. 4, 5). However, the lack of sensitivity and the loss of lower detection limits usually afforded by Southern hybridization observed after hybridizing samples concentrated from raw ground beef with prior incubation was somewhat mysterious. Robust blots were observed at every inoculum, but detection limits did not proceed past a 1/10 dilution of the upper level inoculum (10^6 CFU/ml). Nevertheless, Chen et al. (1998), in work to amplify and hybridize sequences from verotoxigenic *E. coli* (VTEC), recorded no improvement in detection limit by Southern hybridization or colony blots after visualization of amplicons. These researchers hypothesized a lack of colony isolation in samples as the most likely explanation for poor results from DNA Southern and slot-blot hybridization.

PCR detection sensitivities in this study are comparable, and in some cases better than, to detection limits reported by other researchers using an IMS or metal hydroxide concentration method to remove bacteria from a food matrix. McIngvale et al. (2002) reported a detection limit of $4x10^4$ CFU/ml after growth in TSB for eight hours following a 1 CFU/g inoculation into a 25 g autoclaved ground beef sample for *stx*-II mRNA detection. However, we were able to gain PCR detection at 10^2 CFU/ml following growth in TSB starting with the same inoculum level and the same primers. Hsih and Tsen (2001), in attempts to detect *L. monocytogenes* and *S. typhimurium* from dairy products and beef following an IMS concentration and subsequent PCR, reported detection limits of 10^3-10^4 CFU/ml for each pathogen from a no growth homogenate. Tortorello et al. (1998) achieved IMS-PCR detection of *E. coli* O157:H7 in apple juice at a limit of 10^3 CFU/ml without enrichment prior to IMS.

Lucore et al. (2000) reported a detection limit of 10^2 CFU/25 ml sample for *L. monocytogenes* and *S. enterica*, serovar Enteriditis, using metal hydroxides to concentrate cells directly inoculated into (no growth) 25 ml samples of whole milk. Liu et al. (2003) reported chemiluminescence biosensor detection limits of *E. coli* O157:H7 following IMS from raw ground beef, lettuce, and chicken carcasses to be $3.2X10^2$ CFU/ml, $5.5X10^2$ CFU/ml, and $4.4X10^2$ CFU/ml, respectively. Following an IMS-based concentration of *E. coli* O157:H7 from raw chicken carcasses, researchers were able to detect as low as 10^3 CFU/ml by PCR (Chandler et al., 2001). McKillip et al. (2002) reported a PCR detection limit of a 1:8 dilution of DNA concentrated from 10^6 CFU/ml following growth in autoclaved skim milk. We report a similar detection limit from DNA concentrated from cells grown in raw ground beef.

However, detection of DNA from a 1:10 dilution from 10^6 CFU/ml cells following incubation in a food matrix was achieved only after DNA hybridization.

The data presented demonstrate only minor differences between the application of 2x TiOH and saline to concentrate and detect *E. coli* O157:H7 from ground beef. Detection of PCR amplicons of serially diluted DNA was observed at higher dilutions from cells concentrated by 2x TiOH compared to saline-concentrated cells. Observed differences between cell recoveries and PCR detection limits from autoclaved ground beef versus raw ground beef lead us to also conclude that model food systems cannot be trusted to always provide accurate descriptions of real world conditions. The use of a model food system to draw general conclusions about the behavior of a specific bacterium in a food under "natural" conditions may not be appropriate. This conclusion is supported by our study, which found a drop in percent of cells recovered from raw ground beef versus cooked ground beef, a loss of PCR detection at lower DNA concentrations from raw beef samples without growth, and a loss of Southern hybridization detection from 1/10 dilutions of DNA from concentrated cells even after growth. Raw ground beef likely contains components inhibitory both to concentration and detection that are not present in cooked ground beef, presumably due to their destruction via exposure to heat. Further, the effects of growth in a food matrix on bacterial cells in terms of their ability to dissociate from the matrix and be recovered in a viable state for detection/enumeration guide us to two conclusions: that research is needed both to understand the interactions that occur between bacterial cells and a food matrix

during prolonged exposure and to develop methods that might overcome these interactions for more consistent effective recovery of cells.

While we as of yet do not fully understand the mode of action of these metal hydroxides in terms of their ability to bind to and immobilize bacterial cells, it has been speculated that covalent bonds are formed between the hydroxyl groups of the metal hydroxide and various suitable components of amino acid side chains on the outer bacterial cell membrane (Kennedy et al., 1976). This represents one area of interest in terms of future research; work to understand the biochemical and stearic interactions between the metal hydroxides and cells should be completed in order that optimizations above and beyond that of this study may be investigated. Future research should also be carried out to investigate some potential explanations for observed phenomena, particularly the loss of detection sensitivity in samples concentrated from ground beef after enrichment. It may be that some component of the ground beef, not previously recognized as an inhibitor, was purified throughout the concentration and DNA extraction process and thus was able to effectively inhibit amplification.

The use of metal hydroxides for the immobilization of viable *E. coli* O157:H7 cells could prove a powerful technique for researchers and industry personnel who desire a rapid concentration method that is less costly than other methods commercially available to recover *E. coli* O157:H7 from ground beef. This may be of importance considering that *E. coli* O157:H7 remains classified as a zero-tolerance organism in raw ground beef.

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Table 2.1: Primary Concentration of *E. coli* O157:H7 From Autoclaved Ground Beef				
	No Growth: Meat Enrichment Mean % Loss/Recovery[b] (Std. Dev.)		From Growth: Meat Enrichment Mean % Loss/Recovery[b] (Std. Dev.)	
Inoculum[a] (CFU/ml)	Supernatant	Pellet	Supernatant	Pellet
10^2	1.78 (0.54)	90.81 (7.54)	4.87 (3.61)	84.86 (23.95)
10^4	1.99 (1.10)	109.75 (14.83)	1.69 (0.79)	117.60 (14.48)
10^6	2.56 (1.68)	100.14 (8.34)	1.32 (0.52)	122.06 (29.89)

a. Total CFU treated as 100%. Plating on TSA prior to concentration confirmed inoculum.
b. Percent loss or recovery is mean ± standard deviation of three replicate samples. Percent loss was calculated as described: (Tube y Total CFU Supernatant/Tube y Total CFU Input)*100%. Similarly, % recovery calculated as follows: (Tube y Total CFU Pellet/Tube y Total CFU Input)*100%.

Table 2.2: Comparison of 2x TiOH vs. 2x ZrOH for Recovery of *E. coli* O157:H7 From Autoclaved Ground Beef					
No Growth: 2° Pellet Mean % Recovery[b] (Std. Dev.)					
Inoculum[a] (CFU/ml)	1x TiOH	1x ZrOH	2x TiOH	2x ZrOH	0.9% NaCl Control
10^6	46.56^x (3.67)	54.63^x (6.77)	59.59^x (10.52)	33.42^y (8.47)	69.60^x (9.96)

a. Total CFU treated as 100%. Plating on TSA prior to concentration confirmed inoculum.
b. Percent recovery (post 2° concn.) is mean ± standard deviation of three replicate samples. Percent recovery was calculated as described: (Tube y Total Pellet CFU/Tube y Total CFU Input)*100%. Different superscript letters (x, y) denote statistically significant differences ($P \leq 0.05$) between treatment (TiOH, 2x TiOH, ZrOH, 2x ZrOH, Control) recovery values within an inoculum.

