Development of Antibody- and Phage-Based Lateral Flow Test Strips for the Detection of the Bovine Meat and Bone Meal in Animal Feeds and the *Bacillus anthracis* Spores in Water and Foods

by

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Abstract

The outbreak of bovine spongiform encephalopathy (BSE) in 1980s and 1990s, peaked in 1992, mainly took place in most European countries, especially in the United Kingdom, caused widespread panic in the world. It was generally accepted that the most likely route of infection of cattle with BSE is by consumption of feeds containing low levels of processed animal proteins (PAPs), including bovine meat and bone meal (MBM). At present, classical microscopy is the only official method for the detection of PAPs in animal feeds in the European Union. Nevertheless, the method is laborious and time-consuming, and the analytical results depend largely on the experience of the microscopist.

The need for a simple, rapid, specific, sensitive, accurate and inexpensive method to detect *Bacillus anthracis* (BA) spores became obvious after the Fall 2001 anthrax attacks which caused public fears. Immunoassay and polymerase chain reaction (PCR) are generally used for the detection of BA spores. PCR requires considerable effort in sample processing prior to analysis. Therefore, better detection methods need to be developed.

The research described in this dissertation focused on developing antibody- and phage-based lateral flow test strip (LFTS) assay to detect the bovine MBM in animal feeds and the BA spores in water and foods, respectively. Bovine MBM proteins (bMBMP) were extracted from bovine MBM using a special extraction protocol, i.e., bovine MBM was pretreated in 1.0 M NaOH solution under the constant agitation at 37 ^{O}C for 48 h, and after pH adjustment and centrifugation, the precipitate was extracted by sterile deionized water. The anti-bMBMP and

anti-pBA13 (BA 13 phage) antibodies were produced, and antibodies were purified by saturated ammonium sulfate precipitation followed by Protein A affinity column. Anti-bMBMP antibodies were conjugated to colloidal gold and form gold-anti-bMBMP antibody complex, which was deposited in the conjugate pad of LFTS. In addition, the anti-bMBMP antibody was also used as capture agents on the test line of LFTS, which can detect the bovine MBM in animal feeds. The anti-bMBMP antibody was successfully utilized to construct a LFTS which is sensitive and specific to bovine MBM in animal feeds. The detection limit of anti-bMBMP antibody is less than 0.1% (w/w), which reaches the requirement of industry use. The cross-reaction of the anti-bMBMP antibody with the proteins in the animal feeds can be eliminated or significantly reduced after immunoabsorption.

For the development of the phage-based LFTS, *B. anthracis* 13 phage (pBA13) or JRB7 phage (pJRB7) was conjugated to colloidal gold and form gold-pBA13 or gold-pJRB7 complex which was used as recognition element to detect BA spores in water and foods. This complex was deposited in conjugate pad to bind the BA spores in the sample. In the LFTS, the pBA13 or pJRB7 was immobilized on glutaraldehyde-modified nitrocellulose membrane (NCM) as test line and anti-pBA13 antibody was directly immobilized on NCM as control line for detection. The detection limit of the developed phage-based LFTS is 10^7 spores/ml.

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List of Abbreviations

anti-bMBMP Anti-Bovine Meat and Bone Meal Protein

anti-bMBMPAb Anti-Bovine Meat and Bone Meal Protein Antibody

anti-MBMPAbs Anti-Meat and Bone Meal Protein Antibodies

anti-pBA13 Anti-*Bacillus anthracis* 13 Phage

anti-pJRB7 Anti-JRB7 Phage

anti-pBA13Abs Anti-*Bacillus anthracis* 13 Phage Antibodies

ATA Anthrax Toxin Activator

AuNP Gold Nanoparticle

bMBM Bovine Meat and Bone Meal

bMBMP Bovine Meat and Bone Meal Protein

BA *Bacillus anthracis*

BA13 *Bacillus anthracis* 13

BSA Bovine Serum Albumin

BSE Bovine Spongiform Encephalopathy

BclA *Bacillus* Collagen-Like Protein of *anthracis*

CJD Creutzfeldt-Jakob Disease

CWD Chronic Wasting Disease

DAO Diaminooctane

ELISA Enzyme-Linked Immunosorbent Assay

EF	Edema Factor
EU	European Union
FCM	Flow Cytometry
GA	Glutaraldehyde
GSS	Gerstmann-Sträussler-Schenker
ICS	Immunochromatographic Strip
LF	Lethal Factor
LFICS	Lateral Flow Immunochromatography Strip
LFTS	Lateral Flow Test Strip
LOD	Limit of Detection
mAbs	Monoclonal Antibodies
MALDI	Matrix-Assisted Laser Desorption/Ionization
MBM	Meat and Bone Meal
MBMP	Meat and Bone Meal Protein
MCD	Mad-Cow Disease
MMBM	Meat Meal or Meat and Bone Meal
MPLS	Modified Partial Least Squares
nvCJD	new variant Creutzfeldt-Jakob Disease
NASBA	Nucleic Acid Sequence-Based Amplification
NC	Nitrocellulose
NCM	Nitrocellulose Membrane
NIR	Near Infrared
NIRM	Near Infrared Microscopy

NIRS Near Infrared Spectroscopy

O.D. Optical Density

$O.D._{405nm}$ Optical Density at 405 nm

$O.D._{520nm}$ Optical Density at 520 nm

pAbs Polyclonal Antibodies

pBA Phage *Bacillus anthracis* or *Bacillus anthracis* Phage

pBA13 Phage *Bacillus anthracis* 13 or *Bacillus anthracis* 13 Phage

pJRB7 Phage JRB7 or JRB7 Phage

p-NPP *p*-Nitrophenyl Phosphate

PA Protective Antigen

PAPs Processed Animal Proteins

PBS Phosphate Buffered Saline

PBST Phosphate Buffered Saline plus 0.05% Tween-20

PCA Principal Component Analysis

PCR Polymerase Chain Reaction

PEG Polyoxyethylene Glycol

PLS Partial Least Squares

PLS-DA Partial Least Squares-Discriminant Analysis

PMSF Phenylmethylsulfonyl Fluoride, or Alpha-Toluene Sulfonyl Fluoride

PrP Prion Protein

PVA Polyvinyl Alcohol

QD Quantum Dot

rt-PCR Reverse Transcriptase Polymerase Chain Reaction

RBC Rabbit Blood Collection

RIIs Residual Insoluble Impurities

RPE R-Phycoerythrin

RT Room temperature

RT-PCR Real-Time Polymerase Chain Reaction

sBA Spore of *Bacillus anthracis*

SADB Striping Antibody Dilution Buffer

SDS Sodium Dodecyl Sulfate

SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SECV Standard Error of Cross-Validation

SPR Surface Plasmon Resonance

TEM Transmission Electron Microscope

TME Ttransmissible Mink Encephalopathy,

TSE Transmissible Spongiform Encephalopathy

vCJD variant Creutzfeldt-Jakob Disease

WHO World Health Organization

Chapter One Introduction

1.1 Detection of Bovine Meat and Bone Meal in Animal Feeds

1.1.1 Bovine Spongiform Encephalopathy

Transmissible spongiform encephalopathies (TSEs) are a group of rare, fatal and transmissible neurodegenerative diseases of animals and humans (Liberski 1993; Belay 1999; Whitley and others 2000; Hueston and Bryant 2005; Jennelle and others 2009). The typical characterization of the disease is the spongy degeneration of the brain (Liberski 1993; Belay 1999). Known animal TSEs include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, chronic wasting disease (CWD) in North American mule deer and elk, transmissible mink encephalopathy (TME) in mink, exotic ungulate encephalopathy in Nyala, kudu, gemsbok and oryx, and feline spongiform encephalopathy in cats (puma, cheetah) (Hart 1995; Haywood 1997; Whitley and others 2000; Hueston and Bryant 2005; Jennelle and others 2009). Known human TSEs mainly include kuru, Creutzfelt-Jakob disease (CJD), variant Creutzfelt-Jakob disease (vCJD), Gerstmann-Sträussler-Schenker (GSS) syndrome mainly occurring in persons with an apparent hereditary predisposition, and fatal insomnia which occurs in familial and sporadic forms (Liberski 1993; Hart 1995; Haywood 1997; Belay 1999; Hueston and Bryant 2005). CJD is the most common of all the human TSEs and is the disease most frequently mistaken for vCJD (Whitley and others 2000; Jennelle and others 2009).

BSE, commonly known as mad-cow disease (MCD), is a transmissible, neurodegenerative, fatal brain disease of cattle, which causes a spongy degeneration in the brain and spinal cord of

cattle (Özel and others 1994; Brown 2008; Giles and others 2008; Jennelle and others 2009). Lesions are typically characterized by sponge-like changes visible with an ordinary microscope (Özel and others 1994; Giles and others 2008). BSE has an incubation period of four to five years, usually affecting adult cattle at a peak age onset of four to five years, all breeds being equally susceptible, and it is fatal for cattle within weeks to months while the cattle is onset (Hart 1995; Whitley and others 2000; Haywood 1997; Hueston and Bryant 2005; Jennelle and others 2009). BSE is considered a worldwide problem with a significant potential threat to human health (Özel and others 1994; Haywood 1997; Brown 2008; Giles and others 2008).

The emergence of BSE in cattle was first recognized in the United Kingdom in 1986, the peak period of time was from 1988 to 1992, and it has now been found in at least 26 countries throughout the world (Hart 1995; Brown 2008). BSE is one of several different forms of transmissible brain disease affecting a number of animal species (Liberski 1993; Hart 1995; Haywood 1997; Brown 2008). A neurological disease in household cats, ruminant animals and feline species in zoos has been linked to BSE (Hart 1995; Haywood 1997; Brown 2008).

Although about a quarter of century passed after the emergence of BSE, the real origin of BSE disease remains unknown and the nature of the BSE agent is still being debated (Liberski 1993; Prusiner 1994; Haywood 1997; Brown 2008). Most scientists believed that the disease may be transmitted to human beings who eat the brain or spinal cord of infected carcasses. There have been several theories or hypotheses which are adopted to explain BSE infectious agent (Haywood 1997; Brown 2008). For instance, the prion theory is most widely accepted by most scientists (Hart 1995; Haywood 1997; Brown 2008). Based on the prion theory, the infectious agent in BSE is believed to be a specific type of misfolded and self-replicating protein, which is termed as a prion. The prion proteins (PrP) carry the disease between individuals and cause

deterioration of the brain (Prusiner 1994; Haywood 1997; Giles and others 2008). Another theory suggests that the BSE infectious agent is virus or virus-like and possesses nucleic acids which carry genetic information (Özel and Diringer 1994; Özel and others 1994; Prusiner 1994; Haywood 1997). Comparatively, over the past decade the collected evidences significantly support the prion theory, in which the prion is transmitted through the consumption of BSE-contaminated meat and bone meal (MBM) supplements in cattle feed (Özel and Diringer 1994; Özel and others 1994; Hart 1995; Haywood 1997; Brown 2008). The ability of the BSE agent to form multiple strains, however, is more readily explained by a virus-like agent (Özel and others 1994; Haywood 1997; Brown 2008).

vCJD as a newly recognized form of CJD in human beings was first reported in the United Kingdom in March 1996. It was found that in contrast to the classical forms of CJD, vCJD easily influences younger patients (average age 29 years, as opposed to 65 years), has a relatively longer duration of illness (median of 14 months as opposed to 4.5 months) and is strongly linked to exposure, probably through food, to BSE (Davies and others 1993; Hart 1995; Brown 2008). Recent studies have confirmed that vCJD is distinct from sporadic and acquired CJD (Brown 2008; Jennelle and others 2009).

1.1.2 Bovine Meat and Bone Meal (MBM)

Rendered animal tissue, which is processed at high temperature and is also known as meat and bone meal (MBM), is a product of the rendering industry (Taylor and Woodgate 2003; Adedokun and Adela 2005; Garcia and Phillips 2008). It was reported that the main components of MBM are typically about 50% protein, 35% ash, 8-12% fat, and 4-7% moisture (Hendriks and others 2002; Garcia and Phillips 2008). MBM was primarily used as a dietary supplement to

improve the amino acid profile of the feed for farm animals, in particular as a protein supplement to provide essential amino acids to lactating and fast-growing animals (Pearl 2004; Adedokun and Adela 2005; Garcia and Phillips 2008; Fumière and others 2009). It could be used directly as feed or as an ingredient in animal feeds. Many scientists believed that feeding of MBM to cattle could be responsible for the spread of BSE (Momcilovic and Rasooly 2000; Gizzi and others 2003; Taylor and Woodgate 2003). According to World Health Organization (WHO) MBM is officially prohibited to be employed in feeds for ruminant animals, and even all farm animals in most countries. However, in some areas MBM is still used as feeds for monogastric animals. It is widely used in the United States as a low-cost meat in dog food and cat food (Taylor and Woodgate 2003; Adedokun and Adela 2005; Fumière and others 2009).

In many European countries, some MBM is also used as ingredients in pet food but the vast majority is now used as a fossil-fuel replacement for renewable energy generation, as a fuel in cement kilns, landfilling or incineration (Hendriks and others 2002; Pearl 2004; Garcia and Phillips 2008). MBM has about two thirds energy value of fossil fuels such as coal. In United Kingdom MBM is widely used for the generation of renewable electricity, which became particularly prominent after many cattle had to be slaughtered during the BSE crisis (Pearl 2004; Garcia and Phillips 2008). MBM is increasingly utilized in cement kilns as an environmentally sustainable replacement for coal (Hendriks and others 2002; Pearl 2004; Fumière and others 2009).

1.1.3 Detection of Bovine Meat and Bone Meal

To avoid MBM entering the ruminant food chain, the United Kingdom in 1988 enforced a ban which prohibited ruminant material (for instance derived from cattle, sheep, goats or deer)

used as feed for other ruminants (Gizzi and others 2003; Fumière and others 2009). This ban was later extended in the United Kingdom to include all mammalian proteins to all farmed livestock (excluding fish and horses). A ban prohibiting the use of mammalian-derived protein in ruminant feed was introduced to the European Union (EU) in 1994 and to the United States of America (USA) in 1997 (Gizzi and others 2003; van Raamsdonk and others 2004; Fumière and others 2009). Since 2000 a European ban has been extended to include the proteins derived from mammals, birds and fish for all farmed animals to be used in the production of food (Fumière and others 2009).

In order to enforce current legislation, it is necessary to be able to detect prohibited proteins, and identify prohibited and authorized proteins in animal feeds, especially in ruminant feed. Microscopic analysis, which in essence utilizes the microscope at lower (8x-50x) or higher magnification (40x-400x) to identify and detect the color and morphology of the bone fragments in feed material, is currently the only officially recognized method in the EU for the detection of constituents of animal origin in animal feeds (Gizzi and others 2003; van Raamsdonk and others 2004; Fumière and others 2009). However, microscopic analysis requires an experienced analyst, cannot be utilized on liquid samples, cannot detect soft tissues, and is a time-consuming and costly method requiring the use of organic solvents for sedimentation of bone fragments (Gizzi and others 2003; Fumière and others 2009). Enzyme-linked immunosorbent assay (ELISA), near infrared spectroscopy (NIRS), near infrared microscopy (NIRM), polymerase chain reaction (PCR) and real-time PCR were developed to detect and identify animal tissues and MBM in feeds (Gizzi and others 2003; van Raamsdonk and others 2004; Fumière and others 2009). Generally speaking, ELISA is an easy and simple method for bovine MBM detection via an antibody-based detection system. The disadvantages of the ELISA method are that ruminant

products such as milk and milk products have interferences for the results of ELISA tests, and that there exists cross-reaction with other proteins such as plant proteins (Gizzi and others 2003; Fumière and others 2009). NIRS and NIRM require large comparative databases and costly equipment. Particularly for NIRS, accuracy has been a problem (van Raamsdonk and others 2004; Fumière and others 2009). PCR techniques theoretically permit the identification of species-specific nucleic acid, particularly deoxyribonucleic acid (DNA). However, an important limitation of the PCR approach used for the detection of MBM in animal feeds is that the authorized feed ingredients such as milk, blood, fat, hydrolyzed proteins produced from ruminant hides and skins or egg products may contain target DNA which cause false results (Gizzi and others 2003; Fumière and others 2009).

1.2 Detection of *Bacillus anthracis* Spores

1.2.1 *Bacillus anthracis*

Bacillus anthracis, the causative agent of anthrax disease (Rosen and others 1997; Pellegrino and others 1998; Swartz 2001; Higgins and others 2003; Wang and others 2004), is a Gram-positive, aerobic, rod-shaped bacterium. The members of the genetically closely related *Bacillus* genus, including *B. anthracis*, *B. cereus*, and *B. thuringiensis*, collectively form the *B. cereus* group of bacteria, which exist ubiquitously in nature (Errington 1993; Nicholson and others 2000; Wang and others 2009a, 2009b). One of the distinct features among members of the *B. cereus* group is plasmids that encode for virulence factors (Green and others 1985; Helgason and others 2000). Fully virulent bacilli carry two plasmids, pXO1 and pXO2. The former contains genes to produce the edema factor and lethal factor toxins, and the latter contains genes to produce a poly-c-D-glutamic acid capsule (Mikesell and others 1983; Green and others 1985;

Park and others 2006; Wang and others 2009a). The pXO1 plasmid of *B. anthracis* encodes for protective antigen (PA), edema factor (EF), and lethal factor (LF); the three proteins can interact synergistically to form edema toxin (PA and EF) and lethal toxin (PA and LF) (Abrami and others 2005, Park and others 2006). Fully virulent isolates of *B. anthracis* also harbor the pXO2 plasmid that encodes for an anti-phagocytic poly-D-glutamic acid capsule (Green and others 1985; Park and others 2006). The *B. anthracis* Ames strain possesses both virulence plasmids, whereas minimally virulent Sterne and Pasteur strains lack pXO2 or pXO1, respectively (Park and others 2006; Wang and others 2009a).

1.2.2 *Bacillus anthracis* spore

Whenever vegetative cells of *B. anthracis* are deprived of nutrients, *B. anthracis* spores are formed. These spores are highly resistant to normally destructive environmental factors to living cells, such as heat (extreme temperature), radiation, toxic chemicals, desiccation, and physical damages, and can remain viable for many years (Williams and others 2003; Kuehn and others 2009; Wang and others 2009a). The resistance of *B. anthracis* spores to the harsh conditions allows them to persist in a dormant state for extended periods of time in the environment. These remarkable characteristics make them suitable for biological warfare (Rosen and others 1997; Pellegrino and others 1998; Nicholson and others 2000; Park and others 2006; Swiecki and others 2006; Wang and others 2009a, 2009b).

The threat of a biological attack with *Bacillus anthracis* spores has been of particular concern all over the world during the past decade, especially after the anthrax attack that occurred in the USA following the attack on the World Trade Center on September 11, 2001 (Hartley and Baeumner 2003; Brigati and others 2004; Zhang and others 2006; Wang and others 2009a,

2009b). Based on the method of contact with *B. anthracis* spores, infection can lead to cutaneous, gastrointestinal, or pulmonary anthrax (Hartley and Baeumner 2003; Brigati and others 2004; Swiecki and others 2006; Wang and others 2009a). The severity of the disease is dependent on the spore's ability to evade the host's immune responses, resume vegetative growth, and secrete edema and lethal toxin (Brigati and others 2004; Wang and others 2009b). Pulmonary anthrax is the form most commonly associated with bioterrorism because spores can be aerosolized and their size is optimal for deposition in the lungs (Brigati and others 2004; Wang and others 2009a). After inhalation the *B. anthracis* spores are transported by alveolar macrophages to lymph nodes where germination occurs (Walt and Franz 2000; Brigati and others 2004; Wang and others 2009a, 2009b). The bacteria then replicate to very high numbers in the blood and secrete the toxin which ultimately leads to death of the host (Walt and Franz 2000). Inhalation of more than $8 \times 10^3 \sim 1 \times 10^4$ *B. anthracis* spores can lead to death unless medical treatment is received within 24-48 h (Walt and Franz 2000; Wang and others 2009a). Therefore, the rapid and accurate detection of *B. anthracis* spores in the environment prior to infection is extremely important for human safety and national security (Walt and Franz 2000; Brigati and others 2004; Park and others 2006; Wang and others 2009a, 2009b).

1.2.3 Detection of *Bacillus anthracis* Spore

Various methods have been exploited to detect and identify *B. anthracis* spores, such as bacteriological assays, immunological assays, PCR-based methods and other advanced approaches (Baeumner and others 2004; Edwards and others 2006; Wang and others 2009b). These techniques, however, have significant limitations in that they tend to be time-consuming or low in sensitivity. Over the last decade, with the progress in the development of biosensors

and their applications in biology, several sensor platforms that can be used for the direct, rapid and real-time detection and identification of *B. anthracis* spores have been developed (Edwards and others 2006). These detectors include various sensor devices such as quartz crystal microbalance (QCM) (Ivnitski and others 1999; Vaughan and others 2003; Skottrup and others 2008), evanescent wave fiber-optic biosensor (Tims and Lim 2004), electrochemiluminescence sensor (Bruno and Kiel 2004; Lazcka and others 2007), SPR-based sensors (Lazcka and others 2007; Skottrup and others 2008; Wang and others 2009a), electrical or electrochemical impedance sensor (Lazcka and others 2007; Yang and Bashir 2008), and cantilever sensors (Campbel and Mutharasan 2006; Fritz 2008; Skottrup and others 2008).

