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DETECTION OF *Mycobacterium avium* subsp. *paratuberculosis* IgG BY A CONDUCTOMETRIC BIOSENSOR: AN AID IN DIAGNOSIS OF JOHNE'S DISEASE

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By

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ABSTRACT

DETECTION OF *Mycobacterium avium* subsp. *paratuberculosis* IgG BY A CONDUCTOMETRIC BIOSENSOR: AN AID IN DIAGNOSIS OF JOHNE'S DISEASE

By

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The development of a non-laboratory based Johne's disease (JD) diagnostic assay, which is rapid, user-friendly, and supports routine herd testing, would help control the spread of this costly disease of cattle, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The objective of this study was to develop a conductometric biosensor as an on-site, user-friendly, rapid, and inexpensive JD diagnostic assay. First, an immunomigration conductometric biosensor that could detect MAP IgG in bovine serum was designed and fabricated. The MAP IgG detection was measured as electrical resistance. Next, the biosensor elements were optimized to improve accuracy. Finally, the biosensor was tested with field samples and results were compared to a MAP antibody detection ELISA, which is commonly used for JD diagnosis. In the proof of concept study, there was significant difference ($P < 0.05$) between resistance values of the JD positive and JD negative serum samples. Results from the biosensor were obtained in 2 minutes. The assay was simple to run and could be easily transported to and used in a field setting. The assay's precision was improved after optimization. The biosensor had a moderate agreement with ELISA in JD diagnosis (Kappa = 0.41), as well as good sensitivity (71.43%) and specificity (70%). Based on this study, a conductometric biosensor has promise as a diagnostic assay for JD.

DEDICATION

With utmost gratitude, I dedicate this work to
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KEY TO ABBREVATIONS

IgG :	Immunoglobulin G
IgG*:	Serum IgG
AB/IgG:	Anti-bovine IgG
MAP:	*Mycobacterium avium* subsp. *paratuberculosis*
MAPPD:	*Mycobacterium avium* purified protein derivative
JD:	Johne's disease
ELISA:	Enzyme-linked immunosorbent assay
OD:	Optical density
PCR:	Polymerase chain reaction
BVDV:	Bovine viral diarrhea virus
BLV:	Bovine leucosis virus
Pani:	Polyaniline
BSE:	Biological sensing element
%CV:	Coefficient of variation
SD:	Standard deviation

INTRODUCTION

Johne's disease (JD) is a chronic gastrointestinal disease of animals, especially domestic ruminants. It is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and young animals are most susceptible to MAP infection. JD animals shed viable MAP in their milk and feces. The disease causes a significant economic impact on the global cattle industry (Sweeney 1996), mainly from the effects of reduced milk production (Losinger 2005). In the US, economic losses have been estimated to exceed $1.5 billion per year (Stabel 1998). JD raises public health concerns. MAP infections have been reported in some Crohn's disease patients (Naser *et al.* 2004; Sechi *et al.* 2005); these studies hypothesized that MAP plays a role in the etiology of Crohn's disease. The economic losses from JD and the knowledge that MAP may be a zoonotic pathogen have increased the urgency to control MAP in domestic animals. Effective control of JD has been challenging. Limitations in currently available diagnostic tests contribute to this challenge.

Diagnosis of JD is aimed at detecting MAP or its DNA in feces, tissues, and occasionally milk, or detecting an immune response to MAP. Culture is most commonly used in MAP detection (Whitlock *et al.* 2000); Polymerase Chain Reaction (PCR) is used for MAP DNA detection (Sevilla *et al.* 2005); and Enzyme-Linked Immunosorbent Assay (ELISA) is the most common assay used to detect an immune response to MAP in the form of MAP specific antibodies (Kalis *et al.* 2002). Culturing is expensive and requires 7-12 weeks for completion (Kalis *et al.* 1999; Whitlock *et al.* 2000); PCR requires specialized equipment and skilled training, limiting its availability to most developed countries; and ELISA also require specialized skills and equipment. These

currently used diagnostic tests may not be easily adapted for on-site diagnosis and not readily accessible to cattle farmers in the developing countries. The development of new JD diagnostic assays, which are adaptable to the field and potentially useful in developing countries, would be beneficial in furthering JD control efforts.

A conductometric biosensor is an analytical device that contains a transducer, which interprets specific biological recognition reactions (i.e. antigen-antibody binding) as electrical conductance. A transducer, such as polyaniline is placed close to or integrated with the biological element (i.e. antibody). Polyaniline, a conductive polymer, relays any antigen-antibody binding as a measured electrical quantity. Conductometric biosensors have been used in the detection of *E. coli* O157:H7 (Muhammad-Tahir and Alocilja 2003), *Bacillus cereus* (Pal *et al.* 2007), and Bovine viral diarrhea virus (Muhammad-Tahir *et al.* 2005). The assay is rapid, user-friendly, inexpensive, and can be applied to on-site disease diagnosis (Muhammad-Tahir *et al.* 2005; Muhammad-Tahir and Alocilja 2003; Pal *et al.* 2007). This relatively new technology has not been applied towards JD diagnosis.

The overall objective of this study was to develop a rapid, accurate, and easy-to-use assay, which could be non-laboratory based, for JD diagnosis in cattle. The specific aims were to design and fabricate a conductometric biosensor that could detect MAP IgG, optimize the assay for on-site diagnosis of JD, and evaluate the assay's sensitivity and specificity, using a currently available MAP immuno-diagnostic assay (ELISA) as a gold standard. The success of this study would support more frequent and widespread testing of animals, leading to improved JD control.

CHAPTER ONE

REVIEW OF JOHNE'S DISEASE AND THE RELEVANCE OF BIOSENSORS IN INFECTIOUS DISEASE DIAGNOSIS

ABTRACT

Johne's disease is an infectious gastrointestinal disease of ruminants that causes significant economic loss in the cattle industry and public health concerns. Control of this disease has been challenging and could be improved by development of inexpensive and easily performed diagnostic tests. This review was aimed at understanding the opportunities of biosensors in Johne's disease diagnosis. Johne's disease etiology, hosts, transmission, prevalence, economic and public health importance, diagnosis, as well as management and control are covered. Different types of biosensors and transducers as well as the role of biosensors in infectious disease diagnosis are also covered. The information covered in this review supports the development of a conductometric biosensor to be used as a tool to further improve JD control strategies.

I. JOHNE'S DISEASE

A. Etiology

Johne's disease (JD), sometimes referred to as paratuberculosis, is a severe, infectious, and chronic gastrointestinal disease caused by *Mycobacterium avium subsp. paratuberculosis* (MAP). MAP is genetically related to *Mycobacterium avium* but differs in its phenotypic characteristics: growth speed on media, media morphology, mycobactin dependence, and definitive hosts. Hence, specific proteins of *M. avium* could be used as MAP antigens in immuno-diagnostic assays.

B. Susceptible hosts

Primarily a disease of ruminants, JD has also been reported in primates (Zwick *et al.* 2002), and numerous non-ruminant species including rabbits (Judge *et al.* 2006), fox, stoat, weasel, crow and wood mouse (Beard *et al.* 2001). MAP infection has been reported in humans (Scanu *et al.* 2007), but the ability of this organism to elicit a disease in humans has not been confirmed.

C. Transmission

JD infected animals shed viable MAP in feces and milk. In cattle, calves become infected in-utero or by ingesting MAP during feeding. Calves most commonly become infected in the first months of life, but the infection remains unrecognized in animals until about age 2 - 5 years (preclinical phase of JD). During the preclinical phase of JD, animals may shed MAP in feces or milk, providing opportunities for new infections among calves. Other transmissions routes such as the semen of infected bulls, embryo

transfers, and veterinary procedures like rectal examination have been suspected (Sweeney 1996), but there is no available evidence to support JD transmission via these routes.

MAP adapts some potential survival tactics: dormancy; biofilm formation; aerosolization; and interactions with protozoa, nematodes, and insects, that enhance their survivability in the environment for at least a year (Rowe and Grant 2006; Whittington *et al.* 2004). The long environmental survivability of MAP increases the odds of new infections, making control even more challenging.

D. Prevalence

JD is prevalent worldwide. The limited or non-reported accounts of this disease in most countries of the world do not suggest lack of JD in those countries but could be attributed to the countries' low interest in paratuberculosis research. In countries where the cattle industry is rarely commercialized, the economics of JD is hardly appreciated.

The influence of transnational livestock trade has caused the spread of JD beyond continents. In Africa, JD has been reported in Morocco (Delisle *et al.* 1992) and South Africa (Bauerfeind *et al.* 1996; Delisle *et al.* 1992; Michel and Bastianello 2000). In Asia, the list includes China (Cheng *et al.* 2005), India (Singh *et al.* 2007), South Korea (Park *et al.* 2006), and Nepal. The disease has also been reported in Australian cattle (Pitt *et al.* 2002) as well as sheep (Dhand *et al.* 2007). Among the reportedly affected countries within Europe are Spain (Dieguez *et al.* 2007), Austria (Khol *et al.* 2007), Netherlands (Muskens *et al.* 2000; van Weering *et al.* 2007), and the UK (Hayton 2007). In the American continents, the list includes Canada (Scott *et al.* 2007; Tiwari *et al.* 2006),

Argentina (Cirone *et al.* 2006), Chile (van Schaik *et al.* 2007), and the US (Crossley *et al.* 2005; Roussel *et al.* 2005). In the US, a national survey reported MAP infection in at least 68.1% of dairy herds (USDA 2008). In the state of Michigan, an earlier survey reported a dairy herd prevalence of 54% (Johnson-Ifearulundu and Kaneene 1999) but a recent survey reported a herd prevalence of 48.1% (Pillars *et al.* 2007). Although the prevalence of JD may decrease in areas with active control programs, its prevalence could increase in areas with limited opportunities for effective diagnosis and control.

E. Economic importance

Economic losses from JD are primarily due to decreased milk production, unrealized income related to premature culling, reduced carcass dressing weight, and death. Other additional costs are diagnostic testing, implementation of control measures, and accelerated cull and replacement rates. The complicated pathogenesis, control and management of JD make the evaluation its overall economic effects difficult. Economic losses in the US have been estimated to exceed $1.5 billion per year (Stabel 1998), mainly from the effects of reduced milk production (Losinger 2005).

F. Public health concern

MAP infection has been reported in humans (Scanu *et al.* 2007), especially in the intestinal tissues (Sechi *et al.* 2005) and blood (Naser *et al.* 2004) of Crohn's disease patients; these studies hypothesized that MAP plays a role in the etiology of human Crohn's disease. However, a study by Cellier *et al.* (1998) failed to isolate MAP DNA from Crohn's disease patients. MAP has not been found exclusively in Crohn's disease

patients, nor is it the exclusive bacteria found in Crohn's disease patients (Ryan *et al.* 2004; Shanahan and O'Mahony 2005). These studies did not support the MAP etiology of Crohn's disease hypothesis. MAP is not currently recognized as a zoonotic pathogen, because the available information is insufficient to prove or disprove that MAP is a cause of Crohn's disease, but the hypothesis is plausible (Grant 2005).

