ABSTRACT

STEVENS, KELLY, A. The role of molecular methods in the detection of pathogens in food. (Under the direction of Dr. Lee-Ann Jaykus.)

The research described here addresses several issues associated with testing food samples for the presence of pathogens. Based on three different perspectives, the following major objectives are noted: (i) the use of molecular amplification methods to detect *Listeria monocytogenes* and *Salmonella enterica* serovar enteritidis directly from food samples, bypassing the need for cultural enrichment; (ii) evaluation of automated ribotyping for the differentiation of *Salmonella enterica* serovar Typhimurium strains; and (iii) development of a decision model to evaluate a pathogen testing decision in industry.

In the first study, we developed and evaluated a method for the direct detection of foodborne pathogens without prior cultural enrichment. Eleven-gram samples of plain nonfat yogurt or mild cheddar cheese were seeded with *L. monocytogenes* or *S. enterica* Enteritidis at levels of 10^2 - 10^6 CFU per sample. Samples were then processed for bacterial concentration using high-speed centrifugation (9,700 x g) followed by detection using both cultural and molecular methods. Molecular detection limits of 10^3 and 10^1 CFU per 11 g sample were achieved for *L. monocytogenes* and serovar. Enteritidis, respectively, in both product types and without prior cultural enrichment.

In a second and related study, alternative nucleic acid preparation methods were evaluated to improve the direct detection of *Listeria monocytogenes* from a frankfurter matrix. Using a combined concentration / extraction sample preparation, 11-g samples were concentrated 100-fold to 100 µl with recovery of target nucleic acids which were

further purified by column chromatography and specific bacterial rRNA isolation using two magnetic bead-based technologies, i.e., MICROB*Enrich*® and MICROB*Express*®. PCR detection limits were 10^5 CFU/11g sample and RT-PCR detection limits were 10^3 CFU/11g. Detection limits were improved an additional 10-fold (to 10^2 CFU/11g) when extracted RNA was further purified using MICROB*Express*®.

The third study evaluated the RiboPrinter® microbial characterization unit for its ability to differentiate thirty-nine isolates of multi-drug resistant *Salmonella enterica* serovar. Typhimurium. Phenotypically, the isolates varied by phage type and marginally by antibiotic resistance pattern. However, the strains could not be meaningfully differentiated from one another using ribotyping, suggesting that in this case, phenotypic methods may be more discriminatory than this molecular typing method.

In the final study, decision analysis tools were used to develop a model addressing the issues encountered when making a testing decision. From a food processors perspective, three potential consequences of foodborne pathogen contamination were considered as elements of the decision, e.g., no consequences, regulatory recall without disease, and disease outbreak. Accordingly, the inputs of the model were (i) costs associated with food-borne contamination (business and health related costs); (ii) reliability of testing; and (iii) prevalence of contamination. In general, the model indicated that testing for highly prevalent pathogens may provide an improvement in food safety but end product testing for pathogens of low prevalence should be carefully considered and may not be justified due to limited return on investment.

These efforts represent continued progress in harnessing the power and diversity of molecular methods for the identification and characterization of foodborne pathogens. The development of systematic approaches to making testing decisions is also justified and needed by the industry. Taken together, these studies add to our understanding of the impediments to application of rapid methods for the detection of foodborne pathogens, and provide some solutions to facilitate the practical use of these methods in the future.

THE ROLE OF MOLECULAR METHODS IN THE DETECTION OF PATHOGENS IN FOOD

By

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CHAPTER 1

LITERATURE REVIEW

1.1 ABSTRACT

The use of many rapid detection technologies could be expanded if the bacteria were separated, concentrated, and purified from the sample matrix before detection. Specific advantages of bacterial concentration might include facilitating the detection of multiple bacterial strains; removal of matrix-associated assay inhibitors; and provision of adequate sample size reduction to allow for the use of representative food sample sizes and/or small media volumes. Furthermore, bacterial concentration could aid in improving sampling techniques needed to detect low levels of pathogens or sporadic contamination, which may perhaps reduce or even eliminate the need for cultural enrichment prior to detection. Although bacterial concentration methods such as centrifugation, filtration, and immunomagnetic separation have been reported for food systems, none of these is ideal and in many cases a technique optimized for one food system or microorganism is not readily adaptable to others. Indeed, the separation and subsequent concentration of bacterial cells from a food sample during sample preparation continues to be a stumbling block in the advancement of molecular methods for the detection of foodborne pathogens. The purpose of this review is to provide a detailed understanding of the science, possibilities and limitations of separating and concentrating bacterial cells from the food matrix in an effort to further improve on our ability to harness molecular methods for the rapid detection of foodborne pathogens.

1.2 INTRODUCTION

The identification of pathogenic organisms in foods and the environment has become increasingly important. While many methods of detection are available, food and environmental microbiologists must often choose between enumeration and identification without the option of both. Enumerative methods are usually based on the ability of the normal healthy bacterial cells to multiply in nutrient-rich medium, although selective agents are occasionally added to favor the growth of a specific group of organisms such as coliforms or enterococci. These methods therefore measure the total number of organisms or a group of organisms in the sample tested and as such, are relatively non-specific. The most commonly used non-specific methods, both quantitative and semi-quantitative, are plate count methods including the aerobic plate count (APC), coliform, and yeast and mold counts; bioluminescence assays; and impedance or conductance measurements (Dziezak, 1987).

There are many options available to identify specific microorganisms, particularly pathogens. Pathogen detection methods are widely used in clinical microbiology, but for environmental applications, these methods often rely on cultural enrichment to increase the numbers of the target microorganism and allow for resuscitation of injured organisms. When followed by selective and differential plating, these methods allow the analyst to discriminate the target organism from the background microflora, but are almost always non-enumerative (i.e., presence/absence). The combination of cultural enrichment and selective plating results in a lengthy assay, frequently extending beyond four days for even preliminary results. Most rapid methods developments have sought to shorten detection time by replacing the selective

and differential plating steps with methods such as DNA hybridization, agglutination, and enzyme immunoassay (Dziezak, 1987). These approaches have shortened the time to detection, but because these methods detect at best 10^3-10^4 CFU of the target pathogen, lengthy cultural enrichment steps are still necessary. Furthermore, cultural confirmation for presumptively positive results is almost always required.

Enzymatic nucleic acid amplification methods such as the polymerase chain reaction (PCR) and nucleic acid sequence-based amplification (NASBA) offer several potential advantages for the rapid and reliable detection of microbial pathogens in foods. These *in vitro* nucleic acid amplification methods are capable of enriching a single specific DNA or RNA sequence up to a million-fold in a few hours and provide a theoretical detection limit of one single bacterial cell (Erlich et al., 1991, Atlas and Bej, 1994). The primary advantage of this technology is its potential to replace cultural enrichment with specific nucleic acid sequence enrichment, thereby decreasing detection time. Furthermore, these methods have high specificity of detection, can facilitate the identification of microorganisms that are difficult to culture, and have the potential to reduce the overall cost of testing (Bej and Mahbubani, 1994).

Despite these advantages, most applications of nucleic acid amplification for the detection of pathogenic microorganisms in food and environmental samples remain in developmental stages with significant methodological hurdles. Widespread use of PCR in food and environmental microbiology has been limited by high sample volumes ($\geq$25 ml or gm) compared to small amplification volumes (10-50 µl), residual food components that inhibit enzymatic reactions, and low levels of contaminating pathogens (Bej and Mahbubani, 1994). All of these issues work to reduce test specificity and

sensitivity, resulting in less than optimal detection results. Indeed, researchers working in the field are rarely able to achieve detection limits less than 10^2-10^3 CFU of the target pathogen per gram of food product, levels that are only slightly better than ELISA and DNA hybridization. By and large, cultural enrichments are still necessary to provide sufficient target amplification even before the application of nucleic acid amplification.

It has been suggested that the uses of many rapid detection technologies could be expanded if the bacteria were separated, concentrated, and purified from the sample matrix before detection (Swaminathan and Feng, 1994, Wilson, 1997, deBoer and Beumer, 1999). This approach commonly precedes the detection of viral and parasitic agents in food and environmental samples, and would offer such advantages as facilitating the detection of multiple bacterial strains; removal of matrix-associated reaction inhibitors; and provision of adequate sample size reductions to allow for the use of representative food sample sizes and/or smaller media volumes (Jaykus, 2001). Furthermore, bacterial concentration could aid in improving sampling techniques needed to detect low levels of pathogens or sporadic contamination, which may perhaps reduce the need for cultural enrichment prior to detection. Although methods such as centrifugation, filtration, and immunomagnetic separation have been reported for bacterial concentration in food or environmental systems, none of these is ideal and in many cases a technique optimized for one matrix or microorganism is not readily adaptable to others. In short, the separation and subsequent concentration of bacterial cells from a food or environmental sample during sample preparation continues to be a stumbling block in the advancement of molecular methods for the detection of pathogens. The purpose of this review is to provide a detailed understanding of the

science, possibilities and limitations of separating and concentrating bacterial cells from the food matrix in an effort to further improve on our ability to harness molecular methods for the rapid detection of foodborne pathogens.

1.2.1 The Process of Pathogen Separation, Concentration and Purification

The ultimate goal of emerging methods for pathogen detection is to significantly reduce detection time. Given the hurdles to the practical use of some of the newer molecular detection methods, the technique chosen for pathogen separation and concentration must adequately address three issues that plague environmental and food microbiologists. These issues include (1) separation of pathogens from sample particulates; (2) removal of inhibitory compounds associated with the matrix; and (3) provision of sample size reduction with recovery of virtually all of the pathogens, preferably without disrupting bacterial cell viability.

It would be fair to state that environmental microbiologists, particularly virologists and parasitologists, are perhaps further along in the process of developing pathogen concentration methods. This is because, unlike bacterial pathogens that can usually be amplified by cultural methods, the epidemiogically significant enteric viruses and parasitic protozoa are inert in the sample matrix and even *in vitro* amplification is a challenge. Accordingly, most detection methods for these pathogens are designed to separate organisms from the matrix, providing a low-volume aqueous solution that is free of inhibitory compounds but with high recovery of the infectious agent(s). For viruses, sample manipulations have been based on the tendency of the non-enveloped enteric viruses to behave as proteins in solutions, and their ability to remain infectious even after exposure to organic solvents or to extremes of pH (Jaykus, 2001).

Accordingly, concentration schemes usually employ physical and chemical separation methods such as adsorption, elution, filtration, precipitation, and solvent extraction.

Separation can be defined as the removal of a select population from a complex mixture, while concentration is defined as a sample preparation process that seeks to reduce sample volume while simultaneously recovering all of the initial bacterial population of interest. When applied to food and environmental microbiology, the analyst wishes to separate the bacterial population from the sample matrix; in so doing, the food components are discarded and the remaining bacterial cells may or may not be concentrated in the process (Figure 1.1). Separation and concentration of bacterial pathogens from foods is perhaps more complex than concentration of viral and protozoal pathogens because most bacterial cells are more fragile than these other pathogen types. For instance, virus concentration methods routinely use solvent extraction or high levels of detergent solutions to remove contaminating food components, but unlike their relatively harmless effects on viruses, these substances will destroy bacterial cell viability. A critical need is to develop bacterial concentration methods that do not destroy the bacteria yet are effective in sample preparation.

Methods of bacterial separation and concentration can be categorized as chemical, physical, physico-chemical, or biological approaches, keeping in mind that many methods actually apply any number of these general principles in combination. Furthermore, separation and concentration schemes can be used singly or combination, but in all cases the goal is to provide a sample of extremely small volume with high recovery of viable target bacteria and removal of inhibitory compounds. In the following sections, we will outline the principles of these separation approaches and

provide examples in which they have been applied to the concentration and purification of bacteria from the food matrix.

1.3 CHEMICAL METHODS

1.3.1 Principles of adsorption and desorption

Adsorption, or the assimilation of dissolved matter by the surface of a solid material, is mediated by nonspecific and reversible physicochemical interactions including Van der Waal's forces, electrostatic interactions, hydrophobic interactions, and hydrogen bonding (Glantz et al., 1999, Lukasik et al., 2001, Zarate et al., 2002). Bacterial adsorption to food particulates or other surfaces is mediated by the bacterial cell wall constituents including teichoic acids, proteins and carbohydrate moieties (Meylheuc et al., 2001). When extracting bacteria from food samples, the attractive forces between the bacterial cells and the food components must be disrupted during the separation and prevented from reoccurring, without impacting bacterial cell viability.

Most microorganisms have a net negative charge at a pH of $\geq$ 5.0, largely due to ionization of the carboxyl (-COOH) and amino ($-NH_3$) groups of the cell wall (Thomas, 1988, Payne and Kroll, 1991). This negative charge means that bacteria can potentially be adsorbed to positively charged compounds such as food components and ion exchange resins (Kennedy et al., 1976, Thomas, 1988, Payne and Kroll, 1991). These chemical interactions can be exploited when attempting to develop effective pathogen separation and concentration methods. For instance, enteric viruses can be adsorbed onto food particles by lowering the pH of the food, effectively promoting interactions between the virus particles and the food matrix. The acidified food suspension can be

centrifuged and the supernatant discarded, effectively reducing overall sample volume with recovery of virus.

The opposite of adsorption is desorption, or the process of removing the adsorbed substance from the surface of the solid matrix. Sometimes this method is also referred to as elution. This process is mediated by the same nonspecific and reversible physicochemical interactions between the pathogen surface and the solid matrix. In the case of enteric viruses adsorbed to the surface of a food matrix, exposure to high pH and high salt concentration facilitates desorption of the viruses from the particulates due to disruption of the attractive chemical forces promoted by these conditions. In this case, the alkaline mixture can be centrifuged, the pellet discarded, and the remaining particulate-free supernatant represents a "cleaner" virus-containing suspension. Because bacteria are more fragile than non-enveloped enteric viruses, the rather extreme pH and salt conditions utilized for virus absorption and elution may not necessarily lend themselves to the concentration of bacterial cells from foods. However, the general principles of adsorption and desorption may still be applied to the separation of bacterial cells from complex sample matrices. In addition, genus or species level differences in bacterial cell wall composition may also facilitate the development of separation methods based on differential adsorption and elution (Payne and Kroll, 1991).

1.3.2 Adsorption onto solid surfaces

1.3.2.1 Food components

Historically, the adsorption of bacteria to food products such as meat and produce has been associated with a decrease in microbial recovery during sample

processing for subsequent pathogen detection. For instance, it has been demonstrated that bacteria may selectively adsorb to meat constituents such as collagen. Rodrigues-Szulc et al (1996) used the enzymes collagenase and trypsin to degrade the connective tissue on the surface of raw beef samples to release bound bacteria prior to separation and concentration using differential centrifugation. Sodium chloride was added to prevent reattachment of the bacteria that had been released by the enzyme treatment. The combination of enzyme treatment and centrifugation improved bacterial recoveries by as much as 1.7 $\log_{10}$. The authors, however, found that further alteration of pH or ionic strength had little effect on the degree of detachment of *Escherichia coli* O157:H7 cells from beef tissue.

The ability of bacterial pathogens to adhere to produce surfaces continues to be an impediment to effective bacterial concentration. Kenney et al.(2001) reported that *E. coli* O157:H7 cells may penetrate the surface of apples by as much as 6 µm and that bacterial cells remaining after washing appeared to be sealed within cracks and crevices, making removal difficult. Similar findings have been reported in other produce items. For instance, Takeuchi and Frank (2001) found that *E. coli* O157:H7 cells could penetrate up to 10 µm into lettuce leaves, either through damaged tissue or into stomata. The transfer of *Salmonella* cells to the interior of cantaloupe during cutting and preparation was shown to be due to the multidimensional nature of the rind resulting in microenvironments to which bacteria could bind (Ukuku and Sapers, 2001). Bacterial surface charge, hydrophobicity, and the presence of extracellular polysaccharides are important factors in bacterial attachment and detachment from produce surfaces (Ukuku and Fett, 2002). Lukasik et al. (2001) investigated recovery

methods for bacterial and viral pathogens from the surfaces of strawberries and tomatoes, finding that it was necessary to disrupt the hydrophobic and electrostatic interactions between the target organism and the produce surface in order to achieve optimal elution. When a combination of phosphate buffered saline (PBS) and 0.1% Tween 80 was used as the elution buffer, bacterial recoveries increased an average of 2-fold when compared to a PBS control. It is clear that the phenomenon of bacterial adsorption to the surface of the food matrix reduces bacterial concentration efficiency but it may also be used to enhance bacterial recoveries, although this is a largely untapped area of investigation.

1.3.2.2 Biofilms

No discussion of bacterial adhesion would be complete without mentioning biofilms. Biofilms are biologically active matrices containing microorganisms and associated extracellular polymeric materials which adhere to solid surfaces (Kumar and Anand, 1998). Biofilms represent a natural form of cell immobilization and cellular adhesion (Kumar and Anand, 1998). Bacterial adhesion in biofilms is affected by numerous variables including nutrient availability, type and growth stage of the microorganism(s), pH, temperature and the nature of the surface itself. The forces involved in biofilm formation are similar to those involved in bacterial adsorption described earlier, and include hydrophobic interactions, dipole-dipole interactions, ionic and covalent bonding (Kumar and Anand, 1998). An understanding of the processes involved in biofilm formation and disruption may prove useful in the development of methods to concentrate bacteria from food matrices.

1.3.2.3 Ion exchange resins

It follows that the selective adsorption of bacteria to an ion exchange resin could theoretically allow for the separation of bacteria from a food sample or food homogenate (Table 1.1). Ion exchange resins are typically used in water and protein purification and are comprised of small porous polymer beads that have charged groups attached which can be acidic or basic in nature. Exchange resins have a binding capacity of up to 10^{10} CFU/gram of resin under optimized conditions (Payne and Kroll, 1991). Bacterial release from the resins can be accomplished by alteration of pH as many (but certainly not all) bacterial species are tolerant to wide fluctuations in pH for short periods of time (Payne and Kroll, 1991). When cell viability does not need to be confirmed, as may be the case with some molecular detection methods, the use of even higher pH values may further improve separation of bacteria from the sample matrix (Lindahl and Bakken, 1995). Cationic exchange resins have been used to extract bacterial cells and bacterial DNA from soil samples. For instance, the efficacy of the cationic exchange resins on the recovery of *Pseudomonas cepacia* from soil was reported to be approximately 35% (Jacobsen and Rasmussen, 1992). These resins apparently worked by exchanging polyvalent cations on the soil particles with monovalent cations in the resin, resulting in the release of bacterial cells bound to soil particles and the subsequent binding of the soil particles to the cationic exchange resin (Jacobsen and Rasmussen, 1992). While not yet applied to food matrices, this method may prove to be effective in removing matrix-associated inhibitors with recovery of target bacteria.

1.3.2.4 Lectins

Lectins are carbohydrate-binding proteins that can reportedly adsorb bacterial cells (Table 1.2) (Payne and Kroll, 1991, Patchett et al., 1991). They are ubiquitous in nature and have been isolated from many biological sources including plants, viruses, microorganisms, and animals (Patchett et al., 1991). Lectin proteins can be purified from biological sources using a variety of protein purification techniques including salt-induced crystallization, ethanol precipitation, ion exchange chromatography and affinity chromatography (Patchett et al., 1991, Payne et al., 1992). Lectins bind selectively to the surface components of bacterial cells, in particular, to the N-acetyl glucosamine residue of the peptidoglycan layer. Some authors have found that Gram-positive bacterial cells are bound preferentially (Payne et al., 1992). The binding of bacterial cells to lectins does not appear to exhibit genus or species specificity (Payne et al., 1992). Isolated lectins can be adsorbed onto agarose beads and packed into affinity columns or they can be adsorbed onto magnetic microspheres and used in magnetic capture approaches. Release of the bound bacterial cells is variable and may be difficult to achieve (Payne and Kroll, 1991). Regardless, lectin-based methods have been used to separate bacteria from broth cultures and food samples, usually from dilute food samples and cultures when the organisms are in high concentration (Patchett et al., 1991, Payne et al., 1992). Binding efficacy varies based on lectin source, target organism, and food matrix. For example, the binding efficiency of *Agaricus bisporis* lectin to *Listeria monocytogenes* in broth ranged from 64 to 100% and decreased binding efficiency, ranging from <13 to 50%, was exhibited when the same organism was seeded into ground beef and milk (Payne et al., 1992). Issues associated with

binding specificity, overall recovery efficiency, and food matrix interference still need to be addressed.

1.3.2.5 Dielectrophoresis

Dielectrophoresis is the study of the motion of particles in the presence of a non-uniform electric field Gascoyne et al., 1992, Betts, 1995). The dielectrophoretic process works by using high frequency electric fields (0.1 – 10 MHz) to attract suspended cells to electrodes, independently of the applied field (Coakley, 1997). Cells with different physical and chemical properties will act differently in the electric field, allowing for separation of different cell types (Coakley, 1997). Dielectrophoesis has been effective in separating mammalian cell populations based on cell type, size, morphology and even life stage of the cell (Gascoyne et al., 1992). Recent applications of dielectrophoreis to bacterial cell populations has shown that cells are separated by associating or clumping at the electrode and can be subsequently eluted off via a conductivity gradient (Wang et al., 2000, Ukuku and Fett, 2002). Markx et. al. (1996) used dielectrophoresis to separate *Bacillus subtilus*, *E. coli* and *Micrococcus luteus* in pure cultures and in mixtures from a model liquid system at a concentration of 10^7 CFU/ml. The authors did not quantify the efficacy of the separations, however microscopic examination allowed them to estimate that approximately 50% of the cells were released from the electrode. The use of dielectrophoresis as a means of separating and concentrating bacterial cells from food systems is limited by the need for liquid samples, small sample size, and low conductivity. There is little information about the effect of dielectrophoresis on cell viability, and better quantification of recovery efficiencies is needed before this method can be widely applied.

1.3.2.6 Aqueous Two-Phase Partitioning

Polymer aqueous two-phase partitioning has been purported as a simple and relatively easy technique for separating and concentrating microorganisms (Table 1.2) (Payne and Kroll, 1991, Lantz et al., 1994a). This method is used to partition solutes between two immiscible liquid phases that are combinations of aqueous solutions (Catsimpoolas, 1976). The two phases (usually polyethylene glycol and dextrans) generally have molecular weight differences and a small difference in surface tension in order to facilitate separation, but are not toxic to bacterial cells (Payne and Kroll, 1991). Cells will accumulate either at the interface between the phases or in one of the two bulk phases, depending upon the surface characteristics of the bacteria and their interaction with the polymers (Catsimpoolas, 1976, Payne and Kroll, 1991, Pedersen et al., 1998). The affinity of an individual ion for a specific phase creates an electrostatic potential difference between the phases, which can attract positive or negatively charged cells (Catimpoolas, 1976, Walter, 1977, Pedersen et al., 1998). Bacterial cells carry a net surface charge, which is usually negative, and therefore would be attracted to the phase of opposite charge (Catsimpoolas, 1976). Osmotic pressure of two-phase systems tends to be low, and the inclusion of salts and phosphate buffer is generally recommended to avoid impacting cell viability; this also improves partitioning. Additional chemical modification of the polymers in the two-phase system can be done to optimize the affinity of a phase for a particular cell type (Magnusson and Johansson, 1977).

Magnusson and Johansson (1977) used esterified polyethylene glycol (PEG) (altering the hydrophobicity of the phase) to separate SR and R mutants of *S. enterica*

serovar. Typhimurium however they did not report on percent recovery or the efficiency of the separation. More recently, others have shown that aqueous two-phase systems can be useful tools for partitioning PCR inhibitors and bacterial cells into separate phases. Similar systems have been developed to partition bacterial cells from matrix-associated PCR inhibitors. For instance, Lantz et. al. (1994a) used an aqueous two-phase system to separate *L. monocytogenes* from inoculated cheese homogenates, finding that the bacterial DNA could be detected by PCR from the dextran phase of the two-phase system, but not from the PEG phase. Reextraction of the PEG phase with additional dextran resulted in positive PCR results. These investigators concluded that incomplete partitioning of *L. monocytogenes* occurred using this aqueous two-phase system method, although potential PCR inhibitors were removed when they partitioned into the discarded PEG phase (Lantz et al., 1994a). The authors reported detection limits of 10^4 CFU/ g of *L. monocytogenes* in seeded Danish blue Castillo soft cheese (Lantz et al., 1994b). *L. monocytogenes* and *Salmonella enterica* serovar. Berta spiked into smoked sausage have also been separated from this complex food matrix using a two-phase system comprised of PEG and 5% dextran (Pedersen et al., 1998). In this case, the food particles partitioned into the dextran-rich bottom phase and the microorganisms into the top phase containing PEG. Phase partitioning could be changed by manipulation of polymer selection, polymer concentration, pH, and electrostatic potential between the phases (Pedersen et al., 1998). The best results were achieved for a two-phase system containing 7% PEG and 5% dextran, from which the authors were able to recover 56% of the input serovar. Berta from the hydrophobic

upper phase (pH 6) and 90% of the input *L. monocytogenes* from the bottom phase (pH 3).

