DEVELOPMENT OF IMMUNOMAGNETIC CAPTURE (IMC) BASED TECHNIQUES FOR THE DETECTION OF *SALMONELLA* ON POULTRY CARCASSES

by

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Abstract

Current detection methods require at least one 24-48 hour enrichment step for the detection of *Salmonella*. This poses a problem because product often needs to be shipped before microbial contamination levels can be adequately ascertained. Therefore, the need for more rapid methods of *Salmonella* detection becomes apparent.

The purpose of this thesis was to determine if an immunologically-based method, Immunomagnetic Capture(IMC) -ELISA and molecular-based detection methods, PCR and Taqman® PCR employing IMC without enrichment, could detect at least 10^2 cfu/ml of *S.* Typhimurium in broiler carcass rinse fluid (CRF) samples.

IMC-ELISA, IMC-PCR, and IMC-Taqman® PCR were initially tested using 0 to 10^6 cfu/ml of pure culture *S.* Typhimurium. Each detection method was tested using artificially contaminated CRF samples. Finally, standardized IMC-ELISA, IMC-PCR, and IMC-Taqman® PCR methods were tested using commercial CRF samples. Salmonella concentrations were verified using a traditional plate method.

IMC-ELISA produced consistent results when detecting at least 10^4 to 10^6 cfu/mL of pure culture *S.* Typhimurium. IMC-ELISA was not able to produce repeatable results when testing artificially contaminated CRF samples. *S.* Typhimurium was not detected in commercial CRF samples which by virtue of direct plating on XLT-4 were found to contain essentially no *Salmonella* (<1 cfu/ml).

IMC-PCR was able to consistently detect 10^2 cfu/ml, whereas IMC-Taqman® PCR was able to detect 10^1 cfu/ml of pure culture *S.* Typhimurium. IMC-PCR, required four hours to

complete, and it consistently detected 10^4 cfu/ml of *S.* Typhimurium in artificially contaminated CRF samples. IMC-Taqman® PCR took 3 hours to perform and was able to detect 10^3 cfu/ml *S.* Typhimurium in artificially contaminated CRF samples. The sensitivity, as well as the decreased time requirements of these detection methods, would suggest their usefulness in a commercial processing setting.

Dedication

I would like to dedicate this work to my husband, David F. Sharp Jr.
and my parents Steven and Brenda Hurley.
I thank God for their support and influence in my life and work.

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List of Abbreviations

AC	Artificially contaminated
ATCC	American Type Culture Collection
BS	Bismuth sulfite agar
BPW	Buffered peptone water
CRF	Carcass rinse fluid
EDTA	Ethylenediaminetetraacetic acid
HACCP	Hazard Analysis Critical Control Point
HE	Hektoen enteric agar
IMC-ELISA	Immunomagnetic-Capture Enzyme-linked Immunosorbant assay
IMC-PCR	Immunomagnetic-Capture Polymerase Chain Reaction
IMC-TAQMAN® PCR	Immunomagnetic-Capture Taqman® Polymerase Chain Reaction
MMWR	Morbidity and Mortality Weekly Report
PBS	Phosphate-buffered saline
PC	Pure culture
RT-PCR	Reverse transcription polymerase chain reaction
RV	Rappaport-Vassiliadis medium
TT	Tetrathionate broth
USDA	United States Department of Agriculture
XLT-4	Xylose lysine

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CHAPTER 1: LITERATURE REVIEW

1.1 Foodborne disease prevalence

Microbial contamination of foods has been a major source of food borne disease in human beings. The incidence of food poisoning cases per year has ranged from 6.5 to 33 million in the United States, with up to 5,000 deaths per year. The annual cost of food borne illnesses has been estimated at $5 to $6 billion in the United States, and $1 to $2 billion in Canada (McNab, 1998).

Salmonella is one of the leading causes of food borne illness (Hogue *et al*., 1998). Annually, approximately 40,000 cases of salmonellosis are reported in the United States, but it is estimated that 2 to 4 million cases of salmonellosis may actually occur in the United States (MMWR, March 12, 1999).

Currently, approximately 2,213 *Salmonella* species strains have been classified serologically (Coquard *et al*., 1999). They have also been classified based on their adaptation to either human or animal hosts (Acha and Szyfres, 1987). *S. typhi*, which causes human typhoid fever, and *S. paratyphi* A, which causes paratyphoid fever in humans are classified as Group 1. Group 2 includes *S. choleraesuis, S. dublin,* and *S. sendai* which infrequently cause disease in humans but do cause disease in animals. Group 3 includes the remaining species, the most important of which are *S. enteritidis* and *S.* Typhimurium, which cause a typhoid-like disease in mice and gastroenteritis in humans (Acha and Szyfres, 1987).

1.2 Importance of developing rapid detection methods for processing plants

Current large scale agricultural practices have provided a greater opportunity for pathogenic bacteria to affect a larger number of people in a very short period of time. Although *Salmonella* contamination can occur anywhere between the "farm and the table," as much as a tenfold increase in prevalence of *Salmonella* in foods of animal origin has been reported to occur during processing (Lillard, 1990). Clouser *et al*. (1995) reported a significant increase ($p=0.05$) in the contamination level of turkey carcasses from 21% before defeathering to 71% after defeathering. This suggested that a low prevalence of *Salmonella* in a flock can result in a significant level of cross contamination during processing. In addition, concentrated handling of

a larger number of birds in a small number of processing plants increases the chance of cross contamination.

From 1986 to 1996, the number of poultry slaughter plants increased only 6%, whereas the number of poultry slaughtered increased 38% during the same time period. In 1986, approximately 330 poultry processing plants slaughtered 5 billion birds, whereas in 1996, 350 poultry processing plants slaughtered approximately 8 billion birds. This increased load on poultry processing facilities appears to have increased *Salmonella* cross contamination levels (Hogue *et al.*, 1998). These not-so-ideal processing conditions have required modifications of safety standards as well as continuous monitoring of food products.

Slaughter plants and plants producing raw ground products are required to meet certain performance standards with regard to *Salmonella* contamination. For instance, the performance standard, which is a maximum acceptable limit, for broilers is 20.0% (1 bird out of 5 with detectable *Salmonella* contamination). As processing plants meet the above standard and a less than 1:5 contamination level is achieved, the standard will become more stringent to enhance the quality of food further. In the past, monitoring final products for pathogens such as *Salmonella* has been considered the best way of protecting the public against potential illness. Hazard Analysis and Critical Control Point (HACCP) legislation has improved the surveillance by changing from simply an endpoint assessment of the cleanliness of the product (testing final product for contamination) to testing products on the processing line (USDA, 1996). The new HACCP regulations require confirmation of the safety of food products at various points during processing.

Poultry processing generally consists of several steps. Initially chickens are killed, bled, de-feathered, eviscerated and prepared for further processing (averages 15 minutes). The carcasses are then channeled into the chiller (time spent there varies depending on the type of desired end product, up to 72 minutes). The level of microbial contamination is determined by a whole bird rinse after the carcass has been in the chiller. Carcasses can be ready for shipment anywhere between 30 and 90 minutes to 24 hours after slaughter, depending on how they are to be shipped. Regulations state that poultry carcasses must be sampled (nondestructive whole bird rinse) after the chill tank at the end of the drip line or the last readily accessible point prior to packing or cut up (Hogue *et al.*, 1998). Depending on how the carcasses will be shipped (parts or whole), the amount of time required for the final stages of processing may only be a few

hours. Therefore, it is necessary that the detection methods be rapid in order to validate and evaluate the specific HACCP programs within each processing facility. As regulations become more stringent, rapid methods may be a required to determine the safety of products before they are shipped from processing facilities.

1.3 Advantages and disadvantages of rapid detection methods

Detection methods for *Salmonella* can be divided into two categories, conventional and rapid techniques. Conventional culture methods usually involve a time consuming enrichment step and sometimes a pre-enrichment step to stabilize damaged cells. Three to five days are typically required for isolation and identification because of the need for culture on selective media and confirmation of the suspect isolate by biochemical and immunological tests. Rapid methods often rely on at least one enrichment step with immunological detection of antigens or amplification of unique segments of nucleic acid by polymerase chain reaction (PCR) technology (Chen *et al*., 1997). Decreased sensitivity of rapid immunological techniques for *Salmonella* detection, when compared to conventional methods appears to be a major caveat.

1.3.1 Enzyme-linked immunosorbent assays

Antigen capture, enzyme-linked immunosorbent assay (ELISA), which requires steps to increase the amount of antigen available for detection, has been the basis for most rapid detection methods. In 1977, Krysinski and Heimsch reported on the application of a 48 hr enzyme immunoassay using an antiserum prepared against the flagellum of *S.* Typhimurium. The limit of detection for this technique was 10^5 cfu/ml. However, false-positive reactions occurred when they substituted pooled *Salmonella* flagellar antisera for the purified monovalent anti-*S.* Typhimurium flagellar serum. To decrease this cross-reactivity, Minnich (1978) fractionated the pooled Spicer-Edwards antisera, used by Krysinski and Heimsch (1977), as well as specially prepared polyvalent high-titer flagellar antisera to obtain IgG fractions. In a further refinement, Swaminathan and Ayres (1980) used protein-A affinity chromatography to purify the IgG fraction of *Salmonella* polyvalent flagellar antiserum. This modification resulted in a further reduction of cross-reactivity. Minnich *et al*. (1982) then developed an automated microtiter ELISA system which used alkaline phosphatase instead of peroxidase to

reduce the effect of endogenous bacterial peroxidases. The detection method was used on cultures subject to pre-enrichment only, which significantly reduced the time needed for detection.

Even with the above modifications, cross reactivity continued to be a problem. Robinson *et al*. (1983) and Smith and Jones (1983) employed a monoclonal antibody of mouse myeloma origin (MOPC 467) to detect *Salmonella* in infant foods and milk. MOPC 467 recognized a common antigenic determinant present on several *Salmonella* serotypes. This eliminated most of the cross reactivity problems associated with using mixtures of polyclonal antibodies, but MOPC 467 was found to react with varying degrees of avidity to different serotypes (Smith and Jones, 1983). It was also found to cross react with a number of other enteric organisms. Swaminathan *et al*. (1985) improved on this technique by using a mixture of monoclonal antibodies with increased antigenic coverage.

As improvements with cross reactivity were achieved, various immunological based-detection methods for *Salmonella* from food began to evolve. Prusak-Sochaczewski and Luong (1989) were able to detect *Salmonella* in artificially contaminated food samples inoculated with only 25 cfu of *Salmonella*, using an avidin and biotin based enzyme immunoassay. *Salmonella*-TEK™ (Organon Teknika, Inc.) generated a relatively low number of false negative reactions (0.4%), but a significant number of false positive reactions (6.5%) in broiler whole carcass rinse samples. An antibody immobilization test known as the 1-2 Test^{Tm} (Biocontrol Inc., California) generated 7.2% false-negative and 1.4% false-positive reactions (Bailey *et al*., 1991). In both assays, overnight enrichments had to be performed, which extended the total assay time to 48 to 53 hours. Using similar assays, Keller *et al*. (1993) found that a 24-hour enrichment at 37°C of artificially-contaminated eggs was necessary.

To improve rapidity and sensitivity, Tsai and Slavik (1993) developed a fluorescence concentration immunoassay combining intact *Salmonella* cells and 96-well fluoricon plates with an 18 hour direct enrichment in selenite cystine broth. *Salmonella* from the whole carcass rinse samples were detected in less than 24 hours using this assay (Tsai and Slavik, 1993). Wyatt *et al*. (1993) developed a sandwich ELISA using polyclonal antibodies for the capture stage and a cocktail of monoclonal antibodies for

the detection stage. One step pre-enrichment followed by a sandwich ELISA was capable of detecting several serotypes of *Salmonella*. Even with pre-enrichment steps, a microtitration plate ELISA testing macerated giblets generated an unacceptable 7% false-positive reaction and a 26% false-negative reaction (Wyatt *et al*., 1995) with a limit of detection of 10^3 cfu/ml. A six hour pre-enrichment step with 27-hour incubation produced an acceptable limit of detection for *Salmonella* using a dulcitol-1-phosphate dehydrogenase sandwich ELISA. Including the time needed for the ELISA, a total of 36 hours was required, which disqualified this assay for practical application in processing facilties (Tian *et al.,* 1996). Keith (1997) compared an automated, qualitative enzyme-linked fluorescent immunoassay to a conventional plate method for *Salmonella* detection in artificially contaminated milk, whey, and carbohydrate-based products. The overall detection rate of the automated system was 96%, which was comparable to the conventional rate. Sensitivity was slightly decreased and the automated system still required more than two days. The Threshold Immunoassay system which used a solution-based binding of the biotin and fluorescein labeled antibodies to *Salmonella*, detected concentrations as low as 119 cfu/ml. This method involved a filtration-capture of the immunocomplex on a biotin-coated nitrocellulose membrane. A pH change at the silicon surface caused the production of an electronic signal which was proportional to the bacterial concentration. Initially, cellular debris in chicken carcass rinses interfered with the sensitivity of this immunoassay, but the problem was resolved by using a larger-pore biotin-coated nitrocellulose membrane for the capture of the immunocomplex. The drawback to the use of a larger-pore membrane was that it decreased the sensitivity of the detection system (Dill *et al*., 1999). As with previously explained methods, sensitivity was lost when the test became more rapid.

1.3.2 Polymerase chain reaction-based detection methods

There have been a growing number of detection methods utilizing PCR because of its sensitivity and reliability. Nucleic acid based methods such as PCR are not dependent on substrate utilization or the expression of antigens. Therefore, phenotypic variations seen in both biochemical tests and lack of detectable antigens do not effect the results (Hoorfar *et al.,* 1999).

A number of researchers have developed detection methods for *Salmonella* in various types of food samples, all requiring at least a four hour pre-enrichment before PCR. With an overnight sample enrichment prior to testing, Bailey *et al*. (1991) using the GENE-TRAK test which is based on a colorimetric deoxyribonucleic acid (DNA) hybridization, was able to detect *Salmonella* with a low incidence of false-positives (1.4%) and false-negatives (2.5%). Aabo *et al*. (1993) demonstrated a high level of specificity for PCR-based assays by testing one hundred and sixteen serovars of *Salmonella* pure cultures. No PCR products were seen in any of the 86 non-*Salmonella* Enterobacteriacea organisms tested. A PCR-based test for *Salmonella* in stomached chicken meat was developed by Fluit *et al*. (1993) that involved amplifying a portion of sequence of *Salmonella, oriC* gene. Twenty-four hour sample enrichment was required to obtain a sensitivity of 0.1 cfu/g. Jitrapakdee *et al*. (1995) were able to detect 52 of the most prevalent serovars of *Salmonella* in a frozen chicken model using a PCR assay, that required a 6 hour enrichment. The limit of detection in contaminated frozen chicken was 3 cfu/g of chicken meat.

Investigators employing PCR have concentrated on different areas of the *Salmonella* genome. Cohen *et al*. (1996) utilized primers complementary to part of the *fim*A gene of *S.* Typhimurium. These primers successfully amplified a collection of 376 strains of *Salmonella* isolated from animals and humans but did not amplify 40 non-*Salmonella* strains. A primer set from the *omp*C gene of *Salmonella* was shown to selectively amplify 40 common *Salmonella* serovars, but none of the 24 non-*Salmonella* species tested. Stefanovicora *et al*. (1998) developed a PCR method employing primers ST11 and ST15 with results identical to those of standard biochemical and serological methods, in terms of distinguishing between *Salmonella* and other Enterobacteriacae. Commercial poultry, eggs, pork, meat, milk, fish, feed, and pure cultures were evaluated. The time needed for confirmation of *Salmonella* was trimmed to 6 hours. Lin and Tsen (1999) detected *Salmonella* with no false positive results in a PCR-based test for the *mdh* gene which codes for malic acid dehydrogenase of *S.* Typhimurium. This method required an 8 to 12 hour enrichment step involving growth in lactose-tetrathionate broth. Wang *et al*. (1997) were able to detect as few as 40 *Salmonella* cfu/ml using a universal PCR detection protocol that was also able to detect 12 other common types of bacteria.

