Role of surface antigens of Mycobacterium spp. in Diagnosis

A Dissertation

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DEDICATION

This dissertation is dedicated to my wife (Geetika) and daughter (Anaya) since I couldn't be successful without their support, love, patience and encouragement.

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ABSTRACT

Mycobacterial species are ubiquitous in nature and a worldwide concern for human and animal health. The major mycobacterial infections in animals are Johne's disease (JD) and bovine tuberculosis (bTB). Controlling these infections is difficult due to the lack of highly sensitive and sensitive diagnostic test. Currently available diagnostic tests have to be carried out in laboratory settings with well experienced and trained examiners. My goal is to develop a sensitive on-site (in-field) device for diagnosis of Johne's disease and bovine tuberculosis. The specific aims of this thesis were (1) to review currently-used or recently developed diagnostic tests for mycobacterial infections, (2) to optimize a milk-based enzyme linked immunosorbant assay (ELISA) for diagnosis of JD, (3) to evaluate a serum-based ELISA for detection of bTB in red deer and (4) to develop a bead-based microfluidic assay as a prototype of on-site diagnostic device for JD.

Previous reports on currently-used or recently developed diagnostic tests for mycobacterial infections were reviewed to summarize challenges and opportunities in development of sensitive on-site diagnostic devices. Most of the current serological tests for mycobacterial infections utilize crude extract of the pathogen or single molecule causing low diagnostic specificity and specificity. Use of multiple antigens was shown to be effective in improving test accuracy.

Ethanol extract of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) was used to optimize a serum ELISA to test milk samples for JD. Using the optimized conditions, the average of ELISA values in the JD-positive milk samples was found to be significantly higher than that in the JD-negative milk samples.

Using ethanol extract from *Mycobacterium bovi*s (MB), an ELISA was developed to detect anti-MB antibodies in serum of farmed red deer. The tentative diagnostic sensitivity and specificity was estimated to be 90% and 93.3%, respectively.

Magnetic beads coated with ethanol extract of MAP were used to develop a microfluidic immunoassay for diagnosis of JD. The antigen-coated magnetic beads were tested in the microfluidic system using bovine serum samples and a high level of antibody binding in JD-positive serum was observed.

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CHAPTER 1

INTRODUCTION

Opportunities for improved serodiagnosis of human tuberculosis, bovine tuberculosis and paratuberculosis.

Mycobacterial species are causing serious concerns in livestock and wild animals worldwide. For example, Johne's disease (JD) or paratuberculosis is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). It is chronic infectious enteritis of domestic and wild ruminants, causing reduction in milk production, malnutrition, weight loss and eventually death. The causative agent of bovine tuberculosis (bTB), *Mycobacterium bovi*s (MB), infects not only cattle but also humans and various wild animals. During 2005 – 2008, 128 countries have reported the presence of MB infection and /or clinical disease in their cattle population. bTb eradication programs in USA and Great Britain have been spending more than $ 40 and £ 100 million per annum, respectively. Since mycobacterial infections are chronic in nature and there is lack of treatment measures, the best strategy to control the infection is through early identification of the infected animals and removal from the herd. Better diagnostic measures are required for effective control programs. In the past, diagnostic tests are based on antigens which were cross reactive among the closely related mycobacterial species and thus, had poor specificity. Also, diagnostic tests based on direct observation of the bacilli have poor sensitivity since they require a large number bacilli in the specimen used for the test. Also, current diagnostic tests are not suitable to be used in resource limited setting where mycobacterial infections are endemic, due to requirement of proper laboratory settings. Diagnosis of

mycobacterial species based on species specific lipidic antigens has been validated in many recent studies. In the following chapter, we focused on summarizing the available diagnostic tests, discussion on recent development in the technology of diagnostics and provided a perspective on the future of lab-free diagnostic devices for mycobacterial infections.

Optimization of serum EVELISA for milk testing of Johne's disease.

An important first step towards the control of JD is the identification of infected cattle. Another step would be to minimize the spread of MAP infections by applying regulations to prevent movement of animals from infected herds to JD-negative herds. These approaches of JD control require identification of MAP-infected animals with a diagnostic test. However, the currently available diagnostic tests have poor sensitivity. A consensus report recommended the use of enzyme linked immunosorbent assay (ELISA) for the control of JD in dairy herds to reduce the economic impact of the infection. Milk ELISA has been shown to have similar sensitivity as compared to the serum ELISA and has the potential to be used to identify cattle at high risk for MAP infection without costly visits for collection of serum and fecal samples used in traditional programs. Furthermore, herd-level estimates of MAP infection determined by routine, high-throughput analysis of available milk samples could be used to quantify and monitor the risk of individual cattle within the National Dairy Herd Information Association enrolled herds. Dairy owners have already invested in milk sample collection, so additional testing can benefit them in lower cost per test. Keeping in view the advantages of milk testing, we used antigens extracted by briefly vortexing the MAP bacilli in ethanol solution to develop a novel milk based ELISA. Using the optimized conditions for detection of antibodies in milk, 57 milk samples (37 animals – JD-positive by the fecal PCR test; 20 animals – negative and from

JD-free herds) from Holstein dairy cattle were tested. The in-house milk based ELISA showed improved performance when compared with a commercially available ELISA test.

Use of ethanol vortex enzyme linked immunosorbent assay for diagnosis of bovine tuberculosis in farmed red deer (Cervus elaphus):

The infection of MB in wildlife species can cause problems of biodiversity and species conservation. Also, some of the wildlife species can act as reservoir of the MB infection and spill it over to the domestic livestock population. Since postmortem examination of each suspected animal is not practical, serological tests for the detection of bTB have been recommended. Previous studies have developed diagnostic tests based on crude extracts and specific molecules in a single or multiple antigen formats, but still these tests have poor specificity. Co-infection with MAP has been found as a confounding factor for the poor performance of these tests. We used ethanol extract of MB and developed a serum based ELISA test for the detection of anti-MB antibodies. To detect the presence of anti-MB antibodies, a total of 50 red deer samples from 4 different studies in New Zealand were tested using heat killed MAP (sheep strain) as absorbent of cross reactive antibodies. The tentative diagnostic sensitivity and specificity was estimated to be 90% and 93.3%, respectively. The results from this study suggest that ethanol extract based ELISA could be used to diagnose bTB in red deer with minimum false positive results.

Bead-based microfluidic immunoassay for diagnosis of Johne's disease:

Microfluidics or Lab-on-a-chip technology, has been used in various analytical processes, including electrophoresis, single-cell analysis, biochemical assays, and immune assays. The technology offers opportunities for the development of point-of-care diagnostic devices for

various infectious diseases. In the recent years, rapid diagnostic tests have been developed based on multiple antigens, which are highly purified. However, these molecules are complicated and costly to isolate. Ethanol extract coated magnetic beads were used in flow cytometric and microfluidic systems to develop an onsite diagnostic device for JD. A total of 155 well classified serum samples from JD-positive and JD-negative cattle were tested in the bead-based flow cytometric assay. The specificity for this assay was estimated to be 98%. The sensitivity was estimated to be 45.7%, 48.6% and 85.7% for low, medium and high shedders, respectively. Antigen coated magnetic beads were also tested in the microfluidic system using 5 serum samples and a high level of correlation (linear regression, $r^2 = 0.994$) was observed with the bead-based flow cytometric assay. The results from the study suggest that this technology could form a basis for onsite diagnosis of JD.

CHAPTER 2

OPPORTUNITIES FOR IMPROVED SERODIAGNOSIS OF HUMAN TUBERCULOSIS, BOVINE TUBERCULOSIS AND PARATUBERCULOSIS

A version of this chapter hasn't been published anywhere but is submitted to the special issue on Mycobacterial diseases to be published by the Journal of Veterinary Medicine International.

The manuscript based on this chapter has been reviewed by 3 reviewers and it was recommended for minor revisions. The reviewers have asked us to divide the various sections into sub-sections and put more emphasis on recent developments in the field of lab-on-a-chip technology for infectious diseases.

Opportunities for improved serodiagnosis of human tuberculosis, bovine tuberculosis and paratuberculosis

Ashutosh Wadhwa, Graham J. Hickling and Shigetoshi Eda

2.1 Abstract: Current serodiagnostic methods for tuberculosis (TB), bovine tuberculosis (bTB) and Johne's disease (JD) exhibit low sensitivity and/or specificity, due in part to potential exposure of test subjects to other environmental mycobacteria. A review of recent attempts to improve diagnostic test performance indicates that the use of multiple antigens can improve the efficacy of serodiagnosis of these mycobacterial diseases. Most recent studies that have aimed to develop improved serodiagnostic tests have focused on protein antigens. Since mycobacteria are known to produce a variety of species-specific lipidic molecules, efforts to identify lipidic diagnostic antigens may be a useful contribution to the development of more specific tests for TB, bTB and JD. Accurate laboratory-free diagnostic devices would be particularly valuable in understanding the epidemiology of the mycobacterial infections and in facilitating their control.

The emergence of new technologies such as microfluidics and 'Lab-on-Chip' is creating promising opportunities for development of laboratory-free diagnostic devices for these mycobacterial infections.

Key words: *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium avium* subspecies *paratuberculosis*, onsite diagnosis, immunoassay.

2.2 Introduction

Mycobacterial infections are a leading cause of health concerns in humans and animals worldwide. *Mycobacterium tuberculosis* (MTB), *Mycobacterium bovis* (MB) and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) are the causative agents of human tuberculosis (TB), bovine tuberculosis (bTB) and Johne's diseases (JD), respectively. In 2009, more than 9 million cases of TB were reported, causing 1.8 million deaths [1]. Multidrug resistant TB strains and co-infections of TB and HIV are emerging problems globally [2-4]. Despite much progress in eradicating bTB in developed countries, this disease is nevertheless responsible for US$ 3 billion economic losses globally [5], and remains prevalent in some wildlife species [6-7]. MAP is present in 68 % of US dairies [8], with JD responsible for an annual $220 million economic loss to the US dairy industry [9].

Control measures for these mycobacterial diseases revolve around understanding their epidemiology and improving treatment/vaccination protocols, however a major bottleneck has been the lack of efficient diagnostic methods [2, 10-12]. Consequently, there would be much benefit to the development of rapid and accurate diagnosis of TB at point-of-care [3], however,

efficient laboratory-free (lab-free) diagnostic devices for TB are not yet available [13-14]. Similarly, the most common current diagnostic test for bTB, the tuberculin skin test (TST), is not practical for controlling of bTB in wild animals, so a lab-free diagnostic device would also be helpful in this context. Diagnosis of JD is currently conducted annually or bi-annually in diagnostic laboratories. If a lab-free diagnostic device becomes available, it would reduce the long time interval and cost of diagnosis. Thus, there would be great value in lab-free diagnostic technologies for TB, bTB and JD [13, 15].

Here we briefly review currently-available and recently-developed diagnostic methods for these three mycobacterial diseases, and highlight the potential benefits of lab-free diagnosis. Since serodiagnosis has been the most favored format for development of lab-free diagnostic method, we focus this review on methods of serodiagnosis over other diagnostic methods such as bacterial culture and nucleic acid amplification that are necessarily laboratory-based.

2.3 Human tuberculosis

Human tuberculosis (TB) is caused primarily by MTB, and occasionally by MB and *M. africanum* (in this paper we focus on MTB). TB is a leading cause of morbidity and mortality throughout the world [16]. One-third of the world's population is infected by MTB although only 5-10 % of infected individuals develop an active, life-threatening form of the disease. In 2009, 9.4 million cases of TB were reported with 1.8 million deaths worldwide [1-2]. MTB is transmitted through aerosols from infected individuals and attacks the pulmonary system. It also affects brain, spinal cord, circulatory, gastrointestinal, uro-genital systems; skin, bones and joints. Active TB is a progressive disease that progressively impedes vital organ function, leading to severe morbidity and death [17].

Depending on the pathogenesis, infectivity, immune response and effectiveness of treatment, TB can be divided into 3 major forms. The first is the active form of TB (TBA), which results in rapid development of clinical signs in patients following contact with MTB. TBA develops in only 5% of infected with MTB; the remainder develops a strong acquired immune response showing no clinical signs, termed latent TB (TBL) [18]. Infection will reactivate in a small proportion (1-2%) of individuals with TBL, who then develop clinical disease. The TBL group, which constitutes one-third of the world's population, is most important in terms of controlling MTB infections [19]. The third form is multidrug resistant TB (MDRTB), which constitutes approximately 5% of the global TB cases [20]. MDRTB is caused by organisms resistant to, at least, isoniazid and rifampin [21]. The overall prevalence of MDRTB in developed nations is much lower than that in developing nations, but can be high in immigrant populations and among prisoners and immuno-compromised individuals [22, 23]. In the last two decades, the emergence of HIV infection led to the recognition that TB/HIV co-infection promotes both the reactivation of TBA from TBL and also the rapid progression of primary TB following recent exposure to MTB [22].

Controlling TB depends on the following factors - case detection, treatment of individuals with TBA, improving anti-TB therapy to prevent resistance, identification of TBL, and better vaccination strategies for susceptible individuals [16]. All these factors regarding control revolve around the need for a better understanding of the epidemiology of the TB infection [23] and the development of evidence-based approaches for its diagnosis [24]. Efficient diagnosis of TB is particularly important in third world nations that lack proper diagnostic resources at primary health care centers. In these nations, TBL and MDRTB often remain undiagnosed, which facilitates further transmission.

Presently, there are a number of alternative diagnostic approaches towards diagnosis of TB, and of TB co-infection with other emerging infectious diseases. Radiographic imaging is still widely used to diagnose TB, however previous studies have shown that there are no definitive diagnostic patterns, so that the method can be used only for screening of TB cases; further bacteriological examinations are required for confirmation [19, 25]. Smear microscopy of stained sputum or other clinical material is the most common test for TBA. This relatively inexpensive method can be carried out rapidly in low resource settings, however it lacks sensitivity and requires a large number of bacilli (5,000-10,000 organisms/sample) [19, 26] in the clinical specimen, which is often not the case in children, advanced stage TBA patients and individuals co-infected with HIV. Fluorescent microcopy is more sensitive, but its application is limited by cost and by issues relating to the use of mercury vapor lamps in conventional fluorescent microscopes [27]. Nucleic acid amplification (NAA) assays have been found useful for diagnosis of TBA and MDRTB infections, as they have high specificity and sensitivity, and can provide results within few hours. Unfortunately, these assays are costly, require a laboratory with trained staff, and suffer from poor specificity under field conditions [19, 28-29].

Bacterial culture is considered the gold standard for TBA diagnosis, having almost 98% specificity, and is also useful in diagnosis of MDRTB. However, the bacterial culture method suffers from low sensitivity (26-42%), a long growth period (6-8 weeks), the requirement of trained personnel, and the high cost of the culture examination. The need for technical expertise can be particularly problematic in developing nations. Attempts are being made to improve this assay by incorporating a number of growth indicators but culture still requires optimization at various levels - detections time, susceptibility testing against anti-TB drugs and need for disposal of radioactive waste [19, 30-31]. Parsons et al. (2011) has suggested new technologies, including

urine antigen detection, assays based on volatile markers, bead based and flow cytometric based assays [3], but these assays require optimization and establishment of clinical utility.

