RAPID DETECTION OF *SALMONELLA* WITHOUT ENRICHMENT

by

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ABSTRACT

Rapid Detection of Salmonella

Without Enrichment

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Salmonella is one of the leading foodborne pathogens causing illness today. Because of this, Salmonella rapid detection methods are under immense study for use in food. The traditional method, using the Food and Drug Administration-approved Bacterial Analytical Manual procedure, takes 4-6 days for Salmonella detection in food. Other rapid methods still take at least 16 h for detection due to their enrichment steps.

The hypothesis of this study was that the use of immobilized antibodies coupled with polymerase chain reaction (PCR) can be used for the rapid capture and detection of Salmonella spp. in food without the need for pre-enrichment. The rapid detection system was developed using immobilized anti-Salmonella antibody beads to capture and separate Salmonella from food without using an enrichment step. Detection of the immunocaptured Salmonella was done through PCR or an enzyme-linked immunosorbent assay (ELISA)-based system entitled Rapid Immuno-Capture (RIC).
The detection limit for buffer, chicken rinse, and shell eggs with static antibody capture PCR and RIC, was determined by inoculation of the *Salmonella*-free samples. The RIC assay detected *Salmonella* spp. in buffer at concentrations as low as $4 \times 10^1$ CFU/ml, and in chicken rinse and shell eggs, the assay detected *Salmonella* at $4 \times 10^3$ CFU/ml. The antibody capture with PCR detected *Salmonella* in buffer at concentrations as low as $4 \times 10^2$ CFU/ml, in chicken rinse at concentrations as low as $4 \times 10^5$ CFU/ml, and in shell egg at bacteria concentrations of $4 \times 10^6$ CFU/ml.
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Emily J. Harrington
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LIST OF SYMBOLS AND NOTATIONS

$1^\circ = \text{primary}$
$2^\circ = \text{secondary}$
$3^\circ = \text{tertiary}$
$\text{Ab} = \text{antibody}$
$\text{ADH} = \text{adipic acid dihydrazide}$
$\text{AOAC} = \text{Association of Analytical Communities International}$
$\text{AP} = \text{alkaline phosphatase}$
$\text{APTES} = 3\text{-aminopropyltriethoxysilane}$
$\text{ATP} = \text{adenosine 5'\text{-}triphosphate}$
$\text{ATCC} = \text{American Type Culture Collection}$
$\text{BAM} = \text{FDA Bacteriological Analytical Manual}$
$\text{bp} = \text{base pairs}$
$\text{cAMP} = \text{cyclic adenosine monophosphate}$
$\text{CDC} = \text{Centers for Disease Control}$
$\text{ddH}_2\text{O} = \text{double distilled water}$
$\text{DNA} = \text{deoxyribonucleic acid}$
$\text{dNTP} = \text{deoxy nucleotide triphosphate}$
$\text{EDTA} = \text{ethylenediaminetetraacetate}$
$\text{ELISA} = \text{enzyme-linked immunosorbent assay}$
$\text{FDA} = \text{Food and Drug Administration}$
FoodNet = Emerging Infections Program Foodborne Disease Active Surveillance Network

GC% = guanine and cytosine %

IMS = immunomagnetic separation

NCHS = National Center for Health Statistics

PBS = phosphate-buffered saline

PBST = phosphate-buffered saline containing 0.02% (vol/vol) Tween 20

PMN = polymorphonuclear

pNPP = p-nitrophenyl phosphate

RIC = Rapid Immuno-Capture

rRNA = ribosomal ribonucleic acid

SPI-1 = *Salmonella* pathogenicity island-1

SPI-2 = *Salmonella* pathogenicity island-2

TNBS = 2,4,6-trinitrobenzene sulfonic acid

T_m = melting temperature

Tris = tris hydroxymethyl aminomethane hydrochloride

TTSS = type III secretion systems

U.S. = United States of America

VRBA = violet red bile agar

WHO = World Health Organization
Food safety is an important issue in today's world. Outbreaks of foodborne illness caused by *Escherichia coli*, *Listeria monocytogenes*, *Salmonella*, and other foodborne pathogens have not only affected consumer health, but food recalls have been detrimental to food producers. Safe food has the potential to help the economy through health and productivity. On the other hand, bacterial contaminated food creates a downward spiral for the economy. The general health decreases causing an increase in cost for health care and an uncertain consumer society toward food products. Food becomes a risk factor (35).

With this increase in awareness of the importance of food safety comes the importance of food microbial detection. The traditional method for *Salmonella* detection in the U.S. is a culture-based method which takes 4 days for a confirmed negative result and up to 6 days to confirm a positive bacterial contamination (2). Other more rapid methods for *Salmonella*, including the enzyme-linked immunosorbent assay (ELISA) test and the commercial PCR tests, require 20 to 48 h incubation periods (5, 12). Not only do the methods for traditional *Salmonella* detection take up to 6 days, other foodborne pathogens, including *Campylobacter jejuni*, *L. monocytogenes*, and *E. coli*, take up to 7 days or more (2). The time spent on incubation to enhance bacterial numbers and separate food particles from the bacteria could be better utilized for distribution of the food product into the hands of hungry consumers. A significant draw back to using the PCR method of detection for food, despite its rapid detection abilities, is the inhibitory effect of a number of compounds in the food that vary with form and processing batch. These effects may also be concentrated by using a typical extraction method (34).
The Centers for Disease Control and Prevention’s (CDC) Emerging Infections Program Foodborne Disease Active Surveillance Network (FoodNet) stated that during 2002, 16,580 laboratory-diagnosed cases of 10 foodborne diseases under surveillance in the 9 selected U.S. states were identified, and 6,028 of the cases were due to Salmonella infection (8). Between 1996 and 2002, the bacterial pathogens with the highest relative incidence included Campylobacter, Salmonella, and Shigella.

In January 2000, Healthy People 2010 was introduced by the U.S. Department of Health and Human Services as a nationwide health promotion and disease prevention agenda (26). The National Center for Health Statistics (NCHS) defined Healthy People 2010 as “a set of health objectives for the Nation to achieve over the first decade of the new century” (27). The objectives focus on 28 areas including food safety (26). The food safety national health objectives for incidence reduction to be attained by 2010 target 4 foodborne diseases: Campylobacter, E. coli, Listeria, and Salmonella (7). Of these 4 pathogens, Salmonella has the largest average incidence reduction to attain by 2010 (7, 8). Because of this incidence reduction expectation, more pathogen control and more rapid pathogen detection measures need to be implemented.

Increased and improved microbial detection technology at the industrial level, such as more rapid detection measures for foodborne pathogens, could save the food industry money. Most food industry facilities require “in plant” storage of food products due to the time needed for pathogen testing. Enhanced microbial detection technology would allow food that is normally held in storage for days to be released for market in hours. Through prevention, the rapid detection abilities also reduce the costly effects of food recalls due to pathogenic bacteria.
Previous methods for detection of *Salmonella* in food involve enrichment steps to allow for enough growth to be able to detect its presence. The aim of this research is to eliminate the pre-enrichment step which will provide for even more rapid detection of *Salmonella* in food. The method uses immobilized antibodies on glass beads (3-mm diam) for capture of the bacteria followed by either an ELISA-based method or PCR for detection or identification, respectively.
LITERATURE REVIEW

Salmonella spp. and Pathogenicity

Salmonella spp. is a Gram-negative, non-endospore-forming, facultative anaerobic, motile rod-shaped bacteria. Two exceptions exist for motility: Salmonella gallinarum and Salmonella pullorum are nonmotile (37). Currently, there are more than 2,400 different serotypes of Salmonella (11). Salmonella spp. can be divided into the broad groups of typhoidal and nontyphoidal. Normal habitation of these non-typhoidal bacteria is found in the intestinal tract of humans and animals. The Salmonella sp. that causes typhoid colonizes only humans (1).

Many Salmonella isolates have been named after towns or by individuals who first discovered them (1). Salmonella received its name after the discoverer, Dr. Daniel Salmon (14). Naming of Salmonella is complex due to the evolving nomenclature throughout time. These changes have been due to advancement in technology, from early biochemical and serological characteristics to newer numerical taxonomy and DNA-based classification systems (11). One of the earlier systems still in existence, the Kauffinan-White scheme, used biochemical reactions to identify 5 defined subgenera (I to V) (11). Along the same lines, another biochemical classification system was brought about that separated Salmonella into 3 species: Salmonella typhi, Salmonella choleraesuis, and Salmonella enteritidis (11). Based on numerical taxonomy and DNA relatedness, yet another system emerged that suggested a single species (S. choleraesuis) that included seven subspecies: choleraesuis, salamae, arizonae, diarizonae, houtenae, bongori, and indica (11). Due to possibilities of confusion, it was proposed to change the
type species in the former scheme from *S. choleraesuis* to *Salmonella enterica*, keeping the seven subspecies the same (11). A newer DNA-based system, which has been adopted as the official nomenclature system for the CDC, separates *Salmonella* into 2 species: *S. bongori*, and *S. enterica* (1, 11). Both species contain multiple serovars, but *S. enterica* also includes six subspecies (*enterica, salamae, arizonae, diarizonae, houtenae, indica*) (1, 11).

All *Salmonella* spp. are considered pathogenic and may cause salmonellosis or typhoid (enteric) fever depending on the serovar and strain. *S. typhi* and *Salmonella paratyphi* cause the systemic, bacteremic illnesses. Whereas, the non-typhoid *Salmonella* that usually cause food-poisoning in humans bring about salmonellosis.