Table 2.3: Secondary Concentration Using 2x TiOH: No Growth Meat Homogenates From Autoclaved Ground Beef				
	Supernatant Loss % Loss[b] (Std. Dev.)		Pellet Recovery % Recovery[b] (Std. Dev.)	
Inocula (CFU/ml)[a]	Control (0.9% NaCl)	2x TiOH	Control (0.9% NaCl)	2x TiOH
10^2	$11.44^{x,u}$ (1.16)	$8.61^{x,v}$ (0.43)	$67.42^{x,v}$ (5.48)	$63.93^{y,u}$ (0.90)
10^4	$14.51^{x,u}$ (4.14)	$12.96^{x,v}$ (8.17)	$77.75^{x,v}$ (8.09)	$71.37^{x,u}$ (28.75)
10^6	$12.97^{x,u}$ (3.07)	$10.92^{x,v}$ (2.79)	$56.57^{x,v}$ (13.56)	$64.28^{x,u}$ (3.62)

a. Total CFU treated as 100%. Plating on TSA prior to concentration confirmed inoculum.
b. Percent loss or recovery (post 2° concn.) is mean ± standard deviation of three replicate samples. Percent loss was calculated as described: (Tube y Total CFU Supernatant/Tube y Total CFU Input)*100%. Similarly, % recovery calculated as follows: (Tube y Total CFU Pellet/Tube y Total CFU Input)*100%. Different superscript letters denote statistically significant differences ($P \leq 0.05$) between treatment (2x TiOH, Control) recovery values (x, y) and between inocula within a treatment (ova), respectively.

Table 2.4: Secondary Concentration Using 2x TiOH: From Growth Meat Homogenates From Autoclaved Ground Beef				
	Supernatant Loss % Loss[b] (Std. Dev.)		Pellet Recovery % Recovery[b] (Std. Dev.)	
Inocula (CFU/ml)[a]	Control (0.9% NaCl)	2x TiOH	Control (0.9% NaCl)	2x TiOH
10^2	$28.73^{x,u}$ (6.41)	$9.58^{y,v}$ (1.90)	$55.42^{x,u}$ (7.05)	$52.36^{x,v}$ (16.84)
10^4	$19.83^{x,u}$ (5.69)	$6.71^{y,v}$ (2.66)	$60.73^{x,u}$ (9.94)	$57.74^{x,v}$ (13.38)
10^6	$16.27^{x,u}$ (4.90)	$13.45^{x,u}$ (2.04)	$60.08^{x,u}$ (19.02)	$42.36^{x,v}$ (12.72)

a. Total CFU treated as 100%. Plating on TSA prior to concentration confirmed inoculum.
b. Percent loss or recovery (post 2° concn.) is mean ± standard deviation of three replicate samples. Percent loss was calculated as described: (Tube y Total CFU Supernatant/Tube y Total CFU Input)*100%. Similarly, % recovery calculated as follows: (Tube y Total CFU Pellet/Tube y Total CFU Input)*100%. Different superscript letters denote statistically significant differences ($P \leq 0.05$) between treatment (2x TiOH, Control) recovery values (x, y) and between inocula within a treatment (u, v), respectively.

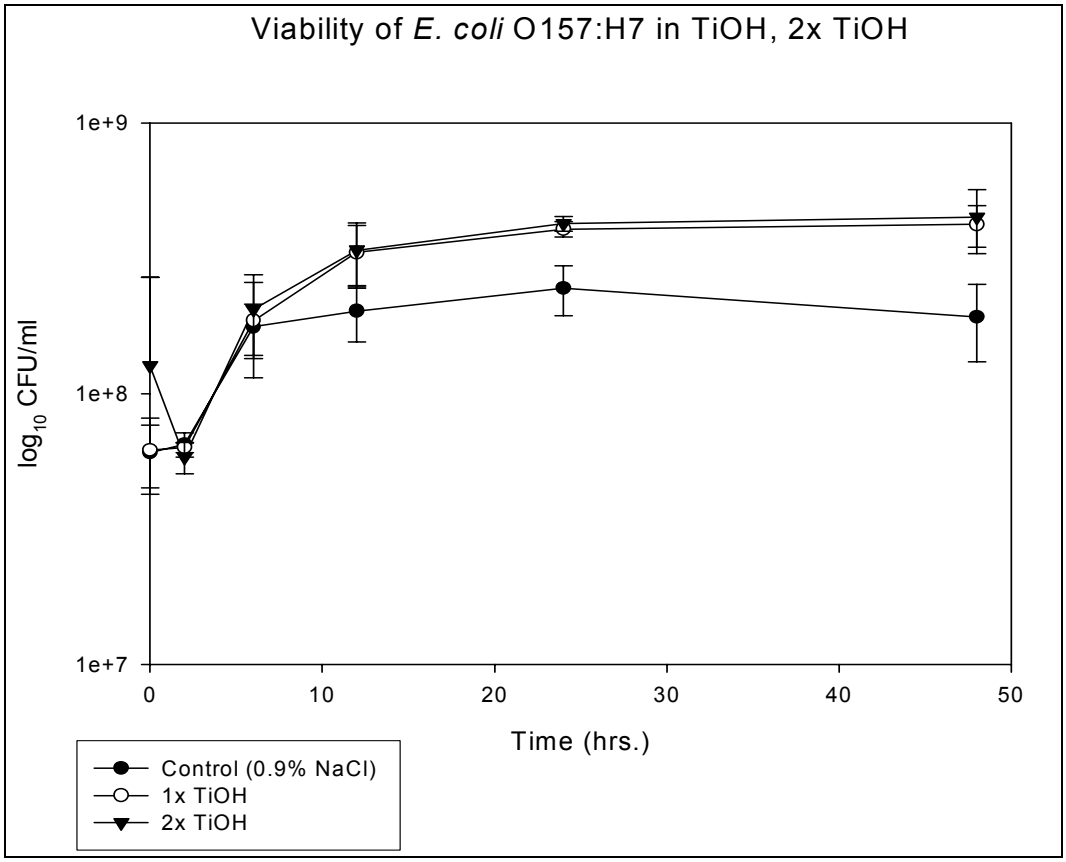
Table 2.5: Secondary Concentration Using 2x TiOH: "No Growth" Raw Ground Beef Homogenates				
	Supt. Loss, 2° Concentration % Loss[b] (Std. Dev.)		Pellet Recovery, 2° Concentration % Recovery[b] (Std. Dev.)	
Inocula (CFU/ml)[a]	Control (0.9% NaCl)	2x TiOH	Control (0.9% NaCl)	2x TiOH
10^2	$18.17^{x,v}$ (3.87)	$16.22^{x,u}$ (6.64)	$70.82^{x,v}$ (10.60)	$66.16^{x,u}$ (9.43)
10^4	$13.53^{x,v}$ (4.87)	$14.08^{x,u}$ (5.85)	$49.61^{x,u}$ (3.92)	$46.71^{x,u}$ (5.13)
10^6	$13.17^{x,v}$ (1.75)	$11.79^{x,u}$ (1.84)	$47.65^{x,u}$ (7.46)	$51.90^{x,u}$ (19.96)

a. Total CFU treated as 100%. Plating on TSA prior to concentration confirmed inoculum.
b. Percent loss or recovery (post 2° concn.) is mean ± standard deviation of three replicate samples. Percent loss was calculated as described: (Tube y Total CFU Supernatant/Tube y Total CFU Input)*100%. Similarly, % recovery calculated as follows: (Tube y Total CFU Pellet/Tube y Total CFU Input)*100%. Different superscript letters (x, y) denote statistically significant differences ($P \leq 0.05$) between treatment (2x TiOH, Control) recovery values (x, y) and between inocula (u, v), respectively.

