IDENTIFICATION OF DIAGNOSTIC MOLECULAR MARKERS FOR DETECTION OF BOVINE TUBERCULOSIS

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ABSTRACT

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Bovine tuberculosis (bTB) is distributed worldwide, creating a public health risk and causing substantial economic loss to cattle producers. Hence, control of bTB is important for both animal and human health. The tuberculin skin tests (TST) and whole blood interferon gamma ELISA (IFN-γ assay) are the principal antemortem diagnostic assays used worldwide for bTB surveillance and in the "test and slaughter" based bTB control and eradication programs. The currently used TST and IFN-γ assays are less than perfect in test sensitivity and specificity, consequently false negative and false positive test results are common. Presently, in the state of Michigan, only 1-2% of the antemortem test-positive cattle culled for postmortem examination have bTB. To reduce the number of healthy cattle slaughtered for confirmation of bTB, diagnostic procedures capable of differentiating test-false positive cattle from true bTB infected cattle are needed. The objectives for the studies presented in this dissertation were: 1) identify differences in gene expression profiles between cattle that have bTB and those that are antemortem test-false positive, 2) analyze the differences in gene expression profiles to select potential molecular markers capable of differentiating bTB infected cattle from antemortem test-false positive cattle, 3) begin a validation process to confirm that the selected molecular markers are able to differentiate bTB infected cattle from antemortem test-false positive cattle.

To accomplish those objectives, microarray hybridization experiments were used to examine gene expression profiles from small groups of bTB infected and antemortem test-false

positive cattle. Quantitative real-time PCR (qPCR) assays were used to validate data derived from the microarray hybridizations and to test selected molecular markers for disease on larger groups of bTB infected and antemortem test-false positive cattle.

In the initial study, cattle were grouped as single antemortem test-false positive (SFP), double antemortem test-false positive (DFP), and postmortem confirmed bTB infected (bTB). Whole blood from the cattle was stimulated for 4 hours with purified protein derivative made from cultures of *Mycobacterium bovis* (bPPD). Unique gene expression profiles were identified from the microarray hybridizations and qPCR assays confirmed that the gene expression profiles differentiated cattle in the bTB group from cattle in the DFP group, but not the SFP group.

In subsequent studies, the period of antigen stimulation of whole blood was extended to overnight; the microarray hybridization library was changed to provide thousands of additional genes for testing. Also, a new group of cattle was created that consisted of animals that were from bTB free herds and were single antemortem test-false positive (SR). The overall results showed that differential gene expression profiles exist between test-false positive and true bTB infected cattle. Furthermore, a predictor model was constructed using seven genes (IL-1R2, ATR, BOLA-DRB4, CCNG1, CXCL2, IL-10 and TARS). This model was tested in a preliminary study and showed high sensitivity (95%), specificity (92.9%), positive, and negative predictive values (90.5% and 96.3% respectively) for diagnosis of bTB. In conclusion, there is great potential for using several genes identified in this group of studies as the basis for a differential diagnostic test for detection of bTB, which may limit the unnecessary culling of test-false positive cattle as bTB suspects.

DEDICATION

To my very special family, Michael, Anna and Etta,
for the love, strength, and support that I could not do without,
I hope I made you proud.

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APCs	antigen presenting cells
aPPD	avian purified protein derivative
ATR	ataxia telangiectasia and Rad3 related protein
BCG	bacille-Calmette-Guerin
BH	Benjamini-Hochberg correction
BLAST	basic local alignment search tool
BOLA-DRB4	MHC class II DR beta4
bPPD	bovine purified protein derivative
bTB	bovine tuberculosis
CCNG1	cyclin G1
CCT	comparative cervical tuberculin test
cDNA	complementary single stranded deoxyribonucleic acid
CFT	caudal fold tuberculin test
CIT	single cervical intradermal test
CMI	cell mediated immune response
CXCL2	C-X-C ligand 2
DAVID	database for annotation, visualization and integrated discovery
DCPAH	Diagnostic Center for Population and Animal Health
DEPC	diethylpyrocarbonate
DFP	double test-false positive
DFP-non-ex	double test-false positive- non bTB exposed
DIVA	differentiate infected from vaccinated animal

DNA	deoxyribonucleic acid
DNR	Department of Natural Resources
DPP	dual-path platform
DTH	delayed type hypersensitivity
ESTs	expressed sequence tags
ELIZA	enzyme-linked immunosorbent assay
EU	European Union
FC	fold change
FDR	false discovery rate
FPA	fluorescence polarization assay
FP-ex	test-false positive-bTB exposed
GO	gene ontology
IFN-γ	interferon-gamma
IFN-γ assay	whole blood interferon gamma ELISA assay
IL	interleukin
IL1R2	Interleukin-1 receptor 2
TARS	threonyl-tRNA synthetase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBAA	latex bead agglutination assay
LDA	linear discriminate analysis
LIMMA	linear models for microarray
LOOCV	leave one out cross validation
LPT	lymphocytes proliferation test
M. avium	*Mycobacterium avium*

M. bovis	*Mycobacterium bovis*
MAANOVA	microarray analysis of variance
MAPIA	multi-antigen printed immunoassay
MHC	major histocompatibility complex
MSU	Michigan State University
NK	natural killer
NTC	No template control
NVSL	National Veterinary Services Laboratories
OIE	Office international des epizooties
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
RT	rapid test
SDHA	Succinate dehydrogenase complex subunit A
Se	sensitivity
SFP	single test-false positive
SICTT	single intradermal comparative tuberculin test
Sp	specificity
SR	single reactor
TB	tuberculosis
Th	T helper
TLR	toll-like receptors
TNF-α	tumor necrosis factor alpha