The tuberculin skin test (TST) – based on detection of delayed-type hypersensitivity after an intradermal injection of purified protein derivative (PPD) extracted from heat killed MTB – has been in use for almost a century. The primary roles of TST are to identify TBL individuals and to monitor recent infection in high risk groups. Some limitations of TST include a high frequency of false reactions, the need for a follow-up visit after 2-3 days of PPD inoculation, misleading results due to confounding factors (e.g. age, HIV infection, and infection with other mycobacterial species or cancer) and positive reactions in TBA patients [19, 27, 32]. Based on the identification of MTB-specific antigens using molecular techniques, detection of cell mediated immune (CMI) response against MTB infection has improved the diagnosis of TB. These assays measure the production of cytokines (mainly interferon-gamma [IFN-γ]) produced by T cells of MTB-infected individuals. Initial IFN-γ assays were based on PPD antigen but later the antigen was replaced by MTB specific antigens, such as early-secreted antigenic target (ESAT-6) and culture filtrate protein (CFP-10) [33]. IFN-γ assays do provide an improved diagnosis of TBL; however, since they detect the presence of the host's CMI response towards MTB antigens, fresh blood samples are required for the test. Inability to differentiate between TBA and TBL, poor reproducibility, and reduced efficacy in children are additional problems of the CMI-based diagnostic tests [34]. In developing countries, TST is still preferred over IFN-γ assay due to its lower cost; but suffers from low efficacy in children, poor reproducibility, and reduced diagnostic accuracy for TBL [34-36].

In circumstances where medical resources (facilities and health care providers) are limited, serodiagnostic methods for detection of anti-MTB antibodies have some advantages (i.e.,

simplicity, low cost, and requirement of minimum medical resources) over aforementioned diagnostic methods [37]. Several target molecules (antigens) have been used to detect the humoral responses (anti-MTB antibodies) in TB patients. Early assays used PPD or other crude molecules as antigens for capturing anti-MTB antibodies; however, these showed poor specificity as dominant antibody responses are against cross-reactive antigens (i.e., antigens commonly found in MTB and also in other mycobacteria) [38]. As molecular techniques have improved, many antigens have been evaluated in serological tests, especially in the format of the enzyme linked immunosorbant assay (ELISA). Some major antigens used in such tests are discussed below.

Antigen 5, also known as 38 kDa antigen, is the best studied and most available antigen for MTB diagnosis due to its expression in the *E. coli* system. Many attempts to develop an improved serological assay for TB have used this antigen [39-40]. Early studies reported 89% sensitivity and 100% specificity in TBA patients [41]. Later studies showed even higher sensitivity, proving a correlation between antibody level and bacterial load [42-45]. As summarized in a review article [46], detection of antibodies against Antigen85 complex in ELISA formats achieves 50% sensitivity; however, this complex is highly cross-reactive and may causes false positive results in individuals infected with atypical mycobacteria. A cell wall component, called a cord factor (trehalose-6,6'-dimycolate), used as antigen in ELISA format achieved 84% sensitivity with 100% specificity [47]. However in a following study, it was shown that anti-cord factor antibodies decline after anti-tuberculous chemotherapy, which makes it difficult to determine the status of the infection [48]. Studies of the serodiagnostic potential of ESAT-6 [49-50] and CFP-10 [49-52] have also been conducted. One showed low sensitivity (67%) and specificity (51%) for ESAT-6 [49]. Low sensitivity (48-63%) also has been reported

for CFP-10 [49, 53]. In high incidence areas, neither ESAT-6 nor CFP-10 antigens are useful in differentiating between TBA and TBL [49]. Another antigen, Kp 90, has been used in ELISA format to detect IgA antibodies against the protein; the results, when compared with NAA and other serological assays, indicated that anti Kp 90 antibodies were detected in 78% of serum samples and 69% of samples from synovial, cerebrospinal and abscess body fluids. [54].

Antigen 60 (A60) is the main thermostable component of PPD [55-56]. Many studies have used this antigen and found almost 100% specificity [57], with sensitivity ranging from 68-91% [58-59]. Unfortunately, this molecule has also been found in *Norcardia* and *Corynebacterium* species [60]. A 30 kDa antigen (isolated from a culture filtrate of MTB, Antigen 85B) was used in dot immunoassay and the result compared with that of standard plate ELISA. The specificities of the dot immunoassay and ELISA were 92% and 97%, respectively, and the sensitivities in the assays were 69% and 78%, respectively [61]. Further studies showed that this antigen not only diagnosed TBA but also detected non-the protective immune response of a healthy household contact group [62].

Malate synthase (MS), a 81 kDa protein (present in MTB culture filtrates, cell wall, and cytoplasmic subcellular fractions) is an enzyme of the glyoxylate pathway used by MTB during intracellular replication in macrophages [63]. Studies with an MS-based assay have shown a sensitivity of 73% and specificity of 98% in smear positive patients, suggesting that MS is a potential candidate for TB diagnosis [53, 64]. The cell wall of MTB also contains lipoarbinomannan (LAM), however its use as antigen in diagnostic tests is limited due to immune complex formation [3]. LAM antigen is found in urine of TBA patients and tests based on detecting the LAM in urine samples have been developed [65-67].

Steingart et al. (2007) conducted an intensive meta-analysis of 67 studies published 1990-2006 on commercial serological tests for TBA (e.g. Detect-TB, and a-TB ELISA, ICT TB test) [37]. Antigens used in the commercial tests include Antigen 60, 38-Kda protein, LAM, and Kp-90. The meta-analysis revealed that estimated diagnostic sensitivities (0-100%) and specificities (31-100%) in the studies were inconsistent and imprecise, which is consistent with a WHO report in 2008 [68].

In patients co-infected with HIV and MTB, the level of antibody production to TB antigens differs from that of HIV-negative TB patients. For example, an ELISA based on MS/MPT51 antigens showed positive reactions in approximately 80% of HIV positive, TB positive patients and in 42% of HIV-negative, TB-positive patients. [69]. Wanchu et al. (2005) suggested that better diagnosis of TB will require a focus on development of multi-antigen based tests and identification of novel MTB proteins that increase in HIV patients [70].

The studies described above indicate that an improved diagnostic test is needed that is better able to differentiate the various forms of mycobacterial infection and to diagnose TB in the presence of HIV infection. Furthermore, since most deaths due to TB occur in developing countries that lack proper laboratory facilities and specialist training, it is important to develop a simple, rapid and cost-effective test. The Xpert MTB/RIF assay has been recently used as point-of-care diagnosis for MDRTB and drug sensitive TB [71-72]. Although simple to perform and highly sensitive, this assay is costly [73]. McNerney and Daley (2011) have summarized the importance of point-of-care diagnosis [74] and suggest three important areas in which progress should be made to achieve better point-of-care for TB. The first is through identification of biological, metabolic and pathogen derived markers that will assist in understanding the disease. The second is the development of effective technologies like immunochromatography and

nanotechnology. The third is to better understand the economical and logistic constraints on the implementation of new tests [74]. In summary, there is an urgent need to develop a lab-free diagnostic device for TB that will decrease disease transmission rate, reduce death rates and permit faster initiation of treatment.

2.4 Bovine tuberculosis

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* (MB), is an infectious, chronic but progressive disease characterized by the formation of granulomatous lesions with varying degrees of necrosis, calcification and encapsulation [11]. MB is known to infect and cause tuberculosis in a wide range of wild animals, livestock animals, and humans. Although bovine bTB has been mostly eradicated in the livestock industry of developed countries, the disease in wildlife still poses a risk to livestock, tourism economy, and wildlife conservation [11]. Infected wildlife species include white-tailed deer (*Odocoileus virginianus*) in several states of the USA, Eurasian badgers (*Meles meles*) in Great Britain and the Republic of Ireland, and brushtail possums (*Trichosurus vulpecula*) in New Zealand [6]. Global economic losses from bTB total US$ 3 billion annually. In the USA, US$ 40 million and in Great Britain £100 million were spent on bTB management in the year 2008-2009 alone [5]. In developing countries, bTB still causes serious concerns not only for wildlife, but also for public health, food safety, and the economy of livestock industries.

Despite progress towards national eradication of bTB from livestock [5], a few U.S. states including Michigan and Minnesota continue to struggle with bTB in their wildlife [7, 75]. While many mammal species can become infected with MB, the white-tailed deer is one of the very few wildlife species that can act as a maintenance host for this organism [76]. In states with

infected wild deer, bTB is also found in cattle operations, with transmission from the wild deer being suspected. Mandatory testing of cattle has cost $3.25 million per year in Minnesota [75]. bTB in wild, white-tailed deer in northeastern Michigan was first recognized in 1994 [77]. The Michigan Department of Natural Resources tested deer samples from 1999 until March 2009 and has found 632 positive samples of the 178,502 samples tested [78]. Current testing procedures for wild deer rely almost exclusively on inspection for gross lesions during postmortem of hunter-collected carcasses, which is thought to achieve only about 50% sensitivity.

In addition to the risk of infection from resident wildlife, the United States imports an average of 1.4 million beef calves annually from Mexico [79]. In spite of all efforts, a few of these animals are infected with bTB and thus are a possible source of infection to the cattle in the US. These infected Mexican cattle can be difficult to detect by screening at the border, because of lack of reactivity (i.e. false negative reactions) [79]. It has been suggested that regionalization would facilitate trade of animals and assist in bTB control by acting as biosecurity barrier against the spread of infection and by managing surveillance programs [80]. There are currently no effective vaccines or medications for bTB in animals, and therefore the most effective method of disease control is to test and cull infected animals. Given the current lack of accurate live-animal diagnostic tests for bTB, this test-and-slaughter approach can result in a large number of healthy animals being depopulated [81].

More accurate diagnosis of bTB would provide an opportunity to reduce the unnecessary sacrifice of healthy animals and would also help to more effectively control bTB. At present, postmortem diagnosis based on examination of gross lesions, followed by histopathology and culture, is widely used for surveillance of bTB in wild animals, but this method is time-consuming and cannot diagnose an early infection [82]. The ante mortem diagnostic method

currently prescribed by OIE is the intradermal tuberculin skin test (TST) [83]. The TST is by far the most effective test used in the eradication of bTB in the developing countries. The test is performed by injecting a small volume of bovine tuberculin in the skin of the animal and palpating a change in the thickness of the skin at the site of injection after 48-72 hours. The tuberculin used in most of the countries is derived from cultures of MB AN5, a field strain isolated in England circa 1948 [5, 82, 84]. The TST is, however, susceptible to causing false positive reactions due to exposure of some animals to environmental mycobacteria such as *M. avium* and MAP [82, 85-87]. TST can also cause false negative reactions due to immunosuppression, desensitization towards tuberculin, sub-potent use of tuberculin, and time since exposure to field strain [82]. Steps have been taken to improve the specificity by using specific antigens, such as ESAT-6 [88] and a cocktail of ESAT-6/CFP-10/ MPB83, however these studies still need to be validated at a larger scale [5].

Revisiting the animal after 2-3 days application of the TST to check their reaction is labor intensive and usually impractical for wildlife species. The alternative IFN-γ assay is an *in vitro* blood test based on measuring the CMI response of the animals infected with MB [89]. The IFN-γ assay is performed mainly using PPD as antigen but recent studies have also used ESAT-6 and CFP-10 [90-93]. IFN-γ assay has some advantages over the TST — early detection and no need of revisiting the animal. A problem with the IFN-γ assay is that it is a costly process and requires well trained personnel to carry out the test [84, 94]. Bacteriological culture of clinical samples (i.e., milk, blood, nasal swab and cattle tissues) is considered to be the gold standard for bTB diagnosis but is a long process that may take several weeks [82, 95]. Nucleic acid amplification methods (e.g. PCR) have been also used for bTB diagnosis but these methods are

costly, less sensitive than the bacteriological culture test, and require a trained technician to perform the test [82, 95-97].

Another type of immunological test is based on detection of humoral immune response (i.e., antibody production). The major advantages of the antibody-detection tests are that they are inexpensive and relatively easy to perform. However, sensitivity of the antibody-detection tests remains a concern. Several attempts have been made to develop ELISA tests for detection of antibody response against MB infections. PPD was used as an antigen to measure antibody response in animals with MB infection [98-99], but the cross reactivity of PPD with closely related mycobacterial species has always been a concern. Auer (1987) used sonicated preparation of MB as antigen and reported low specificity [100]. Further studies used a specific protein isolated from MB bacillus Calmette-Guerin (BCG) strain, MPB70, as an antigen for developing assays for the diagnosis of bTB. The use of MPB70 suggested a better specificity but had poor sensitivity [101-102]. Ag85 complex consists of the major secretion products of MB BCG strain and have 3 major components 85A (31 kDa), 85B (30 kDa) and 85C (31.5 kDa). The Ag85 complex is strongly immunogenic and has been used for development of assays to diagnose TB and bTB. However, low sensitivity was reported from studies using Ag85 in ELISA format and attributed to false positive reactions caused by infections with environmental mycobacteria [46, 102-103]. MPB83 has been used as antigen in many studies and is a very promising candidate for bTB serodiagnosis [104]. As discussed in the TB section, LAM, ESAT-6 and CFP-10 were also used as antigens to detect antibody response against MB [105-110]. Further, as molecular biology tools have improved, recombinant proteins have come to be used as antigens for diagnosis of bTB. Since recombinant proteins can be produced at large scale, they are cost-effective and provide consistency in their quality as diagnostic antigen [111-113]. One of the

promising antibody-based detection assays, Multi-Antigen Print Immuno-Assay (MAPIA), is based on immobilization of antigens onto nitrocellulose membranes by semi-automated microspraying, followed by standard chromogenic immune development. This serodiagnostic test uses a cocktail of multi-antigens, such as MPB83/70, ESAT-6, and CFP10 [114].

In a recent study, seroreactivity with MPB83 in deer was 89%; however, the MAPIA showed that 26% of the results were false positives in deer [115]. Based on these MAPIA results, a new version of an immunochromatographic test format for rapid diagnosis of MB infection, called rapid test (RT), was developed using colloidal gold conjugated to protein A. RT uses a recombinant proteins of MPB83 and TBF10 printed onto a membrane either separately as two bands or as a combination of the two antigens in one test line [116]. Diagnostic sensitivity of the RT in experimentally-infected deer was 79%, whereas that in naturally-infected deer was 67% [115]. Some recent studies have concluded that ESAT-6 and CFP10 (used either individually or as cocktail) are better candidates for diagnosis of bTB [117-119].

MAPIA and RT can be conducted in field and so can contribute to effective testing/control of bTB, especially in wild animals. However, interpretation of the test results in MAPIA and RT relies on observation of color development on a strip, which may vary depending on examiners. Higher accuracy and consistency could be achieved via a lab-free diagnostic device that outputs numerical data based on level of antibody binding to MB antigen(s). Further, as we discussed above, more efforts need to be directed towards identifying a better antigen (or a combination of antigens) to further improve diagnostic sensitivity and specificity.

2.5 Johne's disease

Johne's disease (JD) or paratuberculosis is a chronic infectious enteritis of domestic and wild ruminants, causing reduction in milk production, malnutrition, weight loss and eventually death [120-121]. The agent is *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a hardy, slow growing, gram-positive, and acid-fast bacteria. H. A Johne and L. Frothingham initially reported the disease in Germany in 1894, however, it was not until 1910 F. W. Trowt successfully fulfilled Koch's postulates by growing MAP in the lab and reproducing the disease in experimentally infected cattle [122]. JD is prevalent worldwide and has a significant impact on global animal husbandry. In the US, MAP is found in 68 % of dairies [8], with average herd-level prevalence of JD is estimated to be 22%. MAPs impact on the dairy industry in the United State (US) is significant, causing an estimated annual loss of $ 220 million [9]. Economic losses associated with JD arise from decreased milk production, reduced fertility and higher rate of culling [123]. In addition to the economic impact of JD to dairy industry, it is possible that MAP plays a role in Crohn's disease, which is an inflammatory bowel disease in humans [124]. These economic and possible health concerns create an urgent need for improved control of JD. As no practical treatment is available for JD, a better understanding of the transmission, detection and management of the disease are the recommended procedures for its control [125]. Epidemiological studies of MAP have been hampered by the fact that current diagnostic tests suffer from lack of sensitivity and are incapable of detecting latent MAP infections.