Pathogenicity occurs when the bacteria invade the intestinal epithelium and multiplies within the mucosal cells causing an infection and inflammation. In the case of the systemic illness, the bacteria penetrate the intestinal mucosa where they gain access to the bloodstream and are taken into tissues including the liver, the spleen, and bone marrow. In those tissues, the *Salmonella* multiply during an incubation period of 7 to 28 days and then are released into other areas, such as the kidneys and the gall bladder (11). For salmonellosis, the invasion of the bacteria into the intestinal epithelium not only causes inflammation but also diarrhea due to a polymorphonuclear (PMN) leukocyte influx into the infected mucosa and the leukocytic release of prostaglandins, which activates the adenyl cyclase, resulting in increased intestinal fluid secretion (11). The infectious dose for non-typhoidal *Salmonella* can be as few as 15-20 cells depending on the age and health of the individual and the strain of the bacteria (41). On the other hand, the infectious dose for typhoidal *Salmonella* is thought to be around $10^5$ organisms (1).
Two toxins, enterotoxin and cytotoxin, are supposed virulence factors from *Salmonella*. The release of enterotoxin into the host cell causes an activation of adenyl cyclase in the epithelial cell membrane and an increase in the cytoplasmic concentration of cyclic adenosine monophosphate (cAMP) in the host cells. The suppressed absorption of sodium ions in the intestinal villa in concert with the secretion of chloride ions in the intestinal mucosa result in an increase in intestinal fluid causing diarrhea (11). The thermolabile cytotoxic protein, cytotoxin, inhibits protein synthesis and causes cell lysis of the host cells, which in turn allows for the unhindered spread of *Salmonella* into the host tissue (11).

Pathogenicity occurs when certain genes are expressed creating invasive products. Studies have shown invasive appendages develop on salmonellae surfaces when in contact with epithelial cells. These appendages are used to attach the *Salmonella* to the host. Once the *Salmonella* have colonized to the epithelial cells, the appendages are shed (11). Along with several chromosomal locations that are homologs to *Escherichia coli*, *Salmonella enterica* chromosome contains inv/spa genes mapped near 59 min, whose products are required for the invasion of epithelial cells (37). The *Salmonella* pathogenicity island-1 (SPI-1), which contains the invasive genes, is conserved among *Salmonella* spp. through time allowing its genes to be practical targets to use for PCR DNA identification and detection (11).

Type III secretion systems (TTSS) encoded by SPI-1 and SPI-2 are important in virulence strategies for *S. enterica*. SPI-1 is related to functions involving epithelial cell invasion, inflammation, and stimulation of diarrhea. On the other hand, SPI-2 was found to be related to the intracellular survival and propagation of *Salmonella* (19).
Because *S. enteritidis* is one of the most common of the *Salmonella* serotypes found in the United States, much work is focused on the rapid detection of this organism (6). *S. enteritidis* is in serogroup D of the species and subspecies *S. enterica enterica*, and it is considered nontyphoidal (1). *S. enteritidis* scheme includes more than 50 recognized phage types (28).

**Occurrence of Foodborne Disease**

More than 5 million people a year are infected with bacterial gastrointestinal foodborne pathogens, and there have been an estimated 45,826 hospitalizations and 1,458 deaths a year due to these bacteria (24). Of those enteric pathogenic infections, salmonellosis is worldwide one of the most frequently reported foodborne diseases (35). In the last decade, the reported human cases have shown a dramatic relative increase of *S. enteritidis*. The increasing trend over time was shown in many different countries despite the fact that their may be an inconsistency in efficiency of disease surveillance systems (35).

An estimated 1 in 10,000 hen eggs is contaminated with *S. enteritidis* (1). Other foods that may harvest *Salmonella* include foods of animal origin, most commonly poultry. Foods that contain these ingredients or that may have been cross-contaminated with any of the raw foods of animal origin could possibly contain *Salmonella*. Salmonellosis, the infection caused by *Salmonella*, usually has symptoms including diarrhea, fever, and abdominal cramps that may last 4 to 7 days. The onset of this infection develops 12 to 72 h after consumption for most persons. The infection usually clears-up completely within a few months, but a small number of people infected with
Salmonella may develop Reiter’s syndrome. This disorder may last from a few months to years and includes symptoms such as joint pain, eye irritation, and painful urination. Reiter’s syndrome may lead to chronic arthritis later on in life (6).

Antibiotic resistance is also a concern. Salmonella and Campylobacter have developed resistance to ciprofloxacin, one of the leading drugs to treat enteric infections (25). Patients who are immuno-compromised or at the extremes of age are particularly susceptible to the foodborne bacterial infections and most often require antibiotic treatment (25). This shows the importance of prevention rather than treatment alone.

Developing countries, including South America, Africa, the Indian subcontinent, and South East Asia are influenced greatly by foodborne illness. Unlike developed countries which show only mild to moderate gastroenteritis and <0.5% death when infected, the developing countries have severe gastroenteritis and up to 30% mortality (28). The poor in these developing countries are the most susceptible to ill health, and due to malnutrition, they are unable to combat the symptoms, such as diarrhea which may have a long duration and lead to death (35).

Safety Measures

In May 2000, the World Health Organization (WHO) stated that food safety is one of its top 11 priorities (35). Prevention of the foodborne illnesses has to be involved in every step of food production; from farm to table.

Adjusting the pH and water activity helps reduce the growth of microorganisms, such as Salmonella, in food. Growth for this organism is poor below the pH of 5.0, and 0.93 is the minimum water activity limit for Salmonella growth (11, 13). Salmonella spp.
growth can also be controlled through physical control measures including temperatures above 60°C for 60 s for most Salmonella and irradiation (ionizing radiation) (13). Pasteurization temperatures and sterilization temperatures will also destroy pathogenic organisms.

Food producers have also taken on more responsibility of food safety by implementing Hazard Analysis Critical Control Points (HACCP). The HACCP program plays an important role in the reduction of foodborne illness when used correctly. This program includes the following 7 steps, which are stated in brief (30):

1. Identify the potential hazard associated with food production.
2. Determine the points/procedures/operational steps that can be controlled to eliminate or minimize hazard.
3. Establish target levels and tolerances for CCP.
4. Establish monitoring system for CCP (scheduled testing or observations).
5. Establish corrective action if CCP not under control.
6. Establish procedures for verification.
7. Establish documentation concerning all procedures and records.

At the consumer level, education and awareness of possible bacterial contamination is important. The U. S. Food and Drug Administration (FDA) as well as the university extension services provide information to consumers on safe food handling. For example, keep hot foods hot (above 140°F, 60°C) and cold foods cold (less than 41°F, 5°C) (17). Also, perishable foods should not be left out more than 2 h at room temperature and not more than 1 h when the temperature is above 90°F (17). When cooking, comminuted meats should be cooked to 155°F (68°C) and ground poultry to 165°F (74°C). Poultry should be cooked to an internal temperature of 165°F (74°C). Reheated foods should be brought to a temperature of at least 165°F (74°C) (40).
Methods of Bacterial Detection for

Salmonella spp.

Traditional methods

The FDA Bacteriological Analytical Manual (BAM) specifies the regulations and methods for detection of microbes. For Salmonella, the process takes approximately 6 days including enrichment, selective enrichment, biochemical and seriological tests (2).

Rapid methods approved or tested by the
Association of Analytical Communities International

ATP bioluminescence

Adenosine 5′-triphosphate (ATP) is a component of all forms of life on earth, and can be measured to detect for the presence of microorganisms. The detection occurs by the reaction of luciferin, luciferase, ATP, and magnesium to ultimately produce light which is measured by standard fluorimetry (18).

Food, such as meat or milk, intrinsically contains ATP. To overcome this obstacle, methods of heat treatment, centrifugation, and filtration were used to separate the food components containing ATP from the bacteria. Use of the firefly ATP bioluminescent assay allows for rapid and sensitive detection/enumeration of microorganisms (29). PATH-STIK, a product from Celsis (Chicago, IL) is a current Association of Analytical Communities International (AOAC) performance tested ATP bioluminescence method for the detection of Salmonella spp. (3).
Antibody-based tests

A number of commercial enzyme-linked immunosorbent assays (ELISA) have been developed that utilize monoclonal or polyclonal specific antibodies coupled with enzyme-linked fluorescence to presumptively detect *Salmonella* spp. The AOAC has made many ELISA tests official methods including (but not all inclusive): 1-2 Test® (BioControl Systems Inc., Bellevue, WA) with a sensitivity ranging from 1 to 5 CFU/25g sample, *Salmonella* Tek (Organon Teknika, Durham, NC), VIDAS® *Salmonella* (SLM) (bioMerieux, Hazelwood, MO) which uses a reagent strip with antibodies, Tecra® *Salmonella* VIA™, and Tecra® UNIQUE™ *Salmonella* (Tecra Diagnostics, Sydney, Australia) (3, 42, 45). Tecra® *Salmonella* VIA™ test uses a sandwich-type immunoassay with polyclonal antibodies. This test declares presumptive positive results in 42 to 52 h. The Tecra® UNIQUE™ *Salmonella* procedure, with available presumptive positive results in only 22 h, uses an antibody-coated dipstick in combination with immunoenrichment and immunoassay detection (12). All commercially available ELISA procedures mentioned require at least one enrichment step.

In addition to the antibody based ELISA technique, the use of immunomagnetic beads for the detection of foodborne pathogens has been commercialized. Two commercial AOAC performance tested methods include: Dynabeads® anti-*Salmonella* Kit (Dynal Biotech, Lake Success, NY) and Salmonella Screen/ Salmonella Verify™ (Vicam, Watertown, MA) (3). Immunomagnetic separation is usually used in conjunction with culture-based or PCR-based detection (22, 33). Magnetic particles coated in monoclonal or polyclonal antibodies are exposed to the food samples or
enrichment cultures and incubated for a short period (about 10 min). The magnetic particles are then separated from the sample, washed, and as in the case of the culture method, resuspended in media and plated onto culture plates (33). For the use in PCR, the target bacteria from the food samples are specifically caught in antibody-coated magnetic beads, washed to remove food debris, and prepared for PCR (22). In both situations, the original food sample was enriched for a period of time ranging from 16 to 20 h before testing occurred. The sample sizes for the food tested in both the culture-based and PCR immunomagnetic bead separation were 25 g samples pre-enriched in 225 mL of buffered peptone water. Jentkova et al. reported a PCR sensitivity of 1-5 CFU per 25 g egg sample only after the 16 h pre-enrichment homogenization step (22). The sensitivity for the minced meat was $1-5 \times 10^3$ CFU per 25 g sample, again, only after the pre-enrichment step (22). The use of immunomagnetic bead separation is beneficial in rapid detection because it concentrates target bacteria while reducing the amount of inhibitive food debris, but methods thus far researched involve time consuming pre-enrichment steps (22).

