DEVELOPMENT OF A RAPID DETECTION METHOD OF *SALMONELLA* Spp. ON

CHICKEN SKIN BY REAL-TIME POLYMERASE CHAIN REACTION

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DEVELOPMENT OF A RAPID DETECTION METHOD OF *SALMONELLA* Spp. ON CHICKEN SKIN BY REAL-TIME POLYMERASE CHAIN REACTION

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DISSERTATION ABSTRACT

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Salmonella is a major contributor to food-borne illness with contaminated poultry meat being recognized as a chief source of the infection. The handling of raw poultry has been reported as one of the most frequent causes of *Salmonella* ingestion and human sickness. The use of magnetic particles (beads) coated with a highly specific antibody have become common and serve as a valuable tool in the detection of *Salmonella* and other food borne pathogens in complex food matrices. With the speed, cultural precision and sensitivity of this method, it is extremely likely that it will replace or modify the tiresome traditional protocol in the recognition of *Salmonella* serovars. This method has the capacity of concentrating low levels of the bacteria while simultaneously removing them from the food sample. With so many contaminants and numerous other bacteria

being present, this allows for specific enrichment of the target microbe for greater detection potential. Real-time Polymerase Chain Reaction allows confirmation of the target bacteria presences on the chicken skin and has been used extensively as an alternative method of detection. If the contaminants and food components such as fat remain, the sensitivity and accuracy of real-time PCR is severely decreased.

Magnetite (Fe_3O_4) and silica-magnetite composites were produced within the lab and conjugated with anti-*Salmonella* antibody for its isolation from raw chicken skin. Real-time PCR was used for positive identification. These magnetic beads have shown great potential to meet the demand for rapid and efficient detection techniques in food safety. Preliminary test show an 80% detection rate when inoculating *Salmonella* at a level of 13 cells per 16 square inch, with potential of detection at even lower levels in a twelve hour period. Success would mean a significant reduction in recalls, since the product should still be present in the plant. With the preparation of magnetic composites being carried out as a bench top procedure, commercial bead use is not needed, thus tremendously reducing cost.

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CHAPTER I. INTRODUCTION

Foodborne illnesses are a serious public health concern that affects the United States and world as a whole. It is reported that an estimated 76 million people contract a foodborne illness every year with some 325,000 being hospitalized resulting in approximately 5,000 deaths in the United States (Center for Disease Control 2004). Of major concern is the presence of *Salmonella* in food, where it is believed to be the highest of any reported gastrointestinal infection (CDC 2005). Mead and others 1999 reported that *Salmonella* is responsible for more than 1 million outbreaks that result in over 500 deaths per year. In the United States, an estimated 1.4 million people contract salmonellosis, while costing nearly 4 billion dollars a year (Mead and others 1999; El-Gazzar and Marth 1992). Furthermore, one of the most frequent causes of infection is through the handling of raw poultry carcasses and products, along with the consumption of undercooked poultry (Whyte and others 2002; Panisello and others 2000). Symptoms of salmonellosis may include a mild upset stomach with fever and vomiting to a serious condition of meningitis and death (Olsen and others 2000). The less severe symptoms tend to present themselves in 12 to 72 hours. Meat and poultry products are contaminated with *Salmonella* during slaughtering and continuing processing methods. These methods provide many opportunities for contamination as well as cross-contamination within a production batch. According to Borch and Arinder (2002), during slaughter, pathogenic bacteria may contaminate the carcass and spread to cut raw meat

intended for further processing. If this raw meat is not properly processed, ready to eat meats will remain contaminated. This renders a problem in the ready-to-eat meat industry in that these meats are very unlikely to be reheated or heated to a kill temperature prior to consumption (Li and others 2005). With the use of water for constant carcass rinsing as a means of bacterial removal, the threat of cross contamination is also very likely. In that chilled chlorinated water rinses are decontamination steps, the level of chlorine allowed provides only an approximate 90% reduction in the bacterial level (Tsai and others1992). With only a few cells required to result in salmonellosis, a 90% reduction is not sufficient. Wilson (2002) reports, that few viable Salmonellae cells may be present on chicken carcasses. With this being the case, poultry processors and retailers see this as a major situation, but know that it is extremely difficult and almost impossible to raise chickens free of this bacteria (Corry and others 1995). Serovars of *Salmonella enterica* are the focal microbes in foodborne illness outbreaks of human gastrointestinal disease (Schrank and others 2001). However, *Salmonella* Entreritidis and *Salmonella* Typhimurium are among the serovars that are of the most human concern in poultry products (Schrank and others 2001). For this reason, it is important for the poultry industry to employ techniques for the prevention as well as the detection of *Salmonella* in an attempt to prevent outbreaks of *Salmonella* infection and as a means of industry risk assessment. This is the reason strict hygienic practices and foodborne disease prevention systems, such as Hazard Analysis Critical Control Point, have become mandatory and government regulated for the meat industry (Khawla and others 2004).

It is believed that the initial infection of this microbe is through oral ingestion of the bacteria with the digestive tract mainly through M and epithelial cells (Sadeyen and

others 2004; Jepson and Clark 2001; Philpott and others 2001). Once this happens, a transient colonization of the spleen and liver can evolve into either a systemic typhoid or enteric salmonellosis infection based on the host serovar makeup (Kaufman and others 2001). In chickens this same mechanism takes place with the bacteria being able to stay in the digestive tract for months without any clinical signs of its presence (Sadeyen and others 2004; Barrow and others 1987). This bacteria will then pass through in the feces resulting in a cross contamination of other birds with continued infection during slaughtering (Sadeyen and others 2004).

Due to the relatively high prevalence of *Salmonella* spp. in meat and poultry as well as the high incidence of disease caused by these microbes, a rapid, sensitive and reliable method for detection is of urgent need in the food industry to combat further foodborne outbreaks (Fratamico 2003; Hein and others 2006; Bhagwat 2003). Myint and others (2005) advocate that new methods of detection should be standardized rapid, sensitive and specifically suitable for identifying naturally occurring contamination in food products. This supports the idea that the viability of bacteria from samples spiked with stock cultures may differ from that of naturally occcuring contamination in that these bacteria have been exposed to a variety of unfavorable conditions where they may have encountered some injury during the total processing (Myint and others 2005; Gouws and others 1998; Soumet and others 1994). According to Mizumoto and others (2003), salmonellosis has been reported by public health authorities throughout the world since the mid to late 1980's. Even with intensive eradication efforts, *Salmonella* contamination of foods still remains a persistent problem in the food industry. Traditional and conventional methods of detection have proven to be labor intensive and very time

consuming, requiring 5-7 days at a minimum with poor sensitivity at low levels of contamination (Myint and other 2005; D'Aoust and others 1992). With time of detection being very critical, several methods have been developed to detect the presence of potentially low levels of *Salmonella* in foods on the basis of immunoassays, nucleic acid hybridization and polymerase chain reaction (PCR) (Fratamico 2003; Whyte and others 2002).

Immunoassay tests fall into many categories such as radio and fluorescent, but enzyme-linked immunosorbent assay (ELISA) has been recognized as the most promising in the food industry. This method relies on the binding of an antigen on the bacterium to a specific monoclonal or polyclonal antibody (Fratamico 2003). These antibodies are specific to either O-antigen or H-antigen (NG and others 1996, Wang and others 1996, Jaradat and Zawistowski 1996). The problem with these methods is that they are prone to producing false-positive results due to the cross-reactivity of the polyclonal antibodies with other enterobacteria (Curiale and others 1990).

Nucleic acid-based systems consist of polymerase chain reaction (PCR), pulse-field gel electrophoresis (PFGE) and DNA hybridization. All of these techniques involve the manipulation of the bacterial DNA. PCR has proved to be of most importance in the food industry, where it involves the amplification of the targeted bacterial DNA segments with a heat stable polymerase and two corresponding primers (Gasanov and others 2005). The primary advantage of PCR tests is an increase in sensitivity with a reduction in time required to process samples compared to that of culture methods (Lampel and others 2000). With the general occurrence of low levels of *Salmonella* in foods, an enrichment process is very necessary. Once there has been adequate enrichment, the PCR process

undergoes three stages consisting of denaturation of the DNA, annealing of the specified primer region and polymerization of that region (Olsen and others 1995).

The focal point of this research project was to develop a rapid, sensitive and specific method for the detection and determination of *Salmonella* spp. on the surface of contaminated chicken skin. This study involved a cocktail contamination of the serovars: *Salmonella* Typhimurium, *Salmonella* Entreritidis and *Salmonella* Mission, where the skin was placed in an enrichment medium for six hours. Centrifugation was used as means of concentrating the target microbes for PCR determination. Therefore the primary objective of the study was to standardize protocol for the in-plant determination of *Salmonella* contamination on rinsed de-feathered slaughtered chickens during processing at levels of 200 CFU per 16 in^2 (4 x 4 in) of skin.

CHAPTER II. LITERATURE REVIEW

1. FOODBORNE ILLNESS

Foodborne illnesses affect millions of people every year and present a serious health problem to the world, as new food processes and products continue to emerge and present new challenges to food safety. Estimates show, in the United States alone, some 76 million persons contract foodborne illnesses, 325,000 are hospitalized and 5,000 die each year due to foodborne illnesses (Mead and others 1999). These figures indicate that a person stands a 25% chance of contracting a food-related illness every year (Doyle 2000). It is believed that over 400 million episodes of foodborne diarrhea occur annually (WHO, 1997). With such outstanding numbers, the economic burden is estimated at 6.7 billion dollars per year in patient related cost for treatments of bacterial infections, but in all aspects results in a total of 23 billion annually in the USA (Buzby 1996; Jones and Gerber 2001). In 2004, *Salmonella*, *Shigella*, *Campylobacter*, and *E. coli* were the most common foodborne pathogens costing 6.9 billion (Allos and others 2004). In a 2004 report by the Food Net, a total of 15,806 cases were observed where *Salmonella* accounted for 6,464; *Campylobacter* accounted for 5,665; *Shigella* accounted for 2,231; *and E. coli* O157:H7 accounted for 401 (CDC 2005). With such alarming figures, it is believed that these numbers can be controlled with the application of many new technologies as well as early detection (Tauxe 2001 and 2002).

2. *SALMONELLA* CHARACTERISTICS/HABITAT

It can be seen that *Salmonella* is a major threat to human health every year and must be controlled at all key food production steps to ensure a safe and wholesome product for consumers (Cerro and others 2002). For this to be effective, the control has to be rapid and dependable for the detection, isolation, identification as well as characterization of problematic pathogens (Cerro and others 2002). Each year, approximately 1 million outbreaks of salmonellosis are reported in the United States, resulting in more than 500 deaths (Patel and others, 2006; Mead and others 1999). With many cases not reported, the CDC (2005) estimates numbers may be as high as 2 to 4 million annually. Salmonellosis in most cases is due to the consumption of contaminated eggs, poultry, pork, beef and milk products (Patel and others 2006).

Salmonella are motile, gram-negative, non-spore forming bacteria that are, facultative anaerobic and rod shaped. This bacterium belongs to the family *Enterobacteriaceae* better known as the enterics. It has been established that this species, of microbes has some 2,324 serotypes with serovars Typhimurium, Enteritidis, Heidelberg, Javiana, and Newport, accounting for the majority of foodborne incidences (CDC 2005). There were some 2,501 serotypes identified up to 2004 (WHO 2005). Related to human infection, *Salmonella* can be divided into three groups causing human infection: typhoid fever, bacteremia and enterocolitis (Santos and others 2001). Of the three, enterocolitis has been the most common threat of human infection within the United States. *Salmonella* is estimated to be the source of some 1,412,498 illnesses per year, and is the single most cause of death from foodborne illness associated with viruses, parasites or bacteria (Mead and others 1999). In a ten year span 1987-97, 61 percent of

the human infections were caused by five *Salmonella* serotypes, including Typhimurium (23%), Enteritidis (21%), Heidelberg (8%), Newport (5%) and Hadar (4%) (Olsen and others 2001). Observations have shown that in the United States, outbreaks have occurred from the ingestion of chicken, beef, turkey and eggs (Tauxe and others 2002). These products may contain the microbe from an infected animal or from fecal contamination occurring during processing. Thus the presence and adaptation of the microbe into the food chain is directly related to its familiarity with livestock and domesticated fowl. Therefore, pre-harvest control strategies and other preventive measures are of great importance in the elimination of salmonellosis.

The bacterium has been found in the intestinal tract of most animals (warm and cold blooded) as well as humans. Some species have shown to be adaptive to a particular host while some are ubiquitous in nature. *Salmonella* have been spread throughout the natural environment (soil, water, food plants) through human and animal excretions. This organism does not seem to multiply significantly in the natural environment but may survive for extended periods in soil and water if conditions remain favorable. *Salmonella* are excreted in feces which become the source of contamination. It is this contamination that may produce salmonellosis as a result of ingestion the bacteria in a contaminated food or the handling of raw poultry. Symptoms can be severe or mild depending on the immune response of the host. Thus, the elderly as well as the very young (infant) or anyone with a compromised immune system are very susceptible to the severe effects of this disease of which may result in death. These symptoms generally develop in 12-14 h post ingestion with nausea, vomiting, abdominal pain, headache, chills and most commonly diarrhea. Victims tend to be weak and faint with

fever as symptoms last some 2-3 days. In the most severe case, hospitalization will be necessary where the infection can continue to cause problems for years.

3. ECOLOGY/EPIDEMIOLOGY

This bacterium is found widespread in the environment and found very prevalently on farms, sewage and in any material that is subject to fecal contamination. Salmonellosis is a concern in all countries and appears to affect all domesticated animals, and some can be infected with no symptoms. All members of the genus are considered to be a threat to foodborne illness with some serotypes causing more severe disease than others.

In cattle the most common serovars have been Dublin and Typhimurium which account for 52% of *Salmonella* isolates from cattle (Ferris and others 1996). There has been an increase in the antibiotic resistant of serovar Newport and Typhimurium has shown much resistance (Wray and others 2000). Serovars Choleraesuis, Derby, Typhimurium, Agona, Brandenburg and Mbandaka are the prevelent strains in swine (Fedorka-Cray and others 2000). Their isolations are very geographical in nature (Davies and others 1997). Enteritidis and Typhimurium have been the most isolated in poultry (Boyce and others 1996; Altekruse and others 1993). These are the most common islolated serovars of *Salmonella* in cattle, swine, and poultry but not all that have been found to cause foodborne illness.

Routes of these infections to animals are numerous, but are believed to be more commonly from animal feed and the environment. The microbe can be found in animal feed and feed ingredients that contain animal and vegetable proteins (Davies and others

2000). In many instances, the contamination occurs during storage of feeds, because the bacteria can survive for years in coolers that have housed previous contaminated feed (Davies and others 2000). Contamination of animal feeds can also occur from wild birds, rodents and cats that may have access to the feed or as a result of inadequate cleaning and disinfection. Heat treatment of animal feed has been one method of control, yet the threat of post contamination is a major risk. The use of biologically active competitive exclusion products by the poultry industry has also been a means of controlling *Salmonella* in poultry flocks (Schneitz and others 2000). Within the environment, *Salmonella* is widespread and an environmental organism whose dissemination is likely to continue and increase in the future (Murray 2000). One major problem is the contamination of farm buildings that occur after an outbreak of disease, colonization of animals, water contaminated from cleaning or just the presence of wild animals and birds (Wray and others 2003). Contamination of farm buildings can be aggravated by the use of high-pressure hoses for cleaning which causes aerosols and stirs up dust laden with *Salmonella* (Wray and others 2003). The bacterium has been found to have the ability to survive 3-4 months in infected feces and slurries in temperate climates and even longer in the hotter climates (Wray and others 2003). However, if properly composted, the high temperatures will rapidly kill the pathogen (Poppe 2000). Survival in slurry has been found to last up to 286 days but is very dependent on the initial number of organisms, storage temperature and the serovar (Wray and others 2003). When sewage sludge is used as fertilizer, many samples have been tested to find the pathogen present within the pasture (Wray and others 2003). Disposal problems have been the cause of fecal contamination. Contaminated drinking water may be the biggest threat to wide and rapid

spread of the bacterium throughout a flock or farm because animals might defecate in their drinking water (Wray and others 2003). The threat of other animals passing the bacterium to one another needs special attention. Most farm animals are likely to acquire the pathogen from an animal of their species, but isolation of the *Salmonella* has occurred from a wide range of mammals, birds and arthropods (Murray 2000).

The control of *Salmonella* is a serious challenge in animal production because of its epidemiology and the many routes of transmission. To establish these parameters of flock protection, an adequate knowledge of the ecology and epidemiology of this bacterium must be understood to establish control measures. From this many things have emerged, such as: good manufacturing practices (GMP's) and hazard analysis critical control points (HACCP). These programs along with proper hygiene from the farm to the table may be the key to the reduction in the occurrence of the bacteria and incidence of foodborne infection.

4. MORPHOLOGY

As stated earlier *Salmonella* is a gram negative rod, non-spore former that is unable to ferment sucrose, lactose, or salicin. However, it will ferment glucose with the production of gas. Amino acids generally serve as their nitrogen source but, nitrogen can be acquired from nitrates, nitrites or NH_3. *Salmonella* requires a neutral pH for optimal growth but, it can survive over a pH range of 4.0 to 9.0. The primary reservoir is the intestinal tract, which includes poultry, reptiles and livestock. Once ingested in the body, *Salmonella* are taken up by gut epithelial cells in a process known as bacterial-mediated endocytosis. Here proteins that are encoded by Pathogenicity Island I are

injected into the secretion system of the cell. This promotes the uptake of the bacteria into an intracellular vesicle for replication. Once the cells have multiplied, the cells burst allowing the bacteria to spread to the lamina propia. In immuno-strong people the bacteria are attacked by phagocytic cells and killed. This is not seen in the immuno-compromised in that the phagocytic process of killing is impaired and thus salmonellosis takes place resulting in systemic disease.

Salmonella can be differentiated by three major antigens (somatic, surface and flagella). Somatic antigens are heat stable and resistant to alcohol. Surface antigens have the characteristic of anti-agglutination and only occur in three serovars of this genus. Flagella antigens have heat-labile proteins and are agglutinated (closely packed together) by heat. Pathogenicity depends on the bacterial ability to invade cells, complete a lipopolysaccharide coat, replicate intra-cellular and produce toxins (Chopra and others 1994; Finlay and others 1989; Finlay and others 1992)

4.1 Host Defenses

Normal gastric acidity tends to be lethal to *Salmonella* and, this is the first line of defense to colonization of the intestinal tract (Giannella and others 1973). This is found in healthy individuals where the ingested *Salmonella* are reduced in the stomach, resulting in few to no bacteria entering the intestines (Giannella and others 1973). This along with the natural intestinal microflora and the small intestine quick flushing sweeps any existing bacteria out of body quickly (Giannella 1979). One other defense mechanism would be the secretion of mucosal antibodies in an attempt to protect the intestines (Giannella 1979). Thus, when these defenses have been disrupted (immune

compromised), the host is then susceptible to salmonellosis.

