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AUTHOR'S ACKNOWLEDGEMENTS

I would like to first thank the faculty and staff of the Virginia Tech Food Science and Technology department. Without their help I'd be completely lost. I would like to thank my committee members; Dr. Joe Eifert, Dr. Renee Boyer, Dr. Stephen Melville, and Dr. Susan Sumner, for their endless knowledge and advice given to me throughout the endeavors of my research. I would like to specially thank the married "dynamic duo" Joell and Dr. Joe Eifert for their extreme patience and willingness to answer all of my silly and stupid questions. Without their tolerance, this project may have never been completed (at times I really thought I'd never finish). I would also like to acknowledge Kristi Decourcy for her help with my microscope problems. Thank you so much for dealing with my never ending microscope dilemmas. And a special thank you to Dr. Wesley Black, without your donation of a plasmid, I'd still be at the beginning of this all.

I extend my thanks to my fellow students within the Food Science department. I would like to thank Dina Romano for going on countless adventures to places other than the office and lab to get a short glimpse of what the world looks like beyond the FST building. I would also like to thank her for being one of the best friends and the best influences that one person could ever have. I would like to also thank my pseudo-office mates John Koontz and Kevin Holland for their uncanny ability to make me laugh continuously. Who ever knew that watching way too much "You Tube" and "wiki-ing" everything would have gotten us this far? And don't worry; I will always strive to say things that are "quote worthy".

I would lastly like to give my deepest thanks to my family, my wonderful husband in particular. Without you, I would be nothing. You have been there to push me (sometimes literally) through this all. Thank you for never letting me give up. I would also like to thank my mother-in-law, Jill and father-in-law, Patrick. You were there for me, even when others were not. You always made me strive for things greater than what I thought I was capable of accomplishing. I would also like to thank my sister. Sam, I did this all for you, to show you what we; no, YOU are capable of. Please don't ever think that you are anything less than amazing.

Thank you for being you. I love you all.

DEDICATION

I dedicate this work to my friends and family, especially my husband. Without their undying support I would have never made it this far.

Chapter I- Introduction of Fluorescent Microspheres as Surrogates for
Salmonella enterica serotype Typhimurium in Recovery Studies from Stainless
Steel

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Keywords:

Salmonella Typhimurium, microspheres, bacterial recovery, stainless steel

CHAPTER I

INTRODUCTION AND JUSTIFICATION

The transmission of *Salmonella* to humans can occur through several avenues, including: consumption of contaminated foods (produce, eggs, poultry and other meats); direct contact with animals and their environment; and also cross contamination through direct contact of foods to contaminated surfaces (5). Food contact surfaces, such as stainless steel, are a key mechanism for pathogens to contaminate food products. Even with proper hygienic and sanitation practices, bacterial attachment to and retention on the surface of stainless steel may still occur (33).

With the growing concern over food safety and preventing foodborne illnesses, further research to prevent, detect, and control foodborne pathogenic microorganisms is greatly needed. Historically, surrogate and indicator microorganisms have been used to study and predict the presence or fate of pathogenic microorganisms in the environment. Non-pathogenic surrogate microorganisms have been used for several reasons, including, decreased time required for growth and detection, simpler or more cost-effective analysis, and utility in areas where pathogenic organisms cannot be tolerated. In recent years, non-biological surrogates, such as fluorescent microspheres, have been used in the field of food science to investigate mechanisms of association between bacteria and foods (50), mechanisms of contamination (9), biofilm formation (34), and filtration studies (13). Microspheres are available from many companies.

Microspheres are microscopic polystyrene spheres that are uniform in size and shape, are able to hold charges and surface functional groups, and they will react to fluorescent light (22). Molecular Probes (Invitrogen) (28) states that their microspheres are produced from high-quality, ultra clean polystyrene and are internally labeled with proprietary dyes.

Currently there have been no reports on the use of non-biological surrogates to study the fate of *Salmonella enterica* serotype Typhimurium in a food processing environment. Selected microspheres may be useful in many quantitative and qualitative studies to understand the transmission, movement, attachment, and detachment of microorganisms to or from foods and food-contact surfaces. Since a population of microspheres cannot change biologically, they would be useful for studies that have a goal of determining the fate of microorganisms within a food facility or a quantitative analysis. This project will explore the optimum recovery method for *Salmonella enterica* serotype Typhimurium, the optimum recovery method for selected microspheres, and the optimum recovery of a cocktail of the two inoculated onto stainless steel using a rinse method, a one-ply composite tissue method, or a sonicating brush method. The ability to recover and quantify *S. Typhimurium* will be compared to the ability to recover and quantify fluorescent microspheres that have been applied to stainless steel. The overall goal of this study is to evaluate the use (fluorescent) microspheres as surrogates for *S. Typhimurium* in recovery studies from a food-contact surface (stainless steel).