The ImmunoFlow technology, which has not been AOAC approved, utilizes immobilized antibodies on glass beads to capture bacteria from samples. The fluidized bed allows for large or small samples making the volume independent. Detection of bacteria using an ELISA technique is possible without an enrichment step, but specificity of the immobilized antibody directly affects the method (46). This method could be modified to allow for specificity through the use of the PCR, thus providing a specific rapid detection method without an enrichment step.
Nucleic acid-based tests

DNA-DNA hybridization offers a more rapid detection time of foodborne pathogens, higher detection sensitivity, and allows for a more specific identification of the bacteria species (39). Target genes can be focused toward random DNA fragments, toxin genes, or even rRNA genes. The capability is unending (39).

One concern for the use of DNA probes for the detection of pathogens is the use of radioactive probes. Developing countries are not well equipped for the disposal of such probes, but with new technology, non-radioactive probes have been developed. Two commonly used commercial systems recognized as AOAC official methods of Salmonella detection are the GENE TRAK® Salmonella Assay and the GENE TRAK® Salmonella DLP Assay, both by Neogen Corporation (Lansing, MI) (3).

The polymerase chain reaction allows primers specific to a certain target DNA sequence to hybridize to the DNA amplifying that particular DNA sequence exponentially. The process involves the denaturation of the DNA, then the annealing of the primers to the target DNA sequence, and finally, the extension or polymerization of the specific DNA sequence.

Three PCR-based systems are available commercially: Probelia™ Salmonella spp. of Sanofi Diagnostic Pasteur (Marnes La Coquette, France), Taqman™ Salmonella PCR Assay of Perkin Elmer (Norwalk, CT), and BAX™ for Screening/ Salmonella (Wilmington, DE). Probelia™ uses a post-PCR DNA hybridization process with Salmonella–specific oligonucleotide peroxidase-labeled probes (44). The Taqman™ Salmonella PCR Assay relies on the release of fluorescent dye for the detection of PCR product. This fluorescence-based assay utilizes the ability of DNA polymerase to cleave
nucleotides from a double-fluorescence-labeled probe (20). The BAX™ System with Automated Detection PCR Assay for Screening for Salmonella (Qualicon, Inc., Wilmington, DE) is the only PCR-based performance tested method with AOAC approval (2). The BAX™ system provides all the needed reagents, except for the target DNA, in tablet form to help prevent contamination of samples by environmental DNA. The system amplifies the target DNA with the use of primers, and the results are analyzed on agarose gel (38). Bennet et al. reported a BAX™ system detection limit of <50 cells of Salmonella in various food products, including meat, poultry, and milk products, after a 26-h pre-enrichment step (5).

With prevention of foodborne illness a priority, the need for foodborne pathogen detection is urgent. Rapid detection systems for Salmonella have improved in the recent years, but because pre-enrichment steps are still used, a need still exists to lessen the amount of time to obtain results. Reduction or even elimination of the enrichment period would greatly advance Salmonella rapid detection systems for food. The aim of this work is to eliminate the time consuming pre-enrichment step in Salmonella detection in food samples to allow for a more rapid detection time.
HYPOTHESIS AND OBJECTIVES

Hypothesis

The use of immobilized antibodies coupled with PCR can be used for the rapid capture and detection of *Salmonella* spp. in food without the need for pre-enrichment.

Objectives

1. To compare antibody capture of *Salmonella* spp. in pure culture with enzyme-linked immunosorbent assay (ELISA) technique to PCR DNA identification.
2. To investigate the antibody capture of *Salmonella* spp. in food samples including eggs and cooked chicken with DNA identification using PCR. Various levels of *Salmonella* will be added to samples to determine assay detection limit.
3. To investigate the antibody capture of *Salmonella* spp. with DNA identification in raw food samples.
MATERIALS AND METHODS

Bacterial Strains, Growth, and Preparation of Working Stock

Fourteen strains of *S. enteritidis* (American Type Culture Collection, ATCC 8326, 31194, 13314, 4931, 49214, 49215, 49216, 49214, 49218, 49219, 49220, 49221, 49222, 49223) were. All bacterial cultures were stored frozen in liquid nitrogen (-196°C).

The *S. enteritidis* culture was kept overnight at -20°C prior to a rapid thaw and inoculation into 10 ml sterile nutrient broth (Difco, Detroit, MI). Cells were incubated overnight in a 35°C incubator shaking at 180 rpm to allow for $10^9$ CFU/ml growth.

The overnight culture was precipitated twice through centrifugation ($3,000 \times g$ for 5 min at 4°C), and the pellet was resuspended with either 50 mM Tris pH 7.2 or phosphate-buffered saline (PBS) pH 7.4 to the original culture volume. After a third centrifugation, the pellet was resuspended with either 50 mM Tris pH 7.2, phosphate-buffered saline (PBS) pH 7.4, or 60 mM carbonate buffer pH 9.6 at the original culture volume for use as working stocks.

Antibodies

All the antibodies (Abs) used were IgG. The primary (1°) polyclonal Ab, goat anti-*Salmonella*, with a concentration of 5.4 mg protein/ml, used for immobilization onto the 3-mm glass beads was obtained from OEM Concepts (Toms River, NJ, product G5-
V61, lot 500-30778). The secondary (2°) polyclonal Ab, rabbit anti-Salmonella, with a concentration of 4.5 mg protein/ml was also obtained from OEM Concepts (Toms River, NJ, product R4-V61, lot 101-26361). The tertiary (3°) Ab, anti-rabbit IgG conjugated to alkaline phosphatase (AP) with a concentration of 1.2 mg protein/ml, was obtained from Sigma (Saint Louis, MO, product A3687, lot 011K9209). Another 3° Ab, anti-goat IgG conjugated to AP (concentration 1.2 mg protein/ml), was used with the goat anti-Salmonella Ab. All Abs were stored at 4°C.

Ab ELISA titer

The optimum Ab concentration to use against S. enteritidis was determined for each lot number of Ab and Ab type (1°, 2°, 3°) by performing a titer using an ELISA. The method was modified from Weimer et al. (46). The 1° and 2° Abs were tested against S. enteritidis ATCC 8326 at concentrations ranging from 3.6 x 10^{-6} to 3.6 x 10^{-15} M for the 1° Ab and 3.0 x 10^{-6} to 3.0 x 10^{-15} M for the 2° Ab. The appropriate 3° Ab was tested against the 1° and 2° Abs at concentrations ranging from 4.8 x 10^{-10} to 8.1 x 10^{-11} M. S. enteritidis ATCC 8326 was used in the test plate and no bacteria was used in the negative control plate.

The culture working stock was prepared using 60 mM carbonate buffer pH 9.6. Plates with 96 wells (polystyrene, Fisher Scientific, Pittsburg, PA) were coated either with approximately 10^8 CFU of bacteria/well (75 µl/well) resuspended in the 60 mM carbonate buffer pH 9.6 (test wells) or with 60 mM carbonate buffer pH 9.6 (negative wells). These plates were covered with parafilm and incubated overnight at 4°C. After overnight incubation, the 96-well plates were washed 4 times with 250 µl PBS containing
0.02\% (vol/vol) Tween 20, pH 7.2 (PBST) and thoroughly shaken to remove excess wash. Filter sterilized (0.45 micron) bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) at a 2\% solution in PBS was added to each well (150 \mu l/well). The plate was sealed with parafilm and incubated at 37°C for 1 h to block the plate. The plate was then washed again 4 times with 250 \mu l PBST.

Aliquots (75 \mu l) of each concentration of the 1° Ab or the 2° Ab were added to each well and plates were incubated at 37°C for 1 hr followed by washing 4 times with 250 \mu l PBST. The appropriate 3° Ab, at the concentrations described above, was added to the plates (75 \mu l/well), sealed with parafilm, and incubated 30 min at room temperature. After incubation, the plates were washed 4 times with 250 \mu l PBST. The substrate, p-nitrophenyl phosphate (pNPP) (Sigma, St. Louis, MO) dissolved in 0.1 M glycine buffer, 1 mM MgCl₂, 1 mM ZnCl₂, pH 10.4 (1 mg/ml), was added in the dark to the plates at 75 \mu l/well. The plates were sealed with parafilm, wrapped with foil, and incubated for 20 min at 37°C. Upon completion of the incubation, the color development in the wells was measured using HtSoft™ in a BioAssay 7000 plate reader (Perkin-Elmer, Norwalk, CT) at a wavelength of 405 nm. The signal-to-noise ratio was calculated by dividing the absorbance with the negative control (a well with no bacteria adsorbed).

**Anti-Salmonella Cross-Reactivity ELISA**

The activity of the 1° and 2° Abs to recognize each of the 14 strains of *S. enteritidis* was determined in an ELISA format with limited Ab dilutions. A working solution of each of the 14 *S. enteritidis* strains was prepared as described above and
dispensed into separate wells of a 96-well plate. Primary Ab (75 µl) at 1 of 3 concentrations (3.6 x 10^-6 M, 3.6 x 10^-7 M, 3.6 x 10^-8 M) and 2° Ab (75 µl) at 1 of 3 concentrations (3.0 x 10^-6 M, 3.0 x 10^-7 M, 3.0 x 10^-8 M) was added to each well as described above. The appropriate 3° Ab (75 µl of 4.8 x 10^-10 M) was added to each sample well. All 14 S. enteritidis strains described previously were tested at 10^9 CFU/ml.

The controls included 3 negative controls and a positive control. One negative control was coated with 60 mM carbonate buffer, followed by the addition of 1° or 2° Ab, the 3° Ab, and then the substrate. This control included all the factor level combinations except the bacteria. Another negative control consisted of a 60 mM carbonate buffer coat, followed by the 3° Ab, and finally the substrate. The third negative control consisted of only buffers, a 60 mM carbonate coat and the PBST. The positive control was coated in 2° Ab, followed by the 3° Ab, and finally the substrate as described above. A signal-to-noise value was calculated for each sample as described above.