A biosensor consists typically of a bio-receptor, transducer, and recording device. The receptor recognition (sensing) towards target pathogen mainly relies on either immunosensing or nucleic acid detection. The principle of the immunosensors is based on the interaction between the antigens present on the target cells (sample) and the antibodies immobilized on the surface of the sensor platform. The resulting antigen-antibody complexes or conjugates can be detected via various sensing approaches such as fluorescence (Lazcka and others 2007), quartz crystalline microbalance (QCM) (Ivnitski and others 1999; Skottrup and others 2008), electrical or electrochemical impedance (Lazcka and others 2007; Yang and Bashir 2008), magnetoresistivity (Mujika and others 2009), cantilever (Fritz 2008; Skottrup and others 2008), and surface plasmon resonance (SPR) (Lazcka and others 2007; Skottrup and others 2008; Wang and others 2009a). The principle of the nucleic acid-based sensors is based on the detection of DNA or RNA originating from target cells. Because cells contain a low copy number of nucleic acids, the sensor generally requires a special step to amplify target nucleic acids by using polymerase chain reaction (PCR), reverse transcriptase PCR (rt-PCR) or a nucleic acid sequence-based

amplification (NASBA) reaction (Baeumner and others 2004). Additionally, there are several intricate strategies for amplifying signals that report the hybridization between probe and target DNA, such as using redox probes (Millan and Mikkelsen 1993; Millan and others 1994; Xu and others 2001), enzyme labels (de Lumley-Woodyear and others 1996), intercalators (Takenaka and others 2000), and nanoparticle labels (Fritzsche 2001; Huhtinen and others 2004; Bi and others 2009). The target DNA or RNA can also be detected by using numerous physical sensing methods. In general, the ultimate performance of a biosensor is mainly determined by the efficiency of the biochemical reaction, concentration of target analytes, and detection or transduction approaches of the biosensor (Heo and Hua 2009).

The need for a continuous monitoring system for *B. anthracis* spores was highlighted after the anthrax attack in the Fall of 2001 (Briagti and others 2004; Chesnokova and others 2009). Immunoassay- and biosensor-based detection systems are the best prospects for continuous monitoring systems, but they require specific, selective, and stable diagnostic probes with which the pathogen can be detected. Current monitoring systems mainly use the antibody-based recognition element (probe) to detect *B. anthracis* spores, but the long-term use under extreme conditions such as high temperature and low pH is limited (Briagti and others 2004; Chesnokova and others 2009). Briagti and others (2004) developed phage-derived probes by selecting clones that bound to spores of *B. anthracis* (Sterne strain) from a landscape phage library with random octapeptides. These phage-derived probes can be used as robust substitutes for antibodies.

A brief literature review on the detection methods of *B. anthracis* spores can be seen in Chapter 2.

1.3 Objectives of Research

The first overall goal of the application-based research described in this dissertation was to design, develop and assess the antibody-based lateral flow test strip (LFTS) assay to detect bovine MBM in animal feeds. The objectives utilized to reach this goal were: (i) to extract bovine MBM proteins from bovine MBM; (ii) to purify and characterize the bovine MBM proteins extracted; (iii) to produce anti-bovine MBM protein antibody; (iv) to design and develop an antibody-based LFTS assay to detect bovine MBM in animal feeds; and (v) to assess the detection limit and specificity of the LFTS assay. The second overall goal was to design, develop and assess the phage-based LFTS assay to detect *B. anthracis* spores. The objectives employed to achieve this goal were: (i) to produce anti-*B. anthracis* 13 phage (pBA13) antibody by using purified pBA13; (ii) to design and develop an phage-based LFTS assay to detect *B. anthracis* spores; and (iii) to assess the performance of the phage-based LFTS assay.

Chapter Two Literature Review

2.1 Detection of Bovine Meat and Bone Meal in Animal Feeds

The epidemic of bovine spongiform encephalopathy (BSE) mainly took place in many European countries, especially in the United Kingdom. After the outbreak of BSE the European Union immediately took steps, including enforcing laws and regulations, to avoid the transmission of BSE through the food chain (Momcilovic and Rasooly 2000; Gizzi and others 2003). Presently, with exception for fish meal, all of the processed animal proteins (PAPs), including meat and bone meal (MBM), are prohibited to use as feed ingredients for all farmed animals (Fumière and others 2009). Enforcement of these regulations and laws relies on analytical methods to identify the presence of prohibited proteins in animal feeds, especially ruminant feeds. The methods for the detection and identification of MBM in animal feeds mainly include classical microscopy, near infrared microscopy (NIRM), near infrared spectroscopy (NIRS), molecular biology techniques such as enzyme-linked immunosorbent assay (ELISA), lateral flow test strip (LFTS) and polymerase chain reaction (PCR), and combined methods (Momcilovic and Rasooly 2000; Gizzi and others 2003; Fumière and others 2009). A brief review for these methods is as follows.

2.1.1 Classical Microscopy

Classical microscopy is currently the only official method for the detection of PAPs in feeds in the European Union (Gizzi and others 2003; van Raamsdonk and others 2004; Fumière and others 2009). The method depends largely on the presence of bones in the sample, and is not affected by heat-treatment applied during rendering. In essence, this method is based on the colorful and morphological identification of bone fragments in feeds and allows identification of the constituents of animal origin and differentiation between fish and terrestrial material and mammalian versus non-mammalian material (Gizzi and others 2003; Fumière and others 2009). After sedimentation of bone fragments in an organic solvent (tetrachloroethylene, $Cl_2C = CCl_2$, relative density d = 1.62), at lower magnifications (8x-50x), the general appearance and color of the sediment is the first indication of the origin of the MBM (Gizzi and others 2003). Mammal bone particles are white or cream, whereas poultry bones generally have a darker color and are more splintered and sharply pointed in shape. Compared to the transparent fish bones, mammal and poultry bones are all opaque. The small, parallel-sided and hyaline fish bones can be seen in sediments of fish tissue (Gizzi and others 2003; van Raamsdonk and others 2004). At higher magnifications (40x-400x), mammal bone particles show a more or less globular appearance with elliptical to almost globular lacunae. Canaliculae may be visible depending on the quality and opaqueness of the bone particle. The direction in circular lamellae is sometimes visible (Gizzi and others 2003; Fumière and others 2009). Poultry particles usually show a more splintered (sharp edged) appearance, which is caused by the different structure of the air filled bones. Lacunae are more globular and denser compared to mammal bone particles. Canaliculae are not visible. Fish bones often show parallel sides (Gizzi and others 2003). Lacunae in fish bones are usually elongated with a clear fusiform net of canaliculae. There is a large diversity

among fish species, i.e., in cod, the lacunae are linear without visible canaliculae (Gizzi and others 2003; Fumière and others 2009).

In ideal conditions, classical microscopy can be adopted to distinguish and quantify between mammalian bones and poultry bones. With the sedimentation step in an organic solvent, the limit of detection (LOD) of the technique is very low. Nevertheless, the determination of the species origin is limited and the quantification requires the use of factors introducing sources of errors. The method is also time-consuming, laborious, requires skill and incurs considerable cost and delay (Gizzi and others 2003; van Raamsdonk and others 2004). In addition, the analytical results of classical microscopy depend largely on the experience of the microscopist. Furthermore, this method cannot be used on liquid samples, and cannot detect soft tissues.

2.1.2 Near Infrared Microscopy (NIRM)

The principle of near infrared microscopy (NIRM) method is to discriminate the origin of the feed compound making up the samples by using the infrared spectra of individual particles. The NIRM method follows exactly the same protocol for sample preparation as classical microscopy.

The first NIRM method using near infrared (NIR) microscopy was developed in 1998 (Piraux and others 1999, 2000). Later on the method was dramatically improved, mainly including the development of a protocol focusing on the sediment part of the sample which contains mainly denser particles such as bones (Baeten and others 2001a, 2004, 2005 ; von Holst and others 2008). With currently available NIRM method, the particles from the animal feed are analyzed one by one sequentially, which is a time-consuming process (Baeten and others 2002). In order to overcome this drawback, the second NIRM method using NIR imaging system was developed in 2000, which can also be employed to analyze the raw and sediment fraction. This system can

be used for the analysis of about 300-500 particles simultaneously and reduces significantly the analytical time (Gizzi and others 2003; Baeten and others 2005a, 2005b, 2007 ; Fernández and others 2005).

2.1.3 Near Infrared Spectroscopy (NIRS)

Near infrared spectroscopy (NIRS) is one of the most widely used analytical techniques in the feed sector. It is routinely adopted to determine chemical (i.e., water, protein, fat, sugar, starch, fiber and ash) and biological (i.e., digestibility and energy values) parameters in animal feeding and production (Givens and Deaville 1999; Gizzi and others 2003; Berzaghi and others 2009; Fumière and others 2009).

In principle, NIRS is one of the techniques belonging to vibrational spectroscopy. Its radiation (750 - 2500 nm) interacts with organic matter, and the absorption spectrum is rich in chemical and physical information of organic molecules (Givens and Deaville 1999; Berzaghi and others 2009). The main absorption bands of water are at 1940 and 1450 nm, of aliphatic C-H bonds at 2310, 1725, 1400 and 1210 nm, of O-H bonds around 2100 and 1600 nm and N-H bonds at 2180 and 2055 nm. The NIRS method is based on absorption of light (absorbance) at selective wavelengths of the electromagnetic spectrum by the organic molecules constituting the analyzed samples (Gizzi and others 2003; Berzaghi and others 2009; Fumière and others 2009). In order to extract valuable information on the chemical properties of samples from the absorption spectrum, it is necessary to mathematically process spectral data by chemometric tools (Barnes and others 1989). The most important part in the development of an NIRS method is building the predicting model generally called calibration (Blanco and others 1997; Berzaghi and others 2009). Many studies on NIRS demonstrated that this method can be exploited to identify and/or

quantify the animal ingredients in animal feeds (Baeten and Dardenne 2001b; De la Haba and others 2007).

Murray and others (2001) demonstrated the use of NIRS for detecting MBM in fish meal. Their research involved testing some 46 pure fish meal samples and 90 fish meal samples spiked at 3%, 6% and 9% with MBM, and underlined the potential of NIRS to differentiate between animal protein from two different species (Murray and others 2001).

Contaminating levels of meat meal and/or MBM (MMBM) in fishmeal were detected and quantified by the NIRS approach (Yang and others 2008). In this research, a partial least squares (PLS) discriminant analysis and a modified partial least squares (MPLS) quantitative analysis, using visible and NIRS, were developed using a calibration set of 186 samples including 90 samples of pure fishmeal and 96 samples adulterated with MMBM at levels ranging from 10 to 320g/kg (Yang and others 2008). An external validation set, comprised of 39 pure samples and 54 adulterated samples, was used to validate the calibration model. Fishmeal adulterated with MMBM was successfully detected by a PLS discriminant analysis model. External validation demonstrated that all samples were correctly distinguished. The MMBM in fishmeal was also successfully predicted by a MPLS quantitative model, with standard error of cross-validation (SECV) of 27.89g/kg and ratio of the standard deviation of the validation set to the standard error of prediction (RPD) of 3.37. The calibration and validation results confirm that NIRS could provide the feed industry and inspection bodies with a rapid, non-destructive and non-invasive technique for the detection and quantification of MMBM in fishmeal (Yang and others 2008).

NIRS combined with chemometrics was employed to discriminate between fishmeal, meat meal and soya meal samples (Cozzolino and others 2009). Briefly, commercial feed samples were scanned in the near infrared (NIR) region (1100 - 2500 nm) in a monochromatic instrument

in reflectance mode. On the basis of their NIR spectra, principal component analysis (PCA) and linear discriminant analysis were used to classify samples. Full cross-validation was used in the development of classification models. As a result, 85.7% of the fishmeal samples and 100% of the meat meal and soya meal samples were correctly classified by using partial least squares-discriminant analysis (PLS-DA), which demonstrated the usefulness of the NIR spectra method combined with chemometrics as an objective and rapid method to classify fishmeal, meat meal and soya meal samples. NIRS method can easily be performed in feed mills and may be most useful for initial screening at early stages in the food production chain, enabling more costly methods to be used selectively for suspected specimens (Cozzolino and others 2009).

Compared to other analytical approaches, one of the main features of the NIRS method is the possibility to analyze a large quantity of specimens. The advantages of the NIRS technique mainly include rapidity, minimal sample preparation, non-destructive analysis, good and repeatable signal intensity. The major disadvantages of the NIRS technique are that the limit of detection (LOD) is higher than 1% and the method cannot be used alone as legal evidence. In addition, the method is indirect and therefore requires large numbers of reference values on authenticated samples to form a calibration or discriminant model. Nevertheless, NIRS can be used as a first line screening technique to routinely screen samples for MBM contamination, and more costly methods such as PCR techniques can be adopted to confirm suspect samples.

2.1.4 Immunochemical Assay

The immunochemical technique is based on the specific interaction between the antibody and the antigen. There exist different designs for the detection of this interaction. In the field of the detection of PAPs or MBM in animal feeds, the most commonly used methods are enzyme-

linked immunosorbent assay (ELISA) technique, and the lateral flow "dipstick" or lateral flow test strip (LFTS) technology.

2.1.4.1 Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA method which is based on the specific recognition through the antibody-antigen affinity has been used for meat speciation for more than 20 years (Hitchcock and Crimes 1985). Raw and moderately cooked meat in food can already be determined by several commercial ELISA kits. The research showed that when the ELISA method was used for the detection and identification of the meat or the MBM in feeds, its response strength is dramatically influenced by heat treatment or sterilization conditions such as temperature, pressure and duration of the treatment. For instance, the response of the ELISA was very low when pork had been heated above 120 ^{O}C (Hofmann and others 1995; Pallaroni and others 2001; von Holst and thers 2001), which to a certain extent is used as proof that PAPs containing porcine material have been heat treated according to the legislation.

Ansfield and others (1994, 2000a, 2000b) adopted a sensitive ELISA method to detect the ruminant proteins and identify animal species in rendered animal material or in compound animal feedstuffs heated to more than 130 ^{O}C by raising antibodies against thermaostable antigens which are assumed to withstand severe rendering processes. The special feature of this method is that a special extraction technique was developed. Briefly, for pure MBM the extraction technique involved heat-treatment followd by a two-step ammonium sulphate precipitation procedure. Some interfering proteins such as gelatin, which could have significant impact on the immunological recognition, was removed in the first-step precipitation. The goal of the second-step precipitation is to concentrate the target proteins on which a double sandwich ELISA is applied. For compound animal feeds the procedure is much more complicated, which

needs additional steps to remove other components in order to limit false-positive and false-negative as much as possible. For instance, an additional heat-treatment step is adopted to suppress the effects of plant proteins, fats and oils in the compound animal feeds, because the antibodies may interact with these components in the compound feeds, which lead to false-positive results (Ansfield and others 2000a, 2000b).

Kim and others (2004) developed monoclonal antibodies (mAb) for the detection of prohibited MBM in animal feeds. Heat-stable cow h-caldesmon was purified from bovine intestinal smooth muscle. The mAb 5E12 was raised against h-caldesmon. When this antibody with 100-fold dilution was applied in an ELISA, the limit of detection for bovine MBM mixed in animal feeds is 0.05%, which demonstrated strong affinity toward bovine smooth muscle autoclaved at 130 ^{O}C.

Ofori and Hsieh (2007) developed a sandwich ELISA to detect bovine blood materials in animal feeds. The assay is based on the detection of a 60 kDa thermostable protein in bovine blood. Hsieh and others (2007) in the begining developed mAbs specific to thermostatble proteins in animal feeds. Two of the mAbs such as Bb6G12 and Bb3D6, were used for the sandwich ELISA detection. The mAb Bb6G12 was used as the capture antibody, and biotinylated mAb Bb3D6 was adopted as the detecting antibody. The results showed that this assay specifically provides reliable and sensitive (0.05-0.5% v/v) detection of bovine blood materials in animal feeds.

ELISA kits for the detection of MBM in animal feeds can be found in the market, such as MELISA-TEK kit from the ELISA Technologies Inc. (Gainesville, FL, USA). The ELISA kit is mainly a qualitative method for the detection of species-specific muscle proteins in MBMs and animal feeds. This assay is based on mAbs sensitive to troponin-I, a thermostable muscle specific

antigen. There are two main features for this commercial product. One is that it detects only muscle tissue, which is able to discriminate ruminant and pork Troponin-I from other animal troponins. The other is that it can determine the species content of thermally processed products including those processed under sterilization conditions exceeding 133 ^{O}C and 3 bars of pressure.

In general, ELISAs aim to identify species-specific antigens, without the requirement for bones, via an antibody-based detection system. The drawbacks of the ELISA method include interference from ruminant products such as milk, inhibition by gelatin, and cross-reactivity with other proteins such as plant proteins.

2.1.4.2 Lateral Flow Test Strip (LFTS)

Lateral flow test strips (LFTS) or lateral flow tests (LFT) are also called lateral flow immunochromatographic assays (LFICA), or immunochromatographic strip (ICS) tests or simply strip-tests. They have been a popular platform for diagnostic tests since their introduction in the late 1980s.

In essence, LFTS can be used for the specific qualitative or semi-quantitative detection of many analytes including antigens, antibodies, and even nucleic acids. One or several analytes can be tested simultaneously on the same strip. When LFTS is adopted in clinical diagnostic, whole blood, serum, urine, plasma, saliva, feces, exudates (from wounds or lesions) can all be used as specimens. When LFTS is employed in environmental or other non-clinical applications, the samples may be derived from soils, dust, vegetation, food, animal feed, or environmental swabs such as from food processing plants.

A typical test strip consists of the following components: sample pad, conjugate or reagent pad, detection conjugate, solid-phase membrane, capture (test) and control reagent lines, and absorbent pad. These components of the strip are usually fixed to an inert backing material and

may be presented in a simple dipstick format or within a plastic casing with a sample port and reaction window showing the capture and control zones.

When a test is carried out, a sample is applied on the sample pad at one end of the strip. The sample may be employed alone or mixed with a buffer specific to the test. This buffer may simply be a diluent/running buffer or it may be much more complex and have specific components or properties required to make the strip perform properly, such as a cell lyses buffer. With the addition of the sample the detector molecules in conjugate pad are mobilized, in which the detector molecules mix and bind to the analyte in the sample if the analyte is present in the sample. Then the capillary action draws the fluid mixture into the membrane. The sample/detector molecule mixture continues to move up the membrane until it reaches the test line which is immobilized with a second analyte specific binding agent such as antibody. The control line should always show as a visible line to be valid for the test. If the test is positive, colored (typically pink or purple) lines develop along with the test and control lines. In a negative result, only the control line shows color. If only the test line appears, or if no line appears, the test is invalid.

LFTS is a simple test format, which requires the user to put the test strip in the specimen or add directly onto the strip itself and read the results after a specified period of time. Results can be read in as few as 2 min, but usually around 15 min is more common.

Klein and others (2005) developed LFTS to detect ruminant by-product material in animal feeds and feed ingredients. The test is designed for field use, e.g. at a feed mill, and yields a qualitative (presence/absence) result in 15–20 min. The objective of the study was to validate the lateral-flow test for detection of ruminant by-product material in a variety of finished animal

feeds and feed ingredients. Results indicate that the test is specific for ruminant material and can detect as little as 1% ruminant material in these commodities.

Neogen Corporation (Lansing, MI, USA) has developed the "Reveal for Ruminant" test kit for testing MBM in animal compound feeds (Fumière and others 2009). This assay targets the bovine heat-stable muscle protein troponin I. Two assays are available and are dedicated to the analyses of different types of samples such as feeds and feed ingredients or animal meals. The detection sensitivity can be lower to the level 0.5% bovine MBM contaminated in animal feeds. The other components in samples can affect the specificity and cause false positives (Fumière and others 2009). Some false positive results may be due to the presence of animal fats from rendering industry, especially in pig feeds which the animal fat is frequently used. Other false positive results are also related to beet pulp or citrus pulp used as ingredients in compound feeds. However, these "false" results do not pose any major problems when integrating the method in a global control system, mainly for screening purposes (Fumière and others 2009). Positive samples would then need to be tested by a confirmatory method (Gizzi and others 2003; Fumière and others 2009).

Strategic Diagnostics Inc. (Newark, DE, USA) marketed "FeedCheck" test kit for testing connective tissue (Fumière and others 2009). The test results show a good sensitivity of the animal target. But there exists a cross-reaction with fishmeals at the levels as low as 1.5%, which limits its use in the feeds containing fishmeal.

LFTS assay can be considered as a screening method. Other more accurate techniques such as PCR need to be applied to those positive samples for confirmation test. LFTS test can be performed easily and rapidly in that it does not require any costly equipments and highly trained staff. However, its sensitivity and specificity are significantly affected by the quality of the

antibody used, and extraction protocol of the antigen (proteins) from such a small amount of compound feed.

2.1.5 Polymerase Chain Reaction (PCR)

Proteins and nucleic acids are two major analyses on which molecular biology detection techniques can be focused. These compounds are always present in rendered animal by-products, but detection depends on how the assays are conducted and on the previous thermal treatment imposed.

It is widely accepted at present that gene amplification is one of the most efficient ways to detect a well-defined DNA target, and that PCR, including classical PCR and real-time PCR (RT-PCR) is the most popular one.

Classical PCR consists mainly of six components: (1) DNA template which is from the target source; (2) Oligonucleotide primers that are specifically made for the DNA sequence; (3) DNA polymerase (Taq) which initiates the reaction; (4) Deoxynucleotides (A (Adenine), T (Thymine), G (Guanine) and C (Cytosine)) that are free bases as the "building materials"; (5) Magnesium ions; and (6) Salt buffer.

Classical PCR is designed to take a sequence of DNA and generate a large number of copies of the gene. Essentially, the mechanism of the regular PCR is composed of three major steps which are repeated for 30 - 40 cycles. This is done on an automated thermocycler, which can heat and cool the tubes with the reaction mixture in a very short period of time, and theoretically the number of targets should be doubled at each cycle. The first step is the DNA denaturation stage, in which double strands DNA (helix) are denaturized and become two single stranded DNA at the high temperature between 94 ^{O}C and 100 ^{O}C for 1 to 3 min. The second step is the

annealing stage in which the specific primers bind to DNA templates in complementary manner and this process is carried out at between 50 ^{O}C and 60 ^{O}C for about 45 to 50 sec. The final step is the extension stage at about 72 ^{O}C for about 2 min in which the free nucleotide bases in the reactant attach to the primer and extend the strands under the influence of polymerase. In this process, two primers with 18 - 24 nucleotides are involved, they attach to the DNA template in opposite direction, and therefore the extension is proceeding in continuous way on both strands. In classical PCR operations the reaction is repeated about 30 - 40 times to exponentially increase the number of the gene copies generated (Kubista and others 2006).