Table 2.6: Secondary Concentration Using 2x TiOH: "From Growth" Raw Ground Beef Enrichments				
	Supt. Loss, 2° Concentration % Loss[b] (Std. Dev.)		Pellet Recovery, 2° Concentration % Recovery[b] (Std. Dev.)	
Inocula (CFU/ml)[a]	Control (0.9% NaCl)	2x TiOH	Control (0.9% NaCl)	2x TiOH
10^2	$5.76^{x,v}$ (1.74)	$8.61^{x,u}$ (1.96)	$15.88^{x,u}$ (6.10)	$55.57^{y,u}$ (23.84)
10^4	$11.39^{x,v}$ (7.27)	$6.00^{x,u}$ (3.95)	$41.33^{x,u}$ (15.64)	$35.48^{x,u}$ (2.32)
10^6	$5.88^{x,v}$ (0.99)	$9.57^{x,u}$ (0.67)	$74.98^{y,v}$ (5.81)	$38.16^{x,u}$ (12.63)

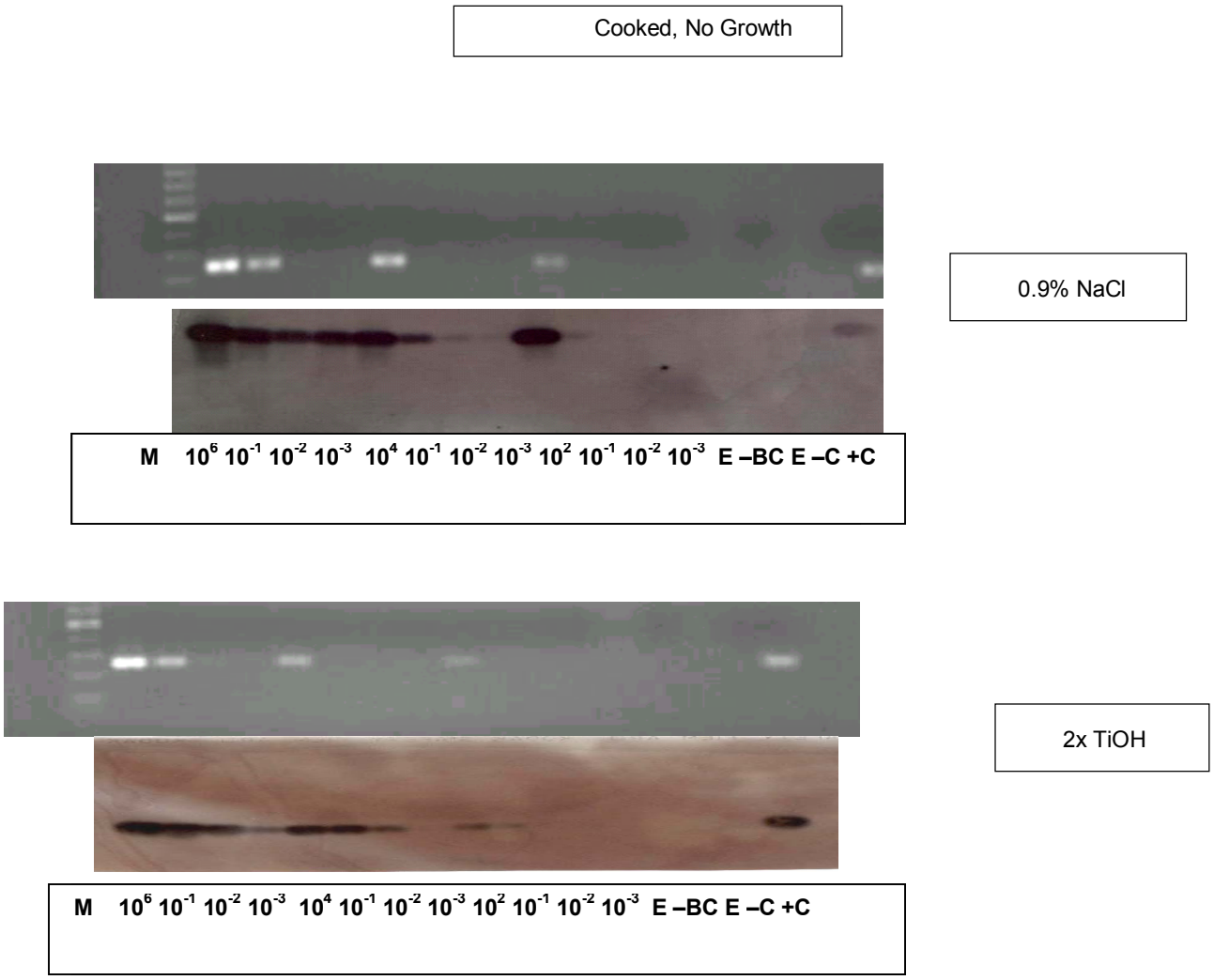
a. Total CFU treated as 100%. Plating on TSA prior to concentration confirmed inoculum.
b. Percent loss or recovery (post 2° concn.) is mean ± standard deviation of three replicate samples. Percent loss was calculated as described: (Tube y Total CFU Supernatant/Tube y Total CFU Input)*100%. Similarly, % recovery calculated as follows: (Tube y Total CFU Pellet/Tube y Total CFU Input)*100%. Different superscript letters (x, y) denote statistically significant differences ($P \leq 0.05$) between treatment (2x TiOH, Control) recovery values (x, y) and between inocula (u, v), respectively.