**Ab Immobilization**

**Preparation of APTES beads**

Preparation of APTES (3-aminopropyltriethoxysilane) beads procedure was modified from Walsh and Swaisgood (43). Borosilicate glass beads (3-mm diam, 7.5 x 10^-4 m^2/g) were boiled with concentrated nitric acid in a boiling water bath for a minimum of 1 h. The nitric acid was decanted, and the beads were pre-washed with tap water followed by washing with 10 times the bead volume of double distilled water (ddH₂O). APTES (Sigma, St. Louis, MO), 13% solution in ddH₂O, was added to the beads until the beads were completely covered. The pH of the solution was adjusted to
4.0-5.0 using 6 N HCl and 1 N NaOH. The beads with APTES were placed in a 70°C water bath for a minimum of 3 h, with swirling every h (Fig. 1).

The APTES treated beads were taken out of the water bath to cool and then washed with 2 times the bead volume of ddH$_2$O. After washing, the beads were placed in an 80-100°C oven to dry for 12 h. Three to four beads were set aside in a test tube for a quality control test. The remainder of the beads were washed with 10 times the bead volume with ddH$_2$O. Again, the beads were placed in an 80-100°C oven to dry for 12 h. The beads were then placed in a closed container and stored at room temperature until used.

The beads for the quality control test, including the negative control (acid washed beads) and the APTES beads, were put in separate test tubes. Sodium tetraborate (0.1 M, 1 ml) and 20 µl of 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Sigma, St. Louis, MO) were added to each tube. The beads and solution were incubated for 30 min in the dark. After incubation, both sets of beads were rinsed with ddH$_2$O. The color of the beads was inspected. The presence of a yellow color was indicative of APTES treated beads.

![Diagram](image)

FIGURE 1. *Derivatization of 3-mm glass beads with 3-aminopropyltriethoxy silane (APTES).*
Antibody immobilization onto APTES beads

Attachment of a dextran spacer was in a coupling arrangement by a method that utilized an oxidized carbohydrate linkage in the dextran to a solution phase hydrazide functional group via a Schiff’s base formation (23). The carbohydrates on the Ab were also oxidized to enable coupling to the hydrazide-activated support. In an opaque covered 1 L flask, 1.5 g of dextran (~40,000 mol wt, Sigma, St. Louis, MO), 2.5 g sodium periodate, NaIO₄ (Fisher, Pittsburgh, PA), and 75 ml ddH₂O were incubated while shaking at 150 rpm at room temperature for 1 h to oxidize the dextran (Fig. 2). All subsequent shaker incubations were performed at room temperature.

APTES beads (100 g of the 3-mm diam beads) were added to the flask containing dextran and NaIO₄ and allowed to react in complete darkness for 1 h on a low speed shaker set at 150 rpm (Fig. 3). The beads were then washed with ddH₂O at 10 times the present bead volume on a funnel. Sodium phosphate (75 ml of 50 mM Na₂PO₄•7 H₂O pH 7.2) 75 ml and 6.53 g adipic acid dihydrazide (Sigma, St. Louis, MO) were added to the beads in a clean flask, and the pH was checked to ensure that it was approximately 7.0. The covered flask was again placed on a low speed shaker for about 2 h (Fig. 4).

![Dextran (glucose polymer) oxidation](image)

**FIGURE 2. Oxidation of dextran for attachment to APTES beads.**
FIGURE 3. *Dextran spacer derivatized onto APTES glass beads.*

FIGURE 4. *Addition of adipic acid dihydrazide to the dextran-coated APTES beads.*
The 1° Ab solution was prepared by combining 5 mg of sodium periodate (meta) (Sigma, St. Louis, MO) with 500 µg of the 1° Ab in a volume of 1 ml with PBS pH 7.4 in an eppendorf tube. The tube was vortexed at high speed to dissolve the reagents and then covered with foil and placed on a low speed shaker for 30 min for the oxidation reaction to occur (Fig. 5). After the incubation, the 1° Ab solution was added to a Pierce KwikSep Dextran Plastic Desalting Column (Rockford, IL) and eluted with PBS pH 7.4. A volume of 9 ml of eluant was collected. A sample of the eluant was checked for the presence of protein by a spectrophotometric reading taken at 280 nm. A positive absorbance compared to PBS alone indicated that Ab was present.

After the beads finished shaking for the 2 h to immobilize the adipic acid dihydrazide, they were reduced with 100 mg of sodium borohydride, NaBH₄ (Fisher, Pittsburgh, PA) and allowed to incubate with shaking for another 30 min (Fig. 6). The beads were then washed on a filter funnel with 1 liter of ddH₂O, followed by 200 ml 50 mM Na₂PO₄•7 H₂O pH 7.2 containing 1 M NaCl, and finally 200 ml of PBS pH 7.4. After washing, the beads were placed in a clean flask and the 500 µg of oxidized Ab

![Antibody Oxidized Antibody](image)

**FIGURE 5.** Oxidation of the 1° Ab to be attached to the beads with sodium periodate.
solution was added. The flask was again covered and placed on a low speed shaker for 1 h (Fig. 7). After shaking, the beads were stored overnight at 4°C.

The Ab-coated beads were then reduced with 100 mg of NaBH₄ (Sigma, St. Louis, MO) and placed on a low speed shaker for 30 min (Fig. 8). When reduced, the beads were washed on filter funnel with 1 liter of 50 mM Na₂PO₄•7 H₂O pH 7.2, followed by 200 ml 50 mM Na₂PO₄•7 H₂O pH 7.2 containing 1 M NaCl, and 200 ml of 50 mM Tris buffer pH 7.2. The beads were then placed in a sealable sterile container, and 100 ml of filtered (0.45 µm filter) 2% BSA in Tris buffer pH 7.2 containing 0.02% sodium azide was added. The closed container of immobilized Ab-coated beads was then placed on a low speed (150 rpm) shaker for 2 h. After shaking, the
container of immobilized anti-Salmonella beads was stored at 4°C. Bead activity was confirmed through a Rapid Immuno-Capture (RIC) test as described subsequently.

![Diagram of Oxidized Antibody and Reduced Dextran Bead](image)

**FIGURE 7.** *Addition of the oxidized Ab to the reduced dextran bead.*
FIGURE 8. *Reduction of the bead solution to immobilize the Ab.*
BAM Detection of *Salmonella*

The procedure for *Salmonella* spp. detection was followed as described in the FDA Bacteriological Analytical Manual (BAM) that included pre-enrichment, isolation, and presumptive positive testing of shell eggs and chicken breast rinse (2).

In addition to the BAM procedure for *Salmonella* spp. detection, Violet Red Bile Agar (VRBA) was used in the BAM procedure for coliform enumeration. The method was slightly modified from the FDA approved BAM method (16). The raw food samples: shell eggs and chicken breasts, were prepared and blended as stated in the *Salmonella* spp. detection BAM method (2). Samples were diluted 1:10 in buffered peptone water and allowed to stand at room temperature for 15 min. The pH of the food dilution was checked to ensure that it was in the range 5.5 to 7.6 and adjusted with sterile 1 N NaOH or 1 N HCL to a pH of 7.0 if outside that range. Decimal dilutions including 1:10 and 1:1000 were also prepared in buffered peptone water. The three dilution levels were then distributed in 1 ml amounts onto duplicate sterile Petri plates (16). The VRBA was added as indicated in the coliform enumeration BAM procedure (16). Incubation and enumeration were also performed as stated in the coliform enumeration BAM procedure (9). The coliform enumeration only occurred in the *Salmonella* spp. detection experiments with raw food samples.

**Rapid Immuno-Capture (RIC)**

**RIC methods**

Bacteria were grown as stated previously and a stock solution was prepared in the
respective buffer or food sample. Serial dilutions of *Salmonella enteritidis* ATCC 8326 were made in 50-ml amounts in CFU/ml. Sample dilutions included the following: $4 \times 10^1$ CFU/ml, $4 \times 10^2$ CFU/ml, $4 \times 10^3$ CFU/ml, $4 \times 10^4$ CFU/ml, $4 \times 10^5$ CFU/ml, and $4 \times 10^6$ CFU/ml. The food samples were a mixture of 25 ml (or g) of food with 25 ml of 50 mM Tris pH 7.2 to equal 50 ml total volumes.

Sixty immobilized Ab beads specific for *Salmonella* were washed with $2 \times 25$ ml of 50 mM Tris pH 7.2 on a Buchner funnel. The beads were placed in the 50 ml of sample and incubated for 20 min at room temperature on a 150 rpm shaker.

The beads were then washed with $4 \times 25$ ml of 50 mM Tris pH 7.2 on a Buchner funnel, and placed in a 15-ml tube. Every experiment had a negative bead control in which 60 beads were washed and then were incubated in 50 ml 50 mM Tris pH 7.2 as described above.

After the Ab-bead capture, $3.0 \times 10^{-7}$ M of $2^\circ$ Ab solution (diluted in 50 mM Tris pH 7.2) was added to the 60 beads in a 15-ml tube. The mixture was then incubated at room temperature for 20 min at 150 rpm. After incubation, the beads were transferred to a Buchner filter and washed with $4 \times 50$ ml 50 mM Tris pH 7.2. The beads were then placed in a clean 15 ml test tube and $4.8 \times 10^{-10}$ M of $3^\circ$ Ab solution (diluted in 50 mM Tris pH 7.2) was added. The samples were again incubated at room temperature for 20 min at 150 rpm. The beads were then transferred to a Buchner filter and washed with $4 \times 50$ ml 50 mM Tris pH 7.2. The beads were then placed in a 48-well plate (10 beads/well). In the dark, 600 µl of pNPP substrate, prepared as in the Ab ELISA titer procedure, was added to each well containing the beads. The plate was wrapped in foil to protect it from light and incubated at room temperature for 20 min at 150 rpm. Substrate
from each well, 250 µl, was transferred to an empty adjacent well. Each RIC experiment had a negative bead control, which included the 2° and 3° Abs and substrate without the addition of Salmonella. The plate was then read on a BioAssay 7000 plate reader (Perkin-Elmer, Norwalk, CT) at 405 nm using the HtSoft™ program. Absorbance values from each of the dilution levels were compared to either the negative bead control absorbance values or the negative food sample control using Student’s t-test. The most dilute sample with a significant p-value \( \alpha = 0.05 \) was indicative of the detection limit.

