ABSTRACT

ISONHOOD, Jr., JAMES, H. Sample processing strategies for optimal PCR detection of pathogens in foods. (Under the direction of Dr. MaryAnne Drake and Dr. Lee-Ann Jaykus)

The research in this manuscript highlights new and improved methods to concentrate pathogens from a complex food matrix and detect them via PCR. These contributions to the science of rapid pathogen detection are unique in that they are designed to measure the efficacy of the concentration or capture technique without using a pre-enrichment step. To address the potential of pathogen concentration to facilitate PCR detection, the following objectives were performed: (i) evaluating the performance of differential centrifugation as a means to concentrate and clarify the food sample for rapid PCR detection of *Listeria monocytogenes*, without pre-enrichment, (ii) investigating the efficacy of a novel immuno-capture device to capture *E. coli* O157:H7 and *Salmonella typhimurium* from foods.

For the first objective, we investigated filtration followed by a two-step, differential centrifugation as a means to concentrate bacteria and remove a large portion of the food sample prior to DNA extraction, PCR amplification, and Southern hybridization of *L. monocytogenes* targeting a unique region of 16S rDNA. Simple high speed centrifugation (11,950 x g) was also investigated to test the efficacy of our two-step method. Our method incorporated use of a 11g sample of ready-to-eat deli salad diluted 1:10 with 99ml 0.9% sterile normal saline. The two-step method was able to reduce the sample volume by approximately 10-fold rather than only 5-fold for simple high speed centrifugation. The two-step method was 1,000 fold (10^6 to 10^3 CFU/g) more sensitive than when using high speed centrifugation alone, and bacterial recoveries indicated that both methods produced similar recoveries. Following DNA extraction, PCR amplification, and Southern hybridization,

detection was achieved at input levels of 10^5 CFU/g for chicken salad, 10^4 CFU/g for macaroni salad, and 10^3 CFU/g for potato and seafood salads, with no pre-enrichment.

In our second objective, we evaluated the efficacy of a novel immuno-capture system (Pathatrix™) to capture *S. typhimurium* in buffered peptone water (BPW), ground turkey and nonfat dry milk and *E. coli* O157:H7 from BPW, ground beef and romaine lettuce. The Pathatrix system is unique in that it is designed to sample an entire 25g sample by circulating the homogenized 250ml volume across a surface of immunomagnetic beads. The samples were seeded with bacteria at levels ranging from 10^6 to 10^0 CFU/25g. The *E. coli* O157 format was able to capture 100% of input *E. coli* O157:H7 and PCR amplification was able to detect the pathogen at 10^0CFU/25g. The *Salmonella* format was not as robust, only capturing approximately < 1 % of cell input, but was able to produce a significant PCR detection limit of 10^2CFU/g when Pathatrix was preceded with the two-step centrifugation method. The two-step centrifugation further clarified the sample and improved detection by 1000-fold (10^5 to 10^2 CFU/25g).

This research provides further clues to expedite sample processing throughput prior to employing rapid methods for detection of pathogens in foods. It is our hope that knowledge of these techniques can help reduce or eliminate the need for preenrichment when screening food systems via PCR and other rapid methods.

SAMPLE PROCESSING STRATEGIES FOR OPTIMAL PCR DETECTION OF PATHOGENS IN FOODS

By

James H. Isonhood, Jr.

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Food Science

Raleigh

2005

Approved by:

Dr. MaryAnne Drake Chair of Advisory Committee	Dr. Craig Altier
Dr. Donn R. Ward	Dr. Lee-Ann Jaykus

BIOGRAPHY

James Harold Isonhood, Jr. was born in New Orleans, LA on the West Bank to two loving and caring parents Jimmy and Judy Isonhood. He is the older brother of two wonderful sisters. After graduating with his B.S. and M.S. degrees in Food Science at Mississippi State University, he moved far from home to the east coast here in Raleigh, North Carolina to attend North Carolina State University for his Ph.D. Shortly before starting his Ph.D., he married the lovely and talented Stacey Ross. He and Stacey have a loving little eight year old dog, Gus. The author is athletic enjoying water and snow skiing, wakeboarding, scuba diving and gymnastics. He enjoys the great outdoors which include camping, canoeing, fishing, and hunting. He often plays "Rock-n-Roll" very loudly on his electric guitar to entertain his family. He will begin work at Schreiber Foods Inc. as a Research Scientist in the Process Cheese Group. He wishes all of his friends and graduate students at North Carolina State and Mississippi State the best!

ACKNOWLEDGEMENTS

I would like to express my most sincere appreciation and gratitude to my advisor Dr. MaryAnne Drake her guidance and support. I also want to thank Dr. Lee-Ann Jaykus who played a significant support role in direction of my research. I also want to send my appreciation to my committee members, Drs. Donn Ward and Craig Altier for their support during my degree program. I would also like to thank the Drake and Jaykus labs for making the 3rd floor of Schaub and E-wing of the Flex building a fun place to be.

The encouragement of my parents, Jimmy and Judy, and their kind loving words and advice helped make this experience much easier. I love you Mom and Dad. I especially want to thank my wife Stacey for putting up with the long hours spent in the lab and really being there for me during the tough times. This experience wouldn't be the same without you Stacey.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

ESCHERICHIA COLI O157:H7 AND DEVELOPMENT OF RAPID METHODS FOR *E. COLI* O157:H7 DETECTION

1.1. INTRODUCTION

E. coli O157:H7 was first recognized as a pathogen in 1982 following two outbreaks in Oregon and Michigan (Jay, 2000). Not all *Escherichia coli* are pathogenic and these organisms are commonly found in the intestinal tracts as part of the normal facultative anaerobic microflora of humans and warm-blooded animals. *Escherichia coli* strains that can cause diarrheal illness are assigned to six specific virulence groups based on virulence properties, mechanisms of pathogenicity, clinical syndromes, and distinct O:H serogroups. These categories include: enteropathogenic *E. coli* strains (EPEC), enterotoxigenic *E. coli* strains (ETEC), enteroinvasive *E. coli* strains (EIEC), diffuse-adhering *E. coli* strains (DAEC), enteroaggregative *E. coli* strains (EAggEC), and enterohemorrhagic *E. coli* strains (EHEC) (Doyle et al., 1997).

EPEC

EPEC are mainly associated with infantile diarrhea. Many adults are carriers of EPEC, but do not show symptoms of illness. Even so, these organisms are capable of causing severe diarrhea. The original definition of EPEC was "diarrheagenic *E. coli* belonging to serogroups epidemiologically incriminated as pathogens but whose pathogenic mechanisms have not been proven to be relatd to either heat-labile enterotoxin, heat-stable enterotoxin, or Shigella-like invasiveness." More recently EPEC has been shown to induce

attaching and effacing (AE) lesions and some have been shown to produce shiga toxins (Stx) as well as heat-labile and heat-stable toxins (Doyle et al., 1997) Some common serotypes in this group include O18ab, O19ac, O55, O86, O111, O114, O119, O125, O126, O127, O128ab, O142, and O158 (Jay, 2000).

ETEC

ETEC are a major cause of infantile diarrhea in developing countries and are also commonly implicated in cases of traveler's diarrhea. These bacteria adhere to the proximal small intestine by fimbrial colonization factors (e.g., CFA/I and CFA/II), but do not invade, where they release heat-labile and heat-stable enterotoxins which cause fluid accumulation and resulting diarrhea (Doyle et al., 1997). Common serotypes of ETEC are O6, O8, O15, O20, O25, O27, O63, O78, O80, O85, O101, O115, O128ac, O139, O141, O147, O148, O149, O153, O159, O167 (Jay, 2000).

EIEC

Humans are a major reservoir for EIEC, which cause a disease similar to *Shigella dysenteriae* by invading and multiplying across colonic epithelial cells. This mode of attack results in death of epithelial cells and nonbloody diarrhea. Similar to *Shigella* spp., the invasive capacity of EIEC originates from the presence of a 140 MDa plasmid which encodes several outer membrane proteins (OMPs) associated with invasiveness. The OMPs and O antigens in EIEC are closely related with respect to antigenicity. Common serogroups of EIEC include O28ac, O29, O112, O124, O136, O143, O144, O152, O164, and O167 (Doyle et al., 1997).

DAEC

DAEC has been identified in cases of diarrhea in children in Mexico. The disease has been marked by mild diarrhea without blood or fecal leukocytes. The organism is capable of covering HEp-2 or HeLa cells uniformly in a diffuse-adherent pattern. In general, DAEC do not produce heat-labile or heat-stable toxins or significant levels of Shiga toxins (Stxs). Another defining characteristic is that DAEC do not invade epithelial cells or have EPEC adherence factor plasmids (Doyle et al., 1997).

EAggEC

EAggEC have been recently associated with persistant diarrhea in infants and children worldwide that can last >14 days. This group is similar to ETEC in the way they bind to intestine mucosal cells but are dissimilar in that they bind in small clumps. They are unique in the way they consistently form a unique pattern of aggregative adherence to HEp-2 cells. The observed appearance has been described as stacked bricks on the surface of HEp-2 cells (Doyle et al., 1997). EAggEC are noninvasive and some produce a heat-stable enterotoxin EAST1 (Jay, 2000). Its significance as an agent of diarrheal disease is still unknown (Doyle et al., 1997) The serotypes found among this group include O3, O4, O6, O7, O17, O44, O51, O68, O73, O75, O77, O78, O85, O111, O127, O142, and O162 (Jay, 2000).

EHEC

The most frequently isolated serotype in this goup is *E. coli* O157:H7. The organism produces a toxin which is almost identical to the Shiga toxin produced by *Shigella dysenteriae*. Disease caused by this microorganism can occur as mild diarrhea or hemorrhagic colitis (HC). In serious cases (2-15%) hemorrhagic colitis can develop into

hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpurea (TTP) (Doyle et al., 1997; Todd and Dundas, 2001). The mortality rate of these complications is 3-17% and up to 30% in outbreaks (Todd and Dundas, 2001).

EHEC strains infect by attaching to small intestine mucosal cells by changing their surface structure by means of attachment and effacement. This occurs when the finger-like projections (microvilli) on the mucosal surface become elongated when the bacteria are in propinquity but not attached to the mucosa; these are lost when the bacteria are attached. Following invasion of mucosal cells, EHEC do not spread to adjacent cells. Non-O157 STEC have also been isolated from cases involving diarrhea and HUS (Law, 2000). Other serotypes of EHEC include O26:H11, O103, 0104, O111, and sorbitol-fermenting O157:H- (Doyle et al., 1997).

1.2. CHARACTERISTICS OF EHEC

E. coli O157:H7 was first recognized as a pathogen in 1982 following two outbreaks in Oregon and Michigan. The isolates were clinically different from EPEC and thus a new group of *E. coli*, EHEC, was formed. Distinguishing characteristics of *E. coli* O157:H7 from other groups of *E. coli* include its inability to ferment sorbitol, produce β-glucuronidase, and grow at or above 44.5°C. Most *E. coli* (93 %) of human origin can ferment sorbitol within 24 h, but *E. coli* O157:H7 does not. Most strains (93 %) also have the enzyme β-glucuronidase, which is the basis for detection of *E. coli* in a rapid fluorogenic assay. The assay utilizes 4-methylumbelliferyl β-D-glucuronide (MUG) as an indicator which is hydrolyzed by the enzyme β-glucuronidase to form a fluorogenic product (Padhye and Doyle, 1992). While these traits do hold true for most 0157 strains, sorbitol-fermenting, β-

glucuronidase producing *E. coli* O157 have been isolated and are estimated to account for 50 % of *E. coli* O157 infections in Germany. These unusual variants are thought to be a distinct clone of *E. coli* O157 (Law, 2000). Gunzer et al. (1992) isolated sorbitol fermenting, β-glucuronidase positive O157 strains in 17 of 44 patients with diarrhea (18) and HUS (26) (Gunzer et al., 1992). Non O157 EHEC is also sorbitol positive and β-glucuronidase positive; making specific identification and differentiation of these EHEC strains more difficult.

E. coli O157:H7 growth studies in trypticase soy broth indicate that the organism grows well between 30-42°C, having generation times between 0.49 h and 0.64 h at 37°C and 42°C, respectively (Padhye and Doyle, 1992). Poor growth of the organism has been observed at 44-45°C, and no growth occurred within 48 h at 10 or 45.5°C. Procedures used to detect fecal coliforms in food use incubation temperatures of 44-45°C; thus *E. coli* O157:H7 would probably not be detected during normal screening for fecal coliforms using such procedures (Padhye and Doyle, 1992).

1.3. OUTBREAKS

A series of outbreaks involving *E. coli* O157:H7 have occurred since its identification as a pathogen in 1982. Between 1982 and 2002, a total of 350 outbreaks have been reported in 49 states resulting in 1,493 (17.4%) hospitalizations, 354 (4.1%) cases of HUS, and 40 (0.5%) deaths (Rangel et al., 2005). These outbreaks have increased from about 2 per year from 1982 to 1992 to about 17 in 1993 and 30 in 1994. From 1994 to 1997 the average number of outbreaks per year was 31 and in 1998 there were 42 confirmed outbreaks (CDC, 2000). The numbers seem to level off in 1999 with 38 reported outbreaks, but five (13 %) of

these outbreaks involved two or more states compared to only two (4 %) of those reported in 1998. Then at the turn of the century, in 2000, the number peaked at 46 outbreaks (Rangel et al., 2005). This increase in the number of reported outbreaks and multistate outbreaks may be due to public health departments' increased use of pulsed-field gel electrophoresis (PFGE) for subtyping strains. Widely dispersed cases and small clusters within states may be linked by using PFGE. These are cases that in the past may have not been known to be related, perhaps providing further explanation as to the increased reporting of outbreaks (CDC, 2000). Unfortunately some cases go unreported since infected persons having mild or no symptoms and those with nonbloody diarrhea are not likely to seek medical attention (Doyle et al., 1997). According to the Centers for Disease Control and Prevention, *E. coli* O157:H7 causes an estimated 73,500 cases of infection, 2,000 hospitalizations, and 60 deaths in the U.S. each year (CDC, 2001).

The majority of outbreaks occur during the warmer summer months. Within the United States, from 1982 to 2002, about 89% of outbreaks occurred from May to November (Rangel et al., 2005). In 1997 almost half (45.7 %) of the 1,167 recorded *E. coli* O157:H7 cases occurred in the months of July, August, and September (Jay, 2000). Possible reasons for increased summer illness include: (i) increased incidence of the pathogen in cattle or livestock during the summer; (ii) increased human exposure to ground beef or other contaminated foods during the "cook-out" months; and/or (iii) increased improper handling or undercooking of products like ground beef during warm summer months than other months (Doyle et al., 2001).

Of the 350 reported outbreaks (1982 - 2002), the majority of transmission routes were as follows: 183 (52%) foodborne, 74 (21%) unknown, 50 (14%) person-to-person, 21 (6%)

recreational water, 11 (3%) animal contact, 10 (3%) drinking water, and 1 (0.3%) laboratory-related transmission route. Of the foodborne outbreaks, ground beef was the most common vehicle at 41%, followed by 23% unknown, 21% produce, 6% other beef, 5% other foods, and 4% in dairy products (Rangel et al., 2005). Most foods implicated have been associated with raw or undercooked foods of bovine origin such as raw milk or ground beef. Other foods associated with *E. coli* O157:H7 infection are vegetables, apple cider, cantaloupe, mayonnaise-containing salad dressing, and salami. Outbreaks involving alfalfa sprouts, fruit salads, and coleslaw have also occurred over the years. In recent years contamination of raw fruits and vegetables has become an important issue due to the lack of further processing of these foods.

The first outbreak of *E. coli* O157:H7 infection occurred in Oregon in 1982 rendering 26 individuals ill and 19 hospitalized. Illness for all patients was accompanied with bloody diarrhea and severe abdominal pain. The outbreak was associated with eating undercooked hamburgers at a fast-food restaurant chain. Another outbreak ensued three months later with the same fast-food restaurant chain. *E. coli* O157:H7 was again implicated as the causative agent and was isolated from patients and a frozen ground beef patty (Doyle et al., 2001).

Contamination of municipal water by *E. coli* O157:H7 occurred in December and January of 1990 in Cabool, Mo. Of the 243 people infected, 86 had bloody diarrhea, 32 were hospitalized, and 4 died. Another *E. coli* O157:H7 outbreak was associated with a lakeside park near Portland, Ore in the summer of 1991. Twenty-one cases were identified from 19 housholds. All patients were children and seven were hospitalized. Swallowing lake water was associated with illness and those ill tended to spend more time in the water. Many of the bathers were toddlers and not yet toilet trained (Doyle et al., 2001).

In southeastern Massachusetts in the fall of 1991, an outbreak involving consumption of unpasteurized apple cider occurred. Twenty-three cases of *E. coli* O157:H7 infection from a total of 13 families were reported and six patients were hospitalized. Investigation at the site where the apple cider was processed indicated that the apples were pressed without washing, the cider was not pasteurized, and no preservatives were used. Before this outbreak, apple cider was considered safe for consumption due to its high acidity and low pH (< 4.0) (Doyle et al., 2001).

The largest multistate outbreak of *E. coli* O157:H7 occurred in four western U.S. states in early 1993. The age of patients ranged from 4 months to 88 years and 90 % of patients had eaten at a single fast-food restaurant chain. One hundred seventy-eight were hospitalized, 56 developed HUS, and 4 children died. The cause of the outbreak was due to consumption of hamburgers that were insufficiently cooked by the fast-food restaurant chain. Investigations at the restaurant revealed that 10 of 16 hamburgers cooked had internal temperatures of below 60° C, which was much lower than the minimum temperature of 68.3°C allowed by the state of Washington (Doyle et al., 2001). The consumption of dry fermented salami was implicated in a multistate outbreak in 1994. Studies later confirmed that *E. coli* O157:H7 can survive the fermentation, drying, and storage of fermented sausage (Doyle et al., 2001).

In Japan, a large outbreak involving 10,000 cases of *E. coli* O157:H7 was associated with the consumption of radish sprouts from May to August 1996. Four separate clusters were identified with the largest involving 6,259 primary school children in Sakai City, Osaka Prefecture, in which 92 patients developed HUS (WHO, 1996; Watanabe et al., 1999).

In 1997, the consumption of alfalfa sprouts was directly related to the illnesses of 60 persons caused by *E. coli* O157:H7. Of those that were ill, 44 had bloody diarrhea and 25 were hospitalized. Two had hemolytic uremic syndrome (HUS) and one developed thrombotic thrombocytopenic purpura. In a case-control study, eighteen of 30 case-patients had eaten alfalfa sprouts within 7 days of the onset of illness (CDC, 1997).

In the fall of 2000, 51 people became ill from *E. coli* O157:H7 after visiting a petting farm in Pensylvania. Patients ranged in age from 1 to 54 (median 4) and symptoms included bloody diarrhea, fever, and vomiting. Sixteen patients, all under the age of ten, were hospitalized. Eight patients developed HUS and one patient experienced renal failure and required renal transplantation. Increased risk of illness was associated with contact with calves and their environment, while hand-washing was protective (Crump et al., 2002).

1.3.1. Recent Outbreaks

In the summer months of 2002, a multistate outbreak linked by PulseNet, the National Molecular Subtyping Network for Foodborne Disease Surveillance, involved 28 cases in Colorado and six other states. Seven of the patients were hospitalized and five developed HUS. Ground beef was implicated in the outbreak and a nationwide recall of 18.6 million pounds of ground beef was issued (CDC, 2002).

1.4. PATHOGENICITY OF EHEC

Shiga toxin-producing *Escherichia coli* (STEC) include *E. coli* strains that produce Shiga toxin (Stx) or possess the Stx encoding gene, *stx*. STEC strains causing HC and HUS have been collectively referred to as enterohaemorrhagic *E. coli* (EHEC) (Cobbold and Desmarchelier, 2001). EHEC is a subset of STEC, with the term EHEC being used to describe STEC strains that have caused HC, produce Shiga toxins, carry the pO157 plasmid, and produce A/E lesions. Some common serogroups of STEC commonly classified as EHEC are O157, O26, and O111. Strains of STEC that contain virulence markers but have not been linked with HC are not classified as EHEC. EHEC produce potent cytotoxins known as Shiga toxins (Stx), which were originally named Verotoxins ((VT) VT1 and VT2) due to their cytopathic effect on Vero cells, an African green monkey kidney cell line. VT1 is genetically and immunologically related to Stx produced by *Shigella dysenteriae* type 1. As a result, researchers named the toxins produced by EHEC as Shiga-like toxins (SLTs). The two terms VT and SLT have since been used interchangeably throughout the literature. Calderwood et al. (1996) proposed renaming the toxins Shiga toxins (Stx) and so this nomenclature will be used throughout this review. The shiga toxins have been associated with human diarrhea, HC, and HUS, but the ability of an organism to produce Stx alone is not believed to result in disease (Law, 2000; Doyle et al., 2001). Other virulence factors which are characteristic of EHEC are therefore thought to contribute to disease. The pathogenicity of EHEC is not fully understood, though histopathology of HC and HUS patients, tissue culture studies, animal model studies, and genetic studies have provided evidence of virulence factors which possibly interact and contribute to disease (Doyle et al.,

2001). Such factors include the shiga toxins, attaching and effacing lesions, fimbrial adhesins, and the adhesin intimin.

1.4.1. Shiga Toxins

Shiga toxin is a holotoxin which is composed of an A-subunit of approximately 32 kDa in association with a pentamer of 7.7 kDa B-subunits. The A subunit is the enzymatic component and cleaves a single adenine residue from near the 3′ end of the 28S rRNA portion of the eukaryotic ribosomal complex. This action causes the inhibition of peptide elongation by blocking elongation factor-1 dependent aminoacyl-tRNA binding. The interaction or binding of the Shiga toxin to mammalian cells occurs by association of B-subunits with a membrane glycolipid of the globo-series, globotriaosylceramide (Gb_3). Entry into Gb_3 containing cells is supposedly mediated by receptor-dependent endocytosis (Tesh and O'Brien, 1991).

Two antigenically distinct toxin forms that are called Stx1 and Stx2 are the primary toxins produced by EHEC and encoded for by genes located on the organism's chromosome. Several subgroups of Stx2 have been identified as Stx2, Stx2c, Stx2d, and Stx2e. Each of these subgroups differs in their amino acid sequences of the A and B subunits of the Stx2 toxin, suggesting great heterogeneity among Stx2 group (Doyle et al., 2001). The Stx2 variant, Stx2e is more toxic to Vero cells than to HeLa cells. Stx2 and Stx2e are neutralized by antisera against Stx2 but not by anti-Stx toxin (Jay, 2000). The Stx2e was identified in association with edema in piglets (Doyle et al., 2001). Calderwood et al. (1987) elucidated the nucleotide sequence of the *sltA* and *sltB* genes that encode Stx. The genes for the A and B subunits form an operon. The amino acid sequence of the B subunit of Stx was identical to

the B subunit of Shiga toxin. The peptide sequence of the A subunit was identical to the A subunit of ricin, a plant toxin. This provides evidence that some prokaryotic toxins may be evolutionarily linked to eukaryotic enzymes (Calderwood et al., 1987).

Shiga toxins are thought to be the main factors causing HC and HUS (Law, 2000). Human stool cultures from patients (7 with HUS, 1 healthy, and 2 with enteritis) indicated that non-O157 STEC represented between 0.03 and 68.1 % of the coliform flora (Beutin et al., 1996). Among the non-O157 isolates the toxin phenotype is variable and isolates producing only Stx1 are commonly found. The production of Stx2 is one of the defining characteristics of *E. coli* O157:H7, since most strains produce Stx2 only (Law, 2000). Some epidemiological evidence indicates that isolates producing Stx2 are more commonly associated with serious disease than those producing only Stx1 or Stx1 and Stx2 (Scotland et al., 1987). Louise and Obrig (1992) hypothesized that lipopolysaccharide (LPS; endotoxin) may combine with Shiga toxin to facilitate vascular damage commonly observed in cases of HUS. They exposed the two toxins to human umbilical vein endothelial cells (HUVEC). When tested alone, LPS was not cytotoxic at concentrations at or below 10 μg/ml and Shiga toxin expressed dose dependent cytotoxic effects. Results indicated that when LPS and Stx were added to HUVEC in combination, there was a synergistic cytotoxic effect and this effect was enhanced with preincubation of LPS (Louise and Obrig, 1992).

Stx1 and Stx2 have similar structures and modes of action, but their toxicities appear to be different (Paton and Paton, 1998). When the toxins were compared using human renal microvascular endothelial cells, the accepted target of Shiga toxins, Stx2 was 1,000 times more cytotoxic than Stx1. Researchers using a mouse model revealed further evidence of the potency of Stx2. An Stx1 and 2 positive *E. coli* O157 isolates were injected into the mouse

resulting in fatal cortical tubular necrosis. Passive immunization with a monoclonal antibody to Stx2 prevented death while immunization with antibody against Stx1 did not prevent death. An *E. coli* strain (K-12) genetically engineered to contain Stx2 caused death in mice, while the same strain engineered with Stx1 did not (Wadolkowski et al., 1990).

Environmental factors have been shown to impact Shiga toxin production. Shiga toxin production was shown to increase with aeration (Weeratna and Doyle, 1991; Weagant et al., 1994; McIngvale et al., 2002). This fact is also supported by Palumbo et al. (1995) who reported that increased Stx production was based on the time and temperature that supported the optimal cell growth. Since aeration increases cell growth rate of *E. coli* O157:H7, one could deduce that greater development of Stx toxin would be produced under such conditions.

1.4.2. Attaching and Effacing Lesions

A very important trait characterizing EHEC is its ability to produce attaching and effacing (A/E) lesions on a variety of cell types. The ability to produce A/E lesions was first observed in EPEC strains. The lesions are characterized by degeneration and effacement of intestinal epithelial cell microvilli, intimate attachment of the bacteria to the cell surface, and assembly of highly organized cytoskeletal structures in the cells beneath the adherent bacteria. Various components make up the cytoskeletal structure including actin, talin, ezrin, and α-actinin (Doyle, 1997).

The molecular events involved in formation of A/E lesions have been elucidated in recent studies. In EPEC, all genes required for the generation of A/E lesions are located on a 35.5-kb "pathogenicity island" known as the locus for enterocyte effacement (LEE). This

locus has been sequenced in both EPEC and *E. coli* O157 (Law, 2000). In EHEC, pathogenicity islands are found of different sizes, and research suggests that genes associated with virulence are not limited to the largest islands (Perna et al., 2001) The LEE of *E. coli* O157 is slightly larger than the EPEC LEE, but has similarities in structure, organization, and gene arrangement (Paton and Paton, 1998). Secretion of the LEE-encoded proteins EspA, EspB, and EspD, necessary for initiation of signal transduction, is carried out by a type III secretion system. The LEE also contains the *eaeA* gene, encoding intimin, which is a 939-amino-acid outer membrane protein (OMP) that regulates intimate attachment to the enterocyte, and *Tir* (previously Hp90) which encodes the protein Tir (translocated intimin receptor) that functions as a receptor for intimin (Perna et al., 1998; Paton and Paton, 1998; Law, 2000).

1.4.3. Adhesion

It is speculated that EHEC must establish an intimate adherence with intestinal epithelial cells within the colon or possibly the distal small intestine following passage through the stomach in order to initiate illness (Paton and Paton, 1998). Several factors involved in attachment to epithelial cell lines and the mechanisms that control those factors have been investigated with varying results. Karch et al. (1987) studied a 60-MDa pO157 plasmid of *E. coli* O157:H7 which expresses a fimbrial adhesin that confers bacterial attachment to Henle-407 but not to Hep-2 cells. Conversely, Junkins and Doyle (1989) showed that a plasmidless derivative of O157:H7 adhered to Henle 407 cells three times better than to the parental strain. Further studies have observed O157:H7 adherence to Hep-2 cells (Law, 2000). Another study by Sherman et al. (1987) reported that only one of five

strains that possessed the 60 MDa plasmid was piliated, yet all strains adhered to Henle 407 and Hep-2 cells. They concluded that pili were not solely responsible for attachment of *E. coli* O157:H7 to epithelial cells (Sherman et al., 1987). *In vivo* studies of *E. coli* O157 infection using rabbits and gnotobiotic piglets indicated that the severity or incidence of diarrhea and intestinal pathology was not affected by the presence or absence of the pO157 plasmid (Law, 2000).

There is sufficient evidence suggesting the carriage of *eaeA*, which codes for a 97-kDa surface-exposed protein known as Intimin needed for intimate attachment of EHEC to epithelial cells, is required by EHEC strains to cause severe human illness such as HC and HUS. *In vivo* studies involving calves and gnotobiotic piglets showed decreased virulence in intimin-deficient *E. coli* O157 mutants, which were unable to colonize the intestine of these animals when compared to the parent strain (Law, 2000). Wieler et al. (1996) discovered that the majority (122 of 174) of STEC strains isolated from diarrhetic calves possessed *eae* genes, indicating the importance of the *eae* gene as a virulence marker for bovine STEC strains. All STEC infections in the calves studied were reported to cause AE lesions. However, the researchers found that only 65.6% of *eae*-positive strains produced a positive fluorescence actin staining (FAS) reaction in HEp2 cells, suggesting that screening for the *eae* gene may not be a sufficient method for the assessment of AE-positive STEC strains (Wieler et al., 1996).

1.5. PREVALENCE

Cattle are thought to be the main reservoir for *E. coli* O157:H7 and thus it is believed that cattle contribute greatly to the contamination of meats (Chapman et al., 1993). Wells et al. (1991) examined 1,266 fecal specimens from healthy cattle during an investigation of two sporadic cases of *E. coli* O157:H7 associated HUS, which had resulted from raw milk consumption. The researchers collected samples from cattle on 22 farms, in a stockyard, and in a packing house. In addition, three raw hamburger specimens from a restaurant and 23 raw milk samples from two farms were collected. *E. coli* O157:H7 was isolated from 16 heifers or calves and 1 adult cow on 22 farms, 1 stockyard calf, 2 beef specimens, and 1 raw milk sample. Other STEC, other than O157, were found on 8 of 10 farms and the stockyard. Overall, the results revealed that 8% of adult cows and 19% of heifers and calves were positive for STEC (Wells et al., 1991).

In England, Chapman et al. (1993) isolated *E. coli* O157 from 84 (4%) of 2103 bovine rectal swabs and 78 of these were STEC positive. *E. coli* O157 was recovered from 7 (30%) of 23 carcasses of rectal swab-positive cattle and from 2 (8%) of 25 carcasses of rectal swab-negative cattle (Chapman et al., 1993). During a four month monitoring period, Chapman et al. (1994) detected *E. coli* O157:H7 in only 84 (8.2%) of 1024 rectal swabs. In Turkey, Yilmaz et al. (2002) investigated the frequency of *E. coli* O157:H7 in cattle. *E. coli* O157:H7 was isolated from all breeds, but most O157 isolates were recovered from Holstein and Swiss Brown breeds. *E. coli* O157:H7 was isolated most frequently from 2-year-old cattle and from male cattle, and isolates of *E. coli* O157:H7 was isolated in four of the five abattoirs that were surveyed (Yilmaz et al. 2002).