Figure 2.1: Viability of *E. coli* O157:H7 in TiOH, 2x TiOH, and Saline[a]



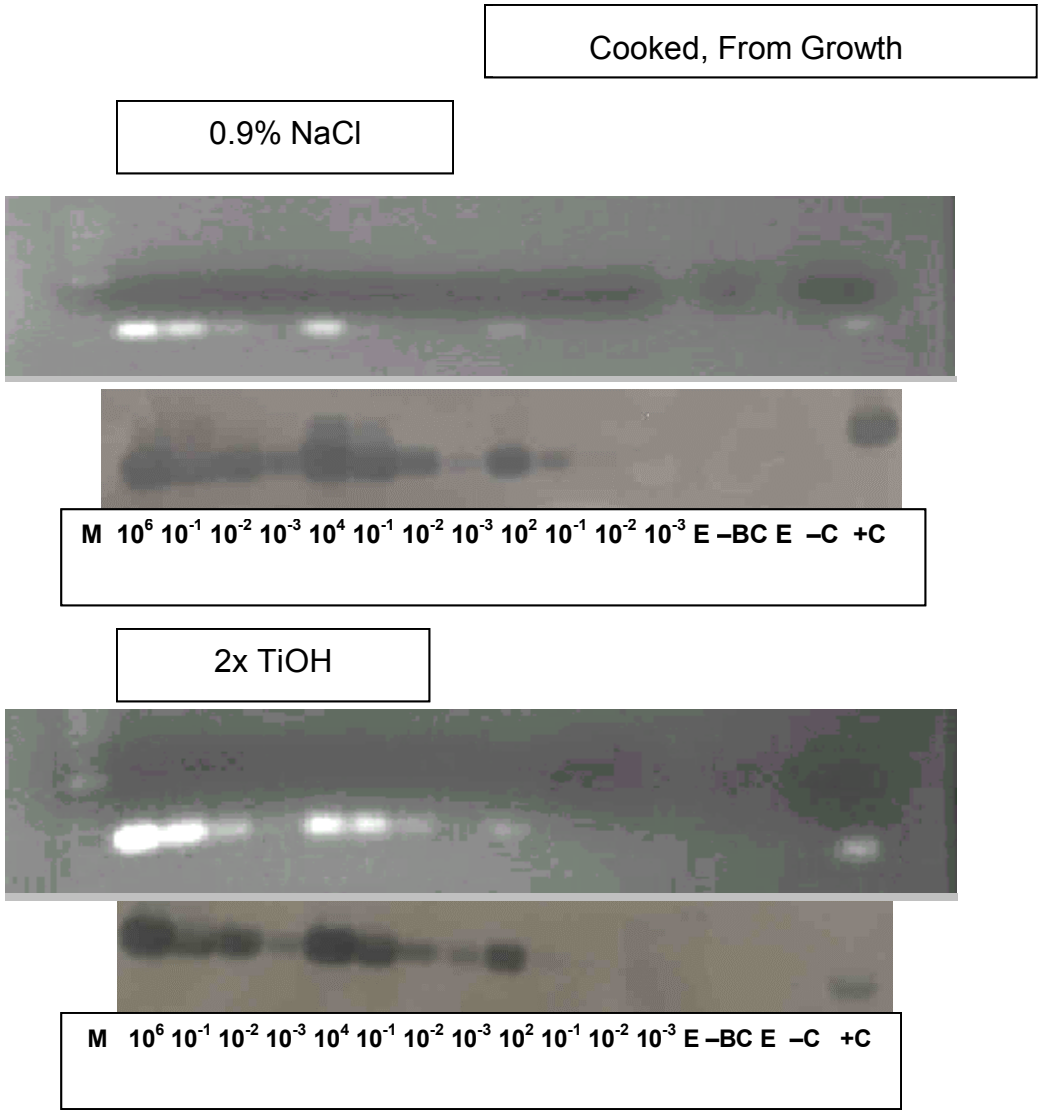
a. Points represent mean values of three replicate samples. Bars represent standard deviations of mean values.

Figure 2.2: Detection of *E. coli* O157:H7 From No Growth Meat Homogenates Using Cooked Ground Beef[a]



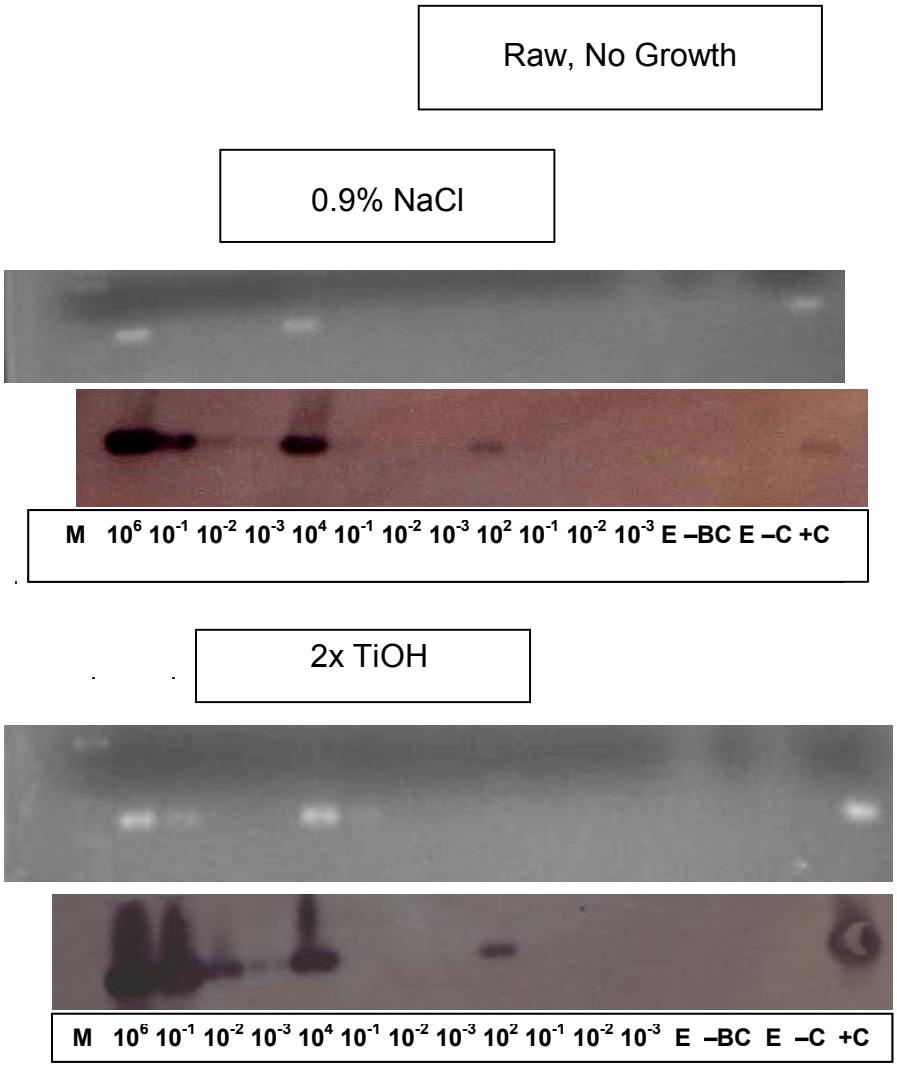
a. PCR detection was conducted in triplicate at each inoculum. Lanes correspond to DNA load taken from sample concentrate or dilution. Dilutions were prepared serially. M: Molecular ladder, -BC: negative beef control, -C: negative control; +C: positive control. Beef controls were processed identically to samples; -C replaced template DNA with 1 µl water and +C replaced template DNA with 1 µl broth culture *E. coli* O157:H7. Corresponding blots are placed below PCR gels.