In general, limited data is available on the recovery efficiency of microorganisms seeded into foods and partitioned using these two-phase systems and the method needs further analysis to determine if it is appropriate, particularly for foods containing low numbers of the target microorganism. Available data suggests that partitioning is frequently incomplete, and that bacterial loss to the opposite phase may be significant (Lantz et al., 1994a, Pedersen et al., 1998). Two-phase systems also may be difficult to standardize due to sensitivity to temperature, mixing, and chemical composition (Catsimpoolas, 1976). Cell partitioning efficiency in the two-phase system may be adversely affected by the presence of fats or other food components (Payne and Kroll, 1991, Catsimpoolas, 1976). Further work should focus on determining the efficacy and improving the ruggedness of the method, as well as optimization of the protocol for use in food systems.

1.4 PHYSICAL METHODS

1.4.1 Principles of physical separation methods

Centrifugation is a separation method that uses rotation about a fixed axis to produce a centrifugal force. This force propels particles suspended in a liquid medium to sediment, with the rate of sedimentation dependent upon a variety of physical factors (Catsimpoolas, 1976). The Stokes equation can be used to describe the settling of cells in a centrifugal field such that the rate of sedimentation depends on the particle diameter, particle density, solution density, volume, angle and speed of rotation. The sedimentation rate is zero when the particle density is the same as the liquid density.

The sedimentation rate decreases as the liquid viscosity increases and increases as the gravitational force increases (Catsimpoolas, 1976). By manipulating variables such as solution density and particle size, alternative centrifugation methods such as density gradient and differential centrifugation, have been developed.

Centrifugation is generally classified by the relative centrifugal force (relative to the earth's gravitational force) applied. By convention, low speed centrifugation has a maximum relative centrifugal force of 8,000 *x g*. Relative centrifugal forces between 8,000 and 60,000 *x g* are classified as high speed centrifugation. Ultracentrifugation utilizes the highest relative centrifugal force with a maximum of 700,000 *x g*. Centrifugation applied at extremely low speeds (< 1,000 *x g*) promotes sedimentation of food particulates, leaving bacterial cells in the supernatant. When applied at slightly higher *g*-forces (1,000-8,000 *x g*), bacterial cells will sediment, as well as other particles of equal or greater density (Table 1.3).

Filtration is another physical method that can be used to separate microorganisms from a food sample. During filtration, a food product or a food product homogenate is passed through a filter and microorganisms are retained on the filter while the surrounding food sample filtrate is discarded. If desired, the organisms can theoretically be released from the filter using principles of elution. Filter type, pore shape and pore dimensions all contribute to the ability to elute microorganisms from the filter. Alternatively, filters of extremely large pore size (such as cheesecloth or filter bags) can be used to trap particulates while the bacteria flow through the filter and are recovered with the filtrate. In this case, assuring that the bacteria do not bind to the discarded particulates is critical. Historically, filtration has been limited to low

particulate foods that will not clog the filter and by the volume of sample that can be passed through the filter (i.e., sample filterability) (Payne and Kroll, 1991). Sample pre-treatment with enzymes and detergents can increase sample filterability but may adversely affect cell viability (Payne and Kroll, 1991).

1.4.2 Centrifugation methods

1.4.2.1 Simple high-speed centrifugation (<60,000 x g)

Many investigators have used fairly simple centrifugation methods to concentrate bacterial cells from microbiological media before extracting nucleic acids and detecting by DNA hybridization or PCR. For instance, Darby et al. (1970) centrifuged at 23,000 x *g* to harvest *Clostridium welchii* cells from 20 liters of growth medium, and the same centrifugation speed was used to remove these cells from a food sample for subsequent isolation and analysis of nucleic acids and polysaccharides. In pure culture, Fliss et al. (1991) concentrated *Lactobacillus* and *Lactococcus* species in broth culture by centrifuging at 10,000 x *g* before suspending in acetone and proceeding with lysis of the cells. These investigators were able to get excellent yields of high quality nucleic acids for subsequent PCR detection. In a clinical application, Tjhie et al. (1994) used high-speed centrifugation to concentrate bacteria in clinical (blood) specimens before resuspending in a small volume of buffer and performing an enzymatic lysis of *Mycoplasma pneumoniae*. These investigators reported PCR detection limits of 10 CFU/ml using their method.

1.4.2.2 Differential centrifugation

Differential centrifugation is based on differences in the sedimentation rate of particles of differing sizes and densities. This method has been used to separate cells

primarily based on cell size differences using stepwise increases in centrifugation speed (Catsimpoolas, 1976). At each step, the particles of higher density are separated from those that are less dense. The speed of centrifugation is increased until the target particle settles, after which the final supernatant is removed and the pellet is resuspended for further assay. The main advantages of differential centrifugation are that it is rapid, easy, and requires fairly low gravitational forces, while its major disadvantage is that the centrifugal force required to pellet the bacterial cells is frequently sufficient to pellet food components that may inhibit detection methods applied downstream (Catsimpoolas, 1976). The most common application of differential centrifugation is the use of low-speed centrifugation to eliminate heavier particles in foods followed by a higher-speed centrifugation to sediment bacterial cells. For example, Neiderhauser et al. (1992) improved PCR detection limits for *L. monocytogenes* in meat homogenates by 1000-fold after spinning at 100 x *g* to eliminate large food particles, followed by a second centrifugation at 3000 x *g* to collect the bacteria. They reported PCR detection limits of 10^3 CFU *L. monocytogenes*/g meat. This approach was also applied to dairy products by Meyer et al. (1991) who used low speed centrifugation (500 *x g*) after enzymatic digestion of soft cheese to facilitate the removal of fat, followed by high-speed centrifugation (10,000 *x g*) to pellet *E. coli* O157:H7 cells, obtaining PCR detection limits of 10^3 CFU/g. Wegmuller et al. (1993) applied a digestion buffer to various dairy products and then used low-speed centrifugation (3,000 *x g*) to separate the sample into a fat layer, a water layer, and a pellet. The bacteria-containing pellet was lysed, extracted for nucleic acid isolation and subjected to PCR with detection limits of 10^4 CFU *Campylobacter*/ml. Firrao and

Locci (1993) used low speed centrifugation (2,000 *x g*) followed by high speed centrifugation (13,000 *x g*) to separate phytopathogenic mycoplasma-like organisms directly from homogenized plants, and were able to identify the organisms by PCR, although no detection limits were reported. Differential pelleting also can be used as a primary separation procedure in the analysis of multiple cell types, in which case the resulting pellet and/or supernatant can be further fractionated using a second round of differential centrifugation, density gradient centrifugation, or other fractionation technique (Catsimpoolas, 1976).

1.4.2.3 Density gradient centrifugation

In general, differences in density between the cell and the surrounding medium play a minimal role in separation (Catsimpoolas, 1976). However, density gradient centrifugation techniques rely upon a suspending solution that decreases in density from the bottom (highest density) to the top (lowest density) of the tube. This method has been used to separate bacteria that are not adhered to particles. Basically, cells and particulates will migrate to the portion of the tube that is at equilibrium with its own density and form a band, which can be removed for further analysis. Materials commonly used to generate density gradients include sucrose, Ficoll, iodinated media such as Metrzamide and Nycodenz, and Percoll (colloidal suspension of polyvinylpyrrolidone-coated silica particles) (Catsimpoolas, 1976, Lindqvist, 1997). Linqvist (1997) used a combination of cultural enrichment and buoyant density centrifugation with Percoll gradients to separate and concentrate *E. coli* O157:H7 from beef samples. A detection limit of 10^3 CFU/g of meat sample could be obtained without prior cultural enrichment; this was decreased to 0.5 to 5 CFU/g of meat sample if

detection was preceded by cultural enrichment (Lindqvist, 1997). Linqvist (1997) also reported using buoyant density gradient centrifugation to concentrate *E. coli* O157:H7 from beef homogenates, obtaining recovery of 20 to 45% of the input bacterial cells with a corresponding 20 to 40-fold sample volume reduction. PCR detection limits were 10 CFU per amplification volume. Lindahl and Bakken (1995) used Nycodenz gradients to separate *E. coli*, *Bacillus subtilus* and indigenous soil microflora from γ – irradiated, sterile soil. The authors determined that the concentration of divalent cations in the cell suspensions was low enough to allow repulsion between the negatively charged bacteria and soil colloids, thus preventing reattachment during the procedure (Lindahl and Bakken, 1995). Reibach et al. (1981) used differential Percoll gradients to isolate bacteroids (rod-shaped bacteria in the root nodules of nitrogen fixing plants) of soybean plants based on the density difference between the bacteroids and the plant cells. The authors were able to recover ~90% of the initial microbial load from the Percoll gradients (Reibach et al., 1981).

Cell viability must be considered when using density gradient centrifugation since it can be significantly impacted if the osmotic strength of the gradient is too high or too low in comparison to the osmotic strength of the cell. Recovery of cells from a gradient may also be negatively impacted if the gradient viscosity is too high or if repeated washings are necessary to remove the separated cells from the gradient material (Catsimpoolas, 1976). Many foods tend to have density gradients of their own which also interferes with bacterial separation from the food (Payne and Kroll, 1991). Foods containing a high concentration of fats tend to trap bacteria at the fat interface, preventing bacterial separation (Payne and Kroll, 1991).

1.4.2.4 Coagulation and Flocculation

The efficiency of centrifugation can be improved if the particle diameter is increased, and this can be accomplished by either coagulation or flocculation. Coagulation is facilitated by the removal of electrostatic charges (e.g., usually by pH change), which allows particles to adhere to one another, thereby facilitating sedimentation by lower centrifugation speeds. Flocculation is achieved by adding small amounts of high molecular weight, charged materials which bridge oppositely charged particles to produce a loose aggregate which may be readily removed by centrifugation or filtration. These principles have been routinely applied when attempting to concentrate viruses and parasitic protozoa from food and environmental samples, but they should likewise be applicable to bacterial concentration.

While centrifugation methods have met with some success, these are far from optimal. It has been noted that concentration of matrix-associated PCR inhibitors is likely to occur when centrifugation is the only method of sample preparation (Jaykus et al., 1993). For this reason, centrifugation is usually applied in conjunction with other methods. Furthermore, foods that contain fat, such as meat or dairy products, may trap bacteria in the fat globules, preventing sedimentation during centrifugation. Highly viscous foods pose similar problems. Additionally, foods with large particles may entrap bacteria as they settle, even at low centrifugation speeds (Lantz et al., 1994b). It is clear that the interplay between bacterial adsorption and desorption to the food matrix must be considered in conjunction with centrifugation in the development of effective bacterial concentration methods.

1.4.3 Filtration methods

Physical concentration methods such as filtration tend to be less selective and have the added advantage of removing food components that may interfere with subsequent pathogen detection (Table 1.4). The commercially available Iso-Grid method (Neogen Corporation, Lansing, MI) for the detection and quantification of microorganisms is a dual filtration procedure (Payne and Kroll, 1991). The food sample or sample homogenate is first passed through a 5 μm pre-filter to remove gross food particulates. Microorganisms that are bound to food particles may be retained along with the food particle at this pre-filter stage, which can subsequently result in an underestimate of bacterial load. The sample is then passed through a 0.45 μm filter that is hydrophobic and demarcated with a grid pattern. The filter is designed to minimize the spread of colonies and is divided into sections of a known area, to facilitate counting after incubation on the surface of a solid agar plate (Payne and Kroll, 1991). The Iso-Grid methods for *E. coli* O157:H7, *Salmonella*, yeast and mold, coliform / *E. coli* and total aerobic plate count have received AOAC approval. Additional Iso-Grid methods are available for *Listeria* spp., *L. monocytogenes*, *Staphylococcus aureus*, fecal streptococci, total Gram-negative bacteria, *Vibrio parahaemolyticus*, *Yersinia enterocolitica* and *Pseudomonas* spp. Since bacteria tend to have a net negative charge, electropositively charged filters can also be used to separate bacteria from food samples on the basis of charge. While bacterial adsorption to these filters is usually quite efficient, desorption rates are relatively poor (Payne and Kroll, 1991). Thomas (1988) used electropositively charged filters to remove yeast contaminants from wine, finding that recovery varied with filter type and recovery method.

Filtration itself can be followed by detection techniques including staining and epifluorescent microscopy for direct enumeration, or PCR for detection. Walls et al. (1990) used a combination of membrane filtration and epifluorescent microscopy to separate bacteria from a beef homogenate. Bacterial counts by traditional plating techniques and by epifluorescent microscopy were similar, however the overall efficiency of the membrane filtration treatment was not evaluated in this study (Walls et al., 1990). Oyofo and Rollins (1993) investigated the effect of filter type (cellulose, hydrophobic and hydrophilic filters) on recovery and subsequent PCR detection of *Campylobacter* spp. isolated from environmental water samples. The investigators found that five of the nine filters used interfered with PCR amplification. The only filters that did not interfere were the 0.2 – 0.5 μm hydrophobic polytetrafluoroethylene filters and the 0.5 μm polyvinyldiene difluoride hydrophilic (HVLP) filters (Oyofo and Rollins, 1993). The authors proposed that interference with PCR was a direct result of nucleic acids binding to the filters, making them unavailable for amplification (Oyofo and Rollins, 1993).

Other researchers have combined filtration with other methods to process food samples in preparation for detection. For instance, Fernandez-Astorga et al. (1996) pre-treated milk samples with trypsin and/or Triton X-100 before filtering through an Isopore polycarbonate black membrane filter for subsequent enumeration by direct epifluorescence technology (DEFT). Although the direct filtration method was faster, the results were difficult to interpret due to the simultaneous concentration of extraneous matter on the filters. Wang et al. (1992) used Whatman filter paper #4 to remove gross particulate matter from meat and cheese samples, followed by heating the

filtrate to lyse the cells and release the nucleic acids for subsequent detection by PCR. This group was able to detect <10 CFU *L monocytogenes*/g from artificially-contaminated meat products, without prior cultural enrichment, but were unable to get detection from soft-ripened cheese samples processed in a similar manner. In clinical specimens, Bernhardt et al. (1991) processed artificially inoculated blood samples using an aqueous two-phase partitioning step to remove the erythrocytes, followed by filtration through a 0.22 μm filter to trap the bacteria. The authors reported 57 to 90% percent recovery of seeded microorganisms using their combined centrifugation and filtration method (Bernhardt et al., 1991). Kirk and Rowe (1994) used 0.4 μm-pore-size polycarbonate membrane filters to remove *Campylobacter jejuni* and *C. coli* from water samples. When filtration was followed by sonication to release the cells from the filter, and subsequent nucleic acid amplification, these investigators were able to detect as few as 10 CFU *Campylobacter*/ml but only after a prior cultural enrichment step.

When taken together, the use of filtration for bacterial concentration from food samples has been largely unsuccessful and is limited because large particles tend to clog the filters, compounds inhibitory to PCR may also be concentrated with the bacteria, and some filters actually inhibit nucleic acid amplification (Oyofo and Rollins, 1993). In addition, filter pore size must be small enough for efficient trapping of the bacteria, creating limitations related to the type of filters and volumes of samples that can be processed (Sharpe, 1977). Furthermore, microorganisms may become attached to the upper surface of the filter or may become trapped within the filter pores (Thomas, 1988). Issues impacting the recovery of the microorganisms from the filter continue to present a challenge, and it can generally be concluded that bacterial elution from filters

is almost always incomplete. The act of filtration itself may also impair the ability of microorganisms to grow on solid media (Thomas, 1988).

1.4.4 Flow Cytometry

While not a bacterial concentration method per se, flow cytometry is an optically-based method for analyzing individual cells in complex matrices and has occasionally been applied to food systems. Fluorescently-stained microorganisms pass through a beam of laser light and a "signature" pattern is achieved by the combination of both the adsorption and scattering of the light (Breeuwer et al., 1995, deBoer and Beumer, 1999). The method has been suggested for the routine detection of yeasts in fermented milk products and bacterial contamination in frozen vegetables (Laplace-Builhe et al., 1993). Flow cytometry has also been used to distinguish between live and dead yeast cells by dual staining (Hutter and Eipel, 1979) and to differentiate between spores and vegetative cells of *Bacillus* spp. (Muldrow et l., 1982). Donnelly and Baigent (1986) reported its use to detect *L. monocytogenes* in seeded and naturally contaminated milk. Overall, detection limits for this method were estimated to be as few as 10^2 yeast cells or approximately 10^2-10^3 bacterial cells/ml. Detection can be completed in a few minutes and some believe that the method is suitable for detecting low numbers of specific microorganisms in fluids or rinses (deBoer and Beumer, 1999, Marie et al., 1999). In a pure culture system, McClelland and Pinder (1994) were able to detect specific serotypes of *Salmonella* at levels of 10^4 CFU/ml within 30 minutes. When applied to eggs and milk, it was possible to detect *Salmonella enterica* serovar. Typhimurium at 10^3 CFU/ml using monoclonal antibodies and 1 CFU/ml after a 6 hour enrichment, although confirmation by traditional cultural methods was required. The

main drawbacks of flow cytometry are the high cost of the equipment and the need for specialized training of personnel. Additional methodological problems include interference by non-specific fluorescence or by particulate matter, less than optimal detection limits, difficulty in applying the method to solid or particulate food samples, and the inability to differentiate between viable and dead cells unless specialized staining is used (Van der Zee et al., 1997, Quintero-Betancourt et al., 2002). Destruction of cellular viability may also occur during sample processing. All told, the method is not very promising for routine use by food microbiologists.

1.4.5 Ultrasound

Ultrasound waves (sonication) have been used to promote detachment of microorganisms from surfaces such as filters, produce and seeds (Thomas, 1988, Kirk and Rowe, 1994, Scouten et al., 2002, Seymour et al., 2002). Ultrasound separations result in clumps of target cells that are removed from the system by physical manipulation, in this case via changes in the frequency (Coakley, 1997, Limaye and Coakley, 1998, Coakley et al., 2000). Limaye and Croakley (1998) achieved 96% recovery of *Saccharomyces cerevisiae* and 72% recovery of *E. coli* in a 5 ml culture system after application of ultrasound. Recovery efficiency was dependent upon cell concentration, with poor recoveries noted at populations $<10^7$ CFU/ml. Using a combination of filtration and ultrasound, Hawkes and Coakley (1995) were able to achieve a 5-fold reduction in sample volume and 99.9% recovery of yeast cells when a high innoculum was used. The use of ultrasound technology for separation and concentration of bacterial cells is limited by the need for high cell concentrations, low

sample volume, and limited data on recovery efficiency or the effect of ultrasound on bacterial cell viability.

1.5 PHYSICO-CHEMICAL METHODS

1.5.1 Principles of Physico-Chemical Methods

Many of the methods that show promise for use in bacterial separation and concentration combine physical and chemical principles. Of particular interest in recent years has been bacterial immobilization using metal hydroxides (Table 1.1). This method is most consistent with the principle of flocculation, which is achieved by the addition of a high molecular weight, charged material, which bridges oppositely charged particles to produce a loose aggregate. This aggregate is easily removed by physical methods such as low-speed centrifugation or filtration, thereby facilitating separation and/or concentration based on both physical and chemical principles.

1.5.2 Metal hydroxides

Kennedy et. al. (1976) first demonstrated the use of titaneous and zirconium hydroxides to immobilize bacterial cells for subsequent use in enzymatic reactors. The proposed method did not negatively impact bacterial cell viability yet enabled the authors to eliminate tedious enzyme purification procedures and therefore enhanced the ease of product recovery. These investigators hypothesized that replacement of hydroxyl groups on the surface of the metal hydroxide complexes with suitable ligands from bacterial cells resulted in the formation of covalent bonds and subsequent adsorption of the organism to the metal hydroxide.

The use of metal hydroxides to concentrate bacteria in clinical samples was later reported by Ibrahim et al (1985a). Their initial experiments used titanous hydroxide to

immobilize various Gram-negative (*Salmonella* spp., *Citrobacter freundii, E. coli, Serratia marcescens, Shigella dysenteriae*) and Gram-positive (*Bacillus cereus, Staphylococcus* spp., *Streptococcus faecalis*) organisms, with recovery rates varying from 90–98%. In a later study, these same investigators (used titanous hydroxide as an immobilization platform for subsequent solid-phase ELISA detection of *Salmonella*, reporting 100 to 160-fold improved detection limits when applied to food enrichment broths (Ibrahim et al., 1985b). In a related study, Berry and Siragusa (1997) utilized hydroxyapatite to immobilize and concentrate *E. coli, E. coli O157:H7, L. monocytogenes, S. enterica* serovar. Typhimurium*, S. aureus*, and *Pseudomonas fluorescens* from growth or enrichment media, with recoveries ranging from 45% for *E. coli* O157:H7 to 99% for *S. aureus*. When the method was applied to meat slurries, recoveries were more variable (9.5-99%).

Lucore et. al. (2000) built upon the foundation laid by Kennedy et. al. (1976)and Ibrahim et al. (1985a,b) by using metal hydroxide immobilization to concentrate bacteria from pure culture and from several food commodities. Using reconstituted non-fat dry milk (NFDM) as a model, two foodborne pathogens (*L. monocytogenes* and *S. enterica* serovar. Enteritidis) were concentrated from 25 ml samples by the sequential steps of clarification and high-speed centrifugation followed by immobilization with zirconium hydroxide and low-speed centrifugation. Sample volume reduction after immobilization with zirconium hydroxide was 50-fold, with total bacterial recoveries ranging from 78-96% of input for serovar. Enteritidis and 65-96% of input for *L. monocytogenes*. Immobilized bacteria remained viable and could be enumerated by standard cultural procedures. When followed by RNA extraction and subsequent

detection by RT-PCR, detection limits of 10^1-10^2 CFU/25 ml reconstituted NFDM were achieved for both organisms. The bacterial immobilization step was relatively non-specific, resulting in recovery of >50% of the input cells when evaluated on a panel of representative (pathogenic and non-pathogenic) bacterial strains of significance to foods. Furthermore, the method was adaptable to more complex dairy products such as whole milk and ice cream. Overall, bacterial immobilization using metal hydroxides was easy, rapid and inexpensive and the method has since been used by others (McKillip et al., 2000). Recently, Cullison and Jaykus (2002) have effectively coupled the zirconium hydroxide suspensions to magnetized carbonyl iron, allowing the hydroxide-bacteria complexes to be separated by exposure to a magnetic field and facilitating bacterial concentration without the need for low-speed centrifugation.

1.6 BIOLOGICAL METHODS

1.6.1 Immunoaffinity

In 1979, Professor John Ugelstad of Norway developed a method for the production of uniformly sized polystyrene. Further research efforts resulted in "superparamagnetic" particles, i.e., particles that are magnetized only in the presence of a magnetic field, and which demagnetize and are readily resuspended upon removal of the field. Soon thereafter the concept of immunoaffinity was born, in which antibodies are attached to the surface of these superparamagnetic particles, allowing specific capture and isolation of intact cells directly from a complex sample suspension without the need for column immobilization or centrifugation. This discovery, along with related research, revolutionized the isolation and separation of many biological materials and the method is now generically referred to as immunomagnetic separation

(IMS). IMS was originally developed for the isolation of blood cells (Lea et al., 1985, Gaudernack et al., 1986), but the technique has since been applied to pathogen isolation from food samples (Skerve et al., 1990, Cudjoe et al., 1991) and was later commercialized in the 1990's.