LITERATURE REVIEW

***Salmonella enterica* Typhimurium**

Characteristics of the organism

The genus *Salmonella* is separated into seven subgroups. These seven subgroups are then serotyped further according to somatic O, surface Vi, phase flagellar, and phase 2 flagellar antigens (17). *Salmonella enterica* serotype Typhimurium belongs to subgroup 1 serotype 1,4,5,12:i:1,2; however, it is typically referred to as *S. Typhimurium* by the Centers for Disease Control and Prevention and the academic community (17). *Salmonella* spp. are gram-negative, nonsporeforming, facultatively anaerobic rods belonging to the *Enterobacteriaceae* family (12). In general, *Salmonella* spp., including *S. Typhimurium*, are motile by peritrichous flagella; however, some variants like *S. pullorum* and *S. gallinarum* do not produce flagella and therefore are immotile (12). This genus is chemoorganotropic, meaning that they are capable of degrading organic material and compounds for energy. It is catalase positive, oxidase negative, and generally produces hydrogen sulfide. *Salmonella* use citrate as their sole carbon source and can decarboxylate lysine and ornithine (12). Because of these characteristics, *Salmonella* spp. are best grown and isolated from triple sugar iron (TSI) agar, Hektoen enteric agar, and lysine iron media. An enrichment broth (typically selenite cystine) added to the primary media can assist in the recovery of small numbers of *Salmonella*. This step is

not typically done unless during an illness outbreak investigation, when screening for carriers, and other clinically necessary situations (17).

Salmonella spp. are exceptionally tolerant and adaptive to harsh and excessive environmental conditions. *Salmonella* are mesophiles that grow in an optimum temperature range of 30 to 42°C; however, the organism also exhibits psychrotropic properties when growing in foods at temperatures from 2 to 4°C (12). *Salmonella* are also extremely adaptable to pH, capable of growing at a pH range of 4.6 to 9.5, with growth between 6.5 to 7.5 (12). They are also tolerant of salt concentrations of 3 to 4%; this tolerance increases as temperature increases, typically in the range of 10°C to 30°C (12).

Distribution

Salmonella is ubiquitous to natural environments and found readily in uncooked meat, poultry, fish, and shellfish; it has been of growing concern to the food industry. For example, salmonellae are prevalent in raw poultry products such as chicken, turkey, and other avian species. This large distribution throughout the poultry industry is attributed to the migration and feeding habits of the carriers; as well as the entry of feral carriers, like rodents, into poultry houses and processing facilities. *Salmonella* can be difficult to eradicate it from food processing environments and products (12).

Specifically, *Salmonella* Enteritidis can proliferate within the shells of eggs. *S. Enteritidis* contaminate the egg itself is due to transovarian transmission which occurs prior to shell formation (7). This is of great concern to the poultry industry

due to the general healthy appearance of the infected hens (7). *Salmonella* spp. are also pervasive throughout the bovine and swine meat industries.

Contamination of these meat products is due to environmental sources of contamination, feed contamination, and parental transmission of infection (12). Infected cattle can excrete up to 10^8 CFU/g of feces and further contaminate the environment and other uninfected animals (54). Some cattle are capable of being active carriers of *Salmonella*; these animals do not exhibit clinical symptoms, but excrete the organism, and therefore become a problem to food production (54). Swine have also been shown to shed *S. Typhimurium* through their feces for up to 28 weeks after initial infection (56). Although ovine species have been shown to be effected by salmonellosis, it is typically a host-specific and does not cross over to humans (54).

Most recently, *Salmonella* contamination of fresh fruits and vegetables have been associated with human salmonellosis. Fresh fruits and vegetables can be contaminated in various ways. Contamination can be introduced through the roots, stem scars, and leaves of several different fruits and vegetables (26, 29, 55). It is thought that the source of most of the contaminated produce is through untreated irrigation waters (12, 29, 55).

Through all of these original sources of contamination, pre- and post-processing facilities may become contaminated with *Salmonella*. Even though regular cleaning and sanitation procedures within a food processing facility are important, contamination must first be addressed at the basic level: prevention.

Salmonellosis

Salmonellosis is the disease that is caused by *Salmonella* species (20).

Salmonellosis infections can lead to numerous clinical conditions, such as enteric (typhoid) fever, uncomplicated enterocolitis, and systemic infections by nontyphoid microorganisms (2). *Salmonella* serotypes are responsible for an estimated 2 to 4 million cases of foodborne illness each year in the United States alone (20).

Those infected with enteric fever (either from the typhi or paratyphi strain) exhibit severe and serious symptoms. Symptoms typically appear after 7 to 28 days after initial infection and they may include diarrhea, lingering and spiking fever, abdominal pain, headache, and the feeling of being drained of all strength.

Typically, an asymptomatic chronic carrier state follows the acute phase (2). The chronic effects of enteric fever are arthritic symptoms that can occur three to four weeks after onset of acute symptoms (20).

All foodborne salmonellosis infections are non-typhoidal and typically start to present symptoms anywhere from 8 to 72 hours after ingestion of the pathogen (2). This illness is characteristically self-limiting (2). Symptoms of nontyphoid salmonellosis are non-bloody diarrhea and abdominal pain which occur within five days of the onset of the disease. This type of salmonellosis can also worsen into systemic infections and several other chronic conditions (2). Chronic conditions can include aseptic reactive arthritis, Reiter's syndrome, and ankylosing spondylitis (12).

The treatment for enteric (typhoidal) fever is based on supportive therapies and/or antibiotic remedies. These antibiotics include, but are not

limited to chloramphenicol, ampicillin, or trimethoprim-sulfamethoxazole (2). Antibiotic treatment, unfortunately, has proven to have limited effectiveness because in recent years, many typhoid and paratyphoid microorganisms have become resistant to antibiotic treatments (2). Rates of antibiotic resistance for several other serotypes of *Salmonella* have also been increasing throughout past years (20). It is rare for non-typhoidal salmonellosis to be treated with antibiotics, yet there are considerable proportions of the *Salmonella enterica* serotype Typhimurium that are resistant to multiple antibiotics (6). In particular *S. Typhimurium* definitive type 104 (DT104) is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline, all very commonly prescribed antibiotic treatments (2).