All replications were evaluated separately due to testing variability between experiments.

**Sample preparation for detection limit using RIC in buffer, chicken rinse, and eggs**

Tris buffer, 50 mM pH 7.2, along with boneless skinless chicken breasts and eggs (purchased locally) were used to determine the detection limits of the RIC and Ab-capture/PCR methods.

The RIC detection limit from Ab-capture of *S. enteritidis* ATCC 8326 in 50 mM Tris pH 7.2 buffer was determined using 50 ml of four dilution levels: \( 4 \times 10^1 \) CFU/ml, \( 4 \times 10^2 \) CFU/ml, \( 4 \times 10^4 \) CFU/ml, and \( 4 \times 10^6 \) CFU/ml. The test was replicated three times, and each replication contained a RIC negative bead control and the four dilution levels. The negative bead control and each dilution level had six RIC absorbance readings in the plate reader. Four of the readings were used for evaluation.

Boneless skinless chicken breasts (purchased locally) used for determining detection limits were cooked to an internal temperature of 74°C to kill any prior *Salmonella* on the chicken. Samples were stored at -20°C until ready for use. The cooked chicken breast was placed in a filtered stomacher bag with buffered peptone
water at a 1-to-1 weight to volume ratio. The chicken breast and buffered peptone water were then stomached for 15 min. The rinse from the sample was used for both RIC and antibody capture/PCR testing. Dilutions of *S. enteritidis* were added to the rinse for all samples except the controls. The FDA approved BAM procedure for *Salmonella* spp. detection was also performed on the chicken rinse with no artificial inoculation of *Salmonella* to ensure no *Salmonella* was present in the cooked chicken samples (2).

The RIC detection limit from Ab bead static capture of *S. enteritidis* ATCC 8326 in cooked chicken rinse samples was determined using the preparation technique described previously and four dilution levels in 50-ml amounts. These dilutions included: $4 \times 10^3$ CFU/ml, $4 \times 10^4$ CFU/ml, $4 \times 10^5$ CFU/ml, and $4 \times 10^6$ CFU/ml. The test was replicated three times, and each replication contained a RIC negative bead control, a chicken control, and the four dilution levels. The chicken rinse controls were the Ab beads exposed to chicken rinse with no artificial inoculation of *Salmonella* during the static capture procedure. The controls and each dilution level had six RIC absorbance readings in the plate reader. Five of the readings were used for evaluation. The detection limit was determined by comparing each dilution level to the chicken control using Student’s t-test.

The egg samples used for determining detection limit were externally washed and soaked as stated for shell egg preparation in BAM (2). The eggs were aseptically cracked and homogenized for use (2). To ensure that no *Salmonella* was present in the original egg samples, the BAM method was performed (2).

The egg sample RIC detection limit was determined using the egg preparation as described above along with the same replications and dilution levels as stated in the RIC
detection limit from Ab-capture of *S. enteritidis* ATCC 8326 in cooked chicken rinse. Each replication contained a RIC negative bead control and an egg control. The egg control was the Ab beads exposed to egg with no artificial inoculation of *Salmonella* during the static capture procedure. As described above, the controls and dilution levels each had six RIC absorbance readings in the plate reader. Five of the readings were used for evaluation. The detection limit was determined by comparing each dilution level to the egg control using Student’s t-test.

**Immuno-Capture with PCR Identification**

**Immuno-capture with PCR methods**

For PCR analysis, a pure culture of *Salmonella* was used as a positive control along with the Ab-captured *Salmonella* samples. For the pure culture, one milliliter of sterile ddH₂O was added to the pelleted cells (10⁸ CFU/ml) that had been washed twice with 50 mM Tris buffer pH 7.2. Then the pelleted cells in sterile ddH₂O were vortexed to resuspend the bacteria. For the Ab-captured *Salmonella* samples, *S. enteritidis* samples were prepared as described for RIC and incubate with 60 beads for 20 min. The beads were washed with Tris pH 7.2 and 600 µl of sterile ddH₂O (enough to cover 60 beads) was added. The samples were vortexed for 20 s. Both pure culture and beads were boiled at 96°C for 10 min to lyse the cells. After lysis, the solutions were put on ice until use in PCR.

Amplification mixtures were prepared using puReTaq™ Ready-To-Go Polymerase Chain Reaction Beads obtained from Amersham Biosciences (Buckinghamshire, England). These beads provided the necessary reagents (stabilizers,
BSA, 200 mM of each dNTP, 10 mM Tris-HCl pH 9.0, 50 mM KCl, and 1.5 mM MgCl$_2$, ~2.5 units of puReTaq™ DNA polymerase) to carry out a 25-µl PCR reaction. The negative PCR control contained the puReTaq™ Ready-To-Go PCR Bead, 23 µl of sterile ddH$_2$O, and 1 µl (20 pmol solutions) of each primer specific for Salmonella spp. All other samples contained 18 µl of sterile ddH$_2$O, 1 µl each of primer, and 5 µl of the cell lysis solution as template DNA to equal a reaction volume of 25 µl. Three controls were run along the test samples: a negative PCR control, a negative bead control, and a positive PCR control. The negative PCR control, which only contained the puReTaq™ Ready-To-Go PCR Bead components, primers, and sterile ddH$_2$O, showed that there were no PCR reaction contaminants. To show that there was no experimental cross contamination, the negative bead control containing puReTaq™ Ready-To-Go PCR Bead components, primers, sterile ddH$_2$O, and solution from the bead sample with no bacteria capture was used. The positive PCR control, containing a pure culture of the bacteria Salmonella at a $10^8$ CFU/ml dilution in addition to the same components as the negative PCR control, was used to show optimum PCR conditions.

Primers for the reaction were specific to the invA gene fragment in Salmonella, and the segment chosen was 284 base pairs (bp) long (31). Primer 139 5’-GTGAAATTATCGCCACGTTGAGCAA- 3’ and primer 141 5’-TCATCGCACC TCAAAGGAACC-3’ were obtained from Qiagen Operon (Alameda, CA) (13) (Appendix B).

The PCR procedure was modified from Scholz et al. (36). The PCR took place in an automated temperature control PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA). Initial denaturation was set at 95°C for 5 min, followed by 35
amplification cycles each consisting of denaturation at 95°C for 30 s, annealing of the primers to the target DNA at 64°C for 30 s, and extension at 72°C for 30 s. The final extension was at 72°C for 7 min. Upon completion, the samples were held at 4°C until analysis by gel electrophoresis.

Agarose gels were prepared from 1.8% agarose in 1 × Tris-borate ehtylenediaminetetraacetate (Tris-borate EDTA) buffer pH 8.3. A 100 bp ladder (Promega Corporation, Madison, WI) was used as the marker. To the 25 µl PCR products, 1.5 µl of 10 x Blue Juice™ Gel Loading Buffer from Invitrogen Corporation (Carlsbad, CA) was added to each sample. The samples ran through the 8-cm gel in the apparatus for 1 h at 80 volts. Ethidium bromide was added to the gel to intercalate through the DNA for visualization via ultraviolet light.

Sample preparation for detection limit using immuno-capture with PCR in buffer, chicken rinse, and eggs

The PCR detection limit from Ab-capture of *S. enteritidis* ATCC 8326 in 50 mM Tris buffer pH 7.2, chicken rinse samples, and shell egg samples was determined using 50 ml amounts of 4 dilution levels chosen from preliminary data. The test was replicated 3 times and the dilution levels used for the Tris buffer pH 7.2, $4 \times 10^1$ CFU/ml, $4 \times 10^2$ CFU/ml, $4 \times 10^4$ CFU/ml, and $4 \times 10^6$ CFU/ml, and the dilution levels used for the chicken rinse and shell egg samples, $4 \times 10^3$ to $4 \times 10^6$ CFU/ml, were duplicated for each replicate. In addition to the duplicate bacterial static capture test sample dilutions, each PCR test also contained a negative PCR control, duplicate negative bead controls, and a positive PCR control. The food samples also had negative food controls. The chicken rinse and shell egg controls were the Ab beads exposed to chicken rinse or shell
egg with no artificial inoculation of *Salmonella* during the static capture procedure. Samples for Tris buffer pH 7.2, chicken rinse, and shell egg were prepared as described previously. The BAM *Salmonella* detection procedure was also performed to verify no *Salmonella* was present before artificial inoculation (2).

**Detection of *Salmonella* in Raw Food Samples**

Raw food samples were purchased from the same local grocery on the same day. The three egg brands had “sell by” dates of September 22, October 25, and October 10. For *Salmonella* detection in raw food samples, the shell egg was prepared as described previously, and the boneless skinless chicken breast was untreated (uncooked). The raw chicken rinse sample preparation was the same as the cooked chicken breast rinse sample as stated above. Three brands of boneless skinless chicken breast and three brands of shell eggs were tested for the presence of *Salmonella*. No additional *Salmonella* was added to the samples.

The raw food samples: boneless skinless chicken breast rinse and shell eggs were tested for *Salmonella* using the Ab-capture method followed by PCR. Both the chicken and egg had three different brands chosen from a local grocery. Of each raw chicken breast brand, three breasts were used for sampling. Duplicate samples of each breast were tested. The shell egg brands each had six eggs pooled together for duplicate sampling of each brand. Controls for the test included, a negative PCR control, duplicate negative bead controls, and a positive PCR control.

The BAM methods for *Salmonella* spp. detection and for coliform enumeration were performed on each of the three breasts in each raw chicken brand and were
performed on the three shell egg brands. VRBA was used to enumerate the coliforms present, including *Salmonella*, in the raw food samples: chicken breasts and shell eggs (16).

