Chapter II- Fluorescent Microspheres as Surrogates for *Salmonella enterica* serotype Typhimurium in Recovery Studies from Stainless Steel

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ABSTRACT

To compare the optimum recoveries of an inoculation of *Salmonella enterica* serotype Typhimurium, fluorescent microspheres (1.0 µm diameter, carboxylate-modified, crimson FluoSpheres®, Molecular Probes, Eugene, OR), or a combination of both from stainless steel, three recovery methods, including a standard rinse, a one-ply composite tissue (Kimwipe®) or a sonicating brush were used. Findings were used to assess the effectiveness of fluorescent microspheres as surrogates for *S.* Typhimurium. For each method, ten coupons (304 grade, 2.5 x 8 cm) were inoculated with either 100 µl of a *S.* Typhimurium culture, or a solution of fluorescent microspheres, or both, at approximate concentrations of 10^6. After drying for one hour, coupons were sampled using either a rinse of 100 ml of phosphate buffered saline solution (PBS) for one min, a Kimwipe® tissue method, or submerged in PBS and subjected to a sonicating brush for one min. Following treatments, PBS solutions were analyzed using duplicate plate counting (*Salmonella*) or hemacytometry (microspheres). For microspheres and *Salmonella*, recovery by sonicating brush > rinse > Kimwipe® method. Additionally, the retention of microspheres on the steel ranged from 16 to 25% (mean from five coupons each recovery method). Microspheres yielded a significantly higher recovery rate (11 – 60%) than *Salmonella* (~1%) for each recovery method, therefore the microspheres used in this study, are not appropriate surrogates for *S.* Typhimurium for future recovery studies on stainless steel. However, due to their low standard deviations for their mean

percent recovery, they hold the opportunity to provide better accuracy and reproducibility.

INTRODUCTION

The Food and Drug Administration's Center for Food Safety and Applied Nutrition (FDA/CFSAN) estimate that 2 to 4 million cases of salmonellosis occur in the United States every year, of these cases only a fraction are treated *(10)*. In 2006, 6,655 laboratory confirmed cases of salmonellosis occurred, of these cases approximately 20% were caused by *Salmonella enterica* serotype Typhimurium *(4)*. When comparing the rate of occurrence since 2003 to a baseline period of 1996 to 1998, *S.* Typhimurium infections have remained steady*(4)*.

Because bacteria, such as *Salmonella* are easily transferable from food contact surfaces to food, investigating these routes of contamination is important. Food contact surfaces, such as stainless steel are prevalent throughout food processing environments. Even with effective cleaning and sanitation procedures in place, bacterial attachment and retention to these surfaces may still occur *(16)*. There is a need for prevention, detection, and enumeration of foodborne pathogenic microorganisms within food processing environments.

Since pathogenic species are not tolerated in food processing environments, surrogates are needed to perform research under typical processing conditions to accurately assess the pathogenic loads and destinations of facility. Fluorescent microspheres have been previously used to study mechanisms of association between bacteria and foods *(22)*, mechanisms of contamination *(5)*, biofilm formation *(17)*, and filtration studies *(8)*. The FDA stipulates that for a surrogate to be used within the food industry, it must first

meet certain criteria *(9)*. A proper surrogate must be non-pathogenic, have similar behavior to that of the target organism, stable, easily prepared, easily enumerated, and has a population that remains consistent until utilized *(9)*. Fluorescent microspheres are a likely surrogate for pathogenic bacteria because of their ease in detection, similar physical dimensions, ability to have their surface properties manipulated, and their stability in systems.

This study aims to assess the potential for fluorescent microspheres as a surrogate for *Salmonella enterica* serotype Typhimurium for recovery studies on stainless steel, as well as to assess the effectiveness of three recovery methods for both fluorescent microspheres and *S.* Typhimurium. This study did not attempt to compare the different surface coatings and properties available to modify microspheres, rather it assessed if unmodified microspheres were appropriate surrogates at a basic level. To assess microspheres usefulness as a surrogate, three recovery methods were performed. The three methods used were a simple rinse procedure, recovery using a one-ply composite tissue (KimWipe®), and a sonicating brush method.

MATERIALS AND METHODS

Cultures and culture maintenance

An animal-passage isolate of wild-type strain SR-11 *Salmonella enterica* serotype Typhimurium x3181 culture was acquired from Robert Curtiss, Department of Biology, Washington University [as listed in Brown et al. 2001 *(2)*, originally obtained from Gulig and Curtiss 1987 *(12)*, (St. Louis, MO)]. Cultures were stored at -80°C in an ultra low-temperature freezer in 30% glycerol/70% tryptic soy broth (TSB, Difco, Franklin Lakes, NJ) until use. Cultures were revived in TSB at 35±2°C for 24±2 hours and sustained on tryptic soy agar (TSA, Difco, Franklin Lakes, NJ) slants held at 4±2°C before preparation of electo-competent cells.