There is an abundance of literature on the use of IMS, and most applications for foodborne pathogen detection rely on monoclonal antibodies attached to magnetic spheres for the selective isolation of target cells from a mixed population. This selective separation relies upon the specificity of monoclonal antibodies directed against a specific and unique cell surface antigen. The efficacy of IMS is dependent upon the specific monoclonal antibody, the size and surface area of the antibody-coated particles, the recovery procedure and sample matrix interference (Gee, 1998, Rochelleet al., 1999). Monosized superparamagnetic polymer particles known as "Dynabeads" are available commercially from Dynal (Oslo, Norway). These particles have a narrow size distribution and consist of iron oxide grains (γ-Fe_2O_3 (magnetite) or Fe_3O_4) evenly distributed throughout the particle (Ugelstad et al., 1993). IMS has been shown to be an effective tool for the separation and isolation of target foodborne pathogens including *L. monocytogenes*, *Bacillus stearothermophilus*, *Vibrio parahaemolyticus, E. coli* O157:H7, *Salmonella* spp., *Cryptosporidium*, and enteric viruses from mixed cell populations (Skerve et al., 1990, Tomoyasu, 1992, Fluit et al., 1993, Yu and Bruno, 1996, Schwab et al., 1996, Blake and Weimer, 1997). IMS is included as a standard method for the detection of *Salmonella* spp. and *E. coli* O157:H7 in foods (AOAC, 1995). In general, detection limits of 10^3 CFU/ml of culture or food homogenate have been reported for IMS; these detection limits are lower if IMS is preceded by cultural

enrichment (Table 1.5). Since foodborne pathogens are usually present in small numbers, this means that cultural enrichment remains necessary prior to IMS and detection.

Magnetic separation principles actually can be applied to a wide range of other receptor – ligand interactions (Cullison and Jaykus, 2002). Alternatively, the beads can be packed in other forms. For instance, Molloy et al (1995) coated polystyrene beads with antibody fragments produced against *Pseudomonas aeruginosa* and packed them into minicolumns for separation purposes (Molloy et al., 1995). The immobilized beads with antibody fragments retained 95% of culture suspension at a concentration of 10^7 CFU/ml, but only 75% of the bacterial cells were retained when a fat-free milk suspension seeded at the level of 10^6 CFU/ml was processed through the column (Molloy et al., 1995). The authors theorized that the decreased efficiency of binding may have been the result of the soluble milk proteins covering the antibody fragments, preventing association between the bacteria and the antibody binding sites. Flow through immunoassay (FIA) is an IMS-based method that uses antibodies immobilized onto some type of solid support such as beads or membranes and is amenable to automation (Bouvrette and Luong, 1995, Bereczki and Horvath, 1999). When flow-injection techniques were used in combination with antibody coated membrane filters, Abdel-Hamid et. al (1999) were able to detect 100 CFU/ml of inactivated *E. coli* and *Salmonella* in less than 30 minutes from a culture system. Work by Weimer et. al. (2001) used a fluidized bed of large sized beads (>3 mm) with bound antibodies to separate bacterial cells from environmental water samples. Based on microscopic examination, these investigators reported that 13% of *Bacillus globigii* spores were

retained by the immobilized beads. The use of beads larger than those typically used in IMS may help reduce interference and clogging caused by food particulates.

It is clear that IMS has several advantages over other sample preparation methods, including effective separation of target bacteria from competitive microflora, the removal of food components and potential inhibitors of molecular detection methods, and the reduction of sample volume. However, despite its promise, the method still requires fairly high cell loads to be effective, which to date has necessitated the continued use of cultural enrichment. IMS is highly specific, which may be an advantage for some applications but not for others. Also, bound organisms may be lost during the subsequent washing procedures, and matrix particulates and other components may interfere with IMS through either non-specific binding or bacterial adherence. In addition, IMS can also be quite costly and usually only small volumes are processed at a time.

1.6.2 Bacteriophage

The potential of bacteriophage to selectively separate and concentrate bacterial cells was first demonstrated by Bennett et al. (1997). These investigators used a *Salmonella*-specific lytic bacteriophage immobilized to microtitre plates to separate and concentrate *Salmonella* in pure culture, generating a PCR-detectable product at concentrations of 10^7 CFU/ml and higher. Bacteriophage-based detection methods for *E. coli* O157:H7 and *Salmonella* species from foods are commercially available (Goodridge et al., 1999, Favrin et al., 2001). The bacterial ice nucleation method (BIND®, IDEXX, Inc., Westbrook, ME) detects *Salmonella* cells by infecting them with genetically modified bacteriophage encoding an ice nucleation gene (Irwin et al.,

2000). Transfected *Salmonella* cell supernatants freeze when supercooled, causing a color reaction (Irwin et al., 2000). Detection limits for these assays are reported to be as low as 2 – 16 CFU/ml after a brief cultural enrichment. While not a concentration method per se, bacteriophage-based detection assays illustrate the potential application of this technology for the separation and concentration of food-borne pathogens.

Although there is only limited information to date, bacteriophage systems appear to be highly species-specific, require a large concentration of the target organism and are not compatible with culture-based identification systems due to the lytic nature of the bacteriophage. Additional research will be necessary to expand the range and specificity of candidate phage, to optimize phage binding efficiency and immobilization, to further apply these methods to food systems, and to improve overall detection limits prior to widespread application of this technology.

1.7 SUMMARY

Without question, the major impediment to our ability to detect foodborne pathogens in "real" or near "real"-time is the continued reliance on cultural enrichment. Indeed, decreasing or eliminating the need for enrichment would allow food and environmental microbiologists the opportunity to truly harness the power of the emerging molecular detection methods. It should be clear from the preceding discussion that further research into effective matrix preparation, specifically targeting bacterial concentration and purification methods, will be necessary if we are to move forward toward achievement of this goal. The ideal method would be able to both concentrate pathogens and remove matrix-associated inhibitors; further, it would be universal (e.g., applicable to multiple food types and microorganisms), simple, rapid,

and inexpensive. This should be done in a manner that minimizes the chance for false positive results that might occur because of cross-reactivity with residual matrix components or because of the detection of dead target cells. Furthermore, it would be best if these assays were available on a commercial basis both inexpensively and with full certification from organizations such as the Association of Official Analytical Chemists (AOAC). Each of the techniques described in this review meet some but certainly not all of these criteria. The need for combined and/or sequential methods is apparent; novel as-yet unreported approaches are also needed. It is our hope that this review will stimulate creative thought and additional research so that the possibility of near real-time detection of foodborne pathogens is not a distant hope but a realistic and attainable goal.

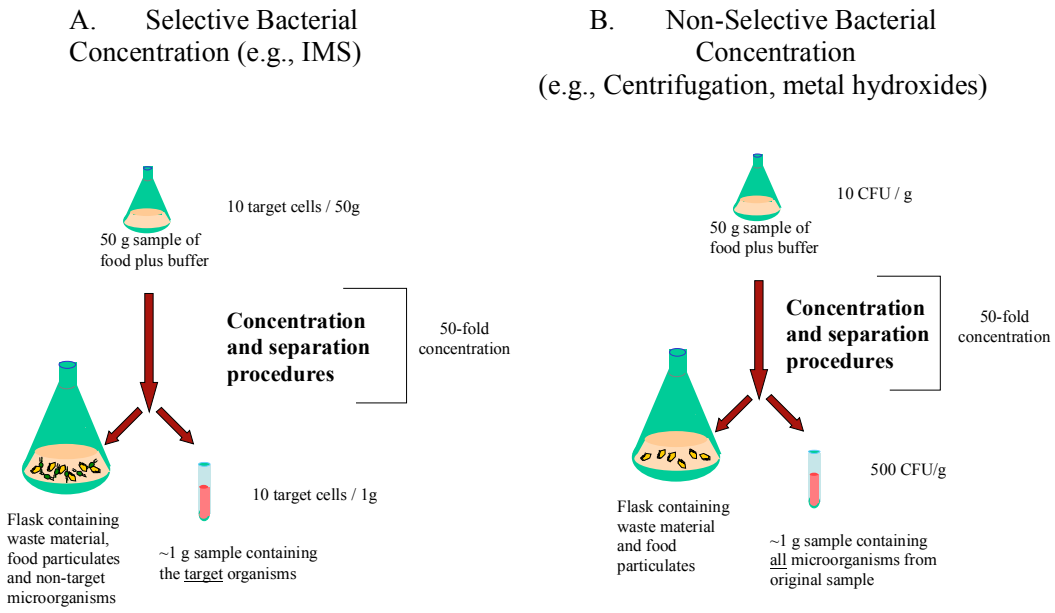

Figure 1.1. Schematic of bacterial separation and concentration principles.

TABLE 1.1: BACTERIAL CONCENTRATION APPROACHES – ADSORPTION AND IMMOBILIZATION

METHOD/EXAMPLES	PRINCIPLE/APPLICATION	ADVANTAGES/EFFICACY	COMMENTS	REF
Ion Exchange Resins	Cationic exchange resins bind bacteria by ion exchange; release of bacteria from resin accomplished by pH manipulation	Rapid; Relatively Inexpensive; Relatively non-specific	Sample pre-treatment to remove debris recommended; pH manipulations needed for desorption destroys cell viability	
Example	*Soil; Pseudomonas cepacia*	*35% recovery*	*Used in combination with differential centrifugation (960 x g followed by 22,100 x g).*	*Jacobsen and Rasmussen, 1992*
Metal Hydroxides	Hydroxides of zirconium, titaneous, or hydroxyapatite; Used in conjunction with centrifugation	Rapid; Inexpensive; Simple; Non-specific; Amenable to large sample sizes	Sample pre-treatment to remove debris required; appears to work best on simpler sample matrices	
Example	*Nonfat dry milk; L. monocytogenes and serovar Enteritidis; zirconium hydroxide*	*65 – 96% recovery*	*Used in combination with differential centrifugation*	*Lucore et al., 2000*
	Nonfat dry milk; L. monocytogenes, serovar Enteritidis, Listeria monocytogenes, Bacillus cereus spores	*75 - >90% recovery*	*Used in combination with centrifugation; zirconium hydroxides were combined with magnetized carbonyl ion*	*Cullison and Jaykus, 2002*
	Beef; E. coli	*9 – 99% recovery*	*Used in combination with fluorescent staining*	*Berry and Siragusa, 1997*

TABLE 1.2: BACTERIAL CONCENTRATION APPROACHES – LECTINS AND AQUEOUS TWO-PHASE PARTITIONING

METHOD/EXAMPLES	PRINCIPLE/APPLICATION	ADVANTAGES/EFFICACY	COMMENTS	REF
Aqueous Two-Phase Partitioning	Cells partition in one of two immiscible liquid phases (PEG and dextrans) based on charge	Rapid; Inexpensive; Simple; Relatively non-specific	Partitioning frequently incomplete; Composition of the phases may impact cell viability; fat interferes with separation	
Example	*Separation of serovar Typhimurium mutants*	*Recovery efficiency not reported*	*Two-phase system contained a mixture of PEG 6000 and dextran T 500.*	*Magnusson and Johanssoon, 1977*
	Soft cheese; L. monocytogenes	*PCR detection limit of 10^4 CFU/g of cheese*	*Improved PCR detection from soft cheese matrix when two-phase system used; PEG and dextran*	*Lantz et al., 1994a*
	Sausage; L. monocytogenes and serovar Berta	*56 – 90% recovery*	*Phase partitioning manipulated using pH, polymer concentration, and addition of salt*	*Oedersen et al., 1998*
Affinity Separation	Immobilization of molecules (lectins) with high affinity for bacteria to a solid support such as agarose beads, affinity columns, or magnetic particles	Rapid; Simple; Specificity unknown	Expensive; Sample pre-treatment to remove debris recommended; release of bound cells may be inefficient; best applied to small sample volumes	
Example	*Broth; L. monocytogenes and Salmonella spp.*	*33 – 215% recovery*	*Affinity chromatography format*	*Patchett et al., 1991*
	Ground beef and milk; L. monocytogenes	*<13 – 50% recovery*	*Lectins were immobilized onto magnetic particles*	*Payne et al., 1992*

TABLE 1.3: BACTERIAL CONCENTRATION APPROACHES - CENTRIFUGATION

METHOD / EXAMPLES	PRINCIPLE / APPLICATION	ADVANTAGES and EFFICACY	LIMITATION	REF.
Simple Centrifugation	Low Speed (<1,000 x g) sediments debris High speed (>8,000 x g) sediments bacteria Used with or without coagulation or flocculation	Rapid; Inexpensive; Simple; Non-specific; Amenable to large sample sizes	Bacteria adhere to and sediment with matrix components; best if preceded by an elution step	
Example	*Seafood and soft cheese; E. coli, Listeria spp., Bacillus spp., Shigella spp., Yersinia spp. and Salmonella spp.*	*Detection limit > 5 x 10^4 CFU / PCR reaction*	*9,000 x g followed by PCR*	*Wang et al., 1997*
Differential Centrifugation	Low speed centrifugation followed by high speed centrifugation; Used with or without coagulation or flocculation	Rapid; Inexpensive; Simple; Non-specific; Amenable to large sample sizes	Bacteria adhere to and sediment with matrix components; few products available to promote desorption without destroying cell viability	
Example	*Meat homogenate and L. monocytogenes* *Soft cheese and E. coli O157:H7*	*1,000- fold improvement in PCR detection limit* *PCR detection limit of 10^3 CFU/g*	*100 x g followed by 3,000 x g* *500 x g followed by 10,000 x g; in combination with enzyme digestion*	*Neiderhauser et al., 1992* *Meyer et al., 1991*
	Raw and cooked beef; E. coli, serovar Enteritidis, S. aureus and L. innocua	*Used confocal microscopy to visualize total number of bacteria*	*1,600 x g for 2 minutes followed by 1,600 x g for 20 minutes; in combination with enzyme digestion*	*Thomas, 1988*
Density Gradient Centrifugation	Cell separation by centrifugation within a density gradient; Requires use of chemical additives to establish a gradient	Can be designed to separate different species from one another	Expensive; difficult to perform; Osmotic strength of gradient destroys cell viability; fat entraps bacteria at interfaces	
Example	*Beef homogenate and E. coli O157:H7*	*20 – 45 % recovery*	*16,200 x g*	*Lindqvist, 1997*

TABLE 1.4: BACTERIAL CONCENTRATION APPROACHES - FILTRATION

METHOD/EXAMPLES	PRINCIPLE/APPLICATION	ADVANTAGES/EFFICACY	COMMENTS	REF
Crude Filtration	Cheesecloth; Filter paper; Filter homogenizer bags	Rapid; Inexpensive; Simple; Non-specific; Amenable to large sample sizes	High particulate foods clog filters; bacterial cells can absorb to the filter or retentate	
Example	*Cheese and L. monocytogenes*	*PCR detection limit of 5 – 15 CFU/100ml of cheese homogenate*	*Used a sieve and separatory funnel in combination with centrifugation and enzyme digestion*	*Uyttendaele et al., 2000*
	Minced beef and natural microflora	*Recovery efficacy not evaluated*	*Combination of homogenizer bag, enzyme treatment, ultra-brief centrifugation and 0.6µm polycarbonate filter*	*Oyofo and Rollins, 1993*
	Milk and E. coli	*10 – 95% recovery*	*Used in combination with enzyme treatments*	*Fernandez-Astorga et al., 1996*
Electro-positive / negative Filtration	Bacteria tend to have a net negative charge, so electropositive filters often used; sample prefiltration to remove debris frequently required	Rapid; Inexpensive; Simple; Non-specific	Rapid filter clogging even if samples are pre-filtered; applicable to small volumes only; desorption of bacteria from filters frequently inefficient	
Example	*Yeast and Lactic acid bacteria from wine and beverages*	*74 - 100 % recovery*	*Filters were directly plated, elution efficacy from the filters was not evaluated*	*Thomas, 1988*

TABLE 1.5: BACTERIAL CONCENTRATION APPROACHES - IMS

METHOD/EXAMPLES	PRINCIPLE/APPLICATION	ADVANTAGES/EFFICACY	COMMENTS	REF
Immunoseparation	Immobilization of monoclonal antibodies to a solid support such as polystyrene beads or magnetic particles	Rapid; Simple; Highly specific; Standard method for some foods	Expensive; Sample pre-treatment to remove debris recommended; best applied to small sample volumes	
Example	*Beef; E. coli O157:H7*	*77 – 121% recovery*	*Used in conjunction with many other concentration and detection methods to avoid lengthy enrichments*	*Fratamico et al., 1992. Restaino et al., 1992, Pyle et al., 1999.*
	Poultry and beef; Salmonella spp.	*31% recovery*	*High non-specific binding*	*Ripabelli et al., 1999, Cudjoe et al., 1995*
	Fish; E. coli O157:H7 and Salmonella spp.	*Detection limit 100 – 1,000 CFU/g*	*Direct detection without cultural enrichment*	*Yu and Bruno, 1996*
	Cheese and L. monocytogenes	*Detection limit 0.5 – 1.5 CFU/g cheese*	*Direct detection without cultural enrichment*	*Uyttendaele et al., 2000*
	Cheese and L. monocytogenes	*5 – 15% recovery, non-specific binding*	*Compatible with molecular detection methods*	*Skerve et al., 1990, Fluit et al., 1993*
	Skim milk; Bacillus stearothermophilus spores	*Detection limit 10^3 CFU/ml*	*Direct detection without cultural enrichment*	*Blake and Weimer, 1997*

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CHAPTER 2

DIRECT DETECTION OF BACTERIAL PATHOGENS IN REPRESENTATIVE DAIRY PRODUCTS USING A COMBINED BACTERIAL CONCENTRATION-PCR APPROACH

2.1 ABSTRACT

It has been suggested that the efficacy of rapid pathogen detection could be expanded if the bacteria were concentrated from the sample matrix before detection. The purpose of this study was to develop a concentration-detection strategy for two representative bacterial pathogens in a complex dairy food matrix. Eleven-gram samples of plain nonfat yogurt or mild cheddar cheese were seeded with *L. monocytogenes* or *S. enterica* Enteritidis at levels of 10^2 - 10^6 CFU per sample. Samples were then processed for bacterial concentration using high-speed centrifugation (9,700 x g) followed by detection using both cultural and molecular methods. Recovery efficiency was calculated based on direct plating of the retained centrifugation pellets and confirmed by enumeration of bacteria remaining in the discarded supernatants. Bacterial recoveries after centrifugation ranged from 53 - >100% and 69 - >100% for serovar. Enteritidis and *L. monocytogenes,* respectively, in both product types. There were no significant differences in recovery efficiency at different inocula levels, and losses to discarded supernatants were always <5 %, regardless of dairy product or pathogen. When followed by pathogen detection using PCR, detection limits of 10^3 and 10^2 CFU per 11 g sample were achieved for *L. monocytogenes* and serovar. Enteritidis, respectively, in both product types and without

prior cultural enrichment. This study represents progress toward the rapid and efficient direct detection (without cultural enrichment) of pathogens from complex food matrices at detection limits approaching those that might be anticipated in naturally contaminated products.

2.2 INTRODUCTION

Enzymatic nucleic acid amplification methods such as the polymerase chain reaction (PCR) and nucleic acid sequence-based amplification (NASBA) offer several advantages for the rapid and reliable detection of microbial pathogens in foods. A primary advantage of these technologies is the potential to replace cultural enrichment with specific nucleic acid sequence enrichment, thereby decreasing detection time. Unfortunately, large sample volumes ($\geq$25 ml or g) compared to small amplification volumes (10-50 µl), residual food components that inhibit enzymatic reactions, low levels of contaminating pathogens, and the presence of competitive microflora which may interfere with amplification and detection reactions have been consistent stumbling blocks to the widespread use of nucleic acid amplification for pathogen detection in foods (Bej and Mahbubani 1994). Because these issues together result in reduced test specificity and sensitivity, researchers working in the field are rarely able to achieve detection limits less than 10^2-10^3 CFU of the target pathogen per gram of food product, levels that are only slightly better than ELISA and DNA hybridization (Lantz *et al.* 1994). By and large, cultural enrichments are still necessary to provide sufficient target amplification even before the application of nucleic acid amplification.

It has been suggested that the adoption of many rapid detection technologies could be expanded if the bacteria were separated, concentrated, and purified from the

sample matrix before detection (Swaminathan and Feng 1994; de Boer and Beumer 1999; Wilson 1997). This approach commonly precedes the detection of viral and parasitic agents in food and environmental samples (Jaykus *et al*. 2001) and would offer such advantages as facilitating the detection of multiple pathogens; removal of matrix-associated reaction inhibitors; and provision of adequate sample size reductions to allow for the use of representative food sample sizes and/or smaller media volumes. Furthermore, prior bacterial concentration could facilitate the detection of low levels of pathogens or sporadic contamination, and perhaps reduce or even eliminate the need for cultural enrichment prior to detection. Although methods such as centrifugation, filtration, and immunomagnetic separation have been reported for bacterial concentration in food systems, none of these is ideal and in many cases a technique optimized for one food matrix or microorganism is not readily adaptable to others (Payne and Kroll 1991; Lantz *et al*. 1994).

In development of rapid detection methods, fermented dairy products have been particularly challenging because they are compositionally complex, and contain food-associated components and high levels of background microflora that often interfere with detection assays, resulting in less than optimal detection limits. In general, authors have found differences of ten-fold or more in detection limits when results from seeded dairy matrices are compared to results from pure culture, even after the incorporation of procedural modifications such as immunomagnetic separation, increased magnesium concentration to improve amplification efficiency, and prior DNA purification (Wernars *et al.* 1991; DeRidder 1993; Fluit *et al.* 1993; Bickley *et al.* 1996; Herman and Wilson 1997). For instance, Wernars *et al.* (1991) found that, depending on the type of cheese

analyzed, PCR detection limits increased from 10^3 CFU to as high as 10^8 CFU when *L. monocytogenes* was detected in pure culture versus the cheese matrix. The purpose of this study was to develop a simple method to non-selectively concentrate bacteria and remove inhibitors from complex fermented dairy food matrices in preparation for direct (without prior cultural enrichment) detection of target bacterial pathogens using nucleic acid amplification methods.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial Cultures and Recovery Media.

Stock cultures of *Salmonella enterica* serovar. Enteritidis (ATCC 13076) and *L. monocytogenes* (ATCC 19115) were obtained courtesy of Dr. Brian Sheldon, Department of Poultry Science, North Carolina State University. Stock cultures were propagated aerobically overnight at 37°C in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI) before experiments. In seeding experiments, serial ten-fold dilutions were done in 0.1% peptone (Difco) and plating for recovery performed using the spread plate technique. Oxford Medium Base (Difco, Detroit, MI) supplemented with Oxford Medium Supplement (Difco) was used for the enumeration of *L. monocytogenes* and XLD Agar (Difco) media was used for the enumeration of serovar. Enteritidis. Oxford medium and XLD plates were incubated for 24-48 hours at 37°C prior to counting colonies. Recovery of endogenous bacterial populations in cheddar cheese was done using Elliker's agar (Difco). Endogenous bacteria associated with yogurt were enumerated on Yogurt Lactic Acid (YLA) agar (Matalon and Sandine, 1986), produced by the addition of 0.1% Tween 80 (v/v) (Sigma Chemical Co., St. Louis, MO) to Elliker's agar.

2.3.2 Bacterial concentration applied to Plain Nonfat Yogurt and Cheddar Cheese.

The bacterial concentration scheme is outlined in Fig. 2.1. Eleven-g samples of plain, nonfat yogurt or mild cheddar cheese were mixed with 25 ml sterile saline and 8 ml 25% w/v sodium citrate (Fisher Scientific, Fair Lawn, NJ) as previously described (Lucore *et al.* 2000). Each sample was supplemented with polyethylene glycol (PEG) 8000 (Fisher Scientific, Fair Lawn, NJ) to reach a final concentration of 4% and stomached for 2 min at room temperature. In order to remove large matrix- associated particulates, a filter stomacher bag (Nasco, Ft. Atkinson, WI) was used for mild cheddar cheese samples, whereas non-fat yogurt samples were strained through sterile cheesecloth after stomaching. The samples were centrifuged at 9,700 *x g* (Sorvall RC-5B, Dupont Company, Wilmington, DE) for 15 minutes at 4°C. The resulting supernatant (~28 – 32ml) was decanted and the remaining pellet (~1-5g) was resuspended in 5 ml 0.9% saline with thorough mixing by vortex. Both the discarded supernatant and the bacteria-containing pellet were plated for recovery. Percent recovery or loss after centrifugation was evaluated using two separate formulae and as previously described (Lucore *et al.* 2000). In the first, recovery was based on direct plating of the precipitated pellet and was calculated as follows: [% recovery = (total population in pellet after centrifugation) * 100/(total population in sample before centrifugation)]. In the second, percent loss to supernatant was calculated based on direct plating of the supernatant as follows: [% loss to supernatant = total population in supernatant after centrifugation) x 100/(total population in sample before centrifugation)]. This was transformed an estimate of overall % recovery using the

following formula: [% recovery = (100 - % loss to supernatant)]. All experiments were done in triplicate. When statistical comparisons were necessary, analysis of variance and the Tukey-Kramer multiple comparisons test were done on % recovery data using the InStat 2 Statistical Analysis Package (GraphPad Software, San Diego, CA).