In a method which required overnight incubation, PCR and restriction endonuclease enzyme digests were used by Cocolin *et al*. (1998) to detect and identify *S.* Typhimurium in minced pork, beef, poultry, fermented sausages, and fish meat. Manzano *et al*. (1998) tested 75 food samples (5 different types) using PCR amplification of a 389 bp section of the *inv*A gene, but again, an overnight enrichment was essential for this assay to generate positive results. Kantama *et al*. (1998) were able to detect the most common *Salmonella* serotypes using two sets of primers in a nested PCR reaction. The assay was able to distinguish many of the non-*Salmonella* tested. *Citrobacter* and *Shigella* generated amplicons, but their PCR products were different in size from the amplicons from *Salmonella*. Sensitivity in the first and second round PCR reactions were 7 pg and 80 fg of DNA, respectively.

Rijpens *et al.* (1999) using immunomagnetic separation and alkaline lysis sample preparation, were able to detect an average of 5.9 stressed *Salmonella* in 25 g of food product (pasteurized egg yolk, egg yolk powder, ice-cream, whole egg, egg white, and cheeses) using a PCR assay with a 16 hour pre-enrichment. Khan *et al*. (1999) also developed a PCR method capable of detecting *S.* Typhimurium DT104 using gene specific primers. They accurately identified twenty-two ACSSuT resistant DT104 isolates. Nogva and Lillehaug (1999) adapted a 5'-nuclease-based kit to quantify *Salmonella* in pure cultures. Requiring a total of 28 hours, they were able to detect viable *S. enteritidis* PT4 in artificially contaminated minced beef and whole egg samples with a reverse transcription-polymerase chain reaction (RT-PCR) method. Burtscher *et al*. (1999) used a 16 hour overnight enrichment, one minicolumn, PCR, and gel electrophoresis to detect as few as 10 cfu/ml of *S.* Typhimurium from organic waste.

PCR based assays usually require electrophoresis to view the results, which add time and the need for additional technical training (Yu & Bruno, 1996). However, methods based on observation of fluorescence do not require electrophoresis. Desjardin *et al*. (1998) compared a standard PCR assay to an automated PCR assay that allowed quantification using the ABI Prism 7700® Sequence Detection System (Taqman®). Chen *et al.* (1997a) with the TaqMan LS-50B PR detection system, a fluorogenic polymerase chain reaction assay which does not require electrophoresis, were able to detect a low number (exact concentrations were not indicated) of *Salmonella* in artificially

contaminated chicken carcass rinses. The test required an overnight pre-enrichment at 37°C. When comparing this method to the conventional modified semi-solid Rappaport Vassiliadis culture method, the correlation was ≥ 98% (Chen *et al*., 1997a). Similar fluorescence-based assays were developed by Chen *et al*. (1997b) and Heid *et al.* (1996).

Carlson *et al*. (1999) developed a multiplex PCR containing primers for targeted sequences of *S.* Typhimurium DT104 exhibiting the ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline) or ASSuT resistance phenotypes. Modification of the PCR to include a fluorogenic assay required a total of 3-4 hours for accurate detection of broth cultured *S.* Typhimurium DT104. PCR reactions were monitored by using the portable Advanced Nucleic Acid analyzer. Contaminated food samples were not tested. Trials performed by Belgrader *et al.* (1999) indicated the ability to detect 500, 50, and 5 *Erwinia* cells, needing 7, 8, and 9 minutes respectively for a positive signal. This real-time PCR utilized the Taqman assay (fluorogenic 5'-nuclease assay) and a spectrofluorometric thermal cycler.

1.3.3 Immunomagnetic capture

Several researchers have used immunomagnetic polystyrene beads to concentrate *Salmonella* prior to PCR being performed. Immunomagnetic separation was shown to give similar numbers of true positive *Salmonella* when compared to the standard enrichment methods (Mansfield and Forsythe, 1993) (Fluit *et al*., 1993). Mansfield and Forsythe (1996) compared selective enrichments in conventional broths to immunomagnetic separation using Dynabeads® coated with anti-*Salmonella* antibodies. The results confirmed the use of immunomagnetic separation as an alternative to enrichment broths for *Salmonella* isolation.

Yu and Bruno (1996) coupled immunomagnetic separation with electrochemiluminescence to detect *Salmonella*. Electrochemiluminescence is the result of a change in the oxidized and reduced states of heavy metal chelates such as ruthenium (II) trisbipyridal. During the oxidation reaction, the reduced compound is converted to its oxidized form, releasing energy. This conversion results in a light emission at 620 nm. Capture antibodies conjugated to immunomagnetic beads were labeled with the heavy metal chelate. After concentration of the magnetic beads from the food samples, an

ORIGEN analyzer (Igen Corp, Gaithersburg, MD) was used to measure light emission. The detection limit of this assay was 10^3 cfu/ml in food samples and results were available in 1 hour.

Cudjoe and Krona (1997) compared the Dynal core method using anti-*Salmonella* Dynabeads® to the conventional ISO 6579 reference method. In ten different food matrices inoculated at low levels (1-5 cells/25 g) with twenty different *Salmonella* serovariants, the Dynal core method showed a 90% concordance with the ISO reference method and actually identified two more *Salmonella* positive samples. Results from the Dynal® core method correlated 100% with results from the standard culture technique used for foods and with the modified semisolid Rappaport-Vassiliadis method used for feeds and environmental samples. This study included 308 naturally contaminated samples, including 46 cheese and egg products, 183 animal feeds, and 79 environmental swabs (Shaw *et al*., 1998). Wang and Slavik (1999) were able to detect an average of 2.3 X 10^4 cfu/ml in chicken rinses by combining immunomagnetic separation with flow cytometry, along with an 18 hour overnight enrichment step at 37°C.

1.4 Summary

The high processing volume in poultry facilities and wider distribution of poultry products has increased the chances of affecting a larger segment of the population. The government has been expected to lower the acceptable rate of contamination of processed chicken from the current 20% to 10% in the near future (Major, 2000). These factors have necessitated the development of more rapid and accurate detection methods for *Salmonella* contamination of poultry products. Previous methods for *Salmonella* detection in carcass rinses have not demonstrated the needed sensitivity and in addition have required at least one enrichment step of 6-18 hours.

New rapid methods of detection of *Salmonella* contamination in foods should not only be rapid, but also their implementation by processors and regulators should be feasible. Cost-effectiveness, speed, and minimal required training are mandatory attributes of such tests if they are to be readily adopted for routine use.

1.5 Hypotheses

Based on current literature and preliminary experiments, IMC-ELISA and IMC-PCR tests were chosen as potential rapid methods for the detection of *Salmonella* in carcass rinse samples.

1.5.1 IMC-ELISA Hypothesis

It is hypothesized that IMC-ELISA (without enrichment) should be able to detect at least 10^2 cfu/ml of *Salmonella* in less than 5 hours in broiler carcass rinse samples.

This system has several advantages that may make it useful in a poultry-processing facility. IMC-ELISA requires approximately 4 to 5 hours, which is faster than current rapid detection methods that involve preenrichment and enrichment steps. Only a few materials are required for IMC-ELISA, which adds to the simplicity of the procedure and a large number (90-96) samples can be tested simultaneously. Immunoassays have been shown to be specific and highly sensitive (Ibrahim, 1986).

1.5.2 IMC-PCR and IMC-Taqman® PCR Hypothesis

It is hypothesized that IMC-PCR and IMC-Taqman® PCR (without enrichment) should be able to detect at least 10^2 cfu/ml of *Salmonella* in less than 4 hours in broiler carcass rinse samples.

PCR-based assays are specific, reliable and rapid. The time needed to conduct the IMC-PCR assay could be decreased to a little more than 3 hours after the optimum conditions had been established and incorporation of a flourogenic procedure (Taqman®). This type of assay has the advantage of being easy to conduct and requires only minimal materials. Standard PCR is relatively less expensive and highly specific primers can be created to increase sensitivity. Even a low level of contamination is likely to be detected using a PCR-based assay because this assay generates DNA to a detectable level.

CHAPTER 2:

DEVELOPMENT AND APPLICATION OF IMC-ELISA TO DETECT *SALMONELLA* IN POULTRY CARCASS RINSE FLUID SAMPLES.

2.1 Abstract

An immunomagnetic capture enzyme-linked immunosorbent assay (IMC-ELISA) method, requiring no pre-enrichment or enrichment step, was developed to detect *Salmonella* in contaminated carcass rinse samples. Antibody conjugated paramagnetic polystyrene beads were used to concentrate and recover naladixic acid resistant *Salmonella* Typhimurium from test samples. This was followed by ELISA to determine the amount of *Salmonella* present. IMC-ELISA was able to detect *Salmonella* in serial dilutions of pure cultures ranging from 10^4 to 10^6 cfu/ml. Artificially contaminated carcass rinse fluid samples yielded similar results. Both pure cultures and artificially contaminated carcass rinses were used to generate standard curves. IMC-ELISA results from unknown commercial CRF samples indicated the presence of *Salmonella* or at least that of poly-O antigens. IMC-ELISA was able to differentiate between *Salmonella* and six other common bacterial contaminants of chicken products. However, *Enterobacter cloacae* and *Klebsiella pneumoniae* at 10^6 cfu/ml generated false positive reactions. Based on these findings, after further optimization to decrease cross-reactivity, IMC-ELISA would appear to have potential as a rapid near on-line detection method for *Salmonella* in poultry processing.

2.2 Introduction

Salmonella is one of the leading causes of food borne illness in the United States (Lillard, 1990). It has been shown that a ten-fold increase in *Salmonella* contamination occurs during the processing of poultry carcasses (Lillard, 1990). Increased mechanization and volume in poultry processing has necessitated the development of rapid detection methods for food borne pathogens like *Salmonella*. Conventional culture methods used for detection of *Salmonella* in food are time consuming due to the need for pre-enrichment and enrichment steps. This limits their practical value for rapid surveillance and verification of wholesomeness. Methods that

generate results within a few hours instead of a few days would greatly reduce the need for future possible product recalls, reprocessing, and most importantly the incidence of food borne illness in consumers. Rapid methods would also assist in the evaluation and validation of HACCP programs within processing facilities.

Enzyme linked immunosorbent assays (ELISA) have been used by several investigators to detect *Salmonella* in food samples and processing fluids. These methods typically require at least one enrichment step prior to running the actual ELISA. In addition, varying amounts of cross reactivity have been observed with each combination of antibodies and type of sample tested. Prusak-Sochaczewski and Luong (1989) used an avidin-biotin based enzyme immunoassay and were able to detect *Salmonella* in food samples inoculated with only 25 *Salmonella* colony forming units (cfu). *Salmonella*-Tek®, another enzyme immunoassay manufactured by Organon Teknika® (Durham, NC) had a relatively low false negative rate of 0.4%, but had a high false positive rate of 5.0% for broiler whole carcass rinses. Overnight enrichments were performed prior to the use of the *Salmonella*-Tek® assay which extended the total time of detection to 48 to 53 hours. Tsai and Slavik (1993) developed a 96 well, fluorescence concentration immunoassay for whole carcass rinse samples which was able to detect 1.25×10^4 cfu/ml of *Salmonella* after only 18 hours of enrichment. Still another detection method marketed as the Threshold Immunoassay manufactured by Molecular Devices Corporation (Sunnyvale, CA) utilized solution-based binding of biotin and fluorescein labeled antibodies to *Salmonella* followed by filtration-capture of the immunocomplex on a biotin-coated nitrocellulose membrane. This method was found to be capable of detecting levels as low as 119 cfu/ml. Initially, cellular debris in chicken carcass rinse samples interfered with the sensitivity of the immunoassay, but this was resolved by using a larger pore biotin-coated nitrocellulose membrane for the capture of immunocomplexes. The use of a larger pore size did however decrease the sensitivity of the detection system (Dill *et al*., 1999).

Immunomagnetic capture as a rapid method of concentrating *Salmonella* in various sample types has also been investigated. Mansfield and Forsythe (1993) found that IMC separation followed by PCR, enabled detection of similar numbers of *Salmonella* positive samples in less time than standard enrichment methods. In a subsequent study (Mansfield and Forsythe, 1996) a number of selective enrichment broths were compared with immunomagnetic separation using Dynabeads® coated with anti-*Salmonella* antibodies. Standard plating on

selective agar along with serology tests and biochemical kits were used to confirm the identification of *Salmonella* colonies. Concentration of *Salmonella* using Dynabeads® proved to be a viable alternative to conventional enrichment for *Salmonella.* At about the same time, Cudjoe *et al*. (1995) developed an assay combining IMC and ELISA technologies which was capable of detecting 10^5 cfu/ml of *Salmonella* after 20 hours of enrichment with a total assay time of just under 26 hours. Most recently, Tan and Shelef (1999) developed an immunomagnetic bead-based automated method capable of detecting 2-25 cfu of *Salmonella* in 25 g of dry eggs or chicken or 1 ml of liquid eggs, shelled eggs, or skim milk within 36 hours. This method involved pre-enrichment of the food samples, followed by immunomagnetic capture. The paramagnetic beads with bound *Salmonella* were then added to a selective liquid medium containing an H_2S indicator. Following incubation, *Salmonella* levels were determined colorimetrically as a function of H_2S production.

The objective of this research was to develop an IMC-ELISA based detection method for *Salmonella* that would require no pre-enrichment or enrichment step and that could detect as few as 10^2 cfu/ml of *Salmonella* in chicken carcass rinse fluid samples with a total assay time of less than 5 hours.

2.3 Material and Methods

Antibodies

The following primary, secondary, and tertiary antibody concentrations were evaluated in extensive preliminary experiments to determine the least cross-reactive combination in ELISA. Primary antibody: *Salmonella* O antiserum produced in rabbits (1:200, 1:400, 1:10); secondary antibodies: chicken anti-rabbit IgG (1:2000, 1:200, 1:1000, 1:10,000); alkaline phosphatase conjugated donkey anti-rabbit (1:25,000, 1:20,000, 1:2000); tertiary antibody: peroxidase-conjugated donkey anti-chicken IgG (1:25,000, 1:20,000). The concentration of Dynal® anti-*Salmonella* conjugated beads remained constant throughout these experiments. Background levels for the ELISA were determined using the absorbence of wells containing only the antibodies, substrate, and stop solution.

Antibody-conjugated paramagnetic beads

Affinity purified goat antibody against *Salmonella* common structural antigens (Kirkegaard and Perry Laboratories Inc.) was conjugated to the Tosyl-activated Dynabeads M-280 Dynal® (Sacramento, CA). These beads were tested against serial dilutions of *S.* Typhimurium in pure culture. Experiment trials analyzing the capture rate of the Dynal® anti-*Salmonella* coated beads and the laboratory antibody coated goat anti-*Salmonella* coated beads were performed. The amount of Dynal® anti-*Salmonella* beads required for each 1 ml of sample was determined by varying the amount of beads (5 µl to 20 µl) over a range of *S.* Typhimurium dilutions (10^1 to 10^6 cfu/ml).

Blocking proteins

Several types of proteins were evaluated for their blocking capabilities in the ELISA assay. These included: nonfat skim milk (3%), bovine serum albumin (3%), gelatin (.5%), gelatin (.2%), fish gelatin (.25%), donkey serum (3%) adsorbed with killed *S.* Typhimurium, and heat inactivated fetal calf serum (3%).

Preparation of *Salmonella* Typhimurium from pure culture

Log phase cultures of naladixic acid resistant *Salmonella* Typhimurium obtained from N. Sriranganathan, Virginia Tech were prepared from frozen stock. Serial dilutions were then made in 1.0 ml of cold buffered peptone water (BPW, whole carcass rinse buffer, USDA, 1996). Samples used for IMC-ELISA evaluation contained final concentrations of 10^1, 10^2, 10^3, 10^4,10^5, and 10^6 cfu/ml as determined by standard dilution plate method. Six separate runs were performed to validate the assay.

Preparation of artificially contaminated chicken carcass rinse samples

Whole chicken carcasses were purchased at a local retailer. Each was placed in a large (3500 ml) sterile bag and 400 ml of pre-chilled BPW was poured into the carcass cavity. The carcass was rocked slowly back and forth for one minute so that all surfaces were rinsed with buffered peptone water. It was then removed from the bag and multiple 1.0 ml aliquots of the carcass rinse fluid (CRF) were removed and frozen at – 80°C. (*Salmonella* Analysis, Food Safety and Inspection Service, 1997). Serial dilutions were then prepared as previously described for pure cultures with 1.0 ml of thawed CRF being substituted for BPW. These samples were then tested using the IMC-ELISA in three separate runs.