Preparation of electro-competent cells

Cells of *S.* Typhimurium x3181 were made electro-competent using methods from Bioprotocol *(1)*. An aliquot of *S.* Typhimurium culture grown in Luria-Bertani broth (LB broth, Difco, Franklin Lakes, NJ) for 24±2 hours at 35±2°C was allowed to grow to an optical density 0.7-0.8 (600 nm) in 2X YT Broth (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) for approximately 5 hours. Culture was then chilled on ice for 20 minutes. A cell suspension of 50 mL was then centrifuged for 7 minutes at 5,000 rpm. The supernatant was decanted and the pellet was resuspended in a 10% glycerol solution and then centrifuged for 5 minutes at ~5,000 rpm. The supernatant was again poured off

and three times the pellet was resuspended in 10% glycerol and centrifuged for 10 minutes at ~1,500 g. Lastly, the pellet was resuspended in 10% glycerol and 50 µl aliquots were placed into sterile 1.5 mL microcentrifuge tubes, quickly frozen in an ethanol/dry ice bath and stored in an ultra low-temperature freezer (~80°C) until use *(1)*.

Preparation of transformed cells

The pWB250 plasmid was obtained from Dr. Wesley Black (Department of Biological Sciences, Virginia Tech, Blacksburg, VA). This plasmid carries a high copy ColE1 origin of replication, kanamycin resistance (50-100 µg/mL), and GFP as a translocational fusion to *lac*Zα expressed from the *lac* promoter *(6)*. The plasmid was held in a Qiagen mini-prep elution buffer [(10 mM Tris-Cl pH 8.5), Qiagen, Valencia, CA] until use. This plasmid was created by a Mx8 phage integrase and attachment site (*intP*) was amplified by PCR, and then was blunt end ligated into PZErO-2 (Invitrogen, Carlsbad, CA) *(6)*. This was then digested with Bg1I and DraIII and blunted with T4 DNA polymerase to create the vector pWB200. An enhanced green fluorescent protein (GFPmut 1 variant) was isolated from pEGFP-1 (Clontech/BD Biosciences, San Jose, CA) as a BamHI/NotI fragment and was ligated into the same sites of the pWB200 vector to create the finished pWB250 plasmid *(6)*.

Electro-competent cells and plasmids were thawed on ice. A 0.1 µL aliquot of plasmid solution was added to cells and this suspension was incubated on ice for one minute. The mixture was then transferred to a cold cuvette and

pulsed at 2.5 kV, 25 μF, and a capacitance extender to 200 Ω for 5.1 seconds (Bio-Rad GenePulser Xcell, Bio-Rad Laboratories, Hercules, CA). Immediately following the pulse, mixture was removed and 1 mL of SOC [98.0 mL SOB (2.0 g/100 mL tryptone, 0.5 g/100 mL yeast extract, 0.2 mL/100 mL 5M NaCl, 0.25 mL/100 mL 1M KCl) and 2.0 mL 2M glucose] broth was added to the cuvette and gently mixed. The cells were then transferred to a 15 mL centrifuge tube and incubated for 1 hour at 35±2°C while shaking at 225 rpm. After incubation, suspension was inoculated on Luria-Bertani agar (LB agar, Difco, Franklin Lakes, NJ) supplemented with 100 μg/mL kanamycin (Fischer Scientific, Pittsburgh, PA) for 24±2 hours at 35±2°C. Colonies were further tested for fluorescence and were confirmed as *Salmonella* by growth and morphology on Hektoen-Enteric (HE, Difco, Franklin Lakes, NJ) agar and biochemical tests using API 20E strips. After confirmation, cells were frozen in 30% glycerol/ 70% TSB, frozen, and stored in an -80°C ultra-low freezer until use.

Preparation of inoculum

Salmonella Typhimurium

Transformed *S.* Typhimurium were cultivated in 10 mL of TSB for 24±2 hours at 35±2°C from a LB plate supplemented with 100 μg/mL of kanamycin. At 24±2 hour intervals, the culture was transferred via a loop-wise inoculation into 10 mL of TSB. Standard plate count method was used to assess the initial concentration (~1.0 x 10^8 CFU/mL after a 24±2 hour incubation period at 35±2°C). Cultures were periodically plated on HE agar and isolated colonies

from LB-kanamycin plates were identified as *Salmonella* using an API 20E biochemical test kit (bioMerieux, Hazelwood, MO).

Fluorescent Microspheres

Carboxylate-modified crimson (625 nm excitation/645 nm emission) fluorescent microspheres (FluoSpheres®, Molecular Probes, Eugene, OR), 1.0µm in diameter, were suspended in a distilled water and 2 mM azide solution (original concentration of 2.7 x 10^{10} particles/mL). The solution was sonicated and serially diluted in PBS solution until final concentration was approximately 10^{7}particles/mL. Remaining portion of microspheres were stored at 4±2°C, in the dark.