4.2 Outbreaks

Outbreaks of salmonellosis have generally occurred at banquets or restaurant style settings. In 1994, the largest outbreak occurred from a different venue involving ice cream that was made from contaminated milk, where some 224,000 people became infected with the bacterium (Jay 2000). This occurrence was the result of milk being transported in a tanker previously containing liquid eggs contaminated with *Salmonella* Enteritidis. Cases were found in 41 states. It is believed that improper sanitation also contributed to the outbreak. In1985, the second largest *Salmonella* outbreak occurred involving 200,000 people. Milk was the vehicle of transmission which was attributed to improperly pasteurized milk (Ryan and others 1987). The U.S. Food and Drug Administration (USFDA) inspectors discovered that modification of the pasteurization equipment, which was to facilitate the running off of raw milk, resulted in the cross contamination of the pasteurized milk with raw milk. In 1974, at a Navajo Indian Reservation, the third largest outbreak occurred where 3,400 people became sick after eating potato salad stored improperly at a barbecue event.

5. METHODS OF DETECTION AND CULTURING

5.1 Traditional Standard Methods

Traditional methods for the detection of *Salmonella* have been widely studied and are highly dependable for their sensitivity and accuracy. Traditional methods involve the use of culturing media whether it is differential or selective and enrichment broths. Pre-enrichment is a resuscitation and multiplication process for sub-lethally damaged cells and generally lasts 16 to 20 h but can last 18 to 48 h for selective enrichment. This allows the number of *Salmonella* present in food to out-number the other organisms. Thus, plating on selective and differential media easily recognizes and identifies colonies of *Salmonella*. Growth for recognition and identification usually takes 24 to 48 h, with subsequent serological or biochemical identification lasting another 4 to 48 h for suspected colonies (Litchfield 1973). Positive identification may take several days, but is essential in serious outbreaks.

5.2 Sampling

With food distribution on such a large scale, it is essential that sampling be adequate and representative. This must be adequate in that a pathogen may be distributed abundantly over several lots or sparsely over one lot. Aseptic techniques must be used to deliver a sample that is intact and in the exact condition as found. Sterility of the equipment and the personnel doing the sampling is critical in maintaining the suspected environment. This not only allows for the conditions to be maintained but prevents the introduction of any outside bacteria (cross contamination) that may negatively affect the results. The FDA recommends that 25 g or mL of the sample be added to 225 mL of the

enrichment broth. The sample should then be homogenized to ensure that the bacteria are removed from the food matrix and completely dispersed in the enrichment medium.

5.3 Pre-enrichment

The objective of pre-enrichment is the recovery of the microbe and may involve the re-hydration of the bacteria. This is needed because of sub-lethal injuries that occur during extreme conditions of some food processes. Recovery and repair of damaged cells to a viable state is essential in this step in order to determine if the bacteria of interest were present in food. Here the medium serves as a nutrient source for the enumeration and proliferation of the injured and non-injured cells. This is generally a non-selective media in that a selective media may create an environment that is detrimental to damaged cells. This could also cause an overgrowth of other bacteria that may inhibit the growth of *Salmonella*. Length (time) of the incubation and temperature are very important in the pre-enrichment process to facilitate adequate repair and recovery of *Salmonella* cells. Therefore, the FDA recommends a 18 to 24 h incubation period at 37ºC. Trypticase soy broth (TSB), brain heart infusion broth (BHI), lactose broth (LB), and buffered peptone water (BPW) appear to be the most popular for recovery purposes (Andrews 1985). Due to the fact, that injury repair can occur in the absence of cell wall and protein synthesis, and that not all cells in the population suffer the same degree of injury, pre-enrichment is vital in bacteria identification.

In the selection of a nutrient broth for cell resuscitation, a non-selective broth such as trypticase soy broth or buffered peptone water is usually selected. These cells may have been injured due to heat, radiation, dehydration and other less than ideal situations.

However, in specimens containing large numbers of competing bacteria, a selective medium should be chosen. This will prevent the overpopulation of unwanted bacteria that may inhibit the growth of the target bacteria. Samples where this can occur are food samples, soil samples, unprocessed raw ingredients and fecal samples. Incubation time as well as temperature helps aid in this selection process and enrichment.

5.4 Selective Enrichment

Selective enrichment is a pivotal step in detection of *Salmonella* in a food matrix. With *Salmonella* generally representing a small population of the microflora in foods, most if not all may be lost during selective enrichment. This is also why pre-enrichment is very important, because in the those possible low numbers of *Salmonella* some may be injured cells that have to be repair and recovered for better detection efficiency. As with pre-enrichment media, there are several selective enrichment media. They are categorized into three groups: selenite broth (Leifson 1936), tetrathionate broth (Muller 1923; Kauffmann 1930), and Rappaport. These media either contain selenite, tetrathionate, brilliant green, bile salts, or malachite green respectfully. Magnesium chloride is also present in variable amounts.

Selenite broth was developed by Leifson (1936), for the cultivation of *Salmonella* that were present in very small numbers. Enzymatic digestion of casein and enzymatic digestion of animal tissue are used as nitrogen and vitamin sources. This broth is designed to inhibit the growth of coliform bacteria and enterococci in the first 6 to 12 h of incubation after which the inhibitory effect declines. Lactose serves as the digested carbohydrate source, resulting in acid production used in maintenance of a neutral pH.

Its main function in the medium is as a pH indicator. Sodium phosphate serves as a buffering agent to help in pH stability of the broth. As the lactose is depleted and the buffering capacity is negated the pH rises and the selectivity of the broth decreases. *Salmonella* is inhibited by selenite but to a small extent in comparison to other bacteria.

Rappaport broth is the newest of the three broths and was introduced by Rappaport in 1956. It was first developed for the enrichment of *Salmonella* Paratyphi and other serotypes known to be resistant to brilliant green (Busse 1995). This broth contains high levels of malachite green and magnesium chloride supporting a pH of 5.2, which is lower than selenite and tetrathionate media. Malachite green and magnesium chloride inhibit the intestinal microorganisms, but have little effect on the growth and multiplication of *Salmonella*. This is due to the presence of magnesium chloride in the media. Vassiladis and others (1976) modified the medium by reducing the concentration of the dye to one third of its original concentration. Thus, the name Rappaport-Vassiladis broth was given to the medium. Rappaport-Vassiladis medium (RV) has been regarded by the AOAC as the medium of choice over selenite and tetrathionate broths for raw flesh products, highly contaminated foods and animal feeds. It has also been recommended for the analysis of low microbial load foods by FDA (2003). In many countries, it is used in combination with selenite broth and has been found to be more efficient in positive determination than tetrathionate. June and others (1995) found that when used in combination with tetrathionate, detection for *Salmonella* was even more sensitive. Davies and others (2001) also found this to be true, but also discovered that delayed secondary enrichment at 42^{o}C rather than 37^{o}C increased the ability to detect *Salmonella*. Maddox and others (1991) showed that the detection of the microbe was 6% higher when

using a secondary enrichment recovery method. This higher incubation temperature was also shown favorable by Vassiladis and others (1976) at 43°C, but was later reduced to 42°C by Baird and others (1989). It is now recommended that Rappaport-Vassiladis medium be used over selenite cystine broth or in combination with the two for the analysis of almost all foods (FDA 2003). Worcman-Barninka and others (2001) indicated that modified semisolid Rappapport-Vassiladis (MSRV) could be used in a more rapid selection process that detected 96.1% of *Salmonella* positive food samples as compared to 84.6% with current FDA protocol (Andrews and others 1998). This work showed 95.5% sensitivity and 96.8% specificity with *Salmonella* contaminated food.

Tetrathionate is a product of thiosulphate and iodine being present in the media. Iodine oxidizes thiosulphate into tetrathionate. This is why thiosulphate is added at high amounts to the medium. These two compounds restrict the growth of coliforms and enteric bacteria. *Salmonella* is a tetrathionate-reducing bacterium which is why it is able to grow in this medium. Calcium carbonate served as the buffering agent for the acidic environment produced, and the bile salts suppress the activity of any bacteria not normally found in the microflora of the intestines. Gram-positive bacteria are inhibited by brilliant green which is not necessarily found in tetrathionate broth (Busse 1995).

By-passing the pre-enrichment step and using direct selective enrichment for the analysis of certain foods, the time needed to complete the conventional culture method would be reduced by one whole day. However, the recovery of *Salmonella* from raw meats and dried egg albumen was significantly decreased by by-passing the pre-enrichment step (Andrews 1985).

Motility enrichment is an old technique (Craigie 1931) and was originally

performed in U-tubes where one side was inoculated and after a certain incubation period fast moving bacteria could be isolated from the other side. Goosens and others (1984) replaced U-tubes with Petri dishes. Motility enrichment on Modified Semisolid Rappaport-Vassiliadis medium (MSRV) was introduced by De Smedt and others (1986) and has been regarded as a very effective procedure for the isolation of *Salmonella* from foods (Boer 1998). In this medium, motility of competitive bacteria is inhibited while *Salmonella* move into fresh media leaving their competitors behind.

5.5 Agar Plating Media

Agar plating media for the isolation of *Salmonella* were developed and based on the selective agents used for identification (Busse 1995). These media consist of the bile salt agars, the brilliant green agar (BGA), and bismuth sulfite agar (BSA) (Busse 1995). Bile salt agars have the widest variation and include deoxycholate citrate agar (DCA), xylose lysine desoxycholate citrate agar (XLD), *Salmonella-Shigella* agar (SS) and Hektoen Enteric agar (HE) (Busse 1995). Bile salt as well as citrate are the selective agents and exist in these agars at various amounts. Thiosulphate is added as a secondary selective agent but primarily as an indicator of hydrogen sulfide active colonies (Busse 1995). In a study by Koivuner and others (2001), superior selection and differentiation was acheived using XLD and BGA with $MgCl_2$ in a detection range of 3×10^0 to 1.1×10^3 CFU/100ml of waste water based on most probable number analysis. Tate and others (1990) used xylosine lysine tergitol (XLT4) and found it to give improved recovery of *Salmonella* from poultry. Miller and others (1991) went on to support this and found further recovery improvement and identification using XLT4. The difference in XLT4

and XLD is that XLT4 uses tergitol rather than deoxycholate as an inhibitor of coliforms. The use of antibiotics has proven even more beneficial in the recovery and identification of *Salmonella* colonies present in food stuffs. Novobiocin has been used as a deterrent in the contamination of other species in pathogen infested food. Komatsu (1981) showed an increase from 50 to 82% of recovery of *Salmonella* and a reduction from 38 to 5% for false positives with the addition of novobiocin to XLD, XLT4, BG and HE media.

Brilliant green media consist of brilliant green agar (BGA) and mannitol lysine crystal violet brilliant green agar (MLCB). *Salmonella* is distinguishable on BGA by the formation of pink colonies and brilliant green is the selective agent. This is due to the microbe's inability to ferment lactose and sucrose (Busse 1995). MLCB works on the basis of mannitol fermentation, lysine decarboxylation and hydrogen sulfide production. It is usually used in combination with Rappaport-Vassiladis soya (RVS) broth because of the high selectivity of MLCB (Busse 1995). MLCB appears to be the superior of the two in that some enterobacteria will grow on BGA but not detected on MLCB (Busse 1995).

Bismuth sulphite agar (BSA) has been found very useful in the detection of *Salmonella* Typhi. Ammonium bismuth citrate and sodium sulphite are present in the medium and combine to produce a bismuth sulphite indicator (Busse 1995). The production of hydrogen sulfide and the reduction of bismuth indicate the presence of *Salmonella* (Busse 1995).

For all tests and media to be successful, the skill of the analyst must be very efficient and consistent so that sensitivity and specificity of the culture data is never compromised. This requires great knowledge of the colony morphology as it pertains to the many media used in identification of *Salmonella* whether on differential or selective.

This tends to create bias because different labs prefer different media and develop their own strategies, sometime resulting in reductions of recovery. This accompanied with the fact that the methods of detection are long and tedious and often takes several days, fuels the need for more rapid detectable methods. These rapid methods must be cost efficient and reliable as well as reproducible in the detection of pathogens. Traditional and conventional methods have proven to be dependable but exercise way too much man power and time.

6. RAPID METHODS

There is an increasing need for rapid test methods to certify the quality and safety of food products with development of innovative strategies for the detection and identification of foodborne pathogens (Glynn and others 2006; Naravaneni and others 2005). This is very important to human health because many high risk disease causing pathogens are passed through contaminated foods. As a rapid detection method, we are looking for technology that can produce dependable results in a few hours as compared to days. As a result, research has taken a journey into areas that are based on molecular techniques for this cause. Therefore much attention is being made on enrichment times focusing on the incubation periods for pre and selective enrichment. Most of the research is geared toward combining the two enrichment steps with focus on recovery. Thus if this is accomplished, molecular techniques such as polymerase chain reaction, immunoassay, and biosensors may be used for quick determination.

6.1 Nucleic Acid Diagnosis

6.1.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a nucleic acid based method and has been the most popular platform applied in this area of assay for foodborne pathogens (Glynn and others 2006). This technique involves the amplification of a specified DNA segment with the use of a heat stable polymerase and two synthetic oligonucleotides better known as primers (del Cerro and others 2002; Patel and others 2006; Malorny and others 2003). PCR is a repetitive cycling process that involves the denaturizing (lysis) of the target DNA, the annealing (binding) of the primers for hybridization to the opposite strands of DNA and the polymerization (amplification) of the targeted DNA. This cycling occurs for approximately 30 cycles. Once amplification has been achieved, the specified segment of DNA is analyzed by agarose gel electrophoresis where ethidium bromide is the selective agent. With it being important to know if the cells of a pathogen are alive and viable, the PCR technique cannot distinguish the difference. It also has the problem of the components within the food matrix. Food matrices pose a real threat in the PCR process in that the matrix has been found to inhibit the procedure blocking amplification of the targeted DNA. To combat this problem other steps are usually incorporated prior in an attempt to remove the inhibitory substances. Due to the lack of international validation and standard protocols, as well as variable quality reagents and equipment, the methodology has difficulties moving from expert to common laboratories (Malorny and others 2003). This can be seen in the published PCR base methods where the detection of *Salmonella* differs in specificity, detection limit as well as sample treatment (Aabo and others 1993; Baumler and others 1997; Burkhalter and others 1995; Cohen and others

1994; Jones and others 1993; Kwang and others 1996). On most occasions, an internal amplification control is rarely included in the diagnosis as an indication of false negatives (Malorny and others 2003). There are several methods of PCR including: traditional, real-time, nested, multi-plex and others incorporating the use of other rapid methods such as immunoassays. One other limitation is that despite the number of validation studies, few report on the sensitivity and specificity for the detection in naturally contaminated food samples (Oliveria and others 2003; Soumet and others 1994 and 1997; Myint and others 2006). PCR does have the primary advantages of increased sensitivity and reduced time in sample processing in the laboratory as compared to standard conventional culture methods (Lampel and others 2000; Whyte and others 2002). It also has the advantage of being able to be applied to mixed microbial specimens without prior isolation of individual species of bacteria at minute genetic quantities (Schrank and others 2001). PCR can be used to amplify genes specific to taxonomic groups of bacteria and also detect genes involved in the virulence of foodborne bacteria (Finlay and others 1988; Bej and others 1994; Naravaneni and others 2005).

6.1.2 Real-Time Polymerase Chain Reaction

In real-time PCR, the potential of false positive results is limited in that there is not post PCR sample handling preventing potential contamination due to PCR product carryover (Fratamico 2003). Real-time PCR methods involve the attachment of a fluorescence dye which serves as an indicator in the presence of the target DNA and is monitored during the process (Hein and others 2006). SYBR Green I-based real-time PCR detection has been used in the detection of *Salmonella* while being used with the

same primer pair as in conventional PCR and proven to be a less expensive approach for real-time PCR (Hein and others 2006). It does lack the additional specificity found when using a more probe detection format (Eyigor and others 2002). These fluorescent technologies employed are either nonspecific by using dyes such as SYBR greeen I or SYBR gold (Glynn and others 2006). These are minor groove binding dyes and intercalate into the PCR product during amplification (Glynn and others 2006). They can also be specific by using probes to detect specific sequence amplification in the PCR (Glynn and others 2006). A number of different fluorescent probe chemistries have been employed in real-time PCR assays including TaqMan (5' exonuclease) probes, fluorescent resonance energy transfer (FRET) probes, molecular beacons and scorpion probes (Glynn and others 2006). Mechanisms for fluorescent signal generation are different for probe chemistries; the fluorescent signal generated is directly proportional to the amount of PCR product generated (Bustin 2002; McKillip and others 2004; Glynn and others 2006). SYBR green real-time PCR techniques have reported the detection of *Salmonella* with an assay time of approximately 2 h when applied directly to samples without pre-enrichment (Fukushima and others 2003; Jothikumar and others 2003). Wang and others (2004) detected levels of 1-5 CFU with this technology in enriched milk and meat samples. Real-time PCR assays for *Salmonella* have also been developed using molecular beacon technology targeting the invA gene (Chen and others 2000; Liming and others 2004; Wan and other 2004).

6.1.3 Multiplex and Other Strategies

Multiplex PCR assays have also been developed for the simultaneous detection of two or more foodborne pathogens (Glynn and others 2006). Li and others (2004) showed that after 24 h of enrichment that the simultaneous detection of *Salmonella, E. coli* O157:H7 and *Shigella* in apple cider was possible. Kawasaki and others (2005) were able to detect, at a level of 1 CFU/g in pork, the presence of *Salmonella, L. monocytogenes* and *E. coli* O157:H7 after proper culture-based enrichment. Jothikumar and others (2003) reported that *Salmonella* was detected at a level of 2.5 cells and *L. monocytogenes* was detected at a level of 1 cell using multiplex real-time PCR with SYBR green detection technology targeting the fimI gene of *Salmonella* and the hly gene of *L. monocytogenes* in a liquid culture. Wang and others (2004) were able to detect slightly higher levels of both bacteria in raw meat samples with a total assay time of 10 h including 6 to 8 h of pre-enrichment.