Cattle from 29 pens of five Midwestern feedlots were sampled for the presence of *E. coli* O157:H7 (Smith et al., 2001). The researchers isolated *E. coli* O157:H7 from the feces of 719 of 3,162 (23%) cattle examined, which included at least one animal from each of the 29 pens. The percentage of *E. coli* O157:H7 isolations did not differ between feedlots, but did differ within feedlots. The isolates were more commonly associated with cattle kept in muddy pens (Smith et al., 2001). These surveys seem to indicate that the prevalence of *E. coli* O157:H7 depends on a variety of factors such as the number of cattle within a confined area, the sex and breed, and the condition of their pens. In addition, cattle are often transient carriers of *E. coli* O157:H7 and thus shedding of this organism can occur sporadically and over varying periods of time.

1.6. GROWTH AND SURVIVAL IN FOODS

Food model systems using nutrient rich broth have been used to characterize the growth and survival of *E. coli* O157:H7 in foods. Kauppi et al. (1996) tested STEC for their ability to grow in different media at low temperatures. The average generation time was higher in brain heart infusion broth (BHI)(13 h) compared to trypticase soy broth (TSB)(10 h) and milk (11 h) at 9.5°C. At temperatures near refrigeration (6.5°C), the STEC strains did not grow in BHI or TSB, but were able to grow in milk with a generation time of about 83 h (Kauppi et al., 1996). Most studies have found that EHEC remain viable in foods at 5° C and can grow at temperatures of 8°C or above, verifying the danger associated with temperature abused foods (Buchanan and Bagi, 1994; Rajkowski and Marmer, 1995; Palumbo et al., 1995; Palumbo et al., 1997). Palumbo et al. (1997) investigated the effects of low temperature and background microflora on the growth and Stx production of EHEC in beef

and milk. Growth occurred in strains held at 8° C when no or low background microflora were present. Strains remained viable at 5° C and in the presence of high background microflora. Shiga toxin production was detected in low amounts that did not exceed the levels obtained from broth cultures at 37° C or 5° C (Palumbo et al., 1997).

Decreased toxin production has been observed in some food substrates. *E. coli* O157:H7 grown at 37°C in milk did not produce as much toxin as the same strain grown in BHI broth under the same conditions (Palumbo et al., 1997).

E. coli O157:H7 has the ability to grow over a broad range of pH (4.5 to 9) values (Zilberstein et al., 1984; Hersh et al., 1996). Since many food environments are either acidified or have an inherently low pH, the ability of EHEC to survive at low pH presents a significant food safety threat. Lin et al. (1995) reported that *E. coli* had a minimum growth pH of 4.4, which was slightly higher than that of *Salmonella typhimium* (pH 4.0). Previous research has indicated that bacterial exposure to a sublethal stress can result in subsequent increased resistance to that particular stress or increased resistance to other stresses (Jenkins et al., 1988, 1990; Farber and Pagotto, 1992; Buncic and Avery, 1998; Leenanon and Drake, 2001). Acid adaptation can increase the heat and freeze-thaw resistance of *E. coli* O157:H7 and nonpathogenic *E. coli*. Cold stress decreased heat resistance of *E. coli* O157:H7 and nonpathogenic *E. coli*, but allowed for greater survival during freezing and thawing (Leenanon and Drake, 2001).

Several studies have investigated the growth of *E. coli* O157:H7 in ground beef (Palumbo et al., 1997; Ajjarapu and Shelef, 1999; Cheng and Kaspar, 1998; Ansay et al., 1999; Uyttendaele et al., 2001; Tamplin, 2002). These studies demonstrated that *E. coli* O157:H7 can survive freezing and refrigeration temperatures (5°C) and is able to grow when

placed under abusive temperatures (≥8°C). In ground beef, the exponential growth rate (EGR) and maximum population density (MPD) of *E. coli* O157:H7 increased with decreasing fat content (Tamplin, 2002). Two native ground beef isolates (unspeciated) were added to sterilized ground beef along with *E. coli* O157:H7; there was a decrease in the EGR and MPD of *E. coli* O157:H7 as the competitive population increased (Tamplin, 2002).

The ability of *E. coli* O157:H7 to grow in fermented products such as sausage (Pond et al., 2001; Uyttendaele et al., 2001), Lebanon bologna (Chikthimmah et al., 2001), cheese (Saad et al., 2001), and buttermilk (McIngvale et al., 2000) has been studied. The sausage fermentation process was able to produce a mean log reduction within a range of 0.3 to 1.33, while the drying stage reduced the population by 1.37 to 2.70 $\log_{10}$. Since Lebanon bologna is not processed above 48.8°C, Chikthimmah et al. (2001) investigated effects of curring salts (NaCl and Na NO_2) on the destruction of *E. coli* O157:H7 during processing. High levels of NaCl (5%) inhibited lactic acid bacteria, resulting in a higher pH (5.0) and better survival of *E. coli* O157:H7. The NaCl concentration had no direct effect on *E. coli* O157:H7, but inactivation of the organism increased significantly at all NaCl concentrations when the pH was lowered from 4.7 to 4.3 and the product heated from 37.7°C to 46.1°C in 5.5 h. (Chikthimmah et al., 2001). Minas cheese, a fresh Brazilian cheese, provides a suitable environment for pathogens, such as *E. coli* O157:H7. Saad et al. (2001) found that cheeses without inoculated lactic acid cultures allowed for a 2 $\log_{10}$ increase in *E. coli* O157:H7 population which was maintatined over the storage period. Minas cheese inoculated with lactic acid bacteria controlled the growth of *E. coli* O157:H7 to only 0.5 $\log_{10}$ over a 24 hour period, and this level remained unchanged thereafter (Saad et al., 2001). Acidic condiments have also been investigated (Tsai and Ingham, 1997). *E. coli* O157:H7 did not survive in

mustard or sweet pickle relish for more than one hour when stored at 5 or 23°C. However, *E. coli* O157:H7 was recovered for up to 7 days in ketchup stored at 5°C (Tsai and Ingham, 1997).

1.7. DETECTION

Traditional cultural methods for the detection of EHEC in foods require selective enrichment, selective and differential plating, followed by phenotypic identification using biochemical tests and subsequent serotype identification. The fundamental tests used for isolation and detection are found in the *Bacteriological Analytical Manual* (BAM). Within BAM, three selective agars are identified: sorbitol-MacConkey agar (SMAC), Hemorrhagic colitis agar (HC), and tellurite-cefixime-sorbitol MacConkey agar (TC SMAC). The use of SMAC involves incubation at 35°C for 18 h. Most *E. coli* O157:H7 strains are sorbitol-negative and will form pale colonies as opposed to most other *E. coli* strains and enterics, which are sorbitol-positive and appear bright pink. Confirmation of sorbitol-negative colonies can be tested for β-glucuronidase (GUD) activity (GUD cleaves the substrate 4-methylumbelliferyl β-D-glucuronide (MUG)) by spotting cultures on HC agar with MUG, and then select MUG-negative colonies. For identification of *E. coli* O157:H7, for sorbitol-negative, MUG-negative colonies are tested for agglutination with O157 and H7 antisera. It would be prudent to mention that the presence of high levels of coliforms may mask the presence of O157:H7 when using SMAC. False positives on SMAC may result when *Escherichia hermanii* and some other enterics are present, or in the presence of *Citrobacter freundii*, which may agglutinate O157 antiserum. Another method, selective plating for the detection for EHEC uses HC supplemented with MUG (note: MUG can be substituted with

the colorimetric substrate BCIG). When low numbers of *E. coli* O157:H7 are suspected, the food may be enriched in modified trypticase soy broth containing novobiocin before being plated on selective media. Another procedure uses Tellurite-Cefixime-SMAC as the selective plating media. Recovery of *E. coli* O157:H7 from foods was improved using TC-SMAC compared to using HC agar (Hitchins et al., 1995). There exists a small population of non O157 isolates that do not ferment sorbitol, and therefore the incorporation of the MUG assay should be included in the analysis to distinguish *E. coli* O157:H7 from these atypical *E. coli* isolates. Selective enrichment followed by immunomagnetic separation may also be performed using anti-O157 beads (Dynabeads; Dynal). This method may result in improved assay sensitivity due to pathogen pre-concentration after enrichment and prior to selective plating on agar such as TC-SMAC (Hitchins et al., 1995).

When presumptive colonies are isolated after enrichment and selective plating, biochemical confirmation is warranted. In particular, EHEC strains will be indole-positive by the spot test. The API or VITEK assays are also useful tests to identify isolates as *E. coli* from TSA-YE slants. Subsequent to biochemical confirmation, latex agglutination using the Prolex *E. coli* O157 Latex Test Reagent kit or RIM *E. coli* O157:H7 Latex Test, an approach for serological identification. A rapid alternative to serological typing involves use of the serotype-specific DNA probe, PF-27. This 18-base oligonucleotide probe targets a unique region of the *uidA* gene in *E. coli* O157:H7. A study several enteric species on and other STEC (in total 280 bacterial isolates), demonstrated that PF-27 was highly specific only for the O157:H7 serotype (Hitchins et al., 1995). Only if both the latex and indole test are positive, should the isolates be tested for the presence of *stx1* and *stx2* genes by colony

hybridization or polymerase chain reaction. Detection of toxins produced by *E. coli* O157:H7 can be performed using tissue culture assays. (Hitchins et al., 1995).

Specifically, the potential for EHEC isolates to produce toxins may be evaluated using the rapid method of colony hybridization by oligonucleotide DNA probes that are specific for the *stx1* and *stx2* genes. Another rapid method utilizes primers specific to the *stx1* and *stx2* genes which can be incorporated into a PCR assay (Hitchins et al., 1995). There are several strains of Stx producing *E. coli* (STEC) other than O157, however, only those strains that have been clinically associated with HC are designated as EHEC. Of the EHEC strains, *E. coli* O157:H7 is the prototypic EHEC and most commonly implicated in illness worldwide (Feng and Weagant, 2002). Therefore O157:H7 specific DNA probe and multiplex PCR (5P PCR) is needed to detect the O157:H7 serotype and its toxigenic non-motile variants. The probe targets a +93 base mutation in the *uidA* gene that encodes for ß-glucuronidase, which is highly conserved in *E. coli* O157:H7 and its toxigenic non-motile variants. The 5P PCR couples *uidA* with primers specific for *stx1*, *stx2*, gamma variant of *eae* that is found in *E. coli* O157:H7, and the *ehxA* enterohemolysin genes to form an assay that simultaneously detect these virulence traits in Stx-producing *E. coli* and also determines if the strain is of the O157:H7 serotype (Feng and Weagant, 2002).

The method used for isolation of *E. coli* O157:H7 from raw and ready-to-eat meat products is described in the USDA/FSIS Microbiology Laboratory Guidebook. The format involves enrichment in a selective broth medium, application of a rapid screening test, immunomagnetic separation (IMS) in paramagnetic columns, and plating on a highly selective medium. Raw ground beef is randomly collected from the package in 65 g sub-samples for a composite sample consisting of 325 g. Enrichment is done in mEC + n broth

(modified EC broth with novobiocin) for 20 to 24 h at 35°C. Positive, negative, and uninoculated controls are prepared for each group of samples. An ELISA-based *E. coli* O157:H7/NM screening test can be done after enrichment, in which case positive samples are considered presumptive and are subject to further confirmation. Negative samples, however, are confirmed after such screening (Cray et al., 1998).

Isolation and confirmation steps can be taken from the enrichment culture in the stomacher bag. In this case, 5 ml of enrichment broth is processed by immunomagnetic separation using Dynal *E. coli* O157:H7 beads, along with appropriate positive and negative controls. The product of IMS is then spread plated on Rainbow agar plates and incubated 24-26 h at 35°C (Cray et al., 1998). *E. coli* O157:H7 colonies appear black or grey on Rainbow agar, but the colonies may have a bluish hue when surrounded by pink or magenta colonies. Positive *E. coli* O157 :H7 isolates (up to 5 per plate) are tested using latex agglutination assays for O157. For biochemical testing, Vitek methods are frequently used. To differentiate *E. coli* O157 from competitive organisms, triple sugar iron agar (TSI), cellobiose, and the motility test are particularly important. For toxin and toxin gene confirmation, various test kits (i.e. Meridian Premier EHEC Kit) based on enzyme linked immunosorbant assay (ELISA) are available. If such tests do not demonstrate the presence of Shiga toxins, detection of one or more toxin genes should be accomplished using PCR. If the cultures are positive for *E. coli* O157:H7, then pulsed field gel electrophoresis (PFGE) can be performed for potential epidemiological association (Cray et al., 1998).

There has been much work in recent years to identify optimal selective approach for presumptive identification of *E. coli* O157:H7, particularly with respect to the recovery of sublethally injured cells. Szabo et al. (1986) evaluated the use of hemorrhagic colitis (HC)

medium for direct overnight isolation of EHEC strains. Recovery of these strains from artificially contaminated ground beef was greater than 90 %. *E. coli* O157:H7 colonies grown on HC medium are blue, do not fluoresce under UV light, and are red following exposure to indole reagent (Szabo et al., 1986). Silk and Donnelly (1997) reported better recovery of injured *E. coli* O157:H7 from apple cider by surface plating on trypticase soy agar (TSA) incubated 2 h at 25° C followed by overlaying with violet red bile agar (VRBA) or sorbitol MacConkey agar (SMAC), compared to the use of other selective or differential media. A modified resuscitation method was also employed using the above method preceded by preincubation of 250 µl trypticase soy broth (TSB) with 1 µl sample for 2 h at 25° C prior to SMAC plating. Despite better recovery by incorporating a resuscitation step, detection was still less efficient than when compared to that obtained using nonselective TSA (Silk and Donnelly, 1997).

Haro-Kudo et al. (1999) determined the best media for use in enrichment procedures for the isolation of *E. coli* O157:H7. The most effective conditions were incubation at 42° C for 18 h in modified TSB (TSB supplemented with bile salts 3, dipotassium phosphate and novobiocin) or modified EC broth with novobiocin (mEC + N) for ground beef and radish sprouts (Haro-Kudo et al., 1999). The use of EHEC agar, a variation of sheep blood agar (washed sheep red blood cells added at 5% (v/v) to trypticase soy agar (TSA) supplemented with 10 mmol/L (final concentration) calcium chloride), for isolation of STEC from foods was assessed by Hudson et al. (2000). The three STEC serotypes O157:H7, O26, and O113:H21 were isolated from raw mince, pasteurized milk, and salami following enrichment in mEC + N and Imbentin (a surfactant). The researchers were able to detect low numbers of cells in pasteurized milk (1 CFU/25 ml), but the method proved less sensitive in salami (10-

1000 CFU/ml), depending on the serotype. It was difficult to isolate STEC strains from raw minced beef because enterohaemolysin-producing colonies were outnumbered by other colonies. In general, recovery of STEC was better in cooked meat products and salami than it was from raw minced meat using this EHEC agar method (Hudson et al., 2000). Lehmacher et al. (1998) detected low numbers (2 to 6 CFU/g) of STEC from spiked stool and raw milk samples by plating samples on blood agar supplemented with vancomycin, cefixime, and cefsulodin (BVCC) after enrichment with mTSB supplemented with novobiocin (Lehmacher et al., 1998).

Many of the rapid kits available for the detection of *E. coli* O157:H7 require an enrichment step to boost the target cells to detectable levels. Blais et al. (1997a) studied enrichment of *Escherichia coli* O157:H7 in meat using modified *E. coli* (EC) broth with novobiocin (mEC + n) and the combined effects of agitation and incubation temperature (37° C or 42° C). Results indicated that 42° C without shaking was most effective in suppressing the growth of competitive ground beef microflora while allowing ample growth of *E. coli* O157:H7 (Blais et al., 1997a).

1.8. METHODS TO SEPARATE BACTERIA FROM FOODS

1.8.1. Immunomagnetic Separation (IMS)

Immunomagnetic separation (IMS) allows for separation of a target microorganism from a population of other competing bacteria providing for easier identification on selective media (Wright et al., 1994). Magnetic beads (2.8 μm diameter) are covalently coated with affinity purified antibodies against specific surface markers on the microorganism. The

beads are stored in phosphate buffered saline (pH 7.4 with 0.1% (human or bovine) serum albumin (HSA/BSA) and 0.02% sodium azide) (Dynal Biotech). Dynabeads®, a commercial IMS bead product, are cited in the 8th edition of the Bacteriological Analytical Manual (Feng and Weagant, 2002).

The use of IMS-PCR has proven to be effective in facilitating the detection of *E. coli* O157:H7 in contaminated ground beef and dairy products, for which detection limits of 1 CFU/g or /ml have been reported (Ogunjimi and Choudary, 1999; McIngvale et al., 2002). Tortorello et al. (1998) compared six methods for the detection of *E. coli* O157:H7 in apple juice, including direct fluorescent antibody (DFA), antibody-direct epifluorescent filter technique (Ab-DEFT), direct selective plating on sorbitol MacConkey agar (SMAC), immunomagnetic separation coupled to either selective plating (IMS-SMAC) or the polymerase chain reaction (IMS-PCR), and flow cytometry (FC). The most sensitive (0.1 CFU/ml) and consistent detection of the slowest growing O157 strain was obtained by IMS-SMAC and IMS-PCR following 8 h of nonselective cultural enrichment. Without enrichment, the level of detection was not as sensitive (IMS-SMAC, 10 CFU/ml and IMS-PCR 10^3 CFU/ml) (Tortorello et al., 1998).

Karch et al. (1996) compared immunomagnetic separation (IMS) to DNA-based methods and direct culture methods for isolation of *E. coli* O157:H7 strains from patients with HUS. They concluded that IMS was more sensitive (10^2 CFU/g with 10^7 coliform background microflora) than direct culture and had an advantage of being less time-consuming and less labor-intensive compared to molecular methods (Karch et al., 1996). Pyle et al. (1999) explored the use of IMS combined with the cyanoditolyl tetrazolium chloride (CTC) incubation, used to determine respiratory activity, and its ability to rapidly

detect viable *E. coli* O157:H7 compared to plating on SMAC. Following IMS, the bacteria were stained with CTC, exposed to an anti-O157 fluorescein isothiocyanate conjugated antibody (FAB) and filtered for microscopic enumeration using solid-phase laser cytometry. This method detected higher numbers (mean recovery ratios) of target bacteria when compared to plating on SMAC preceded by IMS (improvement of mean recovery ratio- beef, 6.0 times; peptone, 3.0 times; water, 2.4 times); detection limits for ground beef were around 10 CFU/g and for liquid samples were <10 CFU/ml (Pyle et al., 1999).

Bolton et al. (1996) studied detection of *E. coli* O157:H7 in raw meat products using enrichment and four subculture procedures. Enrichment in modified tryptone broth at 42° C for 6 h, IMS, and subculture on to cefixime, tellurite SMAC (ctSMAC) was the most sensitive and selective protocol. This procedure was used in combination with a most probable number technique to assess the number of *E. coli* O157 in naturally contaminated raw meat samples and was affective in the range of < 0.3 to 2300 CFU/g (Bolton et al., 1996). Barkocy-Gallagher et al. (2002) utilized nonselective enrichment prior to IMS to selectively concentrate *E. coli* O157:H7 prior to plating onto ctSMAC and Rainbow agar supplemented with novobiocin and potassium tellurite. Both selective media performed comparably well to traditional methods for recovery of *E. coli* O157:H7 from hide, carcass, and fecal material (Barkocy-Gallagher et al., 2002). Immunomagnetic flow cytometry (IMFC), a combination of flow cytometry and IMS, was shown to be an effective method for detecting low numbers of *E. coli* O157:H7 in artificially contaminated ground beef, apple juice, and raw milk. After a 6 h enrichment and IMFC, total analysis time was only 7 h and the method was able to detect 4 *E. coli* O157:H7 cells/g of ground beef (Seo et al., 1998).

Immunomagnetic separation (IMS) using anti-*E. coli* O157 Dynabeads® was utilized by Restaino et al. (1996) to confirm suspect colonies obtained from a food sample by using nonselective enrichment and an antibody-direct epifluorescent filter technique (Ab-DEFT). A 10 h enrichment in modified buffered peptone water followed by Ab-DEFT (<1h) allowed detection of *E. coli* O157 artificially inoculated into beef patties at 0.1 CFU/g. The IMS method using anti-*E. coli* O157 Dynabeads was then used to confirm presumptively positive cultures of *E. coli* O157 after 24 h (Restaino et al., 1996). Chapman et al. (1994) compared IMS to direct culture on cefixime rhamnose sorbitol MacConkey agar (CR-SMAC) and cefixime tellurite sorbitol MaConkey agar (CT-SMAC) for isolation of *E. coli* O157:H7 from bovine feces. Prior to IMS (Dynabeads® anti *E. coli* O157) an enrichment step was performed using modified buffered peptone water. When the sensitivities of the methodologies were tested on bovine fecal samples, the enrichment culture IMS was 100-fold more sensitive than direct culture on either medium (Chapman et al., 1994).

1.8.2. Metal Hydroxide Immobilization

Zirconium chloride in water at pH 7.2 results in formation of multiple tetrameric complexes in which zirconium ions are connected by hydroxide bridges. The gelatinous precipitate (zirconium hydroxide) is effective in non-specifically immobilizing bacteria due to the formation of covalent bonds between the hydroxyl groups of the metal hydroxide and the amino acid side chains that are abundant on the surface of bacterial cells (Kennedy et al., 1976). Lucore et al. (2000) studied the effectiveness of metal hydroxides (zirconium hydroxide, hafnium hydroxide, and titanous hydroxide) to concentrate pathogens in reconstituted nonfat dry milk prior to detection by cultural and molecular methods.

Following concentration and immobilization steps using zirconium hydroxide and low-speed centrifugation, the sample volume was reduced by 50-fold. The two pathogens tested, *S. enterica* serovar Enteritidis and *L. monocytogenes*, were recovered at a range of 78 to 96 % of input to 65 to 96 % of input, respectively. The bacterial-immobilization step was reported to be relatively nonspecific (Lucore et al., 2000) and may provide a less expensive alternative to IMS. The technique has been applied to the concentration of *E. coli* O157:H7 from dairy foods (McKillip et al., 2000b). Recently, Cullison and Jaykus (2002) reported successful magnetization of metal hydroxides. The researchers inoculated nonfat dry milk with 10^3 to 10^6 CFU/25 ml of *S. enterica* serovar Enteritidis, *L. monocytogenes*, or *Bacillus cereus* spores. The magnetized carbonyl iron-zirconium hydroxide mixture allowed bacterial recovery to exceed 75% to >90%, when assessed on the basis of loss to discarded supernatants, and this metal hydroxide was relatively nontoxic to *S.* Enteritidis and *L. monocytogenes* upon further investigation (Cullison and Jaykus, 2002).

The technique has also been successfully used to prepare food samples for pathogen detection using PCR and RT-PCR (McKillip et al., 2000b; Lucore et al., 2000). While bacterial concentration using titanous hydroxide did not improve total DNA yield of *E. coli* O157:H7 inoculated into skim milk, nonfat dry milk, reconstituted whey powder, and cheese, the purity of the DNA obtained following bacterial concentration was improved. *E. coli* O157:H7 inoculated into reconstituted whey powder at 10^6 CFU/ml could not be detected after simple nucleic acid extration, but when a bacterial concentration step was incorporated, a band was visualized from an inoculum as low as 10^4 CFU/ml (McKillip et al., 2000b). Lucore et al. (2000) reported detection limits of 10^1 to 10^2 CFU/25 ml for reconstituted nonfat dry milk (11% w/v), $\geq 10^2$ CFU/ml whole milk, and $\geq 10^1$ CFU/ml for ice cream for *S.*

enterica serovar Enteritidis and *L. monocytogenes* using a metal hydroxide immobilization method and RT-PCR targeting rRNA genes (Lucore et al., 2000).

1.8.3. ELISA

Weeratna and Doyle (1991) investigated simple methods for rapid detection of the toxin Stx1 in beef and milk by using a sandwich ELISA. The sensitivity of the ELISA assay was 0.5 ng of Stx1 per ml of milk and 1.0 ng of Stx1 per g of ground beef (Weeratna and Doyle, 1991). Novicki et al. (2000) explored the use of a two-step method using a chromogenic selective-differential medium for the isolation of selected *E. coli* serotypes to be used in combination with an enzyme-linked immunosorbent assay (ELISA) that was specific for Stx1 and Stx2. The two-step method was much more sensitive (76.5 % for EHEC and 100 % for O157) than the use of selective media alone, such as sorbitol MacConkey agar (SMAC) (23.5 % for EHEC and 50 % for O157). Weimer et al. (2001) utilized a high-flow-rate fluidized bed (Immunoflow) to capture and concentrate bacteria prior to further analysis by ELISA, thereby eliminating the need for an enrichment step. Antibodies for *E. coli* O157:H7, *Bacillus globigii*, bovine serum albumin, or ovalbumin were covalently coupled to glass and ceramic beads. The lower limit of detection was one cell for *E. coli* O157:H7 and this was independent of sample size (only a 4L volume is mentioned of phosphate-buffered saline (PBS) containing 0.02% (vol/vol) Tween 20 (pH 7.2) (PBST) and a meat extract each spiked with 10^4 total cells). The maximum signal was observed at 10^4 cells for meat extract and 10^3 cells for PBST. The anti-*E. coli* O157:H7 modified beads decreased in capture ability after 2 days. The use of Immunoflow capture allowed for

detection via a standard ELISA of the target organism in a 30 min format. (Weimer et al., 2001).

1.9. RAPID METHODS

1.9.1. PCR

The polymerase chain reaction (PCR) technique provides a means for rapid and selective detection of microorganisms by amplifying specific gene fragments. The PCR reaction consists of three steps: (i) denaturation of double-stranded DNA; (ii) annealing of short DNA fragments (primers) to single DNA strands; (iii) extension of the primers with a thermostable DNA-polymerase (Taq polymerase or T^{th} polymerase). After one complete cycle, the DNA is once again denatured followed by annealing and extension generating new copies of the original strands and of those strands made in the first cycle. As the process continues in the presence of excess primer and dNTP's, there is an exponential increase in the number of copies of target DNA (Scheu et al., 1998).

1.9.1.1. PCR with EHEC

Meng et al. (1998) investigated the presense of virulence genes (EHEC – *hly*A, *eae*, *stx*1, *stx*2) in EHEC and STEC isolated from human patients, cattle, sheep and foods using PCR assays. The results indicated that all isolates of O157:H7 carried EHEC-*hly*A, *eae*, and one or both *stx* genes. The EPEC, EIEC, ETEC, and the *E. coli* K12 strain did not carry these virulence genes except for eight EPEC isolates positive for *eae*. This suggested that

STEC genes EHEC-*hly*A and *eae* could provide markers to differentiate EHEC from less pathogenic STEC, and other pathogenic or non-pathogenic *E. coli* (Meng et al., 1998).

Typically, studies involving PCR detection of EHEC will use targets such as the *stx* and *eae* genes. Multiplex PCR is a form of PCR in which two or more targets are amplified in the same reaction (Henegariu et al., 1997). Gannon et al. (1992) evaluated two oligonucleotide primers for use in detecting *stx*1 and *stx*2 genes in multiplex PCR assays. The first pair produced a 600-bp PCR product targeting the *stx*1 gene and the second pair produced an 800-bp PCR product targeting *stx*2. When combined, the two primers were able to detect all STEC strains tested. An enrichment step (6 h at 42° C) in trypticase soy broth was used prior to the PCR assay and allowed for detection of as little as 1 CFU/g inoculum from ground beef (Gannon et al., 1992). Read et al. (1992) also utilized primer pairs targeting Stx1, Stx2, and Stx2v genes. All 223 STEC isolates, covering over 50 different serotypes, tested positive using the PCR assay, and the only other foodborne pathogen that tested positive for the assay was *Shigella dysenteriae* type 1 (Read et al., 1992).

Using oligonucleotides for Stx1 and Stx2 in a multiplex PCR assay, Venkateswaran et al. (1997) recorded a detection threshold of 10^2 CFU per PCR reaction (10 µl PCR mixture) of *E. coli* O157:H7 in sliced beef, but 10^3 CFU/g was required for consistent target amplification. A 6 h enrichment step in nonspecific medium and a two-step filtration procedure was used to prevent inhibition (Venkateswaren et al., 1997). Pollard et al. (1990) amplified regions coding for *stx*1 and *stx*2 in STEC strains confirmed positive for Shigatoxins using traditional tissue culture assays (Pollard et al., 1990). Lindqvist et al. (1998) employed a multiplex PCR targeting the Stx1 and Stx2 genes on an enriched beef homogenate (6h) followed by buoyant density centrifugation as a rapid screening method for

detection of *E. coli* O157:H7 and STEC in domestic and imported beef in Sweden. Paton et al. (1993) utilized oligonucleotide primers for PCR amplification of a 212- or 215-bp region of Stx genes from crude fecal culture extracts. The genes were then typed by hybridization of PCR products to Stx1 or Stx2 specific oligonucleotide probes. Using this procedure, the investigators were able to detect less than 10 STEC cells per ml of culture amongst a background microflora of more than 10^9 organisms per ml (Paton et al., 1993).

Gannon et al. (1993) studied variations of the *eae* gene within various STEC strains. The researchers were able to generate five *Hae*III digestion profiles by amplification of a 2.3-kb DNA fragment from the 5' end of *eae* gene. The *Hae*III profiles for O serogroups, such as 26, 103, and 157, were different from each other, but were consistent among strains within O serogroups (Gannon et al. 1993). Meng et al. (1997) performed a multiplex PCR assay using primers amplifying a DNA sequence upstream of *E. coli* O157:H7 *eaeA* gene and genes encoding Stx1 and Stx2. Using this assay, *E. coli* O157:H7 could be detected and at the same time distinguished from other EHEC strains (O55:H7, O55:NM), and other STEC strains (Meng et al., 1997). Cebula et al. (1995) utilized the *uidA* gene (encoding β-glucuronidase) in a mismatch-multiplex PCR assay to simultaneously identify *E. coli* O157:H7 and the types of Stx(s) encoded. Although *E. coli* O157:H7 isolates do not exhibit gluconidase activity, they still carry the *uidA* gene. A highly conserved base change (G residue rather than a T) possessed by wild-type *E. coli* at position 92 was observed in the *uidA* gene of *E. coli* O157:H7. The primers were made specifically for this unique *uidA* gene and coupled with primers for Stx1 and Stx2, the assay allowed for identification of *E. coli* O157 strains and strain Stx type (Cebula et al., 1995). Chen et al. (1998) employed magnetic capture-hybridization PCR as a sensitive and specific method for detecting STEC.

This technique involved the use of biotin labeled probes to capture DNA segments that contain specific regions of *stx*1 and *stx*2 by DNA-DNA hybridization. These hybrids were then isolated by streptavidin-coated magnetic beads and collected by a magnetic particle separator followed by conventional PCR amplification. A 7 h enrichment step allowed for detection of 10^2 CFU/ml, while a 10 h enrichment improved detection limits to 10^0 CFU/ml. When applied to detection of STEC in ground beef, a 15 h nonselective enrichment step facilitated detection of as little as 10^0 CFU/g (Chen et al., 1998).