Commercial carcass rinse samples

CRF samples from a commercial processing facility containing unknown amounts of *Salmonella* were shipped overnight on ice. Fifteen out of the 17 samples submitted were randomly selected from which multiple 1.0 ml aliquots were taken and frozen at -80°C. Prior to freezing, 3 samples were randomly chosen and 100 µl from each were spread onto Hektoen enteric (HE) agar, bismuth sulfite (BS) agar, and xylose lysine tergitol (XLT-4) agar plate. These were incubated for 24 hours at 35°C. Colonies were then counted to determine the number of cfu/ml. After freezing, the same three samples were thawed and 100 µl were again plated to determine the viable cfu/ml after one freeze-thaw cycle. Finally, thawed samples were tested using the IMC-ELISA in 3 separate runs.

IMC ELISA

IMC-ELISA was performed using the following procedures modified from Yu and Bruno (1996). For the standard curve (positive control) samples, 1.0 ml aliquots of serial dilutions of 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 cfu/ml *Salmonella* were centrifuged at 4,000 x g for 20 minutes. Artificially contaminated CRF and commercial CRF were treated in the same manner. Each pellet of bacteria was resuspended in 300 µl of phosphate-buffered saline (PBS). One hundred-fifty micrograms/ml (15µl) of antibody-coated beads (Dynal® anti-*Salmonella* paramagnetic

beads) were added and incubated at room temperature with gentle rotation for 20 minutes using a Dynal® sample mixer. A MPC-M® magnet was then used to secure the beads and the supernatant was removed. The beads were washed twice with 300 µl of PBS, and resuspended in 50 µl of PBS. The beads were placed in a 96 well polystyrene plate (VWR Scientific) which had been previously blocked for at least 24 hours at 4°C with 3% fetal calf serum. Then 100 µl of a 1:400 dilution of rabbit anti-*Salmonella* primary antibody (Difco) were added and mixed by repeated pipetting. The plates were incubated at room temperature (25°C) for 30 minutes with gentle shaking. This incubation was followed by three washes with 200 µl of PBS. This entailed gentle hand agitation for 1 minute followed by placement of the magnet (MPC magnet from Dynal) under the plate, waiting 1 minute, then inverting the plate and magnet to remove the wash liquid containing unbound antibody. After washing, 100 µl of 1:2000 dilution of alkaline phosphatase-conjugated donkey-anti-rabbit IgG (Bethyl Laboratories) were added to each well. The plate was again incubated for 30 minute at room temperature on a rotating shaker, and the beads were washed 3 times with PBS as previously described. One hundred microliters of alkaline phosphatase substrate, (5-bromo-4-chloro-3-indolyl-phosphate, Kirkegaard and Perry Laboratories, Gaithersburg, MD) and 100 µl of 2.5% EDTA was added to each well and incubated for 30 minutes at room temperature. Absorbence values were determined using a spectrophotometer, Vmax, kinetic microplate reader (Molecular Devices Corporation, Melno Park, CA).

Analysis of non-specific binding using common poultry contaminants

Echerichia coli, Citrobacter freundi, Enterobacter cloacae, Klebsiella pneumoniae, Proteus mirabilis, Shigella sonnei, Vibrio alginolyticus, and *Yersinia enterocolitica* were grown separately on LB plates. Cells were harvested and resuspended in BPW with 2% glycerol. Two hundred and fifty microliter aliquots were frozen overnight at –80°C. After thawing, serial dilutions were plated on LB plates and incubated at 37°C overnight to determine the concentration of cells in the frozen aliquots. For specificity trials, pure cultures were serially diluted to a final concentration of 1 X 10^6 cfu/ml and then run in duplicate using the IMC-ELISA.

Statistical analysis

Mean absorbence and standard errors of the means were calculated using Jump® statistical software. Best fit curves were then generated for pure culture and artificially contaminated CRF using Microsoft Excel® Estimated contamination levels of the commercial carcass rinse samples were determined based on the slope of the standard curve for the artificially contaminated CRF samples.

Direct plating and enrichment

A standard spread plate method was initially employed to determine the cfu/ml of *Salmonella* in log phase cultures. Once this was established and 1 ml aliquots prepared, 100 µl from dilutions containing 10^0, 10^1, 10^2, cfu/ml, 50 µl from those containing 10^3 cfu/ml and 20 µl from those containing10^4, 10^5, and10^6 cfu/ml were spread onto on to HE agar, BS agar, and XLT-4 agar. *Salmonella* appeared as blue/green colonies with black centers on HE agar. *Salmonella* appeared as brown, gray, or black colonies with or without a metallic sheen on BS agar. Colonies on XLT-4 appeared light brown with black centers. Occasionally, colonies were entirely black.

Enrichment was performed in the following manner. One ml of each sample was added to 9 ml BPW in sterile screw cap 15-ml plastic tubes. The tubes were incubated at room temperature for 60 minutes. Depending on the initial cfu/ml, a specified volume (100 µl, 50 µl, 20 µl) was spread onto HE agar, BS agar, and XLT-4 agar plates. After an additional 24 hours of incubation at 35°C, 0.1 ml was transferred to Rappaport-Vassiliadis (RV) medium and 1.0 ml was transferred to tetrathionate (TT) broth. The tubes were then incubated in a 43°C water bath for 24 hours. Finally, depending on the original sample concentration, 100 µl , 50 µl, or 20 µl were spread onto HE, BS, and XLT-4 agar plates and incubated at 35°C for 24 hours. Typical *Salmonella* colonies were then enumerated (Bacteriological Analytical Manual, AOAC International, 1998).

Direct plating and enrichment for artificially contaminated CRF samples was performed in a manner identical to that for pure cultures.

For commercial CRF samples, direct plating and enrichment was performed in the following manner. One hundred µl from each of the 15 samples were plated onto HE, BS, and XLT-4 agar plates. All plates were incubated for 24 hours at 35°C and the colonies were counted. Enrichment of the commercial CRF samples was identical to that used for pure cultures and artificially contaminated CRF samples.

2.4 Results

Optimization of IMC-ELISA Parameters

Early trials designed to optimize antigen capture and antibody binding suggested that there was a high degree of cross reactivity between antibody conjugated beads and the other antibodies used in the assay. Initially it was suspected that this was due to excessive amounts of primary, secondary or tertiary antibody. This was based on the observation that wells containing the lowest concentrations of *Salmonella* showed color reactions within 7 minutes and wells containing no *Salmonella* at all had absorbance values similar to those which contained *Salmonella*. Absorbance readings were negative in the wells containing unconjugated beads but proportional increases were not observed with successively higher concentrations of *Salmonella*. Reducing the amounts of these antibodies in various test combinations did not decrease the apparent level of cross-reactivity. In fact, due to the extremely high level of background, the use of the tertiary antibody was discontinued. ELISA results were found to be most consistent using concentrations of 1:400 and 1:2000 for the primary and secondary antibodies, respectively.

Experiments analyzing the capture rate of the Dynal® anti-*Salmonella* coated beads and the laboratory antibody coated goat anti-*Salmonella* beads indicated that the commercial beads were superior. It was determined that 15 µl of Dynal® beads had a capture rate of > 90% when the concentration of *Salmonella* was between 10^3 to 10^6 and less than 40% for concentrations between 10^1 and 10^2 cfu/ml. Twenty µl of beads did not significantly increase the capture rate for any of the *Salmonella* concentrations tested.

Experiments in which the concentrations of primary antibody (rabbit anti-*Salmonella*) and secondary antibody (alkaline phosphatase conjugated donkey anti-*Salmonella*) were varied

along with the type of blocking protein revealed that heat inactivated fetal calf serum was the most effective in reducing background.

Based on these experimental results, the following reagents and concentrations were considered optimal for IMC-ELISA: 15 µl of Dynal® anti-*Salmonella* beads; blocking with heat inactivated fetal calf serum (3%), primary antibody, rabbit anti-*Salmonella* (1:400 dilution); secondary antibody, alkaline phosphatase conjugated donkey anti-rabbit (1:2000 dilution); substrate, BluePhos Microwell substrate system. Incubation times and plate washing were as described in the Materials and Methods.

Detection of *Salmonella* in BPW and CRF using IMC-ELISA

Employing the optimized assay, BPW samples containing known amounts of *Salmonella* were tested. Raw absorbance values with means and standard errors (SEM) for *S.* Typhimurium (0 to 10^6 cfu/ml) are presented in Table 2.1. Mean absorbance as a function of $\log_{10}$ *Salmonella* concentration is shown in Figure 2.1. This relationship was found to be curvelinear and best described by a second order polynomial equation (R^2 =.89). At dilutions between 10^0 and 10^3, the SEM values indicates enough overlap of absorbance means to indicate a low assay sensitivity. When *Salmonella* concentrations were in the 10^4 to 10^6 range, the sensitivity appeared more acceptable.

The raw absorbance values, means, and standard errors for artificially contaminated CRF samples are presented in Table 2.2. A curvelinear relationship (2^o polynomial) between absorbance and $\log_{10}$ *Salmonella* concentration was also found for these samples (Figure 2.2). This relationship, although of statistical importance, ignores the fact that there was little direct correlation between absorbance and $\log_{10}$ cfu at dilutions between 10^0 and 10^3. Again, the sensitivity of this assay based on deviation from the best fit curve, would appear to be greatest in the 10^4 to 10^6 cfu range. A Students t test analysis (Jump Software®) comparing mean absorbances by dilution for pure cultures in BPW and artificially contaminated CRF indicated no significant differences.

Table 2.3 presents the raw absorbance values, means and standard errors, and estimated *Salmonella* concentrations for "unknown" commercial CRF samples. Estimated concentrations of *Salmonella* were calculated based on the curvelinear equation generated for artificially

contaminated CRF. Thirteen out of fifteen of the samples had high absorbance values which fell within the proposed range of sensitivity (10^4–10^6 cfu/ml) of the assay.

Absorbance values for other Enterobacteriacae are shown in Table 2.4. *Enterobacter cloacae* and *Klebsiella pneumoniae* absorbance values were as high as those for *S.* Typhimurium at 10^6 cfu/ml indicating clear problems with the assay specificity.

Direct plating and enrichment of *Salmonella* from BPW and CRF

Results of the direct plate count of pure *S.* Typhimurium in BPW and artificially contaminated CRF samples are presented in Table 2.5. At concentrations between 10^4 and 10^6 cfu/ml, colonies were too numerous to count for both the pure culture dilutions in BPW and the artificially contaminated CRF samples. For dilutions between 10^1 and 10^3, colony counts were determined and suggested that fewer live *Salmonella* were actually available for immunomagnetic capture in the artificially contaminated CRF compared to pure cultures diluted in BPW. Direct plating of the fifteen commercial carcass rinse samples did not reveal any evidence of viable *Salmonella*.

Enrichment of pure cultures and artificially contaminated CRF prior to plating produced colony numbers too numerous to count. Enrichment followed by plating on XLT-4 agar confirmed the lack of viable *Salmonella* in the 15 commercial CRF samples.

Salmonella (cfu/ml)	Run number						Mean ± Standard error of the mean
	1	2	3	4	5	6	
1.0×10^6	0.268	0.12	0.132	0.128	0.185	0.507	0.223 ± 0.061
1.0×10^5	0.109	0.064	0.073	0.025	0.07	0.186	0.087 ± 0.022
1.0×10^4	0.081	0.037	0.049	0.032	0.026	0.133	0.059 ± 0.016
1.0×10^3	0.092	0.041	0.032	0.002	0.021	0.096	0.047 ± 0.015
1.0×10^2	0.07	0.047	0.015	0.012	0.008	0.092	0.040 ± 0.014
1.0×10^1	0.065	0.065	0.035	0.027	0.026	0.054	0.045 ± 0.007
0	0.093	0.036	0.016	0.008	0.046	0.057	0.042 ± 0.012

Table 2.1: Absorbance values for various concentrations of *Salmonella* in BPW as measured by IMC-ELISA

Salmonella (cfu/ml)	Run number			Mean ± Standard error of the mean
	1	2	3	
1.0×10^6	0.184	0.086	0.117	0.129 ± 0.028
1.0×10^5	0.096	0.047	0.083	0.075 ± 0.014
1.0×10^4	0.033	0.029	0.084	0.048 ± 0.017
1.0×10^3	0.033	0.021	0.006	0.020 ± 0.007
1.0×10^2	0.034	0.03	0.12	0.061 ± 0.029
1.0×10^1	0.027	0.052	0.056	0.045 ± 0.009
0	0.036	0.014	0.028	0.026 ± 0.006

Table 2.2: Absorbance values for various concentrations of *Salmonella* in artificially contaminated CRF as measured by IMC-ELISA

Sample number	Run number			Mean ± Standard error of the mean	Estimated cfu/ml
	1	2	3		
1	0.092	0.229	0.398	0.239 ± 0.088	>1 X 10^7
2	0.084	0.151	0.171	0.135 ± 0.026	1.5 X 10^6
3	0.035	0	0.103	0.046 ± 0.030	1.5 X 10^3
4	0.086	0.115	0.412	0.204 ± 0.104	>1 X 10^7
5	0.127	0.106	0.451	0.228 ± 0.111	>1 X 10^7
6	0.029	0.055	0.107	0.063 ± 0.022	1.2 X 10^4
7	0.024	0.06	0.162	0.063 ± 0.024	1.2 X 10^4
8	0.839	-0.016	0.084	0.254 ± 0.300	>1 X 10^7
9	0.058	0.013	0.351	0.140 ± 0.105	1.5 X 10^6
10	0.109	0.202	0.368	0.226 ± 0.075	>1 X 10^7
11	0.051	0.083	0.141	0.091 ± 0.026	1.5 X 10^5
12	0.005	0.047	0.117	0.056 ± 0.032	1.8 X 10^3
13	0.025	0.06	0.135	0.073 ± 0.032	1.5 X 10^4
14	0.062	0.043	0.169	0.091 ± 0.039	1.5 X 10^5
15	0.092	0.137	0.252	0.160 ± 0.047	>1 X 10^7

Table 2.3: Absorbance values and estimated number of *Salmonella* in "unknown" commercial CRF as measured by IMC-ELISA

Bacteria name	Absorbance (620nm)
Escherichia coli	0.095
Citrobacter freundi	0.062
Enterobacter cloacae	0.177
Klebsiella pneumoniae	0.173
Proteus mirabilis	0.013
Shigella sonnei	0.022
Vibrio alginolyticus	0.061
Yersinia enterocolitica	0.088

Table 2.4: IMC-ELISA absorbance values with the background subtracted.

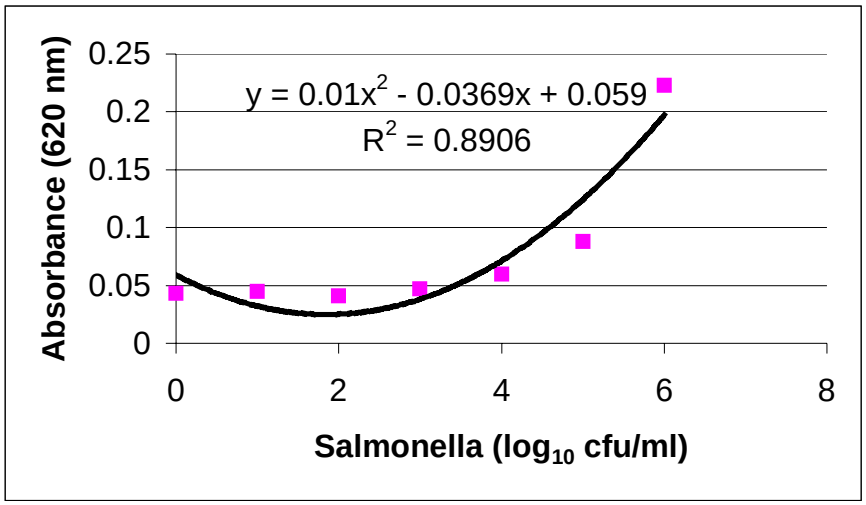


Figure 2.1: Absorbance as a function of log cfu/ml of *Salmonella* (pure culture) in BPW.