Preparation of stainless steel coupons

Inoculum was applied to a 2 by 2 cm area of a 2.5 x 8 cm type 304, unpolished, #2B mill finish stainless steel coupon (McMaster-Carr, Atlanta, GA). Prior to first use, each coupon was sanitized by soaking in an acetone bath for 60 minutes to degrease, rinsed with deionized water, and then placing in glass beakers (containing an ultrasonic cleaning detergent [Fisher Brand, Fisher Scientific, Pittsburgh, PA]). Beakers were sonicated in an ultrasonic water bath (Fisher Brand, 150-300 W, 40 Hz) for 45 minutes. Following the bath, coupons were rinsed repeatedly with tap water, as well as deionized water to remove any remaining detergent debris or residue. Coupons were then placed in a wire test tube rack with flame sterilized forceps, covered with aluminum foil, and steam

sterilized in an autoclave for 45 minutes. The coupons were then dried in a hot air drying oven at approximately 80°C *(14)*.

Before inoculation, coupons were soaked in acetone and sonicated for 30 minutes. After sonication, coupons were rinsed in distilled water. Coupons were then sonicated in ultrasonic cleaning detergent for 30 minutes. After treatment, the coupons were thoroughly rinsed in tap water, followed by distilled water to remove any residual detergent. Coupons were then soaked in 70% ethanol for five minutes and were then rinsed with distilled water and allowed to dry under a laminar flow hood.

To mark a 2 by 2 cm inoculation area a permanent ink marker (Sharpie®, Sanford Corporation, Oak Brook, IL) was used. Each inoculation surface was approximately 1.5 cm from the end of the coupons. After desired area was obtained, coupons were placed in covered petri dishes (Fisher Brand, Fisher Scientific, Pittsburgh, PA) and exposed to shortwave ultraviolet light (254 nm) in a laminar hood for 1 hour on each side of the coupon.

Application of inoculum on stainless steel

For studies using either *S.* Typhimurium or fluorescent microspheres separately, a 100 µL aliquot (10^6) of inoculum was placed in the center of the 2 by 2 cm marked area to be tested and spread with a sterile plastic spreader. The coupons were then to dry for one hour at 20°C under a drying hood. For the study using an inoculum of *S.* Typhimurium and fluorescent microspheres, 50µL aliquots (10^6) of each were used. Two repetitions of five coupons were used for

each of the three recovery methods for *Salmonella* and microspheres. Initial concentrations of inocula were confirmed prior to recovery by standard plate count method onto TSA and evaluated using a hemacytometer and fluorescent microscopy. Fluorescent microspheres were serially diluted ten-fold and concentrations were assessed using a hemacytometer and fluorescent microscopy.

Sampling/ recovery procedures

Each of the following methods was performed on five coupons per method, with two trials. Three separate inocula were used, one containing a culture of *S.* Typhimurium, one containing fluorescent microspheres, and the last containing both *S.* Typhimurium and fluorescent microspheres. Inocula containing *S.* Typhimurium were serially diluted using PBS, and were enumerated using a standard plate count method on TSA. Samples were also enumerated using a hemacytometer. Fluorescent microspheres were enumerated exclusively using a hemacytometer. A total of five coupons from each sampling method (approximately 2-3 coupons from each trial) were examined using fluorescent microscopy.

Rinse method

Inoculated coupons were aseptically immersed into 100 mL of sterile phosphate buffered saline solution (PBS) using flame sterilized forceps (70% ethanol). The lid of each container (125 mL sterile specimen cup) was tightly

capped and the containers were shaken in a 90 degree arc for 25 times in one minute *(14)*. The solutions were then enumerated by fluorescent microscopy and/or standard plate count method.

Sonicating brush method

Sonicating brush heads (Phillips Oral Healthcare Inc., Snoqualmie, WA) were cut to a uniform length of 7 mm at the ends to 8 mm at the middle of the brush head to eliminate irregularity of bristle length. The brush heads were examined before use to make certain that the length was uniform and the plane was level *(14)*.

Using a base unit of the Sonicare® Elite 7300 (Phillips Oral Healthcare Inc., Snoqualmie, WA), a platform was constructed so that when the brush-head is attached to the base, the angle at which the brush-head was parallel to that of a stainless steel coupon within a container. The approximate distance from the bristle tip to the coupon was 2-3 mm. The brush-head was not allowed to contact the coupon or the container. The coupon was submerged in 100 mL of sterile PBS using flame sterilized forceps (in 70% ethanol). The brush-head was then oriented parallel to the inoculated area of the coupon and was allowed to sonicate for one minute. Following sonication, the solutions were enumerated by fluorescent microscopy and/or standard plate count method.

One-ply composite tissue method

Using sterile gloves, a Kimwipe® was folded twice from the side and top edges so as to measure 5.5 cm by 5.5 cm *(16)*. The Kimwipe® was placed in one mL of sterile PBS within a sterile plastic bag. The Kimwipe® was squeezed to remove excess solution and the marked area of the coupon was swabbed 10 times vertically and 10 times horizontally with the Kimwipe's® folded exterior surface. The Kimwipe® was returned to the sterile plastic bag, an additional 10 mL of sterile PBS was added, and stomached for one minute and hand massaged for 30 seconds. After stomaching, the Kimwipe® was unfolded within the bag and the solution was enumerated by fluorescent microscopy or standard plate count method *(25)*.