To speed up analysis, PCR including real-time PCR have been applied in various stages of the diagnostic procedure: confirmation of suspected colonies grown on agar plates, analysis of enrichment broths and direct analysis of suspected food stuffs (Hein and others 2006). This is an in vitro amplication base method, which involves the amplication of a specific sequence of DNA specific for a gene of the target bacteria. Numerous methods have been developed in the detection of *Salmonella* in food. Jin and others (2004) demonstrated that *Salmonella* Typhimurium could be detected at a limit of 100 CFU in a pure culture to 200 CFU from positive chicken meat samples. Their gene of amplification was the ogdH gene. Ferretti and others (2001) have optimized a more sensitive PCR assay involving a detection limit of 1-10 cfu in salami. The total assay

time is 12 h including 6 h of culture enrichment, DNA purification, PCR amplification and analysis. This PCR assay focused on the invA gene. Malorny and others (2003) validated a PCR assay showing selectivity on 242 *Salmonella* strains (inclusivity 99.6%) and 122 non-*Salmonella* strains (exclusivity 100%) where the primer set amplified a 284 base pair sequence of the invA gene. This project did incorporate an internal amplification control. Schrank and others (2001) reported the development of a PCR method coupled with the incubation in a selective broth and rapid DNA preparation for the detection of *Salmonella enterica* serovars in samples directly from the poultry industry. To date, several PCR commercial available kits are available for the detection of *Salmonella* in many foods.

Acceptance and application of nucleic acid technologies has been limited due to the lack of standardization and validation of diagnostic PCR assay protocols (Malorny and others 2003). For this to happen there first must be a sample specific method development and validation that takes into consideration the effect of sampling, sample preparation and preparation of the amplification mixture on test performance (Glynn and others 2006). Then there must be the establishment of an internal quality assurance scheme in the form of an internal amplification control to combat the problem and possibilities of false negative results (Hoorfar and others 2004; Glynn and others 2006). Once these are accomplished, the participation of external quality assurance programs implemented as inter-laboratory trials to validate PCR pathogen detection protocols and methods (Hoorfar and others 2004). False positive results, due to the amplification original dead cells present in the food sample, are also a major concern. Scheu and others (1998) believe that this is circumvented in the enrichment process prior to PCR analysis.

This is totally avoided by amplification techniques that involve RNA. McKillip and others (1998) verified that in vitro amplification of RNA ensure the detection of viable cells. It is believed that due to the fact that RNA is more difficult to isolate, there will be a decrease in the analytical detection limit of the targeted pathogen's RNA. These along with the ever problematic inhibitory ability of the food matrices, will continue to make application of PCR assays for pathogen identification a real challenge, due to the fact that certain foods are more problematic than others (Rossen and others 1992; Bickley and others 1996). Thus, there must be a step for the removal of these inhibitory substances if proper and adequate DNA amplification is to be determined in PCR analysis (Glynn and others 2006).

Recent research has focused on the use of immunomagnetic separation (IMS) to speed up the selective enrichment process prior to PCR analysis. This will not only cut the analysis time but will also aid in the removal of inhibitors that affect the PCR analysis (Glynn and others 2006). This method incorporates the use of magnetic beads that have been conjugated with the antibody that selects for the targeted bacteria in the food sample and as the sample enrich a complex is formed of the targeted organism (Mercanoglu and others 2005). These complexes are captured magnetically and used for further analysis testing using nucleic acid amplification and culture plating (Lamoureux and others 1997; Rijpens and others 1999; Hudson and others 2001; Mercanoglu and others 2005). This method is simple and generally requires no more than 1 h to conduct. As can be seen, this method increases all aspects of the assay by improving sensitivity, selectivity, and processing time.

6.2 **Biosensor/Microarrays**

Biosensors are devices that incorporate the use of technology (probe/receptor and transducer) that use biological derived material that has been immobilized on a detection platform in determination of one or more targeted analytes being present (Mascini 2005). In the food safety and detection venue the ideal biosensor must be self contained, automated and capable of pathogen detection without enrichment directly from a food sample (Ivnitski and others 2000). It must also be able to distinguish the difference between dead and live cells. This technology can be based in three categories: metabolism, antibody and DNA. Biosensors may be classified as optical, mass and electrochemically. Optical biosensors use fluorescence produced by the bacteria under a UV source (Ivnitski and others 2000), some are also based on bioluminescence where enzymes catalyze reactions that give off excited photons. These types of sensors tend to be rapid, direct and highly specific. Electrochemical react off electrical signals generated between the target bacteria and the indicating element. Nucleic acid based biosensors are now on the market and have found their way into the food industry. Quartz crystal microbalances (QCM) and optical detection systems are the two most popular. QCM has been used in combination with PCR in the detection of *E. coli* cells in water at a level of 1-10 cells per 100ml (Mo and others 2002). Optical based sensors being used incorporate what is known as surface plasmon resonance (SPR) where the bio-molecular interactions on a surface in real-time is monitored (Rand and others 2002). This technique is quite promising in that no labeling of a target molecule is needed. These type detectors generally monitor antigen and antibody interaction. This system has seen some development in the detection of *L. monocytogenes* (Leonard and others 2004). This

technique in the measuring of DNA-DNA and DNA-RNA has been studied with defined protocol but the detection and identification of foodborne pathogens has yet to be established (Wang and others 2004; Mannelli and others 2005). Yao and others (2003) have reported the use of advanced visualization and signal amplification technologies in the monitoring of single molecular interactions in real-time. Some nucleic acid targets on a biosensor platform have been seen at high sensitivity levels (3×10^6) target molecules when evaluated with PCR products (Storhoff and others 2004). In a study by Fuentes and others (2005), where a biosensor-based nucleic acid detection method was used involved concentrating the target nucleic acid using DNA probes bound to supramagnetic nanoparticles prior to detection. This method was able to detect a minimum of 2 molecules of target cDNA in a 2.5 million fold excess of nonhomologous DNA.

Microarrays are comprised of large numbers of probes (oligonucleotides or cDNA) immobilized on a solid surface such as specially treated glass (Glynn and others 2006). Hybridization is achieved by applying a labeled nucleic acid of target in a liquid state to the microarray surface. After adequate contact time and washing steps the target nucleic acid left bound to the probes on the microarray are visualized with the use of a scanner (Glynn and others 2006). However, few research has been done in the identification of food pathogens. Wu and others (2003) used this technology in the molecular identification of *E. coli* O157:H7, and Volokhov and others (2003) in the identification of *Campylobacter* spp. from cultures following PCR amplification of target genes. Application without in vitro amplification has been investigated in the direct detection of bacterial RNA using oligonucleotides as the probes. Small and others (2001) showed a sensitivity level of cells (7.5×10^6 CFU) from environmental bacterial isolates.

Anthony and others (2005) demonstrated a detection limit of 1.6 x 10^4 CFU of *S. aureus* rRNA following column purification. These studies also incorporated a chaperone probe system to increase sensitivity. This technology is being incorporated with signal amplification for the identification and subtyping of bacteria and pathogens. Once this technology is completely evaluated, it will be incorporated with many nucleic acid diagnoses.

The developments in biosensor and microarray technology have shown promising advances in the detection and identification of foodborne pathogens. With this technology being designed to have optimum as well as detection at minute levels of target nucleic acid, the sample being free of contaminants is very important (Fung 2002). This will show particular relevance in the food industry if 1 viable cell is to be detected in 25 g of food. This level is not yet applicable with this technology but these challenges must be met before this technology can adequately be considered as a legitimate option of pathogen detection.

STATEMENT OF RESEARCH OBJECTIVES

The objective of this research was to develop a protocol for the rapid detection of *Salmonella* spp. using polyclonal antibodies in conjunction with real-time polymerase chain reaction.

The specific objectives of this study were:

1. to determine the most effective medium for enrichment of *Salmonella* spp. at a very low initial population and work in conjunction with PCR
2. to evaluate the efficiency of centrifugation as a method of concentrating *Salmonella*
3. to evaluate an immuno-magnetic separation technique as a means of removing the microbe from an inhibitory food matrix (magnetic beads)
4. to compare the efficiency of conventional PCR with real-time PCR
5. to determine an incubation time adequate for the detection of *Salmonella* spp.
6. to evaluate the binding capacity and adherence of the antibody to the magnetic bead for *Salmonella* spp. capture
7. to determine the best incubation period and temperature for the magnetic capture of *Salmonella* spp. along with optimal amount of magnetic beads

CHAPTER III. MATERIALS AND METHODS

1. BACTERIAL CULTURING

Salmonella enterica Typhimurium, Enteritidis, and Mission were the three serovars used for this project. *Salmonella* spp. were grown in Trypticase® soy broth (TSB, Difco Laboratories, Detroit, MI) by transferring one colony into 5 mL of TSB. The bacterial cultures were incubated in an orbital shaker (Orbital Shaker, Marietta, OH) incubator at 37ºC and 100 rpm for 16 h. One ml of each serovar was combined into a cocktail and washed twice with sterile Butterfield phosphate buffer (BPB) by mixing and performing centrifugation at 5,000×*g* for 3 min. The bacterial pellet was re-suspended in BPB, and the bacterial population was estimated from the absorbance at $O.D._{640nm}$ using a preconstructed equation. The bacterial population was confirmed by plating *Salmonella* spp. on Trypticase® Soy Agar (TSA). Once the population was determined, the remaining culture was cocktailed and prepared for sample inoculation at the desired level.

2. CHICKEN SKIN PREPARATION

Chicken skins were obtained from Koch Foods in Montgomery, Alabama. Chicken skin samples were packaged in 40 lb bundles and stored under dry ice for the trip to Auburn University. Upon arrival, the skins were repackaged in zip-lock freezer bags and stored at -22^{o}C until needed. Forty-eight hours prior to the experiment, chicken skins were thawed under refrigerated conditions and cut into 4 x 4 in squares and stored

for 24 h at 4^{o}C.

3. CHICKEN SKIN INOCULATION

The bacterial (cocktail) suspension was diluted in TSB to cell counts of 10^3, 10^2 and 10^1 CFU/mL. Chicken skins (16 square inches) were inoculated by applying 200 µL of each cell count and spreading the cells over the square, in order to obtain a contamination level of the skins at approximately 200, 20, and 2 CFU per square. Inoculated skins were allowed to stand at room temperature for 30 min to promote good adherence of the *Salmonella* spp. to the skin. After 30 min, the chicken skins were placed in stomacher bags with 100 mL of TSB and blended in a Seward® 400 Circulator stomacher (Seward Company, Seward, England) at 260 rpm for 2 min. The entire content of each bag was poured into 500 mL flasks in which 1 mL of naladixic acid was added. Naladixic acid was added because all serovars in the inoculum are naladixic acid resistant. The presence of naladixic acid will kill and suppress the growth of the natural microflora present on the chicken skins while allowing *Salmonella* spp. to grow. All flasks were subsequently placed in an incubator orbital shaker at 37ºC for 2 h. Following 2 h of incubation, the chicken skins were removed and the TSB for each sample was poured into two 50 mL centrifuge tubes. Each sample was centrifuged at 7000 *xg* for 10 min in a swing bucket rotor (SH-3000TC). The supernatant (except for the remaining 5 mL) and any solidified lipid were removed. The remaining 5 mL was vortexed and 5 mL of fresh TSB was added to obtain a homogeneous cocktail. Both corresponding 5 mL samples were combined and placed back into the incubator at 37 ºC for another 4 h of incubation. After incubation, samples were plated on Tryptic soy agar (TSA) containing 100 ppm of naladixic acid in order to determine bacterial population for 6 h of

incubation. The cocktail was centrifuged again, and the supernatant was removed leaving approximately 500 μL above the bacterial pellet. The remaining 500 μL was vortexed and then used subsequently for polymerase chain reaction (PCR) analyses.

4. ANTI-*SALMONELLA* ANTIBODY PRODUCTION

4.1 Antigen Preparation

The outer membrane proteins were produced in the same manner that Meenakshi and colleagues (1999) demonstrated with some minor changes. Cells of serovar Typhimurium were used for antigen preparation. *Salmonella* Typhimurium after being incubated in TSB at 37ºC for 16 h was washed twice with saline solution. These cells were re-suspended in 10 mM Hepes buffer (pH 7.4) and sonicated at 20 kHz on ice for 1 min. Samples were sonicated five times. After the final sonication, samples were then centrifuged at 1,700 *xg* (4ºC) for 20 min. The supernatant was centrifuged at 100,000 *xg* (4ºC) for 60 min. The supernatant was discarded, and the clear gelatin pellet was re-suspended in 2% (w/v) sodium lauryl sarcosinate, 10 mM Hepes buffer and allowed to stand at room temperature for 1 h. The mixture was centrifuged at 100,000 *xg* (4ºC) for 60 min; the pellet collected and washed twice with deionized (DI) water and repeat centrifuged under the same parameters. The pellet (outer membrane proteins) was collected and dissolved into 6 mol/L guanidine thiocyanate (Sigma Chemical Co., St. Louis, MO) and allowed to stand at room temperature for 60 min. The solution was centrifuged at 300,000 *xg* to remove insoluble material. The supernatant was dialyzed at 4^{o}C in deionized water overnight. The deionized water was changed approximately every 8 h until dialysis was complete (Kerr and others 1992). Protein concentration was

determined by the Bradford method, and protein purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Molecular weight of protein bands was determined by reference standard of molecular weight markers (Sigma).

4.2 Polyclonal Antibody Production

A New Zealand white rabbit approximately 3 kg in weight was used to produce anti- *S*. Typhimurium serum. Purified outer membrane proteins produced were emulsified with RAS-R730 (Corixa, Hamilton, MT) at 285 μg/mL. Rabbits were then immunized with this emulsion by an intradermal injection of 50 μL aliquots at six sites on the back near the spinal cord and a 300 μL aliquot was injected into both rear legs. This procedure was repeated after four weeks to ensure adequate antibody production. Blood serum was obtained at seven day intervals from the main artery running through the rabbit's ear. Collected serum was centrifuged at 5,000 *xg* (4^{o}C) for 20 min and the supernatant (anti- *S*. Typhimurium serum) was kept. For further purification, polyclonal antibodies were extracted from the serum by precipitating the supernatant with a 20-50% saturated ammonium sulfate solution. The precipitate was then collected and re-suspended in PBS for dialysis with 8h buffer changes for 24 h. Further purification was carried out by affinity chromatography with the use of a protein A column. The rabbit IgGs were eluted with 0.1 M citrate buffer (pH 3.0). To measure protein, the Bradford method was used and purity was determined by SDS-PAGE. Specificity of the antibodies was determined by indirect ELISA.

4.3 Monoclonal Antibody Production

A mouse, BALB/cAnNHsd female mouse (Harlan Sprague Dawley Inc., Indianapolis, IN), was used to produce anti- *S.* Typhimurium serum. The purified outer membrane proteins of *S.* Typhimurium were emulsified with RIBI's adjuvant system (RAS-R700) (Corixa, Hamilton, MT) at 100 μg/mL. Female mouse was immunized with the emulsion of the outer membrane proteins by subcutaneous injection of 100 μL into each rear leg on the ventral side near the axillary and inguinal lymphatics. The initial RAS-R700 was used for the follow-up boosters. The mouse was boosted every 3 weeks for a total of 12 weeks. Blood was collected every 7 days after each booster, and the titer of serum was determined. The procedures described by Kohler and Milstein (1975) were followed with minor modifications. Five days following the final booster, spleen cells were collected to fuse with murine myeloma cells. This was performed at a ratio of 1:2 (spleen cells/myeloma cells) with 50% polyethylene glycol (PEG). Fused cells were suspended in hypoxanthine-aminopterin-thymidine (HAT) selective medium (Dulbecco's modified Eagle medium containing 200 μM hypoxanthine, 0.8 μM aminopterin, and 32 μM thymidine) and seeded into 96-well cell culture plates. After 10 to 14 days, the positive wells were analyzed for production of anti-*Salmonella* Typhimurium antibodies by indirect ELISA. Hybridoma secreting antibodies that reacted with *S.* Typhimurium were selected for expansion and cloning using limiting dilution methods as described by Harlow and Lane, 1988. Monoclonal antibodies were produced from selected cell lines through mouse ascites fluid and purified by affinity chromatography (Biological Duo-Flow System Bio-Rad) with protein A affinity column. Purified antibodies were dialyzed with 0.01 M phosphate-buffered saline (PBS, pH 7.0) for 24 h with changes at

approximately 8 h at 4^{o}C. To measure the concentration of IgG, the Bradford method was used, and purity was determined by SDS-PAGE. To determine isotype of the monoclonal antibodies a Mouse Monoclonal Antibody Isotyping Kit (Sigma) was used. Purified IgG was stored at -80ºC in buffer (0.1 M Tris + 2 mM $MgCl_2$ + 20 mM Glycine + 30 mM sodium azide, pH 8.0, 50% glycerol).

5. INDIRECT ELISA ASSAY

A 96-well assay plate was coated with 100 μL of 10^9 CFU/mL *Salmonella* cells at 37°C for 2 h. Subsequently, coated plates were washed three times with 200 μL of PBST (0.05 % Tween-20 and 0.02% sodium azide). Wells were blocked by adding 200μL of 1% bovine serum albumin in PBS at 25°C for another 1 h. A serial diluted 100 μL antibody solution wERE then added to each well, and allowed to incubate at 25°C for 2 h. Plates were washed again three times with the PBST wash solution ; 100 μL of diluted secondary antibody in PBS were added to each plate and incubated for 1 h. After incubation, plates were washed four times with PBST and 100 μL of *p*-nitrophenyl phosphate (*p*-NPP) (40 mg dissolved in 10 mL of 10 mM Diethanolamine + 0.5 mM $MgCl_2$, pH 9.0) solution were added to every well. Plates were then incubated in the dark at room temperature for color development. Absorbance is measured every five minutes at $O.D._{405nm}$ on a microplate reader (ThermoLabsystems, Helsinki, Finland) and recorded for analysis.

6. MAGNETIC BEAD SYNTHESIS

6.1 Bead Formation and Design

Magnetite (Fe_3O_4) was produced by a modified method developed by Sugimato (Sugimoto and Matijevic, 1980). In an Erlenmeyer flask, 4.5 L of 0.42 M iron (II) sulfate was degassed. A three holed #9 ½ stopper fitted with a thermometer, a plastic propeller stick of the overhead stirrer, and a nitrogen inlet, was used to seal the flask. The flask was heated on a hot plate, and nitrogen gas was carefully released into the flask and exhausted from the propeller stick. Once iron (II) solution reached 95^{o}C, 1.5 L of degassed (65^{o}C) preheated 0.8 M of potassium nitrate and 3.4 M of potassium hydroxide solution were added to the flask. This solution was maintained between 92-96^{o}C for an hour with stirring and constant purging with nitrogen gas. The mixture was then allowed to cool to room temperature, and the flask was placed on a 4 × 4 × ¼ in (N42 strength) neodymium magnetic plate for 10 min. Supernatant was removed by suction. The formed black magnetite was washed several times in deionized water, while being held in place by the magnetic plate. Supernatant was continually removed by suction until supernatant was below pH 7.0. Beads (black magnetite) were stored in the deionized water, yielding approximately 1.5 L in bed volume. Magnetic beads were further examined by transmitting electron microscopy (TEM) (Figure 10).

