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Comparison of Antibodies Raised Against Heat- and Gamma Radiation-Killed Bacteria

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Abstract

For antibody generation, pathogenic bacteria are often heat-treated prior to inoculation into host animals in order to prevent infection and subsequently, premature death of the host. Inoculation of host rabbits with gamma radiation-killed pathogenic bacteria was employed with the hopes of generating antibodies that would have higher affinity, relative to antibodies raised against thermally denatured microorganisms, to live pathogens. The two antibody sets, raised against either heat-killed or irradiated bacterial cells, were compared for immunological response with live, heat-treated, chemically-treated (i.e., bleached), and irradiated *Escherichia coli* O157:H7 and *Salmonella* bacteria. With the exception of the chemically-treated cell immunological response, both antibody sets yielded similar responses—low for irradiated cells, moderate for live cells, and high for heat-treated cells. The results suggested that thermal or chemical treatment of live pathogen containing food samples will elicit higher immunological responses with either antibody system, indicating potential application for detection of the presence of live bacteria in non-irradiated food systems. In addition, these findings also indicated that an immunoassay analysis of irradiated foods may lead to low signals that might be interpreted as indicating the presence of live cells (i.e., may lead to a false negative result).

Keywords: Antibodies; Antigen binding; Cell surface; Immunochemistry; Lipopolysaccharide

Abbreviations: AP: Alkaline Phosphatase; IMB: Immunomagnetic Beads; ELIME: Enzyme-Linked Immunomagnetic Electrochemistry

Introduction

Among the rapid methods for detecting bacteria that have recently emerged [1-3], many employ antibodies to achieve immunological selectivity of specific analytes as differentiated from matrix and background flora in food samples. Rapid methods produced in our laboratory [4-10] can be grouped with such immunoassay-based rapid methods. The assessment of viability has become a pertinent issue and has been addressed in investigations aimed at the detachment or destruction of pathogenic bacterial contaminants on animal carcasses. These investigations have assessed the efficacy of numerous methods of washing carcasses with various chemical agents including organic acid, chlorine, and trisodium phosphate solutions [11-13].

Unfortunately, the ability to distinguish between live and dead microorganisms is problematic for immunoassays. Though techniques exist for the rapid determination of cell viability [14], the application of biorecognition elements (e.g. antibodies) with the ability to differentiate live from dead bacteria could eliminate the need for more time-consuming methods such as enrichment culture or conduct imetric or impedance measurements [15]. Although specific detection of targeted bacteria may be achieved with immunological methods, affinity of antibodies raised to bacterial pathogens may be further improved if host animals used to generate antibodies are inoculated with cells that more closely resemble the topographical features associated with the state (i.e., live or intact dead cells) of the bacterial target. To that end, antibodies were raised in host animals inoculated with gamma irradiated bacterial cells. Although too much irradiation can be deleterious to potential antigens [16], there is precedence for the generation of effective antibodies against lethally irradiated bacteria [17].

This investigation focuses upon quantifying immunological responses of two antibody systems interacting with live and dead bacterial analyte *Salmonella enterica Serovar typhimurium* (*S.* Typhimurium). One antibody system utilized purified immunoglobulin G fractions of custom produced antisera initially raised to *Salmonella* killed by gamma irradiation whereas the other utilized commercially available affinity purified antibodies raised against heat-killed *Salmonella*. Both antibody systems were utilized in parallel sandwich immunoassays (enzyme-linked immunosorbent assay or ELISA format) for the comparison of immunological responses elicited by chemical (bleach)-killed, heat-killed, gamma radiation-killed, and live *S. typhimurium* cells. Incorporation of the knowledge of differential responses into immunological-based methods for determining populations of viable and non-viable cells in a single sample is suggested.