If it were true that MAP is a cause of Crohn's disease, the infection of humans with MAP is either food borne (milk, other dairy products, beef) or waterborne (Grant 2005). Viable MAP has been cultured from raw milk of clinical (Taylor *et al.* 1981) and subclinical (Sweeney *et al.* 1992) diseased cows as well as some retail pasteurized milk (Ellingson *et al.* 2005). MAP genetic components have been detected in cheese (Clark *et al.* 2006) but not in ground beef (Jaravata *et al.* 2007). A significant association between consumption of handmade cheese and MAP infection has been reported (Scanu *et al.* 2007). Consumption of MAP-contaminated milk or cheese could be the transmission modes of MAP to Crohn's disease patients.

It is scientifically unclear whether Crohn's disease increases human's susceptibility to MAP infection or the infection predisposes humans to Crohn's disease. Given that MAP infections in most animal species have elicited intestinal lesions similar to Crohn's disease, the public health concerns of JD is warranted.

G. Diagnosis

Diagnosis of JD is aimed at detecting MAP in feces, tissues, and occasionally milk; or an immune response to MAP. MAP detection is most commonly done by culture. There are several culture systems available: Herrold's egg yolk agar is a solid system; BACTEC 460 and TREK Esp II are liquid culture systems. Although culture is effective in JD diagnosis, it is expensive and detection takes between 7-12 weeks (Kalis *et al.* 1999; Whitlock *et al.* 2000).

Polymerase Chain Reaction (PCR) is a MAP DNA detection test and most PCR assays target specific DNA sequence (IS900) (Sevilla 2005), which is common but not unique to MAP. PCR has been used as a confirmatory diagnostic test, following positive bacteriologic culture. The use of PCR as an independent test for JD diagnosis is gaining in popularity as technologies improve. PCR may not be wholly reliable as an independent test because DNA of a non-viable MAP could be interpreted as positive for JD. The test requires specialized equipment and skilled training, limiting its wide use.

There are two broad categories of MAP immune response detection assays: cell-mediated response and humoral immune response assays. Cell-mediated immune responses (CMI) are mediated by T lymphocytes; they are the first and strongest host responses to MAP infection (Collins 1996). Assays for cell-mediated immunity include an intradermal skin test and a gamma interferon test. The skin test measures delayed-type hypersensitivity after intradermal injection of MAP antigen (Johnin). The gamma interferon test uses an extract of *Mycobacterium avium* (antigenically similar to MAP) as the stimulating antigen and detects gamma interferon released from cells as the measure of CMI. These tests have either low specificity or are logistically challenging to perform.

The most commonly used humoral immunodiagnostic tests for MAP antibodies detection are Enzyme-linked immunosorbent assay (ELISA), agar gel immunodiffusion, and complement fixation test (Kalis *et al.* 2002); ELISA is the most frequently and widely used test. It is less expensive and its results could provide information on an animal's stage of infection. However, MAP antibodies appear late in the course of infection, although before the onset of clinical signs (Collins 1996), limiting the ability of detecting animals in early stages of infection. Also ELISA requires specialized skills, equipment, and may not be readily accessible to the cattle farmers in the developing countries of the world.

H. Management and control

The most effective means of JD's management is its prevention. Treatment has been found to be uneconomical and JD may be classified as an untreatable disease. Recently, there have been evidences that live and killed mycobacterial vaccines reduce JD incidence and MAP shedding rates in Australian sheep (Emery and Whittington 2004; Reddacliff *et al.* 2006). In cattle, vaccination of calves with recombinant MAP proteins was reported to induce differential immune responses, protecting calves against infection by oral challenge (Kathaperumal *et al.* 2008). The immune response tends to reduce but not eliminate JD incidence. Although current and future vaccines may be useful in JD control, they will not likely replace other preventive farm management practices.

Introduction of an MAP-infected animal into a JD-free herd is the most common method of JD transmission between farms; practices that prevent such introduction are valuable for optimum management (Wells and Wagner 2000). These practices are

purchasing replacement animals from JD-free herd and preventing accidental introduction of MAP-contaminated manure through manure spread, manure run-offs from nearby farms or contaminated forage.

In infected farms, management practices are focused on preventing the exposure of calves to feces and milk of infected cows. These practices include calving in a clean environment; and afterwards separating calves to individual pens or hutches; raising calves away from adult herd and manure run-offs; and preventing manure contamination of feed and water. Others are preventing the feeding of calves with pooled colostrums, foster-feeding orphaned neonates and calves from JD-positive with colostrum and milk from JD-negative dam, and more importantly, culling identified JD-positive animals from the herd.

In summary, strategic control of JD becomes necessary due to JD's associated economic losses and potential public health concerns. The development of an on-site, user-friendly, and inexpensive JD diagnostic test is important for effective disease control. The attributes of such a test will support pre-purchase testing and routine herd testing, especially in places with limited resources to the current available diagnostic tests. Such diagnostic test would influence the making of timely decisions, like choice of replacement animals, manure and waste handling, isolation of calves, choice of colostrum and milk for feeding calves, and prompt culling of infected animals.

II. BIOSENSORS

A. Definition

A biosensor is an analytical device that contains a transducer, integrated with or placed close to a biological sensing element (BSE) (i.e. antibody) such that a specific biological recognition (i.e. antigen-antibody binding) reaction produces a measurable signal change in a physicochemical detector component (Lazcka *et al.* 2007).

The biosensor is comprised of 3 components:

- BSE (DNA probes, antigens, antibodies, toxins, tissues, enzymes, etc),
- Transducer (couples BSE to detector element), and
- Detector element (works in a physicochemical way: optical, piezoelectric electrochemical, thermometric, or magnetic (Lazcka *et al.* 2007)).

The device is designed to analyze a desired component of an analyte (i.e serum), which influences the choice of BSE that can form a specific complex with it. Antibodies are commonly used for BSE due to their high specificity (Sergeyeva *et al.* 1996). The transducer is integrated with or placed close to the BSE prior to the introduction of the analyte. BSE and transducer can be coupled in several ways: membrane entrapment, physical adsorption, matrix entrapment, and covalent bonding (Mohanty and Koucianos 2006). After the analyte introduction, transducer conveys the formed specific complex into a measurable signal change in a detector element (Figure 1.1).

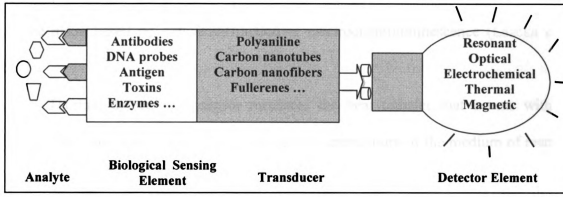

Figure 1.1. Schematic of biosensor detection principle

B. Types of biosensor

A biosensor can be classified based on either the BSE or the transducer components and sometimes a combination of both. Examples of classification based on BSE include antibody-based, DNA-based, Enzyme-based, and antigen-based biosensors. Examples of classification based on transducer include resonant, optical, thermal, Ion-Sensitive Field Effect Transistors (ISFETs), and electrochemical biosensors. Electrochemical biosensors are further classified as amperometric, potentiometric, and conductometric biosensors.

In a resonant biosensor, an acoustic wave transducer is coupled with the BSE. The attachment of analyte molecule to the membrane forms a complex with the BSE on the membrane causing changes in membrane mass, which subsequently changes the resonant frequency of the transducer and this frequency change can be measured (Mohanty and Koucianos 2006).

In an optical biosensor, light is the output signal that is measured. The biosensor can be made based on optical diffraction or electrochemiluminescence (Lazcka *et al.* 2007).

Thermal detection biosensor measures the heat transfer that occurs with the analyte-BSE complex, which in turn changes the temperature of the medium of reaction. In this biosensor, immobilized enzyme molecules are coupled with temperature sensors that measure temperature difference via a thermistor (Mosbach 1991).

Ion sensitive field effect transistor (ISFET) biosensor is a semiconductor FETs having an ion-sensitive surface. The surface electrical potential changes when the ions and semiconductor interact. Primarily, this biosensor is used to detect changes in pH (Yuqing *et al.* 2003).

Electrochemical biosensors measure changes in the electrical properties of a solution following the production or consumption of ions or electrons by chemical reactions. Electrochemical biosensors are further classified based on the measured electrical parameters: amperometric, potentiometric, and conductometric. Amperometric biosensors measure current, potentiometric biosensors measure oxidation/reduction reaction, and conductometric biosensors measure electrical conductance/resistance.

C. Conductometric biosensors

Conductometric biosensors have been applied in various biological and biomedical sciences. The applications include determination of glucose and urea in blood (Shulga *et al.* 1994), heavy metal ions and pesticides in water (Chouteau *et al.* 2005), and detection of disease pathogens (Muhammad-Tahir *et al.* 2005; Muhammad-Tahir and

Alocilja 2003; Pal *et al.* 2007). There has been a considerable interest in using conductive polymers (polyaniline, polypyrrole, polyacetylene, and polythiophene) in the development of conductometric biosensors (Hoa *et al.* 1992; Sergeyeva *et al.* 1996). Conductive polymers are transducers in conductometric biosensors. Polyaniline has been among the most extensively used conductive polymers, due to its strong bio-molecular interactions (Imisides *et al.* 1996), excellent environmental stability, and good conductivity (Syed and Dinesan 1991). Polyaniline have been known for over a century in their 'aniline black' form, an undesirable black deposit formed on the anode during electrolysis involving aniline. Polyaniline are usually prepared by direct oxidation polymerization of aniline in the presence of a chemical oxidant, or by electrochemical polymerization of different electrode materials (Kang *et al.* 1998). Polyaniline is capable of exhibiting many intrinsic redox states. The intrinsic redox states can vary from that of the fully oxidized Pernigraniline to that of the fully reduced Leucomeraldine. In between these forms are the 50% and 75% intrinsically oxidized polymers, Emeraldine and Nigraniline respectively (Kang *et al.* 1998). The structural and chemical flexibility surrounding the amine groups of polyaniline allows for the ease in the structure modification like the attachment of protein molecules to its polymer backbone. Polyaniline has been successfully used in the fabrication of several conductometric biosensors (Muhammad-Tahir *et al.* 2005; Muhammad-Tahir and Alocilja 2003; Pal *et al.* 2007; Yang *et al.* 2007).