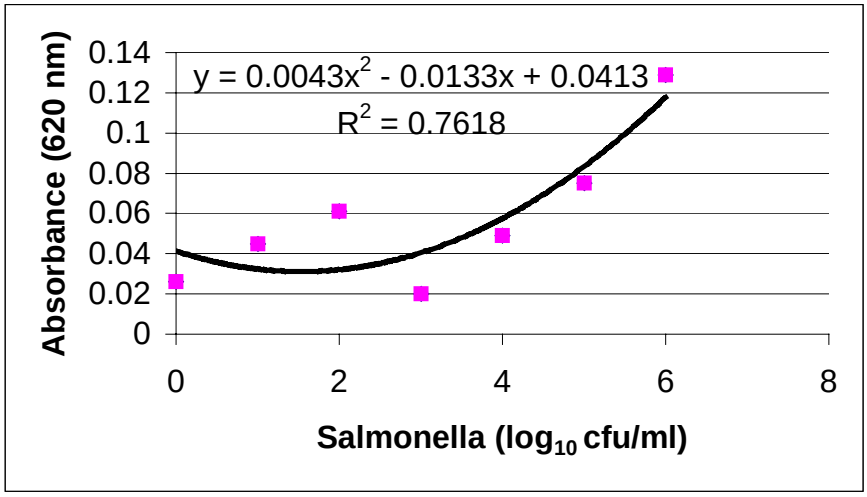


Figure 2.2: Absorbance as a function of log *Salmonella* cfu/ml in artificially contaminated CRF.

Sample type	Estimated *Salmonella* Concentration (cfu/ml)	Plate count at 24 hrs. (cfu/ml)
PC	1.0×10^3	1.81×10^3
	1.0×10^2	1.9×10^2
	1.0×10^1	1.0×10^1
	0	0
AC	1.0×10^3	4.4×10^2
	1.0×10^2	7.0×10^1
	1.0×10^1	1.0×10^1
	0	0

Table 2.5 Direct plate (XLT-4) counts of pure culture (PC) *S.* Typhimurium and artificially contaminated CRF samples (AC)

2.5 Discussion

The adoption of any detection method for food borne pathogens in a commercial setting requires that the method be easy to use, cost effective, sensitive, and specific. Along these lines, IMC-ELISA does have several advantages: 1) it permits testing of a large number of samples in a relatively short period of time i.e., 4-5 hours, 2) it requires only a small sample volume e.g., 1.0 ml, and 3) it appears to be cost effective. In regards to cost, the conjugated-paramagnetic beads are the most expensive component of IMC-ELISA at approximately $1.16 per sample. Including other disposables, i.e., antibodies, miscellaneous reagents, and 96-well plates, the total cost per sample would be approximately $2.00. This compares with $7.00 per sample with methods like Vidas® (bioMerieux, Inc., Hazelwood, MO) assay system that are currently being used in a number of commercial poultry processing plants. Such detection methods often require additional verification of suspected *Salmonellas* by PCR at a cost of approximately $13.00 per sample (D. Kull, pers. comm.).

At the current stage of development, IMC-ELISA without prior sample enrichment does however have some serious limitations. For the assay to be of commercial value, the detection limit needs to be much lower than that found in our studies e.g., 10^4-10^6cfu/ml. It has been shown that levels as low as 10^2 cfu/ml, can be indicative of substantial cross-carcass

contamination. The lack of sensitivity with lower concentrations of *Salmonella* (10^0-10^3 cfu/ml) may likely be related to background obscuration associated with cross-reactivity between assay reagents and/or CRF components like proteins, fats, and cellular debris. Attempts to increase sensitivity by including a tertiary antibody-conjugate in the assay were counterproductive. We therefore attempted to optimize the assay conditions using only a primary antibody, and secondary conjugate along with the Dynal® anti-*Salmonella* beads. Despite numerous variations of antibody and bead concentrations, considerable background (mean background absorbance values: .042 for BPW, .026 for CRF) still remained. On the other hand, unlike some assays (Tsai and Slavik, 1993) our data seems to suggest that the cross-reactivity associated with CRF components is minimal. It is presumably due to such antibody and CRF component interactions, that enzyme immunoassays like *Salmonella*-TEK® (Organon Teknika, Durham, NC) and 1-2 Test® (Biocontrol, Bothell, WA) exhibit a fair percentage of false-positives and negatives (Bailey *et al*., 1991). Further tests are therefore necessary to determine if IMC-ELISA is truly superior with respect to CRF associated background and whether additional reagent variations including changes in the antibody source could help improve sensitivity.

In regards to specificity, IMC-ELISA without enrichment also has some problems. Cross-reactivity with other members of the family Enterobacteriaceae is clearly apparent. Other bacteria yet untested, may also cross-react. This is most likely due to non-specific binding to the conjugated paramagnetic beads and thus suggests the need for additional studies evaluating other, more specific monoclonal conjugates.

Finally, some explanation of the disparity between IMC-ELISA results and direct plating of artificially contaminated CRF and commercial CRF is necessary. According to IMC-ELISA, commercial CRF samples contained significant numbers of *Salmonella* yet, direct plating revealed no contamination. Two explanations for these results are plausible: 1) additional proteins, fats, and cellular debris, by virtue of type or concentration, may have been present in the commercial samples and not in the CRF samples prepared in our laboratory, 2) sodium tri-phosphate, an antibacterial rinse present in the commercial samples, may have inhibited bacterial growth on the plates but not affected the detection of live or dead *Salmonella* by IMC-ELISA. Of the two, the later is the most likely.

In summary, it would appear that IMC-ELISA without prior enrichment does have potential application for the detection of *Salmonella* in commercial poultry processing. It

certainly meets some of the desired criteria, but falls fatally short with regard to its level of detection. Although currently AOAC (http://www.aoac.org/) approved for use with enriched samples, continued work to improve its sensitivity will obviously be necessary if it is to be of any use as a near-on-line detection method.

CHAPTER 3:

DEVELOPMENT AND APPLICATION OF IMC-PCR AND IMC-TAQMAN® PCR TO DETECT SALMONELLA IN POULTRY CARCASS RINSE SAMPLES.

3.1 Abstract

In order to evaluate the applicability of IMC-PCR and IMC-Taqman® PCR to poultry processing facilities, dilutions of pure broth culture of *Salmonella* Typhimurium, artificially contaminated carcass rinse fluid (CRF), and commercial CRF were tested. In a preliminary study, it was shown that IMC-PCR and IMC-Taqman® PCR were able to detect 10^6 cfu/ml in artificially contaminated commercial CRF. Experimental results showed that both detection methods were highly specific, as they were able to differentiate *Salmonella* from other members of family Enterobacteriaceae. The results also demonstrated that IMC-PCR was less sensitive (10^4 cfu/ml) compared to IMC-Taqman® PCR, which was able to consistently detect as low as 10^3 cfu/ml of *Salmonell*a in artificially contaminated CRF samples. In a few artificially contaminated CRF samples, IMC-Taqman® PCR was able to detect concentrations lower than 10^3 cfu/ml of *Salmonella*. From the experimental results, it can be concluded that IMC-Taqman® PCR was more sensitive than IMC-PCR in artificially contaminated CRF. Results of a paired T test analysis (p=.2227) show that there were no significant differences in the detection limits between IMC-Taqman® PCR results from the artificially contaminated CRF and *Salmonella* culture dilutions. In addition, IMC-PCR was more time consuming (4 hours) compared to IMC-Taqman® PCR, which only required three hours. These results not only confirm findings of others but also suggest that both detection methods have attributes which may make them useful in poultry processing facilities.

3.2 Introduction

There are a growing number of bacterial detection methods utilizing polymerase chain reaction (PCR) technology because of its sensitivity and specificity. PCR, a method which detects the presence of nucleic acids, is neither dependent on substrate utilization nor the

expression of antigens. Therefore, phenotypic variations seen in both biochemical tests and lack of detectable antigens in antibody based assays do not affect the results of such nucleic acid based PCR assays (Hoorfar *et al*., 1999).

A number of researchers have developed PCR-based methods for the detection of *Salmonella* in various types of food samples. Most, if not all, have required at least a 4 to 24 hour pre-enrichment prior to PCR. Aabo *et al*. (1993) tested 116 serovars of *Salmonella* cultures using a PCR method. The method was able to distinguish *Salmonella* serovars from 86 other non-*Salmonella* species belonging to the family Enterobacteriaceae. A similar PCR-based detection method for *Salmonella* in stomached chicken meat was developed by Fluit *et al.* (1993) that involved amplifying a portion of the origin of replication of *Salmonella*, *oriC* gene. However, a 24 hour sample enrichment was required to obtain a sensitivity of 0.1 cfu/g detection limit. Jitrapakdee *et al*. (1995) developed a PCR method for the detection of 52 of the most prevalent serovars of *Salmonella* isolated from poultry. The assay was extremely sensitive and the limit of detection in contaminated frozen chicken was 3.0 cfu/g of chicken meat. However, a 6 hour enrichment step was required prior to the PCR assay.

Investigators have developed PCR detection methods focused on the genome of *Salmonella* species. Cohen *et al*. (1996) successfully utilized primers complementary to part of the *fim*A gene of *S.* Typhimurium. Stefanovicora *et al.* (1998) developed a PCR method employing primers ST11 and ST15. Their results completely agreed with those of conventional biochemical and serological classification in terms of distinguishing between *Salmonella* species and other *Enterobacteriacae*. Lin and Tsen (1999) developed a PCR-based test for the *mdh* gene which codes for malic acid dehydrogenase in *S.* Typhimurium, however, this required an 8 to 12 hour enrichment step Wang *et al*. (1997) were able to detect as few as 40 *Salmonella* cells using a universal PCR detection protocol, but this assay lacked specificity in that amplification also occurred with 12 other common types of bacteria. Manzano *et al*. (1998) tested 75 food samples (5 different types) using PCR amplification of a 389 base pair section of the *inv*A gene. One disadvantage of this method was that it required an overnight enrichment to generate positive results. Kantama *et al*. (1998) were able to detect the most common *Salmonella* serotypes using two sets of primers in a nested PCR reaction. These same primers also amplified DNA from *Citrobacter* and *Shigella* species. However, the amplicons from *Citrobacter* and *Shigella* could easily be differentiated from *Salmonella* products by gel electrophoresis to determine amplicon

sizes (Rijpens *et al*., 1999). Khan *et al*. (1999) developed a PCR-based method capable of detecting *S.* Typhimurium DT104. They were able to identify twenty-two ACSSuT resistant DT104 isolates accurately using gene specific primers.

Additional PCR methods have been developed and determined to be highly specific for *Salmonella*, however, assays continued to require enrichment steps, which lengthened the time required for the detection of *Salmonella*. Nogva and Lillehaug (1999) adapted a 5'-nuclease-based kit to quantify *Salmonella* in pure cultures. This method was based on a reverse transcription-polymerase chain reaction (RT-PCR) and their results completely concurred with those of conventional biochemical and serological classification. One of the negative aspects of this assay was that it required a total of 28 hours to complete. A RT-PCR detection method developed by Burtscher *et al*. (1999) was able to detect viable *S.* Enteritidis PT4 in artificially contaminated minced beef and whole egg samples. However, the method required a 16 hour overnight enrichment to detect 10 cfu/ml of *S.* Typhimurium from organic waste using one minicolumn, PCR, and gel electrophoresis. Chen *et al*. (1997a) used the TaqMan LS-50B PR detection system, which allowed for simultaneous amplification and detection of *Salmonella* in artificially contaminated CRF. The actual concentrations of *Salmonella* used in their studies were not indicated, making conclusions difficult. The test required an overnight pre-enrichment at 37°C prior to PCR. There was a ≥ 98% correlation between this method and that of the conventional modified semi-solid rappaport vassiliadis culture method (Chen *et al*., 1997a). Other researchers have developed PCR detection methods with similar outcomes (Chen *et al*., 1997b; Heid *et al*., 1996; Carlson *et al*., 1999).

In order to minimize the amount of time required for detection, researchers have used immunomagnetic separation prior to PCR to rapidly concentrate bacteria from samples instead of an overnight enrichment step. Mansfield and Forsythe (1993) used immunomagnetic separation to rapidly detect *Salmonella* from field samples and compared immunomagnetic separation to standard enrichment methods. Mansfield and Forsythe (1996) compared conventional enrichment with immunomagnetic separation using Dynabeads® coated with anti-*Salmonella* antibodies. The results validated the use of immunomagnetic separation as an alternative to enrichment for *Salmonella* isolation.

The high output of modern poultry processing facilities and rapid distribution of potentially contaminated products over a wide geographical area has increased the chance of

major food poisoning outbreaks. Hazard Analysis Critical Control Point (HACCP) regulations are used to monitor the safety of food products as they are processed. With the current HACCP regulations in place, the responsibility for controlling foodborne pathogens resides squarely with the industry. As the processors demonstrate their ability to meet the HACCP regulations and have less than or equal 20% (one in five) contamination levels, the government is expected to lower its acceptable rate of contamination of processed chicken from 20 to 10 % in the near future (Major, 2000). These factors necessitate the development of more rapid and accurate detection methods for food borne pathogens like *Salmonella*. Previous published methods for *Salmonella* detection in CRF lack sensitivity, require at least one enrichment step, and are time consuming.

The primary objective of this thesis research was to optimize IMC-PCR and IMC-Taqman® PCR to detect *S.* Typhimurium in CRF without enrichment. Initially, it was evaluated using pure culture dilutions of *S.* Typhimurium. The second objective was to determine the sensitivity and specificity of IMC-PCR and IMC-Taqman® PCR for the detection of *S.* Typhimurium in artificially contaminated CRF. Finally, the last objective was to evaluate the optimized IMC-PCR and IMC-Taqman® PCR assays' ability to detect *Salmonella* in commercial CRF.

3.3 Material and Methods

Preparation of *S.* Typhimurium from pure culture

Log phase cultures of naladixic acid resistant *S.* Typhimurium obtained from N. Sriranganathan, Virginia Tech were prepared from frozen stock. Serial dilutions were then made in 1.0 ml of cold buffered peptone water (BPW, whole carcass rinse buffer, USDA, 1996). Samples used for IMC-PCR and IMC-Taqman® PCR evaluations contained final concentrations of 10^1, 10^2, 10^3, 10^4,10^5, and 10^6 cfu/ml as determined by standard dilution plate method.

Preparation of artificially contaminated chicken CRF

Whole chicken carcasses were purchased at a local retailer. Each was placed in a large (3500 ml) sterile bag and 400 ml of pre-chilled BPW was poured into the carcass cavity. The carcass was rocked slowly back and forth for one minute so that all surfaces were rinsed with buffered peptone water. The carcass was then removed from the bag and multiple 1.0 ml aliquots of the carcass rinse fluid (CRF) were collected and stored frozen at – 80°C until needed. (*Salmonella* Analysis, Food Safety and Inspection Service, 1997). Serial dilutions were then prepared as previously described for pure cultures with 1.0 ml of thawed CRF instead of BPW. These samples were then tested using the IMC-PCR and IMC-Taqman® PCR in three separate runs.

Commercial CRF

CRF samples from a commercial processing facility containing unknown amounts of *Salmonella* were shipped overnight on ice. Fifteen out of the 17 samples submitted were randomly selected from which multiple 1.0 ml aliquots were taken and frozen at -80°C. Prior to freezing, 3 samples were randomly chosen and 100 µl from each were spread onto Hektoen enteric (HE) agar, bismuth sulfite (BS) agar, and xylose lysine tergitol (XLT-4) agar plate. These were incubated for 24 hours at 35°C. Colonies were then counted to determine the number of cfu/ml. After freezing, the same three samples were thawed and 100 µl were again plated to determine the viable cfu/ml after one freeze-thaw cycle. Finally, thawed samples were tested using the IMC-PCR and IMC-Taqman® PCR in 3 separate runs.