Treg	regulatory T cells
TST	tuberculin skin tests
UK	United Kingdom
USA	United States of America
WHO	World Health Organization

INTRODUCTION

The bovine tuberculosis (bTB) eradication program began in the United States (USA) in the early 20th century and, within a few decades, the herd prevalence of bTB in the USA was reduced from 5% in 1917 to less than 0.5% by 1941. The herd prevalence of bTB has remained low in the USA for the last 70 years and currently is estimated at 0.001% (Anon, 2009b). The last bTB cow in Michigan was found in 1974, and Michigan was granted bTB-free status in 1979 (Corso et al., 1997). In 1994, a bTB infected white-tailed deer was identified in the Northeastern Lower Peninsula of Michigan (Schmitt et al., 1997). Surveillance of hunter harvested deer from that region of the state in 1995-1996 resulted in an estimated prevalence of bTB in deer of 3.5%, and an estimated risk for transmission of bTB to cattle of only 0.1% (Corso et al., 1997). However, in 1998, bTB was found in a herd of cattle in the same region of Michigan that the infected deer had been found (Kaneene et al., 2006). Intensive surveillance and disease control efforts by the Michigan Department of Natural Resources (DNR) has kept the prevalence of bTB in deer at about 2% in the "bTB area", but those efforts have not kept the disease from periodically spilling over to cattle herds in the area (O'Brien et al., 2006). Re-emergence of bTB in any state is of great concern because it poses a potential zoonotic risk to the general public, disrupts cattle movement, and causes quarantine and depopulation of herds - events that can have devastating economic effects on dairy and beef producers.

Michigan lost its official bTB-free status in cattle in 1998 and began a long and costly bTB control/eradication effort. Control of bTB throughout the USA is implemented through routine gross inspection of carcasses at slaughter and through antemortem testing of cattle before shows, sale, or interstate movement. The primary antemortem test for bTB used in the USA is

the intradermal caudal fold skin test (CFT). For cattle that show a positive reaction on the CFT, a secondary (confirmatory) test is done. The secondary test may be either the intradermal comparative cervical skin test (CCT) or the whole blood interferon gamma assay (IFN-γ assay), (Anon, 2004). When cattle show positive reactions on two successive tests they are examined postmortem for presence of *Mycobacterium bovis* (*M. bovis*) and their herd of origin is quarantined until the postmortem tests are completed. Once bTB is identified in an animal, cattle herds in the immediate vicinity (usually within a 10 mile radius) are tested. All cattle that have moved into or out of the bTB positive herd are identified and traced to herd of origin or herd of destination so that those herds can be tested. If multiple herds in an area are found to have bTB infected cattle, movement of cattle from the area is controlled and the entire area undergoes yearly testing. The process of testing, quarantine, restrictions on cattle movement, and indemnification of cattle for postmortem examination is costly for the animal owner and the state.

Problem statement

Identification of bTB positive cattle herds in Northeastern Michigan in the late 1990s lead to an aggressive statewide bTB testing program, lasting from 2000 to 2003 and resulting in all cattle, bison and goat herds being tested. The testing established that bTB infection in cattle was concentrated in the "bTB area", which is a 5 county area (designated as DNR Management Unit 452) in the Northeast corner of Michigan's Lower Peninsula. As of 2008, 1.4 million bTB tests have been performed (Anon, 2008c) in Michigan, postmortem examinations have been done on about 2,200 cattle, and 125 bTB positive cattle have been identified from 44 herds. Of those 125 bTB positive cattle, 89% were from the "bTB area" and the majority of those bTB positive cattle (74%) were found from 2000 to 2003 (Anon, 2008c). Since that initial period of extensive

testing, bTB prevalence for the entire state of Michigan has remained at about the national level of less than 0.001% (1 in 100,000).

Test sensitivity and/or specificity of the intradermal skin tests and IFN-γ assay have been evaluated in Michigan. Using data from whole herd tests for 7 bTB positive herds identified prior to 2002 in comparison with the results of the intradermal skin tests with results from *M. bovis* culture and/or polymerase chain reaction (PCR) as gold standards, the estimated sensitivity for the CFT, serial CFT with CCT (CFTCCT$_{SER}$), and gross necropsy was determined to be 93.0%, 88.4% and 86.1% respectively (Norby et al., 2004). Point estimations for sensitivity and specificity also were calculated, resulting in test sensitivity and specificity values of 85.4% and 93.9%, respectively, for the CFT and 75.8% and 98.6%, respectively, for the CFTCCT$_{SER}$ (Norby, 2003). Test specificity of the IFN-γ assay is estimated to be 96.9% in the "bTB area" and 90.8% outside the "bTB area" (Larry Judge personal communication, and (Palmer and Waters, 2006).

Even though specificity of the antemortem tests is reasonably good when used in areas with low disease prevalence (like Michigan), the proportion of test-false positive cattle (no evidence of disease at postmortem) is high compared to the proportion of truly infected cattle. Cumulative data from Michigan's bTB program shows that currently only 1-2% of cattle that test as reactors on two successive tests (CFT/CCT or CFT/IFN-γ) are confirmed as positive for bTB at postmortem. The cause of false positive reactions is unknown, but has been speculated to be caused by exposure of cattle with environmental mycobacteria or with *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*), the cause of Johne's disease.

