DEVELOPMENT OF MICROSCOPIC IMAGING SYSTEM FOR RAPID

DETECTION OF *SALMONELLA* IN RAW CHICKEN

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DEVELOPMENT OF MICROSCOPIC IMAGING SYSTEM FOR RAPID DETECTION OF *SALMONELLA* IN RAW CHICKEN

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DISSERTATION ABSTRACT

DEVELOPMENT OF MICROSCOPIC IMAGING SYSTEM FOR RAPID DETECTION OF *SALMONELLA* IN RAW CHICKEN

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Doctor of Philosophy, Aug 10, 2009
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The overall objective of this study was to develop a new *Salmonella* detection method on poultry by combining a biosensor with a light microscopic imaging system. In the first study, brain heart infusion (BHI) broth was the most efficient enrichment medium for *Salmonella* growth which increased the *Salmonella* population by 4-logs in 6 h, whereas, brilliant green (BG) broth was the most efficient medium for enriching *Salmonella* in chilled poultry. The polyclonal antibody (pAb) was specific to entire *Salmonella* strains tested, except *S*. Diarizonae. In the second study, the gold and polystyrene sensor platforms immobilized with pAb possessed significantly high binding efficiencies with *Salmonella*, showing 66 ± 24 CFU/0.0013 mm^2 and 57 ± 16 CFU/0.0013 mm^2, respectively ($p < 0.05$). However, the protein A and lysine treatments

on gold or polystyrene sensor platform did not enhance antibody immobilization. The optimum concentration of pAb, temperature, and the range of pH in PBS were 100 μg/mL, 30 °C, and pH 7.0-8.0, respectively. The detection limit of the gold biosensor with light microscopic imaging system (GB-LMI) was 2.0 ± 1.0 CFU/0.0013 mm^2 with inoculation of 10^3 CFU. The GB-LMI showed that the detected number of *Salmonella* enriched in BHI medium for 6 h with the initial population of 10^2 CFU and 10^3 CFU was significantly higher than that of enriched in BG medium, (i.e., 75 ± 15 CFU/0.013 mm^2 for 6 h-10^3 in BHI and 51 ± 8 CFU/0.013 mm^2 for 6 h-10^3 in BG). Therefore, the GB-LMI method could detect *Salmonella* on chicken skin inoculated within 4.5 h after 4 h-enrichment in BHI and BG media. In the third study, the optimum quantity of pAb for magnetic beads conjugation, time, reaction temperature, buffer type and pH were 0.44 mg per mL MBs, 20 min, 30 °C, and pH 6.5-7.5 in PBS buffer, respectively. The conjunction of GB-LMI and IMS method did not increase the number of *Salmonella* captured on gold biosensor significantly (32.0 ± 0.9 cells/0.013 mm^2 for chicken sample enriched in BHI for 6 h at the inoculation of 10^2 CFU/16 in^2 and 74.0 ± 9.5 cells/0.013 mm^2 for chicken sample enriched in BHI for 6 h at the inoculation of 10^3 CFU/16 in^2). However, the method improved the resolution of microscopic images with enhancing the reliability and accuracy of GB-LMI detection.

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I. INTRODUCTION

Salmonella is the most prevalent foodborne pathogen in the U.S which ranks higher in incidence than *Camplyobacter*, *E. coli* O157:H7, *Listeria monocytogenes*, *Shigella*, *Vibrio*, *Yersinia*, *Cryptosporidium*, and *Cyclospora* (Mead and others 1999; Tirado and Schmidt 2001; CDC 2006). It is estimated that there are approximately 1.4 million *Salmonella* infections each year in the United States (Voetsch and others 2004; Hargen and others 2008). There are that have been more than 2,500 serovars or serotypes *Salmonella* identified, and all are considered to be pathogenic (CDC 2006). The disease caused by *Salmonella* infection is called salmonellosis.

The general symptoms of salmonellosis are characterized as fever, headaches, abdominal pain, diarrhea or constipation, vomiting and nausea (Bopp and others 2003; Currie and others 2005; Montville and Matthews 2005). The general incubation period for salmonellosis is assumed to be 8 to 72 h, and the infections dosage is reported in the range of 10^3 to 10^6 organisms depending on the host, the *Salmonella* strain, and the food matrix (Balser and Newman 1982). Outbreaks of salmonellosis are frequently associated with food and food products including meats, poultry and eggs, milk and dairy products. Outbreaks of salmonellosis associated with minimally processed fruits and vegetables are reported to be increasing (Berends and others 1998 ; Hald and Wegener 1999). The

poultry products are identified as one of the predominant sources of *Salmonella* infection in humans, and poultry has gained more attention due to the increased consumption of poultry (St Louis and others 1988; Tietjen and Fung 1995; Myint and others 2006).

Conventional detection methods for *Salmonella* involve procedures which include pre-enrichment for 16 to 24 h, enrichment for 18 to 24 h, and the plating on either selective or differential media for 24 h, followed by the confirmation of the colonies biochemically and serologically (Ng and others 1996). Although, the conventional method is considered as an effective, sensitive, inexpensive and accurate method, the conventional method is a laborious and time-consuming process requiring a minimum of 4 to 6 days to confirm the results (Mallinson and Snoeyenbos 1989; Uyttendaele and others 2003; Myint and others 2006). Moreover, in recent year food regulatory agencies are focused more on controlling *Salmonella* at the point of process with Critical Control Points (CCPs) rather than controlling the finished end-product (Ricci and others 2007). Therefore, more rapid, sensitive, specific, simple and accurate detection methods are preferred to the conventional detection methods, allowing the *Salmonella* detection in food products within 24 h (Domínguez and others 2002; Eijkelkamp and others 2009). A number of rapid methods for *Salmonella* detection in food and food products have been developed and the major rapid methods that are being studied are Polymerase Chain Reaction (PCR) and Enzyme-Linked Immunosorbent Assay (ELISA).

Since the PCR method is rapid, sensitive, and specific for *Salmonella*, PCR has been used in several studies for the rapid detection of *Salmonella* (Chen and others 1997; Kimura and others 1999; Nogva and Lillehaug 1999; Hoofer and others 2004; Nam and others 2005; Patel and others 2006). According to previous studies (Soumet and others

1994; Soumet and others 1997; Li and Mustapha 2002; Eyigor and Carli 2003; de Medici and others 2003; Wang and others 2004), the detection limit of *Salmonella* in poultry and meat products by PCR method is approximately 10^3 CFU/mL. Eyigor and Carli (2003) reported that they were able to detect as few as 6 CFU/mL in spiked poultry products. Despite the relatively high sensitivity and specificity of the PCR method, the method has limitations. First, the reliability of results is greatly dependent on the purity of the DNA extracted from microorganisms, and obtaining a pure DNA from a mixture of natural ingredients such as polysaccharides, fats, proteins or salts is very difficult (Justé and others 2008). Second, the PCR method requires a high level of expertise in molecular biology as well as expensive instruments (Hargen and others 2008). Third, the PCR method cannot distinguish between live or dead microorganisms and can result in false positive results because DNA can be extracted from both live and dead microorganisms (Rudi and others 2005).

ELISA, on the other hand, is based on the interaction of specific antibodies against target microorganisms. The general detection limits of ELISA for *Salmonella* in food products are reported from 10^3 to 10^5 CFU/ mL in 24 h (Candish 1991; Mansfield and Forsythe 2000). Although the sensitivity of ELISA is considered as less than the sensitivity of the PCR method, ELISA is still considered as one of the most promising methods to detect *Salmonella* (Scheu and others 1998). However, ELISA also possesses limitations in that ELISA involves several incubations and washing steps and requires specific monoclonal and polyclonal antibodies to have the interaction with target microorganisms. Therefore, more research is needed to improve the PCR and ELISA

methods and more research is needed to develop new rapid methods for the detection of *Salmonella* and other foodborne pathogens.

Rapid detection methods using biosensors have gained attention since 1990, and currently there are four categories of biosensors available based on the transduction methods including electrochemical, optical, piezoelectric, and thermometric biosensors (Lazcka and others 2007; Ricci and others 2007). Among the applications of biosensors for microbial detection, the widely used biosensors include Surface Plasmon Resonance (SPR) biosensor, a type of optical biosensors, and Quartz Crystal Microbalance (QCM) biosensor, a type of piezoelectric biosensors. The principles of the two biosensors are based on the detection of small changes caused by the interactions between the target microorganisms and the antibodies immobilized on the sensor platform.

The biosensors are simpler and more rapid than PCR and ELISA because biosensors do not require a DNA purification step as in PCR or several washing and incubation steps as in ELISA. In addition, the biosensors are easy to apply because the biosensors do not require any specifically trained operator, once developed. However, the general detection limits of both biosensors range from 10^5 to 10^6 CFU/mL (Jianming and others 1997; Su and Li 2005; Oh and others 2005; Mazumdar and others 2007), indicating a lower detection limits than PCR or ELISA methods. Other limitations of biosensors include expensive instrument for the SPR biosensor, non-specific bonding, and sensitive interference from the environment (Ricci and others 2007). The development of new biosensors having improved sensitivity, specificity, low cost operation, and less sensitivity from environment interference for *Salmonella* detection would be beneficial to the meats and poultry industries.

The overall objective of this study was to develop a new method which combined a biosensor with a light microscopic imaging system for the rapid *Salmonella* detection within 8 h in poultry products. The biosensors immobilized with specific antibodies will bind *Salmonella* specifically in chicken products, and the bound *Salmonella* will be visualized and enumerated automatically using a light microscopic imaging system. The combination of a biosensor and microscopic imaging system should enhance the sensitivity and specificity for detecting *Salmonella* on poultry products. Other advantages of this method are the simplicity and ease of operation, requiring no expert personnel for routine operation in poultry plants. Therefore, the proposed detection method with automatic counting system in this study should provide the food industry with substantial benefits for rapid detection of *Salmonella* in poultry products.

II. LITERATURE REVIEW

More than 200 foodborne diseases are transmitted by bacteria, viruses, parasites, toxins, metals, and prions. The number of reported illnesses due to foodborne diseases in the United States each year is approximately 76 million, including 325,000 hospitalizations and 5,020 deaths (1,810 deaths due to known pathogens and 3,210 deaths due to unknown pathogens) (Mead and others 1999; CDC 2006). In 1996, the Centers for Disease Control and Prevention (CDC) established the FoodNet surveillance to control foodborne disease systematically and identified the top nine leading pathogens of foodborne diseases as *Camplyobactor*, *Esherichia coli* O157:H7, *Listeria*, *Salmonella*, *Shigella*, *Vibrio*, *Yersinia*, *Cryptosporidium*, and *Cyclospora* (CDC 2006). Among the nine pathogens mentioned above, *Salmonella* is the largest number of outbreak pathogen (55%), the main cause of severe illness (26%), and the leading cause of foodborne death (31%) (Mead and others 1999; Tirado and Schmidt 2001; CDC 2006). Therefore, among the top nine pathogens, *Salmonella* is the most important leading cause of foodborne illness in the United States.

1. *Salmonella*

Salmonella is named after D.E. Salmon, the first scientist to identify *Salmonella choleraesuis* from porcine intestine in 1884. *Salmonella* is classified as Genus III in the family of Enterobacteriaceae along with *E. coli* and *Shigella* strains. *Salmonella* strains are facultative anaerobes and Gram-negative rods. The width of *Salmonella* ranges from approximately 0.7-1.5 μm and its length is 2-5 μm. Most *Salmonella* strains are motile with peritrichous flagella, with the exception of *S.* Gallinarum and *S.* Pullorum (Cox and others 2000). *Salmonella* is mesophilic with an optimum growth temperature range between 8 ºC and 45 ºC (optimum temperature 37 ºC) (Gutherie 1992), although some strains, for example *S*. Typhimurium, can grow at even 6.2 ºC (Jay 2000). The optimum pH ranges for *Salmonella* are between pH 6.5 and 7.5, however it can grow at pH values up to 9.5 and down to 4.0 (Gutherie 1992). *Salmonella* uses glucose and citrate as its major carbon sources and *Salmonella* is non or slow lactose fermenters with some strains fermenting it. *Salmonella* produces hydrogen sulfide (H_2S) from thiosulfate (D'Aoust 1989; Bopp and others 2003).

Nomenclature of *Salmonella*

Salmonella was initially divided into three distinct subspecies including *S. choleraesuis*, *S. typhosa* (*S. typhi*), and *S. Kauffmannii* containing all the other serovars (Su and Chiu 2007). In 1966, Kauffman first proposed the one serotype-one species concept on the basis of the serological identification of somatic (O) and flagellar (H) antigens for nomenclature of *Salmonella* (Brenner and others 2000). In 1970, another proposal of *Salmonella* nomenclature was proposed to divide the serotypes into

subgenera based on the clinical roles, biochemical characteristics and genomic relevance of a strain (e.g., *S. kauffmannii* serovar typhi) (Su and Chiu 2007). In 1987, Le Minor and Popoff classified *Salmonella* as the single *Salmonella* species and seven subspecies on the basis of DNA-DNA hybridization experiments (Brenner and others 2000). Because there are several nomenclature systems for *Salmonella*, there is significant confusion in communication among scientists, health officials and even the public. Moreover, the recent development of genetic and molecular biology techniques is able to identify *Salmonella* more specifically, based on biochemical and serological characteristics. Therefore, more systematic and uniform nomenclature for *Salmonella* is needed.

According to the CDC system, nomenclature of *Salmonella* is based on the recommendation of the WHO Collaborating Centre. *Salmonella* was initially divided into two species: *S. enterica,* and *S. bongori.* However, *S. subterranean* was approved by the Judical Commision of the International Committee on the Systematics of Prokaryotes in 2005. *S. enterica* is further subdivided into six different subspecies, based on biochemical and genomic relevance including *S. enterica* serovar *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI) (Table 1) (Su and Chiu 2007). Among the six subspecies, approximately 99% of *Salmonella* subspecies causing disease in humans and domestic animals belong to the subspecies *enterica* (I) while the other subspecies (II, IIIa, IIIb, IV, and VI) and *S. bongori* are found in cold blooded animals and the environment (Farmer III and others 1984).

Based on the Kauffman-White scheme, there are more than 2500 types of serovars or serotypes within the six subspecies of *S. enterica* (Su and Chiu 2007).

Serotypes or serovars can be defined based on the carbohydrate (polysaccharides) in the lipopolysaccharide (LPS) structure of the microorganism, which gives various antigenic polymorphisms even within the same subspecies. The polymorphism of LPS is related to the polymorphic characteristics of the *rfb* gene on the chromosome, which encodes the enzymes to synthesize the sugars and organize them into the LPS structure in the cell wall (Fierer and Guiney 2001). Although lipid A is the core structure in the LPS, the polysaccharide side chains are so highly varied that the serovars of *Salmonella* can be divided into three types including somatic (O), capsular (Vi), and flagellar (H) antigen (Clarke and Gyles 1993; Popoff 2001) (Figure 1). Somatic antigen is further divided into five groups including A, B, C, D and E groups. More than 95% of *Salmonella* related to human infections are under somatic antigen groups. Somatic antigens are heat stable and alcohol resistant, whereas flagellar antigens are heat-labile proteins.

To avoid confusion between serovars (serotypes) and species, the serovar name is not usually italicized and starts with a capital letter (for example, *Salmonella* serotype or ser. Enteritidis, Typhimurium, and Typhi). As shown in Table 1, there are currently 2,541 serovars in *Salmonella* (Su and Chiu 2007). To express the 2,541 types of serovars in *Salmonella*, each *Salmonella* serovar needs to be written as the distinctive antigenic formula including the O antigen and the H antigen. The notation used to denote the serotype is given by the major antigenic groups separated by colons:O-antigens:phase 1H-antigens: phase 2H-antigens (Old 1992). For example, *Salmonella enterica* serovar Typhimurium can be expressed as the antigenic formula (1, 4, 5, 12: i: 1, 2), which means O antigens identified in 1, 4, 5, and 12 , H antigens identified i in phase 1, and1 and 2 in phase 2 (Lindquist 2006).

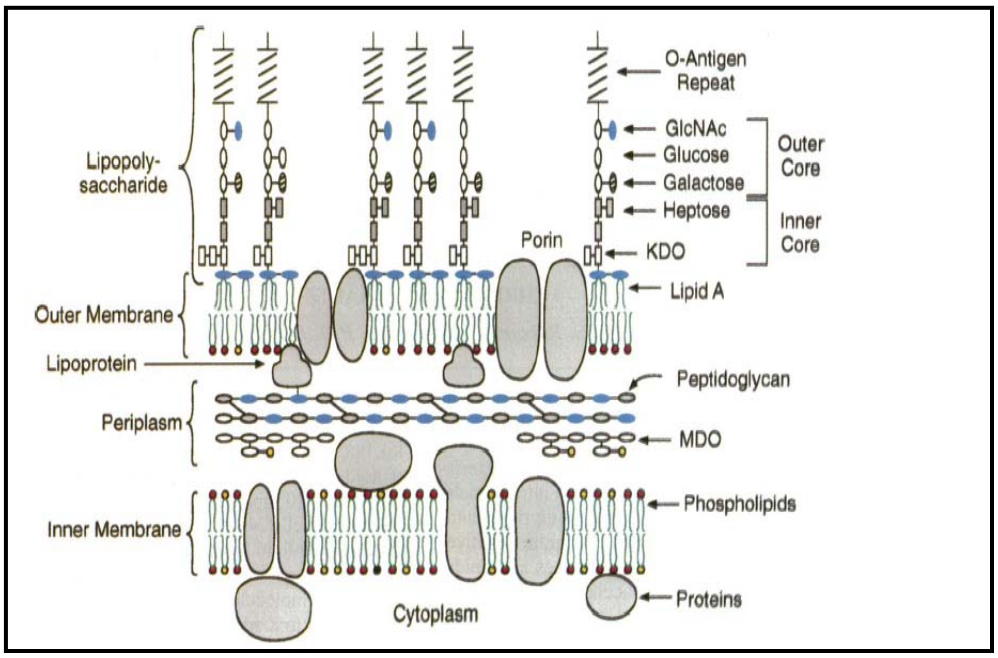

Figure 1-Molecular representation of inner and outer membrane of a gram-negative bacterium depicting the location of the O-antigen. (Adapted from Raetz 1993).

Table 1. Current *Salmonella* Nomenclature recommended by CDC

Taxonomic position and nomenclature				
Genus (capitalized, italic)	Species (italic)	Subspecies (or italic)	Serovars (capitalized, not italic)	No. of serovars in subspecies
Salmonella	*enterica*	*enterica* (I)	Typhimurium	1504
		salamae (II)	9,46:z:z39	502
		arizonae (IIIa)	43:z29:-	95
		diarizonae (IIIb)	6,7:1,v:1,5,7	333
		houtenae (IV)	21:m,t:-	72
		indica (VI)	59:z36:-	13
	bongori	Subspecies V	13,22: z39:-	22
	subterranea			

(Adapted from Su and Chiu 2007).

Salmonellosis

Salmonellosis is the foodborne disease caused by *Salmonella* and approximately 95% of salmonellosis cases is food related (Mead and others 1999; Santos and others 2003; Layton and Galyov 2007). The main syndromes of salmonellosis can be classified as typhoid or enteric (paratyphoid) fever and gastroenteritis (non-typhoidal). Typhoid, or enteric fever, is caused by the infection of exclusively human pathogens such as *S. enterica* serovars Typhi and Paratyphi. Typhoid fever causes systematic illness by reaching the lymph system after penetrating the epithelial cells of the small intestine in the host. Clinical symptoms of typhoid fever are characterized as prolonged high fever, headaches, abdominal pain, transient diarrhea or constipation, occasionally a macula-papolar rash, and death in rare cases (Bopp and others 2003; Montville and Matthews 2005). Each year, approximately 400 Americans suffer from Typhoid fever, which is mainly related to travel to developing countries (CDC 2004). The disease can be cured by taking antibiotics such as ampicillin, gentamicin, trimethoprim/sulfamethoxazole, or ciprofloxacin. However, the mortality rate can be as high as 10-15% without any proper treatment (Ohl and Miller 2001).

Gastroenteritis infection occurs much more frequently in the United States than typhoid fever. Approximately 1.4 million cases of gastroenteritis infections are reported in the United States annually, and the estimated annual cost for medical treatment and lost productivity is estimated to be 0.5 to 2.3 billion dollars (Mead and others 1999). Gastroenteritis infection is mainly caused by five serotypes: *S. enterica* serovars Typhimurium (20%), Enteritidis (15%), Newport (10%), Javiana (7%), and Heidelberg (5%), which together account for 56% of all human cases (CDC 2006).

Gastroenteritis infection has a self-limiting infection system, because *Salmonella* can only reach into the lamina propria of epithelial cells (Ohl and Miller 2001). The symptoms of gastroenteritis infection include diarrhea (88%), fever (80%), abdominal cramps (65%), nausea (42%), occasional vomiting (35%), and headache (29%) with 33% bloody diarrhea (Currie and others 2005). The general incubation period is 8 to 72 h. The reported infections dosage is generally assumed to be 10^3-10^6 organisms depending on the age and health of the host, the strain type, and the ingested food matrix (Balser and Newman 1982). If the foods contain large amounts of fat or protein, these food components can provide protection for *Salmonella* to pass through strong gastric acid in the stomach. With regards to hosts age and health, children less than 10 years old and elderly people are more susceptible to infection than adults. People taking antacid therapy, antibiotic therapy, and immunosuppressive therapy such as for cancer or HIV infection are also at a higher risk for infection (Gutherie 1992; CDC 2006). In most cases, antibiotic treatment is not necessary; however, antibiotic treatment is necessary in cases where the pathogen is severely virulent to host, causing septicemia, reactive arthritis, Reiter's syndrome, appendicitis, endocarditis, meningitis, peritonitis, and urinary tract infection (Bell 2002; Currie and others 2005).

Pathogenicity of *Salmonella*

The success of pathogenic *Salmonella* in the host depends on its adaptation in a new harsh environment and avoidance or neutralization of specific and non-specific defense mechanisms of the host (Finlay and Falkow 1989; Grassl and Finlay 2008). When *Salmonella* enters the host's body with ingested food, strong gastric acid plays an important role as an initial barrier against *Salmonella.* However, highly adaptive

pathogenic *Salmonella* can survive under potentially harsh conditions of gastric acid in the stomach by adapting itself to its new environment rapidly by triggering the Adaptive Acid Tolerance response (ATR) which induces proteins that can protect it from the harsh environment (Finlay and Falkow 1989; Garcia-del and others 1993; Ohl and Miller 2001). Once *Salmonella* survives the harsh conditions present in the host's stomach, *Salmonella* will confront other defense mechanisms in the host, including the innate immune system and the specific immune system (adaptive immune system).

After *Salmonella* becomes highly adaptive and defensive against the host, the *Salmonella* still requires two other essential abilities to have pathogenic characteristics: it must be able to enter non-phagocytic cells (M cells or enterocytes) in the intestinal epithelium, because the intracellular environment provides a favorable niche for bacteria to multiply (Galán 1996; Zhou and Galán 2001) and it must survive and replicate within macrophages (Buchmeier and Heffron 1989). These two essential abilities required to express pathogenic properties are mainly related to the virulence of *Salmonella*, which are encoded by genes on the chromosome in *Salmonella* pathogenicity islands (SPIs).

Salmonella pathogenicity islands (SPIs) are specific regions of DNA on the chromosome of *Salmonella* that contains the virulence-associated genes. Twelve *Salmonella* pathogenicity islands (SPIs) have recently been reported in *Salmonella*. SPI-1 (40 kb DNA region) promotes epithelial cell invasion by encoding a type-III-secretion system (TTSS) which encodes proteins to form needle-like structures of *Salmonella* such as fimbriae and flagella (Fierer and Guiney 2001; Ohl and Miller 2001; Santos and others 2003). TTSS structural genes including *inv*G, *prg*H and *prg*K encode at least 13 effector proteins including AvrA, SipA, SipB, SipC, SipD, SlrP, SopA, SopB, SopD and SopE

(Zhou and Galán 2001). SPI-2 (40 kb DNA region) encodes TTSS-2 to deliver effector proteins for growth in epithelial cells and survival in macrophages (Fierer and Guiney 2001). SPI-3 (17 kb) promotes survival within macrophages and growth in low magnesium ion environments with the help of *mgt*C and *mis* gene expression. SPI-4 promotes survival in intramacrophage and toxin secretion with the help of *spi4R* and *spi4D* gene expression. SPI-5 promotes intestinal fluid secretion and inflammation with the help of *sopB, pipA, pipB* and *pipD* gene expression (Fierer and Guiney 2001; Hensel 2004; Soto and others 2006).

Outbreak of Salmonellosis

Outbreaks of salmonellosis are associated with the consumption of meat, poultry, milk, and dairy products contaminated by animal faeces or by cross- contamination from foods containing *Salmonella*. Although outbreaks of salmonellosis are increasingly related to minimally processed fruit and vegetables such as seed sprouts (Mahon and others 1997; Guo and others 2001), watermelons (Blostein 1993), unpasteurized fruit juices (Guo and others 2001), mango (Sivapalasingam and others 2003), tomatoes, and spinach (CDC 2004), the major reservoir and vehicle of salmonellosis in humans is meat, raw eggs and poultry products (Cason and others 1997; D'Aoust 1997; Berends and others 1998 ; Hald and Wegener 1999).

However, poultry products have drawn attention, because poultry consumption has consistently increased since 1976 whereas beef consumption has consistently decreased (56.8 lbs of poultry per capita and 64.5 lbs of beef per capita annually; poultry per capita consumption 2002). Therefore, outbreaks of salmonellosis are mainly

associated with poultry and poultry products. According to a study (Rose and others 2002), they collected chicken samples randomly from federally inspected processing facilities in the United States over two years and isolated *Salmonella* from ground chicken samples (14.4%) and broiler carcasses (10.2%). According to another study (White and others 2001), *Salmone*lla was positive among the 35% of the ground chicken samples analyzed in the study.

2. Conventional Detection Method

The conventional isolation and identification method for *Salmonella* has been considered as the most common technique. The conventional method includes pre-enrichment (16-24 h) and enrichment (18-24 h) to increase the population of *Salmonella* that might have been injured during food processing or storage. Following the enrichment step the food sample is plated on selective or differential media (24 h) for isolation and identification of *Salmonella* (Ng and others 1996). In biochemical screening, triple sugar iron agar (TSI) and lysine iron agar (LIA) are commonly used for identification of *Salmonella* in mixed food culture (Moats 1981; USFDA 2003). Biochemically screened isolates may be confirmed by serological tests recommended by USFDA (2003). Examples of serological testing include the serological polyvalent flagellar (H) test, serological somatic (O) test, serological capsular (Vi) test, and the Spicer-Edwards serological test.