Material and Methods

Materials

Materials used in this research included: *S.* Typhimurium ATCC strain14028 and brain heart infusion (Difco Laboratories, Detroit, MI), *E. coli* O157:H7 B1409 (Centers for Disease Control, Atlanta, GA), affinity purified goat anti-heat-killed *E. coli* O157:H7antibody and alkaline phosphatase (AP)-conjugate, goat anti-heat-killed *Salmonella* antibody and AP-conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), rabbit anti-irradiation-killed *Salmonella* antibody (protein A purified polyclonal antibodies) and AP-conjugate (custom produced by Rockland, Gilbertsville, PA), goat anti-*Salmonella*M-280

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immuno magnetic beads (IMB) and Magnetic Particle Concentrator (MPC-M; Dynal Inc., Lake Success, NY), graphite ink silk screen printed on Mylar (The Motson Co., Inc. Flourtown, PA), Alnico magnets (Edmund Scientific Co., Barrington, NJ), 1-naphthyl phosphate and platinum wire (Aldrich, Milwaukee, WI), 1% Blocker casein (Pierce, Rockford, IL), double coated plastic tape (Tesa Tape Inc., Charlotte, NC), Falcon 3915 Pro-bind™ polystyrene flat-bottomed assay plates (Becton Dickinson & Co., Lincoln Park, NJ), Tween 20, bovine serum albumin (Fraction V, initially heat-shocked fractionate) and Sigma Fast™ *p*-nitrophenyl phosphate tablet sets (Sigma, St. Louis, MO), and tryptic soy broth and Butterfield's buffer (Fisher Scientific, Pittsburgh, PA). Clorox bleach (containing 5.25% sodium hypochlorite) was obtained from a local supermarket. Other chemicals used were of reagent grade.

Apparatus

All reactions with shaking were performed on a Vortex-Genie 2™ (Scientific Industries, Bohemia, NY) at a "shake" to low vortex setting. Bacteria samples were counted on a Petroff-Hausser bacteria counting chamber (Thomas Scientific, Swedesboro, NJ). Electrochemistry of samples was performed in a custom-built multi-well electrode/magnet assembly that was constructed of polymethyl methacrylate blocks, Alnico magnets, double-sided tape, and graphite ink strip electrodes as previously described [18]. All electrochemical measurements were obtained with a BAS 100B/W electrochemical analyzer (Bioanalytical Systems, Inc., West Lafayette, IN) and accompanying BAS 100W software (version 2.0). An Ag/AgCl reference electrode (Vycor™ tipped, Bioanalytical Systems, Inc.), wrapped with a platinum wire that served as a counter electrode, was inserted into the tested solutions during electrochemical measurements. Colorimetric measurements were obtained using a Bio-Tek EL 312e microplate auto reader (Bio-Tek Instruments Inc., Winooski, VT) in the single wavelength mode (λ=405 nm). Solutions in the microtiter plates were shaken for 10 s, on the medium shake setting, immediately prior to measurement of absorbance.

Growth and enumeration of live *E. coli* O157:H7

A loopful of *E. coli* O157:H7 cells collected from a slant was inoculated into 25 ml of brain heart infusion broth and incubated with shaking (160 rpm) at 37°C for 18 h. The cells (~10^9/ml) were then placed on ice for no longer than 5 hours prior to use.

A single Bactrol disk containing *S. typhimurium* ATCC® 14028 was inoculated into 10 ml of tryptic soy broth and incubated at 37°C for 6 h. The inoculant (1 ml) was transferred to a 500 ml baffled shake flask containing 100 ml of tryptic soy broth and shaken (150 rpm) overnight (~17 h) at 37°C. The cells were pelleted by centrifugation at a relative centrifugal force (RCF) of 1,725 for 30 min and the supernatant was discarded. The pelleted cells were resuspended in 100 ml of sterile glass-distilled water, centrifuged (as above), and the supernatant was discarded. The cells again were resuspended in 100 ml sterile glass-distilled water and aliquots were removed for enumeration by standard pour plate in tryptic soy agar (37°C overnight) to determine total viable bacteria and 100-fold diluted for enumeration by counting chamber for a total cell count as previously described [5].