The products of the classical PCR are verified by using agarose or separide gel. For the agarose gel the main disadvantages are poor precision, low sensitivity, low resolution, short dynamic range (less than 2 log), non-automated, size-based discrimination only, the results are not expressed as numbers, and Ethidium bromide staining is not very quantitative. So the modified PCR, which is generally called the real-time PCR (RT-PCR) and has almost the same mechanism as classical PCR, was developed and is widely used now. The main advantages of the RT-PCR detection are highly sensitive, dynamic, time efficient, cost effective and flexible. The main difference between RT-PCR and classical PCR is that the amplified DNA is detected as the amplification takes place in RT-PCR while the classical PCR can only detect the finally amplified target after DNA amplification (Kubista and others 2006).

On the basis of the RT-PCR protocol, Romero and others (2004) developed a semiquantitative method for the detection of ruminant DNA, targeting a 88 bp segment of the ruminant short interspersed nuclear element Bov-A2. This method is specific for ruminants and is able to detect as little as 10 fg of bovine DNA. Although autoclaving decreased the amount of detectable DNA, positive signals were observed in feedstuff containing 1% bovine material.

Fumière and others (2009) studied the application of PCR approach in the detection of MBM in animal feeds. The aim of the research was to determine if PCR is a suitable detection technique on properly heat-treated MBM. Briefly, five MBM samples sterilized at the temperatures 125°C, 130°C, 135°C, 138°C and 141°C were obtained from the same rendering plant, in which the DNAs in five samples were highly degraded to small segments, mitochondrial-encoded targets of sizes ranging from 60 bp to 70 bp were carefully selected for several species (bovine, porcine, ovine and equine targets) or animal groups (fish) (Fumière and others 2009). For these targets with extremely small size, the RT-PCR instead of classical PCR format was performed in order to avoid agarose gel electrophoresis which is inconvenient on such small fragments. Unlike the commonly used RT-PCT format, the RT-PCR adopted in this research was carried out with appropriate Taqman probes (Fumière and others 2009). The main feature of this RT-PCR format is that the production of amplicon during the whole amplification reaction can be followed by measuring light emission which occurs only when the Taqman probe to which a fluorescent dye is attached recognizes a complementary strand (Gibson and others 1996; Fumière and others 2009). The specificity of this RT-PCR format is much better than that of classical PCR because the measured signal is not only dependent on the chosen primers, but a second selectivity step is introduced with the probe generating the fluorescent signal. A classical RT-PCR protocol with small bovine and porcine targets was used for the above-mentioned five MBM samples produced at five given temperatures. The results showed that the DNA of both species was clearly detectable in all samples, which demonstrates that with the considered MBM production process, irrespective of the temperature up to 141°C, sufficient DNA was present to perform PCR (Fumière and others 2009). However, for the targets with the size of 275 bp and 350 bp, the detection failed for all five samples (Fumière and others 2009), which is similar to

another case (Tartaglia and others 1998). Tartaglia and others used the targets with the size of 250 bp and 350 bp.

On the basis of the results of RT-PCR for the detection of animal DNA segments in MBM, Fumière and others (2009) extended their research to the detection in compound feeds. The limit of detection reached 0.05% (Fumière and others 2009). These results demonstrated that although DNA extraction protocol has influence on the early occurrence of signal formation, in essence, successful detection is far more dependent on the selected type of target (i.e., size and nature) and on how the amplification reaction is achieved (i.e., hybridization elongation temperature or primer concentration) (Tartaglia and others 1998; Fumière and others 2009).

An important limitation of the PCR approach used for the detection of MBM or PAPs in animal feeds is the fact that animal DNA (belonging to a species or a group of species) detected in a feed sample does not necessarily come from MBM or PAPs in animal feeds (Gizzi and others 2003; Fumière and others 2009). In fact, the authorized feed ingredients such as milk, blood, fat, hydrolyzed proteins produced from ruminant hides and skins or egg products may contain target DNA. The practical impact of this limitation in routine control is not well known yet and may be limited as some of these products are rather expensive so that they are not widely used in feeds (Gizzi and others 2003; Fumière and others 2009). However, the experimental results from Bellorini and others (2005) demonstrated that ruminant fat (tallow) could be identified by PCR due to DNA traces present in the residual insoluble impurities (RIIs) of the fat (Bellorini and others 2005; Fumière and others 2009). The identification of tallow by PCR was even possible when the tallow contained less than 0.15% RIIs, and when the tallow was mixed to porcine fat (lard) at a concentration of 2% (Bellorini and others 2005; Fumière and others 2009). The research results from other researchers showed that the presence in feeds of animal fat such

as tallow from the rendering industry might lead to false positive results from a legal point of view when checking for the presence of prehibited MBM while analytically the method is correct (Prado and others 2007; Fumière and others 2009).

The PCR technique can be applied to liquids, on samples ground down to very tiny particles and on animal by-products devoid of bones. In addition, the main value of the PCR approach is its potential for making clear identifications, not only at species level but also at higher taxon levels (i.e., ruminants or mammalian). This will be of great interest when MBM is once again authorized in animal feeds, but with appropriate measures to prevent 'cannibalism' (ban on intra-species recycling) (Gizzi and others 2003; Fumière and others 2009).

Compared to other methods mentioned above, the results detected by using the PCR approach have high forensic value. PCR is also a relatively costly method, not only in regard to the reagents but also regarding equipment, particularly when a real-time thermocycler is required (Gizzi and others 2003; Fumière and others 2009).

2.1.6 Combination of Methods

The comparison of the respective performance characteristics of the methods described above demonstrated that there is no ultimate approach that would fulfill all requirements (i.e., detection at low level, identification at species level and quantification of PAPs) for the detection and identification of MBM in animal feeds. Methods based on the amplification of the DNA are a promising solution, but as they detect any source of DNA, positive results of MBM can be due to authorized feed ingredients such as milk, blood, or fats. NIRM, on the other hand, can detect MBM particles in general without being able to assign it to an animal species. In order to overcome the main drawback of the PCR concerning the positive results due to authorized

ingredients, a strategy combining the NIRM which detects and isolates the particles of MBM origin together with a DNA extraction protocol adapted to a single particle allowing species identification by RT-PCR was developed (Fumière and others 2008, 2009). The main challenge of this combined approach was how to extract enough DNA to be amplified by RT-PCR from such a small amount of material. A rapid protocol (less than 1 hour), using a special buffer extracting a DNA ready to be utilized in a PCR, and allowing the analysis of the DNA coming from a single PAPs particle with five different targets was developed (Fumière and others 2009). Moreover, species-specific spectral databases were built by using the NIRM spectra collected from single particle and authenticated by PCR results (Fumière and others 2005, 2007). The databases are now applied to find species-specific spectral markers.

The results demonstrated that the strategy also improved the specificity potential of the NIRM models and gave indications about the species origin of the animal particles prior to its PCR analysis. However, some problems of cleaning of the particles need to be solved in order to be absolutely sure that the DNA extracts come only from the particles and not from traces of authorized and target DNA containing feed ingredients attached to the surface of the particle (Fumière and others 2009).

2.2 Detection of *Bacillus anthracis* Spores

2.2.1 Introduction

The anthrax attacks took place in Fall 2001 caused widespread panic in USA. The need for a simple, rapid, specific, sensitive, accurate and inexpensive method to detect *Bacillus anthracis* spores became obvious. Currently, mass spectroscopy, immunological assay, molecular biology technique and other advanced methods are commonly adopted to detect *B. anthracis* spores in

water, air and foods. A brief review on the detection methods of *B. anthracis* spores is as follows.

2.2.2 Detection Methods

2.2.2.1 Mass Spectroscopy

Whiteaker and others (2004) utilized mass spectroscopy to detect the *B. anthracis* spores in raw milk or milk products. In their study, the anti-*B. anthracis* antibodies immobilized onto the beads were employed to concentrate the spores, which can be later detected and characterized by Matrix-Assisted Laser Desorption/Ionization (MALDI) spectroscopy based on the presence of their previously characterized small acid-soluble proteins. This approach can provide a very rapid assessment of whether milk or milk products have been purposefully contaminated with *B. anthracis* spores.

2.2.2.2 Immunological Assay

Swiecki and others (2006) used BclA (*Bacillus* Collagen-Like Protein of *anthracis*, a major component of the exosporium (Réty and others 2005)) as immunogen and produced mAbs that were able to distinguish *B. anthracis* spores from other *Bacillus* spores. It was found that these mAbs did not react with vegetative forms. In addition, some mAbs can distinguish *B. anthracis* spores from spores of distantly related species in a highly specific manner, but others can discriminate among strains that are the closest relatives of *B. anthracis*. These Abs provide a rapid and reliable means of detecting *B. anthracis* spores, for probing the structure and function of the exosporium, and in analyzing the life cycle of *B. anthracis* (Swiecki and others 2006).

Kuehn and others (2009) raised antibodies against anthrose tetrasaccharide to detect *B. anthracis* spores. In their experiments, mice, rabbits and chicken were immunized with inactivated *B. anthracis* spores and, in addition, mice and rabbits were immunized with the tetrasaccharide β-Ant-(1→3)-α-L-Rhap-(1→3)-α-L-Rhap-(1→2)-L-Rhap which is an important component of the exosporium glycoprotein BclA and contains a newly discovered sugar anthrose 2-*O*-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-β-D-glucose. The BclA protein is a major component of the exosporium of *B. anthracis* spores and is decorated by the tetrasaccharide (Kuehn and others 2009). The anthrose-containing tetrasaccharide chain, as a key biomarker, was exploited to detect *B. anthracis* spores in that the chain is highly specific for *B. anthracis*.

Wang and others (2009b) utilized the same mAbs to detect *B. anthracis* spores and vegetative cells. This work was based on the fact that EA1 protein is a major S-layer component of *B. anthracis* vegetative cells, and it also persistently exists in the spore preparations and binds tightly to the spore surfaces even after rigorous washing. In their research, fully washed *B. anthracis* spores were used as an immunogen. Three high affinity and specificity mAbs, confirmed to direct against EA1 protein, were attained. The experimental results demonstrated that the three mAbs can recognize the surface of *B. anthracis* spores and intact vegetable cells with high affinity and species-specificity.

2.2.2.3 Molecular Biology Technique

Molecular biology technique is based on changes in proteomic and genomic expression patterns due to infection by a pathogenic agent. The PCR method or a nucleic acid sequence-

based amplification (NASBA) reaction is usually used to amplify the target DNA or RNA for reliable detection (Baeumner and others 2004).

Carl and others (1992) utilized PCR to identify *Bacillus anthracis* spores. In their study, a 1247 bp fragment from the gene that encodes the edema factor of *B. anthracis* was amplified by PCR. As a result, as few as 10^3 copies of a plasmid containing the edema factor gene and as few as 2×10^4 spores could be detected. In addition, it was also demonstrated that subjecting the product of this PCR to a second PCR which was designed to amplify a 208 bp fragment nested within the 1247 bp product could improve the detection to a single plasmid copy PCR and to two *B. anthracis* spores per PCR (Carl and others 1992).

Baeumner and others (2004) developed a biosensor used for the rapid detection of viable *B. anthracis* spores. The main feature of this biosensor is that a nucleic acid sequence-based amplification (NASBA) reaction was combined with a simple membrane-strip-based biosensor assay. Consequently, the biosensor can be utilized to detect a small number (ten) of viable *B. anthracis* spores, and furthermore, only the viable spores were detected. The principle of this biosensor is to identify a unique mRNA sequence from one of the anthrax toxin genes, the protective antigen (PA), encoded on the toxin plasmid, pXO1, and thus provides high specificity toward *B. anthracis*. The same research group employed the anthrax toxin activator (ATA) mRNA to develop a biosensor used for the detection of a single *B. anthracis* spore within 12 h (Baeumner and others 2004). The comparison of these two cases demonstrated that the change of the target sequence, from the ATA mRNA to the PA mRNA, dramatically reduces the overall assay time from 12 h to about 4 h. The results of specificity analysis revealed no cross-reactivity with 11 tested organisms including closely related species such as *B. cereus, B. megaterium, B. subtilis, B. thuringiensis,* and others such as *Lactococcus lactis, Lactobacillus plantarum*, and

Chlostridium butyricum. In addition, no false positive signals were obtained from nonviable spores.

2.2.2.4 Other Advanced Methods

Williams and others (2003) developed several peptide ligands that can bind specifically to *B. anthracis* spores. Based on Williams group's results, Park and others (2006) developed a simple, rapid, specific, and accurate approach used for the detection of *B. anthracis* spores by exploiting specific capture peptides conjugated with fluorescent quantum dots (QDs). QDs have ideal characteristics for dense spectral multiplexing, a narrow emission range and a long lifetime, and have the potential to simplify the multiplexed analysis using different QDs (McBride and others 2003; Makrides and others 2005). Fluorescence confocal microscopy and flow cytometry were adopted to examined specific binding of three capture peptides conjugated with QDs to two attenuated *B. anthracis* strains: *B. anthracis* ΔSterne (pXO1-, pXO2-) and *B. anthracis* Sterne 34F2 (pXO1+, pXO2-). *B. cereus* and *B. thuringiensis*, which are the most similar to *B. anthracis* based on their genome sequences, were used as control strains to be compared during the detection of *B. anthracis* (Radnedge and others 2003; Park and others 2006). A spectrofluorometric assay system was also developed for more convenient and high-throughput detection of *B. anthracis* spores (Park and others 2006). The advantage of the peptide-QDs conjugate approach is that *B. anthracis* spores can be distinguished from the spores of other members of *B. cereus* group of bacteria using these peptide-QD conjugates by flow cytometric and confocal laser scanning microscopic analyses. Additionally, spectrofluorometric analysis of spore-peptide-QD conjugates was used for more convenient high-throughput detection of *B. anthracis* spores. *B. anthracis* spores could be detected in less than 1 h by using this method

(Park and others 2006). Furthermore, in order to improve the specificity and avoid false-positive signal caused by the presence of *B. thuringiensis* spores, the B-Negative peptide, which can only bind to *B. thuringiensis*, conjugated with another type of QD that fluoresces at different wavelength was also developed. In the presence of mixed *B. anthracis* and *B. thuringiensis* spores, the BABA peptide conjugated with QD525 and the B-Negative peptide conjugated with QD585 were able to bind to the former and the latter, specifically and respectively, thus allowing the clear detection of *B. anthracis* spores against *B. thuringiensis* spores by using two QD-labeling systems (Park and others 2006). This capture peptide-conjugated QD system is useful for the detection of *B. anthracis* spores.

The accurate detection and identification of *B. anthracis* and *B. anthracis* spores are still heavily dependent on the traditional culture method (Leise and others 1959) and PCR (Carl and others 1992; Patra and others 2002, Wang and others 2009b), but these methods typically involve extensive sample preparation, and are time-consuming and high cost. Recently spore-based detection approaches are more and more popular in that surface antigens can be rapidly detected on intact spores using relatively simple labeling procedures (Edwards and others 2006). Many of these systems, however, are not selective against other members of the *B. cereus* group (Stopa 2000; Kamboj and others 2006). Brigati and others (2004) developed diagnostic probes for *B. anthracis* spores selected from a landscape phage library. Through a phage display screening process (bio-panning), short peptide fragments that exhibited species-specific binding to *Bacillus* spores were discovered (Turnbough 2003; Williams and others 2003; Brigati and others 2004), and numerous reports showed them to be sensitive and selective (Pai and others 2005; Acharya and others 2007). Generally, for the detection of pathogens, peptides are attractive candidates because of their structural simplicity and durability, as well as their desirable binding properties

as selected by the phage display process. Moreover, such ligands can also be synthesized to include reactive moieties for straightforward attachment onto sensing platforms. Take ATYPLPIRGGGC (abbreviated ATYP) as an example of one such peptide (Brigati and others 2004). ATYP was conjugated to R-phycoerythrin (RPE) through its terminal cysteine residue and found by flow cytometry (FCM) to have nanomolar sensitivity for *B. anthracis* spores and minimal cross-reactivity with closely related species, *B. cereus* and *B. thuringiensis*. However, like other rapid-response detectors, ATYP-RPE could not distinguish among spores from different strains of *B. anthracis* (Williams and others 2003).

In general, for the detection and identification of *B. anthracis spores*, PCR, a primer-mediated enzymatic DNA amplification method, requires considerable effort in sample processing prior to analysis. For the analysis part itself, the PCR-based detection may take 2 to 5 h or longer (Oggioni and others 2002; Hartley and Baeumner 2003; Higgins and others 2003; Wang and others 2004; Vahedi and others 2009). Immunoassays, which rely on the interaction between antibodies and *B. anthracis* cell surface antigens, can detect 10^3 spores in 12 h (Stopa 2000; Hartley and Baeumner 2003; McBride and others 2003; Rivera and others 2003; Wang and others 2006). Immunoassay is relatively fast compared to traditional culture method, but most of the antibody-based detection methods lack accuracy and have limited sensitivity (Swartz 2001; Hartley and Baeumner 2003; Kwak and others 2004), which lead to unacceptably high levels of both false-positive and false-negative responses (Hartley and Baeumner 2003; Wang and othes 2009b). Therefore, better detection systems or methods need to be developed.

Chapter Three

Development of an Antibody-Based Lateral Flow Test Strip for the Detection of Bovine MBM in Animal Feeds

3.1 Introduction

Rapid membrane-based test techniques could be applied in a wide variety of fields such as in agriculture (food safety, plant and crop diseases), clinical sciences (allergies, cardiac markers, degenerative diseases, drugs of abuse, fertility, forensic, immunotyping, infectious diseases, serological tests, sexually transmitted diseases, stress indicators, toxicology, tumor markers), and environment (biological contamination, environmental contamination). The use of the rapid membrane-based test technology will be increased as these low-cost alternatives to expensive instrumented methods of testing become more sensitive and more specific.

Rapid tests have two formats, i.e., lateral flow and flow-through, which are based on the same principles. The lateral-flow format is by far more commonly adopted in that it is easier to manufacture and use. The main features of this rapid test include ease of use, small sample volume, speed, flexibility, reliability, and low sales price. A rapid test can be formatted either as freestanding dipsticks or as devices enclosed within plastic housings, and is an inexpensive, disposable, and membrane-based assay that provides visual evidence of the presence of an analyte or analytes of interest in a liquid sample. Typically, 200 μl or less of liquid sample is employed to carry out the test, which is usually completed within 2-5 min. In clinical assays, the

samples may be blood, serum, saliva, urine or other body fluids. In nonclinical tests, the samples may be prepared from food, plants, soil, or dust with a small volume of solution. The sample is directly applied to the membrane-based test strip, and no instrumentation is required to perform such tests, which can be used in clinics, laboratories, field locations, and the home—often by inexperienced personnel.

The design of a lateral flow test strip (LFTS), also called lateral flow immunochromatography strips (LFICS) is based on the binding specificity between antibody and antigen. The typical construction of LFTS is demonstrated in Figure 3.1, which consists mainly of membrane, capture (test) line (it will be called test line in the following description), control line, sample pad, conjugate pad, and absorbent pad. A nitrocellulose membrane (NCM), as a base substrate, is the most frequently used material in a membrane-based assay. Other membranes manufactured from nylon 6,6, modified polyethersulfone, or polyvinylidene fluoride (PVDF) may also be used. An antibody or antigen is usually immobilized onto the membrane as the test line and control line. A pad (often glass fiber) containing dried conjugate is attached to the membrane strip. For the majority of currently available tests, this conjugate pad contains gold nanoparticles (AuNPs) attached to antibodies or antigens specific to the analyte being detected. A sample pad, usually paper or cellulose fibers, is attached to the conjugate pad. When applied to the sample pad, the liquid sample migrates through the conjugate pad by capillary diffusion, rehydrating the gold conjugate and allowing the interaction of the analyte with the conjugate. The complex of gold conjugate and analyte then moves onto the membrane strip towards the test line, and is captured, and it shows the signal in the form of a sharp red line. A second line, control line, is also formed on the membrane from the binding to excess gold conjugate, indicating the test is complete.

The LFTS format in Figure 3.1 is versatile. By switching the antibodies and making small adjustments to the chemistry of the strip format, the same test design can be used for many applications. For instance, in the detection of bovine MBM in animal feeds using LFTS, the conjugate in the conjugate pad was made by 40 nm AuNPs and rabbit anti-bovine meat and bone meal protein (anti-bovine MBMP) antibody, and anti-rabbit IgG and rabbit anti-bovine MBMP antibody were immobilized onto the NCM as control line and test line, respectively. In the detection of *Bacillus antharcis* spores using LFTS, however, the conjugate was made by the AuNPs and anti-*B. anthracis* spore phage (or phage *B. anthracis*, pBA), and pBA and anti-pBA antibody were immobilized on the NCM as test line and control line, respectively.

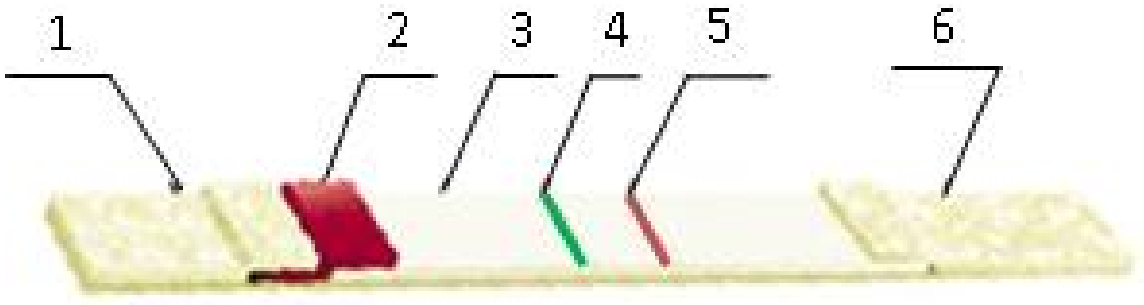


Figure 3.1 Construction of a lateral flow test strip
(adopted from www.devicelink.com/ivdt/archive/00/03/004.html, on 3/24/2006, with modifications)
1 sample pad, 2 gold conjugate pad, 3 nitrocellulose membrane,
4 test line, 5 control line, and 6 absorbent pad.