Pathogenesis

Those who are most susceptible to salmonellosis are newborns, infants, the elderly, and the immunocompromised. Ingestion of as few as 10 colony forming units (CFU) of *S. Typhimurium* from common foods, such as cheddar cheese or chocolate, may cause illness. Factors which determine pathogenesis include the immunological heterogeneity within human populations, the virulence of the infectious strains, as well as the chemical composition of the contaminated foods (2).

Salmonellosis is caused by the penetration and passage of *Salmonella* cells from the gut lumen into the epithelium of the small intestines, predominately from a contaminated food source (20). The *Salmonella* must first successfully

out-compete the native microflora within the intestines to properly attach to the intestinal wall. Once the pathogen has attached, signaling occurs between the pathogen and the host cell, resulting in the invasion of intestinal cells. This invasion, rather than the enterotoxin that is produced, is responsible for the diarrheic response of the body.

The virulence of different serovars varies because of several distinct factors. Serovars such as Typhimurium, Dublin, Gallinarum-Pullorum, Enteritidis, Choleraesuis, and Abortusovis have been found to carry virulence plasmids (12). These plasmids are typically anywhere from 30 to 60 megadaltons in size, are capable of aiding in the systemic spread and infection of other tissues (other than the intestines) in the body, and enable the pathogen to rapidly multiply within the host, overwhelming its defense mechanisms (12). *Salmonella's* virulence is also attributed to the presence of siderophores. Siderophores are responsible for the retrieval of essential iron from the host tissue (12). Without this iron in the host tissues, many cellular functions cannot continue and will ultimately result in cell death (12). In addition to plasmids and siderophores, *Salmonella* also utilizes capsular polysaccharides, differing lengths of the lipopolysaccharide membrane (LPS) protruding from the outer membrane (used as a defense to the pathogen from host immune system attacks), porins (act as transmembrane channels), and diarrheagenic enterotoxins (12).

Foodborne transmission

The consumption of contaminated foods is the most prevalent route of transmission of salmonellosis to humans. Foods may become contaminated through direct contact with an infected animal or from their environment (5). The occurrence of *Salmonella* can contaminate food anywhere along the farm to fork continuum (12, 49). Ready-to-eat products are typically contaminated during post-processing steps (40). Post-processing contamination is largely contributed to poor handling practices (14). This factor is extremely important because of the growing number of ready-eat-products on the market and the convenience they provide to consumers. Improper cleaning and sanitation for preparation utensils and areas of home kitchens can lead to the transmission of *Salmonella* from contaminated surfaces to ready-eat-products. *Salmonella* may persist for up to 1 to 2 hours prior to inoculation (42) and even as long as 12 hours on stainless steel, a common food contact surface (14). Typical food items that have been linked to salmonellosis in the past include, milk, eggs; raw meats such as, poultry, bovine, and swine; salad dressings, peanut butter, and chocolate (20). The contamination of poultry products and fresh produce have been increasing in frequency (5).

Presence in food and food processing environment

One of the largest foodborne *Salmonella* outbreaks to date occurred in 1985 and involved over 16,000 cases in 6 states (20). The outbreak was attributed to the consumption of low fat and whole milk from a single Chicago

dairy (20). The cause of this outbreak was linked to improper pasteurization procedure (20). It was found that the pasteurization equipment had been augmented in a way so that the raw milk came in contact with the pasteurized milk, causing contamination (20).

In 2006, there was an outbreak of *S. Typhimurium* from fresh tomatoes (19). These tomatoes were consumed in restaurants across 21 states, causing 183 cases of illness (19). The FDA effectively traced the source of the tomatoes to the Eastern shore of Virginia (21) and told the public that all contaminated tomatoes had been destroyed, consumed, or perished; assuring that this outbreak was not ongoing (19). This outbreak accounted for 14% of the cases associated with *Salmonella* in 2006 (5).

The Foodborne Diseases Active Surveillance Network (FoodNet) found that in 2006 a total of 17,252 laboratory-confirmed cases of foodborne illness occurred in the United States (5). Of these cases, nearly 6,000 were caused by various *Salmonella* serotypes (5). *S. Typhimurium* was the leading cause of salmonellosis, causing 64% of reported infections (5). Based on previous incidence of occurrence per year, the numbers of infections caused by *S. Typhimurium* significantly decreased (5). It should be noted however, even with this significant drop of cases per year, *S. Typhimurium* is still the number one cause of reported salmonellosis (5).

Bacterial attachment to food contact surfaces

The attachment of bacteria on food contact surfaces is known to increase the risk of foodborne illness from the cross-contamination of foods (33). Some bacteria use attachment as a means to survive in nature and often in environments outside of their natural niche (35). Stainless steel surfaces are extremely prevalent in food processing and packaging facilities. If contaminated, these surfaces pose a great risk of contaminating pre- and post-processed food products. Pathogens such as *Salmonella* Enteritidis, *Staphylococcus aureus*, and *Campylobacter jejuni* are capable of surviving from 40 to 96 hours on stainless steel (33). Maxcy found that even after a water washing, used to simulate a standard washing component of a sanitation process, 52% of a *S. aureus* inoculum remained on the surface of stainless steel. When surfactants (anionic or ionic) at a 0.1% concentration, were added to the rinse, 19% of the original inoculum survived the washing treatment (39).