Beads were coated with silica (sodium silicate) as modified by Taylor and others (2000) and Butterworth and others (1996). One hundred grams of sodium silicate solution (40-42˚ Bé) was dissolved in 1 L of deionized water. To 800 mL of sodium silicate solution, (50 g of washed dry weight Dowex-50 that was regenerated in 1.0 M of hydrochloric acid) was added and stirred slowly for one minute. The resin was then

removed by vacuum filtration and pH was adjusted to 9.5 with the unfiltered sodium silicate solution. Sodium silicate was added to the beads and stirred. As the slurry stirred, 100 mL of 1.0 M of tetramethylammonium hydroxide (TMA) was added, with the pH of the mixture being slowly adjusted to 10.0 with 0.5 M hydrochloric acid. This procedure took approximately 1 h, but the mixture was allowed to react for an additional 2 h while stirring. The beads were once again washed with deionized water until the pH of the supernatant was neutralized. These coated beads (50 µL bed volume) were reacted with 1 mL of 1.0, 2.0, 3.0, and 4.0 M hydrochloric acid in microcentrifuge tubes for two hours and then examined. Coated beads (100 mL bed volume) were suspended in 1 L of 95% ethanol. To this 10 mL of 3-Aminopropyl-trimethoxysilane (APTMOS) was added, to deposit the silicate and bond the H_3N^+- group on the nano-particles (Liao and others 2007). The mixture was stirred at room temperature for two hours, and then transferred to a 90^{o}C water bath with stirring until reaching 70^{o}C for 10 min. Beads were then washed twice with ethanol, twice with deionized water, and once with 10 mM pyridine-NaOH buffer. The beads were re-suspended in 800 mL of the same buffer to which 200 mL of 25% glutaraldehyde was added to introduce an aldehyde group to the ammonium group on the beads (Liao et al., 2007). After 2 h of interaction with stirring at room temperature, the beads were washed with deionized water until the pH was neutralized and re-suspended in 1 L of ethanol. Acetate anhydride (10%) was added to the beads and stirred at room temperature for 30 min in order to block the free ammonium groups on the beads. The glyoxyl beads were washed with deionized water and stored at 4^{o}C until needed for conjugation.

6.2 Conjugation of Antibody

Prior to conjugation, 0.2 M disodium and 2 M cyanoborohydride were prepared and allowed to stand overnight. The amount of needed magnetic beads was then determined and washed with 3 times its bed volume with 0.2 M disodium phosphate. This was repeated twice. Coupling buffer was prepared by combining 1 mL of 2 M cyanoborohydride to 100 mL of disodium phosphate. Antibody was added to this buffer at a minimum of 1 mg/mL of bead volume and read at $O.D._{280nm}$. At this concentration, an OD reading of approximately 0.6 will be obtained with plain coupling buffer used as a blank. Antibody mixture was added to magnetic beads and conjugation reaction was allowed to occur until O.D. reading stopped decreasing. The reaction was allowed to couple for 30 min. Coupling buffer was removed and beads were washed twice at 2x the bead volume with phosphate buffer. To the final wash solution, 12 mg of ethanolamine was added for every mL of bead volume to block the un-reacted glyoxal sites on the beads. This reaction was allowed proceed for 1 h. Beads were washed again in phosphate buffer with sodium azide and stored at 4^oC for use.

7. BACTERIAL ENUMERATION

7.1 Spread Plate from Bead Attachment

Conjugated beads were allowed to interact with concentrated cells (500 μL) and used for further PCR analysis. They were removed from the supernatant, where beads and supernatant were maintained for bacterial enumeration. Supernatant was serial diluted and spread plated on TSA containing nalidixic acid at 100 ppm. Plates were incubated at 37^oC for 24 h and then colonies were counted for population determination.

Beads were placed in 1 mL of citric acid buffer (1M, ph 3.0) with some agitation for 5 min. To the mixture, 95 μL of 11 M TRIS was added. The beads were removed and the solution was serial diluted. These serial diluted solutions were spread plated and incubated at 37^{o}C for 24 h. Bacterial colonies were counted for microbial population determination.

8. PCR DETECTION

8.1 Bioinformatics/Computational Biology and Primer Design

Genetic information on *Salmonella* spp. was obtained from Entrez of the National Center for Biotechnology Information (NCBI Entrez) http://www.ncbi.nlm.nih.gov/entrez/ Workbench 3.2 host at San Diego Supercomputer Center (SDSC), http://workbench.sdsc.edu/ and The Institute For Genomic Research (TIGR), http://www.tigr.org.

DNA and protein sequencing data were analyzed with the Basic Local Alignment Search Tool (BLAST) service provided by NCBI http://www.ncbi.nlm.nih.gov/blast/ or ClustalW provided by SDSC. Sequencing confirmation performed by the Auburn University Genetic Analysis Lab and visualized with Chromas version 1.61 (Technelysium Pty Ltd., Austria) or Vector NTI version 10.1 for Windows (Invitrogen, Carlsbad, CA). Promoter regions and transcription start sites were predicted with geWorkbench developed by Columbia University. DNA restriction cutting cites were analyzed with NEB Cutter provided by New England Lab (NEB, Ipswich, MA, http://tools.neb.com/NEBcutter2/index.php). PCR primers were designed with the help of OligoAnalyzer 3.0 provided by Integrated DNA Technologies (IDT, Coralville, IA)

hosted at (httpps://www.idtdna.com/analyzer/Applications/OligoAnalyer/). PCR reactions were simulated in the web-based UCSC In-Silico PCR (http://genome.ucsc.edu/cgi-bin/hgPcr), or In silico amplifiction hosted at (http://insilico.ehu.es/PCR/*),* depending on the virtual DNA templates provided.

Primers were designed based on the sequencing of the STN gene of the *Salmonella* spp. of which is universal for all strains. Primers were designed manually where the sequence of interest was downloaded and analyzed by SDSC ClustalW. Primers were picked based on a predicted annealing capacity (temperature), and PCR reactions were simulated as previously mentioned**.**

8.2 Primer Specificity/Sensitivity

Primer specificity was analyzed by conducting PCR on several strains of the *Salmonella*, *Listeria*, *Escherichia*, *Citrobacter*, *Klebsiella*, *Morganella*, *Shigella*, *Yersinia*, *Vibrio*, *Xanthomonas* and *Staphylococcus*. All were analyzed on agarose gel by electrophoresis after PCR with the images recorded using a Kodak Gel Logic 200 Imaging System (Eastman Kodak, New Haven, CT). Primer sensitivity was analyzed by serial diluting the target bacteria at 10^5, 10^6, 10^7, 10^8 and 10^9 CFU/mL. Target bacteria were grown for 16 h in TSB and washed twice in BPB at a centrifugation rate of *5,000xg* for 3 min at 4ºC. Bacteria population was determine by $O.D._{640nm}$ and calculated by a pre-constructed equation for *Salmonella*. Once the bacterial number was determined, the original bacterial suspension was serial diluted in fresh TSB at 10^5-10^9 CFU/ml at 10 fold dilutions. A negative control was used by substituting the DNA (bacteria) with purified water, and the positive control was produced with pure chromosomal DNA (TY2) for the

same substitution. Dilutions were PCR amplified in duplicates and analyzed by agarose gel electrophoresis and images recorded as in primer specificity. The actual bacterial population of the bacterial suspension was analyzed by spread plate methods. Plates were incubated for 24 h at 37ºC and colonies were counted on the suitable dilution.

8.3 Convention PCR Protocol

In a sterile 0.5 ml tube, the following materials are mixed in the corresponding ratio:

Conventional PCR

5X buffer TGO (10 μL)

Primer 1&2 mixed (5 μL)

dNTP (1 μL)

Sterilize Deionized H_2O (32 μL)

Template DNA (bacteria) (10 μl)

The positive control replaced template DNA with pure chromosomal DNA (DNA-TY2) and the negative control replaced this DNA with H_2O with 1 μL of each. Samples were placed in the PCR machine for a hot start at 90^{o}C for 1 min. The machine was paused at this point, and 1 μL of heat stable polymerase TGO was added to each amplification tube. Machine was allowed to complete the PCR process. The sample goes through an initial cycle consisting of 2.5 min at 94^{o}C, 30 s at 60.1^{o}C and 72^{o}C for 20 s. The next 35 cycles were at 94^{o}C (Denaturization Temperature) for 30 s, 60.1^{o}C (Annealing temperature) for 30 s and 72^{o}C (Amplification Temperature) for 20 s. The final cycle held the samples at 72^{o}C for 5 min. Amplification was then complete and samples are now ready for analysis

by electrophoresis in an agarose gel (Sambrook and Russell 2001).

An annealing temperature was determined by running a temperature gradient (52^{o}C to 72^{o}C) with the PCR machine where a set of DNA samples were used. This gradient was run under the same conditions as previously mentioned for same number of cycles. The initial cycle samples were held at 94^{o}C for 4.5 min instead of 2.5. A temperature of 60.1^{o}C yielded a well define band at the 400 base pair limit for these primers.

8.4 Real-Time PCR Protocol

In a sterile 0.5 ml tube in a 24 well plate, the following materials are mixed in the corresponding ratio:

Real-Time PCR

Cyber green premix (25 μL)

Primer 1&2 mixed (2 μL)

Sterilize Deionized H_2O (21 μL)

Template DNA (bacteria) (2 μL)

Samples were placed in the PCR machine where they were maintained at 50^{o}C for 2 min. An initial cycle for 8.25 min at 95^{o}C was performed with the next 40 being at 95^{o}C for 15 s, and 60^{o}C for 60 s. Once complete the samples were analyzeds by electrophoresis in an agarose gel (Sambrook and Russell 2001).

9. ELECTROPHORESIS (Agarose Gel)

Samples were mixed with 5 μL of 10X identification dye for analysis by gel electrophoresis. The gel was made by adding 3.75 g of agarose into 250 mL of TAE buffer (1.5%) with heating to dissolve the agarose into the buffer. Ethidium bromide was added at 3 μL per 100 mL of TAE buffer, once it has cooled to approximately 60^{o}C. The gel was then mixed well, and poured on a gel plate containing well combs for sample loading. It was poured at approximately 5 mm in thickness and allowed to set for 45 min for solidification. Once adequate solidification had occurred, the gel was then submerged in the electrophoresis chamber. TAE buffer was added to completely submerge the gel. Samples were mixed with 10X loading buffer (Promega, madison, WI), where wells were loaded at 10 μL per well. Standard DNA marker was added to show bands at appropriate base markings. The chamber was run at high voltage (75 volts for 5 min then 150 volts for approximately 40 min). Once completed, the separated DNA was visualized at UV $_{240nm}$ and captured by an automated Kodak Gel Logic 200 Imaging Machine (Eastman Kodak Company, New Haven, CT). The size of the target DNA of the samples was analyzed by comparing the markers on the same gel with that of the standard.

CHAPTER IV. RESULTS AND DISCUSSION

1. GENERAL

PCR is an emerging method of choice for detection when a rapid approach is needed. Combining it with other methods such as enrichment has changed the detection time from days to hours. Many studies have incorporated extraction methods that involve removing bacteria from the food matrix or targeted DNA from the mixture which in turn reduces the detection level making the PCR method more sensitive and efficient. These extraction methods tend to be tedious and time consuming, and in some instance, very expensive. One, such method, has been the use of a immunochemical method known as magnetic bead removal. Hudson and others, 2001, reported by incorporating magnetic beads into food matrix, as little as 2 CFU/25g of sample could be detected within 24 h. If this is true, then it is conceivable that a sample containing more *Salmonella* might be analyzed for *Salmonella* in a shorter time.

2. MEDIA RESULTS/COMPARISONS

In order to populate low levels of *Salmonella* in a food matrix to a detectable level, medium enrichment is critical. To determine and gain further knowledge of an appropriate media, various non-selective and selective media were compared. Universal preenrichment broth (UPB), Tryptic soy broth (TSB), half strength TSB (1/2 TSB),

lactose broth (LB), brain heart infusion broth (BHI), nutrient broth (NB), and buffered peptone water (BPW) were the non-selective media evaluated. Brilliant green broth (BG), Rappaport-Vassiliadis R10 broth (RV), salmosyst broth, selenite broth (SB), and selenite cystine broth (SC) were the selective media. *S. enterica* Typhimurium at 10^3 CFU/mL population was inoculated at 10 fold dilution in each broth yielding a 10^2cfu/mL population before incubation. After 6 h of incubation at 37^{o}C, the bacterial population was confirmed on bismuth sulfide agar. Of the non-selective enrichment media, BHI produced the highest increase for the non-selective (3.58×10^6 CFU/mL) with BG producing the highest for selective (4.81×10^6 CFU/mL) (Table 1). Based on these results, BHI and BG media were selected for this research.

Because the chicken skin would naturally harbor many kinds of bacteria, a selective medium would be required to selectively cultivate *Salmonella* over other bacteria on the chicken skin. For this reason, the selective medium BG was chosen for this research, rather than the non-selective medium BHI. After several runs with conventional PCR, it showed that BG contained some inhabitants that blocked *Salmonella* detection even at high concentrations. BG's color posed a detection interference for real time evaluation with compounds not removed from bacterial washing made conventional detection impossible. To alleviate this problem, TSB was chosen and our bacteria would be a cocktail of antibiotic (naladixic acid) resistant (Mission, Enteridis and Typhimurium) serovars. TSB is a non-selective medium and had a slightly lower population after 6 h of incubation at (2.74×10^6 CFU/mL) (Table 1) but showed similar growth on chicken skin at (3.44×10^5 CFU/mL) (Table 2). In comparison to BHI and BG on chicken skin the bacteria enumerated in the exact same manner as it

did in pure medium (Table 1 and 2). TSB was chosen over BHI because of the salt content. BHI has two times the amount of salt as in TSB. With TSB being a non-selective medium, the micro flora issue was still a threat on the enrichment of the target bacteria. As a result, it was made selective by adding naladixic acid at 100ppm.

3. EFFICACY of ONE STEP ENRICHMENT in TSB

When the bacterial cocktail was inoculated on the chicken skin, 200 µl at 10^1, 10^2, 10^3 CFU/mL levels were added. Each chicken skin was immersed in 100 mL TSB resulting the initial population levels of 0 .02, 0.2 and 2 CFU/mL, respectfully. *Salmonella* at 0.02 CFU/mL increased to approximately 10 CFU/mL in 4 h. After 6 h of enrichment in TSB, the population reached in excess of 10^2 CFU/mL (Table 3), which is in excess of reported PCR detection limits. Thus, a concentrated centrifuged sample would result in an even higher population. Centrifuged sample populations ranged from 10^3 - 10^4 CFU/mL (Table 3).

Note: 100 mL of enrichment media after 6 h of incubation was centrifuged and re-suspended in 200 µL of PBS. Theoretically, *Salmonella* population should result in a 100 fold increase in population. By comparing the bacterial concentration before centrifugation and after centrifugation, the concentration seems to increase only 30 to 50 times. This difference is probably due to the loss of bacteria during transfer of bacterial suspension into centrifugation tubes, aspiration of the supernatant and pipetting errors during pellet re-suspension.

4. SENSITIVITY TEST of MONOCLONAL AND POLYCLONAL ANTIBODIES by INDIRECT ELISA

Antibodies were designated mAb IB4 for monoclonal antibodies and pAb S48 for polyclonal antibodies. In a previous lab study it was found that fusion occurred in 700 growth-positive hybridomas, where only120 showed positive affinity for *S. enterica* Typhimurium in indirect ELISA. 1B4 and 7B10 cell lines were the positive hybridomas that produced precise antibodies for *S. enterica* Typhimurium. Thus, the 1B4 cell line was cloned and injected into the mouse for mAb production from acities. After blood collection and Affinity Protein A column purification of mAb and pAb, the purity was checked by SDS-PAGE (12% gel). SDS-PAGE showed very distinctive bands indicating highly purified antibodies.

To determine the binding efficiency, antibodies were placed in concentrations of *Salmonella enterica* Typhimurium that ranged from 10^8-10^2 CFU/mL. Efficiency of mAb 1B4 decreased rapidly as the concentration decreased 10^8-10^6 CFU/mL with a gradual decrease to10^2 CFU/mL, where it leveled and remained unchanged. ELISA absorbance readings showed a significant difference between 0 and 10^3 CFU/mL ($p<0.05$). Based on these findings, 10^3 CFU/mL was the detection limit of the mAb (1B4). Polyclonal antibodies (S48) proved to be very similar in detection limit against the same concentrations of *Salmonella enterica* Typhimurium. As a means of interference, *E. coli* O157:H7 (10^8 CFU/mL) was added to the different concentrations of *Salmonella enterica* Typhimurium. No deterrence in the efficiency of the binding mAb (1B4) was noticed. From this, it can be assumed that the presence of other bacteria would not affect the binding of the target bacteria to the antibody. This is ideal for a food

matrix, especially chicken skin where *Salmonella* is not the only species present. It is assumed that pAb (S48) would have similar results.

5. SPECIFICITY of mAb 1B4 and pAb S48

In a previous lab study to test the specificity of mAb (1B4) and pAb (S48), 13 bacterial species and strains were tested by indirect ELISA. Among the 13 tested bacteria, the mAb (1B4) showed high affinity for only *S. enterica* Typhimurium and *S. enterica* Paratyphi. This was not the case for pAb (S48), where it showed an affinity for all tested *Salmonella* except Montevideo. S48 also showed a strong cross affinity with *E. coli* O157:H7. Our ELISA results showed that *E. coli* O157:H7 and *S. enterica* Typhimurium had the strongest affinity for this antibody with *S. enterica* Mission and *S. enterica* Enteritidis having basically the same affinity. Over a 20 min interaction interval, the affinity was very strong. This suggests that 20 min of bacteria and antibody contact is sufficient for optimal adherence.

These results indicate that mAb 1B4 has a high binding specificity for *S. enterica* Typhimurium and *S. enterica* Paratyphi where background would not be a factor, but is very limited for the entire species. This would be excellent if these two strains were the only pathogens within the *Salmonella* species. This may be due to the much larger sized OMP used to produce the mAb, and may suggest that the larger the OMP the more specific the monoclonal antibody can become. In the poultry industry this would not be ideal in that Typhimurim and Paratyphi would have to be the dominant strains present or be able to become enriched to a level of 10^3 CFU/mL. In other words, they may be present but Mission and Enteritidis could dominate the population and give a false

presence. This would result in a misdiagnosis. For this reason, pAb (S48) was chosen as the antibody of detection for chicken skin.

Even though there is some reactivity with *E. coli* O157:H7, PCR should differentiate the two because of primer specificity. With PCR being dependent on the genetic make-up of the bacteria, *E. coli* would never be a factor when the target bacterium is *Salmonella* or if the case was vice versa. In a multi-plex or nested PCR system, the ability of the pAb (S48) to attach to *Salmonella* and *E. coli* O157:H7 would be beneficial, since these are two of the most problematic pathogens in the food industry.

