DEVELOPMENT OF A BIOSENSOR FOR THE RAPID DETECTION OF

SALMONELLA TYPHIMURIUM IN MILK

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DEVELOPMENT OF A BIOSENSOR FOR THE RAPID DETECTION OF *SALMONELLA* TYPHIMURIUM IN MILK

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DISSERTATION ABSTRACT

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Foodborne illnesses are still serious problems in the United States. Methods for rapid detection of foodborne pathogens are needed to protect public health. The recent advances in biosensor technology enabled the rapid detection of pathogens. The purpose of this research was to develop a biosensor for the rapid detection of *Salmonella* in milk.

In the first study, a 55 kD purified outer membrane protein (OMP) of *S. enterica* Typhimurium was used to produce monoclonal and polyclonal antibodies. The *S. enterica* Typhimurium specific monoclonal antibody (mAb) 1B4 was immobilized on a sensor platform as a bacterial capture agent, and the captured *S. enterica* Typhimurium was visualized by a light microscope. The developed biosensor has a detection limit of 10^2 cfu/ml.

In the second study, one step enrichment in conjunction with a centrifugation technique was investigated. In order to increase the relatively low number of *Salmonella* in milk, 7 non-selective and 5 selective enrichment media were compared. Among these media, brain heart infusion broth (BHI) and brilliant green broth (BG) were the most efficient. These media were able to increase *Salmonella* concentration from 0.01 cfu/ml to above 10^2 cfu/ml in 6 h. The centrifugation technique used in this study was able to concentrate bacteria up to 50 times. In conjunction with centrifugation, enrichment in BG or BHI for 4 h could increase *Salmonella* concentration from 0.01 cfu/ml to a detectable level. The milk content, excessive amounts of other bacteria, and enrichment broth content did not affect the detection sensitivity and specificity of the developed biosensor.

The developed biosensor was found to be a rapid and sensitive method for the detection of *S. enterica* Typhimurium in milk. Total time needed for the detection of 0.1 cfu/ml *S. enterica* Typhimurium in milk was 5 h.

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CHAPTER I. INTRODUCTION

Foodborne illnesses affect millions of American each year. The presence of *Salmonella* in foods is a great concern to the food industry, the public, and the regulatory agencies. In the United States, the number of cases of foodborne illnesses due to *Salmonella* is the highest of any reported gastrointestinal infection (CDC 2005). Each year, an estimated 1.4 million people contract salmonellosis in the United States (Mead and others 1999). Outbreaks of salmonellosis associated with milk continue to occur (Ryan and others 1987, Olsen and others 2004). The medical cost of all foodborne illnesses in the US is 5 – 6 billion dollars each year and salmonellosis alone accounts for 1 billion dollars. Rapid and sensitive methods for the detection of relatively low incidence of *Salmonella* in foods, including milk, are of urgent need.

Several methods have been developed to detect the presence of potentially low levels of *Salmonella* in foods. These methods include culturing techniques, bacteriophages (Goodridge and Griffiths 2002; Hirsh and Martin 1983, 1984), nucleic acid-based methods (Fitts and others 1983, Fitts 1985, Fluit and others 1993, McElroy and others 1996, Kwang and others 1996), immunoassays (Mattingly 1984, Ibrahim and others 1986, Hadfield and others 1987), and biosensors (Ye and others 1997, Babacan and others 2002, Bokken and others 2003, Kramer and Lim 2004, Taitt and others 2004). Standard culture methods for *Salmonella* detection involve multi-steps which are labor intensive and time-consuming, taking 4 to 5 days for detection and confirmation (FDA 1992). Furthermore, it has been reported that some of the routinely used selective

enrichment broths are inhibitory towards *Salmonella* (Van der Zee 1994). The nucleic acid-based methods, and bacteriophage methods have shown considerable success by reducing the detection time to 2-3 days, but none of them has been identified as fast, specific, and user-friendly for routine use in screening food samples.

Immunoassay tests have been regarded as an effective tool for microorganism detection and identification. Several formats of immunoassays have been developed, including radioimmunoassay (RIA), fluorescent immunoassay (FIA), and enzyme linked immunosorbent assay (ELISA). Among these, ELISA was regarded as the most promising one. Several types of enzyme-linked immunosorbent assays (ELISAs) have been developed, using either polyclonal antibodies (pAb) or monoclonal antibodies (mAb) that are able to detect most *Salmonella* serotypes. The majority of the developed antibodies are specific to either O-antigen or H-antigen (NG and others 1996, Wang and others 1996, Jaradat and Zawistowski 1996). Some of these methods produce false-positive results due to the cross-reactivity with other enterobacteria (Curiale and others 1990).

Recently, the use of biosensors to detect microorganisms has attracted the public's attention. Most of the currently developed biosensors for bacterial pathogen detection are based on the specific antigen-antibody binding reactions, where the antibody is immobilized on the sensor platform to capture the bacteria that are of interest. Then the detection is measured through electrochemical, optical, or piezoelectrical signals. These biosensors include piezoelectrical biosensors (Prusak-Sachaczewski and others 1990, Si and others 1996, Ye and others 1997, Babacan and others 2002), fiber optic biosensors (Kramer and Lim 2004), optical surface plasmon resonance biosensors (Bokken and

others 2003), bienzyme electrochemical biosensors (Yang and others 2001). The detection limit of these biosensors is high, ranging from 10^5 cfu/ml to 10^7 cfu/ml. Therefore, more sensitive biosensors are in urgent need to detect relatively low numbers of pathogens in foods.

The advancement in biosensor technology requires highly specific antibodies to reduce detection interference. *Salmonella* are antigenically complex and serotypes have been differentiated by somatic lipopolysaccharide (LPS) or flagellar protein antigens (Le Minor 1984). The outer membrane proteins (OMPs) of *Salmonella* are also known to have an important role in evoking immune responses (Kim and others 1991; Meenakshi and others 1999). There appears to be a potential to develop species or serotype specific antibodies from *Salmonella* OMPs.

Due to the low incidence of *Salmonella* in foods, preenrichment, enrichment, and sometimes even a post-enrichment were involved in rapid detection methods (Flowers and others 1988). One step enrichment has been investigated prior to PCR, ELISA, and biosensor assays for the detection of pathogens in foods. Different enrichment media have been investigated on their efficacy of rapid culturing of pathogens in foods (Kwang and others 1996, Eyigor and others 2002, Fluit and others 1993, Prusak-Sochaczewski and Luong 1989). The total detection time required by their methods was more than 24 h. Lack of unanimity on the selection of media for one step enrichment is still a problem.

Methods for separation and concentration of *Salmonella* from foods using immunomagnetic separation (IMS), filtration, or centrifugation have been investigated. IMS uses antibody coated magnetic particles to separate and concentrate microbes from foods (Fluit and others 1993, Cerro and others 2002). The membrane filtration technique

for the capture of microbes has been investigated since 1951 (Goetz and Tsuneshi). The hydrophobic grid membrane filter (HGMF) technique has been used for bacterial enumeration since 1974 (Sharpe and Michaud). Centrifugation has been studied as a means of concentration of microbes by researchers and regarded as an effective way of concentrating microbes (Hawa and others 1984, McElroy and others 1995, McElroy and others 1996, Cerro and others 2002).

In this study, purified *Salmonella* OMP were used as antigens for the production of specific pAb and mAb. The *Salmonella* specific mAb was used to construct a novel biosensor, which combines an antibody immobilized sensor platform with microscopic imaging systems to accurately and sensitively detect *Salmonella* in milk. One step enrichment in conjunction with centrifugation was investigated to quickly culture and concentrate *Salmonella* to a detectable level.

CHAPTER II. LITERATURE REVIEW

Salmonella general characteristics

Foodborne illnesses are a worldwide serious public health problem. The Centers for Disease Control and Prevention (CDC) estimates that 76 million persons contract foodborne illnesses, 325,000 are hospitalized and 5,000 die each year due to foodborne illnesses in the United States (Mead and others, 1999). Foodborne illnesses have been estimated to cost as much as $23 billion annually in this country (Jones and Gerber, 2001). The annual cost of foodborne illnesses caused by the 4 most common bacterial pathogens alone (*Salmonella*, *Shigella*, *Campylobacter*, and *E*. coli) is $6.9 billion (Allos and others, 2004). In 2004, a total of 15,806 laboratory-diagnosed cases of infections in the FoodNet surveillance areas were reported and the top nine pathogens were identified as *Salmonella*, 6,464; *Campylobacter*, 5,665; *Shigella*, 2,231; *Cryptosporidium*, 613; STEC O157, 401; *Yersinia,* 173;Vibrio, 124; *Listeria*, 120; and *Cyclospora*, 15 (CDC, 2005). These data indicates that *Salmonella* is still the leading cause of foodborne illnesses in the US.

Each year, approximately 40,000 cases of salmonellosis are reported in the United States. Because many milder cases are not reported, the actual number of infections may be thirty or more times greater which would be about 2 to 4 million each year (CDC 2005). It is estimated that approximately 600 people die each year with acute salmonellosis. *Salmonella* is a group of bacteria and has over 2300 serotypes. In 2004,

five serotypes accounted for 56% of infections. They are as follows: Typhimurium, 20%; Enteritidis, 15%; Newport, 10%; Javiana, 7%; and Heidelberg, 5% (CDC, 2005).

Salmonella has been known to cause gastrointestinal illness in humans for over 100 years. They were discovered by an American scientist named Salmon, for whom they are named. They are gram-negative, non-spore forming rods, motile (with few exceptions), and microscopic living creatures. They are widely distributed in nature. Humans and animals are their primary reservoirs. They live in the intestinal tract of humans and other animals, including birds. They can pass from the feces of humans and animals to other people or other animals. Salmonellosis results mainly from the ingestion of *Salmonella* contaminated foods. The infective dose varies with different strains, host health and age with the elderly, infants and immunocompromised people being more easily infected. Contaminated foods are often of animal origin, such as beef, poultry, milk, or eggs, but all foods, including vegetables can become contaminated.

S. Typhi and the paratyphoid bacteria cause septicemic and typhoid or typhoid-like fever. Other forms of salmonellosis generally produce milder symptoms, which include diarrhea, fever, and abdominal cramps 12 to 72 hours after ingestion of contaminated foods. The illness usually lasts 4 to 7 days, and most people recover without treatment. However, in some people, such as infants, the elderly, and the infirm, the diarrhea may be so severe that the patient needs to be hospitalized. In these cases, the *Salmonella* infection may spread from the intestines to the blood stream, and then to other body sites and result in death. A small number of people who are infected with *Salmonella* will develop Reiter's syndrome, which includes pains in joints, irritation of

the eyes, and painful urination. It can last for months or years, and can lead to chronic arthritis.

Salmonellosis outbreaks

Large outbreaks of salmonellosis typically occur at banquets or similar circumstances. However, the two largest outbreaks occurred under unusual circumstances. The largest outbreak occurred in 1994, which involved more than 224,000 people (Jay 2000). The vehicle food was ice cream produced from contaminated milk. The contamination occurred in milk transported in a tanker truck which previously hauled liquid eggs. *S*. Enteritidis was identified as the etiological agent.

The second largest outbreak occurred in 1985 which involved around 200,000 people (Ryan and others 1987). The vehicle food was milk pasteurized in a Chicago dairy plant. *S*. Typhimurium was identified as the etiological agent. The U.S. Food and Drug Administration (USFDA) inspectors discovered that modification of the pasteurization equipment, which was to facilitate the running off of raw milk, resulted in the cross contamination of the pasteurized milk with raw milk.

Currently developed methods

1. Standard culture methods

Standard culture methods for the detection of *Salmonella* in foods include: preenrichment (16-20 h) to allow the resuscitation and multiplication of sub-lethally damaged cells; selective enrichment (18-48 h) to increase the ratio of *Salmonella* to other organisms; and plating on selective/differential media (24-48 h) to enable the recognition

of *Salmonella* colonies; and subsequent serological or biochemical identification (4-48 h) of suspected colonies (Litchfield, 1973).

1.1. Sampling methods

Because interpretations of a large consignment of food are based on a relatively small sample of the lot, representative sampling is essential and the adequacy and condition of the sample are of great importance when pathogens are sparsely distributed in the foods. The use of sterile sampling equipment and aseptic techniques should under no circumstances be compromised. Samples should be delivered to the laboratory promptly maintaining as nearly as possible the original storage condition. Usually a 25 g analytical unit is diluted at a 1:9 sample/broth ratio and mixed in a homogenizer, stomacher or pulsifier. Samples may be composit. The mixing processes are used to ensure that the bacteria are completely released from the food matrix and adequately dispersed before the pre-enrichment stage.

1.2. Preenrichment media

The first step of conventional culture methods for the detection of *Salmonella* is preenrichment. One of its primary functions is to rehydrate bacteria that have been dehydrated during the food processing steps. Preenrichment also repairs damaged cells to a stable physiological condition. The preenrichment medium serves as a nutrient source for the proliferation of the rejuvenated cells. Because non-selective, highly nutritious media are used at this stage, there is a risk of overgrowth of other contaminants which could actually inhibit the growth of *Salmonella*. Therefore, the timing and temperature are important at this stage to ensure recover of *Salmonella*. Usually an 18-24 h incubation at 37 ºC is used.