Overall hypothesis and study objectives

The overall hypothesis for this study is that, after antigen stimulation of white blood cells, the gene expression profiles of cattle with bTB will differ from cattle free of bTB that test positive in 1 or 2 antemortem diagnostic tests (test-false positive reactors). If true, then identifying such differences should allow identification of specific molecular markers that can be used to differentiate the test-false positive reactors from true bTB infected cattle.

To test this hypothesis, the studies presented here had two main objectives:

1. Compare the gene expression profiles of antigen stimulated peripheral blood mononuclear cells (PBMC) from test-false positive (reactors) cattle with those from true bTB positive cattle using microarray hybridization studies. From those comparisons, identify genes that are uniquely up-regulated or down-regulated within groups of cattle infected with bTB or cattle that test-false positive and are not infected with bTB.
2. Validate the differential transcription of genes using quantitative real-time PCR (qPCR), and then test the validated genes for potential use in a novel diagnostic assay for detection of bTB.

Overview of this dissertation

A review of literature on bTB infection, use and development of bTB diagnostic tests, and the current and future challenges faced by bTB control programs locally and globally is presented in Chapter 1.

The differential gene expression profiles of PBMC from cattle that were test-false positive for bTB were compared with those from cattle that had bTB. The PBMC in whole blood were stimulated with tuberculin for 4 hours before cellular ribonucleic acid (RNA) was harvested for use in the immunogenic BOTL5 microarray library. The differential gene

expression profiles observed were promising for development of a diagnostic assay that could discriminate cattle with bTB from cattle that do not have bTB. Results of this study are summarized in Chapter 2.

The results from the study described in Chapter 2 encouraged a similar study that examined gene expression profiles of PBMC after overnight stimulation with tuberculin. The study used a larger microarray library (the BLOPlus), which allowed identification of a panel of molecular markers with potential to differentiate test-false positive and true bTB infected cattle. The results of that study are presented in Chapter 3.

The potential molecular markers identified in Chapter 3 were tested using larger numbers of cattle then were used in Chapters 2 or 3. Several classifiers or predictor models for group classification (infected or not infected) of individual cattle were built based on the expression level of those markers. Estimation of specificity and sensitivity of the models for diagnostic purposes also was assessed. The results of that study are presented in Chapter 4.

Finally, a summary of the conclusions made from the studies presented in this dissertation, and thoughts on directions for future research, are presented in Chapter 5.

CHAPTER ONE

Literature Review

General information on bovine tuberculosis

Bovine tuberculosis (bTB) is caused by infection with *Mycobacterium bovis (M. bovis)*. *M. bovis* belongs to the *Mycobacterium tuberculosis* complex that includes *Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium canettii, Mycobacterium microti, Mycobacterium pinnipedii* and *Mycobacterium caprae*. All members of this complex are important animal and human pathogens that cause tuberculosis in their host species (Oreilly and Daborn, 1995; Thoen et al., 2009). Tuberculosis is an infectious disease that is characterized by formation of nodular granulomas known as tubercles (McMurray, 1996).

M. bovis has a very broad host range, and may infect most warm-blooded animals including humans (Oreilly and Daborn, 1995). Although cattle are considered the true hosts of *M. bovis*, bTB has been reported in many animals within the Orders Artiodactyla, Carnivora, Primates, Rodentia, Lagomorpha, Marsupialia and others (Gavier-Widen et al., 2009; Kovalev, 1980). Many of the animals that can be infected with *M. bovis* are considered spill-over hosts, meaning that an animal can acquire bTB but the disease is not sustained in a population of that species. In most instances, a spill-over host acquires the disease by close contact with bTB infected animals, or through ingestion of infected carcasses. A few wildlife species have been confirmed as maintenance/reservoir hosts where the disease is sustained in the population and can spread infection to other wildlife or domestic animals (Corner, 2007; de Lisle et al., 2002; Michel et al., 2006). Currently, the known wildlife reservoirs of bTB include the African buffalo in South Africa (De Vos et al., 2001; Keet et al., 1996), wood bison and North American bison in

Canada (Nishi et al., 2006), white-tailed deer in Michigan (O'Brien et al., 2008; O'Brien et al., 2004; O'Brien et al., 2006; Schmitt et al., 2002), red deer in Spain (Vincent et al., 2006), Eurasian badgers in the United Kingdom (UK) and Ireland (Barrow, 1981; Little et al., 1982), brushtail possums in New Zealand (Coleman et al., 1999; de Lisle et al., 2001) and wild boar in Europe (Naranjo et al., 2008; Parra et al., 2003; Vincent et al., 2006). *M. bovis* infection also is recognized as a serious emerging disease of exotic wildlife species in zoological collections (Chambers, 2009; Thoen et al., 2009).

In addition to direct contact with an infected animal, transmission of bTB can occur through contact with contaminated environmental sources (Ayele et al., 2004). Infected animals frequently shed *M. bovis* in exhaled air, sputa discharge, feces, milk, urine, vaginal and uterine discharges, and from abscessed peripheral lymph nodes that discharge through the skin (Neill et al., 1991). *M. bovis* can survive for long periods of time in soil, on pastures, and in feces, urine or water under limited exposure to direct sunlight (Courtenay et al., 2006; Young et al., 2005). In cattle, aerosol transmission currently is accepted as the most common route of infection, as lesions are most frequently seen in bronchial, mediastinal, and retropharyngeal lymph nodes (Menzies and Neill, 2000; Neill et al., 1994; Whipple et al., 1996).