2.3.3 DNA Extraction

Centrifugal pellets (1 – 5g) were resuspended in DNAzol® BD Reagent (Invitrogen, Carlsbad, CA) in a volume ratio of 1:2 (pellet : DNA) and the DNA extracted as per manufacturer's instructions. Briefly, 400 µl of isopropanol was added to 1.5 ml of the resuspended pellet, mixed and centrifuged at 6,000 x g. The resulting pellet was washed with 1ml of DNAzol® BD and 1 ml of 75% ethanol. The final pellet was resuspended in 200 µl sterile deionized water. Additional purification of the DNA extract was achieved by centrifugation through a QIAshredder® (QIAGEN Inc, Valencia, CA) column for 2 min at 11,750 x g (Sair *et al.* 2003). The resulting filtrate was retained for nucleic acid amplification.

2.3.4 PCR Amplification

PCR reactions were performed using the GeneAmp® PCR kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Each 100µl reaction contained 1x PCR buffer, 200µM each dATP, dCTP, dTTP, and dGTP, 0.5mM dithiothreitol, and 2µl of DNA extract. For serovar Enteritidis, of 0.1 µM of each primer (Table 2.1), 2.5 units Ampli*Taq* Gold DNA Polymerase (Applied Biosystems), and 1.5 mM magnesium chloride was used. For *L. monocytogenes*, 0.2µM of each primer (Table 2.1), 2.5 units Ampli*Taq* DNA polymerase, and 3 mM magnesium chloride was used. The amplification mixtures were overlaid with 50µl of mineral oil.

PCR amplification for *L. monocytogenes* consisted of one cycle at 94°for 2 minutes, followed by 40 cycles of 95°C for 40 seconds, 60°for 30 seconds, 72°C for 1 minute, and a final extension of 72°C for 10 minutes. PCR amplification for serovar Enteritidis consisted of one cycle at 95°for 5 minutes, followed by 35 cycles of 95°C for 90 seconds, 58°C for 80 seconds, 72°C for 2 minutes, and a final extension of 72°C for 7 minutes. Temperature cycling was done in a Perkin Elmer DNA Thermal Cycler (Perkin Elmer, Norwalk, CT). The 858 bp fragment PCR product from *L. monocytogenes* and the 389 bp PCR product from serovar Enteritidis were separated and visualized under ultraviolet light after agarose (1%) gel electrophoresis and ethidium bromide staining.

2.3.5 PCR Enhancement Agents

The inhibitory effect of the sample matrices on PCR amplification was initially evaluated by serial dilution PCR on DNA extracted from yogurt or cheese samples seeded with *L. monocytogenes* and serovar. Enteritidis as previously described (Jaykus *et al*. 1996). Various chemical additives were tested in an effort to overcome this matrix-associated PCR inhibition. Specifically, dimethyl sulfoxide (DMSO) (3% per reaction) (Sigma, St. Louis, MO), PEG 8000 (2.5% per reaction) (Sigma), dithiothreitol (DTT) (0.5mM per reaction) (Sigma), and glycerol (8% per reaction) (Sigma) were all evaluated. An alternative second generation *Taq* DNA polymerase, Ampli*Taq* Gold, was also evaluated.

2.3.6 Southern Blot Hybridization

To confirm the identity of PCR amplicons, agarose gels were transferred to positively charged nylon membranes (Roche, Indianapolis, IN) using the method of

Southern and hybridized with a digoxigenin (DIG)-labeled DNA probe (DIG Oligonucleotide Tailing Kit, Roche) according to manufacturer's instructions. The membranes were prehybridized at 55°C with ExpressHyb Hybridization Solution (BD Biosciences Clontech, Palo Alto, CA) followed by hybridization at 55°C using the same solution supplemented with 50 pmol of DIG-labeled probe. The membranes were washed in series at room temperature and detection was achieved using the DIG Nucleic Acid Detection Kit (Roche), which is based on an enzyme-catalyzed colorimetric reaction using 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium salt as reagents to produce an insoluble blue precipitate.

2.4 RESULTS

Initial studies focused on the ability to concentrate endogenous bacteria from unseeded 11 g samples of plain nonfat yogurt or mild cheddar cheese using centrifugation. Various centrifugation speeds ranging from 750 *x g* to 9,700 *x g* were evaluated and in general, the higher the centrifugation speed, the more effective the concentration, as evidenced by a decrease in the percent of bacteria lost to discarded supernatants. At the optimal centrifugation speed of 9,700 *x g*, recoveries of endogenous bacteria based on direct plating of the centrifugal pellet ranged from 52 – 81% for unseeded non-fat yogurt and from 87 – 124% for mild cheddar cheese; loss to discarded supernatants were always less than 1% (data not shown). Final pellet volumes were approximately 4-5 g in size, representing a two-fold sample volume reduction after completion of the centrifugation step.

To ascertain the efficiency of the concentration step in the recovery of *L. monocytogenes* and serovar. Enteritidis seeded into non-fat yogurt and mild cheddar

cheese, seeding experiments were conducted at levels of $10^2 – 10^6$ CFU pathogen/11 g food sample. Bacterial recoveries based on direct plating of the centrifugal pellet ranged from 53 - 145% and 71 - 128% for serovar Enteritidis and *L. monocytogenes,* respectively, in the nonfat yogurt samples; and from 77- 135% and 69 - 122% for serovar. Enteritidis and *L. monocytogenes* respectively, in the cheddar cheese samples (Table 2.2). Consistent with other investigators (Boulanger and Edelstein 1995; Lucore *et al.* 2000; Uyttendaele *et al.* 2000; Cullison and Jaykus 2002), differences in recoveries based on direct plating of the pellet were more variable and statistically different than if recoveries were calculated based on the percent loss to supernatant. When recovery results based on direct plating of the pellet were transformed to log format, the counts never varied by more than 0.5 $\log_{10}$, an acceptable within-method degree of variability (Kramer and Gilbert 1978; Alonso-Calleja *et al.* 2002). Bacterial loss to discarded supernatants was always less than 5%, regardless of pathogen or dairy product tested.

PCR optimization was done initially on pure cultures of serovar. Enteritidis and *L. monocytogenes*, and detection limits of 10 CFU/reaction were consistently achieved for both pathogens (data not shown). However, when PCR conditions used for pure cultures were applied to DNA extracted from the food matrix, detection limits dropped dramatically. In an effort to overcome matrix-associated inhibition, PCR enhancement agents, including PEG, DMSO, DTT, and glycerol were evaluated using dilution series PCR (Jaykus *et al.* 1996). Glycerol and DMSO had either no effect or a negative effect on both the intensity of the amplicon signals and the detection limits of the PCR. Addition of PEG resulted in more intense bands for *L. monocytogenes*. However,

supplementing amplification reactions with DTT resulted in either more intense bands or improved detection sensitivity for both *L. monocytogenes* and serovar Enteritidis extracted from the cheese matrix (Figure 2.2). Although the use of second-generation *Taq* DNA polymerase was found to improve detection sensitivity for food samples seeded with serovar Enteritidis, no appreciable improvement in amplification efficiency was observed for samples seeded with *L. monocytogenes*. Similar data was observed for the yogurt matrix (data not shown).

In an effort to ascertain the overall PCR detection limits of the combined concentration-detection method, plain nonfat yogurt and cheddar cheese were each seeded with *L. monocytogenes* or serovar Enteritidis at levels of 10^2 - 10^6 CFU per 11-g sample, processed for bacterial concentration using centrifugation, followed by DNA extraction, PCR amplification, and Southern hybridization. The centrifugation step resulted in a 2-fold sample volume concentration, while the DNA extraction step resulted in an additional 10-fold sample volume reduction; in all, an 11-g sample was concentrated 20-fold to a final volume of 500 µl. PCR detection limits of 10^3 and 10^1 CFU per 11-g sample were achieved for *L. monocytogenes* and serovar. Enteritidis, respectively, in both product types and without prior cultural enrichment (Fig. 2.3 and Fig. 2.4). When detection limits were interpreted on the basis of individual amplification reactives, 10^1 and 10^{-1} CFU/amplification reaction could be achieved for these pathogens, respectively.

2.5 DISCUSSION

Fermented dairy food products are compositionally complex and represent one of the more challenging matrices for which to develop molecular-based pathogen

detection strategies. For instance, there are numerous reports of dairy matrix-associated PCR inhibition (Niederhauser *et al.* 1992; Rossen *et al.* 1992; Herman and DeRidder 1993; Makino *et al.* 1995). Decreased sensitivity of the PCR reactions may also be the result of inefficient DNA extraction and / or high levels of background and competing microflora, both of which can be problematic for compositionally complex dairy products (Wegmüller *et al.* 1993; Wilson 1997). Regardless of these difficulties, there have been numerous reports on the development and application of nucleic acid amplification-based assays to detect foodborne pathogens in a variety of foods, including dairy products (Rossen *et al.* 1992; Wilson 1997).

Centrifugation has been used, with limited success, to concentrate bacteria from both cultural enrichments and directly from the dairy food matrix prior to PCR detection. Typically, researchers attempting direct detection, (without cultural enrichment), of pathogens from food matrices report poorer detection limits in comparison to methods that are preceded by cultural enrichment. For example, when centrifugation was used in combination with enzymatic digestion, investigators reported PCR detection limits for *Escherichia coli* O157:H7 and *Campylobacter* species from dairy products ranging from $10^3 – 10^4$ CFU/g (Meyer *et al.* 1991; Wegmuller *et al.* 1993). Makino et. al. (1995) reported a PCR detection limit of 10^3 CFU/g for the direct detection of *L. monocytogenes* from cheese after using several sample preparation steps, including centrifugation and enzyme digestion. Uyttendaele *et al*. (2000) evaluated immunomagnetic separation (IMS) for the recovery of *L. monocytogenes* from cheese, noting that the cheese matrix interfered with both the efficiency of antibody-antigen binding and the recovery of the beads. These authors reported that IMS resulted in

losses of 1.1 – 1.4 $\log_{10}$ of the target organism and were only able to achieve detection limits of 10^2-10^3 CFU/ml when attempting direct detection of *L. monocytogenes* from a mixed population. Herman and DeRidder (1993) reported PCR detection limits ranging from 10 to 10^6 CFU / g for *L. monocytogenes* seeded into various diluted cheeses, depending on the type of cheese evaluated. In our study, we report detection limits of 10 CFU/11-g of serovar. Enteritidis and 10^3 CFU/11-g of *L. monocytogenes* from both cheese and yogurt matrices, without prior cultural enrichment. These detection limits are comparable if not better than those reported in the literature for the direct detection of pathogens from cheese, and only slightly less sensitive than detection limits for traditional methods that rely on cultural enrichment.

There were several optimizations that, when applied together, allowed us to achieve improved detection limits using our combined sample preparation-PCR detection approach. For instance, in the initial sample preparation phase, we were able to incorporate filtration and improved precipitation efficiency to facilitate the separation of bacteria from the food matrix. A simple filtration step using either sterile cheesecloth or a filter stomacher bag allowed for the removal of large food particulates that would otherwise co-sediment with the bacteria during the centrifugation step. Polyethylene glycol (PEG) was added to the yogurt and cheese samples during the homogenization step to facilitate precipitation. We speculate that PEG promoted protein precipitation, with the bacteria in the food suspensions co-precipitating in a manner similar to organic flocculation (Sobsey *et al*. 1975). The combination of simple filtration of gross particulates and the addition of PEG allowed efficient bacterial sedimentation to occur at lower centrifugal forces, leaving unprecipitated food

components to be discarded in the filter or the supernatant. The net result was a smaller bacteria-containing pellet and removal of unwanted food-related materials by filtration and subsequent centrifugation, all with recovery of close to 100% of the bacterial population.

Likewise, the optimization of DNA extraction and PCR amplification conditions was necessary in order to achieve improved detection limits of *L. monocytogenes* and serovar Enteritidis in the complex dairy matrix. For instance, the incorporation of an additional DNA purification step, in the form of the QiaShredder column, produced DNA that could be enzymatically amplified without significant inhibition. The addition of DTT and in some cases, a second generation *Taq* DNA polymerase, also improved PCR detection limits by as much as 100-fold. It has been hypothesized that PCR enhancement agents work on two levels, e.g., they help to reduce nucleic acid secondary structure, making the DNA more accessible to the polymerase, and they serve as scavengers of inhibitory compounds, preventing their binding to and subsequent inactivation of *Taq* DNA polymerase (Wilson 1997). All told, these additions to the protocol further facilitated detection of these two pathogens in yogurt and cheese samples.

We can conclude that, with careful attention to sample preparation and nucleic acid amplification optimization, the direct detection of pathogens from foods is possible and that the sensitivity of this detection is slowly approaching that of other rapid methods. With that said, there is no "silver bullet" with respect to matrix preparation, and it is likely that future methods will be both matrix- and perhaps organism-specific. For instance, the fact that our detection limits were so much better for *Salmonella* than

for *L. monocytogenes*, despite the fact that bacterial recoveries after concentration were virtually identical, suggests that additional refinements to the amplification protocols will be necessary. In fact, we have anecdotal evidence that different gene targets and amplification primers vary widely in their amplification efficiency when applied to compositionally complex sample matrices (data not shown). It is our hope that the method reported here represents as step towards developing effective combinations of sample concentration, purification, and nucleic acid amplification, which may eventually be used to reduce or eliminate lengthy cultural enrichments.

Table 2.1. Primers and Probes

Organism	Primer	Sequence (5' – 3')	Location (bp)	Reference
Listeria monocytogenes *858 bp* *hly*A	α -1	CCT AAG ACG CCA ATC GAA AAG AAA	196 - 219	Norton *et al.* 2000
	β- 1	TAG TTC TAC ATC ACC TGA GAC AGA	1053 - 1030	Norton *et al.* 2000
	α – 1 (probe)	GAA AAA TAT GCT CAA GCT TAT CCA AAT GTA	622 - 651	Bsat and Batt, 1993
Salmonella enterica **Enteritidis** *389 bp* *inv* A	Salm 3	GCT GCG CGC GAA CGG CGA AG	586 - 605	Manzano *et al.*, 1998
	Salm 4	TCC CGG CAG AGT TCC CAT T	954 – 972	Manzano *et al.*, 1998
	Salm (probe)	TTT GTG AAC TTT ATT GGC GG	697 - 716	Manzano *et al.*, 1998

Table 2.2: Recovery Efficiency of *Listeria monocytogenes* and *Salmonella enterica* Enteritidis after Concentration by Centrifugation

	Listeria monocytogenes		***Salmonella enterica* serovar. Enteritidis**	
	% Recovery (based on loss to supernatant)[A]	% Recovery-Pellet (based on direct plating of pellet) [B]	% Recovery (based on loss to supernatant)[A]	% Recovery (based on direct plating of pellet)[B]
Mild Cheddar Cheese				
10^6 / 11g	96 ± 1^{XY}	122 ± 82^{X}	98 ± 2^{X}	93 ± 45^{X}
10^4 / 11g	98 ± 1^{Y}	75 ± 16^{X}	98 ± 3^{X}	77 ± 53^{X}
10^2 / 11g	96 ± 1^{X}	69 ± 15^{X}	100 ± 1^{X}	135 ± 33^{X}
Non-Fat Yogurt				
10^6 / 11g	99 ± 1^{X}	75 ± 14^{X}	99 ± 1^{X}	100 ± 23^{XY}
10^4 / 11g	99 ± 1^{X}	71 ± 13^{X}	98 ± 1^{X}	53 ± 19^{Y}
10^2 / 11g	98 ± 2^{X}	128 ± 52^{X}	100 ± 1^{X}	145 ± 25^{X}

[A] % Recovery (based on loss to supernatant) = 100 - [(total population in supernatant after centrifugation) x 100/(total population in sample before centrifugation)]

[B] % Recovery (based on direct plating of pellet) = [(total population in pellet after centrifugation) * 100/(total population in sample before centrifugation)]

Different superscript letters (x and y) identify statistically significant differences ($p \leq 0.05$) in percent recovery at different input levels of each organism. Statistically significant differences ($p \leq 0.05$) were observed in all comparisons between percent recovery values when calculations based on loss to the supernatant versus direct plating of the pellet were compared.

11-g sample
25 ml 0.9%NaCl
8 ml 25% Sodium Citrate
4% PEG 8000

↓

Homogenize

Stomach / 2 min

↓

Centrifugation

9,700 *x g* / 15 min / 4C
4 – 5g pellet (2-fold concentration)

↓

Pellet Reconstitution
1:2 ratio of pellet to DNAzol® BD
DNA Extraction
(500µl, 10-fold concentration)

↓

PCR

Figure 2.1. Sample Concentration, Purification and Detection Flow Diagram

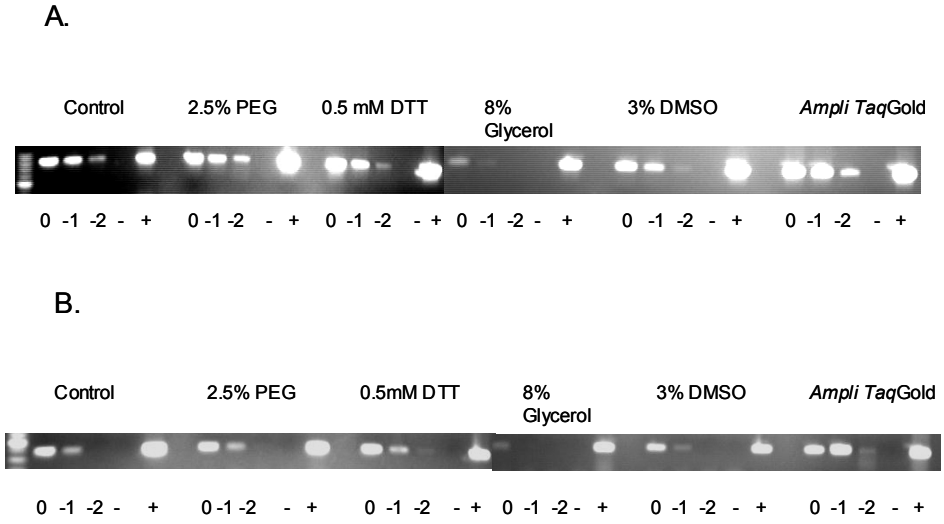

Figure 2.2. Effect of PCR enhancement agents (2.5% PEG, 0.5mM DTT, 8% glycerol, 3% DMSO and Ampli*Taq* Gold) on the detection of serovar Enteritidis (A) and *L. monocytogenes* (B) in artificially contaminated mild cheddar cheese after centrifugation, DNA extraction, and PCR amplification. Eleven g samples of mild cheddar cheese were inoculated with 10^6 CFU of servoar Enteritids or *L. monocytogenes* and processed for bacterial concentration followed by DNA isolation. Prior to PCR, DNA extracts were diluted up to 100 fold. Undiluted, 10-fold (-1) and 100-fold (-2) diluted extracts were then amplified and detected by agarose gel electrophoresis. The corresponding dilution is given below each gel lane. Lanes: (M), marker, (U), uninoculated 11g sample of mild cheddar cheese processed for bacterial concentration, (-), complete PCR cocktail without sample (i.e., water); (+), positive control reaction for amplification (i.e., DNA extracted from approximately 10^8 CFU of serovar Enteritidis or *L. monocytogenes* in pure culture).

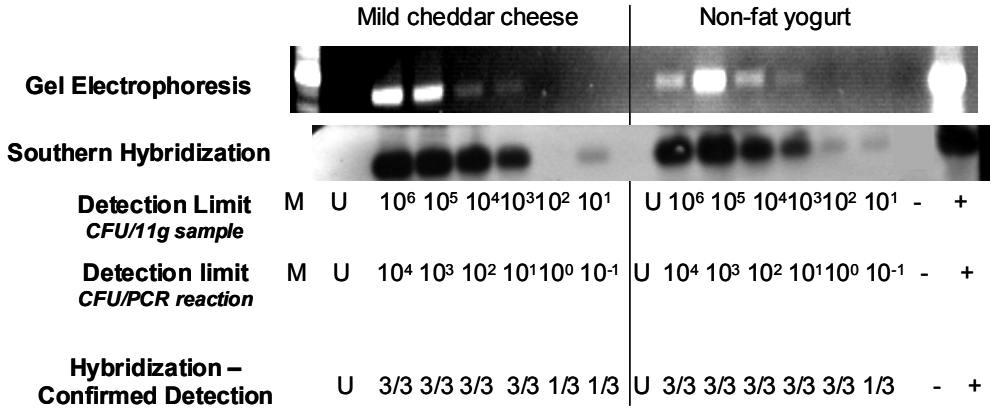

Figure 2.3. Detection of *Salmonella enterica* serovar enteritidis in artificially contaminated mild cheddar cheese and non-fat yogurt. Eleven g samples were inoculated with 10^1 to 10^6 CFU of servoar Enteritids and processed for bacterial concentration followed by DNA isolation, PCR amplification and Southern hybridization. The corresponding initial inoculum level (CFU/11g of mild cheddar cheese or non-fat yogurt and CFU /PCR reaction) is given below each gel lane. Each inoculum level was run in triplicate and the number of hybridization confirmed positive results per triplicate run is indicated on the bottom row below the corresponding inoculum level. Lanes: (M), marker, (U), uninoculated 11g sample of mild cheddar cheese or non-fat yogurt processed for bacterial concentration, (-), complete PCR cocktail without sample (i.e., water); (+), positive control reaction for amplification (i.e., DNA extracted from approximately 10^8 CFU of serovar Enteritidis in pure culture).

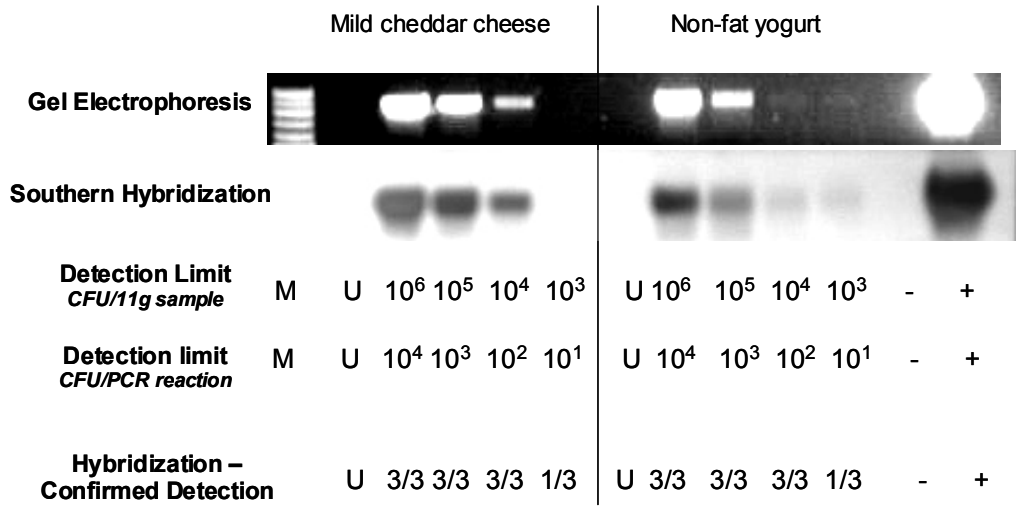

Figure 2.4. Detection of *Listeria monocytogenes* in artificially contaminated mild cheddar cheese and non-fat yogurt. Eleven g samples were inoculated with 10^3 to 10^6 CFU of *L. monocytogenes* and processed for bacterial concentration followed by DNA isolation, PCR amplification and Southern hybridization. The corresponding initial inoculum level (CFU/11g of mild cheddar cheese or non-fat yogurt and CFU /PCR reaction) is given below each gel lane. Each inoculum level was run in triplicate and the number of hybridization confirmed positive results per triplicate run is indicated on the bottom row below the corresponding inoculum level. Lanes: (M), marker, (U), uninoculated 11g sample of mild cheddar cheese or non-fat yogurt processed for bacterial concentration, (-), complete PCR cocktail without sample (i.e., water); (+), positive control reaction for amplification (i.e., DNA extracted from approximately 10^8 CFU of *L. monocytogenes* in pure culture).