Preenrichment media vary widely in nutritive values. Lactose broth (LB), trypticase soy broth (TSB), reconstituted nonfat dry milk, brain heart infusion broth (BHI) and buffered peptone water (BPW) are considered to be nutritionally complex and are recommended by various organizations (Andrews 1985). However, some research data indicate that less nutritive broth, such as M-9 broth, would favor the recovery of sublethally injured *Salmonella*.

1.3. Selective enrichment media

The second step of conventional methods for the detection of *Salmonella* is selective enrichment. Normally, *Salmonella* is only a small portion among competing microorganisms in foods and can be lost during selective enrichment. Therefore, successful selective enrichment is the decisive step in the detection of *Salmonella*. Numerous selective enrichments have been developed and great efforts have been made in recent years to improve media and procedures for selective enrichment. Media containing either selenite or tetrathionate plus brilliant green and bile salt or malachite green in combination with high amounts of magnesium chloride are the major selective enrichment media for *Salmonella*. These media can be distinguished into three groups: selenite broth (Leifson 1936), tetrathionate broth (Muller 1923, Kauffmann 1930), and the Rappaport family.

Selenite broth, originated by Leifson (1936), is used as a selective enrichment for the cultivation of *Salmonella* that may be present in small numbers, competing with other microbes. Enzymatic digestion of casein and enzymatic digestion of animal tissue are used as nitrogen and vitamin sources. Selenite is the selective agent, which inhibits the growth of coliform bacteria and enterococci in the first 6-12 hours of incubation, its

inhibitory effect then gradually declines. Lactose is the fermentable carbohydrate. A rise in pH decreases selectivity of selenite. The acid produced by lactose fermentation helps to maintain a neutral pH. Sodium phosphate is also used as a buffering agent to maintain neutral pH of the broth. *Salmonella, Proteus* and *Pseudomonas* are only slightly inhibited by selenite. Selenite cystine broth is based on the formulation of selenite broth with the addition of L-cystine, which is a reducing agent.

Tetrathionate is produced from thiosulfate by adding iodine to the medium. Tetrathionate and thiosulfate suppress coliform and other enteric bacteria, whereas tetrathionate-reducing bacteria, such as *Salmonella* and *Proteus*, can grow in this medium. Calcium carbonate neutralizes the acidic products which are formed from the decomposition of tetrathionate. Bile salts inhibit bacteria which do not normally inhabit in the intestine. Brilliant green suppresses the Gram-positive bacteria. Novobiocin may be added at 40 mg/l to suppress the growth of *Proteus* (Jeffries 1959).

Rappaport broth was proposed by Rappaport and others (1956) for the selective enrichment of *Salmonella* (with exception of *S.* Typhosa). The original Rappaport medium contains high amounts of malachite green and magnesium chloride and has a very low pH of 5.2. Malachite green and magnesium chloride inhibit the growth of the other intestinal microorganisms, but do not affect the proliferation of most *Salmonella*. Magnisium chloride was shown to counteract the toxic effect of the dye for *Salmonella*. *S.* Typhosa and *Shigella* are inhibited by this broth. Vassiliadis and others (1976) modified the medium by reducing the concentration of the dye. Rappaport-Vassiliadis medium (RV) has been regarded as a useful media beside selenite and tetrathionate broths. Based on the completion of AOAC precollaborative and collaborative studies,

Rappaport-Vassiliadis medium is being recommended for the analysis of high microbial and low microbial load foods by FDA (2003). FDA also recommends that Rappaport-Vassiliadis medium replaces selenite cystine broth for the analysis of almost all foods (FDA 2003). Vassiliadis and coworker (1976) recommended incubation of RV at 43 ºC to achieve maximum selectivity. However, any deviation above 43 ºC may be lethal for *Salmonella*. For this reason, the ISO recommendation to incubate at 42 ºC is followed by most researchers (Busse 1995).

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

Motility enrichment is an old technique (Craigie 1931) and was originally performed in U-tubes where one side was inoculated and after a certain incubation period fast moving bacteria could be isolated from the other side. Goosens and others (1984) replaced U-tubes with Petri dishes. Motility enrichment on Modified Semisolid Rappaport-Vassiliadis medium (MSRV) was introduced by De Smedt and others (1986) and has been regarded as a very effective procedure for the isolation of *Salmonella* from foods (Boer 1998). In this media, motility of competitive bacteria is inhibited while *Salmonella* move into fresh media leaving their competitors behind.

1.4. Plating media

Selective plating media for the isolation of *Salmonella* can be divided into three groups according to the selective agents used. These groups are the bile salt agars, the

brilliant green agar (BGA), and bismuth sulfite agar (BSA). The bile salt agars include deoxycholate citrate agar (DCA) originally proposed by Leifson (1935), xylose lysine desoxycholate citrate agar (XLD) (Taylor 1965), Salmonella-Shigella agar (SS) (Pollock and Dahlgren 1974), and Hektoen Enteric agar (HE) (King and Metzger 1968). These media contain various amounts of citrate and bile salts as selective substances. Thiosulphate is added to most of these agars both as a source of hydrogen sulfide to detect hydrogen sulfide positive colonies and as a selective agent (Busse 1995). Tate and others (1990) used XLT4, which is essentially a modification of XLD with the addition of a surfactant, Tergitol, and found it to give improved recovery of *Salmonella* from poultry. Recently, a new plating medium belonging to the bile salt family has been proposed by Rambach (1990). Rambach agar is characterized by a new chromogenic indicator system. *Salmonella* is detected by its ability to ferment propylene glycol and produce acid, appearing as bright red colonies. In addition, chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is included to differentiate *Salmonella* from other β-D-galactosidase positive enterobacteria, such as *E. coli* and other coliform bacteria, which ferment lactose and appear as blue colonies. *Proteus* spp. are negative for both reactions and their colonies are colorless, while *Citrobacter* are positive for both reactions and their colonies are violet from the combination of colors resulting from the two reactions. Studies indicate that most *Salmonella* ferment propylene glycol except for *S.* Typhi and occasional representatives of other serotypes (Gruenenwald and others 1991).

On BGA, *Salmonella* is detected by its inability to ferment lactose or lactose and sucrose, appearing as pink colonies. The mannitol lysine crystal violet brilliant green agar

(MLCB) was introduced by Inoue and others (1968) and its selective components are crystal violet and brilliant green.

Bismuth sulphite agar, originally formulated by Wilson and Blair (1927), contains ammonium bismuth citrate and sodium sulphite. *Salmonella* is detected by H_2S formation and bismuth reduction. Rainbow agar Salmonella (Biolog) can isolate and differentiate the widest range of *Salmonella* species, including *S*. Typhi, and can detect the weakest to the strongest H_2S producers.

1.5. Biochemical and serological confirmation tests

A range of biochemical tests are used to confirm the suspected colonies on selective agars. Of these, triple sugar iron agar (TSI) and lysine iron agar (LIA) are the most commonly used for the identification of *Salmonella* in mixed cultures, while urea broth is used for the identification of *Salmonella* in pure cultures (Moats 1981, USFDA 2003). Serological tests may be used to confirm the biochemically screened isolates. Serological polyvalent flagellar (H) tests, serological somatic (O) tests, and Spicer-Edwards serological tests are recommended by USFDA (2003).

1.6. Conclusion of standard culture methods

Although the conventional methods are effective and have been used for many years for isolating and identifying bacterial pathogens in foods, the procedures are labor intense. These methods provide a theoretical level of sensitivity of one *Salmonella* cell per 25 g of food, but detection can be prevented by the presence of other microbes that can compete with *Salmonella* during cultural enrichment. The requirement for time-consuming enrichment steps and biochemical characterization has lengthened most analysis time to several days. Therefore, these methods are inadequate for making timely

assessments on the microbiological safety of foods. In order to overcome these drawbacks, a variety of so called rapid methods have been investigated.

2. Rapid culture methods

Researchers have attempted to reduce the incubation periods for both the preenrichment and selective enrichment. Through comparing the efficiency of preenriching a variety of foods and feed ingredients for 6 and 24 hr in 7 media, D'Aoust and Maishment (1979) found that none of the 7 preenrichment media were adequate when incubated for only 6 hr. Investigating the effect of reducing the incubation period for selective enrichments, researchers (D'Aoust and others 1983, Rappold and others 1984) found that selective enrichment for 8 h and overnight resulted in similar recovery of *Salmonella*. Therefore, it appears to have some potential for reducing the selective enrichment, but not the preenrichment period of conventional culture methods.

Combining preenrichment and selective enrichment into a single step has been attempted in order to reduce the incubation period of these two steps. Sveum and Hartman (1977) tested enrichment of samples in a media with the addition of timed-release capsules of selective agents. Results indicated that a 24 h incubation period of the capsule media was inadequate when compared to conventional culture methods. Pignato and others (1995) investigated the use of Salmosyst broth in combination with Rambach agar for rapid detection of *Salmonella* in foods. Heat injured *Salmonella* was preenriched in the Salmosyst preenrichment broth (SPB) for 6 h and followed by selective enrichment for 18 h with the addition of selective agent to SPB. Their results indicated that the use of

Salmosyst broth was even better than conventional methods for the recovery of heat injured *Salmonella*.

3. Bacteriophage

In the reporter bacteriophage technology, DNA carrying a reporter gene, such as luciferase (*lux* and *luc*) gene, ice nucleation (*inaW*) gene and the *E. coli* β-galactosidase (*lacZ*) gene, is introduced into a target bacterium via a bacteriophage. Once the bacterium-specific phage invade host cells, the reporter gene is expressed with phage DNA, allowing bacterial cells to be rapidly identified. Since bacteriophages can not replicate without host cells, expression of the reporter gene is indicative of the presence of the infected organism (Goodridge and Griffiths, 2002). Bacteriophages can also be used to detect specific bacteria without reporter genes. Felix O1 bacteriophage lyses most *Salmonella* at concentrations ranging from 10^9 to 10^{12} plaque-forming units. Detection of *Salmonella* was achieved at cell concentrations of $\geq 4 \times 10^4$ cfu/ml by using Felix O1 phage and high performance liquid chromatography (Hirsh and Martin, 1983). Because some *Salmonella* strains did not support bacteriophage replication, the technique was modified by the same group of researchers (Hirsh and Martin, 1984). A large-pore charge-modified filter was used to concentrate cells, which were then eluted into enrichment broth containing brilliant blue dye. After 16 h incubation, centrifugation and resuspension, the cells were inoculated on a nutrient agar plate to which Felix O1 phage had been added. The presence of lacunae after 6 h was indicative of a positive result. However, the application of this method to food is yet to be assessed. One possible reason is that it is difficult to genetically modify bacteriophages and the bacterial hosts

have not been genetically characterized. Another potential problem is that an individual bacteriophage can not detect all isolates of a bacterial species.

4. Nucleic acid-based methods

Nucleic acid based methods include pulsed-field gel electrophoresis (PFGE), DNA hybridization (DNAH), and polymerase chain reaction (PCR). PFGE has been used in the subtyping of bacteria, in which restriction patterns of whole bacterial gene sequences are analyzed and compared. The bacteria are immobilized by mixing the bacterial suspension with melted agarose. Then the bacteria are lysed to release DNA and the released bacterial DNA is cut into a moderate number of DNA fragments by a selected enzyme. The DNA fragments are loaded onto agarose gel and separated by pulsed-field electrophoresis, in which the orientation of the electrical field alternates. Several factors, such as recent point mutation, deletion, insertion, and loss or acquisition of plasmids might result in differences in PFGE banding patterns. PFGE alone is unable to replace conventional phage typing of *Salmonella* (Lukinmaa and others 2004). Some *Salmonella* strains can not be typed by PFGE since the DNA is degraded during electrophoresis (Liesegang and Tschape 2002).

The essential principle of DNAH and PCR methods is the specific formation of double stranded nucleic acid molecules from two complementary, single stranded molecules under defined physical and chemical conditions. Typically, a DNAH assay consists of the following steps. The bacteria are applied to a solid support such as a nitrocellulose membrane filter and are then lysed to release their DNA, and the DNA is denatured into separate strands and fixed to the solid support. The membrane is then

hybridized with a labeled DNA probe. Labeled nucleotides are added to the system for synthesis to occur. These nucleotides can be labeled with a radioisotope, or with a non-isotopic reporter molecule. These hybrids contain one radiolabeled strand that can then be detected by autoradiography (Fitts and others 1983, Fitts 1985). Because of safety concerns and disposal problems associated with radiolabels, they have been replaced with non-isotopic labels (King and others 1989). The ribosomal RNA (rRNA) hybridization has several advantages over DNAH: more sensitive (more copies of rRNA per cell) and no denaturation is required before hybridization (single strand of rRNA) (King and others 1989). Because the detection limit of DNAH is 10^4-10^5 cfu/ml, selective enrichment is needed to bring the bacterial number above the detection limit. This step may take 18-48 h (Swaminathan 1994).