Figure 2.3: Detection of *E. coli* O157:H7 With "From Growth" Meat Homogenates Using Cooked Ground Beef[a]



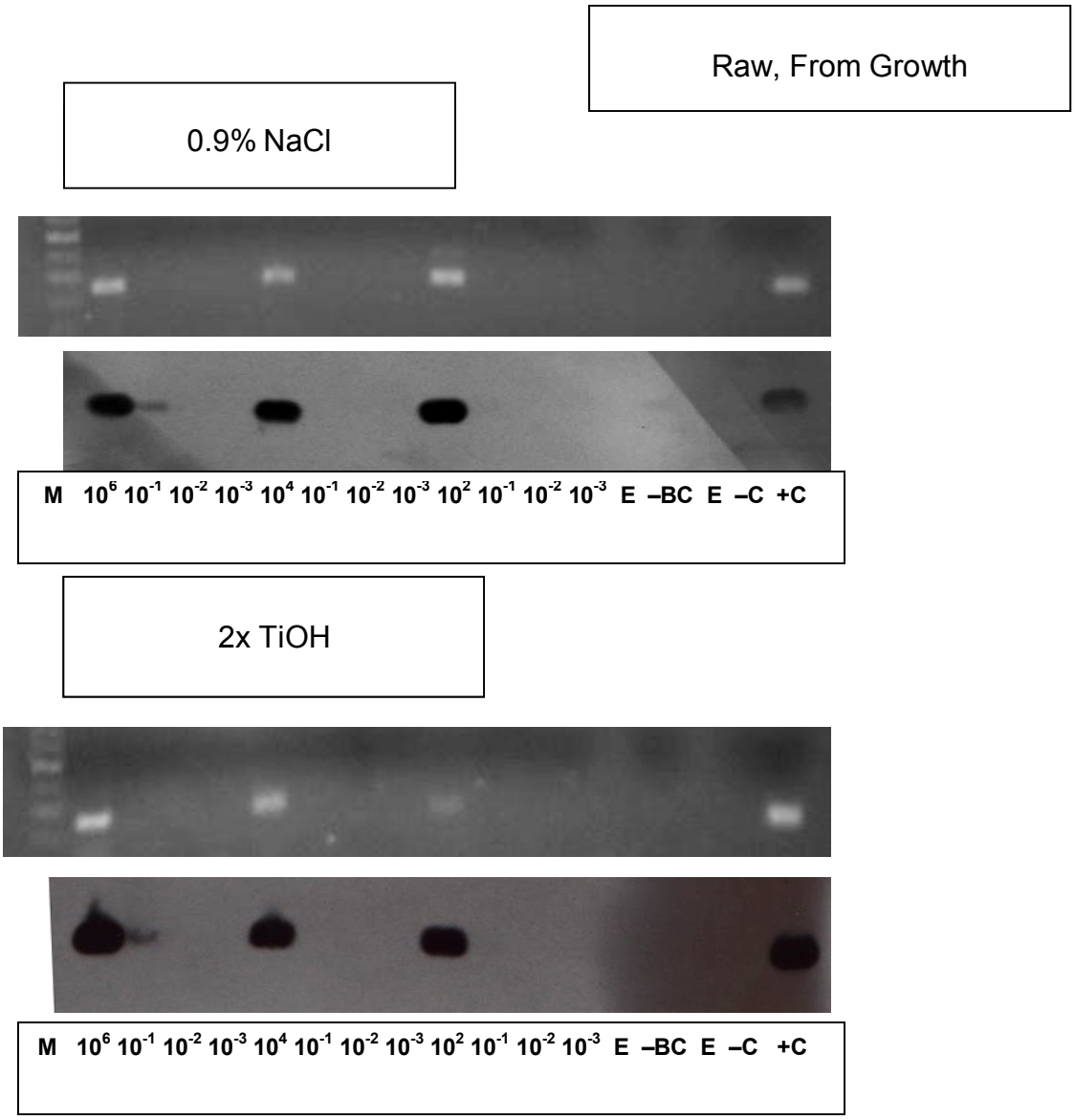
a. PCR detection was conducted in triplicate at each inoculum. Lanes correspond to DNA load taken from sample concentrate or dilution. Dilutions were prepared serially. M: Molecular ladder, -BC: negative beef control, -C: negative control; +C: positive control. Beef controls were processed identically to samples; -C replaced template DNA with 1 µl water and +C replaced template DNA with 1 µl broth culture *E. coli* O157:H7. Corresponding blots are placed under PCR gels.

Figure 2.4: Detection of *E. coli* O157:H7 With "No Growth" Meat Homogenates Using Raw Ground Beef[a]



a. PCR detection was conducted in triplicate at each inoculum. Lanes correspond to DNA load taken from sample concentrate or dilution. Dilutions were prepared serially. M: Molecular ladder, -BC: negative beef control, -C: negative control; +C: positive control. Beef controls were processed identically to samples; -C replaced template DNA with 1 µl water and +C replaced template DNA with 1 µl broth culture *E. coli* O157:H7 GFP. Corresponding blots are placed below PCR gels.