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CHAPTER 3

DIRECT DETECTION OF *LISTERIA MONOCYTOGENES* FROM ARTIFICIALLY CONTAMINATED FRANKFURTERS

3.1 ABSTRACT

A method for the direct detection (without cultural enrichment) of *Listeria monocytogenes* from 11g samples of frankfurters, with particular attention to alternative nucleic acid preparation methods prior to amplification, is described. Frankfurter samples were artificially inoculated with *L. monocytogenes* at levels of 10^1 to 10^6 CFU /11g and processed for initial bacterial concentration using centrifugation, with recoveries ranging from 58 to >100%. Nucleic acids in centrifugal pellets were extracted using a commercial guanidinium isothiocyanate system, further purified by column chromatography and processed for total DNA, total RNA, and specific bacterial rRNA isolation using two magnetic bead-based technologies, i.e., MICROB*Enrich*® and MICROB*Express*®. Overall, 11-g samples were concentrated 100-fold to 100 µl with recovery of target nucleic acids. Using primers targeting rRNA sequences, the nucleic acids were amplified by PCR and RT-PCR, allowing for a direct comparison of detection limits. PCR detection limits were 10^5 CFU/11g sample and RT-PCR detection limits were 10^3 CFU/11g. Detection limits were improved an additional 10-fold (to 10^2 CFU/11g) when extracted RNA was further purified using MICROB*Express*®. Results from this study show that for this particular matrix / pathogen / primer set combination, amplification via RT-PCR yielded more sensitive

detection than DNA based amplification and that further purification of RNA extracts was beneficial for improving RT-PCR detection limits.

3.2 INTRODUCTION

Listeria monocytogenes continues to be an important food-borne pathogen of particular concern in ready–to-eat foods (Gallagher et al. 2003). Conventional methods for the detection of *L. monocytogenes* in foods involve pre-enrichment, selective enrichment, selective plating and biochemical identification, which while straightforward, are laborious, requiring 2-3 days to obtain negative results and several additional days to confirm positive results. Commercially available rapid methods replace the selective plating step, thereby decreasing detection time by approximately one day, although this is irrelevant for presumptively positive samples. The ideal method for the food industry would be rapid (<24 hours), simple, inexpensive, yielding confirmed results that could be used for preventive rather than corrective actions. For instance, a truly rapid method would be an invaluable tool for the identification of contamination in raw ingredients, the processing environment prior to production as well as preventing contaminated product from being released into the marketplace.

Nucleic acid amplification methods such as the polymerase chain reaction (PCR) and nucleic acid sequence-based amplification (NASBA) have the potential to decrease detection time for food-borne pathogens by replacing cultural enrichment with specific nucleic acid enrichment. However, wide spread use of these methods has been limited due several inherent methological issues, including: i) the need for small reaction volumes ($\leq$100 µl) in comparison to large food sample size (usually $\geq$25g); ii) the requirement for at least one target nucleic acid sequence per reaction which equates

to a detection limit of approximately 10^3 CFU/g of food matrix; iii) the potential for amplification inhibition by food components; iv) the presence of competitive microflora which may interfere with effective and efficient amplification; v) low levels and / or sporadic contamination with target pathogens; and vi) the potential for amplification of both live and dead cells. The net result of these limitations has been continued reliance on cultural enrichment to provide sufficient preliminary amplification of live microorganisms to overcome this projected reduction in test sensitivity and specificity.

Methods for the separation and concentration of bacteria from food samples can be a way of increasing the number of target cells in a sample prior to the implementation of detection methods. Many methods, both selective and non-selective, have been applied to the concentration of target organisms from food samples and have been the subject of extensive reviews (Payne and Kroll, 1991, Benoit and Donahue, 2003, Stevens and Jaykus, in press). These methods include, but are not limited to centrifugation, filtration, aqueous two-phase separation, and immunoaffinity. Investigators who have used these methods report several advantages, including removal of matrix associated inhibitors, sample size reduction, and reduced reliance on cultural enrichment resulting in decreased detection time. Unfortunately, no one method has proven ideal, and most need to be optimized for different matrix and pathogen combinations.

Nucleic acid amplification efficiency can also be affected by extraction method, choice of primers, and amplification conditions (McKillip et al. 2000). For instance, nucleic acid extraction efficiency can vary by extraction method and this in turn can have a direct impact on the efficiency of subsequent amplification procedures (Smith et

al. 2003, Xiang et al. 2001). There is evidence to suggest that extraction efficiency and reliability decreases as the number of target cells decreases (McKillip et al, 1999). Extraction efficiency also has been shown to be negatively impacted when cells are suspended in a food matrix, as compared to suspension in a culture medium (McKillip et al. 2000). It should be noted that it has not been experimentally determined that the reduced detection sensitivity that arises when nucleic acids are present in low copy number is due to extraction or amplification issues, or a combination of both. Burtscher and Wuertz (2003) reported a significant difference in amplification efficiency of inoculated organic waste samples depending on the RT-PCR amplification kit used. Primer selection may also impact the sensitivity of the amplification, which may be particularly relevant to the detection of *L. monocytogenes* in foods, for which many different primers have been reported (Levin, 2003).

RT-PCR offers some advantages over PCR in that multiple copies of RNA may be present per cell, increasing the potential number of target sequences that can be amplified, and theoretically increasing test sensitivity. Ribosomal RNA may be particularly appealing in this regard as viable cells may have several thousand copies of rRNA per cell (Waters and McCatchan, 1990, Milner et al., 2001). However, when using rRNA as a target, there is the potential for amplification of non-viable cells. McKillip et al. (1999) reported that rRNA was stable for as long as 48 hours after cell death, but was nonetheless a better indicator of viability than DNA, which was also stable for 48 hours with considerably stronger amplification signals than the corresponding rRNA. Overall, rRNA has been found to be less stable than DNA, and even recent literature has shown mRNA to survive up to 30h post-cell death depending

on the target region, amplification method, environmental conditions and physiological state of the cell (Birch et al, 2001).

Based on the hypothesis that background nucleic acids may affect nucleic acid amplification methods, we sought to use commercially available proprietary kits for nucleic acid purification. Both kits are designed for use with clinical samples for the purification of bacterial mRNA from mixtures containing both mammalian and bacterial RNA. In this study, kits were adapted for use with food matrices and the recovery of bacterial rRNA. The first, MICROB*Enrich*® (Ambion, Austin, TX), was used to separate mammalian RNA from bacterial RNA. This method is designed to selectively concentrate mammalian 18S and 28S rRNAs and polyadenylated mRNAs from mixtures containing both bacterial and mammalian RNA. The mammalian RNA is effectively removed from solution by magnetic capture using a bridging oligonucleotide for annealing with oligo d(T) and with mammalian rRNA sequences. The bound eukaryotic mRNA and rRNA can then be further processed for nucleic acid amplification, or, in our case, discarded. We used the supernatant, from which mammalian RNA was removed, for amplification and detection of bacterial rRNA. It was our hypothesis that this method might effectively remove matrix-associated RNA.

The second kit, MICROB*Express*®, (Ambion, Austin, TX) is procedurally similar to MICROB*Enrich*® in that it also uses magnetic beads and a capture hybridization approach for the separation of bacterial rRNA from mRNA. The magnetic beads are conjugated with capture bridging oligonucleotides which anneal to universal bacterial rRNA probes (16S and 23S) and are captured by oligo d(T). After magnetic capture, the supernatant is discarded and the bead-bound bacterial rRNA can

be used directly in RT-PCR targeting rRNA genes. In this case, we hypothesized that matrix associated inhibitors might be removed in the discarded supernatant.

In this study we compared nucleic acid amplification methods for the direct detection of *L. monocytogenes* from a frankfurter matrix. A single two-step nucleic acid extraction, which could simultaneously separate and isolate DNA and RNA, was performed for each sample. These individual nucleic acid extracts were amplified by DNA PCR and RT-PCR under uniform conditions, allowing for direct comparison of detection limits. Secondly, we investigated whether amplification efficiency could be improved by the further removal of background nucleic acids and / or inhibitors in the final RNA extracts using MICROB*Enrich*® and MICROB*Express*®. Detection limits of PCR and RT-PCR amplification were determined for frankfurter samples seeded with 10^1 to 10^6 CFU / 11g sample and confirmed using Southern hybridization.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial Cultures and Recovery Media

Stock cultures of *L. monocytogenes* NCTC 10527 that was constructed with streptomycin resistance were obtained courtesy of Dr. Sophia Kathariou, Department of Food Science, North Carolina State University. Stock cultures were propagated aerobically overnight at 37°C in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI). In seeding experiments, serial ten-fold dilutions were done in 0.1% peptone (Difco) and plating for recovery performed using the pour plate technique. Trypticase Soy Agar (Difco) supplemented with 0.6% yeast extract (Difco) and streptomycin (Sigma, St. Louis, MO) (0.025mg/ml) (TSAYE-S) was used for the enumeration of streptomycin resistant *L. monocytogenes.* TSAYE-S plates were

incubated for 24-48 hours at 37°C prior to enumeration. Using this media, the inoculated *L. monocytogenes* was effectively recovered while suppressing the growth of matrix-associated competitive microflora.

3.3.2 Sample Preparation

A simple centrifugation scheme was applied in an effort to concentrate microorganisms and remove matrix-associated inhibitors prior to nucleic acid extraction (Figure 3.1). Eleven-g samples of frankfurters were mixed with 25 ml sterile saline and 8 ml 25% w/v sodium citrate (Fisher Scientific, Fair Lawn, NJ) as previously described (Lucore *et al.* 2000). Each sample was supplemented with polyethylene glycol (PEG) 8000 (Fisher Scientific, Fair Lawn, NJ) to reach a final concentration of 4% and stomached for 2 min at room temperature. In order to remove large matrix- associated particulates, a filter stomacher bag (Nasco, Ft. Atkinson, WI) was used with recovery of the filtrate and disposal of the retentate. The filtered samples were centrifuged at 9,700 *x g* (Sorvall RC-5B, Dupont Company, Wilmington, DE) for 15 minutes at 4°C. The resulting supernatant (~25ml) was decanted and the remaining pellet (~1g) was retained. This represented a sample volume reduction of approximately 10-fold. In an effort to ascertain the efficiency of the sample preparation method with respect to bacterial recovery, both the discarded supernatant and the bacteria-containing pellet were serially diluted and plated for recovery. Percent recovery or loss after centrifugation was evaluated using two separate formulae and as previously described (Lucore *et al.* 2000). In the first, recovery was based on direct plating of the precipitated pellet and was calculated as follows: [% recovery = (total population in pellet after centrifugation) * 100/(total population in sample before centrifugation)]. In

the second, percent loss to supernatant was calculated based on direct plating of the supernatant as follows: [% loss to supernatant = total population in supernatant after centrifugation) x 100/(total population in sample before centrifugation)]. All experiments were done in triplicate. When statistical comparisons were necessary, analysis of variance and the Tukey-Kramer multiple comparisons test were done on percent recovery data using the InStat 2 Statistical Analysis Package (GraphPad Software, San Diego, CA).

3.3.3 Nucleic Acid Extraction

RNA and DNA were extracted from the 1g centrifugal pellets using a single Trizol (Invitrogen, Carlsbad, CA) extraction in accordance with manufacturer instructions. Briefly, the 1g pellets were resuspended 1:1 with Trizol reagent. RNA was extracted by adding 200 µl of chloroform per 1.0 ml volume of Trizol followed by centrifugation at 12,000 x *g*. The aqueous layer was removed and the RNA was precipitated by the addition of 10 µg of glycogen (Ambion, Austin, TX) and 500 µl of isopropanol. After centrifugation at 12,000 x *g*, the pellet was resuspended with 50 µl nuclease-free sterile water. DNA was extracted from the remaining non-aqueous Trizol with 300 µl of ethanol and sample centrifugation at 2,000 x *g*. The pellet was washed twice using 0.1M sodium citrate in 10% ethanol followed by washing with 75% ethanol. The final pellet was resuspended with 100 µl of sterile nuclease free water. Additional purification of the RNA and DNA extracts was achieved by centrifugation through a QIAshredder® (QIAGEN Inc, Valencia, CA) column for 2 min at 11,750 x *g*

(Sair *et al*. 2003). The resulting filtrate was retained for nucleic acid amplification or further RNA purification followed by amplification.

3.3.4 RNA Purification

The MICROB*Enrich*® kit (Ambion, Austin, TX) and the MICROBExpress® kit (Ambion) were evaluated for further purification of the extracted RNA. Both kits were used individually and in accordance with manufacturer instructions. The MICROB*Enrich*® kit was used to remove mammalian RNA from the desired bacterial RNA. Briefly, the MICROB*Enrich*® protocol involves the mixture of 10µl extracted RNA with 300µl of binding buffer containing 4µl of capture oligonucleotides, followed by incubation for one hour at 37°C. The mixture was then added to 50µl of oligonucleotide-bound beads and incubated for an additional 15 minutes. The bead complexes were collected by a magnet, the supernatant was retained, and the enriched RNA was reprecipitated at –80°C using 40 - 47µl of 3M sodium acetate, 4µl of 5 mg/ml glycogen, and 1ml of ethanol. The final precipitated RNA was resuspended to a final volume of 15µl in TE buffer.

The MICROB*Express*® kit was procedurally similar in that 10µl extracted RNA was added to 200µl of binding buffer containing 4µl of capture oligonucleotides and incubated for one hour at 37°C. Fifty microliters of oligonucleotide-bound magnetic beads was added to the mixture, incubated for 15 minutes, and the bead complexes were collected by a magnet. The precipitate was resuspended in 10µl of TE buffer and the supernatant was discarded.

3.3.5 Nucleic Acid Amplification

RT-PCR and PCR reactions were performed using the SuperScript® One-Step RT-PCR kit with Platinum® *Taq* (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Each 50µl reaction contained 2µl of nucleic acid extract, 1x reaction mix, 0.2 µM of each primer (primer L1 – 5' - CAC GTG CTA CAA TGG ATA G –3', primer L-2, 5' - AGA ATA GTT TTA TGG GAT TAG- 3') (Wang et al. 1992), 3 mM magnesium sulfate, 5% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO), 2.5 units Ampli*Taq* Gold DNA Polymerase (Applied Biosystems), 2% RT/Platinum *Taq* mix, and 2µl of RNA extract. The amplification mixtures were overlaid with 50µl of mineral oil. RT-PCR amplification for *L. monocytogenes* consisted of a reverse transcriptase step of 50°C for 30 minutes followed by PCR amplification. PCR amplification conditions were the same for both PCR and RT-PCR, and consisted of one cycle at 95°for 2 minutes, followed by 40 cycles of 95°C for 20 seconds, 48°for 20 seconds, 72°C for 40 seconds, and a final extension of 72°C for 3 minutes. Temperature cycling was done in a Perkin Elmer DNA Thermal Cycler (Perkin Elmer, Norwalk, CT). The 67 bp PCR amplicon was separated and visualized under ultraviolet light by agarose (2% Nusieve® GTG® agarose and 1% SeaPlaque® agarose) (Cambrex BioScience, Rockland, ME) gel electrophoresis and ethidium bromide staining.

3.3.6 Southern Blot Hybridization

To confirm the identity of PCR amplicons, agarose gels were transferred to positively charged nylon membranes (Roche, Indianapolis, IN) using the method of Southern and hybridized with a digoxigenin (DIG)-labeled DNA probe (DIG Oligonucleotide Tailing Kit, Roche) according to manufacturer's instructions. The

membranes were prehybridized at 65°C with ExpressHyb Hybridization Solution (BD Biosciences Clontech, Palo Alto, CA) followed by hybridization at 65°C using the same solution supplemented with 100 pmol of DIG-labeled probe (probe RL-3, 5' - GTC GCG AAG CCG CGA GGT – 3') (Wang et al. 1992). The membranes were washed in series at room temperature and detection was done using the DIG Nucleic Acid Detection Kit (Roche), which is based on an enzyme-catalyzed colorimetric reaction using 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium salt as reagents to produce an insoluble blue precipitate.

3.4 RESULTS

To ascertain the efficiency of sample manipulations to concentrate *L. monocytogenes* from frankfurters, seeding experiments were done at levels of $10^3 – 10^6$ CFU *L. monocytogenes* /11 g food sample. Bacterial recoveries were not affected by the use of filter stomacher bags during the sample homogenization step (data not shown). Bacterial recoveries based on direct plating of the centrifugal pellet ranged from 58 - 118%. If recoveries were based on loss to the discarded supernatant, they ranged from 89 to 94% (Table 3.1). Consistent with other investigators (Boulanger and Edelstein 1995; Lucore *et al.* 2000; Uyttendaele *et al.* 2000; Cullison and Jaykus 2002), recoveries based on direct plating of the pellet were more variable than if recoveries were calculated based on the percent loss to supernatant. When direct pellet plating recovery results were transformed to log format, the counts never varied by more than 0.5 $\log_{10}$, an acceptable within-method degree of variability (Kramer and Gilbert 1978; Alonso-Calleja *et al.* 2002). Bacterial loss to discarded supernatants was always less than 12%, which is higher than previous work on commodities such as yogurt and may

possibly be attributed to the higher fat content of the product matrix (Cullison and Jaykus, 2002, Stevens and Jaykus, 2004).

PCR and RT-PCR optimization was initially performed on pure cultures of *L. monocytogenes*. Optimized PCR detection limits were 10^5 CFU/ml (10^3 CFU/reaction), while detection limits of 10^3 CFU/ml (10^1 CFU/reaction) were consistently achieved by RT-PCR (data not shown). Target amplicon was not detected when RNA extracts were subjected to PCR amplification, indicating that DNA contamination was not present (data not shown). In an effort to ascertain the overall PCR and RT-PCR detection limits of the combined concentration-detection method, frankfurter samples were seeded with *L. monocytogenes* at levels ranging from 10^1 - 10^6 CFU per 11-g sample, processed for bacterial concentration using centrifugation, followed by simultaneous DNA and RNA extraction, nucleic acid amplification, and Southern hybridization. The centrifugation step resulted in a 10-fold sample volume reduction, while the nucleic acid extraction step resulted in an additional 10 to 20-fold sample volume reduction; overall, an 11-g sample was concentrated 100 to 200-fold to a final volume of 50 to 100 µl. PCR detection limits of $\geq 10^5$ CFU per 11-g sample ($\geq 10^3$ CFU per reaction) were achieved for *L. monocytogenes* without cultural enrichment (Figure 3.2A). RT-PCR detection limits of $\geq 10^3$ CFU per 11-g sample ($\geq 10^1$ CFU per reaction) without prior cultural enrichment or further RNA purification were achieved (Figure 3.2B).

Subjecting RNA extracts to MICROB*Enrich*® purification did not improve detection sensitivity and detection limits remained at 10^3 CFU/11 g sample (10^1 CFU per reaction) (Figure 3.3B). Detection limits were improved by 10-fold to $\geq 10^2$

CFU/11g ($\geq 10^0$ CFU per reaction) when the MICROB*Express*® kit was used for further RNA purification with consistently stronger amplicon signals observed (Figure 3.3C). The two kits (MICROB*Enrich*® followed by the MICROB*Express*®) could be used in sequence, but there was no additional improvement in detection limits when the sequential procedure was used (data not shown).

3.5 DISCUSSION

Bacterial concentration methods such as centrifugation have been used, with limited success, to concentrate bacteria from both cultural enrichments and directly from food matrices prior to nucleic acid amplification (Stevens and Jaykus, 2004). Typically, researchers attempting direct detection (without cultural enrichment) of pathogens from food matrices report decreased detection limits in comparison to methods that are preceded by cultural enrichment. Most of the literature on the PCR detection of *L. monocytogenes* from meat products report the need for such cultural enrichment (Ingianni et al. 2001, Kanuganti et al. 2002, Jung et al. 2003). For instance, Wang et al. (1992), using the same primers used in this study, reported PCR detection limits of 4 – 40 CFU of *L. monocytogenes* per 25g sample after cultural enrichment of food samples, failing to directly detect *L. monocytogenes* from food samples at the same inoculum levels. Ingianni et al. (2001) tested an array of meat products and were able to achieve sensitive detection only after an enrichment step was incorporated. Uyttendaele et al. (1995) first reported the use of NASBA for the detection of *L. monocytogenes* from meat products and other foods, obtaining detection limits as low as 1-2 CFU/25g sample only after cultural enrichment. Hudson et al. (2001) used a combined centrifugation and immunomagnetic separation (IMS) method coupled with

multiplex PCR for the direct detection of *L. monocytogenes* from ham samples. The authors reported that centrifugation alone was not adequate and that the IMS step was necessary in order to achieve detection. Detection limits were as low as 1 CFU/g which equates to 10^1-10^2 CFU/25 g ham sample. Our method showed approximately the same detection limit as that of Hudson et al. (2001) without the need for expensive IMS reagents.

Direct detection of food-borne pathogens from food matrices necessitates the optimization of nucleic acid extraction, purification, and PCR amplification conditions in order to achieve detection limits which are comparable to those for culture based methods (Payne and Kroll, 1991, Stevens and Jaykus, 2004). We chose to use the method of Sair et al. (2002), which incorporated an additional nucleic acid purification step in the form of the QiaShredder® column, to initially purify the nucleic acids. The MICROB*Enrich*® kit was then used to separate the bacterial RNA from matrix-associated mammalian RNA prior to amplification. We also tried the MICROB*Express*® kit to separate the bacterial rRNA from the bacterial mRNA. The MICROB*Express*® method consistently yielded stronger amplicon signals and improved detection limits by 10-fold. We hypothesize that this was due to the combined effect of specifically concentrating bacterial rRNA and disposal of matrix associated inhibitors that were discarded in the waste supernatant. A similar improvement in detection limits was not observed when the MICROB*Enrich*® kit was used for RNA purification. We suspect that this was due to potential losses in nucleic acid due to the additional RNA precipitation step and failure to remove residual matrix-associated inhibitors. This leads us to conclude that inhibitory compounds include but

are indeed more complex than competitor nucleic acids alone. However, we caution that this study was done on only a single matrix/pathogen/primer combination, and investigation and optimization may be warranted for other applications.

In our study, we report PCR and RT-PCR detection limits for nucleic acids extracted from the same sample. In previous studies which have compared PCR and RT-PCR detection limits head-to-head, extractions have been done on separate samples and by different methods (Burtscher and Wuerts, 2003). Some have suggested that RT-PCR will yield more sensitive detection than PCR by virtue of the difference in target nucleic acid numbers, however this may be at least partially negated by the inefficiencies of the reverse transcription step (Fehlmann et al., 1993). In our case, pure culture optimization experiments indicated that RT-PCR was indeed 100-fold more sensitive than was PCR. This also carried through in the matrix experiments. Interestingly, there is little direct data in the literature comparing PCR and RT-PCR detection limits in more complex sample matrices. Burtscher and Wuertz (2003) evaluated PCR and RT-PCR for the detection of *L. monocytogenes*, as well as other bacterial pathogens, from organic waste following traditional enrichment procedures. Comparable detection limits of <10 cells / g waste sample, prior to cultural enrichment, were found for both amplification methods. Bauer and Patzelt (2003), using a method to simultaneously extract RNA and DNA from forensic clinical samples, also found comparable (10^3 eukaryotic cells per sample) detection limits by PCR and RT-PCR. In comparison, we report direct PCR detection of 10^5 CFU/11g frankfurter sample (10^3 CFU/reaction) and direct RT-PCR detection limits of 10^3 CFU/11g frankfurter sample (10^1 CFU/reaction). However, we cautiously conclude that RT-PCR is more sensitive

than PCR. Specifically, the nucleic acid extraction method employed here, which was designed to isolate both DNA and RNA, was particularly cumbersome for DNA isolation. It may not, therefore, be the most optimal DNA extraction method and had an alternative method been used, PCR detection limits might have been better.