Chemical, heat, and irradiation treatment of bacteria

Aliquots (1 ml) of the live *E. coli* O157:H7 (~10^9 cells/ml) were placed into two 1.5 ml polypropylene tubes. The cells were washed 3X with TBS (25 mM Tris, 150 mM NaCl, pH 7.6) by centrifugation at 3,000 RCF for 2 min, removal of the supernatant, and resuspension of the pellet with 1 ml of TBS. Heat-killed bacteria were prepared by placing one of the sample tubes (capped) in a 100°C water bath for 15 min.

Aliquots (25 ml) of the live *S.* typhimurium (~10^{10} cells/ml) were placed into 4 separate 250 ml round-bottomed polyethylene vessels (capped). One sample vessel was exposed to gamma radiation in a manner consistent with the method reported by Thayer & Boyd [19]. In short, the cells were exposed to 8.5 kGy of gamma radiation using an on-site self-contained ^{137}Cs source. The samples were maintained at 5 ± 1°C during the irradiation process. One vessel (cap loosened) was immersed in a 100°C water bath for 15 min to prepare heat-killed bacteria. Bleach (Clorox; 5 ml) was added to one and sterile glass-distilled water (5 ml) was added to the other three.

Enzyme-linked immunomagnetic electrochemical (ELIME) detection of *S. Typhimurium*

The following method was performed at room temperature. Using reaction volumes suggested by Dynal Inc., 20 µl of IMB were placed in 1.5 ml polypropylene microcentrifuge tubes, 1 ml of bacteria (in TBS) was added, and the mixture was incubated by shaking (sufficient agitation to prevent settling of the IMB) for 30 min. The tubes were placed into the magnetic particle concentrator for 3 min in order to trap the IMB (a portion containing bound bacteria when present) against the walls of the tubes and the liquid was removed by aspiration. During IMB concentration, the particle concentrator was gently inverted several times to retain any IMB located in the cap of the tubes and to "focus" the IMB into a single "spot". The IMB were resuspended by gentle vortexing with 1 ml of AP-conjugated goat anti-*Salmonella* antibody (diluted 1:500 in 1% Blocker™ casein) and reacted for 30 min with gentle shaking. The IMB again were separated using the magnetic particle concentrator for 3 min and the liquid was removed. The IMB then were washed/resuspended twice with 1 ml TBS followed by magnetic separation for 3 min in the magnetic particle concentrator and removal of the liquid after each wash. Finally, the IMB were resuspended with 0.2 ml TBS.

For electrochemical analysis, 200 ml of IMB (with or without bound bacteria) were added to the solution holding block of a multi-well electrode/magnet. The beads were magnetically trapped against the electrodes for 2 minutes and the liquid was removed by aspiration. With the magnetic field applied, 200 ml of 1-naphthyl phosphate (2.7 mM in 0.2 M Tris, pH 9.6) was added to the well and allowed to react for 5 minutes. Production of electroactive 1-napthol was measured using Osteryoung square wave voltammetry (50 to 500 mV, 25 mV sweep width amplitude, 5 Hz frequency, 4 mV step potential, 10^{-6} A/V sensitivity) and the peak current was determined by drawing a tangent line across the base of the peak using the BAS 100W software.

ELISA detection of bacteria

Either anti-*E.coli* O157:H7 antibody and AP-conjugated anti-*E. coli* O157:H7 antibody or two separate antibody systems to *Salmonella* cells (heat-killed or irradiation-killed) and their respective AP conjugates were used in sandwich ELISA assays for comparing immunologic response to chemical (bleach)-killed, heat-killed, irradiation-killed, and/or live bacteria. The room temperature assay consisted of incubating 200 µl of unconjugated capture antibody (15 µg/well, diluted in TBS) in polystyrene microtiter plate wells for 1 h, blocking the wells for 30 min with 300 µl of bovine serum albumin (0.1% in TBS),

1 h incubation with 200 µl of bacteria, 1X wash with blocking solution (300 µl), 1 h incubation with 200 µl AP-conjugated antibody (1 µg/ml; from the same source as the respective capture antibody), 2X washes with TTBS (0.05% Tween 20 in TBS), and colorimetric 'development' with 200 µl of Sigma Fast *p*-nitrophenyl phosphate solution followed by measurement of absorbance at 405 nm. The removal of all solutions from the microtiter plates was effected through aspiration.