Using a PCR method, specific DNA-fragment is amplified during a three-step cyclic process: first, the target DNA is denatured at high temperatures (95-100 ºC), second, two synthetic oligonucleotides (primers) are annealed at opposite strands at a temperature (37-55 ºC) that allows hybridization to the correct target, finally polymerization is performed with the oligonucleotides as primers for the DNA polymerase and the target DNA as a template. When this is repeated over and over with newly synthesized DNA as template in addition to the original target DNA, an exponential amplification of the DNA fragment is obtained.

Many different applications of PCR have been described and some of these applications use the whole gene sequences rather than a selected fragment as a target. Random amplified polymorphic DNA (RAPD) is based on the amplification of DNA with arbitrarily developed primers. RAPD is criticized for its lack of reproducibility

(Lukinmaa and others 2004). The rep-PCR is based on the amplification of the repetitive sequences of bacterial genomes. Restriction fragment length polymorphism PCR (RFLP-PCR) utilizes a restriction enzyme to cut the amplified known sequence and the restriction fragment profiles are compared between different strains. Amplified fragment length polymorphism PCR (AFLP-PCR) is a recently adopted PCR-based typing technique. Two enzymes are used in this method, one frequently cutting and the other one rarely cutting. The disadvantage of AFLP-PCR is that incomplete digestion of DNA may result in an aberrant AFLP pattern. The real time PCR (rt-PCR) technique combines amplification and detection in one single step (Eyigor and others 2002). The detection sensitivity of rt-PCR was determined as low as 6 cfu/ml from artificially spiked poultry intestine samples after 18 h enrichment in tetrathionate broth by Eyigor and coworkers (2002).

Nucleic acid based methods have been commonly used in many research laboratories, the majority of these methods have not been applied in routine food microbiology. Major problems are the detection of dead cells and the presence of enzyme inhibitors in food samples (Olsen, et al, 1995).

5. Immunoassay

Immunoassays are powerful analytical tools that permit the specific and rapid detection of antigens to which antibodies can be produced. Application of immunoassays in *Salmonella* detection could provide a considerable savings in time, effort and materials over conventional cultural methods.

Antigens used for antibody production include lipopolysaccharide (LPS, O antigen), flagellin (H antigen), fimbriae, outer membrane protein (OMP), Capsular antigen (Vi of a few serovars including *S. typhi* and K-antigen of other *Salmonella*), and whole inactivated bacteria. Variation of H-antigen, O-antigen, and capsular antigen is the basis of *Salmonella* serotyping (Mortimer and others 2004).

The LPS of *Salmonella* has three components: a lipid moiety, a core oligosaccharide, and the O-specific side chain. LPS affects virulence by the amount produced, the O side chain length and composition, and the degree of glycosylation. Flagella are antigenic surface structures of *Salmonella*. The LPS antigen had been used to produce polyclonal and monoclonal antibodies for the detection of *Salmonella* (Tsang and others 1987, Luk and Lindberg 1991, Lu and others 1991, Choi and Ng 1992, Wang and others 1996). LPS has been used for serological identification of *Salmonella* infected chicken (van Ziderveld and others 1992) and cattle (Veling and others 2000).

Flagellin is the major structural protein of flagella and carries the serotype-specific H-antigenic determinants. They elicit an early short-lived humoral response in the host upon infection, making them ideal for the detection of infections in livestock through screening anti-salmonella antibodies of the infected individuals. These H antigens probably induce the production of serotype-specific antibodies in the infected host. Antibodies against the conserved N- and C-terminal fagellin domains give rise to cross-reactions between *Salmonella* serotypes and other Enterbacteriaceae (Ibrahim and others 1985). Antibodies against H-antigen have been developed for the detection of salmonella in foods (Swaminathan and Ayres 1980, Wyatt and others 1993).

Fimbriae or pili are thin proteinaceous and filamentous surface organelles produced by most *Salmonella* and some other enteric bacteria. Fimbriae are an important factor in bacterial survival and persistence in the host. They mediate the adhesion of bacteria to epithelial cell surfaces (Kisiela and others 2003). Fimbriae are composed of protein subunits called fimbrins and auxiliary proteins (Peralta and others 1994). The expression of fimbriae by certain strains of salmonella was first described in 1958 (Duguid and Gillies), but only recently has their potential as diagnostic antigens been considered. It is believed that *Salmonella* only express fimbriae in close association with epithelial and other cell types, but rarely express fimbriae in extracellular environments (Thorns, 1995). Therefore this antigen is probably suitable for serologically screening of antibodies produced by *Salmonella* infected live hosts (Thorn and others 1996) but not suitable for identifying *Salmonella* in food. Although much effort has gone into the characterization of fimbriae expressed by *Salmonella*, their application to the detection of specific *Salmonella* has not been fully exploited. One major reason for this has been a lack of understanding of the role of these antigens in the lifecycle of the bacterium.

Four basic types of immunoassay have been developed, including agglutination (Barrow, 1994), radioimmunoassay (RIA) (van Vunaki, 1980), fluorescent immunoassay (FIA), and enzyme immunoassay (EIA) (Candish, 1991).

The agglutination method has been used to detect whole cells by adding specific antisera to a suspension of bacteria at approximately 10^9 cfu/ml. The antibody binds to the bacterial surface antigens forming a network of linkages, which result in visual precipitation of the bacteria. Pure bacteria cultures at high concentrations are required and often many different organisms can cause agglutination. Therefore, this method is

extremely insensitive and nonspecific (Candish 1991). Agglutination tests have also been used for the serological identification of infected chicken (van Zijderveld and others 1992) and cattle (Veling and others 2000). Latex agglutination using different colored beads coated with antibodies specific to different antigens was applied to Salmonella groupings (Hadfield and others 1987).

The first RIA was developed by Yalow and Berson (1959) for clinical studies. RIA is sensitive, which can detect nanograms to picograms of antigens. ^{125}I, ^{14}C and ^{3}H are the commonly used radioactive labels. RIA was used for the detection of *Salmonella* in foods by Ibrahim and others (1985, 1986). RIA has safety problems due to the use of radioactive labels. Also, the need for expensive equipment for measuring radioactivity and the chemical instability of certain radioactive labels are a limitation of RIA (Ibrahim 1986).

Because of the disadvantages of RIA, effort has been attempted to develop alternative labels. Fluorescent molecule and enzymes are proven to be most promising labels. The labeling of antibody with fluorescein isothiocyanate (FITC) was first demonstrated and applied for identifying bacteria in clinical specimens by Coons and others (1942). The first FIA for *Salmonella* detection was reported by Thomason and others (1957). In their study, the fluorescein labeled antibodies were demonstrated to be able to stain O, Vi, and H antigens of *S. typhi*. Later, an indirect FIA (using polyclonal anti-salmonella O antibody and fluorescent goat anti-rabbit serum) for the detection of *Salmonella* in foods was developed (Georgala and Boothroyd 1964, Haglund and others 1964, Silliker and others 1966). Although the FIA technique had been tested in a wide range of foods and feeds, it never enjoyed widespread use. The false positive results

(non-specific staining), false negative results (obscuring of fluorescing cells by food particles), high cost of instrumentation, requirement of confirmation of FIA positive results by culture methods, and the limitation of sample throughput have limited its application (Blackburn 1993). FIA in combination with flow cytometry for the detection of *Salmonella* in milk had been developed (McClelland and Pinder 1994). In order to reduce the high level of background labeling of milk micelles, a milk clearing solution was used to coagulate the particulate matter and then centrifugation was used to remove the coagulant. The clearing solution and centrifugation were effective for whole and reduced fat pasteurized milk but not the ultrahigh-temperature milk (McClelland and Pinder 1994).

The use of enzymes as a label in immunoassays was first introduced by van Weenman and Schuurs (1971) and Engvall and Perlmann (1971).The first EIA for *Salmonella* detection was reported by Krysinski and Heimsch (1977) using a polyclonal antibody specific to flagella of *S.* Typhimurium. Since then, EIA techniques have shown tremendous potential for the detection of pathogens in foods and are the most widely used format for immunoassays. By Enzyme-linked immunosorbent assay (ELISA), one of the reactants is absorbed on the surface of a solid phase such as polyvinyl chloride microplate. Krysinski and Heimsch (1977) were able to detect S. Typhimurium in artificially inoculated foods and feeds in 48 h with a detection sensitivity of 10^5 cfu/ml. False positive results were observed in their study. Efforts have been attempted to reduce the cross-reactivity of the polyclonal antibodies by fractioning the IgGs using gel filtration and affinity chromatography (Swaminathan and Ayres 1980).

Several ELISA formats, which include sandwich ELISA, indirect ELISA, direct ELISA, and competitive ELISA, have been applied in foods (Swaminathan and Feng 1994). Each format of ELISA has its advantages and disadvantages. The competitive ELISA is rapid, involving only one incubation step and one washing step. However, a purified antigen is required to perform the assay. Also, food ingredients may interfere with the assay since the enzyme conjugated antibody reacts directly with the sample. These problems can be avoided in non-competitive ELISA (sandwich, indirect, direct ELISA). Non-competitive ELISA also has the possibility of amplification of the detection signal through binding of several enzyme-labeled antibody molecules to one antigen molecule. Sandwich ELISA is suitable for analyzing food samples because it can avoid the competitive binding of food ingredients and other microbes with the analyst. Also, it is adaptable to a kit format for use. ELISA is undoubtedly a useful format of immunoassay in food microbiology due to its simplicity and ability to simultaneously test multiple samples.

The major problems with ELISA for *Salmonella* detection is the inadequate specificity of the polyclonal antibodies. The development of monoclonal antibodies could lead to improvements in the specificity of ELISA. ELISA involves several steps of incubation which prolonged the detection time. The detection sensitivity of the most developed ELISA is about 10^5 cfu/ml (Candish 1991). Preenrichment or selective enrichment of food samples are needed to increase the bacteria number in order to reach the detection limit.

6. Bacterial concentration methods

The requirement to detect low numbers of *Salmonella* in foods has limited the application of rapid detection methods. Preenrichment, selective enrichment, and sometimes even a post-enrichment are normally involved before applying rapid detection methods. As a consequence, research effort has been put on the development of *Salmonella* concentration methods (Blackburn 1993). Membrane filtration techniques using hydrophobic grid-membrane filter (HGMF), immunomagnetic separation (IMS), and centrifugation have been used by various researchers to separate and concentrate microbes from foods.

Membrane filtration was first used for the capture of bacteria from water samples (Goetz and Tsuneshi 1951). A wide variety of applications has been discovered in the past several decades. The concept of using HGMF as a bacterial enumeration tool was introduced by Sharp and Michaud (1974). HGMF is essentially a conventional membrane imprinted with hydrophobic grids to form individual bacterial growth compartments. The wax grids limit the growth of bacteria within the individual compartments. Because more than one bacteria could be seeded in one compartment during the filtration, the bacterial number on the HGMF is determined by the most probable number (MPN) method. The original HGMF technique for enumeration of bacteria in foods involves a complicated and lengthy incubation procedure, which include preenrichment by placing the filter on a nonselective medium and followed by selective enrichment by transferring the filter to an appropriate selective medium (Brodsky and others 1982). Wu and Fung (2004) have simplified the HGMF bacterial enumeration technique by using HGMF in combination with the thin agar layer (TAL) methods.

IMS technique uses antibody coated magnetic particles to capture, separate, and concentrate microbes from foods. It has been proposed as a method of selective enrichment by several researchers (Luk and Lindberg 1991, Mansfield and Forsythe 1993). In their methods, IMS only replaced the selective enrichment of the standard culture method.

7. Immunosensor

Recently, the use of biosensors to detect microorganisms has attracted the public's attention. The currently developed biosensors for bacterial pathogen detection are mostly based on the antigen-antibody specific binding reaction where the antibody is immobilized on a sensor platform to capture the bacteria that are of interest. Then the detection is measured electrochemically, optically, or piezoelectricly. These biosensors include piezoelectric biosensors (Prusak-Sachaczewski and others 1990, Si and others 1996, Ye and others 1997, Babacan and others 2002), fiber optic biosensor (Kramer and Lim 2004), optical surface plasmon resonance biosensor (Bokken and others 2003), bienzyme electrochemical biosensor (Yang and others 2001). The detection limit of these biosensors is high, ranging from 10^5 cfu/ml to 10^7 cfu/ml. Therefore, more sensitive biosensors are in urgent need to detect relatively low numbers of pathogens in foods.

STATEMENT OF RESEARCH OBJECTIVES

The objective of this research was to develop a sensitive biosensor using *Salmonella* specific mAbs for the rapid detection of *Salmonella* in milk.

The specific objectives for the first study were to:

1. develope *S. enterica* Typhimurium specific mAb and pAb using purified outer membrane proteins as an immunogen.
2. characterize the developed *S. enterica* Typhimurium specific mAb and pAb.
3. develop a biosensor using *S. enterica* Typhimurium specific mAb as a capture antibody and visualize the captured *S. enterica* Typhimurium by a light microscope.

The specific objectives for the second study were to:

1. select an effective media for rapid culturing of low numbers of inoculated *S. enterica* Typhimurium in milk.
2. evaluate the efficacy of centrifugation as an bacterial concentrating method
3. compare different sensor platforms
4. develop an indirect sandwich ELISA to verify the performance of the biosensor.
5. evaluate the performance of the biosensor for the detection of inoculated *S. enterica* Typhimurium in milk..