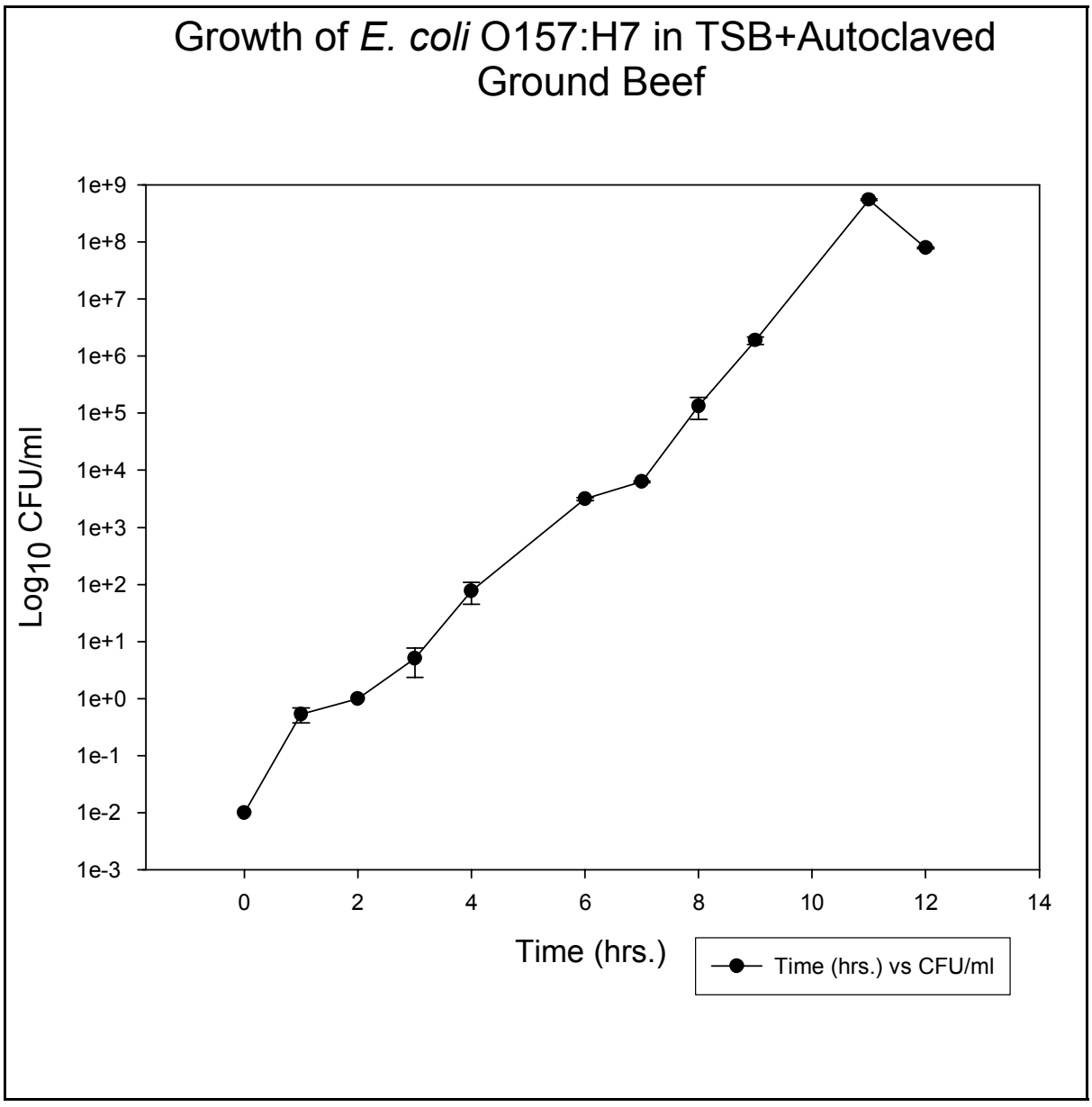
Figure 2.5: Detection of *E. coli* O157:H7 With "From Growth" Meat Homogenates Using Raw Ground Beef[a]



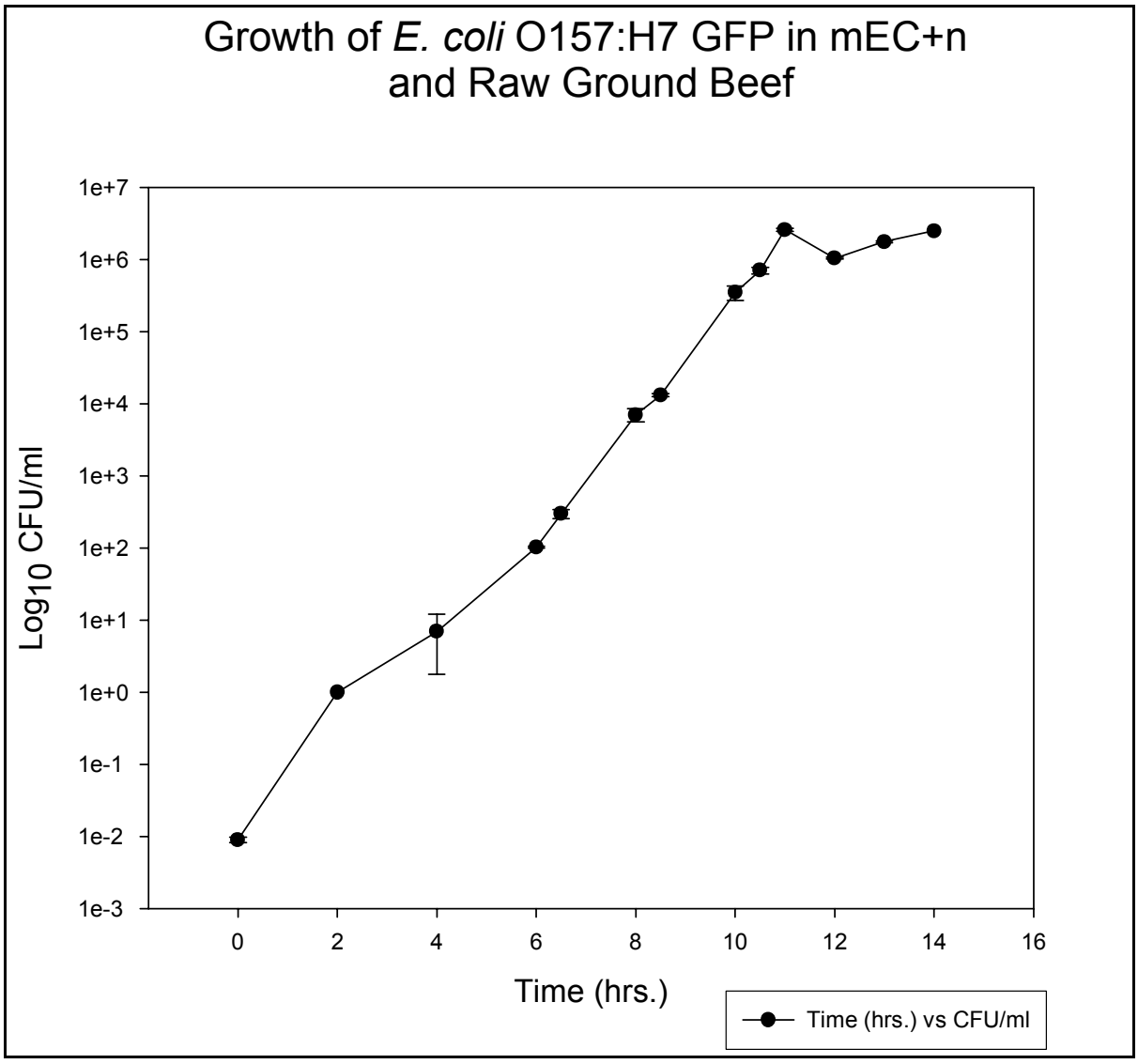
a. PCR detection was conducted in triplicate at each inoculum. Lanes correspond to DNA load taken from sample concentrate or dilution. Dilutions were prepared serially. M: Molecular ladder, -BC: negative beef control, -C: negative control; +C: positive control. Beef controls were processed identically to samples; -C replaced template DNA with 1 µl water and +C replaced template DNA with 1 µl broth culture *E. coli* O157:H7. Corresponding blots are placed below PCR gels.