Results and Discussion

Initial observations were made employing ELIME detection with anti-*Salmonella* (heat-killed) antibodiesfor the comparison of immunological responses of heat-killed versus gamma radiation-killed *S.* typhimurium (Figure 1). The alkaline phosphatase (AP) substrate, 1-naphthyl phosphate, was used to indirectly monitor the antibody-bound paramagnetic bead-based sandwich immunoassay and the resultant electrochemical current versus irradiation-killed *S. Typhimurium* concentration profile was plotted along with data for heat-killed *S. Typhimurium*. The error bars (standard deviation from the mean) in figure 1 represent accumulation of no less than quadruplicate measurements per data point. The heat-killed *S. Typhimurium* data displayed a slope that was significantly (~8-fold) greater than that for the irradiated *S. Typhimurium*. Using the F-value from ANOVA, the probability (P) was calculated that the two slopes were equivalent was P=0.000124. The error bars (standard deviation from the mean) in figure 1 represent accumulation of no less than quadruplicate measurements per data point.

Further investigations employed anti-heat-killed *E. coli* antibodies to compare the immunological response for live and heat-killed *E. coli* O157:H7 from the same stock culture. Using a sandwich ELISA immunoassay, the heat-killed *E. coli* similarly displayed a higher (~2.7-fold) response than the live *E. coli* over the linear portion of the curves

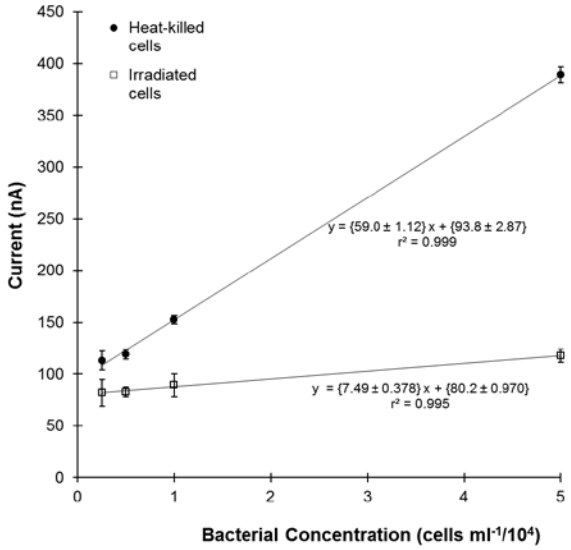

Figure 1: ELIME (enzyme-linked immunomagnetic electrochemical) detection of *S. t*yphimurium cells. Bacteria samples (1 ml) were immunomagnetically captured with anti-heat-killed *Salmonella* antibody-coated magnetic beads, reacted with AP-conjugated anti-heat-killed *Salmonella* antibody, and detected electrochemically after a short reaction with AP substrate as previously described. The above plot shows the electrochemical responses (current) for irradiation-killed (open squares) and heat-killed (closed circles) *S. typhimurium* (bacteria). The error terms in the equations are the standard errors of the slopes and intercepts.