CHAPTER III

DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST *SALMONELLA ENTERICA* TYPHIMURIUM FOR BIOSENSOR APPLICATION

ABSTRACT

For the construction of a biosensor which can specifically detect *Salmonella enterica* Typhimurium, monoclonal antibody (mAb) 1B4 and polyclonal antibody (pAb) S48 were developed against purified outer membrane protein (OMP) of *Salmonella enterica* Typhimurium. Gel electrophoresis data indicated that the apparent molecular weight of OMP was 55 kDa. The mAb 1B4 strongly reacted to *Salmonella enterica* Typhimurium and *Salmonella enterica* Paratyphi and had no cross reaction to other gram negative and gram positive bacteria tested. The pAb S48 also showed high reactivity to *Salmonella enterica* Typhimurium, but had cross reactivity with other tested bacteria. Due to its high specificity and sensitivity against S. *enterica* Typhimurium, mAb 1B4 was immobilized to the biosensor surface. The detection of *Salmonella* by the biosensor was photographed by a light microscope. The detection limit of the biosensor was determined to be 1×10^2 cfu/ml in bacterial culture. The presence of excess amounts of *E. coli* did not interfere with the detection limit of the biosensor constructed in this study. This is the first time the 55 kDa antigenic component purified in this study has been

reported. The biosensor (an immunosensor platform in combination with microscopic imaging systems) could accurately and sensitively detect *Salmonella* within hours.

Keywords: *Salmonella enterica* Typhimurium, biosensor, monoclonal antibody

INTRODUCTION

The presence of *Salmonella* in foods is a great concern to the food industry, the public, and the regulatory agencies. In the United States, the number of cases of foodborne illnesses due to *Salmonella* is the highest of any reported gastrointestinal infections. Although *Salmonella* has more than 2,300 serotypes, *Salmonella enterica* serotype Typhimurium is the most common serotype in the U.S. isolated from people with salmonellosis, a disease caused by the infection of *Salmonella* (CDC 2005).

Several methods have been developed to detect the presence of potentially low levels of *Salmonella* in foods. These methods include culturing techniques, bacteriophages (Goodridge and Griffiths 2002; Hirsh and Martin 1983, 1984), DNA hybridization (Fitts and others 1983, Fitts 1985), polymerase chain reaction (PCR, Fluit and others 1993, McElroy and others 1996, Kwang and others 1996), immunoassays (Mattingly 1984, Ibrahim and others 1986, Hadfield and others 1987), and biosensors (Ye and others 1997, Babacan and others 2002, Bokken and others 2003, Kramer and Lim 2004, Taitt and others 2004). Standard culturing methods for *Salmonella* detection involve multi-steps which are labor intensive and time-consuming, taking 4 to 5 days for detection and confirmation (FDA 1992). Furthermore, it has been reported that some of

the routinely used selective enrichment broths are inhibitory towards *Salmonella* (Van der Zee 1994). The PCR, DNA hybridization, and bacteriophage methods have shown considerable success by reducing the detection time to 2-3 days, but none of them has been identified as fast, specific, and user-friendly for routine use in screening food samples. Therefore, development of rapid detection methods is necessary for the food industry to quickly respond to food and food product contamination. These methods would also allow regulatory agencies to deal with outbreaks or to enforce food regulation laws.

Since the first enzyme immunoassay for *Salmonella* detection was reported in 1977 (Krysinski and Heimsch), immunochemical techniques have been shown to be stable and reliable methods for microorganism detection or identification. Several enzyme-linked immunosorbent assays (ELISAs) have been developed, using either polyclonal antibodies (pAb) or monoclonal antibodies (mAb) that are able to detect most *Salmonella* serotypes. The majority of developed antibodies are specific to the *Salmonella* outer core lipopolysaccharide (LPS, O-antigen) or flagella (H-antigen) (NG and others 1996, Wang and others 1996, Jaradat and Zawistowski 1996). Although immunoassays reduce the total assay time by 1 or 2 days, most of the detection kits actually only replace the agar-plating stage of the culturing assay and even introduce a third, "post enrichment" broth stage before the sample can be assayed by ELISA. In addition, the immunochemically based methods produces many false-positive results due to the cross-reactivity with other enterobacteria (Curiale and others 1990).

Recently, the use of biosensors to detect microorganisms has hit the public. The currently developed biosensors for bacterial pathogen detection are mostly based on the

antigen-antibody specific binding reactions, where antibody is immobilized on sensor platform to capture the bacteria that are of interest. Then the detection is measured electrochemically, optically, or piezoelectrically. These biosensors include piezoelectric biosensors (Prusak-Sachaczewski and others 1990, Si and others 1996, Ye and others 1997, Babacan and others 2002), fiber optic biosensor (Kramer and Lim 2004), optical surface plasmon resonance biosensor (Bokken and others 2003), bienzyme electrochemical biosensor (Yang and others 2001). The detection limit of these biosensors is high, ranging from 10^5 cfu/ml to 10^7 cfu/ml. Therefore, more sensitive biosensors are in urgent need to detect relatively low numbers of pathogens in foods.

The advancement in biosensor technology requires the highly specific antibody to reduce interference of the detection. *Salmonella* are antigenically complex, serotypes have been differentiated by somatic lipopolysaccharide (LPS) or flagellar protein antigens (Le Minor 1984). The outer membrane proteins (OMPs) of *Salmonella* are also known to have an important role in evoking immune responses (Kim and others 1991; Meenakshi and others 1999). There appears to be a potential to develop species or serotype specific antibodies from *Salmonella* OMPs. Therefore, the objective of this study was to develop *Salmonella*-specific antibodies to build a novel biosensor, combining antibody immobilized sensor platform with microscopic imaging systems, to accurately and sensitively detect *Salmonella* within hours.

MATERIALS AND METHODS

Bacteria culture

Salmonella enterica Typhimurium ATCC13311, *E. coli* 43895, *E. coli* 932, and *E. coli* 48-2 were obtained from James Barbaree and Stuart Price laboratories at Auburn University, Auburn, AL. *Salmonella enterica* serovars Paratyphi, Enteritidis, Mission, and Montevideo, *E. coli* O157:H7 204P, *L. monocytogenes* G3982 4b, and *L. innocua* were provided by the Center for Food Safety, University of Georgia (Griffin, GA). *Listeria monocytogenes* Scott A and *Staphylococcus aureus* ATCC 12600 were purchased from the American Type Culture Collection (ATCC), Rockville, MD. *Salmonella* spp., *E. coli* spp., and *Staphylococcus aureus* were grown in Trypticase® Soy Broth (TSB, Difco Laboratories, Detroit, MI) while *Listeria* spp. were grown in TSB with the addition of 0.6% yeast extract (TSBYE). The bacterial cultures were incubated in a gyratory water bath at 37 ºC at 100 rpm for 16 hr. After incubation the cultures were washed twice with phosphate buffered saline (PBS, pH 7.2) by centrifugation at 5,000 × *g* for 20 min. The bacteria were resuspended in PBS and bacterial concentration was estimated from the absorbance of the bacterial suspensions at 640 nm using a standard curve. The bacterial concentrations were confirmed by plating *Salmonella* spp., *E. coli* spp., and *Staphylococcus aureus* on Trypticase® Soy Agar (TSA) and *Listeria* spp. on TSA with addition of 0.6% yeast extract (TSAYE).

Antigen preparation

The outer membrane proteins (OMPs) were prepared as described by Meenakshi and colleagues (1999) with modifications. After *S.* Typhimurium was grown in TSB and

held in a gyratory water bath (100 rpm) at 37 ºC for 16 h, the bacteria were washed twice with normal saline solution through centrifugation at 5,000 × *g* for 10 min at 4 ºC. The cells were suspended in 10 mM Hepes buffer (pH 7.4) and sonicated at 20 kHz five times on ice for 1 min each time at 1 min intervals. The sonicated samples were centrifuged for 1,700 × *g* for 20 min to remove the intact cells and debris. The supernatant, which was designated as sonicate extract, was collected and centrifuged for 100,000 × *g* at 4 ºC for 60 min. The supernatant was discarded, and the clear gel-like pellet was collected and resuspended in 2% (w/v) sodium lauryl sarcosinate detergent in 10 mM Hepes buffer followed by incubation at room temperature for 1 hr. The detergent insoluble fraction was collected by centrifuging for 100,000 × *g* at 4 ºC for 60 min, and the pellet was washed twice with deionized (DI) water through centrifugation for 100,000 × *g* at 4 ºC for 60 min. The OMPs were solubilized by adding 6 mol/l guanidine thiocyanate (Sigma Chemical Co., St. Louis, MO) to the pellet. The solution was mixed at room temperature for 60 min. Insoluble material was removed by ultracentrifugation for 300,000 × *g* at 4 ºC and the remaining solution was dialyzed against deionized (DI) water overnight at 4 ºC with several changes of DI water (Kerr and others 1992). Protein content was measured by the Bradford method using a protein assay kit (Bio-Rad, Hercules, CA). The purity of OMPs was determined by gel electrophoresis. The OMPs were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-Protein II electrophoresis unit with stacking (4% T, 2.67% C) and separating (12% T, 2.67% C) acrylamide gels. Protein bands were stained with Coomassie Brilliant Blue R-250 and the apparent molecular weights of protein bands were calculated using a calibrating curve constructed from molecular weight standards (Sigma).

Monoclonal antibody (mAb) production

The purified OMPs of *S.* Typhimurium were emulsified with RIBI's adjuvant system (RAS-R700) (Corixa, Hamilton, MT) at 100 μg/ml for mice immunization. Each BALB/cAnNHsd female mouse (Harlan Sprague Dawley Inc., Indianapolis, IN) was immunized with 200 μl of the OMPs-adjuvant emulsion through the injection of 100 μl aliquots into each of two legs subcutaneous sites on the ventral side near the axillary and inguinal lymphatics. Three mice were immunized and the same RAS-R700 was used for the initial immunization and booster.

Mice were boosted 4 times after the initial immunization with antigen-adjuvant emulsion at 3-week intervals. Blood was collected 7 days after each booster started at the second booster and the titers of the serum were determined. The procedures described by Kohler and Milstein (1975) were followed with modifications. Spleen cells were harvested 5 days after the final booster to fuse with murine myeloma cells at a ratio of 2:1 (spleen cells/myeloma cells) using 50% polyethylene glycol (PEG). The fused cells were suspended in hypoxanthine-aminopterin-thymidine (HAT) selective medium (Dulbecco's modified Eagle medium containing 200 μM hypoxanthine, 0.8 μM aminopterin, and 32 μM thymidine) and seeded into 96-well cell culture plates. After 10 to 14 days, the supernatant of the growth positive wells were screened for production of anti-*Salmonella* Typhimurium antibodies using indirect ELISA. The hybridoma secreting antibodies that only reacted with *S.* Typhimurium were selected for expansion and cloning using limiting dilution methods (Harlow and Lane 1988). The mAbs was produced from selected cell lines through mouse ascites fluid and purified by affinity chromatography using the Biological Duo-Flow System (Bio-Rad) with a protein A affinity column. The purified

antibody was dialyzed against 0.01 M phosphate-buffered saline (PBS, pH 7.0) at 4 ºC with three changes of the dialysis buffer. The concentration of IgG was determined by Bradford method using a protein assay kit (Bio-Rad) and the purity of the antibody was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The isotype of mAb was determined by a Mouse Monoclonal Antibody Isotyping Kit (Sigma). The purified IgG was stored at -20 ºC in antibody buffer (0.1 M Tris + 2 mM $MgCl_2$ + 20 mM Glycine + 30 mM sodium azide, pH 8.0) with the addition of 50% glycerol.

Polyclonal antibody (pAb) production

New Zealand white rabbits about 3 kg each were used to produce antiserum against *S*. Typhimurium. The purified OMPs of *S.* Typhimurium were emulsified with RAS for rabbit (RAS-R730) at 285 μg/ml. Each rabbit was immunized with 1 ml of the OMPs-adjuvant emulsion through an intradermal injection of 50 μl aliquot into each of six sites on the back close to the spinal cord and 300 μl aliquot intramuscular into each hind leg. The same dosage of OMPs-adjuvant emulsion was used to boost the rabbit at 4-week intervals after initial immunization. Rabbit blood was collected from the central ear artery 7 days after each boost and antiserum was collected from the supernatant by centrifuging the blood for 5,000 × *g* at 4 ºC for 20 min. The polyclonal antibodies in the antiserum were collected from 20-50% saturated ammonium sulfate precipitation and re-suspended for dialysis in PBS for 24 hr with 3 changes of dialysis buffer. The partially purified antibodies were further purified by affinity chromatography using a protein A affinity column (Sigma), where the protein only binds with IgG and its subclasses. The

bound antibodies were eluted with 0.1 M citrate buffer, pH 5.0. The titer and specificity of the purified polyclonal antibodies were determined by indirect ELISA.