6. SDS-PAGE PATTERNS of OMPs

To obtain optimal OMPs separation on SDS-PAGE, a 10% polyacrylamide gel containing 0.1% SDS was used. To determine the approximate molecular weights of the purified protein bands, a standard molecular weight curve was constructed from standards allowing calculation by way of extrapolation. The calculated molecular weight of the major purified protein was approximately 55 kDa. Based on the SDS-PAGE protein patterns, the purity of extracted target protein appears to be very high. This was the only protein pattern noticed on the gel which confirms high purity and a strong possibility of producing very specific pAb or mAb against *Salmonella* from using the OMP as antigen. This is the largest reported OMP to be used as antigen to produce mAbs against *S. enterica* Typhimurium.

7. SAMPLE ENRICHMENT (6 and 8 h)

With such a concentrated sample it is assumed that the bacterial level after two h enrichment with centrifugation would easily be detectable by real-time PCR due to a high sensitivity detection limit. This was not the case. After 6 h of enrichment in TSB, no detection of the bacteria on a consistent basis was noticed. This is due to the contaminants of the chicken skin. One, very noticeable aspect of the sample, was the increase in the soluble lipid in the medium over the 6 h period. The lipid content of the skin is very high and is believed to be one if not the main reason for the lack of detection. The temperature of the incubation process allowed the lipids of the skin to become soluble within the broth medium. Lipids have a tendency to encapsulate material and in this case bacteria. This encapsulation serves as a barrier of protection which results in the bacteria to remain intact to the extent that the primers cannot anneal to the DNA for elongation. It could also be a factor in that the DNA also becomes encapsulated in return, blocking the attachment of primer to the exposed bacterial DNA for multiplication and detection. With lipids being good surfactants, the primer and target DNA bonding could also become tremendously weak. This would interfere with the elongation and multiplication process also.

During the enrichment and incubation periods, the soluble lipid content increased. Upon centrifugation this lipid content tends to solidify. As solidification of the fat occurs, encapsulation of the bacterial is possible, and would result in a reduction in the bacterial population as the solid fat is removed from the sample after centrifugation. Thus, the population could be higher after centrifugation. Since the discarded lipid portion was not analyzed for significant *Salmonella* content, this hypothesis cannot be

substantiated. With this as a possibility the centrifugation process still provided population in a higher detectable range (8.31×10^6 CFU/mL) in comparison to the non-centrifuged sample (1.29×10^5 CFU/mL) (Table 3). For this reason, the highest inoculum level became the new focus of this study. Looking at the bacterial population after a 6 h enrichment at this inoculum level, shows that before centrifugation, the bacterial population easily reached levels of 10^5 CFU/mL (Table 3).

8. PRIMER SPECIFICITY and LEVEL of DETECTION

To ensure that the primers would distinguish the presence of *Salmonella* from other bacteria, conventional PCR was run on several bacterial species and strains. Gel electrophoresis confirmed that the primers only selected strains of *Salmonella*. Strains of *Listeria*, *E. coli* and others were all negative. This shows that the primers are highly specific for *Salmonella* and should work well in an environment that is contaminated with many species. Thus, those strains of most concern to the food industry will be detectable.

In order to determine the minimum bacterial concentration needed for adequate primer detection, PCR was run over a range of concentration levels that ran from 10^0 to 10^9 CFU/mL. Results indicate that the detection level was very low and detection of a population in pure sample was at 10^1 CFU/mL (Figure 3). In some cases a concentration of 10^0 CFU/mL could be detected, but with inconsistent results. This was for real-time PCR only. Conventional PCR showed that it needed a population of 10^6 CFU/mL for consistent detection (Figure 8). It is understandable that the matrix environment of food will play a vital role in this detection limit, which is why the concentration of the bacteria

must be much higher. This shows the significance of enrichment.

9. POLYMERASE CHAIN REACTION

Several changes to protocol were needed to rectify the issues of detection. In the beginning, enrichment and incubation method had to be changed frequently. To deter the issues with lipid content, samples were enriched with and without skin in the incubation process for 2 h. The skin was removed and all samples were centrifuged and the pellets were re-suspended in 100, 20, and 5 ml of TSB and incubated for another 4 h. When the pellet was re-suspended in 100 mL there was a tendency to lose bacteria during the concentration procedure, and with 5 mL, the fat and organic matter left behind tended to be very trashy. Thus, detection was very limited and for the most part not apparent during PCR and gel electrophoresis. It appeared that 20 mL was the better volume for the 2nd enrichment process. Results of gel electrophoresis were always similar to those of figure 9.

After 6 h of enrichment, the lipid and organic matter level was visibly high, resulting in a sample that was not clear of debris and hard to handle. This is why a 2 h enrichment process was necessary to grow the bacteria and discard some of the inhibiting by-product contaminants. This procedure cleaned up the sample, but lipid presence remained an issue.

In an attempt to further remove the sample of soluble lipids and inhibiting components, Tween 20 was administered at 5 levels (0, 0.1, 0.01, 0.001 and 1%). The purpose of the Tween 20 was to serve as a surfactant and un-encapsulate the target cells along with removing more lipid content. In order to ensure that the Tween 20 levels were

not harmful to the bacteria, 10^3 CFU/mL were mixed at each level and allowed to stand for 20 and 60 min (Table 4.). The results showed that Tween 20 had no detrimental effect on the bacteria but when used with the skin samples for PCR purposes, detection was not improved. Tween 20 was only observed with the 6 h enrichment process at the 0.01 % concentration. Conventional and real-time PCR resulted in no detection of the bacteria. Tween 20 may also have had some inhibitory effects but this was not tested.

With 2 h enrichment, Tween 20 and centrifugation not being able to yield a desirable purified sample; magnetic bead technology was incorporated and evaluated. The use of magnetic beads has gain popularity in the capturing of microbes in complex matrices. With the natural contamination of the chicken skin, magnetic beads were used to capture *Salmonella* in order to leave inhibiting material behind. Use of beads has not only proven to be an effective means of sample purity, but has shown success in samples with low levels of the target bacteria present. Thus, the idea is to remove *Salmonella* from the matrix and concentrate it in an pure and clean environment. This magnetic technology shows promise in enrichment, purification and bacterial detection of complex food matrices. Production was inexpensive and all magnetic bead processing took place on site. Also, only a small amount of beads are needed to carry out the bacteria removal protocol.

For this research 40 and 100 µL bead volume was used. In previous lab studies, 40 µL were very sufficient in non fat samples, but in the case of the high lipid chicken skin samples, the volume tended to yield negative results. Volume of 100 µL gave the best results. With this volume, the 6 h enrichment yielded a 75% detection success rate and a 95% success rate at 8 h of enrichment. Beads at 100 µL could extract from the

sample an average of 10^6 CFU/mL of the target bacteria after 8 h of enrichment (Table 5). This far exceeded the detection level. Figures 1 and 2, show the real-time PCR results of chicken skins testing positive for the presence of *Salmonella* spp. with 6 and 8 h of enrichment after magnetic bead separation. It can be seen that gel electrophoresis of the real time PCR product confirm the presence of *Salmonella* spp. on the skins for both enrichment times (Figures 6 and 7). Conventional PCR for the 6 h enrichment showed no detection for the bacteria (Figure 9) and similar results were noticed for the 8 h samples. This shows that real-time PCR is the more sensitive of the techniques.

Temperature of bead incubation and contact time with constant motion proved to be critical in the attachment of *Salmonella* to the magnetic beads. Contact times of 5, 20 and 60 min were evaluated. Initially the contact time of 5 min yielded no detection of *Salmonella*. The time was increased to 20 min with inconsistent results, but these times were done in an ambient environment. *Salmonella* spp. and most pathogens are very active at the incubation temperature of 37^{o}C. This had been the temperature for the enrichment process and should be significant for this purpose. It is conceivable that there is some stress on the cells when leaving the enrichment process and entering a different temperature range and this may have contributed to the bead attachment problem. For this reason, the contact time was increased to 60 min inside an incubator at 37^{o}C. Under these conditions optimal bead attachment at 10^6 CFU/mL was achieved and determined by standard plate count (Table 5).

Another critical point was that under these new conditions, the beads were excluded from the PCR process. This was achieved by pH manipulation. Dropping the

pH to 3.0 caused the bacteria to release from the antibody. It was raised back to 7.0 as the bead are extracted together to keep from injuring the bacteria. The remaining solution was used for PCR detection. In previous evaluations, beads were allowed to remain in the analyzed solution for the PCR process. With real-time PCR being based on the detection of fluorescence of the target bacterial DNA, and the magnetic beads being black, it is believed that the fluorescence was being absorbed by the beads. This would give false negative results.

From this data, it appears that it is crucial to remove a very high concentration of bacteria from the sample to achieve positive identification on a significant level. Real time was much more successful at detecting the presence of the bacteria. This may be due to the use of a smaller primer encoding sequence. The primer encoding sequence used for the conventional PCR was double that of real-time. This smaller sequence may have better stability under the PCR conditions as well as a shorten elongation time, of which was probably needed with the lipid content of the chicken skin. With chicken skin being primarily fat, it may contribute to an inability to achieve adequate attachment of the bacteria to the magnetic beads for the 5 and 20 min incubation times. The encapsulation effect of the lipids may have a blocking effect at the attachment sites of the antibody and the bacteria, which is why the longer incubation times are more productive. This in turn requires a higher population for the detection during PCR. The detection percentages given were only from real-time PCR; conventional results were very sporadic and inconclusive. As stated earlier, the conventional appears to need a population of 10^6 CFU/mL in a pure sample. In a high fat food system this number has to be higher. Results would become more positive with increased enrichment time in the aspects of

conventional PCR. With real-time PCR performing better after 8 h of incubation, conventional may need 10 – 12 h. The longer time should allow the bacteria in the medium to completely digest the lipids present in the sample, while subsequent washing should remove the digestive byproducts. This would definitely clean up the sample for the PCR process to be more effective.

As a confirmation of detection specificity, PCR products were analyzed by the Auburn University sequencing lab. After entering the findings in BLAST on the NCBI data base, the bacteria detected were *Salmonella* spp. at a confidence level of 99.9%. This confirms that the PCR reaction amplified the expected region of genomic DNA of *Salmonella*.

CHAPTER V. OVERALL CONCLUSION

Serovars of *Salmonella enterica* are major focus in foodborne outbreaks of human gastrointestinal trauma. *S.* Typhimurium and *S.* Enteritidis have been the more prevalent (Schrank et al., 2001). Poultry has been implicated as a serious source of human infections and intoxication (Cohen et al., 1994; Nguyen et al., 1994; Schrank et al., 2001). To gain an understanding of the time needed to grow the bacteria to an detectable level, a bacteria (cocktail) suspension at the load levels of 10^3, 10^2, and 10^1 CFU/mL were grown over a 6 h period in TSB and plated in 3 different manners (direct spread plate, centrifuge spread plated and pour plated). Table 3 shows the comparison of methods over the six hour period. It can be seen that centrifugation yields the highest concentration of the target bacteria, thus making detection more prominent. Data show that even at the lowest inoculum in 6 h, centrifugation concentrates *Salmonella* to the 10^3 CFU/mL detection level (Table 3). Be mindful that eventhough 10^3 CFU/mL is the inoculum level only 200 μL is initially incubated. This means that out of the 1000 bacterial cells only 20% are inoculated on the sample (200 cells). From this observation, 6 h of growth is adequate incubation time. What cannot be seen is the effect of food matrix on the detection limit. In a pure culture these numbers would always be detected, but when the environment is chicken skin (high lipid), detection becomes more difficult.

It is true that brilliant green broth is a great selective medium for *Salmonella* spp. growth (Table 1). Preliminary studies proved this to be true, resulting in it being chosen

as the medium to enrich *Salmonella* for further PCR analysis. As expected, the medium provided good bacterial growth, but proved to be undetectable after 6 h by conventional PCR. Several attempts were made at the highest inoculum with this medium, but PCR was unable to detect the presence of the *Salmonella* spp. These results did not change with several modifications of the method. Modifications were done in an attempt to produce a clean but usable concentration of the target DNA needed. It is well known that industrial and clinical samples contain many PCR inhibitory substances (Flekna and others 2007; Radstrom and others 2004). This is also true for many of the enrichment media, of which, generate false negatives and are the result of the PCR inhibitory compounds (Flekna and others 2007; Radstrom and others 2004). These compounds are introduced into the DNA-PCR reaction mixture with the biological sample and can be from the enrichment media used or during the DNA isolation process (Flekna and others 2007; Radstrom and others 2004). Even clinical samples that have undergone antiviral treatment have been reported to produce false negatives because of PCR inhibitory agents present (Flekna and others 2007; Yedidag and others, 1996).

Tryptic soy broth (TSB) is a widely used enumeration medium. With the inhibitory effects of the brilliant green broth and the higher salt concentrations of BHI, it was thought that the use TSB was more beneficial. TSB is a good enrichment medium for *Salmonella* spp. and preliminary studies supported these findings. Ellingson and others (2004) reported good success with a 12 h PCR technique that was based on real-time applications and gave both qualitative and quantitative analysis of the pathogen within the food matrix. Previously Trikov and others (1999) reported the same success with a 30 h PCR/enrichment method. Both methods involve a 6 h pre-enrichment step.

This 6 h enrichment process supports our findings for adequate growth for detection. To see the efficacy of TSB, a preliminary 10 h enrichment process was conducted to determine if the targeted pathogen could be detected by conventional PCR from the inoculated chicken skin sample. This proved to be successful but not at 100%. The targeted bacteria could be found at 6 and 8 h of incubation by real-time PCR. For this reason, TSB was a good enrichment medium for this project.

The testing of samples by real-time PCR has been extensively used with success for accurate detection and quantification (Flekna and others 2007; Heid and others, 1996). Since most conventional PCR assays have to be confirmed by gel electrophoresis with ethidium bromide staining, it is labor intensive, time-consuming and difficult to automate (Patel and others, 2006). To alleviate this process, real-time PCR has included its own monitoring method with the incorporation of a fluorescent dye known as SYBR Green (Patel and others, 2006; Bhagwat, 2003 and 2004). Here the target gene (DNA) is amplified causing fluorescence of the dye which is recognized and monitored by the probe moiety (Patel and others, 2006; Tyagi and Kramer, 1996). Real-time PCR coupled with the use of magnetic beads proved to be a promising technique in adequate detection. This research suggests that real-time PCR is much more effective than conventional especially in the analysis of *Salmonella* on chicken skin. Conventional PCR may be more beneficial in non meat applications or low fat foods.

SDS-PAGE of the purified antibody, the remaining serum, coated magnetic beads, antibody fixed beads to show the purity of the antibody and to show that it was attached to the magnetic bead. Figures 4 and 5, show the protein bands of the SDS-PAGE gel and it can clearly be seen that the anti- *S.* Typhimurium antibody is very pure

and that it is attached to the magnetic beads. This shows that the outer membrane proteins produced are high in purity. The lack of other protein bands appearing, also suggest that the polyclonal antibodies generated are highly specific for *Salmonella* spp. This technology along with the magnetic bead innovations proves to be a phenomenal component in the detection of foodborne pathogens in the food industry. With this being the case, foodborne pathogens can be detected before the product can reach the public. Eliminating the need for recalls when the pathogen component of food safety has been breached. HACCP now has a secure technology to stand on and make more industrial SOP's efficient.

The potential of this technology and research is unlimited. Being able to incorporate the DNA of bacteria as a detection module makes way for technologies to intertwine and become more reliable. This will not only solidify a safer food environment but aid in the prevention of terrorist attacks on the food supply.

Table 1. Comparison of Selective and Non-Selective Enrichment Media (6 h incubation)

Media	Type	Concentration (CFU/ml)
TSB	Non-Selective	2.74×10^6
½ TSB	Non-Selective	2.13×10^6
LB	Non-Selective	1.61×10^6
NB	Non-Selective	8.03×10^5
BPW	Non-Selective	2.55×10^6
UPB	Non-Selective	2.86×10^6
BHI	Non-Selective	3.58×10^6
BG	Selective	4.81×10^6
SB	Selective	1.39×10^5
SC	Selective	2.23×10^5
RV	Selective	4.17×10^5
Salmosyst	Selective	7.31×10^5

Note: TSB(Tryptic Soy Broth); ½ TSB (half strength TSB); LB (Lactose Broth); NB (Nutrient Broth); BPW (Buffered Peptone Water); UPB (Universal Pre-enrichment Broth); BHI (Brain Heart Infusion Broth); BG (Brilliant Green Broth); SB (Selenite Broth); SC (Selenite Cytstine Broth); RV(Rappaport-Vassiliadis R10 Broth); and Salmosyst: (Salmocyst Broth). Initial inoculation level here was 10^2 CFU/mL.

Table 2. Comparison of Top Medium for 2, 4, 6 h of Incubation with Inoculated Chicken Skin.

Inoculation CFU/ml	Time (h)	Concentration CFU/ml		
		TSB	BHI	BG
10^3	6	3.44×10^5	1.34×10^6	3.13×10^6
10^3	4	2.17×10^3	1.04×10^4	2.87×10^4
10^3	2	1.63×10^1	4.47×10^2	8.96×10^2
10^2	6	2.41×10^3	1.06×10^4	3.32×10^4
10^2	4	4.24×10^2	1.36×10^3	2.31×10^3
10^2	2	ND*	ND	ND
10^1	6	1.44×10^2	1.09×10^3	2.01×10^3
10^1	4	1.74×10^1	7.64×10^1	9.13×10^1
10^1	2	ND	ND	ND

Note: Inoculation is done at only 20% of the inoculation level. ND* means not detectable by spread or pore plate method

Table 3. Efficacy of One Step Enrichment at 2, 4 and 6 h of Incubation.

Inoculation dose (CFU/mL)		Bacterial concentration (CFU/mL)		
		2 h	4 h	6 h
10^1	Centrifuge	ND*	8.54×10^2	5.92×10^3
	Spread	ND	2.60×10^1	1.86×10^2
	Pour	ND	3.18×10^1	3.02×10^2
10^2	Centrifuge	2.09×10^2	8.56×10^3	6.13×10^5
	Spread	ND	2.72×10^2	1.41×10^4
	Pour	3.54×10^0	2.74×10^2	2.82×10^4
10^3	Centrifuge	1.49×10^3	3.78×10^4	8.31×10^6
	Spread	3.72×10^1	1.12×10^3	1.29×10^5
	Pour	3.90×10^1	1.06×10^3	2.13×10^5

Note: ND* = not detectable by spread or pour plate methods.