Pre-enrichment media

The pre-enrichment step is essential in order to increase the population of *Salmonella* to a detectable level and to resuscitate *Salmonella* that might have been injured during food processing or storage. The injured microorganisms need to be repaired and rejuvenated before division, because severely injured microorganisms can existe in a Viable-But–Non-Culturable (VBNC) state under unfavorable conditions and only grow under favorable conditions (Foster and Spector 1995). Therefore, injured microorganisms grow very slowly and their lag phase is extended (van der Zee 1994).

Pre-enrichment media provide basic nutrients and growth factors for most bacteria to repair and multiply, compensating for damaged or injured microorganisms. The peptone in the mediums provides carbon, nitrogen and energy to the cells while beef and yeast extracts provide sources of amino acids, peptides, nucleotides, organic acids, vitamins, minerals and nitrogen (Prescott and others 1990). Among several pre-enrichment media, lactose broth (LB) first gained popularity; however, LB has a limitation in dropping the pH due to the fermentation of lactose, resulting in inhibiting or even killing *Salmonella* (Hiker 1975). Buffered peptone water (BPW) has been used instead of lactose. In 1984, several researchers found that BPW was more appropriate than LB for *Salmonella* (Juven and others 1984; Fricker 1987), however, LB has been still used for certain serotypes of *Salmonella* isolation.

The most commonly used media for pre-enrichment of *Salmonella* in food samples are buffered peptone water (BPW) and tryptic soy broth (TSB), although some serotypes require a more specific medium (Boer 1998). In this study, buffered peptone

water (BPW), lactose broth (LB), brain heart infusion broth (BHI), universal pre-enrichment broth (UPB), nutrient broth (NB), tryptic soy broth (TSB) and salmoyst were compared for efficiency of non-selective enrichment to bring *Salmonella* from low population to a detectable level in inoculated chicken skin.

Selective enrichment media

To detect *Salmonella* from complex food samples can be difficult, especially when a small number of *Salmonella* coexist with other competing microorganisms. Therefore, selective enrichment is very important for the successful detection of *Salmonella* when present in foods is low numbers. Selective enrichment helps the multiplication of the target microorganisms while inhibiting other microorganisms (Chang and others 1999), because selective enrichment media contain selective compounds that work against competing microorganisms. However, due to their toxicity against competing microorganisms, enrichment media may also affect the target microorganism to a certain extent. To survive and overcome the toxicity of the selective enrichment media, it has been reported that a minimum of 10^5 CFU/ml *Salmonella* is necessary in the initial enrichment (Chen and others 1993; Boer 1998). Recovery rates of microorganisms in various selective enrichment media are also affected by the types of food, the number of *Salmonella* present in the food sample, the types of competing microorganisms, and the times and temperatures of incubation (Cox and others 1982; Blivet and others 1997; Bailey and others 1988).

There are currently three major types of selective-enrichment media: selenite broth (SB), tetrathionate broth (TT), Rappaport family (RV) (Bussse 1995). There are also different formulations within each type of enrichment (Waltman 2000).

Selenite broth, first formulated by Leifson (1936), contains $NaHSeO_3$ (26.5 mM) as a selective agent, which inhibits the growth of coliform bacteria and enterococci by increasing the rate of uptake of selenite for non-*Salmonella*. For non-*Salmonella* microorganisms, the uptaken selenite incorporates in the cell proteins as a sulfur analog and thus delays their multiplication (Singleton and Sainsbury 1987; D'Aoust 1989). North and Bartram (Moats 1981) modified selenite broth by adding L-cystine as a reducing agent into the selenite broth formula, called selenite cystine (SC). Although Rappapor-Vassiliadis (RV) medium is currently recommended as an enrichment medium for *Salmonella* from low and highly contaminated foods, selenite or selenite cystine broth are still useful as selective broths for *Salmonella*, due to the lower toxicity against low numbers of *Salmonella* and certain *Salmonella* serotypes such as *S*. Gallinarum, *S*. Pullorum, *S*. Typhi and *S*. Paratyphi (Fagerberg and Avens 1976; Harvey and Price 1979; Patil and Parhad 1986; Bailey and others 1988; Papadoulou and Xylouri 1989).

Tetrathionate broth (TT), developed by Muller (1923), contains iodine and sodium thiosulphate (as thionate) to form tetrathionate. In 1935, Kauffman modified tetrathionate to tetrathionate brilliant green (TBG) by adding oxbile and brilliant green (MKTT) as selective agents to suppress bacteria such a *Proteus* spp. (Busse 1995). Tetrathionate oxidized from thiosulphate in medium is a selecting agent that inhibits coliform and enteric bacteria unlike *Salmoenella* and *Proteus*. Oxbile salt inhibits other intestinal microorganisms such as *bifidobacterium,* and brilliant green suppresses the

Gram-positive bacteria. Several studies found that tetrathionate enrichment medium was better than selecite enrichment medium to recover *Salmonella* (D'Aoust and others 1992a; Waltman and others 1995; Waltman 2000). Tetrathionate broth (TT/ TBG) is recommended for use at different incubation temperature 35 °C for low contamination food samples and 43 °C for high contamination food samples (Rall and others 2005).

Rappaport broth, developed by Rappaport (1956), contains malachite green and magnesium chloride ($MgCl_2$) as selective agents (Busse 1995). The Rappaport broth has the specificity to multiply *Salmonella* by maintaining at a very low pH of 5.2, and relatively high osmotic pressures due to magnesium chloride. However, due to the toxicity effect of malachite green and magnesium chloride to *Salmonella,* Rappaport broth was modified by Vassiliadis and others (1976) by decreasing the amount of selecting agent to one third. According to several studies (Pietzsch and Burse 1984; Allen and others 1991; Maijala and others 1992; June and others 1996; Fries and Steinhof 1997; Rall and others 2005), Rappaport-Vasiliadis (RV) medium was more efficient than tetrathionate and selenite broth and has been chosen as one of the most effective selective media for *Salmonella*. According to the research (D'Aoust and others 1992b), the selectivity of RV media at temperatures of 42-43 °C was greater than that at lower temperatures (35-37 °C), and prolonged enrichment time (48 h) in selective media did not increase the recovery of *Salmonella*. Goossen and others (1984) developed a semi-solid medium based on RV enrichment medium (MSRV). In several recent comparative studies (O'Donoghue and others 1992; O'Donoghue and Winn 1993; Pless and Reissbrodt 1995), modified semisolid Rappaport-Vassiliadis (MSRV) medium has been considered to be more effective for increasing the population of *Salmonella* from food samples.

Limitations of the conventional detection method

The conventional detection method has been considered as an effective, sensitive, inexpensive and accurate method to isolate and identify target microorganisms. However, the conventional detection method needs several days to confirm the results because it relies on the multiplication of the target microorganisms to form visualable colonies (Ricci and others 2007). Sometimes this method shows poor sensitivity when detecting low-level of microbial contaminated food samples (D'Aoust and others 1992a). Moreover, recent food safety and control issues are focused on controlling the process line at CCPs (Critical Control Points) rather than controlling the end-product (Ricci and others 2007). Therefore, a more rapid, sensitive, specific, simple and accurate detection method is required in order to detect *Salmonella* in food samples within a relatively short period of time.

3. Rapid Detection Method

To overcome the limitations of the conventional detection method, many researchers have tried to find new and more rapid detection methods, including PCR (Polymerase Chain Reaction), ELISA (Enzyme-Linked Immunosorbent Assay) and biosensor as substitutes for conventional detection method. Over 20 years, approximately 2,500 articles have been published relating to pathogen detection, and most detection methods have been focused on the detection of *Salmonella* and *E. coli*, 33% and 27% respectively (Lazcka and others 2007). PCR related detection method has been used most frequently (840 articles among 2,500), followed by the conventional

detection method (750 articles), ELISA (280 articles), biosensors (170 articles) and others (450 articles) (Lazcka and others 2007). To date, PCR and ELISA are the most promising methods as substitutes for conventional detection method, due to their specificity and reliability; they are also less time consuming than the conventional detection method (Bej and others 1991; Leoni and Legnani 2001; Lazcka and others 2007).

Polymerase Chain Reaction (PCR)

Since PCR was developed by Mullis in 1983, the PCR method has been applied in many areas to detect microorganisms. This method consists of isolation, amplication and quantification of a short DNA sequence in target microorganisms. PCR method requires three main components, including free nucleotides, a heat stable polymerase enzyme, and primers. There are several different PCR methods, such as real-time PCR, multiplex PCR, reverse transcriptase PCR (RT-PCR), or coupling PCR with the biosensor or ELISA method (Lazcka and others 2007).

The PCR method is composed of three main steps including denaturing, annealing and polymerization (Figure 2). During denaturation step, as the DNA heated at 94-98 °C, the hydrogen bonds holding the double strands of DNA together are broken, resulting in single strands of DNA. Once temperature is lowered to 50-65 °C, the primer selected as complimentary DNA is attached to a single strand DNA during annealing step. Increasing the temperature to 72-80 °C, free nucleotide is added to the primer to extend the DNA strands by polymerase to yield two identical strands. Finally, two DNA strands

are undergoing the denaturing step for the next cycle, and the cycle is repeated over and over to obtain exponential amplification of identical DNA segment.

Many researchers have used the PCR method for detecting foodborne pathogens such as *Salmonella*, *Listeria*, *Campylobactor* and *E. coli* in dairy, meat and poultry products. According to previous studies (Soumet and others 1994; Li and Mustapha 2002; de Medici and others 2003; Croci and others 2004; Kanki and others 2009), the sensitivity of the PCR method for *Salmonella* detection in poultry products is reported as 10^3 CFU/mL. De Medici and others (2003) detected *Salmonella* from chicken sample using PCR at a detection limit of 10^3 CFU/mL. Soumet and others (1994) also exhibited that detection limit of PCR assay for *Salmonella* in raw sausage meat was 10^3 CFU/mL. However the detection limit of S*almonella* could be decreased as much as 6 CFU/mL in spiked poultry products (Eyigor and Carli 2003).

Although the PCR method has been one of the most promising of the rapid detection methods for microorganisms, there are still some limitations. First, the reliability of results depends on the purity of the DNA extracted from microorganisms; however it is hard to extract pure DNA from a mixture of natural ingredients such as polysaccharides, fats, proteins or salts (Justé and others 2008). Second, the PCR needs a specific primers that this method requires a high level of expertise in molecular biology as well as expensive instruments (Hargen and others 2008). Third, the PCR method does not distinguish live or dead microorganisms because DNA is extracted from even dead microorganisms (Scheu and others 1998; Rudi and others 2005).

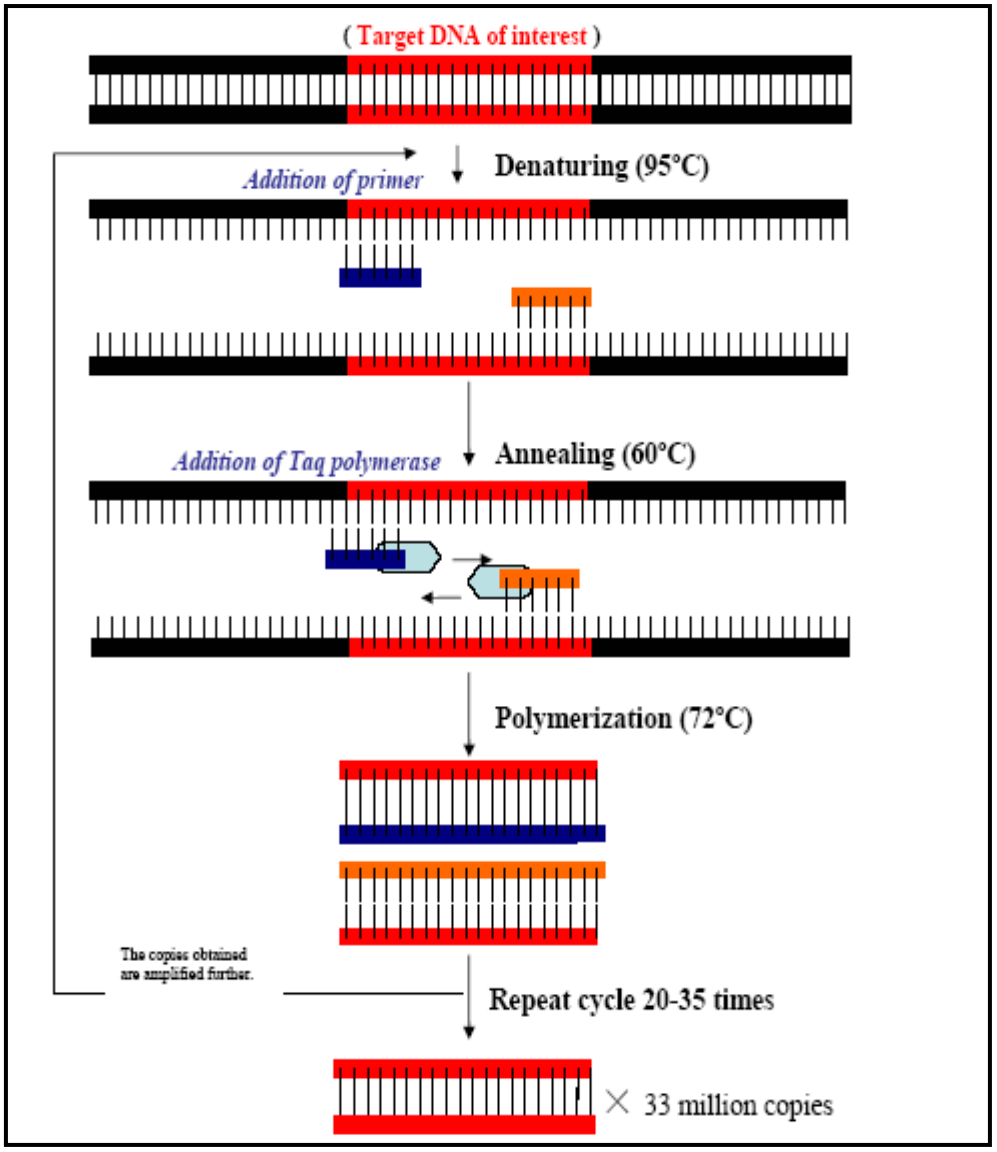

Figure 2-Schematic representation of PCR cycle. (Adapted from Lazcka and others 2007).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA, originated in the late 1950s by Yalow and Berson, is a type of immunoassay that uses specific antibodies or antibody fragments to detect target molecules (antigen) such as microorganisms, toxins, drugs or pesticides. There are four

types of immunoassays including agglutination, radioimmunoassay (RIA), fluorescent immunoassay (FIA) and Enzyme-Linked Immunosorbent Assay (ELISA). Among these, ELISA has gained more popularity than other immunoassays. ELISA has used enzymes as markers including alkaline phosphatase, horseradish peroxidase, β-galactosidase, glucose oxidase, alkaline phosphatase and urease mainly extracted from microorganisms (Plaza and others 2000). The quantity of antigen is calculated by the amount of color change produced. There are three different types of ELISA: indirect ELISA, sandwich ELISA, and competitive ELISA (Figure 3) (Swaminathan and Feng 1994).

A sandwich ELISA (Figure 3) is used to determine the antigen concentration in unknown samples by using two antibodies. One capturing antibody is bound to a plate well and then antigen is added to form a complex with the bound antibody. After unbound antigens are removed with washing, a labeled second antibody is added to bind to the antigen complex, thus forming the "sandwich". The major advantages of this method are that antigen does not need to be purified prior to use, and it is very specific because this method uses two "matched pair" antibodies. However, this method is limited because it requires relatively large quantity of antibodies.

In competitive ELISA (Figure 3), a primary unlabeled antibody is incubated in the presence of its antigen to form antibody-antigen complex and the antibody-antigen complex is added to an antigen coated well. Thus, the more antigens in the sample, the fewer antibodies will be able to bind to the antigen in the well. Then, a secondary antibody conjugated with enzyme is added to bind the primary antibody specifically, resulting in producing color by adding a substrate. Therefore, the higher the original antigen concentration is, the less color is changed.

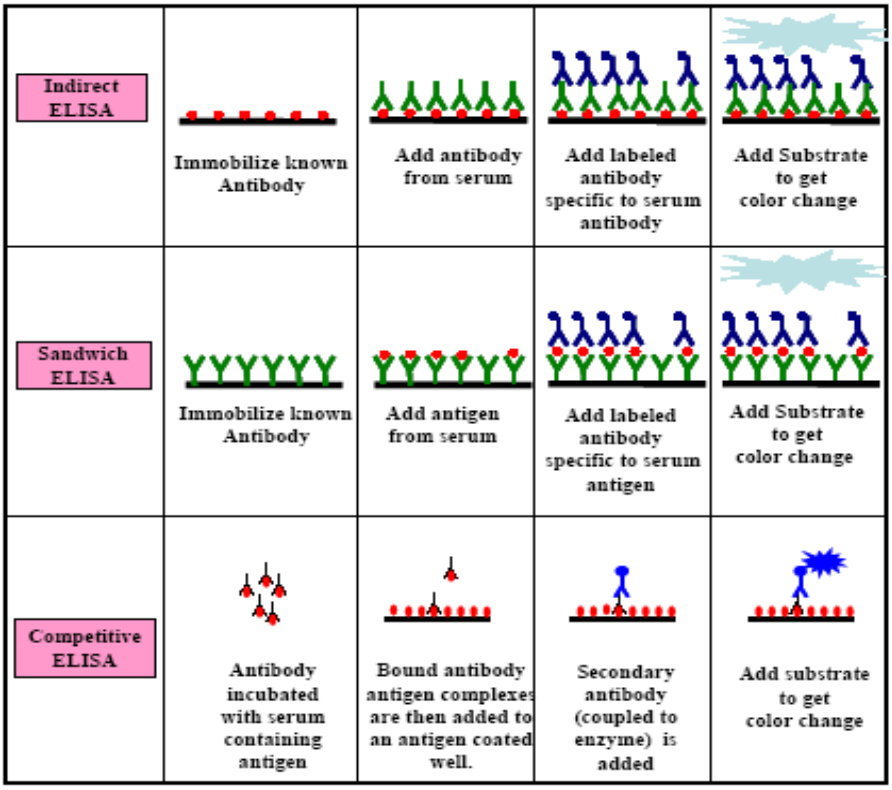

Figure 3-Schematic showing basic steps involved in Indirect ELISA, Sandwich ELISA, and Competitive ELISA. (Adapted from Ramji 2005).

An indirect ELISA (Figure 3) detects specific antibodies whereas sandwich ELISA and competitive ELISA detect soluble antigens. In indirect ELISA, antigen is coated on a plate well and primary antibody is added to bind to it. Then conjugated secondary antibody is added to bind to the primary antibody. The amount of specific antibody is measured by color change of the substrate reacted by the enzyme conjugated to secondary antibody.

Since *Salmonella* was first detected with ELISA by Krysinski and Heimsch (1977), ELISA has become a popular and rapid detection method for *Salmonella* (Lazcka

and others 2007). The general detection limit of *Salmonella* in food samples using ELISA was reported as 10^3-10^5 CFU/ mL in 24 h (Candish 1991; Mansfield and Forsythe 2000a). Although ELISA is one of the most promising methods for detecting *Salmonella*, the method still has some limitations. The limitations include several incubation and washing steps, specific pAb or mAb required to interact with antigen, and cross-reactivity. Therefore, a more simple, rapid, reliable, specific and sensitive detection method is required to overcome its limitations. One of most promising alternative methods for substituting the rapid detection methods is the biosensor method.

4. Biosensor (Immunsensor) Detection Method

Recently, rapid detection method for *Salmonella* has been needed not only for controlling food safety but also for preventing biological attack, because some foodborne pathogens have been considered as biological warfare agents (BWA): *Bacillus anthracis*, *Campylobacter jejuni*, *Clostridium botulinum*, *Esherchia coli*, *Salmonella typhi*, and *Salmonella paratyphi*, etc (Compton 1987; Dando 1994; Ivnitski and others 1999). In addition, many food companies have recently focused not on controlling final product but on controlling every critical control point by HACCP (Hazard Analysis and Critical Control Point) program, which is a systematic and scientific control method for specific hazards during food processing and storage. Therefore, the development of portable, rapid, sensitive, specific, and simple detection methods with minimum sample preparation steps is emphasized (Feng 1996; Deshpande and Rocco 1994).

Definition of Biosensor

Biosensors have recently been defined as "analytical devices that incorporate a biological material (e.g., tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acid, etc.), a biologically derived material (recombinant antibodies, engineered proteins, etc.), or biomimic (e.g., synthetic catalysts, ligand and polymers) with or integrated within physicochemical transducers" for the detection of analytes or target materials (Lazcka and others 2007; Skottrup and others 2008). Especially when antibodies or antibody fragments as a molecular recognition element used to detect specific antigens, we define this device as immunosensor (Ricci and others 2007).

Classification of Biosensors

Biosensors are classified into four different categories by transduction method: electrochemical, optical, piezoelectric, and thermometric (Lazcka and others 2007; Ricci and others 2007). Biosensor methods also can be divided into direct (label-free) and indirect methods (labeled) or competitive and a sandwich methods. This classification is very close to that of ELISA methods because the principle of biosensors (immunosensors) originated from ELISA methods (Figure 4, Ricci and others 2007).

Direct biosensors detect physical and chemical signals directly produced by interaction of analytes and sensing materials. Indirect biosensors detect biochemical signals intensified by labels such as enzymes and fluorescence, which are attached on analytes or sensing materials. The principle of direct competitive biosensor methods is based on direct interaction between antibody and antigen. Immobilized antibodies (Ab) react with free antigens in competition with labeled antigens (Ag*) (Figure 4A),

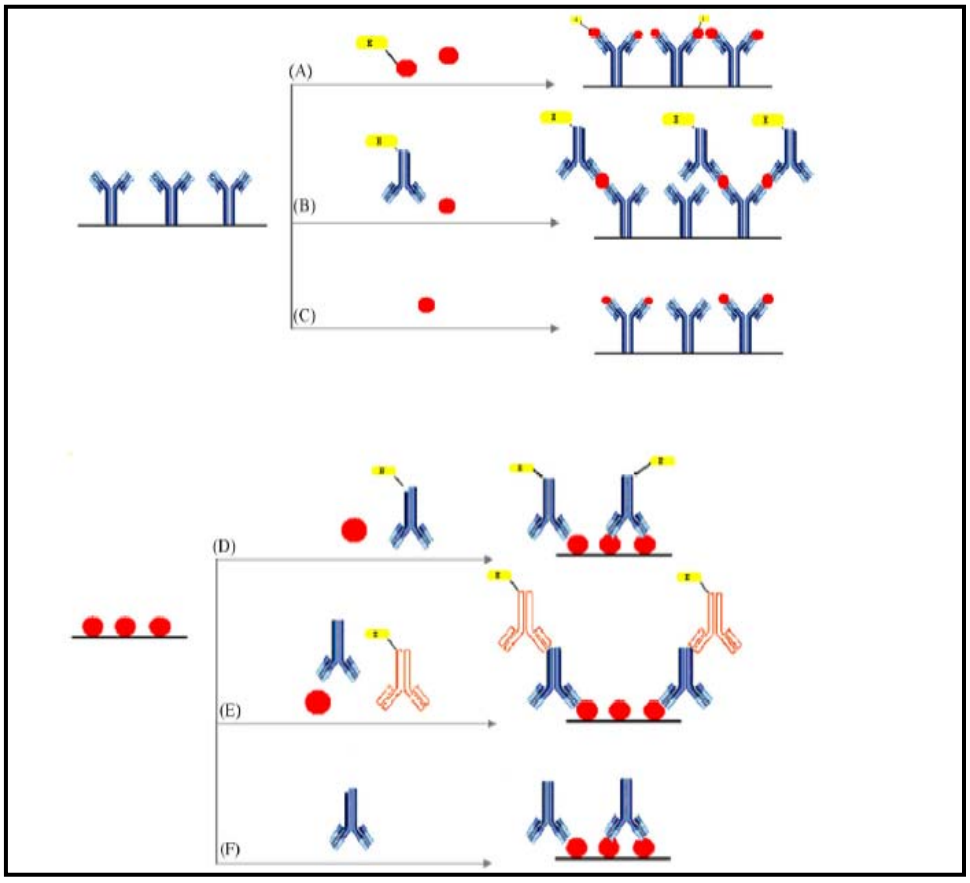

Figure 4-Different type of immunoassay formats. Direct competitive assay using antigen labeled with enzyme; (B) Sandwichassay using detecting antibody labelled with enzyme; (C) Direct assay (for SPR and QCM); (D) Direct competitive assay using primary antibody labeled with enzyme; (E) Indirect competitive assay using a secondary antibody labeled with enzyme; (F) Direct assay for (SPR and QCM) (Adapted from Ricci and others 2007).

or with free antigens (Ag) (Figure 4C). Also immobilized antigens compete with free antigens for labeled free antibodies (Ab*) (Figure 4D), or immobilized antibodies react with antigens (Figure 4F). Indirect competitive methods require secondary antibodies conjugated with enzymes after a primary antibody binds with immobilized antigens (Figure 4E). Sandwich methods also require secondary antibodies, where immobilized

antibodies (Ab) and antigen (Ag) react together and then labeled antibodies (Ab*) are added (Figure 4B) (Ricci and others 2007).

Electrochemical biosensors

Electrochemical biosensors are mainly based on the current (amperometric), potential (potentiometric) or impedience (impediometric) changes due to the interaction of sensing material and analyte at a sensor platform (Lazcka and others 2007). Among the electrochemical sensors, amperometric biosensor has been considered the most appropriate for foodborne pathogen detection by using antibody, due to its high sensitivity, low cost and the possibility of developing a small-sized detector (Delibato and others 2006; Ricci and others 2007). According to one study (Che and others 2001), *Campylbactor jejuni* was detected from chicken carcasses and wash water with the detection limit of 2.1×10^4 CFU/mL using an amperometric biosensor within 2-3 h. another (Chemburu and others 2005) detected *E. coli*, *Campylobactor jejuni* and *Listeria monocytogenesis* in milk and chicken extracts with the detection limit of 50, 50 and 10 CFU/mL, respectively, within 30 min using amperiometric biosensor.

Optical biosensors

Optical biosensors have been used the most widely due to their selectivity and sensitivity (Baeumner and others 2003; Lazcka and others 2007; Ricci and others 2007). Optical biosensors are used as direct detection methods, which measures the direct interactions of antigens and antibodies at sensor platforms without any help from secondary antibodies (Figure 4). The optical biosensors can be categorized by types of transducer such as chemiluminescence, light absorbance, fluorescence, phosphorescence,

or light polarization and rotation. Optical biosensors have been the most popularity (35%) for using in detecting various foodborne pathogens biosensors (Lazcka and others 2007).