D. Biosensor's relevance in infectious disease diagnosis

From the onset of this century, biosensors have been explored in many aspects in disease diagnosis. With the increase in the global fear of terrorism, biosensors are among the current and developing technologies used for monitoring agents of bioterrorism and biowarfare (Lim *et al.* 2005).

Biosensors can play a vital role in the diagnosis of emerging infectious diseases (Pejcic *et al.* 2006). A microchip-based magnetic biosensor has been designed for simplified and rapid point-of-care diagnosis of infectious disease (Aytur *et al.* 2006). Resonant biosensors have been applied in the diagnosis of mumps (Kim *et al.* 2006) and enyzyme-based amperometric biosensors have been used in detecting *Streptococcus pyogenes* and *Staphylococcus aureus* (Safina *et al.* 2005).

In the livestock industry, an amperometric biosensor was used in detecting progesterone levels in cow milk (Pemberton *et al.* 1998). The application of biosensors for the detection and identification of infectious diseases, contaminants, food toxins, and therapeutic drug residues has been proposed (Velasco-Garcia and Mottram 2001).

Applications of conductometric biosensors in disease diagnosis are relatively new. In the last few years, conductometric biosensors have been used in the detection of *E. coli* O157:H7 (Muhammad-Tahir and Alocilja 2003), *Bacillus cereus* (Pal *et al.* 2007), and Bovine viral diarrhea virus (Muhammad-Tahir *et al.* 2005). User-friendliness, rapid detection, environmental stability and inexpensive costs of equipment and test analysis are attributes of a conductometric biosensor. These attributes contribute to the growth of biosensor applications in biomedical sciences.

In summary, conductometric biosensors have attributes that make them attractive for use in infectious diseases diagnosis. Developing such biosensor for Johne's disease diagnosis will provide an inexpensive and user-friendly assay that would improve Johne's disease control programs.

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CHAPTER TWO

DEVELOPMENT OF A CONDUCTOMETRIC BIOSENSOR FOR DETECTION OF *Mycobacterium avium* subsp. *paratuberculosis* IgG

ABSTRACT

Johne's disease (JD) is one of the most costly bacterial diseases in cattle. In the US, economic losses from the disease have been estimated to exceed $1.5 billion per year, mainly from the effects of reduced milk production. Current diagnostic tests for JD are laboratory based; they require specialized equipment and training. Development of rapid and inexpensive diagnostic assays, which could be deployed in the field, would aid in the control of JD. In this study, an inexpensive and user-friendly polyaniline (Pani)-based conductometric biosensor, in an immunomigration format, was developed for the detection of MAP IgG in serum. Immobilized *Mycobacterium avium* purified proteins in the capture membrane were used to detect MAP IgG, previously conjugated with Pani/anti-bovine IgG in the conjugate membrane. After detection, captured Pani closed an electrical circuit between silver electrodes, flanking the capture membrane. The electrical conductance, caused by Pani, was measured as a decrease in electrical resistance. Testing of the biosensor with known JD positive and negative serum samples demonstrated a significant difference in the mean resistance observed between the groups. This study demonstrated that a conductometric biosensor could detect MAP IgG in 2 minutes. Further optimization can support its use in JD management.

I. INTRODUCTION

Johne's disease (JD) is a chronic gastrointestinal disease of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). JD causes significant economic loss in the cattle industry. In the US, economic losses from the disease have been estimated to exceed $1.5 billion per year (Stabel 1998), mainly from the effects of reduced milk production (Losinger 2005). Additional sources of economic losses are unrealized income related to premature culling, reduced meat quantity at slaughter and animal death. Although there is evidence that MAP may be associated with Crohn's disease in humans, MAP is not currently recognized as a zoonotic pathogen (Grant 2005). Economic losses from JD and the knowledge that MAP may be a zoonotic pathogen have increased the urgency to control the spread of MAP in domestic animals. Effective control of JD has been challenging. Limitations in currently available diagnostic tests contribute to this challenge.

Diagnosis of JD is aimed at detecting MAP or its DNA in feces, tissues, and occasionally milk, or detecting an immune response to MAP. Currently, culture is most commonly used in MAP detection (Whitlock *et al.* 2000); Polymerase Chain Reaction (PCR) is used for MAP DNA detection (Sevilla *et al.* 2005); and Enzyme-Linked Immunosorbent Assay (ELISA) is the most common assay used to detect an immune response to MAP in the form of MAP antibodies (Kalis *et al.* 2002). However, culturing is expensive and requires 7-12 weeks for completion (Kalis *et al.* 1999; Whitlock *et al.* 2000); PCR requires specialized equipment and skilled training, limiting its availability to most developed countries; and ELISA also requires specialized skills and equipment. These currently used diagnostic tests may not be easily adapted for on-site diagnosis and

not readily accessible to the developing countries. Development of new JD diagnostic assays, which are adaptable to the field and potentially useful in the developing countries, would be beneficial in furthering JD control efforts.

A conductometric biosensor is an analytical device that contains a transducer, which interprets specific biological recognition reactions (i.e. antigen-antibody binding) as electrical conductance. A transducer, such as polyaniline, is placed close to or integrated with the biological element (i.e. antibody). Polyaniline, a conductive polymer, relays any antigen-antibody binding as a measured electrical quantity. Conductometric biosensors have been used in the detection of *E. coli* O157:H7 (Muhammad-Tahir and Alocilja 2003), *Bacillus cereus* (Pal *et al.* 2007), and Bovine viral diarrhea virus (Muhammad-Tahir *et al.* 2005) but has not been applied towards JD diagnosis. The development of a biosensor as an on-site, user-friendly, and inexpensive JD diagnostic assay would support more frequent and widespread testing of animals.

The objective of this study was to develop a conductometric biosensor for the detection of MAP IgG in cattle serum. Optimization of the developed biosensor for JD diagnosis would support frequent testing of animals especially at the point-of-sale. Hence, making management decisions that would improve JD control.

II. MATERIALS AND METHODS

A. Immunosensor membranes and electrodes

The immunosensor component of the biosensor consists of four individual membranes: sample application, conjugate, capture, and absorption membranes (Hi-Flow Plus Assembly Kit, Millipore, Bedford Massachusetts). These membranes were prepared, fabricated, and assembled to form a functional biosensor. The choice of these membranes was based on previous studies (Kim *et al.* 2000; Muhammad-Tahir and Alocilja 2003; Sergeyeva *et al.* 1996). The sample application membrane, made of cellulose, provides a quick flow of the sample with no or minimal interference; the conjugate membrane, made of fiberglass, adsorbs the polyaniline-conjugated antibody and allows easy flow of fluid; the pore size of the capture membrane, made of nitrocellulose, allows the flow of non-target molecules while providing good adsorption properties for the immobilized molecule; and the absorption membrane, a cellulose membrane, absorbs and retains the fluid from the capture membrane.

For a functional conductometric biosensor, the capture membrane is printed with silver electrodes, yielding a 1 mm wide capture channel. Then, the electrodes are connected to an etched copper wafer, which is connected to an ohmmeter. Silver electrodes and etched copper wafers have been demonstrated to possess good electrical and easy fabrication properties, based on previous studies (Kim *et al.* 2000; Muhammad-Tahir and Alocilja 2003; Sergeyeva *et al.* 1996).

B. Capture membrane preparation

The capture membrane was prepared at 20°C under a clean biosafety cabinet unless otherwise stated. The capture membrane was first flushed with distilled water, to remove any debris, and air-dried for 0.5 h. Then, the membrane was flushed with 10% methanol and air-dried for 0.5 h, to activate the membrane. Next, the membrane was washed with 1.2 ml of 0.5 % glutaraldehyde solution and air-dried for 1 h, to provide a link-on between the hydroxyl group in the ethanol and the biological receptor molecule. *Mycobacterium avium* purified protein derivative (MAPPD) is antigenically similar to MAP. A total volume of 1.2 ml of 1 mg/ml MAPPD (AntelBio, East Lansing, Michigan) was pipetted on the membrane and incubated at 35°C in a closed plastic container for 1 h. Afterwards, the membrane was washed with 1.2 ml of 0.1 M Tris buffer containing 0.1% (v/v) tween-20, to remove all non-specifically absorbed MAPPD. Finally, the membrane was incubated at 35°C in a closed plastic container for 0.75 h, air-dried for 0.5 h, and was set to be fabricated with the other membranes.

C. Polyaniline – Anti bovine IgG conjugation

AquaPass polyaniline (Mitsubishi Rayon Co, Japan) was diluted to 0.001 % with 0.1 M phosphate buffer solution. Purified mouse clone BG-18 monoclonal anti-bovine IgG (Sigma-Aldrich, St Louis, Missouri) was added to the 0.001 % AquaPass polyaniline (Pani) solution to produce a final monoclonal anti-bovine antibody (AB/IgG) concentration of 0.0115 mg/ml. 4 ml of the AB/IgG solution was left to conjugate the Pani in a hybridization oven at 27°C for 1.0 h, to form Pani-AB/IgG conjugate. To inactivate the non-reacted aldehyde group of the conjugate, 0.5 ml of 0.1M Tris buffer

containing 0.1 % casein (pH 9.0) was added to the Pani-AB/IgG conjugate solution and left to react in a hybridization oven at 27°C for 0.5 h.

D. Conjugate membrane immobilization

To immobilize Pani-AB/IgG conjugate on the conjugate membrane, a conjugate membrane was immersed in the Pani-AB/IgG conjugate solution until saturated and then air-dried at 20°C under a clean biosafety cabinet for 0.75 h.

E. Immunosensor fabrication

The capture membrane, besides the prepared portion at the center, has waterproof adhesives at both ends and provides the backing for attachment of the other membranes. The waterproof adhesives were peeled-off and the other membranes were attached to the waterproof ends during fabrication. First, the conjugate membrane was attached to one end of the prepared portion of the capture membrane, and then the application membrane was attached overlaying a portion of the conjugate membrane. The absorption membrane was attached on the opposite end of the capture membrane, to complete the immunosensor fabrication. The fabricated immunosensor was cut into 5 mm-wide immunosensor strips with a pair of scissors. With a silver-microtip conductive pen (MG Chemicals, Surrey, B.C., Canada), silver electrodes were hand-printed on both sides of the capture membrane, such that an approximate 1 mm wide capture channel is produced (Figure 2.1).

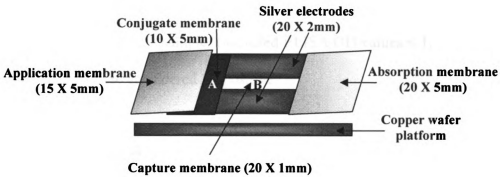

Figure 2.1. Schematic of the fabricated immunosensor strip (A) conjugate membrane containing Pani-AB/IgG (B) capture membrane with immobilized MAPPD

F. Biosensor assembly

Each silver electrode, flanking the capture membrane, was connected to a copper wafer (Figure 2.1); connection was hand-printed using a silver-microtip conductive pen. The two ends of the copper wafer were connected to the detector element, a multimeter (Model: 2880A BK Precision multimeter, Worchester, MA).