Peng et al. suggested that this adhesion to food contact surfaces can be facilitated by extracellular surface structures such as flagella, pili, and extracellular polysaccharides (46). Generally, attachment occurs in a two-step process (37). First the organism comes close enough to the surface to weakly attach by electrostatic forces (58). During this attachment step, cells can be easily removed from the surface. In the second step, the attachment is irreversible (58). Extracellular polysaccharides are produced by the bacteria and form a matrix which firmly attaches itself to the surface (58).

Salmonella and attachment

Some *Salmonella* spp. are capable of producing exopolymeric substances, such as curli and cellulose, to aid in attachment and biofilm formation on surfaces. Through the use of these extracellular appendages, *Salmonella* Typhimurium can exhibit rugose, or rough wrinkled colony morphologies (15). This morphology is often associated with resistance to adverse living conditions such as a low pH or the presence of hydrogen peroxide (15). Rugosity of an organism is correlated with curli and cellulose (15). Curli may work in combination with cellulose and cause cells to adhere to one another, which yields a wrinkled or rugose colony morphology (15). Curli are generally synthesized below 30°C, are active in biofilm formation, and can cause an organism to adhere to a surface or to one another (15). Hammer et al. postulated that curli are the most likely candidate to mediate the initial step of surface attachment (25). Bacteria that possess curli can colonize numerous surfaces such as plant tissue, glass, and more importantly, stainless steel (25).

In 2006, Olivera et al. (45) showed that the degree of adherence of *Salmonella enterica* Enteritidis from four different sources on three different kitchen surfaces tested were statistically significant. They concluded that *Salmonellae*'s adhesion to surfaces is extremely strain dependent, regardless of the similarity between the degrees of hydrophobicity of the strains investigated, and that the non-uniformity of bacterial surfaces can affect the hydrophobicity of a cell from one study to another (45). They also recognized that the differences

in adherence can establish a factor of virulence between the different serotypes tested (45).

More recently, in 2007, a study using *Salmonella* Typhimurium (ATCC 13311), results showed a statistically significant difference in the amount of cells attached to marble and granite surfaces (51). This study concluded that stainless steel; in particular type 304, had greater hydrophobicity than any of the other surfaces tested, greater extent of roughness, and that *S. Typhimurium* was highly hydrophilic (51).

Bacterial recovery from food surfaces

Effective removal and recovery of bacterial pathogens from food contact surfaces is important to accurately determine the amount of contamination present. Several methods are currently used to recover bacteria from surfaces. These methods generally vary depending on the surfaces being tested. Bacterial recovery from stainless steel is most commonly achieved using a swab, sponge, or rinse method (23, 43, 48). However, among these methods of recovering and enumerating bacteria on surfaces, there is no consensus for an acceptable standard (43). Despite this finding, information gained from recovery studies can aid in the assessment for the optimal level of cleaning, sanitation, and disinfection for both processing and packaging equipment and environments in the food industry. Three methods for removing bacteria from stainless steel and other food contact surfaces are further discussed below. Each of these methods could be used to enumerate microorganisms from a sampled surface.

Rinse method

Environmental sampling and testing can be used as an early warning system for the detection of unwanted bacteria. A rinse method is often used to detect and enumerate bacteria present within a food processing or packaging environment. For this method, a known volume of sterile buffered rinse solution is used. The surface of interest is placed within the sterile solution and is either allowed to sit or agitate. After an allotted amount time (typically around one minute), a portion of the solution is serially diluted. From these dilutions, standard plate counts or selective media can be used to determine the approximate number or type of bacteria present on the contaminated sample. If low numbers are expected, a membrane filtration procedure for enumeration may be used. The types and number of microorganisms enumerated can be directly correlated to the potential of spoilage of a food product that comes in contact with the contaminated surface (16).

In a recent study, the efficacy of several microbiological methods for enumeration of turkey carcasses was assessed. Due to their relatively large weight and volume, turkey carcasses are often sampled with sponges and swabs rather than by rinsing. When comparing the recovery of *Salmonella* from whole carcasses, McEvoy et al. reported that the rinse method was more effective than the swabbing methods tested. They attested their findings to heterogeneous distribution of *Salmonella* on the turkey carcasses (41).

Sonicating brush method

Human error is often a significant factor in the recovery of bacteria. Bloomfield et al. (3) declared that the accuracy, precision and repeatability of conventional methods of recovery are greatly affected by the skill and technique of the operators. By using an automated recovery method, these errors can be significantly reduced. Sonication serves as a mechanical method to efficiently remove bacterial cells from surfaces.

Sonicating toothbrushes are used to remove plaque causing bacteria from hard to reach areas of teeth. Sonicating toothbrushes like the Sonicare® Elite, which operate at 260 Hz, can remove plaque on teeth by generating localized hydrodynamic shear force (27). The oscillation of the brush head, when inserted into a fluid/air environment, generates turbulent fluid and bubble activity (27). This force and bubble activity provide for the sonicating toothbrush's superior plaque moving abilities (27). A previous study showed that even from a distance of nearly 4 mm away from the surface of interest (in this case a model dental surface *in vitro*), a sonicating toothbrush was capable of removing the attached oral bacterium *Actinomyces naeslundii* (57).