A multiplex PCR was developed to rapidly detect EHEC from food by simultaneously amplifying *eae*, *stx1*, *stx2* genes and a fragment of the 60-MDa plasmid (Deng and Fratamico, 1996). All EHEC serogroup O157:H7 isolated from meat samples were identified using the multiplex PCR. Additionally, 70 experimentally inoculated ground beef and milk samples (initial inoculum 4 to 9 CFU/g or ml) were identified as positive after 6 h of cultural enrichment (Deng and Fratamico, 1996). Fratamico and Strobaugh (1998a) designed a multiplex PCR for detection of both *E. coli* O157:H7 and *Salmonella* spp. from enrichment cultures. The target genes used for *E. coli* included *stx* and *eaeA*. Multiplex PCR was performed following a 20-24 h (37°C) enrichment step in modified EC broth and subsequent immunomagnetic separation. All targets were amplified even when the target bacterial concentration was as low as ≤1 CFU/g ground beef and bovine fecal sample enrichments or ≤1 CFU/ml beef carcass wash water and apple cider (Fratamico and Strobaugh, 1998a). Fratamico and Strobaugh (1998b) compared the multiplex assay to the direct immunofluorescent filter technique (DIFT) and ELISA in the evaluation of beef carcass wash water for the detection of *E. coli* O157:H7. After a 4 h sample enrichment, detection limits for *E. coli* O157:H7 were 100, 0.1, and 1.0 CFU/ml for ELISA, DIFT, and

multiplex PCR, respectively (Fratamico and Strobaugh, 1998b). Another *E. coli* O157:H7 study (Fratamico et al., 2000) investigated using a plasmid encoded gene (hly_{933}), and chromosomal flagella ($fliC_{h7}$; flagellar structural gene of H7 serogroup), *stx* 1 and 2, and *eaeA* genes in a multiplex PCR. The results from the assay were obtained within 24 h and the sensitivity of the assay was <1 CFU/g of food (ground beef, blue cheese, mussels, and alfalfa sprouts) or bovine feces. Enrichment immediately after inoculating the samples and enrichment of previously frozen or refrigerated samples produced similar levels of detection. The investigators claimed that multiplex assay could shorten the time necessary for confirmation of *E. coli* O157:H7 by 3 to 4 days (Fratamico et al., 2000).

1.9.1.2. PCR Inhibitors

Nutrients and other components within a food matrix can have a drastic, negative effect on the sensitivity of the PCR assay as applied to pathogen detection. Many studies have investigated various sample preparation procedures to overcome this shortfall (Lantz et al., 1994; Wilson, 1997). Rossen et al. (1992) discovered that foods containing high amounts of fat and protein reduced the sensitivity of PCR, but an extraction procedure involving treatment with hot NaOH/SDS significantly reduced these inhibitory effects. They also found that certain culture media commonly used for enrichment such as Fraser, modified *Listeria* enrichment broth (MLEB), modified Rappaport broth (MRB), and Rappaport broths were inhibitory. Compounds found in some DNA extraction procedures such as detergents, lysozyme, NaOH, alcohols, EDGA, and EGTA also had some inhibitory effect (Rossen et al., 1992).

De Boer et al. (1995) experienced inhibition of PCR when trying to detect low levels of pathogenic bacteria and fungi in plant tissue. The inhibitory agents were found to be plant polyphenolic molecules. The problem was completely alleviated with the addition of non-fat milk. These non-fat milk cocktails have been coined with the term BLOTTO (Bovine Lacto Transfer Technique Optimizer) and have been used to prevent non-specific binding of proteins and nucleic acids to nitrocellulose in Western and Southern blotting protocols. This technique has also been historically used to prevent non-specific binding in ELISA (De Boer et al., 1995). Bickley et al. (1996) found that the presence of more than 5% milk inhibited amplification using a standard PCR reaction. This inhibition was not attributed to the fat content of the milk, but to the calcium ions within the milk. The inhibition by calcium ions was partially reversed by increasing the magnesium concentration in the reaction (Bickley et al., 1996).

McKillip et al. (2000b) evaluated the effect of upstream sample processing and DNA extraction efficiency on the PCR-based detection of *E. coli* O157:H7 in artificially contaminated dairy products. These investigators observed better DNA purity after bacterial concentration than after organic solvent extraction alone. PCR detection limits were poor from reconstituted whey powder, but those obtained from milk and cheese ranged from 10^1 to 10^4 CFU/ml (McKillip et al., 2000b). Ogunjimi and Choudary (1999) found that an IMS-PCR assay produced no PCR products from apple juice artificially contaminated with *E. coli* O157:H7. They hypothesized that nonspecific endogenous polyphenols, common in plant products, were binding to antibodies on the surface of the immunobeads and inhibiting capture of the target bacteria. To alleviate the problem, polyvinylpyrrolidone (a synthetic

fining agent) was added to the sample prior to IMS, which restored the sensitivity of the immuno-PCR (Ogunjimi and Choudary, 1999).

Shearer et al. (2001) evaluated a PCR based BAX system that utilized a specific target DNA sequence for the detection of *Salmonella* Enteritidis, *E. coli* O157:H7, *Listeria* spp., and *L. monocytogenes* from fresh fruits and vegetables. The BAX system is a user-friendly system containing all PCR reagents, including the polymerase enzyme, nucleotides, and primers, provided in tablet form. In this study, the BAX system proved more sensitive than the culture-based method for detection of *E. coli* O157:H7 on green pepper, carrot, radish, and sprout samples. Detection limits of samples spiked with a predicted number of less than 10 CFU was possible for most produce samples. However, low numbers of *E. coli* O157 were not consistently recovered from alfalfa sprouts (Shearer et al., 2001).

1.9.2. RT-PCR

The RT-PCR reaction is similar to PCR, except that an RNA template is utilized to make cDNA through the action of a reverse transcriptase. The first step in an RT-PCR assay involves treatment of the extracted nucleic acid with DNase to remove any residual DNA from the reaction mix. Then purified RNA is placed in a small reaction vessel with reverse transcriptase, which acts to transcribe the RNA to a single stranded cDNA. Then the sense strand is used as template to form the antisense DNA and subsequent formation of the double stranded cDNA. The cDNA is then amplified by PCR (Bej et al., 1991).

PCR detection of pathogens can result in false-positive results from the amplification of DNA from dead cells (Klein and Juneja, 1997). The presence of RNA within the cell is a better indicator of active cell metabolism and more importantly, cell viability (Bej et al.,

1996). Reverse transcriptase PCR (RT-PCR) is a useful tool for detecting messenger RNA (mRNA). Prokaryotic mRNA has a short half-life, making it ideal for determining cell viability. However, it is difficult to obtain intact mRNA from the cell, although the problem can be circumvented by rapidly lysing the microorganisms prior to extraction (Scheu et al., 1998). Most mRNA has a half-life of only a few minutes and turnover is rapid in living bacterial cells. While this makes mRNA a good indicator of cell viability, there are some cases in which mRNA associated with dead cells can persist in the environment (Bej et al., 1996; Sheridan et al., 1999).

Sheridan et al. (1998) investigated the detection of different types of RNA as indicators of viable and dead *E. coli* cells. Message rRNA was detected by RT-PCR in cells that had been inactivated by heat or ethanol, but eventually no detection was observed in dead cells held at room temperature. Some mRNA was undetectable after 2 to 16 h following heat or ethanol treatment. However, rRNA was detected at all time points and after cell death. After destruction of bacterial cells following autoclaving, rRNA was not detected by Northern blot analysis or RT-PCR. In contrast, rRNA remained detectable after the 48 h period following moderate heat or UV irradiation (McKillip et al., 1998). In a similar study, McKillip et al. (1999) found less efficient amplification of rRNA compared to DNA at corresponding time points for heat-killed cells of *E. coli* O157:H7 in skim milk by RT-PCR and PCR, respectively. Following a mild heat treatment (60° C for 3 h), *E. coli* O157:H7 rRNA and rDNA remained detectable following the 48 h post-treatment period. The rRNA amplification signals decreased with increased holding time (McKillip et al., 1999). These studies confirm the stability of rRNA following cell death and suggest that

rRNA amplification may not be the most suitable target for the detection of viable pathogens in foods (McKillip et al., 1998; McKillip et al., 1999).

Different mRNA's display different stabilities and subsequent different half-lives. Sheridan et al. (1999) found that *E. coli* cells killed with 50% ethanol could still be detected by RT-PCR targeting *tufA* mRNA when incubated in Luria-Bertani broth at different temperatures. The mRNA was detected for up to 16 h at 15° C or 4° C, but the signal was lost after only 2 h at 37° C (Sheridan et al., 1999). Herman (1997) evaluated the sensitivities of PCR and RT-PCR for detection of viable and dead *L. monocytogenes* by targeting the listeriolysin O mRNA transcript. Incubation of cells in pure ethanol for 30 days decreased PCR sensitivity of DNA 100 times compared to untreated cells. Cells treated at 60°C for 30 min. could be detected by PCR 48 h after treatment, similar to DNA amplification detection limits for untreated cells. When RT-PCR was applied to cells heated to pasteurization temperature, no signal was detected, while a signal was generated for untreated cells (positive control). This study confirmed that even though bacterial cells were destroyed by heat treatments practical to the food industry they could still be detected by PCR, but RT-PCR detection of mRNA was a better indicator of cell viability (Herman, 1997). Szabo and Mackey (1999) employed RT-PCR as a technique to detect mRNA from the *sefA* gene of *Salmonella enteritidis*. Levels of transcription of the *sefA* gene differed based on the physiological state of the cells which in turn influenced RT-PCR detection sensitivity. Therefore an enrichment step was performed to increase the levels of *S. enteritidis* in spiked food samples (minced beef and whole egg) prior to nucleic acid amplification. A minimum of ten cells could be detected following 16-h enrichment using RT-PCR. The RT-PCR assay

was not able to detect heat-inactivated *S. enteritidis*, indicating the usefulness of the assay for the detection of viable microorganisms (Szabo and Mackey, 1999).

Klein and Juneja (1997) studied detection of viable *L. monocytogenes* cells using RT-PCR. They found that the *iap* gene was the most suitable target for detection of *L. monocytogenes* and allowed detection of 10 to 15 CFU/ml from pure culture following 1 h enrichment, PCR, and amplicon detection by Southern hybridization. Approximately 3 CFU/g in artificially contaminated ground beef after a 2 h enrichment period could be detected. The assay was completed in about 54 h (Klein and Juneja, 1997). Kaucner and Stinear (1998) developed an RT-PCR assay for the simultaneous detection of *Giardia* cysts and *Cryptosporidium* oocysts from water samples. An internal positive control was used with the assay to determine the efficiency of mRNA isolation and possible RT-PCR inhibition. Low levels in the order of a single cyst or oocyst could be detected from samples spiked with 100-µl packed pellet volumes of concentrates from creek or river water (Kaucner and Stinear, 1998). McIngvale et al. (2002) investigated the use of RT-PCR for detection of viable STEC. Primers specific to *stx2* mRNA were optimized and used to detect STEC in ground beef and trypticase soy broth (TSB). Cellular mRNA or DNA were extracted at designated time points (4-24 h) and RT-PCR or PCR were conducted. The mRNA target was detected following 12 h in cooked ground beef enrichment broths having an initial inoculum of 1 CFU/g. This indicated that *E. coli stx2* mRNA was a suitable target for detection of viable STEC in ground beef (McIngvale et al., 2002).

1.9.3. TD-PCR

Touchdown (TD) PCR is a simple method used to reduce nonspecific priming and increase specificity even when the degree of primer-template complementarity is not fully known (Hecker and Roux, 1996). During the PCR reaction the melting temperature (Tm) between the primer and the template is of great importance. Temperatures higher than the Tm will yield no product, while temperatures much lower than the T_m will yield unwanted products due to false priming (Roux, 1994). Calculations of T_m for primers can give a wide range of values, some ranging up to 27° C. TD-PCR operates over a broad range and can thus overcome the problem of obtaining an accurate T_m for primer optimization (Hecker and Roux, 1996). The TD-PCR technique involves lowering the annealing temperature by 1° C every second cycle from i.e. 65° C to a 'touchdown' at 55° C, followed by 10 cycles carried out at the final temperature (55° C). Differences in T_m between the correct and incorrect annealings will provide an advantage of 2-fold per cycle, or 4-fold per °C, to the correct product. Therefore a 5° C difference would result in a 4^5 (1024) fold advantage (Don et al., 1991). Once the annealing temperature drops to the level where nonspecific priming would occur, the target product will already have a head start and should outcompete any nonspecific product for resources resulting in a single predominant amplicon (Hecker and Roux, 1996).

Roux (1994) evaluated the priming ability of mismatched primers by comparing conventional and touchdown PCR using a model system having defined base mismatches. False priming resulted in multiple bands with conventional PCR at low annealing temperatures and higher temperature gave fewer bands. One primer pair gave two weak bands at 50° C, with only one of these being correct, while no bands were produced using the

other two primer combinations. However, a single strong band of the correct size was obtained for each of the convergently oriented primer pairs by using a TD-PCR system where the annealing temperature was lowered in one-degree intervals from 55° to 41° C, over 42 cycles (3 cycles/°C) followed by 10 cycles at 40° C. The researchers obtained similar results with 50° to 35° C over 45 cycles followed by a fresh reamplification of 1.0 μl of the product for 30 cycles at 40° or 55° C (Roux, 1994). Hecker and Roux (1996) obtained similar results which indicated that although TD PCR incorporated 30 to 40 cycles having annealing temperatures below 55° C (a temperature causing spurious products with conventional PCR), only target amplicons were observed. Hecker and Roux (1996) also studied a modified TD PCR known as stepdown (SD) PCR which involved the same number of cycles patterned over fewer but larger steps (3° or 5° C). One program tested involved 69° to 55° C in five 3° C steps with 20 more cycles at 55° C. The SD PCR method produced results equivalent to those obtained using TD PCR. The SD PCR does have the advantage of involving fewer steps when programming the thermocycler (Hecker and Roux, 1996).

1.9.4. NASBA

Nucleic acid sequence based amplification (NASBA) is an isothermal RNA amplification process involving the simultaneous action of three enzymes, which are avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H, and T7 RNA polymerase (Uyttendaele et al., 1997). The reaction is carried out at 41°C for 90 min. In the first reaction, a primer containing a T7 RNA promotor sequence anneals to the single stranded RNA (Figure 1). Then the primer is extended via AMV-RT forming an RNA/DNA hybrid. The RNA strand of the hybrid is then cleaved by RNase H. Then, the second primer anneals

to the single stranded cDNA and AMV-RT generates a double stranded cDNA molecule containing a double stranded T7 promotor. Further, T7 RNA polymerase initiates transcription resulting in the generation of multiple anti-sense RNA copies. These RNA copies act as a template during subsequent amplification cycles (Widjojoatmodjo et al., 1999).

The method is specific for RNA and is effective even in samples containing traces of DNA. NASBA technology has been used to detect several pathogens including RNA viruses (Lanciotti and Kerst, 2001). Lanciotti and Kerst (2001) found that NASBA assay resulted in excellent sensitivities and specificities compared to those of virus isolation in cell culture, the TaqMan assay, and standard RT-PCR. Voisset et al. (2000) indicated that NASBA cannot amplify RNA exclusively because an important quantity of homologous plasmid DNA that carried sequences specific to the primers used was able to produce a positive signal. These results indicated that plasmid DNA could not be used as a negative control when using NASBA (Voisset et al., 2000). Uyttendaele et al. (1997) found that amplification of 16S rRNA using NASBA was not a sound method for differentiating between viable and non-viable *Campylobacter jejuni* (Uyttendaele et al., 1997), confirming other studies regarding the stability of rRNA.

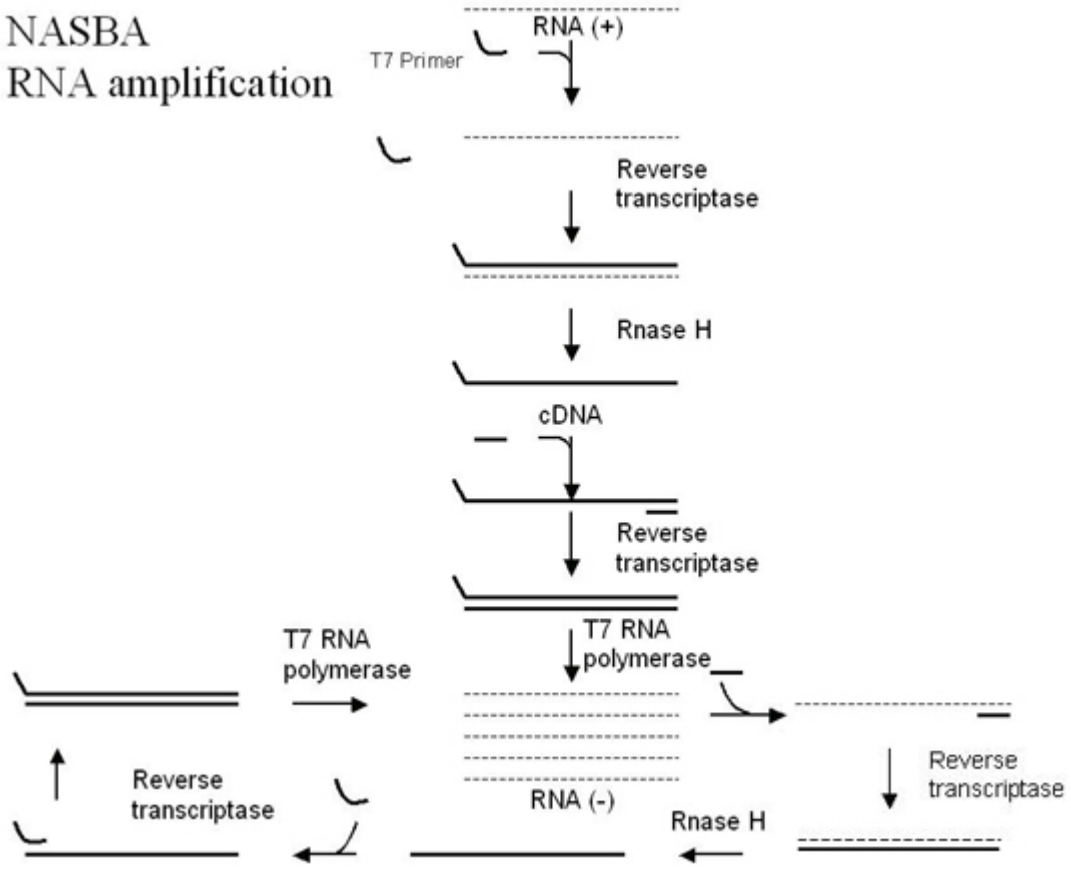


Figure 1.1 Schematic of NASBA
(Jean et al., 2001)

Kievits et al. (1991) evaluated the use of NASBA for detection of HIV-1. The NASBA method allowed amplification ranging from 2 x 10^6 to 5 x 10^7- fold after incubation at 41° C for 2.5 h. The detection limits were 10 HIV-1 target molecules in a model system (Kievits et al., 1991). Blais et al. (1997b) developed a highly specific nucleic acid sequence-based amplification (NASBA) system targeting the mRNA of the *hlyA* gene of *L. monocytogenes*. The researchers reported that the system allowed detection of *L.*

monocytogenes from dairy and egg products at levels of less than 10 CFU/g after 48 h enrichment in modified Listeria enrichment broth (Blais et al, 1997b).

Uyttendaele et al. (1995) evaluated NASBA amplification of rRNA and subsequent detection of amplification products by an enzyme-linked gel assay (ELGA) for detection of *C. jejuni* in foods, and this method was compared to conventional identification methods. They found that high numbers of indigenous microflora detracted from the sensitivity of both methods and resulted in a few false-negative results for both NASBA (1.42 %) and conventional isolation (2.86 %). *C. jejuni* was detectable using NASBA at ratios of 10,000:1 (indigenous flora:target) and the detection limit was less than 10 CFU/10 g. Centrifugation following the enrichment step eradicated inhibition interference by food components. The analysis time using NASBA compared to conventional isolation was shortened from 6 days to 26 h (Uyttendaele et al., 1995).

Simpkins et al. (2000) utilized NASBA to overcome the problems associated with RT-PCR in detecting mRNA in the presence of a DNA background. The target mRNA was transcribed from the bacterial *hsp*70 homologue *dnaK*. Detection of viable *Salmonella enterica* by amplification of mRNA was consistent and significantly different results were obtained for viable versus heat-killed cells. NASBA amplification of RNase treated extracts resulted in no signal despite the high concentrations of DNA confirmed by PCR. This was a good indicator that NASBA was not affected by background DNA (Simpkins et al., 2000). Jean et al. (2001) evaluated the NASBA technique for the detection of hepatitis A virus(HAV) in foods and compared it to traditional RT-PCR. NASBA allowed for detection of as low as 0.4 ng of target RNA/ml compared to 4 ng/ml for RT-PCR. Using crude HAV lysate, NASBA had a detection limit of 2 PFU (4 x 10^2 PFU/ml), while RT-PCR had a

detection limit of 50 PFU (1 x 10^4 PFU/ml). No interference occurred during amplification of HAV RNA, even in the presence of excess nontarget RNA or DNA. The NASBA system successfully detected HAV from experimentally inoculated samples of wastewater, lettuce, and blueberries. Several advantages of NASBA compared to RT-PCR include sensitivity, speed, and simplicity, making the NASBA system a powerful tool for detection of viral RNA (Jean et al., 2001).

1.10. FLUORESCENT PROBES USED IN REAL-TIME PCR ASSAYS

1.10.1. FRET

Fluorescence resonance energy transfer (FRET) is a process in which the energy from an excited fluorophore is transferred to an acceptor moiety at distances up to 70-100Å (Nazarenko et al., 1997). In other words, FRET is the emission or transfer of light energy from an unquenched fluorophore. Several FRET formats have been developed. A post-PCR probing step is required for some methods, while other methods are carried out in a closed tube format. Two commercialized FRET assays utilizing the closed tube format include molecular beacons and TaqMan. Drawbacks to these methods are that DNA is not detected directly, and the generated signal is dependent on the efficiency of the probe hybridization (Nazarenko et al., 1997).

In the intact TaqMan probe, the fluorophore on the left side (short-wavelength) is quenched by the fluorophore on the right side (long-wavelength) via fluorescence resonance energy transfer (FRET). Once the probe has hybridized to the target, the 5′ nuclease activity of Taq polymerase degrades the probe. Following degradation, the FRET occurring between

the two fluorophores is disrupted and the fluorescence from the short-wavelength fluorophore is increased, which results in a decrease in the signal from the long-wavelength fluorophore (Fig. 2) (Molecular Probes, Inc, 2001).

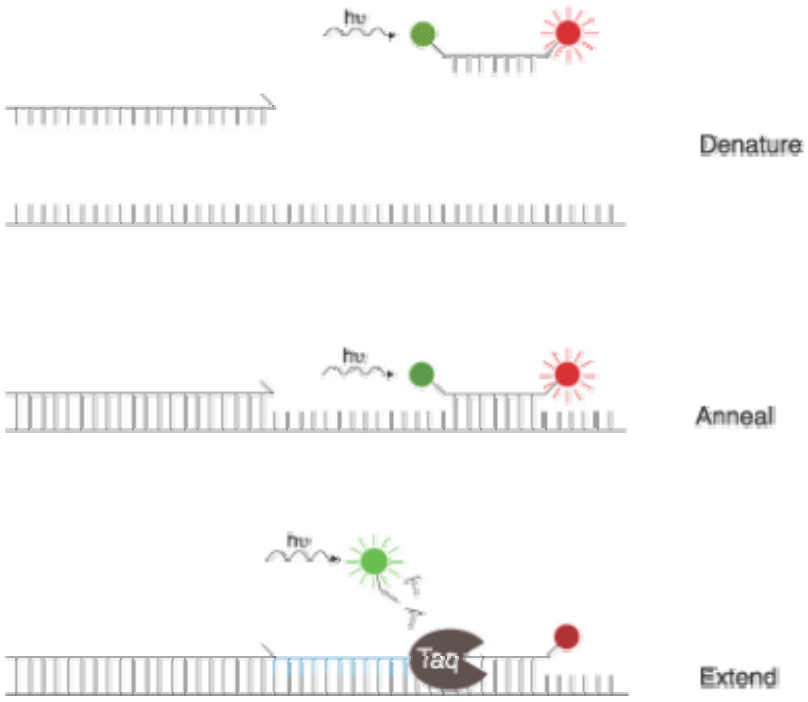


Figure 1.2. Summary of the TaqMan assay, a FRET method.
(Molecular Probes, Inc., 2001)

Taylor et al. (2001) utilized sequence capture (purification technique) combined with rapid-cycle PCR and real-time fluorescence using a *Mycobacterium tuberculosis* complex-specific Cy5-labeled fluorescence resonance energy transfer (FRET) probe in a LightCycler LC32 to detect *Mycobacterium bovis* in lymph node specimens from 38 cattle with bovine tuberculosis lesions, and compared this to a conventional PCR method. In comparison, conventional PCR allowed detection of 26 of 28 culture-positive specimens (93 %) in about 9 h, while the LightCycler PCR detected 20 of 28 culture-positive specimens (71 %) in only 30

min. The lower sensitivity by the FRET probe was due to disruption of fluorescent signals by the red iron oxide in the sequence capture beads. The fluorescent signal was significantly improved by detachment of captured mycobacterial DNA from the magnetic beads by heating and centrifugation prior to amplification. Huang et al. (2001) used a dual probe FRET PCR in an open tube format to detect *Chlamydia* by targeting *omp1* DNA sequences. FRET PCR allowed for linear quantification of chlamydiae with as low as single copies of the DNA template. When using FRET PCR, it was important to place the probes at least 60 bp downstream of the primer annealing to the same DNA strand and to obtain fluorescence within 3 s of equilibrium at the annealing temperature. Failure to follow such guidelines results in the delay of signal appearance by up to 10 cycles (Huang et al., 2001). While some FRET assays have used two single fluorescence-labeled oligonucleotides, Taylor et al. (2001) chose only a single-oligonucleotide FRET probe because the target was small and GC rich and the single-probe format has been used with greater sensitivity than the double-probe approach. The highly conserved housekeeping gene, *rpoB*, was targeted by Qi et al. (2001) in a real-time PCR assay performed on a LightCycler via FRET to detect *Bacillus anthracis*. The assay was specific for 144 strains of *B. anthracis* from different geographical locations and did not cross-react with other related bacilli (175 strains), except for one strain, due to a similarity in nucleotide sequence. This PCR assay could be applied to purified DNA or crude vegetative cell isolates for detection in less than 1 h (Qi et al., 2001).

1.10.2. Molecular Beacons

The molecular beacon concept was devised as a nonradioactive assay for RNA detection (Liu et al., 2002). Molecular beacons are useful in situations where it is either not

possible or not desirable to isolate probe-target hybrids from a surplus of the hybridization probes, such as in real-time monitoring of DNA/RNA amplification reactions or detection of RNA within living cells (Tan et al., 2000) They can also be applied to traditional PCR.

Molecular beacons are single stranded nucleic acid molecules that form a stem-and-loop or hairpin structure. The loop portion of the sequence is the probe, which is complementary to the target nucleic acid sequence. The stem is formed by two complementary arm sequences that lie on either side of the probe sequence. The stem sequences are independent of the target sequence. A fluorescent component is covalently linked to the end of one arm and a non-fluorescent quenching component to the end of the other arm. The stem maintains close proximity between the two arms causing the fluorescence of the fluorophore to be quenched by fluorescence resonance energy transfer. The energy produced by the fluorophore is transferred to the quencher as heat rather than being emitted in the form of light. In this state, the fluorophore is said to be quenched and does not fluoresce. In the event that the probe encounters and binds to a target sequence, a hybrid is formed that is more stable and is stronger than the forces holding the two arm sequences. This action results in unfolding of the secondary or 'hairpin' structure with separation of the quencher and fluorophore as it becomes bound to the target sequence. Now that the fluorophore is no longer in close proximity to the quencher, it will fluoresce when illuminated with ultraviolet light (Tyagi and Kramer, 1996; Tan et al., 2000; Liu et al., 2002). The conformational change will only occur when the probe is bound to the target, which must be perfectly complementary, having no mismatched nucleotides or deletions (Tyagi and Kramer, 1996). In real-time assays, the PCR reaction must be monitored at low

temperatures, since the molecular beacon will dissociate at higher temperatures (Tan et al., 2000).

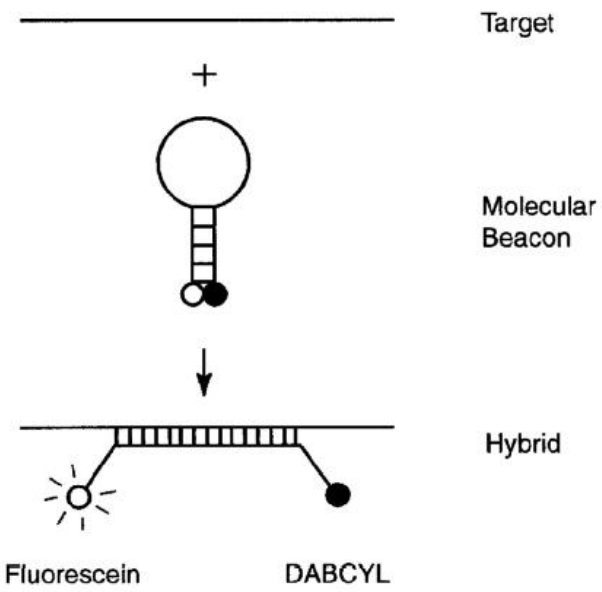


Figure 1.3. Molecular beacon hybridizing to target. (Tyagi and Kramer, 1996)

A variation of the molecular beacon technology involves the addition of two different fluorophores (F_1 and F_2) to each end of the stem structure (Zhang et al., 2001). The F_1 (fluorophore) and F_2 (acts like a quencher) are selected in such a fashion that fluorescence resonance energy transfer (FRET) will occur when the two are in proximity. When the beacon is unbound and in the stem and loop confirmation F_1 will be quenched by F_2 and F_2 will be observed. Once the beacon binds to the target, the conformational change results in the fluorescence of F_1 dominating, while F_2 diminishes or disappears. Using this format, the change of two fluorescence intensities can be monitored rather than the usual one fluorophore and one quencher model. An advantage of using molecular beacons with two fluorophores is that they are more sensitive to the concentration of target DNA than single

fluorophore molecular beacons. Another advantage would include better quantification, less dependence on optical geometry, and less interference due to photobleaching that could affect optical measurements. This technique could be very useful in DNA/RNA interaction studies and protein-DNA/RNA interaction studies (Zhang et al., 2001).

Molecular beacons have been referenced for a variety of applications, such as real-time monitoring of polymerase chain reactions, detection of DNA/RNA hybridization in living cells, DNA/RNA biosensors, monitoring of enzyme cleavage, investigation of the progression of HIV-1 disease, the study of DNA-protein interactions, and bacterial detection (Zhang et al., 2001). Molecular beacons have an advantage over conventional nucleic acid probes because they have a higher degree of specificity with better signal-to-noise ratios (Park et al., 2000).