All samples were in 50-ml total volumes (CFU/50ml). The food samples were a mixture of 25 ml (or g) of food with 25 ml of 50 mM Tris pH 7.2 and assayed using the Ab-capture method followed by PCR as described previously.
RESULTS AND DISCUSSION

Ab ELISA Titer

The Ab titer of the 1° Ab, goat anti-Salmonella, and the 3° Ab, anti-goat IgG conjugated to AP, against S. enteritidis ATCC 8326 resulted in optimum concentrations at $3.6 \times 10^{-7}$ M and $4.8 \times 10^{-10}$ M, respectively (Fig. 9). In the same respect, the 2° Ab, rabbit anti-Salmonella, and the 3° Ab, anti-rabbit IgG conjugated to AP, against S. enteritidis ATCC 8326. The signal-to-noise ratio was calculated by dividing the average absorbance in samples to the negative control.

FIGURE 9. ELISA assay to determine the optimum concentration of 1° Ab, goat anti-Salmonella, and 3° Ab, anti-goat IgG conjugated to AP, against S. enteritidis ATCC 8326. The signal-to-noise ratio was calculated by dividing the average absorbance in samples to the negative control.
enteritidis ATCC 8326 resulted in optimum dilution levels at $3.0 \times 10^{-7}$ M and $4.8 \times 10^{-10}$ M (Fig. 10). This combination resulted in a signal to noise value of approximately 15. From these results, the greatest signal-to-noise values were observed with the highest concentration of $3^\circ$ Abs which represented a 1:16,500 dilution of the purchased $3^\circ$ Ab solutions. The $1^\circ$ and $2^\circ$ antibodies were optimum at the lower concentrations of $3.6 \times 10^{-7}$ M and $3.0 \times 10^{-7}$ M, respectively, which represented a 1:100 dilution of the purchase Ab solutions.

FIGURE 10. ELISA assay to determine the optimum concentration of $2^\circ$ Ab, rabbit anti-Salmonella, and $3^\circ$ Ab, anti-rabbit IgG conjugated to AP, against S. enteritidis ATCC 8326. The signal-to-noise ratio was calculated by dividing the average absorbance in samples to the negative control.
Anti-Salmonella Cross-Reactivity ELISA

*S. enteritidis* was chosen as the *Salmonella* testing strain due to the known binding and cross-reactivity of the 1° Ab, goat anti-*Salmonella* (Fig. A-1). The cross-reactivity chart provided by OEM (Toms River, NJ) depicts the ability of the 1° Ab to not only bind *S. enteritidis* but to also bind many other *Salmonella* strains (Appendix A).

An ELISA assay using limited concentrations of the 1° and 2° Abs with a fixed concentration of 3° Ab (4.8 x 10^{-10} M) was done to confirm the capture of all 14 *S. enteritidis* strains. Each of the 14 *S. enteritidis* strains, except for ATCC 31194, had optimum 1° Ab levels at 3.6 x 10^{-7} M. *S. enteritidis* ATCC 31194 had an optimum 1° Ab level at 3.6 x 10^{-6} M (Fig. 11). This Ab cross-reactivity shows that the 1° Ab attached to the beads to make anti-*Salmonella* beads will capture different *S. enteritidis* strains. In the cross-reactivity of the 14 *S. enteritidis* strains against the 2° Ab, all of the strains, except ATCC 13314 and 49220, had optimum 2° Ab levels at 3.0 x 10^{-7} M (Fig. 12). The

**FIGURE 11.** *ELISA assay to confirm the binding of the 1° Ab, goat anti-Salmonella, at three dilutions (■ 3.6 x 10^{-6} M, □ 3.6 x 10^{-7} M, ▪ 3.6 x 10^{-8} M), to 14 strains of *S. enteritidis*. Anti-goat IgG was the tertiary Ab used at a concentration of 4.8 x 10^{-10} M. The signal-to-noise ratio was calculated by dividing the average absorbance in samples to the negative control (ELISA without bacteria).**
FIGURE 12. Cross-reactivity of 14 S. enteritidis strains with three dilutions of 2° rabbit anti-Salmonella Ab (3.0 \times 10^{-6} M, 3.0 \times 10^{-7} M, 3.0 \times 10^{-8} M). Anti-rabbit IgG was the 3° Ab used at a concentration of 4.8 \times 10^{-10} M. The signal-to-noise ratio was calculated by dividing the average absorbance in samples to the negative control (ELISA without bacteria).

other two strains were optimum at 3.0 \times 10^{-6} M 2° Ab (Fig. 12). The optimum 2° Ab level of 3.0 \times 10^{-7} M was used for all of the RIC experiments performed. Because the signal to noise ratio was fairly similar among S. enteritidis strains, S. enteritidis ATCC 8326 was chosen as a representative strain for continued study with the Salmonella detection assays. The signal-to-noise ratio was calculated by dividing the absorbance with the negative control (a well with no bacteria adsorbed).

**Rapid Immuno-Capture (RIC)**

The RIC procedure that used S. enteritidis ATCC 8326 in 50 mM Tris pH 7.2 buffer successfully detected S. enteritidis in samples at concentrations as low as 4 \times 10^{1} CFU/ml (Table 1). A pairwise comparison was made between the absorbance values at each dilution and the negative controls and each bacteria level was significantly different from the negative control (p < 0.05) in each of the three replications.

The RIC procedure for the cooked chicken samples was successful at detecting
Table 1. *RIC detection limit of S. enteritidis ATCC 8326 serial dilutions in 50 mM Tris pH 7.2 shown by significant p-values (α = 0.05).*

<table>
<thead>
<tr>
<th>Artificial inoculation level (CFU/ml)</th>
<th>Absorbance at 405 nm</th>
<th>Mean absorbance ± SD</th>
<th>p-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 x 10⁶</td>
<td>0.212</td>
<td>0.171</td>
<td>0.157</td>
</tr>
<tr>
<td>4 x 10⁴</td>
<td>0.153</td>
<td>0.163</td>
<td>0.156</td>
</tr>
<tr>
<td>4 x 10²</td>
<td>0.158</td>
<td>0.156</td>
<td>0.164</td>
</tr>
<tr>
<td>4 x 10¹</td>
<td>0.194</td>
<td>0.170</td>
<td>0.156</td>
</tr>
<tr>
<td>Negative bead control</td>
<td>0.131</td>
<td>0.113</td>
<td>0.107</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 x 10⁶</td>
<td>0.237</td>
<td>0.199</td>
<td>0.158</td>
</tr>
<tr>
<td>4 x 10⁴</td>
<td>0.220</td>
<td>0.198</td>
<td>0.155</td>
</tr>
<tr>
<td>4 x 10²</td>
<td>0.204</td>
<td>0.191</td>
<td>0.170</td>
</tr>
<tr>
<td>4 x 10¹</td>
<td>0.252</td>
<td>0.165</td>
<td>0.168</td>
</tr>
<tr>
<td>Negative bead control</td>
<td>0.079</td>
<td>0.071</td>
<td>0.073</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 x 10⁶</td>
<td>0.294</td>
<td>0.232</td>
<td>0.177</td>
</tr>
<tr>
<td>4 x 10⁴</td>
<td>0.244</td>
<td>0.229</td>
<td>0.173</td>
</tr>
<tr>
<td>4 x 10²</td>
<td>0.250</td>
<td>0.202</td>
<td>0.149</td>
</tr>
<tr>
<td>4 x 10¹</td>
<td>0.220</td>
<td>0.211</td>
<td>0.143</td>
</tr>
<tr>
<td>Negative bead control</td>
<td>0.191</td>
<td>0.148</td>
<td>0.124</td>
</tr>
</tbody>
</table>

a pairwise comparison between the dilution level and the negative bead control

*S. enteritidis* in samples at concentrations as low as 4 x 10³ CFU/ml (Table 2). This detection limit without a pre-enrichment step is noteworthy in the realm of antibody tests. Currently antibody tests, such as the 1-2 Test®, have a detection limit of 1 to 5 CFU/25 g sample, but only after an enrichment step (45).

The RIC assay using shell eggs was successful at detecting *S. enteritidis* in samples at bacteria concentrations as low as 4 x 10³ CFU/ml in two of the three replicates and successful at detecting bacteria as low as 4 x 10⁴ CFU/ml in one of the three replicates (Table 3). The lowest concentration tested was 4 x 10³ CFU/ml. Because eggs contain a number of proteins and enzymes, it is quite possible that adsorbed egg components nonspecifically attached to the 1° Ab blocking the site for *Salmonella* attachment. This would lead to the higher detection limit.
Table 2. RIC detection limit of *S. enteritidis* ATCC 8326 serial dilutions in rinse from cooked boneless, skinless chicken breasts shown by significant p-values ($\alpha = 0.05$).

<table>
<thead>
<tr>
<th>Artificial inoculation level (CFU/ml)</th>
<th>Absorbance at 405 nm</th>
<th>Mean absorbance ± SD</th>
<th>p-value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$4 \times 10^6$</td>
<td>0.170</td>
<td>0.135</td>
<td>0.108</td>
</tr>
<tr>
<td>$4 \times 10^5$</td>
<td>0.177</td>
<td>0.149</td>
<td>0.116</td>
</tr>
<tr>
<td>$4 \times 10^4$</td>
<td>0.164</td>
<td>0.141</td>
<td>0.129</td>
</tr>
<tr>
<td>$4 \times 10^3$</td>
<td>0.099</td>
<td>0.131</td>
<td>0.129</td>
</tr>
<tr>
<td>Chicken control</td>
<td>0.067</td>
<td>0.069</td>
<td>0.067</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$4 \times 10^6$</td>
<td>0.278</td>
<td>0.221</td>
<td>0.169</td>
</tr>
<tr>
<td>$4 \times 10^5$</td>
<td>0.226</td>
<td>0.197</td>
<td>0.144</td>
</tr>
<tr>
<td>$4 \times 10^4$</td>
<td>0.226</td>
<td>0.185</td>
<td>0.127</td>
</tr>
<tr>
<td>$4 \times 10^3$</td>
<td>0.289</td>
<td>0.193</td>
<td>0.188</td>
</tr>
<tr>
<td>Chicken control</td>
<td>0.071</td>
<td>0.073</td>
<td>0.070</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$4 \times 10^6$</td>
<td>0.226</td>
<td>0.179</td>
<td>0.137</td>
</tr>
<tr>
<td>$4 \times 10^5$</td>
<td>0.184</td>
<td>0.161</td>
<td>0.120</td>
</tr>
<tr>
<td>$4 \times 10^4$</td>
<td>0.197</td>
<td>0.154</td>
<td>0.113</td>
</tr>
<tr>
<td>$4 \times 10^3$</td>
<td>0.258</td>
<td>0.160</td>
<td>0.163</td>
</tr>
<tr>
<td>Chicken control</td>
<td>0.071</td>
<td>0.073</td>
<td>0.073</td>
</tr>
</tbody>
</table>

$^a$pairwise comparison between the dilution level and the chicken control

Table 3. RIC detection limit of *S. enteritidis* ATCC 8326 serial dilutions in shell eggs shown by significant p-values ($\alpha = 0.05$).