Zoonoses and public health impacts of bTB

M. bovis is a zoonotic organism that causes many cases of human tuberculosis (TB) worldwide (Cosivi et al., 1998). The true prevalence of human TB caused by *M. bovis* is likely underestimated because speciation of mycobacteria from humans is not routinely done in many countries, particularly in developing countries where TB is common (de Kantor et al., 2010; Thoen et al., 2006). In 2006, bTB was classified as a neglected zoonotic disease by the World Health Organization (WHO) (Anon, 2006). Risk of zoonotic bTB infection is much higher in

HIV endemic areas of the world, especially where bTB infection in cattle is common. Studies in Tanzania and Uganda showed that 10-30% of the human TB isolates are *M. bovis* (Cleaveland et al., 2007; Kazwala et al., 2001). Cousins (2001) reported that more than 94% of the world's human population lives in countries with limited or no bTB control, which would greatly increase the risk of zoonotic infection with bTB.

M. bovis currently is regarded as a significant re-emerging cause of extra-pulmonary TB in humans, as reported in Mexico (Cicero et al., 2009) and the USA (Hlavsa et al., 2008; LoBue et al., 2003). Oral transmission of *M. bovis* through ingestion of contaminated milk and dairy products is an important cause of extra-pulmonary TB in humans. Death caused by bTB in human populations has decreased drastically. This decrease has occurred due to improvement in medical treatment, and more importantly due to implementation of bTB eradication programs in cattle worldwide and pasteurization of milk. Unfortunately, consumption of unpasteurized milk and milk products is still common practice in many undeveloped regions of the world, and occasionally in certain areas of developed countries.

In the USA, about 1% of human TB cases occur as extrapulmonary disease caused by *M. bovis*; and many of those cases can be linked to ingestion of unpasteurized dairy products of Mexican origin (Hlavsa et al., 2008; LoBue et al., 2003). Recent reports of human bTB cases from the UK are also linked to consumption of unpasteurized dairy products such as artisan cheeses made from unpasteurized milk (Rowe and Donaghy, 2008). It is important to rule out *M. bovis* as the cause of extra-pulmonary TB because *M. bovis* is resistant to pyrazinamide (Scorpio et al., 1997), a drug used during the first months of treatment of infection with *M. tuberculosis*. Use of pyrazinamide is not appropriate for treatment of *M. bovis* infections because this drug may interfere with timely successful treatment (Cicero et al., 2009).

Airborne aerosol plays an important role in cattle to human transmission. Occupational risk of bTB infection for people working closely with livestock, such as slaughter house workers, farmers, and veterinarians is well recognized. There are reports of bTB infection of abattoir workers in Australia (Georghiou et al., 1989; Robinson et al., 1988), bTB exposure of workers during depopulation of farmed red deer and elk in Canada (Fanning and Edwards, 1991; Liss, 1994), and infection of young children living on a bTB infected farm in the UK (Smith et al., 2004). With the finding of bTB in gaming wildlife, such as in Michigan and Africa, there becomes a potential risk for disease transmission to hunters when they field dress infected animals or consume improperly cooked wild game. An example of disease transmission from wildlife is the case of a hunter in Michigan contracting bTB via a finger injury that occurred while field dressing a bTB infected white-tailed deer (Wilkins et al., 2008).

Social-economic impact of bTB

Global estimates of bTB economic losses are about $3 billion annually, with 50 million cattle worldwide infected with bTB (Garnier et al., 2003; Steele, 1995). Direct economic losses come from condemnation of bTB infected carcasses, and equally important indirect losses come from bTB control and testing efforts. The current 'test and slaughter' based bTB control programs implemented in many developed countries are very costly. In 2008/09 it was estimated that control programs cost NZ$82 million for New Zealand, US$40 million for USA, and £100 million for the UK (Anon, 2009c, 2010; Schiller et al., 2010a). Where bTB control programs are implemented to safeguard the public interest, finding bTB infection in cattle herds can be a devastating event for farm owners. Beginning with a presumptive positive antemortem bTB test, the cattle herd will be quarantined. A confirmed bTB positive animal, identified on a postmortem examination, could mean depopulation of the whole herd. Aside from potential

financial losses, a diagnosis of bTB in a herd may lead to loss of social status causing additional stress and emotional upset for producers and their families.

Control measures for bTB infection in wildlife species that directly impact the cattle industries are limited at this time and often focus on planned reduction of infected populations, such as has been attempted with brushtail possums in New Zealand and white-tailed deer in Michigan. Infection of protected wildlife species with bTB in their natural setting could lead to higher than normal yearly death losses, endangering the survival of the species. Currently, the consequences and long term impact of wildlife bTB infection to ecosystems are not well understood. Certainly, there is a concern that widespread bTB in Kruger National Park in South Africa could negatively impact populations of endangered species (Michel et al., 2010). Cattle industries worldwide are an important source of food for humans, both from meat and dairy products. The beef and dairy industries are at risk when local wildlife populations become infected with bTB. Without implementation of proper disease control measures in wildlife and domestic cattle populations, a sustainable food supply is at risk (Michel et al., 2010).

Pathogenesis of bTB

In cattle, bTB is commonly found as a chronic disease with little to no clinical signs. Occasionally, a more progressive form of the disease occurs with clinical signs of coughing, evidence of dyspnea, and productive broncho-pneumonia (Thoen et al., 2009). Definitive information on the pathogenesis of *M. bovis* is incomplete. Most of the knowledge on pathogenesis has been acquired through experimental cattle infected with *M. bovis*, and some from field cases of natural infection. An advanced understanding of pathogenesis of *M. tuberculosis* in human TB cases, and a wealth of information from experimental animal models, has helped provide insight into the pathogenesis of *M. bovis* in cattle (Pollock et al., 2001).