3.6 CONCLUSION

Sensitive direct detection of pathogenic microorganisms from food matrices continues to be a challenge for food microbiologists. In this study we evaluated molecular techniques to improve detection limits for the direct detection of *L. monocytogenes* from a frankfurter matrix. Under uniform extraction and amplification conditions, RT-PCR was found to be 100 times more sensitive than PCR. RT-PCR detection limits, and hence amplification efficiency, could be further improved upon by the use of an additional nucleic acid purification technique aimed at removal of competitor RNA and matrix associated inhibitors. Use of the MICROB*Express*® kit on bacterial RNA extracts improved detection limits by 10-fold and consistently yielded stronger amplification signals. Further research in the areas of extraction efficiency and further purification of extracted nucleic acid may result in continued improvements in the direct and sensitive detection of target pathogens from food matrices.

Table 3.1: Recovery Efficiency of *Listeria monocytogenes* seeded into a hotdog matrix after Concentration by Centrifugation

	% Recovery (based on loss to supernatant)[A]	% Recovery-Pellet (based on direct plating of pellet)[B]
10^6 / g	92 ± 4 (8% LTS)	118 ± 17^{X}
10^5 / g	89 ± 7 (11% LTS)	84 ± 8^{X}
10^4 / g	94 ± 2 (5% LTS)	78 ± 19^{XY}
10^3 / g	90 ± 1 (10% LTS)	58 ± 13^{XY}

[A] % Recovery (based on loss to supernatant) = 100 - [(total population in supernatant after centrifugation) x 100/(total population in sample before centrifugation)]

[B] % Recovery (based on direct plating of pellet) = [(total population in pellet after centrifugation) * 100/(total population in sample before centrifugation)]

Different superscript letters (x and y) identify statistically significant differences ($p \leq 0.05$) in percent recovery at different input levels of each organism. Statistically significant differences ($p \leq 0.05$) were observed in all comparisons between percent recovery values when calculations based on loss to the supernatant versus direct plating of the pellet were compared.

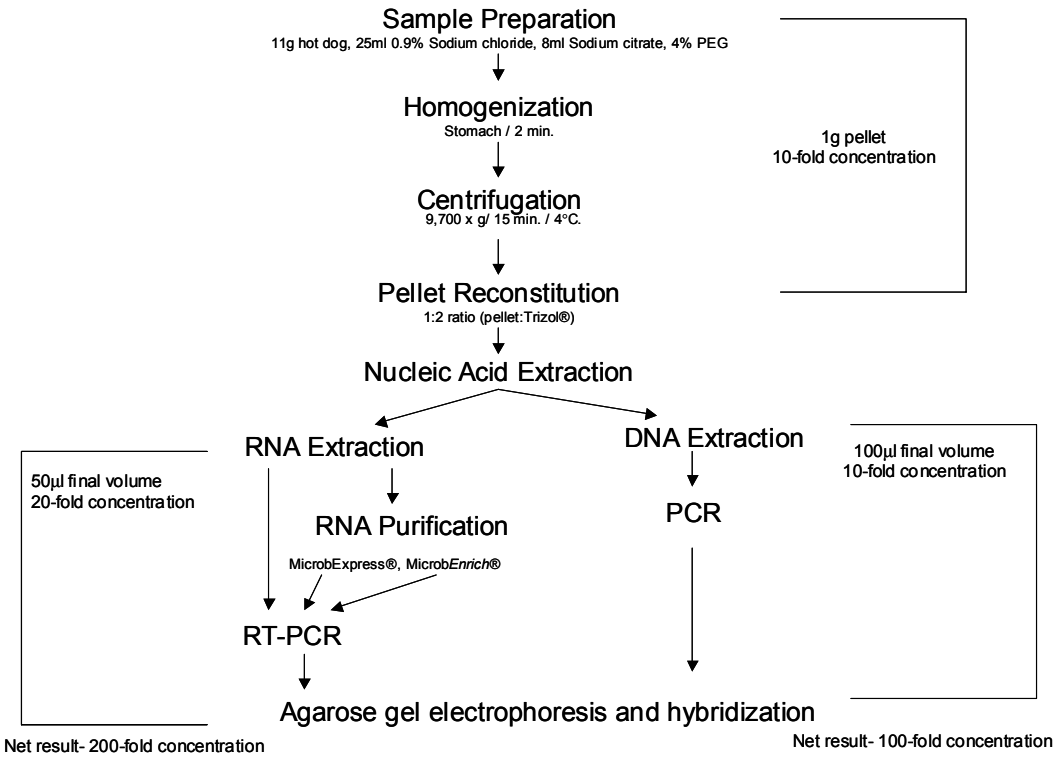

Figure 3.1. Sample Concentration, Purification and Detection Flow Diagram

A

Detection in Food (CFU/11g)	U	10^6	10^5	10^4	10^3	10^2	10^1	-	+
Detection level per amplification reactive (CFU/reaction)	U	10^4	10^3	10^2	10^1	10^0	10^{-1}	-	+
Number positive samples	0/3	3/3	2/3	0/3	0/3	0/3	0/3	0/3	3/3

B

Detection in Food (CFU/11g)	U	10^6	10^5	10^4	10^3	10^2	10^1	-	+
Detection level per amplification reactive (CFU/reaction)	U	10^4	10^3	10^2	10^1	10^0	10^{-1}	-	+
Number positive samples	0/3	3/3	3/3	2/3	1/3	0/3	0/3	0/3	3/3

Figure 3.2. Detection of *Listeria monocytogenes* in artificially contaminated frankfurters. Eleven g samples were inoculated with 10^1 to 10^6 CFU of *L. monocytogenes* and processed for bacterial concentration followed by nucleic acid isolation, amplification (Panel A, PCR; Panel B, RT-PCR) and Southern hybridization. The corresponding initial inoculum level (CFU/11g of hotdog and CFU /PCR reaction) is given below each gel lane. Each inoculum level was run in triplicate and the number of hybridization confirmed positive results per triplicate run is indicated on the bottom row below the corresponding inoculum level. Lanes: (U), uninoculated 11g sample of frankfurter processed for bacterial concentration, (-), complete reaction cocktail without sample (i.e., water); (+), positive control reaction for amplification (i.e., DNA (Panel A) or RNA (Panel B) extracted from approximately 10^8 CFU of *L. monocytogenes* in pure culture).

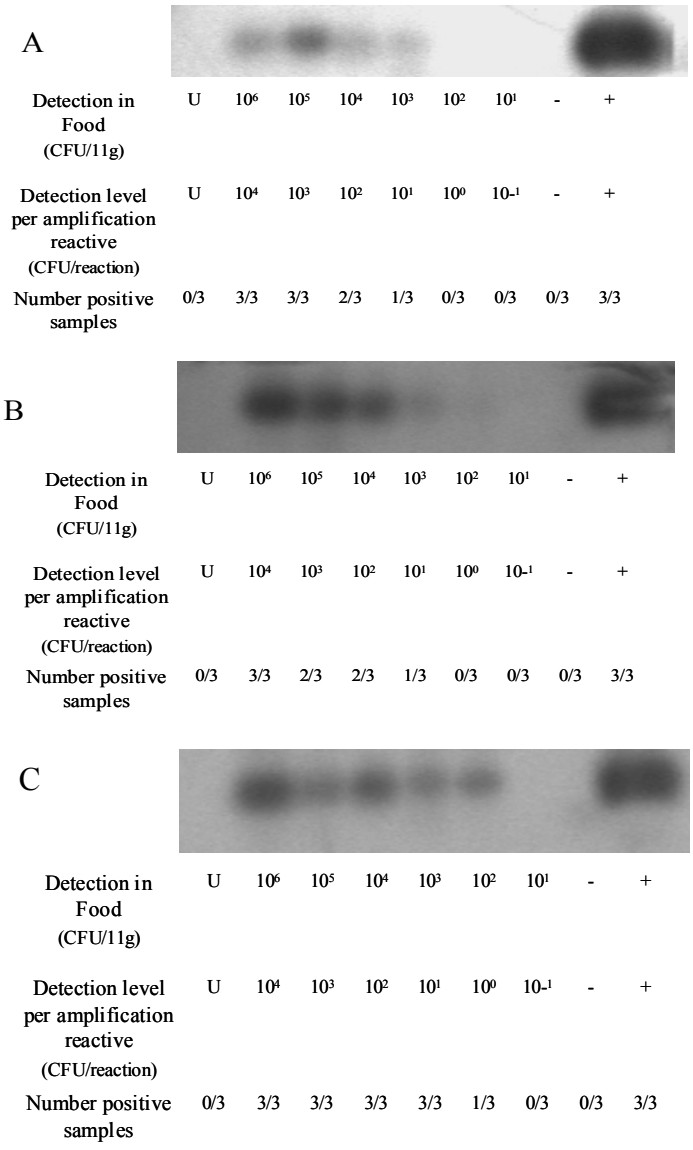

Figure 3.3 RT-PCR detection of *L. monocytogenes* in artificially contaminated frankfurters. Eleven g samples were inoculated with 10^1 to 10^6 CFU of *L. monocytogenes* and processed for bacterial concentration followed by nucleic acid isolation by guanidinium isothiocyanate (GITC, Panel A), GITC followed by MICROB*Enrich*® (Panel B), or GITC followed by MICROB*Express*® (Panel C), RT-PCR amplification and Southern hybridization. The corresponding initial inoculum level (CFU/11g of hotdog and CFU / reaction) is given below each corresponding gel lane. Each inoculum level was run in triplicate and the number of hybridization confirmed positive results per triplicate run is indicated on the bottom row below the corresponding inoculum level. Lanes: (U), uninoculated 11g sample of frankfurter processed for bacterial concentration, (-), complete PCR cocktail without sample (i.e., water); (+), positive control reaction for amplification (i.e., RNA extracted from approximately 10^8 CFU of *L. monocytogenes* in pure culture). (Shown again for comparative purposes).

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CHAPTER 4

EVALUATION OF AUTOMATED RIBOTYPING FOR THE CHARACTERIZATION OF MULTI-DRUG RESISTANT *SALMONELLA ENTERICA* SEROVAR. TYPHIMURIUM STRAINS

4.1 ABSTRACT

Automated ribotyping using the RiboPrinter® microbial characterization unit was evaluated for its ability to differentiate thirty-nine isolates of multi-drug resistant *Salmonella enterica* serovar. Typhimurium. Riboprint patterns were generated by the automated system using the restriction enzyme *Pvu*II followed by agarose gel electrophoresis and Southern hybridization, probing with the rRNA operon genes of *Escherichia coli*. Phenotypically, the isolates varied by phage type and marginally by antibiotic resistance pattern. However, the strains could not be meaningfully differentiated from one another using ribotyping. Specifically, the 39 isolates were grouped into three ribogroups, two of which (130 – S1 and 130 – S7) were virtually identical. Statistical analysis indicated no significant differences among riboprint patterns of the 39 isolates. While recognized as a valuable test for genetic typing, phenotypic methods such as phage typing and antibiotic susceptibility patterns may be more discriminatory, depending on the application

4.2 INTRODUCTION

Salmonella species cause an estimated 1.4 million cases of food-borne illness annually (Mead et al., 1999). Intestinal colonization of food animals has contributed to the association between *Salmonella* in humans and the consumption of meat and poultry products. In recent years, an increased prevalence of antibiotic resistant *Salmonella* strains, including stains resistant to multiple antibiotics, has been observed (Gebreyes et al., 2000). It has been suggested that this increased resistance may be attributed to selective pressure resulting from the use of antibiotics for therapeutic and non-therapeutic applications in both livestock and humans. The implications of multi-drug resistant Salmonella for human health are significant (White et al., 2001).

Epidemiological investigations dictate the need for determining the relatedness of bacterial isolates at levels beyond the traditional phenotypic based classification schemes. Methods for the subtyping and identification of *Salmonella* species include phenotypic methods, serological testing, phage typing, antimicrobial susceptibility testing, and genotyping, with serotyping remaining the most common means of subtyping *Salmonella* isolates (Bailey et al., 2002). Molecular methods have the potential to provide detailed information on the genetic relatedness of strains, therefore providing critical information on strain relatedness and probable transmission dynamics. Genotyping methods such as pulsed field gel electrophoresis (PFGE), randomly amplified polymorphic DNA analysis (RAPD), restriction fragment length polymorphism (RFLP) and riboprinting (both manual and automated) are powerful tools for the identification and differentiation of bacterial strains. PFGE is considered the "gold standard" and is the method used by regulatory agencies during outbreak

investigations. The question remains as to which genotyping method is the most discriminatory and most appropriate for any one particular application.

The RiboPrinter® microbial characterization system (Qualicon, Inc., Wilmington, DE) is designed to automatically generate ribotyping data. For conventional ribotyping, chromosomal DNA is extracted and then digested with the restriction enzyme *Eco*R1. Restriction fragments are separated electrophoretically followed by Southern hybridization with an *Escherichia coli* rRNA operon probe. The automated RiboPrinter® simplifies this by using robotics in the form of a liquid dispensing and dilution (LDD) and membrane-processing (MP) pipettes to transfer chromosomal DNA samples, starting with DNA extraction and digestion, subsequent gel loading all the way through the generation of final data without any human manipulations. The only manual steps are the initial steps involved in sample preparation including the harvesting of cells using a small plastic pick, suspension of cells in a sample buffer, transfer of cell suspension to a sample carrier, heat inactivation and addition of lysing agents all of which is performed immediately prior to loading the sample carrier into the RiboPrinter® system. The system also utilizes custom software to generate images of the generated fragment pattern, which are then normalized for band intensity and relative band position compared to molecular weight markers. Finally, images are characterized and identified (if possible) automatically using an *EcoR*1 database included in the system. Automated ribotyping utilizes a standardized procedure. Unfortunately, recent studies have shown that *EcoR*1 may not be suitable for ribotyping *Salmonella* species (Bailey et al., 2002, Liesegang et al., 2002, Reche et al., 2003). These studies have indicated that the restriction enzyme *Pvu*II might be a more

appropriate, yielding more discriminatory results for *Salmonella* typing. Recent changes to the RiboPrinter® platform have made it possible to use different restriction enzymes, however the database upon which comparisons can be made is somewhat limited. In this pilot study, we evaluated the automated RiboPrinter® system for the discrimination of thirty-nine previously characterized multi-drug resistant strains of *Salmonella enterica* serovar Typhimurium using the restriction enzyme *Pvu*II. Specifically, we evaluated discriminatory power, reproducibility, and adequacy of the *Pvu*II RiboPrinter® database and compared this to conventional phenotypic and serological analysis.

4.3 MATERIALS AND METHODS

Thirty-nine isolates of *Salmonella enterica* serovar Typhimurium were obtained courtesy of W. Gebreyes and C. Altier (NCSU Veterinary School). Strain history, including phage type, antibiotic resistant pattern and isolation source were previously identified and are detailed in Table 4.1. Ribotyping of the *Salmonella* isolates was performed using the automated RiboPrinter® microbial characterization system (Qualicon, Inc., Wilmington, DE). The procedure followed that provided by the manufacturer. In brief, individual cultures were streaked onto Brain Heart Infusion (BHI) (Difco, Landsing, MI) and incubated for 18 – 24 hours at 37ºC. Colonies were picked from individual culture plates, placed into tubes containing lysing buffer, and heated for 10 minutes at 80ºC in the manufacturer supplied processing module in order to inactivate endogenous DNA-degrading enzymes. Two lytic enzymes (A&B), supplied by the manufacturer, were added to each sample, which were then loaded onto the RiboPrinter® system. The remainder of the steps were completely automated. The

DNA was digested using the restriction enzyme *PVU* II (Qualicon, Wilmington, DE) followed by agarose gel separation of the restriction fragments and Southern hybridization. Riboprints were generated using a chemiluminescent probe formed from the rRNA operon of *Escherichia coli*. The RiboPrinter software normalizes the fragment pattern data for band intensity and relative band position in comparison to incorporated molecular weight markers. Patterns were grouped automatically by the system software into ribogroups based on clusters of isolates with 90 – 93% homology. Similarity values for samples are automatically generated by the RiboPrinter. The RiboPrinter software utilizes a custom proprietary mathematical algorithm which is based on individual band intensities and band distances and common statistical distances. The systems uses a cut-off of ≥ 0.85 for identification (genus – species) and ≥ 0.90 for characterization at the strain level and clustering of isolates into related groups known as RiboGroups.

4.4 RESULTS AND DISCUSSION

The restriction enzyme chosen for the characterization of *Salmonella* isolates will impact the discriminatory power of the data obtained. When initiating this study, we evaluated the restriction enzymes *Eco*RI, *Pst*II and *Pvu*II to determine which enzyme gave the largest number of bands, suggesting the highest potential discriminatory power. Based on preliminary data, *Pvu*II was determined to be the best available restriction enzyme (data not shown). This finding was in accordance with previous studies (Kumau et al., 2002, Hosoglu et al., 2003, Clark et al., 2003). For instance, Oscar (1998) studied the identification and characterization of 117 *Salmonella* isolates by automated ribotyping with the restriction enzyme *Eco*RI, finding that the

RiboPrinter® was limited in its ability to correctly identify the *Salmonella* isolates. When RiboPrinter® results using *Pvu*II were compared to serological results for 108 isolates, the RiboPrinter® was able to detect 31 ribotypes whereas serological testing was only able to detect 22 distinct antigen groups of *Salmonella* from the same sample set. The author concluded that ribotyping using the RiboPrinter® was more discriminatory than serological typing for this set of *Salmonella* isolates. Bailey et al (2002) evaluated the use of the restriction enzyme *Pvu*II for the automated ribotyping of 259 *Salmonella* isolates, finding 80% concordance between RiboPrint® type and the USDA identified serotype. The authors concluded that the percent correlation between the RiboPrinter® identification and the USDA identification would increase as the RiboPrinter® PvuII database was expanded (Bailey et al., 2002).

Genetic fingerprint analysis of the thirty-nine strains did not reveal significant differences (> 0.85) amongst the thirty-nine isolates. The isolates were grouped into 3 ribogroups, with all but one of the isolates grouped into only two of the ribogroups (Table 4.1). Two ribogroup patterns (130 – S1 and 130 –S7) were particularly similar to each other (Figure 4.1).

Ribogroups are defined by the RiboPrinter® system and are dynamic entities. As the database becomes more populated, ribogroup assignments may shift so that members in a given ribogroup maintain 90 – 93% similarity. Ribogroup patterns 130-S1 and 130-S7 appeared virtually alike. Further analysis of the samples contained within these two Ribogroups indicated that all of the samples were within the similarity standard as defined by Qualicon $\geq$ 90% allowing for manual merging of the two Ribogroups. Manual merging of the two Ribogroups, 130-S1 and 130-S7, resulted in a

Ribogroup with a mean similarity of 0.96 and all samples within the newly created RiboGroup having a similarity value of ≥ 0.90 (Figure 4.2), therefore meeting all of the criteria as defined by Qualicon for RiboGroups and manual merging. .

Thirty-seven of the 39 samples were ribotyped in duplicate in order to assess the repeatability of the process. Replicate samples were grouped in the same ribogroup 57% of the time, with the patterns of the two ribogroups being very similar. Replicate samples were found to have an average similarity value of 94%, with a range of 0.86 – 0.99, based on the characteristic banding patterns. Seventeen of the isolates had their replicates initially assigned to two different ribogroups. However, manual merging of ribogroups 130-S1 and 130-S7 as described above, resulted in only one of the isolates being assigned to two different ribogroups.

The high reproducibility of automated ribotyping has been reported in the literature, with >95% reproducibility for *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella* isolates (Pfaller et al., 1996, Oscar, 1998, Clark et al., 2003). Replicate samples that were assigned to separate ribogroups exhibited minor difference including a one band difference or a difference in band intensities. These slight differences may have been a result of differing initial cell densities used during the sample preparation step (Clark et al., 2003). Some authors have attempted to overcome this source of variability by altering the manufacturers protocol to include a measurement of optical density prior to cell lysis (Fontana et al., 2003). The slight pattern difference may not be considered significant enough to designate the strains as different when using manual ribotyping (Tenovar et al., 1995).

In general, ribotyping results were not able to discriminate strains in accordance with phenotypic typing methods, specifically antibiotic typing and phage typing. Analysis of ribogroups for samples designated into a single ribogroup by replicate analysis showed that ribogroup S7 contained 10 isolates from phage types DT104, DT193, DT12 and DT21 while ribogroup S1 contained 12 isolates, 11 of which were DT104. Both ribogroups S1 and S7 contained isolates from all of the antibiotic resistance patterns.

Advantages to using the automated RiboPrinter system include a high degree of automation, ease of use, and rapid turn around time for sample processing. Thirty-two samples can be processed within an eight-hour time frame with minimal human manipulation. As with PFGE, automated riboprinting has a standardized protocol allowing for lab to lab comparison of results. Disadvantages for the widespread use of the RiboPrinter include high initial capital expenditure for the unit (~175,000) and high reagent cost per sample (~$50), although the latter is comparable to other genotyping methods. Additional disadvantages include a lack of extensive data for alternative restriction enzymes and the potential to be too discriminatory. However, the automated system requires that samples be run in batches of 8 (at a reagent cost of $400 per run). In addition, users must run a set of four quality control organisms every four batches to ensure appropriate quality assurance. All told, automated ribotyping can be a valuable tool for the genetic characterization of isolates, however, riboprinting may have limited capabilities to discriminate specific phenotypic characteristics and should therefore be used in conjunction with phenotypic and serological testing results in order to adequately differentiate isolates.