Any immunochemical technique is mainly based on the interaction between antibody and antigen, in which a specific antibody will bind with its specific antigen to give an exclusive antibody-antigen complex. Antigen is a substance that when introduced into the body stimulates the production of an antibody. Common antigens include bacteria, toxins, foreign blood cells,

and the cells of transplanted organs. Antigens are usually proteins or polysaccharides with molecular weights of at least 8,000 to 10,000 Daltons. Proteins, peptides, carbohydrates, nucleic acids, lipids and many other naturally occurring or synthetic compounds can act as successful immunogens. Peptides and non protein antigens usually need to be conjugated to a carrier protein (bovine serum albumin (BSA) or keyhole limpet hemocyanin) to become good immunogens. This is due to their small size and to the need for a carrier needed to provide the class II T receptor binding sites. Additionally these may need to be administered with an adjuvant to ensure a high quality/quantity response. Adjuvants are non-specific stimulators of the immune response. They allow smaller doses of antigen to be used and the antibody response is more persistent. Thus, given enough time, just about any foreign substance will be identified by the immune system and evoke specific antibody production. However, this specific immune response is highly variable and depends in part on the size, structure and composition of the antigens. Antigens that elicit strong immune responses are said to be strongly immunogenic.

The quality of the antibody employed in LFTS technique greatly influences the quality of the detection. A monoclonal antibody (mAb) is usually used for the gold conjugate in order to enhance the specificity of the detection, and a polyclonal antibody (pAb) is immobilized on the membrane to increase the sensitivity of the detection. Challenges in the development of LFTS technique mainly include extraction and purification of antigen for antibody production, specificity and sensitivity of antibody, conjugation between AuNPs and antibody or antigen, and membrane selection. In this study, several extraction approaches were used to extract typical proteins from bovine MBM. Based on the results of characterization of the antigens, typical antigens were utilized to produce the antibody. The NCM was directly used for the

immobilization of the anti-MBMP antibody and the anti-rabbit IgG on the test and control line, respectively.

3.2 Materials and Methods

3.2.1 Materials

Tris (Tris[hydroxymethyl]aminomethane, ultrapure, MB grade) was purchased from USB Corporation (Cleveland, OH, USA). PMSF (phenylmethylsulfonyl fluoride, or alpha-toluene sulfonyl fluoride), guanidine hydrochloride and sodium azide (natriumazid) were purchased from ACROS ORGANICS (Fair Lawn, NJ, USA). Sodium hydroxide, sodium chloride, SDS (sodium dodecyl sulfate (electrophoresis)), potassium hydroxide, potassium carbonate, methanol, Triton X-100 (electrophoresis grade), ammonium sulfate, and hydrochloric acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Borax (sodium tetraborate, decahydrate), sodium phosphate dibasic, 2-mercaptoethanol, and Freund's Adjuvants (complete and incomplete), PVA (polyvinyl alcohol, 99+%, hydrolyzed) were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI, USA). Gold colloid was purchased from Millenia Diagnostics Inc. (San Diego, CA, USA). BSA (bovine serum albumin, reagent grade powder) was purchased from Equitech-Bio Inc. (Kerrville, TX, USA). Bovine meat and bone meal (MBM) was provided by TBC and BHT Packing Co. (Birmingham, AL, USA). *p*-Nitrophenyl phosphate (p-NPP) was purchased from PIERCE (Lubbock, TX, USA). Glycerol was purchased from EMD Chemicals Inc. (Darmstadt, Germany) and nitrocellulose membrane (NCM, HF240MC100, Hi-Flow Plus 240 membrane cards 60 mm × 301 mm) was purchased from Millipore Corporation (Billerica, MA, USA).

3.2.2 Methods

3.2.2.1 Extraction of Protein

The following methods were used to extract water-soluble protein from pure bovine meat and bone meal (bMBM), and animal feeds.

Method One: Tris Buffer as Extraction Solvent

Five grams of bovine MBM were added to 20 ml of Tris buffer (50 mM Tris-HCl, 0.01% sodium azide, 0.25 mM PMSF, pH 7.5), which was homogenized (three times, each time for 40 s, room temperature (RT)) for protein extraction with a homogenizer (Polytron, Brinkmann Instruments, Westbury, NY, USA). The crude extract was centrifuged at 12,000 x g for 10 min at 4 ^{O}C. The supernatant was filtered through glass wool (Owens-Corning Fiberglas Corporation, NY, USA) to obtain the extract. The total protein concentration of the extract was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). The extract was stored at 4 ^{O}C for further use.

Method Two: Water as Extraction Solvent

The extraction protocol was the same as that used in Method One, but water was used as the extractant.

Method Three: 6.0 M Guanidine as Extraction Solvent

Five grams of bovine MBM were added to 30 ml of 6.0 M guanidine extraction solution and then the sample was homogenized as described in method one. The crude extract was centrifuged at 12,000 x g for 30 min at 4 ^{O}C. The further treatment was the same as Method One.

Method Four: NaOH as Pretreatment Solution and Water as Extraction Solvent

Twenty-five grams of the bovine MBM were added to 100 ml of 1.0 M NaOH solution, which was put into a shaker incubator (Environ Shaker, Lab-Line Instruments Inc., Melrose

Park, IL, USA) at 250 rpm for 48 h at 37 ^{O}C. Then, the sample was centrifuged at 12,000 x g for 10 min at 4 ^{O}C, and the supernatant was discarded. After adding 50 ml of sterile deionized water to the precipitate, the suspension was incubated at 37 ^{O}C or RT for 4 h with stirring and the pH of the suspension was adjusted to 7.0. The following extraction protocol and total protein concentration assay were the same as described in the Method One.

Method Five: Boiled in NaOH, H_2O or Tris Buffer and Extracted in Water

Twenty-five grams of the bovine MBM were added to 200 or 400 ml of boiling NaOH solution with the designated concentrations (0.1, 0.5, 1.0 M), the boiling H_2O or the boiling Tris buffer (pH 7.5), which was continuously boiled for the designated periods of time (5, 10 or 15 min) and then samples were cooled down to RT. After centrifugation at 12,000 x g for 10 min at 4 ^{O}C, the supernatants were discarded. Then, 50 ml of sterile deionized water were added to each precipitate and the pH of the suspension was adjusted to 7.0 as described above. The following extraction protocol and total protein concentration assay were the same as described in the Method One.

3.2.2.2 Purification of Protein

An affinity column was made following the procedures of "Affi-Gel Hz Hydrazide Gel Scale-Up Instruction" and "Affi-Gel Hz Immunoaffinity Kit Instruction Manual" (Econo-Pac® 10 DG disposable chromatography columns, 30 × 10 ml column, Bio-Rad Laboratories, Hercules, CA, USA) and used for the purification of protein.

Cobalt bead and nickel bead affinity columns were also employed to purify MBM extracts, respectively. For the protein purification using cobalt bead affinity column, the cobalt beads were washed with buffer (50 mM Tris, 100 mM NaCl, 5 mM imidazole, pH 7.0) and then added

to the crude bovine MBM protein (MBMP) extract in 6.0 M guanidine extraction buffer at 1:10 (v/v) ratio with gently shaking overnight at RT. Then 2-mercaptoethanol was added to the concentraton at 10 mM with gentle shaking for 30 min at RT. The protein-bound beads were sequentially washed with 4 bed volumes of washing buffer (binding buffer (50 mM phosphate buffer, pH 7.0) plus 10% glycerol (4:1, v/v); binding buffer plus 500 mM sodium chloride; and binding buffer). Then, the protein-bound beads were packed into a microcentrifuge column. After washing the column twice with 500 µl of binding buffer a time, the column was eluted with elution buffer (50 mM phosphate buffer, 50 mM imidazole, pH 7.0). The concentrations of the collected fractions were measured by Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA), and the proteins were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For the protein purification using nickel beads, the protocol used is similar to that of cobalt beads. The main differences are that for the nickel bead method the pH of binding buffer (50 mM phosphate buffer) used is 7.4, and there is no 10 mM of 2-mercaptoethanol treatment.

3.2.2.3 Characterization of Protein

Protein extracts were analyzed by SDS-PAGE using a Mini-PROTEIN II electrophoresis cell (Bio-Rad Laboratories). Total proteins extracted from the sample were separated on the 12% separating gel and 4% stacking gel and then stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA).

3.2.2.4 Production of Anti-Bovine Meat and Bone Meal Protein Antibody

The rabbit anti-bovine meat and bone meal protein (anti-bMBMP) antibodies used in this study were produced by immunizing the purified bovine MBM proteins (bMBMP) in Freund's adjuvant. The immunization schedule in Table 3.1 was performed.

Table 3.1 Schedule for the Production of Anti-Bovine MBM Protein Antibody

Day	Date	Procedure
0	03/17/2008	Primary Immunization
31	04/17/2008	1st Boost
39	04/25/2008	Rabbit Blood Collection (RBC)
59	05/15/2008	2nd Boost
67	05/23/2008	RBC
91	06/16/2008	3rd Boost
100	06/25/2008	RBC
121	07/16/2008	4th Boost
129	07/24/2008	RBC
154	08/18/2008	5th Boost
163	08/27/2008	RBC
183	09/17/2008	6th Boost
191	09/25/2008	RBC
209	10/13/2008	7th Boost
217	10/21/2008	RBC
219	10/23/2008	Final RBC

The procedure used a New Zealand white rabbit with a weight of about 3 kg to produce antiserum against the proteins extracted from bovine meat and bone meal (bMBM), called bovine MBM protein (bMBMP). One ml aliquot of purified bMBMP with about 2 mg/ml proteins was emulsified with complete Freund's adjuvant (1:1 v/v). The rabbit was immunized with 1 ml of the protein-adjuvant emulsion through a subcutaneous injection of 100 µl aliquot into each of six sites on the back close to

the spinal cord and 200 µl aliquot into each thigh muscle. For boosting, the same dosage of protein-adjuvant (incomplete) emulsion was used. The rabbit was boosted 7 times at 30-day intervals after initial immunization. Rabbit blood was collected from the central ear artery at the eighth day after each boost, and stored at 4 ^{O}C for further IgG purification.

3.2.2.5 Purification of Anti-Bovine Meat and Bone Meal Protein Antibody

After the rabbit blood was stored at 4 ^{O}C for 2-4 h, serum was collected from the supernatant by centrifuging the blood at 2,500 x g for 25 min at 4 ^{O}C. The IgG was precipitated with 20~50% saturated ammonium sulfate ($(NH_4)_2SO_4$) solution. After centrifugation at 10,000 x g for 15 min at 4 ^{O}C, the supernatant was discarded. The precipitate was re-suspended in 20 mM phosphate buffer with pH 7.0, and the sample was dialyzed in 20 mM phosphate buffer, pH 7.0 for overnight at 4 ^{O}C with one time change of dialysis buffer. After centrifugation at 12,000 x g for 10 min at 4 ^{O}C, the IgG in the supernatant was further purified by a Protein A affinity column in which the Protein A only bound with IgG and its subclasses. The anti-bovine meat and bone meal protein (anti-bovine MBM protein) antibody flowed through the Protein A column at 1 ml/min. The Protein A column was washed with 20 mM phosphate buffer, pH 8.0 until optical density ($O.D._{280\ nm}$) was close to zero at the column exit. The IgGs were eluted with three bed volumes of elution buffer (0.5 M citric acid, pH 3.5). The eluent containing the antibody was neutralized to pH 7.0-7.4 with 1.0 M NaOH, and dialyzed in 20 mM phosphate buffer (pH 7.0) overnight at 4 ^{O}C with at least one time change of dialysis buffer. The dialyzed sample was centrifuged at 12,000 x g for 10 min at 4 ^{O}C. The concentration of the antibody was determined by the Bradford protein assay kit (Bio-Rad) and the purity of the antibody was checked by SDS-PAGE. The reactivity and specificity of the purified polyclonal antibodies (pABs) were

determined by indirect ELISA. The purified antibody was stored at -20 ^{O}C or -70 ^{O}C for future use.

A small Protein A column was made using commercial Protein A product, which was employed to purify the anti-bovine MBM protein antibody produced after the 1st boost bovine MBM protein, and part of the anti-bovine MBM protein antibody produced after the 2nd boost bovine MBM protein. A large Protein A column was made using Protein A produced in our lab, which was exploited to purify the anti-bovine MBM protein antibody produced after the 3rd - 7th boost bovine MBM protein and part of the anti-bovine MBM protein antibody produced after the 2nd boost bovine MBM protein.

3.2.2.6 Characterization of Anti-Bovine Meat and Bone Meal Protein Antibody

3.2.2.6.1 Purity Determination of Anti-Bovine Meat and Bone Meal Protein Antibody

SDS-PAGE was used to determine the purity of the anti-bMBMP antibody. All the gels (12%) used for running SDS-PAGE in this study were made in our lab. Fermentas Benchmark protein ladder was utilized to determine protein molecular weights. The samples to be loaded into the gel well were diluted to 500 µg/ml by using sterile water and sample buffer, and boiled for 2.5 minutes. The boiled samples (10-15 µl) were added into each well of the gel. After running SDS-PAGE at 100 V for 15 min followed by 200 V for 35-40 min, the gel was stained with Coomassie Blue solution for 25 min at RT, and then destained in destaining solution I for 2 h followed by destaining solution II overnight at RT.

3.2.2.6.2 Sensitivity and Specificity of Anti-Bovine Meat and Bone Meal Protein Antibody

The reactivity, sensitivity, and specificity of the anti-bovine MBM protein antibody were determined by the enzyme-linked immunosorbent assay (ELISA).

For determinating the reactivity of the anti-bMBM protein antibody, the samples, including the extracted bMBM protein, negative (BSA) and a positive control, were prepared in coating buffer (phosphate buffered saline, PBS, pH 7.4) to the concentration of 50 μg/ml, then 100 μl of each sample were added to a well of a 96-well ELISA assay plate (Costar, Corning Incorporated, Corning, NY, USA) in duplicate. The plate was covered and incubated at 25-30 ^{O}C for 2 h. Subsequently, the coated plate was washed three times with PBST buffer (PBS, 0.05% Tween-20 and 0.01% sodium azide (NaN_3), pH 7.4) using an automated microplate washer (StatFax-2600, Awareness Technology, Inc., Palm City, FL, USA) to remove the excess bovine MBM protein. The plate was blocked by adding 200 μl of blocking buffer (1% BSA in PBS plus 0.1% NaN_3, pH 7.4) to each well. The plate was covered and incubated at 25-30 ^{O}C for 1 h. After the excess blocking solution was removed and the plate was washed three times with PBST buffer, 100 μl of each diluted anti-bovine MBM protein antibody solution (as primary antibody) using 2-fold dilution factors were added to each well of the plate. The plate was covered and incubated at 25-30 ^{O}C for 2 h and then the plate was washed three times with PBST as described above. One hundred μl of the diluted secondary antibody (goat anti-rabbit IgG, alkaline phosphatase conjugated, Sigma-Aldrich Inc., St. Louis, MO, USA, 3000-fold dilution, v/v in PBS, pH 7.4) were added to each well of the plate and incubated at 25-30 ^{O}C for 1 h. Then, the plate was washed four times with PBST washing buffer as described above, and 100 μl of fresh prepared substrate solution (*p*-nitrophenyl phosphate (*p*-NPP), 20 mg *p*-NPP in 10 ml of 10 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.5) were added to each well of the plate. The plate was incubated in the dark at RT for color development and the absorbance was measured every 15 min at 405 nm ($O.D._{405nm}$) using a microplate reader (Thermo Laboratory System, Helsinki, Finland).

For the sensitivity determination of the anti-bovine MBM protein antibody, the protocol used was similar to that for the reactivity determination as described above. The protein samples extracted from bovine MBM using Method Four of MBM protein extraction, negative (BSA) and positive control were diluted in PBS to different concentrations of 64, 50, 32, 16, 8, 4, 2, 1, 0.5, 0.25 µg/ml. An aliquot (100 µl) of each diluted protein sample, negative and positive control was added to a well in a 96-well ELISA assay plate in duplicate, and the plate was incubated at 25 ^{O}C for 2 h. Subsequently, the coated plate was washed three times with PBST washing buffer. The plate was blocked by adding 200 µl of blocking buffer to each well and incubated at 25 ^{O}C for 1 h. After the blocked plate was washed three times with PBST washing buffer, 100 µl of an appropriately diluted primary antibody solutions (e.g., 1:500, 1:1000, 1:2000, v/v in PBS, pH 7.4) were added to each well, and the plate was incubated at 25 ^{O}C for 2 h. The following treatments for the samples in the plate were the same as in the reactivity determination as described above.

For the specificity determination of the anti-bovine MBM protein antibody the protocol was similar to that for reactivity determination as described above. More than 80 protein samples (including un-boiled and boiled samples), extracted from 37 animal feeds and feed's ingredients by using Tris buffer (50 mM Tris-HCl, 0.01% sodium azide, 0.25 mM PMSF, pH 7.5) or/and sterile deionized water, were diluted in PBS to the 50 µg/ml protein, and 100 µl of the diluted samples, negative (BSA, 50 µg/ml) and positive control (bMBM extract, 50 µg/ml) were added to a well of a 96-well assay plate in duplicate, and the plates were incubated at 25 ^{O}C for 2 h. The following treatments for the samples in the plate were the same as in the sensitivity determination as described above.

3.2.2.7 Immunoabsorption of Anti-Bovine Meat and Bone Meal Protein Antibody

The immunoabsorption method was adopted to reduce or eliminate the cross-reaction between anti-bMBM protein antibody and the proteins extracted from animal feeds. The proteins were extracted from animal feeds without bovine MBM by using different extraction approaches, and centrifuged at 20,000 x g for 20 min at 4 ^{O}C. Then the extracted sample was gently mixed with anti-bMBM protein antibody at 30:1 ratio at RT for 5.5 h. After centrifugation at 20,000 x g for 30 min at 4 ^{O}C the supernatant was collected and precipitated at 50% saturated $(NH_4)_2SO_4$ solution as described above. The anti-bMBM protein antibody was re-suspended in 20 mM phosphate buffer with pH 7.0, dialyzed in 20 mM phosphate buffer (pH 7.0) with at least one time change of dialysis buffer, and centrifuged at 12,000 xg for 10 min at 4 ^{O}C. The concentration, purity, reactivity, sensitivity and specificity of the anti-bMBM protein antibody after immunoabsorption were conducted using the methods described previously.

3.2.2.8 Colloidal Gold Conjugation to Anti-Bovine Meat and Bone Meal Protein Antibody

Colloidal gold particles with the size about 40 nm in a suspension were adjusted to pH 9.0. Anti-bMBM protein antibody at 0.3 mg/ml was dialyzed in 2 mM borate buffer at pH 9.0 for 24 h at 4 ^{O}C prior to conjugation. After centrifugation at 10,000 x g for 1 h at 4 ^{O}C, 0.5 ml of the dialyzed anti-bMBM protein antibody with the concentration of 0.3 mg/ml was conjugated to 10 ml of the colloidal gold particles at pH 9.0 and blocked with 1.0 ml of 10% BSA at pH 9.0. The conjugated sample was centrifuged at 10,000 x g for 1 h at 4 ^{O}C to remove unbound anti-bMBM protein antibody. The gold colloid-conjugated antibodies were purified by gradient centrifugation [10-25% glycerol in buffer A (20 mM Tris/HCl, 0.137 M NaCl, 0.1% BSA and 0.01% sodium azide, TBS, pH 8.2)] at 4,500 x g for 1 h at 4 ^{O}C to remove any small clusters.

The purified conjugate was dialyzed in buffer A to remove glycerol, and concentrated by centrifugation at 4,500 x g for 40 min at 4º C. The supernatant was discarded and the conjugate pellet was re-suspended in buffer A (pH 8.2) and the concentration was adjusted to 3.0 (optical density at 520 nm, $O.D._{520nm}$ =3.0).

3.2.2.9 Lateral Flow Test Strip

The LFTS was constructed using the following conditions: nitrocellulose membrane (NCM, Millipore Corporation, Billerica, MA, USA) was used as the solid phase for the assay. The test line was stripped with 0.7 mg/ml of anti-bMBM protein antibody diluted in striping antibody dilution buffer (SADB: 20 mM Na_2HPO_4, pH 7.4, 10% CH_3OH) at 4.5 µl/cm. The control line was stripped with 0.7 mg/ml of goat anti-rabbit IgG (alkaline phosphatase conjugated antibody) diluted in SADB at 4.5 µl/cm. The colloidal gold-anti-bMBM protein antibody conjugate with $O.D._{520nm}$ =3.0 was adjusted to $O.D._{520nm}$ =1.0 with 12% sucrose and conjugate dilution buffer, stripped on conjugate pad, and air-dried for more than 24 h at RT. Stripped NCM was blocked using lateral flow blocking solution (PBS, pH 7.4; 0.1% PVA; 0.01% SDS), and rinsed using rinsing buffer (5 mM Na_2HPO_4, pH 7.4; 0.01% SDS) and air-dried for at least 24 h at 4 ^{O}C prior to assembling into the test cards. The sample pads, stripped conjugate pads, membranes prepared as stated above, and absorbent pads were assembled into cassettes, and stored at 4 ^{O}C until use.

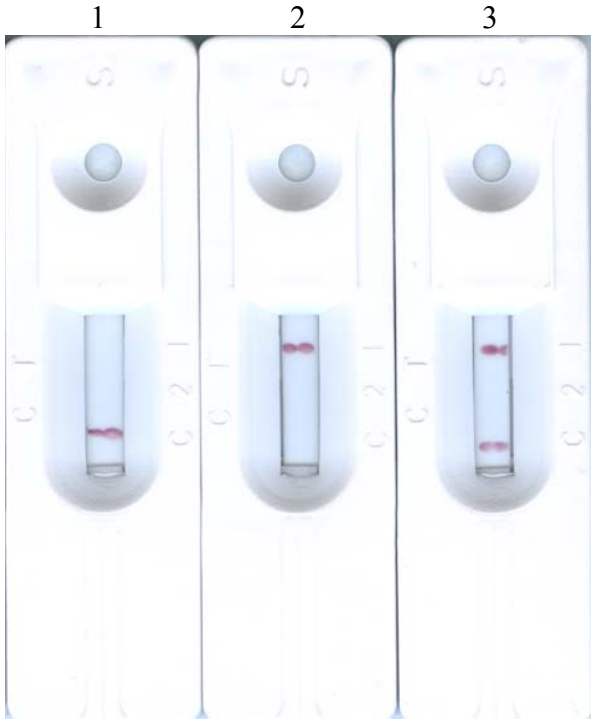
3.2.2.10 Performane of Lateral Flow Test Strip

To perform the test strip, 200 µl of sterile deionized water, PBS, TBS and the protein samples extracted from the pure bMBM and animal compound feeds were added to the sample pad,

allowed to move down the NCM, and the results were recorded after 15 min development. An example of LFTS testing is shown in Figure 3.2.