Among the optical biosensors, surface plasmon reasonance (SPR) is currently the most commonly used detection method for foodborne pathogens (Koubová and others 2001; Bokken and others 2003; Thomas and others 2006; Ricci and others 2007). The basic principle of the SPR method is based on a small change in refractive index (RI) caused by interaction of biomolecule (antigen and antibody complex) on thin biological film near the surface and the change is directly proportional to the concentration of the target analytes (Darren and others 1998; Sakai and others 1998; Oh and others 2004a; Oh and others 2004b; Oh and others 2005; Ricci and others 2007). According to study (Koubová and others 2001), the detection limit of SPR immunosensors for *Salmonella* was 10^6 cells/ mL using a gold surface immobilized with mAb. Moreover, the other study (Thomas and others 2006) showed that compared to commercial ELISA kit, SPR was able to detect mouse monoclonal antibodies against *Salmonella* in egg yolk sample with significantly higher percentage. Another study (Bokken and others 2003) showed that SPR biosensor method was able to detect 53 different *Salmonella* serovars using pAbs, and the detection limit was 1.7×10^3 CFU/10 μL of test portion. In further study, protein G was applied onto a sensor platform to improve the orientation of antibody, and the detection limit was 10^5 CFU/mL (Oh and others 2005). A study (Mazumdar and others 2007) used SPR biosensor (sandwich type) to detect *Salmonella enterica* serovar Typhimurium in milk and buffer with a detection limit of 1.25×10^5 cells/mL within 1 h.

Piezoelectric (PZ) biosensors

The principle of PZ biosensors is based on the measurement of the mass changes and physical properties on the thin layer of quartz crystal surface by binding with microorganisms and antibodies coated on quartz crystal (Ivnitski and others 1999; Ricci and others 2007). Antibodies against target microorganisms are coated on the quartz crystal surface, which is highly stable and precise oscillator. Then, the quartz crystal surface coated with antibodies is placed in a solution containing target microorganisms. As the bacteria will bind to the antibodies coated on quartz crystal surface, the mass of the crystal will increase while resonance frequency of oscillation will decrease proportionally. One approach that has received increasing attention is a quartz crystal microbalance (QCM) biosensor, which is able to detect *Salmonella* by antibodies immobilized onto a quartz crystal microbalance (QCM).

Using the QCM biosensor method, antibodies should be immobilized onto a gold coated quartz crystal surface or quartz crystal surface by various coupling methods, including biotin-avidin interaction (Prusak-Sochaczewski and Loung 1990; Prusak-Sochaczewski and others 1990; Mimunni and others 1994), glutaraldehyde cross-linking, physical adsorption, thin silane layers, antibody thiolation with protein A (Konig and Graetzel 1994) and protein G (Mimunni and others 1994), polyethylenimine-glutaraldehyde (PEG) and dithiobissuccinimidyl propionate (DSP) coupling (Katz 1990; Hermanson and others 1992), and Langmuir Blodgett (LB) (Pathirana and others 2000).

There are several studies to detect *Salmonella* using QCM biosensor. In one study, QCM biosensor could detect *S. enterica* serovar Typhimurium in meat samples at

10^5-10^6 CFU/mL, and by incorporation anti-*Salmonella*-magnetic beads as separator and concentrator for extracting *Salmonella* from the sample, QCM biosensor could lower the detection limit to 10^2 CFU/mL (Su and Li 2005). A study detected *Salmonella* in milk using QCM biosensor, where a gold coated quartz crystal surface was immobilized with antibodies by covalent cross-link with thiol, and the detection range was 1.2×10^7- 4.8×10^7 CFU/mL (Park and others 2000). Another study demonstrated that a quartz crystal surface was more successful when immobilized with antibodies by PEG rather than DSP, and the detection range for *S. enterica* serovar Typhimurium was 5.3×10^5-1.2×10^9 CFU/mL within 25 min (Jianming and others 1997).

Limitations of biosensor method

Since biosensors were introduced in 1962 by Clark and Lyons, thousands of papers have been published about the application of biosensors for detecting microorganisms. Biosensors can mainly be categorized into electrochemical, optical, and piezoelectric methods. However, SPR and QCM are more suitable for detecting microorganisms in food samples than any other biosensors due to theirs reproducibility, reliability, speed, and sensitivity (Janschoff and others 2000; Skládal 2003). Even though the specificity and sensitivity of QCM biosensor has lower than that of ELISA and SPR biosensor, the QCM biosensor has still gained attention due to its relatively inexpensiveness, simple operation, and the possibility of a portable machine (Janschoff and others 2000).

However, biosensors have their limitations in application for detection of microorganisms in food samples. For example, the fact that SPR and QCM biosensor

does not use any enzymatic labeled antibodies is an advantage over the ELISA; however, this could be a disadvantage because enzymatic label is essential for obtaining detection limits which are far below those achieve with SPR. Other limitation is that both biosensors still have non-specific bindings in food mixture on sensor platform rather than target microorganisms. Those non-specific bindings cause to increase detection signal, because both method depend on weight change on sensor platform. Another limitation regards the expensive instrument and lacks of portability due to its size, although QCM biosensor is relatively simple and less expensive than SPR. Lastly, SPR and QCM biosensor are more affected by environmental conditions than other methods and the results obtained a lower sensitivity (Ricci and others 2007).

5. Immobilization of Antibodies on Solid Supports Antibodies

Antibodies

Antibodies are produced by lymphocytes of vertebrates as defense mechanisms against the invasion of antigens such as bacteria, viruses, prions, and other foreign materials. The basic unit of antibody structure contains four polypeptide chains (Figure 5); two identical light (L) chains and two identical heavy chains (H), where each light chain binds with a heavy chain with a disulfide bond. Antibodies are divided into five major classes, including immunoglobulin G (IgG), IgA, IgM, IgE, and IgD, and each class is divided into subclasses depending on the number of disulfide bonds and the length of the hinge region of the antibodies. The most commonly used antibody in immunoassay is the

IgG class (Kindt and others 2007). The IgG is composed of the two variables, including an antigen binding fragment (Fab), and a constant fragment (Fc).

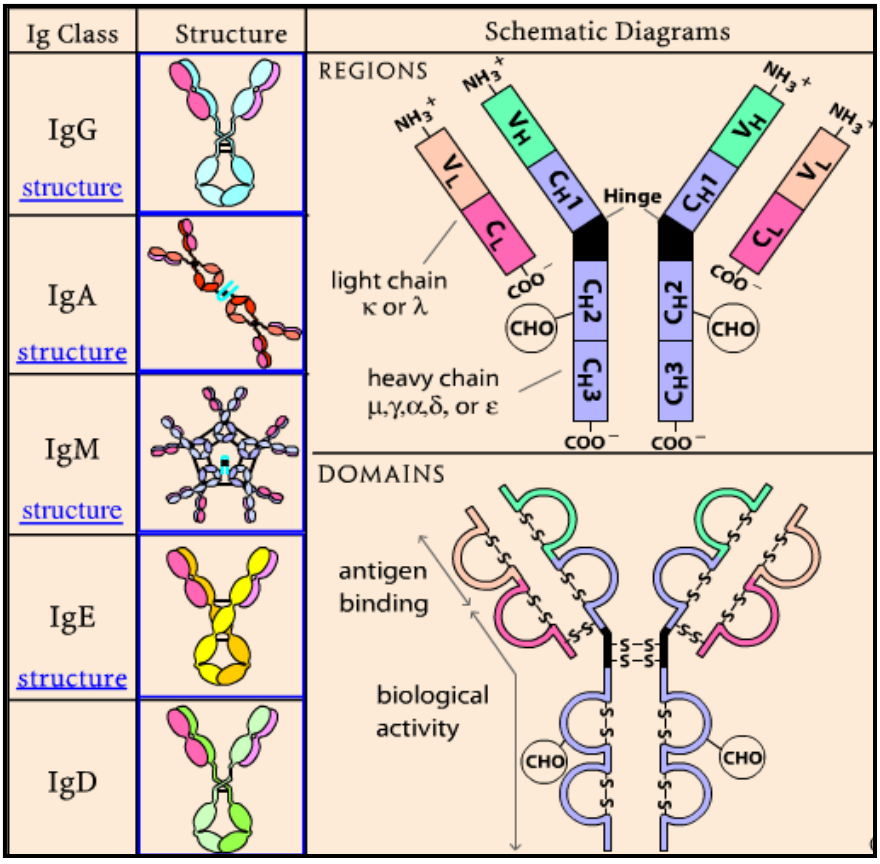

Figure 5-The structure of antibodies. (Adapted from Kindt and others 2007).

Antibodies bind to epitopes (or antigenic determinants) on antigens by intermolecular forces: hydrogen bonding, electrostatic interactions, van der Waals forces, and hydrophobic interactions (Deshpande 1996). The strength of interaction between antibody and antigen is called affinity or avidity. Affinity describes the strength of interaction between antibody and antigen at a single antigenic site whereas avidity describes the overall stability or strength between antibody and antigen at multivalent

antigenic sites. Multivalent antigen has multiple epitopes on an antigen recognized by multiple antibodies and the multivalent antigen can bind to multiple antibodies with more stabilized strength. When an antibody binds to an epitope on an antigen, it is called highly specific. In contrast, when an antibody can bind to epitopes on other antigen, it is said to have cross-reactivity. The specificity property of antibodies is widely applied in various types of immunoassays that use either monoclonal antibodies or polyclonal antibodies (Deshpande 1996).

Polyclonal antibodies (pAbs) are produced by immunizing a mammal (e.g. goat or rabbit) with antigens and extracting the antibodies from the serum. By contrast, monoclonal antibodies (mAbs) are derived from a single clone of antibody-producing B cells fused to myeloma cell, so called "hybridomas". Since pAbs are composed of a heterogenous population of antibodies with various specificity and affinity to antigens whereas mAbs have homogeneous antibodies against a single epitope or antigenic determinant, polyclonal antibodies are less specific against a target antigen than mAbs. Although the preparation of mAbs is much more complicated and difficult than that of pAbs, a powerful advantage of mAbs is to provide constant monoclonal antibodies from immortal hybridomas (Campbell 1991; Plaza and others 2000).

Antibody immobilization

The orientation of immobilized antibodies on solid sensor platforms is critical for detection sensitivity because it ensures higher antigen binding capacity. Thus, the main factor in antibody efficiency is related to specific anchoring of antibodies on solid sensor platforms with desirable antibody orientation, which means antigen binding sites in

antibodies should be free and open (Oh and others 2004b; Oh and others 2005; Jung and others 2008; Skottrup and others 2008)

There are two different immobilization methods for antibodies on solid sensor platforms: direct physical adsorption and covalent attachment (Jung and others 2008). Direct physical adsorption involves hydrophilic and/or hydrophobic interaction between antibodies and solid sensor platforms such as polystyrene, nitrocellulose, nylon and metal. Covalent attachment involves in chemical binding between antibody and chemical materials. Although direct physical adsorption immobilization is already used widely in the ELISA method due to its simple, fast and easy application, adsorbed antibodies are sometimes so randomly oriented that they may compromise their antigen binding ability. In addition, the continuous washing step in ELISA may cause antibodies leaching from the solid support due to the weak bonding of antibodies and the solid support (Butler and others 1993; Tombelli and Mascini 2000). The percent of binding rate between antigens and antibodies immobilized by the direct adsorption method was reported less than 5 to 10 % (Butler and others 1993; Cho and others 2007).

In contrast, covalent attachment of antibodies is a more stable attachment than physical adsorption due to a bifunctional cross-linker in that one functional group reacts with a solid sensor platform and another group interacts with antibody (Lundström 1994; Disley and others 1998; Zhou and Galán 2001). Moreover, covalent bonds of antibodies may allow optimizing the orientation on solid sensor platforms increasing an operational stability during analysis (Lundström 1994; Caruso and others 1996; Lu and others 1996a; Tronin and others 1996). However, there are still some problems with improper orientation of antibodies and loss of their biological activities.

In order to solve the problem, protein A, G, and their derivatives have recently been applied on solid sensor platforms to interact with Fc region of antibodies to increase the proper orientation and anchoring (Kaku and others 1989; Lu and others 1996b; Danczyk and others 2003; Jung and others 2008; Skottrup and others 2008). According to these studies, the immobilization using protein A increased antibody sensitivity 10 times more than did random immobilization (Lu and others 1996b). Another study showed that the chemically thiolated protein G gold surface increased the binding ability of antibodies with antigens (Lee and others 2005).

Protein A and G are cell wall proteins and are isolated from *Stapylococcus aureus, Streptococcus aureus*, respectively (Akerstrom and Bjorck 1986; Deshpande 1996), however their binding affinity is slightly different depending on species and antibody class. For example, protein G shows more versatile and effective binding ability for IgG in human, however it does not react with other immunoglobulins in human including IgM, D, A and E (Lu and others 1996a). On the other hand, protein A is most effective to interact with IgG in human whereas is least effective in certain animal IgGs such as goat, sheep, and cow and horse, due to their weak binding. In order to overcome the weakness, recombinant protein A/G was developed, which combines the property of protein A and G into a more efficient protein (Lu and others 1996b).

6. Application of Immunomagnetic Beads Separation

Immunomagnetic separation (IMS) involves the use of magnetic beads or magnetic colloids conjugated with a specific antibody against target microorganisms,

which can bind selectively to target microorganisms in an assay. For optimum efficiency, the antibodies should be oriented with their Fc part towards the magnetic beads and Fab part outwards from the beads.

The binding efficiency of magnetic beads is mainly associated with the size of the magnetic beads, the amounts of antibody bound to the magnetic beads, and the types of chemical ligand coupled with capturing antibodies (Che and others 2001; Tu and others 2009). According to the studies (Tu and others 2002; Tu and others 2003; Tu and others 2009), the sizes of magnetic beads ranging from 2.6 to 2.8 μm produced stronger signals than the 1 μm-beads by capturing *Salmonella* Enteritidis using an europium (Eu^{3+}) labeled secondary antibody with time-resolved fluorescence. Che and others (2001) also reported that the 2.8 μm magnetic beads showed the highest capturing ability among the magnetic beads of 1.0, 2.8, and 4.5 μm. Therefore, it was concluded that the 2.8 μm bead sizes were more effective in capturing bacteria than the smaller sized beads (1.0 μm) in the identical density. The amounts of antibodies bound to magnetic beads within the identical size of beads also affected the capturing ability of magnetic beads significantly. As the antibodies coated on the bead increased, the captured cells were increased (Che and others 2001).

There are two formats using immunomagnetic beads (IMBs) in microbial detection, direct and indirect method. In direct method, more popular method, IMB is added to food sample and incubated for 30-60 min. Then, unbound microorganisms are removed by washing and the bound microorganisms by antibodies on magnetic beads are separated from IMBs or colloids. In indirect method, magnetic beads immobilized with secondary antibodies are added to the sample that primary antibodies have been added

before. Magnetic beads immobilized with secondary antibodies bind rapidly and firmly to the primary antibodies on the target microorganisms (Šafařík and Šafaříková 1999).

Since Olsvik and others (1994) applied magnetic beads to separate microorganisms from food sample, the immunomagnetic separation (IMS) method has been used widely for detecting microorganisms (Bennett and others 1996; Himathongkham and others 2007). IMS has been combined with other methods, such as conventional method, ELISA, and PCR to detect *Salmonella* from food samples.

The combination of IMS and conventional method provided the significant increase in sensitivity and specificity for *Salmonella* detection by introducing IMS method between pre-enrichment and selective enrichment (Coleman and others 1995a; Mansfield and Forsythe 1996; Cudjoe and Krona 1997; Ripabelli and others 1997). However the replacement of IMS method for selective or non-selective enrichment process did not increase the number of positive sample (Coleman and others 1995b; Mansfield and Forsythe 1996; Ripabelli and others 1997).

The combination of IMS and ELISA method has been used to detect *Salmonella* in several studies (Cudjoe and others 1995; Holt and others 1995; Gehring and others 1996; Mansfield and Forsythe 2001). According to a study (Mansfield and Forsythe 2001), *Salmonella* inoculated in raw chicken was detected within 27 h with a sensitivity of 10^6 CFU/mL by the combination of IMS and ELISA (IMS-ELISA) method and IMS-ELISA method also recovered *Salmonella* from artificially contaminated chicken samples more frequently (12/15) than conventional RV-XLD (10/15).

The IMS has been combined with PCR method to detect *Salmonella* from food samples in several studies (Soumet and others 1997; Rijpens and others 1999; Jenikova and others 2000; Chen and others 1997; Hish and Tsen 2001; Kumar and others 2005; Notzon and others 2006; Hargen and others 2008). Jeniková and others (2000) found that IMS-PCR method could detect *Salmonella* in egg and meat samples within 24 h with the detection limit of 1-5 cells/25 g egg sample and 1-5 x 10^3 cells/25 g meat samples, respectively. In other study (Chen and Griffiths 2001), the combination of IMS and PCR method could detect *Salmonella* and *E. coli* simultaneously with 7 and 10 h enrichment from initial inoculated population of fifty-seven *Salmonella* and forty-one *E. coli* by using streptavidin–coated magnetic beads. Another study (Kumar and others 2005) reported that the IMS combined with polymerase chain reaction (IMS-PCR) was an effective method to capture *Salmonella* in inoculated meat sample when detected within 6 h with a sensitivity of 10^5 cells.

III. STATEMENT OF RESEARCH OBJECTIVES

The objective of this study was to develop a new detection method by combining a biosensor with light microscopic imaging system for detection of *Salmonella* in poultry products within 8 h.

The specific objectives for the evaluation of enrichment media and for evaluating the specificity of purified antibodies for *Salmonella* were 1) to select an optimum non-selective enrichment medium and selective enrichment medium for promoting growth of *Salmonella* from low populations to detectable levels on inoculated chicken, 2) to produce and purify the polyclonal and monoclonal antibodies against *Salmonella,* and 3) to test the binding activity for monoclonal and polyclonal antibodies and specificity for various bacteria using the indirect ELISA method.

The specific objectives for the development of an optimized gold biosensor for *Salmonella* detection by light microscopic imaging system were 1) to select an optimum sensor platform based on the binding efficiencies with *Salmonella* in pure culture, 2) to compare the binding efficiencies among the selected sensor platforms treated with purified recombinant protein A, commercial protein A, and lysine to increase antibody immobilization on selected sensor platforms, 3) to determine the optimum concentration

of polyclonal antibodies, incubation temperature and pH of buffer to enhance the binding efficiency of *Salmonella* on selected sensor platform, 4) to determine the detection sensitivity (detection limit) of optimized biosensor using light microscopic imaging system, and 5) to apply the biosensor with light microscopic imaging system to detect *Salmonella* on chicken.

The specific objectives for the application of immunomagnetic beads to capture *Salmonella* for detection using gold biosensor with light microscopic imaging system were 1) to prepare the magnetic beads coupled with ligand, 2) to optimize the reaction conditions including the concentration of polyclonal antibodies, reaction time, temperature, types of buffers and pHs for immunomagnetic beads and antibodies conjugation, and 3) to apply immunomagnetic beads to capture *Salmonella* from inoculated chicken skin for detection using the developed biosensor with light microscopic imaging system.

IV. MATERIALS AND METHODS

1. Determination of Enrichment Media and Specificity Test for Purified Antibodies

1.1. Efficiency of non-selective enrichment media and selective enrichment media

A. Bacterial strains and culture conditions

Salmonella enterica serovar Typhimurium ATCC13311 was obtained from James Barbaree at Auburn University (Auburn, AL), and *Salmonella enterica* serovar Enteritidis and Missions were provided by the Center of Food Safety, University of Georgia (Griffin, GA). All strains used in this study were resistant to 100 ppm nalicixic acid. The strains of *Salmonella* were cultivated in Trypticase® Soy Broth (TSB, Difco Laboratories, Sparks, MD). The bacterial cultures were incubated in a gyratory water bath at 37 °C at 100 rpm for 16 h. After incubation, the cultures were washed three times with phosphate buffered saline (PBS, pH 7.2) by centrifugation at 5,000 × g for 4 min. The bacterial cultures were re-suspended in PBS and the bacterial populations were adjusted to 10^9 CFU/mL, based on the absorbance of the bacterial suspension at $O.D._{640\ nm}$ using a pre-constructed standard curve. The adjusted bacterial culture was further diluted to 1,000 CFU/mL.

B. Preparation of non-selective and selective enrichment media

Buffered peptone water (BPW, EMD Science, Darmstadt, Germany), lactose broth (LB, EMD Science, Darmstadt, Germany), brain heart infusion broth (BHI, EMD Science, Darmstadt, Germany), universal pre-enrichment broth (UPB, Difco Laboratories, MD), nutrient broth (NB, EMD Science, Darmstadt, Germany), tryptic soy broth (TSB, Difco Laboratories, Sparks, MD), and salmoyst (EMD Science, Darmstadt, Germany) were prepared as pre-enrichment media as per the manufacturers′ recommendations. Brilliant green broth (BG, Difco Laboratories, Sparks, MD), rappaport-vassiliadis R 10 broth (RV, Difco Laboratories, Sparks, MD), selenite cystine broth (SC, Difco Laboratories, Sparks, MD), selenite broth (SB, Difco Laboratories, Sparks, MD), and tetrathionate brilliant green (TBG, Difco Laboratories, Sparks, MD) were prepared as selective enrichment media.

C. Efficiency of bacterial recovery for non-selective and selective enrichment media from inoculated chicken skin

Chicken skins were randomly collected from Koch Food Company (Montgomery, Al) and cut into 4×4 in^2. Aliquots of 200 μL of *Salmonella* cocktail at selective populations (1,000 CFU/200 μL) were inoculated by spreading onto chicken skins. The chicken skins were held 30 min to allow for bacterial attachment. After drying, each inoculated chicken skin was put into a sterile stomach bag containing 100 mL of non-selective enrichment medium or selective medium broth, blended in a Seward 400 circulator stomacher (Seward Company, Seward, England) at 260 rpm for 2 min, and transferred to an Erlenmeyer flask for incubating at 37 °C in an orbital shaker (250 rpm).

The bacterial populations of each sample were determined by the spread-plate method using Tryptic soy agar (TSA) plates containing 100 ppm nalidixic acid at 2, 4, and 6 h incubation. The bacterial population was recorded as log colony forming units (CFU)/16 in^2 chicken skin for data analysis. The media with higher bacterial populations were chosen for bacterial enrichment on *Salmonella* inoculated chicken skin.

D. Efficiency of *Salmonella* recovery at various populations on BHI, TSB and BG enrichment media

For determining the optimum enrichment medium, the recovery efficiency was studied at various populations of *Salmonella* using three enrichment media. Chicken skins were inoculated with *Salmonella* at 10, 50, 100, 500, and 1,000 CFU/16 in^2 chicken skins. After drying for 30 min, each inoculated chicken skin was put into a sterile stomach bag containing 100 mL of BHI, TSB and BG enrichment broth, blended in a Seward 400 circulator stomacher at 260 rpm for 2 min, and then transferred to an Erlenmeyer flask for incubating at 37 °C in an orbital shaker at 250 rpm. The bacterial populations were determined after initial incubation at 2, 4, and 6 h by the spread-plate method on TSA plates containing 100 ppm nalidixic acid.

E. Effect of chilling on recovery of *Salmonella* from inoculated chicken skin using BHI, TSB and BG enrichment media

Prior to determine the optimum enrichment media for further study, chilling effect on *Salmonella* was studied using selected three enrichment media. Two hundred μL aliquots containing 10, 50, 100, 500, and 1,000 CFU *Salmonella* cocktail, respectively, were inoculated on 16 in^2 chicken skin per sample. After drying for 30 min, each

inoculated chicken skin was put into a sterile stomacher bag containing 100 mL of BHI, TSB and BG enrichment broth, blended in a Seward 400 circulator stomacher at 260 rpm for 2 min, and placed in a refrigerator at 4 °C for 48 h. Then, the sample was transferred an Erlenmeyer flask and was incubated at 37 °C in an orbital shaker at 250 rpm. The bacterial populations in the sample were determined by the spread-plate using TSA plates containing 100 ppm nalidixic acid at 2, 4, and 6 h incubations, and the results were recorded as log (CFU)/16 in^2 chicken skin for analysis. The medium that promoted the most growth of *Salmonella* was chosen as the bacterial enrichment medium for further study.

1.2. Purification of monoclonal and polyclonal antibodies and specificity test using ELISA

A. Purification of monoclonal and polyclonal antibodies

The polyclonal antibody (#48) was produced from a white rabbit (New Zealand) against *Salmonlella enterica* serovar Typhimurium. The white rabbit was immunized and boosted with 1% formalin inactivated bacteria at 10^8 cells at a time in RAS-R730 (Corixa, Hamilton, MT) adjuvent by intradermal injection for the production of antibodies. Rabbit blood was collected after 7 days, centrifuged at 5,000 × g for 20 min, and the collected serum was used for antibody purification. The monoclonal antibody (IB4) was produced from a BALB/cAnNHsd female mouse (Harlan Sprague Dawley Inc., Indianapolis, IN) in the Hybridoma Laboratory, Auburn University.

The purification procedures of polyclonal antibodies and monoclonal antibodies were followed by the modified protocols from Kohler and Milstein (1975). The collected

anti-*Salmonella* rabbit or mouse immunoglobulins (IgGs) were purified by two main steps including saturated ammonium sulfate precipitation and protein A affinity column (Sigma Chemical Co., St. Louis, MO) separation.

For saturated ammonium sulfate precipitation, ammonium sulfate (Fisher Scientific, NJ) was added very slowly into rabbit serum to 50% (w/v) with stirring to dissolve the chemical completely. After placing the sample in a refrigerator for 30 min, it was centrifuged at 10,000 × g for 30 min. Then, the precipitate was re-suspended and dialyzed in a 20 mM sodium phosphate buffer (pH 7.0) at 4 °C overnight.

Further purification was carried out using a protein A affinity column. Citric acid (0.1 M, pH 3.45) was used to elute IgGs from the protein A column and the eluted IgG solution was immediately neutralized with 1 M Tris buffer. The purity of purified antibodies was determined by sodium dodecyl sufate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified IgGs were stored at -80ºC in a buffer (pH 8.0) consisting of 0.1 M Tris, 2 mM $MgCl_2$, 20 mM Glycine, and 30 mM sodium azide for future study.

B. Reactivity test for monoclonal and polyclonal antibodies and specificity test for polyclonal antibodies using indirect ELISA

B.1. Preparation of bacterial cultures

Salmonella spp. (other than three nalidixic resistant strains), two strains of *Esherichia coli*, two strains of *Staphylococcus aureus,* and *Listeria monocytogenes* were obtained from Salmonella Genetic Stock Center (Canada), the center for Food Safety, University of Georgia (Griffin, GA), and the American Type Culture Collection (ATCC, Rockville, MD), respectively. The *Salmonella* spp., *E. coli*, and *S. aureus* were

cultivated in TSB; while *L. monocytogenes* was cultivated in TSB with 0.6% yeast extract (TSBYE). The bacterial cultures were incubated in a gyratory water bath at 37 °C at 100 rpm for 16 h. After incubation, the cultures were washed three times with PBS (pH 7.2) by centrifugation at 5,000 × g for 4 min. The bacterial cultures were re-suspended in PBS, and the bacterial populations were adjusted to 10^9 CFU/mL, based on the absorbance at $O.D._{640\ nm}$ using a pre-constructed standard curve.