Table 4. Tween 20 Study for Effect on *Salmonella* at 20 and 60 Minutes

0%	0.001%	0.01%	0.1%	1.0%	Time (min)
58	72	58	81	68	20
60	73	77	74	74	60
176	182	176	172	186	20
174	195	182	177	190	60
119	113	117	111	128	20
125	123	110	107	134	60
120	122	126	120	138	20
123	132	131	127	143	60

Note: Numbers represent colonies formed after 1 mL of Phosphate Buffer containing 0 to 1% Tween 20 was allowed to interact with 10^3 CFU for 20 and 60 minutes. 100 µL of the 1 mL was plated for bacterial growth and colony formation.

Table 5. Bacterial Concentration Efficacy of Magnetic Bead (8 h)

Bead Attachment (CFU/mL)					
Run #	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
1	3.3×10^6	5.4×10^5	9.9×10^5	4.5×10^6	5.5×10^6
2	1.6×10^7	3.7×10^8	5.3×10^6	2.8×10^6	2.8×10^6
3	4.9×10^6	4.5×10^6	9.1×10^5	1.9×10^6	1.7×10^6
4	6.3×10^6	4.4×10^6	2.7×10^6	4.9×10^6	2.8×10^5
Remaining Solution (CFU/mL)					
Run #	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
1	2.0×10^8	1.1×10^8	1.0×10^8	2.2×10^8	2.1×10^8
2	1.6×10^8	1.9×10^8	3.4×10^8	2.3×10^8	2.7×10^8
3	9.3×10^8	8.6×10^8	3.5×10^8	1.1×10^9	6.5×10^8
4	9.9×10^8	8.5×10^8	2.9×10^8	1.9×10^8	2.4×10^8

Note: These numbers represent the bacterial population after 8 h of enrichment where the sample was centrifuged down to approximately less than 1 mL and magnetic beads were added at 100 µL (bead volume) and allowed to incubate at 37^{o}C for 1 h. Counts are based on CFU/mL and an inoculation level of 10^3 CFU/mL.

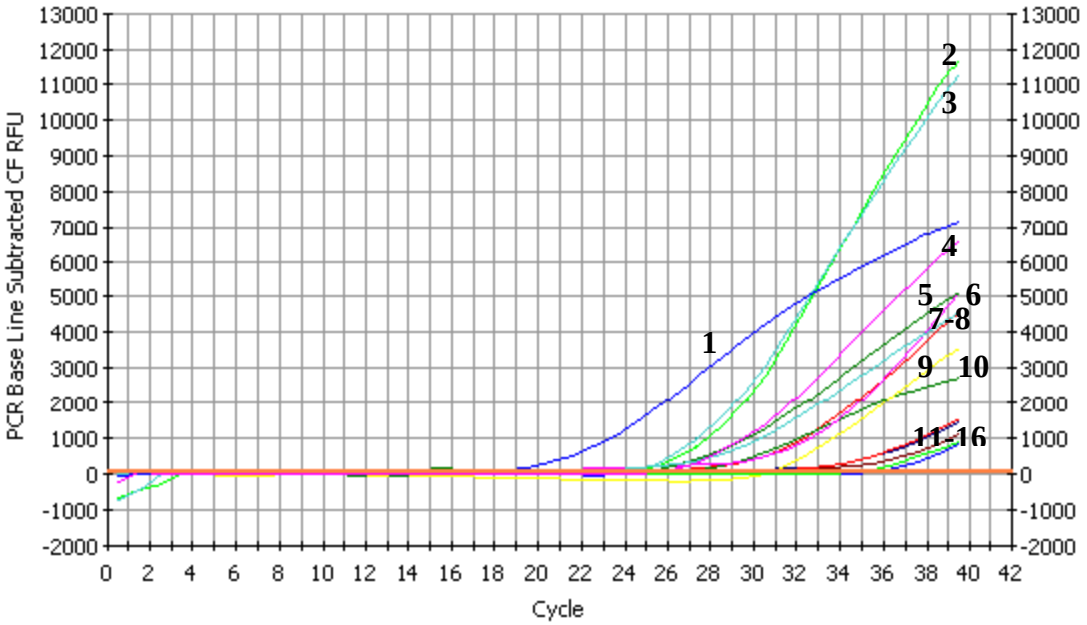


Figure 1. PCR Amp/Cycle for SYBR-490 (6 h). 1-4 are standards from inoculum cocktail at levels of 10^8,10^6, 10^4 and 10^2 CFU/mL respectively,5 and 6 are *S.*Typhimurium at 10^9 CFU/Ml, 7-16 are duplicates for 5 chicken skin samples (ex. 7-8 skin 1, 9-10 skin 2, etc.).

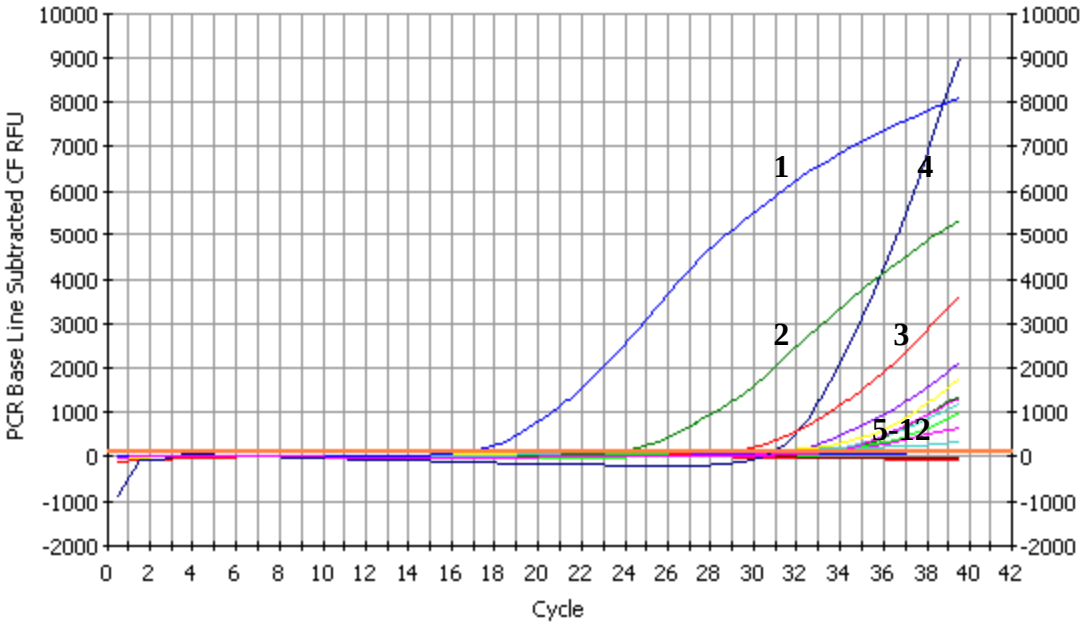


Figure 2. PCR Amp/Cycle for SYBR-490 (8 h). 1-4 are standards from inoculum cocktail at levels of 10^8,10^6, 10^4 and 10^2 CFU/mL, respectively. 5-12 are duplicates for 5 chicken skin samples (ex. 5-6 skin 1, 7-8 skin 2, etc.). 13-14 are skin 5 but were not detected and 15-16 were *E. coli* O157:H7 also not detected.

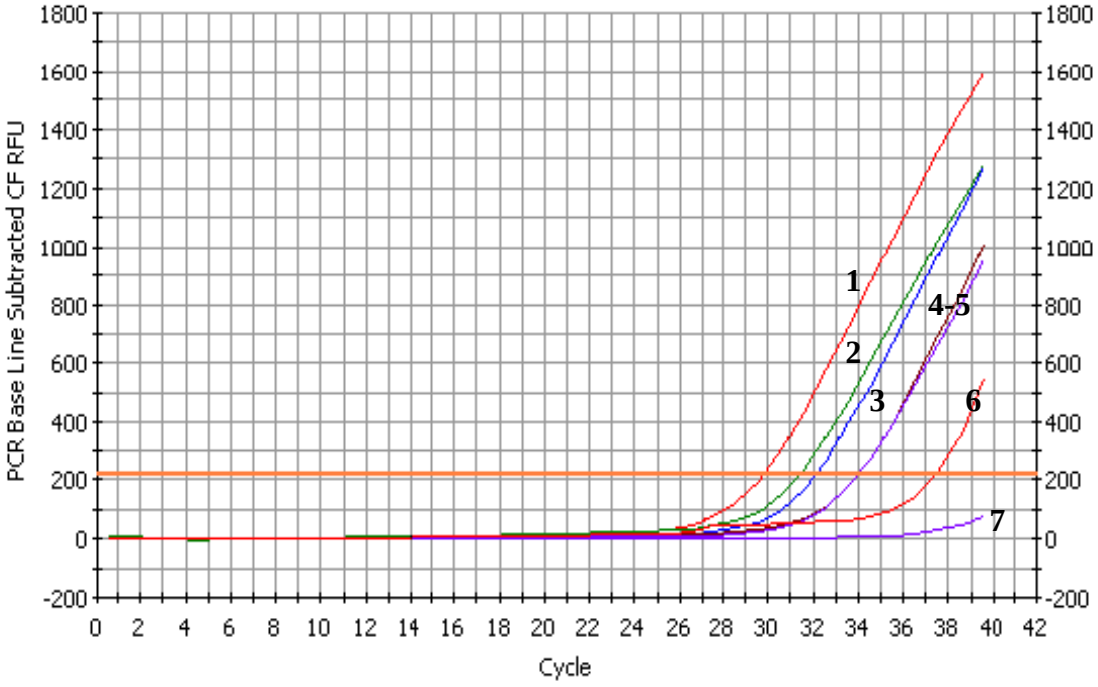
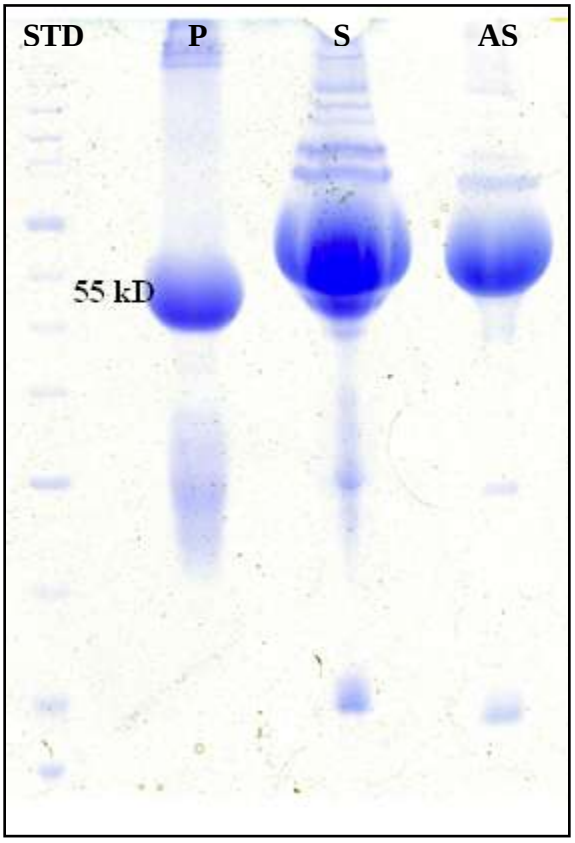


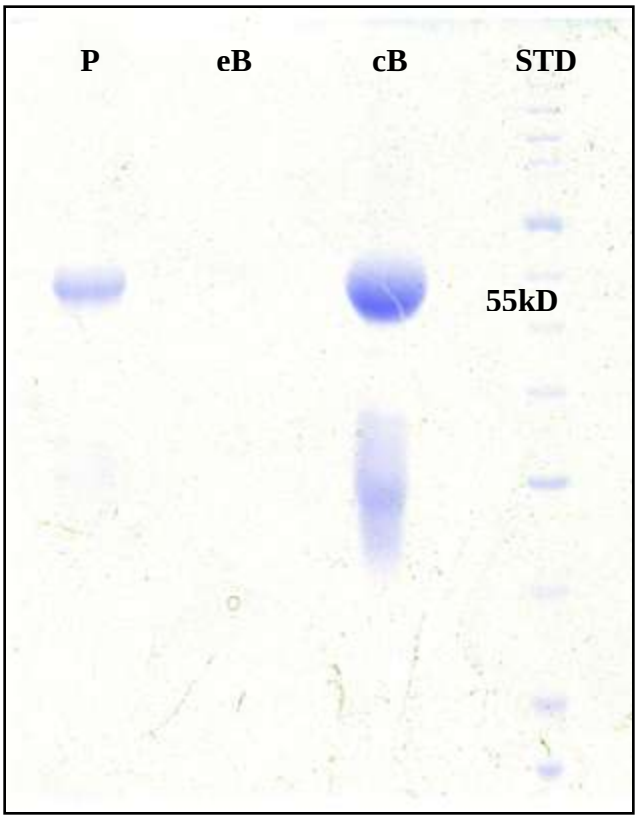
Figure 3. PCR Amp/Cycle for SYBR-490 (Detection Limit). 1-7 are standards from inoculum cocktail at levels of 10^3, 5.0 x10^2, 2.5 x 10^2,10^2, 5.0 x10^1, 10 and 1 CFU/mL, respectively.

Figure 4. SDS-PAGE of Purified Antibody



Note: STD = standard marker, P = Protein A column, S = rabbit serum, AS = ammonium Sulfate (precipitate antibody).

Figure 5. Immobilization of Antibody on Magnetic Beads.



Note: P = Protein A column, eB = magnetic bead without antibody, cB = magnetic bead conjugated with antibody, STD = standard marker.

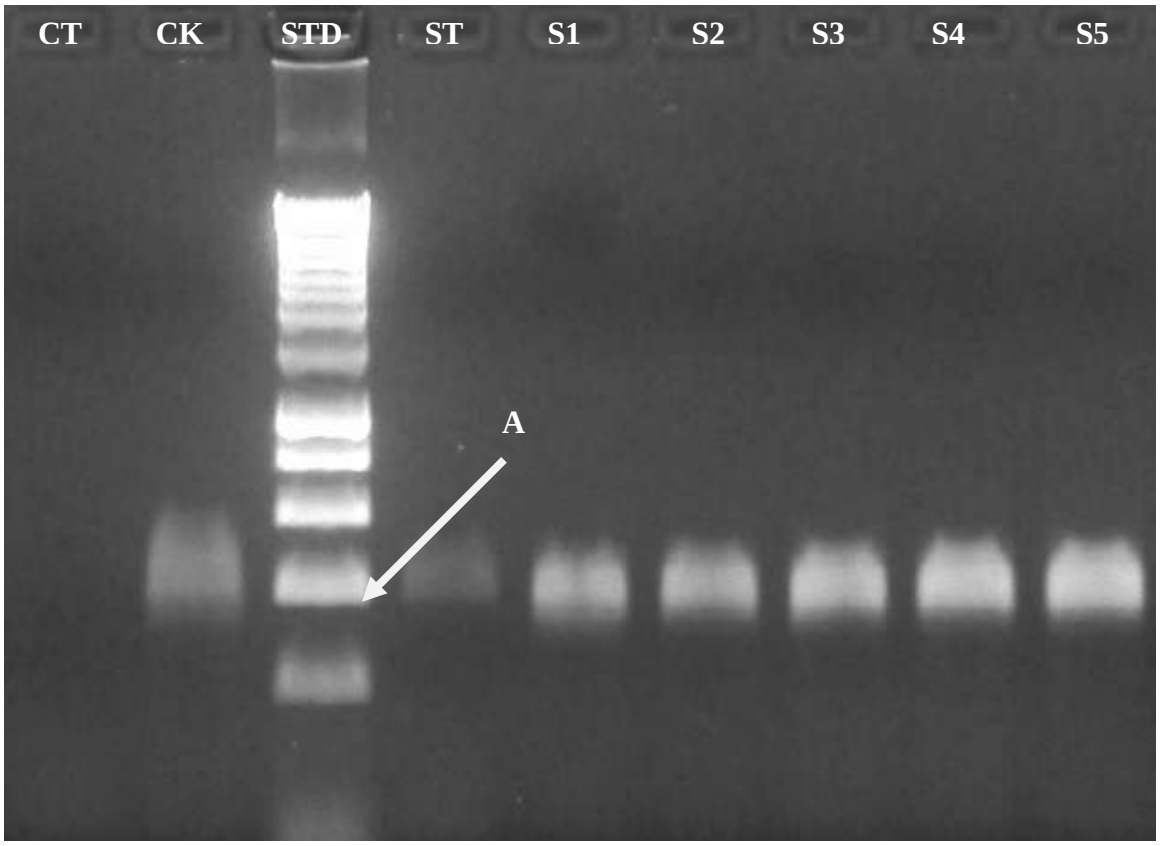


Figure 6. Gel Electrophoresis of real-time PCR product of chicken skin detection for *Salmonella* after 6 h of enrichment in TSB after magnetic bead separation. CT = negative control, CK = bacteria cocktail serovars Typhimurium, Enteritidis and Mission, STD = standard marker, ST = serovar Typhimurium, S1 – S5 = inoculated chicken skin samples, A = 200 bp point on marker.

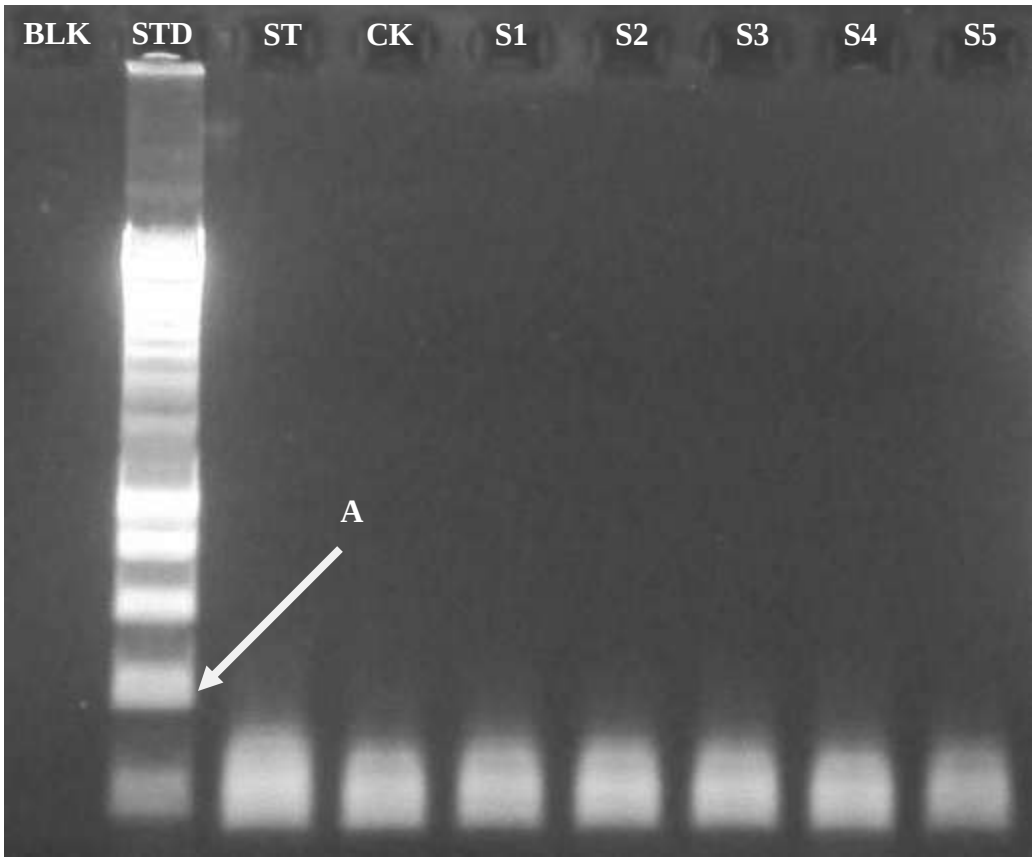


Figure 7. Gel Electrophoresis of real-time PCR product of chicken skin detection for *Salmonella* after 8 h of enrichment in TSB after magnetic bead separation. BLK = negative control, STD = standard marker, ST = serovar Typhimurium, CK = bacteria cocktail serovars Typhimurium, Enteritidis and Mission, S1 – S5 = inoculated chicken skin samples, A = 200 bp point on marker.