Immunomagnetic separation of *Salmonella* from poultry whole CRF samples using paramagnetic beads coated with anti-*Salmonella* antibodies

Each 1.0 ml sample was centrifuged at 4,000 x g for 20 minutes. The supernatants were discarded, and the pellets were resuspended in 300 µl of PBS. One hundred-fifty micrograms (15µl per 1 ml sample) of antibody-coated beads (Dynal® anti-*Salmonella* paramagnetic beads) were then added to each resuspended pellet and incubated at room temperature with gentle

rotation for 20 minutes using a Dynal sample mixer®. Using a MPC-M® magnet to secure the beads, the supernatant was removed and the beads washed twice with 300 µl of PBS. They were then resuspended in 50 µl of PBS in 1.5 ml tubes.

DNA preparation for PCR

The 1.5 ml tubes containing the 50 µl of PBS/beads/ *Salmonella* complex were placed in the MPC-M® magnet and after concentration of the beads, the supernatant was discarded. Two hundred microliters of Instagene Matrix® were added to each tube, vortexed, and incubated at 56°C for 25 minutes. After vortexing, the tubes were placed in a boiling water bath for 8 minutes. Each sample was then vortexed for ten seconds and centrifuged at 13,000 rpm for three minutes. The supernatant containing DNA was transferred to clean tubes and used for PCR reactions or frozen at –20°C for later use. If frozen, the supernatant, was vortexed and centrifuged for five minutes at 16,000 x g to further remove water-insoluble impurities before PCR was conducted (Chen *et al*., 1997). Frozen template was used within a week to guarantee minimal degradation of the DNA.

Development of IMC-PCR and IMC-Taqman® PCR

Many factors were optimized for successful amplification of the desired DNA sequences. The specificity of the PCR reaction was influenced most by the specificity of the primers for the target DNA sequence. After extensive review of current literature, primers were chosen from the sequence of *S.* Typhimurium ATCC 23566 used previously by Kantama *et al*. (1998) when developing a detection probe for *Salmonella* from food. Initially two sets of primers were chosen for IMC-PCR.

The first set of primers for IMC-PCR was:
Forward primer: 5'ATCCCCGCCATCACGCCGCCTAAC3'
Reverse primer: 5'ATTTTGATGGCGCTTACGGGGCTGGATG3'.
The second pair of primers for IMC-PCR was:
Forward primer: 5'CCAAATATCCCCATCAGGCCCAGCAGGTT3'

Reverse primer: 5'GCCAGGGAAAAACTCGCCGCTCATCA3'**.**

Concentration of template DNA and the annealing temperature were optimized first. Concentrations of template DNA extracted from 10^4 to 10^6 cfu/ml of *S*. Typhimurium with annealing temperatures of 45°C, 55°C, and 65°C were tried using 40 cycles. Each cycle consisted of a denaturation step (94°C) for 30 seconds, annealing step (45°C, 55°C, or 65°C) for 30 seconds, and a 1 minute extension (72°C). To generate positive results, the time for each temperature within the 40 cycles was extended. Each set of primers was tested. Optimal conditions for IMC-PCR consisted of 40 cycles of 94°C (denaturation) for 1 minute, 65°C (annealing) for 1 minute, and 72°C (extension) for 2 minutes. A five minute enzyme activation was incorporated before the 40 cycles and a 7 minute extension (72°C) followed the 40 cycles.

After optimization of the standard PCR was complete, only a few modifications were necessary to achieve success with IMC-Taqman® PCR. Primers and probe were chosen from the same sequence as the standard PCR primers. Perkin Elmer's Primer Express® software was used to identify the most appropriate primer and probe sequences. Taqman® chemistry required production of amplicons less than 200 base pairs because the efficiency of this type of reaction increased as the size of the amplicon decreased. The standard PCR primers produced a 554bp amplicon and the amplicons were larger than what was recommended for the Taqman® reaction. Therefore, a new pair of primers and probe were designed and purchased from Perkin Elmer (Foster City, CA) (see IMC-Taqman PCR section).

DNA extracted from whole cell lysates from the serial dilutions containing 0 to 10^6 cfu/ml *S.* Typhimurium were used to generate a standard curve (three replicates). One replicate of each of the standard curve concentrations was included as positive control, while testing artificially contaminated CRF and commercial CRF. This method allowed for relative quantification of the artificially contaminated CRF and the commercial CRF.

IMC-PCR

The second primer pair from our PCR experiments was used in all of the IMC-PCR sample testing, as it was able to generate amplicons consistently. This primer pair generated a 554 bp amplicon. PCR reagents were purchased from Perkin Elmer Biosystems (Foster City,

California). Each PCR reaction contained 5 µl Gold buffer (10X), 5 µl 25 mM $MgCl_2$, 4 µl 10 mM dNTP, 0.25 µl Amplitaq DNA polymerase, 2 µl of each primer, and 11.75 µl dH_2O. Twenty µl (50 to 100 ng) of DNA template from the previous extractions, were used in each reaction. PCR reactions were performed using Perkin Elmer 9700 thermalcycler®. A 5 minute enzyme activation at 95°C was followed by 40 cycles of 94°C for 1 minute, 65°C for 1 minute, 72°C for 2 minutes. The 40 cycles were followed by a 72°C extension for 7 minutes and finally a 4°C hold. Ten µl of each PCR reaction were loaded onto a 1% agarose gel and electrophoresed for one hour at 100 volts. The amplicon sizes were determined by comparing them with a standard 100 base pair molecular weight marker ladder run at the same time.

IMC-Taqman® PCR

Primers and probe for the IMC-Taqman® PCR were chosen from the same DNA sequence used for the design of primers for the conventional PCR. The following sequences were identified using the Perkin-Elmer Primer Analysis® software:

Forward primer: 5'CATCAGGCCCAGCAGGTTAG 3'

Reverse primer: 5' TTATCGCATCCGGCTTGATT 3'

Probe: 5' FAM-TCGCCGACGATATCCGGACGTTTA-TAMRA 3'.

FAM (6-carboxy-fluorescein) was the reporter dye, while TAMRA (6-carboxy-tetramethylrhodamine) was the quencher dye. Primers and probe were purchased from Perkin Elmer Biosystems® (Foster City, CA).

Taqman® PCR reactions were performed using the Perkin Elmer 7700 Sequence Detection System® thermalcycler. Each 50 µl total volume PCR reaction contained 25 µl Universal Taqman Master Mix®, 4.0 µl of 900 nM forward primer, 4.0 µl of 900 nM reverse primer, and 2 µl of 200nM probe. Twenty µl (50 to 100 ng) of DNA template were used in each reaction. The following conditions were used for the Taqman® PCR reactions: 2 minute hold at 50°C, 10 minute hold at 95°C, 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. The 45 cycles were followed by a 4°C hold.

Samples of pure culture of *S.* Typhimurium, artificially contaminated CRF, and commercial CRF were analyzed using IMC-Taqman® PCR. Relative cfu/ml of *Salmonella* in CRF were determined using the standard curve generated with known concentrations of

Salmonella pure culture (0 to 10^6 cfu/ml). Artificially contaminated CRF and commercial CRF were analyzed on three separate runs, along with one set of samples of the pure culture dilutions (standard curve concentrations) to allow for the determination of relative cfu/ml of *Salmonella* in each CRF. Standard curves were generated by plotting the known cfu/ml (concentrations) of *Salmonella* versus the cycle threshold values. The artificially contaminated CRF and commercial CRF were labeled as unknowns. Unknown samples were run with standard pure culture samples and unknown sample concentrations were determined by interpolation of the cycle threshold values against the pure culture concentrations. A paired T-test from Jmp® software (Cary, NC) was used to analyze the data. Two commercial CRF were artificially contaminated with 10^6 cfu/ml *S.* Typhimurium to determine if the CRF contained any Taqman® PCR inhibitors.

Analysis of non-specific binding using common poultry contaminants

Escherichia coli, Citrobacter freundi, Enterobacter cloacae, Klebsiella pneumoniae, Proteus mirabilis, Shigella sonnei, Vibrio alginolyticus, and *Yersinia enterocolitica* were grown separately on LB plates. Cells were harvested and resuspended in BPW with 2% glycerol. Two hundred and fifty µl aliquots were frozen overnight at –80°C. After thawing, serial dilutions were plated on LB plates and incubated at 37°C overnight to determine the concentration of cells in the frozen aliquots. For specificity trials, pure cultures were serially diluted to a final concentration of 1 X 10^6 cfu/ml and then run in duplicate using the IMC-PCR and IMC-Taqman® PCR.

Statistical analysis

Mean absorbence and standard errors of the means were calculated using Jmp® statistical software. Best fit curves were then generated for pure culture and artificially contaminated CRF using Microsoft Excel®. Estimated contamination levels of the commercial CRF were determined based on the slope of the standard curve for the artificially contaminated CRF samples.

Direct plating and enrichment

A standard spread plate method was initially employed to determine the cfu/ml of *Salmonella* in log phase cultures. Once this was established and 1 ml aliquots prepared, 100 µl from dilutions containing 10^0, 10^1, 10^2, cfu/ml, 50 µl from those containing 10^3 cfu/ml and 20 µl from those containing10^4, 10^5, and10^6 cfu/ml were spread onto on to HE agar, BS agar, and XLT-4 agar. *Salmonella* appeared as blue/green colonies with black centers on HE agar. *Salmonella* appeared as brown, gray, or black colonies with or without a metallic sheen on BS agar. Colonies on XLT-4 appeared light brown with black centers. Occasionally, colonies were entirely black.

Enrichment was performed in the following manner. One ml of each sample was added to 9 ml BPW in sterile screw cap 15 ml plastic tubes. The tubes were incubated at room temperature for 60 minutes. Depending on the initial cfu/ml, a specified volume (100 µl, 50 µl, 20 µl) was spread onto HE agar, BS agar, and XLT-4 agar plates. After an additional 24 hours of incubation at 35°C, 0.1 ml was transferred to Rappaport-Vassiliadis (RV) medium and 1.0 ml was transferred to tetrathionate (TT) broth. The tubes were then incubated in a 43°C water bath for 24 hours. Finally, depending on the original sample concentration, 100 µl , 50 µl, or 20 µl were spread onto HE, BS, and XLT-4 agar plates and incubated at 35°C for 24 hours. Typical *Salmonella* colonies were then enumerated (Bacteriological Analytical Manual, AOAC International, 1998).

Direct plating and enrichment for artificially contaminated CRF samples was performed in a manner identical to that for pure cultures.

For commercial CRF samples, direct plating and enrichment was performed in the following manner. One hundred µl from each of the 15 samples were plated onto HE, BS, and XLT-4 agar plates. All plates were incubated for 24 hours at 35°C and the colonies were counted. Enrichment of the commercial CRF samples was identical to that used for pure cultures and artificially contaminated CRF samples.

3.4 Results

Development of IMC-PCR

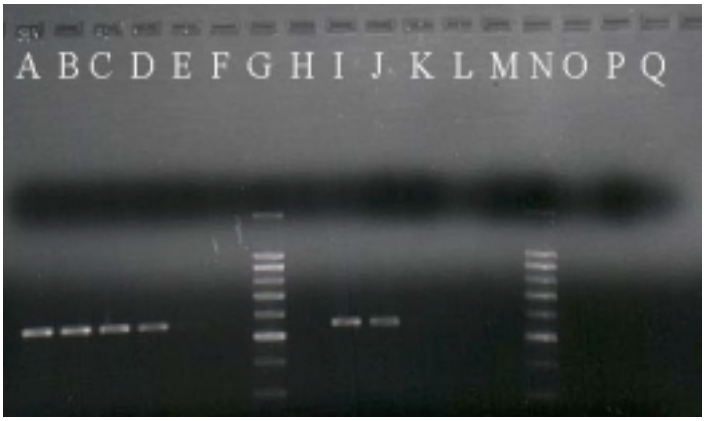
Initial PCR reactions, using annealing temperatures of 45°C, 55°C, and 65°C and 40 cycles of 94°C (denaturation) for 30 seconds, specific annealing temperature for 30 seconds, and 72°C (extension) for 1 minute, did not yield an amplicon. After increasing the times of the three temperatures (denaturation for 1 minute, annealing for 1 minute, extension for 2 minutes) within the 40 cycles, the PCR reactions yielded an amplicon of the expected size. The second set of primers consistently produced amplicons of the expected size whereas the first pair of primers inconsistently produced amplicons. A five-minute enzyme activation at 95°C preceding the 40 cycles was shown to prevent non-specific binding due to gradual activation of the DNA polymerase. A seven-minute extension following the 40 cycles produced optimal results. The annealing temperature of 65°C was shown to be optimal.

IMC-PCR

Standard PCR of dilutions of *S.* Typhimurium pure culture was performed with each set of artificially contaminated CRF samples and commercial CRF samples. Results of the experiments performed on three separate runs are shown in Figures 3.1a, 3.1b, and 3.1c. The results clearly demonstrate that IMC-PCR consistently detected *S.* Typhimurium concentrations as low as 10^3 cfu/ml in pure culture. In two of the three replicates, IMC-PCR was able to detect 10^2 cfu/ml in pure culture (figure 3.1b, lane E and figure 3.1c, lane E). In one of the three replicates, IMC-PCR was able to detect 10^1 cfu/ml (figure 3.1c, lane F) but this was not reproducible. In two of the three trials evaluating artificially contaminated CRF samples, IMC-PCR detected 10^4 cfu/ml (figure 3.1b, lane K and 3.1c, lane K). Figure 3.1c also shows a faint band in lane L, demonstrating the detection of 10^3 cfu/ml in artificially contaminated CRF. The same figure also contains a faint band in lane O, indicating the ability of IMC-PCR to detect 10^1 cfu/ml in artificially contaminated CRF.

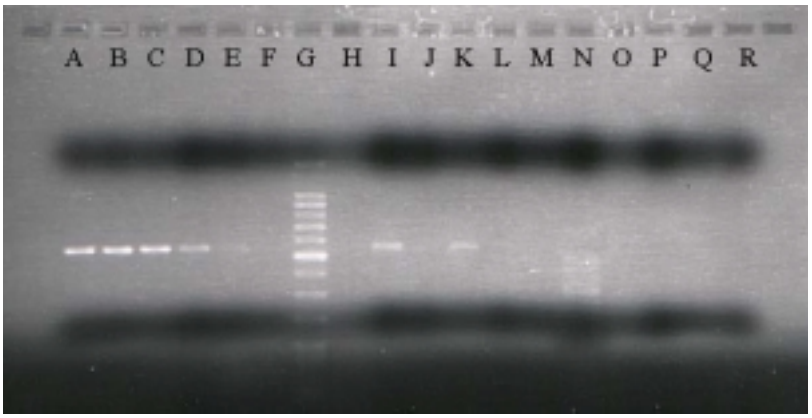
Commercial CRF samples did not yield any amplicons in our IMC-PCR assay (see figure 3.2, lanes G, H, J, K and L and figure 3.3, lanes A, B, C, D, E, F, H, I, J, and K). The 10^3 cfu/ml

pure culture dilution was accidently missed during the PCR reaction set up, so there is no band in lane D of figure 3.2, as it now represents a concentration of 10^2 cfu/ml. IMC-PCR was repeated twice using the same commercial CRF samples. The results were identical to the first set of PCR results (data not shown). IMC-PCR of artificially contaminated (10^6 cfu/ml *S.* Typhimurium*)* commercial CRF samples resulted in amplicons of the expected size of 554 bp in 6 out of 6 reactions (figure 3.3, lanes L and M), suggesting that there were no negative effects of possible PCR inhibitors in the commercial CRF at this concentration level.



A= 10^6 cfu/ml PC
B= 10^5 cfu/ml PC
C= 10^4 cfu/ml PC
D= 10^3 cfu/ml PC
E= 10^2 cfu/ml PC
F= 10^1 cfu/ml PC
G= 100 bp DNA ladder
H= 0 cfu/ml
I= 10^6cfu/ml AC
J= 10^5 cfu/ml AC
K= 10^4 cfu/ml AC
L= 10^3 cfu/ml AC
M= 10^2 cfu/ml AC
N= 100 bp DNA ladder
O= 10^1 cfu/ml AC
P= 0 cfu/ml AC
Q= water control

Figure 3.1a: Detection of *S.* Typhimurium in dilutions of pure culture (PC) and artificially contaminated (AC) CRF samples using IMC-PCR.