Diagnostic tests to detect infection with MAP can be categorized into those that identify the organism and those that identify the immunological response to the organism. Fecal culturing for MAP using Herrold's egg yolk medium (HEYM) has been considered as a gold-standard test for JD diagnosis; however, it takes as long as 16 weeks to see an observable growth. Other

approaches, such as the use of BACTEC radiometric liquid culture [126-127] and MGIT culture medium [128], have been examined to reduce the culture time but these approaches require a specialist and are relatively expensive. Polymerase chain reaction (PCR) based diagnosis using IS900 insertion sequence [129] or F57 DNA fragment [130], on the feces of suspect animals can also be used. This PCR-based approach is much faster but is less sensitive than the culture test because PCR reaction can be inhibited by substances in the feces. Animals develop both CMI and humoral responses against MAP. CMI responses in the early stages of JD include recruitment and proliferation of $CD4^+$ Th1 cells, $CD8^+$ cells, and peripheral blood mononuclear cells. The recruited immunocytes secrete high levels of cytokines, including IFN-γ, which can be detected serologically. In late stage of JD, a shift from the CMI to humoral response (antibody production) is generally observed [131-132]. A CMI-based diagnostic test, the IFN-γ assay, has been evaluated using blood samples of experimentally-infected cattle. The study demonstrated that the IFN- γ assay could detect MAP infections in early stage of JD [133-134]; however, IFN-γ assay is affected by antigen stimulation and blood sampling - storage conditions [135-136]. This suggests that the IFN-γ test requires further optimization.

Three different tests are used to measure antibody response in JD – complement fixation, agar gel immunodiffusion, and ELISA. The complement fixation and agar gel immunodiffusion tests both suffer poor sensitivity [137], and so a recent report has suggested that ELISAs are the best of the three methods for controlling JD in dairy and beef herds [125]. Diagnoses of JD using ELISA have been reported in many previous studies using different antigens [134, 138-148]. The antigens used in these studies have used protoplasmic antigen (PPA) [140-143, 145, 147], lipoarabinomannnan (LAM) [138], culture filtrate of MAP [146] and MAP proteins – 1152 and 1156 [148] for testing antibodies against MAP. Beam et al. (1969) described a crude antigen

mixture termed PPA, which is prepared by thorough physical disruption of mycobacterial bacilli followed by removal of cell debris and cell wall components [149]. Although many investigators have prepared PPA using various preparation protocols, it contains proteins very similar to proteins commonly found in closely related mycobacteria species. LAM is one of the components of the cell wall of mycobacteria species [108] and its core structure is shared among mycobacterial species [150].

Sweeney et al., (1994) tested milk and serum samples in a LAM-based ELISA to detect antibodies for JD diagnosis and found that sensitivity and specificity of the ELISA were similar regardless of the tested samples (i.e. milk and serum) [138]. McKenna et al., (2005) compared diagnostic performance of PPA-based ELISA and LAM-based ELISA using fecal culture test as a gold standard. Sensitivity and specificity of the PPA-based ELISA were higher than that for the LAM-ELISA [151]. PPA and LAM both contain structures common in mycobacterial species, so the use of these molecules as diagnostic antigen can cause false positive reactions in animals infected with environmental mycobacteria other than MAP [152].

Bannantine et al., (2008) tested 18 purified recombinant proteins in ELISA format for serodiagnosis of ovine paratuberculosis. They found that MAP proteins 0862 and 3786 demonstrated the strongest antibody response and MAP protein 2116c the weakest [153]. Shin et al. (2008) used culture filtrate of a MAP strain, JTC, in ELISA format for JD diagnosis and named the method JTC-ELISA [146]. JTC-ELISA showed significantly higher sensitivity than that of commercial ELISA tests and performed effectively on both serum and milk samples. As mentioned above that the recommended control measure for JD is testing herds by ELISA methods but the current ELISA tests have low sensitivity (28 – 44.5%) [142]. We have previously reported that the surface antigens of MAP are capable of detecting anti-MAP

antibodies in serum at early stages of JD [154-157] and developed an ELISA for JD, named ethanol vortex ELISA (EVELISA). The results from EVELISA showed that 97.4% of the JD positive samples had higher antibody binding levels than those of JD negative samples [156-157].

ELISA, as well as other methods for JD diagnosis, needs to be conducted in diagnostic laboratories employing staff with expertise in microbiology, molecular biology, and immunology. This requires a labor-intensive process involving collecting samples into proper containers, indexing, packing, and shipping. Furthermore, cost per sample is relatively high -- testing a sample by current fecal culture, PCR, and ELISA tests cost $16-19, $25 and $5-6, respectively, and this does not include costs associated with site visits and sample collections and shipping [125]. Because of the labor and cost for the current JD diagnosis, screening of cattle herds for JD is generally conducted at an interval of 6 – 12 months. During this interval, non-shedding animals can become shedders and low-shedding animals can become high shedders, thereby spreading MAP infection widely in the herd. This relatively long time interval between JD screening tests, in combination with low sensitivity of current diagnostic tests, may have been a reason that MAP infections remain so widespread in the U.S. dairy and beef industries.

Controlling JD requires a better understanding of the spread of MAP in a dairy herd, which can be achieved by continuous monitoring of the infection using a lab-free diagnostic device. For development of a lab-free diagnostic device, microfulidic technology has begun to be employed in the last decade [14]. Microfluidic devices are state-of-the-art tools for biochemical and immunological analysis that have high sensitivity, require only short periods of time, small amounts of reagents and do not require an expert operator [13, 15, 158]. In our recent study, we developed a prototype of lab-free diagnostic device for JD by using a microfluidic technology and the antigen used in the EVELISA test [159]. The major advantages of this system are its low

cost, ultraportable and disposable immunoreactions chip, and the ability to detect antibodies within 20 min [159].

2.6 Conclusion

As discussed above, current serodiagnosis for TB, bTB and JD suffers low sensitivity and/or false positive reactions (causing low specificity), arising from the potential exposure of tested individuals other nonpathogenic environmental bacteria. Recent studies have indicated that the use of multiple antigens may improve diagnostic accuracy of the serodiagnosis of the mycobacterial diseases. Most recent efforts to develop improved serodiagnostic tests have focused on protein antigens. Since mycobacteria are known to produce a variety of species-specific lipidic molecules, efforts to identify lipidic diagnostic antigens could be a particularly useful contribution to the development of more specific tests for TB, bTB and JD. Accurate lab-free diagnostic devices would be valuable in understanding the epidemiology of the mycobacterial infections and would facilitate their control. The emergence of new technologies such as microfluidics and 'Lab-on-Chip' hold considerable promise for accelerating the development of lab-free diagnostic devices for these mycobacterial infections.

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CHAPTER 3

OPTIMIZATION OF SERUM EVELISA FOR MILK TESTING OF JOHNE'S DISEASE

A version of this chapter hasn't been published anywhere but is submitted to the Journal of Foodborne Pathogens and Disease.

The manuscript based on this chapter has been reviewed by 3 reviewers. It was recommended for major revisions. A revised version has been submitted. The editor of the journal asked us to reduce the word count to 3000 words. The reviewers had asked us to incorporate information on PCR test. It was done as per information provided by the co-author Dr. Todd Byrem and Traci L. Stein. They also asked us to do some optimization experiments. This was taken care by statistical analysis

Optimization of serum EVELISA for milk testing of Johne's disease

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Running title

Milk testing for Johne's disease

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3.1 Abstract

Johne's disease (JD) or paratuberculosis, caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP), is one of the most widespread and economically important diseases of livestock and wild ruminants. Control of JD could be achieved by good herd management practices and diagnosis; however, this approach has been hampered by the fact that the currently available commercial

diagnostic tests suffer low sensitivity. In our previous study, we developed a sensitive serum ELISA test, named EV (ethanol-vortex) ELISA using surface antigens of MAP extracted by a brief agitation of the bacteria in 80% ethanol using a vortex mixer. The objective of this study is to demonstrate that the EVELISA can be used for detection of anti–MAP antibodies in milk samples. In this study, we tested and optimized concentrations of antigen, milk, and secondary antibody for better differentiation of milk samples of cattle with MAP infections from those of cattle in JD–free herds. We evaluated five environmental mycobacteria as absorbents of cross reactive antibodies in milk and found that the mycobacteria had no significant effect on EVELISA results. Using the optimized conditions, a total of 57 milk samples from Holstein dairy cattle (37 animals found positive on the fecal PCR test and 20 animals from JD-free herds) were tested for anti-MAP antibody in milk by using the EVELISA method. The average of ELISA values in the JD-positive milk samples (mean ± SD = 0.355 ± 0.455) was significantly higher than that in the JD-negative milk samples (mean ± SD = 0.071 ± 0.011). These results warrant further studies for evaluation and validation of the EVELISA for milk testing of cattle for JD.

Key Words: *Mycobacterium avium* ssp. *paratuberculosis*, Johne's disease, milk, ELISA.

3.2 Introduction

Johne's disease (JD), caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP), is a chronic infectious enteritis of domestic and wild ruminants. The disease is economically very important causing a loss of 200- 250 million US dollars annually to the US dairy industry (Ott *et al.*, 1999). A regression model estimated that economic losses caused by JD in dairy farms is in the range of

$40 to $227 per cow per year (Ott *et al.*, 1999). Most of the economic losses associated with JD are related to decreased milk production, reduced fertility and higher rate of culling (Nordlund *et al.*, 1996). In addition to the economic impact of JD to dairy industry, a number of reports suggested a possible role of MAP in the inflammatory bowel disease in human, Crohn's disease (Wynne *et al.*, 2011). Also, some recent reports suggested a link between MAP infections and type-I diabetes (Cossu *et al.*, 2011; Sechi *et al.*, 2008). Since MAP bacilli were found in dairy products (Ayele *et al.*, 2005; Ellingson *et al.*, 2005) and meats (Alonso-Hearn *et al.*, 2009), it is important to control JD to avoid the potential human health concerns caused by MAP. A recent report recommended that ELISA should be used for controlling the disease in dairy as well as beef herds (Collins *et al.*, 2006). However, current ELISA tests were reported to have low sensitivity (28 – 44.5%) (Collins *et al.*, 2005). We previously reported that the surface antigens of MAP are capable of detecting anti-MAP antibodies in serum at early stages of JD (Eda *et al.*, 2006; Eda *et al.*, 2005; Scott *et al.*, 2010; Speer *et al.*, 2006) and developed an ELISA for JD, named ethanol vortex ELISA (EVELISA). The results from EVELISA showed that 97.4% of the JD positive samples had higher antibody binding levels than those of JD negative samples (Eda *et al.*, 2006; Scott *et al.*, 2010).

Serum ELISA is similar to milk ELISA in terms of the testing time and cost of the assay. However, since milk samples from individual cows are routinely collected on dairy farms enrolled in the Dairy Herd Improvement Association (DHIA) testing, milk ELISA is less labor-intensive and less invasive for testing dairy cattle as compared to serum ELISA. In this study, we tested if the EVELISA method can be used to detect anti-MAP antibodies in milk samples of dairy cattle naturally infected with MAP.

3.3 Materials and Methods

3.3.1 Milk samples

A total of 57 serum samples used in this study were obtained from two dairy herds in Michigan. Twenty samples were from JD negative herds (JD-negative samples) and 37 samples were from cattle tested positive for JD by a PCR test (JD-positive samples). Ten JD-negative and 10 JD-positive samples were selected for optimization of ELISA conditions and the remaining samples were added for evaluation of the optimized condition. The details of the samples and grouping were described below.

Group N1: A total of 10 Holstein cattle from a dairy herd in Michigan were included in this group. The herd is a level 2 status dairy herd according to the National Voluntary Johne's Program. A herd is classified as level 2, if it is negative for ELISA followed by MAP detection test and its size is less than 100 (USDA, 2010). The animals were tested negative for JD by fecal PCR test (AntelBio, 2010) and ELISA (Parachek, PRIONICS, Schlieren-Zurich, Switzerland). The age and lactation history of the animals in this herd are not available.

Group N2: A total of 10 Holstein dairy cattle from a dairy in Michigan were included in this group. The whole herd was tested negative using the PRIONICS ELISA for 2 consecutive years in 2008 and 2009. The animals in this group ranged from 1-2 lactations and 2-3 years of age.

Group P1: A total of 10 Holstein dairy cattle from a dairy farm in Michigan were included in this group. The animals in this group were in their 1st to 3rd lactation. The age of the animals ranged from 4 to 5 years and they were tested positive for JD by the AntelBio fecal PCR test. The herd level test prevalence of this herd for JD at the time of sample collection (2009) was 8.7 %.

Group P2: Another set of positive samples were from the same herd of Group P1 in Michigan but consisted of a total of 27 Holstein dairy cattle that were tested positive for JD by the

AntelBio fecal PCR. The animals in this group ranged from their 2nd to 7th lactation. The age of the animals in this group ranged from 3 to 9 years.

3.3.2 ELISA procedures

Commercial ELISA: A commercial ELISA (IDEXX Laboratories, Westbrook, ME), termed ELISA-I in this study, was carried out according to the manufacturer's instructions. The ELISA-I was approved by the US Department of Agriculture for detection of anti-MAP antibodies in milk samples as well as serum samples. The milk samples were tested in duplicates for the presence of antibodies against MAP. The ratio of optical densities of samples and positive control (S/P value) was determined and JD status was assigned using the cut-off S/P value of 0.40 as recommended in the manufacturer's instructions.

EVELISA: EVELISA test was conducted as described previously (Eda *et al.*, 2006). In Figures 1A-1C, different dilutions of MAP ethanol extract, milk and secondary antibody were used to optimize the conditions as indicated in the figures. In Figure 1D, milk samples were incubated (room temperature, 30 min) with heat-killed mycobacteria (2 mg/mL; *M. phlei*, *M. chelonae*, *M. fortuitum*, *M. terrae*, and *M. flavescens* provided by Dr. P. Small, Department of Microbiology, the University of Tennessee, Knoxville) prior to EVELISA testing in an attempt to absorb cross-reactive antibodies in milk. In Figure 2, the cut-off value was set as mean + 3 standard deviation (0.1) of the OD values obtained using the 20 negative milk samples (Groups N1 and N2).

3.3.3 Antel Bio Fecal PCR test

For fecal sample processing, 2 g of feces was mixed in 25 mL of 0.2N NaOH for 30 min and coarse sediment removed by centrifugation at 300 x g for 3 minutes. The supernatant was harvested through 2 layers of cheesecloth and MAP concentrated into the pellet fraction by centrifugation for 30 minutes at 4000 x g. After washing, the pellet (30 minutes at 4000 x g) was

transferred into a microcentrifuge tube and disrupted by bead beating for 2 minutes with 0.5 g zirconium beads (0.1 mm) in 200 µL of PBS-1 mM EDTA. The clarified supernatant after centrifugation at 10,000 x g for 10 minutes was diluted 1:50 in PCR-grade water prior to direct analysis by quantitative PCR (qPCR) described below. The DNA primer and probe combination for MAP qPCR was designed by Primer Express 3 software (Life Technologies, Carlsbad, CA) for TaqMan chemistry to recognize a 65 base pair region of the repeat element IS900 in the MAP genome (Acc. No. X16923). The primers and probe sequences used for target amplification and detection were; forward primer, GCC TTC GAC TAC AAC AAG AGC; reverse primer, GCG TCG GGA GTT TGG TAG; and probe with a FAM-based detection dye, GCC GCG CTG ATC CTG CTT ACT. Amplification efficiency during qPCR was also monitored by the inclusion of internal positive control DNA (IPC), plasmid pCR2.1-TOPO containing the human actin gene (Acc. No. NM001101.3). The primers and probe for the amplification and detection of the 55 base pair target region of human actin were; forward primer, GCG CGG CTA CAG CTT CA; reverse primer, CTT AAT GTC ACG CAC GAT TTC C, and probe with a VIC-based detection dye, CAC CAC GGC CGA GC. The content of IPC used for qPCR was determined by titration and yielded cycle threshold (Ct) values of 32 ± 1. Quantitative PCR was performed in an AB 7500 Real-time Thermocycler (Life Technologies, Carlsbad, CA) using a 25-µL reaction volume containing 5% bovine serum albumin. Specifically, each reaction contained 12.5 µL TaqMan Universal Master Mix, 0.9 µM IS900 primers, 0.9 µM IPC primers, 0.1 µM FAM-labeled probe for IS900, 0.1 µM VIC-labeled probe for IPC, 0.125 ng IPC plasmid DNA and 9 µL of diluted sample extract or controls. The qPCR reaction conditions were as follows: 1 cycle of 95°C for 10 minutes; and 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. In addition to the IPC, each

test panel included positive (MAP derived DNA) and negative controls in PCR-grade water. Samples with Ct values less than 36 are considered positive for the presence of MAP.