The outcome of a potential *M. bovis* infection is influenced by many factors, including route of infection, the level of host immunity, and the capacity of the host for clearance of pathogens. Not surprisingly, there is disagreement on what constitutes the minimum infectious dose for *M. bovis*. Most recent investigations have shown that as few as 5000 colony forming units are sufficient to infect cattle orally (Palmer et al., 2004), while Neill et al. suggested that a single bacilli entering by an aerosol route may be sufficient to cause disease in cattle (Neill et al., 1991). Experimental studies have shown a correlation between the amount of the challenge dose and the severity of the disease (Buddle et al., 1994; Neill, 1988). Typically, an aerosol challenge dose of 10^2 to 10^4 is required to simulate a disease process similar to that is seen in field cases (Maue et al., 2004; Rodgers et al., 2007).

In addition to the dose of organisms in the infectious inoculum, several lipid complexes associated with the mycobacteria cell wall are important virulence factors that contribute to the pathogenicity and survival of the organism in the macrophage and anaerobic environment of the granuloma. Lipoarabinomannan (LAM), phenoglycolipid mycosides (PGL-1), trehaloes-6,6-dimycolate (TDM), sulfatides, mycobactins and exochelins are among the known virulence factors that influence *M.bovis* survival and disease outcome (Collins, 2001). Suppressed host immunity caused by malnutrition, stress-associated events such as pregnancy and the post-partum period, and concurrent infections with viruses such as bovine viral diarrhea virus (BVDV) may enhance bTB infection (Houe and Heron, 1993). Increased age appears to correlate with an increase in bTB incidence (Menzies and Neill, 2000; Munroe, 2000). Finally, the *Bos indicus* (Zebu breed) of cattle is reported to be more resistant to bTB infection than the *Bos taurus* breeds (Oreilly and Daborn, 1995), however the genetic component for the resistance has not been defined.

Host immunity to bTB infection

Innate immunity

Infection is established when *M. bovis* bacilli in aerosol droplets are inhaled and become lodged on the respiratory tract or when *M. bovis* bacilli are ingested and enter the alimentary tract (Pritchard, 1988). Macrophages are the key effector cells in a host's innate immune response to bTB infection. The functions of macrophages in an infection range from bacterial destruction to bacterial dissemination. Initial uptake of *M. bovis* primarily happens in tissue macrophages, followed later by dendritic cells and blood-derived macrophages (Denis and Buddle, 2008). Binding of *M. bovis* to various receptors on the macrophage cell surface such as Toll-like receptors (TLR), complement receptors, mannose receptors, and type A scavenger receptors trigger phagocytic uptake of the organism into phagosomes within the macrophage. Normally, the phagosome would fuse with a lysosome to form a phagolysosome, where destruction of the pathogen would occur. However, the *M. bovis* organisms within a phagosome can inhibit fusion of the phagosome with lysosymes, thereby limiting acidification and enabling the organism to survive inside the phagosome (Thoen and Bloom, 1995; van Crevel et al., 2002). This is a self-protection mechanism shared by many pathogenic mycobacteria (Armstrong & Hart, 1975). Preventing intracellular destruction in a phagolysosome allows *M. bovis* to multiply in macrophages.

Release of tumor necrosis factor alpha (TNF-α) and inflammatory chemokines from the infected macrophages causes blood monocytes, neutrophils, natural killer (NK) cells, and other inflammatory cells to migrate to the infection site. The monocytes differentiate into macrophages and ingest *M. bovis* released from infected apoptotic macrophages. Thus, the organism can multiply in a new supply of cells and an inflammatory cascade is triggered at the

local site of infection. At this point in the inflammatory process, it has been proposed that apoptosis of infected cells may occur to limit the outgrowth of intracellular pathogens in macrophages (Rodrigues et al., 2009).

Cells other than macrophages are involved in the initial response to mycobacterial infection. In human TB and in murine models of TB, natural killer (NK) cells play an important protective role by producing interferon-gamma (IFN-γ) and by causing cytolysis of mycobacteria-infected target cells (Yoneda and Ellner, 1998). Human neutrophils can kill *M. tuberculosis* in vitro (Brown et al., 1987) and rabbit neutrophils can produce monocyte recruiting chemokines when stimulated with mycobacteria, suggesting their involvement in the development of the tuberculous granuloma (Antony, 1985). Neutrophils are among the earliest cells associated with the developing granuloma as identified in histopathology of bTB infection (Cassidy et al., 1998). However, the precise role of the neutrophils in bTB remains unknown.

Cell mediated immune response (CMI)

Studies of natural and experimental bTB infection in cattle have shown that a complex spectrum of immune responses is generated following *M. bovis* infection and that cell mediated immune (CMI) responses are dominant (McNair et al., 2001; Neill et al., 1994; Pollock et al., 2001; Wood and Rothel, 1994). Dissemination of *M. bovis* by blood mononuclear cells from the initial site of infection to regional lymph nodes results in activation of T cells. *M. bovis* replicating in macrophages produce secreted antigens and breakdown products that move to the cell surface and are presented to T cells, triggering the adaptive immune response (Cooper, 2009). It is widely accepted that T cells responding to macrophage-presented *M. bovis* antigens undergo clonal expansion, leading to the development of memory T cell populations (Pollock et al., 2001). Immunohistochemical examination of early granulomatous lesions in experimentally

infected cattle confirm that T cells are among the first cells involved in the inflammatory reaction (Cassidy et al., 2001).