Indirect ELISA for specificity tests of pAb and mAb

Each well of a 96-well assay plate (polystyrene plate, Costar, Cambridge, MA) was coated with 100 µl of 10^8 cfu/ml of formalin inactivated or live bacteria at 37 °C for 1 h. The plate was washed 3 times with 200 µl of PBS containing 0.1 % Tween-20 and 0.02% sodium azide (PBST), and then the unbound areas of the wells were blocked with 200 µl of 1% bovine serum albumin (BSA) in PBS at 37 °C for 1 h. Following the addition of 100 µl appropriately diluted Abs to each well, the plate was incubated at 37 °C for 1 h and then washed three times with PBST. After adding 100 µl/well of alkaline phosphatase conjugated goat anti-rabbit /rabbit anti-mouse IgG (Sigma) at 3,000 times dilution in PBS, the plate was incubated at 37 °C for 1 h. The plate was washed 4 times and 100 µl of *p*-nitrophenyl phosphate (*p*-npp, Sigma) solution was added to each well. The plate was incubated at room temperature in the dark for color development. Absorbance at 405 nm was measured by a microplate reader (ThermoLabsystems, Helsinki, Finland) and recorded for analysis.

Preparation of biosensor platform and antibody immobilization

The sensor platform was prepared by cutting microscope cover glass (0.17 mm thickness) into 5 mm squares. The squares were ultrasonically cleaned in DI water and in 95% ethanol. The polished surface of each square was coated with a 140 nm gold by using a Pelco SC-6 sputter coater (Ted Pella Inc., Redding, CA) to provide a favorable layer for antibody immobilization. The monoclonal antibody (1B4) was coated on the sensor platform as the receptor to capture the bacteria. The biosensor platform was

constructed by incubating 100 µl of 24 µg/ml mAb 1B4 on the gold coated platform at room temperature for 1 h in a 6 × 6 mm^2 square well of 96-well Uniplate® (Whatman Inc, Clifton, NY). After washing 3 times with PBST, the platform was incubated with 1% BSA for 30 min to block unbound areas. After washing 3 times with PBS and then 3 times with DI water, the platform was used immediately or dried at room temperature and stored at 4 ºC till use.

Sensor-bacteria binding and light microscopic imaging

The biosensor platforms were incubated with 100 µl of 0, 10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 cfu/ml *Salmonella* Typhimurium suspension at room temperature for 1 h, and washed 4 times with DI water. After the immunosensors were dried at room temperature, the bacteria captured by the biosensor were fixed with OsO_4 for 1 h and photographed using a Nikon Eclipse L150 Industrial light microscope (Nikon Instruments Inc., Melville, NY) with 1,000 times magnification. The experiment was repeated five times, and the images of the bacteria on two representative microscope fields at 0.025 mm^2 from each immunosensor were recorded as a data set. The bacterial number on each field was counted and the detection of *Salmonella* was defined as positive when one or more bacteria were observed on all the images of a data set.

RESULTS AND DISCUSSION

SDS-PAGE patterns of OMPs

The 10% polyacrylamide gel contained 0.1% SDS is optimal for OMPs separation on SDS-PAGE. The apparent molecular weights of protein bands on the gel were calculated through a calibrating curve constructed from the molecular weight standards.

From the protein patterns, the purity of OMPs extract is high and the apparent molecular weight of the major protein of the extracted OMPs was about 55 kDa (Figure 1). Few other proteins had been observed on the gel and the contents were very low compared to the major protein. It indicated that there were high probabilities to obtain very specific pAb or mAb against *Salmonella* by using OMPs as antigen. Compared to Kerr and others (1992) results, who produced mAbs against a 36 kD OMP of *S. enterica* Enteritidis, this is the first time reporting of the use of the 55 kD OMP to produce mAbs against *S. enterica* Typhimurium.

Sensitivity test of mAb 1B4 and pAb by indirect ELISA

One fusion resulted in about 700 growth-positive hybridomas and 120 showed positive reactivity with *S. enterica* Typhimurium cells as determined by indirect ELISA. Among these positive hybridomas, only two hybridoma cell lines 1B4 and 7B10 secreted antibodies specific to *S. enterica* Typhimurium and *S. paratyphi*. After single cell cloning of the 1B4, the cells were used to produce acities from mouse for mAb production. After purification by affinity protein A column, the purity of mAb and pAb was checked by SDS-PAGE with 12% gel and showed very high (data not shown). The isotype of mAb 1B4 was determined to be IgG2a.

The binding efficiency of mAb 1B4 to *Salmonella enterica* Typhimurium decreased rapidly with the decrease of bacterial concentration from 1×10^8 to 1×10^6 cfu/ml with the highest ELISA reading decreasing from above 4.0 to 0.5. The binding efficiency was then decreased slowly from 1×10^6 to 1×10^2 cfu/ml and leveled off from 1×10^2 to 0 cfu/ml (Figure 2). There was a significant difference of ELISA readings between 0 cfu/ml and 1×10^3 cfu/ml ($p<0.05$). Therefore, the detection limit of the mAb

1B4 based indirect ELISA was 1×10^3 cfu/ml. pAb S48 showed similar sensitivity as mAb 1B4 when tested against different concentrations of *Salmonella enterica* Typhimurium (data not shown). *E. coli* O157:H7 was added to different concentrations of *Salmonella* suspensions at 10^8 cfu/ml to evaluate the effect of binding efficiency of mAb 1B4 against *Salmonella enterica* Typhimurium. The results showed that the bindings between 1B4 and *Salmonella* were not affected by other bacterium (Figure 2).

Specificity of mAb 1B4 and pAb S48

The mAb 1B4 and pAb S48 were then tested for specificity with 13 different bacterial species or strains by indirect ELISA. The bacteria were coated to microtiter plate at 1×10^8 cfu/ml. Both mAb and pAb were diluted to 1.0 μg/ml and applied 100 μl to each well based on the results of checker board titration of these two antibodies (results not shown). Among the 13 tested bacteria, the mAb 1B4 only reacted with *S. enterica* Typhimurium and *S. enterica* Paratyphi and showed high reactivity. The pAb S48 is able to react with all tested *Salmonella* except Montevideo, and this antibody also has slight cross-reactivity with *E. coli* O157:H7 (Table 1). These results indicated that mAb 1B4 had higher binding specificity against *Salmonella* and lower background signal than those in pAb. By comparing the mAbs 1B4 produced from *S. enterica* Typhimurium OMPs in this study to the only mAbs developed from *S. enterica* Enteritidis OMPs by Kerr and others (1992), 1B4 was highly specific to *S. enterica* Typhimurium and Paratyphi and had no cross reactions with other bacteria. The mAbs developed by Kerr and others reacted to a wide variety of *Salmonella* and other strains of Enterobacteria, which resulted in false-positive reactions during sample testing. Based on the specificity, mAb 1B4 was selected as the capture antibody to be immobilized onto the biosensor platform

to bind *Salmonella* cells. This would result in reducing the cross reactivity with other bacteria to lower the false positive reaction and increasing detection accuracy. In the sandwich test, the pAb is better than mAb 1B4 used as the second antibody to conjugate with enzymes for enhancing detection signal due to its high reactivity.

Detection of *S. enterica* Typhimurium by biosensor

The mAb 1B4 was immobilized onto the surface of gold coated sensor platform at different concentrations to determine the bacterial capture efficiencies. On each sensor, 100 µl of *S. enterica* Typhimurium suspension at 1×10^8 cfu/ml were added, and the numbers of captured bacteria at different antibody concentrations were calculated from the light microscopic images (Table 2). There were 467 bacteria been detected on the sensor coated with 1B4 at 24 µg/ml, which was the highest number of bacteria captured. The number of captured bacteria decreased with the decreasing of antibody concentration from 24 µg/ml to 0.75 µg/ml. The mAb concentration and captured bacteria number had shown a high second order polynomial relationship ($r^2 = 0.9549$). No bacteria had been observed on the sensors coated with mAb 1B4 at 0.375 and 0 µg/ml. Based on these results, the mAb antibody concentration of 24 µg/ml was selected for the following studies.

The effect of detection sensitivity and specificity of 1B4 to *S. enterica* Typhiumrium by other nonspecific bacteria on sensor platform was also performed by adding *E. coli* O157:H7 to *Salmonella* suspensions at 10^8 cfu/ml. The results showed that the same bacterial numbers were captured on both sensors treated with *S. enterica* Typhiumrium only and *S. enterica* Typhiumrium combined with1×10^8 cfu/ml *E. coli* O157:H7. The population of captured bacteria was about tenfold decrease when the

concentrations of *S. enterica* Typhiumrium suspensions were in tenfold decrease from 1×10^8 to 1×10^6 cfu/ml. Then the captured bacterial numbers drop dramatically to single digit when bacteria concentrations were reduced from 1×10^6 to 1×10^2 cfu/ml. No bacteria were observed on the biosensor in the treatment of *E. coli* O157:H7 at 1×10^8 cfu/ml without *Salmonella* (Table 3, Figure 3). The results also showed that the binding of *S. enterica* Typhiumrium to mAb 1B4 was not affected by *E. coli* O157:H7. Therefore, the binding specificity of mAb 1B4 to *S. enterica* Typhiumrium is high. The bacteria on sensors at 10 cfu/ml of *S. enterica* Typhiumrium treatments, about 50% of the treated sensors were shown positive and only one bacterium was observed on each sensor. Therefore, the detection sensitivity of the sensor was limited to 100 cfu/ml. In contrast, ELISA-based methods using LPS-specific antibodies reported by other researchers have a detection limit of 10^6-10^7 cfu/ml in 17 to 29 hr (Choi and others 1992, NG and others 1996). Electron microscopy (EM) is the other technique that can directly visualize bacteria more detail than does by light microscope, but it requires more complicate sample preparations and trained personnel to operate the EM (Skepper 2000).

CONCLUSION

The mAb 1B4 developed in this study had high specificity and high sensitivity to *S. enterica* Typhiumrium. The 55 kDa OMP purified in this study is the antigen which was first time being reported. The biosensor (an immunosensor combined with microscopic imagining systems) developed in this study can specifically detect *S. enterica* Typhiumrium in 1 h with a sensitivity of 1×10^2 cfu/ml. The whole detection is rapid, cheap, and simple.

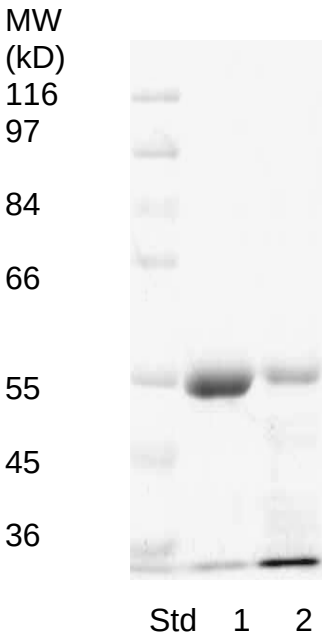

Figure 1. SDS-PAGE of purified *S. enterica* Typhimurium outer membrane protein. Std: molecular weight standard; 1: *S. enterica* Typhimurium outer membrane proteins (OMP); 2: Sonicate extract of *S. enterica* Typhimurium

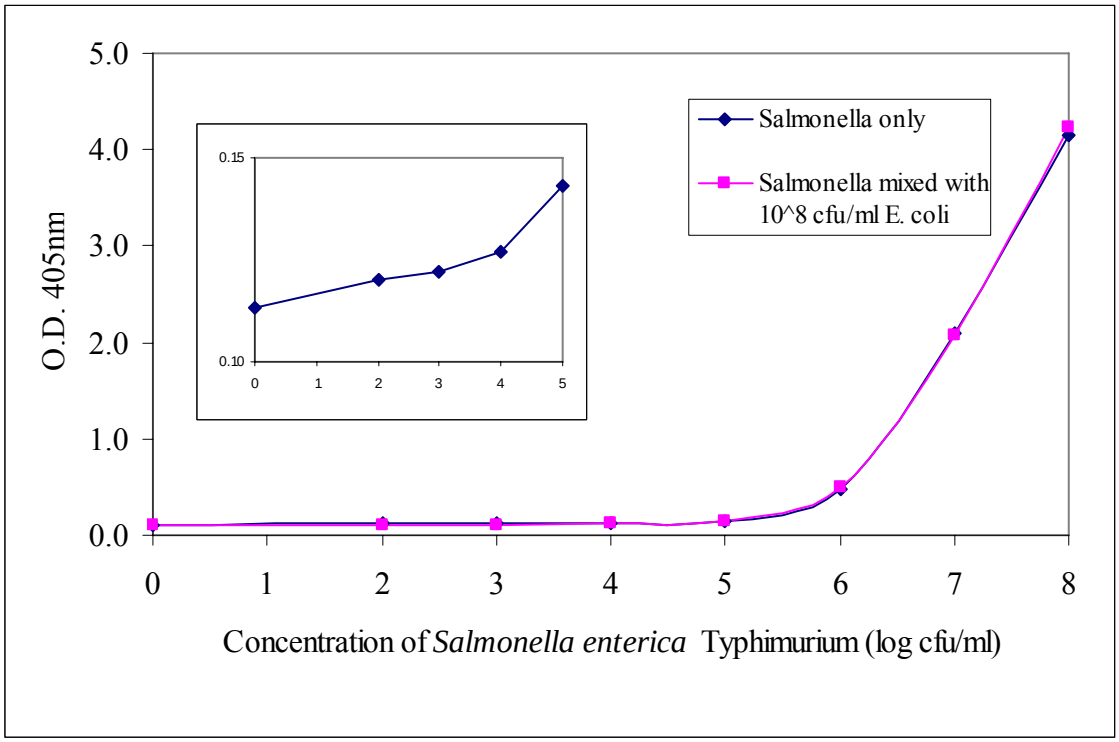

Fig. 2. Detection sensitivity of mAb 1B4 to *Salmonella enterica* Typhimurium by indirect ELISA