3.3.4 Statistical analysis

All experiments were conducted in duplicate or triplicate and repeated at least twice. The statistical difference of antibody binding among various sets of conditions and between Group N1 and Group P1 were evaluated by using Mann-Whitney U test, due to lack of normality in the distribution of the data (calculated by Shapiro - Wilk normality test). The statistical analysis and depiction of box plots were conducted by using statistical software, R.

3.4 Results

3.4.1Optimization of antigen dilutions

Antibody binding was evaluated in EVELISA test with different antigen dilutions – 1:20, 1:40, 1:80 and 1:160. In this experiment, two groups of milk samples, Groups N1 (n=10) and P1 (n=10), were tested in duplicates using a 1:5 dilution for milk samples and a 1:1,000 dilution for secondary antibody. The difference between antibody binding in the two groups was greatest ($P < 0.001$, Mann-Whitney U test) at the 1: 20 antigen dilution (Figure 1-A). For 1: 20 antigen dilution, the average OD values for JD positive and negative milk samples were 0.249 ± 0.068 and 0.078 ± 0.024 (mean ± SD), respectively. Based on this result, the 1:20 dilution of ethanol-extracted antigen was used in the following experiments.

3.4.2 Optimization of milk dilutions

Milk samples of Groups N1 and P1 were tested for antibody binding in the EVELISA test using different dilutions (1:2.5, 1:5, 1:10 and 1:20) and a secondary antibody dilution of 1:1,000. The greatest difference ($P < 0.0001$, Mann-Whitney U test) was observed at the milk dilution of 1:2.5

(Figure 1B); however, levels of antibody binding in JD-negative samples (Group N1) were higher than other dilutions. Therefore, the milk dilution of 1:10 ($P < 0.001$, Mann-Whitney U test) was selected for the following experiments. For 1: 10 dilution of milk, the average OD values for JD positive and negative milk samples were 1.2 ± 0.479 and 0.195 ± 0.146 (mean ± SD), respectively.

3.4.3 Optimization of secondary antibody dilutions

Using the antigen dilution of 1:20 and milk dilution of 1:10, antibody binding was evaluated using different secondary antibody dilutions, 1:500, 1:100, 1:2000 and 1:4000. The difference between antibody binding in the Group N1 and Group P1 was greatest ($P < 0.001$, Mann-Whitney U test) at a 1: 500 dilution of secondary antibody (Figure 1-C) and this dilution was used in the following experiment. For 1: 500 dilution of secondary antibody, the average OD values for JD positive and negative milk samples were 1.338 ± 0.440 and 0.303 ± 0.187 (mean ± SD), respectively.

3.4.4 Comparison of different mycobacteria as absorbent

Antibody binding to MAP antigen was evaluated in EVELISA test using different mycobacteria species as absorbent of cross-reactive antibodies (Figure 1-D). There was no significant difference between antibody binding in the Group N1 and Group P1 using unpaired t - test.

3.4.5 Evaluation of the EVELISA for milk testing

JD-negative milk samples (Groups N1 and N2) and JD-positive milk samples (Groups P1 and P2) were tested by the EVELISA using the optimized dilutions of antigen, milk and secondary antibody. Out of the 37 milk samples in the Groups P1 and P2, 25 samples showed higher levels of antibody binding than the tentative cut-off value determined using results of Groups N1 and

N2 samples (Figure 2). In the same sample set, 21 samples in Groups P1 and P2 were tested positive for JD by using a commercial ELISA test, ELISA-I.

3.5 Discussion

Previous studies on developing milk ELISA for JD have used protoplasmic antigen (PPA) (Collins *et al.*, 2005; Hendrick *et al.*, 2005; Klausen *et al.*, 2003; Sharma *et al.*, 2008; Singh *et al.*, 2007; Wells *et al.*, 2006), lipoarabinomannnan (LAM) (Sweeney *et al.*, 1994), and culture filtrate of MAP (Shin *et al.*, 2008) for testing antibodies in the milk of animals. PPA is a crude antigen mixture prepared by thorough physical disruption of mycobacterial bacilli followed by removal of cell debris and cell wall components (Beam *et al.*, 1969). It is likely that PPA contains proteins that are common or very similar in the closely related mycobacteria species. LAM is one of the components of the cell wall of mycobacteria species (Sugden *et al.*, 1997) and its core structure is shared among mycobacterial species (Mishra *et al.*, 2011). Sweeney *et al*. (1994) tested a LAM-based ELISA to detect antibodies in milk and serum for JD diagnosis and found that sensitivity and specificity of the ELISA were similar regardless of the tested samples (i.e. milk and serum). McKenna *et al*. (2005) compared diagnostic performance of PPA-based ELISA and LAM-based ELISA using fecal culture test as a gold standard. Sensitivity and specificity of the PPA-based ELISA were higher than that for the LAM-ELISA (McKenna *et al.*, 2005). Because PPA and LAM contains structures common in mycobacterial species, use of these molecules as diagnostic antigen can cause false positive reactions in animals infected with environmental mycobacteria other than MAP (Osterstock *et al.*, 2007). In a recent study, Shin *et al*. (2008) used culture filtrate of a MAP strain, JTC, in ELISA format for JD diagnosis and named the method, JTC-ELISA. JTC-ELISA showed significantly higher sensitivity than that of

commercial ELISA tests and performed effectively on both serum and milk samples. In this study, we used antigens extracted from MAP bacilli by using 80% ethanol in the EVELISA for milk testing. The ethanol extract was prepared by gently mixing bacteria in an ethanol solution for a short time period and it is likely that major components are surface antigens. We previously showed that the ethanol extract reacted specifically with serum samples of MAP-infected calves but not with those of calves infected with other species of mycobacteria (Eda *et al.*, 2006). In the ethanol extract, MAP-specific lipidic molecules were detected by thin layer chromatography (data not shown). Thus, the high sensitivity of the EVELISA test may be due to the reduction of non-specific antibody binding. Molecular characterization of the lipidic molecules in the extract has not yet been completed.

Previous studies have used different dilutions of milk for ELISA – 1:20 (Shin *et al.*, 2008), 1:10 (Sweeney *et al.*, 1994), 1:2 (Klausen *et al.*, 2003; Nielsen *et al.*, 2002; van Weering *et al.*, 2007). Singh *et al*. (2007) and Sharma *et al*. (2008) used whey for testing antibodies against MAP at a dilution of 1:10 and 1:8 respectively. For the EVELISA, a 1:10 dilution of milk was found to be optimal for differentiation between JD positive and negative milk samples. Thus, the dilutions used in the previous studies were similar to the dilution optimized in the current study despite the difference of the antigens used in the ELISAs. The secondary antibody in most of the studies (Collins *et al.*, 2005; Hendrick *et al.*, 2005; Huda *et al.*, 2004; Klausen *et al.*, 2003; Nielsen *et al.*, 2002; Sharma *et al.*, 2008; Shin *et al.*, 2008; Singh *et al.*, 2007; Sweeney *et al.*, 1994; van Weering *et al.*, 2007) were diluted in the range of 1:500 to 1:4000. These dilutions are compatible with the dilution optimized in this study (1:500).

In the previous studies on milk ELISA tests for JD, milk samples were preabsorbed with environmental mycobacteria (*M. phlei*) at different dilutions before performing the assay to

prevent cross reaction of non-specific antibodies (Klausen *et al.*, 2003; Nielsen *et al.*, 2002; van Weering *et al.*, 2007). In a recent study, preabsorption of serum samples improved the specificity of the EVELISA when serum samples were tested (Scott *et al.*, 2010). However, in the current study with milk samples, there was no effect of preabsorption on differentiation of JD-positive and JD-negative milk samples. The reason for this difference in serum and milk EVELISA is not known, but it is possible that there was no (or only a low level of) infection of cattle with environmental mycobacteria in the dairy farms where milk samples were collected for this study. There were several previous studies evaluating diagnostic performance of milk ELISA for JD (Collins *et al.*, 2005; Hendrick *et al.*, 2005; Sharma *et al.*, 2008; Shin *et al.*, 2008; Singh *et al.*, 2007; Sweeney *et al.*, 1994; van Weering *et al.*, 2007; Wells *et al.*, 2006). The sensitivity and specificity of milk ELISA in previous studies ranged from 26%-76.9% and 99%-100%, respectively. For example, Hendrick *et al*. (2005) and Collins *et al*. (2005) reported sensitivities of only 40 and 28.6%. Wells *et al*. (2006) reported a sensitivity of only 26% for their milk ELISA test. Singh *et al*. (2007) reported a higher sensitivity for milk ELISA (76.9%). But the number of samples tested was only 26 and also the status of the herds from where the samples were collected was not mentioned. In this study, we tested a total of 57 milk samples (37-positive and 20-negative). Out of the 37 positive samples (see methods section for details), 25 samples were found positive by the EVELISA test compared to 21 samples testing positive by ELISA-I. However, the number of samples in this study is not enough to compare diagnostic accuracy of the ELISA-I and EVELISA tests.

3.6 Conclusion

Previous reports suggested that MAP exists in food materials and may be a cause of human diseases. JD control is, therefore, important for prevention of the potential food-borne diseases. Since milk can be obtained cost-effectively and non-invasively, a sensitive milk ELISA would facilitate JD control in dairy farms. Our data demonstrated that optimized EVELISA test could detect antibodies in JD-positive milk samples and differentiated a majority of JD-positive samples from JD-negative samples. This work warrants further studies to examine if a sensitive milk ELISA test for JD diagnosis can be developed based on the EVELISA optimized for milk testing.

3.7 Acknowledgement

The work was supported by the following grants to S. E: USDA/NRI # 2007-3504-1862, Johne's Disease Integrated Project (JDIP #2008-55620-18710), and AgResearch Extension Innovation Fund of the University of Tennessee Institute of Agriculture.

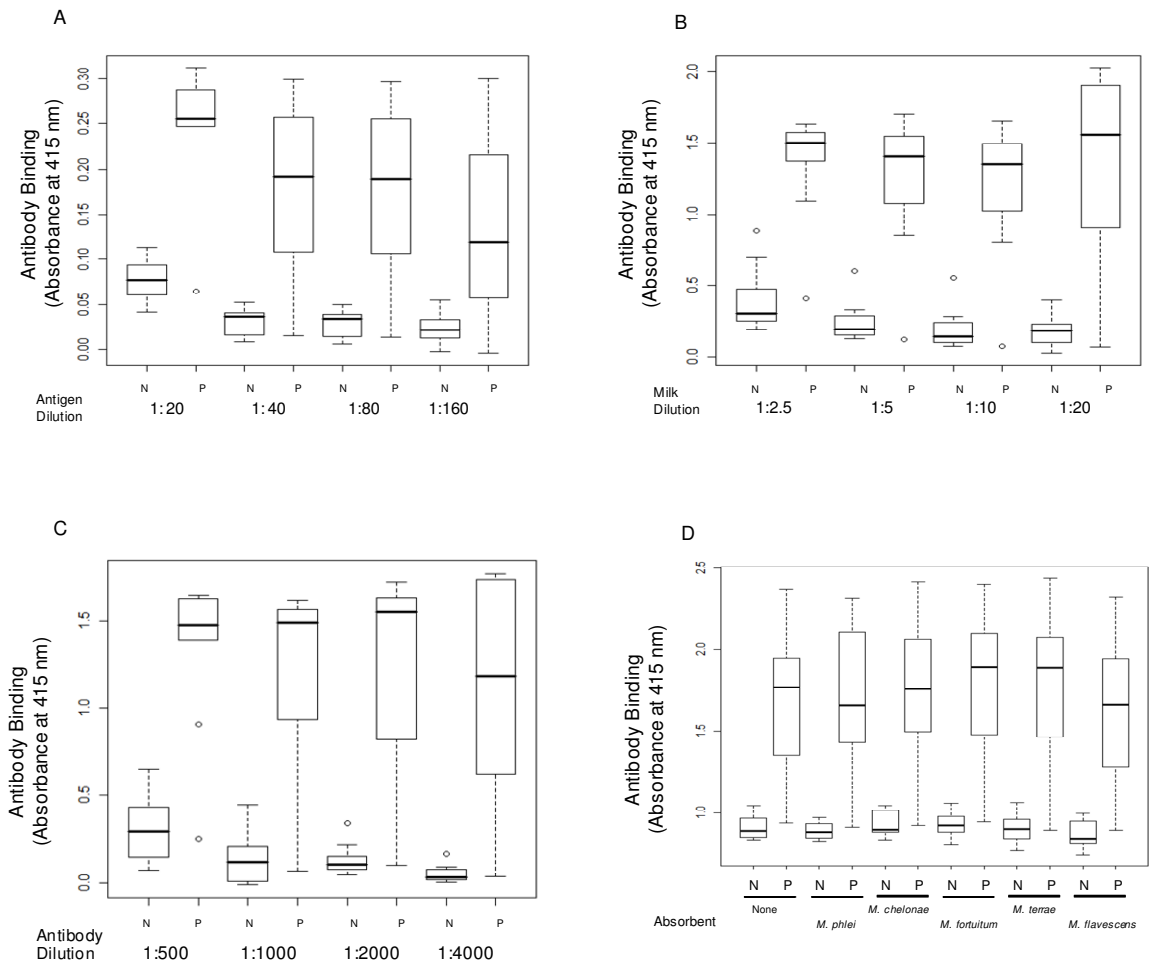


FIGURE 1- Wadhwa *et al.*

Figure 1- Optimization of EVELISA conditions for milk testing: Optimization of the different conditions for the milk EVELISA test were conducted by using different dilutions of antigen (A), milk (B), secondary antibody (C) and statistical analyses were done using Mann – Whitney U. Comparison of different mycobacterial species (D) was conducted for possible use as preaborbent of cross reactive antibodies, the values were log-transformed and then p values for different mycobacteria species were calculated using unpaired t – test. The open circles indicate outliners.