Appendices

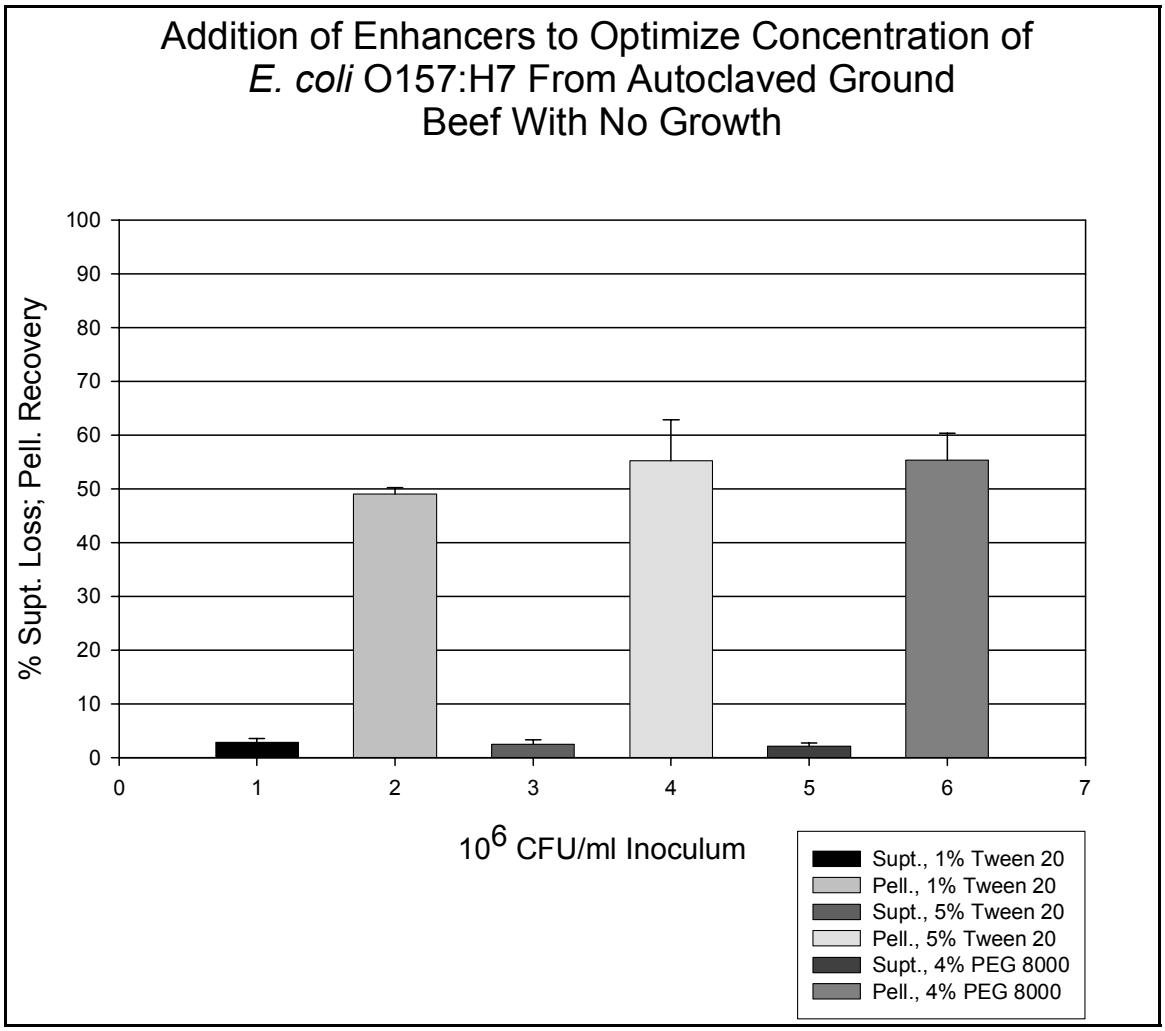
Appendix 1: Growth of *E. coli* O157:H7 in 99 ml TSB+10 g Autoclaved Ground Beef



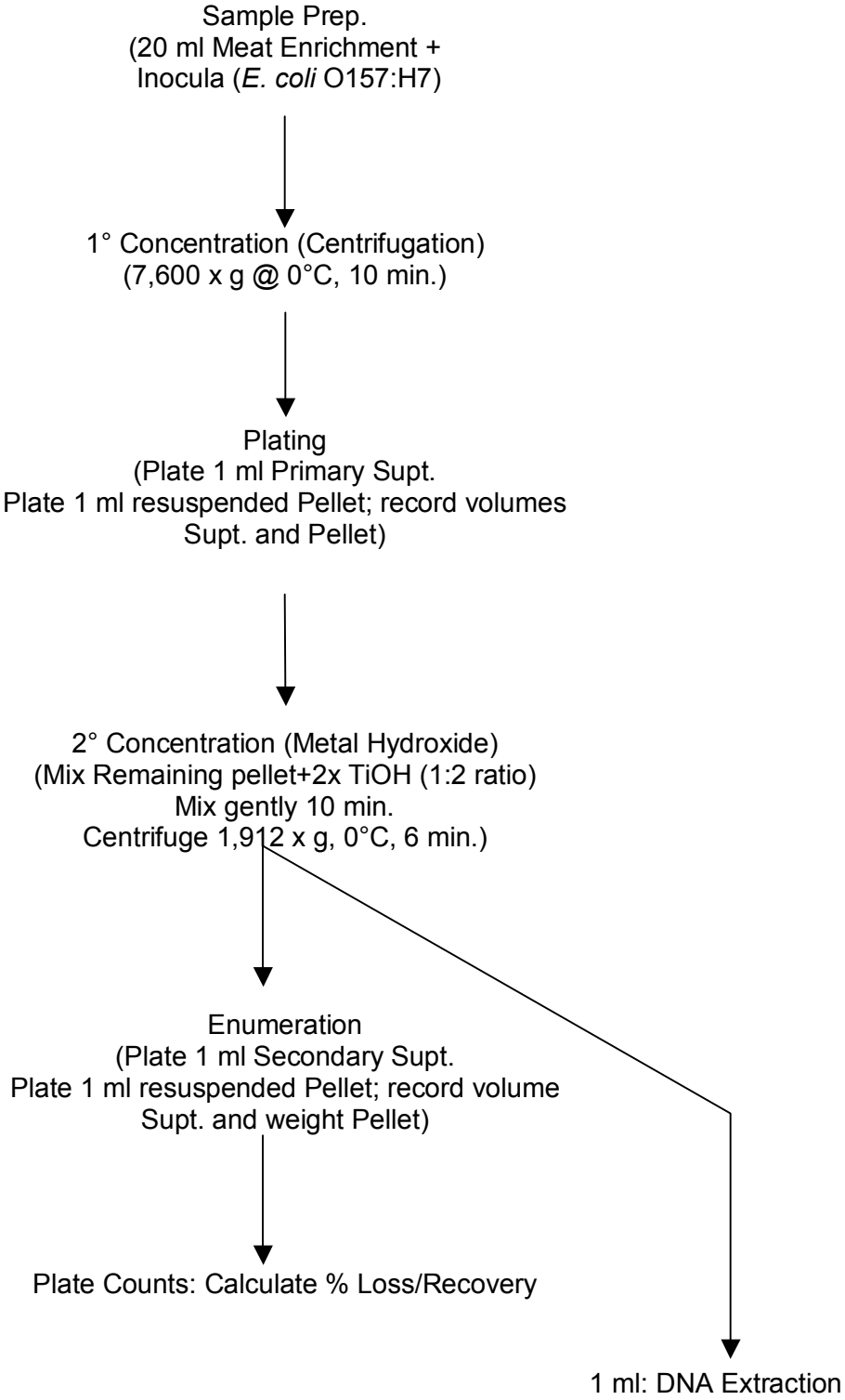
Appendix 2: Growth of *E. coli* O157:H7 GFP in 99 ml mEC+n+10 g Raw Ground Beef