The NASBA detection system is often coupled with molecular beacons in real time assays. During amplification, the molecular beacon anneals to the antisense RNA amplicon generated by NASBA, producing a fluorescent signal that can be monitored in real-time. This homogeneous RNA assay is referred to as AmpliDet RNA (Leone et al., 1998). One major advantage of the assay is that it is isothermal and reactions are carried out in one tube allowing for high-throughput applications for nucleic acid detection. Another advantage is that the real-time assay allows for closed tube format, which minimizes handling of the material thus less risk of carry-over contamination (Leone et al, 1998; Yates et al., 2001; Weusten et al., 2002). For quantification of RNA, a known amount of calibrator RNA is added to the sample to serve as an internal standard. In this assay approach, two molecular beacons are added, each with a highly specific loop sequence for binding to either the sample RNA or calibrator amplicons. As the endogenous RNA and the calibrator RNA are

amplified, two fluorescent signals are generated simultaneously with amplification. The amount of endogenous sample fluorescence is compared to the calibrator RNA fluorescence to quantify the amount of RNA present in the endogenous sample (Weusten et al., 2002). Lanciotti and Kerst (2001) found that the NASBA-beacon assay was very rapid, producing results in less than one hour.

1.11. APPLICATIONS OF FLUORESCENCE DETECTION TECHNOLOGIES

1.11.1. Molecular Beacons

McKillip and Drake (2000a) employed a molecular beacon to detect *E. coli* O157:H7 in artificially contaminated skim milk. The probe was designed to hybridize with the *stx2* gene coding for the A subunit. The degree of fluorescence correlated with the amount of DNA template in each reaction. The fluorescence was measured every two cycles and fluorescence significantly increased above the background levels by cycle 8, 14, or 14 for reactions containing DNA from 10^7-, 10^5- or 10^3- CFU/ml template, respectively. Fortin et al. (2001) developed a fluorogenic, real-time PCR assay for the detection of *E. coli* O157:H7 based on the *rfbE* locus. Their results were similar to McKillip and Drake (2000a) in that they were able to detect the presence of *E. coli* O157:H7 at $>10^2$ CFU/ml in samples. The assay was highly specific and allowed detection of EHEC in contaminated milk and apple juice. Detection of *E. coli* O157:H7 in milk or apple juice was as low as 1 CFU/ml following 6 h of enrichment (Fortin et al., 2001).

Poddar (1999) utilized molecular beacon probes for the detection of adenovirus. The molecular beacon was characterized for its efficiency of quenching and signal to noise ratio by spectrofluorometric analysis of its hybridization with virus-specific complementary single

stranded oligonucleotide target. Detection was carried out after amplification by measuring the fluorescent signal in the post PCR sample using a spectrofluorometer. The detection limit using the molecular beacon probe for adenovirus detection was similar to that obtained using labeled linear probe hybridization following PCR (Poddar, 1999). Park et al. (2000) applied molecular beacon technology to a collection of 23 strains of *Candida*, with the majority being *Candida albicans* and the remaining consisting of *Candida dubliniensis*. The species-specific probes were 100% effective in identifying both species following PCR amplification of the ITS2 region. The results were verified by random amplified polymorphic DNA analysis-based genotyping and by restriction enzyme analysis with enzymes *Bsm*AI and *Nsp*BII, which cleave recognition sequences between the ITS2 regions (Park et al., 2000).

A real-time RT-PCR assay using molecular beacons was used to quantitatively measure the different mRNAs produced by *Mycobacterium tuberculosis* following exposure to selected stresses (Manganelli et al., 1999). Another real-time assay employing molecular beacons demonstrated detection sensitivity as low as 2 CFU/PCR reactions for *Salmonella* species (Chen et al., 2000). Specificity was also demonstrated by producing amplicons for similar species such as *Escherichia coli* and *Citrobacter freundii* (Chen et al., 2000). A general fluorescence PCR assay incorporating a molecular beacon successfully detected *M. tuberculosis* using a fluorometer (Li et al., 2000). The sensitivity was 100% with detection limits ranging from 1-10 cells/ml. A clinical study confirmed the specificity of the assay, which compared well with smear (93.6%) and culture (98.4%) for positive samples. Fluorescence PCR was more specific than the other conventional methods, since more positive samples were detected by fluorescence PCR (62.9%) than by smear (30.3%) and

culture (31.4%) methods (Li et al., 2000). A real-time PCR assay utilizing molecular beacons was employed to detect and quantify *Chlamydophila felis* infection of cats (Helps et al., 2001). The assay was highly reproducible and was able to detect fewer than 10 genomic copies of a specific outer membrane protein gene (Helps et al., 2001).

1.11.2. Taqman

In the Taqman assay, a fluorescent hybridization probe bound to its target amplicon is cleaved through the action of the 5′-nuclease activity of the DNA polymerase. PCR or RT-PCR can be applied to Taqman formats. Usually Taq or Tth polymerase is used, but any enzyme having equivalent 5′-nuclease activity can be used. The first level of specificity is provided by the two template-specific primers on each end of the amplicon (Fig. 2B) that are used as in traditional RT-PCR or PCR. Further specificity is provided by a third oligonucleotide probe that hybridizes to the amplicon during the annealing/extension phase of the PCR (Fig. 2C) (Bustin, 2000). The probe possesses a fluorescent reporter dye at its 5′ end and the emission spectrum is quenched by a second dye at its 3′ end (Livak et al., 1995; Bustin, 2000). If there is no amplification of the amplicon complementary to the probe, then the probe stays unbound. Since the 5′-exonuclease activity of *Taq* polymerase is double-strand-specific, the unbound probe remains intact and no reporter fluorescence is detected. On the other hand, if the target amplicon is amplified, then the probe can hybridize to the amplicon following denaturation. The polymerase extends the primers until it reaches the hybridized probe where it displaces the 5′ end to form a forked structure. The polymerase continues to move from the free end to the bifurcation duplex where cleavage occurs, separating the reporter and quencher dyes allowing for fluorescence emission (Fig. 2D, E).

The greatest detection signal is reached when the reporter and quencher are the furthest apart at the 5′ and 3′ ends of the probe, which might be related to better cleavage efficiency by the polymerase (Bustin, 2000). This is a drawback to the method, since 5'-3' hydrolysis will only occur when the fluorophore and quencher are not too close to each other. The efficiency of energy transfer decreases with the inverse sixth power of the distance between the reporter and quencher. This may result in a high background emission from unhybridized probes (Nazarenko et al., 1997).

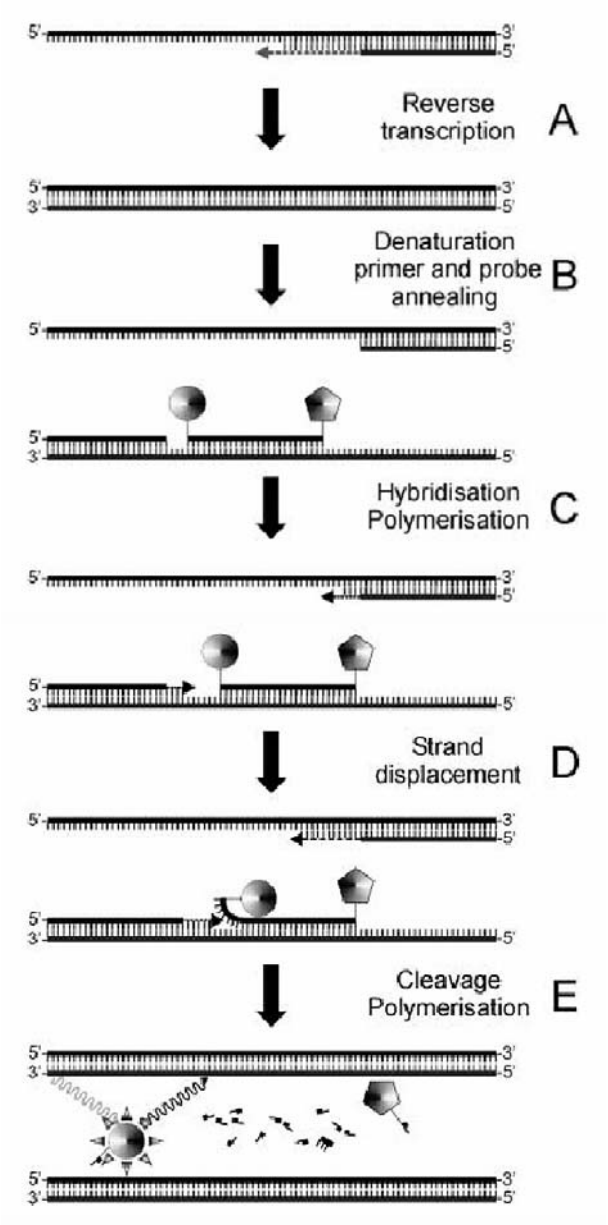


Figure 1.4. The Taqman Assay. (taken from Bustin, 2000)

Temperature considerations in the Taqman assay, the polymerization phase of the PCR must be adjusted to ensure proper probe binding, since the polymerase will only cleave the probe while it is hybridized to its complementary strand. Most probes have a T_m of about 70° C, so optimum conditions for the Taqman assay are 60-62° C, which is the optimum for

probe/target binding and polymerase activity during annealing and polymerization (Bustin, 2000). It is also important to consider the magnesium chloride concentration because optimal concentrations stabilize the hybridization complex of the probe and its target (Batt, 1997). With all other parameters held constant, an increase in magnesium chloride results in an increase in ΔRQ values for a given probe. However, a magnesium concentration that is too high can result in false positive results due to loss of specificity by the fluorogenic probe. The ΔRQ is a measure of the increase in emission intensity of the reporter dye (R) as a product of its release from the fluorogenic probe, and the proximal quencher (Q) over the emission intensity of the quenched reporter on the intact fluorogenic probe. Therefore the extent of cleavage affects the release of the reporter dye and the magnitude of the ΔRQ (Batt, 1997). Probe degradation is also affected by the initial number of target molecules, probe concentration, and number of PCR cycles (Holland et al., 1991).

The TaqMan assay is sensitive, specific, and speedy, having the capacity to detect < 50 cells of a target organism in approximately 100 samples within 2 to 3 h (Batt, 1997). Holland et al. (1991) found the Taqman assay to be sensitive and specific over more cumbersome detection methods. Rapid and specific detection of hepatitis C virus RNA in serum and plasma was achieved within minutes following PCR by using the TaqMan assay. The TaqMan PCR assay achieved similar sensitivity when compared with nested PCR, but with the added benefits of speed, increased throughput, and decreased potential for false-positive results due to cross-contamination of samples by eliminating the need for a second round of amplification (Morris et al., 1996). Although the specificity of the TaqMan assay is high, Morris et al. (1996) emphasized that tests should be performed in duplicate,

especially when testing low-titered samples (i.e. 10^{-4} or 10^{-5} dilutions), since less predictable results can be obtained as the concentration of the template is decreased (Morris et al., 1996).

Verstrepen et al. (2001) found the TaqMan real-time reverse transcription-PCR assay to be faster and more specific for enterovirus detection than traditional cell culture methods. The assay was also able to quantify the enterovirus genome in a linear range reaching at least 5 logs (Verstrepen et al., 2001). Following a 12 h enrichment step in modified EC broth, the 5' nuclease (TaqMan) assay allowed for detection of as few as 0.5 CFU/g of STEC in ground beef (Witham et al., 1996). Oberst et al. (1998) achieved good specificity in detecting *E. coli* O157:H7 using a *eaeA*-based 5' nuclease (TaqMan) assay. This method was only able to detect $\geq 10^3$ CFU/ml in mTSB broth or modified EC broth and $\geq 10^4$ CFU/ml in ground beef-mTSB mixtures. The procedure improved by $\geq 10^2$ after an IMS procedure, followed by a secondary enrichment step and DNA recovery with a QIAamp tissue kit (Qiagen) (Oberst et al., 1998). Kimura et al. (1999) tested the TaqMan *Salmonella* amplification/detection kit from PE Applied Biosystems (Foster City, CA). They evaluated several fecal *Salmonella* strains by inoculating each into raw meat or shrimp. Detection limits were 120 CFU/ml of TSB culture and similar results were obtained from food samples. Conventional culture methods were performed to confirm results and experiments did not yield any false-negative or false-positive results (Kimura et al., 1999). The TaqMan assay was employed for rapid detection of *Clostridium botulinum* Type E by Kimura et al. (2001). These investigators reported that increases in *C. botulinum* DNA could be monitored much earlier than toxin could be detected by the mouse assay. Overall, TaqMan was a rapid, quantitative, and sensitive PCR method for the estimation of botulinal risk in seafood samples. This technique

was capable of quantifying *C. botulinum* DNA within a range of 10^2 to 10^8 CFU/ml or g (Kimura et al., 2001).

Nogva et al. (2000) investigated the use of a magnetic bead-based cell concentration step and DNA purification followed by the Taqman assay to detect *L. monocytogenes* in milk (1.6 ml), water (1.6 ml), and pure culture (0.5 ml) without prior enrichment. The procedure was highly specific, allowing detection of all *L. monocytogenes* isolates (65) and negative results for all other bacteria (18) including other *Listeria* spp (16 isolates from 5 species tested). Detection limits were approximately 6 to 60 CFU/PCR and the method could be completed in 3 h (Nogva et al., 2000). Hristova et al. (2001) utilized specific primers and probes designed to target the 16S ribosomal DNA region of the methyl *tert*-butyl ether (MTBE)-degrading bacterial strain PM1, a member of the *Rubrivivax gelatinosus* subgroup of β-*Proteobacteria*. Using real-time Taqman PCR, a detection limit of 2 PM1 cells/ml in pure culture or 180 PM1 cells/ml in a mixture of PM1 and *E. coli* was obtained (Hristova et al., 2001). Weller et al. (2000) described the use of a fluorogenic (Taqman) PCR assay to detect *Ralsonia solanacearum* strains. Tests performed on pure cultures generated better detection limits ($\geq 10^2$ cells) than those obtained from samples of inoculated potato tissue extracts. Two fluorogenic probes were used in a multiplex PCR reaction and a third fluorogenic probe was used as an internal standard. The probes used were inhibited by undiluted potato extract, explaining the lower sensitivity of Taqman PCR on the potato tissue extracts (Weller et al., 2000). Becker et al. (2000) tested the performance of Taqman 5' nuclease assays for analyzing the rRNA sequence divergence of red-pigmented cyanobacteria isolated from deep lakes. Control of amplification efficiency and the use of specific primers and probes could allow for good quantification of target DNA in the presence of up to 10^4 –

fold excess of phylogenetically similar DNA, and up to 10^7 -fold excess of dissimilar DNA (Becker et al., 2000). Batt (1997) also observed negative effects on the TaqMan assay once the ratio of competing to target organisms was exceeded 10^6, after which nonspecific binding of either PCR primers or fluorogenic probes occurred (Batt, 1997).

The objectives for this study will be to incorporate novel methods for recovery and specific detection of viable *L. monocytogenes*, *E. coli* O157:H7, and *S. typhimurium* from media and selected foodstuffs. Recovery of bacteria will be optimized by differential centrifugation and the novel immuno-capture device, Pathatrix. Detection and quantification of viable *L. monocytogenes* will be determined by plating on TSA supplemented with streptomycin and PCR targeting a 287bp unique region of the 16S rDNA; *E. coli* O157:H7 will be detected by plating on SMAC and by using PCR targeting a 254bp region of the *stx*2 gene; *S. typhimurium* will be recovered using TSA supplemented with kanamycin and PCR detection targeting a 389bp fragment of the *inv*A gene.

1.12. REFERENCES

Ajjarapu, S., and L.A. Shelef. 1999. Fate of pGFp-bearing *Escherichia coli* O157:H7 in ground beef at 2 and 10 C and effects of lactate, diacetate, and citrate. *Appl. Environ. Microbiol*. 65:5394-5397.

Barkocy-Gallagher, G.A., E.D. Berry, M. Rivera-Betancourt, T.M. Arthur, X. Nou, and M. Koohmaraie. 2002. Development of methods for the recovery of *Escherichia coli* O157:H7 and *Salmonella* from beef carcass sponge samples and bovine fecal and hide samples. *J. Food Prot*. 65(10):1527-1534.

Batt, C.A. 1997. Symposium: molecular probes—theory and application (titled) Molecular diagnostics for dairy-borne pathogens. *J. Dairy Sci*. 80:220-229.

Becker, S., P. Böger, R. Oehlmann, and A. Ernst. 2000. PCR bias in ecological analysis: a case study for quantitative Taq nuclease assays in analyses of microbial communities. *Appl. Environ. Microbiol*. 66(11):4945-4953.

Bej, A., M. Mahbubani, and R. Atlas. 1991. Amplification of nucleic acids by polymerase chain reaction (PCR) and other methods and their applications. *Crit. Rev. Biochem. Mol. Biol*. 26:301-334.

Bej, A.K., W.Y. Ng, S. Morgan, D.D. Jones, and M.H. Mahbubani. 1996. Detection of viable *Vibrio cholerae* by reverse-transcriptase polymerase chain reaction (RT-PCR). *Mol. Biotech*. 5:1-10.

Beutin, L., S. Zimmermann, and K. Gleier. 1996. Rapid detection and isolation of Shiga-like toxin (Verocytotoxin)-producing *Escherichia coli* by direct testing of individual enterohemolytic colonies from washed sheep blood agar plates in the VTEC-RPLA assay. *J. Clin. Microbiol*. 34(11):2812-2814.

Bickley, J., J.K. Short, D.G. McDowell, and H.C. Parkes. 1996. Polymerase chain reaction (PCR) detection of *Listeria monocytogenes* in diluted milk and reversal of PCR inhibition caused by calcium ions. *Lett. Appl. Microbiol*. 22:153-158.

Blais, B.W., R.A. Booth, L.M. Phillippe, and H. Yamazaki. 1997a. Effect of temperature and agitation on enrichment of *Escherichia coli* O157:H7 in ground beef using modified EC broth with novobiocin. *Int. J. Food Microbiol*. 36:221-225.

Blais, B.W., G. Turner, R. Sooknanan, and L.T. Malek. 1997b. A nucleic acid sequence-based amplification system for detection of *Listeria monocytogenes hlyA* sequences. *Appl. Environ. Microbiol*. 63(1):310-313.

Bolton, F.J., L. Crozier, and J.K. Williamson. 1996. Isolation of *Escherichia coli* O157:H7 from raw meat products. *Lett. Appl. Microbiol*. 23:317-321.

Buchanan, R.L. and L.K. Bagi. 1994. Expansion of response surface models for the growth of *Escherichia coli* O157:H7 to include sodium nitrite as a variable. *Intl. J. Food Microbiol.* 23:317-322.

Buncic, S., and S. M. Avery. 1998. Effects of cold storage and heat-acid shocks on growth and verotoxin2 production of *Escherichia coli* O157:H7. *Food Microbiol*. 15:319-328.

Bustin, S.A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymersase chain reaction assays. *J. Mol. Endocrin*. 25:169-193.

Calderwood, S.B., F. Auclair, A. Donohue-Rolfe, and G.T. Keusch. 1987. Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. *Biochem*. 84:4364-4368.

Calderwood, S.B., D.W.K. Acheson, G.T. Keusch, T.J. Barrett, P.M. Griffin, N.A. Strockbine, B. Swaminathan, J.B. Kaper, M.M. Levine, B.S. Kaplan, H. Karch, A.D. O'Brien, T.G. Obrig, Y. Takedo, P.I. Tarr, and I.K. Wachsmuth. 1996. Proposed new nomenclature for SLT (VT) family. *ASM News*. 62:118-119.

[CDC] Centers for Disease Control and Prevention. 1997. Outbreaks of *Escherichia coli* O157:H7 infection associated with eating alfalfa sprouts – Michigan and Virginia, June-July 1997. *Morbid. Mortal. Weekly Rep*. 46(32):741-744.

[CDC] Centers for Disease Control and Prevention Outbreak Response and Surveillance Unit. 2000. *E. coli* O157:H7 Outbreak summaries. Outbreak Reports and Publications, 1998-1999.

[CDC] Centers for Disease Control and Prevention. 2001. Outbreaks of *Escherichia coli* O157:H7 infections among children associated with farm visits --- Pennsylvania and Washington, 2000. *Morbid. Mortal. Weekly Rep*. 50(15): 293-297.

[CDC] Centers for Disease Control and Prevention. 2002. Multistate Outbreak of *Escherichia coli* O157:H7 Infections Associated with Eating Ground Beef --- United States, June--July *Morbid. Mortal. Weekly Rep*. 51(29):637-639.

Cebula, T.A., W.L. Payne, and P. Feng. 1995. Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their shiga-like toxin type by mismatch amplification mutation assay-multiplex PCR. *J. Clin. Microbiol*. 33(1):248-250.

Chapman, P.A., C.A. Siddons, D.J. Wright, P. Norman, J. Fox, and E. Crick. 1993. Cattle as a possible source of verocytotoxin-producing *Escherichia coli* O157 infections in man. *Epidemiol. Infect*. 111:439-447.

Chapman, P.A., D.J. Wright, and C.A. Siddons. 1994. A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* O157 from bovine feces. *J. Med. Microbiol*. 40:424-427.

Chen, J., R. Johnson, and M. Griffiths. 1998. Detection of verotoxigenic *Escherichia coli* by magnetic capture-hybridization PCR. *Appl. Environ. Microbiol*. 64(1):147-152.

Chen, W., G. Martinez, and A. Mulchandani. 2000. Molecular beacons: A real-time polymerase chain reaction assay for detecting *Salmonella*. *Anal. Biochem*. 280:166-172.

Cheng, C., and C. W. Kaspar. 1998. Growth and processing conditions affecting acid tolerance in *Escherichia coli* O157:H7. *Food Microbiol*. 15:157-166.

Chikthimmah, N., R.C. Anantheswaran, R.F. Roberts, E.W. Mills, and S.J. Knabel. 2001. Influence of sodium chloride on growth of lactic acid bacteria and subsequent destruction of *Escherichia coli* O157:H7 during processing of Lebanon bologna. *J. Food Prot*. 64(8):1145-1150.

Cobbold, R., and P. Desmarchelier. 2001. Characterisation and clonal relationships of Shiga-toxigenic *Escherichia coli* (STEC) isolated from Australian dairy cattle. *Vet. Microbiol*. 79(4):323-335.

Cray, W.C., D.O. Abbott, F.J. Beacorn, and S.T. Benson. 1998. Detection, isolation and identification of *Escherichia coli* O157:H7 and O157:NM (nonmotile) from meat products. C.5 In USDA/FSIS Microbiology Laboratory Guidebook. Rev #2 2-23-01 pp. 1-13. Hypertext Source: (2005) http://www.fsis.usda.gov/ophs/Microlab/Mlg5.03.pdf

Crump, J.A., A.C. Sulka, A.J. Langer, C. Schaben, A.S. Crielly, R. Gage, M. Baysinger, M. Moll, G. Withers, D.M. Toney, S.B. Hunter, R.M. Hoekstra, S.K. Wong, P.M. Griffin, and T.G. Van Gilder. 2002. An outbreak of *Escherichia coli* O157:H7 infections among visitors to a dairy farm. *New Engl. J. Med.* 347(8):555-560.

Cullison, M.A., and L.A. Jaykus. 2002. Magnetized carbonyl iron and insoluble zirconium hydroxide mixture facilitates bacterial concentration and separation from nonfat dry milk. *J. Food Prot*. 65(11):1806-1810.

De Boer, S.H., L.J. Ward, X. Li, and S. Chittaranjan. 1995. Attenuation of PCR inhibition in the presence of plant compounds by addition of BLOTTO. *Nucl. Acids Res.* 23(13):2567-2568.

Deng, M.Y., and P.M. Fratamico. 1996. A multiplex PCR for rapid identification of shiga-like-toxin-producting *Escherichia coli* O157:H7 isolated from foods. *J. Food Prot*. 59(6):570-576.

Don, R.H., P.T. Cox, B.J. Wainwright, K. Baker, and J.S. Mattick. 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucl. Acids Res.* 19(14):4008.

Doyle, M.P., T. Zhao, J. Meng, and S. Zhao. 1997. *Escherichia coli* O157:H7. In: Food Microbiology: Fundamental and Frontiers. eds. M.P. Doyle, L.R. Beuchat, and T.J. Montville. ASM Press. Washington, D.C. pp. 171-191.

Farber, J.M. and F. Pagotto. 1992. The effect of acid shock on the heat resistance of *Listeria monocytogenes*. *Lett. Appl. Microbiol*. 15:197-201.

Feng, P., and S.D. Weagant. 2002. Chapter 4A: Diarrheagenic *Escherichia coli*. In: FDA Bacteriological Analytical Manual 8th Edition. Revision A. U.S. Food and Drug Administration, Center for Food Safety & Applied Nutrition. Hypertext Source: (2005) http://www.cfsan.fda.gov/~ebam/bam-4a.html.

Fortin, N.Y., A. Mulchandani, and W. Chen. 2001. Use of real-time polymerase chain reaction and molecular beacons for the detection of *Escherichia coli* O157:H7. *Anal. Biochem*. 289:281-288.

Fratamico, P.M. L.K. Bagi, and T. Pepe. 2000. A multiplex polymerase chain reaction assay for rapid detection and identification of *Escherichia coli* O157:H7 in foods and bovine feces. *J. Food Prot*. 63:81032-1037.

Fratamico, P.M., and T.P. Strobaugh. 1998a. Simultaneous detection of *Salmonella* spp. and *Escherichia coli* O157:H7 by multiplex PCR. *J. Ind. Microbiol. Biotechnol.* 21(3):92-98.

Fratamico, P.M., and T.P. Strobaugh. 1998b. Evaluation of an enzyme-linked immunosorbent assay, direct immunofluorescent filter technique, and multiplex polymerase chain reaction for detection of *Escherichia coli* O157:H7 seeded in beef carcass wash water. *J. Food Prot*. 61(8):934-938.

Gannon, V.P.J., R.K. King, J.Y. Kim, and E.J.G. Thomas. 1992. Rapid and sensitive method for detection of shiga-like toxin-producting *Escherichia coli* in ground beef using the polymerase chain reaction. *Appl. Environ. Microbiol*. 58(12):3809-3815.

Gannon, V.P.J., M. Rashed, R.K. King, and E.J.G. Thomas. 1993. Detection and characterization of the *eae* gene of shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. *J. Clin. Microbiol*. 31(5):1268-1274.

Gunzer, F., H. Bohm, H. Russmann, M. Bitzan, S. Aleksic, and H. Karch. 1992. Molecular detection of sorbitol-fermenting *Escherichia coli* O157 in patients with hemolytic-uremic syndrome. *J. Clin. Microbiol*. 30(7):1807-1810.

Hara-Kudo, Y., Y. Onoue, H. Konuma, H. Nakagawa, and S. Kumagai. 1999. Comparison of enrichment procedures for isolation of *Escherichia coli* O157:H7 from ground beef and radish sprouts. Int. J. Food Microbiol. 50:211-214.

Hecker, K.H., and K.H. Roux. 1996. High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR. *Biotechniques*. 20:478-485.

Helps, C., N. Reeves, S. Tasker, and D. Harbour. 2001. Use of real-time quantitative PCR to detect *Chlamydophila felis* infection. *J. Clin. Microbiol*. 39(7):2675-2676.

Henegariu, O., N.A. Heerema, S.R. Dlouhy, G.H. Vance, and P.H. Vogt. 1997. Multiplex PCR: Critical parameters and step-by-step protocol. *Biotechniques*. 23:504-511.

Herman, L. 1997. Detection of viable and dead *Listeria monocytogenes* by PCR. *Food Microbiol*. 14:103-110.

Hersh, B.M., F.T. Farooq, D.N. Barstad, D.L. Blankenhorn, and J.L. Slonczewski. 1996. A Glutamate-dependent acid resistance gene in *Escherichia coli*. *J. Bacteriol*. 178(13):3978-3981

Hitchins, A.D., P. Feng, W.D. Watkins, S.R. Rippey, and L.A. Chandler. 1995. *Escherichia coli* and the coliform bacteria. In: FDA Bacteriological Analytical Manual 7th Edition. Arlington, VA: Association of Official Analytical Chemists. 4.20-4.25.

Holland, P.M., R.D. Abramson, R. Watson, and D.H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'→ 3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci.* 88:7276-7280.

Hristova, K.R., C.M. Lutenegger, and K.M. Scow. 2001. Detection and quantification of methyl *tert*-butyl ether-degrading strain PM1 by real-time Taqman PCR. *Appl. Environ. Microbiol.* 67(11):5154-5160.

Huang, J., F.J. DeGraves, D. Gao, P. Feng, T. Schlapp, and B. Kaltenboeck. 2001. Quantitative detection of *Chlamydia* spp. by fluorescent PCR in the LightCycler. *Biotechniques.* 30(1):150-157.

Hudson, J.A., C. Nicol, J. Capill, and J. Bennett. 2000. Isolation of shiga toxin-producing *Escherichia coli* (STEC) from foods using EHEC agar. *Lett. Appl. Microbiol*. 30:109-113.

Jay, J.M. 2000. Foodborne gastroenteritis caused by *Escherichia coli*. Modern Food Microbiology. ed. D.R. Heldman. Aspen Publishers. Gaithersburg, Maryland. pp. 531-547.

Jean, J., B. Blais, A. Darveau, and I. Fliss. 2001. Detection of Hepatitis A virus by the nucleic acid sequence-based amplification technique and comparison with reverse transcription-PCR. *Appl. Environ. Microbiol*. 67(12):5593-5600.

Jenkins, D.E., Schultz, J.E., and A. Matin. 1988. Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli*. *J. Bacteriol*. 170:3910-3914.

Jenkins, D.E., Chaisson, S.A., and Matin, A. 1990. Starvation-Induced cross protection against osmotic challenge in *Escherichia coli*. *J. Bacteriol*. 172:2779-2781.

Junkins, A.D., and M.P. Doyle. 1989. Comparison of adherence properties of *Escherichia coli* O157:H7 and a 60-megadalton plasmid-cured derivative. *Curr. Microbiol*. 9:21-27.

Karch, H., J. Hassenmann, R. Laufs, A.D.O'Brien, C.O. Tacket, and M.M. Levine. 1987. A plasmid of enterohemorrhagic *Escherichia coli* O157:H7 is required for expression of a new fimbrial antigen and for adhesion to epithelial cells. *Infect. Immun*. 55:455-461.

Karch, H., C. Janetzki-Mittmann, S. Aleksic, and M. Datz. 1996. Isolation of enterohemorrhagic *Escherichia coli* O157 strains from patients with hemolytic-uremic syndrome by using immunomagnetic separation, DNA-based methods, and direct culture. *J. Clin. Microbiol*. 34(3):516-519.

Kaucner, C., and T. Stinear. 1998. Sensitive and rapid detection of viable *Giardia* cysts and *Cryptosporidium parvum* oocysts in large-volume water samples with wound fiberglass cartridge filters and reverse transcription-PCR. *Appl. Environ. Microbiol.* 64(5):1743-1749.