Expansion of T cells can be driven by peptides presented in the context of major histocompatibility complex class I or II (MHC I or II) molecules, and by recognition of non-protein mycobacterial lipids by non-polymorphic MHC molecules like CD1 proteins. All of the main T-cell subsets (γδ T cells, CD4+ and CD8+ αβ T cells) have been shown to participate in the anti-mycobacterial immune response in cattle (Pollock et al., 1996). Studies of circulating T cells in experimentally infected animals suggest that different populations of T cells become involved sequentially as the infection develops; the γδ T cells have a significant initial involvement followed by the CD4+ (MHC II-restricted) and then the CD8+ (MHC I-restricted) αβ T cells later in infection (Orme, 1987; Pollock et al., 1996).

The γδ T cell subset exists in much greater numbers in ruminants than in other animal species. The majority of the circulating γδ T cells in ruminants express a 215 kDa surface molecule designated as WC1 (Wedlock et al., 2002). Studies of WC1+ γδ T cells suggest that they localize to the site of initial infection with *M. bovis*, become involved with recruitment of lymphocytes and monocytes, and contribute to the development of the granuloma in bTB infected cattle (Pollock et al., 2001; Skinner, 2001 ; Smith, 1999). WC1+ γδ T cells are important early sources of IFN-γ (Smyth et al., 2001). In addition, there is evidence that the γδ T cells have a role in linking the innate and adaptive immune systems (Bukowski et al., 1999; Kennedy et al., 2002; Mak and Ferrick, 1998; Pollock et al., 2005).

The CD4+ T cells respond to antigens presented via MHC-II by antigen presenting cells (APCs) such as macrophages and dendritic cells. Of the subset of CD4+ T helper (Th) cells, the interleukin (IL)-2 and IFN-γ producing-Th1 helper cells appear to be most important in the

defense against *M. bovis*. IL-12 and IL-18 produced by APCs are key cytokines that promote polarization of Th0 cells toward Th1 rather than Th2 type. Establishment of a Th1 type of immune response, characterized by IFN-γ production, is essential for activation of macrophage anti-mycobacterial pathways (Cooper, 2009). Within 2-3 weeks after the initial infection, activated Th1 helper cells migrate to the infection site. Th1 helper cells promote containment of the bacilli through release of cytokines (IL-12 and IFN-γ), which stimulate macrophage activation and release of chemokines to recruit other T cells involved in CMI (Kennedy et al., 2002; McNair et al., 2007; Pollock and Neill, 2002; Pollock et al., 1996; Smyth et al., 2001). Animals that can maintain a bias toward a Th1 response often show less severe disease after infection with *M. bovis* (Welsh et al., 2005).

Primary cytokines that play important roles in control of bTB infection are those of the Th1 type, secreted mainly by CD4+ Th1 cells and APCs, and to a lesser extent by NK cells and CD8+ cytotoxic T cells. IL-1β, IL-2, IL-12, IL-18, IFN-γ and TNF-α, are the most important cytokines involved in CMI. IL-1β is produced mainly by activated macrophages and is important in the acute phase response, promoting T cell expression of IL-2 and its receptor (IL-2R or CD25). IL-2 is essential in inducing T cell expansion and generation of memory T cells. IL-12 produced by APCs activates macrophages in an autocrine manner and also activates NK cells. IL-12 also stimulates production of IFN-γ, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF). It is a key mediator in CMI, promoting Th1 polarization and exerting protective effects through induction of IFN-γ. IL-18 also is an important IFN-γ inducing factor. IFN-γ has an important protective role in CMI. The main cell population producing IFN-γ is the CD4+ Th1 helper cells. CD8+ and WC1+ T cells also are potential sources of IFN-γ, although they release the cytokine at much lower levels than the CD4+ Th1

helper cells (Smyth et al., 2001). IFN-γ and TNF-α up-regulate production of reactive oxygen intermediates, nitric oxide, and reactive nitrogen intermediates; which are involved in the killing of mycobacteria within activated macrophages (Flynn and Chan, 2001; Stenger, 1999). TNF-α also promotes granuloma formation.

The CD8+ T cells play an important protective role by killing macrophages that are infected with mycobacteria (Liebana et al., 2000a; Skinner et al., 2003). Binding of CD8+ T cells to the mycobacteria-infected macrophages, followed by the release of perforin and granzymes from cytoplasmic granules within the CD8+ T cells, are crucial for induction of apoptosis. The ability of CD8+ T cells to kill mycobacteria in human TB is associated with granulysin release from cytoplasmic granules of cytolytic T cells (Stenger et al., 1998). Apoptotic macrophages are cleared by phagocytic cells through the process of efferocytosis (Vandivier et al., 2006). The CD8+ T cells also play a role in controlling the chronic phase of the disease and possibly in latent infection. In vitro studies have shown that antigen-stimulated CD8+ T cells are capable of releasing metabolically-active *M. bovis* from infected macrophages (Liebana et al., 2000b), which promote uptake by antigen presenting cells for more efficient antigen presentation.

The Th17 cell is a newly identified subset of effector T cells that are involved in TB infection. Th17 cells produce IL-17 upon antigen stimulation. In the mouse model, Th17 cells were connected to protection from *M. tuberculosis* challenge, suggesting a role in TB immunity (Khader et al., 2007; Khader and Cooper, 2008). In a study of white-tailed deer, IL-17 expression was shown to inversely correlate with pathology in deer that were experimentally challenged with *M. bovis*, which indicated the potential role of Th17 cells in CMI of bTB infection (Thacker et al., 2009).