Fluorescent microscopy enumeration

To determine concentrations, PBS solutions of fluorescent microspheres and *S.* Typhimurium were counted using a Hausser Brightline hemacytometer (Hausser Scientific, Horsham, PA) under a Zeiss Axiovert 200 fluorescent microscope (Carl Zeiss, Thornwood, NY) equipped with a camera (Canon USA). The improved Neubauer rulings, six 1/16th blocks (each of the four corners and center block) were used for counting. The sum of the blocks was then divided by 0.02 (the combined volume of the five blocks) and then multiplied by 1000 (cubic millimeters in one cubic centimeter) *(3)*. For assessment of microsphere concentration, 9 x 1 mm^2 fields were counted. The total sphere count was divided by 9 (number of 1 mm^2 fields counted), and multiplied by 10,000 (scale

factor for 0.1μL to 1.0 mL), and then multiplied by the volume of the solution (10 or 100 mL) collected to attain the concentration of spheres or cells recovered.

Stainless steel coupons were also examined using fluorescent microscopy. The 2 by 2 cm marked off area was studied to count microspheres left on the steel following each recovery method. A series of ten 1,000 μm^2 areas were visualized and counted. The average of the counts was multiplied by 40000 (the number of μm^2 in 2 cm^2), and then multiplied by 10, this was done to calculate the concentration of cells or spheres per mL that remained on the coupon.

Data analysis

To assess the recovery of *S.* Typhimurium and fluorescent microspheres from stainless steel, each sampling procedure was replicated twice with quintuplicate analysis in each replicate resulting in 10 observations. The mean recovery of cells from duplicate plate counts, as well as the mean recovery of microspheres from each sampling method was determined. The mean recovery for both cells and microspheres were transformed to $\log_{10}$ for statistical analysis. The mean percent recovery was calculated by averaging the log means by transforming the means into percentage. To determine the mean percent of retention of the microspheres and *S.* Typhimurium to the stainless steel, the mean recovery per coupons were averaged and then transformed to percentage. The total percent of microspheres and cells were obtained by adding the mean percent recovery and the mean percent of retention.

A one-way analysis of variance (ANOVA) was used to determine significant differences between means for each recovery methods, between means for recovered *S.* Typhimurium and fluorescent microspheres, and between means for cells and microspheres still attached to the surface of stainless steel coupons at a statistical significance of $\alpha = 0.05$. When significant differences were found a Tukey's multiple range test was performed to determine significant differences between mean recoveries within each trial. Calculations were performed with JMP® Statistical Discovery Software, version 5.1 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Recovery of *Salmonella* Typhimurium

The sonicating brush method is the most effective method for recovery of *Salmonella* Typhimurium from stainless steel coupons. Kang et al. (2007) also reported that the sonicating brush method was superior for recovering *Listeria monocytogenes* from stainless steel. Recovery of *S.* Typhimurium from stainless steel, in this study, was much lower than recovery of *Listeria monocytogenes* in the study by Kang, et al. *(14)*. The mean percent recovery of *S.* Typhimurium was 1.2% (Table 1), compared to a 60% recovery of *L. monocytogenes (14)*.

The KimWipe® method yielded a 4.30 mean log_{10} recovery of *S.* Typhimurium cells from the stainless steel coupons (Table 1). This recovery accounted for only 0.8% mean recovery. This level of recovery is similar to that of a previous study where this one-ply composite tissue method recovered 4.5 to 5.0 log_{10} of *L. monocytogenes (25)*. When compared to the other methods used by the previous study (environmental sponge, cotton-tipped swab, and calcium alginate swab) recovery using the one-ply composite tissue method was 1.11 to 2.70 log/cm^2 higher *(25)*. Researchers suggested that the structure of the Kimwipe® provides improved abrasion of the stainless steel surface which effectively removes cells from the surface and that the antistatic coating of the tissue aids in the release of bacteria through reducing electrostatic discharge *(25)*. It was surprising to find that in this current study, the Kimwipe® method was not the most effective.

The rinse method resulted in recovery that was significantly different from recovery using the brush method ($p<0.05$) (Table 1). This method proved to be the least effective in the recovery of cells, yielding only a 0.6% mean recovery. When the mean log_{10} recovery is compared to that reported by Kang, et al. for *L. monocytogenes*, there is a population difference 0.83 to 1.63 in log_{10} recovery *(14)*. Also, mean recovery, after a one hour drying time, was 38% in that study. Low recovery could be attributed to use of a rinse method where there is no physical application of pressure or abrasive action to the inoculated surface to aid in the removal of bacteria.