B.2. Reactivity test for monoclonal and polyclonal antibodies and specificity test for polyclonal antibodies using indirect ELISA

A cocktail of nalidixic acid resistant *Salmonella* (*S*. Thyphimurium, *S*. Enteritidis, and *S*. Missions) was used for the reactivity test for monoclonal antibodies and polyclonal antibodies. The rest of the *Salmonella* spp., *L. monocytogenes*, *E. coli*, and *S. aureus* were used for specificity testing for polyclonal antibodies using indirect ELISA. A 96-well polystyrene assay plate (Costar, Cambridge, MA) was coated with 100 µL of bacterial suspension (1×10^9 CFU/mL) at 37 °C for 1 h. After washing three times with 200 µL PBS containing 0.1% Tween-20 and 0.02% sodium azide (PBST), the plate was blocked with 200 µL of 1% bovine serum albumin (BSA, Equitech-Bio Inc., Kerrvill, TX) for 1 h at room temperature. After washing three times with 200 µL PBST, 100 µL anti-*Salmonella* antibodies (monoclonal or polyclonal antibodies) were added to each well, either serially diluted anti-*Salmonella* antibodies for the reactivity test or 1/800 diluted anti-*Salmonella* antibodies for specificity test. Then, the plate was incubated at room temperature for 2 h and the plate was washed three times with PBS. Next, 100 µL of alkaline phosphatase conjugated secondary antibody diluted at 1/3,000 in PBS was added

to each well, either anti-mouse goat IgG (Sigma-Aldrich Inc., MO) for monoclonal antibodies or anti-rabbit goat IgG (Sigma-Aldrich Inc.) for polyclonal antibodies. The plate was incubated at room temperature for 1 h and washed four times with PBS. Finally, *p*-nitrophenyl phosphate (*p*-npp, Sigma Chemical Co.) in 10 mM diethanolamine buffer (pH 9.5) containing 0.5 mM $MgCl_2$ was added to the wells as a substrate for alkaline phosphatase for color development and the absorbance at $O.D._{405\ nm}$ was measured using a microplate reader (ThermoLabsystems, Helsinki, Finland). The plate was incubated further in the dark at room temperature for 15 min and then the absorbance at $O.D._{405\ nm}$ was measured using a microplate reader. The result was expressed by difference of absorbance between 0 min and 15 min. The antibodies showing higher absorbance difference in the reactivity test were chosen as the primary antibodies for further studies and tested for specificity against various bacteria.

2. Development of Gold Biosensor with Light Microscopic Imaging System for *Salmonella* Detection

2.1. Binding efficiency of gold, polystyrene, glass, and polyvinyl chloride sensor platforms

A. Preparation of selected types of sensor platforms

Glass, gold, polystyrene and polyvinyl chloride were used for sensor platforms. A glass sensor platform was prepared by a cutting microscopic cover glass (0.17 mm thickness) into 5 × 5 mm with micro-dicing saw (MPE INC., Grass valley, CA). After

cutting the microscopic cover glass, the glass was ultrasonically cleaned in an acetone solution and rinsed with alcohol and deionized water. For the gold sensor platform, the clean glass (5 × 5 mm) was sputtered with 140 nm gold by a Pelco SC-6 sputter coater (Ted Pella Inc., Redding, CA). The polystyrene sensor platform was prepared by cutting a flat polystyrene plate (Costar, Cambridge, MA) to 5 × 5 mm. The polyvinyl chloride sensor platform was prepared by cutting the bottom of a microplate (Whatman Inc., Clifton, NY) to 5 × 5 mm.

B. Preparation of antibody immobilized sensor platform

In this study, anti-*Salmonella* polyclonal antibody (rabbit IgG) was used. One hundred μL of purified polyclonal antibodies (6.5 mg/mL) were added onto selected types of sensor platforms including gold, polystyrene, polyvinyl chloride and glass in a 96-well plate (Whatman Inc., Clifton, NY), and the plate was incubated at 22 °C for 2 h. After washing three times with PBS, the sensors were blocked with 100 μL of 1% bovine serum albumin (BSA, Equitech-Bio Inc., Kerrvill, TX) at 22 °C for 30 min. Finally, the sensors were washed three times with PBS and air dried for *Salmonella* immobilization studies.

C. Detection of *Salmonella* on biosensor using light microscopic imaging system

The sensors immobilized with pAbs were incubated with *Salmonella* cocktail at 22 °C for 1 h and washed four times with deionized (DI) water. After binding to the *Salmonella* cocktail, the biosensor was dried at room temperature and fixed with OsO_4 for at least 1 h. The bacteria bound on the biosensor were observed and photographed with a Nikon Eclipse L 150 Industrial light microscope (Nikon Instruments Inc., Melville, NY)

at 1,000 times magnification. The numbers of *Salmonella* on the biosensor were recorded for data analysis.

2.2. Binding efficiency of gold and polystyrene biosensors untreated or treated with lysine, recombinant protein A, commercial protein A

A. Overexpression of recombinant protein A from *E. coli* and purification of recombinant protein A using DEAE column

To produce recombinant Staphylococcal Protein A (SPA), *spa* coding sequences were inserted with plasmid pET20b and transformed into DE3 strains of *Escherichia coli*. The *E. coli* culture were grown in 2 L of ZYM-5052 medium (pH 6.6) containing 1% tryptone, 0.5% yeast extract, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.5% glycerol, 0.05% glucose, 0.2% lactose, trace mineral mixtures (10 μM $FeCl_3$, 4 μM $CaCl_2$, 2.5 μM $MnCl_2$, 2.5 μM $ZnSO_4$, and 0.40 μM each of $CoCl_2$, $CuSO_4$, $NiCl_2$, Na_2MnO_4, Na_2SeO_3, and H_3O_3), and ampicillin (50 μg/mL) at 37 °C with 250 rpm shaking and continuous aeration overnight. The culture was centrifuged at 5,000 × g for 20 min, and the bacterial pellet was suspended in 10 mM Tris buffer (pH 7.5) containing 10 mM EDTA. Then, the pellet suspension was centrifuged at 12,000 × g for 2 min, and the pellet was re-suspended in 10 mL of 10 mM Tris buffer (pH 7.5). Next, 8 M urea was added to the pellet suspension to lyse bacteria and the lysate was centrifuged at 25,000 × g for 20 min. The supernatant was collected and mixed with DEAE-cellulose in a beaker. The mixture was packed in a column for the further purification of SPA.

The column was washed with ten bed volumes of 10 mM Tris (pH 6.8) and equilibrated with five bed volume of 10 mM Tris (pH 7.2). The recombinant protein A was eluted with 50 mM Tris buffer (pH 7.2) containing 0.5 M NaCl. The protein content of each fraction was measured by Lowery assay and the protein was confirmed by SDS-PAGE in 12% gel. Fractions with SPA content were pooled and imidazole was added at the final concentration of 5 mM for further purification.

Each 50 mg of SPA were mixed with 1 ml of CM-cobalt chelated agarose beads (Talon, BD Biosciences) for 2 h. After centrifugation at 700 × g for 3 min, the beads were washed twice with washing buffer (50 mM Tris, 100 mM NaCl, 5 mM imidazole pH 7.0) and packed into a column. The column was washed with 2 bed volumes of washing buffer and the SPA was eluted with the elution buffer (50 mM Tris, 100 mM NaCl, 500 mM imidazole pH 7.8). The protein content of each fraction was measured by Lowery assay and was confirmed by SDS-PAGE in 12% gel. Fractions with pure SPA were pooled and dialyzed in 20 mM phosphate buffer (pH 7.0) at 4 °C overnight with two changes of buffer. After centrifuged at 10,000 × g for 30 min, the SPA in the supernatant was collected and the protein content was measured by Lowery assay. The protein A was ready for use.

B. Preparation of polystyrene and gold sensors treated with protein A and commercial protein A

In the study, the recombinant protein A, purified from our lab and commercial protein A (Sigma Chemical Co. St Louis, MO) were used. The immobilization protocol on sensors was performed following Babacan and other's method (2000) with minor

modifications. Gold and polystyrene sensors were prepared by the method described above. Aliquots of 10 µL purified recombinant protein A (3.0 mg/mL) and 30 µL commercial protein A (1 mg/mL) in 20 mM phosphate buffer (pH 7.4) were applied to gold and polystyrene sensors. For optimizing the adsorption of protein A on sensors, the same ratio of 0.1 M sodium-acetate buffer (pH 4.69) compared to the amount of protein A was added immediately to sensors for adjusting the pH to 5.95. The sensors were incubated at 22 °C for 2 h. The gold and polystyrene sensors treated with protein A or commercial protein A were washed gently with PBS and stored at 4 °C until further study.

C. Preparation of polystyrene and gold sensors treated with poly-L-Lysine

One mL of Poly-L-Lysine (Sigma Chemical Co., St. Louis, MO) solution was mixed with 9 mL DI water in a Petridish and the polystyrene and gold sensors were placed in the diluted Poly-L-Lysine solution for 10 min with gentle agitation using an orbital shaker (50 rpm). The treated sensors were then dried at room temperature for future use.

D. Binding efficiencies of polystyrene and gold sensors untreated or treated with recombinant protein A, commercial protein A and lysine

Regular polystyrene and gold sensors and polystyrene and gold sensors treated with lysine, recombinant protein A, and commercial protein A were used for the bacterial binding efficiency test. One hundred µL of purified pAbs was added onto each sensor in 96-well plate and the sensors were incubated at 22 °C for 2 h. After washing 3 times with PBS, the sensors were blocked by adding 100 µL of 1% BSA and incubated at 22 °C

for 30 min. Then, the sensors were washed three times with PBS and dried for testing of bacterial binding efficiency.

The sensors were applied with 100 µL *Salmonella* cocktail in PBS (pH 7.4) at 22 °C for 1 h, washed 4 times with DI water, and dried at room temperature. Then, the biosensors were fixed with OsO_4 for at least 1 h and the bound bacteria were photographed with a Nikon Eclipse L 150 Industrial light microscope at 1,000 times magnification. The number of *Salmonella* bound on the biosensor was recorded for analysis. The sensor captured with higher population number of *Salmonella* was chosen as the optimum sensor platform.

2.3. Determination of optimum concentration of antibody, incubation temperatures, and pHs of PBS buffer for the gold biosensor detection

A. The effect of concentration of monoclonal and polyclonal antibodies on the binding efficiency of the gold biosensor

An aliquot of either 100 µL pAbs or mAbs (10, 25, 50, 75, 100, 150, 200, 300 µg/mL PBS) was applied to gold sensors and the gold sensors were incubated at 22 °C for 2 h. After washing 3 times with PBS (pH 7.4), 100 µL of 1% BSA was added to block the gold sensors at 22 °C for 30 min. Then, the gold sensors were washed gently three times with PBS (pH 7.4) and dried for immediate use. An aliquot of 100 µL *Salmonella* cocktail was added to the antibody immobilized sensors at 22 °C for 1 h, washed 4 times with DI water, and dried at room temperature. Then, the biosensors were fixed with OsO_4 and the captured bacteria were photographed with a Nikon Eclipse L 150

Industrial light microscope at 1,000 times magnification. The optimal concentration of antibodies for immobilization was the one which had the highest bacterial number.

B. The effect of incubation temperatures on the binding efficiency of the gold biosensor

The gold sensor chip was immobilized with 100 µL pAbs following a method described previously (2.3.A). After immobilized with pAbs, an aliquot of 100 µL of *Salmonella* cocktail in PBS buffer (pH 7.4) was applied onto gold sensor chips and the chips were incubated at 4, 15, 22, 30, 37 and 45 °C for 1 h and washed gently four times with DI water. After drying, the gold biosensor was fixed with OsO_4 at room temperature for 1 h. The bacteria bound on the gold biosensor were counted and photographed with a Nikon Eclipse L 150 Industrial light microscope at 1,000 times magnification.

C. The effect of pHs of PBS on the binding efficiency of the gold biosensor

One hundred µL of pAbs was immobilized on gold biosensor by following the procedures mentioned above. Then, an aliquot of 100 µL *Salmonella* cocktail in PBS at pH of 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.2, 10.2, 11.2, and 12.0 were applied onto gold sensors and the sensors were incubated at 30 °C for 1 h. The gold biosensors were washed four times with DI water and dried at room temperature. The bacteria on the sensors were counted and photographed with a Nikon Eclipse L 150 Industrial light microscope at 1,000 times magnification. An optimal pH for bacterial binding on antibody immobilized gold sensor was the one which had highest bacterial number.

2.4. The detection sensitivity (detection limit) of the optimized gold biosensor with light microscopic imaging system

To investigate the detection limit (sensitivity) of the gold biosensor with light microscopic imaging system, the *Salmonella* cocktail cultured in TSB was used. The gold sensor was immobilized with 100 μL pAbs following a method described previously (2.3.A). After immobilized with pAbs, an aliquot of 100 μL *Salmonella* cocktail at different populations (10^1-10^9 CFU/100 μL) in PBS buffer (pH 7.0-8.0) was applied to the gold sensor and the sensor was incubated at 30 °C for 1 h and washed gently four times with DI water. After drying, the gold biosensor was fixed with OsO_4 at room temperature for 1 h. The bacteria bound on the gold biosensor were counted and photographed with a Nikon Eclipse L 150 Industrial light microscope at 1,000 times magnification.

2.5. Application of the gold biosensor with light microscopic imaging system to detect *Salmonella* on inoculated chicken skin

Chicken skins were randomly collected from the Koch Food Company (Montgomery, AL) and cut into 4×4 in^2. A 200 μL aliquot of *Salmonella* cocktail in PBS buffer (pH 7.4), including 100 ppm nalidixic acid-resistant cultures of *Salmonella enterica* serovar Thyphimurium, Enteritidis, and Missions, was spread onto the chicken skin at designed populations and the chicken skin was dried at room temperature to allow for bacterial attachment. The inoculated chicken skin was then put into a sterile stomach bag containing 100 mL BHI (non-selective medium) or BG (selective medium) and blended in a Seward 400 circulator stomacher at 260 rpm for 2 min. The

homogenized mixture was transferred to an Erlenmeyer flask for incubating at 37 °C in orbital shaker (250 rpm). Then, a 20 mL aliquot was taken at 2, 4, and 6 h during incubation and the 100 μL aliquots were spread on triplicate TSA plates containing 100 ppm nalidixic acid for determining the bacterial population of the *Salmonella* cocktail.

The entire 20 ml samples taken from the BG and BHI media were filtered, washed three times by centrifugation at 4,000 × g for 10 min, and re-suspended in 1 mL PBS buffer. An aliquot of 100 μL centrifuged chicken skin sample was applied onto the gold biosensors immobilized with pAbs and the gold biosensors were incubated at 30 °C for 1 h. Then the gold biosensors were washed four times with DI water and dried at room temperature. The bound bacteria were counted and photographed with a Nikon Eclipse L 150 Industrial light microscope with 1,000 times magnification. Each image taken from the microscope represented 0.013 mm^2 among 25 mm^2 of a gold biosensor, because each image showed one 1,900th of a gold biosensor. The number of captured bacteria for each sensor was obtained from at least 10 fields under the microscope per one gold biosensor and the data were analyzed by ANOVA.

3. Application of Immunomagnetic Beads to the Gold Biosensor with Light Microscopic Imaging System for *Salmonella* Detection

3.1. Preparation of magnetic beads coupled with ligand

A. Preparation of magnetic beads

Magnetic beads (magnetite,Fe_3O_4) was synthesized following Sugimato's protocol with modifications (Taylor and others 2000). A 4.5 L of 0.42 M Iron (II) sulfate-heptahydrate ($FeSO_4$-$6H_2O$) was degassed and put in an Erlenmeyer flask and sealed with a stopper fitted with a thermometer, a plastic propeller stick and a nitrogen gas inlet. The Erlenmeyer flask was heated on a hot plate with aerating nitrogen gas while operating the propeller stick. Once the iron (II) solution reached at 95 °C, degassed 1.5 L of 0.8 M potassium nitrate and 3.4 M potassium hydroxide solution (65 °C) were added to the Erlenmeyer flask. The mixture was heated to reach 92-96 °C and maintained for further 1 h with continuous stirring and constant purging with nitrogen gas. The mixture was allowed to cool to room temperature and it was placed on a 4 × 4 × ¼ in (N42 strength) neodymium magnetic plate for 10 min in order to settle down and form black magnetite beads. The supernatant was removed and the precipitated black magnetite beads were washed several times with deionized (DI) water until the pH of supernatant was close to 7.0. Then, the formed black magnetic beads were examined to measure their sizes and shapes using transmitting electron microscopy (TEM) and stored in DI water for further study.

B. Coating the magnetic beads with sodium silicate

Beads were coated with sodium silicate following the methods developed by Taylor and others (2000). One hundred grams of sodium silicate (40-42˚ Bé) were dissolved in 1 L DI water and 800 mL of sodium silicate solution was transferred to new beaker. Fifty grams of Dowex-50 (Dow Chemical Company, Midland, Michigan) regenerated in 500 mL of 1.0 M hydrochloric acid was added slowly to 800 mL sodium silicate solution with gentle stirring. The mixture of Dowex-50 resins and sodium silicate

solution was filtered to remove the resins, and the pH of the filtered sodium silicate suspension was adjusted to 9.5 using 200 mL of unfiltered sodium silicate solution. The filtered sodium silicate suspension was added to the magnetic beads with stirring. Then, 100 mL of 1.0 M tetramethylammonium hydroxide (TMA) was added to the mixture of filtered sodium silicate and magnetic beads and maintained with gentle stirring for 1 h, while the mixture was adjusted pH 10.0 using 0.5 M hydrochloric acid. After continuous stirring for another 2 h, the coated beads were washed with DI water until the pH of the supernatant was neutralized. To examine the coating efficiency, the 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 Eppendorf tubes up to 2 h.

C. Coupling the coated magnetic beads with ligand

Coated magnetic beads (100 mL bed volume) were suspended in 1 L of 95% ethanol and 10 mL of 3-Aminopropyl-trimethoxysilane (APTMOS) was added to accomplish coupling a ammonium (NH^{3+}) to the coated magnetic beads. The mixture was stirred at room temperature for 2 h and placed a hot water bath at 70 °C for 10 min. Then, the coupled magnetic beads were washed with ethanol and DI water twice, respectively. The NH^{3+} coupled magnetic beads were re-suspended in 800 mL of 10 mM pyridine-NaOH buffer to couple with glutaraldehyde (12-carbon cross-link agent).

Two hundred mL of 25% glutaraldehyde were added to introduce an aldehyde group to the ammonium group coupled on the magnetic beads. After coupling with aldehyde for 2 h, the coupled magnetic beads were washed with DI water until the pH was neutralized and re-suspended in 1 L of ethanol. To block the free ammonium groups

on the coupled magnetic beads, acetate anhydride (10%) was added to the solution of coupled magnetic bead and ethanol and the mixture was stirred continuously at room temperature for 30 min. Finally, the coupled magnetic beads with ligand (MBs) were washed with DI water and stored at 4 °C for further study.

3.2. Optimization of reaction condition for using immunomagnetic beads

A. Determination of optimum concentration of pAbs for conjugating with magnetic beads

To determine the optimum concentration of polyclonal antibodies to be immobilized covalently onto the coupled magnetic beads (MBs), various concentrations of polyclonal antibodies (0.022, 0.11, 0.22, 0.33, 0.44, 0.55, 0.66 and 0.77 mg per mL MBs) were used. To optimize the conjugation reaction, MBs were equilibrated several times with coupling buffer (1 mL of 2 M sodium cyanoborohydride stock solution and 100 mL of 0.2 M disodium phosphate buffer). One mL of MBs was reacted with various concentrations of polyclonal antibodies at room temperature on a rotary shaker for 2 h to form the antibody-conjugated MBs. The excess antibodies were removed by washing several times with coupling buffer. To block the free aldehyde group on antibody-conjugated MBs, an appropriate ethanolamine (12 mg/mL antibodies-conjugated MBs) and 1 mL PBS buffer were added. The blocking reaction was maintained at room temperature for 1 h, and the mixture was washed several times with PBS buffer (pH 7.4). Then, 1% bovine serum albumin (BSA, Equitech-Bio Inc., Kerrvill, TX) in PBS (1 mL/mL antibody-conjugated MBs) was added to block the non-specific binding between the extra site on the MBs and *Salmonella* in the subsequent immunoassay. The blocking

reaction was maintained with gentle shaking at room temperature for 1 h. The mixture was washed three times with PBS buffer and antibody-conjugated magnetic beads (IMBs) were prepared.

An aliquot of 100 µL IMBs was transferred into an Eppendorf tube, and 1 mL of PBS (pH 7.4) was added. An aliquot of 100 µL *Salmonella* cocktail (2.0×10^9 CFU/mL) previously cultured and washed was added to IMBs and incubated with continuous shaking at room temperature for 1 h. The IMBs were settled down using a magnet and the supernatant was removed to waste. To remove unbound *Salmonella* from IMBs, 1 mL PBS buffer (pH 7.4) was re-suspended into IMBs with gentle shaking for 20 s and the IMBs suspension was then transferred to a new Eppendorf tube. The IMBs were settled down using a magnet to remove the supernatant and this step was repeated. The bound *Salmonella* was eluted from the IMBs by adding 850 µL of 0.1 M citric acid (pH 3.8) with vigorous vortexing. After 2 min, the supernatant was transferred to a new Eppendorf tube containing 150 µL of 1 M Tris buffer (PH 10.7). The entire separation of *Salmonella* from IMBs was repeated. Finally, 100 µL of the separated *Salmonella* was spread on triplicate TSA plates containing 100 ppm nalidixic acid. The plates were incubated at 37 °C for 24 h, and the numbers of *Salmonella* colonies were recorded for analysis.

B. Determination of optimum time, temperature, buffer type at various pHs for capturing *Salmonella*

The MBs were conjugated at a concentration of 0.44 mg per mL following the method mentioned above. In order to optimize the reaction time and temperature for

IMBs to *Salmonella* cocktail, various incubation times (5, 10, 20, 30, 40, 50 and 60 min) and reaction temperatures (4, 15, 22, 30, 37 and 45 °C) were compared. Then, PBS and TBS buffers were compared to optimize buffer type at various pH values (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.2, 10.2 and 11.2). An aliquot of 100 µL IMBs mixed with 100 µL of *Salmonella* cocktail in PBS (pH 7.4) either at room temperature for a various times or at various temperatures for 20 min. For comparing buffer types and pHs, 100 µL IMBs were mixed with 100 µL of *Salmonella* cocktail at various pH values in TSB or PSB at 30 °C for 20 min. After incubation, the IMBs were settled using a magnet and washed with PBS buffer (pH 7.4) in order to remove unbound *Salmonella* from the IMBs. Then, bound *Salmonella* were eluted from IMBs using 0.1 M citric acid (pH 3.8) and neutralized by 1 M Tris buffer (pH 10.7). After repeating the entire separation process, 100 µL of the bacterial suspension was spread on triplicate Tryptic soy agar (TSA) plates containing 100 ppm nalidixic acid for incubation to enumerate bacterial colonies.

C. Determination of the quantity of immunomagnetic beads required for capturing various populations of *Salmonella* cocktail

Various quantities of IMBs were compared to determine the efficient quantities of IMBs required for capturing various populations of *Salmonella* cocktail. The IMBs (25, 50, 75, 100, 125, 150, 175 and 200 µL) were mixed with 100 µL of various populations of *Salmonella* cocktail (10^1, 10^3, 10^5, 10^7 CFU/100 µL *Salmonella* cocktail) at 30 °C for 20 min. The IMBs were settled using a magnet and washed with PBS buffer (pH 7.4) in order to remove unbound *Salmonella* from the IMBs. The *Salmonella* bound to the IMBs were eluted from the IMBs using 0.1 M citric acid (pH 3.8) and neutralized by 1 M Tris buffer (pH 10.7). After repeating the entire separation process, 100 µL of the bacterial

suspension was spread on triplicate Tryptic soy agar (TSA) plates containing 100 ppm nalidixic acid for incubation to enumerate bacterial colonies.

3.3 Application of immunomagnetic beads to capture *Salmonella* on chicken skin inoculated using a gold biosensor with light microscopic imaging system

Chicken skin (16 in^2) inoculated at 10^2 and 10^3 CFU of *Salmonella* cocktail was enriched in BHI medium following the method mentioned above (2.5). Twenty mL of the BHI mediums were randomly selected at 2 h intervals during incubation at 37 °C. The entire 20 mL sample was filtered through glass wool, washed three times by centrifugation at 4,000 × g for 10 min, and re-suspended with 1 mL PBS buffer (pH 7.4). An aliquot of 1 mL sample was reacted with the optimum quantity of IMBs (50 µL IMBs for 2 h sample, 100 µL IMBs for 4 h sample, and 200 µL IMBs for 6 h sample) at 30 °C for 20 min. Then, *Salmonella* bound on the IMBs were separated by 0.1 M citric acid (pH 3.8) and neutralized by 1 M Tris buffer (pH 10.7), and the entire separation process was repeated.

An aliquot of 100 µL of suspension containing *Salmonella* eluted from IMBs was applied onto the gold biosensors immobilized with pAbs, and the gold biosensors were incubated at 30 °C for 1 h. Then, the gold biosensor were washed four times with DI water and dried at room temperature. Finally, the gold biosensors were fixed with OsO_4 for at least 1 h and photographed with a Nikon Eclipse L 150 Industrial light microscope at 1,000 times magnification. The number of captured bacteria on the gold biosensors was recorded for analysis.

V. RESULTS AND DISCUSSION

1. Determination of Enrichment Media and Specificity Test for Purified Antibodies

1.1. Efficiency of non-selective and selective enrichment media

A. Comparison of non-selective enrichment media

The bacterial population of *Salmonella* enriched in a series of non-selective enrichment media including TSB, BHI, LB, NB, UPB, BPW and salmoyst media were counted at 2, 4, and 6 h, and compared for the selection of the most efficient enrichment medium (Figure 6). Among the seven non-selective enrichment media, BHI and TSB were chosen as the most effective media based on the results. BHI increased the population of *Salmonella* from 3 logS (=$\log_{10}10^3$ CFU/16 in^2 chicken skin) to 7.3 logS (=$\log_{10}2.0\times10^7$ CFU/16 in^2 chicken skin) and 7.1 logS (=$\log_{10}1.3\times10^7$ CFU/16 in^2 chicken skin) for TSB within 6 h.