Kauppi, K.L., S.R. Tatini, F. Harrell, and P. Feng. 1996. Influence of substrate and low temperature on growth and survival of verotoxigenic *Escherichia coli*. *Food Microbiol*. 13:397-405.

Kennedy, J.F., S.A. Barker, and J.D. Humphreys. 1976. Microbial cells living immobilized on metal hydroxide. *Nature*. 261:242-244.

Kimura, B., S. Kawasaki, T. Fuji, J. Kusunoki, T. Itoh, and S.J.A. Flood. 1999. Evaluation of TaqMan PCR assay for detecting *Salmonella* in raw meat and shrimp. *J. Food Prot.* 62(4):329-335.

Kimura, B., S. Kawasaki, H. Nakano, and T. Fujii. 2001. Rapid, quantitative PCR monitoring of growth of *Clostridium botulinum* Type E in modified-atmosphere-packaged fish. *Appl. Envrion. Microbiol*. 67(1):206-216.

Klein, P.G. and V.K. Juneja. 1997. Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. *Appl. Environ. Microbiol*. 63(11):4441-4448.

Lanciotti, R.S., and A.J. Kerst. 2001. Nucleic acid sequence based amplification assays for rapid detection of West Nile and St. Louis encephalitis Viruses. *J. Clin. Microbiol.* 39(12):4506-4513.

Lantz, P.G., B. Hahn-Hägerdal, and P. Rådström. 1994. Sample preparation methods in PCR-based detection of food pathogens. *Trends Food Sci. Technol*. 5:384-389.

Law, D. 2000. Virulence factors of *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli*. *J. Appl. Microbiol*. 88:729-745.

Leenanon B.R., and Drake M.A. 2001. Effect of stress on subsequent post stress behavior of *Escherichia coli*. *J. Food Prot*. 64:970-974.

Lehmacher, A., H. Meier, S. Aleksic, and J. Bockemühl. 1998. Detection of hemolysin variants of Shiga toxin-producing *Escherichia coli* by PCR and culture on vancomycin-cefixime-cefsulodin blood agar. *Appl. Environ. Microbiol*. 64(7):2449-2453.

Leone, G., H. van Schijndel, B. van Gemen, F.R. Kramer, and C.D. Schoen. 1998. Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nucl. Acids Res*. 26(9):2150-2155.

Li, Q.G., J.X. Liang, G.Y. Luan, Y. Zhang, and K. Wang. 2000. Molecular beacon-based homogeneous fluorescence PCR assay for the diagnosis of infectious diseases. *Anal. Sci.* 16:245-248.

Lin, J., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster. 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium, Shigella flexneri*, and *Escherichia coli*. *J. Bacteriol*. 177:4097-4104.

Lindqvist, R., A.K. Antonsson, B. Norling, L. Persson, A.C.L. Ekström, U. Fäger, E. Eriksson, S. Löfdahl, and P. Norberg. 1998. The prevalence of verocytotoxin-producing *Escherichia coli* (VTEC) and *E. coli* O157:H7 in beef in Sweden determined by PCR assays and an immuno-magnetic separation (IMS) method. *Food Microbiol*. 15:591-601.

Liu, J., P. Feldman, and T.D.Y. Chung. 2002. Real-time monitoring in vitro transcription using molecular beacons. *Anal. Biochem.* 300:40-45.

Livak, K.J., S.J.A. Flood, J. Marmaro, W. Giusti, and Karin Deetz. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Meth. and Applic*. 4:357-362.

Louise, C.B., and T.G. Obrig. 1992. Shiga toxin-associated hemolytic uremic syndrome: combined cytotoxic effects of Shiga toxin and lipopolysaccharide (endotoxin) on human vascular endothelial cells in vitro. *Infect. Immun*. 60(4):1536-1543.

Lucore, L.A., M.A. Cullison, and L.A. Jaykus. 2000. Immobilization with metal hydroxides as a means to concentrate food-borne bacteria for detection by cultural and molecular methods. *Appl. Environ. Microbiol*. 66(5):1769-1776.

Manganelli, R., E. Dubnau, S. Tyagi, F.R. Kramer, and I. Smith. 1999. Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. *Mol. Microbiol*. 31(2):715-724.

McIngvale, S.C., X.Q. Chan, J.L. McKillip, and M.A. Drake. 2000. Survival of *Escherichia coli* O157:H7 in buttermilk as affected by contamination point and storage temperature. *J. Food Prot*. 63(4):441-444.

McIngvale, S.C., D. Elhanafi, and M.A. Drake. 2002. Optimization of reverse transcriptase PCR to detect viable shiga-toxin-producing *Escherichia coli*. *Appl. Environ. Microbiol*. 68(2):799-806.

McKillip, J.L., L.A. Jaykus, and M.A. Drake. 1998. rRNA Stability in Heat-Killed and UV-Irradiated Enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 64(11):4264-4268.

McKillip, J.L., L.A. Jaykus, and M.A. Drake. 1999. Nucleic acid persistence in heat-killed *Escherichia coli* O157:H7 from contaminated skim milk. *J. Food Prot*. 62(8):839-844.

McKillip, J.L. and M.A. Drake. 2000a. Molecular beacon polymerase chain reaction detection of *Escherichia coli* O157:H7 in milk. *J. Food Prot.* 63(7):855-859.

McKillip, J.L., L.A. Jaykus, and M.A. Drake. 2000b. A comparison of methods for the detection of *Escherichia coli* O157:H7 from artificially-contaminated dairy products using PCR. *J. Appl. Microbiol.* 89:49-55.

Meng, J., S. Zhao, M.P. Doyle, S.E. Mitchell, and S. Kresovich. 1997. A multiplex PCR for identifying Shiga-like toxin producing *Escherichia coli* O157:H7. *Lett. Appl. Microbiol*. 24:172-176.

Meng, J., S. Zhao, and M.P. Doyle. 1998. Virulence genes of Shiga toxin-producting *Escherichia coli* isolated from food, animals and humans. *Int. J. Food Microbiol*. 45(3):229-235.

Morris, T., B. Robertson, and M. Gallagher. 1996. Rapid reverse transcription-PCR detection of Hepatitis C virus RNA in serum by using the TaqMan fluorogenic detection system. *J. Clin. Microbiol*. 34(12):2933-2936.

Nazarenko, I.A., S.K. Bhatnagar, and R.J. Hohman. 1997. A closed tube format for amplification and detection of DNA based on energy transfer. *Nucl. Acids Res*. 25(12):2516-2521.

Nogva, H.K., K. Rudi, K. Naterstad, A. Holck, and D. Lillehaug. 2000. Application of 5'-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. *Appl. Environ. Microbiol*. 66(10):4266-4271.

Novicki, T.J., J.A. Daly, S.L. Mottice, and K.C. Carroll. 2000. Comparison of sorbitol MacConkey agar and a two-step method which utilizes enzyme-linked immunosorbent assay

toxin testing and a chromogenic agar to detect and isolate enterohemorrhagic *Escherichia coli*. *J. Clin. Microbiol*. 38(2):547-551.

Oberst, R.D., M.P. Hays, L.K. Bohra, R.K. Phebus, C.T. Yamashiro, C. Paszko-Kolva, S.J.A. Flood, J.M. Sargeant, and J.R. Gillespie. 1998. PCR-based DNA amplification and presumptive detection of *Escherichia coli* O157:H7 with an internal fluorogenic probe and the 5' nuclease (TaqMan) assay. *Appl. Environ. Microbiol*. 64(9):3389-3396.

Ogunjimi, A.A., and P.V. Choudary. 1999. Adsorption of endogenous polyphenols relieves the inhibition by fruit juices and fresh produce of immuno-PCR detection of *Escherichia coli* O157:H7. *FEMS Immun. Med. Microbiol*. 23:213-220.

Padhye, N.V., and M.P. Doyle. 1992. *Escherichia coli* O157:H7: epidemiology, pathogenesis, and methods for detection in food. *J. Food Prot.* 55:535-565.

Palumbo, S.A., J.E. Call, F.J. Schultz, and A. Williams. 1995. Minimum and maximum temperatures for growth and verotoxin production by hemorrhagic strains of *Escherichia coli*. *J. Food Prot*. 58(4):352-356.

Palumbo, S.A., Pickard, A., and Call, J.E. 1997. Population changes and verotoxin production of enterohemorrhagic *Escherichia coli* strains inoculated in milk and ground beef held at low temperatures. *J. Food Prot*. 60(7):746-750.

Park, S., M. Wong, S.A.E. Marras, E.W. Cross, T.E. Kiehn, V. Chaturvedi, S. Tyagi, and D.S. Perlin. 2000. Rapid identification of *Candida dubliniensis* using a species-specific molecular beacon. *J. Clin. Microbiol*. 38(8):2829-2836.

Paton, A.W., J.C. Paton, P.N. Goldwater, and P.A. Manning. 1993. Direct detection of *Escherichia coli* shiga-like toxin genes in primary fecal cultures by polymerase chain reaction. *J. Clin. Microbiol*. 31(11):3063-3067.

Paton, J.C. and A.W. Paton. 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin. Microbiol. Rev*. 11(3): 450-479.

Perna, N.T., G.F. Mayhew, G. Posfai, S. Elliott, M.S. Donnenberg, J.B. Kaper, and F.R. Blattner. 1998. Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. *Infect. Immun*. 66(8):3810-3817.

Perna, N.T., G. Plunkett, V. Burland, B. Mau, J.D. Glasner, D.J. Rose, G.F. Mayhew, P.S. Evans, J. Gregor, H.A. Kirkpatrick, G. Posfal, J. Hackett, S. Kllnk, A. Boutin, Y. Shao, L. Miller, E.J. Grotbeck, N.W. Davis, A. Lim, E.T. Dimalanta, K.D. Potamousl, J. Apodaca, T.S. anantharaman, J. Lin, G. Yen, D.C. Schwartz, R.A. Welch, F.R. Blattner. 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature.* 409:529-533.

Poddar, S.K. 1999. Detection of adenovirus using PCR and molecular beacon. *J. Virol. Meth*. 82:19-26.

Pollard, D.R., W.M. Johnson, H. Lior, S.D. Tyler, and K.R. Rozee. 1990. Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. *J. Clin. Microbiol*. 28:540-545.

Pond, T.J., D.S. Wood, I.M. Mumin, S. Barbut, and M.W. Griffiths. 2001. Modeling the survival of *Escherichia coli* O157:H7 in uncooked, semidry, fermented sausage. *J. Food Prot*. 64(6):759-766.

Pyle, B.H., S.C. Broadaway, and F.A. McFeters. 1999. Sensitive detection of *Escherichia coli* O157:H7 in food and water by immunomagnetic separation and solid-phase laser cytometry. *Appl. Environ. Microbiol*. 65(5):1966-1972.

Qi, Y., G. Patra, X. Liang, L.E. Williams, S. Rose, R.J. Redkar, and V.G. DelVecchio. 2001. Utilization of the *rpoB* gene as a specific chromosomal marker for real-time PCR detection of *Bacillus anthracis*. *Appl. Environ. Microbiol*. 67(8):3720-3727.

Rajkowski, K.T. and B.S. Marmer. 1995. Growth of *Escherichia coli* O157:H7 at fluctuating incubation temperatures. *J. Food Prot.* 58:1307-1313.

Rangel, J.M., P.H. Sparling, C. Crowe, P.M. Griffin, and D.L. Swerdlow. 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. *Emerg. Infect. Dis*. 11(4):603-609.

Read, S.C., R.C. Clarke, A. Martin, S.A. De Grandis, J. Hii, S. McEwen, and C.L. Gyles. 1992. Polymerase chain reaction for detection of verocytotoxigenic *Escherichia coli* isolated from animal and food sources. *Mol. Cell. Probes*. 6:153-161.

Restaino, L., H.J. Castillo, D. Stewart, and M.L. Tortorello. 1996. Antibody-direct epifluorescent filter technique and immunomagnetic separation for 10-h screening and 24-h confirmation of *Escherichia coli* O157:H7 in beef. *J. Food Prot*. 59(10):1072-1075.

Rossen, L., P. Nørskov, K. Holmstrøm, and O.F. Rasmussen. 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *Int. J. Food Microbiol*. 17:37-45.

Roux, K.H. 1994. Using mismatched primer-template pairs in touchdown PCR. *Biotechniques*. 16(5):812-814.

Saad, S.M.I., C. Vanzin, M.N. Oliveira, and F.D.G.M. Franco. 2001. Influence of lactic acid bacteria on survival of *Escherichia coli* O157:H7 in inoculated minas cheese during storage at 8.5°C. *J. Food Prot*. 64(8):1151-1155.

Scheu, P.M., K. Berghof, and U. Stahl. 1998. Detection of pathogenic and spoilage micro-organisms in food with the polymerase chain reaction. *Food Microbiol*. 15:13-31.

Scotland, S.M., G.A., Willshaw, H.R. Smith, and B. Rowe. 1987. Properties of strains of *Escherichia coli* belonging to serogroup O157 with special reference to production of Vero cytotoxin VT1 and VT2. *Epidemiol. Infect.* 99:613-624.

Seo, K.H., R.E. Brackett, and J.F. Frank. 1998. Rapid detection of *Escherichia coli* O157:H7 using immunomagnetic flow cytometry in ground beef, apple juice, and milk. *Int. J. Food Microbiol*. 44:115-123.

Shearer, A.E.H., C.M. Strapp, and R.D. Joerger. 2001. Evaluation of a polymerase chain reaction-based system for detection of *Salmonella* Enteritidis, *Escherichia coli* O157:H7, *Listeria* spp., and *Listeria monocytogenes* on fresh fruits and vegetables. *J. Food Prot*. 64(6):788-795.

Sheridan, G., C. Masters, J. Shallcross, and B. Mackey. 1998. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. *Appl. Environ. Microbiol*. 64:1313-1318.

Sheridan, G.E.C., E.A. Szabo, and B.M. Mackey. 1999. Effect of post-treatment holding conditions on detection of *tufA* mRNA in ethanol-treated *Escherichia coli*: implications for RT-PCR-based indirect viability tests. *Lett. Appl. Microbiol*. 29:375-379.

Sherman, P., R. Soni, M. Petric, and M. Karmali. 1987. Surface properties of verocytotoxin-producing *Escherichia coli* O157:H7. *Infect. Immun*. 55:1824-1829.

Silk, T.M., and C.W. Donnelly. 1997. Increased detection of acid-injured *Escherichia coli* O157:H7 in autoclaved apple cider by using nonselective repair on trypticase soy agar. *J. Food Prot*. 60(12):1483-1486.

Simpkins, S.A., A.B. Chan, J. Hays, B. Popping, and N. Cook. 2000. An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*. *Lett. Appl. Microbiol*. 30:75-79.

Smith, D., M. Blackford, S. Younts, R. Moxley, J. Gray, L. Hungerford, T. Milton, and T. Klopfenstein. 2001. Ecological relationships between the prevalence of cattle shedding *Escherichia coli* O157:H7 and characteristics of the cattle or conditions of the feedlot pen. *J. Food Prot*. 64(12):1899-1903.

Szabo, R.A., E.C.D. Todd, and A. Jean. 1986. Method to isolate *Escherichia coli* O157:H7 from food. *J. Food Prot*. 49(10):768-772.

Szabo, E.A., and B.M. Mackey. 1999. Detection of *Salmonella enteritidis* by reverse transcription-polymerase chain reaction (PCR). *Int. J. Food Microbiol*. 51:113-122.

Tamplin, M.L. 2002. Growth of *Escherichia coli* O157:H7 in raw ground beef stored at 10°C and the influence of competitive bacterial flora, strain variation, and fat level. *J. Food Prot*. 65(10):1535-1540.

Taylor, M.J., M.S. Hughes, R.A. Skuce, and S.D. Neill. 2001. Detection of *Mycobacterium bovis* in bovine clinical specimens using real-time fluorescence and fluorescence resonance energy transfer probe rapid-cycle PCR. *J. Clin. Microbiol*. 39(4):1272-1278.

Tesh, V.L., and A.D. O'Brien. 1991. The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins. *Mol. Microbiol*. 5(8):1817-1822.

Todd, W.T.A., and S. Dundas. 2001. The management of VTEC O157 infection. *Int. J. Food Microbiol*. 66:103-110.

Tortorello, M.L., K.F. Reineke, D.S. Stewart, and R.B. Raybourne. 1998. Comparison of methods for determining the presence of *Escherichia coli* O157:H7 in apple juice. *J. Food Prot*. 61(11):1425-1430.

Tsai, Y.W., and S.C. Ingham. 1997. Survival of *Escherichia coli* O157:H7 and *Salmonella* spp. in acidic condiments. *J. Food Prot.* 60(7):751-755.

Tyagi, S., and F.R. Kramer. 1996. Molecular Beacons: Probes that fluoresce upon hybridization. *Nature Biotech*. 14:303-308.

Uyttendaele, M., R. Schukkink, B. van Gemen, and J. Debevere. 1995. Detection of *Campylobacter jejuni* added to foods by using a combined selective enrichment and nucleic acid sequence-based amplification (NASBA). 1995. *Appl. Environ. Microbiol*. 61(4):1341-1347.

Uyttendaele, M., A. Bastiaansen, and J. Debevere. 1997. Evaluation of the NASBA nucleic acid amplification system for assessment of the viability of *Campylobacter jejuni*. *Int. J. Food Microbiol*. 37:13-20.

Uyttendaele, M., S. Vankeirsbilck, and J. Debevere. 2001. Recovery of heat-stressed *E. coli* O157:H7 from ground beef and survival of *E. coli* O157:H7 in refrigerated and frozen ground beef and in fermented sausage kept at 7 degrees C and 22 degrees C. *Food Microbiol*. 18(5):511-519.

Venkateswaran, K., Y. Kamijoh, E. Ohashi, and H. Nakanishi. 1997. A simple filtration technique to detect enterohemorrhagic *Escherichia coli* O157:H7 and its toxins in beef by multiplex PCR. *Appl. Environ. Microbiol*. 63(10):4127-4131.

Verstrepen, W.A., S. Kuhn, M.M. Kockx, M.E. Van De Vyvere, and A.H. Mertens. 2001. Rapid detection of enterovirus RNA in cerebrospinal fluid specimens with a novel single-tube real-time reverse transcription-PCR assay. *J. Clin. Microbiol*. 39(11):4093-4096.

Voisset, C., B. Mandrand, and G. Paranhos-Baccalà. 2000. RNA amplification technique, NASBA, also amplifies homologous plasmid DNA in non-denaturing conditions. *Biotechniques.* 29(2):236-240.

Wadolkowski, E.A., L.M. Sung, J.A. Buris, J.E. Samuel and A.D. O'Brien. 1990. Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin type II. *Infect. Immun*. 58:3959-3965.

Watanabe, Y., K. Ozasa, J.H. Mermin, P.M. Griffin, K. Masuda, S. Imashuku, and T. Sawada. 1999. Factory outbreak of *Escherichia coli* O157:H7 infection in Japan. *Emerg. Infect. Dis*. 5(3):424-428.

Weagant, S., J. Bryant, and K. Jinneman. 1994. An improved rapid technique for isolation of *Escherichia coli* O157:H7 from foods. *J. Food Prot*. 58:7-12.

Weimer, B.C., M.K. Walsh, C. Beer, R. Koka, and X. Wang. 2001. Solid-phase capture of proteins, spores, and bacteria. *Appl. Environ. Microbiol*. 67(3):1300-1307.

Weller, S.A., J.G. Elphinstone, N.C. Smith, N. Boonham, and D.E. Stead. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Appl. Environ. Microbiol*. 66(7):2853-2858.

Wells, J.G., L.D. Shipman, K.D. Greene, E.G. Sowers, J.H. Green, D.N. Cameron, F.P. Downes, M.L. Martin, P.M. Griffin, S.M. Ostroff, M.E. Potter, R.V. Tauxe, and I.K. Wachsmuth. 1991. Isolation of *Escherichia coli* serotype O157:H7 and other shiga-like-toxin-producing *E. coli* from dairy cattle. *J. Clin. Microbiol*. 29(5):985-989.

Weeratna, R.D., and M.P. Doyle. 1991. Detection and production of verotoxin 1 of *Escherichia coli* O157:H7 in food. *Appl. Environ. Microbiol*. 57(10):2951-2955.

Weusten, J.J.A.M., W.M. Carpay, T.A.M. Oosterlaken, M.C.A. van Zuijlen, and P.A. van de Wiel. 2002. Principles of quantitation of viral loads using nucleic acid sequence-based amplification in combination with homogeneous detection using molecular beacons. *Nucl. Acids Res*. 30(6):e26.

WHO. 1996. Enterohaemorrhagic *Escherichia coli* infection, Japan. WHO *Weekly Epidemiol. Rec.* 30:229-230.

Widjojoatmodjo, M.N., A. Borst, R.A.F. Schukkink, A.T.A. Box, N.M.M. Tacken, B. Van Gemen, J. Verhoef, B. Top, A.D.C. Fluit. 1999. Nucleic acid sequence-based amplification (NASBA) detection of medically important *Candida* species. *J. Microbiol. Meth*. 38:81-90.

Wieler, L.H., E. Vieler, C. Erpenstein, T. Schlapp, H. Steinrück, R. Bauerfeind, A. Byomi, and G. Balier. 1996. Shiga toxin-producing *Escherichia coli* strains from bovines: association of adhesion with carriage of *eae* and other genes. *J. Clin. Microbiol*. 34(12):2980-2984.

Wilson, I.G. 1997. Minireview: inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol*. 63:3741-3751.

Witham, P.K., C.T. Yamashiro, K.J. Livak, and C.A. Batt. 1996. A PCR-based assay for the detection of *Escherichia coli* shiga-like toxin genes in ground beef. *Appl. Environ. Microbiol*. 62(4):1347-1353.

Wright, D.J., P.A. Chapman and C.A. Siddons. 1994. Immunomagnetic separation as a sensitive method for isolating *Escherichia coli* O157 from food samples. *Epidemiol. Infect*. 113:31-39.

Yates, S., M. Penning, J. Goudsmit, I. Frantzen, B. van de Weijer, D. van Strijp, and B. van Gemen. 2001. Quantitative detection of Hepatitis B virus DNA by real-time nucleic acid sequence-based amplification with molecular beacon detection. *J. Clin. Microbiol.* 39(10):3656-3665.

Yilmaz, A., H. Gun, and H. Yilmaz. 2002. Frequency of *Escherichia coli* O157:H7 in Turkish cattle. *J. Food Prot*. 65(10):1637-1640.

Zhang, P., T. Beck, and W. Tan. 2001. Design of a molecular beacon DNA probe with two fluorophores. *Angew. Chem. Int*. 40(2):402-405.

Zilberstein, D., V. Agmon, S. Schuldiner, and E. Padan. 1984. *Escherichia coli* intracellular pH, membrane potential, and cell growth. *J. Bacteriol*. 158:246-252.

CHAPTER 2

UPSTREAM SAMPLE PROCESSING FACILITATES PCR DETECTION OF *LISTERIA MONOCYTOGENES* IN MAYONNAISE-BASED READY-TO-EAT (RTE) SALADS

Jamie Isonhood, MaryAnne Drake*, and Lee-Ann Jaykus

Department of Food Science, North Carolina State University, Raleigh, North Carolina, USA

Keywords: PCR, bacterial concentration, pathogen detection, *Listeria*, deli salad

Running Title: Detection of *Listeria monoctyogenes* in deli salads by PCR

*Author for correspondence.

Tel: (919)513-4598

Fax: (919)513-0014

E-mail: mdrake@ncsu.edu

(Accepted for publication in Food Microbiology)

2.1. ABSTRACT

Sample pretreatment to reduce volume and concentrate target organism(s) prior to molecular detection offers a useful supplement or alternative to cultural enrichment. The purpose of this study was to develop an upstream processing method to facilitate the detection of *Listeria monocytogenes* in ready-to-eat (RTE) salads by PCR. Potato salad, a model RTE commodity, was seeded with *L. monocytogenes* and processed by two alternative upstream sample processing methods (designated one-step and two-step centrifugation), followed by DNA extraction, PCR amplification, and Southern hybridization. The two-step method resulted in 1000-fold improvements in the lower limit of PCR detection, from 10^6 CFU/g (no sample processing) to 10^3 CFU/g. The two-step method was applied for upstream sample processing of four representative deli salad items artificially inoculated with *L. monocytogenes* at levels ranging from 10^1-10^6 CFU/g. Following DNA extraction, PCR amplification, and Southern hybridization, detection was achieved at input levels of 10^5 CFU/g for chicken salad, 10^4 CFU/g for macaroni salad, and 10^3 CFU/g for potato and seafood salads. The two-step method reported here facilitates the production of a final sample concentrate of reduced volume and improved purity which was compatible with PCR amplification. This approach offers further progress in our efforts to reduce or eliminate cultural enrichment in an effort to speed time to results when applying molecular methods to the detection of pathogens in foods.

2.2. INTRODUCTION

The psychrotrophic nature of the pathogen *Listeria monocytogenes* allows it to proliferate in foods and persist in the processing plant environment, making it a difficult pathogen to control (Levin 2003). Because *L. monocytogenes* is an opportunistic pathogen, often causing illness in our most susceptible populations, the United States has implemented a zero tolerance policy for the organism in ready to eat (RTE) foods such as luncheon meats, salad kits, and deli salads (Levin 2003). Several Class I recalls by the U.S. Food and Drug Administration have been made on mayonnaise-based salads (including vegetable, meat, seafood, and pasta salads) contaminated with *L. monocytogenes* (Bornemeier et al. 2003). Unfortunately, detection of this pathogen in contaminated foods often requires 5 to 7 days to complete (Hsih and Tsen 2001, Somer and Kashi 2003). Rapid methods, particularly the polymerase chain reaction (PCR), have shown promise in reducing time to detection, but sample size restrictions and amplification inhibitors can significantly reduce the sensitivity of the PCR (Fluit et al. 1993, Bickley et al. 1996, Scheu et al. 1998, deBoer and Beumer 1999, McKillip et al. 2002, Levin 2003).

To overcome such problems, a variety of biological, physical, and physiochemical methods have been employed to separate and concentrate the target organism(s) from the food matrix prior to the application of detection strategies. Physical methods of separation and concentration include filtration and centrifugation, respectively. Of particular interest has been differential centrifugation, a method based on the principle that particles of different sizes and densities sediment at different rates (Stevens and Jaykus 2004a). In very early studies, Niederhauser et al. (1992) applied differential centrifugation to concentrate and purify *L. monocytogenes* from contaminated raw and cooked meat products after prior

cultural enrichment. Their protocol resulted in a 5-fold sample volume reduction and 1000-fold improvement in PCR detection limits, with final detection limits of 10^3 CFU *L. monocytogenes*/ml of cultural enrichment. Later studies have confirmed the benefits of centrifugation as an upstream sample processing step to prepare the food matrix for pathogen detection by PCR, resulting in improved detection limits (Bansal et al. 1996, Kaclikova et al. 2001, Cui et al. 2003, Somer and Kashi 2003, Taylor et al. 2005).

In the present study, we present data on an upstream sample processing method, applied to various deli salads, which results in sample volume reduction and removal of PCR inhibitors such that direct (without prior cultural enrichment) detection of *L. monocytogenes* is possible. The method is based on simple filtration and centrifugation steps. Two methods (single and two-step centrifugation methods) were compared for concentrating bacterial cells from potato salad, and the optimized two-step method was subsequently tested on other mayonnaise-based deli salads.

2.3. MATERIALS AND METHODS

2.3.1. Bacteria and Cultural Recovery Methods. *L. monocytogenes* 4B1 (parent strain: NCTC 10527), which is streptomycin resistant, was obtained from Dr. Sophia Kathariou, Department of Food Science, North Carolina State University. The culture was transferred daily to brain heart infusion (BHI; Teknova, Hollister, CA) broth and incubated at 37°C. Plating for recovery was done on trypticase soy agar (Hardy Diagnostics, Santa Maria, CA) with yeast extract (BBL, Sparks, MD) containing 0.025 mg/mL streptomycin sulfate (Sigma, St Louis, MO) (TSA-YE-S) using the pour plate method.

2.3.2. Composition of Foods. Four deli salad items (potato, macaroni, chicken, and seafood salads) were purchased from local commercial sources. The composition of each salad matrix follows. Potato salad consisted of potatoes, condiments (mayonnaise, mustard), corn syrup, modified food starch, vinegar, vegetables (celery, onion, pickles, red bell pepper), spices, salt, preservatives (potassium sorbate, sodium benzoate), xanthan gum, and annatto. The ingredients of macaroni salad included cooked elbow macaroni, condiments (mayonnaise, mustard), eggs, sugar, high fructose corn syrup, vinegar, vegetables (carrots, green peppers, onion), salt, preservatives (potassium sorbate), and annatto. Chicken salad contained mechanically deboned chicken along with salad dressing, textured soy flour, vegetables (celery, pickle relish), salt and spices. Seafood salad was produced with imitation crabmeat to which was added shrimp, condiments (mayonnaise), sugar, high fructose corn syrup, vegetables (celery), salt, garlic powder, and preservatives (sodium benzoate and potassium sorbate).

2.3.3. Deli Salad Growth Study. A 99 g sample of deli salad (stored at 5°C) was placed into a sterile filter stomacher bag (Nasco, Whirl-Pak filter bags, Fort Atkinson, WI) and inoculated with an overnight culture (18 h) of *L. monocytogenes* to a final concentration of $1x10^6$ CFU/g. Each sample was hand massaged for 2 minutes to evenly distribute bacteria. Samples were subsequently held at refrigeration temperature (5°C) or room temperature (25°C). For refrigeration temperature, samples were removed on days 0, 3, 7, 10, and 14, while samples were taken at hour 0, 8, 24, and 48 for salads held at 25°C. At the timepoint of sampling, an eleven gram sample was removed and aseptically transferred to a sterile filter stomacher bag. Sterile 0.1% peptone water (99 ml) was added followed by pummeling the sample in a stomacher (Seward, Stomacher 400) at 230 rpm for 2 minutes. The pour plate

method was performed on all samples using TSA-YE supplemented with 0.025 mg/mL streptomycin. All experiments were performed in triplicate.

2.3.4. Upstream Sample Concentration and Purification Methods. Sterile filter stomacher bags (Nasco, Fort Atkinson, WI) were filled with 11.0 g of the salad item and 99 ml of sterile saline (0.9% NaCl). An overnight culture (18 h) of *L. monocytogenes* was then serially diluted and inoculated into the bag to achieve the desired final inoculum level (ranging from 10^6CFU/g-10^1CFU/g). The spiked salad sample was pummeled in a Stomacher 400 (Seward, U.K.) at 230 rpm for 2 min, after which 80 ml of the filtrate (representing about 75% of the total sample volume) was removed for further processing. At this time, one ml of the sample was also removed from the stomacher bag and pour-plated on TSA-YE-S to confirm initial inoculum level.