A= 10^6 cfu/ml PC
B= 10^5 cfu/ml PC
C= 10^4 cfu/ml PC
D= 10^3 cfu/ml PC
E= 10^2 cfu/ml PC
F= 10^1 cfu/ml PC
G= 100 bp DNA ladder
H= 0 cfu/ml
I= 10^6cfu/ml AC
J= 10^5 cfu/ml AC
K= 10^4 cfu/ml AC
L= 10^3 cfu/ml AC
M= 10^2 cfu/ml AC
N= 100 bp DNA ladder
O= 10^1 cfu/ml AC
P= 0 cfu/ml AC
Q= water control
R= empty well

Figure 3.1b: Detection of *S.* Typhimurium in dilutions of pure culture (PC) and artificially contaminated (AC) CRF samples using IMC-PCR.

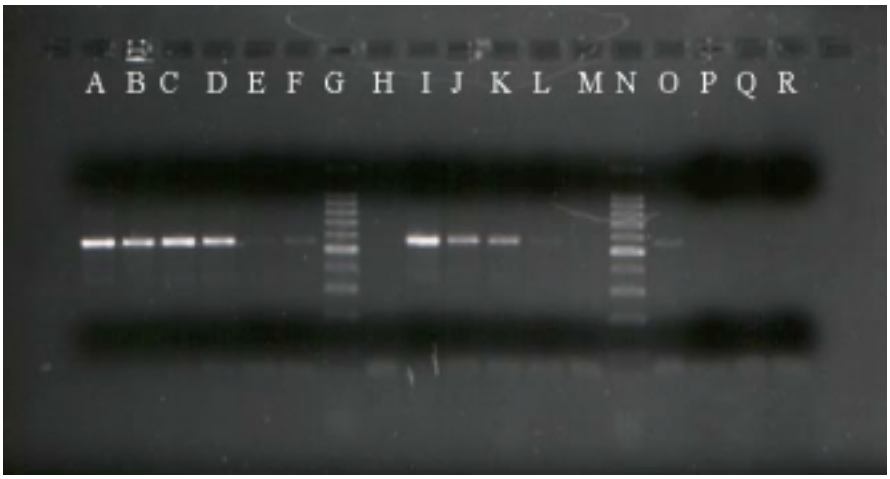


Figure 3.1c: Detection of *S.* Typhimurium in dilutions of pure culture (PC) and artificially contaminated (AC) CRF samples using IMC-PCR.

A= 10^6 cfu/ml PC
B= 10^5 cfu/ml PC
C= 10^4 cfu/ml PC
D= 10^3 cfu/ml PC
E= 10^2 cfu/ml PC
F= 10^1 cfu/ml PC
G= 100 bp DNA ladder
H= 0 cfu/ml
I= 10^6cfu/ml AC
J= 10^5 cfu/ml AC
K= 10^4 cfu/ml AC
L= 10^3 cfu/ml AC
M= 10^2 cfu/ml AC
N= 100 bp DNA ladder
O= 10^1 cfu/ml AC
P= 0 cfu/ml AC
Q= water control
R= empty well

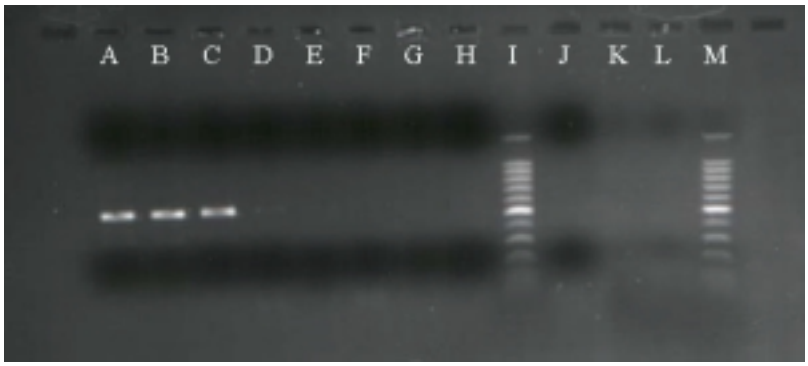
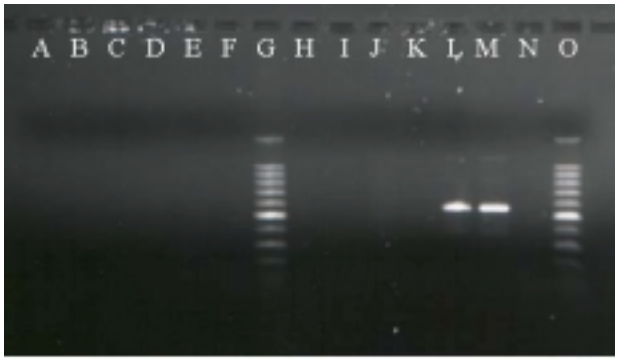


Figure 3.2: Detection of *S.* Typhimurium in dilutions of pure culture (PC) and commercial (CR) CRF samples using IMC-PCR.

A= 10^6 cfu/ml PC
B= 10^5 cfu/ml PC
C= 10^4 cfu/ml PC
D= 10^2 cfu/ml PC
E= 10^1 cfu/ml PC
F= 0 cfu/ml PC
G= CR #1
H= CR #2
I = 100 bp DNA ladder
J= CR #3
K= CR #4
L= CR #5
M= 100 bp DNA ladder



A= CR #6
B= CR #7
C= CR #8
D= CR #9
E= CR #10
F= CR #11
G= 100 bp DNA ladder
H= CR #12
I= CR #13
J= CR #14
K= CR #15
L= CR #16 with 10^6 cfu/ml
M= CR #17 with 10^6 cfu/ml
N= water
O= 100 bp DNA ladder

Figure 3.3: Detection of *S.* Typhimurium in commercial CRF samples and inoculated commercial CRF samples using IMC-PCR.

Analysis of non-specific binding using common poultry contaminants

Agarose gel electrophoresis of PCR products using *Escherichia coli, Citrobacter freundii, Enterobacter cloacoe, Klebsiella pneumoniae, Proteus mirabilis, Shigella sonnei, Vibrio alginolyticus, Yersinia enterocolitica, and Campylobacter jejuni* DNA extracts were all consistently negative, as they did not generate any amplicons (data not shown).

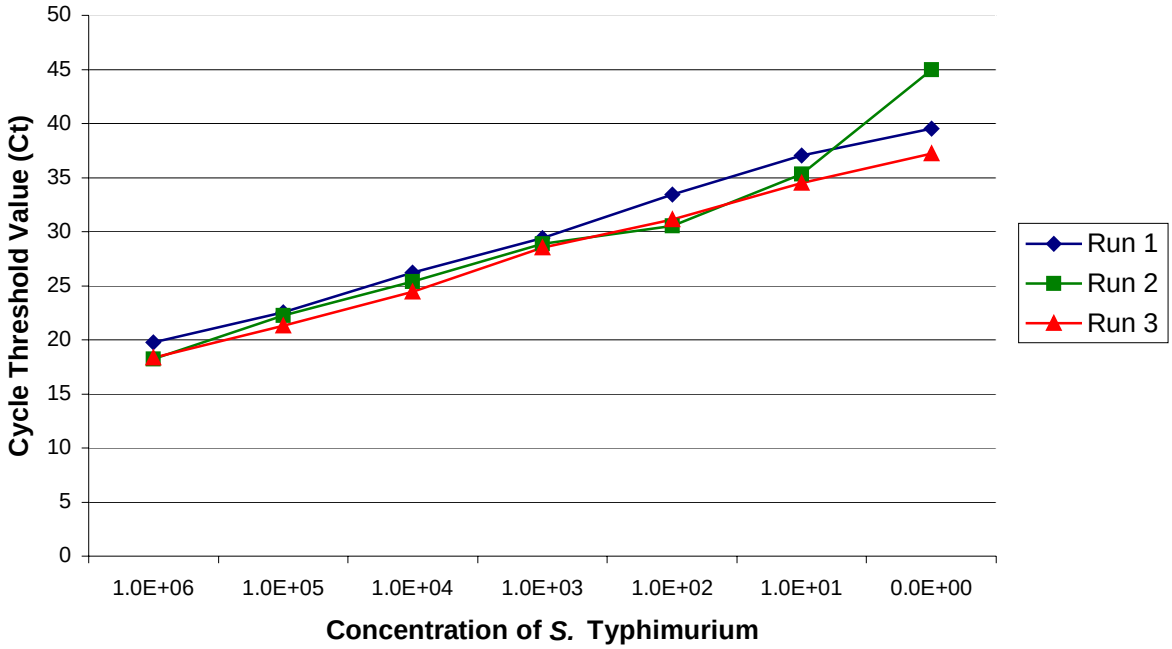
IMC-Taqman® PCR

Figure 3.4 shows the standard curves generated by 3 runs of IMC-Taqman® PCR using pure culture dilutions (0 to 10^6 cfu/ml *S.* Typhimurium). Cycle threshold (Ct) values, located on the y-axis, are the cycle number at which the Perkin Elmer 7700 System® detected an increase of fluorescence over background readings during the PCR reaction. Table 3.1 contains the data used to generate Figure 3.4 as well as the mean Ct values and the standard error of the mean Ct values at each concentration of *S.* Typhimurium. Higher initial amounts of target DNA allowed for greater binding and hydrolysis of the Taqman® probes. Thus, higher concentrations of target

DNA yielded increased emission of fluorescence at lower Ct values than those obtained when samples contained fewer copies of target DNA. The correlation coefficient of R= 0.957 for the three curves indicates the high degree of similarity between the curves. Figure 3.5 is a graph comparing the mean pure culture standard curve to the mean Ct values generated from the artificially contaminated trials using IMC-Taqman® PCR. The correlation coefficient comparing these curves to each other is R=0.989. Again, this indicates that the data generated from the pure culture dilutions is similar to the data from the artificially contaminated CRF samples. Artificially contaminated CRF samples' estimated concentrations and cycle threshold values with their mean values and standard error of the mean values are shown in Table 3.2. A paired T-test from Jmp software used to compare the differences in mean Ct values for pure culture samples and artificially contaminated samples did not indicate any significant differences (p=.2227). These results suggest that there were no significant differences in the amount of *S.* Typhimurium detected in the pure culture samples as compared to the artificially contaminated samples.

Commercial CRF samples with pure culture concentration samples were analyzed using IMC-Taqman® PCR. The Ct value and estimated concentrations, including the mean values, are listed in Table 3.3. This data was collected during two separate runs. Although, these results suggest that there was a low level of *Salmonella* present in some of the samples, there was a high degree of variability in the Ct values generated for each sample from the three runs. IMC-Taqman® PCR was able to detect *Salmonella* from the artificially contaminated (10^6 cfu/ml) commercial CRF samples 16 and 17 (Table 3.3).

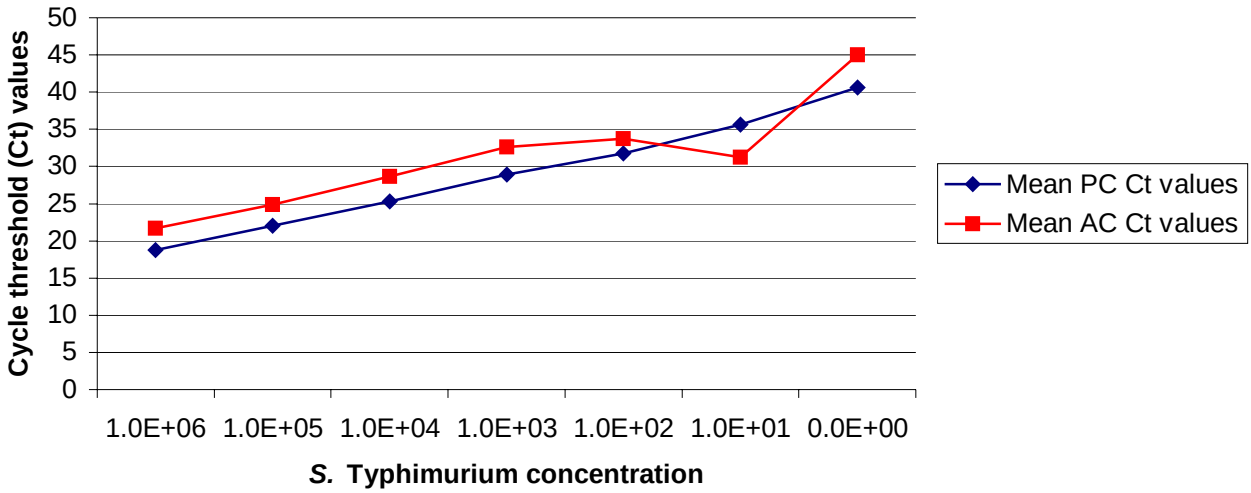
Figure 3.4: Standard curves from pure culture dilutions of *S.* Typhimurium using IMC-Taqman PCR



Salmonella (cfu/ml)	Cycle threshold values			Mean Ct values	Standard error of mean Ct values
	Run 1	Run 2	Run 3		
1.0 X 10^6	19.74	18.23	18.32	18.79	±.489
1.0 X 10^5	22.54	22.26	21.28	22.03	±.382
1.0 X 10^4	26.19	25.36	24.42	25.32	±.511
1.0 X 10^3	29.43	28.85	28.55	28.94	±.258
1.0 X 10^2	33.45	30.56	31.14	31.72	±.883
1.0 X 10^1	37.02	35.33	34.49	35.61	±.744
0	39.53	45	37.2	40.58	±2.31

Table 3.1: IMC-Taqman® PCR cycle threshold (Ct) values and mean Ct values with standard error of the mean values from pure culture dilutions

Figure 3.5:Pure culture (PC) standard curve vs. artificially contaminated standard curve using IMC-Taqman PCR



Initial (cfu/ ml)	Run 1		Run2		Run 3		Mean		SEM of Ct value
	(cfu/ml)	Ct value	(cfu/ml)	Ct value	(cfu/ml)	Ct value	(cfu/ml)	Ct value	
10^6	1.2 X 10^6	21.69	6.7 X 10^5	22.02	2.4 X 10^5	21.31	7.0 X 10^5	21.67	±.205
10^5	1.1 X 10^5	25.03	7.6 X 10^4	25.12	2.3 X 10^4	24.48	6.9 X 10^4	24.87	±.200
10^4	7.5 X 10^4	28.8	9.1 X 10^3	28.13	7.6 X 10^3	29.08	3.1 X 10^4	28.67	±.281
10^3	6.0 X 10^2	32.36	4.1 X 10^2	32.54	4.2 X 10^2	32.99	4.7 X 10^2	32.63	±.187
10^2	1.4 X 10^2	34.44	3.8 X 10^2	32.67	1.8 X 10^2	34.15	2.3 X 10^2	33.75	±.548
10^1	1.7 X 10^3	30.86	3.4 X 10^2	32.76	3.3 X 10^3	30.23	1.7 X 10^3	31.28	±.760
0	0	45	0	45	0	45	0	45	±0

Table 3.2: IMC-Taqman® PCR estimated *Salmonella* concentrations (cfu/ml) and cycle threshold (Ct) values with standard error of the mean Ct values from artificially contaminated CRF samples

Carcass rinse #	Run 1		Run 2		Mean	
	cfu/ml	Ct value	cfu/ml	Ct valued	cfu/ml	Ct value
1	2.9×10^2	34.31	4.8×10^2	29.07	3.8×10^2	31.69
2	1.7×10^2	35.08	4.4×10^2	29.17	3.1×10^2	32.13
3	5.6×10^1	36.8	3.2×10^2	29.48	1.3×10^2	33.14
4	2.3×10^2	34.67	5.4×10^2	28.94	3.9×10^2	31.81
5	3.1×10^2	34.23	3.1×10^2	29.52	3.1×10^2	31.88
6	9.6×10^1	35.98	9.8×10^1	30.71	9.7×10^1	33.34
7	1.2×10^2	35.61	4.6×10^2	29.12	3.5×10^2	32.36
8	2.8×10^2	34.34	8.4×10^2	28.49	5.6×10^2	31.42
9	2.6×10^2	34.45	7.5×10^1	30.99	1.6×10^2	32.72
10	1.8×10^2	35.06	4.3×10^2	29.19	3.1×10^2	32.12
11	1.1×10^2	35.73	3.7×10^2	29.33	2.9×10^2	32.53
12	2.9×10^2	34.3	5.7×10^2	28.88	4.3×10^2	31.59
13	1.2×10^2	35.62	1.3×10^2	30.4	1.3×10^2	33.01
14	0	39.22	6.4×10^2	28.76	3.2×10^2	33.99
15	0	40.52	7.0×10^2	28.68	3.5×10^2	34.60
16	6.6×10^5	19.06	1.4×10^6	20.86	1.0×10^6	19.96
17	8.2×10^5	18.72	6.4×10^4	24.01	4.4×10^5	21.36
CRF 1-15 mean concentration and SEM = $3.0 \times 10^2 \pm 0.326$						
CRF 16 and 17 mean concentration and SEM = $5.0 \times 10^5 \pm 0.280$						

Table 3.3: IMC-Taqman® PCR estimated *Salmonella* concentrations (cfu/ml) from commercial CRF samples

Analysis of non-specific binding using common poultry contaminants

IMC-Taqman® PCR cycle threshold values for *E. coli, C. freundi, E. cloacae, K. pneumoniae, P. mirabilis, S. sonnei, V. alginolyticus,* and *Y. enterocolitica* are shown in Table 3.4. These results indicate a low level of non specific binding which would give a false positive result.