The population of *Salmonella* cocktail enriched in BHI, TSB, and LB were significantly higher than that enriched in salmoyst ($p < 0.05$), and the population of *Salmonella* cocktail enriched in BHI was significantly higher than the population enriched in NB or UPB ($p < 0.05$). The bacterial populations enriched in TSB, BHI,

BPW and LB did not demonstrate any significant differences among the media. However, since the bacterial populations enriched in BHI and TSB were higher than the populations enriched from other media within 6 h, BHI and TSB were selected as the efficient non-selective media for *Salmonella* enrichment for further study.

Boer (1998) selected TSB and BPW as enrichment media for *Salmonella* in typical food samples, although some food samples require specific media. The results in this study are in good agreement with their results that TSB, BHI and BPW exhibited significantly higher populations than those from other media. Stephenson and others (1991) also selected TSB as the most efficient enrichment medium for *S. enterica* serovar Enteritidis from artificially contaminated egg yolk among the tested five media including LB, TSB, BPW, and two modified LB. Since TSB and BHI contain similar nutrient composition such as casein, glucose, disodium phosphate and sodium chloride, except the meat substrate in BHI and soybean in TSB, the bacterial populations enriched in both media did not show significant differences. Although UPB also possesses a similar nutrient composition to TSB and BHI, except magnesium sulfate, the bacterial numbers enriched in UPB were significantly lower than the numbers from TSB or BHI, presumably due to the lower pH of UPB (pH 6.3) compared to TSB or BHI (pH 7.3).

B. Comparison of selective enrichment media

A series of selective enrichment media including BG, SC, SB, RV and TBG were compared to determine the most efficient medium for *Salmonella* enrichment at low population inoculated chicken skin. The bacterial populations of *Salmonella* enriched in selective media are summarized in Figure 7. The bacterial populations in BG were

significantly higher than the bacterial populations in SC (5.5 logS = $\log_{10}2.9\times10^5$ CFU/16 in^2 chicken skin), SB (5.5 logS = $\log_{10}3.1\times10^5$ CFU/16 in^2 chicken skin) or TBG (4.4 logS = $\log_{10}2.5\times10^4$ CFU/16 in^2 chicken skin) ($p < 0.05$). Although the bacterial population in RV (5.9 logS = $\log_{10}9.7\times10^5$ CFU/16 in^2 chicken skin) was not significantly different from the bacterial population of BG; the bacterial population in BG exhibited the highest number among the five selective media. Therefore, BG was selected as the most effective medium, resulting in an increase of the bacterial population from approximately 3 logS (= $\log_{10}10^3$ CFU/16 in^2 chicken skin) to 6.4 logS (= $\log_{10}2.7\times10^6$ CFU /16 in^2 chicken skin) within 6 h-incubation.

Even though RV was not chosen as the most effective medium, RV and BG were shown to be the most effective selective media for *Salmonella* enrichment, as found in numerous other studies (Truscott and Lammerding 1987; Allen and others 1991; Maijala and others 1992; Oboegbulem 1993; June and others 1996).

It has been concluded from other studies that TBG was significantly superior to SC. For example, a study (Rall and others 2005) compared the efficiency of detecting of *Salmonella* in poultry samples using three types of media including SC, TBG, and RV. TBG (58.6%) next to RV was more effective medium for detecting *Salmonella* in poultry samples than SC medium (24.1%). The other studies (Hammack and others 1999; June and others 1995) also found that tetrathionate broth (TT) at 42 °C was better than SC for detecting *Salmonella* from fresh meats and poultry products. Therefore, the fact that population of *Salmonella* in SC was significantly higher than the population in TBG ($p < 0.05$) in this study was not agree with several studies above. This maybe resulted of a higher toxicity of thionate used as selecting agent in the TBG medium than the toxicity of

$NaHSeO_3$ in SC. The other reason is probably related to the temperature effect, because many studies have confirmed that TT or TBG performed the best result at 42 °C. However, since the samples from this study were incubated at 37 °C, TBG was a significantly less effective medium for *Salmonella* enrichment than BG, SC, SB and RV.

C. Efficiency of *Salmonella* recovery at various populations for BHI, TSB and BG enrichment media

To determine the optimum enrichment medium for *Salmonella*, selected enrichment media (BHI, TSB and BG) were studied further for the efficiency of recovery of *Salmonella* at various populations from 2 h up to 6 h incubation (Figure 8). These studied could also provide information about proper incubation time and number to detect *Salmonella* using gold biosensor method with light microscopic imaging system, since the small population of *Salmonella* needs to be reached a detectable level prior to applying.

After 2 h incubation, the populations of *Salmonella* enriched in BHI, TSB, and BG were increased from 3 logS to 3.7 logS (= $\log_{10}5.3\times10^3$ CFU/16 in^2 chicken skin), 3.4 logS (= $\log_{10}2.7\times10^3$ CFU/16 in^2 chicken skin), and 3.5 logS (= $\log_{10}3.3\times10^3$ CFU/16 in^2 chicken skin), respectively. Based on the result (Figure 17), the detection limit of gold biosensor was determined as 3 logS per one test portion (convert to 4.7 logS (= $\log_{10}5.0\times10^4$ CFU/16 in^2 chicken skin). It was concluded that the populations of the three groups enriched for 2 h were not increased sufficiently to apply for the gold biosensor detection method developed in this study. A possible explanation for the low populations is that *Salmonella* might be still in the lag phase during only 2 h incubation

due to their mild physical injury or stress during preparation step. This explanation was supported a study (Chen and others 1993), which the lag phase of bacterial cells injured by heat could be extended to 4 or 5.3 h depending on serotype. Compared that lag phase of active bacterial cells could reach the exponential phase within 30 min, *Salmonella* in this study were probably subjected to be associated with mild physical injury or stress, resulting in longer lag phase at least 2 h.

After 4 h-incubation, the populations of *Salmonella* in BHI, TSB, and BG increased to 5.5 logS (= $\log_{10} 3.3\times10^5$ CFU/16 in^2 chicken skin), 5.2 logS (= $\log_{10} 1.6\times10^5$ CFU/16 in^2 chicken skin), and 5.4 logS (= $\log_{10} 2.8\times10^5$ CFU/16 in^2 chicken skin) at 1,000 cell inoculation, respectively, which are detectable ranges using the developed biosensor. The bacterial populations of BHI, TSB, and BG inoculated at levels of 100, 500 and 1000 cells were significantly increased to levels of 10^4 or 10^5 CFU within the 4 h incubation period ($p < 0.05$). However, the bacterial populations inoculated at the levels of 10 and 50 CFU in chicken skins did not increase to detectable levels.

After incubation for 6 h, the populations from the entire three media were significantly increased to the level of 10^7 CFU/16 in^2 chicken skin ($p < 0.05$). The bacterial population in BHI exhibited the greatest increase at the level of 1,000 CFU inoculation with 7.8 logS (= $\log_{10} 6.4\times10^7$ CFU/16 in^2 chicken skin), as compared to bacterial populations of 7.1 logS (= $\log_{10}1.2\times10^7$ CFU/16 in^2 chicken skin) and 7.2 logS (= $\log_{10}1.6\times10^7$ CFU/16 in^2 chicken skin) in TSB and BG, respectively. Even at the smallest inoculation (10 CFU), the bacterial population of BHI reached up to 10^5 CFU and was detectable when using the developed biosensor. Therefore, BHI medium was

selected as the most efficient enrichment medium for *Salmonella* in chicken skin samples.

D. Chilling effect on *Salmonella* recovery from inoculated chicken skin in BHI, TSB and BG enrichment media

Following the regulations in the US, poultry carcasses should be chilled to 4.4 °C or lower for a certain period of time to ensure a high quality and safe product (Code of federal regulations 1992). Therefore, a similar chilling step was introduced in this study, storing the *Salmonella* cocktail inoculated chicken skins at 4 °C for 48 h. Since the minimum growth temperature of *Salmonella* in poultry was reported at 5 °C (James and others 2006), it was hypothesized that the *Salmonella* cocktails stored at 4 °C should be injured. Therefore, it is required to determine the optimum enrichment medium that which media could provide better efficient condition to grow *Salmonella* injured by chilling.

The populations in BHI, TSB and BG enrichment media after chilling at 4 °C for 48 h are presented in Figure 9. Each bacterial population obtained in Figure 9 was compared to each population obtained in Figure 8. Due to the 48 h chilling effect, the overall growth rate was 10 times lower than previous data (Figure 8). The final population of *Salmonella* was 6.5 logS (= $\log_{10}3.1\times10^6$ CFU/16 in^2 chicken skin) for BHI, 6.3 logS (= $\log_{10}2.1\times10^6$ CFU/16 in^2 chicken skin) for TSB, and 6.4 logS (= $\log_{10}2.3\times10^6$ CFU/16 in^2 chicken skin) for BG, and there were no significant differences among them ($p < 0.05$). All three groups were affected by chilling treatment samples for 48 h, and their populations were significantly decreased ($p < 0.05$), due to a prolonged lag

phase caused by injury from chilling. Among the three media, BHI was the most affected by the chilling treatment and the population number was significantly decreased from 7.8 logS to 6.5 logS ($p < 0.05$). Therefore, BG was the most efficient medium for chilled *Salmonella* recovery, due to least decrease from 7.2 logS to 6.4 logS and second highest number increasing.

Overall, the results from Figure 8 and 9 showed that *Salmonella* cultivated in BHI had the greatest increase up to 7.8 logS from 3 logS, and BG was the most efficient medium for recovering *Salmonella* injured by chilling. Therefore, BHI and BG were chosen as the optimum media for *Salmonella* enrichment in order to increase the population for applying to the gold biosensor method.

1.2. Purification of monoclonal and polyclonal antibodies and Specificity test of polyclonal antibodies using indirect ELISA

A. Purification of monoclonal and polyclonal antibodies

MAbs and pAbs were purified by 50% saturated ammonium sulfate precipitation and protein A affinity column. The purities of mAbs and pAbs were then confirmed by SDS-PAGE (12%) (data not shown). The final antibody concentrations were measured by the Bradford protein assay method and they were 4.0 mg/mL and 6.5 mg/mL for mAbs and pAbs, respectively.

B. Reactivity test of monoclonal and polyclonal antibodies by indirect ELISA

The reactivity of mAb and pAb against *Salmonella* cocktail (1.9×10^8 CFU/well) was tested with a serial dilution of mAb and pAb by indirect ELISA and the results were summarized in Figure 10. PAbs exhibited higher binding ability against *Salmonella*

cocktail, from 1/200 to 1/1,600 dilution of the original concentration of pAbs (6.5 mg/mL). However, mAbs (4.0 mg/mL) did not showing any binding ability against *Salmonella* cocktail. Therefore, 1/800 diluted pAbs were used for further specificity testing against various microorganisms.

The reason for low reactivity of the mAbs is that the binding sites of mAbs may not be recognized by the determinants of antigens (*Salmonella* cocktail). The mAbs are such homogeneous antibodies having one binding site that can only be recognized one epitope of the antigen. The other reason is probably associated with mishandling and prolonged storage. The mAbs were produced for 3 years and could be precipitated or denaturized during a long storage time, even though it stored under the proper conditions. Another reason is that mAbs have limited stability to the changes of pH or salt concentration. Therefore, even minor changes in the ELISA would be able to decrease the reactivity with *Salmonella* when compared to pAbs. The results showed that pAbs had significantly greater reactivity with *Salmonella* than mAbs did ($p < 0.05$).

C. Specificity test of polyclonal antibodies by indirect ELISA

For the specificity test of pAbs (Table 2), various *Salmonella* serotypes, *L. monocytogenes*, *S. aureus* and *E. coli* (1.8×10^8 CFU/well) were tested with 1/800 diluted pAbs from the original sample (6.5 mg/mL). The specificity of pAbs to various bacteria is summarized in Table 2. All *Salmonella* strains, except *S*. Diarizonae, and *E. coli* showed higher binding efficiency with pAb whereas *L. monocytogenes* (H7757), *L. monocytogenes* (H7738), *S. aureus* (ATCC 12600), and *S. aureus* (ATCC 6538) showed significantly lower binding efficiency. This presumably provides that the detection

accuracy of *Salmonella* may be decreased if *Salmonella* coexist with *E. coli* in chicken samples. Overall, the purified pAbs had specificity and high binding efficiency with all of *Salmonella* strains, except *S*. Diarizonae, and *E. coli*.

2. Development of Gold Biosensor with a Light Microscopic Imaging System for *Salmonella* Detection

2.1. Binding efficiency of gold, polyvinylchloride, polystyrene, and glass sensor platforms

There are several types of solid sensor platforms widely used in the literature; plastic surfaces (polystyrene, polyvinylchloride, and silicon), metallic surfaces (gold and silver), and membranes (nitrocellulose and nylon) (Jung and others 2008). Among several types of sensor platforms, polystyrene has been widely used for ELISA assay because polystyrene is inexpensive, convenient and inert. Gold has been also used commonly in QCM and SPR biosensors (Butler and others 1993; Oh and others 2005). Therefore, four popular sensor platforms including gold (AU), polyvinyl chloride (PVC), polystyrene (PS) and glass (GL) were selected for the test in this study to determine the optimum sensor platform for a biosensor. The comparison of the binding efficiency for the four types of sensor platforms is presented in Figure 11.

The binding efficiency of AU and PS were significantly greater than the binding efficiency of GL and PVC ($p < 0.05$), which were 66 ± 24 CFU/ mm^2 and 57 ± 16 mm^2 respectively. Since there was no significant difference between AU and PS, PS and AU

were selected as the efficient sensor platforms to immobilize pAbs through physical adsorption, as reported in other research (Butler and others 1993; Jung and others 2008).

2.2. Binding efficiency of polystyrene and gold sensors untreated or treated with recombinant protein A, commercial protein A and lysine

The binding efficiencies of regular AU and PS sensor platforms with antibodies were compared with the binding efficiencies of AU and PS sensor platform treated with recombinant protein A, commercial protein A and lysine (Figure 12). The number of *Salmonella* captured on PS treated with lysine (LC-PS) and commercial protein A (CPA-PS), and *Salmonella* captured on regular polystyrene (R-PS) were significantly greater than the numbers on polystyrene treated with recombinant protein A (PPA-PS) ($p < 0.05$). The average numbers of *Salmonella* captured on LC-PS, CPA-PS, R-PS, and PPA-PS were 87 ± 28 CFU/0.013 mm^2, 77 ± 11 CFU/0.013 mm^2, 71 ± 12 CFU/0.013 mm^2, and 52 ± 6 CFU/0.013 mm^2, respectively.

The number of *Salmonella* captured on gold treated with lysine (LC-AU), and regular gold (R-AU) were significantly greater than the numbers on gold treated with commercial protein A (CPA-AU) or gold treated with purified recombinant protein A (PPA-AU) ($p < 0.05$). The captured populations were 98 ± 16 CFU/0.013 mm^2 for LC-AU, 83 ± 11 CFU/0.013 mm^2 for R-AU, 61 ± 9 CFU/0.013 mm^2 for CPA-AU, and 59 ± 12 CFU/0.013 mm^2 for PPA-AU.

For the optimum binding to the biosensor, the antibodies should be oriented on the sensor properly, and the binding site should be exposed to antigens through several immobilization methods, such as a covalent attachment with cross-linker (Zhou and

Synder 2003; Yuk and Ha 2005) and the application of protein A, G, and other derivatives on the sensor platforms (Kaku and others 1989; Lu and others 1996a; 1996b; Danczyk and others 2003; Jung and others 2008; Skottrup and others 2008).

According to research (Kaku and others 1989; Lu and others 1996a; 1996b; Danczyk and others 2003), the application of protein A onto the sensor platforms is one of the most effective methods to increase the binding efficiency of the sensor. Lu and others (1996b) demonstrated that the immobilization of protein A onto platforms enhanced the binding efficiency of the sensor platform up to 10 times greater than the random immobilization. More recently, Fower and others (2007) also reported that the chemically thiolated protein G onto the gold surface significantly improved the binding efficiency of antibodies with the antigen. More studies reported that the efficiency of antibody immobilization of the cysteine-tagged protein G layer exhibited a binding efficiency that was 4 times greater than that of protein G (Lee and others 2007), and that protein A on the gold sensor increased the immobilization of the antibody up to 42.1% from 31.6 % in polyethylenimine (PEI) coated gold sensor (Babacan and others 2000). However, the results in this study did not support previous findings, exhibiting that the *Salmonella* binding efficiency of AU and PS treated with protein A was not improved.

Lysine coating on the sensors was applied to enhance the efficiency of *Salmonella* binding. The results showed a significant increase in the binding efficiency of *Salmonella* on AU and PS treated with lysine. The greater number of captured *Salmonella* on the lysine treated sensors than those treated with the recombinant protein A were probably attributed to the "sticky" property of lysine. However, lysine treatment has its limitations in use, even though the lysine treatment significantly increased the

number of captured *Salmonella* on the sensors ($p < 0.05$). Due to the glue property, lysine treatment increased the attachment of unwanted impure particles on the sensor platforms. Further, the "messy" sensor surface treated with lysine might cause interference when observing *Salmonella* on chicken skin inoculated under the microscopic imaging system. Thus, it was difficult to prepare consistent and reliable sensors.

Although there was no significant difference between R-PS and R-AU, R-AU was chosen the best sensor platforms among those. In case of PS sensor platforms, *Salmonella* tended to bind to the edge of the sensor platforms and this caused serious problems under the microscope.

2.3. Determination of optimum concentration of antibody, incubation temperature, and pH of buffer for gold biosensor detection

For the successful binding of antibodies and target microorganisms on gold biosensor, the optimized reaction condition is critical for obtaining desirable results. Therefore, several factors need to be considered prior to applying gold biosensor for bacterial detection on chicken samples. The factors for optimizing reaction conditions include the concentrations of the antibody, incubation temperature, and pH of PBS buffer.

The detection of *Salmonella* on the gold biosensor from selected concentrations of pAbs and mAbs is presented in Figures 13 and 14, respectively. The binding efficiency of pAbs for *Salmonella* cocktail on the gold biosensor was significantly higher than the binding efficiency of mAbs ($p < 0.05$). The higher binding efficiency of pAbs for *Salmonella* was also supported by the previous ELISA test as presented in Figure 9.

The average populations of *Salmonella* detected at the selected pAbs concentrations of 10, 25, 50, 75, 100, 150, 200, and 300 μg/mL were 9 ± 5 CFU/0.013 mm^2, 40 ± 10 CFU/0.013 mm^2, 82 ± 3 CFU/0.013 mm^2, 74 ± 3 CFU/0.013 mm^2, 104 ± 11 CFU/0.013 mm^2, 91 ± 21 CFU/0.013 mm^2, 76 ± 3 CFU/0.013 mm^2, 30 ± 20 CFU/0.013 mm^2, respectively (Fig. 13). The concentrations of pAbs at 50, 75, 100, 150 and 200 μg/mL exhibited significantly higher binding efficiencies with *Salmonella* than those at 10, 25, and 300 μg/mL ($p < 0.05$). Although there were no significant differences in binding efficiencies among the concentrations of 50, 75, 100, 150 and 200 μg/mL, the pAbs concentration of 100 μg/mL was determined as the optimum concentration based on the highest bacterial population detected on the gold sensor. The low binding efficiency of pAbs at the concentration of 300 μg/mL might be attributed to the steric hindrance (steric forces) among immobilized antibodies.

The optimum incubation temperature for binding between *Salmonella* and pAbs previously immobilized on gold biosensor is presented in Figure 15. The incubation temperatures at 30 and 37 °C exhibited significantly higher binding efficiency than those at other selected temperatures ($p < 0.05$), and the captured *Salmonella* were 85 ± 9 CFU/0.013 mm^2 for 30 °C and 71 ± 6 CFU/0.013 mm^2 for 37 °C. The binding efficiency at 45 °C was significantly decreased, exhibiting the captured number of 38 ± 11 CFU/0.013 mm^2. The result was in agreement with a study (Park and others 2000), where it was found that the binding efficiency between an antibody and *Salmonella* was abruptly decreased by incubation temperature at greater than 40 °C. Babacan and others (2000) also reported that the low temperature-long time incubation was better for non-

covalent binding, including the ELISA test and biosensor. Therefore, the optimum incubation temperature for efficient binding was determined to be 30 °C in this study.

Regarding the pH of PBS buffer, the optimum pH for maximum binding efficiency was determined as pH 8.0 (Figure 16). As the pH increased, the binding efficiency of *Salmonella* on the gold sensor platform also increased up to pH 8.0. The binding efficiency was maintained between pH 8.0 and 10.2, however suddenly decreased beyond pH 10.2. Therefore, the optimum pH range of the PSB buffer was determined to be from 7.0 to 8.0.

2.4. The detection sensitivity (detection limit) of optimized gold biosensor with light microscopic imaging system

The detection limit of gold biosensor with light microscope imaging system (GB-LMI) was examined by using different populations of *Salmonella* cocktail cultivated in TSB, based on the optimized conditions above. Therefore, the binding efficiency of GB-LMI was determined by the bacterial population detected on the gold biosensor (Figure 17). The *Salmonella* populations detected by GB-LMI were 2 ± 1 CFU/0.013 mm^2 for the inoculated population of 10^3 CFU per gold biosensor, 6 ± 2 CFU/0.013 mm^2 for the inoculated population of 10^4 CFU per gold biosensor, 14 ± 6 CFU/0.013 mm^2 for the inoculated population of 10^5 CFU per gold biosensor, 53 ± 10 CFU/0.013 mm^2 for the inoculated population of 10^6 CFU per gold biosensor, and 111 ±19 CFU/0.013 mm^2 for the inoculated population of 10^7 CFU per gold biosensor. The inoculated populations of *Salmonella* at 10^8 CFU and 10^9 CFU per gold biosensor were difficult to count because of the large number of *Salmonella* attached on the gold biosensor (Figure 18-D). Therefore,

the detection limit of GB-LMI was determined as 2.0 ± 1 CFU with the inoculation of 10^3 CFU per gold biosensor.

2.5. Application of gold biosensor with light microscopic imaging system to detect *Salmonella* on inoculated chicken skin

The GB-LMI method was applied to the detection of *Salmonella* on inoculated chicken skin. The population of *Salmonella* detected every 2 h are presented in Figure 19. The detected population of *Salmonella* enriched in BHI medium was higher than the number enriched in BG for every 2 h-time interval sample. The detected number of *Salmonella* enriched in BHI medium for 6 h with the initial population of 10^2 CFU and 10^3 CFU was significantly higher than the detected number of *Salmonella* enriched in BG medium for 6 h (i.e., 75 ± 15 CFU/0.013 mm^2 for chicken sample enriched in BHI for 6 h after the initial inoculation of 10^3 CFU/16 in^2, 51 ± 8 CFU/0.013 mm^2 for chicken sample enriched in BG for 6 h after the initial inoculation of 10^3 CFU/16 in^2, 27 ± 1 CFU/0.013 mm^2 for chicken sample enriched in BHI for 6 h after the initial inoculation of 10^2 CFU/16 in^2, and 17 ± 11 CFU/0.013 mm^2 for chicken sample enriched in BG for 6 h after the initial inoculation of 10^2 CFU/16 in^2. Since GB-LMI method could be used to detect *Salmonella* at the minimum population of 10^3 CFU per gold biosensor, the bacteria on samples from 2-h incubation were not detected due to the low population of *Salmonella*. Therefore, it was concluded that GB-LMI method could detect *Salmonella* on inoculated chicken skin after 4 h enrichment in BHI and BG media and the total detection time of GB-LMI required was 4.5 h.

3. Application of Immunomagnetic Beads to Gold Biosensor with Light Microscopic Imaging System for *Salmonella* Detection

3.1. Preparation of the magnetic beads coupled with ligand

Magnetic beads (magnetites) synthesized from Iron sulfate-heptahydrate were coated with sodium silicate to allow for stability in acid or alkali solution. The coating efficiency was determined using hydrochloric acid. The beads coated with sodium silicate did not exhibited yellowish color at levels of 1 to 4 M hydrochloric acid solution, confirming that the magnetic beads were properly coated (data was not shown). The size of the coated magnetic beads was in the range of 10 to 100 nm from the observation under the TEM (Figure 20). Compared to commercial brand (size; 800-10,000 nm, shape: round), the prepared magnetic beads in this study were approximately 100 times smaller than the commercial magnetic beads and showed almost rectangular shape. Due to smaller size, it was speculated that the prepared magnetic beads provided a greater surface area for antibodies to be conjugated than commercial beads within an identical volume of magnetic beads, which might result in a higher efficiency in capturing *Salmonella*.

3.2. Optimization of reaction condition for using immunomagnetic beads

A. Determination of optimum concentration of pAbs for conjugating with magnetic beads

To determine the optimum concentrations of pAbs, various concentrations of pAbs were conjugated with MBs and the results are summarized in Figure 21. As the concentration of pAbs increased, the number of captured *Salmonella* also increased until

the pAbs concentration reached 0.44 mg per mL MB. The capturing abilities of pAbs at the concentration of 0.44 and 0.55 mg per mL MB were significantly higher than the other concentrations of pAbs ($p < 0.05$). Since there was no significant difference between the captured *Salmonella* at 0.44 and 0.55 mg per mL MB, the low concentration (0.44 mg per mL MB) was determined to be the optimum pAbs concentration.

In commercial beads (Dynabeads®) used to separate *Salmonella* from food sample mixtures, the amount of antibodies required for conjugation with MBs were 0.2 - 0.5 μg in 25 μL MB (Coleman and others 1995a). The difference in the concentrations of antibodies between the commercial beads and the MBs prepared in this study could be attributed to the different sizes of the beads. The MBs prepared in this study were much smaller in the size than the commercial beads, presumably requiring more antibodies to cover the larger surface area of the MBs.

B. Determination of optimum reaction time, temperature, buffer type at various pHs for capturing *Salmonella*

Several factors such as reaction time, temperature, and buffer type at various pH values were investigated for optimizing immunomagnetic separation (IMS). The effect of reaction time on IMS is presented in Figure 22. The number of *Salmonella* captured on IMB was increased with the increase in reaction time to 20 min, exhibiting the highest captured number of *Salmonella* (2.0×10^7 CFU). However the number of *Salmonella* was steady after 20 min. Therefore, the optimum reaction time for the IMB to bind *Salmonella* was chosen as 20 min.

Regarding the reaction temperature, the number of *Salmonella* captured at selected temperatures of 15, 22, 30, and 37 °C were significantly higher than the number of *Salmonella* captured at 4 and 45 °C ($p < 0.05$) (Figure 23). These results are in agreement with the study conducted by Park and others (2000). In their study, the interaction between *Salmonella* and antibodies coated on sensor platform was increased until 35 °C, however their binding was abruptly decreased over 35 °C. The main reason for the reduced number of captured *Salmonella* at temperature higher than 37 °C was probably attributed to the reduced affinity between the antibody and *Salmonella* caused by the conformational changes of pAbs conjugated on the MB at higher temperatures and/or microbial inactivation (Park and others 2000). Therefore, the optimum temperature for reaction was determined as 30 °C.