Regulatory T cells (Treg) have been shown to limit protective immunity in many infectious diseases including human TB. The CD4+CD25+ Treg cell numbers were elevated in blood and infection sites in TB patients, suggestive of suppressed host immunity (Chen et al., 2007; Guyot-Revol et al., 2006) . Li et al (2007, 2008) showed that Treg cells inhibit induction of IFN-γ in both CD4+ T cells and γδ T cells in response to mycobacterial antigen (Li et al., 2007; Li and Wu, 2008). The role of Treg cells in bTB is unknown. In an experimental white-tailed deer study, involvement of Treg cells was not found after BCG vaccination or after *M. bovis* challenge (Thacker et al., 2009).

Humoral response

Past reports indicate that only a small proportion of cattle with bTB lesions have a measurable antibody response (McNair et al., 2001). In an experimental infection study, B-cells were not found within the granuloma until 42 days post infection, which agreed with observations that antibody production is delayed in bTB infection (Cassidy et al., 2001). It is generally considered that antibodies are not produced in appreciable quantity until the more advanced stages of infection. Anti-mycobacterial antibody is predominantly the IgG1 isotype and development of an antibody response correlates positively with development of lesions (Welsh et al., 2005). Antibody directed against the bacterial MPB70 protein is detected mainly in cattle with advanced bTB, further suggesting that the antibody response is associated with lesion development and may be a useful indicator of disease status (Lightbody et al., 2000).

Anergy

Under certain circumstances in advanced and/or disseminated tuberculosis, cattle become immunologically anergic. Anergic animals lack a detectable CMI response, but may have high levels of circulating antibody (Lepper et al., 1977; Neill et al., 1994; Plackett et al., 1989).

Anergic cattle are not considered an issue in countries with active bTB control programs, where diseased cattle are generally identified and removed shortly after infection. However, in countries where advanced bTB infection is common, the proportion of anergic animals may be appreciable. Failure to identify and remove bTB infected anergic animals can have serious consequences, as those animals are a source of infection within the herd. In human TB, anergy is more prevalent in advanced cases of disease (Toossi, 1996). As in cattle, the mechanisms of tuberculosis-associated anergy in humans are not well understood.

Latent bTB infection

Latent TB infection occurs when a human or animal is infected with mycobacteria, but does not have active disease, and is not infectious. Latent infection occurs when mycobacteria are effectively contained and persist without disease progression for prolonged periods of time. Latent infection is common in human TB, and it is believed that latent bTB infection also occurs in cattle (Morrison et al., 2005; Pollock and Neill, 2002; Van Rhijn et al., 2008; Vordermeier et al., 2008). Philips et al. (2003) suggested that up to 30% of cattle in a bTB positive herd can become infected. Cattle with a robust innate immune response can clear the infection, while other cattle with a less effective immune response fail to clear the infection and may progress to a latent infection or toward a disease state (Phillips et al., 2003). During early infection, and possibly during latent infection, the pathological changes caused by the bacilli are not detected by visual inspection, and *M. bovis* numbers may be insufficient for successful bacterial culture. However, cellular immune responses, as measured by intradermal skin tests or the IFN-γ assay, may be used to detect infection before development of pathological lesions or before culture of *M. bovis* from tissue samples is successful (Pollock et al., 2000; Pollock and Neill, 2002; Vordermeier et al., 2008).

The populations of cattle that tested positive by antemortem bTB tests, but lack visual or microscopic lesions, and are *M. bovis* culture negative at postmortem, are generally classified as test-false positive. This classification may not be correct and additional testing may be needed especially for cattle with a history of exposure to diseased cattle. Identification and removal of this population is critical in bTB control programs, as these animals can serve as reservoir for source of infection to other herdmates (Vordermeier et al., 2008).

Delayed type hypersensitivity (DTH) and granuloma lesions

Delayed-type hypersensitivity (DTH) is a major mechanism of defense against intracellular pathogens, such as *M. bovis* (Kobayashi et al., 2001). The escape of *M. bovis* from the initial intracellular phagolytic process in macrophages leads to development of a memory T cell response. DTH is characterized by CMI responses caused by sensitized T cells, with initiation by CD4+ T cells and direct cell cytotoxicity by CD8+ T cells. DTH is mediated by Th1 cells secreting predominantly INF-γ and IL-2. In previously sensitized hosts, the tuberculin test induces a DTH response that results in recruitment of T cells and macrophages to the site of intradermal deposition of antigen. Those cell types become activated and produce an inflammatory response seen as a marked swelling at the site within 24 to 48 hours.

Early lesion development in an infected host is marked by macrophage differentiation into multinucleated giant cells, epitheliod cells, and foamy macrophages at the site of infection. As bacilli escape from the edge of a lesion, they are ingested by macrophages attracted to the site and again multiply within those macrophages, promoting a further DTH response. This on-going process leads to continuous remodeling and tissue destruction at the infection site. Eventually, a granuloma forms that has a necrotic center surrounded by infected macrophages, enclosed by foamy macrophages and other mononuclear phagocytes, and encased by an extensive fibrous

capsule. This fibrous capsule helps exclude lymphocytes from the central core of the structure, and reduces penetration of blood vessels into the granuloma. Within the granuloma, the balance between killing and survival of the pathogen determines the outcome of clearing the infection, containment of infection, or progression to an advanced disease state (Russell, 2007; Russell et al., 2009; Saunders and Britton, 2007).