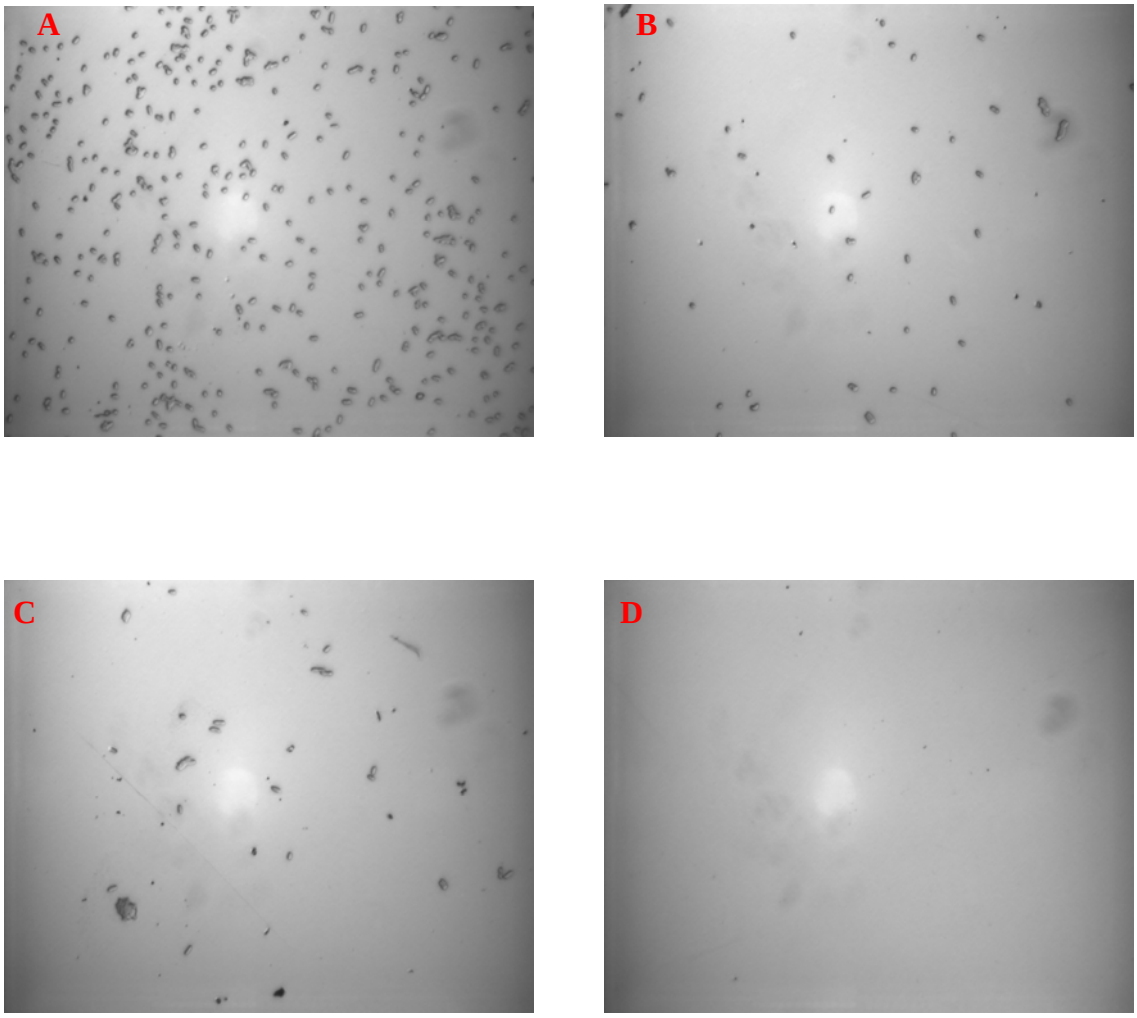

Fig. 3. Photograph of biosensor captured *S. enterica* Typhimurium at 10^8 cfu/ml (A), 10^7 cfu/ml (B), 10^6 cfu/ml (C), and blank(D)

Table 1. Specificity of mAbs and pAbs for *S. enterica* Typhimurium assayed by indirect ELISA (n=6, mean ± SD)

Bacteria	Absorbance$_{405nm}$	
	mAb 1B4	pAb
S. enterica Typhimurium	4.214 ± 0.089	4.248 ± 0.051
S. enterica Paratyphi	3.788 ± 0.116	3.972 ± 0.063
S. enterica Enteritidis	0.023 ± 0.003	0.430 ± 0.040
S. enterica Mission	0.010 ± 0.003	2.519 ± 0.098
S. enterica Montevideo	0.038 ± 0.005	0.185 ± 0.006
E. coli 43895	0.024 ± 0.004	0.140 ± 0.012
E. coli 932	0.043 ± 0.005	0.177 ± 0.050
E. coli 48-2	0.031 ± 0.001	0.125 ± 0.002
E. coli O157:H7 204P	0.029 ± 0.001	0.391 ± 0.008
*Staphylococcus aureus*ATCC12600	0.092 ± 0.002	0.185 ± 0.006
Listeria innocua	0.011 ± 0.002	0.129 ± 0.006
L. monocytogenes Scott A	0.012 ± 0.003	0.097 ± 0.011
L. monocytogenes G3982 4b	0.022 ± 0.001	0.087 ± 0.080

Table 2. The binding of *Salmonella enterica* Typhimurium* on mAb 1B4 immobilized biosensor platforms at various mAb concentrations.

Salmonella	mAb Concentration (μg/ml)							
	24	12	6	3	1.5	0.75	0.375	0
Bound bacteria	467	378	300	214	45	1	0	0

*The bacterial concentration was 1 x 10^8 cfu/ml.

Table 3. Numbers of bacteria bound to biosensors at various *S. enterica* Typhimurium concentrations as well as mixed with *E. coli* O157:H7.

S. enterica Typhimurium (cfu/ml)	Bacterial solution	
	S. enterica Typhimurium	*S. enterica* Typhimurium mixed with 1×10^8 cfu/ml *E. coli* O157:H7
1×10^8	467	467
1×10^7	46	40
1×10^6	8	6
1×10^5	3	4
1×10^4	2	2
1×10^3	2	2
1×10^2	1	1
1×10^1	0	1
0	0	0

Number in this table is the mean of six replications

CHAPTER IV

RAPID DETECTION OF *SALMONELLA* IN MILK USING A BIOSENSOR

ABSTRACT

In the previous study, a *Salmonella* Typhimurium-specific biosensor with a detection limit of 1×10^2 cfu/ml in pure culture and in a mixture with *E. coli* was developed. Due to the low number of *Salmonella* in foods, a one step enrichment in different non-selective enrichment or selective enrichment media with different incubation time, in combination with separation and concentration of bacteria by centrifugation, was evaluated for the rapid detection of *S. enterica* Typhimurium in milk. Among the seven preenrichment media (tryptic soy broth, half strength tryptic soy broth, lactose broth, nutrient broth, universal preenrichment broth, buffered peptone water, and brain heart infusion broth) tested, *S. enterica* Typhimurium grew best in brain heart infusion broth. Among the five selective enrichment media (selenite cystine borth, selenite broth, Rappaport-Vassiliadis R10 broth, Salmosyst broth, and brilliant green broth), *Salmonella* Typhimurium grew best in brilliant green broth. Therefore, brain heart infusion broth and brilliant green broth were selected for further study. *S. enterica* Typhimurium inoculated milk (0.1, 1, 10 cfu/ml) was enriched (1:9 milk to broth ratio) in the two media for 2, 4, and 6 h. Enrichment in the two media gave similar results. In combination with centrifugation, the bacterial number was able to reach the biosensor detection limit in 4 h for milk samples inoculated with 0.1 cfu/ml *Salmonella*

Typhimurium and in 2 h for milk samples inoculated with 1 cfu/ml *S. enterica* Typhimurium. Centrifugation was able to increase bacterial concentration to biosensor detection limits without enrichment for milk containing 10 cfu/ml *Salmonela* Typhimurium. Different sensor platforms, including glass, polystyrene, polyvinyl chloride, and glass coated with gold were compared in this study. Among these platforms, gold showed better consistency regarding sensitivity and more even distribution of captured bacteria. The surface of the gold platform was further processed with a self assembly monolayer (SAM) using 1mM $C_{11}H_{22}O_2S$ and 1 mM $C_{18}H_{17}N_3$.HCl. Compared with the gold coated platform, SAM did not improve the detection sensitivity. The light microscopic images showed that milk content did not interfere with the detection of *Salmonella* using the developed biosensor.

Keywords: *Salmonella* Typhimurium, enrichment, centrifugation, biosensor

INTRODUCTION

Outbreaks of salmonellosis associated with milk continue to occur (Ryan and others 1987, Jay 2000, Olsen and others 2004). Several rapid *Salmonella* detection methods have been developed for the detection of *Salmonella* in foods. These methods include polymerase chain reaction (PCR), DNA hybridization (DNAH), enzyme-linked immunosorbent assay (ELISA), and biosensors. The detection sensitivity of these rapid methods is about 10^5 to10^7 cfu/ml in foods. Due to the low incidence of *Salmonella* in foods, preenrichment, enrichment, and sometimes even a post-enrichment were involved in the rapid detection methods (Flowers and others 1988).

One step enrichment has been investigated prior to PCR, ELISA, and biosensor assays for the detection of pathogens in foods. Kwang and others (1996) used 4-6 h enrichment in a non-selective medium, trypticase soy broth (TSB), for the detection of inoculated *S. enterica* Typhimurium in ground beef before conducting PCR. One step enrichment in tetrathionate broth (TT) for 18 h was employed by Eyigor and others (2002) for the detection of inoculated *S. enterica* Enteritidis in poultry samples before PCR. Buffered peptone water (BPW), lactose broth (LB), and selenite cystine broth (SC) were used in one step enrichment (6 h) for the detection of inoculated *Salmonella* in chicken meat before conducting PCR (Fluit and others 1993).

Preenrichment in nutrient broth (NB) followed by enrichment in Rappaport-Vassiliadis (RV) medium was used before ELISA for the detection of *S. enterica* Typhimurium (Prusak-Sochaczewski and Luong 1989). The total detection time required by their method was about 24 h. Preenrichment in BPW and then enrichment in Salmosyst before ELISA (Salmonella-TEK test kit) was regarded as the quickest method

by the author, which took 31 h for the total analysis time, by van Poucke (1990). One step enrichment in non-selective medium, brain heart infusion broth (BHI) for 14 to 16 h before immunoassay was evaluated by NG and others (1996). The total assay time required by their method was 30 h and the detection sensitivity of their developed sandwich ELISA was 10^5 to 10^7 cfu/ml.

The preenrichment media recommended by US Food and Drug Administration (USFDA 2003) for *Salmonella* isolation and identification in various foods include brilliant green water (BGW), lactose broth (LB), nutrient broth (NB), skim milk, brain heart infusion broth (BHI), trypticase soy broth (TSB) and universal preenrichment broth (UPB). LB has been recommended for the preenrichment of liquid milk (skim milk, 2% fat milk, whole, and butter milk) for 24 ± 2 h by the USFDA (2003). Lack of unanimity on the selection of media for one step enrichment is still a problem.

Methods for separation and concentration of *Salmonella* from foods using immunomagnetic separation (IMS), filtration, or centrifugation have been investigated. IMS uses antibody coated magnetic particles to separate and concentrate microbes from foods. This technique has been investigated before performing PCR (Fluit and others 1993, Cerro and others 2002), used as an alternative to enrichment broth in conventional culture method (Mansfield and Forsythe 1993), and used in between preenrichment and enrichment steps (Cudjoe and Krona 1997). The membrane filtration technique for the capture of microbes has been investigated since 1951 (Goetz and Tsuneshi). The hydrophobic grid membrane filter (HGMF) technique has been investigated for bacterial enumeration since 1974 (Sharpe and Michaud). Centrifugation has been studied as a means of concentrating microbes by researchers and is regarded as an effective way of

concentrating microbes (Hawa and others 1984, McElroy and others 1995, McElroy and others 1996, Cerro and others 2002).

Although various biosensors have been developed and regarded as highly sensitive with a detection limit of about 10^3 to 10^7 cfu/ml (Bokken and others 2003, Taitt and others 2004, Yang and others 2001), few of them could be successfully used for the detection of pathogens in foods, due to the complex nature of the food matrix (Prusak-Sochaczewski and others 1990). The objectives of this study were to select either a preenrichment or an enrichment media that could be used in one step enrichment for rapid culturing of inoculated *S. enterica* Typhimurium in milk; to evaluate centrifugation as a microorganism concentration method; to evaluate the performance of different sensor platforms; to evaluate the detection sensitivity of the developed biosensor for the detection of inoculated *S. enterica* Typhimurium in milk; and to compare biosensor and indirect sandwich ELISA.