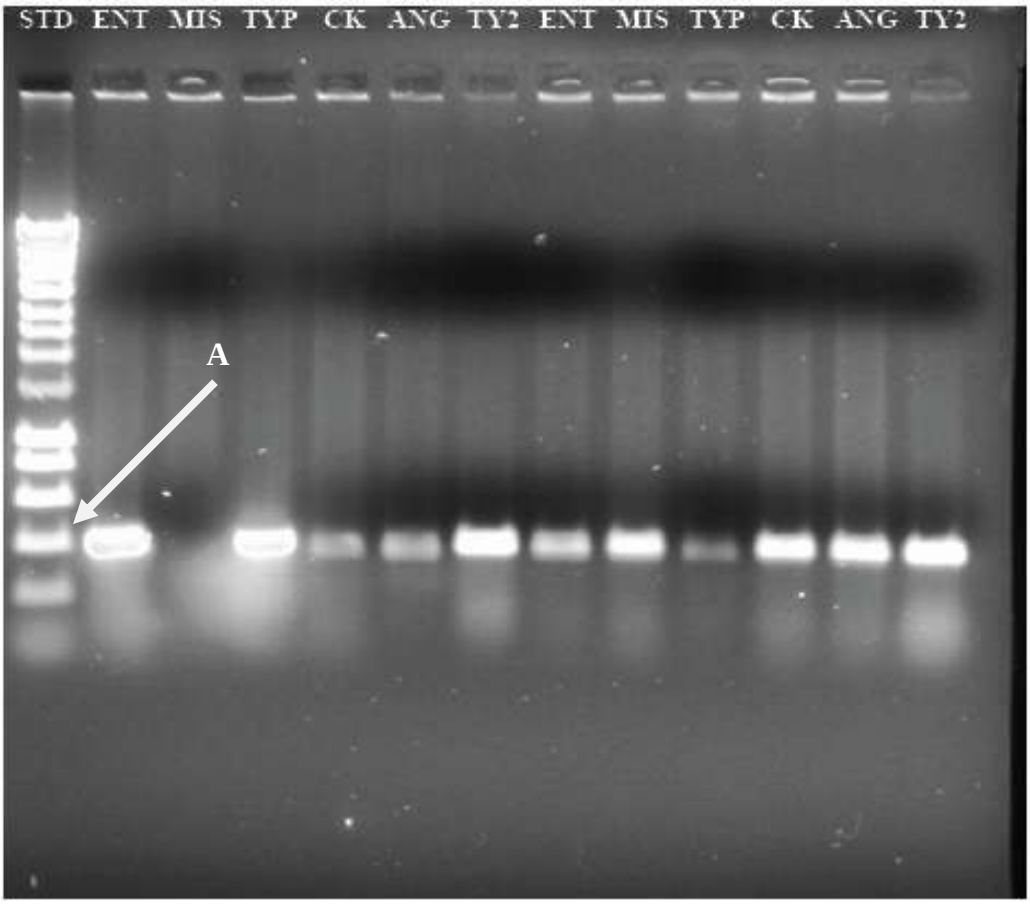


Figure 8. PCR analysis of pure cultures of *Salmonella* serovars Enteritdis, Mission, Typhimurium, cocktail of previous three, serovar Agona, serovar Typhi. Done at concentration levels of 10^6 and 10^9 CFU/mL, A = 400 bp point on marker.

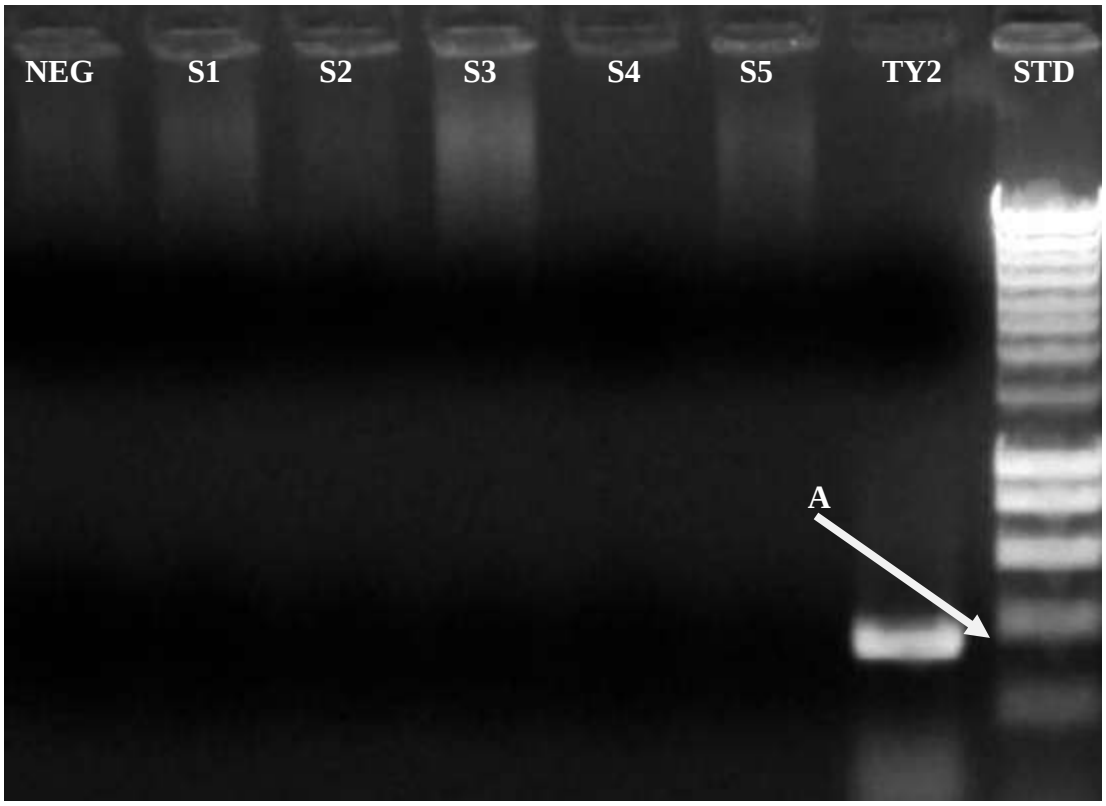


Figure 9. Gel Electrophoresis of conventional PCR product of chicken skin detection for *Salmonella* after 6 h of enrichment in TSB after magnetic bead separation. NEG = negative control, S1 – S5 = inoculated chicken skin samples, TY2 = serovar Typhi, STD = standard marker, A = 400 bp point on marker.

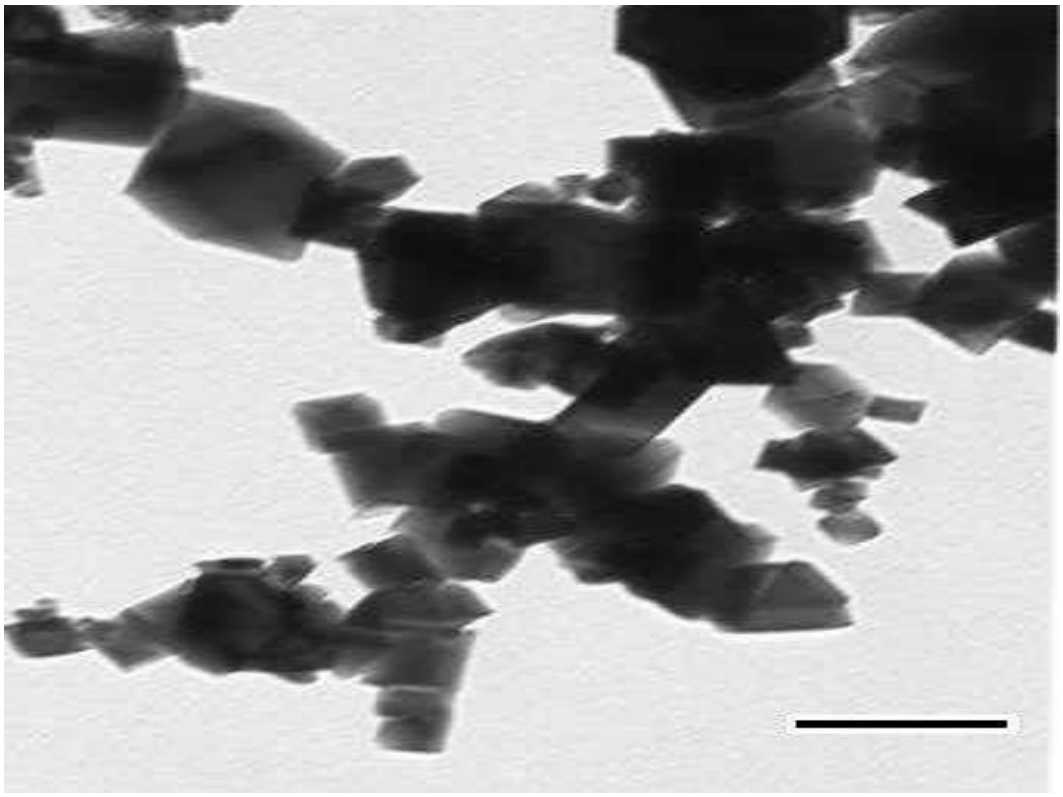


Figure 10. Transmitting electron microscopic image of magnetic nano particles. Magnetite was dispersed on formvar filmed grid and observed under TEM (Philips 301). The scale bar represents 100 nm.

CHAPTER VI. REFERENCES

Aabo S, Rasmussen OF, Roseen L, Sorensen PD, Olsen JE. 1993. Salmonella identification by polymerase chain reaction. Mol Cell Probes 7:171-78.

Allos BM, Moore MR, Griffin PM, Tauxe RV. 2004. Surveillance for sporadic foodborne disease in the 21st century: the FoodNet perspective. Clin Infect Dis 38(Suppl 3):S115--20.

Andrews WH, June GA, Sherrod PA, Hammack TS, Amaguana RM. 1998. FDA Bacteriological Analytical Manual. 8th Edn. Salmonella. AOAC Int (Chapter 5).

Andrews WH. 1985. A review of culture methods and their relation to rapid methods for the detection of *Salmonella* in foods. Food Tech 39:77-82.

Anthony RM, Schuitema ARJ, Oskam L, Klatser PR. 2005. Direct detection of Staphylococcus aureus mRNA using a flow-through microarray. J Microbiol Methods 60:47-54.

Barrow PA, Huggins MB, Lovell MA, Simpson JM. 1987. Observation on he pathogenesis of experimental Salmonella enterica serovar Typhimurium infection in chickens. Res Vet Sci 42:194-199.

Baumler AJ, Heffron F, Reissbrodt R. 1997. Rapid detection of Salmonella enterica with primers specific for iroB. J Clin Microbiol 35:1224-230.

Bej K, Mahbubani MH, Boyce MJ, Atlas RM. 1994. Detection of Salmonella spp. in oysters by PCR. Appl Environ Microbiol 60:368-73.

Bhagwat AA. 2003. Simultaneous detection of Escherichia coli O157:H7, Listeria monocytogenes and Salmonella strains by real-time PCR. Int J Food Microbiol 84:217-24.

Bhagwatt AA. 2004. Rapid detection of Salmonella from vegetable rinse-water using real-time PCR. Food Microbiol 21:73-78.

Bickley J, Short JK, McDowell DG, Parkes HC. 1996. Polymerase chain reaction (PCR) detection of Listeria monocytogenes in diluted milk and reversal of PCR inhibition caused by calcium ions. Lett Appl Microbiol 22:153-58.

Boer E de. 1998. Update on dedia for isolation of Enterobacteriaceae from foods. Int J Food Microbiol 45:53-53.

Borch E, Arinder P. 2002. Bacteriological safety issues in red meat and ready-to-eat meat products, as well as control measures. Meat Sci 62(3):381-90.

Bouvet P, Grimont P. 2000 Donnees de surveillance 1999 du Centre National de reference des Salmonella et Shigella. Epideiol Hebdomadaire 12:49-52.

Burkhalter PW, Muller C, Luthy J, Candrian U. 1995. Detection of Salmonella spp. in eggs: DNA analyses, culture techniques, and serology. J AOAC Int 78:1531-537.

Busse M. 1995. Media for salmonella. Intl J Food Microbiol 26:117-131.

Bustin SA. 2002. Quantification of mRNA using real-time reverse-transcription PCR (RT-PCR): trends and problems. J Mol Endoc 29:23-29.

Butterworth MD, Bell SA, Armes SP & Simpson AW. 1996. Synthesis and Characterization of Polypyrrole- Magnetite-Silica Particles. Journal of Colloid and Interface Science 183(1):91-99.

Buzby JC, Roberts T. 1996. ERS updates US foodborne disease cost for seven pathogens. Food Rev 19:29-25.

CDC. 2004. Preliminary foodnet data on the incidence of infection with pathogens transmitted commonly through food-selected sites, United States, 2003, MMWR 53:338-43.

CDC. 2005. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food—10 sites, United States, 2004. MMWR 54(14):352-356.

Cerro Adel, Soto SM, Landeras E, Gonzalez-Hevia MA, Guijarro JA, Mendoza MC. 2002. PCR-based procedures in detection and DNA-fingerprinting of *Salmonella* form samples of animal origin. Food Microbiol 19:567-75.

Chen W, Martinez G, Mulchandani A, 2000. Molecular beacons: a real-time polymerase chain reaction assay for detecting Salmonella. Anal Biochem 280:166-72.

Chopra AK, Peterson JW, Chary P. 1994. Molecular characterization of an enterotoxin from Salmonella typhimurium. Microb Pathogen 16:85.

Cohen ND, Neibergs HL, Wallis DE, Simpson RB, McGruder ED, Hargis BM. 1994. Genus-specific detection of salmonellae in equine feces by use of the polymerase chain reaction. Am J Vet Res 55:1049-54.

Corry JE, James C, James SJ, Hinton M. 1995. Salmonella, Campylobacter and Escherichia coli O157:H7 decontamination techniques for the future. Int J Food Microbiol 28:187-96.

Cortez AL, Carvalho A, Ikuno A, Burger KP, Vidal-Martins A. 2006. Res Vet Sci 81:340-44.

Curiale MS, McIver D, Weathersby S, Planar C. 1990. Detection of salmonella and other enterobacteriaceae by commercial Deoxyrobonucleic Acid Hybridization and Enzyme Immunoassay Kits. J Food Prot 53:1037-46.

Crigie J. 1931. Studies on serological reactions of flagella of B. typhosus. J Immunol 21:417-511.

D'Aoust JY, Maishment C. 1979. Preenrichment conditions for effective recovery of Salmonella in foods and feed ingredients. J Food Prot 42:153-7.

D'Aoust JY, Sewell A, Boville A. 1983. Rapid cultural methods for detection of Salmonella in foods and feed ingredients. J Food Prot 46:851-6.

D'Aoust JY, Sewell A, Warburton DW. 1992. A comparison of standard cultural methods for the detection of foodborne Salmonella. Int J Food Microbiol 16:41-50.

Davies RH, Hinton MH. 2000. Salmonella in animal feed, p 285-300. In C. Wray and A. Wray (ed), Salmonella in domestic animals. Cabi, Wallingford, England.

Davies R, Breslin M, Corry JE, Hudson W, Allen VM. 2001. Observation on the distribution and control of Salmonella species in two integrated broiler companies. Vet Rec 149:227-232.

De Smedt J, Bolderdijk R, Rappold H, Lautenschlaeger D. 1986. Rapid Salmonella detection in foods by motility enrichment on a Modified Semi-solid Rappaport-Vassiliadis medium. J Food Prot 49:510-4.

El-Gazzar RE, Marth EH. 1992. Foodborne disease: investigative procedures and economic assessment. J Environ Health 55:24-27.

Ellingson JL, Anderson JL, Carlson SA, Sharma VK. 2004. Twelve hour real-time PCR technique for the sensitive and specific detection of Salmonella in raw and ready-to-eat meat products. Mol Cell Probes 18:51-57.

Eyigor A , Carli KT, Unal CB. 2002. Implementation of real-time PCR to tetrathionate broth enrichment step of *Salmonella* detection in poultry. Lett Appl Microbiol 34:37-41.

FAO/WHO. 1997. Risk management and food safety. Report of a Joint FAO/WHO Consultation. Rome, Italy. 27-31 January 1997. Rome.FAO.

Ferretti R, Mannazzu I, Cocolin L, Comi G, Clementi F. 2001. Twelve-hour PCR-based method for the detection of Salmonella spp. in food. Appl Environ Microbiol 67:977-78.

Finlay PL, Falkow S. 1988. Virulence Factors associated with Salmonella species. Microbiol Sci 5:324-28.

Finlay RB, Heffron F, Falkow S. 1989. Epithelial cell surfaces induce Salmonella proteins required for bacterial adherence and invasion. Science 243:940.

Finlay BB, Leung KY, Rosenshine I. 1992. Salmonella interactions with the epithelial cell. A model to study the biology of intracellular parasitism. ASM News 58:486.

Flekna G, Schneeweiss W, Smulders FJM, Wagner M, Hein I. 2007. Real-time PCR method with statistical analysis to compare the potential of DNA isolation methods to remove PCR inhibitors from samples for diagnostic PCR. Mol Cell Probes 21:282-87.

FoodNet C. 2006. Preliminary FoodNet data on the incidence of infection with pathogens tranmitted commonly through food—10 states, United States MMWR 55(14)392-95.

Fratamico PM. 2003. Comparison of culture, PCR, TaqMan Salmonella, and Transia Card Salmonella assays for detection of Salmonella spp. in naturally-contaminated ground chicken, ground turkey, and ground beef. Mol Cell Probes 17:215-221.