3.2.2.10.1 Limit of Detection

The protein samples tested were prepared in two ways. One was the proteins were extracted separately from bMBM and animal feed (no bMBM), and then mixed to designated concentrations. The other was the protein was extracted directly from the mixtures of bMBM and animal feed at the designated bMBM content. In this study, the latter way was adopted. The protein samples extracted were added to the sample pads to determine the limit of detection (LOD). The LOD of LFTS was referred to as the lowest concentration of bMBMP or the lowest amount of bMBM in the samples that can be detected by a test strip.



1 control line only, 2 test line only, 3 control and test lines

Figure 3.2 LFTS testing

3.2.2.10.2 Specificity

Forty-three protein samples were extracted from 37 different protein sources by using Tris buffer (50 mM Tris-HCl, 0.01% sodium azide, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5) or/and sterile deionized water. The first 37 protein samples were also boiled for 20 min in a water bath before ELISAs were run, called protein samples (boiled). The concentration of the protein samples (boiled) was not measured, which is considered that the total protein concentration before and after the boiling should be the same. These samples were also tested with the LFTS.

3.3 Results and Discussion

3.3.1 Extraction and Purification of Bovine Meat and Bone Meal Protein

Meat and bone meal (MBM) is composed of various proportions of bone and soft tissue particles. The soft tissue fraction contains more than 80 % MBM protein, and these proteins are more soluble than the proteins of the bone fraction (Garcia and Phillips 2008). MBM protein (MBMP) is heterogeneous at macro- and microscope scales, poorly soluble, and interspersed with non-protein substances. The solubility of MBMP is low. Under mild conditions only a small portion of MBMP can be solubilized. The most aggressive non-hydrolytic conditions fail to solubilize more than 55% of the protein (Garcia and Phillips 2008). Amino acid analysis reveals that a large portion of MBMP is collagen. The extract of MBMP has some of the functional properties of gelatin. Collagen accounts for approximately 17% of the protein in soft tissue particles and 26% of that in bone particles (Garcia and Phillips 2008). The extractable fractions of MBMP are highly polydisperse and have weight-average molecular masses of 71.1-86.7 kDa (Garcia and Phillips 2008).

Different proteins can be obtained by using different extraction solvents, or by using different extraction conditions (e.g., temperature and pH) for the same extraction solvent. In this study, the first four methods for protein extraction were employed to extract MBMP in order to obtain some proteins which can be used for the production of anti-bMBM protein antibody. The pure bMBM samples were provided by TBC and BHT Packing Co. (Birmingham, AL, USA).The results show that the proteins extracted by Method One (Tris buffer, pH 7.5) are mainly gelatin. It is difficult to see clear bands on SDS-PAGE for these protein samples. An affinity column was made following the procedures of "Affi-Gel Hz Hydrazide Gel Scale-Up Instruction" and "Affi-Gel Hz Immunoaffinity Kit Instruction Manual" to completely or partially remove gelatin in the samples to purify the protein, but the results were still not good. Low quantity of MBMP was obtained when deionized water or sterile deionized water was utilized to extract MBMP from pure bovine MBM at RT (Method Two of protein extraction), which showed that the solubility in water of bovine MBMP is very low. When 6.0 M guanidine was used as an extraction solvent (Method Three of protein extraction), the amount of MBMP obtained was more than that extracted in Method Two, but still low. After cobalt or nickel bead purification and visualized in SDS-PAGE, it was found that the results were still not satisfied. The main purpose of Method Four for protein extraction is to remove gelatin or collagen in pure bovine MBM by the pretreatment of NaOH solution. Various NaOH solutions at 0.1, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 M NaOH were used for the pretreatments at 37 ^{O}C for 2 days or other designated time under agitation. The results demonstrated that gelatin in the bMBM cannot completely be removed by the pretreatment using 0.1, 0.25 and 0.5 M NaOH for less than 2 days, and the proteins extracted using sterile deionized water following the pretreatment still contained a lot of gelatin, which can significantly influence the quality of the antibody. The higher concentration of NaOH such as

1.5 M leads to very low MBM protein concentration after water extraction. As a result, 1.0 M NaOH solution was chosen as the pretreatment solution. The bMBM protein pretreated in 1.0 M NaOH solution at 37 ^{O}C for 2 days under agitation followed by protein extraction in sterile deionized water, and the extracted proteins were dialyzed and centrifuged at 20,000 x g for 10 min at 4 ^{O}C. The supernantant collected was used for the production of anti-bMBM protein antibody.

The disadvantage of Method Four of protein extraction is the long pretreatment time. To overcome this disadvantage, the pretreatment was carried out at a higher temperature, i.e., 100 ^{O}C, in which the pretreatment can be finished at 5-15 min (Method Five of protein extraction).

It should be stressed that after the pure bovine MBM was pretreated in 1.0 M NaOH solution at 100 ^{O}C, the components of the proteins extracted by sterile deionized water is not completely the same as those pretreated at 37 ^{O}C, which means that the temperature used with the NaOH pretreatment has impacts on the components of extracted proteins.

3.3.2 Characterization of Bovine Meat and Bone Meal Protein

The results of SDS-PAGE on the protein extracted from bovine MBM using Method Four of protein extraction is shown in Figure 3.3.

It can be seen from Figure 3.3 that there exist two obvious bands with molecular weights of 120 and 41 KDa. In addition, there are some light bands with different molecular weights. When the bovine MBM proteins extracted were used to raise antibodies for immunoassay development, a good immune response was observed.

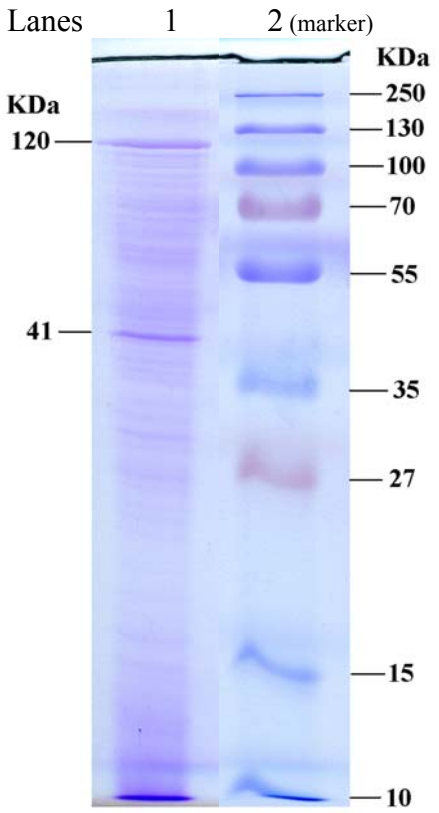


Figure 3.3 SDS-PAGE on bovine MBM protein

Lane 1: bovine MBM protein used for production of anti-bovine MBM protein antibody
Lane 2: protein standard (PageRuler™ Prestained Protein Ladder Plus, Fermentas Life Sciences)

3.3.3 Purification of Anti-Bovine Meat and Bone Meal Protein Antibody

The rabbit anti-bovine MBM protein antibody was purified through ammonium sulfate precipitation followed by Protein A affinity column. The results of SDS-PAGE on the purified antibody were shown in Figure 3.4. Lane 1 is rabbit serum; Lane 2 is the anti-bovine MBM protein antibody purified by 20-50% saturated $(NH_4)_2SO_4$ precipitation; Lane 3 is flow-through fraction of Protein A affinity column purification; Lane 4 is the anti-bovine MBM protein antibody purified by 20-50% saturated $(NH_4)_2SO_4$ precipitation followed by Protein A affinity column; Lane 5 is protein standard (PageRuler™ Prestained Protein Ladder Plus, Fermentas Life

Sciences). Lane 1 shows the amount of the antibodies and other proteins that were present before the purification by 20-50% saturated $(NH_4)_2SO_4$ precipitation. Lane 2 shows the proteins that were subsequently removed by the Protein A affinity column.

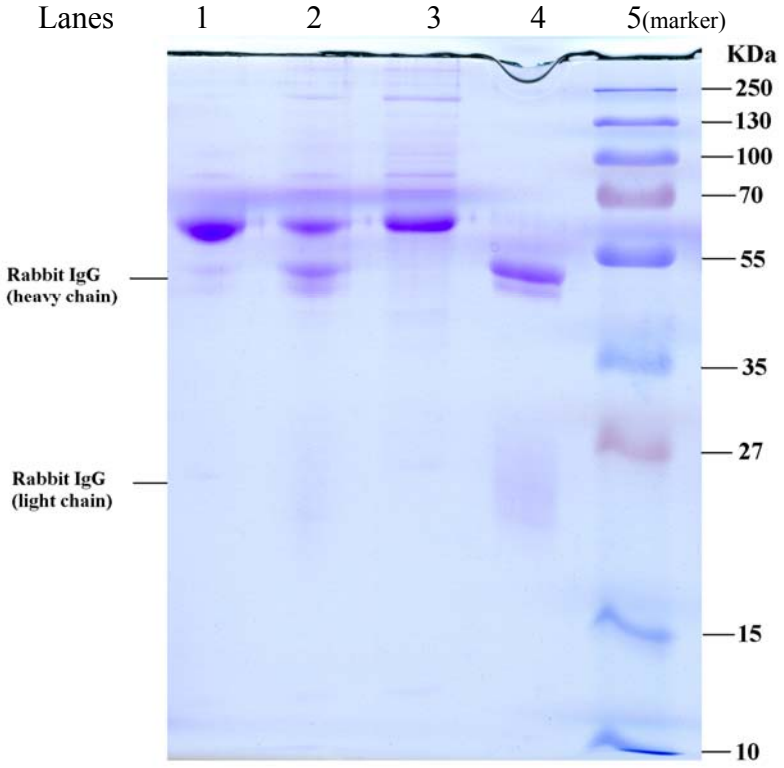


Figure 3.4 SDS-PAGE on anti-bovine MBM protein antibody purification

Lane 1: rabbit serum
Lane 2: purified by 20-50% saturated $(NH_4)_2SO_4$ precipitation
Lane 3: flow-through fraction of Protein A affinity column purification
Lane 4: purified by 20-50% saturated $(NH_4)_2SO_4$ precipitation followed by Protein A affinity column
Lane 5: protein standard (PageRuler™ Prestained Protein Ladder Plus, Fermentas Life Sciences)

By comparing Lane 1 and Lanes 2 through 4, it is obvious that many non-specific proteins were removed from the serum, which demonstrated that the purification by saturated $(NH_4)_2SO_4$ precipitation plus Protein A affinity column was very effective at removing additional proteins

that could have potentially interfered with the colloidal gold conjugation to the antibodies. Figure 3.4 shows that the purity of the purified antibody is very high. This antibody appears to have good prospects to be detected by the LFTS method.

3.3.4 Characterization of Anti-Bovine MBM Protein Antibody

3.3.4.1 Reactivity of Anti-Bovine MBM Protein Antibody

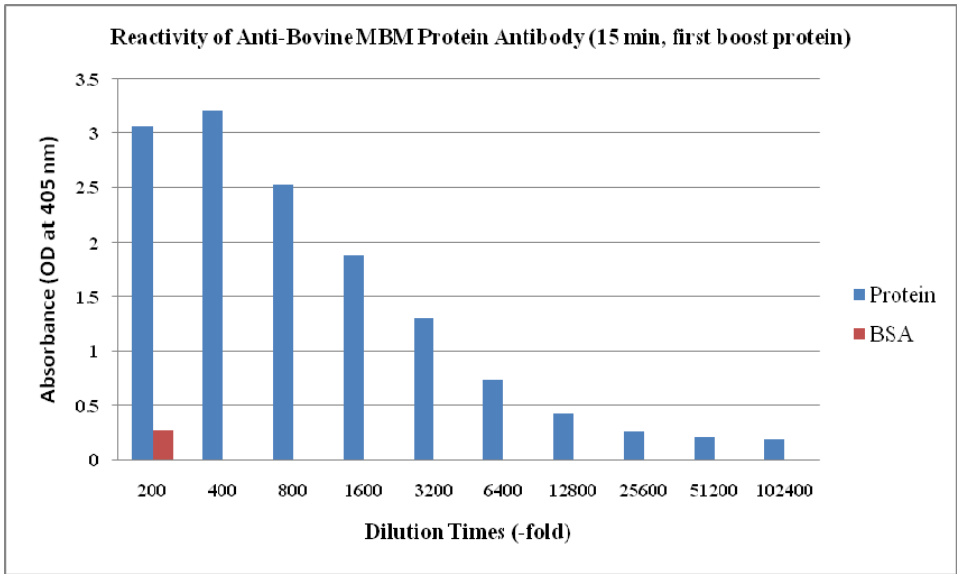
The rabbit had good immunogenic responses to bovine MBM protein antigen as demonstrated in ELISA results in Figure 3.5 (A and B). Based on a common rule, any ELISA reading above 1.000 at 30 min is considered an excellent titer of the antibody. Figure 3.5 shows the ELISA results for the anti-bovine MBM protein polyclonal antibody (pAb) produced from the 1st boost bovine MBM protein and purified by $(NH_4)_2SO_4$ precipitation followed by Protein A affinity column. The rabbit produced an excellent concentration of antibodies towards bovine MBM protein at an antibody dilution of 1: 6,400.

For the final bleed, the rabbit produced an excellent concentration of antibodies towards bovine MBM protein, which is shown in Figure 3.6 (A-D). The total 104 ml of 5311.76 µg/ml purified anti-bovine MBM protein antibodies were obtained from the final serum and used for the development of the detection of bovine MBM in animal feeds using LFTS.

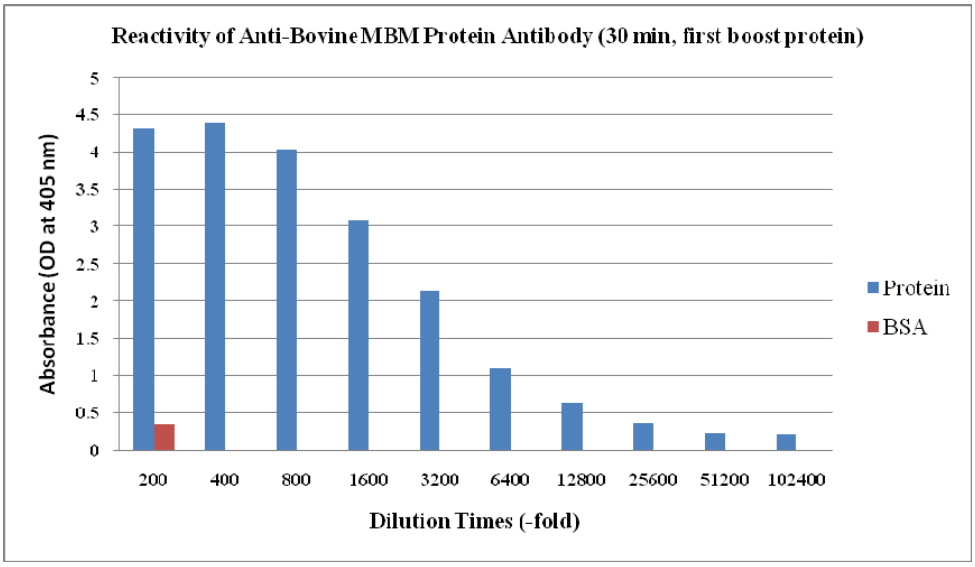
3.3.4.2 Sensitivity of Anti-Bovine MBM Protein Antibody

The sensitivity of anti-bovine MBM protein antibody is shown in Figure 3.7. Generally, the concentration of bovine MBM protein extracted from pure (100%) bovine MBM and employed to produce anti-bovine MBM protein antibody is around 2 mg/ml. Based on the ELISA results in Figure 3.7, the sensitivities (or limit of detection) of anti-bovine MBM protein antibody at

dilution factors 1:500 and 1:1000 are less than 0.1% (w/w), which reaches the requirement for industry use.

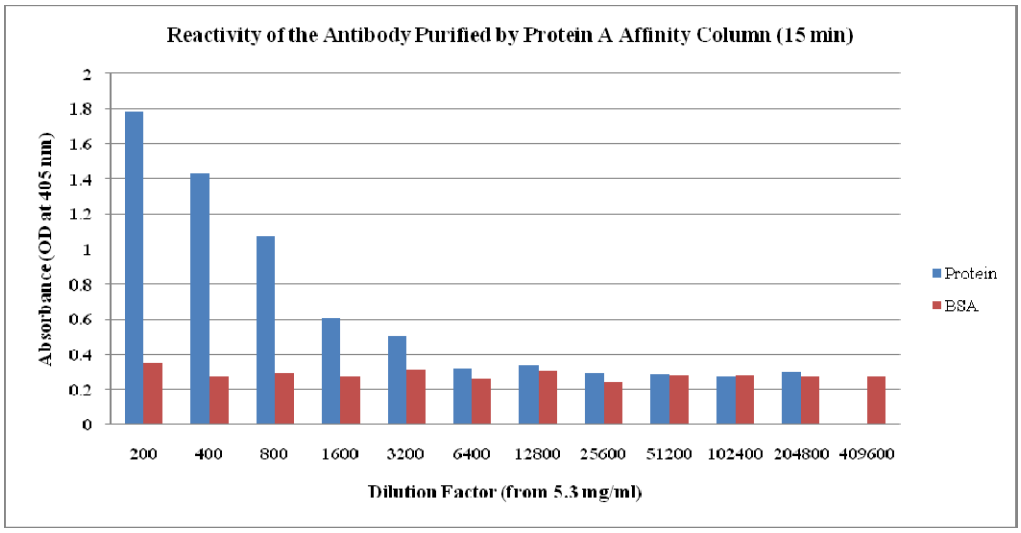


(A)

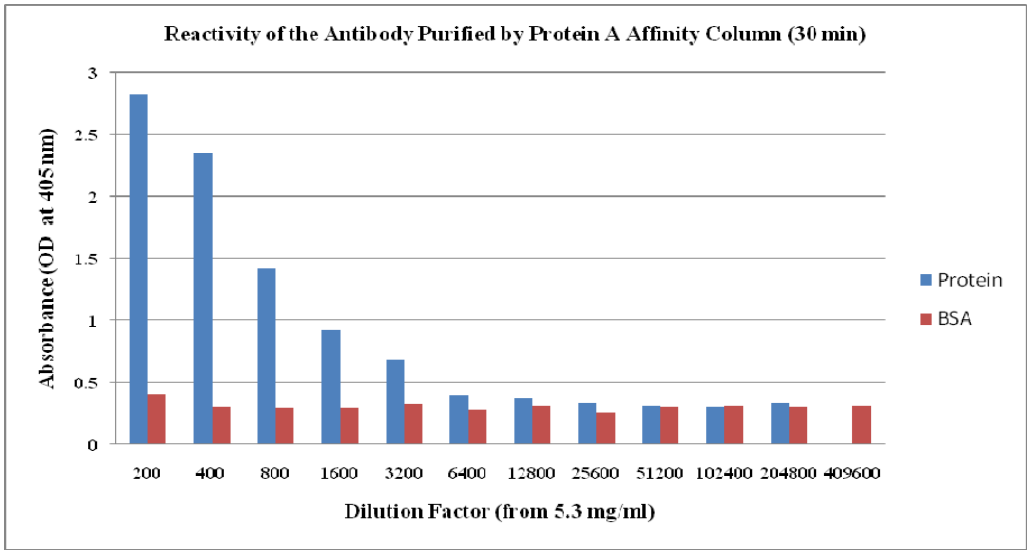


(B)

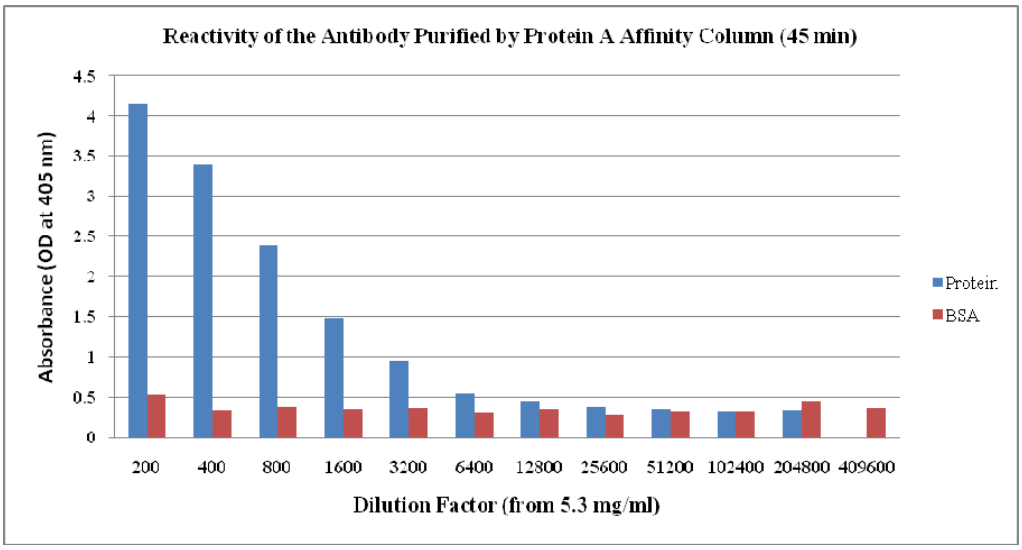
Figure 3.5 Reactivity of Anti-Bovine MBM Protein Antibody (1st boost MBM protein)
(Abs: produced from the 1st boost MBM protein, purified by $(NH_4)_2SO_4$ followed by Protein A affinity column)