In early studies, two different protocols, a single step centrifugation and a two-step centrifugation method, were evaluated to concentrate bacterial cells prior to DNA amplification, using potato salad as a model RTE food (Figure 2.1). The single step method involved direct concentration of bacterial cells by centrifuging the 80 ml potato salad filtrate at 11,950 x g for 10 min at 5°C (Figure 2.1). The two-step method incorporated one centrifugation step (119 x g for 15 min at 5°C) to remove large food particulates and a second centrifugation step (11,950 x g for 10 min at 5°C) to concentrate the bacterial cells in the supernatant that was recovered after the first centrifugation (Figure 2.1).

Cells recovered in the precipitate and supernatant of each centrifugation were enumerated by pour plating on TSA-YE-S. Percent recovery (or loss) was calculated as previously described (Lucore et al. 2000, Stevens and Jaykus 2004b). Specifically, [percent recovery = (total population in recovered pellet or recovered supernatant after centrifugation * 100)/

(total population in sample before centrifugation)]; [percent loss = (total population in discarded supernatant or discarded pellet after centrifugation * 100) / (total population in sample before centrifugation)].

2.3.5. Isolation of Nucleic Acids. DNA extraction was done on resuspended cell pellets obtained after each sample preparation technique using a phenol-free guanidine detergent lysing solution (DNAzol BD, Invitrogen Life Technologies, Carlsbad, CA). The approximate pellet size for the single step method was 2.5 g; that for the two-step method was 0.5 g. DNA extraction was carried out according to manufacturer instructions. DNAzol BD was added to the pellet at a 2:1 (w/v, pellet:DNAzol) ratio and the final DNA pellet obtained after extraction was resuspended in 1.4 ml of water for the single step method, and in 200 µl of water for the two-step method. The resuspension volumes used were necessitated by residual matrix-associated materials that remained after the one and two-step upstream processing methods. The resuspended DNA was serially diluted and subjected to dilution series PCR amplification.

2.3.6. PCR Amplifications. For PCR, primers targeting a unique region of the 16S rDNA were utilized, creating a 287 bp product. Each reaction included 20 pmol of forward primer (5'-GGC TAA TAC CGA ATG ATg AA-3') and reverse primer (5'-AAG CAG TTA CTC TTA TCC T-3') (Somer and Kashi 2003). The PCR reaction components (50 µl reaction volume) included 43 µl supermix (Taq polymerase, dNTPs, $MgCl_2$, and rxn buffer) (Promega, Madison, WI), 20 pmol (2 µl) of each primer, and 5 U of Taq polymerase (Promega, Madison, Wisconsin).

All reactions were done in a thermal cycler (MJ Research PT-200 Peltier Thermal Cycler, Watertown, MA) using the following program: 95°C for 3 min; 40 cycles of 95°C for

30 s, 55°C for 1 min, 72°C for 40 s; and 72°C for 7 min. The PCR products were visualized using gel electrophoresis and confirmed by chemiluminescent Southern blot hybridization. When DNA was extracted from pure cultures of *L. monocytogenes*, consistent PCR detection was possible at 10^2 CFU/ml (equivalent to 10^0 CFU/amplification reaction), with sporadic detection possible at 10^1 CFU/ml (data not shown).

2.3.7. Southern Blot Hybridization. The alkaline transfer method was used for Southern blotting (Sambrook and Russell 2001). Biotinylated probes were created with the Random Primer Biotin Labeling kit with Streptavidin-AP (Perkin Elmer Life and Analytical Sciences, Boston, MA) according to manufacturer's instructions and following the method of Taylor et al. (2005). Briefly, PCR product generated from DNA extracted from a pure culture of *L. monocytogenes* 4B1 was purified using the Mo Bio UltraClean DNA purification system (Mo Bio Laboratories, Inc. Solana Beach, CA) in accordance with manufacturer instruction. One-half of the of the resuspended DNA (10 µl) was diluted in 9 µl of water, heated at 95°C for 3 min, rapidly chilled on ice, and then mixed with random primers, biotinylated nucleotides, and Klenow enzyme. After incubation at 37°C for one hour, probe was diluted 10-fold with water and stored at -20°C until use. DNA hybridization as recommended by the Random Primer Biotin Labeling kit using a probe concentration of 25 ng/ml. Hybridization was carried out at 65°C overnight. The membrane was washed in series at 65°C, soaked in streptavidin-alkaline phosphatase conjugate buffer for one h at 27°C, washed in series, and subsequent chemiluminescent detection was done using CDP-Star reagent with visualization on Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

2.3.8. Statistical Analysis. All experiments were done in triplicate. Statistical analysis of percent recovery and percent loss was done using SAS (version 8.2, Cary, NC) as evaluated

by analysis of variance (PROC GLM) with least-squares means in a completely randomized design ($p<0.05$).

2.4. RESULTS

2.4.1. Preliminary Experiments. Aerobic plate counts of indigenous microflora ranged from 10^1-10^2 CFU/g for potato salad and chicken salad, and from 10^3-10^4 CFU/g for macaroni and seafood salads, as evaluated on TSA-YE. The growth of indigenous microflora was effectively controlled when supplementing TSA-YE with 0.025 mg/ml streptomycin, without impacting the recovery of added *L. monocytogenes* (data not shown). There was no substantial growth or inactivation of *L. monocytogenes* in any of the deli salad items tested over the one hour sample preparation period which preceded DNA extraction (data not shown). *L. monocytogenes* survival/growth in mayonnaise-based deli salads at refrigeration or room temperature are listed in Figures 2.2 and 2.3. In this study, pH was a good indicator of bacterial survival since the salad with the lowest pH (pH = 4.73, macaroni salad) resulted in rapid decline of *L. monocytogenes*, while in the salad with the highest pH (pH = 5.98, seafood salad), *L. monocytogenes* remained stable at 5°C and increased at 25°C. *L. monocytogenes* decreased one log in potato (pH = 4.91) and chicken (pH = 4.77) salad over the two week period at 5°C and remained stable at 25°C.

2.4.2. Comparison of the Two Upstream Sample Preparation Methods. PCR detection was not possible when DNA was directly extracted from 1 g of potato, macaroni, chicken, or seafood salad artificially inoculated with *L. monocytogenes* at a level of 10^6 CFU/g. When attempting to detect the DNA extracted from 1:10 homogenates without prior upstream

sample processing, we were able to achieve detection only at inoculum levels of $\geq 10^5$ CFU/g (macaroni and seafood salads) to $\geq 10^6$ CFU/g (potato and chicken salads) (data not shown).

In early experiments, the single and two-step methods were compared with respect to bacterial recovery and PCR detection limits using potato salad as the model RTE commodity. Potato salad was seeded with *L. monocytogenes* at a level of 10^7 CFU/g and processed by each of the two methods. For the one-step method, recoveries ranged from 83-93% based on direct plating the final pellet. The combined recovery after the two-step method ranged from 52-81%. Although less of the input *L. monocytogenes* appeared to be recovered using the two-step method as compared to the single-step method, there were no statistically significant differences in recoveries between the two methods when calculations were based on direct plating of the pellet obtained after centrifugation. However, the percent loss to discarded supernatant was much less (9%) for the one-step as opposed to the two-step (30%) method (Table 2).

The two-step method had the added advantage of reducing the sample volume by almost 10-fold (from 11g to 1g) as compared to only a 5-fold (from 11g to 2.5g) sample size reduction with the one-step method. Furthermore, the final DNA extract volume reductions were 400-fold (from 80 ml to 0.2 ml) for the two-step method but only 40-fold (from 80 ml to 2.0 ml) for the single step centrifugation method. When potato salad samples were seeded with *L. monocytogenes* at levels ranging from 10^1 to 10^6 CFU/g, processed by either the single or two-step centrifugation methods, and subjected to PCR amplification and Southern hybridization, detection on using the one-step method was approximately 1000-fold less sensitive than that for the two-step method (Figure 2.4). Specifically, the lowest detection limit for the one-step method was 10^6 CFU/g, while we were able to achieve a lower limit of

detection corresponding to 10^3 CFU/g when preceding PCR with the two-step upstream sample processing method. Because the two-step method resulted in smaller volumes with enhanced PCR detection limits, this upstream sample processing method was selected for application to a panel of deli salads.

2.4.3. Application of the Optimized Method to Four Salad Items. The four salad items (potato, macaroni, chicken, and seafood salads) were seeded with 10-fold serial dilutions of *L. monocytogenes* to reach inoculum levels ranging from 10^1 to 10^6 CFU/g, followed by upstream sample processing using the two-step method, with subsequent DNA extraction and PCR detection. Pellets were plated for recovery after centrifugation, as well for detection using PCR and Southern hybridization (Table 3). Mean recoveries based on direct plating of the final pellet were 68% (potato salad), 49% (seafood salad), 36% (chicken salad), and 34% (macaroni salad). The recoveries for the latter two items were statistically significantly lower (less efficient recovery) when compared to the potato salad.

PCR amplification was done on the undiluted DNA and on serial dilutions of the DNA for each product at each *L. monocytogenes* seeding level. With the exception of chicken salad, detection was possible on the undiluted DNA extract, suggesting that the upstream sample processing method followed by DNA extraction effectively removed PCR inhibitors (data not shown). *L. monocytogenes* could be detected in potato salad when inoculated with *L. monocytogenes* at levels of 10^3 CFU/g, with consistent detection (3/3 samples positive by PCR) at initial inoculum levels of 10^4 CFU/g (Figure 2.5). Similarly, consistent detection of *L. monocytogenes* in artificially contaminated seafood salads was possible at 10^3 CFU/g. The assay was less sensitive when applied to macaroni and chicken salads, with consistent PCR detection occurring when these salad items were seeded with *L.*

monocytogenes at levels between 10^5 - 10^6 CFU/g, with lower limits of detection of 10^4 CFU/g and 10^5 CFU/g, respectively (Figure 2.5). In general, higher cell recoveries by cultural methods (potato=67%, seafood=49%) were associated with better PCR detection limits (potato, seafood 10^3CFU/g).

2.5. DISCUSSION

In this study, two upstream sample processing methods were compared for their ability to facilitate the detection of *L. monocytogenes* in a model RTE food product. Clearly, the two-step method out-performed the one-step method with respect to sample volume reduction, removal of PCR inhibitory compounds, and improvement of PCR detection limits. Specifically, prior to DNA extraction, the two-step centrifugation method provided a 10-fold sample volume reduction (as compared to five-fold for the one-step method); after DNA extraction, total sample volume reductions were as high as 400-fold. The two-step method also improved PCR detection limits by 1000-fold compared to the one-step method.

Many investigators have harnessed PCR for the rapid detection of foodborne pathogens. It is generally recognized that some form of upstream sample processing, be it concentration/purification of bacterial cells, DNA extraction, or both, is needed to overcome PCR inhibition and address sample size issues. In many instances, centrifugation is a common sample preparation method. For instance, Lindqvist et al. (1997) showed that centrifugation could help remove food-related inhibitors, since amplicons could not be obtained by direct PCR amplification of a cheese homogenate containing 2 x 10^5 CFU/ml *Shigella flexneri*, but centrifugation of the homogenate at 16,200 x g for 1 min prior to PCR facilitated detection. It is critical to note that centrifugation must be adjusted to balance

pathogen yield with best attainable quality of the resulting sample concentrate. This is not an easy feat and frequently requires optimization on a product-specific basis (McKillip et al. 2000).

Few investigators have sought to replace cultural enrichment with upstream sample processing (bacterial concentration) in an effort to eliminate or at least reduce the length of cultural enrichment. Taylor et al. (2005) used a two-step centrifugation approach to pre-concentrate *E. coli* O157:H7 from ground beef, reporting detection limits (10^3 CFU/g) similar to the ones reported here. In a method similar to our approach, Cui et al. (2003) evaluated four different centrifugation speeds (50, 100, 200, and 400 x g) to optimize recovery of *E. coli* O157:H7 from ground beef, finding significantly better retention of the bacteria in the supernatant at the lower g-forces, but better removal of matrix-associated particulates at the higher centrifugation speeds. Using their two-step upstream sample processing method followed by DNA extraction and PCR detection, these investigators were able to produce a 0.5-1.0 g pellet from an initial 25 g ground beef sample with recovery of 75% of the initial inoculum, achieving consistent PCR detection at levels of 10^3 CFU/g *E. coli* O157:H7 without prior enrichment. Cui et al. (2003), like others (Uyttendaele et al.1998, Ferretti et al. 2001, McIngvale et al. 2002, Panicker et al. 2004, Wang et al. 2004) advocated that by preceding detection with a 4-6 hour cultural enrichment, PCR detection limits corresponding to 1 CFU/g could be achieved. For example, Moon et al. (2004) utilized centrifugation and heat treatment following an abbreviated enrichment to detect 1 CFU of *E. coli* O157:H7 or *L. monocytogenes* in spiked milk, chicken, ham, and pork. Ellingson et al. (2004) centrifuged a 15 ml subsample of a 6 hour enrichment of raw and RTE meat products to detect 1 CFU/ml *Salmonella* by real-time PCR within 12 hours. Indeed, it may be that

"flash" enrichment will become the next feasible approach to further improve detection limits and reduce testing time. This approach has the added advantage of confirming viability of the detected cells.

Only a few investigators have sought to use PCR to detect *L. monocytogenes* from RTE salads items (Somer and Kashi 2003, Dickinson et al., 1995, Rossen et al. 1992, Levin 2003). Early on, Rossen et al. (1992) reported that the undiluted chicken salad matrix was particularly inhibitory to PCR, and these investigators circumvented the problem by incorporating enrichment steps. Dickinson et al. (1995) were able to concentrate *L. monocytogenes* from coleslaw by centrifuging a 1 ml subsample of homogenate diluted 1:10, achieving a PCR detection limit of 10^3 CFU/g. By shortening enrichment times to a total of 24 hours, Somer and Kashi (2003) were able to detect *L. monocytogenes* in dairy and meat products by multiplex PCR at detection limits as low as 1 to 5 CFU/25 g. When applied to various salad items, the method performed in a manner equivalent to culturally-based USDA-recommended procedures.

It is well recognized that matrix-associated inhibitory compounds can dramatically impact detection limits when applying PCR to the detection of foodborne pathogens. While some of these inhibitory components have been previously identified (Demeke and Adams 1992, Rossen et al. 1992, Wilson 1997), many remain either anecdotal or uncharacterized. Deli salads might be considered a worst-case scenario because these food items contain many ingredients. Indeed, in this study, PCR detection of *L. monocytogenes* was not consistently possible, even at high seeding levels (>10^5 CFU/g), without preceding amplification with both upstream sample processing and DNA extraction. In some cases, particularly for chicken salad, 10-fold serial dilution of the extracted DNA was necessary in order to

consistently obtain PCR amplicons, suggesting the presence of residual matrix-associated inhibitors even after concentration and DNA extraction. However, the upstream sample processing method did reduce sample volume while concentrating target bacteria, thereby removing many of the food-related inhibitors and consequently improving PCR detection limits.

From our study, we can conclude that the need to use a representative food sample size (25 g or larger) can be at least partially addressed by using upstream sample processing methods that result in concentration of the pathogen and removal of a portion of the matrix, followed by DNA extraction and PCR detection. In this case, simple filtration and centrifugation provided an easy and inexpensive approach to upstream sample processing. In accordance with previous studies with other foods (Cui et al. 2003, Taylor et al. 2005), our results suggest that *L. monocytogenes* detection limits from the RTE salad matrix can be improved if such sample processing strategies are used. Indeed, for this matrix, crude filtration in conjunction with the two-step centrifugation method allowed for the production of a final sample concentrate of reduced volume and improved purity which was compatible with PCR amplification. This simple method can be quickly adapted in most laboratories and offers another step in our efforts to reduce or eliminate cultural enrichment in an effort to speed time to results when applying molecular methods to the detection of pathogens that may contaminate foods.

2.6. ACKNOWLEDGEMENTS

This work was supported in part by USDA CSREES NRI, Ensuring Food Safety Division Grant #2002-35201-11670. This manuscript is paper number FSR 05-** in the Journal

Series of the Department of Food Science, North Carolina State University, Raleigh, NC. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service nor criticism of similar ones not mentioned.

2.7. REFERENCES

Bansal, N.S., McDonell, F.H.Y., Smith, A., Arnold, G. and Ibrahim, G.F. (1996) Multiplex PCR assay for the routine detection of *Listeria* in food. *Int. J. Food Microbiol*. 33, 293-300.

Bornemeier, V.L., Albrecht, J.A. and Sumner, S.S. (2003) Survey of mayonnaise-based salads for microbial safety and quality. *Food Prot. Trends*. 23(5), 387-392.

Cui, S., Schroeder, C.M., Zhang, D.Y. and Meng, J. (2003) Rapid sample preparation method for PCR-based detection of *Escherichia coli* O157:H7 in ground beef. *J. Appl. Microbiol.* 95, 129-134.

deBoer, E. and Beumer, R.R. (1999) Methodology for detection and typing of foodborne microorganisms. *Int. J. Food Microbiol*. 50, 119-130.

Demeke, T. and Adams, R.P. (1992) The effects of plant polysaccharides and buffer additives on PCR. *Biotechniques.* 12, 332-334.

Dickinson, J.H., Kroll, R.G. and Grant, K.A. (1995) The direct application of the polymerase chain reaction to DNA extracted from foods. *Lett. Appl. Microbiol.* 20, 212-216.

Ellingson, J.L.E., Anderson, J.L., Carlson, S.A. and Sharma, V.K. (2004) Twelve hour real-time PCR technique for the sensitive and specific detection of *Salmonella* in raw and ready-to-eat meat products. *Mol. Cell Probes.* 18(1), 51-57.

Ferretti, R., Mannazzu, I., Cocolin, L., Comi, G. and Clementi, F. (2001) Twelve-hour PCR-based method for detection of *Salmonella* spp. in food. *Appl. Environ. Microbiol.* 67(2), 977-978.

Fluit, A.C., Torensma, R., Visser, M.J.C., Aarsman, C.J.M., Poppelier, M.J.J.G., Keller, B.H.I., Klapwijk, P. and Verhoef, J. (1993) Detection of *Listeria monocytogenes* in cheese with the magnetic immuno-polymerase chain reaction assay. *Appl. Environ. Microbiol.* 59, 1289-1293.

Hsih, H.Y. and Tsen, H.Y. (2001) Combination of immunomagnetic separation and polymerase chain reaction for the simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. in food samples. *J. Food Prot.* 64(11), 1744-1750.

Kaclikova, E., Kuchta, T., Kay, H. and Gray D. (2001) Separation of *Listeria* from cheese and enrichment media using antibody-coated microbeads and centrifugation. *J. Microbiol. Meth*. 46, 63-67.

Leggitt, P.R. and Jaykus, L.A. (2000) Detection methods for human enteric viruses in representative foods. *J. Food Prot.* 63(12), 1738-1744.

Levin, R.E. (2003) Application of the polymerase chain reaction for detection of *Listeria monocytogenes* in foods: A review of methodology. *Food Biotech*. 17(2), 99-116.

Lindqvist, R., Norling, B. and Lambertz, S.T. (1997) A rapid sample preparation method for PCR detection of food pathogens based on buoyant density centrifugation. *Lett. Appl. Microbiol.* 24, 306-310.

Lucore, L.A., Cullison, M.A. and Jaykus, L.A. (2000) Immobilization with metal hydroxides as a means to concentrate food-borne bacteria for detection by cultural and molecular methods. *Appl. Environ. Microbiol*. 66(5), 1769-1776.

McIngvale, S.C., Elhanafi, D. and Drake, M.A. (2002) Optimization of reverse transcriptase PCR to detect viable shiga-toxin-producing *Escherichia coli*. *Appl. Environ. Microbiol.* 68(2), 799-806.

McKillip, J.L., Jaykus, L.A. and Drake, M.A. (2000) A comparison of methods for the detection of *Escherichia coli* O157:H7 from artificially-contaminated dairy products using PCR. *J. Appl. Microbiol.* 89, 49-55.

McKillip, J.L., Jaykus, L.A. and Drake, M.A. (2002) Influence of growth in a food medium on the detection of *Escherichia coli* O157:H7 by polymerase chain reaction. *J. Food Prot.* 65(11), 1775-1779.

Moon, G.S., Kim, W.J. and Shin, W.S. (2004) Optimization of rapid detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* by PCR and application to field test. *J. Food Prot.* 67(8), 1634-1640.

Niederhauser, C., Candrian, U., Hofelein, C., Jermini, M., Buhler, H.P. and Luthy, J. (1992) Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. *Appl. Environ. Microbiol.* 58(5), 1564-1568.

Panicker, G., Myers, M.L. and Bej, A.K. (2004) Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. *Appl. Environ. Microbiol*. 70(1), 498-507.

Rossen, L., Norskov, P., Holmstrom, K. and Rasmussen, O.F. (1992) Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *Intl. J. Food Microbiol*. 17, 37-45.

Sambrook, J. and Russell, D.W. (ed.). (2001) *Molecular Cloning:a Laboratory Manual*. 3rd Edt. Cold Spring Harbor: New York.

Scheu, P.M., Berghof, K. and Stahl, U. (1998) Detection of pathogenic and spoilage microorganisms in food with the polymerase chain reaction. *Food Microbiol.* 15, 13-31.

Somer, L. and Kashi, Y. (2003) A PCR method based on 16S rRNA sequence for simultaneous detection of the genus *Listeria* and the species *Listeria monocytogenes* in food products. *J. Food Prot.* 66(9), 1658-1665.

Stevens, K.A. and Jaykus, L.A. (2004a) Bacterial separation and concentration from complex sample matrices: A review. *Crit. Rev. Microbiol*. 30(1), 7-24.

Stevens, K.A. and Jaykus, L.A. (2004b) Direct detection of bacterial pathogens in representative dairy products using a combined bacterial concentration-PCR approach. *J. Appl. Microbiol*. 97(6), 1-8.

Taylor, M.T., Drake, M.A. and Jaykus, L.A. (2005) Development of an alternative method to concentrate *E. coli* O157:H7 from meat homogenates to facilitate detection by cultural and molecular methods. *J. Food Prot*. 68(2), 225-232.

Uyttendaele, M., Grangette, C., Rogerie, F., Pasteau, S., Debevere, J. and Lange, M. (1998) Influence of cold stress on the preliminary enrichment time needed for detection of Enterohemorrhagic *Escherichia coli* in ground beef by PCR. *Appl. Environ. Microbiol.* 64(5), 1640-1643.

Wang, X., Jothikumar, N. and Griffiths, M.W. (2004) Enrichment and DNA extraction protocols for the simultaneous detection of *Salmonella* and *Listeria monocytogenes* in raw sausage meat with multiplex real-time PCR. *J. Food Prot.* 67(1), 189-192.

Wilson, I.G. (1997) Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63(10), 3741-3751.

Figure Legends

Figure 2.1. Schematic of 1-step and 2-step centrifugation upstream sample processing methods, followed by subsequent DNA extraction.

Figure 2.2. Growth/Survival of *L. monocytogenes* in potato, macaroni, chicken, and seafood salads stored at 5°C over a 2 week period.

Figure 2.3. Growth/Survival of *L. monocytogenes* in potato, macaroni, chicken, and seafood salads stored at 27°C over a 48h period.

Figure 2.4. Comparison of PCR detection limits for potato salad artificially inoculated with *L. monocytogenes* and processed using the single-step centrifugation method (A) or the two-step centrifugation method (B) followed by DNA extraction, PCR amplification and subsequent gel electrophoresis (top) and Southern hybridization (bottom). Each band represents DNA extracted from 11 g potato salad samples inoculated with *L. monocytogenes* to achieve levels of contamination between 10^1 and 10^6 CFU/g. Each experiment at each seeding level was done in triplicate. Inoculation level and detection limits are given below each lane. Lanes: M (marker), + (positive control reaction for detection of *L. monocytogenes*), -- (water control), and U (uninoculated potato salad).

Figure 2.5. PCR detection of *L. monocytogenes* from artificially contaminated potato salad (A), macaroni salad (B), chicken salad (C), and seafood salad (D) processed by the two-step centrifugation method followed by DNA extraction, PCR amplification and subsequent gel electrophoresis (top) and Southern hybridization (bottom). Each band represents DNA extracted from 11 g of deli salad item inoculated with *L. monocytogenes* to achieve levels of contamination between 10^1 and 10^6 CFU/g. Each experiment at each seeding level was done in triplicate. Inoculation level and detection limits are given below each lane. Lanes: M (marker), + (positive control reaction for detection of *L. monocytogenes*), -- (water control), and U (uninoculated deli salad).

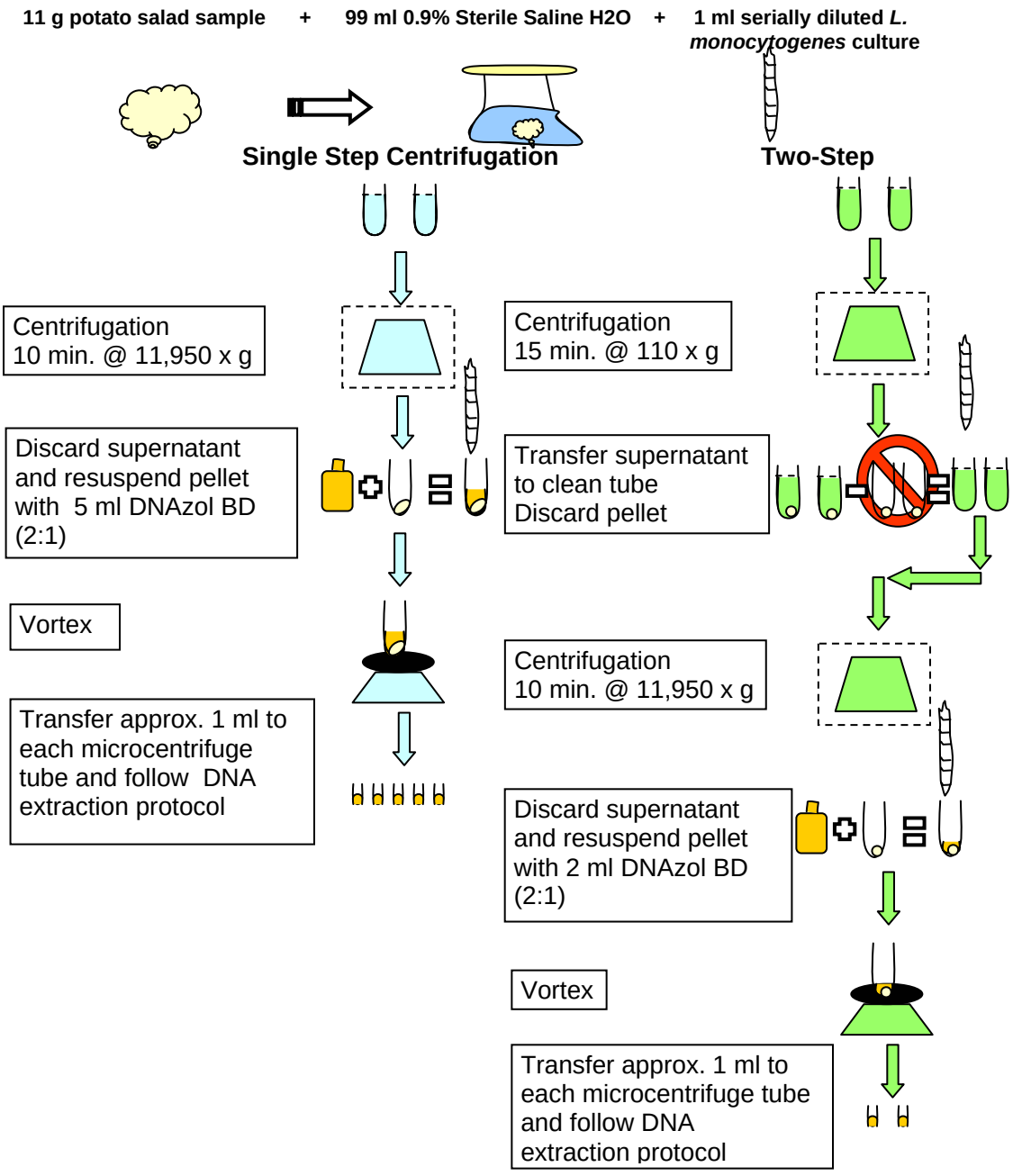


Figure 2.1. Schematic of 1-step and 2-step centrifugation upstream sample processing methods, followed by subsequent DNA extraction.

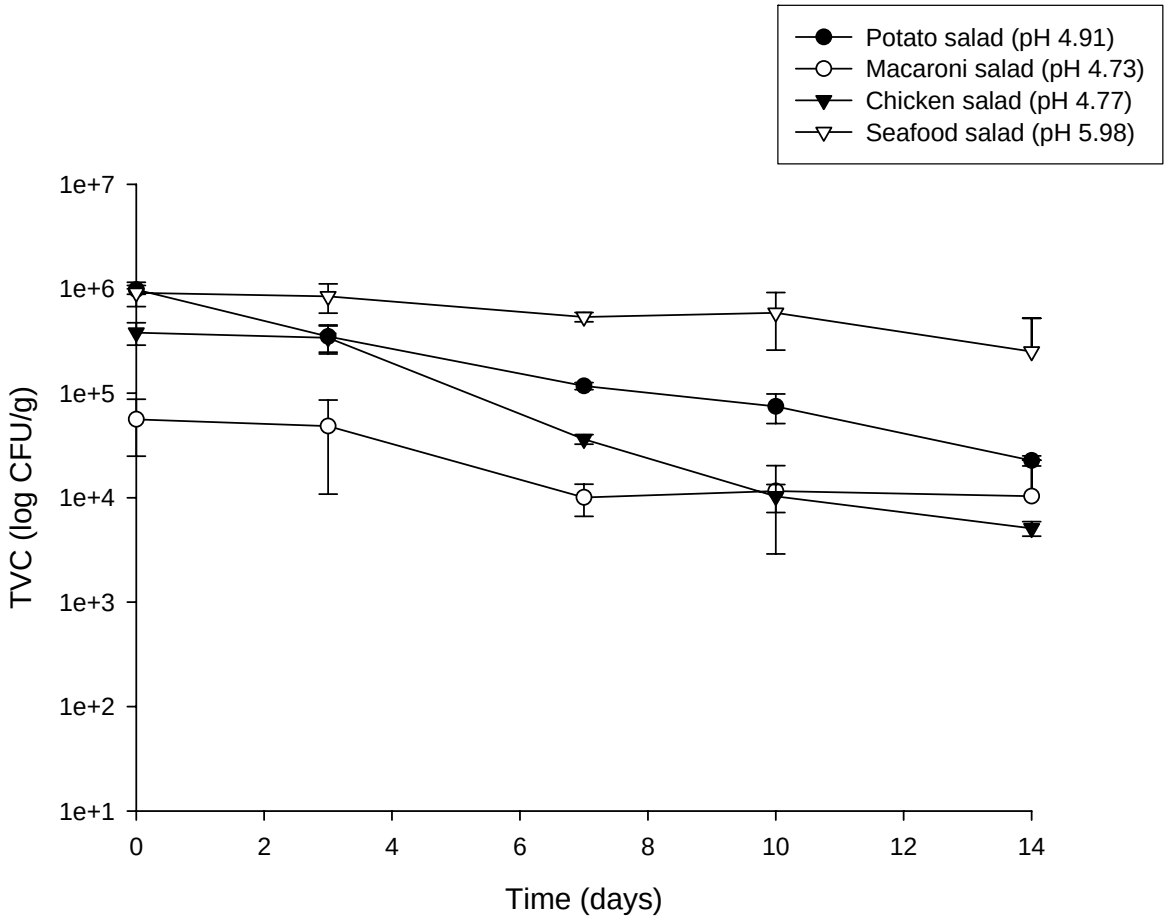


Figure 2.2. Growth/Survival of L. monocytogenes in potato, macaroni, chicken, and seafood salads stored at 5°C over a 2 week period.