G. Sample

The developed biosensor was tested with 6 bovine serum samples: 3 JD positive and 3 JD negative samples. The positive samples were collected from clinical JD cows housed at the Michigan State University Veterinary Research Farm, while the negative samples were collected from cows at the Michigan State University Dairy Teaching and Research Center, who had been tested negative for JD a minimum of three times. JD status of the samples was determined by a commercially available MAP ELISA (PARACHEK, Prionics, Schlieren-Zurich, Switzerland), performed at the Diagnostic

Center for Population and Animal Health, Michigan State University. The ELISA interpretation was based on the optical density (OD) values, a reflection of the MAP antibody concentration in each sample. Corrected ELISA OD values < 1.0 are considered JD negative and > 1.0 are considered JD positive.

H. Mechanism of detection

One hundred micro liter of sample is applied to the application membrane and is drawn into the immunosensor strip by capillary action. The sample passes the conjugate membrane, where serum IgG (IgG*), both MAP and non-MAP, are bound to the Pani-AB/IgG conjugate, forming Pani-AB/IgG-IgG* complex (Figure 2.2). The complex is drawn into the capture membrane, where immobilized MAPPD captures the MAP specific IgG* (JD positive serum) and allow the non-MAP IgG* to flow to the absorption membrane. As more and more MAP IgG* are captured, the Pani in the Pani-AB/IgG-IgG complex forms a bridge between the silver electrodes, flanking the capture membrane. Pani causes an electrical conductance through the electrodes; a higher electrical conductance is recorded as a reduced resistance.

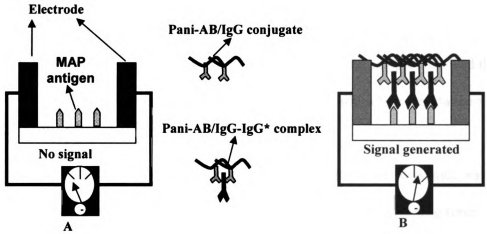

Figure 2.2. Cross-section of a capture membrane before (A) and after (B) positive JD sample application

I. Signal measurement

After each sample application, the resistance value (Kilo ohms) was recorded at 2, 4, and 6 minutes. Three replications were performed for each sample.

J. Statistical analyses

A 2-way ANOVA was used to analyze if the mean resistance values were significantly different among the sample groups, adjusting for the effects of different ELISA OD values and different reading times. Holm-Sidak test, a multiple comparison procedure, was used to isolate which group(s) differed from the others. The statistical analyses were performed with SigmaStat 3.1 software. Intra-assay coefficient of variation of the biosensor was calculated to evaluate the precision of the biosensor assay.

III. RESULTS

Results for each sample tested in the biosensor at different time points are shown in Table 2.1. The conductometric biosensor evaluation of the samples showed that at each 2-minute interval, the JD positive samples had numerically lower resistance values than the JD negative samples.

A 2-way ANOVA showed that the difference in mean resistance values of the samples was significantly affected by the ELISA OD values, the reading times, as well as their interactions ($P < 0.05$) (Figure 2.3). Hence, the effect of ELSA OD values on the biosensor resistance depended on what time the reading was taken.

The Holm-Sidak test showed that at 2 minutes, the mean resistance value of each of the JD positive samples (16.83, 13.80, and 9.78) was significantly different ($P < 0.05$) from the mean resistance of each of the JD negative samples (0.14, -0.20, and -0.48). At 4 minutes, the difference in mean resistance was statistically significant only between sample 16.83 and sample -0.48. And at 6 minutes, the difference in mean resistance between the JD positive samples and the JD negative samples was not statistically significant. The intra-assay coefficient of variation of the biosensor at 2 minutes was 14.48%.

Table 2.1. Conductometric biosensor analysis of bovine serum samples at 3 time intervals

Sample ELISA OD values	Conductometric biosensor mean (n=3) resistance (Kilo ohms) at 3 time intervals		
	2 min Mean ± SD	4 min Mean ± SD	6 min Mean ± SD
16.83**	43.47 ± 4.76[a]	75.63 ± 32.20[a]	66.63 ± 24.66[a]
13.80**	70.33 ± 3.95[a]	93.43 ± 33.50[ab]	81.20 ± 33.98[a]
9.78**	95.43 ± 12.58[a]	97.60 ± 30.19[ab]	97.13 ± 24.94[a]
0.14*	437.00 ± 33.29[b]	114.73 ± 23.97[ab]	112.83 ± 20.87[a]
-0.20*	448.37 ± 99.41[c]	125.83 ± 19.69[ab]	117.73 ± 20.85[a]
-0.48*	672.33 ± 101.93[c]	228.53 ± 162.9[b]	152.13 ± 20.33[a]

** = Johne's disease (JD) positive, * = JD negative, SD = standard deviation, Different superscripts [a b c] within columns indicate significant differences between the mean resistance of the samples ($p < 0.05$)

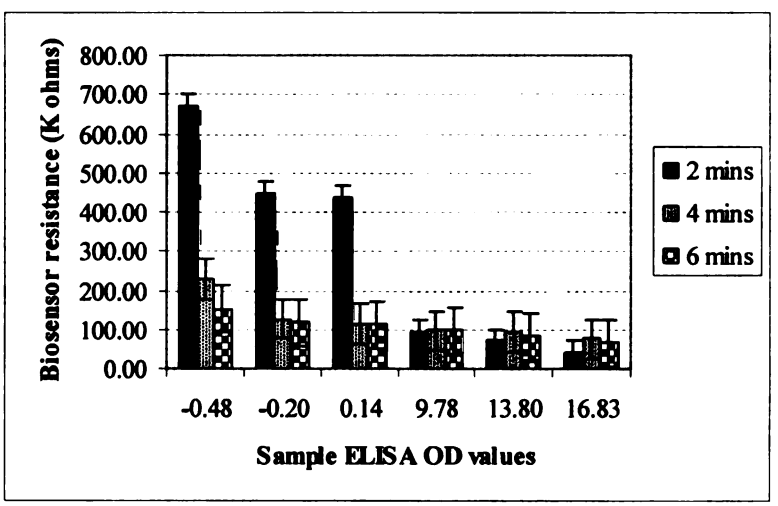

Figure 2.3. Influence of biosensor reading time and ELISA OD on the biosensor resistance; ELISA OD < 1 = JD negative, >1 = JD positive

IV. DISCUSSION

In this study, a conductometric biosensor was successfully designed and fabricated to detect MAP IgG* in serum samples. At the conjugate membrane, both JD negative and JD positive samples potentially formed Pani-AB/IgG-IgG* complex, but the difference in resistance values showed that among JD positive samples (ELISA OD >1), more Pani-AB/IgG-IgG* complexes were captured at the capture membrane. The captured complexes could be attributed to the ability of the MAPPD (MAP antigen) on the capture membrane to immobilize MAP IgG* in the positive samples while IgG* in JD negative samples continued unbound to the absorption membrane. The polyaniline, in the Pani-AB/IgG-IgG* complex, due to its conductive property, resulted in a lower electrical resistance in JD positive samples. Generally, the biosensor electrical resistance decreased as the ELISA OD increased from JD negative to JD positive samples. The relationship between the ELISA OD (antibodies concentration) and the biosensor values (Figure 2.3) suggest that the biosensors could become a quantitative measure.

The difference in biosensor values was statistically significant at 2 but not at 4 and 6 minutes. It is not clear why the resistance in negative samples dropped after 2 minutes. One possibility is the absorption membrane's inability to completely pull Pani-AB/IgG-IgG* complex from the capture membrane. In this study, serum (0.1ml) migrated to the absorption membrane in about 2 minutes by capillary action but the subsequent flow of Pani-AB/IgG-IgG* complex into the capture membrane appeared stagnated. The stagnated Pani-AB/IgG-IgG* complex could have lowered the resistance in readings taken after 2 minutes. This could explain why there was no significant difference in mean resistance values of the samples at 4 and 6 minutes.

Another issue was that the conductometric biosensor had high variance within each sample. The biosensor's intra-assay coefficient of variation (%CV) at 2 minutes was 14.48%. A reasonable target for %CV in routine diagnostic testing is 10-15% but a value of 10% or less is considered satisfactory (Murray *et al.* 1993). The free-hand application of silver electrodes on the capture membrane introduced variability in the width of the capture channels from one biosensor strip to the other. The non-uniform channels could introduce variability in fluid flow, causing faster Pani bridging on narrower channels. Such variability could affect the result within each sample and may be responsible for the high variance within tests in the biosensor. A possible way to limit this variability is to have a uniform screen-printing of silver electrodes on the capture membrane. Other potential sources of variability include choice of MAP antigen, MAPPD concentration, AB/IgG concentration, and polyaniline concentration. Future optimization research would address these variability sources.

V. CONCLUSION

The fabricated conductometric biosensor developed in this study can differentiate between MAP antibodies concentration of JD positive and negative samples in 2 minutes. The rapidity of this biosensor agreed with previous studies (Muhammad-Tahir *et al.* 2005; Muhammad-Tahir and Alocilja 2003). The developed assay was user-friendly, inexpensive, and portable. Further optimization is needed to decrease the biosensor's intra-assay %CV, improving its level of precision. The optimized biosensor would support frequent testing of animals, thus improving JD control.

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CHAPTER THREE

OPTIMIZATION OF A CONDUCTOMETRIC BIOSENSOR FOR DETECTION OF *Mycobacterium avium* subsp. *paratuberculosis* IgG

ABSTRACT

Johne's disease (JD) is an important infectious disease of cattle worldwide, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). In an effort to address some limitations of the currently available JD diagnostic assays, a user-friendly and inexpensive conductometric biosensor has been developed to detect MAP IgG. Optimization of the biosensor is needed to support more frequent testing of animals, which could improve JD control. In this study, two aspects of the previously developed conductometric biosensor were optimized for better detection of MAP IgG. First, to address variability in fabrication of the biosensor electrodes, the previous hand-printed electrodes were replaced with uniform screen-printed electrodes. Second, the optimal polyaniline/anti-bovine IgG conjugate concentrations needed for optimum detection by the biosensor was studied. Three anti-bovine IgG conjugate concentrations (0.046 mg/ml, 0.0115 mg/ml, and 0.0046 mg/ml) were evaluated. Results from this study demonstrated that uniform screen-printed electrode could reduce the intra-assay coefficient of variation and the biosensor optimally detected MAP IgG using anti-bovine IgG concentration of 0.0115 mg/ml. Further optimization of the other parts of the biosensor can support its use in JD control programs.