Kang, et al. (31) used a sonicating brush, among other methods, to compare quantitative recovery of *Listeria monocytogenes* from stainless steel surfaces. A cocktail of four serotypes of *L. monocytogenes* were mixed in equivalent concentrations and inoculated onto type 304 stainless steel coupons (31). After 1 h exposure, coupons were sampled either by contact with the bristles of a sonicating brush head for 1 min, indirect contact (2-4 mm) with a

sonicating brush head for 1 min, sonication in an ultrasonic water bath (40 kHz), swabbing using a pre-moistened Dacron swab, rinsing with phosphate buffered saline, or direct contact onto tryptic soy agar containing 0.6% yeast extract (TSA+YE) plates for 10 sec (31). The three sonication methods yielded higher cell recoveries than the other three methods ($P < 0.05$) (31). Brushing the coupons with the sonicating brush head (contact or non-contact) yielded the highest recovery level (~60%) of the inoculum (31).

One-ply composite tissue method

Recently, Vorst et al. (2004) compared several accepted methods of bacterial recovery for *Listeria monocytogenes* from stainless steel. The most successful method utilized ordinary one-ply composite tissue, better known as a Kimwipe®. The one-ply composite tissue performed better than well accepted methods such as the sterile environmental sponge, sterile cotton-tipped swab, and sterile calcium alginate fiber-tipped swab. The one-ply composite tissue recovered populations from 1.11 to 2.70 log/cm² higher than the other methods. The differences between this new method and older methods were statistically significant and showed that even the most commonly recommended protocol for environmental sampling, the environmental sponge method, was the least effective in quantitatively recovering *L. monocytogenes* (53). The rationale for this difference is that unlike other conventional methods, such as an environmental sponge or swab, the one-ply tissue does not allow for the

entrapment of bacterial cells. The one-ply composite tissue method is also ideal for the detection of low levels of bacterial contamination (53).

Surrogates for food borne pathogenic microorganisms

The presence of non-pathogenic microorganisms may suggest the presence of a pathogen. These non-pathogenic microorganisms are called indicator organisms. Mossel and others define indicator organisms as markers whose presence in numbers exceeding given numerical limits indicate the possible occurrence of ecologically similar pathogens. For instance, the presence of non-pathogenic *Escherichia coli* may indicate fecal contamination within a food processing facility (47). The use of indicator organisms is extremely dependent upon microbiological criteria in place for a certain food product (18). Non-pathogenic microorganisms have also been used as surrogates for pathogens in food processing environments.

To assess the efficacy of *Mycobacterium terrae* as a surrogate for *Mycobacterium tuberculosis*, Griffith et al. compared both bacteria's resistance to disinfection procedures. *M. terrae* was slightly more resistant to disinfectants, but should be considered a valuable surrogate since it poses little threat to researchers, is easily manipulated, and has a shorter incubation period than *M. tuberculosis* (24). *E. coli* K12 has long been used as a non-pathogenic surrogate for *E. coli* O157:H7, but it has also been recently used successfully as a surrogate for *S. Enteritidis* in a study investigating thermal death times in liquid

eggs (30). Currently, there are no studies reporting the use of non-pathogenic microorganisms as surrogates for *S. Typhimurium*.

The FDA has suggested criteria for surrogates used within the food industry. They have stated that an ideal surrogate would be one that is nonpathogenic, has similar behavior to that of the target organism, stable, easily prepared, easily enumerated, shares similar attachment characteristics of the target, and has a population that is consistent until utilized (18). Fluorescent microspheres, with the appropriate characteristics mimicking that of the target organism, could prove to be extremely useful as a bacterial surrogate.

Microspheres as surrogates

Fluorescent microspheres have been used extensively throughout chemical, water and sewage treatment research (1, 38), and only recently as bacterial surrogates (50). Microspheres are microscopic polystyrene spheres that are uniform in size and shape, are able to hold charges and surface functional groups, and they will react to fluorescent light (22). Fluorescent microspheres are often used as tracers for certain chemicals through a system to determine where chemicals of interest end in the system. These microspheres are capable of holding coatings to mimic the surfaces of numerous types of materials and objects. Studies have used microspheres with varying hydrophobicity, surface properties, antigens, polysaccharides, and enzymes attached to their surface (11, 13, 32, 38). Fluorescent microspheres often hold dyes that are spectrally distinct from one another. These dyes have certain

approximate excitation and emission wavelengths so that they are easily discernable (22). These dyes exhibit a wide spectrum of colors (from blue to infrared) when their excitation or emission wavelengths are met (from 350 nm to greater than 680 nm). Microspheres are also available in a wide variety of diameters, ranging from 0.2 μm to 15 μm . With increasing diameter, the size uniformity of the suspension increases as well (22).

In a recent study, Baeza et al. (1) investigated the potential of fluorescent microspheres to act as surrogates for *Cryptosporidium parvum*. They showed that unmodified fluorescent microspheres are capable of producing similar behavior to *C. parvum* oocysts when exposed to ozone followed by chlorine exposure. When exposed to these two sequential disinfectants, the microspheres exhibited structural damage due to exposure to ozone. They concluded that microspheres were promising as a non-biological surrogate, and were capable of producing similar behavior to that of *C. parvum* oocysts.

Chancellor et. al. (9) found that fluorescent microspheres were appropriate biomarkers for hepatitis A virus in a green onion system. They showed that under low- and high-powered microscopy with fluorescent filters that the microspheres were clearly visible within the onion for up to 8 weeks after the initial inoculation. The results revealed that the fluorescent microsphere biomarkers could be trapped within the green onion and can be transported from inoculated nutrient solution to the growing green onion.