MATERIAL AND METHODS

Milk inoculation and one step enrichment

Pasteurized Vitamin D whole milk (Barbar's™) was purchased from a local grocery store in Auburn. Milk was cooked in a water bath at 98 ºC for 5 min to kill background microorganisms. Non-selective enrichment media, which included tryptic soy broth (TSB), half strength TSB (1/2 TSB), lactose broth (LB), nutrient broth (NB), universal preenrichment broth (UPB), buffered peptone water (BPW), and brain heart infusion broth (BHI); and five enrichment broth, which included brilliant green broth (BG), selenite broth (SB), selenite cystine broth (SC), Rappaport-Vassiliadis R10 broth

(RV), and Salmosyst broth, were prepared according to manufacturer's recommendation. For comparing the efficiency of different non-selective enrichment and selective enrichment media, washed overnight culture of *Salmonella* Typhimurim was diluted in phosphate buffered saline (PBS) to 10^4 cfu/ml. Ten ml of the diluted bacterial culture was inoculated into 90 ml of milk in a sterile milk dilution bottle and mixed well to give bacterial concentration of 10^3 cfu/ml in milk. Then, 2 ml of the inoculated milk were added to 18 ml of each of the seven preenrichment and enrichment media (1:9 milk to broth ratio) and incubated in a gyratory water bath at 37 ºC for 6 h with constant shaking. After incubation the bacterial number was checked by plating on bismuth sulfite agar. The background bacterial number of milk was checked by plating on bismuth sulfite agar and plate count agar.

After the selection of the two optimal non-selective enrichment and selective enrichment media, direct enrichment in the selected media, in combination with centrifugation, was used to determine the incubation time needed to bring bacterial concentration to the detection limit of the biosensor. *S. enterica* Typhimurium was inoculated into milk at 10, 1, and 0.1 cfu/ml. Then 25 ml of the inoculated milk was added to 225 ml of each of the selected media and incubated at 37 ºC for 2, 4, and 6 h. The bacteria number in the media was checked after 2, 4, and 6 h incubation.

Centrifugation

Centrifugation was used to concentrate bacteria from one step enriched media. Fifty ml of enriched milk at each incubation period (2, 4, and 6 h) were added into a 50 ml sterile polypropylene conical centrifuge tubes (Fisher Scientific, Suwanee, GA) and centrifuged at 5,000 × *g* for 10 min at 4 ºC. The supernatant was aspirated and the pellet

was resuspended in 1 ml PBS. The bacterial concentration in the resuspended pellet was checked by plating on bismuth sulfite agar.

Indirect sandwich ELISA

Each well of a 96-well polystyrene microtiter plate (Costar, Cambridge, MA) was coated with 100 µl of 6 µg/ml mAb 1B4 and incubated at 37 ºC for 1hr. The plate was washed 3 times with 200 µl of PBST, then the unbound areas of the wells were blocked with 200 µl of 1% BSA-PBS at 37 ºC for 1 h. After the plate was washed once with PBST, 100 µl of inoculated milk containing *Salmonella* Typhimurium 10 time serial diluted from 10^2 to 10^8 cfu/ml were added to each well and incubated at 37 ºC for 1 h. The plate was washed three times followed by adding 100 µl of pAb S48 (10 µg/ml) to each well and incubating at 37 ºC for 1 hr. After the plate was washed three times, 100 µl of 1:3000 diluted goat anti-rabbit IgG alkaline phosphatase conjugate (GAR-AP) was added to each well and incubated at 37 ºC for 1 hr. After the plate was washed 4 times, 100 µl of *p*-nitrophenyl phosphate (*p*-npp, Sigma Chemical Co, St. Louis, MO) solution (4 mg/ml) was added to each well and incubated at room temperature in darkness for 15 min. Absorbance was measured at 405 nm by a microplate reader (ThermoLabsystems, Helsinki, Finland).

Different sensor platform comparison

Four sensor platforms, which include glass, polystyrene, polyvinyl chloride, and gold, were compared. Glass platforms were prepared by cutting microscope cover glass (0.17 mm thickness) into 5×5 mm^2. Polystyrene platforms were prepared from flat polystyrene plate (Costar). Polyvinyl chloride platforms were cut from the bottom of microtiter plate (Costar). Gold platforms were prepared as described in the previous

study. Briefly, 100 nm of gold was sputter-coated on the surface of microscope cover glass, which was cut into 5 × 5 mm^2. The mAb 1B4 was immobilized on the four sensor platforms by incubating each of the sensor platforms in 100 µl of 24 µg/ml 1B4 solution at room temperature for 2 h. Then the sensor platform was washed 3 times with PBST, and incubated with 1% BSA-PBS for 30 min to block unbound areas. After washing 3 times with PBS, the platforms were either used immediately, or dried and stored at 4 ºC till use.

Preparation of self-assembly monolayer (SAM)

The gold platform was immersed in 1 mM 3-Mercaptopropionic acid 2-ethylhexyl ester ($C_{11}H_{22}O_2S$) dissolved in deoxygenated anhydrous ethanol (SAM solution) at room temperature for 16 h to form SAM on the surface. After the platform was removed from the SAM solution, it was thoroughly rinsed in anhydrous ethanol and then dried in a stream of nitrogen. The dried platform was immediately immersed in 1 mM 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, $C_8H_{17}N_3$.HCl) dissolved in acetonitrile at room temperature for 8 h to chemically activate the tail groups (carboxy groups) of SAM on the gold surface. After the platform was removed from EDC solution and rinsed thoroughly in acetonitrile and dried in a stream of nitrogen, mAb 1B4 was immobilized on SAM surface using the procedures described above.

Detection of *S. enterica* Typhimurium in inoculated milk using developed biosensor

Each of the prepared sensor platforms was incubated with 100 µl of milk inoculated with *S. enterica* Typhiumrium from 10^1 to 10^8 cfu/ml in a square well microtiter plate at room temperature for 1 h. To evaluate the effect of the background bacteria on the detection of *S. enterica* Typhimurium using the biosensor, *E coli* was

inoculated into milk at 10^8 cfu/ml before inoculating different concentrations of *Salmonella* into milk. The unbound bacteria were removed by washing the sensor platform three times with PBST then four times with DI water. After the biosensors were dried at room temperature, the bacteria captured by the biosensor were fixed with OsO_4 for 1 hr and photographed using a Nikon Eclipse L150 Industrial light microscope (Nikon Instruments Inc., Melville, NY) with 1,000 times magnification. Each image represents 0.025 mm^2 area of the biosensor. The experiment was repeated five times, and the number of the captured bacteria on the representative microscopic images was recorded. The bacterial number on each field was counted and the detection of *Salmonella* was defined as positive when one or more bacteria were observed on each of the images.

RESULTS AND DISCUSSION

Media comparison

In order to rapidly bring low *Salmonella* concentration in milk to the detectable level, one step enrichment in different non-selective and selective media was compared. The non-selective enrichment media included tryptic soy broth (TSB), half strength TSB (1/2 TSB), lactose broth (LB), nutrient broth (NB), universal preenrichment broth (UPB), buffered peptone water (BPW), and brain heart infusion broth (BHI). The selective enrichment media included brilliant green broth (BG), selenite broth (SB), selenite cystine broth (SC), Rappaport-Vassiliadis R10 broth (RV), and Salmosyst broth. Barbar's™ Vitamin D whole milk was inoculated with *S. enterica* Typhimurium at 10^3 cfu/ml. Due to the 1:9 ratio of milk to broth, the initial bacterial concentration in broth was 10^2 cfu/ml before incubation. The bacterial number in the media was checked by

plating on bismuth sulfide agar after 6 h incubation. Among the seven non-selective enrichment media, *Salmonella* grew best in BHI, in which the *Salmonella* number increased from 1×10^2 cfu/ml to 3.7×10^6 cfu/ml after 6 h incubation (Figure1). Among the five selective enrichment media, *Salmonella* grew best in BG, in which *Salmonella* number increased from 1×10^2 cfu/ml to 4.8×10^6 cfu/ml after 6 h incubation. Based on this result, BHI and BG were selected for further study.

Efficacy of one step enrichment in BG and BHI

BG, selective medium, and BHI, non-selective medium, have similar efficacy for the enrichment of *Salmonella* in this study (Table 1). When *S. enterica* Typhimurium was inoculated in milk at 0.1 cfu/ml, the initial bacterial number in broth was 0.01 cfu/ml due to 1:9 ratio of milk to broth. Enrichment in BG and BHI for 4 h increased *Salmonella* number from 0.01 cfu/ml in broth to less than 10 cfu/ml in broth. Six hour enrichment in broth increased *Salmonella* number to over 10^2 cfu/ml, which is the detection limit of the developed biosensor as determined in Chapter IV. When *S. enterica* Typhimurium was inoculated in milk at 1.0 cfu/ml, *Salmonella* number increased from 0.1 cfu/ml in broth to over 10^2 cfu /ml in broth within 4 h enrichment in both BG and BHI. When *S. enterica* Typhimurium was inoculated in milk at 10 cfu/ml, *Salmonella* number increased from 1 cfu/ml in broth to over 10^3 cfu/ml in 4 h enrichment in both BG and BHI. **Concentration of *Salmonella* from one step enrichment media by centrifugation**

Fifty ml of enrichment media at 2, 4, and 6 h incubation was centrifuged and resuspended in 1 ml PBS. Theoretically, *Salmonella* should be concentrated 50 times. By comparing the bacterial concentration before centrifugation and after centrifugation, we found that *Salmonella* concentration increased 30 to 50 times by centrifugation (Table 2).

The difference between the theoretical and the actual concentration times may be due to the loss of bacteria during aspiration of the supernatant and/or inaccurate pellet resuspension volume.

Comparison of different sensor platform

The four different sensor platforms, gold, polyvinyl chloride (PVC), polystyrene (PS), and glass, were used in this study. When different concentrations of *Salmonella* (10 to 10^8 cfu/ml) were tested using 4 different sensor platforms, the gold platform performed better than the other three types of platforms (Table 3). PVC and PS, which are the commonly used solid phases in immunoassays, did not show better antibody immobilization capability than the gold platform. In the PVC platform, bacteria tended to bind to the edge of the platform, which made it difficult to focus and visualize the captured bacteria. The SAM did not increase the detection sensitivity of the gold platform.

Indirect sandwich ELISA

Indirect sandwich ELISA was tested against *Salmonella* inoculated milk with or without an excessive amount of *E. coli* (10^8 cfu/ml). The ELISA response (O.D. $_{405\ nm}$ reading) decreased rapidly (from above 2 to 0.6) when inoculated *Salmonella* concentration decreased from 10^8 to 10^5 cfu/ml (Figure 2). ELISA response gradually decreased from 0.6 to around 0.4 when *Salmonella* concentration decreased from 10^5 to 10^2 cfu/ml. The detection limit of ELISA, defined as the mean O.D. value of the negative control (milk or *E. coli* inoculated milk without *Salmonella*) plus 2 standard deviations, was 10^2 cfu/ml. The presence of 10^8 cfu/ml *E. coli* in milk did not interfere with the detection of *Salmonella* by indirect sandwich ELISA.

Sensitivity of Biosensor

The biosensor was used to test against *Salmonella* inoculated milk with or without excessive amounts of *E. coli* (10^8 cfu/ml). The number of the captured *Salmonella* dramatically decreased when inoculated *Salmonella* concentration decreased from 10^8 to 10^5 cfu/ml, then gradually decreased when inoculated *Salmonella* concentration decreased from 10^5 to 10^2 cfu/ml (Table 3). These results have shown good correlation with the results of the indirect sandwich ELISA.

Although small particles of milk content sometimes were visible under the light microscope, their shape and size were distinctly different from captured *Salmonella*. Therefore, these particles would not affect the enumeration of *Salmonella* from the biosensor. Excessive amount of *E. coli* did not interfere with the detection sensitivity of *Salmonella* by the biosensor.

When the biosensor was used to test the enriched samples, 6 h incubation was needed to bring *Salmonella* concentration from 0.1 cfu/ml to the detectable level (> 10^2 cfu/ml) of the biosensor. However, when the biosensor was used to test the enriched plus centrifuged samples, 4 h incubation was needed to bring *Salmonella* concentration from 0.1 cfu/ml to the detectable level of the biosensor. The ingredients of the culture media and milk content did not interfere with the detection of *Salmonella* by the biosensor. Centrifugation step removed most of the milk content and resulted in clean background on the biosensor. Since 1 h was needed to perform biosensor testing, the entire time needed for detecting 0.1 cfu/ml *Salmonella* in milk was 5 h when centrifugation was employed to concentrate *Salmonella* from enrichment broth.