I. INTRODUCTION

A conductometric biosensor is an analytical device that contains a transducer, which interprets specific biological recognition reactions (i.e. antigen-antibody binding) as electrical conductance. A transducer, such as polyaniline is placed close to or integrated with the biological element (i.e. antibody). Polyaniline, a conductive polymer, relays any antigen-antibody binding as a measured electrical quantity. Among infectious pathogens, conductometric biosensors have been used to detect *E. coli* O157:H7 (Muhammad-Tahir and Alocilja 2003), *Bacillus cereus* (Pal *et al.* 2007), Bovine viral diarrhea virus (Muhammad-Tahir *et al.* 2005), and IgG to *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative organism of Johne's disease (JD) (Okafor 2008b). The developed biosensor for MAP IgG detection possesses certain attributes like affordability, user-friendliness, rapidity in detection, and on-site adaptability, which could make it a useful assay for JD control. Further optimization of this biosensor could improve its accuracy and usefulness as a diagnostic assay for JD.

JD is a chronic gastrointestinal disease of ruminants whose economic impact in the cattle industry and a public health concern has increased the urgency of its control in animals. In the US, economic losses from the disease have been estimated to exceed $1.5 billion per year (Stabel 1998), mainly from the effects of reduced milk production (Losinger 2005). Although there is evidence that MAP may be associated with Crohn's disease in humans, MAP is not currently recognized as a zoonotic pathogen (Grant 2005). Current diagnostic tests for Johne's disease are laboratory based; they require specialized

equipment and training. Development of rapid and inexpensive diagnostic assays, which could be deployed in the field, could aid in the control of JD.

The objectives of this study were to decrease the intra-assay variability of the previously developed conductometric biosensor and also optimize the biosensor's detection of MAP IgG. The decrease in the biosensor's intra-assay variability as well as its optimization could increase its sensitivity and specificity in JD diagnosis, support frequent testing of animals, and improve JD control.

II. MATERIALS AND METHODS

A. Immunosensor membranes

The immunosensor section of the biosensor consists of four individual membranes: sample application, conjugate, capture, and absorption membranes (Hi-Flow Plus Assembly Kit, Millipore, Bedford Massachusetts). These membranes were individually prepared, fabricated, and assembled to form a functional biosensor. Any unused prepared or fabricated membrane was stored at 4°C. The suitability of the immunosensor membranes and the silver electrodes for a biosensor assay was detailed in a previous study (Okafor 2008b). Besides the procedures of electrode screen printing and assembled membrane cutting, all other protocols were as described in a previous study (Okafor 2008b).

B. Capture membrane preparation

The capture membrane was prepared at 20°C, under a clean biosafety cabinet unless otherwise stated. First, silver polymer paste (Gwent Electronic Materials Ltd, UK) was uniformly screen-printed on the capture membrane, according to the manufacturing specifications. The 20 μm-thick silver electrode films provided a uniform 1 mm wide channel on the capture membrane, for a later antigen immobilization (Figure 3.1). Next, the membrane was flushed with distilled water, to remove any debris, and air-dried for 0.5 h. Then, the membrane was flushed with 10% methanol and air-dried for 0.5 h, to activate the membrane. The membrane was washed with 1.2 ml of 0.5 % glutaraldehyde solution and air-dried for 1 h, to provide a link-on between the hydroxyl group in the ethanol and the biological receptor molecule. *Mycobacterium avium* purified protein

derivative (MAPPD) is antigenically similar to MAP. A total volume of 1.2 ml of 1 mg/ml MAPPD (AntelBio, East Lansing, Michigan) was pipetted on the membrane and incubated at 35°C in a closed plastic container for 1 h. Afterwards, the membrane was washed with 1.2 ml of 0.1 M Tris buffer containing 0.1% (v/v) tween-20, to remove all non-specifically absorbed MAPPD. Finally, the membrane was incubated at 35°C in a closed plastic container for 0.75 h, air-dried for 0.5 h, and was set to be fabricated with the other membranes.

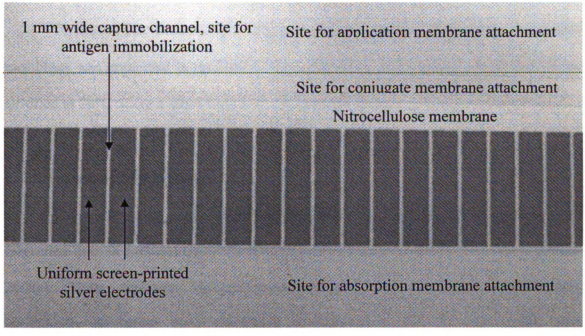

Figure 3.1. A silver screen-printed capture membrane before immunosensor

C. Optimization of anti-bovine antibody concentrations in polyaniline conjugate

AquaPass polyaniline (Mitsubishi Rayon Co, Japan) was diluted to 0.001 % with 0.1 M phosphate buffer solution. Purified mouse clone BG-18 monoclonal anti-bovine IgG (Sigma-Aldrich, St Louis, Missouri) was added to 0.001 % AquaPass polyaniline

solution (Pani) to produce 3 final monoclonal anti-bovine IgG (AB/IgG) concentrations: 0.046 mg/ml, 0.0115 mg/ml, and 0.0046 mg/ml. A 4 ml of each AB/IgG concentration in Pani solution was left to conjugate in a hybridization oven at 27°C for 1.0 h, to form Pani-AB/IgG conjugate. To inactivate the non-reacted aldehyde group of the conjugate, 0.5 ml of 0.1M Tris buffer containing 0.1 % casein (pH 9.0) was added to the each Pani-AB/IgG conjugate solution and left to react in a hybridization oven at 27°C for 0.5 h.

D. Conjugate membrane immobilization

To immobilize Pani-AB/IgG conjugate on the conjugate membrane, a conjugate membrane was immersed in the Pani-AB/IgG conjugate solution until saturated and then air-dried at 20°C under a clean biosafety cabinet for 0.75 h.

E. Immunosensor fabrication

The membranes: sample application, conjugate, capture, and absorption, were fabricated into an immunosensor (Figure 3.2), as explained in a previous study (Okafor 2008b). Three separate immunosensors (varying AB/IgG concentrations) were individually fabricated. Each fabricated immunosensor was cut into 5 mm wide disposable strips (Figure 3.3) with a programmable shear (Matrix 2360, Kinematic Automation Inc.). A roller presses the membrane into the programmable shear for automated cutting of the strips.

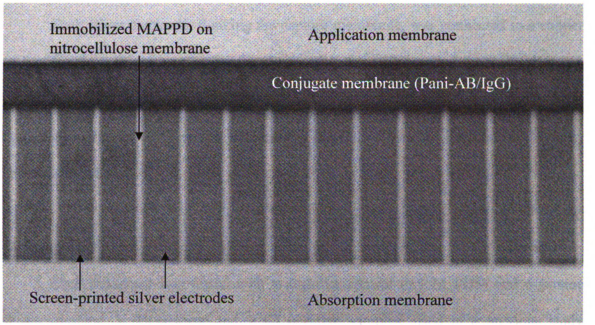

Figure 3.2. The fabricated immunosensor before cutting

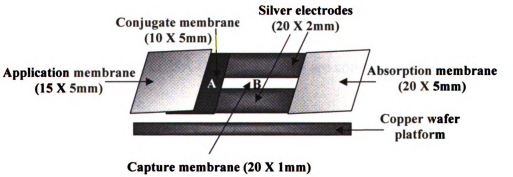

Figure 3.3. Schematic diagram of the immunosensor strip (A) Conjugate membrane containing Pani-AB/IgG (B) Capture membrane with immobilized MAPPD

F. Biosensor assembly

Each silver electrode, flanking the capture membrane, was connected to a copper wafer (Figure 3.3); connection was hand-printed using a silver-microtip conductive pen (MG Chemicals, Surrey, B.C., Canada). The two ends of the copper wafer were connected to the detector element, a multimeter (Model: 2880A BK Precision multimeter, Worchester, MA).

G. Sample

Each biosensor was tested with a negative control (0.1 M PBS) and 6 bovine serum samples: 3 JD positive and 3 JD negative samples, used in a previous study (Okafor 2008b). The positive samples were collected from clinical JD cows housed at the Michigan State University Veterinary Research Farm, while the negative samples were collected from cows at the Michigan State University Dairy Teaching and Research Center, who had been tested negative for JD a minimum of three times. JD status of the samples was determined by a commercially available MAP ELISA (PARACHEK, Prionics, Schlieren-Zurich, Switzerland), performed at the Diagnostic Center for Population and Animal Health, Michigan State University. The ELISA interpretation was based on the optical density (OD) values, a reflection of the MAP antibody concentration in each sample. Corrected ELISA OD values < 1.0 are considered JD negative and > 1.0 are considered JD positive.

H. Mechanism of detection

One hundred micro liter of sample is applied to the application membrane and is drawn into the immunosensor strip by capillary action. The sample passes the conjugate membrane, where serum IgG (IgG*), both MAP and non-MAP, are bound to the Pani-AB/IgG conjugate, forming Pani-AB/IgG-IgG* complex (Figure 3.4). The complex is drawn into the capture membrane, where immobilized MAPPD capture the MAP specific IgG* in the complex (for JD positive serum) and allow the non-MAP IgG* to flow to the absorption membrane. As more and more MAP IgG* are captured, the Pani in the Pani-AB/IgG-IgG* complex forms a bridge between the silver electrodes, flanking the capture membrane. The Pani causes an electrical conductance through the electrodes; a higher electrical conductance reflects a reduced resistance.

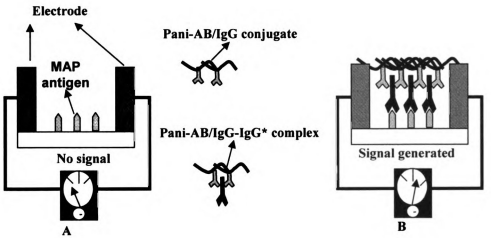

Figure 3.4. Cross-section of a capture membrane before (A) and after (B) positive JD sample application

I. Signal measurement

After each sample application, the resistance value (kilo ohms) was recorded at 2 minutes. Each sample was tested on the three biosensors (varying AB/IgG concentrations) and three replications were performed.

J. Statistical analyses

For each biosensor, the intra-assay coefficient of variation (%CV) was calculated, to evaluate if the uniformly screen-printed electrode affected the precision of the biosensor assay. A 2-way ANOVA was used to analyze if the mean resistance values among the sample groups were significantly different, adjusting for the effects of different ELISA OD values and different AB/IgG concentrations; Holm-Sidak test, a multiple comparison procedure, was used to isolate which group(s) differed from the others. The statistical analyses were performed with SigmaStat 3.1 software.