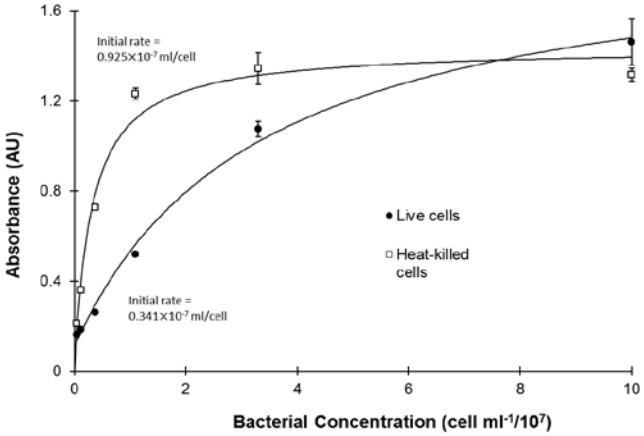

Figure 2: ELISA detection of *Escherichia coli* O157:H7 cells. In a sandwich immunoassay, live (closed circles) and heat-killed *E. coli* O157:H7 (open squares) bacteria were reacted with anti-heat-killed *E. coli* O157:H7 capture antibody (15 µg/ml) bound to polystyrene microplate wells followed by reaction with AP-conjugated anti-heat-killed *E. coli* O157:H7 antibody and colorimetric substrate. Formation of colored product was measured and represented above as absorbance (at 405 nm) versus bacteria concentration.(Initial rates were determined using LINEST in Microsoft Excel as applied to the first four data points).

(Figure 2). Using the F-value from ANOVA, the probability (P) was calculated that the two initial rates were equivalent was P=0.000160. The error bars (standard deviation of the mean) represent triplicate measurements per data point.

The results from figure 1 indicated that an equal number of *S. Typhimurium* cells, rendered non-viable by either exposure to high temperature or gamma irradiation, elicited statistically different immunological responses when tested with an identical method that used the same antibody system (antibodies raised against heat-killed *Salmonella*). Whereas the results shown in figure 2 demonstrated higher response for heat-killed as opposed to live *E. coli* O157:H7 as determined by a sandwich ELISA immunoassay that used anti-heat-killed *E. coli* O157:H7 antibodies. Taken together, these observations prompted an investigation in which chemical (bleach)-, irradiation-, and heat-killed *S. Typhimurium*, along with live *S. Typhimurium*, were analyzed using antibodies raised to heat-killed *Salmonella* as compared to antibodies raised against irradiation-killed *S. Typhimurium* in parallel sandwich immunoassays in an ELISA format (Figure 3A and B). The measurements displayed in figure 3 represent acquisition of data points in duplicate; nonetheless, though very small, vertical error bars were incorporated into figure 3. As with the electrochemical results obtained with the ELIME experiment in figure 1, the colorimetric results in figure 3A indicate a higher response for heat-killed as opposed to irradiation-killed *S. Typhimurium* using the antibodies raised against heat-killed *Salmonella*. Interestingly, similar results were obtained with the antibody system that included antibodies raised against irradiation-killed *Salmonella* (Figure 3B). The live *S. Typhimurium* cells elicited a somewhat lower response as opposed to the heat-killed *S. Typhimurium* with either antibody system. Bleach treatment elicited an immunological response as high as heat treatment (curves overlap) for the *S. Typhimurium* cells as reacted with antibodies raised to heat-killed bacteria. Interestingly, a similar study [20] demonstrated considerably higher immunogenic capacity for chemical (acetone) as opposed to gamma irradiation treated *Salmonella* Gallinarum-Pullorum. Using the antibodies raised against irradiated bacteria, the bleach treated cells