<table>
<thead>
<tr>
<th>Artificial inoculation level (CFU/ml)</th>
<th>Absorbance at 405 nm</th>
<th>Mean absorbance ± SD</th>
<th>p-value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$4 \times 10^6$</td>
<td>0.335</td>
<td>0.291</td>
<td>0.216</td>
</tr>
<tr>
<td>$4 \times 10^5$</td>
<td>0.319</td>
<td>0.277</td>
<td>0.226</td>
</tr>
<tr>
<td>$4 \times 10^4$</td>
<td>0.290</td>
<td>0.275</td>
<td>0.206</td>
</tr>
<tr>
<td>$4 \times 10^3$</td>
<td>0.392</td>
<td>0.259</td>
<td>0.274</td>
</tr>
<tr>
<td>Egg control</td>
<td>0.191</td>
<td>0.149</td>
<td>0.142</td>
</tr>
<tr>
<td>2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>$4 \times 10^6$</td>
<td>0.492</td>
<td>0.423</td>
<td>0.359</td>
</tr>
<tr>
<td>$4 \times 10^5$</td>
<td>0.441</td>
<td>0.422</td>
<td>0.329</td>
</tr>
<tr>
<td>$4 \times 10^4$</td>
<td>0.395</td>
<td>0.356</td>
<td>0.247</td>
</tr>
<tr>
<td>$4 \times 10^3$</td>
<td>0.498</td>
<td>0.356</td>
<td>0.386</td>
</tr>
<tr>
<td>Egg control</td>
<td>0.123</td>
<td>0.125</td>
<td>0.125</td>
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<tr>
<td>3</td>
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<td></td>
<td></td>
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<tr>
<td>$4 \times 10^6$</td>
<td>0.109</td>
<td>0.106</td>
<td>0.102</td>
</tr>
<tr>
<td>$4 \times 10^5$</td>
<td>0.085</td>
<td>0.082</td>
<td>0.081</td>
</tr>
<tr>
<td>$4 \times 10^4$</td>
<td>0.089</td>
<td>0.079</td>
<td>0.078</td>
</tr>
<tr>
<td>$4 \times 10^3$</td>
<td>0.101</td>
<td>0.079</td>
<td>0.082</td>
</tr>
<tr>
<td>Egg control</td>
<td>0.069</td>
<td>0.067</td>
<td>0.068</td>
</tr>
</tbody>
</table>

$^a$pairwise comparison between the dilution level and the egg control
The anti-Salmonella bead static capture of *S. enteritidis* ATCC 8326 in 50 mM Tris pH 7.2 followed by PCR showed the identification of *Salmonella* in samples with concentrations as low as $4 \times 10^2$ CFU/ml (Table 4). A similar study which used immunomagnetic separation (IMS) and multiplex PCR in phosphate-buffered saline solution had a *Salmonella* detection limit of $10^0$ CFU/ml after a 20-h enrichment (21).

The sensitivity of the immuno-capture with PCR assay for the artificially inoculated cooked chicken breast rinse sample was less than that observed with buffer or the RIC with chicken breast rinse. The anti-Salmonella bead static capture of *S. enteritidis* ATCC 8326 followed by PCR identified *S. enteritidis* in samples containing $4 \times 10^5$ CFU/ml and $4 \times 10^6$ CFU/ml (Table 4). Since the sensitivity with PCR identification was less than

<table>
<thead>
<tr>
<th>Bacteria suspension medium</th>
<th>Artificial inoculation level (CFU/ml)</th>
<th>PCR product formed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris pH 7.2</td>
<td>$10^6$, $10^4$, $10^2$, $10^1$</td>
<td>+</td>
</tr>
<tr>
<td>Rinse (25 ml) from boneless, skinless cooked chicken breast</td>
<td>$10^6$, $10^5$, $10^4$, $10^3$</td>
<td>+, -</td>
</tr>
<tr>
<td>Shell egg (25 g)</td>
<td>$10^6$, $10^5$, $10^4$, $10^3$</td>
<td>+, -</td>
</tr>
</tbody>
</table>

*average of 3 replications
that observed with the RIC method, presumably the bacteria were immuno-captured onto the beads, but the limitation in the PCR assay reduced sensitivity. Alternatively, unknown compounds in the rinse inhibited the PCR reaction. This is reasonable, considering the RIC assay in Tris pH 7.2 was at least $10^1$ CFU/ml. Concentrating the DNA and the use of real-time PCR may improve the sensitivity. Bennet et al. reported a detection limit of $<50$ cells per 25 g of chicken meat using the BAX™ system (5). This detection limit was only obtained after a 20-h pre-enrichment period (5). Another detection limit of $1-5 \times 10^3$ CFU was reported for minced meat, but again only after a pre-enrichment step (22). The immuno-capture PCR assay used no broth enrichment step and only took about 3 h to perform.

The inoculated shell egg antibody static capture followed by PCR identified *S. enteritidis* only in samples containing at least $4 \times 10^6$ CFU/ml (Table 4). The significant detection decrease compared to that of pure culture may be due to the limitations associated with end-point PCR or the possibility of a PCR inhibitor in the cell lysis solution from the egg sample. Jenfková et al. (22) reported a PCR sensitivity of 1-5 CFU per 25-g egg sample, but only after a 16-h pre-enrichment homogenization step and an immunomagnetic separation step.

**Detection of *Salmonella* in Raw Food Samples**

Anti-*Salmonella* bead static capture followed by PCR did not detect *Salmonella* in either of the raw food samples: chicken breast and shell egg. All of the chicken breast samples showed positive BAM results for *Salmonella* spp. detection and all had bacterial coliform counts of approximately $10^5$ CFU/ml (Table 5).
Table 5. PCR, VRBA, and BAM Salmonella spp. detection results from raw food samples: rinse from boneless, skinless chicken breasts and shell eggs.

<table>
<thead>
<tr>
<th>Food type</th>
<th>Sample</th>
<th>PCR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>VRBA (CFU/ml)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BAM&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rinse (25 ml) from boneless, skinless chicken breast</td>
<td>Brand 1, breast 1</td>
<td>-</td>
<td>$10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Brand 1, breast 2</td>
<td>-</td>
<td>$10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Brand 1, breast 3</td>
<td>-</td>
<td>$10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Brand 2, breast 1</td>
<td>-</td>
<td>$10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Brand 2, breast 2</td>
<td>-</td>
<td>$10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Brand 2, breast 3</td>
<td>-</td>
<td>$10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Brand 3, breast 1</td>
<td>-</td>
<td>$10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Brand 3, breast 2</td>
<td>-</td>
<td>$10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Brand 3, breast 3</td>
<td>-</td>
<td>$10^5$</td>
<td>+</td>
</tr>
<tr>
<td>Shell egg (25 g)</td>
<td>Brand 1</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Brand 2</td>
<td>-</td>
<td>28</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Brand 3</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>immuno-capture followed by PCR  
<sup>b</sup>Sample coliform counts after incubation on VRBA  
<sup>c</sup>FDA BAM Salmonella detection

The VRBA coliform counts for the raw boneless skinless chicken breast rinses ranged from $\sim 1 \times 10^5$ CFU/ml to $\sim 6 \times 10^5$ CFU/ml. The boneless skinless chicken breast brands, though purchased the same day, differed in appearance from a light pink color typical of fresh chicken for one brand to a green/grayish shiny color for another brand. The similar numerical growth of these coliforms was probably due to the competitive nature of the organisms and the supply of nutrients (Table 5). It must be noted that VRBA contains a neutral red pH indicator to reveal the fermentation of lactose (16). A contemporary definition states that a typical Salmonella isolate would produce gas and (11). The FDA approved VRBA for coliform enumeration used in this research showed acid in a glucose-based media rather than lactose (11). Many other studies have shown some Salmonella to be lactose positive that competitive organisms and possibly some atypical Salmonella were present on the food.
Because raw chicken contains many other bacteria including *Campylobacter*, and the VRBA is known for its enumeration of non-*Salmonella* coliform, we can assume that the bacterial load of *Salmonella* was under $10^5$ CFU/ml which was less than the detectable limit for the test. Not only may the number of *Salmonella* have been less than $10^5$ CFU/ml, but due to the presence of other coliforms, the *Salmonella* bead Ab binding sites used for capture could have not only bound *Salmonella* but also other bacteria, such as *Campylobacter* and *E. coli* (Appendix A). The presence of competitive organisms in samples reduced the capture of the target organism, *Salmonella*, which in turn decreased the detection ability of the PCR system. The BAM procedure was used as a comparison of the traditional culture method to the rapid detection method using Ab-capture and PCR.