When GFP transformed *S.* Typhimurium were viewed using a hemacytometer and on stainless steel using fluorescent microscopy, the cell did not fluoresce brightly enough to accurately quantify recovered cells or those retained on the coupon. Therefore, quantifying the recovery of *S.* Typhimurium was done solely by duplicate standard plate counts on TSA. Cells that retained the plasmid may have not been able to efficiently express GFP due to the disruption of cell processes and weakening through the drying procedure.

According to a previous study by Vogler (1998), surfaces exhibiting a water contact angle higher than 65° are considered to be hydrophobic and those having a water contact angle of below 65° are hydrophilic *(24).* In a recent study, *S.* Typhimurium ATCC 13311 was shown to have a contact water angle of 17.1° and type 304 steel showed a water contact angle of 81.2° *(23)*. *L. monocytogenes* also shown to be a hydrophilic bacterium, with a water contact angle of 26.3° *(18)*. Despite the fact that the hydrophobicity of *Salmonella* is

strongly strain dependent *(19)* and the hydrophobicity of both the strain of *S.* Typhimurium and stainless steel used in this study were not assessed, a difference between water contact angles of 9.2° between *S.* Typhimurium and *L. monocytogenes*, may explain the differences in the recoveries of the two bacteria. Because *S.* Typhimurium was more hydrophilic than *L. monocytogenes*, the number of *Salmonella* cells that adhered to the steel may have been significantly greater than the amount of *L. monocytogenes*. When compared to other organisms, such as *L. monocytogenes*, *Salmonella* Typhimurium has a stronger initial attachment to stainless steel *(15)*, this strong attachment may also explain the differences in percent recovery between studies involving *L. monocytogenes* and those of the current study.

Factors other than differing hydrophobicity may also influence the degree of attachment of cells to stainless steel *(21)*. *S.* Typhimurium and *L. monocytogenes* have different surface characteristics. *Salmonella* spp. are characterized as a gram-negative bacteria *(7)*, unable to produce a peptidoglycan cell wall, unlike *Listeria (11)*. This difference in extracellular components may also have an effect on the recoveries of these two organisms *(20)*.

Recovery of fluorescent microspheres

The sonicating brush method yielded the highest recovery of fluorescent microspheres, with populations 0.78 $\log_{10}$/mL higher when compared to the Kimwipe® method, which was the least effective method. Differences between

the Kimwipe® method and the other methods used were statistically significant ($p<0.05$) (Table 2). Microspheres attached to the coupons were enumerated after each recovery method. The mean proportion retained on the steel ranged from 16.3 to 25.5%. The brush method yielded the highest percentage (84.6%) of microspheres that could be enumerated (60.4% recovered and 24.1% retained on steel). The inability to account for 100% of the spheres was attributed to several phenomena. The microspheres may have been removed from the inoculation area of the steel coupons during the recovery methods and may have reattached to portions that were not visualized (this is especially possible for the rinse method and brush method) and enumerated by microscopy or the microspheres were trapped within the Kimwipe® used during testing. This entrapment of the microspheres within the Kimwipe® could account for the low mean percent recovery (10.9%) of the inoculum population.

Vorst et al. found that the entrapment of cells within sampling devices did not apply to the one-ply composite tissue method because of it's lack of a large porous structure *(25)*. Despite this finding, the microspheres may have had a higher affinity for attachment to the various sampling devices used. The Kimwipe®'s fibrous texture could have provided desirable areas for attachment.

Recovery of *Salmonella* Typhimurium and fluorescent microspheres

Through investigating the recoveries of both *S.* Typhimurium and fluorescent microspheres from stainless steel it was found that the fluorescent microspheres had at least a 1.5 log_{10} difference higher recovery than *S.*

Typhimurium (Table 3). The recovery of microspheres was more consistant than the recovery of *Salmonella* (Table 3). These differences further illustrate the consistency that microspheres provide. These differences could be attributed to biofactors that *S.* Typhimurium possesses, such as curli. Although the strain used was not tested for the ability to produce curli in this study, previous studies confirm the production of curli by this strain *(2)*. Hammer et al. hypothesized that these curli are the most likely candidate that mediate the initial step of surface attachment. At room temperature there is a high possibility of colonization of stainless steel because this is the optimum temperature for curli production *(13)*.

It should be noted that the atmospheric conditions (relative humidity, temperature, etc.) of this study were not held constant; therefore the drying of inoculum on the coupons was not consistent during the one hour drying period. The inoculum of both *S.* Typhimurium and microspheres on the coupons for the first replication appeared visually to be wet after the one hour drying time had lapsed. This may have led to the high recovery of both the *Salmonella* and the fluorescent microspheres for this trial. When comparing $\log_{10}$ values it must be understood that counting one additional CFU or microsphere can raise the calculated $\log_{10}$ concentration/mL by approximately 0.05.