In 2005, a study was conducted using fluorescent microspheres as a bacterial surrogate in growing plants. Solomon and Matthews reported that using

microspheres purely for their high fluorescence was an unsuccessful approach (50). When comparing the uptake of microspheres to that of *E. coli* O157:H7, there was a significant difference in recovery from within the plant. Although the microspheres were recovered at much higher numbers than that of the *E. coli*, the study provided much needed information about the uptake of this pathogen in plants, showing that there are no specific biofactors or chemistries that mediate entry and that it is most likely a passive process (50). This study also illustrated the need for modifying microspheres to match the biological characteristics of the target organism to get similar results between microspheres and bacteria.

Through the use of surrogates it could be possible to replace the use of pathogens when studying environmental interactions where the use of pathogens would not be acceptable. An advantage of the use of microspheres within research areas is that they may not require extensive microbiological expertise. In addition, some fluorescent microspheres are biodegradable and non-biohazardous and will not cause contamination of future experiments (44). The use of fluorescent microspheres in a food processing environment, in place of pathogenic microorganisms, would not compromise the microbiological safety of the process environment.

Enumeration using fluorescent microscopy

Fluorescent microscopy relies on the excitation of a molecule at a specific wavelength and corresponding fluorescence at another, typically higher, wavelength for visualization. These molecules are called fluorophores or

fluorochromes. To visualize the higher wavelength, filters are typically employed (52). There has been a steady increase in the use of fluorescent microscopy in past years throughout the scientific community. Through the use of fluorescent probes, plasmids, and dyes, the visualization of cells and other small particles has been made easier.

Although scientists have been using fluorescent microscopy for almost a century, it was not until 1994 when Chalfie et al. successfully visualized green fluorescent protein in living organisms. Because cells are transparent and hard to visualize using microscopy, the use of fluorescent proteins has become increasingly prevalent. Green fluorescent protein (GFP) has been used extensively as a visualizing agent. GFP is a protein of 238 amino acids purified from jellyfish (*Aequorea victoria*) and has an excitation wavelength of 488 nm and a peak emission wavelength of 509 nm, which can be seen under blue light (8). Plasmids containing GFP are easily transformed across a bacteria's cell surface. To better isolate cells containing GFP, plasmids are often fitted with a selective marker, such as resistance to antibiotics (e.g. kanamycin) (10). Through the utilization of GFP in bacterial cells, enumeration of bacteria via microscopy has become less problematic.

The use of hemacytometry has typically focused around the enumeration of blood cells (36). Hemacytometers employ the use of a slide with a slight depression that holds a known volume of liquid. In this depression there is a series of nine 1 mm² grids that aid in the counting of particles. When the cover slip is placed over the depression and liquid is injected onto the slip, capillary

action allows only for a set amount of liquid to enter the slide. Depending on the expected concentration, a number of grids are counted and then the total count is divided by the corresponding total sample volume over the counted grids. This concentration can then be converted to particles present per milliliter, if desired

(4). Hemacytometry presents an opportunity for easy enumeration of both fluorescent microspheres and *S. Typhimurium* cells expressing GFP.

REFERENCES

1. Baeza, C., and J. Ducoste. 2004. A non-biological surrogate for sequential disinfection processes. *Water Res.* 38:3400-3410.
2. Bailey, J. S., and J. J. Maurer. 2005. *Salmonella* Species. p. 85-99. In T.J. Montville, and K.R. Matthews (ed.), Food Microbiology An Introduction ASM Press, Washington D.C.
3. Bloomfield, S. F., M. Arthur, B. Van Klingerren, W. Pullen, J. T. Holah, and R. Elton. 1994. An evaluation of the repeatability and reproducibility of a surface test for the activity of disinfectants. *J. Appl. Bacteriol.* . 76:86-94.
4. Caprette, D. R. Using a Counting Chamber. Available at: <http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html>. Accessed February 5, 2008.
5. CDC-MMWR. Preliminary FoodNet Data on the Incidence of Infection with Pathogens Transmitted Commonly Through Food --- 10 States, United States, 2006. Available at: <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5614a4.htm>. Accessed May 1 2007.
6. CDC-MMWR. Summary of Notifiable Diseases --- United States, 2005. Available at: <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5453a1.htm>. Accessed April 30, 2007.
7. CDC. *Salmonella* Enteritidis. Available at: http://www.cdc.gov/ncidod/dbmd/diseaseinfo/salment_g.htm. Accessed February 29, 2008.
8. Chalfie, M., G. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science.* 263:802-805.
9. Chancellor, D. D., S. Tyagi, M. C. Bazaco, S. Bacvinskas, M. B. Chancellor, V. M. Dato, and F. de Miguel. 2006. Green onions: potential mechanism for hepatitis A contamination. *J. Food Prot.* 69:1468-72.
10. Clontech. pEGFP-C1 Vector Information. Available at: http://www.clontech.com/images/pt/dis_vectors/PT3028-5.pdf. Accessed March 12, 2008.
11. Cummings, J. H., S. Milojevic, M. Harding, W. A. Coward, G. R. Gibson, R. Louise Botham, S. G. Ring, E. P. Wraight, M. A. Stockham, M. C. Allwood, and J. M. Newton. 1996. In vivo studies of amylose- and ethylcellulose-coated