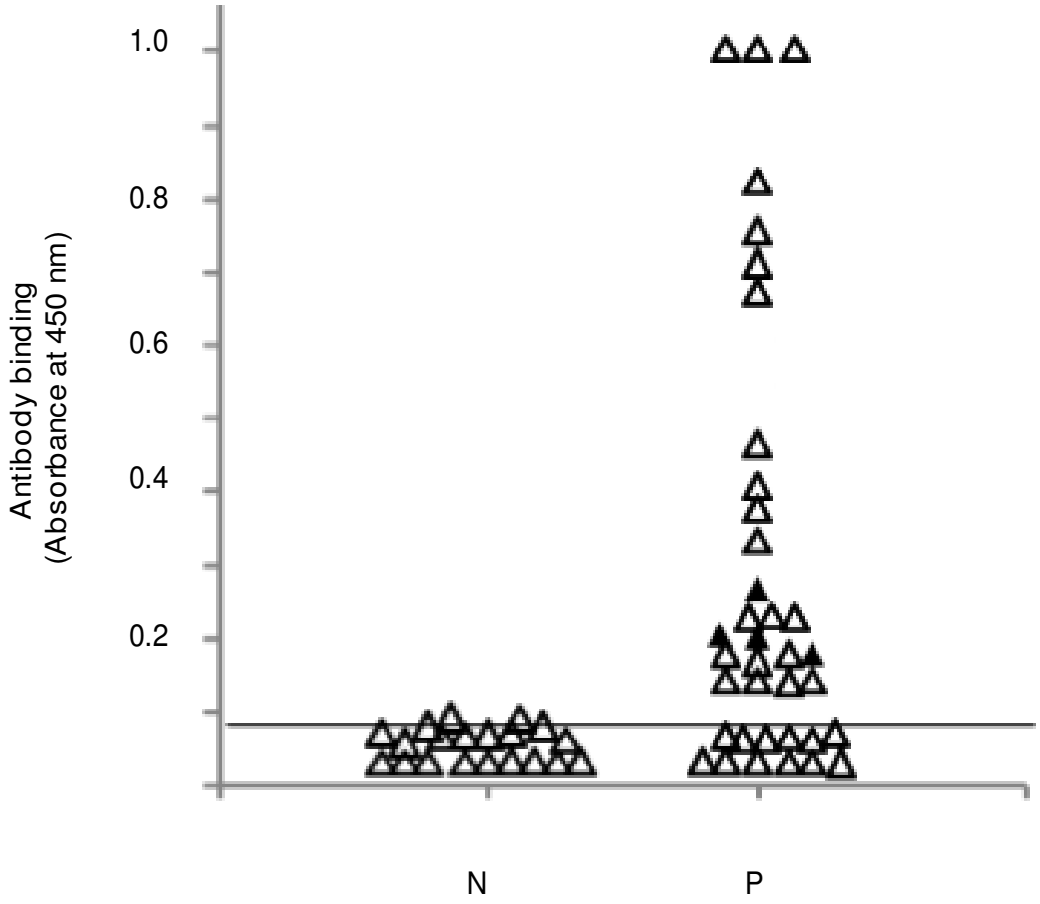


FIGURE 2 – Wadhwa *et al.*

Figure 2 – EVELISA results on 57 milk samples: Antibody binding in 57 milk samples in Groups N1 & N2 (N) and P1 & P2 (P) were tested by using the optimized EVLISA test. The horizontal line represents a tentative cut-off value (0.105, mean + 3 standard deviation of the OD values obtained using milk samples of Groups N1 and N2) for milk EVELISA test. The 4 shaded triangles represent milk samples that tested JD positive by milk EVELISA test but not by ELISA-I. All of the samples tested negative by the EVELISA test were also tested negative by the ELISA-I. The 3 positive samples plotted at the OD value of 1.0 showed OD values of 1.18, 1.50, and 1.90.

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CHAPTER 4

USE OF ETHANOL VORTEX ENZYME LINKED IMMUNOSORBENT ASSAY FOR DIAGNOSIS OF BOVINE TUBERCULOSIS IN FARMED RED DEER (*CERVUS ELAPHUS*)

A version of this chapter hasn't been published anywhere but has been submitted to the Journal of Veterinary Diagnostic Investigation.

Use of ethanol vortex enzyme linked immunosorbent assay for diagnosis of bovine tuberculosis in farmed red deer (*Cervus elaphus*)

Ashutosh Wadhwa, Rachel E. Johnson, Colin G. Mackintosh, J. F. T. Griffin, W. Ray Waters, John P. Bannantine and Shigetoshi Eda

Running Title:

ELISA for diagnosis of bovine tuberculosis

4.1 Abstract:

Bovine tuberculosis (bTB) has posed threat due to its presence in the wildlife species and possibility to spill the infection to the domestic animals. Control programs of bTB depend on testing and slaughtering the positive animals; however, the currently available diagnostic tests suffer poor specificity, due to possible confounding factors like infection and vaccination with *Mycobacterium avium* ssp. *paratuberculosis* (MAP). In our previous study, we developed a sensitive enzyme linked immunosorbent assay (ELISA), called ethanol vortex ELISA (EVELISA), using surface antigens of MAP by briefly agitating the bacilli in 80% ethanol solution. The objective of this study is to examine whether EVELISA technique could be used to

detect anti- *Mycobacterium bovis* (MB) antibodies in the serum of infected farmed red deer (*Cervus elaphus*). In this study, we tested a total of 50 red deer serum samples, divided in 4 groups - animals uninfected with both MB and MAP (n = 15), experimentally infected with MB (n = 15), naturally infected with MB (n = 5) and animals vaccinated against MAP (n = 15). The presence of anti-MB antibodies was tested using ethanol extract of MB. Without absorption of anti-MAP cross reactive antibodies, it was found that 13 out of the 15 animals vaccinated for MAP showed high antibody binding. Using heat killed MAP as absorbent of cross reactive antibodies, the tentative diagnostic sensitivity and specificity of the EVELISA was estimated to be 90% and 93.3%, respectively. The results from this study suggest that EVELISA may form a basis for a sensitive and specific test for the diagnosis of bTB.

Key words: Bovine tuberculosis; ELISA; *Mycobacterium bovis*; Red deer

4.2 Introduction

Bovine tuberculosis (bTB), mainly caused by *Mycobacterium bovis* (MB), is an infectious, chronic disease of zoonotic and economic importance. It is characterized by formation of typical granulomatous lesions with varying degrees of necrosis, calcification and encapsulation. [2,11,19,21,31] bTB has been identified in a wide range of wildlife species, domestic animals and humans. [20,24] Global economic loss due to bTB is estimated to be about US$ 3 billion annually. [27] In the recent years, importance of bTB in the wildlife species has increased not only due to its role as potential reservoir for domestic animals but also as a threat to the wildlife. [5] In the state of Michigan, 633 free- ranging white tailed deer have been confirmed positive for MB infection out

of the 178,199 tested. [23] Since, there are no effective treatments and vaccines for bTB, eradication of the disease remain the only control measure.

Effective eradication programs to control bTB focus on test- slaughter policy of the wildlife reservoirs and/or abattoir surveillance. [12,22] One of the major antemortem tests for bTB is the tuberculin skin test (TST) using purified protein derivatives (PPD). [11,18] But, the TST suffers low specificity and is impractical for wildlife species due to the need of recapturing - testing animals 48 – 72 hours after the injection of PPD. [4-5] Assays like interferon-γ which measures cell mediated immune response have also been evaluated for bTB diagnosis. [25] But these assays require fresh blood samples and also have not been validated for diagnosis of bTB in wildlife species. [15] Antibody- based assays for detection of bTB have shown promising results due to their flexibility and cost effectiveness. Prior studies on development of antibody based assays have used cross reactive preparations of MB, such as crude cell sonicate [8], culture filtrate [26], PPD [14], and lipoarbinomannan (LAM) [30]. Specific molecules like ESAT-6, CFP10, MPB83 and MPB70 have also been used for detection of anti-MB antibodies. [13,32,37] Recent studies have demonstrated the advantages of multi-antigen techniques, such as multi-antigen print immune-assay (MAPIA) [35] and lateral flow rapid test (RT) [15], have been tested using cocktails of antigens like ESAT-6, CFP10 and MPB83. Although, these studies have shown promising results in detecting antibodies against MB, but their results were confounded due to presence of anti - *Mycobacterium avium* ssp. *paratuberculosis* (MAP) antibodies due to infection and/or vaccination. [3] We have previously developed a novel enzyme linked immunosorbent assay (ELISA), called as ethanol vortex ELISA (EVELISA) using surface antigens of MAP for detecting anti-MAP antibodies in serum at early stages of Johne's disease (JD). [6-7,28-29] The aim of the present work was to assess the performance of EVELISA optimized to diagnose bTB

using serum samples from various groups of red deer (*Cervus elaphus*) including naturally and experimentally infected animals with MB and MAP.

4.3 Materials and Methods

4.3.1. Samples

In order to evaluate the performance of EVELISA, a total of 50 red deer sera were obtained from 4 different studies in New Zealand. Group 1 consisted of 15 deer of various ages which were not vaccinated or challenged with neither of MB and MAP. All the animals in this group were negative for MB by isolation methods using lymph node samples and also for MAP using an ELISA test (paralisa).[16] Group 2 consisted of 15 deer of different ages which were experimentally challenged using 0.2 mL volume of 500 CFU of MB into the left tonsillar crypt of anesthetized deer. [17] From all the animals in this group, MB was isolated from gross lesions or pooled lymph node samples (head, thoracic or intestinal lymph nodes) after 27 weeks of experimental challenge. Out of the 15 samples in this group, 7 samples were positive for MAP using paralisa test. Group 3 consisted of 5 deer samples which were naturally infected with MB (confirmed by MB culture). Out of these 5 samples, 2 samples were positive for MAP using paralisa test. Finally, Group 4 consisted of 15 sera samples from deer vaccinated with MAP using a method previously described. [16] All the samples in this group were from animals sourced from a property with no history of bTB or paratuberculosis. MAP was isolated from all the deer in this group using culture method after 50 weeks post challenge and 12 out of the 15 samples in this group were positive using paralisa test.

4.3.2 Bacterial species and strains

Virulent strain of MB (HC2005T), which was originally isolated from MB infected dairy cow, was cultured in Middlebrook's 7H9 medium [a] with addition of 0.05% Tween 80 [b], 10% oleic acid-albumin-dextrose-NaCl [c] at 37^0C until used for antigen preparation. For antigen preparation MB bacilli was harvested from stationary phase cultures, suspended in 80% ethanol and agitated by vortex to dislodge surface antigens. MAP (sheep strain) was kindly provided by Dr. John. P. Bannantine (Bacterial Diseases of Livestock Research Unit, National Animal Disease Center, USDA, Ames, IA).

4.3.3 EVELISA

Dislodged MB antigen was diluted in the ethanol solution and immobilized on wells of a 96-well plate by evaporation. The antigen-coated plate was incubated with 150 μL of buffer B (10 mM phosphate buffered saline, pH 7.0, containing 0.05v/v% Tween 20 and 10v/v% SuperBlock [d]) at room temperature for 30 min. The plate was then washed 4 times with 200 μL of PBST (10 mM phosphate buffered saline, pH 7.0, containing 0.05% Tween 20). Fifty μL of serum sample (with or without preabsorption of cross-reactive antibodies with heat-killed MAP [sheep strain, 4 mg/mL] for 30 minutes) was then inoculated and incubated at room temperature for one hour. After washing the wells four times with 200 μL of PBST, each well was then inoculated with 50 μL of horseradish peroxidase (HRP)-conjugated anti-deer immunoglobulin G (IgG) heavy and light chains [e] (diluted in buffer B), and incubated at room temperature for one hour. After washing the wells four times with 200 μL of PBST, 100 μL of tetramethylbenzidine (TMB) solution [f] was used to develop color reaction according to manufacturer's instruction and optical density (OD) of the solution was determined by a microplate reader [g] at 450 nm for 10 min after terminating the reaction by adding 100 μL of 2M sulfuric acid.

4.3.4 Statistical Analysis

The cut-off value was estimated using 2 graph receiver operating characteristic to maximize sensitivity and specificity. [10] The test sensitivity was determined by dividing the number of EVELISA test positive animals by the total number of MB culture positive animals (Groups 2 and 3), with the result expressed as a percentage. The test specificity was determined by dividing the number of EVELISA test negative animals by the total number of bTB free animals (Groups 1 and 4), with the result expressed as a percentage.

4.4 Results

The diagnostic performance of EVELISA test to detect anti-MB antibodies in the sera of red deer was evaluated using ethanol extract of MB and compared with or without the use of MAP as absorbent of cross-reactive antibodies (Figure 3A-B). When antibody binding to MB antigen was evaluated without pre-absorption with MAP, MB positive samples (Groups 2 and 3) showed higher antibody binding levels than those of uninfected samples (Group 1), except for one sample in Group 2 (Figure 3A). However, the samples in Group 4 (experimentally infected with MAP) showed high antibody binding levels, suggesting presence of cross-reactive antibodies in MAP-infected animals.

In Figure 3B, antibody binding was tested after absorbing cross-reactive antibodies with heat-killed MAP bacilli. Antibody binding levels in groups 2-4 were significantly reduced. Using a cut-off value of 0.065 (determined by two-graph receiver operating characteristic analysis), the sensitivity and specificity were estimated to be 90% and 93.3%, respectively. Out of the 15 animals in Group 1 (uninfected), 14 animals showed negative reaction with the EVELISA test.

Out of the 15 animals in Group 2 (experimentally challenged with MB), 13 animals were found positive by EVLISA test. Group 3 consisted of 5 animals with natural infection of MB and all of these animals were found positive by our test. The most significant difference was that antibody binding levels in Group 4 (n=15, vaccinated with MAP) were reduced to the same levels as Group 1 (uninfected), whereas antibody binding levels in the group was indistinguishable from those in MB positive groups (Groups 2 and 3, Figure 3A).

4.5 Discussion

Previous studies on developing ELISA for bTB in deer have used PPD (bovine and avian), MPB70 [11,13,36], LAM [33-34]. Griffin *et al*. (2004) have described the use of bovine PPD in ELISA format and suggested a sensitivity of 70%. They have also reported an ancillary blood test (BTB) which is composite test of both lymphocyte transformation assay and the bovine PPD ELISA. The BTB showed a high sensitivity of 94%; however, the test is a costly assay as compared to ELISA and is used as an ancillary test to examine TST-reactors rather than for whole herd testing. LAM has also been used as an antigen in development of antibody based assays and to evaluate antibody response kinetics on experimental inoculation but it is cross reactive to non-tuberculous mycobacteria. [34] Waters *et al*. (2005) suggested the use of purified proteins to improve the specificity of antibody based assays. [33] Recently, few rapid tests have been developed – MAPIA, RT and dual-path-platform (DPP) VetTB assay – using MB-specific antigens such as MPB83, ESAT6 and CFP-10 antigens. [9,15,35] Buddle *et al*. (2010) compared the 2 lateral flow tests – CervidTB STAT-PAK and DPP VetTB assay and reported specificities of 83.8% and 91.4%, respectively. [3] Boadella *et al*. (2011) reported a sensitivity of 51% and specificity of 96% for fallow deer using bovine PPD ELISA. They also tested DPP VetTB assay and depending on the

cut-off value selected, the sensitivity and specificity ranged from 62-71% and 88-95%, respectively. [1] Although these rapid tests show improved sensitivity and specificity, preparation of the highly purified recombinant proteins can be costly. In this study, we used MB antigens prepared simply by agitating the bacteria in an ethanol solution. Our previous studies showed that ethanol extract of MAP contains MAP-specific antigens and can be used to diagnose Johne's disease with high sensitivity.[6] We recently showed that absorption of cross reactive antibodies in serum samples with *Mycobacterium phlei* improved specificity of the EVELISA test for Johne's disease.[28] This is similar to the observation in this study that specificity of EVELISA test could be improved by absorption of cross reactive antibodies by using environmental mycobacteria. After absorption of cross reactive antibodies with MAP, tentative sensitivity and specificity of the EVELISA test for bTB were estimated to be 90% and 93.3%, respectively. Forty out of 15 samples from animals vaccinated with MAP (Group 4) were tested negative by the EVELISA test, indicating that a majority of antibodies reacting with MAP were removed by the absorption. Thus, this study suggested that the EVELISA could diagnose bTB in red deer with minimum false positive results caused by antibodies reacting with MAP, encouraging further studies to validate the test using a larger number of samples obtained from red deer farms.

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4.7 Sources and Manufacturers:

a. Middlebrook's 7H9 medium, Becton Dickinson, Cockeysville, MD).

b. Tween 80, Fisher Scientific, Fair Lawn, NJ.

c. Oleic acid-albumin-dextrose-NaCl Becton Dickinson, Microbiology Systems, Franklin Lakes, NJ.

d. SuperBlock, PIERCE Biotechnology, Rockford, IL.

e. Horseradish peroxidase (HRP) - conjugated anti-deer immunoglobulin G (IgG) heavy and light chains, Kirkegaard and Perry Laboratories, Gaithersburg, MD.

f. Tetramethylbenzidine (TMB) solution, THERMO Scientific, Rockford, IL.

g. Microplate reader, Model 680, BioRad, Hercules, CA.