Recovery of microspheres was statistically greater ($p<0.05$) than recovery of *S.* Typhimurium for all methods tested. This difference is a direct result of the superior recovery that microspheres allow. Although the microspheres were recovered in much higher numbers, there were still microspheres that were not accounted for. The discrepancies between the amounts of spheres left on the

steel and those that were enumerated could be attributed to counting errors. Because such a small area of the steel was counted, there could be significant error associated. It may be possible that the areas counted were not accurately representative of the total quantity of microspheres retained on the steel. It may be valuable to enumerate a larger area of the inoculated area to obtain a more accurate count of the microspheres maintained on the steel.

Because of the inability of the *S.* Typhimurium cells to be visualized on the stainless steel coupons a representative image of what the microspheres and *S.* Typhimurium inoculated coupon would look like was created (Figure 3). A 24±2 hour culture of *S.* Typhimurium in TSB was stained with acridine orange (Invitrogen, Molecular Probes, Eugene, Oregon) by adding 0.5 mL and allowing it to sit at room temperature for 30 minutes. After the staining period, a 50 µl aliquot of cells, as well as a 50 µl aliquot of microspheres were inoculated and evenly distributed onto a stainless steel coupon and allowed to dry for one hour. After the one hour drying time the coupon was then visualized by fluorescent microscopy. The image captured shows the difficulty of visual quantitative analysis of *S.* Typhimurium on stainless steel. It is difficult to distinguish separate cells from one another, which could have significant effects on mean percent retention calculations. Unfortunately, this method of staining does not differentiate between living and dead cells.

When assessing the recovery of fluorescent microspheres and *Salmonella enterica* serotype Typhimurium using three recovery methods, microspheres yielded the highest recovered populations. When comparing the recovery

between methods, the brush method had the most successful recovery, followed by the rinse method, and lastly the Kimwipe® method had the lowest overall population recovery. Through comparing the standard deviations of the mean log recoveries for both the microspheres and *S.* Typhimurium, the microspheres provide a more accurate and higher recovery. Overall, the recovery of microspheres was considerably higher than that of *S.* Typhimurium, with significant differences between all methods. It was also determined that the counting method for the retention of microspheres held large inconsistencies. A more reliable method would require a larger surface area to be enumerated. Ultimately, it can be concluded that the microspheres, used in this study, are not appropriate surrogates for *Salmonella enterica* serotype Typhimurium. With appropriate modifications in size and surface properties, to better mimic the target bacteria, microspheres could serve as effective models for other studies with pathogenic microorganisms.

Potential extensions of current study

There are numerous studies that could be performed using the results of this experiment as a guide. If the microspheres were altered and specific coatings were to be added to better mimic the surface properties of *S.* Typhimurium; perhaps an addition of a lipid polysaccharide membrane, purified flagellum, or even curli to reproduce rugose colony morphologies, the results might prove beneficial. If the environmental conditions, such as temperature and humidity, were held constant, then the magnitude of the drying for the inocula

and cell or sphere attachment may be more reproducible. Assessing the hydrophobicity of the stainless steel coupons, microspheres, and the bacterial strains being investigated would also prove beneficial for further studies.

When investigating the question of how effective current cleaning and sanitation procedures, in a food production and processing environment, many factors need to be addressed. One important issue is that cleaning and sanitation procedures often leave bacterial populations weakened or injured. These populations are often not easily cultivated, but may still remain viable. These bacteria may eventually become a contaminate in final production and packaging steps, as well as within the final product*(26)*. This in turn may result in recalls that cost companies millions of dollars in economic loss. Microspheres that are appropriately modified to the microorganisms of interest may prove as an effective surrogate for total enumeration of possible bacterial contaminants. Through further studies that include inoculation of a test surface with a known concentration of microspheres prior to standard cleaning and disinfection procedures, it may be possible to assess the effectiveness of those procedures through quantifying the microspheres recovered. Furthermore, it may be possible to calculate the amount of microspheres left behind, which could be indicative of the number of residual bacteria on the cleaned surface.

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TABLES AND FIGURES

Table 1. Mean recovery of *Salmonella* Typhimurium from stainless steel coupons using three sampling methods (Rinse, Kimwipe®, and Brush)

Method	Mean recovery[*] (log_{10})±SD[**]	Mean % Recovery
Rinse	3.67±0.53[b]	0.6
KimWipe®	4.30±0.79[ab]	0.8
Brush	4.58±0.83[a]	1.2

(n=10)

*Means not connected by the same letter are significantly different

**SD = Standard deviation

Table 2. Mean recovery of fluorescent microspheres from, and retention on stainless steel coupons using three sampling methods (Rinse, Kimwipe®, and Brush)

Method	Mean recovery* (log_{10})±SD**	Mean % Recovery	Mean % Retained on steel	Total % Recovered and Retained
Rinse	5.86±0.18[a]	40.9	25.5	66.4
KimWipe®	5.28±0.24[b]	10.9	16.3	27.2
Brush	6.06±0.10[a]	60.4	24.1	84.6

(n=10)