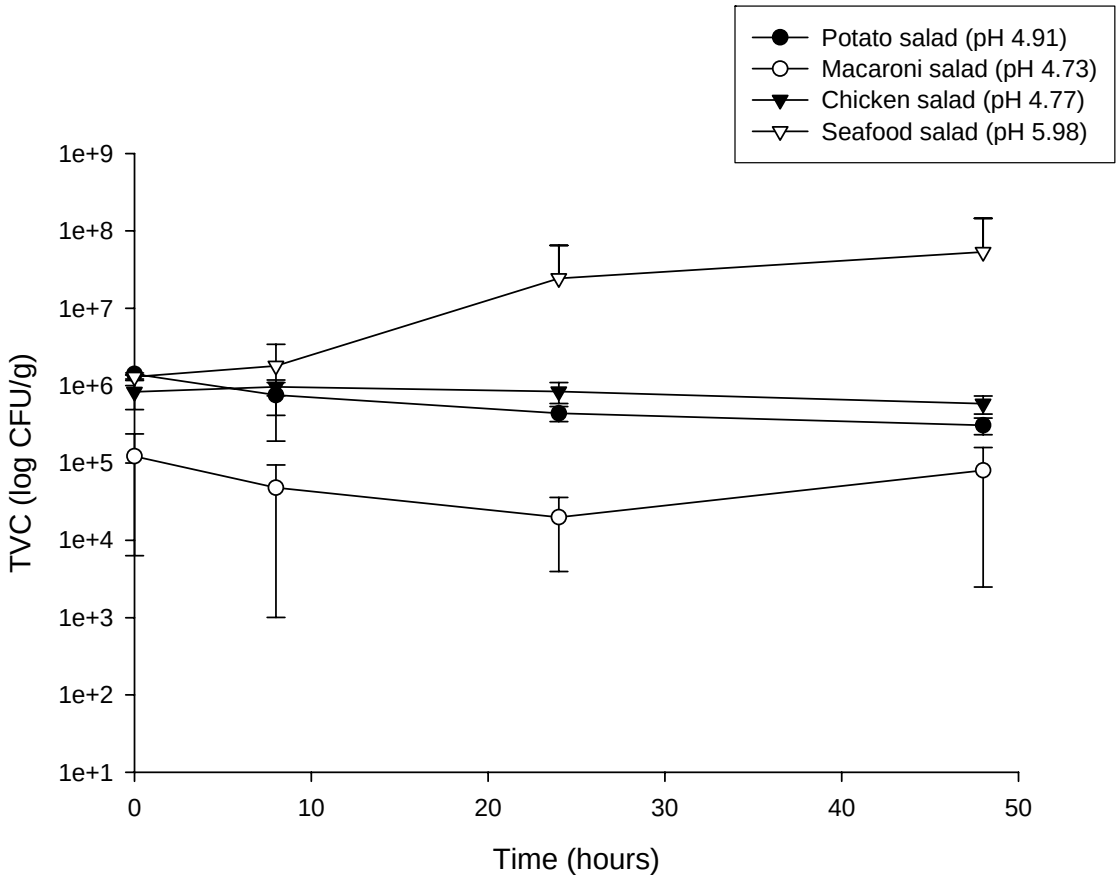


Figure 2.3. Growth/Survival of *L. monocytogenes* in potato, macaroni, chicken, and seafood salads stored at 27°C over a 48h period.

Table 2.1. Comparison of *L. monocytogenes* recoveries from artificially inoculated potato salad using two upstream sample processing methods.

	Step 1 – low speed centrifugation (15 min. @ 119 x g)		Step 2 – high speed centrifugation (10 min. @ 11,950 x g)		
	% Loss (Pellet) [a]	% Recovery (Supernatant) [b]	% Loss (Supernatant)	% Recovery (Pellet)	Cumulative % Recovery (Based on inoculum) [c]
Single Step	N/A	N/A	9.0 (1.4)	88.7 (5.0)	88.7 (5.0)
Two Step	22.0 (2.6)	83.3 (8.9)	9.7 (4.0)	81.0 (13.2)	68.0 (14.9)

Percent recovery/loss is mean (top) ± standard deviation (bottom, parenthetically) of three trials; initial inoculation done to reach level of 10^7CFU/g

[a]Percent loss was calculated as follows: [percent loss = (total population in discarded supernatant or discarded pellet after centrifugation * 100) / (total population in supernatant before centrifugation)]

[b]Percent recovery was calculated as follows: [percent recovery = (total population in recovered pellet or recovered supernatant after centrifugation * 100)/ (total population in sample before centrifugation)].

[c]Percent combined recovery was calculated as: [combined recovery = (% Step 1 * % recovery step 2)/100]

Table 2.2. Percent cell recoveries for *L. monocytogenes* from four different deli salads using the two-step upstream processing method.

	Step 1 – low speed centrifugation (15 min. @ 119 x g)		Step 2 – high speed centrifugation (10 min. @ 11,950 x g)		
Deli Salad	% Loss (Pellet)[a]	% Recovery (Supernatant)[b]	% Loss (Supernatant)	% Recovery (Pellet)	Cumulative % Recovery (Based on Inoculum)[c, d]
Potato Salad	22.0 (2.6)	83.3 (8.9)	9.7 (4.0)	81.0[a] (13.2)	68.0[ab] (14.9)
Macaroni Salad	24.9 (2.4)	38.7 (3.3)	6.2 (0.3)	88.8[a] (0.1)	34.7[d] (3.0)
Chicken Salad	24.9 (2.7)	40.2 (4.1)	5.3 (5.2)	88.7[a] (22.4)	36.3[cd] (13.2)
Seafood Salad	22.2 (3.9)	85.8 (5.1)	2.4 (0.3)	57.4[bc] (5.8)	49.0[bcd] (2.6)

Percent recovery/loss is mean (top) ± standard deviation (bottom, parenthetically) of three trials; initial inoculation done to reach level of 10^7CFU/g

[a]Percent loss was calculated as follows: [percent loss = (total population in discarded supernatant or discarded pellet after centrifugation * 100) / (total population in supernatant before centrifugation)]

[b]Percent recovery was calculated as follows: [percent recovery = (total population in recovered pellet or recovered supernatant after centrifugation * 100)/ (total population in sample before centrifugation)].

[c]Percent combined recovery was calculated as: [combined recovery = (% Step 1 * % recovery step 2)/100]

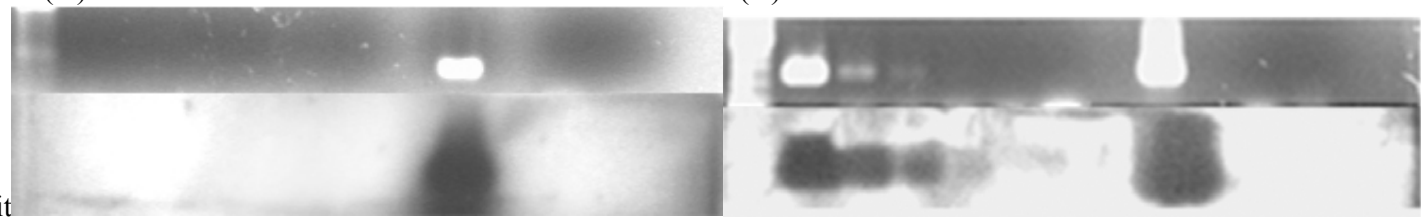
[d]Means in the final two columns not followed by the same letter represent a statistically significant difference

Single Step

Two-Step

(A)

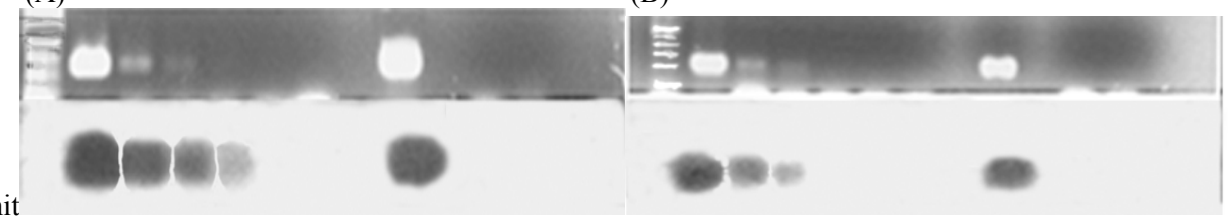
(B)



Detection limit CFU/g sample	M	10^6	10^5	10^4	10^3	10^2	10^1	+	--	U	M	10^6	10^5	10^4	10^3	10^2	10^1	+	--	U
Detection limit CFU/PCR rxn.	M	10^4	10^3	10^2	10^1	10^0	10^{-1}	+	--	U	M	10^4	10^3	10^2	10^1	10^0	10^{-1}	+	--	U
Hybridization – Confirmed detection	M	1/3	0/3	0/3	0/3	0/3	0/3				M	3/3	3/3	3/3	2/3	0/3	0/3			

Figure 2.4.

(A) (B)

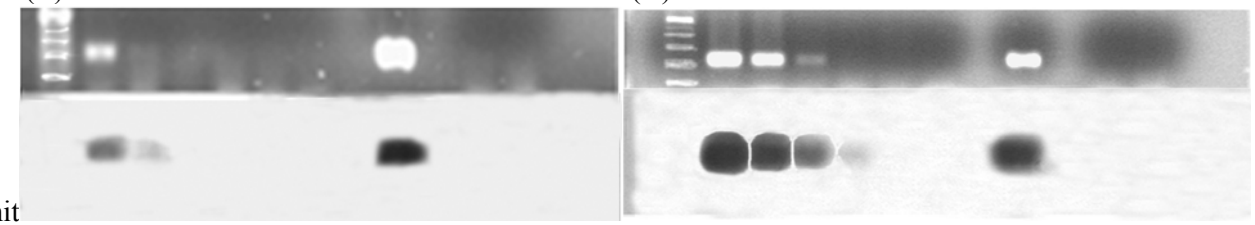


Detection Limit
CFU/g sample M 10^6 10^5 10^4 10^3 10^2 10^1 + -- U M 10^6 10^5 10^4 10^3 10^2 10^1 + -- U

Detection limit
CFU/PCR rxn. M 10^4 10^3 10^2 10^1 10^0 10^{-1} + -- U M 10^4 10^3 10^2 10^1 10^0 10^{-1} + -- U

Hybridization M 3/3 3/3 3/3 2/3 0/3 0/3 M 3/3 3/3 2/3 0/3 0/3 0/3
Confirmed detection

(C) (D)



Detection Limit
CFU/g sample M 10^6 10^5 10^4 10^3 10^2 10^1 + -- U M 10^6 10^5 10^4 10^3 10^2 10^1 + -- U

Detection limit
CFU/PCR rxn. M 10^4 10^3 10^2 10^1 10^0 10^{-1} + -- U M 10^4 10^3 10^2 10^1 10^0 10^{-1} + -- U

Hybridization M 3/3 1/3 0/3 0/3 0/3 0/3 M 3/3 3/3 3/3 3/3 0/3 0/3
Confirmed detection

Figure 2.5.

CHAPTER 3

DETECTION OF *E. COLI* O157:H7 AND *SALMONELLA TYPHIMURIUM* FROM FOOD USING A NOVEL IMMUNO-CAPTURE SYSTEM AND PCR

J.H. Isonhood, M.A. Drake*, and L.A. Jaykus

Department of Food Science, North Carolina State University, Raleigh, North Carolina, USA

Keywords: PCR, bacterial concentration, pathogen detection, immunomagnetic separation, *E. coli* O157:H7, *Salmonella typhimurium*

Running Title: Immuno-Capture-PCR Detection of Pathogens from food

*Author for correspondence.

Tel: (919)513-4598

Fax: (919)513-0014

E-mail: mdrake@ncsu.edu

(To be submitted to Food Microbiology)

3.1. ABSTRACT

Since pathogen numbers are often low in foods, it is imperative to screen a large and representative amount of food. The Pathatrix system is designed to recirculate a 250 ml sample volume (consisting of 25g of food and 225ml buffer) and capture the target pathogen using immunomagnetic beads, making the system ideal for pre-PCR processing. The efficacy of the system was tested on *S. typhimurium* in buffered peptone water (BPW), ground turkey and nonfat dry milk; and *E. coli* O157:H7 in BPW, ground beef and romaine lettuce spiked at 10^6 to 10^0 CFU/25g without prior enrichment. Recoveries, as evaluated by plating, were better for *E. coli* O157:H7 than for *S. typhimurium* ($p<0.05$), presumably due to the better avidity of the *E. coli* O157:H7 immuomagnetic beads. *E. coli* O157:H7 was reliably detected by PCR at all levels (10^6 to 10^0 CFU/25g) across all matrices tested following Pathatrix processing. Clarification of the sample matrix (for ground turkey) by centrifugation prior to Pathatrix processing improved *S. typhimurium* PCR detection from 10^5 (Pathatrix only) to 10^2 CFU/25g. The PCR detection limit for *S. typhimurium* from broth and nonfat dry milk was 10^1 and 10^4CFU/25g (Pathatrix only), respectively. Pathatrix offers a cost-effective method for processing large sample volumes and can facilitate the sensitive detection of *E. coli* O157:H7 in foods.

3.2. INTRODUCTION

The PCR has been a powerful tool to facilitate the rapid detection of pathogens. However, several factors common to clinical, environmental, and food samples can decrease the efficiency of the PCR reaction (Wilson, 1997; Radstrom et al., 2003). These factors include relatively large sample volumes compared to small PCR amplification volumes, sample components which inhibit the PCR reaction, low levels of contaminating pathogens, and the presence of competitive microflora that may interfere with the amplification reaction (Bej and Mahbubani, 1994). Current methodologies rely on a 24 hour enrichment step to overcome these problems and have proven successful, but alternatives are readily being sought to circumvent this lengthy process. A large number of target cells (10^5 cells/ml) is required for reliable detection by PCR from foods. To reach this concentration of target cells**,** cultural enrichment of a sample containing 1 cell/25g would require a net gain of about 10^7, equivalent to 22 doublings (similar to overnight enrichment) **(**Sharpe, 2003**).** Under ideal conditions, if all inhibitors were removed and the DNA were resuspended in small volumes, a five-minute PCR cycle could reach the necessary concentration gain for detection in about two hours (Sharpe, 2003). However, without prior enrichment, detection of pathogens is rarely achieved at levels below $10^2 – 10^3$ CFU per gram of food (Lantz et al., 1994, Stevens and Jaykus, 2004b).

Concentration and separation of pathogens from the food matrix has been the focus of many studies investigating ways to improve sample assay detection limits and speed time to results. Several investigators have studied immunomagnetic separation (IMS) as a means to selectively separate and concentrate pathogens from food (Tortorello et al., 1998; Fratamico and Strobaugh, 1998; Oberst et al., 1998; Ogunjimi and Choudary, 1999; Hsih and Tsen,

2001; McIngvale et al., 2002) and environmental samples (Enroth and Engstrand, 1995; Bopp et al., 2003). This technique involves the use of a polyclonal or monoclonal antibody to selectively capture the pathogen of interest, which are then concentrated by addition of a magnetic field (Levin, 2003). Pathogen detection utilizing IMS has improved sample concentration, but falls short because most protocols are only able to process a small 1ml sample volume (Cullison and Jaykus, 2002).

To address the issue of large sample volumes, the Pathatrix system developed by Matrix Microscience (Golden, CO, USA) is an AOAC approved method (Parton and Scott, 2002) that consists of incubation pots, capture phase, and a peristaltic pump. This combined system can expose an entire 250 ml (25g or mL sample in 225 mL buffer) sample to specific antibody coated capture beads twice every two minutes across a specified incubation time. The advantages of this system is that it allows for processing of a large and realistic volume of samples, immunomagnetic capture. In addition, since immunocapture is done at sample enrichment temperatures, pathogen proliferation can occur simultaneous to capture. At the end of the cycle, the capture phase is washed, re-capured, and resuspended in the desired volume for either plating or DNA/RNA extraction. Pathatrix can detect *E. coli* O157:H7 at <10 CFU/25g sample after sample pre-enrichment for 4 hours followed by a 1 h Pathatrix run (Parton and Scott, 2002; Wu et al., 2004). An eight hour format utilizing pre-enrichment (4-6 h) has been developed for detection of *E. coli* O157:H7 in raw ground beef, however, overnight enrichment is still required for other pathogens to achieve the same level of detection.

The purpose of this research was to evaluate the Pathatrix unit as a method to provide upstream sample processing prior to PCR detection. Specifically, we sought to use the

method to circumvent cultural enrichment, allowing for direct PCR detection. *E.coli* O157:H7 and *Salmonella typhimurium* were the target pathogens, and these were tested in broth buffer and epidemiologically relevant food matrices.

3.3. MATERIALS AND METHODS

3.3.1. Bacteria and Culture Recovery. *Salmonella typhimurium* MA1567 *leu-3242 leuA3241*::MudJ (kanamycin resistant) was obtained from Dr. Driss Elhanafi, Department of Food Science, North Carolina State University and transferred daily into brain heart infusion (BHI; Teknova, Hollister, CA) broth supplemented with 50μg/ml kanamycin (Sigma, St Louis, MO) and incubated at 37°C (Elhanafi et al., 2000). *Escherichia coli* O157:H7 ATCC 43895 (raw hamburger isolate) was transferred daily in BHI and incubated at 37°C. Stock cultures were maintained at -80°C in 15% glycerol. Two 18 h broth passages of each organism were conducted prior to any experiments. All experiments for each strain were performed using cells in early stationary phase (18h culture, based on growth curves conducted at 37°C). Trypticase Soy Agar (TSA; Hardy Diagnostics, Santa Maria, CA) with kanamycin (TSA-K) or Sorbitol MacConkey Agar (SMAC) was used for pathogen recovery after sample processing using the spread plate method. Preliminary experiments indicated that TSA-K was effective in eliminating background microflora, however, low levels (at $<10^2$ CFU/25g for lettuce, < 5 CFU/25g for ground beef) of background microflora, all sorbitol positive (pink colonies) bacteria, were recovered on SMAC plates when directly plated (rather than diluted, as would be done for higher inoculums) from foods.

3.3.2. Model matrices. *S. typhimurium* recovery and detection were evaluated from BPW, ground turkey, and nonfat dry milk. *E. coli* O157H7 recovery and detection were evaluated from BPW, ground beef, and romaine lettuce. Ground turkey (lean 9% fat), romaine lettuce, ground beef (8% fat), and nonfat dry milk were purchased from local retail establishments. Fresh ground turkey and ground beef were weighed into 25g portions, wrapped in aluminum foil, and stored at -20°C until needed. The frozen ground beef or turkey was thawed at 5°C approximately 24h prior to experiments and transferred to a filter stomacher bag with 225ml of BPW. The BPW, ground beef, ground turkey, and NFDM were inoculated, in separate experiments, with a serially diluted 18h culture of *E. coli* O157:H7or *S. typhimurium* at the desired concentration of cells (10^6-10^0 CFU/25g). For romaine lettuce, a 25 g sample was weighed, the *E. coli* O157:H7 was evenly spread across the leaf surface at the desired inoculum level, allowed to dry for 30 min. under a sterile laminar flow hood, followed by aseptically transferring the lettuce to a filter stomacher bag with 225 ml BPW.

3.3.3. Centrifugation and Pathatrix Processing. To investigate the recovery and detection limit for each pathogen in the absence of a food matrix using the Pathatrix system, preliminary studies were conducted on *E. coli* O157:H7 and *S. typhimurium* using 250 ml sterile buffered peptone water (BPW; ingredients: peptone, sodium chloride (Fisher Scientific), sodium phosphate (dibasic), and potassium phosphate (monobasic) (Sigma, St Louis, MO)). For food samples, a 25g sample of food was added to a filter stomacher bag (Nasco, Fort Atkinson, WI) and then 225g of sterile BPW was added to reach a final sample volume of 250 ml. A 50µl volume of the surfactant Triton X-100 was added to ground beef and turkey samples prior to Pathatrix. Serially diluted culture was added to reach the desired sample concentration (10^6-10^0 CFU/25g sample) followed by mixing in a Stomacher 400

(Seward, U.K.) at 230 rpm for 2 min. For each of the Pathatrix experiments, the spiked sample was processed using the Pathatrix system for 2 h, with 100µl of antibody-bound beads rather than the recommended 50µl. Other processes were performed according to manufacturer's directions.

Since preliminary data indicated that *Salmonella* PCR detection from ground turkey was significantly less (4 log difference) than detection from BPW, upstream sample processing via centrifugation was used to help clean the sample and optimize Pathatrix cell recovery and detection. For ground turkey, a two-step centrifugation was applied to 216 ml of filtrate (representing approximately 86% of the sample (remainder of sample was turkey meat)) aseptically transferred to a 500ml centrifuge bottle (Isonhood et al., 2005). The resulting pellet was resuspended in 50 ml 0.1% sterile peptone water and mixed for 20s by shaking the centrifuge bottle. Cell recovery was determined via spread plate and the resuspended pellet was then poured into a filter stomacher bag. The centrifuge bottle was washed twice with additional sterile 0.1% peptone water, and poured into the stomacher bag until the final sample was 250ml in volume. Based on preliminary studies, a 100µl rather than a 50µl volume of beads was added to each sample, and the sample was processed for 2 h in Pathatrix at 37°C, according to manufacturer's instructions (Figure 1). When centrifugation was not used, samples were processed in Pathatrix using BPW as the resuspensional diluent medium.

After Pathatrix processing, the captured beads were washed with 100ml BPW, eluted from the column, concentrated in the vessel via magnet, and the fluid carefully decanted by pipette. The beads were resuspended in 1.1ml BPW and mixed. The sample was divided in

half, 0.5ml for DNA extraction and 0.5 ml for spread plate enumeration on either TSA-K or SMAC.

3.3.4. DNA Extraction. The concentrated bead sample (0.5ml) was placed in a 2.0ml microcentrifuge tube and mixed with 1.0ml DNAzol BD according to manufacturer's instructions. Final DNA pellets were resuspended in 25µl molecular grade water. The beads were inhibitory to PCR as observed in preliminary BPW experiments and were thus removed via magnetic rack (DYNAL, MPC-M, Oslo, Norway) and the DNA rich supernatant transferred to a sterile microcentrifuge tube. The resuspended DNA was serially diluted 1:10 (undiluted through 1:1000) and PCR was conducted. Samples containing lower concentrations ($<10^4$CFU/25g) of cells were diluted 1:3 prior to PCR to dilute any potential inhibitors without dramatically increasing sample volume or reducing detection sensitivity.

3.3.5. PCR Amplification. For *E. coli* O157:H7, PCR primers targeted a 254bp region of the *stx*2 gene (B subunit). Each reaction contained 20 pmol of the forward primer TXBF (5′-TGTTTATGGCGGTTTTATTTG-3′) and reverse primer TXBR (5′-ATTATTAAACTGCACTTCAG-3′) (McIngvale et al., 2002, Taylor et al., 2005). For *S. typhimurium*, primers were specific for a 389bp fragment of *inv* A gene and each reaction included 20 pmol of the forward primer Salm 3 (5′-GCTGCGCGCGAACGGCGAAG-3′) and reverse primer Salm 4 (5′-TCCCGGCAGAGTTCCCATT-3′) (Manzano et al., 1998; Stevens and Jaykus, 2004). Amplification reactions for both pathogens consisted of (50 µl reaction volume) 43 µl supermix (Taq polymerase, dNTPs, $MgCl_2$, and rxn buffer) (Promega, Madison, WI), 20 pmol (2 µl) of each primer, and 2µl of template. Optimization in broth indicated that additional Taq polymerase (Promega, Madison, WI) was beneficial for

amplification of *stx*2, and was thus added for *E. coli* O157:H7 experiments and supermix volume was adjusted to account for the increase in volume.

The thermal cycler (MJ Research PT-200 Peltier Thermal Cycler, Watertown, MA) program for amplification of *E. coli* O157:H7 targeting *stx*2 was: 94°C for 2 min; 36 cycles of 94°C for 1min., 54°C for 90s, 72°C for 2min.; and 72°C for 10min (Taylor et al., 2005). Amplification of *S. typhimurium* targeting *inv* A consisted of 95°C for 5 min; 40 cycles of 95°C for 90s, 58°C for 80s, 72°C for 2min.; and 72°C for 7min (Stevens and Jaykus, 2004). Visualization of the 254bp amplicon from *E. coli* O157:H7 and the 389bp amplicon from *S. typhimurium* amplifications was done using (1%) agarose gel electrophoresis and ethidium bromide staining under UV light. All amplicons were confirmed by chemiluminescent Southern hybridization.

3.3.6. Southern Blot Hybridization. The alkaline transfer method was used for Southern blotting (Sambrook and Russell 2001). Biotinylated probes were created with the Random Primer Biotin Labeling kit with Streptavidin-AP (Perkin Elmer Life and Analytical Sciences, Boston, MA) according to manufacturer's instructions and following the method of Taylor et al. (2005). Briefly, PCR product generated from DNA extracted from a pure culture of *E. coli* O157:H7 or *S. typhimurium* was purified using the Mo Bio UltraClean DNA purification system (Mo Bio Laboratories, Inc. Solana Beach, CA) in accordance with manufacturer instruction. One-half of the of the resuspended DNA (10 µl) was diluted in 9 µl of water, heated at 95°C for 3 min, rapidly chilled on ice, and then mixed with random primers, biotinylated nucleotides, and Klenow enzyme. After incubation at 37°C for one hour, probe was diluted 10-fold with water and stored at -20°C until use. DNA hybridization was done as recommended by the Random Primer Biotin Labeling kit using a probe concentration of 25

ng/ml. Hybridization was carried out at 65°C overnight. The membrane was washed in series at 65°C, soaked in streptavidin-alkaline phosphatase conjugate buffer for one h at 27°C, washed in series, and subsequent chemiluminescent detection was done using CDP-Star reagent with visualization on Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

3.3.7. Statistical Analysis. All experiments were performed in triplicate. Statistical analysis of log means was done using SAS (Statistical Analysis Software, version 8.2, Cary, NC, USA) as evaluated by analysis of variance (PROC GLM) with least squares means in a completely randomized design ($p<0.05$).

3.4. RESULTS

3.4.1. Preliminary Experiments. Immunomagnetic bead concentrations (50, 75, and 100 μl) were tested in Pathatrix at low inoculum levels ($10^3 – 10^2$) of *S. typhimurium* in broth. There was a linear, 2-fold increase in cell recovery (spread plates) as the volume of beads was increased (data not shown). Therefore, we chose to increase our bead concentration to 100 μl for each sample. As recommended by the manufacturer, we also increased our Pathatrix run time from 30min. to 2h to optimize recovery. This procedure was later applied in the *E. coli* O157 study as well. An insignificant amount of growth or approximately a 1-log increase in target bacterial titer was observed during the run time in a rich medium (BHI) for both pathogens (Figure 2). Enrichment with BPW produced similar results to BHI, which was in agreement with Wu et al. (2004) in finding no significant difference between modified EC broth with novobiocin and BPW. When processing food samples, a similar recovery of background microflora was recovered after Pathatrix as was recovered from the food before processing the sample **(**data not shown**).** The TSA-K and SMAC was effective in eliminating

background microflora on spread plates **(**data not shown**).** However lower inocula (10^3CFU/25g) revealed a population (10^2 CFU/25g sample of lettuce 10^1CFU/25g for ground beef) of sorbitol positive cultures present in the foods, and particularly true with romaine lettuce. This indicated to us that the immunomagnetic separation was only somewhat effective in eliminating nonspecific bacteria in the sample.

3.4.2. Evaluation of Cell Recovery on Spread Plates. Each of the food samples evaluated was spiked with 10-fold serial dilutions of an overnight culture of *Salmonella* or *E. coli* O157:H7 to reach cell numbers ranging from 10^6-10^0 CFU/25g sample. Since the *Salmonella* recoveries from ground turkey were about 1% of sample input, we employed the two-step method in hopes of eliminating some of the components in the food matrix that might interfere with antibody-antigen binding. This upstream sample processing method resulted in greater than a 1-log increase in cell recovery was observed for each level of the spiked sample, compared to the sample with no centrifugation (data not shown). When preceding Pathatrix with two-step centrifugation, the cell recoveries were more similar to those of broth, but the additional centrifugation step increased processing time by about 45 minutes. Commercial grade A milk powder was directly processed in the Pathatrix system, and for this food no pre-processing was investigated. The plate counts for the milk powder were similar to the ground turkey not processed by two-step centrifugation in that they were about 1-log less than samples that were processed by two-step centrifugation (Table 1).

The cell recoveries for *E. coli* O157:H7 were much improved compared to those of *Salmonella*. *E. coli* O157:H7 recoveries were approximately 2 logs greater than *Salmonella* (Tables 1,2)**.** Therefore, *E. coli* O157:H7 recoveries (Table 2) were greater than 100% for all foods tested rather than being 1% or less for *S. typhimurium* (Table 1). The only explanation

that can be offered for this higher recovery is that the *E. coli* O157 antibody on the immunomagnetic beads had a greater affinity or avidity for *E. coli* O157:H7 than did the *Salmonella* specific antibodies for *S. typhimurium*.

3.4.3. PCR Results. The PCR detection limits were correlated with capture efficiency, such that the better the capture ability, the lower the PCR detection limits. Therefore, similar to cell recoveries, PCR detection of *S. typhimurium* was not as sensitive as detection of *E. coli* O157:H7 (Figures 3, 4). In broth, *S. typhimurium* detection limits were 10^1 CFU/250ml sample and *E. coli* O157:H7 detection limits were 10^0 CFU/250ml sample. The PCR detection for *S. typhimurium* was much less efficient when foods were tested, with detection limits of 10^4 CFU/25g in NFDM and 10^2 CFU/25g in ground turkey. Two-step sample processing prior to Pathatrix improved PCR detection limits by 1000-fold (10^5 to 10^2). Although it is reported that *E. coli* O157 beads have greater avidity compared to the *Salmonella* beads (personal communication), our research demonstrates that there indeed is a relationship between the food matrix and bead avidity. For example, the food matrix played a minimal role in the ability of the immunomagnetic beads to capture and detect *E. coli* O157:H7 (Figure 3), but this was not the case for *Salmonella*.