Reactivity of the Antibody Purified by Protein A Affinity Column (15 min)
Absorbance (OD at 405 nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
200
400
800
1600
3200
6400
12800
25600
51200
102400
204800
409600
Dilution Factor (from 5.3 mg/ml)
Protein
BSA

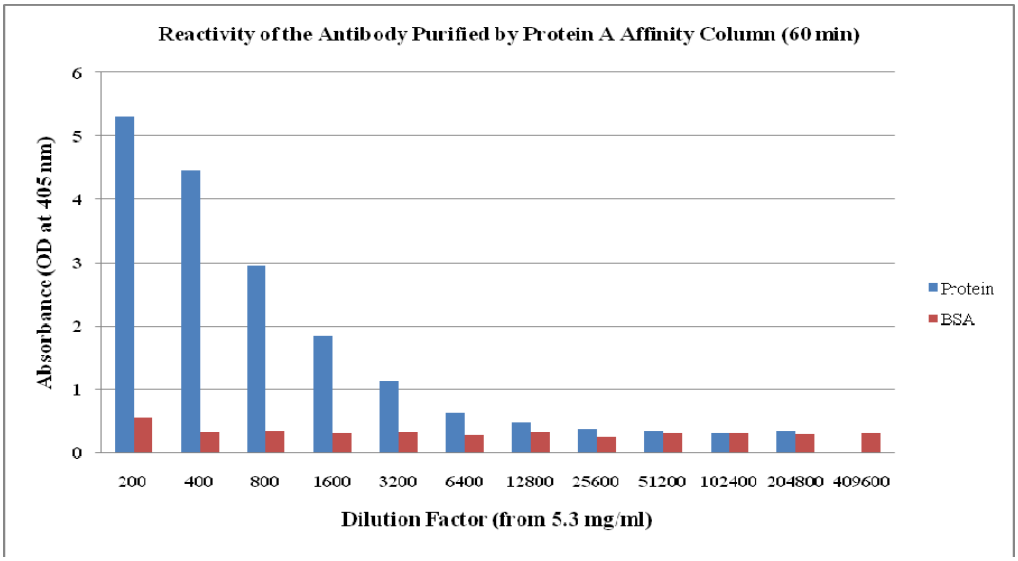
(A)


Reactivity of the Antibody Purified by Protein A Affinity Column (30 min)
Absorbance (OD at 405 nm)
3
2.5
2
1.5
1
0.5
0
200
400
800
1600
3200
6400
12800
25600
51200
102400
204800
409600
Dilution Factor (from 5.3 mg/ml)
Protein
BSA

(B)

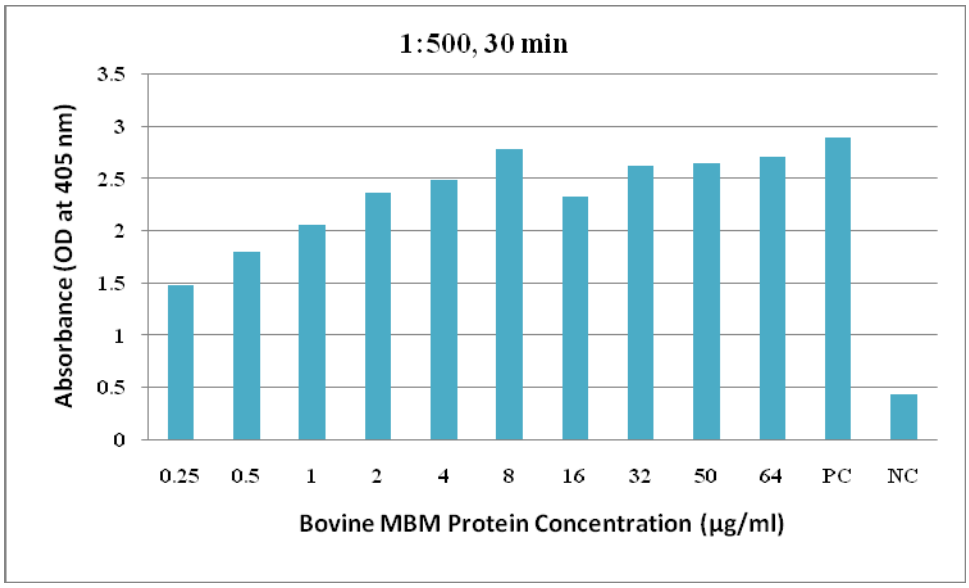


(C)

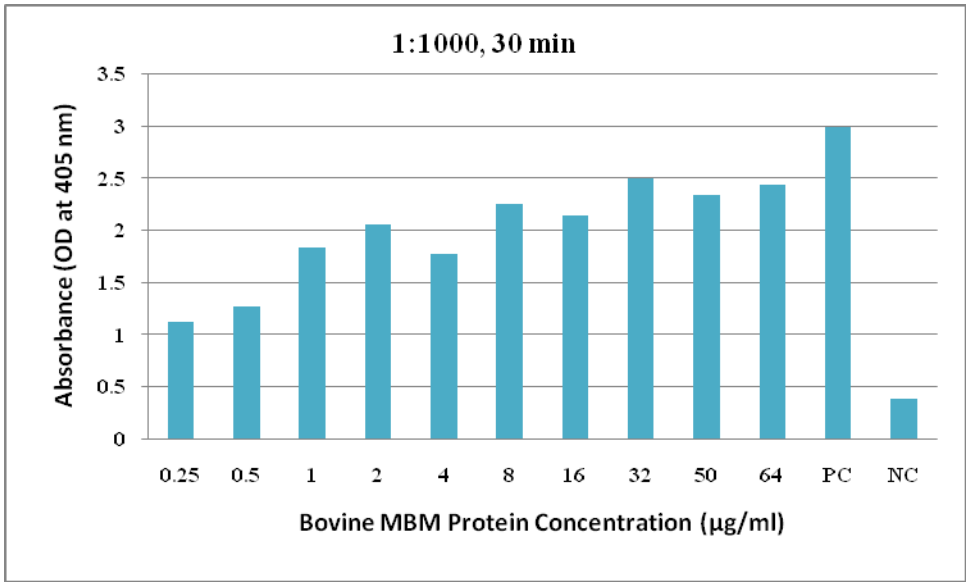


(D)

Figure 3.6 Reactivity of Anti-Bovine MBM Protein Antibody
A: 15 min; B: 30 min; C: 45 min; D: 60 min; PC: positive control



(A)



(B)

Figure 3.7 Sensitivity of Anti-Bovine MBM Protein Antibody

A: 1:500, 30 min; B: 1:1,000, 30 min; PC: positive control; NC: negative control

3.3.4.3 Specificity of Anti-Bovine MBM Protein Antibody

The specificity of anti-bovine MBM protein antibody is shown in Table 3.2. It can be seen that for the anti-bovine MBM protein antibody with 500-fold dilution, the ELISA results for the first 37 protein samples at 15 min demonstrated that there exists cross-reaction between the antibody and other ingredients such as TBC3 (pure bovine MBM) and SH pellets. At 30 min, ELISA results from ingredients such as ground corn, rice, alfalfa, bull grower (B15) (X) mediated, poultry by-products meal, porcine MBM, Nutrebeef cattle (BREEDER complete cube), TBC3 (bovine MBM) and SH pellets shown the cross-reaction. For the anti-bovine MBM protein antibody with 1,000-fold dilution, only TBC3 ELISA results are positive, which demonstrates that the developed antibody can be employed to detect the bovine MBM in animal feed with 1,000-fold dilution by the ELISA method.

Table 3.2 Specificity of the Purified Anti-Bovine MBM Protein Antibody

Protein Sources	Protein Concentration before boiling (μg/ml)	Ab			
		1:500		1:1000	
		Protein (un-boiled)	Protein (boiled)	Protein (un-boiled)	Protein (boiled)
		30 min	30 min	30 min	30 min
Negative Control (BSA)	1000	0.484	0.467	0.461	0.446
Positive Control	1900	3.623	3.521	2.862	2.502
Ground Corn	1410.59	1.156	0.826	0.791	0.684
Rice	609.25	1.007	1.084	0.867	0.772
Rice Bran	2075.35	0.68	0.579	0.536	0.447
Oats	1096.77	0.753	0.986	0.583	0.644
Soybean Meal (1)	11255.295	0.896	0.678	0.503	0.472
Soybean Meal (3)	1011.89	0.654	0.675	0.475	0.409
Peanut Hull	237.37	0.845	1.628	0.596	0.794
Midds	4770.555	0.704	0.594	0.446	0.391
Cotton seed Meal	1726.35	0.92	0.736	0.479	0.495
Alfalfa	786.07	1.272	1.009	0.812	0.758
Dairy supplement	1705.055	0.787	0.724	0.626	0.463
NB finisher-Bull	1676.0	0.83	0.785	0.689	0.606

NB finisher-cattle	2309.805	0.873	0.738	0.698	0.532
NB finisher-goat	1444.115	0.785	0.612	0.596	0.574
Pasture Cube cattle	703.02	0.716	0.686	0.594	0.463
Super-20 dairy pel (x)	1643.885	0.688	0.713	0.556	0.546
Mighty Cow Im BYP 18 dairy feed (x)	1694.47	0.673	0.613	0.513	0.449
Nurebeef cattle transition pellet (mediated)	933.41	0.893	0.694	0.595	0.483
Bull grower (B15) (X) mediated	1566.0	1.101	0.779	0.7	0.569
Southwind 12% Horse & cattle pellet (x)	1082.06	0.75	0.64	0.544	0.511
Fish Meal (05/09/2008)	1221.0	0.628	0.644	0.498	0.441
Dried whey	3253.75	0.695	0.598	0.479	0.45
Poultry Bleeding Meal	132.5625	0.83	1.013	0.624	0.752
Nutrebeef Breeding Herd	547.445	0.857	0.909	0.573	0.661
Poultry By-products Meal	1974.125	1.452	1.496	0.885	1.057
Porcine MBM	1030.375	1.177	1.068	0.798	0.889
TBC 3	783.335	2.352	2.287	1.769	1.66
Brewers Rice	974.25	0.892	1.194	0.665	0.732
Nutrebeef cattle (BREEDER complete cube)	848.75	1.123	1.051	0.891	0.857
Nutrebeef Bull (DEVELOPER PELLET 12)	1769.375	0.849	0.667	0.53	0.462
SH pellets (02/12/2004)	1632.345	1.58	1.056	1.008	0.777
Naturewise Goat PELLETS (Cargill, Mont.)	1307.0	0.754	0.618	0.566	0.475
Cow Intestine	4056.48	0.535	0.454	0.417	0.323
Hog Intestine	5173.2	0.592	0.701	0.439	0.286
Chicken Intestine	7308	0.548	0.419	0.417	0.341
Chicken Gizzard	5773.2	0.521	0.395	0.416	0.336
Beef	7765.2	0.478	0.534	0.38	0.404
TBC3 (boiling 10 min using 1.0 M NaOH, then extraction by H_2O)	96.61		3.189		2.899
TBC3 (boiling 5 min using 1.0 M NaOH, then extraction by H_2O)	231.07		3.119		2.797
TBC3 (boiling 10 min using 0.5 M NaOH, then extraction by H_2O)	277.86		3.609		2.711
TBC3 (boiling 5 min using 0.5 M NaOH, then extraction by H_2O)	494		3.208		2.724
TBC3 (boiling 10 min using 0.1 M NaOH, then extraction by H_2O)	1967.09		2.581		1.963
TBC3 (boiling 5 min using 0.1 M NaOH, then extraction by H_2O)	1286.51		2.272		1.709

Comparing Methods Four and Five of protein extraction described in the protein extraction section, pretreatment time of the sample in Method Five is significantly reduced. The ELISA results of the protein samples extracted from bovine MBM using Method Five were also shown in Table 3.2. Positive results were demonstrated, which means that the pretreatment approach in Method Five may be exploited to replace the one in Method Four for the preparation of the protein samples to be tested.

3.3.5 Immunoabsorption of Anti-Bovine MBM Protein Antibody

Immunoabsorption is referred to as the removal of a specific group of antibodies by antigens or the removal of antigen by interaction with specific antibodies.

In this study, the proteins extracted from pure animal feed by using the Method Five of protein extraction were utilized to immune-absorb the anti-bovine MBM protein antibody in order to remove some specific groups of the antibody. The ELISA results of the immuno-absorbed antibody demonstrated that the cross-reaction still exists more or less, which means that the Method Five of the protein extraction is not a good substitution for Method Four for protein extraction. Compared to the commercial LFTS kit method, specific chemical(s) or chemical components used as effective extraction solvents need to be found to help to extract the protein from the animal compound feed, in which no cross-reaction exists.

3.3.6 Colloidal Gold Conjugation to Anti-Bovine Meat and Bone Meal Protein Antibody

A transmission electron microscope (TEM) picture of commercial colloidal gold adopted in this study was shown in Figure 3.8, which demonstrated that the gold nanoparticles (AuNPs) have the size ~35-40 nm. The AuNPs with different sizes made in our lab were also utilized to

conjugate anti-bovine MBM protein antibody. The results indicated that the AuNPs with the size 20-50 nm show the similar results to commercial AuNPs for the anti-bovine MBM protein antibody conjugateion.

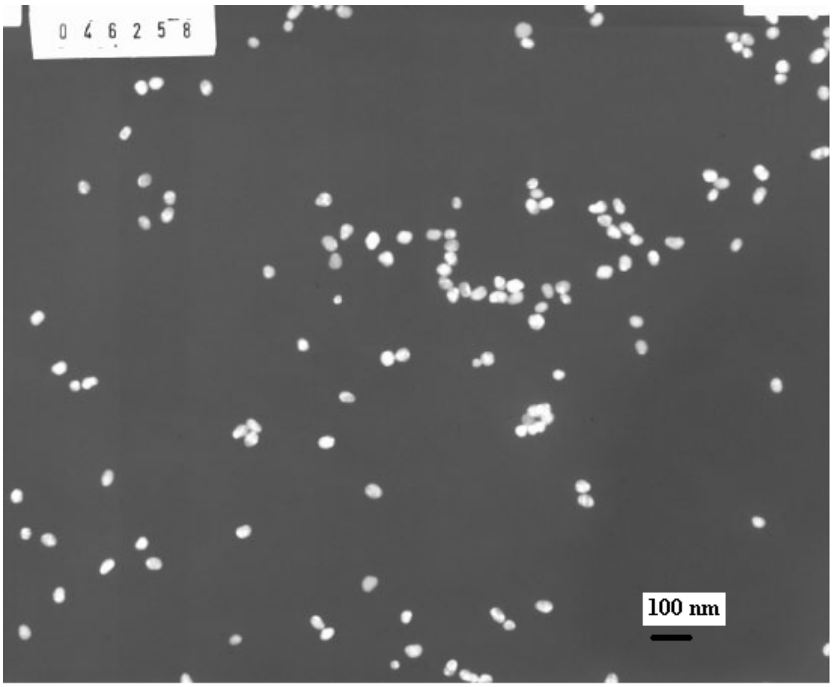


Figure 3.8 TEM picture of commercial colloidal gold with the size 35-40 nm

3.3.7 Performance of Lateral Flow Test Strip

The test scale used for all LFTS testing was: 0 = no test line, +1 = very faint test line, +2 = test line less intense than control line, +3 = test line as intense as control line, +4 = test line more intense than control line, and +5 = test line very intense.

3.3.7.1 Limit of Detection

The LFTS developed in this study was enclosed within a plastic housing, called the LFTS card in the following description.

Three LFTS cards were used for blank testing. They contained the control line, test line, and control line plus test line, respectively. 200 µl of sterile deionized water, PBS or TBS was applied to the sample pad of the LFTS card drop by drop (70 µl plus 70 µl plus 60 µl, 1 min internal). The strips only produced a strong positive control signal indicating the anti-bovine MBM protein antibody was successfully conjugated to colloidal gold particles, and no visual signal on the test line. The strength of the positive signal on the control line is directly related to the amount of the anti-rabbit IgG immobilized.

Two hundred µl of the bovine MBM protein samples extracted from pure bovine MBM using Method Four of protein extraction and diluted to the designated concentrations, were applied to the sample pad as described above. The results showed when the protein sample was 10,000-fold diluted, the positive test signals could still be seen on the test line and control line. These results indicate that the limit of detection for this case is 0.01% or less, and that this rapid test can offer sensitivities similar to or better than ELISA method.

Two hundred µl of the protein samples, extracted from 100% animal feed using Method Five of protein extraction in which the feed in pretreatment was boiled for 5 min in 1.0 M NaOH solution, were applied to the sample pad as mentioned above. The results show a positive signal on the control line, and negative or positive signal on the test line, which demonstrated that there exists cross-reactivity between the protein in animal feed and the anti-bovine MBM protein antibody. For the protein sample pre-treated for 10 min in 1.0 M NaOH solution, the cross-reaction also occurred. In addition, as 0.5 M NaOH solution was adopted in pretreatment, the animal feed sample had to be boiled at least 15 min in order to reduce the cross-reaction. On the basis of these results, the following protein samples were extracted from the solid mixture of 100% animal feed and pure bovine MBM by boiling the mixed samples in 1.0 M NaOH solution

for 5 min: 90%, 95%, 97%, 99%, 99.5%, and 99.9% animal feed. LFTS testing results show that even though the extracted protein sample was 60-fold diluted, positive test signals on test line and control line can be obtained. This demonstrated that for this case the limit of detection is 0.1% or less. However, considering the cross-reaction between the proteins extracted from 100% animal feed and the antibody, this low detection limit is not reliable.

3.3.7.2 Specificity

Part of the protein samples in Table 3.2, including the samples which demonstrated cross-reaction with the anti-bovine MBM protein antibody more or less on the basis of ELISA results, was employed to test the specificity of the LFTS. Two hundred µl of the protein samples without dilution were applied onto the sample pad. It can be seen from Table 3.3 that the proteins extracted from pure peanut hull, Alfalfa and SH pellets have the cross-reaction with the antibody and after the immunoabsorption of anti-bovine MBM protein antibody, these cross-reactions were eliminated.

3.4 Conclusion

The antibody-based LFTS was successfully developed to detect bovine MBM in animal feeds by using gold-anti-bovine MBM protein antibody complex as a detector (recognition element), anti-bovine MBM protein antibody as a test line agent and anti-rabbit IgG as a control line agent. Prior to the immunoabsorption of the anti-bovine MBM protein antibody, this antibody had varying degrees of cross-reactivity with the protein extracted from animal feeds. This was eliminated or significantly reduced via the immunoabsorption. The limit of detection of the LFTS is 0.1% or less.

Table 3.3 Specificity of Lateral Flow Test Strip

Protein Sources	Protein Concentration before boiling (μg/ml)	Antibody			
		Before Immunoabsorption		After Immunoabsorption	
		15 min		15 min	
		Protein (un-boiled)	Protein (boiled)	Protein (un-boiled)	Protein (boiled)
Negative Control (BSA)	1000	0	0	0	0
Positive Control	1900	+4	+4	+4	+4
Ground Corn	1410.59	0	0	0	0
Rice	609.25	0	0	0	0
Soybean Meal (1)	11255.295	0	0	0	0
Peanut Hull	237.37	+1	+1	0	0
Alfalfa	786.07	+1	+1	0	0
Dairy supplement	1705.055	0	0	0	0
Fish Meal (05/09/2008)	1221.0	0	0	0	0
Dried whey	3253.75	0	0	0	0
Poultry By-products Meal	1974.125	0	0	0	0
TBC 3	783.335	+3	+3	+3	+3
SH pellets (02/12/2004)	1632.345	+2	+2	0	0
TBC3 (boiling 10 min using 1.0 M NaOH, then extraction by H_2O)	96.61		+3		+3
TBC3 (boiling 5 min using 1.0 M NaOH, then extraction by H_2O)	231.07		+3		+3
TBC3 (boiling 10 min using 0.5 M NaOH, then extraction by H_2O)	277.86		+3		+3
TBC3 (boiling 5 min using 0.5 M NaOH, then extraction by H_2O)	494		+3		+3
TBC3 (boiling 10 min using 0.1 M NaOH, then extraction by H_2O)	1967.09		+2		+2
TBC3 (boiling 5 min using 0.1 M NaOH, then extraction by H_2O)	1286.51		+2		+2

Chapter Four

Development of a Phage-Based Lateral Flow Test Strip for the Detection of *Bacillus anthracis* Spores in Water and Foods

4.1 Introduction

Compared to the antibody-based lateral flow test strip (LFTS) in Chapter Three, the phage-based LFTS is discussed in Chapter Four. *Bacillus anthracis* 13 phage (phage *B. anthracis* 13, pBA13) and JRB7 phage (phage JRB7, pJRB7), provided by Dr. James Barbaree's Lab at the Department of Biological Science of Auburn University, were specific to *B. anthracis* (BA) spores. These two phage types (pBA13 and pJRB7) were used as recognition elements, respectively. The gold-pBA13 or gold-pJRB7 conjugate or complex was utilized to detect the BA spores in the sample. The pBA13 or pJRB7 was also immobilized onto the nitrocellulose membrane (NCM) to form a test line and the anti-pBA13 antibody produced in this research was immobilized onto the membrane to form the control line. After samples were applied to the test strip, the gold conjugated phages bound the spores to form pBA13-BA spores or gold pJRB7-BA spores complexes moved down the membrane for detection. The schematic diagram of the construction of phage-based LFTS is shown in Figure 4.1 (adopted from www.devicelink.com/ivdt/archive/00/03/004.html, on 3/24/2006, with modifications).