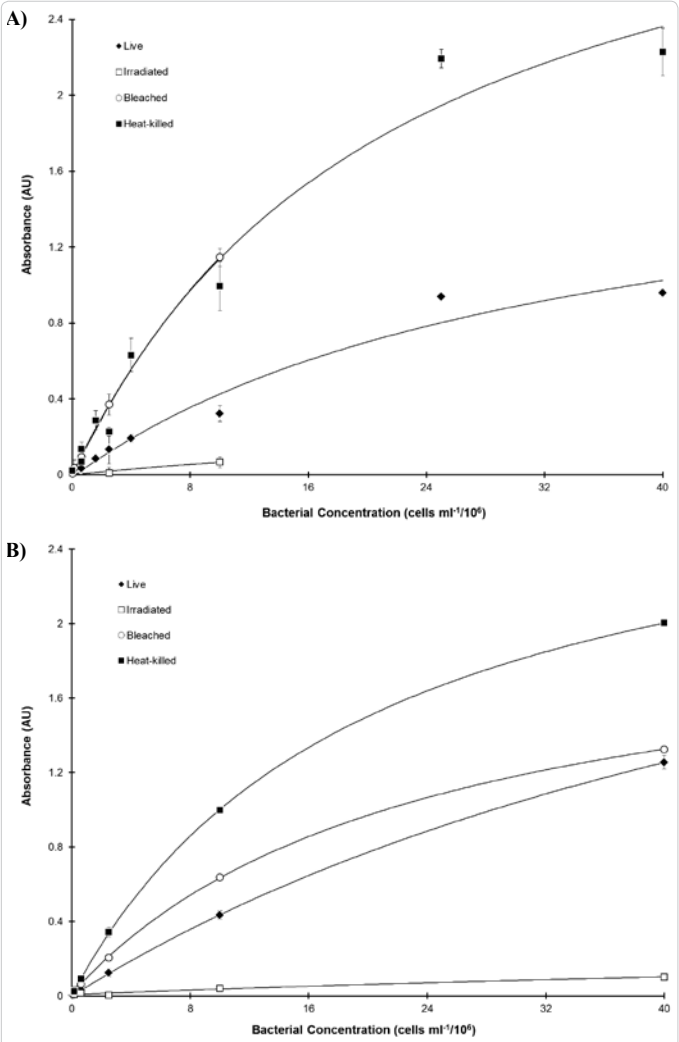

Figure 3: Comparison of antibody systems in the ELISA detection of *S. Typhimurium*. In a sandwich immunoassay, chemical (bleach/sodium hypochlorite; open circles), heat-killed (closed squares), irradiation-killed (open squares), and live (closed diamonds) *S. Typhimurium* (bacteria) were reacted with two separate antibody systems, one raised against heat-killed (A) and the other raised against irradiation-killed (B) *Salmonella*. Formation of colored product was measured and represented above as absorbance (at 405 nm) versus bacteria concentration.

elicited a more moderate response. Irradiation treated cells yielded low level immunological responses when assayed with either antibody system.

It has also been demonstrated that live and heat-killed bacterial immunogens can elicit the formation of distinct sets of antibodies in the same hosts [21]. Results of this investigation support previous citings [22,23] that heat treatment of bacteria (particularly *E. coli* O157:H7 as investigated in this study) may facilitate either the release of antigenic cell surface structures, for example lipopolysaccharides (LPS), or expose cryptic or core antigens (such as LPS) to immunological interaction. It is possible that LPS are simply "shed" from bacteria and the relatively small fragments elicit higher immunological responses due to vastly improved reaction kinetics. Unfortunately, this phenomenon may lead to false positive results when the immunological method detects non-viable (e.g. heat killed) cells in the presence or even the absence of live cells. Furthermore, the age of the microorganisms may be related to their shedding or exposure of LPS.

Wash water samples from the rinsing of slaughtered animal carcasses with high pressure anti-microbial chemical containing sprays, may contain both live and chemically-killed bacteria. These studies suggests that the presence of live bacteria that may remain in such a sample may be determined, and perhaps quantified through an immunological detection scheme that includes measurements before and after heat as well as chemical (e.g. bleach) treatment. Chemically treated, as opposed to gamma-irradiated, foods containing targeted pathogenic bacteria may demonstrate differentiable immunogenic response ratios before and after heat treatment of the bacterial samples. Such a differential response may yield predictable ratios that indicate the presence of live cells that have survived bactericidal treatment. In addition, it would be important if the irradiation-killed bacteria was either comprised of relatively virulent enterotoxins that may cause intoxication or simply present in high numbers. Both of these scenarios may present a low immunological response that could be construed as a false negative result and could lead to either intoxication or overconfidence in pretreatments (e.g. spray washing) employed to lower bacteria counts on foods.

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