*Means not connected by the same letter are significantly different

**SD = Standard deviation

Table 3. Mean recovery of fluorescent microspheres and *Salmonella* Typhimurium (when inoculated together) from, and retention on stainless steel coupons using three sampling methods (Rinse, Kimwipe®, Brush).*

Method	Mean recovery (log_{10})±SD**	Mean % Recovery***	Mean % Retained on Steel	Total % Recovered and Retained
Rinse				
Microspheres	5.97±0.23	75.7 [a]	4.0	76.9
Salmonella	4.38±1.08	25.3[b]	——	——
KimWipe®				
Microspheres	5.44±0.30	23.7 [a]	20.4	44.1
Salmonella	3.89±0.53	0.9[b]	——	——
Brush				
Microspheres	6.06±0.25	95.9 [a]	22.1	118.0
Salmonella	4.61±1.24	44.5[b]	——	——

(n=10)

* Spheres and bacteria inoculated together on each coupon

**SD = Standard deviation

*** Mean percent recoveries not connected by the same letter are significantly different

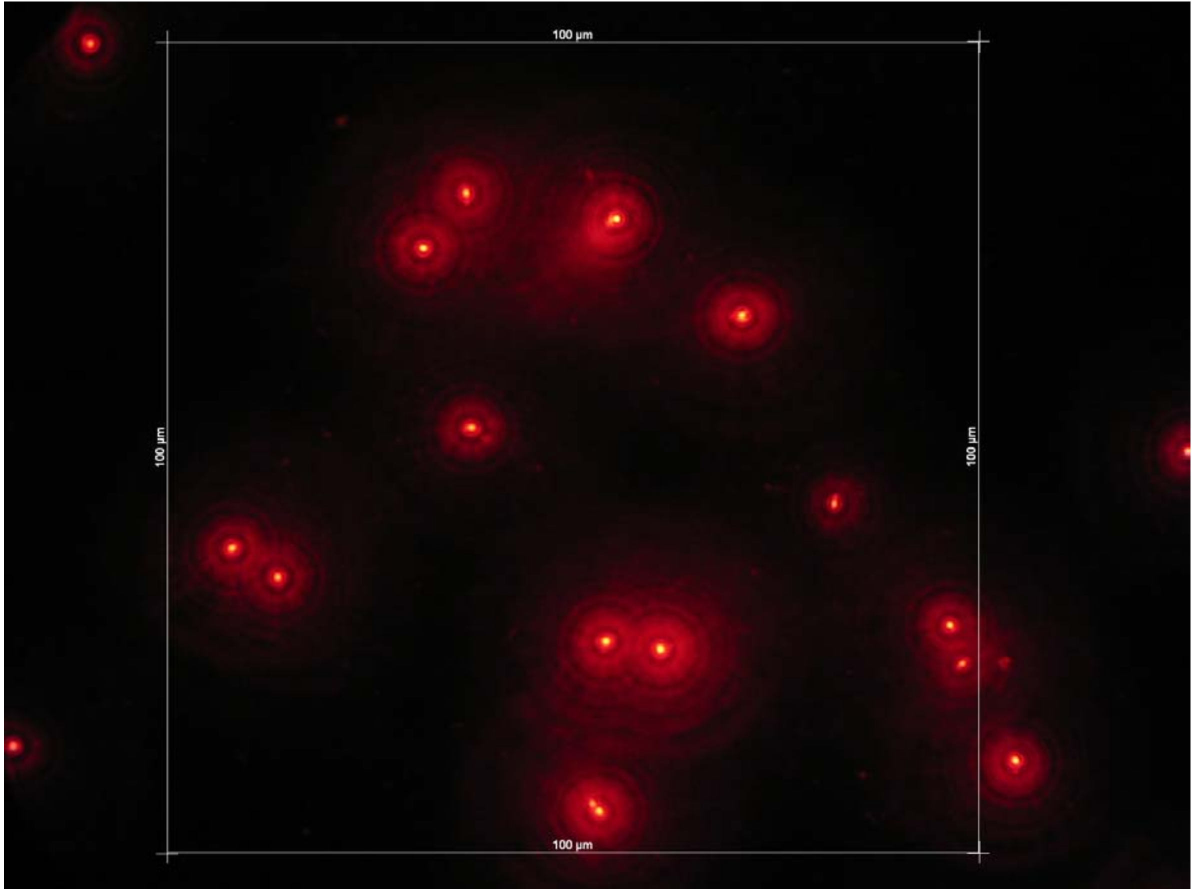

Figure 1. Representative image of retained fluorescent microspheres (625 nm/645 nm) on type 304 stainless steel. (Boxed area is 100μm^2)