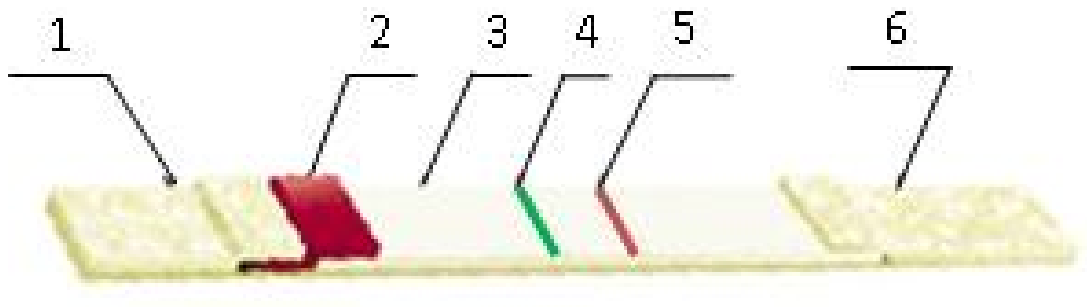


Figure 4.1 Construction of phage-based LFTS

(adopted from www.devicelink.com/ivdt/archive/00/03/004.html, on 3/24/2006, with modifications)

1 sample pad, 2 gold conjugate pad (**phage-based probe**, gold-pBA13 or gold-pJRB7 conjugate),

3 nitrocellulose membrane, 4 test line (pBA13 or pJRB7),

5 control line (anti-pBA13 antibody), and 6 absorbent pad

4.2 Materials and Methods

4.2.1 Materials

The compound 1,8-diaminooctane (DAO, H_2N-$(CH_2)_8$-NH_2) was purchased from ACROS ORGANICS (Fair Lawn, NJ, USA). Acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA). UltraBind™ US450 (modified polyethersulfone, affinity membrane 0.45µ, 20 cm × 20 cm sheets), Biodyne® C 0.45µ (nylon 6, 6, 7 cm × 8.5 cm, P/N 60315), BioTrace™ PVDF (polyvinylidene fluoride, transfer membrane 0.45µm, 7 cm × 8.5 cm sheets, P/N 66594) were purchased from Pall Corporation, Life Sciences (Ann Arbor, MI, USA). *B. anthracis* 13 phage (or phage *B. anthracis* 13, pBA13), JRB7 phage (or phage JRB7, pJRB7), and *B. anthracis* spores were provided by Dr. James Barbaree's Lab at the Department of Biological Science of Auburn University. The sources of other materials used are the same as described in section 3.2.1.

4.2.2 Methods

4.2.2.1 Production of Anti-*Bacillus anthracis* 13 Phage Antibody

The anti-*Bacillus anthracis* 13 phage (anti-pBA13) polyclonal antibodies used in this study were produced in our lab by using seven immunizations of purified *B. anthracis* 13 phage (pBA13) in rabbit using Freund's adjuvant. The purified pBA13 antigen was used as immunogens. The M13 phage with peptide sequence of "XXXXSMXARDYR" was screened from PhD12 library (New England Biolabs, Ipswich, MA, USA) against the spores of *B. anthracis* Sterne strain.

Table 4.1 Schedule for the Production of anti-pBA13 Antibody

Day	Date	Procedure
0	03/17/2008	Primary Immunization
31	04/17/2008	1st Boost
39	04/25/2008	Rabbit Blood Collection (RBC)
59	05/15/2008	2nd Boost
67	05/23/2008	RBC
91	06/16/2008	3rd Boost
100	06/25/2008	RBC
121	07/16/2008	4th Boost
129	07/24/2008	RBC
154	08/18/2008	5th Boost
163	08/27/2008	RBC
183	09/17/2008	6th Boost
191	09/25/2008	RBC
209	10/13/2008	7th Boost
217	10/21/2008	RBC
219	10/23/2008	Final RBC

Rabbit immunizations or boosts used (Table 4.1) were conducted by using 1 ml of 1×10^{13} virions/ml pBA13. The protocol adopted is similar to that in section 3.2.2.4. A New Zealand white rabbit of about 3 kg was used to produce antiserum against the purified pBA13 antigen.

The purified pBA13 with the concentration of 1×10^{13} virions/ml was emulsified with complete Freund's adjuvant (1:1 v/v) for primary immunization. The following treatments were the same as the production of anti-bovine MBM protein antibody in section 3.2.2.4.

4.2.2.2 Purification of Anti-*Bacillus anthracis* 13 Phage Antibody

The rabbit blood collected was stored at 4 ^{O}C for 2-4 h and then centrifuged at 2,500 x g for 1 h at 4 ^{O}C. The supernatant was collected and the antibody was precipitated at 20-50% saturated ammonium sulfate ($(NH_4)_2SO_4$). After centrifugation at 10,000 x g for 15 min at 4 ^{O}C, the supernatant was discarded. The pellet or precipitate (antibody) was suspended in 20 mM phosphate buffer with pH 7.0. After dialysis in 20 mM phosphate buffer pH 7.0 overnight with at least one time change of dialysis buffer, the antibody was centrifuged at 12,000 x g for 10 min at 4 ^{O}C. The supernatant was collected and stored at -20 ^{O}C for further purification. The anti-pBA13Abs purified from 20-50% saturated $(NH_4)_2SO_4$ flowed through a Protein A affinity column at 1 ml/min. The column was washed with 20 mM phosphate buffer with pH 8.0 until $O.D._{280\ nm}$ at the exit of the column was very close to zero, and then eluted with three bed volumes of Protein A elution buffer (0.5 M citric acid, pH 3.5). The eluent containing the antibody was neutralized to pH 7.0 to 7.4 with 1.0 M NaOH, dialyzed in 20 mM phosphate buffer (pH 7.0) overnight at 4 ^{O}C. The sample was centrifuged at 12,000 x g for 10 min at 4 ^{O}C, and the supernatant contained antibody was collected for further use.

4.2.2.3 Purity Determination of Anti-*Bacillus anthracis* 13 Phage Antibody

All the gels (12%) used for running SDS-PAGE in this study were made in our lab. Fermentas Benchmark protein ladder was utilized to determine protein molecular weights. The

samples were diluted to 500 μg/ml by using deionized water and sample buffer, and boiled for 2.5 min. An aliquot of 15 μl of each boiled sample was added into each well of the gel. After running SDS-PAGE at 100 V for 15 min followed by 200 V for 35-40 min, the gel was stained with Coomassie Blue R-250 staining solution for 25 min at room temperature. This was then destained in destaining solution I (25% methanol, 10% acitic acid) for 2 h followed by destaining solution II (5% methanol, 7% acitic acid) for overnight at room temperature.

4.2.2.4 Reactivity of Anti-*Bacillus anthracis* 13 Phage Antibody

The protocol of the reactivity test for rabbit anti-pBA13Abs is similar to that of rabbit anti-bovine MBM protein antibody described in Chapter Three with some modifications.

Purified pBA13 was diluted to 1×10^{11} virions/ml with PBS, pH 7.4 and 100 μl of the diluted pBA13 were added to a well of a 96-well ELISA plate. The BSA at 5 μg/well was used as a negative control. The plate was incubated at 30 ^{O}C for 2 h and then washed three times using washing buffer (PBST: PBS plus 0.05% Tween-20 and 0.01% sodium azide (NaN_3), pH 7.4). The plate was blocked by adding 200 μl of 1% BSA in PBS plus 0.1% NaN_3, pH 7.4 to each well and incubated at 30 ^{O}C for 1 h. After washing three times with PBST, 100 μl of each 2-fold series diluted anti-pBA13Abs were added to each well and the plate was incubated at 30 ^{O}C for 2 h.

The plate was washed three times with PBST as described above, and 100 μl of 3,000-fold diluted goat anti-rabbit IgG alkaline phosphatase conjugated antibody (Sigma-Aldrich) were added to each well. After incubation at 30 ^{O}C for 1 h following washing four times with PBST, 100 μl of fresh prepared substrate of *p*-nitrophenyl phosphate (*p*-NPP, New England Biolabs, Ipswich, MA, USA) at 2 mg/ml were added to each well of the plate. The plate was incubated in

the dark at room temperature for color development and the absorbance at 405 nm ($O.D._{405\ nm}$) was measured 15 min intervals using a microplate reader (Thermo Laboratory System, Helsinki, Finland) up to 60 min and recorded for analysis.

4.2.2.5 Interaction between *Bacillus anthracis* 13 Phage or JRB7 Phage and BA Spores

For the phage-based LFTS test, adsorption time for the interaction between the sample (*B. anthracis* spores) and the detector (colloidal gold-pBA13 or colloidal gold-pJRB7 conjugate) is an important factor. It is expected that the adsorption time should be short. In this research all the pBA13, pJRB7, and *B. anthracis* spores used were provided by Dr. James Barbaree's Lab at the Department of Biological Science of Auburn University. Prior to the construction of the phage-based LFTS, the interaction between pBA13 or pJRB7 and *Bacillus anthracis* spores was tested by ELISA. The protocol of the ELISA employed is similar to that in 4.2.2.4 section with some modifications.

Purified pBA13 and pJRB7 were diluted to 1×10^{11} virions/ml with coating buffer (PBS, pH 7.4). BSA was diluted to 50 µg/ml in PBS (pH 7.4) and used as negative control. One hundred µl of 5×10^{8} spores/ml *B. anthracis* spores and the diluted BSA were added to a well of the 96-well ELISA plate. The plate was incubated at 28 ^{O}C for 2 h and then washed three times using washing buffer (PBST: PBS plus 0.05% Tween-20 and 0.01% sodium azide (NaN_3), pH 7.4). The plate was blocked by adding 200 µl of 1% BSA in PBS plus 0.1% NaN_3, pH 7.4 to each well and incubated at 28 ^{O}C for 1 h. After washing three times with PBST, 100 µl of the diluted pBA13 and pJRB7 were added to a well of the 96-well ELISA plate, respectively. The plate was incubated at 28 ^{O}C for 2 h. After washing three times with PBST, 100 µl of the anti-pBA13Abs

with 1,000-fold diluted in PBS were added to each well of the plate. The plate was incubated at 28 ^{O}C for 2 h. The following treatments were the same as section 4.2.2.4.

4.2.2.6 Colloidal Gold Conjugation to *Bacillus anthracis* 13 Phage or JRB7 Phage

The protocol of colloidal gold conjugation to pBA13 is similar to that of colloidal gold conjugation to anti-bovine MBM protein antibody with some modifications. A suspension of 40 nm colloidal gold particles was adjusted to pH 9.0. The pBA13 was dialyzed in 2 mM borate buffer at pH 9.0 for 24 h at 4 ^{O}C prior to the conjugation. Eighty μl of pBA13 with the concentration of 1×10^{13} virions/ml was conjugated with 8 ml of the colloidal gold particles at pH 9.0 and blocked with 0.8 ml of 10% BSA or 1% PEG at pH 9.0. The conjugate was purified twice. One is centrifugation at 6,000 x g for 1 h at 4 ^{O}C in order to remove excess pBA13. The other was centrifugation with the gradient (5-25% glycerol in buffer A (Tris/HCl buffered saline at pH 8.2 with 1% BSA and 0.1% sodium azide)) at 4,500 x g for 1 h at 4 ^{O}C in order to remove any small clusters. The purified conjugate was dialyzed in 2 mM borate buffer at pH 9.0 to remove glycerol, and then concentrated by centrifugation at 4,500 x g for 40 minutes at 4º C. The supernatant was discarded and the conjugate pellet was then re-suspended in 2 mM borate buffer (pH 9.0) to $O.D._{520nm}$ = 3.0.

Filamentous JRB7 phage (or phage JRB7, pJRB7), selected specifically for *B. anthracis* spores (clone JRB7, displaying the foreign peptide EPRLSPHS on its outer coating) (Brigati and others 2004; Wan and others 2007), was also utilized to conjugate to colloidal gold particles and form gold-pJRB7 conjugate (a recognition element or binding probe) to detect *B. anthracis* spores. The protocol employed is the same as that of gold-pBA13 conjugate. In addition, pJRB7 was also immobilized on the test line.

4.2.2.7 Phage-Based Lateral Flow Test Strip

The phage-based LFTS was constructed using similar conditions to those in section 3.2.2.9 with some modifications: nitrocellulose membrane (NCM, Millipore Corporation) or other kinds of membranes were used as the solid phase for the assay. The membrane part to which the test line lies was modified by chemicals so that pBA13 or pJRB7 could indirectly be immobilized onto the membrane. After drying for a designated period of time at room temperature (RT), the test line was immobilized with 3.0 µl/cm of pBA13 or pJRB7 with the concentration of $\sim 10^{11}$ virions/ml or 10^{12} virions/ml (in PBS at pH 7.4, TBS at pH 8.2, or sterile water). Control line reagent was immobilized with rabbit anti-pBA13Abs at a concentration of 0.7 mg/ml and 3.0 µl/cm. The colloidal gold-pBA13 or gold-pJRB7 conjugate was adjusted to $O.D._{520nm}$ =1.0 by using 12% sucrose and conjugate dilution buffer, deposited on a conjugate pad, and air-dried for more than 24 h at RT. The immobilized NCM was blocked using lateral flow blocking solution and air-dried for 24 h at 4 ^{O}C prior to assembling into test cards. The sample pads, conjugate pads, membranes, and absorbent pads were assembled into cassettes together, pouched with desiccant and stored at 4 ^{O}C for use.

4.2.2.8 Phage-Based Lateral Flow Test Strip Testing

To test the phage-based LFTS, 200 µl of sterile water, 2 mM borax with pH 9.0, PBS with pH 7.4, TBS with pH 8.2 and the samples containing *B. anthracis* spores were applied to the sample pad, allowed to laterally flow down the membrane, and the results were recorded at 15 min.

4.2.2.9 Modification of Nitrocellulose Membrane

After obtaining negative test results for all five adsorption times (15, 30, 60, 90 and 120 min), one of the possible reasons is that the efficiency of pBA13 or pJRB7 immobilization on nitrocellulose (NC) is low. Two ways were tried to fix this issue. One was to use different membranes for pBA13 immobilization. The other was to modify NCM using chemical(s) so that pBA13 or pJRB7 could bind to the chemical and be indirectly immobilized onto the NCM.

I. Selection of the Membrane

Four different membranes were used including NCM (HF240MC100, Hi-Flow Plus 240 membrane, Millipore Corporation), UltraBind™ US450 (modified polyethersulfone, affinity membrane 0.45µ, Pall Corporation, Life Sciences), Biodyne® C 0.45µ (nylon 6, 6, P/N 60315, Pall Corporation, Life Sciences), BioTrace™ PVDF (polyvinylidene fluoride, transfer membrane 0.45µm, P/N 66594, Pall Corporation, Life Sciences). For NCM and UltraBind ™ US450, the treatment conditions were as follows. The membrane was cut to 2.5 × 1.6 cm, soaked into 8 ml of TBS buffer (pH 8.2) at RT for 2 min, and air-dried at RT for 30 min (not completely dried). Then 16 µl of 2% (v/v) glutaraldehyde in TBS (pH 8.2) was applied onto the surface of the membrane as the test line along the width of the membrane. After the membrane was dried at RT for 1.5 h, 10 µl of pBA13 or pJRB7 were applied to the same location of the membrane (test line), and 3 µl of anti-pBA13 antibodies was also applied to the membrane as the control line. The test strips can be used after drying at RT for at least 24 h. For Biodyne® C 0.45µ and BioTrace™ PVDF, the treatment conditions of the membrane were the same as NCM and UltraBind™ US450 except that the membranes were wetted in 100% methanol at RT for 15 seconds prior to treatment.

II. Modification of Nitrocellulose Membrane

Several modified NCM were also tried to indirectly immobilize pBA13 or pJRB7:

Case One: NCM (card or pure membrane) + 1% (DAO) + 1% GA + pBA13 or pJRB7

1% (w/v) 1,8-diaminooctane (DAO, H_2N-$(CH_2)_8$-NH_2) was adopted to modify NCM (Masson and others 1993). NCM was soaked in 1% (w/v) DAO in deionized water at RT for 30 min with constant agitation (KJ-201BD OCILLATOR, 100 rpm). After rinsing with deionized water, the modified NCM was washed with 1.0 M acetic acid at RT for 24 h with constant agitation followed by deionized water at RT for 2 h with constant agitation. The membrane was immersed in 1% (v/v) glutaraldehyde (GA) in 0.5 M $NaHCO_3/Na_2CO_3$ (pH 10) at RT for 15 min, and washed with deionized water at RT for 3 h with constant agitation at 100 rpm. After drying at RT for 4.5 h, 5 µl of pBA13 or pJRB7 (~10^{11} or ~10^{12} virions/ml) were applied onto the NCM as the test line, and 5 µl of anti-pBA13 antibodies were also applied to the membrane as the control line. The membrane was dried at RT for 11.5 h. After the NCM was treated with blocking buffer (PBS, pH 7.4; 0.1% PVA; 0.01% SDS) and rinsed with rinsing buffer (5 mM Na_2HPO_4, pH 7.4; 0.01% SDS), the membrane was completely dried at RT. The test strips were tested by using sterile deionized water, PBS, TBS, and *B. anthracis* spores. The test results were recorded at 15 min.

Case Two: NCM + 1% BSA + 15% GA + pBA13 or pJRB7

BSA (1%, w/v) in deionized water was utilized to modify NCM. NCM was cut into 2.5 × 1.6 cm, and 5 µl of BSA was immobilized onto the NCM as the test line. After drying at RT for 30 min or 5.5 h, 5 µl of 15% (v/v) GA in deionized water were applied to the test line. The NCM was dried at RT for 30 min or 12 h, then 5 µl of pBA13 or pJRB7 (~10^{12} virions/ml) were applied onto the NCM as the test line, and 5 µl of anti-pBA13 antibodies were also applied to the

membrane as the control line. The membrane was dried at RT for 4 h or 12 h. The test strips were tested by using sterile deionized water, PBS, TBS, and *B. anthracis* spore samples. The test results were recorded after 15 min.

Case Three: NCM + 1% BSA conjugated with 15% GA + pBA13 or pJRB7

BSA (1%, w/v) in deionized water and 15% (v/v) GA in deionized water were conjugated at 25 ^{O}C for 1 h with agitation. The conjugate was dialyzed in PBS (pH 7.4) overnight at 4 ^{O}C with at least one time change of dialysis buffer to remove the excess GA in BSA-GA conjugate. After centrifugation, 5 µl of the dialyzed conjugate was immobilized onto the NCM for test line. The membrane was dried at RT for 30 min or 5.5 h, 5 µl of pBA13 or pJRB7 (~10^{12} virions/ml) were applied onto the NCM as the test line, and 5 µl of anti-pBA13 antibodies were applied to the membrane as the control line. The membrane was dried at RT for 4 h or 12 h. The test strips were tested by using sterile deionized water, PBS, TBS and *B. anthracis* spores. The test results were recorded after 15 min.

Case Four: NCM + BSA conjugated with GA and pBA13 or pJRB7

One ml of BSA (2 mg/ml) in deionized water and 1 ml of pBA13 or pJRB7 (~5×10^{12} virions/ml) were mixed. One ml of 20 mM GA in deionized water was added to the mixture, which was incubated at 25 ^{O}C for 1 h with constant agitation for conjugation. BSA-GA-pBA13 or BSA-GA-pJRB7 conjugate was dialyzed in PBS (pH 7.4) overnight at 4 ^{O}C. After centrifugation the supernatant with conjugate was collected and 5 µl of the dialyzed conjugate was immobilized onto the NCM as the test line, and 5 µl of anti-pBA13 antibodies was applied to the membrane as the control line. The membrane was dried at RT for 4 h. The test strips were tested as described above.

Case Five: NCM + GA + pBA13 or pJRB7

NCM was used as the solid phase in this assay. The treatment conditions of NCM are similar to that mentioned above with some modifications. Briefly, NCM with 2.5 × 1.6 cm was immersed into 8 ml of PBS buffer (pH 7.4) at RT for 2 min, and air-dried at RT for 30 min (not completely dried). Then, 5, 10 or 16 µl of GA with the designated concentrations (1, 2, 5, 10, 15, 20, and 25% (v/v) in deionized water, PBS (pH 7.4) or TBS (pH 8.2)) were applied onto the NCM as the test line. After the membrane was dried at RT or 37 ^{O}C for 30 min or 5.5 h, 5 or 10 µl of pBA13 or pJRB7 with the concentration of ~10^{11} or ~10^{12} virions/ml were applied to the test line, and 5 µl of anti-pBA13 antibodies were applied to the membrane as the control line. The membranes were dried at RT for the various designated periods of time (4, 12, 17, 24 h or longer). The test strips were tested by using sterile deionized water, PBS, TBS and *B. anthracis* spores. The test results were recorded after 15 min.

4.3 Results and Discussion

4.3.1 Purification of Anti-*Bacillus anthracis* 13 Phage Antibody

The SDS-PAGE results of anti-pBA13 antibody purification are shown in Figure 4.2. Lane 5 shows the proteins of rabbit serum before the purification. Lane 4 shows the proteins that were purified by 20-50% saturated ammonium sulfate ($(NH_4)_2SO_4$). Lane 3 shows the flow through proteins of 20-50% saturated $(NH_4)_2SO_4$ precipiated rabbit serum that passed the Protein A affinity column. Lane 2 is the rabbit IgG purified by 20-50% saturated $(NH_4)_2SO_4$ followed by Protein A affinity column. By comparing Lane 5 to Lanes 2-4, it shows that the purification efficiency of rabbit IgG by $(NH_4)_2SO_4$ precipitation followed by Protein A affinity column was very high.