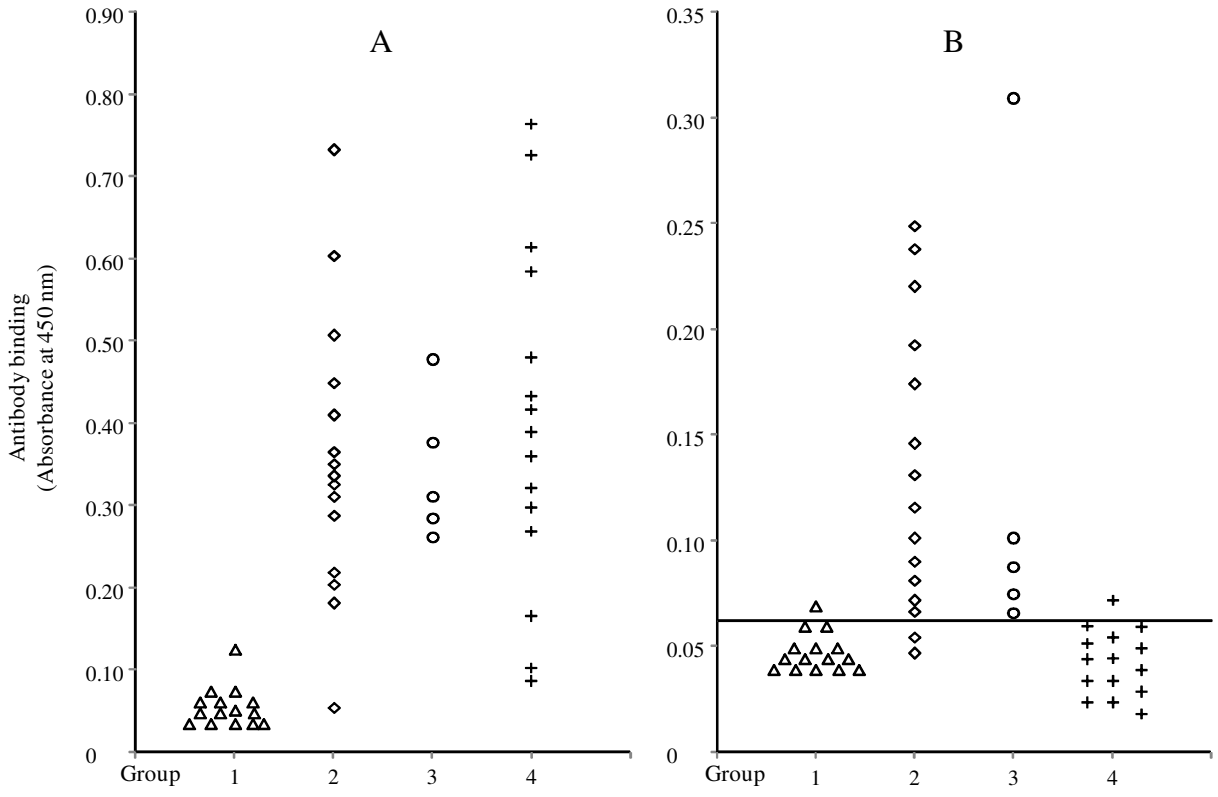


Figure 3- Wadhwa *et al*.

Figure 3 – Diagnostic performance of EVELISA test on 50 red deer serum samples. Antibody binding was tested using 50 red deer serum samples, without (A) and with (B) absorption of cross reactive antibodies against MAP. The samples were divided in 4 groups (1- Unvaccinated – unchallenged animals (n = 15); 2- Experimentally challenged with MB (n = 15); 3- Naturally infected with MB (n = 5; 4- vaccinated with MAP (n = 15)). Each marker represents an average of duplicate measurement. To estimate tentative diagnostic sensitivity and specificity of the EVELISA test, cut-off value of 0.65 (horizontal line) was used (B).

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CHAPTER 5

BEAD-BASED MICROFLUIDIC IMMUNOASSAY FOR DIAGNOSIS OF JOHNE'S DISEASE

A version of this chapter hasn't been published anywhere but we will be submitting it to the Journal of Immunological Methods.

The manuscript based on this chapter is being reviewed by our collaborator Dr. Robert S. Foote and Dr. Robert W. Shaw.

Bead-based microfluidic immunoassay for diagnosis of Johne's disease

Ashutosh Wadhwa, Robert S. Foote**,** Robert W. Shaw, and Shigetoshi Eda

5.1 ABSTRACT

Microfluidics technology offers a platform for development of point-of-care diagnostic devices for various infectious diseases. In this study, we examined whether serodiagnosis of Johne's disease (JD) can be conducted in a bead-based microfluidic assay system. Magnetic micro-beads were coated with antigens of the causative agent of JD, *Mycobacterium avium* subsp. *paratuberculosis*. The antigen-coated beads were incubated with serum samples of JD-positive or negative serum samples and then with a fluorescently-labeled secondary antibody (SAB). To confirm binding of serum antibodies to the antigen, the beads were subjected to flow cytometric analysis. Different conditions (dilutions of serum and SAB, types of SAB, and types of magnetic beads) were optimized for a great degree of differentiation between the JD-negative and JD-positive samples. Using the optimized conditions, we tested a well-classified set of 155 serum samples from JD negative and JD-positive cattle by using the bead-based flow cytometric assay. Of 105 JD-positive samples, 63 samples (60%) showed higher antibody binding levels than a cut-off value determined by using antibody binding levels of JD-negative samples. In contrast,

only 43-49 JD-positive samples showed higher antibody binding levels than the cut-off value when the samples were tested by commercially-available immunoassays. Microfluidic assays were performed by magnetically immobilizing a number of beads within a microchannel of a glass microchip and detecting antibody on the collected beads by laser-induced fluorescence. Antigen-coated magnetic beads treated with bovine serum sample and fluorescently-labeled SAB were loaded into a microchannel to measure the fluorescence (reflecting level of antibody binding) on the beads in the microfluidic system. When the results of five bovine serum samples obtained with the system were compared to those obtained with the flow cytometer, a high level of correlation (linear regression, $r^2 = 0.994$) was observed. In a further experiment, we magnetically immobilized antigen-coated beads in a microchannel, reacted the beads with serum and SAB in the channel, and detected antibody binding to the beads in the microfluidic system. A strong antibody binding in JD-positive serum was detected, whereas there was only negligible binding in negative control experiments. Our data suggest that the bead-based microfluidic system may form a basis for development of an on-site serodiagnosis of JD.
Key Words: *Mycobacterium avium* ssp. *paratuberculosis*, Johne's disease, microfluidics, lab-on-a-chip.

5.2 Introduction

Johne's disease (JD) caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is prevalent worldwide and has significant impact on the global animal husbandry industry. For example, annual economic loss due to JD in US dairy was estimated to be $ 220 million (Ott et al., 1999). The economic concerns clearly indicate an urgent need for control of the disease. A recent report recommended that a serological test, called enzyme linked immunosorbant assay

(ELISA), should be used for controlling the disease in dairy as well as beef herds (Collins et al., 2006). However, ELISA test needs to be conducted in diagnostic laboratories, causing cost/labors associated with shipment of samples and a long-time interval (6-12 months) in JD screening. Therefore, development of on-site (laboratory-free) diagnostic device would contribute to cost-effective JD diagnosis and control.

Microfluidic devices are state-of-the-art tools for biochemical and immunological analysis that have high sensitivity and require only short periods of time and small amounts of reagents (Whitesides, 2006). Because of these advantages, microfulidic technology has been used for development of immunoassays for on-site diagnosis of various diseases (Ng et al., 2010). However, to date, there is no report for development of microfluidic system for diagnosis of JD. In this study, we examined whether a bead-based microfluidic system can be used to diagnose JD by detecting specific serum antibodies in animals infected with MAP.

5.3 Materials and methods

5.3.1. Bacterial strains and isolates

The Linda strain of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was obtained from Dr. John P. Bannantine in the United States Department of Agriculture (Ames, IA) and was cultured in Middlebrook 7H9 medium (Becton Dickinson Microbiology Systems, Franklin Lakes, NJ) with 10% oleic acid-albumin-dextrose-NaCl (Becton Dickinson Microbiology Systems, Franklin Lakes, NJ) and 2 mg/L of mycobactin J (Allied Monitor, Fayette, MO). The cultures were

maintained at 37°C without shaking until they reached an optical density of approximately 0.7 at 600 nm.

5.3.2. Serum Samples

5.3.2.1. Group I:

Four serum samples were obtained from female Holstein cattle (2.8-5.0 years old) in a dairy farm in Minnesota. Two of the four cattle (ID 11 and 167) were tested positive for JD by a commercial ELISA test (IDEXX, Westbrook, ME) and fecal culture test at the US Department of Agriculture (Ames, Iowa). The other two cattle (ID 7 and 18) were tested negative for JD by the two diagnostic tests. Also, fetal bovine serum (FBS, Thermo Scientific, South Logan, UT) was used as a negative control.

5.3.2.2. Group II:

One hundred fifty five serum samples (50 µL each) were kindly provided by Dr. Michael Collins, University of Wisconsin Madison. These samples were classified as JD-negative animals (N, n=50), low shedders (L, n=35), medium shedders (M, n=35), and high shedders (H, n=35) as described previously (Collins et al., 2005). MAP-infected animals shed MAP organisms in their feces and the level of shedding (low-high) increases as JD progresses (Sweeney, 2011). These samples were tested by three commercial ELISA tests (IDEXX [ELISA-A], Biocor [ELISA-B] and Pouquire [ELISA-C]) (Collins et al., 2005). All serum samples were separated into aliquots of 20 to 1,000 µL and stored at -20 ^{0} C for short-term storage (< 6 months) or at -80 ^{0} C for long-term storage (> 6 months).

5.3.3. Secondary antibodies

Three different types of secondary antibodies - goat anti-bovine IgG (H+L) conjugated with DyLight488 (SAB1), goat anti-bovine IgG (H+L) conjugated with FITC (SAB2) and goat anti-bovine IgG_1 conjugated with FITC (SAB3) - were obtained from Jackson Immuno Research Inc. (West Grove, PA) and used to label antibodies bound to MAP antigen.

5.3.4. Magnetic micro-beads

Four different types of magnetic beads – 2.74 μm plain polystyrene surface beads (BEAD1), 5 μm plain surface beads (BEAD2), 2 μm COOH-surface beads (BEAD3) and 2 μm NH_3-surface beads (BEAD4) obtained from Spherotech Inc. (Lake Forest, IL) and were used to immobilize MAP antigen.

5.3.5. Preparation of MAP antigen coated magnetic beads and off- chip antibody binding assay

MAP bacilli (100 μL of confluent culture) cultured in the Middlebrook's medium were harvested in a microfuge tube and centrifuged at 3500 xg for 10 minutes. The bacterial pellet was suspended in 80% ethanol solution at 80 mg wet weight of bacteria/ml, agitated by using a vortex mixer and centrifuged at 10,000 xg for 10 minutes. The supernatant (ethanol extract) was used as MAP antigen. The antigen was coated on the surface of magnetic beads (400 μL) by incubating the beads with 400 μL of the ethanol extract for overnight at room temperature. After washing with PBS (phosphate-buffered saline [pH 7.0]), the antigen-coated beads were incubated with 400 μL of buffer B (PBS containing 10 % SuperBlock [PIERCE Biotechnology, Rockford, IL] and 0.05 % Tween 20 [ACROS Organics, Morris Plains, NJ]) to block uncoated surface. The antigen-coated beads were then incubated with bovine serum (50 μL, diluted in buffer B) for 30 min at room temperature, washed twice with 200 μL of buffer B, and incubated with SAB (50 μL, diluted in buffer B) for 30 min at room temperature. After the reactions,

magnetic beads were subjected to analysis of antibody binding using a flow cytometer or microfulidic system as described below. The schematic of this procedure is presented in Figure 4.

5.3.6. Flow cytometric method (FCM) for analysis of antibody binding

After the antigen coating and antibody binding described above, magnetic beads were suspended in 1ml of PBS and loaded to a flow cytometrer (LSR II, BD Bioscience, San Diego, CA) for analysis of antibody binding. On the flow cytometer, photomultiplier tube voltages were set to 691 eV for forward scatter channel, 307 eV for side scatter and 590 eV for FL1 channel. The threshold was set at 200 on side scatter channel. Data of mean fluorescent (FL1) intensity (proportional to the level of antibody binding) were collected for 10,000 beads and analyzed by using DiVa software (BD Bioscience).

5.3.7. Microfluidic system

Microfluidic chips were fabricated as described previously (W. H. Ko, 1989; Jacobson et al., 1994). Briefly, a microchannel (100 µm deep x 200 µm wide at half-depth x 4 cm long) was etched in glass substrates (White Crown B-270; Telic, Santa Monica, CA) by photolithography and chemical wet etching, followed by bonding to a glass coverplate to enclose the channels. Access to the ends of the channel was provided by holes (2 mm diameter, ~5 µl capacity) drilled in the etched substrate prior to bonding the coverplate. As shown in the schematic of Figure 5, a permanent magnet (neodymium disk magnet, 1/2 “diameter x 1/16” thick, K&J Magnetics) was placed on the top of the microchip to attract magnetic beads in the microchannel. The proximal edge of the magnet was placed 1.0 cm from the entrance to the channel. A single layer of black electrical tape was placed between the magnet and the microchip to prevent light reflection from the magnet surface. Suspensions of magnetic beads or reagents were pipetted into the microchip addition port and allowed to flow into the channel. Optionally, a microsyringe can be attached to

the exit hole to draw fluids into the channel. Magnetic beads were found to collect as a closely-packed layer (Figure. 7) on the upper surface of the channel directly under the proximal edge of the magnet and remained stably immobilized during subsequent washing and reagent addition steps. Fluorescence from the magnetic beads was detected using a confocal optical system (Figure. 5) comprising a 10x objective and Nikon filter cube for fluorescein excitation/emission. The 488nm line from a 100W argon ion laser was used for excitation. The microchip was mounted on an x-y translation stage (not shown) to adjust the position for optimum excitation of the immobilized beads. Emitted fluorescence was collected with the objective, passed through the dichroic mirror of the filter cube and further filtered spatially (1mm pinhole filter) and spectrally (488 notch filter and bandpass filter) prior to detection with a photomultiplier tube (PMT). Electrical signals from the PMT were amplified and analyzed by LabView software (National Instruments, Austin, TX). Images of fluorescence on the magnetic beads were captured using a Nikon TE300 inverted microscope equipped with a 10X objective, FITC filter cube and CCD camera (model TE/CCD, Princeton Instruments). IPLab Spectrum software (Signal Analytics) was used for camera control and image processing.

5.3.8. Detection of antibody binding in the microfluidic system

5.3.8.1. Off-chip protocol:

Antibody binding to antigen-coated magnetic beads was conducted off-chip as described above. The chip microchannel was first filled with PBS and aliquots (5 µl) containing approximately $1.5*10^7$ treated beads were then loaded into the microchannel and immobilized in the channel

using a permanent magnet. Fluorescence associated with the beads was quantified using the fluorescence detection system described above. For comparison of samples the same approximate number of beads was used for each assay. Because the focused laser beam interrogates only immobilized beads within the focal spot in the channel, the assay should not be sensitive to variations in the number of beads introduced into the channel, provided that sufficient beads are used to fill the focal spot in each assay. The microchannel was rinsed to remove beads after each assay and refilled with PBS before adding the next sample.