Table 4.1 - *Salmonella enterica* serovar Typhimurium isolates; antibiotic resistance pattern phage type, isolation source and initial Ribogroup assignment

Strain #	Resistance Pattern	Phage Type	Isolation Source	Ribogroup
UAS05	AmCmStSuTe	DT104	Nursery	138-130-S1 138-130-S1
UBC36	AmKmStSuTe	DT104	Nursery	138-130-S1 138-130-S1
UBC54	AmKmStSuTe	DT104	Nursery	138-130-S1 138-130-S1
UBE1	AmKmStSuTe	DT193	Nursery	138-130-S7 138-130-S7
UBE12	AmKmStSuTe	DT193	Nursery	138-130-S1 138-130-S7
UBE13	AmKmStSuTe	DT193	Nursery	138-130-S7 138-130-S1
UBE18	AmKmStSuTe	DT12	Nursery	138-130-S7 138-130-S7
UBE20	AmKmStSuTe	DT12	Nursery	138-130-S7 138-130-S7
UBE23	AmKmStSuTe	DT193	Nursery	138-130-S7 138-130-S7
UBE27	AmKmStSuTe	DT193	Nursery	138-130-S7 138-130-S7
UBE30	AmKmStSuTe	DT193	Nursery	138-130-S7 138-130-S7
UBE32	AmKmStSuTe	DT193	Nursery	138-130-S1 138-130-S7
UBE36	AmKmStSuTe	DT193	Nursery	138-130-S1 138-130-S7
UBE39	AmKmStSuTe	DT12	Nursery	138-130-S1 138-130-S7
UBE4	AmKmStSuTe	DT12	Nursery	138-130-S7 138-130-S1
UBE40	AmKmStSuTe	DT12	Nursery	138-130-S7 138-130-S7
UBE5	AmKmStSuTe	DT193	Nursery	138-130-S7 138-130-S1
UBE7	AmKmStSuTe	DT193	Nursery	138-130-S7 138-130-S7
UBE9	AmKmStSuTe	DT12	Nursery	138-130-S7 138-130-S7

Table 4.1 - *continued*

Strain #	Resistance Pattern	Phage Type	Isolation Source	Ribogroup
UBF07	AmCmStSuTe	DT104	Finisher	138-130-S1 138-130-S1
UBF1	AmCmStSuTe	DT104	Finisher	138-141-S1 138-130-S1
UBF10	AmCmStSuTe	DT104	Finisher	138-130-S1 138-130-S7
UBF11	AmCmStSuTe	DT104	Finisher	138-130-S7 138-130-S1
UBF12	AmCmStSuTe	DT104	Finisher	138-130-S7 138-130-S1
UBF13	AmCmStSuTe	DT104	Finisher	138-130-S1 138-130-S1
UBF14	AmCmStSuTe	DT104	Finisher	138-130-S1 138-130-S1
UBF15	AmCmStSuTe	DT104	Finisher	138-130-S1 138-130-S7
UBF16	AmCmStSuTe	DT104	Finisher	138-130-S1
UBF18	AmCmStSuTe	DT104	Finisher	138-130-S1 138-130-S1
UBF19	AmCmStSuTe	DT104	Finisher	138-130-S1 138-130-S7 138-130-S7
UBF2	AmCmStSuTe	DT104	Finisher	138-130-S1 138-130-S7
UBF3	AmCmStSuTe	DT104	Finisher	138-130-S1 138-130-S1
UBF4	AmCmStSuTe	DT104	Finisher	138-130-S1 138-130-S1
UBF8	AmCmStSuTe	DT104	Finisher	138-130-S7 138-130-S7
UBF9	AmCmStSuTe	DT104	Finisher	138-130-S7 138-130-S1
UBS08	AmCmStSuTe	DT104	Finisher	138-130-S7 138-130-S1
UBS09	AmCmStSuTe	DT21	Finisher	138-130-S7
UBS10	AmKmStSuTe	RDNC	Finisher	138-130-S1 138-130-S1
UBU04	StSu	DT104	Finisher	138-130-S7 138-130-S7

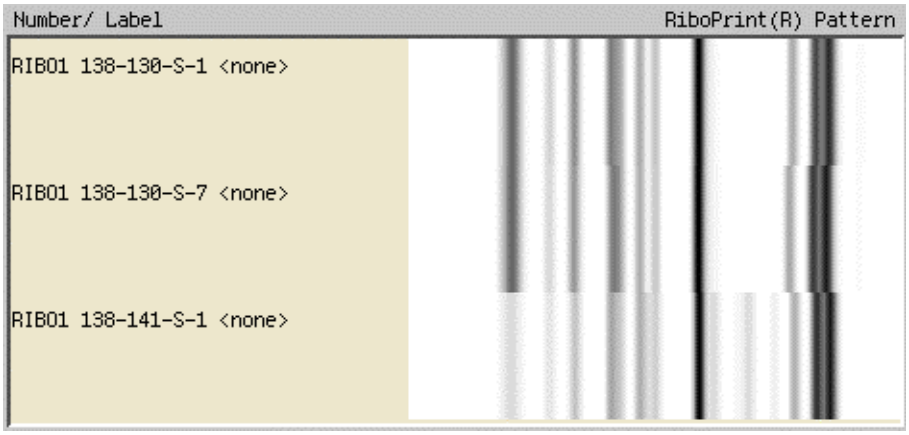

Figure 4.1 - Ribogroup patterns (as assigned by the automated Riboprinter® system)

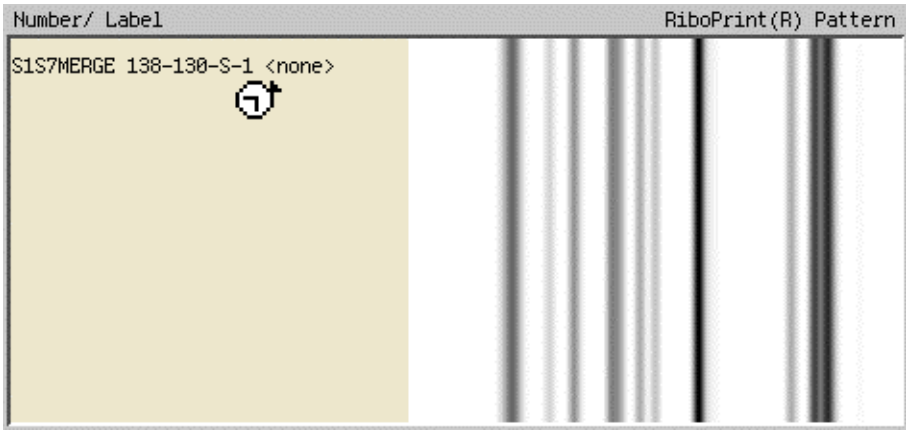

Figure 4.2 - Ribogroup pattern for merged ribogroups 130-S1 and 130-S7

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CHAPTER 5

APPLICATION OF DECISION ANALYSIS TOOLS TO THE DECISION OF WHETHER TO TEST FOR THE PRESENCE OF *LISTERIA MONOCYTOGENES* IN SMOKED FISH

5.1 ABSTRACT

Food processors have many options to minimize the risk of contamination, including finished product testing for pathogens. The purpose of this study was to develop a model addressing the issues encountered when making a testing decision. *L. monocytogenes* contamination in smoked fish and a commercial PCR-based method were used in the model. Three potential consequences of contamination were considered as elements of the decision, e.g., no consequences, regulatory recall without disease, and disease outbreak. Accordingly, the inputs of the model were (i) costs associated with food-borne contamination (business and health related costs); (ii) reliability of testing; and (iii) prevalence of contamination. Prevalence of contamination, test sensitivity, and test specificity values were estimated in distribution form from the literature. Information on costs associated with contamination was obtained in consultation with food safety experts. The elements of the decision were structured into a logical format using decision trees (DecisionTools®, Palisade Corp., Newfield, NY). Each branch of the decision tree was solved using the corresponding estimates for probability of contamination, costs incurred, and positive predictive value. Sensitivity analysis indicated that the decision to test or not to test for *L. monocytogenes* in smoked fish is most influenced by the positive predictive value. Product value inputs

were also important to the decision. In general, the model indicated that testing for highly prevalent pathogens may provide an increase in food safety but end product testing for pathogens of low prevalence should be carefully considered and may not be justified due to limited return on investment.

5.2 INTRODUCTION

Listeria monocytogenes is an environmentally ubiquitous, psychrotrophic food-borne pathogen. It is of particular concern for ready-to-eat products such as smoked fish that do not receive a terminal heating step prior to consumption, and which may have a particularly long shelf-life. Foodborne illness due to *L. monocytogenes* can be severe for select at-risk populations including the young, elderly, immunocompromised, and fetuses / newborns. Symptoms range from mild and flu-like to meningitis, septicemia, and spontaneous abortion. For normal populations, ingestion of the organism, even at high levels, usually has no consequence. It is estimated that each year *L. monocytogenes* contaminated food causes approximately 2,500 cases of foodborne listeriosis, however this organism has the second highest case fatality rate and the highest hospitalization rate of all of the foodborne pathogens (Mead et al., 1999, Gallagher and Kause, 2003).

Microbiological testing of finished product does not ensure food safety and has inherent statistical limitations. False positive and false negative test results can be misleading, resulting in either overestimation or underestimation of product safety. For instance, it cannot be assumed that if a food sample tests negative for the presence of a pathogen, that the sample or even the lot from which that sample was taken is free of the pathogen and therefore safe. In fact, pathogen contamination in foods is more likely

to be detected when the prevalence of the pathogen is high. In addition, sampling plans for the detection of food-borne pathogens assume that the distribution of the organisms in the food is random. However, non-random distribution of pathogens is more likely. In short, it is not possible to ensure food safety by testing due to the impossibly large number of samples that would need to be screened in order to assure that low levels of contamination are reliably detected. It must also be remembered that many food-borne pathogens are highly infectious, meaning that only a small number of cells are needed to cause illness. The interactions between food contamination, pathogen test results and food-borne illness are extremely complex and an understanding of when testing no longer adds value to a food safety program is greatly needed.

Due to nature and severity of illness caused by *L. monocytogenes*, FSIS has established a "zero tolerance" policy for the presence of this organism in ready-to-eat foods. This means that no detectable level of *L. monocytogenes* is permitted in these products. Destructive microbial testing at the processing facility can be used to determine if *L. monocytogenes* is present in a food product. Limitations that should be considered prior to implementing a lot acceptance sampling plan include; 1) the inability to screen 100% of product produced; 2) non-uniform distribution of a pathogen throughout the lot of food; 3) high cost and lengthy timeframe for testing; 4) false positive and false negative rates; and 5) test detection limits. Prevalence of the target organism in the food product will also impact the ability of the test method to accurately detect the target pathogen.

The application of risk assessment tools to the understanding and management of food safety issues is relatively recent (Jaykus, 1996, Lammerding and Paoli, 1997,

Cassin et. al., 1998, and Gallagher et. al., 2003). In general, the focus of recent risk assessments has been to identify stages in the production and processing of food that may result in pathogen contamination, and to use this information to model potential mitigation strategies and their possible efficacy. Although the draft *Escherichia coli* O157:H7 risk assessment evaluates the relative test sensitivity of different cultural methods and the effect of sample size on detection limits in clinical (fecal) specimens, it makes no conclusions about the value of testing (USDA – FSIS, 2001). As a whole, the microbial risk assessments available in the literature do not address the value of testing finished product nor do they address the elements involved in making the decision of whether or not to test for the presence of pathogenic bacteria in finished product.

Decision analysis is commonly used in fields such as business, economics and pharmaceuticals as a systematic approach to solving complex problems (Clemen and Reilly, 2001, Holloway, 1979). Decision analysis tools allow decision makers to use a logical framework to structure a decision, typically using decision "trees" to develop a decision model. The purpose of this study was to develop a model addressing the issues encountered when making a testing decision. This study focused on the issues relevant to the ability of testing methodologies to accurately detect the presence or absence of *L. monocytogenes* in model ready to eat smoked fish product.

5.3 MATERIALS AND METHODS

The DecisionTools® (Palisade Corp., Newfield, NY) suite of programs was used to develop and solve this decision problem. The PrecisionTree component of the program was used to construct the decision tree and calculate the estimated monetary values for each branch of the tree. Sensitivity analysis in the form of Tornado diagrams

were automatically generated using the TopRank component of the DecisionTools® software. Variables in the testing decision for this model included the value of product prior to sale (since product that tests positive must be destroyed), estimated costs associated with recall, estimated costs associated with food-borne illness, legal costs associated with product recall, legal costs associated with food-borne illness, costs of micobiological testing, prevalence of *L. monocytogenes* in smoked fish, test sensitivity, and test specificity. All of this information was used to solve the decision tree and determine the optimal decision for the testing decision (Table 5.1, 5.2, and 5.3).

Financial costs associated with recalls, including cost associated with the recall process, product value, business cost, and legal costs, are not readily available in the literature. Data for this portion of the model was generated using expert judgment from an industry contact. The research literature was reviewed for the following data inputs: 1) prevalence of *Listeria monocytogenes* in smoked fish (Gombas et al., 2003, Hoffman and Wiedmann, 2001, Norton et al., 2000, Norton et al., 2001); 2) sensitivity and specificity of the BAX method for the identification of *L. monocytogenes* in smoked fish samples (Norton et al., 2000, Hoffman and Wiedmann 2001); 3) the economic value of lost life (Shorgren et al, Buzby et al., 1996, Kuchler and Golan, 1996); and 4) costs associated with cases of listeriosis (Buzby et al., 1996, Kuchler, and Golan, 2001, SaraLee 2003). The combination of *Listeria monocytogenes* and smoked fish were chosen for this model due to the availability of data in the research literature and the relevance of this pathogen – product combination with respect to foodborne disease.

Issues associated with sampling such as sampling plans, pathogen distribution, number of samples needed for statistical significance, number of samples tested per lot, and costs associated with test and hold programs were not addressed in this model.

5.4 RESULTS AND DISCUSSION

In order to construct a decision tree for the testing decision, one must first understand the potential consequences of *L. monocytogenes* contamination in smoked fish products which are outlined in Figure 5.1. The four potential consequences include the following: (i) no consequence; (ii) endemic disease; (iii) regulatory recall (no disease); and (iv) disease outbreak. Because the situation cannot be quantified and is likely to go unrecognized, the possibility of endemic disease was not considered in this model.

Food contaminated with bacterial food-borne pathogens could have three possible outcomes when consumed, each with different financial implications (Figure 5.1). The first and most desirable outcome to the processor would be that the contaminated food was consumed but with no resulting illness, in which case there would be no cost to the processor. The second possible outcome would be detection of pathogen contamination by regulatory agencies. This outcome is possible even when finished product testing is implemented, due to inherent limitations in sampling programs and pathogen distribution. If pathogens were detected in ready to eat foods such as smoked fish by regulatory agencies, the product would be subject to immediate recall irrespective of associated illness. Potential costs of a regulatory recall without foodborne illness would include shipping and transportation, publicity, product rework or destruction and lost revenue. These costs were structured in proportion to the cost of the product being

recalled (Table 5.1). The least desirable outcome would be that contaminated food causes illness. Since food associated with an outbreak is also recalled, this outcome would have all of the costs associated with a recall plus legal, business, and health related costs of an outbreak (Table 5.2). Legal costs associated with food-borne illness may include legal fees, court fees, and increased liability insurance. Business costs, including the damage to a processors reputation as a result of a recall or outbreak is not easily quantified, but can be seen in measures such as decreased sales. Health related costs are incurred only when food-borne illness results from the consumption of contaminated food and include the cost of an illness and the cost of a lost life. Using the base values as an example, it is apparent that although the probability of an illness related recall is low (.001%) (Evancho, 2003), the financial consequences can be substantial (Figure 5.2).

Once the potential consequences are defined, the decision tree can be built. The decision tree presented in Figure 5.2 was designed to evaluate the testing decision from the processors perspective. The numbers at the end of each branch represent the potential cost to the processor for each scenario. For example, the branches labeled "illness outbreak", imply that smoked fish contaminated with *L. monocytogenes* was consumed and caused illness, with an estimated monetary cost at 0.5 million dollars per outbreak episode.

In terms of microbiological methods of detection, we chose the PCR-based BAX® system for the detection of *Listeria monocytogenes* is an AOAC-approved testing method that is widely used by the industry (Figure 5.3). BAX® sensitivity and specificity data has been reported in the literature, particularly for the detection of *L.*

monocytogenes from naturally contaminated smoked fish (Table 5.3). There is also ample prevalence data for *L. monocytogenes* in smoked fish (Table 5.3). The sensitivity, specificity and prevalence data were used to calculate point estimates for the positive predictive value for the test. The application of Bayes theorem to this calculation resulted in an estimate that *L. monocytogenes* was actually present only 52% of the time that the test was positive, 48% of the time, these would be false positive test results.

Initial one-way sensitivity analysis for the testing decision involving the regulatory recall situation only was done using the default values as determined by the TopRank program. TopRank default settings for sensitivity analysis are uniform distribution, upper and lower bounds within +/- 10% of the base values, and use of four intermediate values (uniformly distributed) within those preset upper and lower bounds. The resulting sensitivity analysis indicated that in the case of regulatory recalls, the testing decision was most sensitive to the positive predictive value of the test (Figure 5.3). Business and legal cost inputs also were important to the decision. Changing the program default setting from uniform distribution to normal distribution for all of the inputs did not alter the resulting sensitivity analysis (data not shown).

The sensitivity analysis described above was refined using the uniform distribution for all inputs and using the upper and lower bounds as indicated in Table 5.1 in place of the default settings of +/- 10%. The resulting sensitivity analysis (Figure 5.4) indicated that legal costs were most important to the decision, followed by positive predictive value and business costs. In point of fact, there is a large amount of uncertainty in costing estimates, as indicated in our case by the large range in values.

This may contribute to the significant impact costing has on the decision and suggests that more definitive costing estimates and / or restructuring of the decision may be warranted. It might be advantageous to restructure the decision such that costing does not unjustly influence it, perhaps allowing, for widespread use of the model by processors regardless of lot size and / or product value.

In the first two analyses described above, positive predictive value was used as an input. Positive predictive value is calculated from *L. monocytogenes* prevalence, test sensitivity and test specificity (Table 5.3). It made sense to determine what influence, if any, the individual variables used to calculate positive predictive value had on the testing decision. For this analysis, a uniform distribution and the actual upper and lower bounds indicated in Table 5.1 were used in TopRank. One-way sensitivity analysis indicated that once again, legal costs were most important to the decision, followed by test specificity, business costs, *L. monocytogenes* prevalence, and test sensitivity (Figure 5.5). Clearly, the test specificity and the *L. monocytogenes* prevalence were the two most important issues related to test validity. This makes sense because poor (low) specificity suggests a high rate of false positive results, which would later be confirmed as the negatives. This would of course be modulated by prevalence such that extremely low prevalence of contamination might be unduly influenced by a high false positive rate as compared to higher prevalence situations.

Initial one-way sensitivity analysis for the testing decision involving an illness associated recall only was done using a uniform distribution and the actual upper and lower bounds as indicated in Table 5.2. As in Figure 5.4, positive predictive value was used as an input. The resulting sensitivity analysis indicated that the testing decision

again was most sensitive to the positive predictive value of the test, followed closely by legal costs. Business related costs, health associated costs were also important to the decision (Figure 5.6). When the variables used to calculate positive predictive value were used as inputs, legal costs became more important to the testing decision, followed by test specificity, business costs, prevalence of *L. monocytogenes*, test sensitivity and health associated costs (Figure 5.7). Clearly, the results from sensitivity analysis for illness associated recalls parallel the results from the regulatory recall testing decision scenario.

5.5 CONCLUSIONS AND FUTURE WORK

A formal decision analysis model such as the one presented in this paper has not been reported in the literature for a food-borne pathogen testing decision. This decision tree would be of value to processors when faced with the dilemma of whether or not to test for the presence of microbial pathogens in finished product. It could provide a logical systematic method to make decisions based on data, including pathogen prevalence and test reliability. By incorporating individual estimates for the cost of testing, as well as recall and outbreak-associated costs, which are likely to differ among companies, the food processor can tailor the tool to his/her specific needs. The decision tree serves as a visual diagram of the potential decisions and consequences associated with the testing decision. Sensitivity analysis provides guidance to the decision maker as to which factors most heavily influence the decision and associated outcomes.

Preliminary sensitivity analysis indicated that the positive predictive value of testing frequently influences the decision to test. It seems plausible that testing for highly prevalent bacterial pathogens would potentially improve food safety by

increasing the likelihood of detecting the pathogen and hence facilitating segregation or removal of contaminated food from the market. Conversely, testing for a pathogen of low prevalence may not be justified due to limited return on investment with no substantial improvement in food safety. Sensitivity analysis also indicated that the quantity and value of the product produced expressed as business and recall costs, will also influence the testing decision.

A theoretical goal for this type of exercise would be to produce a model that could be used universally and so would not be unduly influenced by product value or lot size. To do this, business cost and recall costs might be better presented in a proportional manner, thereby preventing bias due to varying processor size. Costs associated with outbreaks also need to be more rigorously estimated. Outbreak costs are substantial and are related to the proportion of the lot contaminated as well as the likelihood of consumption with subsequent disease. Once again, these costs need to be addressed in the model without undue influence on the final model outcome. In addition, users of the model should be able to apply it to a testing decision for pathogens other that *L. monocytogenes* and a wide variety of products. It is anticipated that such a model could be used for any microorganism that is detected and regulated by qualitative (presence / absence) assay.

Some additional work is needed in order to refine the cost inputs. MonteCarlo simulation of the inputs was not performed in this project, however such simulations may provide additional insight into the key drivers of the testing decision. Another intriguing possibility for the refinement of this model is to include information pertaining to the issues associated with sampling, including sampling plan design,

pathogen distribution, statistical significance, and costs associated with test and hold programs. The removal of costing bias in conjunction with the potential linkage to sampling inputs may provide a model with direct applicability to the needs of food processors.

Table 5.1: Cost Inputs for Regulatory Recall

Cost Input	Base Value	Lower Bound	Upper Bound	Reference
Pounds of smoked fish produced per year	500,000	60,000	3,500,000	Lappi., 2003
Pounds of smoked fish produced per lot (1 lot per day, 260 days per year)	1000	480	3360	Lappi., 2003
Product cost per pound (producer)	1	.3	1.2	Evancho, G., 2003
Product Value**	1000	144	4032	
Recall costs (as function of product value)	0.2	0.1	0.5	
Cost to recall - regulatory recall***	200	14.4	2016	Evancho, G. 2003
Cost to business reputation - regulatory recall	100000	0	125,000	Evancho, G. 2003
Legal cost - regulatory recall	100,000	0	500,000	SaraLee, 2003
Total cost of regulatory recall****	104,844	40	558,477	

**Product Value = (Pounds of smoked fish produced per year) (Product cost per pound)

***Cost to recall = (Product Value) (Recall costs)

****Total cost of regulatory recall = (Positive predictive value)(Product value + cost to recall + cost to business reputation + legal cost)

Table 5. 2: Cost Inputs – Illness Recall

Cost Input	Base Value	Lower Bound	Upper Bound	Reference
Pounds of smoked fish produced per year	500,000	60,000	3,500,000	Lappi., 2003
Pounds of smoked fish produced per lot (1 lot per day, 260 days per year)	1000	480	3360	Lappi., 2003
Product cost per pound (producer)	0.5	0.3	1	Evancho, G., 2003
Product Value**	250,000	150,000	500,000	
Recall costs (as function of product value)	0.2	0.1	0.5	
Cost to recall - regulatory recall***	50,000	15,000	250,000	Evancho, G. 2003
Cost to business reputation - regulatory recall	240,000	125,000	1,000,000	Evancho, G. 2003
Legal cost - regulatory recall	500,000	100,000	5,000,000	SaraLee, 2003
Estimated cost of food-borne disease – (Listeriosis)	33,600	250	50,000	SaraLee, 2003 Kuchler and Golan, 2001
Estimated value of a statistical loss of life	5,900,000	100,000	8,000,000	Buzby et al., 1996 Shogren et al. 2000 Buzby et al., 1996 Kuchler 1999
Total cost of regulatory recall****	540,000	97,890	7,627,118	

**Product Value = (Pounds of smoked fish produced per year) (Product cost per pound)

***Cost to recall = (Product Value) (Recall costs)

****Total cost of regulatory recall = (Positive predictive value)(Product value + cost to recall + cost to business reputation + legal cost)

Table 5.3: Testing and Prevalence Inputs for Smoked Fish Samples

Input	Lower bound	Upper bound	Base Value	Reference
Prevalence of *L. monocytogenes* in smoked fish	3.8%	7.2 %	4.31%	Gombas et al. 2003 Hoffman and Weidmann, 2001 Norton et al., 2000 Norton et al., 2001
Sensitivity of BAX method	84.8	99%	91.8%	FSIS,1998 Hoffman and Weidmann, 2001 Norton et al., 2000
Specificity of BAX method	90%	99%	96.2%	FSIS, 1998 Hoffman and Weidmann, 2001 Norton et al., 2000
Positive predictive value*	25%	88%	52%	--
Cost per test using BAX method	$17 /test	$36.50 / test	$20 / test	Tortorelli, 2003

* positive predictive value = $\frac{\text{(prevalence) (sensitivity)}}{\text{(prevalence) (sensitivity) + (1 - prevalence) (1 - specificity)}}$