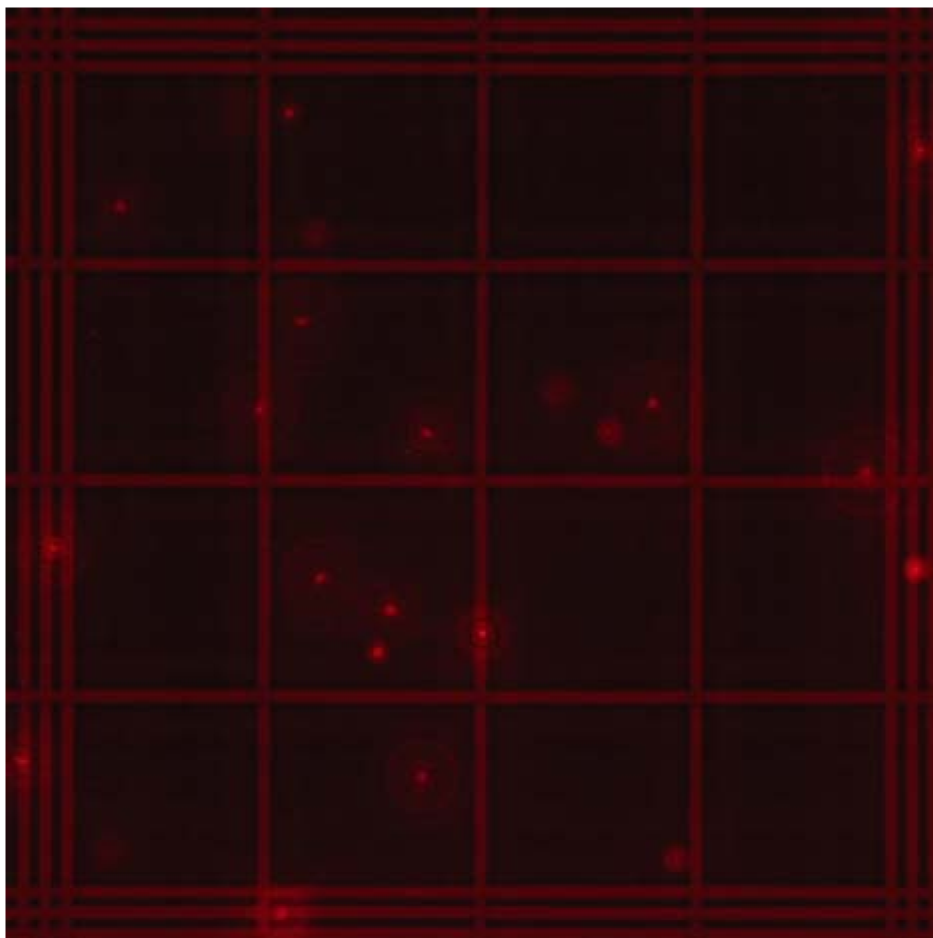

Figure 2. Fluorescent microscopy representative image of fluorescent micropspheres (625 nm/645 nm) on hemacytometer.

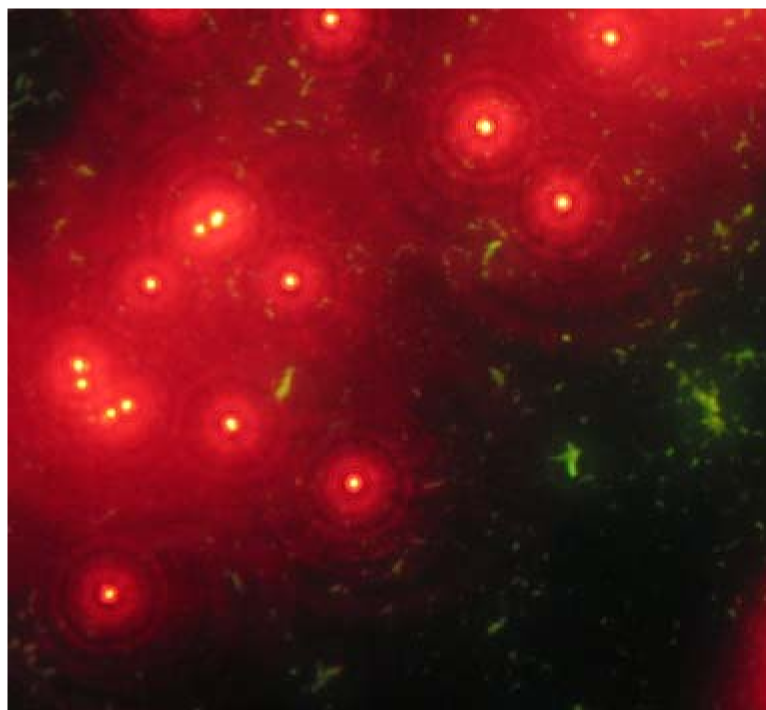

Figure 3. Fluorescent microscopy image of fluorescent microspheres (625 nm/645 nm) and stained *Salmonella* Typhimurium cells

APPENDIX

Appendix A, Figure 1. Modified bristles of the Sonicare® Elite 7300 brush head.

Appendix A, Figure 2. Sonicare® Elite 7300 apparatus for the sonicating brush method.

Appendix A, Figure 3. Position of the brush head relative to the inoculated area on the stainless steel coupon submerged in 100 mL PBS solution used for the sonicating brush method.