5.3.8.2. On-chip protocol:

After immobilizing antigen-coated magnetic beads in the channel, serum sample (Group I, cow ID 11, 1:20 dilution in buffer B) was loaded into the channel by passive flow. After 30 min incubation, PBS containing 0.05% Tween 20 was loaded into the channel to remove antibodies that did not bind to the magnetic beads. SAB 1(1:100 dilution in buffer B) was then loaded into the microchannel and unbound antibodies were removed by loading PBS containing 0.05% Tween 20 into the channel. After the reaction, fluorescence associated with the beads was quantified by the fluorescence detection system described above.

5.4 Results

5.4.1. Optimization of assay conditions in FCM

Antibody binding to MAP antigen-coated beads was evaluated in the FCM assay with different dilutions of serum (1: 20, 1: 50 and 1:100) and secondary antibody (1: 20, 1: 50 and 1:100). In this experiment, we used 2 µm plain surface magnetic beads (BEAD1) and goat anti-bovine IgG

(H+L) conjugated with DyLight488 (SAB1). The difference of antibody binding between JD-negative and JD-positive samples were found greatest with the combination of 1:20 serum dilution and 1: 100 secondary antibody dilution (Figure 6-A). In the following experiment, the serum samples (1:20 dilution) were tested for antibody binding using different types of secondary antibodies (SAB1, SAB2 and SAB3, 1:100 dilution). The greatest difference of antibody binding between JD-negative and JD-positive samples was observed with SAB1 (Figure 6-B) and this secondary antibody was used in the following experiments. Using the optimized dilutions and secondary antibody, antibody binding was evaluated using different types of magnetic beads (BEAD1, BEAD2, BEAD3 and BEAD4). The greatest difference between antibody binding levels in JD-negative and JD-positive samples was observed when BEAD1 was used for the assay (Figure 6-C).

5.4.2. Estimation of sensitivity and specificity of the bead-based antibody binding assay in FCM

By using the optimized assay conditions, 155 bovine serum samples were tested in the FCM. A cut-off-value was determined as mean + 2 standard deviation of data obtained with 50 JD-negative serum samples. Using the cut-off value, specificity of the assay was estimated to be 98.0%. Estimated sensitivities were 45.7, 48.6 and 85.7 % for low, medium and high shedders, respectively (Table 1). The estimated sensitivities were higher than those of commercial ELISA tests, especially in low shedders (45.7% versus 5.7-17.1%).

5.4.3. Detection and quantification of antibody binding in a microfulidic system

To examine if antibody binding can be detected in a microfulidic system, antigen-coated magnetic beads (BEAD1) reacted off-chip with bovine serum and secondary antibody (SAB1) were loaded in to a microchannel and fluorescence associated with the magnetic beads was

observed under a fluorescent microscope. Magnetic beads reacted with a JD-positive serum showed a higher level of fluorescence than that in beads treated with a JD-negative sample (Figure 7). The relative fluorescent intensity of the samples was determined by measuring the average pixel brightness in equivalent regions of interest (ROIs, green rectangles in Figure 7). The reacted beads were also analyzed using the detection system of Figure. 5, as described in Materials and Methods. Higher levels of fluorescence were observed in this system on beads treated with a JD-positive serum (1:20 and 1:50 dilution) when compared with those of beads treated with JD-negative serum or no serum (Figure 8A). In a separate experiment, antigen-coated beads were treated off-chip with JD-positive serum sample (n=2), JD-negative sample (n=2), or fetal bovine serum and subjected to the fluorescence quantification in the microfulidic system and FCM. A strong correlation (r^2=0.994) was observed between the results of the microfluidic system and FCM (Figure 8B), suggesting that the microfluidic system has a comparable analytical sensitivity as the FCM. In the following experiment, we examined if antibody binding can be detected after conducting on-chip antibody reactions. Antigen-coated beads were loaded into a microchannel, immobilized in the channel with a permanent magnet, and reacted sequentially with serum (JD-positive, JD-negative, or fetal bovine serum) and secondary antibody. As shown in Figure 9, significantly higher fluorescence intensity was observed on the beads treated with JD-positive serum than those treated with JD-negative or fetal bovine serum samples.

5.5 Discussion

Microfluidic immunoassay device possesses remarkable features such as high surface-to-volume ratio and nanoliter volume of microchannel. Microchannel can serve as immunoreactor chamber

that leads to significant decrease in analysis time from hours to minutes and with minimal sample/reagent utilization as compared to microwell technology (Terence G. Henares et al., 2008). To further increase the size surface area for antibody reaction, microbeads have been used in microfluidic immunoassays (Lim and Zhang, 2007). Also, microbeads can be washed out of the microchannel after antibody binding reactions and make a microfulidic immunoassay system reusable. Furthermore, by using a set of microbeads coated with different antigen, a single microfulidic system can be used to diagnose different diseases. Based on these advantages, we elected to use magnetic microbeads in this study.

Magnetic microbeads were coated with MAP antigens extracted from MAP bacilli by using 80% ethanol. We previously showed that MAP surface antigens can be used to diagnose JD with higher sensitivity than that of commercial ELISA tests and that such antigen can be extracted by 80% ethanol (Eda et al., 2005; Eda et al., 2006; Speer et al., 2006; Scott et al., 2010). The antigen-coated beads were then treated with bovine serum samples and the optimal serum dilution was found to be 1:20. This dilution is much lower than that (1:100) used in our previous study on ELISA test developed using the ethanol extract of MAP (Eda et al., 2006; Scott et al., 2010). The optimized dilution for SAB (1:100) was also lower than the dilution (1:500) used in the previous study (Eda et al., 2006; Scott et al., 2010). These differences in optimal dilutions may be due to the fact that sensitivity of ELISA (based on enzyme reaction) is generally higher than that of fluorescence-based detection.

Among the three SABs tested in this study, anti-IgG (H+L) antibody conjugated with DyLight488 (SAB1) showed the greatest level of antibody binding (fluorescence intensity)

compared to that of anti-IgG (H+L) antibody labeled with FITC (SAB2). The relatively new fluorescent dye (DyLight488) is claimed to be brighter and photoresistant than FITC and, therefore, the difference between the results of SAB1 and SAB2 is likely attributed to the property of the fluorescent dye. The anti-IgG_1 antibody labeled with FITC (SAB3) showed lower antibody binding (fluorescence intensity) than SAB2 (anti-IgG [H+L] antibody that binds to all immunoglobulin classes and subtypes), suggesting that a significant amount of immunoglobulin molecules other than IgG_1 binds to the MAP antigen.

Using the optimized dilutions and SAB, four different types (size and surface modifications) of magnetic beads were tested in this study. After antibody reactions, the 5-μm beads with plain surface (BEAD2) showed higher fluorescence intensity than 2-μm beads with plain surface (BEAD1); however, the difference between JD-negative and JD-positive samples was more significant in BEAD1. The higher fluorescence intensity is likely due to the larger surface area of BEAD1 since the FCM quantifies fluorescence intensity of each beads. Since fluorescence on islands of immobilized magnetic beads (but not each bead) is quantified in our microfluidic system, BEAD1 is a preferable option than BEAD2. In comparison of BEAD1 with BEAD3 (2 μm beads with amino groups on their surface) and BEAD4 (2 μm beads with carboxyl groups on their surface), BEAD1 showed the greatest differentiation between JD-negative and JD-positive samples. This indicates that the functional groups on the surface of BEAD3 and BEAD4 caused immobilization of MAP antigens that nonspecifically react with antibodies in the JD-negative sample.

By testing 155 serum samples from JD-negative and JD-positive samples using the bead-based assay in FCM, higher sensitivities were observed in JD-positive samples (low, medium and high shedders), especially in low shedders. This observation is similar to our previous study with the ELISA tests developed using ethanol extract of MAP; however, overall sensitivity of the bead-based assay (60.0%) was lower than that of the ELISA test (97.4%) (Eda et al., 2006). A different set of samples were used in this and previous studies and, therefore, direct comparison of the sensitivities would not be appropriate. Also, it is possible that different properties of the surface of the magnetic beads (plain polystylene) and ELISA plate (polystylene treated for higher binding of molecules) caused a difference in amount and/or kind of antigens immobilized onto the surface of the beads and plate.

Using the microfluidic system developed in this study, we showed that antibody binding to MAP-antigen-coated magnetic beads can be detected after off-chip and on-chip antibody reactions. Magnetic bead-based microfuidic systems have been used for development of immunoassays for diagnosis of diseases (GIJS et al., 2010). For example, Lee et al. immobilized dengue virus on magnetic beads to detect anti-dengue virus antibody in serum samples (Lee et al., 2009). The system is fully automated and could detect antibodies in 30 min. However, there has not been a report describing development of microfluidic system for JD. Our results suggest that the microfulidic system developed using ethanol extract of MAP may form a basis for development of on-site diagnostic device for JD.

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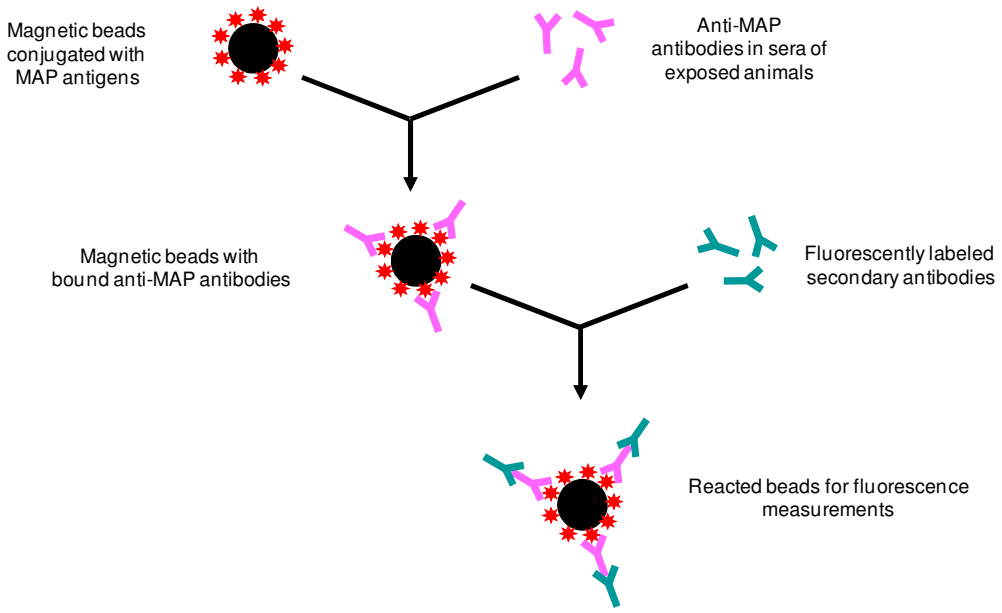


Figure 4. Wadhwa *et al*.

Figure 4. Schematic for immunofluorescence assay using magnetic beads

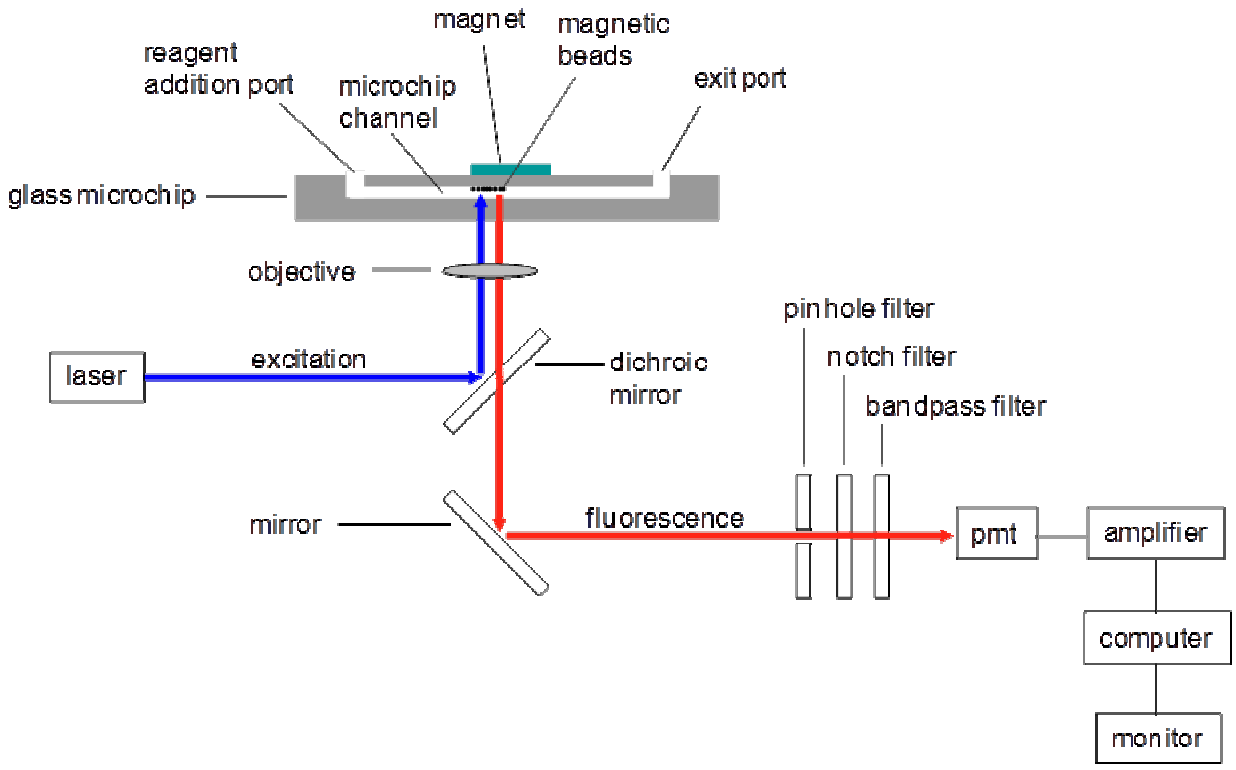


Figure 5. Wadhwa *et al*.

Figure 5. Schematic for the microfluidc system used in this study. The microchip channel is filled with buffer solution. Suspended beads are added to a channel port and migrate through the channel to accumulate under the magnet, where they can be imaged by fluorescence microscopy or semi-quantified by laser-induced fluorescence.

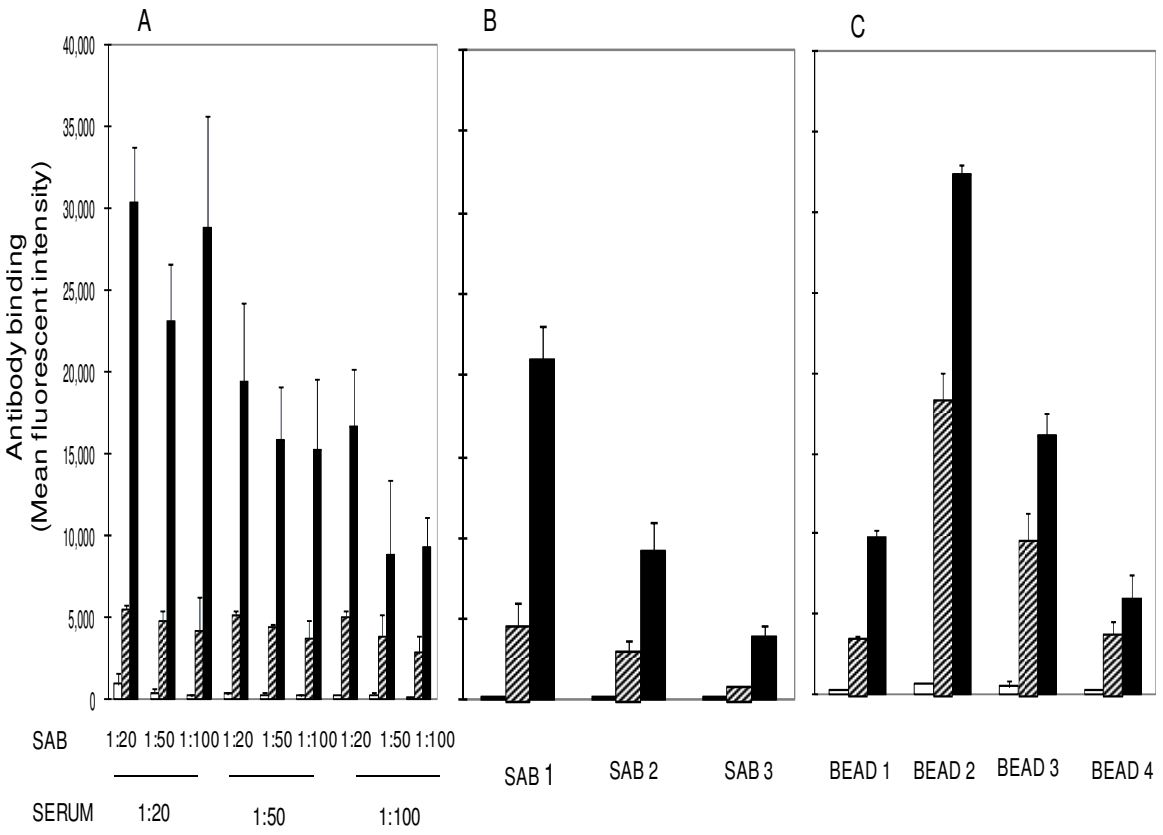


Figure 6. Wadhwa *et al*.