Bacteria name	Concentration (cfu/ml)	Cycle threshold
E. coli	2.8 X 10^1	33.25
C. freundi	1.1 X 10^1	34.61
E. cloacae	1.8	37.06
K. pneumoniae	1.5 X 10^1	34.13
P. mirabilis	6.8	35.21
S. sonnei	6.3	35.32
V. agonilyticus	5.4	35.53
Y. enterocolitica	2.3	36.7

Table 3.4: IMC-Taqman® PCR results of common poultry product contaminants

Direct plating and enrichment of *Salmonella* from BPW and CRF:

Results of the direct plate count of pure *S.* Typhimurium in BPW and artificially contaminated CRF samples are presented in Table 3.5. At concentrations between 10^4 and 10^6 cfu/ml, colonies were too numerous to count for both the pure culture dilutions in BPW and the artificially contaminated CRF samples. For dilutions between 10^1 and 10^3, colony counts were determined and suggested that fewer live *Salmonella* were actually available for immunomagnetic capture in the artificially contaminated CRF compared to pure cultures diluted in BPW. Direct plating of the fifteen commercial CRF samples did not reveal any evidence of viable *Salmonella*.

Enrichment of pure cultures and artificially contaminated CRF prior to plating produced colony numbers too numerous to count. Enrichment followed by plating on XLT-4 agar confirmed the lack of viable *Salmonella* in the 15 commercial CRF samples.

Sample type	Estimated *Salmonella* Concentration (cfu/ml)	Plate count at 24 hrs. (cfu/ml)
PC in BPW	1.0×10^3	1.8×10^3
	1.0×10^2	1.9×10^2
	1.0×10^1	1.0×10^1
	0	0
AC in CRF	1.0×10^3	4.4×10^2
	1.0×10^2	7.0×10^1
	1.0×10^1	1.0×10^1
	0	0

Table 3.5 Direct plate (XLT-4) count of pure culture (PC) *S.* Typhimurium and artificially contaminated CRF samples (AC)

3.5 Discussion

A comparison of the sensitivities, specificities, time requirements, and costs of IMC-PCR and IMC-Taqman® PCR are important considerations when determining a detection methods' applicability in a processing facility environment. While using S. Typhimurium pure culture, IMC-PCR was able to consistently detect 10^2 cfu/ml (4 out of 5 trials). This was the lowest dilution that consistently produced a faint amplicon. The results from IMC-Taqman® PCR showed a general trend of increasing error as indicated by the standard error of the mean values for pure culture dilutions less than 10^3 cfu/ml. However, the standard error values for each of the means do not overlap with other dilution means and their standard errors. Therefore, IMC-Taqman® PCR was able to distinguish between pure culture concentrations as low as 10^1 cfu/ml.

On the other hand, the lowest concentration that IMC-PCR was able to consistently detect in artificially contaminated CRF samples was 10^4 cfu/ml. IMC-Taqman® PCR was more sensitive than IMC-PCR, as it was able to detect as low as 10^3 cfu/ml in artificially contaminated CRF samples. This limit of detection was based on the fact that the mean Ct value for 10^1 cfu/ml was lower than the mean Ct value for 10^3 cfu/ml. The standard errors of the mean values for dilutions less than 10^3 cfu/ml did not reveal distinguishable differences between the lower concentrations of *Salmonella*. In addition, the Ct values for dilutions below 10^3 cfu/ml were not significantly different from each other (p=.33). This analysis seemed to contradict the Jmp® statistical analysis used to compare the differences of the mean values of the pure culture dilutions and artificially contaminated samples which indicated no significant differences. However, the Jmp® analysis compared only the differences between the means generated from each dilution (pure culture and artificially contaminated samples) to each other. It did not analyze the ability of IMC-Taqman® PCR to distinguish between individual dilutions. Therefore, detection limits were based on the amount of the standard error of the mean. The correlation coefficients calculated by the Taqman® software were generated using a similar comparison as the Jmp® results. It compared the similarity of the pure culture Ct values to the artificially contaminated CRF samples Ct values. The high correlation coefficients for the dilutions of pure culture *S.* Typhimurium and the artificially contaminated CRF samples suggested that a $\log_{10}$ plot was the best method for estimating concentrations using IMC-Taqman® PCR.

There are several factors that could have contributed to the inconsistent results of IMC-PCR and IMC-Taqman® PCR. As indicated in the results, IMC-PCR occasionally detected lower than 10^2 cfu/ml pure culture *S.* Typhimurium. The lack of consistency could be due to less efficient capture ability of the immunomagnetic beads at lower concentrations. Debris in the artificially contaminated CRF samples may have also attributed to the decrease in sensitivity of IMC-PCR at concentrations of 10^2 cfu/ml and lower. These same factors, the capture ability of the beads and debris, could be the reason for IMC-Taqman® PCR not being able to consistently detect less than 10^3 cfu/ml in artificially contaminated CRF samples. The estimated contamination levels of the artificially contaminated CRF samples (contaminated with 10^1 cfu/ml) produced by IMC-Taqman® PCR are higher than the pure culture concentration used to inoculate the samples (Table 3.1). A background level of flourescence could have caused the artificially elevated fluorescence readings. With fluorescence-based detection methods there is usually some "background" or "noise". Establishing a "cut-off" cycle threshold value just above the level of background would make it more clear when true amplification occurred. This "cut-off" cycle threshold value can be determined by the negative control trials involving other members of the family Enterobacteriaece (Table 3.4). The concentrations of other Enterobacteriaceae were 10^6 cfu/ml in the control assay. Their mean cycle threshold value (35.23) was higher than the mean cycle threshold value (20.66) for 10^6 cfu/ml of *S.* Typhimurium (see Table 3.3). Background fluorescence could have been the cause of the low level of fluorescence detected in the closely related bacteria samples. Therefore, amplification may have occurred in samples generating cycle threshold values less than 35.23. Primer dimers occurring during the reactions probably did not contribute to a significant amount of the background because the water controls (0 cfu/ml) generated cycle threshold values of 45, indicating no amplification (primers were added to water reactions). Primer dimers occur when the forward and reverse primers bind to each other. In general, the estimated concentrations of the artificially contaminated CRF samples obtained on the first day were higher than the concentrations obtained on the second and third day (Table 3.2). This gradual reduction of estimated concentrations could be due to a small amount of degradation of the flourescent probe. Slight variations between the three days (Table 3.1) could have been caused by pipetting errors i.e., variation in probe volume. However, the consistent decrease of values suggest otherwise.

Results from the commercial CRF samples were contradictory to our observations from the direct plating. IMC-PCR did not generate any amplicons, which agreed with our conventional culture results. As shown in Table 3.3, IMC-Taqman® PCR did generate Ct values that were consistent with the presence of low levels of *Salmonella* (10^2 cfu/ml). The Ct for some of the commercial CRF samples were lower than 35.23, indicating a low level of contamination. Another possible explanation for the appearance of amplification could have been the presence of a substance in the commercial CRF samples that gradually hydrolyzed the probe, which would cause fluorescence to occur. Contamination levels of the commercial CRF samples were unknown and direct plating did not reveal any *Salmonella* even after 24 hours of enrichment in broth. Polyphosphates, common bacteriostatic chemicals used to rinse carcasses could have damaged the cells and made them non-viable. IMC-PCR and IMC- Taqman® PCR did not require the presence of intact cells, only DNA. This fact can be both an advantage and disadvantage.

IMC-PCR and IMC-Taqman® PCR did not require any time consuming pre-enrichment steps to achieve detection of *S.* Typhimurium from pure culture dilutions and artificially contaminated CRF samples. Other researchers have developed methods that required enrichment to obtain similar sensitivities. Fluit *et al*. (1993) developed a PCR-based detection method with a sensitivity of 0.1 cfu/g in stomached chicken which required a 24 hour enrichment. IMC-PCR and IMC-Taqman® PCR's ability to detect low concentrations of *Salmonella* without enrichment steps is likely due to the use of immunomagnetic beads to concentrate the *Salmonella* from 1.0 ml samples. Dynal, the manuafacturer of the immunomagnetic beads, suggests using enrichment steps before adding the beads to the samples. Therefore, others that used Dynal® anti-*Salmonella* beads did not add the beads directly to the samples. This fact makes it difficult to compare IMC-PCR and IMC-Taqman® PCR results with previous studies. Shaw *et al*. (1998) showed that the Dynabeads® method (enrichment included) gave 100% correlation with the results of the standard culture technique used for foods and the modified semisolid Rappaport-Vassiliadis method used for feeds and environmental samples. Using flow cytometry, after an 18hr overnight enrichment, followed by immunomagnetic separation, Wang and Slavik (1999) detected an average of 2.3×10^4 cfu/ml *Salmonella* in chicken washes. According to these results, with enrichment of samples, immunomagnetic separation using Dynal® anti-*Salmonella* beads achieved sensitivities similar to standard culture techniques. The results from this study

showed that without enrichment, both IMC-PCR and IMC-Taqman® PCR were more sensitive (IMC-PCR: 10^4 cfu/ml; IMC-Taqman® PCR: 10^3 cfu/ml) in detecting *Salmonella* in artificially contaminated CRF samples.

IMC-Taqman PCR® was more expensive to perform. The cost/sample for this detection method was $7.00 compared to IMC-PCR cost/sample which was $5.00. Results were obtained within 3 hours when analyzing samples with IMC-Taqman® PCR compared to 4 hours for IMC-PCR.

According to the Food Safety Inspection Service Microbiological Baseline Date Collection Results acquired during the last four years, only 20% of 1,297 broiler carcass rinse samples tested positive for *Salmonella*. The level of contamination was only 20 cfu/ml *Salmonella* in broiler carcass rinse samples (FSIS, 1999). This level of contamination is below the detection limit of IMC-Taqman® PCR in artificially contaminated CRF samples. Previous detection methods using systems similar to Taqman®, have been able to detect concentrations lower than 10^6 cfu/ml by including at least one enrichment period (Chen *et al*., 1997a). Using the AG-9600 Amplisensor Analyzer, a sensitivity as low as 3 cfu per 25 g of food was achieved from overnight pre-enriched samples (chicken carcass rinses, ground beef, ground pork, and raw milk (Chen *et al.,* 1997 b). Therefore, a short enrichment step may be required to increase the sensitivity of IMC-Taqman® PCR to achieve detection of *Salmonella* below 10^3 cfu/ml.

As processing facilities develop the ability to increase processing rates, the likelihood for cross-contamination will increase. After CRF samples are collected, depending on how the carcasses will be shipped (parts or whole), the time required for processing ranges from 30 minutes to 24 hours. Therefore, development of rapid detection methods that can be used within this time window would be ideal. Results of this research indicate that IMC-PCR and IMC-Taqman® PCR could be adapted to test CRF samples in the short amount of time needed for processing poultry. IMC-Taqman® PCR may be more capable of detecting low levels of *Salmonella* in commercial CRF samples.

To thoroughly determine the detection limits in commercial CRF samples, experiments using a larger number of commercial CRF samples should be performed. Additional experiments involving artificial inoculation of commercial CRF samples are needed to determine if a low level of *Salmonella* contamination can be detected from commercial CRF samples consistently. A KNAPSACK analysis of the Ct values generated from more experiments could

assist in determining a more realistic Ct “cut-off” value. A KNAPSACK analysis incorporates the results of multiple methods to determine the best predictive values to assist in obtaining the most accurate results. A small enrichment step may be required to achieve the needed sensitivity for commercial CRF samples. Once the detection method has been optimized, additional primers and probes could be designed and included for the simultaneous detection of other foodborne bacterial pathogens along with *Salmonella*.

CHAPTER 4:

GENERAL CONCLUSIONS

There are many factors that need to be considered, when determining the applicability of a rapid detection method for *Salmonella* contamination in a poultry-processing facility. The most important of these would be: 1) sensitivity of the assay 2) specificity of the assay, 3) time required to complete the assay, 4) cost per sample, and 5) level of personnel training necessary to perform the assay.

Sensitivity: The results from this study with IMC-ELISA without enrichment, suggested a limit of detection of 10^4 to 10^6 cfu/ml of *S.* Typhimurium in both pure culture and artificially contaminated CRF samples. Where as IMC-PCR without enrichment, was able to detect 10^2 cfu/ml in pure culture, but only 10^4 cfu/ml in artificially contaminated CRF samples. On the other hand IMC-Taqman® PCR was able to detect as low as 10^1cfu/ml in pure culture and approximately 10^3 cfu/ml in artificially contaminated CRF. In this set of experiments the highest degree of sensitivity was seen with IMC-Taqman®.

Specificity: The results of IMC-ELISA indicated a high degree of cross-reactivity with other common poultry contaminants. This detection method also resulted in a high level of background. IMC-PCR did not result in any false positive results when tested with the same species of bacteria. When IMC-Taqman® PCR was tested using the same organisms, the results suggested a possible cross-reaction with some of the bacteria. However, this background may be important in determining an appropriate Ct "cut-off" value, which is needed to assess positivity. Based on these results, IMC-PCR would be the most specific detection method.

Time required: None of the assays evaluated in this effort involved pre-enrichment or enrichment steps. However, the immunomagnetic capture used in all three assays required a 20-minute incubation with Dynal® anti-*Salmonella* paramagnetic beads to recover *Salmonella* from the samples. Other immunoassay-based methods have utilized pre-enrichment and/or enrichment steps requiring at least 18 hours. The amount of time required to perform IMC-ELISA was approximately 5 hrs. Where as IMC-PCR and IMC-Taqman® took 4 and 3 hours respectively. Therefore, all three methods, based on the time required for completion, have the potential to be useful in determining the level of *Salmonella* contamination in processed poultry because they would enable evaluations to be made prior to shipping product out of the plant. All three

methods were also less time consuming compared to conventional plating and other PCR detection methods that require 24 to 36 hours due to the need for an enrichment step. The results of this study compared favorably with those of Carlson *et al*. (1999) who developed a PCR-based method specific for *S.* Typhimurium DT 104 using a fluorogenic assay. Even though their assay required only 3 to 4 hours, it was substantially less sensitive when compared to the three assays results reported in this study.

Cost: In regard to the cost of disposable supplies and reagents, IMC-ELISA was the least expensive assay to perform at $2.00 per sample. Estimated initial equipment costs for IMC-ELISA would be the following: plate washer ($4,700.00), spectrophometer ($7,000.00), computer ($2,000.00), and software ($1,500.00). IMC-PCR was more expensive at $5.00 per sample and IMC-Taqman® PCR, was the most expensive at $7.00 per sample. These cost estimates did not include labor, but only the materials and supplies needed to perform the assays. Startup costs for the IMC-PCR assay would include $7,995.00 for a thermalcycler, $1,000.00 for gel rigs, $1,500.00 for a gel documentation system and $2,000.00 for a computer. Initial costs for IMC-Taqman® PCR would be $90,000.00 for the Sequence Detection System® which includes the thermalcycler and the computer. By comparison, standard plating and identification using a system like Vidas® (bioMerieux, Hazelwood, MO) would appear to cost about the same as IMC-Taqman® PCR. Based on this, and the economic consequences of a product recall, it seems that an assay like the IMC-Taqman®PCR would without a doubt be a viable option for "near on-line" *Salmonella* detection.