CONCLUSION

Both BG and BHI are efficient for the enrichment of *Salmonella* prior to detecting *Salmonella* using the developed biosensor. Six hour enrichment in either BG or BHI would be sufficient for the detection of 0.1 cfu/ml *Salmonella* in milk by the developed biosensor. The centrifugation technique used in this study was able to concentrate *Salmonella* up to 50 times. Through a one step 4 h enrichment in BG/BHI followed by centrifugation, the biosensor was able to detect 0.1 cfu/ml *Salmonlla* in milk in 5 h. The indirect ELISA results correlated well with the biosensor results.

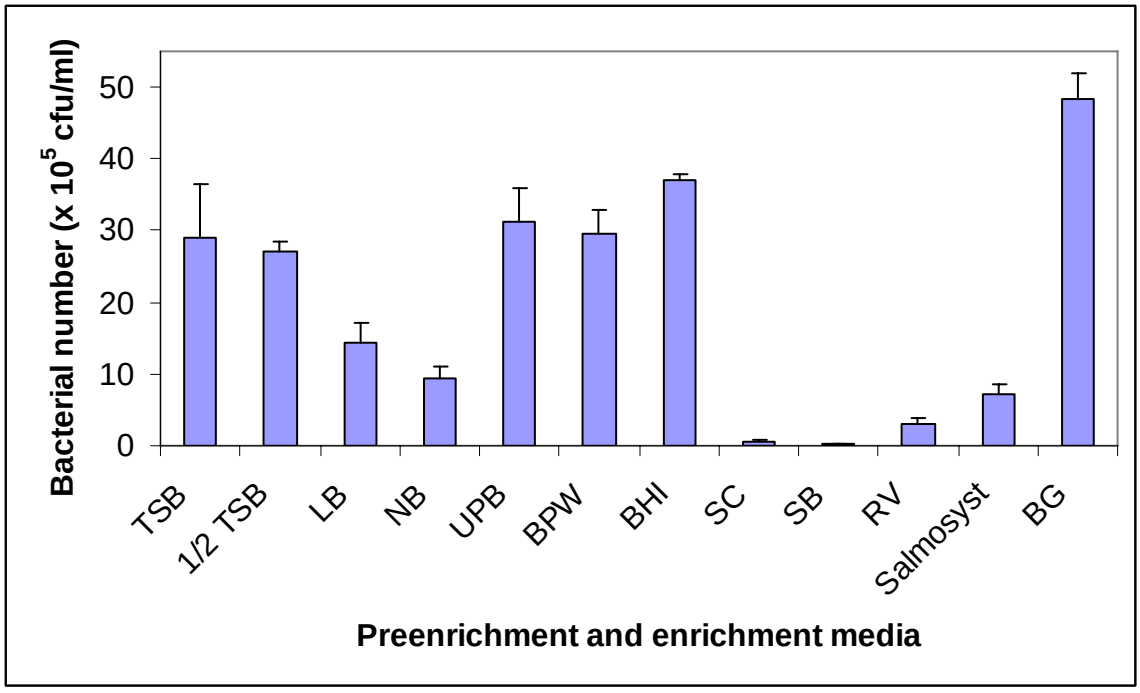

Figure 1. Comparion of different preenrichment and enrichment media for the recovery of inoculated *S. enterica* Typhimurium in milk after 6 h enrichment. TSB: Trypticase soy broth, ½ TSB: half strength TSB, LB: lactose broth, NB: nutrient broth, UPB: universal preenrichment media, BPW: buffered peptone water, BHI: brain heart infusion broth, SC: selenite broth, SB: selenite broth, RV: Rappaport-Vassiliadis R10 broth, Salmosyst: salmosyst broth, and BG: brilliant green broth.

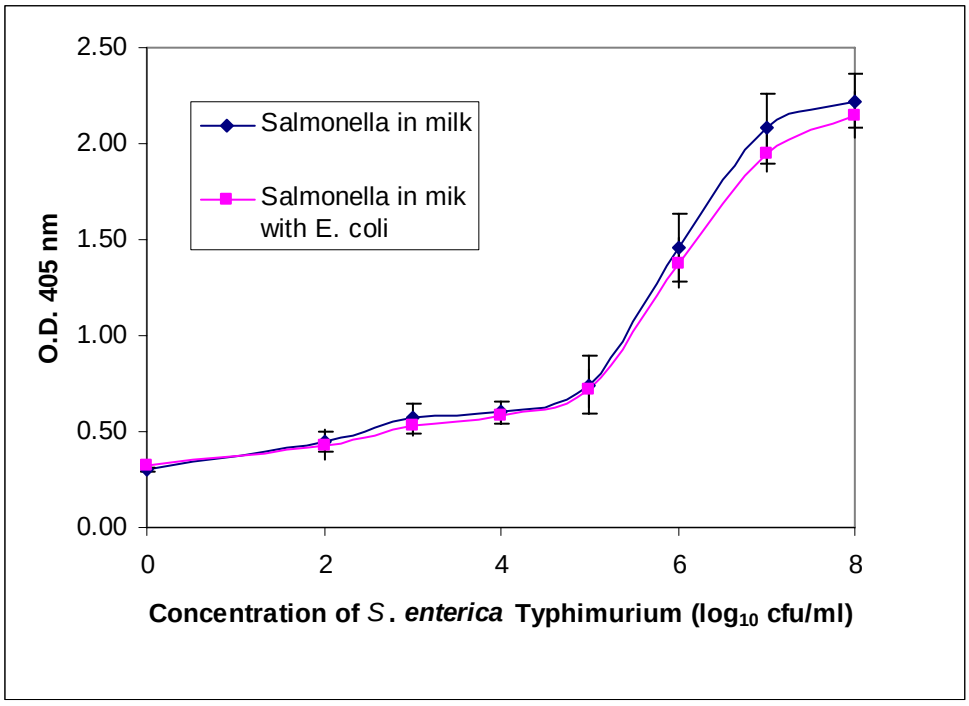

Figure 2. Detection sensitivity of indirect sandwich ELISA using mAb 1B4 as capture antibody. *E. coli* was inoculated at 10^8 cfu/ml in milk.

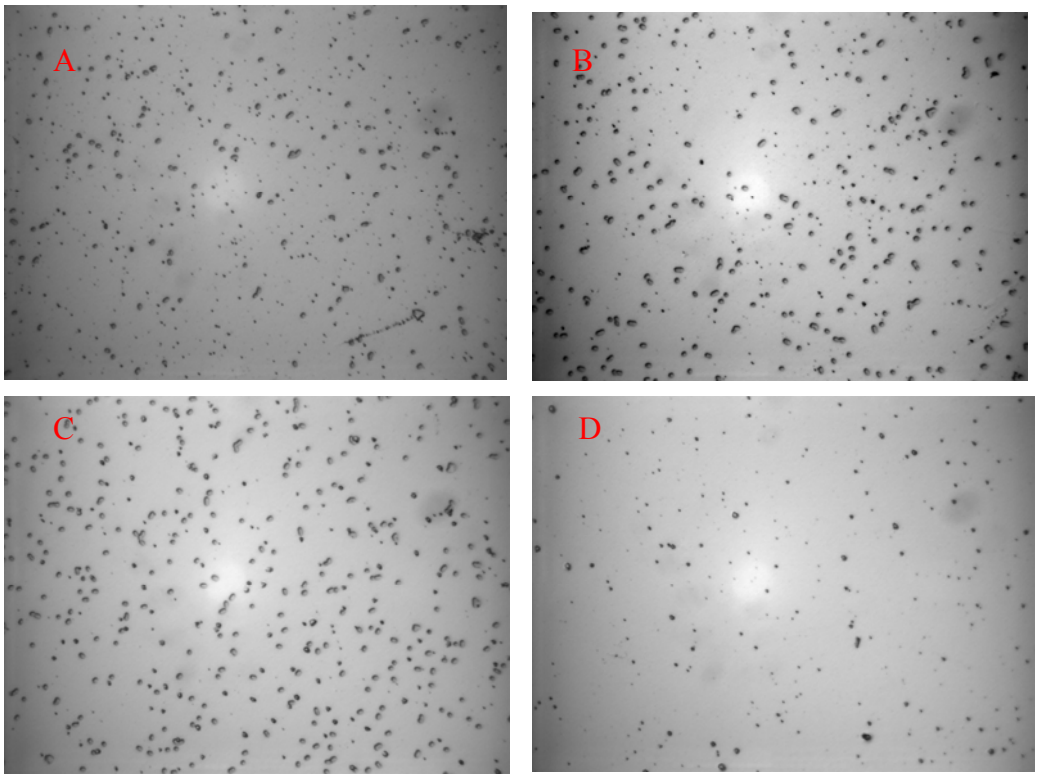

Figure 3. Light microscopic images of captured *S. enterica* Typhimurium in milk, enrichment broth, and centrifuged samples. A: inoculated milk, B: enrichment broth, C: centrifuged sample, D: milk negative control.

Table 1. Efficacy of one step enrichment at 2, 4, and 6 h incubation period before or after centrifugation.

Inoculation dose (cfu/ml)	Media	Bacterial concentration (cfu/ml)					
		Without centrifugation			With centrifugation		
		2 h	4 h	6 h	2 h	4 h	6 h
0.1	BG	ND*	8	5.7×10^2	ND	2.7×10^2	2.0×10^4
	BHI	ND	3	1.6×10^2	ND	1.6×10^2	6.3×10^3
1.0	BG	4	1.5×10^2	1.1×10^4	1.2×10^2	7.8×10^2	3.8×10^5
	BHI	2	1.6×10^2	1.0×10^4	1.1×10^2	6.2×10^2	3.8×10^5
10	BG	25	1.8×10^3	8.1×10^4	8.3×10^2	7.1×10^4	4.0×10^6
	BHI	11	1.4×10^3	6.5×10^4	5.6×10^2	6.1×10^4	3.2×10^6

ND*: not detectable using pour or spread plate method

Table 2. Bacterial concentration efficacy of centrifugation.

Inoculation dose	Media	Enrichment Time		
		2 h	4 h	6 h
0.1 cfu/ml				
	BG	ND*	34	35
	BHI	ND	50	38
1.0 cfu/ml				
	BG	30	52	38
	BHI	50	39	38
10 cfu/ml				
	BG	33	40	48
	BHI	53	43	49

Concentration cofactor shown in this table was calculated through dividing the bacterial concentration after centrifugation by the bacterial concentration before centrifugation.

ND*: Because the bacterial concentration before centrifugation was not detectable using pour or spread plate methods, the ratio of the bacterial concentration after centrifugation and the bacterial concentration before centrifugation could not be calculated.

Table 3. Comparison of four different sensor platforms

S. enterica Typhimurium (cfu/m)	Sensor platform				
	Gold	Glass	PS	PVC	SAM
1×10^{8}	467	295	360	237	430
1×10^{7}	46	20	38	33	48
1×10^{6}	8	2	10	11	7
1×10^{5}	3	2	5	5	3
1×10^{4}	2	1	1	0	1
1×10^{3}	2	0	1	0	1
1×10^{2}	1	0	1	0	1
1×10^{1}	0	0	0	0	0
0	0	0	0	0	0

Bacterial number shown in this table is mean of six replications. PS: polystyrene, PVC: polyvinyl chloride

CHAPTER V. OVERALL CONCLUSIONS

Development of a sensitive and specific biosensor requires highly specific and sensitive monoclonal antibodies. Most of the currently developed antibodies are against either O-antigen or H-antigen. They suffer from different degrees of cross reactions with other enteric bacteria. False positive and false negative results have been reported using the O- or H- antigen specific antibodies. Therefore, the development of highly specific antibodies against other antigens needs to be investigated. The 55 kD OMP of *S. enterica* Typhimurium was extracted and used as an antigen for the production of Salmonella specific antibodies. This is the first time the use of the 55 kD OMP of *S. enterica* Typhimurium for specific antibody production has been reported. The mAb 1B4 developed in this study has high specificity and high sensitivity to *S. enterica* Typhiumrium. The biosensor (an immunosensor combined with microscopic imagining systems) developed in this study can specifically detect *S. enterica* Typhiumrium in 1 h with a sensitivity of 1×10^2 cfu/ml. Therefore, the entire detection method is rapid, cheap, and simple.

In order to culture the relatively low number of *Salmonella* in milk, 7 different non-selective and 4 selective enrichment media were investigated. Among these, BHI and BG were selected as the best non-selective and selective enrichment media respectively. A six hour enrichment in either BG or BHI was able to rapidly increase *S. enterica*

Typhimurium concentration from 0.01 cfu/ml to over 10^2 cfu/ml in broth, which could be detected by the biosensor. The centrifugation technique used in this study was able to concentrate bacteria 50 times. In conjunction with centrifugation, 4 h enrichment was sufficient to bring 0.1 cfu/ml inoculated *S. enterica* Typhimurium to detectable level. Unlike other biosensors, the detection sensitivity and specificity of this biosensor was not affected by milk content.

Further work should focus on the detection of stressed *Salmonella* in milk and other foods, such as meat, poultry and vegetables. Naturally contaminated foods should be tested using the developed biosensor. The test results of the biosensor should be confirmed by traditional culture methods to verify accuracy. Automation of the detection process using the developed biosensor should be investigated.

CHAPTER VI. REFERENCES

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