Figure 6. Results from the optimization of different conditions for bead-based FCM assay by using different dilutions of serum and SAB (A), different types of SAB (B) and different types of magnetic beads (C). The bars represent antibody binding levels in fetal bovine serum (open bar), JD-negative (hatched bar, Group I sample, cow ID 18) and JD-positive serum (solid bar, Group I sample, cow ID 11) samples. Each bar represents mean ± standard deviation of triplicate determinations. Each experiment was repeated twice with similar results.

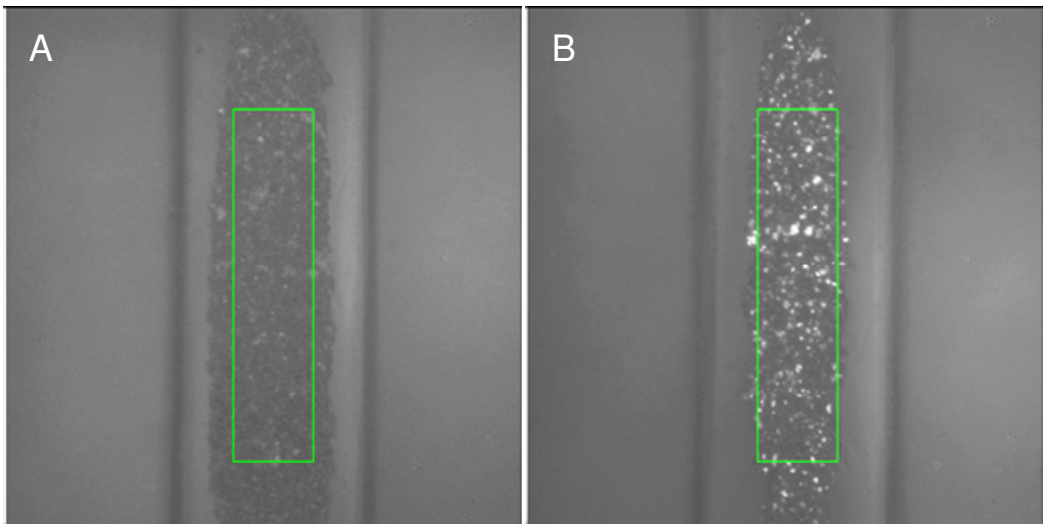


Figure 7: Wadhwa *et al*.

Figure 7: Fluorescence images of immobilized immunoassay beads prepared from JD-negative (**A**, Group I sample, cow ID 18) and JD-positive (**B,** Group I sample, cow ID 11) samples. The CCD images were taken using a Nikon TE 300 inverted microscope with 10x objective and FITC fluorescence filters. Total pixel values in the identically-sized regions of interest (ROI, green outline) were corrected for differences in background and used to measure the relative fluorescence of the negative and positive beads.

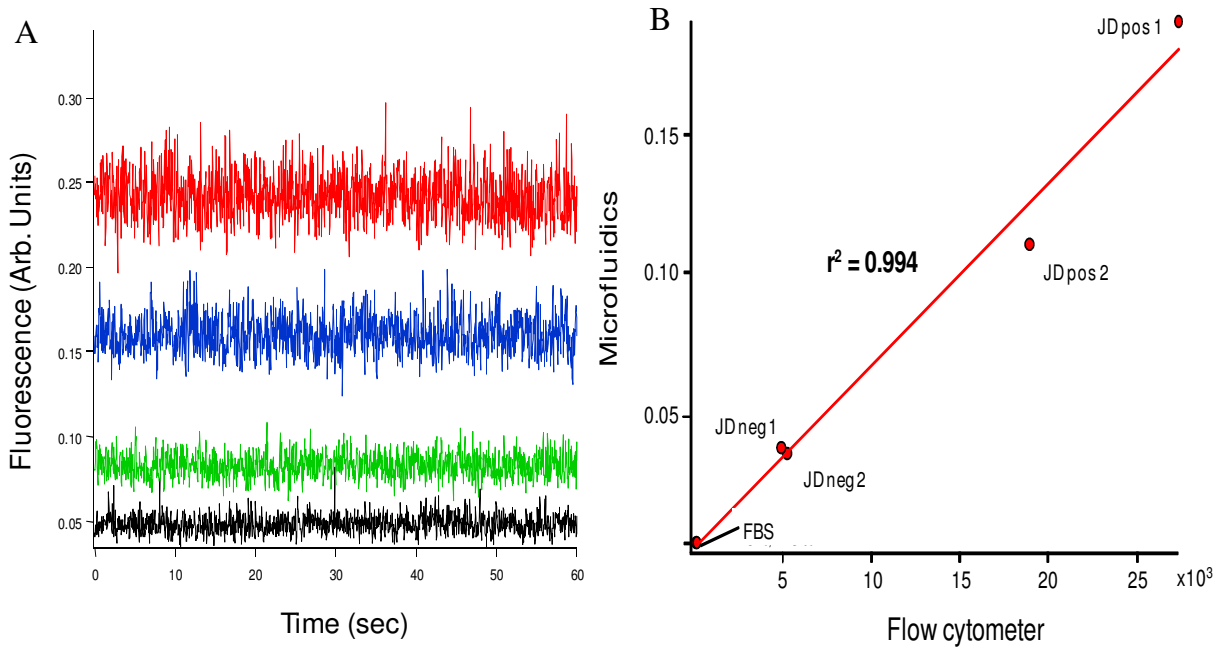


Figure 8. Wadhwa *et al*.

Figure 8. Results from FCM and microfluidics. (A) The results of the assays for three representative serum samples are shown as red - JD-positive serum (1:20 dilution, Group I sample, cow ID 11), blue - JD-positive serum (1:50 dilution, Group I sample, cow ID 11), green line - JD-negative serum (1:20 dilution, Group I sample, cow ID 18) and black line - no serum. (B) The results were compared with the antibody binding levels determined by the microfluidic system and FCM. Group I serum samples were used in this experiment - JD pos 1: cow ID 11; JD pos 2: cow ID 167; JD neg 1: cow ID 7; JD neg 2: cow ID 18 and FBS: fetal bovine serum.

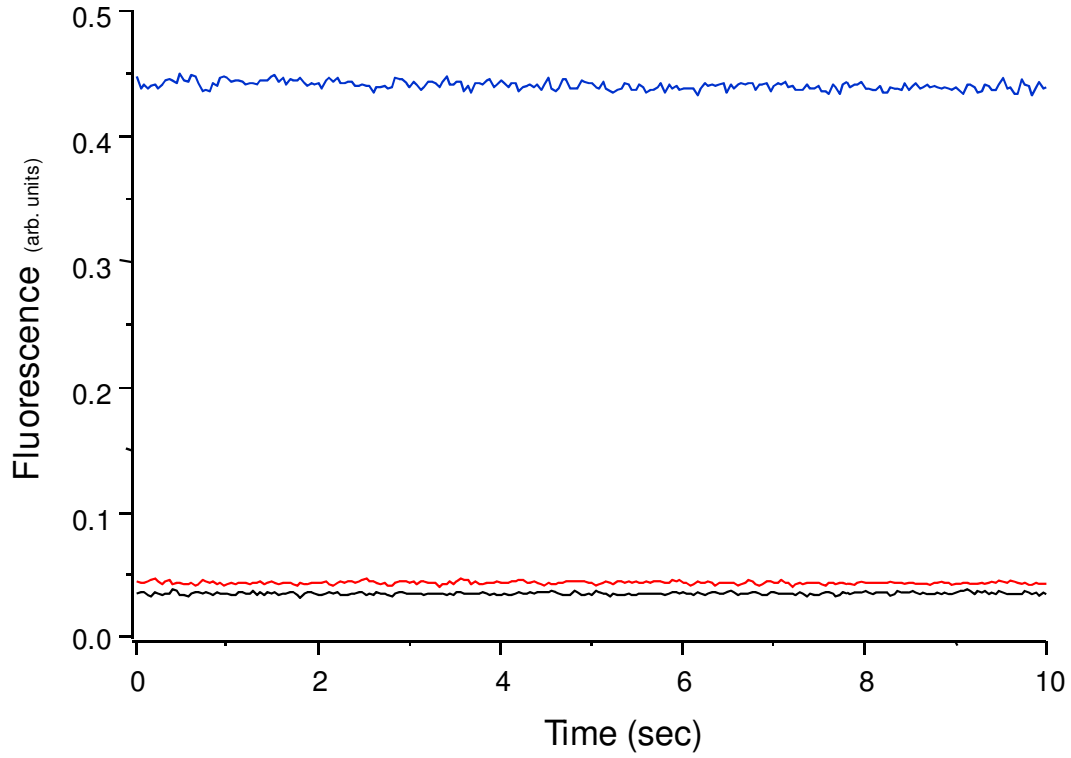


Figure 9. Wadhwa *et al*.

Figure 9. Antibody binding in the microfluidic system using on-chip antibody reaction protocol. Fluorescence of immobilized antigen-conjugated magnetic beads after on-chip incubation with antibody-containing JD-positive serum only (black line, Group I sample, cow ID 11), secondary only (red, SAB1), or the JD-positive serum followed by the secondary antibody (blue).

	Specificity (%)	Sensitivity (%)		
	N	L	M	H
Beads	98	45.7	48.6	85.7
ELISA-A	100	2.9	45.7	80.0
ELISA-B	100	17.1	42.9	80.0
ELISA-C	100	5.7	42.9	74.3

Table 1. Wadhwa *et al*.

Table 1. Comparison of sensitivity and specificity of the bead-based assay with those of commercial ELISA tests (ELISA-A~C). Serum samples (Group II) obtained from JD-negative animals (N, n=50), low shedders (L, n=35), medium shedders (M, n=35), and high shedders (H, n=35) were tested for antibody binding by different serological tests.

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CHAPTER 6

CONCLUSIONS AND OUTLOOK

This dissertation provides an in-depth analysis on the current status of diagnostic techniques for mycobacterial infections. Mycobacterial species are ubiquitous in nature and infect a wide variety of hosts. The infections caused by mycobacterial species are chronic in nature and it requires an early diagnosis. Control measures also require an understanding of the epidemiology of the agent and continuous surveillance of the host species. Previous studies on diagnosis have used various diagnostic approaches – bacterial culture, nucleic acid amplification assays and quantification of the immune responses. Each approach is unique and has its advantages and disadvantages; however, it is well established that the development and validation of the novel diagnostic tests is very challenging. The reasons being long incubation period, unpredictable disease progression, possible infection with closely related strains and use of different specimen often with low bacterial load. Present diagnostic tests suffer with drawbacks of lack of sensitivity – specificity, need for re-testing, higher cost and long turnaround time (up to 16 weeks) of the agent. The major focus of our project was to access the role of surface antigens of mycobacterial species in detection of humoral response using different specimens (milk and serum) and diagnostic formats (enzyme linked immunosorbant assay {ELISA} and lab-on-a-chip{LOC}). This study presents the results from optimization of serum based ethanol vortex ELISA (EVELISA) for milk testing of Johne's disesease (JD) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), development of a serum based EVELISA for testing of

bovine tuberculosis (bTB) in red deer and progress in formation of a bead-based microfluidic immunoassay for diagnosis of JD.

The use of ELISA has been recommended in previous studies to control JD. But the present ELISAs suffer with poor sensitivity. Most of these ELISA tests are based on detection of antibodies in the serum. Regular checking of antibodies present in serum is a tedious and costly process since it requires bleeding the animals and visit of veterinarians to farm. Testing of milk becomes more practical as it is collected on regular basis by the dairy herd improvement associations. Thus, we optimized a previously developed serum based ELISA based on surface antigens in ethanol solution to be used for milk testing (Chapter 3). By testing a set of well characterized milk samples, it was found that the EVELISA test was better than the commercially available ELISA. The results from this study showed that EVELISA could detect more animals which were actually positive and were showing a negative reaction by the commercial ELISA test. By utilizing milk based EVELISA test, better screening of the animals would be possible, reducing the spread of infection with in the herd.

Developed nations have eradicated bTB from their domestic animals but the wildlife species is still infected with the *Mycobacterium bovis* (MB). Domestic livestock population is in continuous contact with the wildlife species which acts as reservoir for MB and can spill over the infection to them. It becomes very important to have a highly sensitive – specific diagnostic test for bTB. Prior to this study, many diagnostic tests have been developed for bTB but they suffer with poor sensitivity as well as specificity. Co-infection with MAP has been previously described as a confounding factor for detection of antibodies in animals infected with MB. In this work, ethanol extract from MB was used to develop a sensitive – specific test for diagnosis of bTB in red deer (Chapter 4). We found that by using heat killed MAP to absorb cross reactive

antibodies; EVELISA can detect anti-MB antibodies. Therefore, ELISA test based on surface antigens from MB in ethanol solution can form a basis for a highly sensitive and specific test for detection of bTB in wildlife species.

Present diagnostic tests are required to be conducted in diagnostic laboratories with expertise staff in molecular biology. This involves a labor and cost intensive process requiring collecting samples and shipping it to the lab. Use of surface antigens of MAP and its application in development of LOC device for diagnosis of JD is also described in this dissertation (Chapter 5). Here, we provide evidence to conclude that species specific surface antigens provide better diagnostic advantages and could be used to form a basis for onsite diagnosis of the mycobacterial infections.

Finally, to expand upon the work in this dissertation, testing of more samples to calculate diagnostic sensitivity and specificity should be performed with EVELISA (both for JD and bTB) and the bead-based onsite diagnostic format. Characterization and isolation of molecules present in the ethanol extract would also be important for further improvement of diagnostic accuracy. Recently, specific lipid molecules (e.g. Para-LP-01) have been isolated and shown to be useful in detection of antibodies against MAP. Therefore, understanding the structure of the specific markers and incorporating them in development of better diagnostic test could be achieved in future. Further, it would be interesting to perform further studies on cellular immune responses against species-specific antigens in the ethanol extract. Indeed, a recent study showed that ethanol extract of MAP induced extensive inflammatory reactions in mouse model.

VITA

Ashutosh Wadhwa received his Bachelor's degree in Veterinary Science and Animal Husbandry (B. V. Sc & A. H.) from the College of Veterinary Science and Animal Husbandry, Mathura, India in 2004. Then, he did a Masters of Veterinary Science (M. V. Sc) in Epidemiology and Preventive Veterinary Medicine at the College of Veterinary and Animal Science, Bikaner, India in 2007. For his doctoral degree, he worked under the guidance of Dr. Shigetoshi Eda on role of surface antigens in diagnosis of mycobacterial infections. His long-term goal is to peruse academic and research career in the field of Immunology, Molecular Epidemiology and Animal Disease Diagnosis.