3.5. DISCUSSION

In this study, we investigated the efficacy of Pathatrix as a pre-PCR concentration and upstream sample preparation step. It is clear that the Pathatrix system offers a unique method to process large (≥ 25g) samples. In all cases, the resulting sample appeared very clean and allowed us to easily extract the DNA and resuspend in a very small volume (concentration factor 1000-fold, from 25g to 0.025ml). Our detection limits were extremely low for *E. coli* O157:H7 at 10^0 CFU/25g, ground beef or romaine lettuce; for *S. typhimurium*, detection limits were 10^2 CFU/25g ground turkey after clarification. These detection limits for *E. coli* O157:H7 are similar to those reported by Wu et al. (2004) of $<10^1$ CFU/25g for *E. coli* O157:H7 from ground beef with a 4.5h pre-enrichment step and a 0.5 h Pathatrix run. However, our method replaced that 4.5h pre-enrichment step and 0.5h in Pathatrix with a 2h Pathatrix run. This manuscript is the first to report results based on Pathatrix enrichment only, meaning no pre-enrichment. Our *S. typhimurium* format was also successful at effectively capturing and detecting cells, considering that our samples did not receive a pre-enrichment step. This study also demonstrates the benefit of a separate clarification step prior to using the Pathatrix system for *Salmonella* capture, particularly when dealing with samples of complex composition, such as NFDM and ground turkey.

For over a decade, immunomagnetic separation has been applied successfully to help separate pathogens from the food matrix and has even been adopted as part of the *E. coli* O157:H7 protocol in the FDA Bacteriological Analytical Manual (BAM). However, the stumbling block for this method is its inability to process a volume greater than 1ml, making a 24h sample preenrichment a necessity (Ogden et al., 2000). In an effort to decrease the time to endpoint detection for methods such as PCR, the elimination of lengthy cultural

enrichment steps would greatly improve sample throughput for food processors and government regulatory agencies. This is a difficult feat, but is partially addressed by the Pathatrix system, in that the entire sample volume is processed by recirculating through a heated incubation pot (enrichment) and a magnetic capture unit (capture), allowing for simultaneous enrichment and capture (Wu et al., 2004).

The Pathatrix system, as a validated AOAC approved technique, addresses many of the needs for researchers wishing to clarify samples for subsequent pathogen detection by PCR, RT-PCR, and ELISA. Specifically, the Pathatrix system is able to separate pathogens from food particulates, remove inhibitory compounds associated with the food matrix, reduce sample size while recovering the pathogen, all done without disrupting cell viability (Stevens and Jaykus, 2004a). While the Pathatrix system certainly is able to fulfill many of these needs, particularly when targeting *E. coli* O157, some improvement can still be obtained by pre-concentrating or clarifying the food sample prior to beginning immunomagnetic capture by Pathatrix. We hypothesize that this may be necessary because some foods may interfere with target cell/bead interaction, preventing contact and binding of the bead with the pathogen, thus reducing or limiting the number of target cells that are recovered in the system.

Other researchers have found reduced cell recoveries when capturing cells in artificially contaminated fat-free milk suspension as compared to a broth model. Using polystyrene beads coated with antibody fragments specific for *Pseudomonas aeruginosa*, Molloy et al. (1995) recovered 95% cells from an overnight culture at 10^7 CFU/ml, but were only able to recover 75% of cells seeded in a fat-free milk suspension at 10^6 CFU/ml. These researchers theorized that the low recoveries from the fat-free milk may have been due to soluble milk proteins covering the antibody fragments, which prevented binding of the cell to

the antibody binding sites. In studies involving ground beef, Ripabelli et al. (1999) reported low *Salmonella* recoveries using IMS, supposedly due to non-specific binding with foods of high fat content. The sample clarification step applied to ground turkey prior to immunomagnetic capture of *Salmonella*, and since we saw improved recoveries with Pathatrix, was intended to remove mainly food components such as protein and fat, we conclude that food matrix was the main variable affecting cell recovery in this case. However, recovery of *E. coli* O157:H7 was not affected by the presence of food matrix during the 2h Pathatrix run. Contrary to our finding, Arthur et al. (2005) studied recovery of *E. coli* O157:H7 in inoculated ground beef and determined that a second filtration step was needed prior to Pathatrix to assure adequate recovery of the beads following IMS. The main difference in the Arthur et al. (2005) study was that there was more ground beef per sample (250ml sample was processed following a 4h enrichment step from a composite sample containing 375g ground beef suspended in 1 L BPW) than was used in our study. In our present study, it is presumed that differences in *E. coli* O157:H7 and *S. typhimurium* recoveries are a matter of antibody avidity. This is perhaps the most logical reason for large differences in recoveries found between *S. typhimurium* and *E. coli* O157:H7 detection from ground beef and ground turkey tested in this study.

The final Pathatrix step involves slowly washing the beads with fresh media, which in theory, should reduce or effectively eliminate inhibitors and wash away microorganisms bound non-specifically. Studies investigating the ability of IMS to eliminate or reduce competitive microflora have resulted in different conclusions. Hanai et al. (1997) found a reduction in competitive microflora when using an IMS system for capturing *Salmonella*. Other researchers using these IMS systems (Cudjoe et al., 1994; Ripabelli et al., 1997;

Ripabelli et al., 1999) found abundant growth of competing microflora after selective plating of IMS concentrates. To reduce this nonspecific binding, Seo et al. (1998) determined that a shorter incubation time helped, while Hepburn et al. (2002) suggested increasing incubation temperature to 42°C to inhibit background microflora and favor growth of *E. coli* O157. Since we did not measure the efficacy of Pathatrix beads to capture cells in the presence of background microflora in a broth system, we cannot state the role of nonspecific microflora in capture efficiency for both target pathogens used in our assay. However, we do know that a significant number (10^2-10^3 CFU/25g) of background microflora were recovered on nonselective media following Pathatrix. Additional limitations or considerations of the method that occur during the capture or Pathatrix run time that can limit pathogen recovery are as follows: association of the pathogen with the food matrix, food components or nonspecific organisms blocking the antibody sites, making them unavailable for capturing the target pathogen, or flow parameters reducing bead/pathogen interaction or binding strength.

This study demonstrates that the Pathatrix method works well as a pre-PCR processing device, in some cases, circumventing the the need to perform prior cultural enrichment. Consistent detection of *E. coli* O157:H7 at very low levels down to 10^0 CFU/25g food sample validates the effectiveness of this system for this organism. With further development, the assay may be able to give results in as little as 7h if linked to real-time PCR. It is our hope that this study will provide useful clues to help other investigators further reduce the time to endpoint detection of pathogens in food in the future. Other pathogens, such as *Salmonella*, can also be effectively processed by Pathatrix but require either additional clarification or a short enrichment step to improve cell recovery and PCR detection limits.

3.6. ACKNOWLEDGEMENTS

Funding provided in part by the California Dairy Research Foundation. The use of tradenames does not imply endorsement nor criticisms of those not mentioned. Paper FSR XXXX of the department of Food Science, North Carolina State University.

3.7. REFERENCES

Arthur, T.M., J.M. Bosilevac, X. Nou, and M. Koohmaraie. 2005. Evaluation of culture- and PCR-based detection methods for *Escherichia coli* O157:H7 in inoculated ground beef. *J. Food Prot*. 68(8):1566-1574.

Bej, A.K., and M.H. Mahbubani. 1994. Detection of foodborne microbial pathogens. *In* PCR Technology: Current Innovations. CRC Press. Boca Raton, FL. pp. 341-365.

Bopp, D.J., B.D. Saunders, A.L. Waring, J.Ackelsberg, N. Dumas, E. Braun-Howland, D. Dziewulski, B.J. Wallace, M. Kelly, T. Halse, K.A. Musser, P.F. Smith, D.L. Morse, and R.J. Limberger. 2003. Detection, isolation, and molecular subtyping of *Escherichia coli* O157:H7 and *Campylobacter jejuni* associated with a large waterborne outbreak. *J. Clin. Microbiol.* 41(1):174-180.

Cudjoe, K.S., R. Krona, and E. Olsen. 1994. IMS: a new selective enrichment technique for detection of *Salmonella* in foods. *Int. J. Food Microbiol*. 23:159-165.

Cullison, M.A., and L.A. Jaykus. 2002. Magnetized carbonyl iron and insoluble zirconium hydroxide mixture facilitates bacterial concentration and separation from nonfat dry milk. *J. Food Prot*. 65(11):1806-1810.

Enroth, H., and L. Engstrand. 1995. Immunomagnetic separation and PCR for detection of *Helicobacter pylori* in water and stool specimens. *J.Clin. Microbiol*. 33(8):2162-2165.

Fratamico, P.M., and T.P. Strobaugh. 1998. Simultaneous detection of *Salmonella* spp. and *Escherichia coli* O157:H7 by multiplex PCR. *J. Ind. Microbiol. Biotechnol.* 21(3):92-98.

Hanai, K., M. Satake, H. Nakanishi, and K. Venkateswaran. 1997. Comparison of commercially available kits with standard methods for detection of *Salmonella* strains in foods. *Appl. Environ. Microbiol*. 63:775-778.

Hepburn, N.F., M. MacRae, M. Johnston, J. Mooney, and I.D. Ogden. 2002. Optimizing enrichment conditions for the isolation of *Escherichia coli* O157 in soils by immunomagnetic separation. *Lett. Appl. Microbiol*. 34:365-369.

Hitchins, A.D., P. Feng, W.D. Watkins, S.R. Rippey, and L.A. Chandler. 1995. *Escherichia coli* and the coliform bacteria. *In* FDA Bacteriological Analytical Manual 7th Edition. Arlington, VA: Association of Official Analytical Chemists. 4.20-4.25.

Hsih, H.Y., and H.Y. Tsen. 2001. Combination of immunomagnetic separation and polymerase chain reaction for the simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. in food samples. *J. Food Prot*. 64(11):1744-1750.

Lantz, P.G., B. Hahn-Hagerdal, and P. Radstrom. 1994. Sample preparation methods in PCR-based detection of food pathogens. *Trends Food Sci. Technol*. 5:384-389.

Levin, R.E. 2003. Application of the polymerase chain reaction for detection of *Listeria monocytogenes* in foods: A review of methodology. *Food Biotech*. 17(2):99-116.

Manzano, M., L. Cocolin, G. Astori, C. Pipan, G.A. Botta, C. Cantoni, and G. Comi. 1998. Development of a PCR microplate-capture hybridization method for simple, fast and sensitive detection of *Salmonella* serovars in food. *Mol. Cell Probes*. 12:227-234.

McIngvale, S.C., D. Elhanafi, and M.A. Drake. 2002. Optimization of reverse transcriptase PCR to detect viable shiga-toxin-producing *Escherichia coli*. *Appl. Environ. Microbiol.* 68(2):799-806.

Molloy, P., L. Brydon, A.J. Porter, and W.J. Harris. 1995. Separation and concentration of bacteria with immobilized antibody fragments. *J. Appl. Bacteriol*. 78:359-365.

Oberst, R.D., M.P. Hays, L.K. Bohra, R.K. Phebus, C.T. Yamashiro, C. Paszko-Kolva, S.J.A. Flood, J.M. Sargeant, and J.R. Gillespie. 1998. PCR-based DNA amplification and presumptive detection of *Escherichia coli* O157:H7 with an internal fluorogenic probe and the 5' nuclease (TaqMan) assay. *Appl. Environ. Microbiol*. 64(9):3389-3396.

Ogden, I.D., M. MacRae, N.F. Hepburn, and N.J.C. Strachan. 2000. Improved isolation of *Escherichia coli* O157 using large enrichment volumes for immunomagnetic separation. *Lett. Appl. Microbiol*. 31:338-341.

Ogunjimi, A.A., and P.V. Choudary. 1999. Adsorption of endogenous polyphenols relieves the inhibition by fruit juices and fresh produce of immuno-PCR detection of *Escherichia coli* O157:H7. *FEMS Immun. Med. Microbiol*. 23:213-220.

Parton, A., and M. Scott. 2002. Pathatrix *E. coli* O157 test AOAC performance tested method validation study. AOAC Validation Study Report. Cert No. 030202 pp. 1-28.

Radstrom, P, C. Lofstrom, M. Lovenklev, R. Knutsson, and P. Wolffs. 2003. Strategies for overcoming PCR inhibition. *In* PCR Primer: A Laboratory Manual 2nd ed. C.W. Dieffenbach and G.S. Dveksler (eds.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 149-161.

Ripabelli, G., M.L. Sammarco, A. Ruberto, G. Iannitto, and G.M. Grasso. 1997. Immunomagnetic separation and conventional culture procedure for detection of naturally occurring *Salmonella* in raw pork sausages and chicken meat. *Lett. Appl. Microbiol*. 24:493-497.

Ripabelli, G., M.L. Summarco, and G.M. Grasso. 1999. Evaluation of immunomagnetic separation and plating media for recovery of *Salmonella* from meat. *J. Food Prot*. 62(2):198-201.

Sharpe, A.N. 2003. Separation and concentration of samples. In *Detecting Pathogens in Food*. Woodhead Publishing. Boca Raton, FL. p. 52-68.
Stevens, K.A. and Jaykus, L.A. (2004a) Bacterial separation and concentration from complex sample matrices: A review. *Crit. Rev. Microbiol*. 30(1):7-24.

Stevens, K.A. and Jaykus, L.A. 2004b. Direct detection of bacterial pathogens in representative dairy products using a combined bacterial concentration-PCR approach. *J. Appl. Microbiol*. 97(6):1-8.

Taylor, M.T., Drake, M.A. and Jaykus, L.A. 2005. Development of an alternative method to concentrate *E. coli* O157:H7 from meat homogenates to facilitate detection by cultural and molecular methods. *J. Food Prot*. 68(2):225-232.

Tortorello, M.L., K.F. Reineke, D.S. Stewart, and R.B. Raybourne. 1998. Comparison of methods for determining the presence of *Escherichia coli* O157:H7 in apple juice. *J. Food Prot.* 61(11):1425-1430.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63(10):3741-3751.

Wu, V.C.H, V. Gill, R. Oberst, R. Phebus, and D.Y.C. Fung. 2004. Rapid protocol (5.25 h) for the detection of *Escherichia coli* O157:H7 in raw ground beef by an immuno-capture system (Pathatrix) in conmbination with Colotrix and CT-SMAC. *J. Rapid Meth. Autom. Microbiol.* 12:57-67.

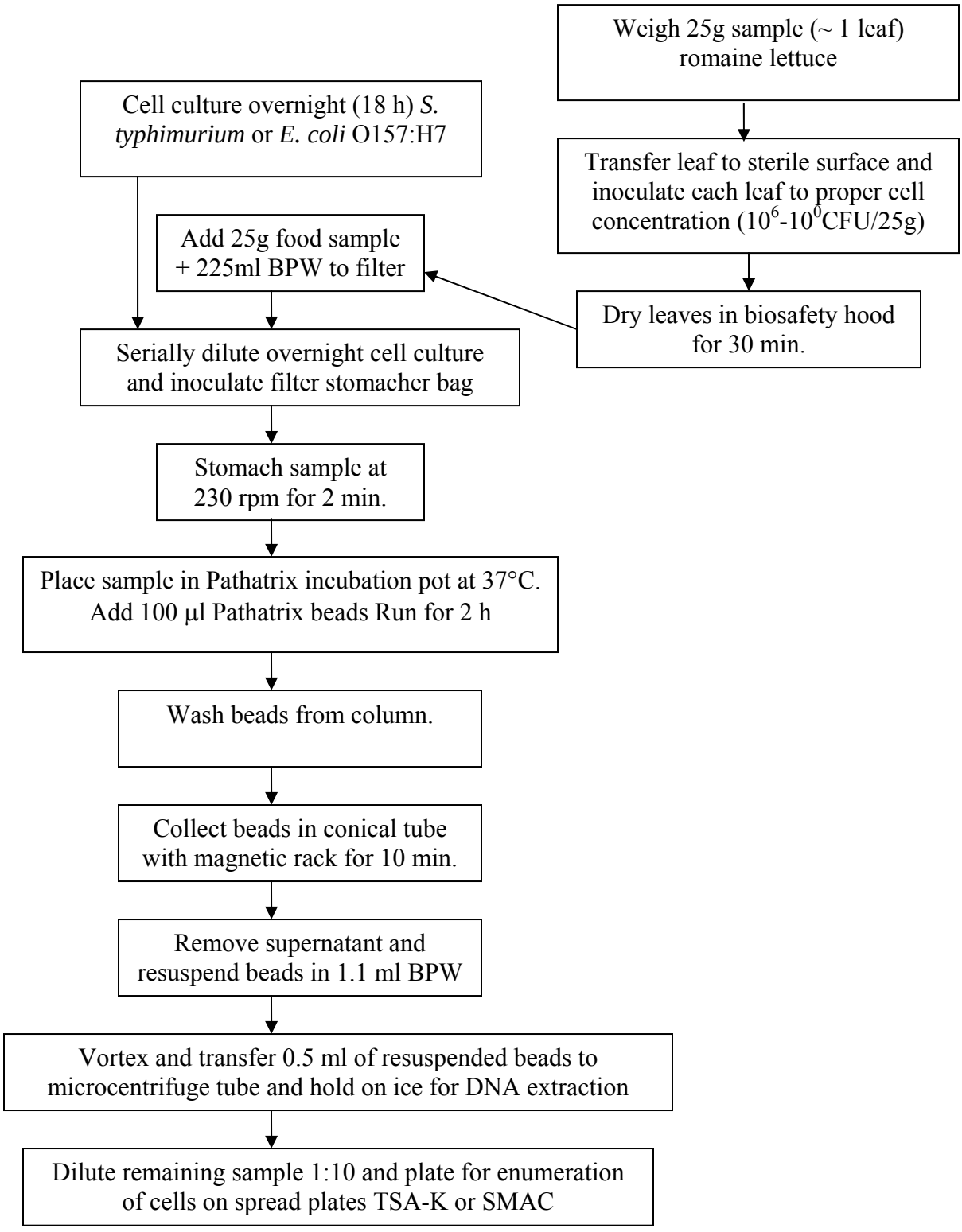


Figure 3.1. Flow Chart of Pathatrix processing.

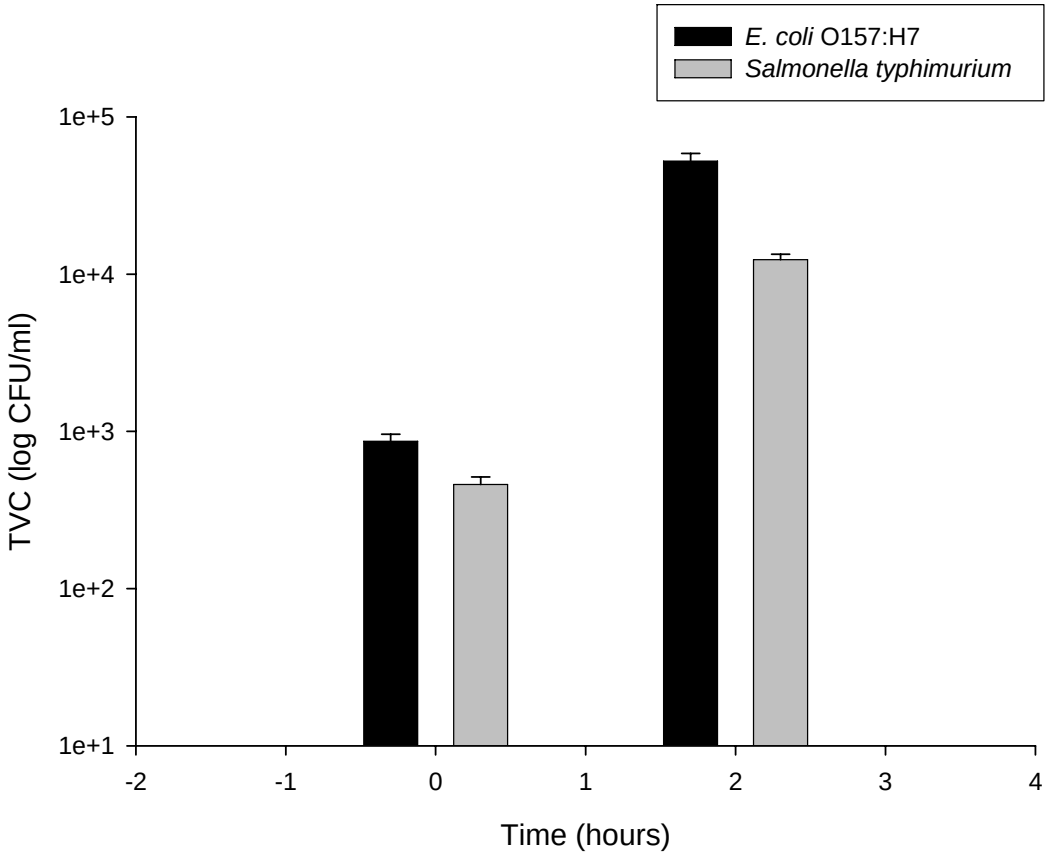


Figure 3.2. Growth of *E. coli* O157 and *S. typhimurium* in BHI broth at 37°C after a 2 h run using Pathatrix.

Table 3.1. Pathatrix cell recovery for *S. typhimurium* (A) and *E. coli* O157:H7 (B) from broth and foods.

(A)

Input CFU/25g sample	Broth	Ground Turkey		Nonfat Dry Milk
		Two-Step	Pathatrix	
Overnight culture (18h)	8.71×10^{8}	1.05×10^{9}	1.05×10^{9}	8.53×10^{8}
10^{6}	1.88×10^{4b}	1.08×10^{6}	1.98×10^{4b}	4.96×10^{3b}
10^{5}	2.82×10^{3cd}	1.25×10^{5}	4.81×10^{3c}	6.15×10^{2d}
10^{4}	3.05×10^{2b}	1.83×10^{4}	4.33×10^{2b}	5.85×10^{1c}
10^{3}	3.02×10^{1b}	2.58×10^{3}	8.87×10^{1b}	1.02×10^{1c}
10^{2}	5.04×10^{0c}	8.43×10^{2}	3.52×10^{1b}	0.00×10^{0c}
10^{1}	0.00×10^{0}	N/A	N/A	N/A
10^{0}	N/A	N/A	N/A	N/A

(B)

Input CFU/25g sample	Broth	Ground Beef	Romaine Lettuce
Overnight culture (18h)	1.91×10^{9}	1.38×10^{9}	9.43×10^{8}
10^{6}	4.50×10^{6a}	2.34×10^{6a}	6.33×10^{6a}
10^{5}	4.75×10^{5b}	5.82×10^{5ab}	1.54×10^{6a}
10^{4}	6.88×10^{4a}	4.01×10^{4a}	9.80×10^{4a}
10^{3}	8.73×10^{3a}	9.89×10^{3a}	7.77×10^{3a}
10^{2}	6.29×10^{2a}	4.55×10^{2a}	3.38×10^{3a}
10^{1}	7.21×10^{1b}	3.09×10^{2a}	6.78×10^{1b}
10^{0}	1.17×10^{1a}	3.52×10^{2a}	9.77×10^{1a}

[a]Means across columns not followed by the same letter represent a statistically significant difference

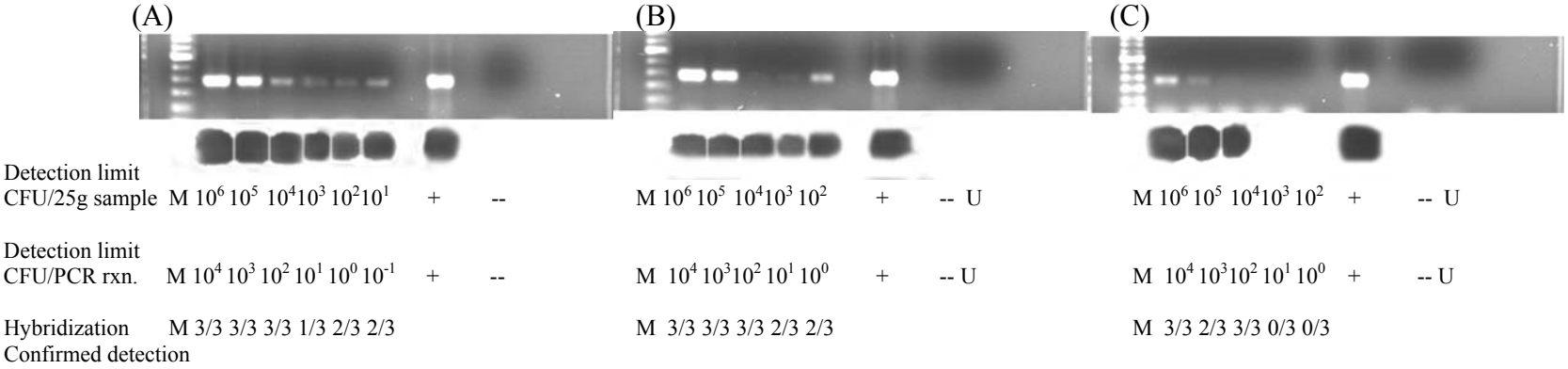


Figure 3.3. *S. typhimurium* detection from Pathatrix following a 2 h Pathatrix run from BPW (A), ground turkey (B), and nonfat dry milk (C) followed by DNA extraction, PCR amplification, gel electrophoresis (top), and Southern hybridization (bottom). Ground turkey was pre-processed using centrifugation and processed in 0.1% peptone. Each band represents DNA extracted from a 25g sample inoculated with *S. typhimurium* to achieve levels of contamination between 10^1 – 10^6CFU/25g. All experiments were performed in triplicate. Inoculation level and detection limits are given below each lane. Lanes: M (marker), + (positive control reaction for detection of *L. monocytogenes*), -- (water control), and U (uninoculated potato salad).

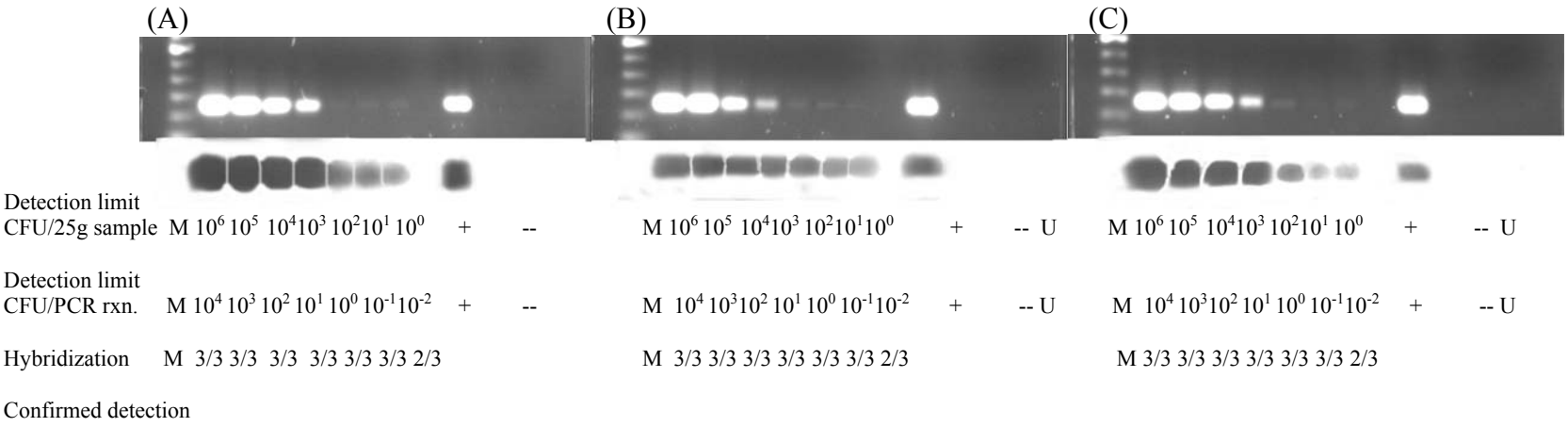


Figure 3.4. ***E. coli*** **O157:H7 detection from Pathatrix following a 2 h Pathatrix run from BPW (A), ground turkey (B), and nonfat dry milk (C) followed by DNA extraction, PCR amplification, gel electrophoresis (top), and Southern hybridization (bottom). Each band represents DNA extracted from a 25g sample inoculated with** ***E. coli*** **O157:H7 to achieve levels of contamination between 10^1 – 10^6CFU/25g. All experiments were performed in triplicate. Inoculation level and detection limits are given below each lane. Lanes: M (marker), + (positive control reaction for detection of** ***L. monocytogenes*****), -- (water control), and U (uninoculated potato salad).**

CHAPTER 4

CONCLUSIONS

The advancement and application of rapid detection method chemistries to rapidly detect pathogens in foods has long relied on 24h pre-enrichment to boost cell numbers and dilute food matrix containing potential inhibitors. The presence of low pathogen numbers spread throughout a large food sample containing a significantly greater number of indigenous microflora compounded with the small sample size required by most rapid methods has necessitated the use of selective pre-enrichment as the most cost-effective, feasible solution. Until now, very few investigators have sought to determine the efficacy of alternative more rapid methods to enhance target pathogen cell numbers while reducing the associated food matrix with no pre-enrichment step. In developing such a method, it is desirable to have a method that is able to concentrate cells and effectively separate them from the matrix. Ideally, the method would be universal (applicable to several food types and pathogens) fast, simple, and inexpensive. Indeed this is a very worthy cause, and rightly so, very challenging. However, several clues and procedures within the scientific literature act as guides making available several formats that might be considered, whether they are physical (filtration, centrifugation), physico-chemical (metal hydroxides), or biological (IMS). Within our laboratories we have investigated all three of these formats, and found, as many other researchers have, that the physical and biological methods are by-far the most effective at meeting the needs of our ideal model.

In the first study, we combined simple filtration with differential separation (centrifugation) to help separate, concentrate, and ultimately clarify a large sample containing a conglomerate of ingredients to a small sample that fits nicely into a DNA

extraction protocol prior to detection of *L. monocytogenes* by PCR. Recoveries were matrix dependent and PCR detection limits were highest when centrifugation enabled high bacterial recovery in the final clarified pellet. The PCR detection limits ranged from 10^5 to 10^3 CFU/g sample and were comparable to other scientific works applying similar methods to other food matrices.

In the second study we investigate a novel, highly touted, AOAC approved system for concentrating pathogens *S. typhimurium* and *E. coli* O157:H7 by IMS, while maintaining the whole sample (25g) approach. The system is unique in that it incorporates an entire 25g sample which can be passed several times across a column of immuno-magnetic beads (IMS). Although the machine is very effective, since it is able to detect $<10^1$ CFU/25g sample, it still requires the help of a short pre-enrichment step to achieve this level of detection. Our approach was unique in that we attempted to by-pass the pre-enrichment step and push the machine to its limits by increasing the run time, number of beads, and in some cases incorporating a clarification step developed in the first study. Overall, this study offers several clues which can and will ultimately enhance design of the Pathatrix system and other research tools aimed at by-passing 24h pre-enrichment allowing for same-day (8h) and perhaps (4h) rapid method protocols for the detection of foodborne pathogens.

In closure of these research efforts, we believe this work will help address and enlighten those seeking to process food samples for use in rapid molecular technologies. The issues addressed here can help focus future work towards developing the ideal system to process food samples for rapid detection of pathogens.