Training: A final consideration should be the level of training required for personnel to be able to perform these assays. Today, ELISA and PCR techniques might be thought of as "cook book" since they are being routinely used in a variety of diagnostic settings. That being said, IMC-ELISA would be the easiest to perform and would require only a minimum skill level. On the other hand, IMC-PCR and IMC-Taqman® PCR would be more difficult because of the need for exceptional technique to prevent DNA cross contamination. Of the three assays, IMC-Taqman® PCR would require the most training due to the more complicated nature of the data analysis.

The Hazard Analysis Critical Control Point (HACCP) process is not an endpoint assessment of product safety. It is a system that allows evaluation at multiple steps during processing. Implementing a detection method at one or more steps would provide essential

contamination information well before product leaves the processing plant. Currently, whole carcass rinses are performed on poultry, after removal from the chill tank and before "cut-up" or packaging of the carcasses, to determine the incidence of carcass contamination with *Salmonella*. This is the most likely processing step at which the detection methods developed could be most readily applied. Another possible control point application may be in the analysis of chill tank water which has been well established as a source of cross contamination. One (IMC-Taqman® PCR) of the three assays is conceivably quantitative, and the results would allow quick intervention to reduce contamination.

Future Work

There are several suggestions that could improve IMC-ELISA, IMC-PCR, and IMC-IMC-Taqman® PCR:

1) Changing the antibodies involved in the capture stage could further optimize IMC-ELISA. Trials using monoclonal antibodies conjugated to the beads may decrease cross-reactivity. Also, coating the antibody conjugated beads with a blocking protein may decrease the affinity of the non-coated areas of the beads for other species of bacteria. As with development of other ELISA-based detection methods, changing the antibodies after concentration of the bacteria could decrease cross-reactivity.
2) Artificially inoculating commercial CRF samples with a range of *Salmonella* dilutions and different types of *Salmonella* would provide more insight into the sensitivity and specificity of IMC-ELISA, IMC-PCR and IMC-Taqman® PCR methods. More samples would also be useful for determining IMC-PCR and IMC-Taqman® PCR sensitivity and specificity.
3) Further analysis under increased stringency to reduce the potential of nonspecific binding should be performed to verify true amplification of only *Salmonella* DNA. Also, degenerate primers specific for the *Salmonella* genus could possibly be developed to detect only *Salmonella*, and not other *Enterobacteriacae*.

4) Development of primers and probes specific for other microorganisms like *Campylobacter*, *Listeria*, and *E. coli*, would allow for simultaneous detection of multiple food borne pathogens in a single assay and provide a more detailed analysis of CRF samples. This would also make the assay more cost effective.

REFERENCES

Aabo, S., Rasmussen, O. F., Rossen, L., Sorensen, P. D. and Olsen, J. E., 1993. *Salmonella* identification by the polymerase chain reaction. *Mol. Cell. Probes* 7:180-182.

Acha, A. G., Szyfres, B., 1987. Zoonoses and communicable disease common to man and animals. Office International des epizooties, Paris, France, 2nd ed., 156-166.

Bailey, J. S., Cox, N. A. and Blankenship, L. C., 1991. A comparison of an enzyme immunoassay, DNA hybridization, antibody immobilization, and conventional methods for recover of naturally occurring *Salmonellae* from processed broiler carcasses. *Food Protection* 54: 354-356.

Belgrader, P., Benett, W., Hadley, D., Richards, J., Stratton, P., Mariella Jr., R., and Milanovich, F., 1999. PCR detection of bacteria in seven minutes. *Science* 284:449-450.

Burtscher, C., Fall, P. A., Wilderer, P. A., Wuertz, S., 1999. Detection of *Salmonella* spp. and *Listeria monocytogenes* in suspended organic waste by nucleic acid extraction and PCR. *Appl. Environ. Microbiol*. 65:2235-2237.

Carlson, S.A., Bolton, L. F., Briggs, C. E. Hurd, H. S., Sharma, V. K., Fedorka-Cray, P.J., and Jones, B. D., 1999. Detection of multiresistant *Salmonella typhimurium* DT 104 using multiplex and fluorogenic PCR. *Mol. Cell. Probes* 13:213-222.

Chen, S., Yee, A., Griffiths, M., Wu, K. Y., wang, C. N. Rahn, K., and De Grandis, S. A., 1997a. A rapid, sensitive and automated method for detection of *Salmonella* species in foods using AG-9600 AmpliSensor Analyzer. *J. Appl. Microbiol*. 83:314-321.

Chen, S. Yee, A., Griffiths, M., Larkin, C., Yamashiro, C. T., Behari, R., PaszkoKolva, C., Rahn, K., and De Grandis, S. A., 1997b. The evaluation of a fluorogenic polymerase chain reaction assay for the detection of *Salmonella* species in food commodities. *Int. J. Food Micro*. 35: 239-250.

Clouser, C. S. Doores. S., Mast, M. G. and Knabel, S. J., 1995. The role of defeathering in the contamination of turkey skin by *Salmonella* species and *Listeria monocytogenes*. *Poultry Sci.* 74: 765-767.

Cocolin, L., Manzano, M. Cantoni, C., Comi, G., 1998. Use of polymerase chain reaction and restriction enzyme analysis to directly detect and identify *Salmonella typhimurium* in food. *J. Applied Microbiol.* 85:673-677.

Cohen, H. J., Mechanda, S. M. and Lin W., 1996. PCR amplification of the *fimA* gene sequence of *Salmonella typhimurium*, a specific method for detection of *Salmonella* spp. *Appl. Environ. Microbiol.* 62:4303-4308.

Coquard, D., Exinger, A., 1999. Routine detection of *Salmonella* species in water: comparative evaluation of the ISO and PROBELIA polymerase chain reaction methods. *J. AOAC International.* 82:871-876.

Cudjoe, K. S., Krona, R., 1997. Detection of *Salmonella* from raw food samples using Dynabeads® anti-*Salmonella* and a conventional reference method. *Inter. J. Food Microbiol.* 37:55-62.

Desjardin, L. E., Chen, Y., Perkins, M. D. Teixeira, L., Cave, M. D., Eisenach, K. D., 1998. Comparison of the ABI 7700 System (Taqman) and competitive PCR for quantification of IS6110 DNA in sputum during treatment of tuberculosis. *J. Clin. Microbiol.* 36:1964-1968.

Dill, Kilian, Stanker, Larry H., and Young, Colin R., 1999. Detection of *salmonella* in poultry using a silicon chip-based biosensor. *J. Biochem. Biophys. Meth.* 41: 61-67.

Fluit, A. C., Widjojoatmodjo, M. N. Box, A. T. A., Torensma, R. and Verhoef, J., 1993. Rapid detection of *Salmonellae* in poultry with the magnetic immuno-polymerase chain reaction assay. *Appl. and Environ. Microbiol*. 59:1342-1346.

Food and Drug Administration. Bacteriological Analytical Manual, 8th ed, 1998. AOAC International. Gaithersburg, MD.

Food Safety and Inspection Service. *Salmonella* Analysis: Collecting Raw Meat and Poultry Samples. October 1997.

Food Safety and Inspection Service. Poultry Microbiological Baseline Data Collection Results. 1999. http://www.fsis.usda.gov/OPHS/baseline/contents.htm.

Heid, C. A., Stevens, J., Livak, K. J., Williams, P. M., 1996. Real time quantitative PCR. *Genome Research*. 6: 986-994.

Hogue, A., T., White, R. L., and Heminover, J. A., 1998. Pathogen reduction and hazard analysis and critical control point (HACCP) systems for meat and poultry. *Veterinary Clinics of North America: Food Animal Practice* 14 (1): 151-162.

Hoorfar, J., Baggesen, D. L., and Porting, P. H., 1999. A PCR-based strategy for simple and rapid identification of rough presumptive *Salmonella* isolates. *J. Microbiol. Meth.* 35: 77-84.

Ibrahim, G., 1986. A review of immunoassays and their application to *Salmonella* detection in foods. *Journal of Food Protection*. 49: 299-310.

Jitrapakdee, S., Tassanakajon, A., Boonsaeng, V., Piankijagum, S., Panyim, S., 1995. A simple, rapid and sensitive detection of *Salmonella* in food by polymerase chain reaction. *Mol. Cell. Probes* 9:375-382.

Kantama, Laksana, Jayanetra, Panida, Pilantanapak, Apiradee, and Atti Charaensub, 1998. Primers for *Salmonella* serovar detection by polymerase chain reaction. *Southeast Asian J. Trop. Med. Pub. Health,* 29:9-18.

Keith, M., 1997. Evaluation of an automated enzyme-linked fluorescent immunoassay system for the detection of *Salmonellae* in foods. *J. Food Protect*. 60:682-685.

Keller, L. H., Benson, C. E., Barcia, V., Nocks, E., Battenfelder, P. and Eckroade, R.J., 1993. Monoclonal antibody-based detection system for *Salmonella enteritidis.* *Avian Dis.* 37:501-507.

Khan, A. A., Nawaz, M. S., Khan, S. A., and Cerniglia, C. E., 1999. Detection of multidrug-resistant *Salmonella* Typhimurium DT104 by multiplex polymerase chain reaction. *FEMS Microbiol. Letts*. 182:355-360.

Krysinski, E. P. and Heimsch, R. E., 1977. Use of enzyme-labeled antibodies to detect *Salmonella* in foods. *App. Environ. Microbiol*. 33:947-952.

Kwang, J., Littledike, E. T. and J. E. Keen., 1996. Use of polymerase chain reaction for *Salmonella* detection. *Lett. Appl. Microbiol.* 22:46-51.

Lillard, H. S., 1990. The impact of commercial processing procedures on the bacterial contamination and cross-contamination of broiler carcasses. *Food Protect*. 53: 202-204.

Lin, Jer-Sheng, and Hau-Yang Tsen., 1999. Development and use of polymerase chain reaction for the specific detection of *Salmonella* Typhimurium in stool and food samples. *Food Protect.* 62: 1103-1110.

Major, M., 2000. A wider margin. *Supermarket Business*. 27:5-7.

Mansfield, L., Forsythe, S., 1993. Immunomagnetic separation as an alternative to enrichment broths for *Salmonella* detection. *Lett. Appl. Microbiol*. 16:122-125.

Mansfield, L., Forsythe, S., 1996. Collaborative ring-trial of Dynabeads® anti-*Salmonella* for immunomagnetic separation of stressed *Salmonella* cells from herbs and spices. *Inter. J. Food Microbiol.* 29:41-47.

Manzano, M., Cocolin, L., Astori, G., Pipan, C., Botta, G. A., Cantoni, C., and Comi, G., 1998. Development of a PCR microplate-capture hybridization method for simple, fast and sensitive detection of *Salmonella* serovars in food. *Mol. Cell. Probes*. 12: 227-234.

McNab, B. W., 1998. A general framework illustrating an approach to quantitative microbial food safety risk assessment. *Food Protect*. 61: 1216-1228.

Minnich, S. A., 1978. The detection of *Salmonella* in foods using enzyme-labeled antibodies. M.S. thesis. Univ. of Idaho., Moscow., ID.

Minnich, S. A. Hartman, P. A. and Heimsch. R. C., 1982. Enzyme imunoassay for detection of *Salmonellae* in foods. *Appl. Environ. Microbiol.* 43: 877.

Nogva, H. K., Lillehaug, D., 1999. Detection and quantification of *Salmonella* in pure cultures using 5'-nuclease polymerase chain reaction. *Inter. J. Food Microbiol*. 51:191-196.

Prusak-Sochaczewski, E., Luong J. H. T., 1989. Utilization of two improved enzyme Immunoassays based on avidin-biotin interaction for the detection of *Salmonella. National Research Council Proceedings, Canada.* 89:321-327.

Rijpens, N., Herman, L., Vereecken, F., Jannes, G., Smedt, J. D., and Zutter, L. D., 1999. Rapid detection of stressed *Salmonella* spp. in dairy and egg products using immunomagnetic separation and PCR. *Inter. J. Food Microbiol*. 46:37-44.

Robinson, B. J., Pretzman, C. I., and Matting, J. A., 1983. An enzyme immunoassay using myeloma protein for detection of *Salmonellae*. *Appl. Environ. Microbiol*. 45:1816.

Shaw, S. J., Blais, B. W., Nundy, D. C., 1998. Performance of Dynabeads® Anti-*Salmonella* system in the detection of *Salmonella* species in foods, animal feeds, and environmental samples. *J. Food. Prot*ect. 61:1507-1510.

Smith, A. M. and Jones, C., 1983. Use of murine myeloma protein M467 for detecting *Salmonella* species in milk. *Appl. Environ. Microbiol.* 46:826.

Stefanovicora, A., Rchakova, H., Skarkova, A., Rijpens, N., and Kuchta, T., 1998. Confirmation of presumptive *Salmonella* colonies by the polymerase chain reaction. *Food Protect.* 61:1381-1383.

Swaminathan, B. and Ayres, J. C., 1980. A direct immunoenzyme method for the detection of *Salmonellae* in foods. *J. Food. Sci*. 45:352.

Swaminathan, B., J., Aleixo, A. G., and Minnich S. A., 1985. Enzyme immunoassays for *Salmonella*: one-day testing is now reality. *Food Technology*. 6:83-89.

Tan, W., Shelef, L. A., 1999. Automated detection of *Salmonella* spp. in foods. *J. Microbiol. Meth.* 37:87-91.

Tian, H, Miyamoto, T., Okabe, T., Kuramitsu, Y., Honjog, K. and Hatano, S., 1996. Rapid detection of *Salmonella* spp. in foods by combination of a new selective enrichment and a sandwich ELISA using two monoclonal antibodies against dulcitol 1-phosphate dehydrogenase. *Food Protect*. 59:1158-1163.

Tsai H. S., Slavik, M., 1993. Rapid fluorescence concentration immunoassay for detection of *Salmonellae* in chicken rinse water. *Food Protect*. 57:190-194.

United States Department of Agriculture, Food Safety Inspection Service. Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems. Final rule. Federal Register. 1996. 61:38805.

Wang, R. F., Cao, W. W. and Cerniglia, C. E., 1997. Universal protocol for PCR detection of 13 species of foodborne pathogens in foods. *J. Appl. Microbiol*. 83:727-736.

Wang, X, Slavik, M. F., 1999. Rapid detection of *Salmonella* in chicken washes by immunomagnetic separation and flow cytometry. *Food Prot*. 62:717-723.

Ward, John W., March 12, 1999. MMWR Weekly. Incidence of foodborne illnesses: preliminary data from foodborne diseases active surveillance network (FoodNet). 48:189-194.

Wyatt, G. M., Langley, M. N., Lee, H. A, and Morgan M. R. A., 1993. Further Studies on the feasibility of the one-day *Salmonella* detection by enzyme-linked immunosorbent assay. *Appl. Environ. Microbiol.* 59:1383-1390.

Wyatt, G. M., Lee, H. A., Dionysiou, S., Morgan, M. R. A., Stokely, D. J. Alhajji a. H, Richards, J., Sillis, a. J. and Jones, P. H., 1995. Comparison of a microtitration plate ELISA with a standard cultural procedure for the detection of *Salmonella* spp. in chicken. *Food Protect.* 59: 238-243.

Yu, H. and Bruno, J. G., 1996. Immunomagnetic-electrochemiluminescent detection of *Escherichia coli O157* and *Salmonella* Typhimurium in foods and environmental water samples. *Appl. Environ. Microbiol*. 62:587-592.

Vita

Jennifer Mae Sharp was born in Richmond, Virginia in 1976. She enjoyed academics as well as sports year round while living in rural Louisa County. In 1994, she enrolled as an animal and poultry science major and a biology minor at Virginia Polytechnic Institute and State University. After finishing her bachelors degree in 1998, she began pursuing a Master of Science degree under the guidance of F.W. Pierson, N. Sriranganathan, and S. Sumner.

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