With respect to the type of buffer and pH, PBS buffer was more effective for capturing *Salmonella* than TBS buffer at all tested pH values (Figure 24). In the TBS buffer, the number of captured *Salmonella* at pH 5.5, 6.5 and 7.5 was significantly higher than that at pH 8.0, 9.0 and 10.0. On the other hand, the number of captured *Salmonella* at pH 6.5 and 7.5 in PBS buffer was significantly higher than those at pH 5.5, 8.0, 9.0 and 10.0. Since the captured number of *Salmonella* in PBS was higher than that in TBS, PBS was selected for IMB separation assay at pH 6.5-7.5.

C. Determination of the quantity of immunomagnetic beads required for capturing various populations of *Salmonella*

Various populations of *Salmonella* cocktail were used to determine the appropriate quantity of IMBs for bacterial detection in chicken samples. To determine

the appropriate quantity of IMBs, the binding efficiency and economic cost were considered as important factors for practical application. The number of captured *Salmonella* at the initial population of 10^1 CFU exhibited the higher captured number at the concentrations of 50 and 200 μL IMBs than the numbers from other concentrations (Figure 25). However, from the economic stand point, the 50 μL IMBs were determined as the optimum quantity of IMBs for capturing 10^1 CFU of *Salmonella*.

In capturing *Salmonella* at 10^3 and 10^5 CFU, the number of captured *Salmonella* was increased to 100 μL IMBs as the quantity of IMB increased. Since the number of captured *Salmonella* was not increased in IMBs higher than 100 μL, the optimum quantity of IMB was determined as 100 μL. In capturing *Salmonella* at 10^7 CFU, the number of captured *Salmonella* was increased gradually as IMBs increased to 200 μL. However, the number of captured *Salmonella* did not increase significantly after at 200 μL IMB or higher (data not presented). Therefore, the optimum quantity of IMBs for high bacterial population detection was determined as 200 μL.

3.3. Application of immunomagnetic beads to capture *Salmonella* on chicken skin inoculated using a gold biosensor with light microscopic imaging system

The *Salmonella* inoculated chicken skin samples were detected using an optimized gold biosensor with light microscopic imaging system (GB-LMI) previously developed in this study. However, the detection of *Salmonella* in chicken sample using a GB-LMI may not always guarantee the desirable efficiencies, mainly because undesirable food particles such as fats, humic acids, bile salts, and lipids can interfere with the binding between *Salmonella* and pAbs immobilized on the gold sensor platforms. In fact,

one of the serious problems of using GB-LMI is the interference with food particles due to the attachment of undesirable food particles on the gold biosensor (Figure 27-A). As a result, there was a significant decrease in the detection specificity, detection limit, accuracy and reliability when the GB-LMI was applied to chicken samples. Although the filtration steps might remove the big, chunky, and undesirable particles in chicken samples to certain degree, there were still substantial amounts of undesirable particles other than *Salmonella* on the gold sensor platform. Therefore, the conjunction of IMS and BG-LMI method (IMS-GB-LMI) was applied in this study for better detection of *Salmonella* in real chicken samples.

Salmonella on chicken skin was detected using BG-LMI after IMS treatment (Figure 26). The detected number of *Salmonella* on the gold biosensor was 4.0 ± 1.7 CFU/0.013 mm^2 for chicken sample enriched in BHI for 4 h after the initial inoculation of 10^2 CFU/16 in^2, 9.0 ± 1.9 CFU/0.013 mm^2 for chicken sample enriched in BHI for 4 h after the initial inoculation of 10^3 CFU/16 in^2, 32.0 ± 9.0 CFU/0.013 mm^2 for chicken sample enriched in BHI for 6 h after the initial inoculation of 10^2 CFU/16 in^2, and 75.0 ± 16.0 CFU/0.013 mm^2 for chicken sample enriched in BHI for 6 h after the initial inoculation of 10^3 CFU/16 in^2. Even though the previous results exhibited approximately 1 to 2 log reductions in bound *Salmonella* in pure culture after IMS treatments, the number of *Salmonella* in chicken sample detected by the IMS-GB-LMI method (Figure 26) exhibited no significant difference in the number of *Salmonella* in chicken samples detected by only the GB-LMI method (Figure 19).

The microscopic images of *Salmonella* bound to the gold biosensor are presented in Figure 27. The GB-LMI method detected not only *Salmonella* but also undesirable

food particles (A), whereas the IMS-GB-LMI method exhibited mainly *Salmonella* (C and D). Therefore, it was concluded that IMS-GB-LMI method improved the detection efficiency by improving the resolution of microscopic images and reducing the interferences.

Considerable research reported that the combination of IMS and conventional method improved the sensitivity and specificity for *Salmonell*a detection. Coleman and others (1995a) combined the IMS method and conventional method to detect *Salmonella* in frozen and chilled chickens. The combination method could detect 26% *Salmonella* positive samples, whereas the conventional method showed only 10% positive samples. The other studies showed that the combination of IMS and conventional method increased the sensitivity and specificity for *Salmonella* detection significantly (Coleman and others 1995b; Mansfield and Forsythe 1996b; Ripabelli and others 1997).

Kumar and others (2005) reported that the IMS combined with polymerase chain reaction (IMS-PCR) was an effective method to capture *Salmonella* in inoculated meat sample when detected within 6 h with a sensitivity of 10^5 cells. In other study (Mansfield and Forsythe 2001), *Salmonella* inoculated in raw chicken was detected within 27 h with a sensitivity of 10^6 CFU/mL by the combination of IMS and ELISA (IMS-ELISA) method.

In summary, the IMS-GB-LMI method did not increase the number of *Salmonella* captured on the gold biosensor significantly. However, this method enhanced the resolution of microscopic images by reducing the attachment of undesirable particles on the gold biosensor, because the small size of the IMBs interacted selectively with

Salmonella rather than any other undesirable particles in chicken samples by forming covalent bond. Therefore, the IMS-GB-LMI method can be useful in detecting *Salmonella* in poultry and meat products commonly coexisted with the undesirable particles with enhancing the reliability and accuracy of GB-LMI method.

VI. OVERALL CONCLUSION

The overall objective of this study was to develop a new method combination of the gold biosensor with light microscopic imaging system for rapid *Salmonella* detection within 8 h in poultry products. *Salmonella* bound on the biosensors was visualized and enumerated using a light microscopic imaging system (GB-LMI). The conjunction of IMS and GB-LMI method enhanced the resolution of the microscopic imaging by reducing the attachment of undesirable particles on the gold biosensor. Therefore, this system for *Salmonella* detection in chicken samples is with high sensitivity and specificity, and easy operation. In addition, this detection method can provide food industry with substantial benefits as a rapid detection method.

The conclusions of the first study include:

(1) BHI and TSB media were selected as the most effective non-selective enrichment media and BG was selected as the most effective selective enrichment medium for *Salmonella* due to greater increasing in *Salmonella* population within 6 h-enrichment than other media.

(2) In the further study for determining the most effective medium among three chosen media (BHI, TSB, and BG media), BHI and BG media were chosen for

the most effective media to increase the population of *Salmonella* up to detectable level.

(3) The purified pAbs have shown higher binding activity with *Salmonella* than mAb. The selected pAbs also showed the specificity against all of the tested *Salmonella* strains, except for *S*. Diarizonae.

The conclusions of the second study include:

(1) The binding efficiency of AU and PS were significantly greater than that of GL and PVC. Since the pre-treatments with lysine and protein A to AU and PS did not improve the binding efficiency of the sensors, AU was finally selected as a sensor platform due to the fracture problem in preparing PS sensor platform.

(2) The optimum concentration of pAbs was 100 μg/mL for antibody immobilization. The optimum incubation temperature and pH were 30 °C, and 7.0 to 8.0 in PBS, respectively.

(3) The detection limit of GB-LMI was 2.0 ± 1 CFU with the inoculation of 10^3 CFU of *Salmonella*.

(4) In the application of GB-LMI to the detection of *Salmonella* on inoculated chicken skin, the detected population of *Salmonella* was higher in BHI enriched sample than that in BG. The GB-LMI method can detect *Salmonella* on inoculated chicken skin (10^2 CFU/16 in^2) after 4 h enrichment in BHI and BG media and the total detection time was 4.5 h.

The conclusions of the third study include:

(1) The optimum quantity of pAbs for conjugation with MBs was 0.44 mg per mL of MBs. The optimum reaction time for the IMB to bind *Salmonella* was chosen as 20 min due to exhibiting the highest captured number of *Salmonella*. The optimum temperature, buffer type, and pH for IMB performance were 30 °C, PBS, and 6.5-7.5, respectively.

(2) The appropriate quantity of IMBs to bind *Salmonella* was determined as 50 μL IMBs for capturing 10 CFU *Salmonella*, 100 μL IMBs for 10^3 and 10^5 *Salmonella,* and 200 μL IMBs for 10^7 CFU *Salmonella.*

(3) The conjunction of IMS and BG-LMI method did not increase the binding efficiency of *Salmonella* significantly; however, this method enhanced the resolution of microscopic images by reducing the attachment of undesirable particles on the gold biosensor. Therefore, the IMS-GB-LMI method can be useful in detecting *Salmonella* in poultry and meat products commonly coexisted with undesirable particles for enhancing the reliability and accuracy of GB-LMI method.

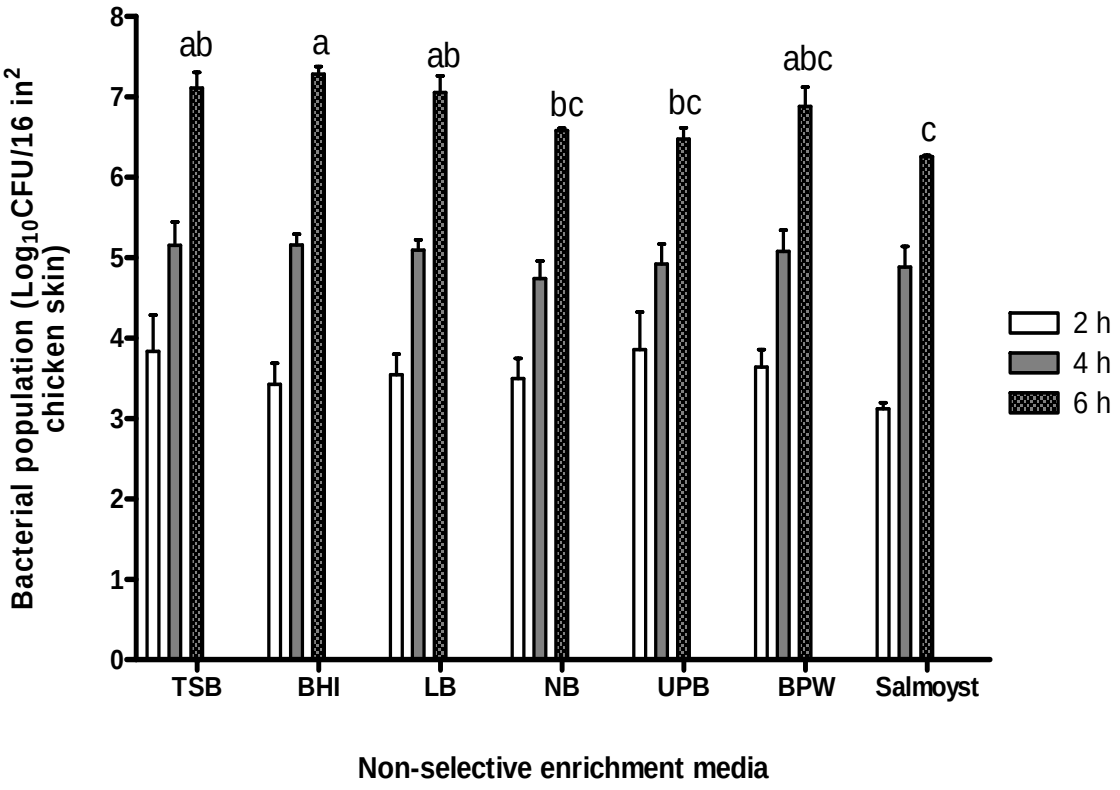

Figure 6-Efficiency of different types of non-selective enrichment media for *Salmonella* recovery in chicken samples inoculated with 1,000 CFU of *Salmonella* cocktail. TSB: tryptic soy broth, BHI: brain heart infusion broth, LB: lactose broth, NB: nutrient broth, UPB: universal pre-enrichment broth Salmoyst: salmoyst broth. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions. Different letters (a,b,c) within the same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviations (N=10).

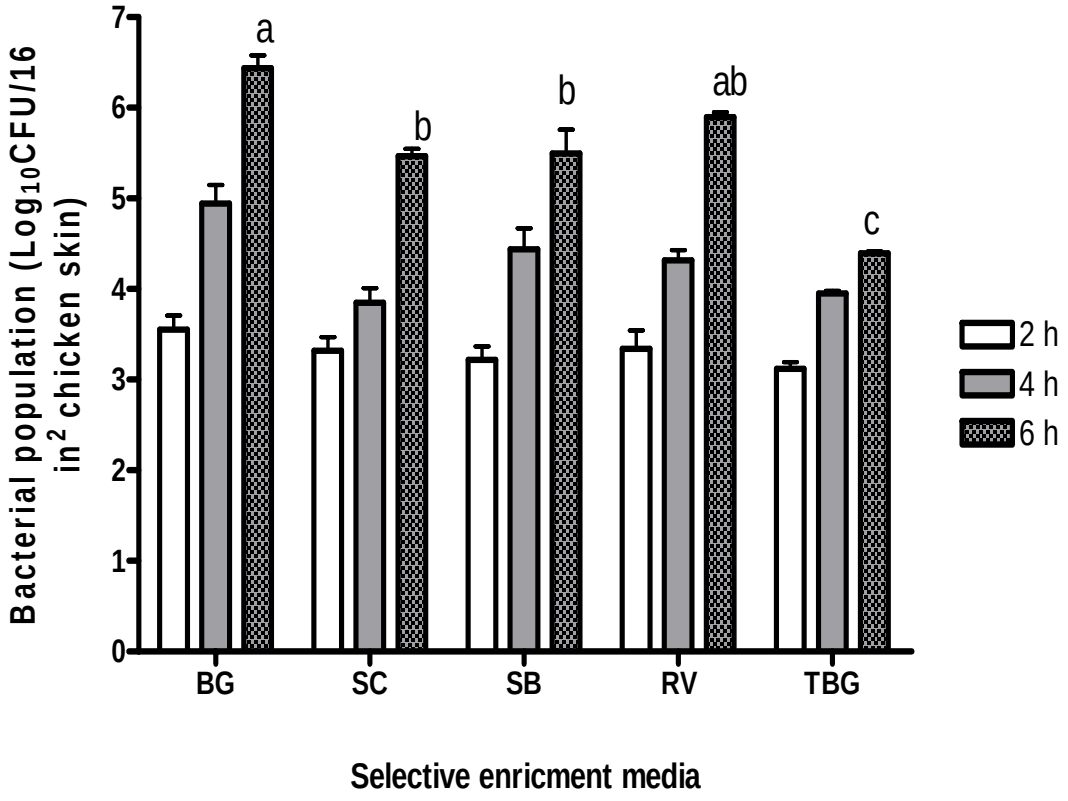

Figure 7-Efficiency of different types of selective enrichment media for *Salmonella* recovery in chicken samples inoculated with 1,000 CFU of *Salmonella* cocktail. BG: brilliant green broth, SC: selenite cystine broth, SB: selenite broth, RV: Rappaport-Vassilidis R10 broth, TBG: Tetrathionate brilliant green. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions. Different letters (a,b,c) within same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviations (N=10).

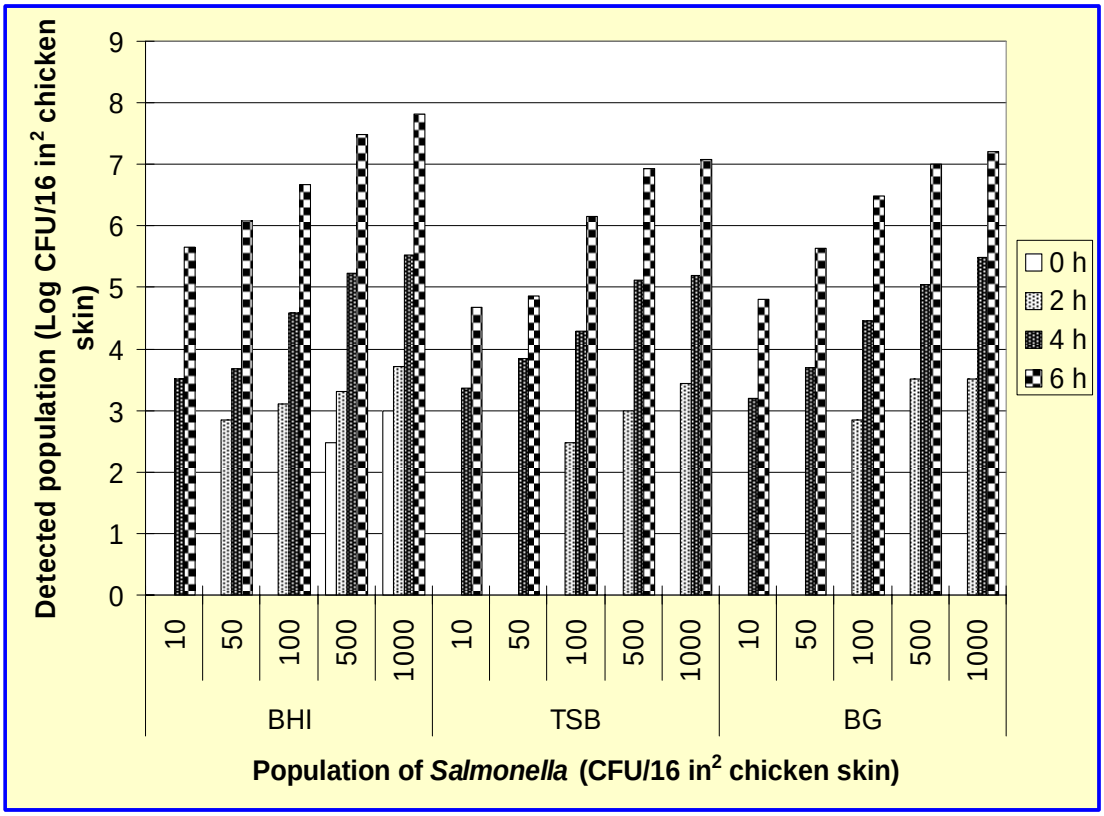

Figure 8-The growth efficiency of *Salmonella* at different populations in selected media. BHI: brain heart infusion broth, TSB: tryptic soy broth and BG: brilliant green broth. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions.

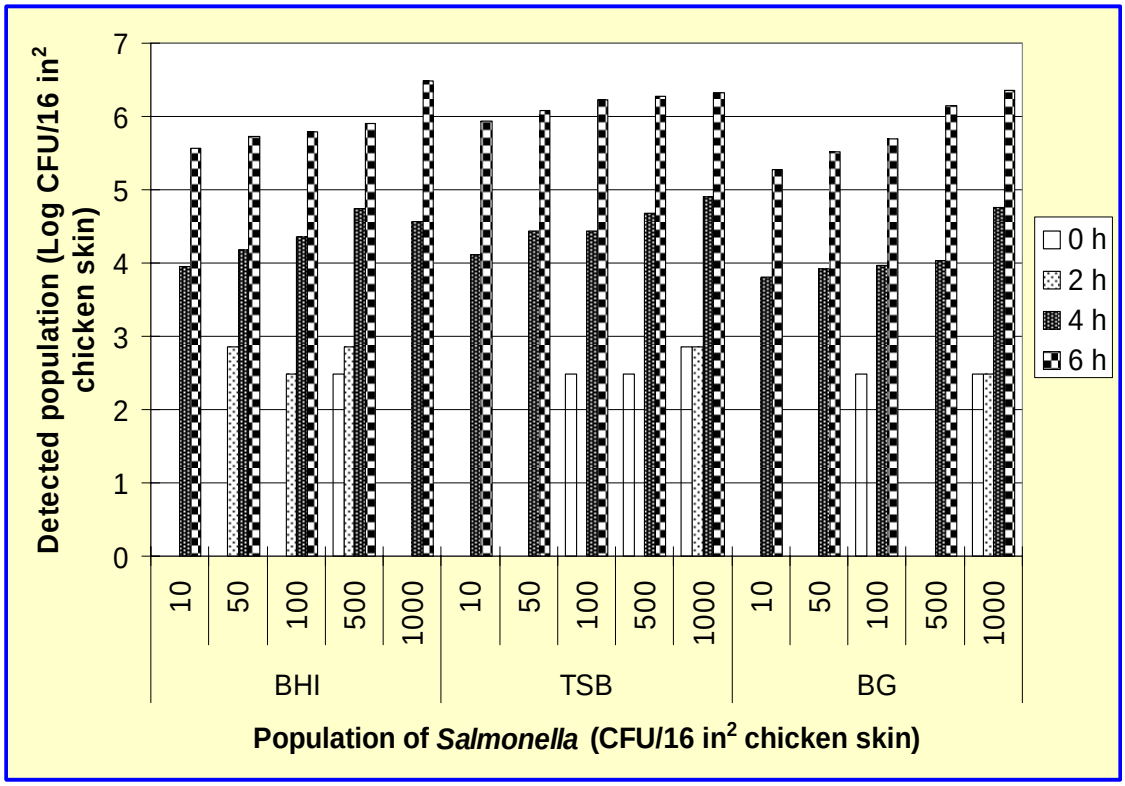

Figure 9-Recovery efficiency of selected media inoculated with *Salmonella* injured from chilling treatment at 4 °C for 48 h. BHI: brain heart infusion broth, TSB: tryptic soy broth and BG: brilliant green broth. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions.

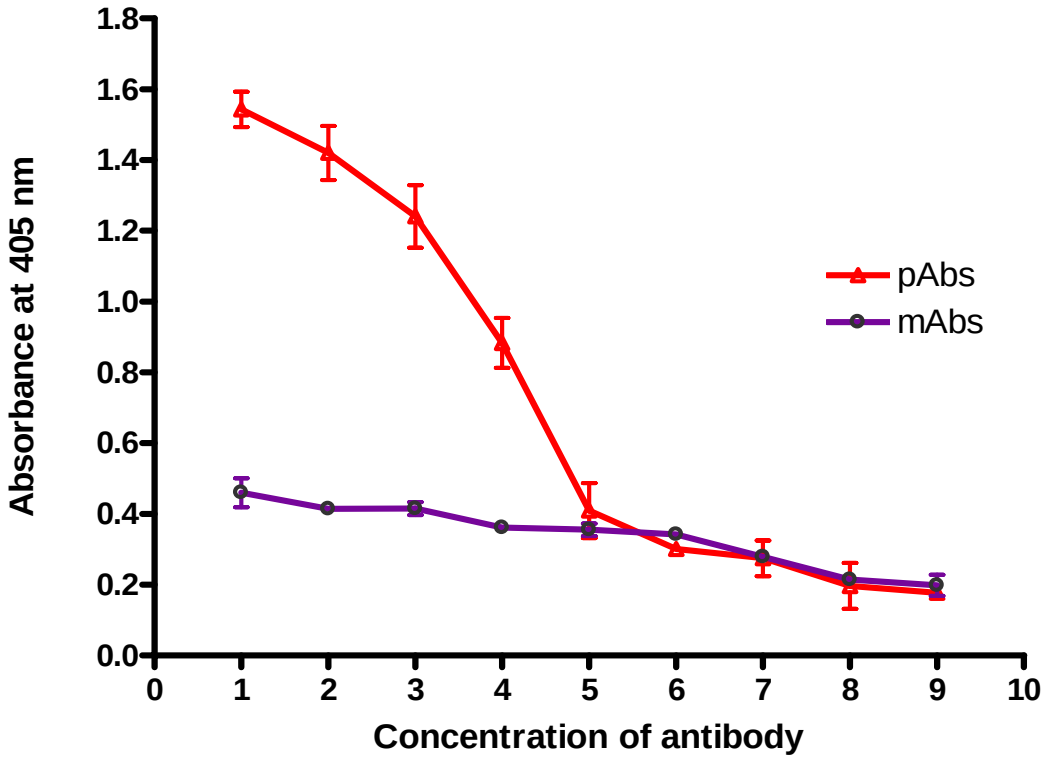

Figure 10-Reactivity of mAb and pAbs to *Salmonella* cocktail by indirect ELISA. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions. The concentration of monoclonal and polyclonal antibodies was 4.0 mg/mL and 6.5 mg/mL respectively. 1: 200X dilute, 2: 400X dilute, 3: 800X dilute, 4: 1600X dilute, 5: 3200X dilute, 6: 6400X dilute, 7: 12800X dilute, 8: 25600X dilute, 9: 51200X dilute, and 10: 102400 dilute from the original antibodies. Vertical bars represent standard deviations (N=3).

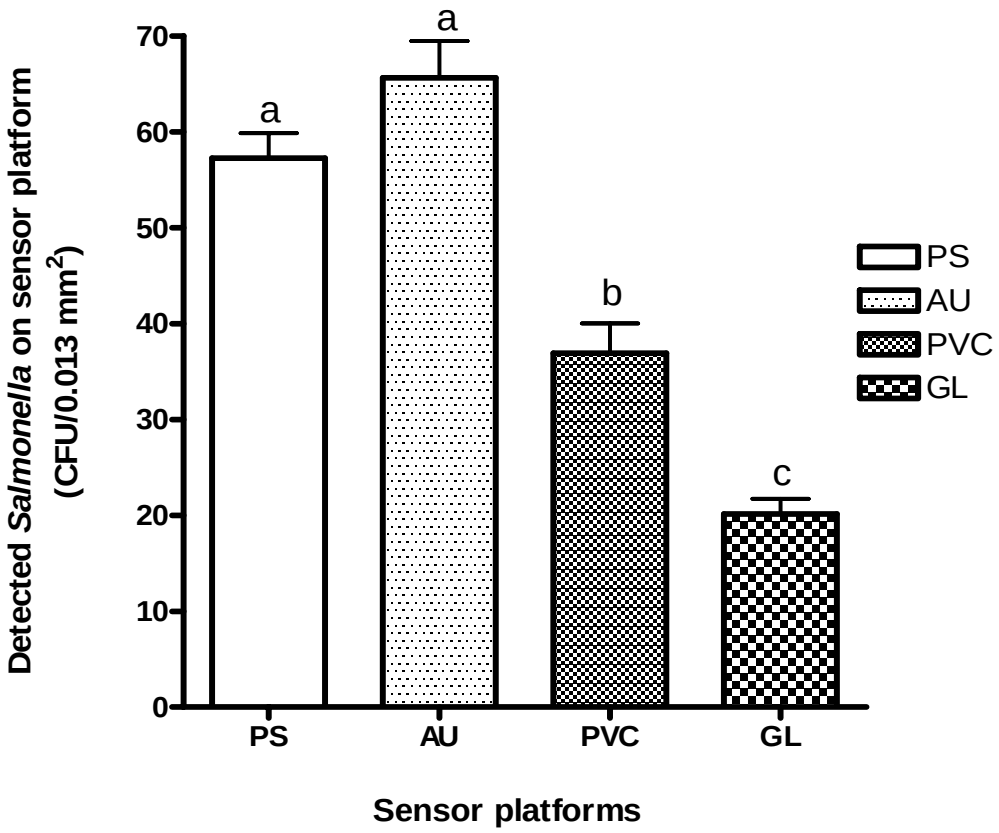

Figure 11-Comparision of binding efficiency on sensor platforms. PS: Polystyrene, AU: Gold, PVC: Polyvinyl chloride, GL: Glass. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions. Inoculated population of *Salmonella* cocktail: 1.0×10^7 CFU/sensor. Different letters (a,b,c) within same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviation (N=10).