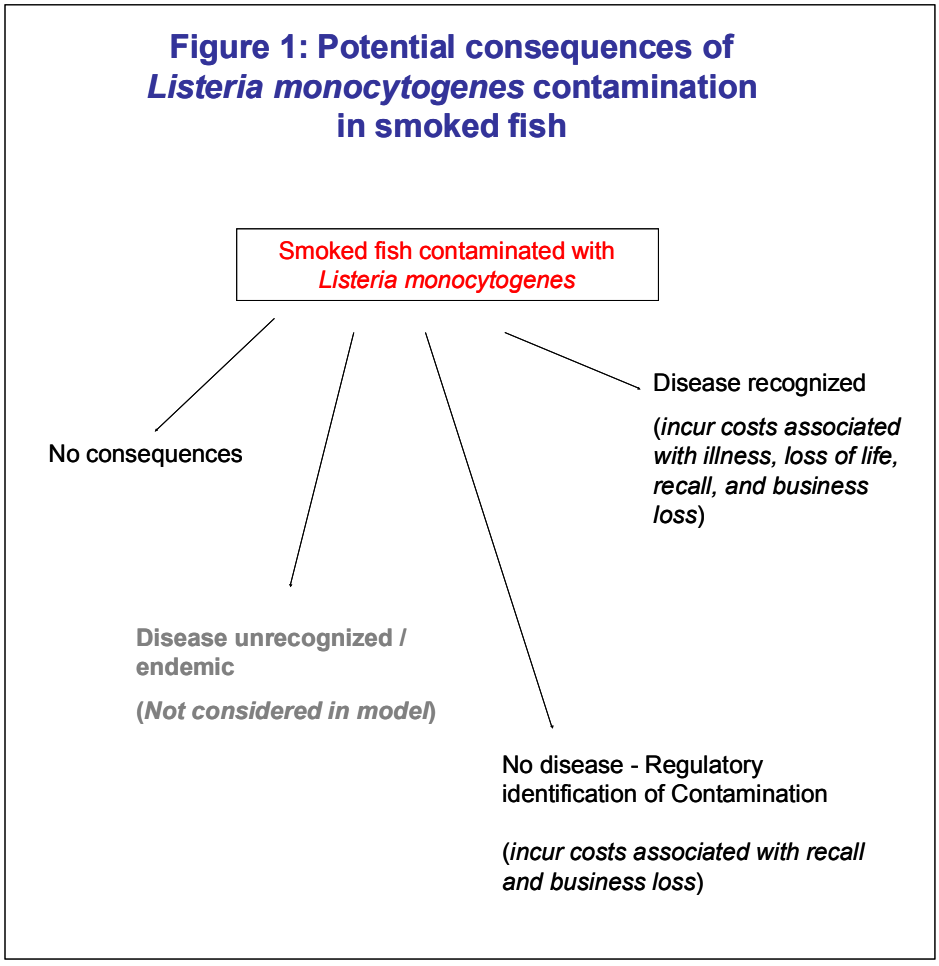

Figure 5.1 – Potential Consequences of *Listeria monocytogenes* in Smoked Fish

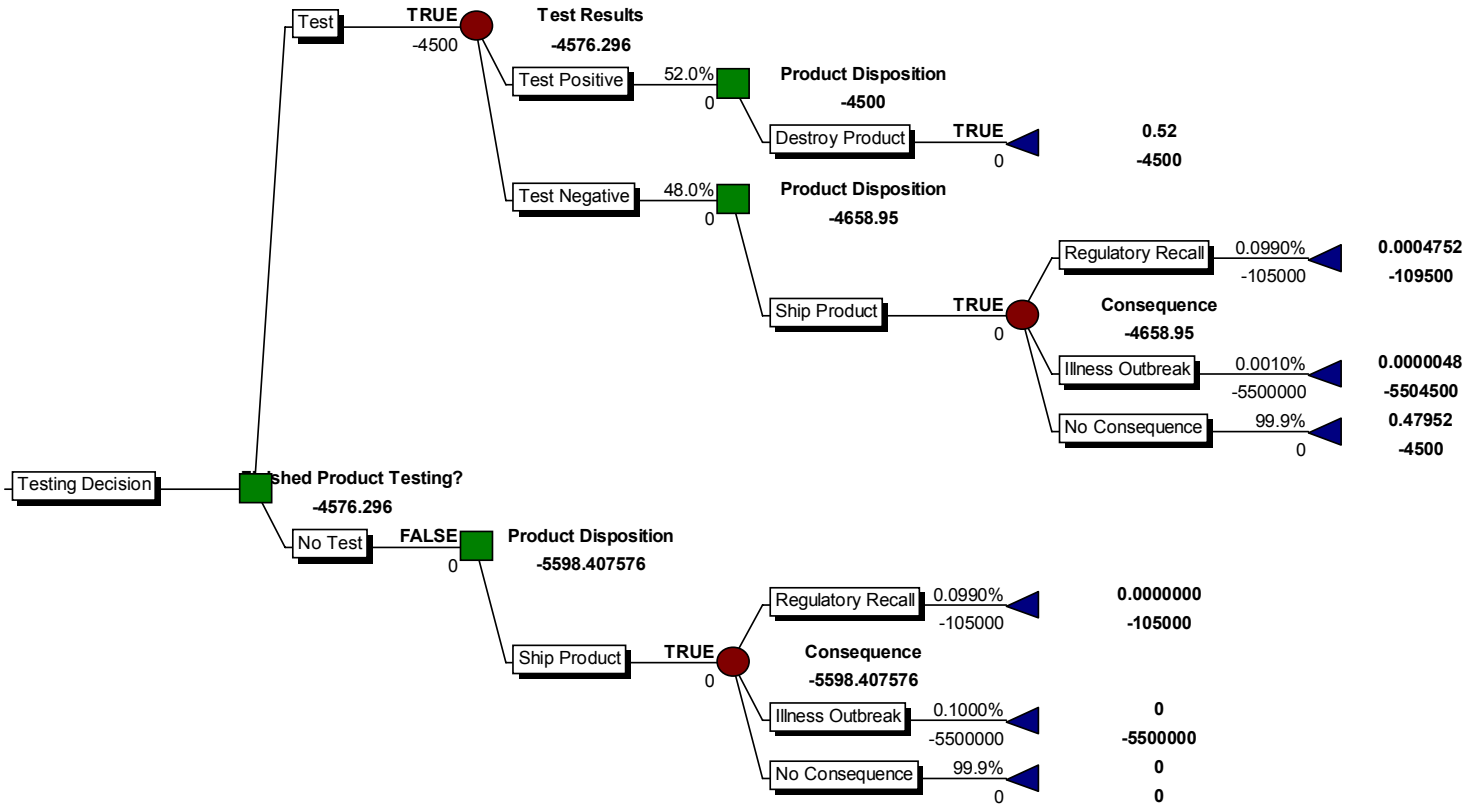

Figure 5.2 – Decision Tree for Testing Decision

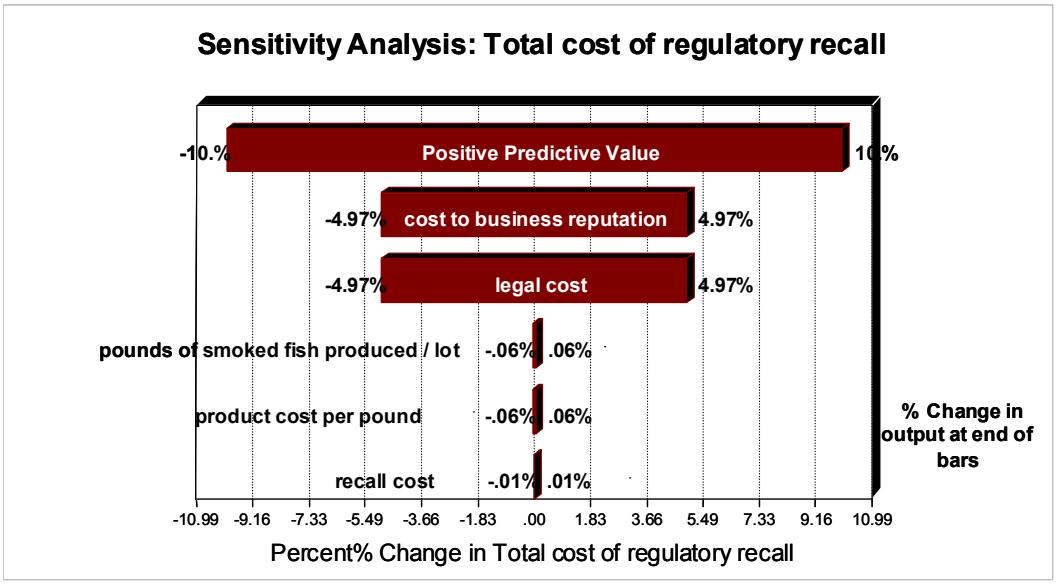

Figure 5.3 - Initial sensitivity analysis for regulatory recall (inputs +/- 10%)

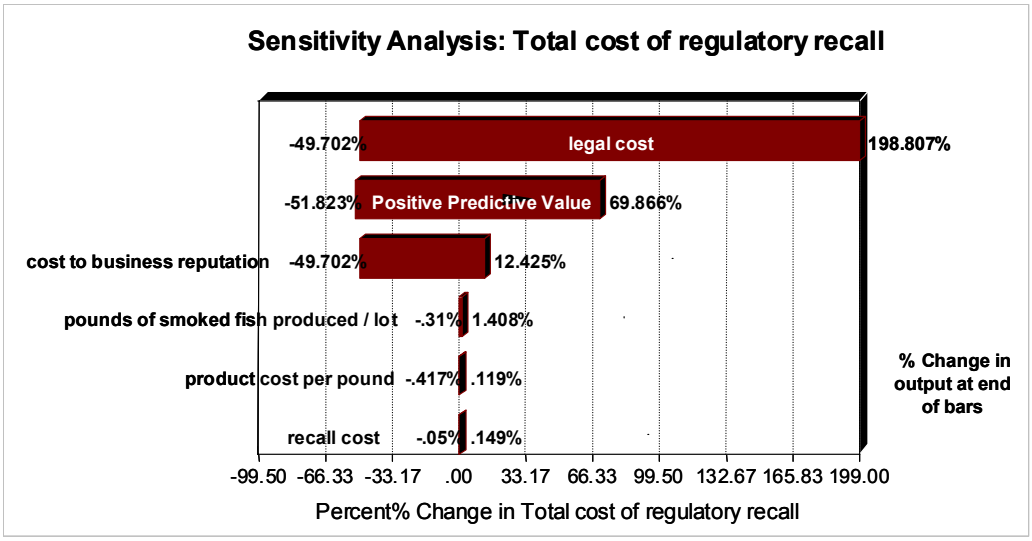

Figure 5.4 - Sensitivity analysis for regulatory recall (upper and lower bounds from data and positive predictive value as input)

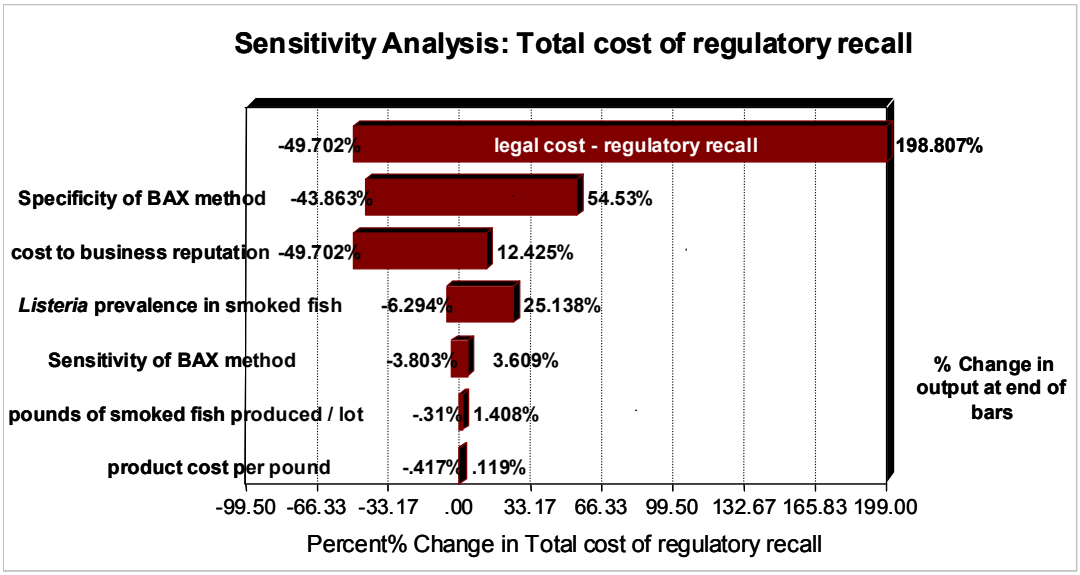

Figure 5.5 - Sensitivity analysis for regulatory recall (upper and lower bounds from data, prevalence, test sensitivity and test specificity as inputs)

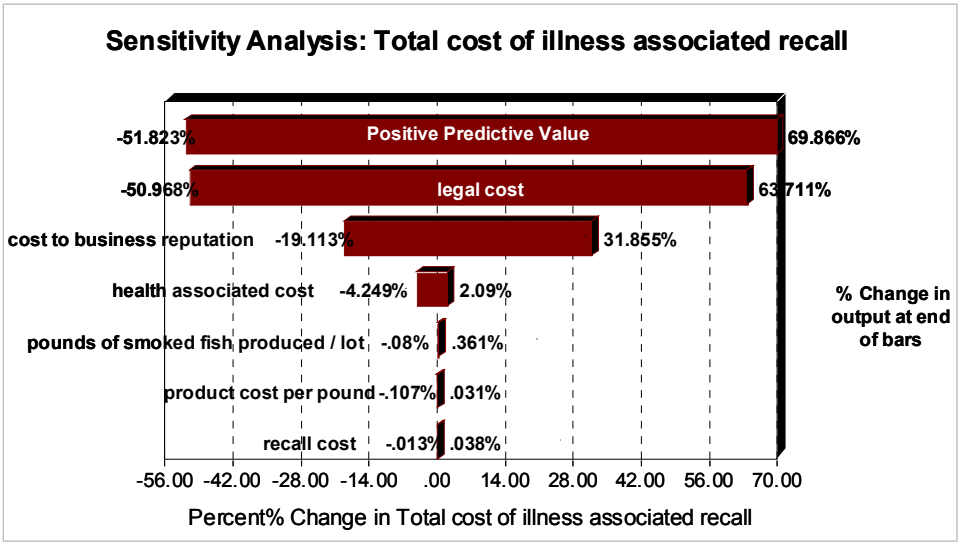

Figure 5.6 - Sensitivity analysis for illness associated recall (upper and lower bounds from data and positive predictive value as input)

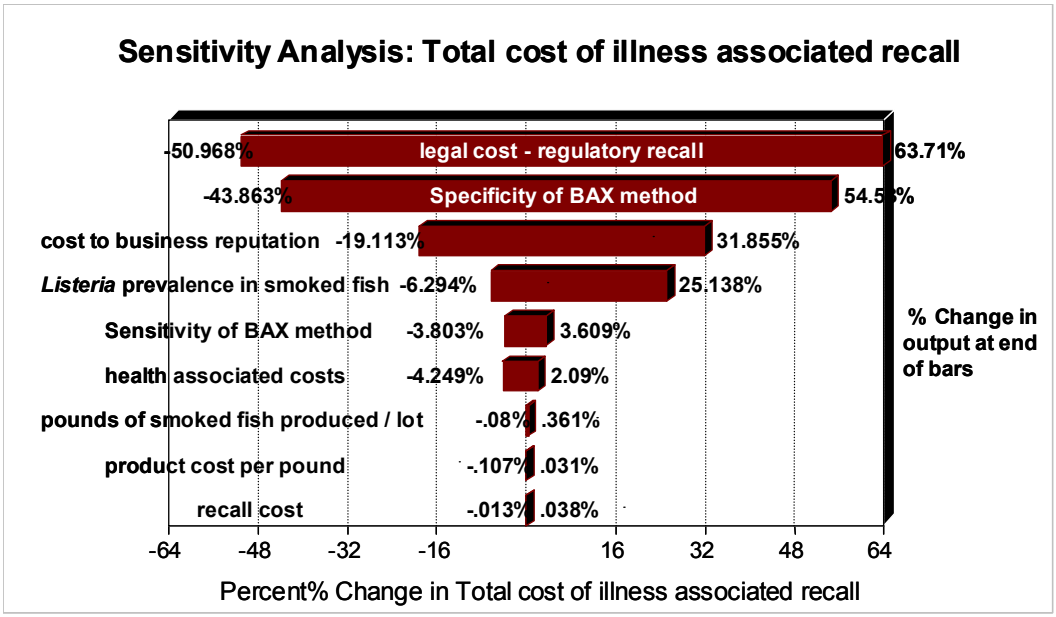

Figure 5.7 - Sensitivity analysis for illness associated recall (upper and lower bounds from data, prevalence, test sensitivity and test specificity as inputs)

5.6 REFERENCES

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CHAPTER 6

CONCLUSIONS

Sensitive direct detection of pathogenic microorganisms from food matrices continues to be a challenge for food microbiologists. Without question, the major impediment to our ability to detect foodborne pathogens in "real" or near "real"-time is the continued reliance on cultural enrichment. Decreasing or eliminating the need for enrichment would allow food and environmental microbiologists the opportunity to truly harness the power of the emerging molecular detection methods. The ideal method would be able to both concentrate pathogens and remove matrix-associated inhibitors; further, it would be universal (e.g., applicable to multiple food types and microorganisms), simple, rapid, and inexpensive. This should be done in a manner that minimizes the chance for false positive results that might occur because of cross-reactivity with residual matrix components or because of the detection of dead target cells. Once the presence of foodborne pathogens is detected, the source of that contamination may also need to be identified. Molecular tools such as automated ribotyping can be used to characterize isolates, and this was investigated and compared to phenotypic based characterization methods. Finally, we approached the testing decision from the perspective of the food processor using a logical, decision analysis approach.

In the first study, careful attention to sample preparation and nucleic acid amplification optimization allowed for the direct detection of *Listeria monocytogenes* and *Salmonella enterica* serovar Enteritidis from complex dairy matrices. The sensitivity of detection achieved in this study, without the use of cultural enrichment,

approaches that of other rapid methods based on cultural enrichment. With that said, there is no "silver bullet" with respect to matrix preparation, and it is likely that future methods will be both matrix- and perhaps organism-specific. Detection limits were better for *Salmonella* than for *L. monocytogenes*, despite the fact that bacterial recoveries after concentration were virtually identical, suggesting that additional refinements to the amplification protocols will be necessary.

In the second study we evaluated alternative nucleic acid purification techniques to improve molecular amplification detection limits for the direct detection of *L. monocytogenes* from a frankfurter matrix. Under uniform extraction and amplification conditions, RT-PCR was found to be 100 times more sensitive than PCR. RT-PCR detection limits, and hence amplification efficiency, could be further improved upon by the use of an additional nucleic acid purification technique aimed at removal of competitor RNA and matrix associated inhibitors. Further research in the areas of nucleic acid extraction efficiency and purification may result in continued improvements in the direct and sensitive detection of target pathogens from food matrices.

The third study evaluated automated ribotyping for its ability to differentiate thirty-nine strains of antibiotic resistant *Salmonella enterica* serovar Typhimurium. Advantages to using the RiboPrinter system include a high degree of automation, ease of use, and rapid turn around time for sample processing. Thirty-two samples can be processed within an eight-hour time frame with minimal human manipulation. As with PFGE, automated riboprinting has a standardized protocol allowing for comparison of results between laboratories. Disadvantages for the widespread use of the RiboPrinter

include high initial capital expenditure for the unit and high reagent cost per sample (~$50), although the latter is comparable to other genotyping methods. Additional disadvantages include a lack of extensive data for alternative restriction enzymes and the potential to be too discriminatory. Automated ribotyping can be a valuable tool for the genetic characterization of isolates, however, in our application riboprinting was limited in its ability to discriminate between the serovar. Typhimurium strains tested. We conclude that in this case, phenotypic and serological testing was more discriminatory than was ribotyping. Furthermore, in this case, there was no relationship between ribotype or phage resistance, nor was there a relationship between ribotype and specific antibiotic susceptibility pattern.

In the final study, a formal decision analysis model was designed for a food-borne pathogen testing decision from a food processor's perspective. The model provided a logical method to make decisions based on data, including pathogen prevalence and test reliability. By replacing estimates on the cost of testing, as well as recall and outbreak-associated costs, the food processor can tailor the tool to his/her specific needs. The decision tree also served as a visual diagram of the potential decisions and associated consequences associated with the testing decision. Sensitivity analysis provided the decision maker with information on which factors most heavily influenced the decision and associated outcomes. Preliminary sensitivity analysis indicated that the positive predictive value of testing significantly influences the decision to test. It seems plausible that testing for highly prevalent bacterial pathogens would potentially improve food safety by increasing the likelihood of recognizing contamination and the subsequent opportunity to prevent contaminated food from reaching the market.

Conversely, testing for a pathogen of low prevalence may not be justified due to limited return on investment without substantial improvement in food safety. Sensitivity analysis also indicated that the quantity and value of the product produced, business and recall costs will also affect the testing decision.

These efforts represent continued progress in harnessing the power and diversity of molecular methods for the identification and characterization of foodborne pathogens. The development of systematic approaches to making testing decisions is also justified and needed by the industry. Taken together, these studies add to our understanding of the impediments to application of rapid methods for the detection of foodborne pathogens, and provide some solutions to facilitate the practical use of these methods in the future.

APPENDICES

APPENDIX 1 – Metal Hydroxide Concentration Flow Chart

Sample Preparation
11-g sample
25 ml 0.9%NaCl
8 ml 25% Sodium Citrate
4% PEG 8000

↓

Homogenize
Stomach / 2 min

↓

Primary Centrifugation
9,700 *x g* / 15 min / 4C
4 – 5g pellet (2-fold concentration)

↓

Immobilization
2X Zirconium Hydroxide (2:1v/w) to pellet
Agitate 10 min at room temperature

↓

Secondary Centrifugation
9,700 *x g* / 15 min / 4C
4 – 5g pellet (2-fold concentration)

↓

Recovery and Enumeration

APPENDIX 2 – Recovery Studies of Mild Cheddar Cheese Using Zirconium Hydroxide

	% Loss to Supernatant		% Recovery
	1° Centrifugation	2°Centrifugation	
Listeria monocytogenes			
High (10^6 cfu/11g)	3.78±0.3	1.21±1.6	102.97±21.8
Medium (10^4 cfu/11g)	2.03±0.4	0.24±0.1	71.67±10.6
Low(10^2 cfu/11g)	4.49±1.2	0.27±0.1	78.97±6.40
Salmonella enteritidis			
High (10^5 cfu/11g)	1.78±1.5	1.01±1.4	118.38±52.7
Medium (10^3 cfu/11g)	1.96±2.9	0.27±0.05	71.05±20.9
Low(10^2 cfu/11g)	0.46±0.4	0.63±0.2	103.40±33.1

APPENDIX 3 – Recovery Studies of Non-Fat Yogurt Using Zirconium Hydroxide

	% Loss to Supernatant		% Recovery
	1° Centrifugation	2°centrifugation	
Listeria monocytogenes			
High (10^6 cfu/11g)	0.56+0.2	1.4±1.0	66.27±8.6
Medium (10^4 cfu/11g)	1.17±0.7	1.74±1.5	66.68±5.8
Low(10^2 cfu/11g)	1.81±1.4	1.86±1.2	141.4±57.0
Salmonella enteritidis			
High (10^5 cfu/11g)	2.04±2.1	0.44±0.3	134.80±78.0
Medium (10^3 cfu/11g)	1.55±0.9	1.70±0.6	113.33±65.3
Low(10^2 cfu/11g)	0.45±0.1	0.76±0.3	166.37±76.5

APPENDIX 4 – Bacterial Concentration Followed by Cultural Enrichment Procedures

Listeria monocytogenes			
Cheddar Cheese		**Non-Fat Yogurt**	
Inoc level	Confirmed Recovery	Inoc level	Confirmed Recovery
10^0 / 11g (5-26 cfu, x=12.8)	4/4 positive	10^0 / 11g (8-14 cfu, x=10.3)	4/4 positive
10^1 / 11g (59-158 cfu, x=96)	4/4 positive	10^1 / 11g (70-151 cfu, x=118.8)	4/4 positive
10^2 / 11g (570-1480 cfu, x=845.3)	4/4 positive	10^2 / 11g (798-1354 cfu, x=959.5)	4/4 positive

Salmonella enteritidis			
Cheddar Cheese		**Non-Fat Yogurt**	
Inoc level	Confirmed Recovery	Inoc level	Confirmed Recovery
10^{-1} / 11g (0-3 cfu, x=1.5)	0/3 positive	10^{-1} / 11g (0-3 cfu, x=1.8)	1/3 positive
10^0 / 11g (7-21 cfu, x=14.7)	1/3 positive	10^0 / 11g (6-26 cfu, x=17.7)	2/3 positive
10^1 / 11g (16-114 cfu, x=74)	3/3 positive	10^1 / 11g (135-185 cfu, x=156.3)	3/3 positive
10^2 / 11g (1030-1600 cfu, x=1300)	3/3 positive	10^2 / 11g (1396-2350 cfu, x=1974.2)	3/3 positive

Pathogens were seeded into 11g non-yogurt or cheddar cheese samples. Seeded samples were prepared using 8ml of 25% sodium citrate, 1.76g PEG, and 25ml normal saline. Samples were centrifuged at 9,700 x g for 15 minutes at 4C using sterile cheesecloth. The pellet was resuspended using 20 ml the appropriate enrichment buffer (as per BAM methodology).