With respect to the eggs, the results from static capture followed by PCR coincided with the BAM *Salmonella* spp. detection results. No *Salmonella* was detected in either test. Egg brand 2 did have results for the VRBA test of 28 CFU/ml (Table 5). Again the VRBA tests for coliforms including *Salmonella*. Either the level of *Salmonella* in the sample was too low for the BAM to detect or no *Salmonella* was in the sample. If it was the former, the lack of detection could have been from iron deficiency do to the ovotransferrin in the egg white which chelates iron causing inhibition of *Salmonella* growth (4). This was probably not the case because in the BAM procedure for *Salmonella* spp. detection, the pre-enrichment broth contains iron. It has been found that iron supplementation could enhance the growth of *Salmonella* in egg contents as long as it was in the optimal range (9).
CONCLUSIONS

Thus far, research using immuno separation methods followed by PCR to detect *Salmonella* in food have used at least one enrichment step before or after the immunocapture (10, 15, 21, 32). This allowed one study to detect 3 CFU/ml of *Salmonella* in food in 20 h (15). By selective capture with anti-*Salmonella* beads, the need for time consuming broth enrichment is eliminated. A presumptive positive test for *Salmonella* can be performed in less than 2 h with the RIC procedure, and a confirmed positive test can be performed in about 3 h using the static capture followed by PCR. The RIC assay can detect *Salmonella* in buffer at concentrations as low as $4 \times 10^1$ CFU/ml and can detect *Salmonella* in chicken rinse and shell eggs at concentrations as low as $4 \times 10^3$ CFU/ml. The Ab capture with PCR can identify *Salmonella* in buffer at concentrations as low as $4 \times 10^2$ CFU/ml, in chicken at concentrations as low as $4 \times 10^5$ CFU/ml and in shell egg at bacteria concentrations of $4 \times 10^6$ CFU/ml.

With its ability to give confirmed results, immuno-capture with PCR assay could one day be applied to food industry HACCP plans for detection of foodborne pathogens. After optimization and standardization, the immuno-capture with PCR assay could be an easy use detection and identification system that would take an h to receive confirmatory results, especially with the use of real time PCR. The time saved from this assay could not only save money through fewer man-hours for performing the detection procedures, but it could save millions of dollars from a recall due to late detection of foodborne pathogen contamination.

Future research in the area of antibody bead static capture with PCR and Rapid Immuno-Capture could be to improve the sensitivity and detection limit of the assays.
Methods for improvement could focus on concentrating the DNA after cell lysis or focus on increasing the DNA amplifications by doubling the PCR primer amounts. Real time PCR could also be applied to the immuno-capture PCR assay to decrease the detection time even more. Because of the cross-reactivity of the *Salmonella* bead antibodies, the immuno-capture PCR assay could be applied to a multiplex system to capture and identify other bacteria such as *E. coli* and *Campylobacter* at the same time. These rapid immuno-detection assays could be applied to many other foodborne pathogens.
REFERENCES


APPENDICES
Appendix A.

Goat Anti-Salmonella Bead Activity
Tests and Cross-reactivity
Anti-Salmonella Bead Activity Tests

After every batch of anti-Salmonella beads was produced, a sample of the beads underwent an activity test. The tests were a miniature version of the Rapid Immuno-Capture (RIC) assay. Instead of 60 beads, 10 beads were tested in triplicate. The triplicate samples of 10 beads each were incubated in 1 ml of $10^8$ CFU/ml Salmonella in 50 mM Tris pH 7.2. The 2° and 3° Abs were applied as described in the RIC procedure. A signal to noise ratio (absorbance at 405 nm of beads in bacterial dilution divided by absorbance at 405 nm of the RIC negative bead control) above 1 was considered positive for activity, and the beads were used for further study in the static capture followed by PCR and the RIC experiments. The five batches used for this study all had positive activity with an average signal to noise ratio of 1.255 and a maximum variance of 0.491 among the batches (Table A-1).

Table A-1. Anti-Salmonella Ab bead activity RIC tests on five bead batches used for the static Ab capture. The signal to noise ratio was the absorbance at 405 nm of a bacterial dilution divided by the absorbance at 405 nm of the RIC negative bead control.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Signal to noise ratioa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.746</td>
</tr>
<tr>
<td>2</td>
<td>1.137</td>
</tr>
<tr>
<td>3</td>
<td>1.090</td>
</tr>
<tr>
<td>4</td>
<td>1.167</td>
</tr>
<tr>
<td>5</td>
<td>1.135</td>
</tr>
</tbody>
</table>

aaverage of triplicate values in one replication

Goat Anti-Salmonella Cross-Reactivity

The 1° and 2° Ab suppliers for this study, OEM, provided a figure showing the cross-reactivity of the 1° goat anti-Salmonella Ab with various bacteria (Figure A-1). The Ab is very cross-reactive and not specific for Salmonella. Because of these
Figure A-1. Cross-reactivity of primary goat anti-Salmonella antibody.

Ab crossreactivity study: OEM goat anti Salmonella
4/4/00 BD

OEM goat anti Salmonella

Bacteria

O.D.
characteristics, the 2° Ab, rabbit anti-Salmonella, used in the RIC assay was chosen because of its specificity for *Salmonella* “O” and “H” antigens.
Appendix B.

Primer Selection
Two primer sets chosen from literature, ST11/ST15 and 139/141, were used for *Salmonella* DNA amplification in this study. The ST11/ST15 primer set was shown in Aabo et al. (1) to detect 144 of 146 *Salmonella* strains and displayed no product for non-*Salmonella* strains tested. This primer set had a 429 base pair (bp) PCR product by amplification of a random fragment. The 139/141 primer set was shown in Rahn et al. (3) to yield a very specific amplification product of 284 bp for only *Salmonella* strains by amplification of the *invA* gene fragment.

For both primer sets, the guanine and cytosine percentage (GC%) was near or above 50%. The guanine and cytosine percentage (GC%) should be at least 50% because these DNA bases have 3 hydrogen bonds which allow for a higher melting temperature rather than the adenine and thyamine bases which only have 2 hydrogen bonds (Table B-1). From the primary secondary structures and the ratings, the 139/141 primer set appears to be a better binding primer with less hairpins and dimers than the ST11/ST15 primer set. Secondary primer structures, such as hairpins, dimers, and palindromes, inhibit proper binding of the primer to the DNA amplification sequence. Primer set

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Sequence 5' to 3'</th>
<th>GC %</th>
<th>Number of Hairpins</th>
<th>Number of Dimers</th>
<th>Number of Palindromes</th>
<th>Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST11</td>
<td>AGCCAACCATTTGCTAATTTGGCGCA</td>
<td>72.33</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>ST15</td>
<td>GGTAGAAATTCCACGCGGTACTG</td>
<td>65.32</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>89</td>
</tr>
<tr>
<td>139</td>
<td>GTGAAATTATCGCCACGTTCCGCGCA</td>
<td>73.43</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>82</td>
</tr>
<tr>
<td>141</td>
<td>TCATCGCACCCTCAAAGGAACC</td>
<td>54.55</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

*primer melting temperature*  
*b*guanine and cytosine percent in primer  
*c, d, e*secondary primer structures  
*f*quantitative prediction of primer efficiency
139/141 also has an overall higher rating or quantitative prediction of primer efficiency, 82 and 100, than the ST11/ST15 primer set (Table B-1).

Thermal cycler conditions for the samples using the 139/141 primer set was modified from Scholz et al. (4). Initial denaturation was set at 95°C for 5 min, followed by 35 amplification cycles each consisting of denaturation at 95°C for 30 s, annealing of the primers to the target DNA at 64°C for 30 s, and extension at 72°C for 30 s. The final extension was at 72°C for 7 min. Upon completion, the samples were held at 4°C until analysis by gel electrophoresis. The thermal cycler conditions for ST11/ST15 primers were performed directly as stated in Gouws et al. (2). All polymerase chain reactions took place in an automated temperature control PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA).

Even though past research has shown the sensitivity of primer set ST11/ST15 toward different bacteria, it was tested for sensitivity toward the 14 S. enteritidis strains supplied by the Utah State University Nutrition and Food Sciences Department. The $10^9$ CFU/ml dilutions all showed PCR products at 429 bp (Fig. B-1, B-2).

The two primer sets were tested for detection limit sensitivity using serial dilutions of S. enteritidis 8326 in physiological saline solution. The ST11/ST15 primer set resulted in a detection limit of $10^4$ CFU/ml, and the 139/141 primer set was able to detect at $10^0$ CFU/ml or 3 cells (Fig. B-3, B-4). Because the 139/141 primer set resulted in a lower detection limit, it was chosen as the primer set for the antibody static capture followed by PCR assays.
Figure B-1. *PCR of first seven S. enteritidis strains at 10⁹ CFU/ml using ST11/ST15 primer set. Lanes: 1, ATCC 8326; 2, ATCC 49214; 3, ATCC 49215; 4, ATCC 49216; 5, ATCC 49217; 6, ATCC 49218; 7, ATCC 49219; 8, Negative PCR control; 9, DNA ladder.*

Figure B-2. *PCR of last seven S. enteritidis strains at 10⁹ CFU/ml using ST11/ST15 primer set. Lanes: 1, ATCC 49220; 2, ATCC 49221; 3, ATCC 49222; 4, ATCC 49223; 5, ATCC 4931; 6, ATCC 13314; 7, ATCC 31194; 8, Negative PCR control; 9, DNA ladder.*
Figure B-3. PCR of S. enteritidis ATCC 8326 dilutions in physiological saline solution using ST11/ST15 primer set. Lanes: 1, DNA ladder; 2, Negative PCR control; 3, \(10^0\) CFU/ml; 4, \(10^1\) CFU/ml; 5, \(10^2\) CFU/ml; 6, \(10^3\) CFU/ml; 7, \(10^4\) CFU/ml; 8, \(10^5\) CFU/ml; 9, \(10^6\) CFU/ml; 10, \(10^7\) CFU/ml; 11, \(10^8\) CFU/ml; 12, \(10^9\) CFU/ml.

Figure B-4. PCR of S. enteritidis ATCC 8326 dilutions in physiological saline solution using J39/J41 primer set. Lanes: 1, DNA ladder; 2, Negative PCR control; 3, \(10^0\) CFU/ml; 4, \(10^1\) CFU/ml; 5, \(10^2\) CFU/ml; 6, \(10^3\) CFU/ml; 7, \(10^4\) CFU/ml; 8, \(10^5\) CFU/ml; 9, \(10^6\) CFU/ml; 10, \(10^7\) CFU/ml; 11, \(10^8\) CFU/ml; 12, \(10^9\) CFU/ml.

References