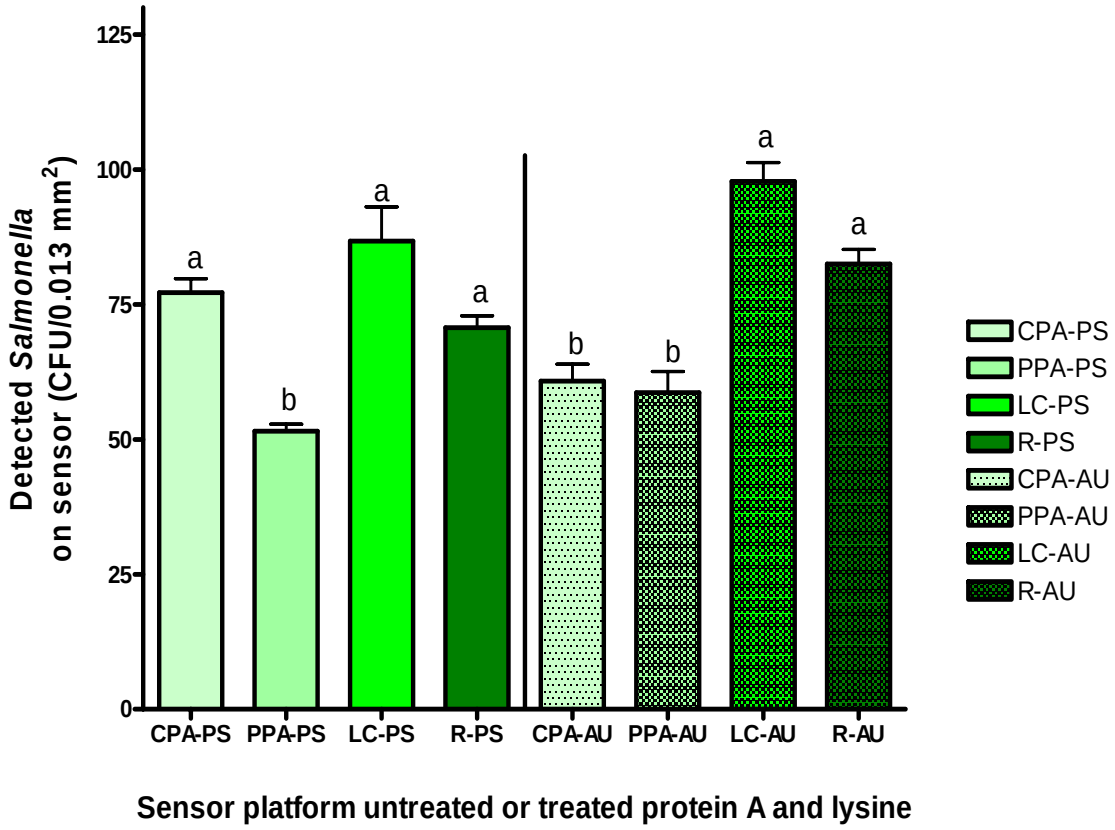

Figure 12-Comparision of antibody binding efficiencies of polystyrene and gold sensor treated with/without recombinant protein A, commercial protein and lysine. CPA-PS: Polystyrene treated with commercial protein A, PPA-PS: Polystyrene treated with purified protein A, LC-PS: Polystyrene treated with lysine, R-PS: Regular polystyrene, CPA-AU: Gold treated with commercial protein A, PPA-AU: Gold treated with purified protein A, LC-AU: Gold treated with lysine, R-AU: Regular gold. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions. Inoculated population of *Salmonella* cocktail: 1.6×10^7 CFU/sensor. Different letters (a,b) within same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviation (N=10).

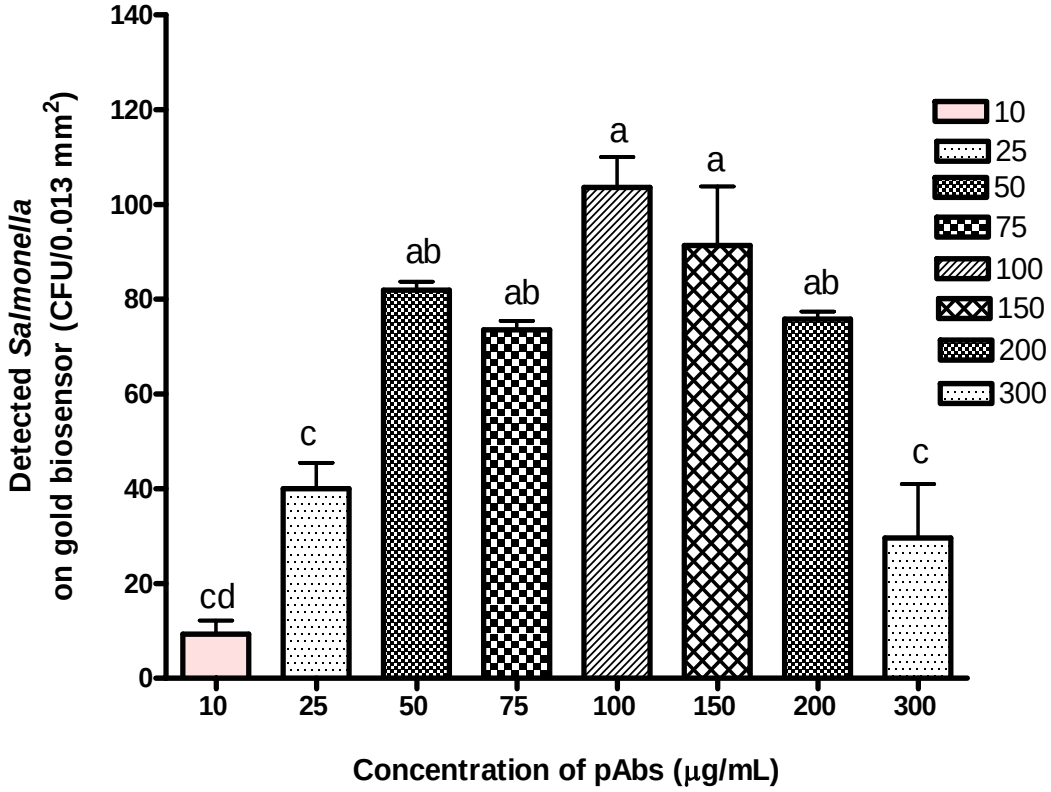

Figure 13-The effect of polyclonal antibodies (pAbs) concentrations on gold biosensor for *Salmonella* detection. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions. Inoculated population of *Salmonella* cocktail: 1.6×10^7 CFU/sensor. Different letters (a,b,c,d) within same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviation (N=10).

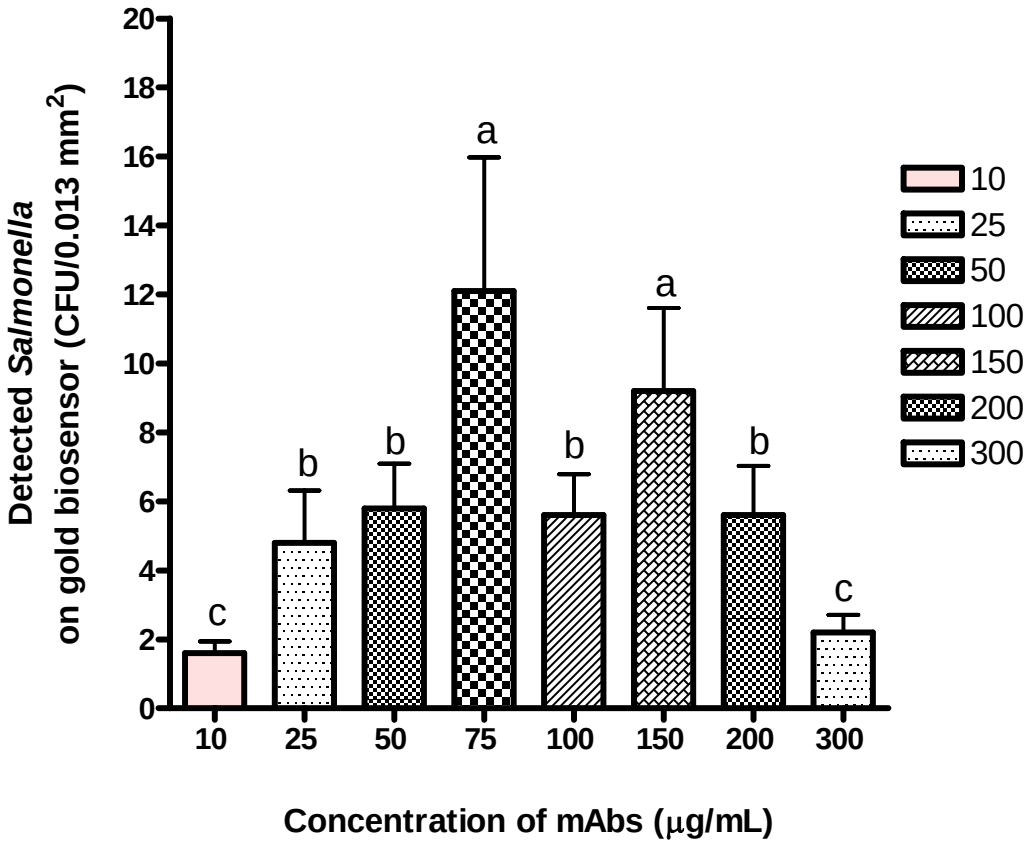

Figure 14-The effect of monoclonal antibodies (mAbs) concentrations on gold biosensor for *Salmonella* detection. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions. Inoculated population of *Salmonella* cocktail: 1.6×10^7 CFU/sensor. Different letters (a,b,c) within same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviation (N=10).

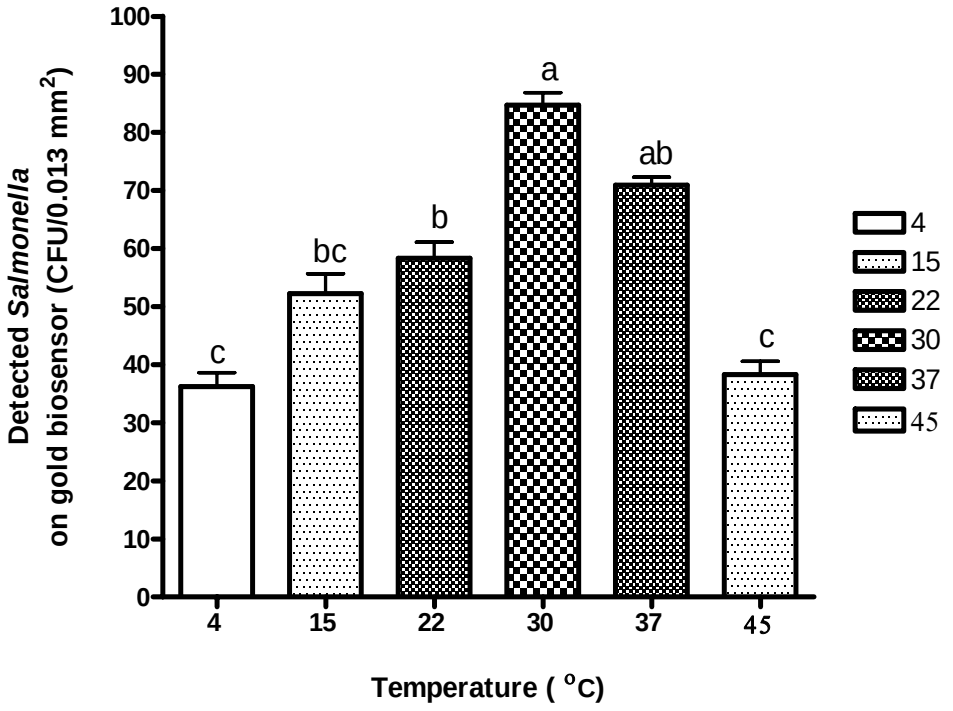

Figure 15-The effect of incubation temperature on antibody immobilized gold biosensor for *Salmonella* detection. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions. Inoculated population of *Salmonella* cocktail: 1.9×10^7 CFU/sensor. Different letters (a,b,c) within same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviation (N=10).

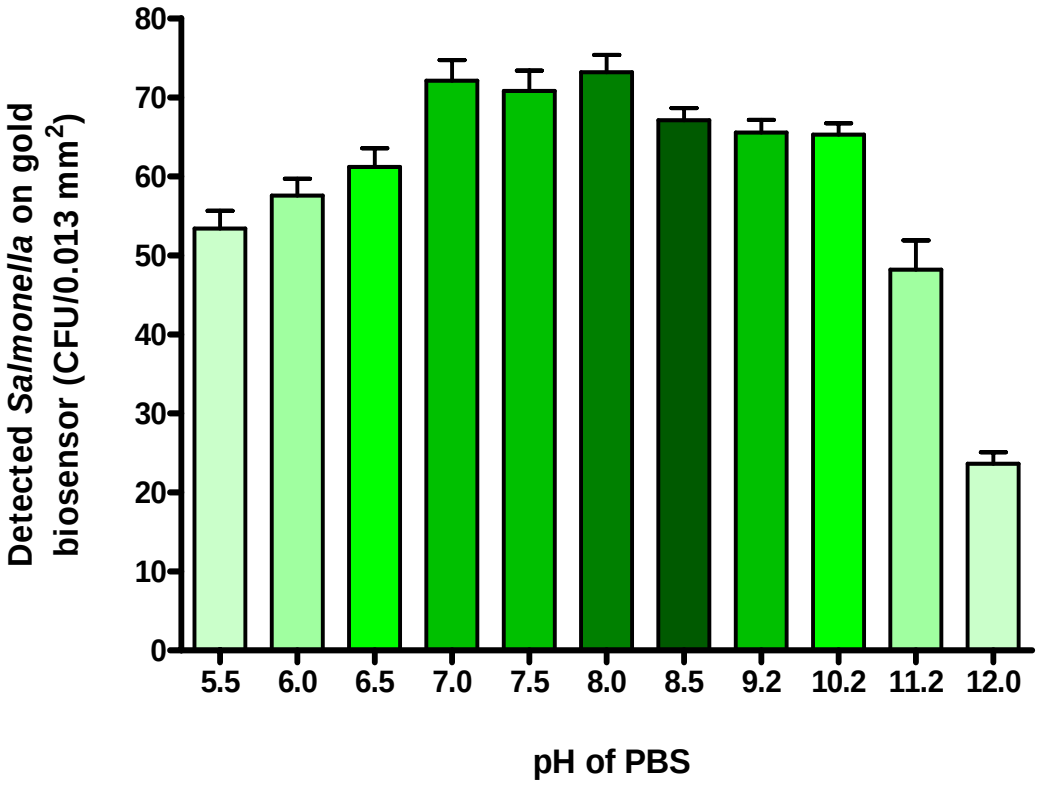

Figure 16-The effect of PBS pH on antibody immobilized gold biosensor for *Salmonella* detection. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions. Inoculated population of *Salmonella* cocktails: 1.9×10^7 CFU/sensor. Vertical bars represent standard deviation (N=10).

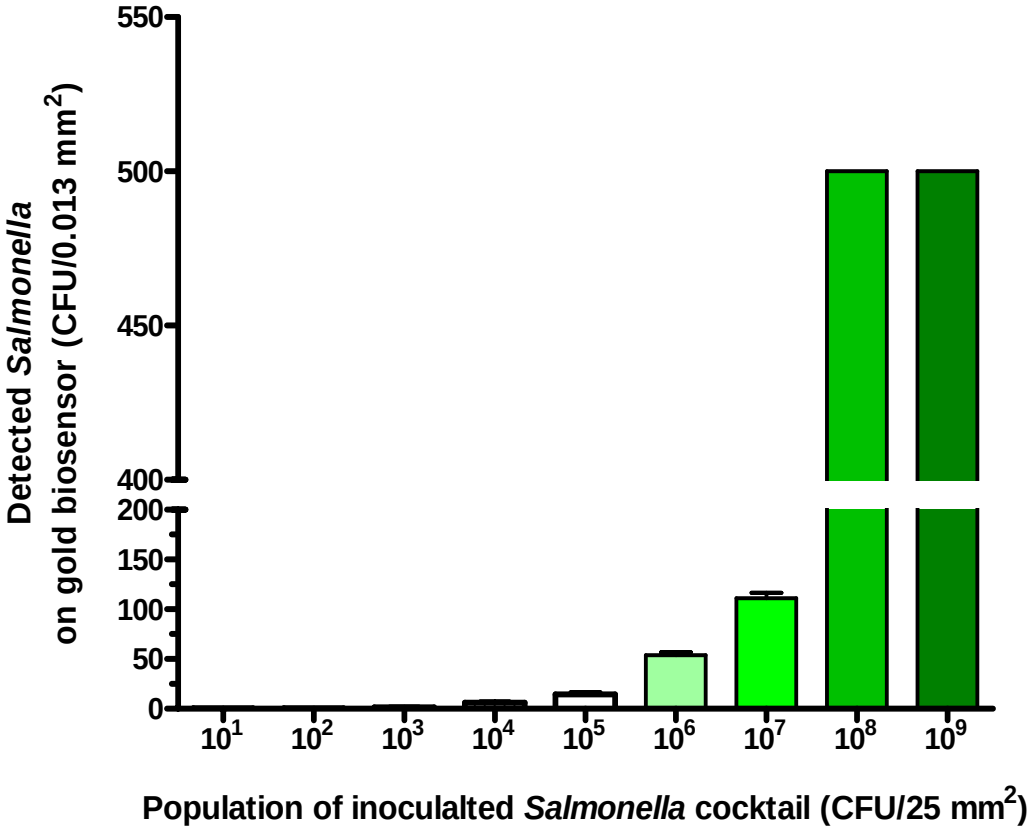

Figure 17-The detected *Salmonella* by gold biosensor with light microscope imaging system at various bacterial populations inoculated chicken skins. Vertical bars represent standard deviation (N=10).

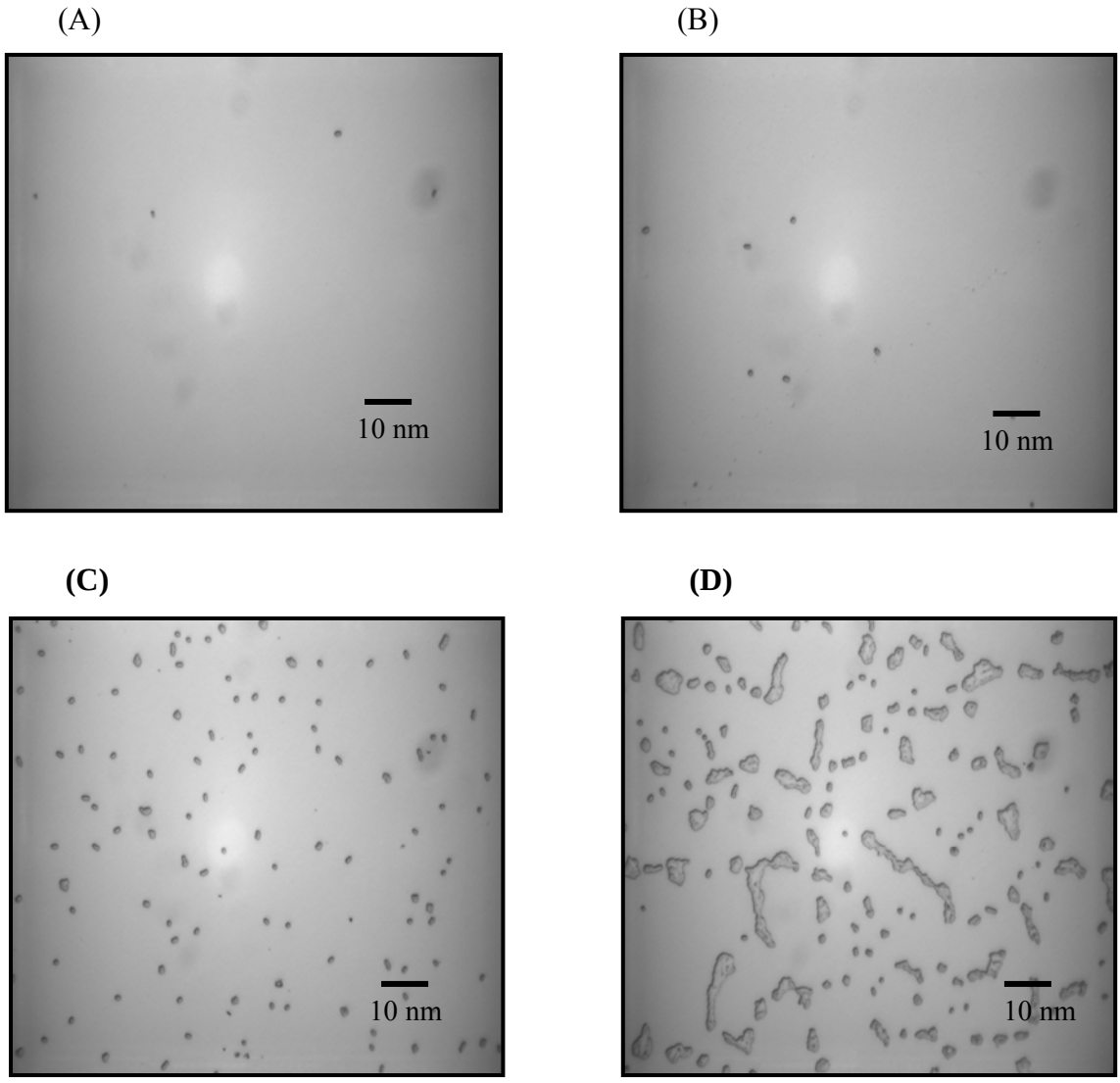

Figure 18-The *Salmonella* bound on gold biosensor photographed by light microscope imaging system at various bacterial populations. The populations of *Salmonella* cocktail were added onto a gold biosensor at 10^3 CFU (A), 10^5 CFU (B), 10^7 CFU (C), and 10^8 CFU (D).

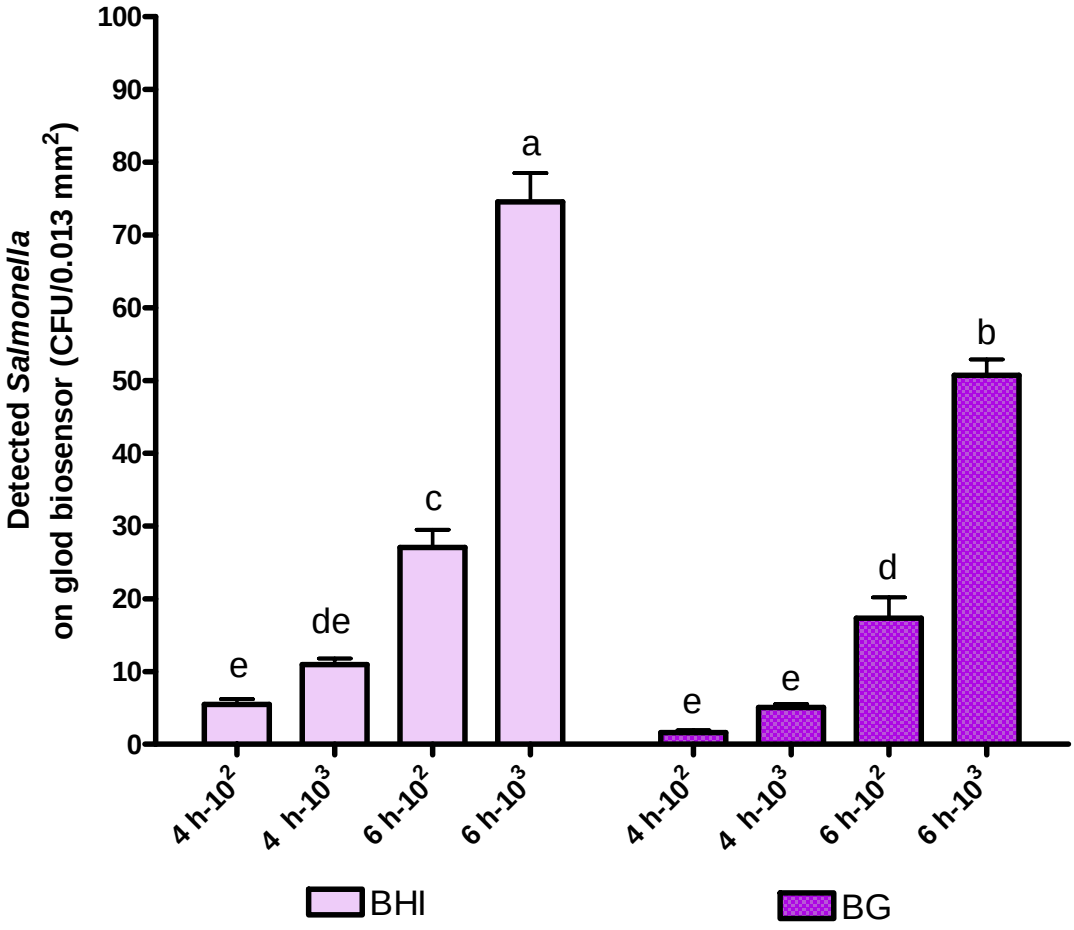

Figure 19-The detected *Salmonella* from inoculated chicken sample enriched in BHI and BG using GB-LMI. *Salmonella* cocktail: *Salmonella enterica* serovar Typhimurium, Enteritidis and Missions. 4 h-10^2 means that the sample was enriched in medium for 4 h after the initial inoculation of 10^2 CFU/16 in^2 chicken skin. 4 h-10^3 means that the sample was enriched in medium for 4 h after the initial inoculation of 10^3 CFU/16 in^2 chicken skin. 6 h-10^2 means that the sample was enriched in medium for 6 h after the initial inoculation of 10^2 CFU/16 in^2 chicken skin. 6 h-10^3 means that the sample was enriched for 6 h after the initial inoculation of 10^3 CFU/16 in^2 chicken skin. Different letters (a,b,c,d,e) within the same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviation (N=15).