III. RESULTS

Results of each sample tested on each AB/IgG concentration of the biosensor are shown in Table 3.1. For each of the three biosensors: 0.046 mg/ml, 0.0115 mg/ml, and 0.0046 mg/ml AB/IgG concentrations, the intra-assay coefficient of variation (%CV) was 4.90%, 3.88%, and 7.62% respectively. The conductometric biosensor evaluation of the samples, for each AB/IgG concentration, showed numerically lower mean resistance values among the JD positive samples than observed in the JD negative samples and negative control.

Table 3.1. Conductometric biosensor analysis of bovine serum samples at different anti-bovine IgG concentrations

ELISA OD values	Conductometric biosensor resistance (K Ohms) at 2 mins for varying AB/IgG concentrations in 0.001 % AquaPass		
	0.046 mg/ml Mean ± SD	0.0115 mg/ml Mean ± SD	0.0046 mg/ml Mean ± SD
16.83 **	6.35 ± 0.12^{a}	6.56 ± 0.31^{a}	22.01 ± 1.70^{a}
13.80**	7.24 ± 0.68^{a}	8.23 ± 0.34^{a}	22.46 ± 0.90^{a}
9.78**	8.30 ± 0.46^{a}	8.80 ± 1.36^{a}	23.19 ± 2.45^{a}
0.14*	9.30 ± 0.33^{ab}	13.70 ± 0.27^{b}	23.04 ± 2.60^{a}
-0.20*	10.25 ± 0.45^{ab}	15.52 ± 0.28^{b}	24.04 ± 2.43^{a}
-0.48*	11.39 ± 0.55^{b}	19.13 ± 0.23^{c}	24.59 ± 0.54^{a}
(-)control PBS	14.94 ± 1.06^{c}	20.73 ± 1.80^{c}	24.28 ± 0.34^{a}

** = Johne's disease (JD) positive, * = JD negative, SD = standard deviation, Different superscripts $^{a\,b\,c}$ within the columns indicate significant differences between the mean resistance of the samples ($p < 0.05$)

A 2-way ANOVA showed that the mean resistance values among the sample groups was significantly difference ($P < 0.05$). The observed resistance values were significantly ($P < 0.05$) affected by the ELISA OD values and the AB/IgG concentrations, both individually and by their interaction.

Holm-Sidak test (Figure 3.5) showed that for 0.0115 mg/ml AB/IgG concentration, the mean resistance values of each of the JD positive ELISA OD values (16.83, 13.80, and 9.76) was significantly different ($P < 0.05$) from each of the JD negative ELISA OD values (0.14, -0.20, and -0.48). However, there was no significant difference ($P < 0.05$) between the 2 groups for 0.046 mg/ml and 0.0046 mg/ml AB/IgG concentrations.

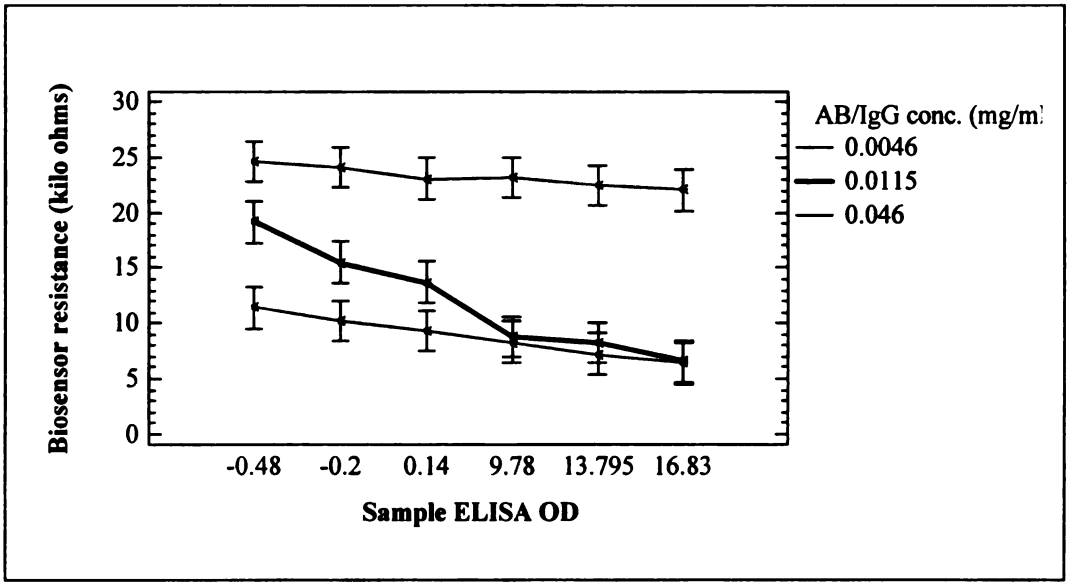

Figure 3.5. The mean biosensor resistance values of the samples at different AB/IgG concentrations. ELISA OD < 1 = JD negative, >1 = JD positive

IV. DISCUSSION

In this study, to address variability in the fabrication of the biosensor electrodes, uniformly screen-printed electrodes were used to replace the hand-printed electrodes, which could have influenced the biosensor's intra-assay % CV of 14.48%, as reported in the previous study (Okafor 2008b). The uniformly screen-printed electrodes yielded a uniform 1 mm-wide capture channel on the capture membrane of the conductometric biosensor. The intra-assay %CV of each of the optimized biosensor was less than 8%. A reasonable target for %CV in routine diagnostic testing is 10-15% but a value of 10% or less is considered satisfactory; lower % CV is a good indication of precision and precision is a required attribute in assay validation (Murray *et al.* 1993). The intra-assay %CV values of these biosensors confirms that 1 mm width uniformity of the capture channel in all biosensor strips, following uniform screen-printing, reduced the intra-assay %CV of the biosensor. Hence, the uniformly screen-printed biosensor improved precision of the assay.

The lower resistance values among JD positive samples were also reported in a previous study (Okafor 2008b). Among JD positive sample many Pani-AB/IgG-IgG* complexes were captured by immobilized MAPPD at the capture membrane, causing a higher electrical conductance recorded as lower resistance. In the JD negative samples, the Pani-AB/IgG-IgG* complexes continued unbound to the absorption membrane, reflecting higher resistance values.

To further optimize the biosensor, the effect of different concentrations of AB/IgG on the biosensor performance was studied. From the Holm-Sidak test, the difference in resistance between JD positive and JD negative samples was significant ($P < 0.05$) for 0.0115 mg/ml but not 0.046 mg/ml and 0.0046 mg/ml AB/IgG concentrations (Figure 3.5). At lower AB/IgG concentration (0.0046 mg/ml), the relatively higher resistance in all samples, indicating low conductance, could be explained by fewer Pani-AB/IgG conjugate. Hence, upon sample application, limited MAP specific Pani-AB/IgG-IgG* complexes are formed in the conjugate membrane, with possible non-conjugated MAP IgG*. When the Pani-AB/IgG-IgG* complexes and likely unbound MAP IgG* are drawn into the capture membrane, immobilized MAPPD would capture the limited MAP specific Pani-AB/IgG-IgG* complexes as well as the non-conjugated MAP IgG*. The few immobilized MAP specific Pani-AB/IgG-IgG* complexes would result in relatively low electrical conductance. So, fewer complexes would reflect a relatively high resistance value. The relative higher resistance values with lower AB/IgG concentrations agree with result of a similar study where a conductometric biosensor was developed to detect Bovine viral diarrhea virus (Muhammad-Tahir and Alocilja 2003).

For all the sample groups, the higher AB/IgG concentration biosensor (0.046 mg/ml) yielded lower resistance values than the other AB/IgG concentration biosensors (Figure 3.5). The excess AB/IgG molecules after Pani-AB/IgG conjugation could be responsible for lower resistance values at higher AB/IgG concentration. Upon sample application, the excess un-conjugated AB/IgG molecules could get attached to serum IgG* to form AB/IgG-IgG* molecules. In the capture membrane, the AB/IgG-IgG* molecules could crowd the capture channel such that the crowded molecules could

provide platform for easier bridging of the electrodes by Pani, inducing higher conductance (lower resistance). This over-crowding effect may be responsible for the low resistance observed at the 0.046 mg/ml AB/IgG concentration. Therefore, given the parameters used in this study, such as the concentration of the immobilized MAPPD on the capture membrane, 0.0115 mg/ml AB/IgG concentration was optimum for the biosensor's detection of MAP IgG.

The relative resistance values recorded in this study for 0.0115 mg/ml AB/IgG concentration was lower in comparison to the previous study (Okafor 2008b). It is not clear why the change in resistance values occurred. The few areas of change in methodology could be responsible. One possible reason could be that the conductive properties of the silver paste used for electrode printing in this study differ from that of the silver ink used in the earlier study (Okafor 2008b). Another reason could be the effect of the roller, in the programmable shear, pressing on the immunosensor membranes during membrane cutting. Pressing on the capture membrane after MAPPD immobilization could push the antigen into the nitrocellulose membrane or distort the antigen's orientation, affecting specific antigen-antibody binding. Cutting of the fabricated immunosensor membranes without tampering with the membrane surfaces might address the resistance change.

Areas for further research are optimizations of MAP antigen types, MAP antigen concentrations, sample processing, and polyaniline concentrations. These optimizations could improve the performance of the biosensor assay in MAP IgG detection.

V. CONCLUSION

In this study, the 20 μm-thick uniformly screen-printed silver electrodes on the capture membrane yielded a uniform 1 mm-wide capture channel, controlled the intra-assay variability, and increased the precision of the biosensor. Also AB/IgG concentration of 0.0115 mg/ml was optimum for the conductometric biosensor in detecting MAP IgG.

Further testing of the optimized conductometric biosensor with larger sample size is needed; application of the biosensor to JD diagnosis, using a similar immunodiagnostic test like ELISA, will provide information on the sensitivity and specificity of the biosensor. Such results could make the biosensor a desirable assay for improving JD control.

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CHAPTER FOUR

COMPARISON OF A CONDUCTOMETRIC BIOSENSOR AND ELISA IN THE DIAGNOSIS OF JOHNE'S DISEASE

ABSTRACT

Johne's disease (JD) is a chronic gastrointestinal disease of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The disease causes significant loss in the dairy cattle industry. The attributes of the currently available diagnostic tests contribute to the challenges of JD control. Conductometric biosensor is a newly developed, non-laboratory based assay for the detection of MAP IgG. The biosensor's attributes: user-friendliness; affordability; and detection rapidity, would support more frequent testing of JD animals, improving disease control. In this study, 17 bovine serum samples, 2 negative controls, and a positive control were tested in triplicates using the biosensor assay. The cut-off limit of the biosensor was determined using the Mean ± 2SD method. The sensitivity and specificity of the conductometric biosensor was evaluated, using conventional ELISA as a gold standard. The agreements by both assays in JD diagnosis were measured using Cohen's kappa analysis (K) and the intra-assay coefficient of variation (%CV) of the biosensor was calculated. The biosensor's sensitivity was (71.43%) and specificity (70%). There was a moderate strength of agreement (K= 0.41) between both assays in JD diagnosis at 95% confidence interval. This study showed that a conductometric biosensor is a valuable assay in JD diagnosis.