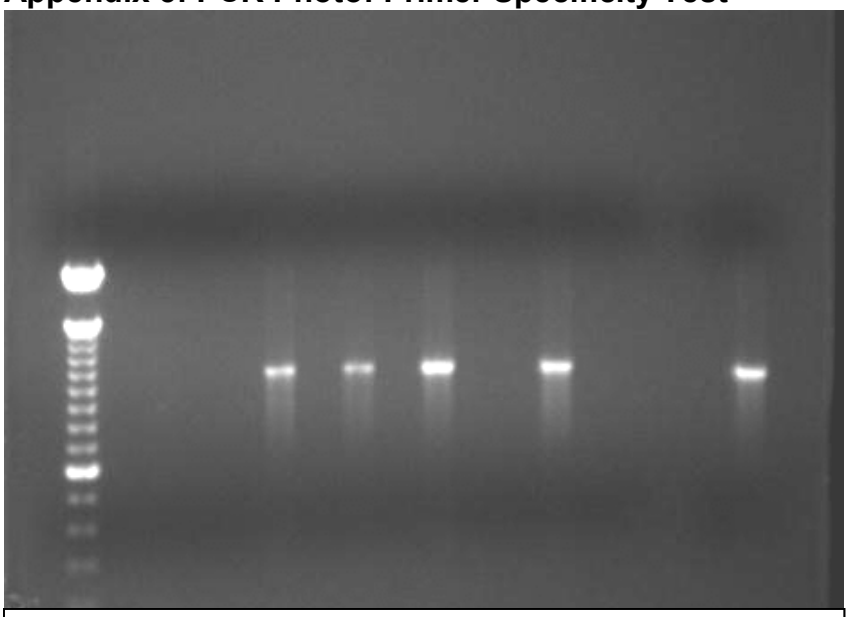
Appendix 3: Addition of Enhancers to Optimize Concentration of *E. coli* O157:H7 From Autoclaved Ground Beef With No Growth



Appendix 4: Flow Diagram of Metal Hydroxide Concentration Method



Appendix 5: PCR Photo: Primer Specificity Test[a]

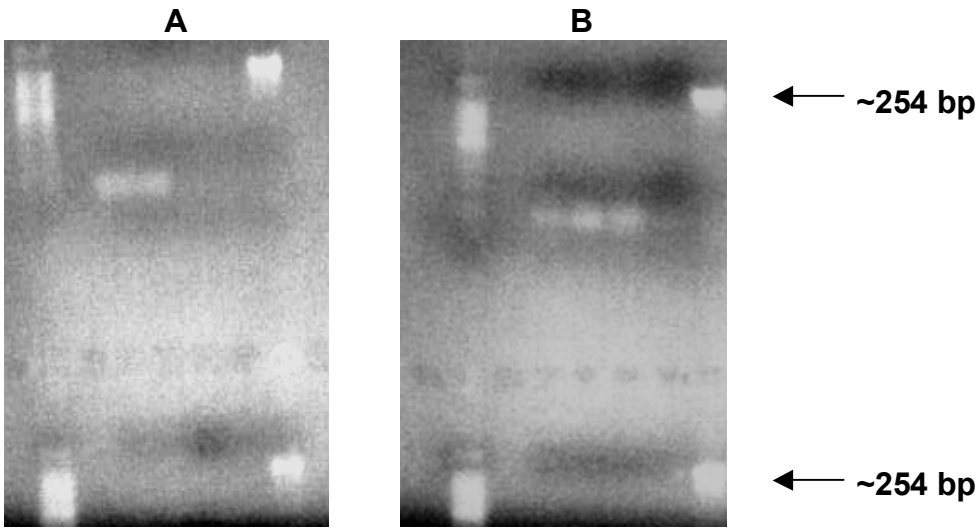


a. Lane assignments: E=empty, M=100 bp marker, -C=negative PCR control, +C=positive PCR control from *E. coli* O157:H7 culture. *stx*-II positive cultures: EC ATCC #43980 (6), EC O157:H- (8), EC O157:H7 (ATCC #43888) (10), *Shigella flexneri* 2a (ATCC #12022) (13). Negative cultures: EC O111:H- (1), EC O26:H11 (2), EC ATCC #43892 (3), *Salmonella enterica* (4), Serovar Enteriditis, *E. aerogenes* (5) EC ATCC #25922 non-pathogenic (7), *E. coli* spp. K-12 (9), *Listeria monocytogenes* (11), *S. typhimurium* (12), *A. hydrophila* (14), *V. vulnificus* (15).

Appendix 6: PCR Photo: Metal Hydroxide Concentration of *E. coli* O157:H7 From Autoclaved Ground Beef Using 2x TiOH or 2x ZrOH[a]

a. Lane Assignments: 1: Marker, 2: 2x TiOH-concentrated 10^6 CFU/ml cells, 3: 1/10 diluted DNA, 4: 1/100 diluted DNA, 5:1/1000 diluted DNA, 6: 2x ZrOH-concentrated 10^6 CFU/ml cells, 7: 1/10 diluted DNA, 8: 1/100 diluted DNA, 9: 1/1000 diluted DNA, 10: Empty, 11: Negative PCR control (no DNA template), 12: Positive PCR control (2 μl *E. coli* O157:H7 broth culture)

Appendix 7: Addition of Enhancement Agents to Aid in PCR Detection of 10^4 CFU/ml *E. coli* O157:H7 Following Metal Hydroxide Concentration[a,b]



Lane Assignments. **A**. **Top**: 1: Marker, 2: Empty, 3: 10^4 CFU/ml+DTT (0.5 mM), 4: 1/10 Diluted DNA+DTT, 5: 1/100 Diluted DNA+DTT, 6:Negative PCR Control (No DNA template), 7: Positive PCR Control (2 μl *E. coli* O157:H7 broth culture). **Bottom**: 1: Marker, 2: Empty, 3: 10^4 CFU/ml+Glycerol (8%), 4: 1/10 Diluted DNA+Glycerol, 5: 1/100 Diluted DNA+Glycerol, 6: Negative PCR Control, 7: Positive PCR Control. **B. Top**: 1: Marker, 2: Empty, 3: 10^4 CFU/ml+PEG 8000 (2.5%), 4: 1/10 Diluted DNA+PEG 8000, 5: 1/100 Diluted DNA+PEG 8000, 6: Negative PCR Control, 7: Positive PCR Control. **Bottom**: 1: Marker, 2: Empty, 3: 10^4 CFU/ml (Control), 4: 1/10 Diluted DNA, 5: 1/100 Diluted DNA, 6: Negative PCR Control, 7: Positive PCR Control.