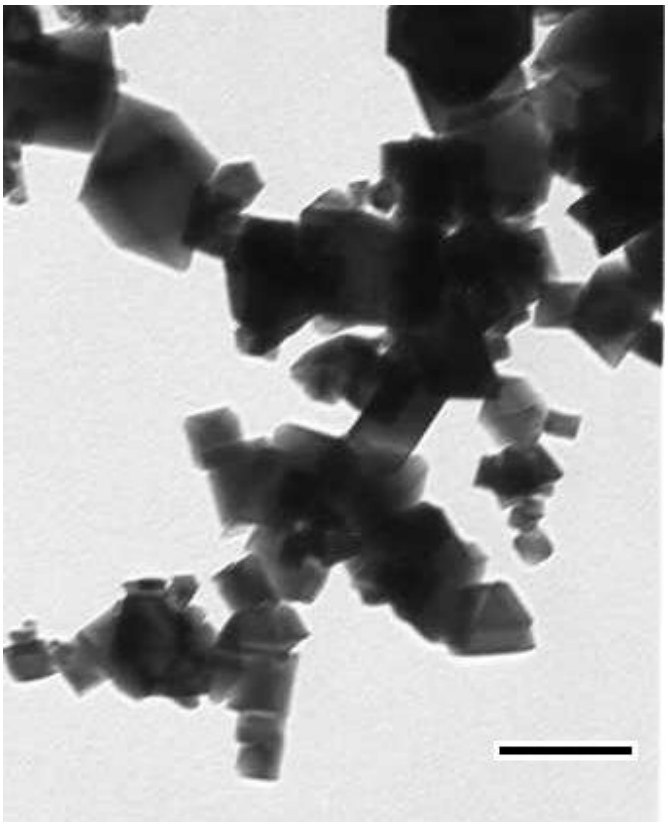

Figure 20-Transmitting electron photography of synthesized magnetic beads. The bar at the right bottom represents 100 nm.

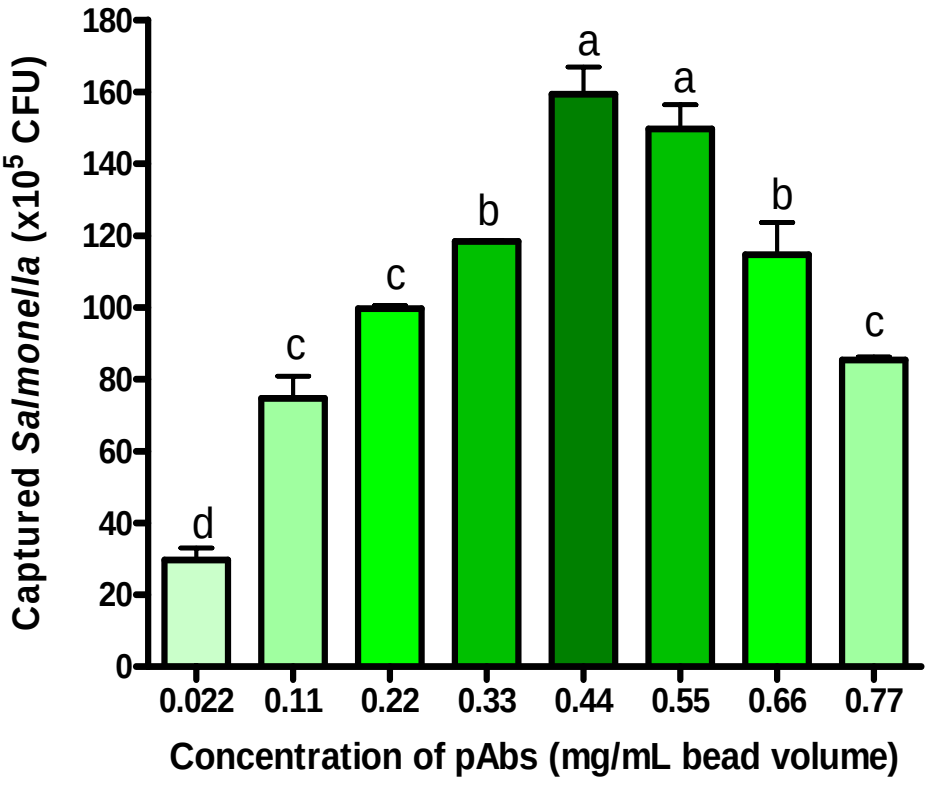

Figure 21-The number of captured *Salmonella* on immunomagnetic beads immobilized at various concentrations of polyclonal antibodies. *Salmonella* cocktail: *Salmonella enterica* serovar Thyphimurium, Enteritidis and Missions *Salmonella* cocktail populations were 2.0×10^9 CFU/each test tube. Different letters (a,b,c,d) within the same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviation (N=3).

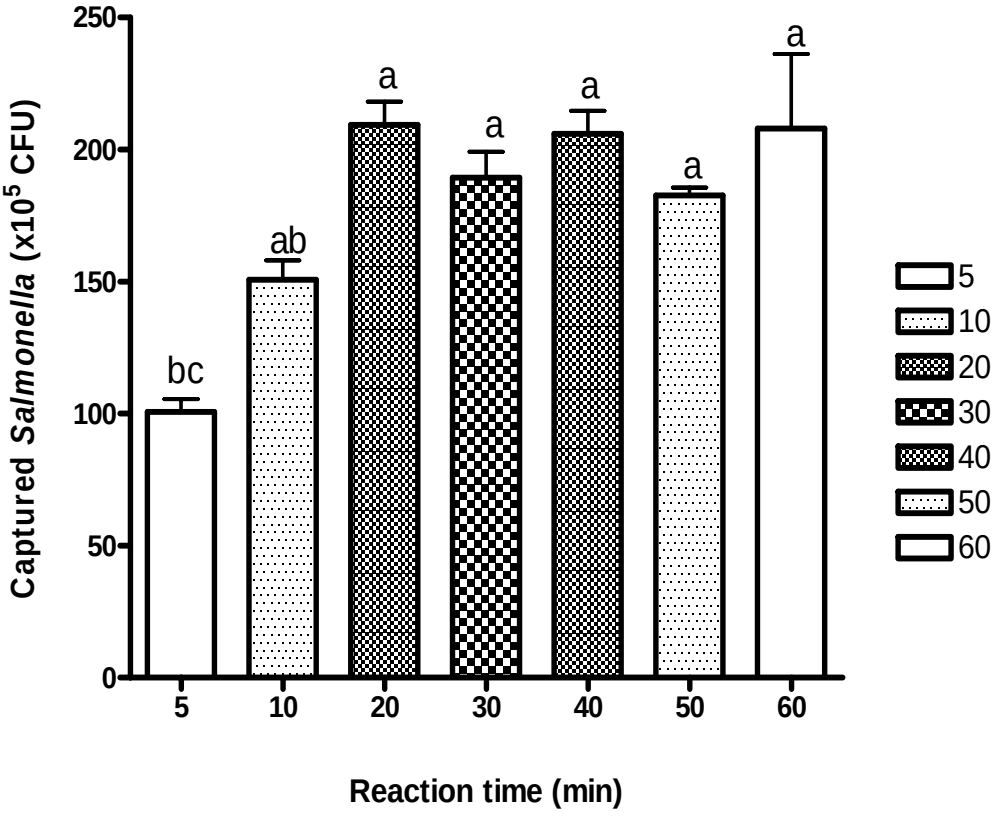

Figure 22-The number of captured *Salmonella* on polyclonal antibodies immobilized immunomagnetic beads at various reaction times. *Salmonella* cocktail: *Salmonella enterica* serovar Thyphimurium, Enteritidis and Missions. *Salmonella* cocktail populations were 2.0×10^9 CFU/each test tube. Different letters (a,b,c) within the same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviation (N=3).

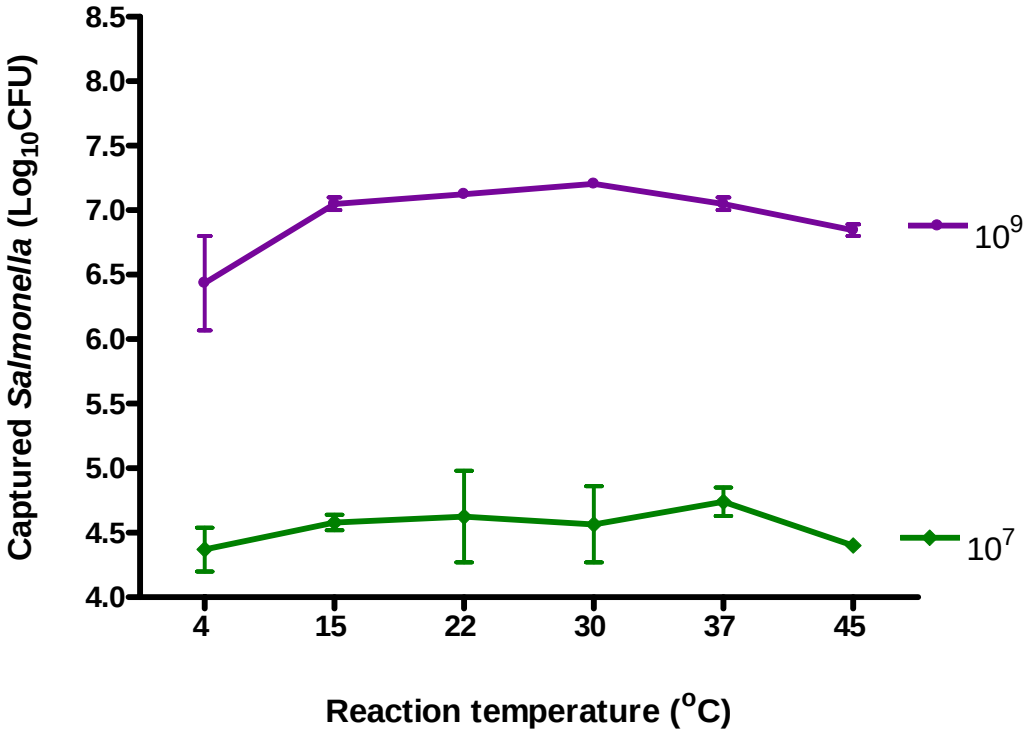

Figure 23-The number of captured *Salmonella* on polyclonal antibodies immobilized immunomagnetic beads at various reaction temperatures. *Salmonella* cocktail: *Salmonella enterica* serovar Thyphimurium, Enteritidis and Missions. Vertical bars represent standard deviation (N=4).

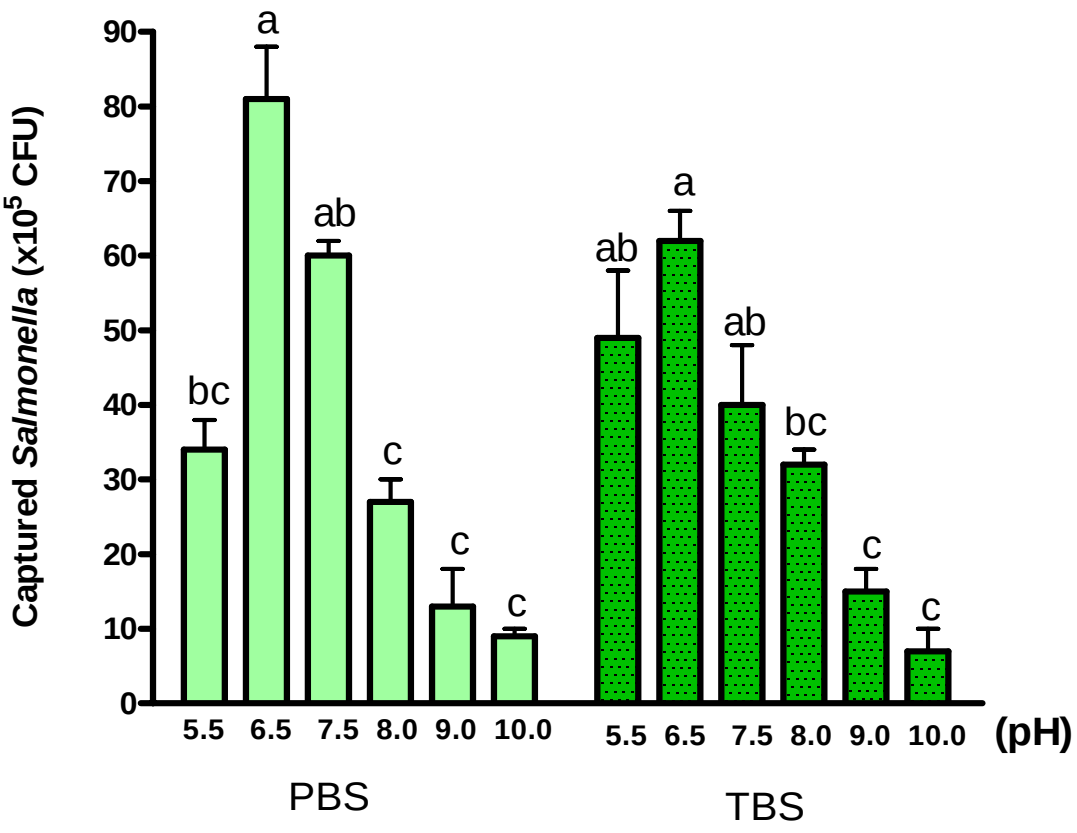

Figure 24-The number of captured *Salmonella* on polyclonal antibodies immobilized immunomagnetic beads at various pHs in PBS or TBS buffer. *Salmonella* cocktail: *Salmonella enterica* serovar Thyphimurium, Enteritidis and Missions. *Salmonella* cocktail populations were 1.5×10^8 CFU/each test tube. Different letters (a,b,c) within the same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviation (N=3).

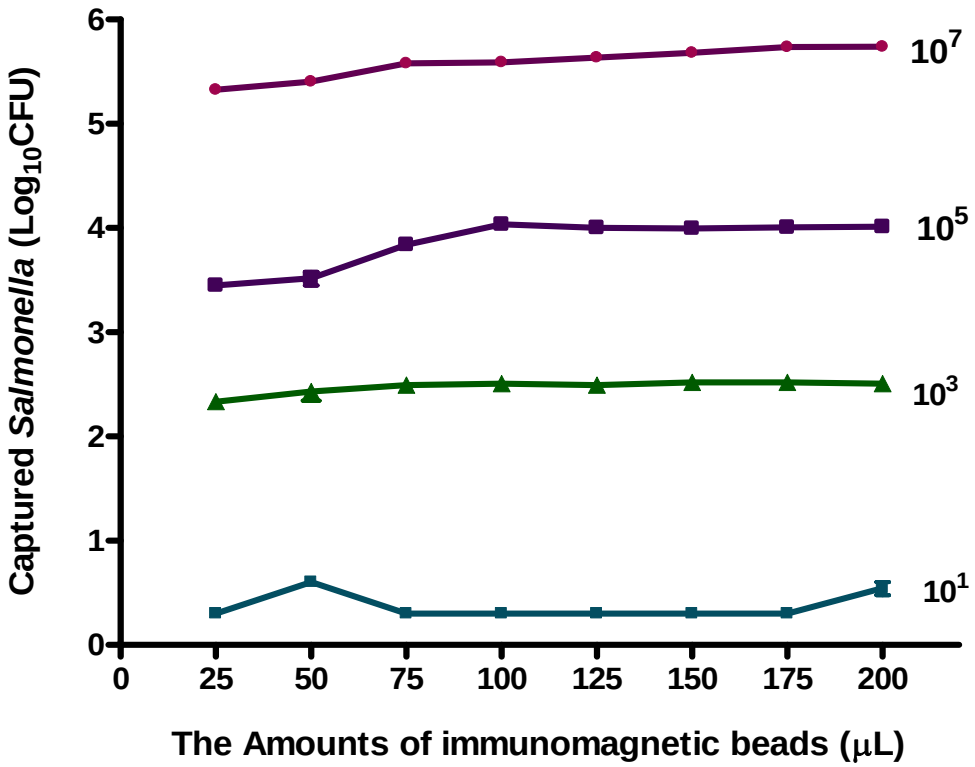

Figure 25-The number of captured *Salmonella* on various amounts of polyclonal antibodies immobilized immunomagnetic beads. *Salmonella* cocktail: *Salmonella enterica* serovar Thyphimurium, Enteritidis and Missions. Vertical bars represent standard deviation (N=3).

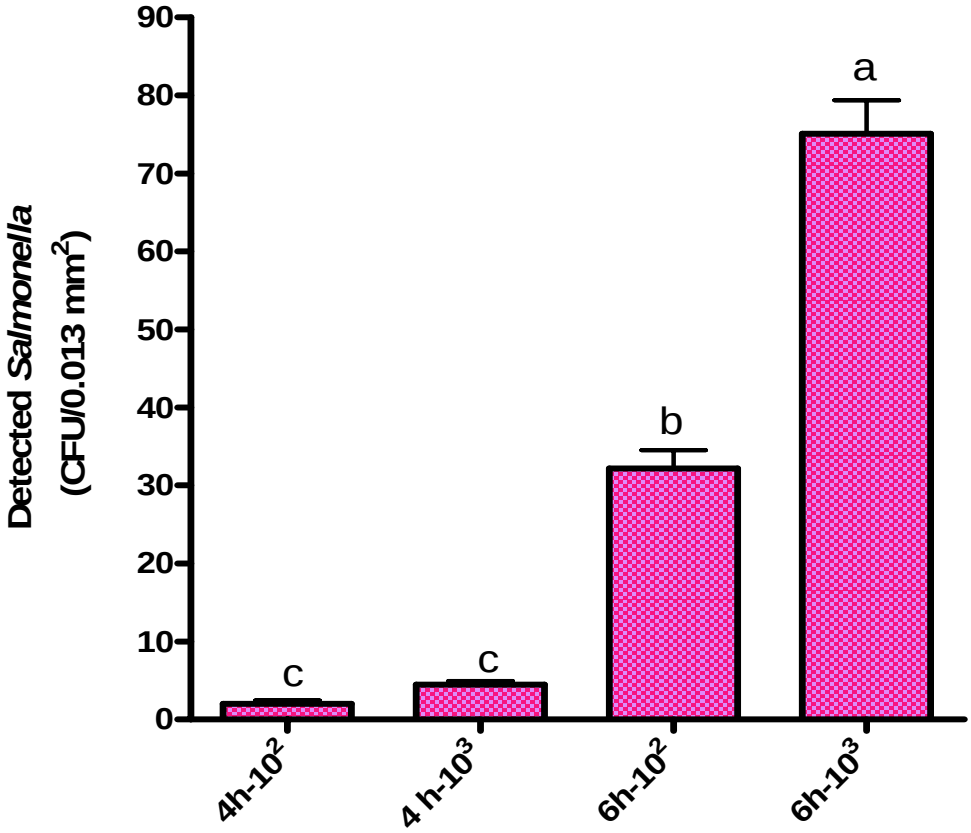

Figure 26-The detected *Salmonella* from inoculated chicken samples by gold biosensor with light microscopic imaging system after immunomagnetic beads separation. *Salmonella* cocktail: *Salmonella enterica* serovar Thyphimurium, Enteritidis and Missions. 4h-10^2 means that the sample was enriched in BHI medium for 4 h after the initial inoculation of 10^2 CFU/16 in^2 chicken skin. 4hr-10^3 means that the sample was enriched in BHI medium for 4 h after the initial inoculation of 10^3 CFU/16 in^2 chicken skin. 6h-10^2 means that the sample was enriched in BHI medium for 6 h after the initial inoculation of 10^2 CFU/16 in^2 chicken skin. 6hr-10^3 means that the sample was enriched in BHI medium for 6 h after the initial inoculation of 10^3 CFU/16 in^2 chicken skin. Different letters (a,b,c) within the same group indicate significantly different means among treatments at $p < 0.05$. Vertical bars represent standard deviation (N=15).

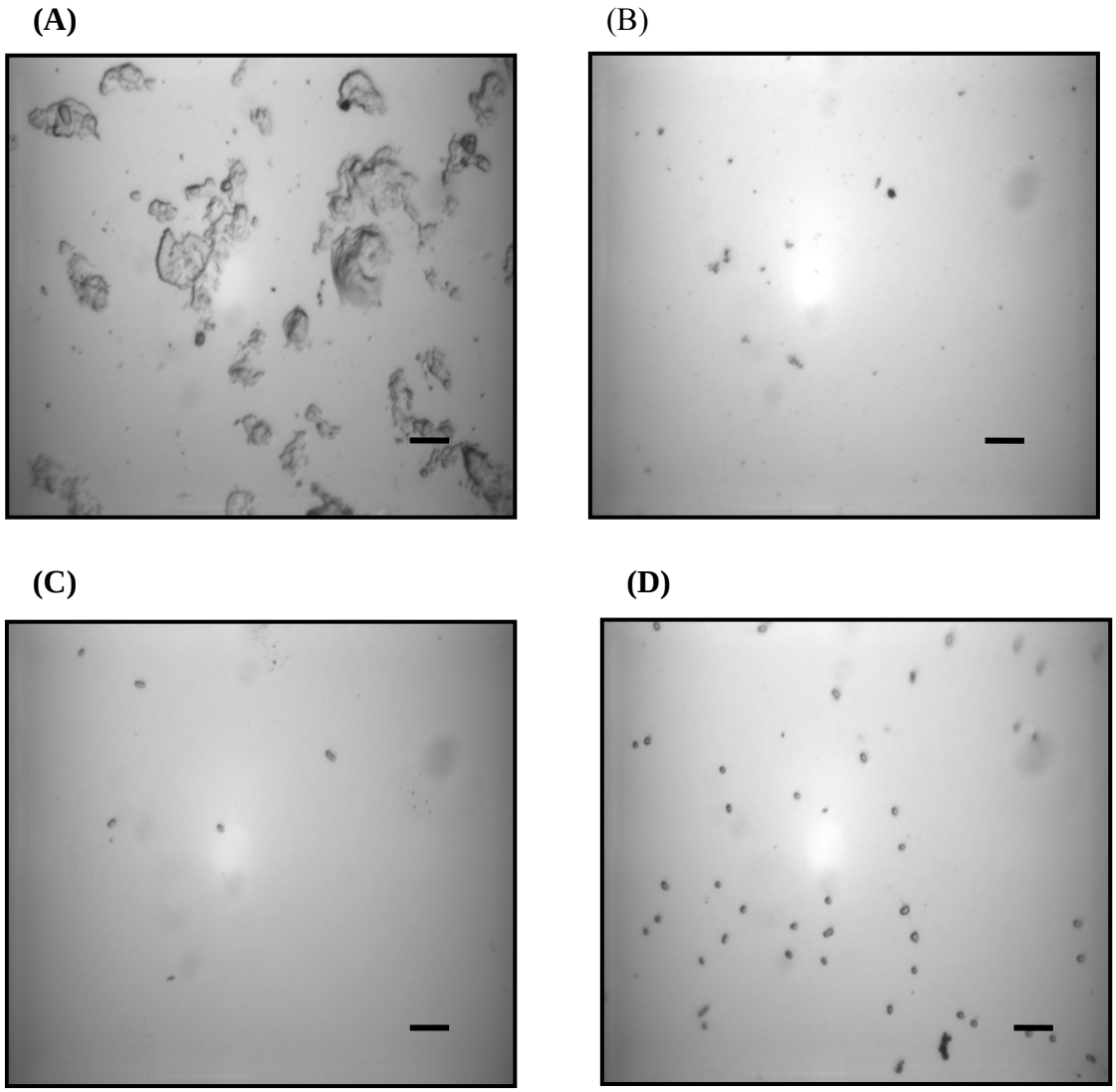

Figure 27-The *Salmonella* bound on gold biosensor photographed by light microscope imaging system at various bacterial treatments. (A) Sample was enriched in BHI medium for 4 h after the initial inoculation of 10^3 CFU/16 in^2 chicken skin and added onto gold biosensor, (B) PBS buffer after treated with IMS was added onto gold biosensor instead of Salmonella culture, (C) Sample was enriched in BHI medium for 4 h after the initial inoculation of 10^3 CFU/16 in^2 chicken skin, treated with IMS and then added onto gold biosensor, (D) Sample was enriched in BHI medium for 6 h after the initial inoculation of 10^3 CFU/16 in^2 chicken skin, treated with IMS and then added onto gold biosensor. Bars mean 10 nm.

Table 2- Specificity of polyclonal antibodies for pathogenic bacteria assayed by indirect ELISA

(n=6, mean ± SD)

Bacteria	Absorbance	Bacteria	Absorbance
S. Abony (SH 465)	1.774 ± 0.013	*S*. Typhimurium (SA 14121)	1.653 ± 0.132
S. Arizonae (SA 4407)	0.852 ± 0.036	*S*. Dublin	1.164 ± 0.152
S. Bongori (SA 4410)	1.008 ± 0.123	*S*. Panama	1.190 ± 0.090
S. Diarizonae (SA 4408)	0.469 ± 0.012**	*S*. Typhi	1.557 ± 0.122
S. Cerevisae (SA 3004)	1.175 ± 0.007	*S*. Enteritidis	1.250 ± 0.110
S. Dublin (RKS 4699)	0.846 ± 0.020	*S*. Montevideo	0.893 ± 0.014
S. Gallinarum (SA 4404)	0.997 ± 0.001	*L.monocytogenes* (H7757)	0.339 ± 0.013**
S. Houtenae (SA 4409)	1.002 ± 0.153	*L.monocytogenes* (H7738)	0.285 ± 0.010**
S. Indica (SA 4411)	1.082 ± 0.050	*E. coli* (GM 2163)	1.470 ± 0.018
S. Heidelberg (SARA 36)	1.166 ± 0.194	*E. coli* O157:H7 (204P)	1.109 ± 0.063
S. Montevideo (SARB 31)	1.171 ± 0.004	*S. aureus*(ATCC 12600)	0.121 ± 0.250**
S. Paratyphi A (R737)	0.874 ± 0.130	*S. aureus*(ATCC 6538)	0.259 ± 0.0146**
S. Salamae (SA 4406)	0.803 ± 0.103	*S. Typhimurium	1.695 ± 0.042
S. Pullorum (SARB 52)	1.288 ± 0.054	*S. Enteritidis	1.721 ± 0.042
S. Mission	1.001 ± 0.100	*S. Missions	1.499 ± 0.158
S. Paratyphimurium UF	1.452 ± 0.221		

* indicates *Salmonella* strains used in this study as nalidixic acid resistant strains.
** indicates bacteria exhibiting the absorbance less than 0.5.
The polyclonal antibodies were diluted at 1/800 from the original concentration (6.5 mg/mL).
The absorbance was measured at 405 nm.
E. coli O157:H7 (204 P)- new measure 0.285 ± 0.012, E. coli (GM 2163)= 0.458 ± 0.111
E. coli K12 (NEB Turbo)- 0.358 ± 0.059 E. coli ER 2738 = 0.377 ± 0.101

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