- [13C]glucose microspheres as a model for drug delivery to the colon. *J. Contr. Release.* 40:123-131.
12. D'Aoust, J.-Y. 1997. *Salmonella* Species. p. 129-158. In M.P. Doyle, L.R. Beuchat, and T.J. Montville (ed.), Food Microbiology Fundamentals and Frontiers ASM Press, Washington D.C.
 13. Dai, X. J., and R. M. Hozalski. 2003. Evaluation of microspheres as surrogates for *Cryptosporidium parvum* oocysts in filtration experiments. *Environ. Sci. Tech.* 37:1037-1042.
 14. De Cesare, A., B. W. Sheldon, K. S. Smith, and L. A. Jaykus. 2003. Survival and persistence of *Campylobacter* and *Salmonella* species under various organic loads on food contact surfaces. *J. Food Prot.* 66:1587-94.
 15. Eriksson de Rezende, C., Y. Anriany, L. E. Carr, S. W. Joseph, and R. M. Weiner. 2005. Capsular Polysaccharide Surrounds Smooth and Rugose Types of *Salmonella enterica* serovar Typhimurium DT104. *Appl. Environ. Microbiol.* 71:7345-7351.
 16. Evancho, G. M., W. H. Sveum, L. J. Moberg, and J. F. Frank. 2001. Microbiological Monitoring of the Food Processing Environment. p. 25-35. In F.P. Downes, and K. Ito (ed.), Compendium of Methods for the Microbiological Examination of Foods American Public Health Association, Washington D.C.
 17. Farmer, J. J. 1995. *Enterobacteriaceae*. p. 438-456. In P.R. Murray, et al. (ed.), Manual of Clinical Microbiology ASM Press, Washington D.C.
 18. FDA/CFSAN. Analysis and Evaluation of Preventive Control Measures for the Control and Reduction/Elimination of Microbial Hazards on Fresh and Fresh-Cut Produce. Available at: <http://www.cfsan.fda.gov/~comm/ift3-toc.html>. Accessed February 21, 2008.
 19. FDA/CFSAN. FDA Notifies Consumers that Tomatoes in Restaurants Linked to *Salmonella* Typhimurium Outbreak Available at: <http://www.fda.gov/bbs/topics/NEWS/2006/NEW01504.html>. Accessed March 12, 2008.
 20. FDA/CFSAN. *Salmonella* spp. Available at: <http://www.cfsan.fda.gov/%7Emow/chap1.html>. Accessed April 30, 2007.
 21. FDA/CFSAN. 2007. How the FDA Works to Keep Produce Safe. *FDA Consumer.* 41.
 22. FMRC. Fluorescent Microsphere Resource Center University of Washington. Available at: <http://fmrc.pulmcc.washington.edu/FMRC.SHTML>. Accessed April 1, 2007.

23. Foschino, R., C. Picozzi, A. Civardi, M. Bandini, and P. Faroldi. 2003. Comparison of surface sampling methods and cleanability assessment of stainless steel surfaces subjected or not to shot peening. *J. Food Eng.* 60:375-381.
24. Griffiths, P. A., J. R. Babb, and A. P. Fraise. 1998. *Mycobacterium terrae*: a potential surrogate for *Mycobacterium tuberculosis* in a standard disinfectant test. *J. Hosp. Infect.* 38:183-192.
25. Hammer, N. D., J. C. Schmidt, and M. R. Chapman. 2007. The curli nucleator protein, CsgB, contains an amyloidogenic domain that directs CsgA polymerization. *Proc. Natl. Acad. Sci. Unit. States Am.* 104:12494-12499.
26. Hedberg, C. W., F. J. Angulo, K. E. White, C. W. Langkop, W. L. Schell, M. G. Stobierski, A. Schuchat, J. M. Besser, S. Dietrich, L. Hessel, P. M. Griffin, J. W. McFarland, and M. T. Osterholm. 1999. Outbreaks of salmonellosis associated with eating uncooked tomatoes: implications for public health. . *Epidemiol. Infect.* 122:385-93.
27. Hope, C. K., and M. Wilson. 2003. Effects of dynamic fluid activity from an electric toothbrush on in vitro oral biofilms. *J. Clin. Periodontol.* 30:624-9.
28. Invitrogen. Section 6.5-Microspheres. Available at: <http://probes.invitrogen.com/handbook/sections/0605.html>. Accessed April 20, 2007.
29. Jablasone, J., L. Y. Brovko, and M. W. Griffiths. 2004. A research note: the potential for transfer of *Salmonella* from irrigation water to tomatoes. *J. Sci. Food Agr.* 84:287-289.
30. Jin, T., H. Zhang, G. Boyd, and J. Tang. 2008. Thermal resistance of *Salmonella* Enteritidis and *Escherichia coli* K12 in liquid egg determined by thermal-death-time disks. *J. Food Eng.* 84:608-614.
31. Kang, D., J. D. Eifert, R. C. Williams, and S. Pao. 2007. Evaluation of quantitative recovery methods for *Listeria monocytogenes* applied to stainless steel. *J. AOAC International.* 90:810-816.
32. Kellar, K. L. 2003. Applications of multiplexed fluorescent microsphere-based assays to studies of infectious disease. *Journal of Clinical Ligand Assay.* 26:76-86.
33. Kusumaningrum, H. D., G. Riboldi, W. C. Hazeleger, and R. R. Beumer. 2003. Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *Int. J. Food. Microbiol.* 85:227-36.