I. INTRODUCTION

Johne's disease (JD) is a chronic gastrointestinal disease of ruminants with a significant economic impact in the cattle industry and a public health concern. In the US, economic losses resulting from decreased milk production, unrealized income related to premature culling, reduced carcass dressing weight and animal death have been estimated to exceed $1.5 billion per year (Stabel 1998). *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative organism of JD, has also been linked to Crohn's disease; evidence of this association is conflicting and MAP is not currently recognized as a zoonotic pathogen (Grant 2005). Economic losses from JD and the knowledge that MAP may be a zoonotic pathogen have increased the urgency to control the spread of MAP in domestic animals. Limitations in currently available diagnostic tests contribute to the challenge of effective JD control.

Diagnosis of JD is aimed at detecting MAP or its DNA in feces, tissues, and occasionally milk, or detecting an immune response to MAP. Currently, culture is most commonly used to detect MAP (Whitlock *et al.* 2000); PCR is used for MAP DNA detection (Sevilla *et al.* 2005); and ELISA is the most common assay used to detect an immune response to MAP in the form of MAP specific antibodies (Kalis *et al.* 2002). However, culturing is expensive and requires 7-12 weeks for completion (Kalis *et al.* 1999; Whitlock *et al.* 2000); PCR and ELISA require specialized equipment and skilled training. These currently used diagnostic tests may not be easily adapted for on-site diagnosis and not readily accessible in the developing countries of the world. The development of new technologies for JD diagnosis, which are adaptable to the field and

potentially useful in developing countries, would be beneficial in furthering JD control efforts.

A conductometric biosensor is an analytical device that contains a transducer, which interprets specific biological recognition reactions (i.e. antigen-antibody binding) as electrical conductance. A transducer, such as polyaniline is placed close to or integrated with the biological element (i.e. antibody). Polyaniline, a conductive polymer, relays any antigen-antibody binding as a measured electrical quantity. Among infectious pathogens, conductometric biosensors have been used in the detection of *E. coli* O157:H7 (Muhammad-Tahir and Alocilja 2003), *Bacillus cereus* (Pal *et al.* 2007), and Bovine viral diarrhea virus (Muhammad-Tahir *et al.* 2005). The affordability, user-friendliness, rapidity in detection, and on-site adaptability of conductometric biosensors could replace the limitations of the current JD diagnostic tests (Okafor 2008a). A conductometric biosensor has been developed (Okafor 2008b) and optimized (Okafor 2008c) for the detection of MAP IgG in bovine serum.

The objectives of this study were to investigate the agreement between the biosensor and ELISA assays in JD diagnosis; evaluate the sensitivity and specificity of an optimized conductometric biosensor in JD diagnosis, using ELISA as the gold standard; and also evaluate the biosensor's intra-assay %CV. A good correlation with ELISA as well as a good level of precision would support evaluation of the biosensor as a JD diagnostic assay. This would provide another tool for testing of animals, thus improving JD control.

II. MATERIALS AND METHODS

A. Immunosensor membranes

The immunosensor section of the biosensor consists of four individual membranes: sample application, conjugate, capture, and absorption membranes (Hi-Flow Plus Assembly Kit, Millipore, Bedford Massachusetts). These membranes were individually prepared, fabricated, and assembled to form a functional biosensor. Any unused prepared or fabricated membrane was stored at 4°C.

B. Capture membrane preparation

The capture membrane was prepared at 20°C, under a clean biosafety cabinet unless otherwise stated. First, silver polymer paste (Gwent Electronic Materials Ltd, UK) was uniformly screen-printed on the capture membrane, according to the manufacturing specifications. The 20 μm-thick silver electrode films provided a uniform 1 mm wide channel on the capture membrane, for a later antigen immobilization (Figure 4.1). Next, the membrane was flushed with distilled water, to remove any debris, and air-dried for 0.5 h. Then, the membrane was flushed with 10% methanol and air-dried for 0.5 h, to activate the membrane. The membrane was washed with 1.2 ml of 0.5 % glutaraldehyde solution and air-dried for 1 h, to provide a link-on between the hydroxyl group in the ethanol and the biological receptor molecule. *Mycobacterium avium* purified protein derivative (MAPPD) is antigenically similar to MAP. A total volume of 1.2 ml of 1 mg/ml MAPPD (AntelBio, East Lansing, Michigan) was pipetted on the membrane and incubated at 35°C in a closed plastic container for 1 h. Afterwards, the membrane was washed with 1.2 ml of 0.1 M Tris buffer containing 0.1% (v/v) tween-20, to remove all

non-specifically absorbed MAPPD. Finally, the membrane was incubated at 35°C in a closed plastic container for 0.75 h, air-dried for 0.5 h, and was set to be fabricated with other membranes.

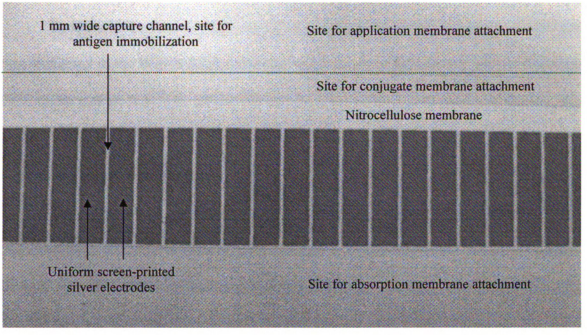

Figure 4.1. A silver screen-printed capture membrane before immunosensor

C. Anti-bovine antibody -polyaniline conjugation

AquaPass polyaniline (Mitsubishi Rayon Co, Japan) was diluted to 0.001 % with 0.1 M phosphate buffer solution. Purified mouse clone BG-18 monoclonal anti- bovine IgG (Sigma-Aldrich, St Louis, Missouri) was added to 0.001 % AquaPass polyaniline solution (Pani) to produce a final monoclonal anti-bovine IgG (AB/IgG) concentration of 0.0115 mg/ml. 4 ml of the AB/IgG concentration solution was left to conjugate in a hybridization oven at 27°C for 1.0 h, to form Pani-AB/IgG conjugate. To inactivate the non-reacted aldehyde group of the conjugate, 0.5 ml of 0.1M Tris buffer containing 0.1 %

casein (pH 9.0) was added to the Pani-AB/IgG conjugate solution and left to react in a hybridization oven at 27°C for 0.5 h.

D. Conjugate membrane immobilization

To immobilize Pani-AB/IgG on the conjugate membrane, a conjugate membrane was immersed in the Pani-AB/IgG conjugate solution until saturated and then air-dried at 20°C under a clean biosafety cabinet for 0.75 h.

E. Immunosensor fabrication

The membranes: sample application, conjugate, capture, and absorption, were fabricated into an immunosensor (Figure 4.2), as explained in a previous study (Okafor 2008b). The fabricated immunosensor was cut into 5 mm wide disposable strips (Figure 4.3) with a programmable shear (Matrix 2360, Kinematic Automation Inc.).

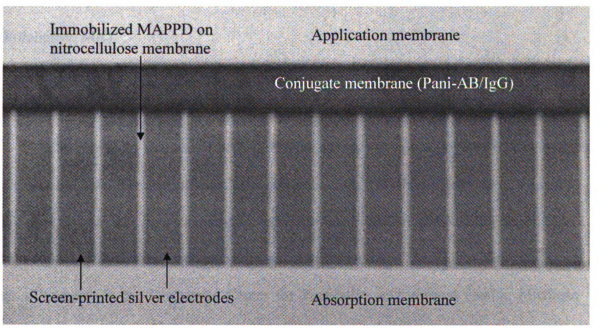

Figure 4.2. The fabricated immunosensor before cutting

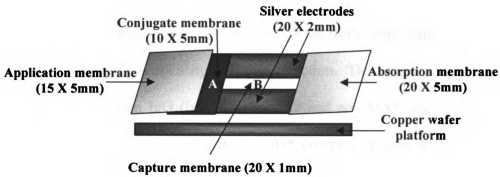

Figure 4.3. Schematic diagram of the immunosensor strip (A) Conjugate membrane containing Pani-AB/IgG (B) Capture membrane with immobilized MAPPD

F. Biosensor assembly

Each silver electrode, flanking the capture membrane, was connected to a copper wafer (Figure 4.3); connection was hand-printed using a silver-microtip conductive pen (MG Chemicals, Surrey, B.C., Canada). The two ends of the copper wafer were connected to the detector element, a multimeter (Model: 2880A BK Precision multimeter, Worchester, MA).

G. Sample

The biosensor was tested with 17 bovine serum samples, a positive control and 2 negative controls. The test samples were collected from a Michigan dairy farm, known to be infected with JD. A commercially available MAP ELISA (PARACHEK, Prionics, Schlieren-Zurich, Switzerland) was used as the gold standard. The ELISA was performed on all samples at the Diagnostic Center for Population and Animal Health, Michigan State University.

H. Conductometeric biosensor mechanism

One hundred micro liter of sample is applied to the application membrane and is drawn into the immunosensor strip by capillary action. The sample passes the conjugate membrane, where serum IgG (IgG*), both MAP and non-MAP, are bound to the Pani-AB/IgG conjugate, forming Pani-AB/IgG-IgG* complex (Figure 4.4). The complex is drawn into the capture membrane, where immobilized MAPPD captures the MAP specific IgG* (JD positive serum) and allow the non-MAP IgG* to flow to the absorption membrane. As more and more MAP specific IgG* are captured, the Pani in Pani-AB/IgG-IgG* complex forms a bridge between the silver electrodes, flanking the capture membrane. The Pani causes an electrical conductance through the electrodes; a higher electrical conductance reflects a reduced resistance.

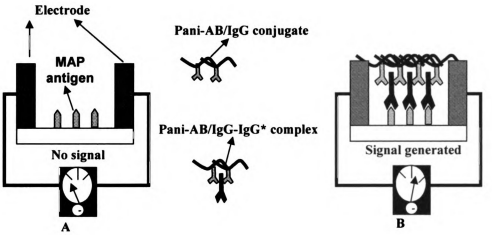

Figure 4.4. Cross-section of a capture membrane before (A) and after (B) positive JD sample application

I. Signal measurement

After each sample application, the resistance value (Kilo ohms) was recorded at 2 minutes. Three replications were performed for each sample.