Fuentes M, Mateo C, Rodriguez A, Casqueiro M, Tercero JC, Riese HH, Fernandez-Lafuente R, Guisan JM. 2005. Detecting minimal traces of DNA using DNA covalently attached to superparamagnetic nanoparticles and direct PCR-ELISA. Biosensers and Bioelectronics 21:1574-1580.

Fukushima H, Tsunomori Y, Seki R. 2003. Duplex real-time SYBR green PCR assays for the detection of 17 species of food or waterborne pathogens in stools. J Clin Microbiol 41:5134-5146.

Fung DY. 2002. Predictions for rapid methods and automation in food microbiology. J AOAC Int 85:1000-1002.

Gasanov U, Hughes D, Hansbro PM. 2005. Methods for the isolation and identification of Listeria spp. and Listeria monocytogenes: a review. FEMS Micro Review. (In Press, Corrected Proof, Available on line http://www.sciencedirect.com.)

Giannella RA. 1979. Importance of the intestinal inflammatory reaction in Salmonella-mediated intestinal secretion. Infect Immune 23:140.

Giannella RA, Broitman SA, Zamcheck N. 1973. Influence of gastric acidity on bacterial and parasitic enteric infections: a perspective. Ann Intern Med 78:271.

Glynn B, Lahiff S, Wernecke M, Barry T, Smith TJ, Maher M. 2006. Current and emerging molecular diagnostic technologies applicable to bacterial food safety. Dairy Tech 59(2):126-129.

Goossens H, Wauters G, De Boeck M, Jansen M, Butzler JP. 1984. Semisolid selective-motility enrichment medium for the isolation of salmonella from fecal specimens. J Clin Microbiol 19:940-1.

Gouws PA, Visser M, Brozel VS. 1998. A polymerase chain reaction procedure for the detection of Salmonella spp. within 24 h. J Food Prot 61:1039-42.

Harlow E, Land D. 1988. Antibodies-A Laboratory Manual. Cod Spring Harbor, NY: Cold Spring Harbor Laboratory. p139-243.

Heid CA, Stevens J, Livak KJ, Williams PM. 1996. Real time quantitative PCR. Genome Methods 6:986-994.

Hein I, Flekna G, Krassnig M, Wagner M. 2006. Real-time PCR for the detection of Salmonella spp. in food: an alternative approach to a conventional PCR system suggested by the FOOD- PCR project. J Microbiol Methods 66:538-47.

Ivnitski D, Abdel-Hamid I, Atanasov P, Wilkins E, Stricker S. 2000. Application of electrochemical biosensors for detection of food pathogenic bacteria. Electroanalysis 12:317-25.

Jaradat ZW, Zawistowski J. 1996. Production and characterization of monoclonal antibodies against the O-5 antigen of *Salmonella* typhimurium lipopolysaccharide. Appl Environ Microbiol 62(1):1-5.

Jay JM. 2000. Foodborne gastroenteritis caused by *Salmonella* and *Shigella*. In: Heldman DR, editor. Modern food microbiology. 6th ed. Gathersburg, Maryland: Aspen Publishers. P511-530.

Jepson MA, Clark MA. 2001. The role of M cells in Salmonella infection. Microbes Infect 3:1183-1190.

Jin UH, Cho SH, Kim MG, Ha SD, Kim KS, Lee KH, Kim KY, Chung DH, Lee YC, Kim CH. 2004. PCR method based on the ogdH gene for the detection of Salmonella spp. from chicken meat samples. J Microbiol 42:216-22.

Jones TF, Gerber DE. 2001. Perceived etiology of food borne illness among public health personnel. Emerg Infect Dis 7:904-905.

Jones DD, Law R, Bej AK. 1993. Detection of Salmonella spp. in oysters using polymerase chain reaction (PCR) and gene probes. J Food Sci 58:1191-197.

Jothikumar N, Wang X, Griffiths MW. 2003. Real-time multiplex SYBR green I-based PCR assay for simultaneous detection of Salmonella serovars and Listeria monocytogenes. J Food Prot 6:2141-145.

Kaufmann S, Raupach B, Finlay BB. 2001. Microbiology and immunology: lessons learned from Salmonella. Microbes Infect 3:1177-1181.

Kauffmann F. 1930. Ein kombiniertes Anreicherungsverfahren für Typhus- und Paratyphusbazillen. - Zbl. Bakt. I. Orig. 119: 148-152.

Kawasaki S, Horikoshi N, Okada Y, Takehita K, Sameshima T, Kawamoto S. 2005. Multiplex PCR for simultaneous detection of Salmonella spp., Listeria monocytogenes and Escherichia coli O157:H7 in meat samples. J Food Prot 68:551-56.

Kerr S, Hall HJ, Mackis DP, Pollock DA, Finlay DA. 1992. Diagnostic application of monoclonal antibodies to outer membrane protein for rapid detection of Salmonella. J Appl Bacteriol 72:302-08.

Kwang J, Littledike ET, Keen JE. 1996. Use of polymerase chain reaction for *Salmonella* detection. Letters Appl Microbiol 22:46-51.

Lamoureux M, MacKay A, Messier S, Fliss I, Blais BW, Holley RA, Simard RE. 1997. Detection of Campylobacter jejuni in food and poultry viscera using immunomagnetic separation and microtitre hybridization. J Appl Microbiol 83:641-51.

Lampel KA, Orlandi PA, Kornegay L. 2000. Improved template preparation method for PCR-based assays for detection of food-borne bacterial pathogens. Appl Environ Microbiol 66(10):4539-542.

Leifson E. 1936. Now cuolture media based on sodium deoxycholate for the isolation of intestinal pathogens and for the enumeration of the colon bacillus in milk and water. J Pathol Bacteriol 40:581-599.

Leifson E. 1936. New selenite enrichment media for the isolation of typhoid and paratyphoid bacilli. Am J Hyg 24:423-432.

Leonard P, Hearty S, Quinn J, O'Kennedy R. 2004. A generic approach for the detection of whole Listeria monocytogenes cells in contaminated samples using surface plasmon resonance. Biosensors and Bioelectronics 19:1331-335.

Li Y, Zhuang S, Mustapha A. 2005. Application of a multiplex PCR for the simultaneous detection of Escherichia coli O157:H7, Salmonella and Shigella in raw and ready-to-eat meat products. Meat Sci 71:402-06.

Li Y, Mustapha A. 2004. Simultaneous detection of Escherichia coli O157:H7, Salmonella and Shigella in apple cider and produce by a multiplex PCR. J Food Prot 67(1):27-33.

Liao Y, Cheng Y, Qingge L. 2007. Preparation of nitrilotriacetic acid/Co^{2+}-linked, silica/boron-coated magnetite nanoparticles for purification of 6 x histidine-tagged proteins. J Chromat A 1143:65-71.

Liming SH, Bhagwat AA. 2004. Application of a molecular beacon--real-time PCR technology to detect Salmonella species contaminating fruits and vegetables. Int J Food Microbiol 95;177-87.

Litchfield JH. 1973. *Salmonella* and the food industry – Methods for isolation, identification and enumeration. CRC Crit Rev Food Tech 3:415-456.

Malorny B, Bunge C, Hoorfar J, Helmuth R. 2003. Multi-center validation of the analytical accuracy of Salmonella PCR: towards an international standard. Appl Environ Microbiol 69:290-96.

Malorny B, Hoorfar J, Hugas M, Heuvelink A, Fach P, Ellerbroek L, Bunge C, Dorn C, Helmuth R. 2003. Interlaboratory diagnostic accuracy of a Salmonella specific PCR-based method. Int J Food Microbiol 89:241-49.

Malorny B, Paccassoni E, Fach P, Bunge C, Martin A, Helmuth R. 2004. Diagnostic real-time PCR for detection of Salmonella in food. Appl Environ Microbiol 79(12):7046-52.

Malorny B, Schroeter A, Bunge C, Hoog B, Steinbeck A, Helmuth R. 2001. Evaluation of the molecular typing methods for Salmonella enterica serovar Typhimurium DT104 isolated in Germany from healthy pigs. Vet Res 32:119-29.

Malorny B, Tassios, PT, Radstrom, P, Cook N, Wagner M. Hoorfar J. 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int J Food Microbiol 83:39-48.

Mannelli I, Minunnia M, Tombellia S, Wang R, Spiritia MM, Mascinia M. 2005. Direct immobilization of DNA probes for the development of affinity biosensors. Bioelectrochemistry 66:129-38.

Mascinia M, Tombellia S, Palchetti I. 2005. New trends in nucleic acid based biosensors. Bioelectrochemistry 67:131-33.

McKillip JL, Jaykus LA, Drake M. 1998. rDNA stability in heat-killed and UV-irradiated enterotoxigenic Staphylococcus aureus and Escherichia coli O157:H7. J Clin Microbiol 64:4264-268.

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV. 1999. Food-related illnesses and death in the United States. Emerg Infect Dis 5:607-25.

Medici D, Croci L, Delibato E, Pasquale S, Filetici E, Toti L. 2003. Evaluation of DNA extraction methods for the use in combination with SYBR Green I real-time PCR to detect Salmonella enterica serotype Enteritidis in Poultry. Appl Environ Microbiol 69(6):3456-61.

Mercanoglu B, Griffiths MW. 2005. Combination of immunomagenetic separation with real-time PCR for rapid detection of Salmonella in milk, ground beef, and alfalfa sprouts. J Food Prot 68:557-61.

Meenakshi M, Bakshi CS, Butchaiah G, Bansal MP, Siddiqui MZ, Singh VP. 1999. Adjuvanted outer membrane protein vaccine protects poultry against infection with Salmonella enteritidis. Vet Res Comm 23:81-90.

Miller RG, Tate CR, Mallinson ET, Scherrer JA. Xylose-lysine-tergitol 4: an improved selective agar medium for the isolation of Salmonella. Poult Sci 70:2429-432.

Mizumoto N, Toyota-Hanatani Y, Sasai K, Tani H, Ekawa T, Ohta H, Baba E. 2004. Detection of specific antibodies against deflagellated Salmonella enteritidis and S. enteritidis FliC-specific 9 kDa polypeptide. Vet Microbiol 99:113-20.

Mo XT, Zhou YP, Lei H, Deng L. 2002. Microbalance-DNA probe method for the detection of specific bacteria in water. Enz Microb Tech 30:583-89.

Muller L.1923. Un nouveau milieu d'enrichissement pour la recherche du bacille typhique et des paratyphiques. Comprend Soc Biol 89:434-7.

Myint MS, Johnson YJ, Tablante NL, Heckert RA. 2006. The effect of pre-enrichment protocol on the sensitivity and specificity of PCR for the detection of naturally contaminated Salmonella in raw poultry compared to conventional culture. Food Microbiol 23:599-604.

Naravaneni R, Jamil K. 2005. Rapid detection of food-borne pathogens by using molecular techniques. J Med Microbiol 54:51-54.

NG SP, Tsui CO, Roberts D, Chau PY, NG MH. 1996. Detection and serogroup differentiation of *Salmonella* spp. in food within 30 hours by enrichment-immunoassay with a T6 monoclonal antibody capture enzyme-linked immunosorbent assay. Appl Environ Microbiol 62:2294-302.

Nguyen AV, Khan MI, Lu Z. 1994. Amplification of Salmonella chromosomal DNA using the polymerase chain reaction. Avian Dis 119-26.

Olsen SJ, MacKinon LC, Goulding JS, Bean NH, Slutsker L. 2000. Surveillance for foodborne disease outbreaks, United States, 1993-1997. Morb Mortal Wkly Rep 49:1-51.

Olsen J E, Aabo S, Hill W, Notermans S, Wernars K, Granum PE, Popovic T, Rasmussen HN, Olsvik O. 1995. Probes and polymerase chain reaction for detection of food-borne bacterial pathogens. Int J Food Microbiol 28:1-78.

Panisello PJ, Rooney R, Quantick PC, Stanwell-Smith R. 2000. Application of foodborne disease outbreak data in the development and maintenance of HAACP systems. Int J Food Microbiol 59:221-34.

Patel JR, Bhagwat AA, Sanglay GC, Solomon MB. 2006. Rapid detection of Salmonella from hydrodynamic pressure-treated poultry using molecular beacon real-time PCR. Food Microbiol 23:39-46.

Philpott DJ, Girardin SE, Sansonetti P. 2001. Innate immune response of epithelial cells following infection with bacterial pathogens. Curr Opin Immunol 13:410-16.

Rand AG, Ye Y, Brown CW, Letcher SV. 2002. Optical biosensors for food pathogen detection. Food Tech 56:32-39.

Radstrom P, Knutsson R, Wolffs P, Lovenklev M, Lofstrom C. 2004. Pre-PCR processing-strategies to generate PCR-compatible samples. Mol Biotechnol 26:133-46.

Rappaport F, Konforte N, navon B. 1956. A new enrichment medium for certain salmonellae. J Clin Pathol 9:261-6.

Rappaport F, Kronforti N. 1959. Selective enrichment medium for paratyphoid bacteria. inhibitory and growth promoting factors. Appl Microbiol 7:63-6.

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

Rossen L, Norskoy P, Holmstrom K, Rasmussen O. 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int J Food Microbiol 22:37-45.

Ryan CA, Nickels MK, Hargrett-Bean NT, Potter ME, Endo T, Mayer L. 1987. Massive outbreaks of antimicrobial-resistant salmonellosis traced to pasteurized milk. JAMA 258:3269-74.

Sadeyen JR, Trotereau J, Philippe V, Marly J, Beaumont C, Barrow PA, Bumstead N, Lalmanach AC. 2004. Salmonella carrier state in chicken: comparison of expression of immune response genes between susceptible and resistant animals. Microbes and Infect 6:1278-86.

Sambrook J, Russel DW. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

Schrank IS, Mores MAZ, Costa JLA, Frazon APG, Soncini R, Schrank A, Vainstein MH, Silva SC. 2001. Influence of enrichment media and application of a PCR based method to detect Salmonella in poultry industry products and clinical samples. Vet Microbiol 82:45-53.

Small J, Call DR, Brockman FJ, Straub TM, Chandler DP. 2001. Direct detection of 16S rRNA in soil extracts by using oligonucleotide microarrays. Appl Environ Microbiol 67:4708-716.

Soumet C, Ermel G, Fach P, Colin P. 1994. Evaluation of different DNA extraction procedures for the detection of Salmonella from chicken products by polymerase chain reaction. Lett Appl Microbiol 19:294-98.

Soumet C, Ermel G, Salvat G, Colin P. 1997. Detection of Salmonella spp. in food products by polymerase chain reaction and hybridization assay in microplate format. Lett Appl Microbiol 24:113-16.

Sugimoto T & Matijevic E. 1980. Formation of uniform spherical magnetite particles by crystallization from ferrous hydroxide gels. Journal of Colloid and InterfaceScience 74(1):227-243.

Tate CR, Miller RG, Mallinson ET, Douglass LW, Johnson RW. 1990. The isolation of salmonella from poultry environmental samples by several enrichment procedures using plating media with and without novobiocin. Poult Sci 69:721-6.

Tauxe, RV. 2001. Food safety and irradiation: protecting the public health from foodborne disease. Emerg Infect Diseases 7(3):516-521.

Tauxe, RV. 2002. Emerging foodborne pathogens. Int J Food Microbiol 78:31-41.

Tauxe, RV. 2002. Surveillance and investigation of foodborne disease; roles for public health in meeting objectives for food safety. Food Cont 13:363-69.

Taylor JI, Hurst CD, Davies MJ, Sachsinger N & Bruce IJ. 2000. Application of magnetite and silica-magnetite composites to the isolation of genomic DNA. Journal of Chromatography A 890(1):159-166.

Trkov M, Majerikova I, Jersek B, Stefanovicova A, Rijpens N, Kuchta T. 1999. Detection of Salmonella in food over 30 h using enrichment and polymerase chain reaction. Food Microbiol 16:393-99.

Tyagi S, Kramer FR. 1996, Molecular beacons-probes that fluoresce upon hybridization. Nat Biotechnol 14:303-08.

[USFDA] U.S. Food and Drug Administration. 2003. Bacteriological Analytical Manual online. 8th edition. U.S. Department of Health and Human Services Food and Drug Administration center for food safety and applied nutrition. Available from:www.cfsan.fda.gov/~ebam/bam-toc.html.

Uyttendaele M, Vanwildemeersch K, Debevere J. 2003. Evaluation of real time PCR vs automated ELISA and a conventional culture method using a semi-solid medium for the detection of Salmonella. Letters Appl Mirobiol 37:386-91.

Vassiliadis P, Pateraki E, Papaiconomou N, Papadakis JA, Trichopoulos D. 1976. Nouveau procédé d'enrichissement de Salmonell. Ann Microbiol Inst Pasteur 127B:195-200.

Volokhov D, Chizhikov V, Chumakov K, Rasooly A. 2003. Microarray-based identification of thermophilic Campylobacter jejuni, C. coli, C. lari and C. upsaliensis. J Clin Microbiol 41:4071-80.

Wan CS, Li J, Luo J. 2004. Detection of the Salmonella invasion gene invA using molecular beacon probe. Acad J First Med College PLA 24:1257-59.

Wang H, Blais BW, Brooks BW, Yamazake H. 1996. Salmonella detection by the polymyxin-cloth enzyme immunoassay using polyclonal and monoclonal detector antibodies. Int J Food Microbiol 29:31-40.

Wang X, Jothikumar N, Griffiths MW. 2004. Enrichment and DNA extraction protocols for the simultaneous detection of Salmonella and Listeria monocytogenes in raw sausage meat with multiplex real-time PCR. J Food Prot 67:189-92.

Wang R, Tombelli S, Minunni M, Spiriti MM, Mascini M. 2004. Immobilisation of DNA probes for the development of SPR-based sensing. Biosensors and Bioelectronics 20:967-74.

Whyte P, McGill K, Collins JD, Gormley E. 2002. The prevalence and PCR detection of Salmonella contamination in raw poultry. Vet Microbiol 89:53-60.

Wilson IG. 2002. Salmonella and Campylobacter contamination of raw retail chickens from different producers: a six year survey. Epidem Infect 129(3):635-45.

Wu CF, Valdes JJ, Bentley WE, Sekowski JW. 2003. DNA microarray for discrimination between pathogenic O157:H7 EDL933 and nonpathogenic Escherichia coli strains. Biosensors and Bioelectronics 19:1-8.

Yao G, Fang X, Yokota H, Yanagida T and Tan W. 2003. Monitoring molecular beacon DNA probe hybridization at the single molecule level. Chem-A Euro J 9:5686-5692.

Yedidag EN, Koffron AJ, Mueller KH, Kaplan B, Kaufman DB, Fryer JP. 1996. Acyclovir triphosphate inhibits the diagnostic polymerase chain reaction for cytomegalovirus. Transplant 62:238-42.