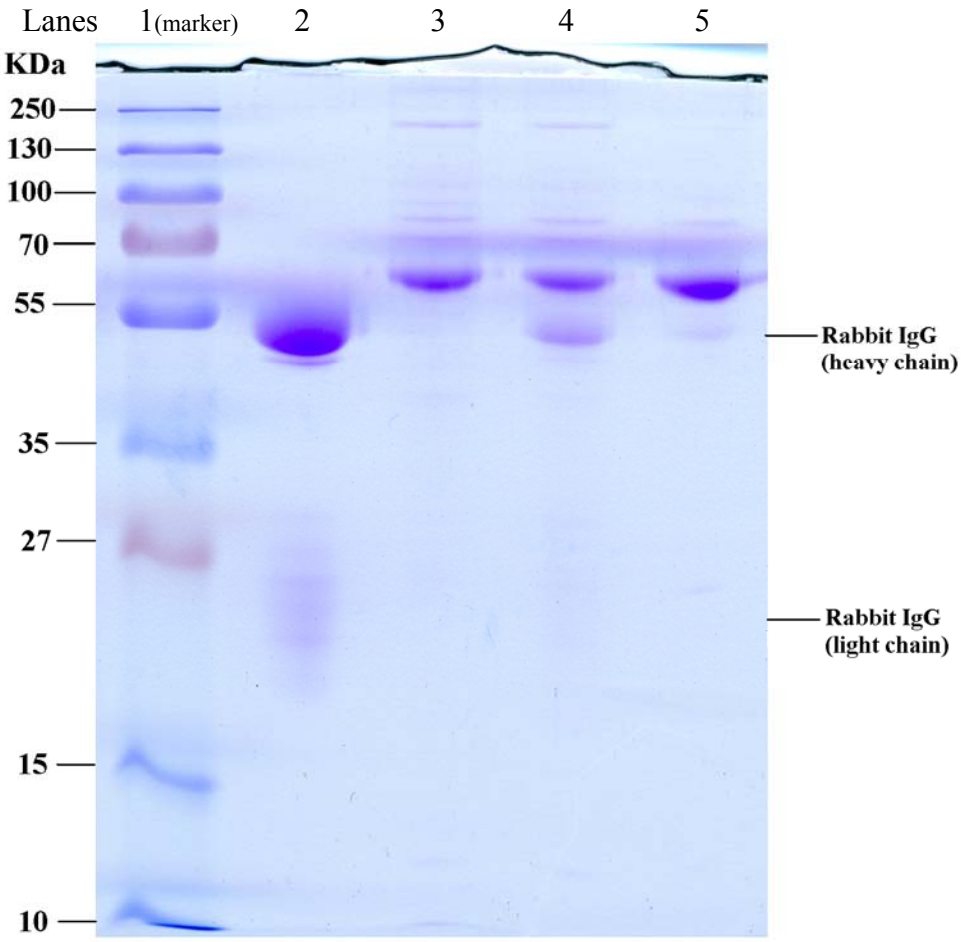


Figure 4.2 SDS-PAGE on anti-*Bacillus anthracis* 13 phage antibody purification

Lane 1: standard marker
Lane 2: antibody purified by Protein A affinity column
Lane 3: flow-through fraction of Protein A affinity column purification
Lane 4: anti-pBA13 antibody purified by 20-50% saturated $(NH_4)_2SO_4$ precipitation
Lane 5: rabbit serum

4.3.2 Reactivity of Anti-BA13 Phage Antibody

The ELISA data demonstrated that the rabbit had good immunogenic responses to pBA13 antigen (Tables 4.2 and 4.3). Based on a common rule, any ELISA reading above 1.000 at 30 min is considered an excellent amount (or titer) of the antibody. Table 4.2 shows the ELISA

results for the anti-pBA13 polyclonal antibody produced from the 1st and 2nd boosts of BA13 phage antigen and purified by 20-50% saturated $(NH_4)_2SO_4$ precipitation followed by Protein A affinity column. The rabbit produced high reactivity antibodies against BA13 phage, even at 25,600 times, the antibody still has high reactivity. Table 4.3 shows the reactivity of the antibody collected and purified from the 7th boost rabbit serum. The anti-pBA13 antibody was only used as the control line to validate the proper operation of the LFTS for *B. anthracis* spore detection.

Table 4.2 Reactivity of Rabbit Anti-BA13 Phage Antibody
(Abs: produced from the 1st and 2nd boost BA13 phage, purified by saturated $(NH_4)_2SO_4$ precipitation followed by Protein A affinity column)

Dilution Times	Reactivity of the Antibody (30 min, $O.D._{.405nm}$)			
	Antibody from the 1st boost pBA13		Antibody from the 2nd boost pBA13	
	BA 13 Phage	BSA	BA 13 Phage	BSA
1:100	4.329			
1:200	4.204		4.045	0.347
1:400	4.221		3.966	
1:800	4.327		3.638	
1:1600	4.037		3.657	
1:3200	4.223		2.376	
1:6400	3.95		2.103	
1:12800	2.002		1.368	
1:25600	1.335		0.864	
1:51200	0.611	0.174	0.531	
1:102400			0.448	
1:204800			0.437	
1:409600			0.332	

Table 4.3 Reactivity of Antibody Purified by Protein A Affinity Column
(Abs: produced from the 7th boost BA13 phage, purified by saturated $(NH_4)_2SO_4$ precipitation followed by Protein A affinity column)

Dilution Times	Reactivity of the Purified Antibody (30 min, $O.D._{405nm}$)	
	Anti-pBA13 antibody produced and purified from the 7th boost pBA13	
	30 min	
	BA 13 Phage	BSA
1:200	3.963	0.498
1:400	3.715	0.364
1:800	3.433	0.281
1:1600	3.217	0.303
1:3200	2.663	0.29
1:6400	1.928	0.248
1:12800	1.414	0.3
1:25600	0.963	0.261
1:51200	0.731	0.305
1:102400	0.434	0.278
1:204800	0.406	0.277
1:409600	0.338	0.298

4.3.3 Interaction between *Bacillus anthracis* 13 Phage or JRB7 Phag and BA Spores

The interaction between pBA13 (or pJRB7) and *B. anthracis* spores using indirect double sandwich ELISA was performed to predict the sensitivity of the phage-based LFTS for *B. anthracis* spores (Table 4.4). The results show that the interaction between the pBA13 or pJRB7 and *B. anthracis* spores was moderate.

Table 4.4 Interaction between pBA13 or pJRB7 and *Bacillus anthracis* Spores

Coating, 2 h	5×10^8 spores/ml	BSA	5×10^8 spores/ml	BSA
Blocking, 1 h	BSA			
Adding Phage	pBA13 (10^{11} virions/ml, 2 h)		pJRB7 (10^{11} virions/ml, 2 h)	
Anti-pBA13 Ab	Dilution: 1:1000 (2 h)		Dilution: 1:1000 (2 h)	
Alkaline Phosphatase conjugated Anti-Rabbit IgG	Dilution: 1:3000 (1 h)		Dilution: 1:3000 (1 h)	
Substrate* color development				
15 min	0.334	0.116	0.422	0.125
30 min	0.589	0.171	0.806	0.248
45 min	0.911	0.235	1.272	0.305
60 min	1.151	0.284	1.601	0.378

*: the substrate is *p*-NPP and the absorbance is measured at 405 nm ($O.D._{405nm}$)

4.3.4 Colloidal Gold Conjugation to *Bacillus anthracis* 13 Phage or JRB7 Phage

In this study the protocol of colloidal gold conjugation to pBA13 or pJRB7 is similar to that of colloidal gold conjugation to anti-bovine MBM protein antibody with some modifications. The main differences include two aspects. One was that comparing to BSA, polyoxyethylene glycol (PEG) is a better agent to block remaining active sites on the colloidal gold surface. The other was that the lower centrifuge speed was necessary to purify the gold-pBA13 conjugate complex in that the size of gold-pBA13 conjugate is larger than that of gold-anti-bovine MBM protein antibody.

Transmission electron microscope (TEM) picture of commercial colloidal gold adopted in this study is shown in Figure 3.8, which demonstrated that the gold nanoparticles (AuNPs) have the size ~35-40 nm. The AuNPs with different sizes made in our lab were also utilized to conjugate

pBA13 or pJRB7. The results indicated that the AuNPs with the size 20-50 nm show similar results to commercial AuNPs for phage conjugation.

The anti-pBA13 antibodies were used only for the control line in this research. In LFTS testing, a dark red or pink control line can be attained, which means that the pBA13 or pJRB7 was successfully conjugated with the colloidal gold.

4.3.5 Modification of Nitrocellulose Membrane

The most commonly used LFTS are constructed using a specific antibody as the biological recognition element (detector) to detect antigens or analytes in the sample. The main disadvantages of this antibody-based LFTS are relatively short shelf-life and they are not easy to be used outside of laboratory. In this research, the specific phage (*B. anthracis* 13 phage, pBA13 or JRB7 phage, pJRB7) was exploited to substitute the antibody in order to lengthen the shelf-life of the LFTS and increase the test efficiency. The results of preliminary experiments demonstrated that it is difficult for the phage to be directly immobilized onto four commercial membranes, such as NCM, UltraBind™ US450, Biodyne® C 0.45μ and BioTrace™ PVDF. The significant difference of the size and structure between the phage and the antibody may cause the different efficiencies of immobilization on membranes. After comprehensively comparing the test results, NCM was still the best choice for the phage-based LFTS. The purpose of this study is to immobilize phages onto the modified and activated NCM.

The principle of modification of NCM in Case One is to utilize the two amino groups (NH_2) of DAO (H_2N-$(CH_2)_8$-NH_2) to cross-link the NCM and GA, in which GA then was linked to pBA13 or pJRB7. The results shown that the NCM became hard after modification by 1% DAO and activation by 1% GA, and the mobility of samples on the NCM treated was very poor. The

modification for this case is as follows. Five µl of 1% DAO in deionized water were applied on the NCM as test line. The membrane was dried at RT for 1 h. Then the test line was rinsed with deionized water for 1 min, and dried at RT for 24 h. 5 µl of 10% GA in deionized water were applied on NCM. After the NCM was dried at RT for 24 h, 10 µl of pBA13 or pJRB7 with ~10^{11} or 10^{12} virions/ml were applied on the NCM. The testing results of LFTS (Figure 4.3) showed that the mobility of the samples on the treated NCM was still not good.

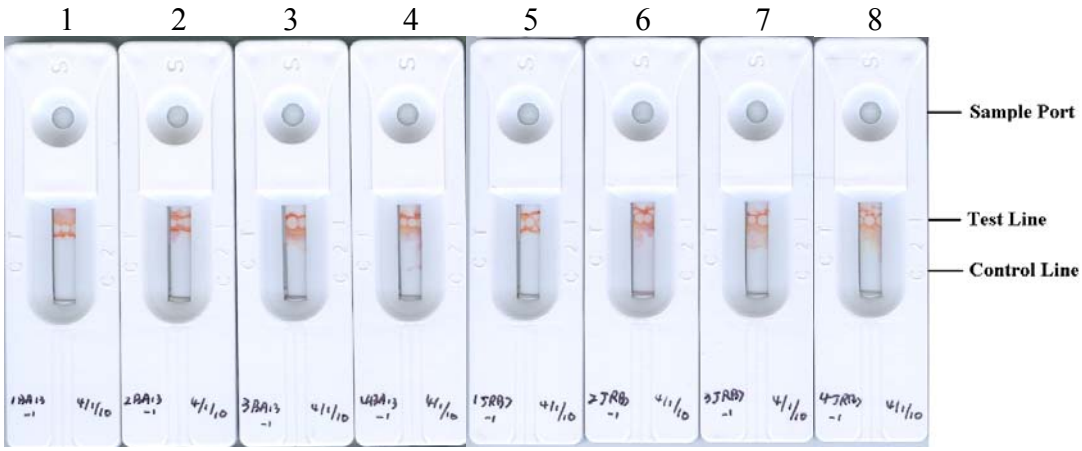


Figure 4.3 LFTS testing for NCM + 1% DAO + 1 or 10% GA + pBA13 or pJRB7

1: NCM + 1% DAO + 1% GA + pBA13 (10^{11} virions/ml)
2: NCM + 1% DAO + 1% GA + pBA13 (10^{12} virions/ml)
3: NCM + 1% DAO + 10% GA + pBA13 (10^{11} virions/ml)
4: NCM + 1% DAO + 10% GA + pBA13 (10^{12} virions/ml)
5: NCM + 1% DAO + 1% GA + pJRB7 (10^{11} virions/ml)
6: NCM + 1% DAO + 1% GA + pJRB7 (10^{12} virions/ml)
7: NCM + 1% DAO + 10% GA + pJRB7 (10^{11} virions/ml)
8: NCM + 1% DAO + 10% GA + pJRB7 (10^{12} virions/ml)

BSA is the most commonly used blocking agent on NCM. So, BSA can be coated onto the NCM. The modification of NCM in Case Two was based on the interaction between BSA and

GA. However, after the *B. anthracis* spores were applied onto the test strip, the red or pink test line did not appear. One explanation is that the time for the interaction between BSA and GA, and GA and pBA13 or pJRB7 is not enough. In order to overcome this drawback, the procedure of constructing the test strip employed in Cases Three and Four was adopted. But the expected visual signal at the test line still did not appear.

In Case Five, GA ($O=CH-(CH_2)_3-CH=O$) was directly used as a cross-linker to link NCM and phage. It was expected that the factors such as the concentration and pH of GA, drying temperature and time will affect the immobilization of pBA13 or pJRB7 onto NCM. The preliminary results demonstrated that 10-15% (v/v) of GA in deionized water or PBS at 7.4, and drying at RT for 24 h or longer are suitable conditions for binding GA onto the NCM.

4.3.6 Performance of Phage-Based Lateral Flow Test Strip

The phage-based LFTS was constructed following the schematic diagram in Figure 4.1. The pBA13 or pJRB7 was directly immobilized onto the test line on NCM without any chemical modification. After getting negative test results for *B. anthracis* spores the following modifications for the above-mentioned LFTS mode were conducted in order to allow a longer time for the interaction between the sample (*B. anthracis* spores) and the detector (colloidal gold-pBA13 or colloidal gold-pJRB7 conjugate), which is shown in Fig. 4.4. In this test, 90 µl of the colloidal gold-pBA13 or colloidal gold-pJRB7 liquid conjugate with $O.D._{520nm}$ 3.0 and 1.2 ml of *B. anthracis* spores with the designated concentration (5×10^8 virions/ml) were put into a 2 ml microcentrifuge tube. After mixing, the tube was incubated on a shaker at RT for designated period of time (15, 30, 60, 90, and 120 min). Then, 200 µl of each sample were added to the

sample pad and allowed to move down the NCM. The results were recorded after 15 min of sample application.

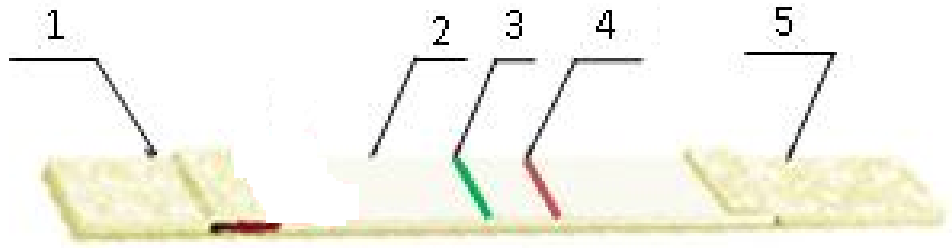


Figure 4.4 Construction of modified phage-based LFTS

(adopted from www.devicelink.com/ivdt/archive/00/03/004.html, on 3/24/2006, with modifications)

1 sample pad, 2 nitrocellulose membrane, 3 test line (pBA13 or pJRB7),
4 control line (anti-pBA13 antibody), 5 absorbing pad

The testing results showed no visual signal at the test line after the *B. anthracis* spores and pBA13 or pJRB7 were incubated at RT for 30 min. However, all the test strips produced a strong signal in the control line indicating that pBA13 or pJRB7 was successfully conjugated to colloidal gold particles.

The results of the additional tests allowing for up to 120 min for gold-conjugated pBA13 or pJRB7 adsorption to *B. anthracis* spores were also negative, which could be a result of few pBA13 or pJRB7 adsorption occurred thereby there was not enough complex on the test line to show signal. Another possible reason is that the pBA13 or pJRB7 cannot be immobilized onto the NCM.

According to the performance of GA, it should be a suitable cross-linker to connect NCM and phage. Further research was focused on the indirect immobilization of phage onto the NCM via

GA. The results showed that the GA concentration and drying time in the NCM modification are very important factors. For NCM with the size of 2.5 × 1.6 cm, 5 µl of 10% (v/v) GA in deionized water was applied onto the NCM as test line. After the membrane was air-dried for at least 7 h, 10 µl of pBA13 or pJRB7 with the concentration of 10^{11} or 10^{12} virions/ml were applied the test line. Three µl of anti-pBA13 antibody were applied onto the NCM as control line. The membrane was air-dried for at least 24 h. The results of the testing showed visual signal at the test line and the control line after 200 µl of of *B. anthracis* spores with the concentration of 10^{7}-10^{8} spores/ml was added to sample pad, shown in Figure 4.5 (for 10^{8} spores/ml of *B. anthracis* spores).

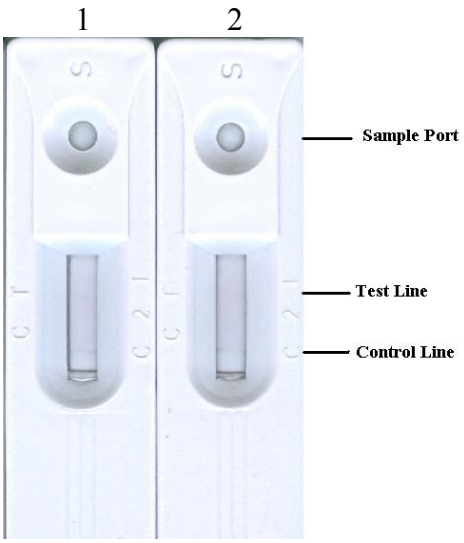


Figure 4.5 LFTS testing for NCM + 10% GA + pBA13 or pJRB7

1: NCM + 10% GA + pBA13 (10^{12} virions/ml); 2: NCM + 10% GA + pJRB7 (10^{12} virions/ml)

4.4 Conclusion

The phage-based LFTS was successfully developed to detect *B. anthracis* spores by using gold-pBA13 or gold-pJRB7 conjugate as recognition element with pBA13 or pJRB7 as test line agent and anti-pBA13 antibody as control line agent. The modification of NCM is necessary in order to increase the immobilization efficiency of pBA13 or pJRB7 onto the NCM. GA is a suitable cross-linker to connect NCM and phage, and the GA concentration at test line and the drying time are two critical factors for successful indirect immobilization of phage. The detection limit of this phage-based LFTS is 10^7 spores/ml.

Chapter Five Project Assessment and Future Research

5.1 Overview of the Project

The overall goals of this research included two aspects. The first goal was to design, develop and assess the antibody-based lateral flow test strip (LFTS) assay to detect bovine meat and bone meal (MBM) in animal feeds. The objectives to realize this goal were: (i) to extract bovine MBM proteins from bovine MBM; (ii) to purify and characterize the bovine MBM proteins; (iii) to produce anti-bovine MBM protein antibody; (iv) to design and develop an antibody-based LFTS to detect bovine MBM in animal feeds; and (v) to assess the antibody-based LFTS assay in terms of the detection limit, specificity, and simplicity of use of LFTS. Several extraction solvents were utilized to extract proteins from bovine MBM. Based on the results of SDS-PAGE for the proteins extracted, the suitable extraction solvent and conditions were determined. A pretreatment step in 1.0 M NaOH solution was adopted to remove gelatins in bovine MBM. After purifying bovine MBM proteins, these proteins were used to immunize the rabbit for producing the anti-bovine MBM proteins antibody. After the immunoabsorption of the antibody with the proteins from animal feeds, the cross-reactivity between the antibody and the animal feed was eliminated or significantly reduced. In the antibody-based LFTS detection, the antibody was conjugated to colloidal gold forming gold-anti-bMBMPAbs complex for the detection of the bovine MBM in animal feeds. The antibody also served as the test line agent to be immobilized onto the test line of the nitrocellulose membrane to capture the gold-bMBMAbs-bMBMP complex. The second goal was to design, develop and assess the phage-based LFTS assay to

detect *B. anthracis* spores in water and foods. The objectives to achieve this goal were: (i) to produce anti-*B. anthracis* 13 phage (pBA13) antibody by using purified pBA13 as an immunogen; (ii) to design and develop an phage-based LFTS assay to detect *B. anthracis* spores; and (iii) to assess the phage-based LFTS assay. The main differences from antibody-based LFTS were that in phage-based LFTS assay pBA13 or pJRB7 specific to *B. anthracis* spores served as recognition element to detect the *B. anthracis* spores, and used as the test agent to be immobilized onto the test line. Two important factors in this assay are the reactivity between the phages and the spores and the immobilization efficiency of the phage onto the membrane.

5.2 Project Assessment

The overall goals of designing, developing, and assessing antibody- and phage-based LFTS assays to detect the bovine MBM in animal feeds and the *B. anthracis* spores in water and foods were achieved with varying degrees of success. A special protocol for protein extraction from bovine MBM was developed, and the gelatin proteins in the bovine MBM were removed by a pretreatment step in 1.0 M NaOH solution. The anti-bovine MBM protein and anti-pBA13 antibodies produced were effectively purified by ammonium sulfate precipitation and Protein A affinity column. A cross-reaction exists between the un-immunoabsorbed anti-bovine MBM antibody and the proteins in animal feeds, but the cross-reaction can be eliminated or significantly reduced by the immunoabsorption process. The LFTS assay developed can detect 0.1% (w/w) bovine MBM. For the development of the phage-based LFTS assay, an effective protocol for the conjugation of colloidal gold to pBA13 or pJRB7 was successfully developed. The pBA13 and pJRB7 cannot be directly immobilized onto the surface of NCM and

glutaraldehyde was used as the cross-linker for indirect phage immobilization on NCM. The limit of detection for this assay is 10^7 spores/ml.

5.3 Recommended Future Research

For the LFTS detection of bovine MBM, monoclonal antibody (mAb) should be produced against bovine MBM protein to eliminate or significantly reduce the cross reaction to improve the LFTS detection capabilities. A new protein extraction protocol should be developed to reduce preparation time of the test sample.

For the LFTS detection of *B. anthracis* spores, the membrane suitable for direct immobilization of the phage should be developed and evaluated. The optimization of the modification and activation conditions for increasing the phage immobilization efficiency on the membrane should be further investigated. In addition, the reactivity between the phage and the spores is very important for the successful development of the phage-based LFTS assay. A more specific and higher reactivity phage to the *B. anthracis* spores should be further screened.

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