J. Statistical analyses

The biosensor's cut-off value was determined using Mean ± 2SD (Girish 2007). ELISA assay was used as the gold standard for the calculation of biosensor's sensitivity and specificity. The biosensor's sensitivity and specificity were calculated using the determined cut-off value. The strength of agreement between the assays was analyzed using Cohen's Kappa analysis. Cohen's Kappa analysis was performed with Graphpad software. Intra-assay coefficient of variation (%CV) was calculated to evaluate the precision of the biosensor assay.

III. RESULTS

With the Mean ± 2SD method, a cut-off value of 12.03 Kilo ohms (KΩ) (< 12.03 = JD positive, >12.03 = JD negative) was generated. The biosensor's sensitivity was 71.43% and specificity 70%. Cohen's kappa value was 0.41 at 95% confidence interval and the intra-assay %CV of the biosensor was 5.91%.

Table 4.1. Comparison between conductometric biosensor and ELISA in Johne's disease diagnosis

Sample ID	Mean ± SD biosensor resistance (KΩ)	Biosensor interpretation	ELISA OD	ELISA interpretation
1	9.59±1.34	Positive	0.00	Negative
2	10.17±0.29	Positive	1.67	Positive
3	10.37±0.30	Positive	9.78	Positive
4	10.70±0.07	Positive	4.58	Positive
5	10.74±0.05	Positive	4.90	Positive
6	10.76±0.06	Positive	5.45	Positive
7	10.98±0.07	Positive	0.03	Negative
8	11.66±0.61	Positive	0.00	Negative
9	12.14±1.10	Negative	0.00	Negative
10	12.27±1.64	Negative	9.30	Positive
11	12.40±0.33	Negative	0.00	Negative
12	12.84±1.56	Negative	0.00	Negative
13	13.30±0.31	Negative	0.00	Negative
14	13.38±0.24	Negative	0.00	Negative
15	13.64±0.55	Negative	0.00	Negative
16	14.80±1.02	Negative	6.11	Positive
17	15.70±3.01	Negative	0.00	Negative
(+) control	5.61±0.30	Positive	16.83	Positive
(-) control	12.77±0.37	Negative	-0.20	Negative
0.1 M PBS (-) control	21.81±1.46	Negative	N/A	N/A

SD = standard deviation, OD = optical density, PBS = Phosphate buffered saline

IV. DISCUSSION

Results from the biosensor assay were obtained in 2 minutes. The assay is inexpensive, user-friendly and could be applied on-site. The biosensor's intra-assay %CV value of 5.91% supports that the biosensor has a good precision level. A reasonable target for %CV in routine diagnostic testing is 10-15% but a value of 10% or less is considered satisfactory; lower % CV is a good indication of precision and precision is a required attribute in assay validation (Murray *et al.* 1993). These attributes would support animal point-of-sale pre-purchase testing and more frequent testing of animals especially in places with limited access to the currently available JD tests, thus improving management strategies towards JD control.

A Cohen's kappa value of 0.41 signified a moderate strength of agreement between the conductometric biosensor and ELISA assays in JD diagnosis. In this study, the ELISA was chosen as the gold standard for comparison purposes because this assay is most commonly used in JD diagnosis in the cattle industry today (Kalis *et al.* 2002). However, ELISA has 30 ± 5% sensitivity and 99.5 ± 1% specificity (Collins *et al.* 2006). It could be possible that the biosensor has a better sensitivity that ELISA. The relatively low sensitivity of ELISA could make it difficult for a generalized evaluation of the biosensor, based on the obtained kappa value in this study. Other JD diagnostic tests like Necropsy, biopsy, and fecal culture have better sensitivity and specificity values. Fecal culture, the least sensitive and specific of the tests, has 60 ± 5% sensitivity and 99.9 ± 0.1% specificity (Collins *et al.* 2006). To further evaluate the sensitivity and specificity of

the biosensor assay, a definitive standard JD diagnostic test like necropsy, biopsy or fecal culture should be used as the gold standard.

Also, the biosensor's cut-off value can be adjusted to improve sensitivity and specificity results, depending on which of the two parameters (sensitivity, specificity) is more important. A cut-off value below 12.03 KΩ will improve the specificity and above 12.03 KΩ could improve the sensitivity of the biosensor assay. A cut-off value below 12.03 KΩ will increase the kappa value, leading to a better strength of agreement between the biosensor and ELISA.

Areas for further research are optimizations of MAP antigen types, MAP antigen concentrations, sample type (milk, whole blood), sample processing, and polyaniline concentrations. These could further improve the biosensor's sensitivity and specificity, making it a desirable assay for JD control.

The long term applications of the conductometric biosensors in veterinary sciences include miniaturization into disposable test kits for JD diagnosis, a prerequisite test for all animals at point-of-sale, the adaptation of the assay for multiplex testing of pathogens like BVDV, Bovine leucosis virus (BLV), *E.coli*, etc, and automation for in-line testing of milk samples for JD in dairy farms. The assay could be developed into an equivalent of ELISA 96 well plates, such that larger sample numbers could be analyzed in minutes. Conductometric biosensors should be applied to the detection of IgG to *Mycobacterium tuberculosis*, the causative organism of bovine tuberculosis and a zoonotic pathogen for human tuberculosis. Such assays would reduce the incidence of bovine tuberculosis, leading to a safer public health. The attributes of the conductometric supports its applications to numerous diseases of veterinary and public health concern.

Developing the assay for the diagnosis of the emerging diseases, such that diagnosis and decisions are made at the transnational borders, would improve food animal defense.

V. CONCLUSION

A conductometric biosensor assay developed and tested in this study had a moderate agreement with a standard ELISA assay used for the diagnosis of JD. The sensitivity and specificity as well as the precision level of the assay were also good. The biosensor assay is a very promising tool in the control of JD in cattle. Additional optimization of the biosensor assay could further improve its sensitivity and specificity, making it a desirable assay for JD control.

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GENERAL DISCUSSION AND CONCLUSION

In this study, a conductometric biosensor was successfully designed and fabricated to detect MAP IgG in serum samples. This study represents the first application of a biosensor towards the diagnosis of Johne's disease. It also expands the application of a conductometric biosensor into the detection of disease specific antibodies, as compared to detection of the actual disease pathogen.

As a proof of concept, a biosensor was initially designed and fabricated to detect MAP IgG. At the conjugate membrane, both JD negative and JD positive samples potentially formed Pani-AB/IgG-IgG* complex, but the difference in resistance values showed that among JD positive samples, more Pani-AB/IgG-IgG* were captured at the capture membrane. The captured Pani-AB/IgG-IgG* could be attributed to the ability of the MAPPD on the capture membrane to immobilize MAP IgG in the positive samples while non-MAP IgG continued unbound to the absorption membrane. Pani, a conductive polymer, bridged the silver electrodes flanking the capture membrane, and resulted in a lower electrical resistance in JD positive samples. The difference in biosensor values was significant ($p < 0.05$) at 2 but not at 4 and 6 minutes. Hence, the biosensor could differentiate between MAP antibody concentration of JD positive and negative samples in 2 minutes. The rapidity of this biosensor agreed with studies that detected *E.coli* O157:H7 (Muhammad-Tahir and Alocilja 2003), BVDV (Muhammad-Tahir *et al.* 2005), and *B. cereus* (Pal *et al.* 2007), using similar conductometric biosensor. The developed assay was simple to run and could be easily transported to and used in the field.

One problem encountered in the proof of concept studies was that the biosensor's intra-assay coefficient of variation (%CV) at 2 minutes was 14.48%. A reasonable target for %CV in routine diagnostic testing is 10-15% but a value of 10% or less is considered satisfactory (Murray *et al.* 1993). The free-hand application of silver electrodes on the capture membrane introduced variability in the width of the capture channels from one biosensor strip to the other. The non-uniform channels could introduce variability in fluid flow, causing faster Pani bridging on narrower channels. Such variability could affect the result within each sample and may be responsible for the high variance within tests in the biosensor. To address the variability in fabrication of the biosensor electrodes, the hand-printed electrodes were replaced with uniformly screen-printed electrodes. The screen-printed electrodes yielded a uniform 1 mm wide capture channel on the capture membrane of the conductometric biosensor. This fabrication change resulted in a consistent intra-assay %CV of less than 8% in future studies, thus addressing the variability encountered in the first study.

To further optimize the biosensor, three different AB/IgG concentrations were evaluated for optimum detection of MAP IgG. The difference in resistance between JD positive and JD negative samples was significant ($P < 0.05$) for 0.0115 mg/ml but not 0.046 mg/ml and 0.0046 mg/ml AB/IgG concentrations. Thus, 0.0115 mg/ml AB/IgG concentration was optimum for the biosensor's detection of MAP IgG and chosen for use in future studies.

When the biosensor was applied to JD diagnosis and compared with ELISA as the gold standard, it had a sensitivity of 71.43% a specificity of 70%, and a good level of precision (intra-assay coefficient of variation 5.91%). ELISA was chosen as the gold

standard for comparison purposes because it is most commonly used in the cattle industry today (Kalis *et al.* 2002). To effectively evaluate the sensitivity and specificity of the biosensor assay, a definitive standard test like necropsy, biopsy evaluations or culture (Collins *et al.* 2006) should be used as the gold standard in the comparison.

Areas for further research are optimizations of MAP antigen types, MAP antigen concentrations, sample type (milk, whole blood), sample processing, and polyaniline concentrations. These could further improve the biosensor's sensitivity and specificity, making it a desirable assay for JD control.

The long term applications of the conductometric biosensors in veterinary sciences include miniaturization into disposable test kits for JD diagnosis, a prerequisite test for animals at point-of-sale, the adaptation of the assay for multiplex testing of pathogens like BVDV, Bovine leucosis virus (BLV), *E.coli*, etc, and automation for in-line testing of milk samples for JD in dairy farms. The assay could be developed into an equivalent of ELISA 96 well plates, such that larger sample numbers could be analyzed in minutes. The attributes of the conductometric biosensor supports its applications to numerous diseases of veterinary and public health concern. Developing the assay for the diagnosis of the emerging diseases, such that diagnosis and decisions are made at the transnational borders, would improve food animal defense.

CONCLUSION

The fabricated conductometric biosensor developed in this study could differentiate between MAP antibodies concentration of JD positive and negative samples in 2 minutes. The assay was user-friendly, inexpensive, and portable for on-site diagnosis. These attributes support animal point-of-sale testing, routine herd testing, and clinical animal testing, especially in places with limited access to the currently available JD diagnostic tests. The test results will provide a basis for timely management decisions that would control the spread of JD among animals. Hence, a conductometric biosensor is a promising diagnostic assay that would improve JD control.

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