34. Langmark, J., M. V. Storey, N. J. Ashbolt, and T. A. Stenstrom. 2005. Accumulation and fate of microorganisms and microspheres in biofilms formed in a pilot-scale water distribution system. *Appl. Environ. Microbiol.* 71:706-12.
35. Lindsay, D., and A. von Holy. 1999. Different responses of planktonic and attached *Bacillus subtilis* and *Pseudomonas fluorescens* to sanitizer treatment. *J. Food Prot.* 62:368-79.
36. Margaret McGinley, L. L. W. J. H. M. D. O. R. 1992. Comparison of various methods for the enumeration of blood cells in urine. *Journal of Clinical Laboratory Analysis.* 6:359-361.
37. Marshall, K. C., R. Stout, and R. Mitchell. 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. *J. Gen. Appl. Microbiol.* 68:337-348.
38. Martins, J. A., D. H. Crout, and P. Critchley. 2004. Cn microspheres as surrogate membranes in glycosidase-catalysed hydrolysis of glycolipids. *Chem. Comm.:*198-199.
39. Maxcy, R. B. 1975. Fate of Bacteria Exposed to Washing and Drying on Stainless Steel. *J. Milk Food Technol.* 38:192-194.
40. Mbandi, E., and L. A. Shelef. 2002. Enhanced antimicrobial effects of combination of lactate and diacetate on *Listeria monocytogenes* and *Salmonella* spp. in beef bologna. *Int.J. Food Microbiol.* 76:191-198.
41. McEvoy, J. M., C. W. Nde, J. S. Sherwood, and C. M. Logue. 2005. An evaluation of sampling methods for the detection of *Escherichia coli* and *Salmonella* on turkey carcasses. *J. Food Prot.* 68:34-9.
42. Moore, C. M., B. W. Sheldon, and L. A. Jaykus. 2003. Transfer of *Salmonella* and *Campylobacter* from stainless steel to romaine lettuce. *J. Food Prot.* 66:2231-6.
43. Moore, G., and C. Griffith. 2002. A comparison of surface sampling methods for detecting coliforms on food contact surfaces. *Food Microbiol.* 19:65-73.
44. Oberyshyn, A. S., and F. M. Robertson. 2001. Novel rapid method for visualization of extent and location of aerosol contamination during high-speed sorting of potentially biohazardous samples. *Cytometry.* 43:217-22.
45. Oliveira, K., T. Oliveira, P. Teixeira, J. Azeredo, M. Henriques, and R. Oliveira. 2006. Comparison of the adhesion ability of different *Salmonella enteritidis* serotypes to materials used in kitchens. *J. Food Prot.* 69:2352-2356.

46. Peng, J. S., W. C. Tsai, and C. C. Chou. 2001. Surface characteristics of *Bacillus cereus* and its adhesion to stainless steel. *Int. J. Food Microbiol.* 65:105-11.
47. Pierson, M. D., and L. M. Smoot. 2005. Indicator Microorganisms and Microbiological Criteria. p. 65-80. *In* T.J. Montville, and K.R. Matthews (ed.), Food Microbiology An Introduction ASM Press, Washington D.C.
48. Rose, L., B. Jensen, A. Peterson, S. N. Banerjee, and M. J. Arduino. 2004. Swab Materials and *Bacillus anthracis* Spore Recovery from Nonporous Surfaces. *Emerg. Infect. Dis.* 10:1023-1029.
49. Small, A., C. James, S. James, R. H. Davies, E. Liebana, M. Howell, M. Hutchison, and S. Buncic. 2006. Presence of Salmonella in the Red Meat Abattoir Lairage after Routine Cleansing and Disinfection and on Carcasses. *J. Food Prot.* 69:2342-2351.
50. Solomon, E. B., and K. R. Matthews. 2005. Use of fluorescent microspheres as a tool to investigate bacterial interactions with growing plants. *J. Food Prot.* 68:870-873.
51. Teixeira, P., J. C. Lima, J. Azeredo, and R. Oliveira. 2007. Note. Colonisation of bench cover materials by *Salmonella* Typhimurium. *Food Sci. Tech. Int.* 13:5-10.
52. Vonesch, C., F. Aguet, J. Vonesch, and M. Unser. 2006. The Colored Revolution of Bioimaging. *IEEE Signal Process Mag.* 23:20-31.
53. Vorst, K. L., E. C. Todd, and E. T. Ryser. 2004. Improved quantitative recovery of *Listeria monocytogenes* from stainless steel surfaces using a one-ply composite tissue. *J. Food Prot.* 67:2212-7.
54. Wallis, T. S. 2006. Host-specificity of *Salmonella* infections in animal species. p. 57-88. *In* P. Mastroeni, and D. Maskell (ed.), Salmonella Infections Clinical, Immunological and Molecular Aspects Cambridge University Press, Cambridge.
55. Weissinger, W. R., W. Chantarapanont, and L. R. Beuchat. 2000. Survival and growth of *Salmonella* baillon in shredded lettuce and diced tomatoes, and effectiveness of chlorinated water as a sanitizer. *Int. J. Food Microbiol.* 62:123-31.
56. Wood, R. L., A. Pospischil, and R. Rose. 1989. Distribution of persistent *Salmonella* typhimurium infection in internal organs of swine. *AJVR: American Journal of Veterinary Research.* 50:1015-1021.

57. Wu-Yuan, C. D., and R. D. Anderson. 1994. Ability of the Sonicare electronic toothbrush to generate dynamic fluid activity that removes bacteria. *J. Clin. Dent.* 5:89-93.
58. Zottola, E. A. 1991. Characterization of the attachment matrix of *Pseudomonas fragi* attached to non-porous surfaces. *Biofouling.* 5:37-55.