The granulomas in bTB cattle are usually non-odoriferous, have a yellowish appearance and are caseo-calcareous, or calcified in consistency. The caseous centre is usually dry, firm and surrounded with a thick fibrous tissues. Lesion size ranges from small enough to be missed by visual inspection, to involvement of the greater part of an organ. Microscopically, lesions are composed of a necrotic center bordered by epitheliod cells, giant multi-nucleated cells, lymphocytes, a few granulocytes and enclosed within layers of fibrous connective tissue (Thoen et al., 1981; Thoen et al., 2009). These granuloma are often paucibacillary (having few organisms) and sometimes with an absence of acid-fast organisms. The appearance of granulomas is normally more purulent in cervids and camelids, and occasionally in cattle. In many free-range wildlife populations, clinical signs of disease are not apparent, even when lesions are well developed. Although clinical signs of disease are not apparent, these animals can be highly contagious (Gavier-Widen et al., 2009; Thoen et al., 2009).

Current bTB antemortem tests

Tuberculin Skin Tests (TST)

Tuberculin skin tests are the principal antemortem tests used in surveillance for bTB, and the only Office International des Epizooties (OIE) approved bTB tests for international trade (Anon, 2008a). The TST are *in-vivo* based assays performed by intradermal injection of tuberculin, which then elicits a DTH response in animals infected with or sensitized to

mycobacteria. DTH results from a CMI response mounted against the injected tuberculin and is characterized by infiltration of white blood cells at the injection site, which leads to measurable swelling and thickening of skin by 72 hours post injection (Monaghan et al., 1994). There are several variations of approved TST currently used in different countries. Those include the caudal fold tuberculin test (CFT), the single cervical intradermal test (CIT), and the comparative cervical tuberculin test (CCT) or single intradermal comparative tuberculin test (SICTT) (Buddle et al., 2009; de la Rua-Domenech et al., 2006; Monaghan et al., 1994; Schiller et al., 2010a).

Tuberculin is prepared from heat-killed cultures of mycobacteria. Although tuberculin is also termed purified protein derivative (PPD), in reality, it is a poorly-defined crude mixture of mycobacterial proteins. The mycobacteria are grown in liquid medium, heat treated by free-flowing steam, filtered and precipitated, followed by washing and suspended into a sterile preparation of tuberculin (Francis et al., 1978; Monaghan et al., 1994). Tuberculin prepared from *M. bovis* is termed bovine PPD (bPPD) and tuberculin from *M. avium* is termed avian PPD (aPPD). The current bPPD used worldwide is prepared from *M. bovis* strain AN5 that was originally isolated in England in 1948 (Paterson, 1948). The *M. bovis* AN5 was selected through repeated subculture on glycerinated-media, similar to the method used for attenuation of *M. bovis* BCG. However, *M. bovis* AN5 was shown to not have extensive gene deletions or an altered gene expression profile (Inwald et al., 2003; Pelayo et al., 2009). Despite this fact, suitability of *M. bovis* AN5 bPPD used for detection of various strains of *M. bovis* worldwide has been questioned (Buddle et al., 2009). Potencies and performance of bPPD varies among different manufacturers and even between different batches from a given manufacturer (Monaghan et al., 1994; Schiller et al., 2010b). Potency of PPD is determined by *in vivo* testing in sensitized guinea pigs or cattle, and standardized against an international reference standard

(Anon, 2008b). Standardization of PPD is critical as variation in PPD performance directly affects the accuracy of the TST.

The CFT is a single injection of bPPD into the skin fold at the base of an animal's tail. The site of injection is visually examined and palpated for inflammation (swelling and redness) approximately 72 hours post injection. An animal showing a reaction against bPPD is classified as a CFT responder (or suspect), otherwise the animal is classified as CFT negative (Monaghan et al., 1994). The CFT is widely used as a primary routine screening test in most bTB control programs worldwide. The sensitivity of CFT ranges from 68-96.8% and specificity can be as high as 96-98.8%. The CFT is widely used because it is amenable to high throughput, it is relatively inexpensive, and the tuberculin reagent is readily available (Monaghan et al., 1994; Schiller et al., 2010a). The limitations of CFT are that it is labor intensive and time consuming because a trained professional must make 2 trips to a farm, one trip to administer the test and a second trip 3 days later to determine the results of the test. Therefore, animals must be restrained twice. Interpretation of results of the test can be dependent on the individual who performs the test; thus, there is potential for human error and bias (especially when testing hundreds or thousands of animals) (de la Rua-Domenech et al., 2006; Schiller et al., 2010a).

The CIT is very similar to CFT, but the injection site is at the mid-cervical area of the neck instead of the caudal skin fold of an animal. Besides visual examination and palpation, skin thickness at the injection site is also measured prior to injection and again after 72 hours. An animal with an increase in skin thickness less than 2 mm with no visible local inflammation is consider negative, an increase in skin thickness more than 4 mm is considered a reactor, and an increased skin thickness between 2-4mm with no visible inflammation is inconclusive (Monaghan et al., 1994; Schiller et al., 2010a). The CIT is widely used in the European Union

(EU) countries as a primary routine test. In general, CIT is regarded as more sensitive than the CFT, thus smaller amounts of tuberculin are needed for the test (Francis et al., 1978). Sensitivity for the CIT ranges from 80-91%, and specificity ranges from 75.5-96.8%. The CIT has the same limitations as CFT, and the CIT is not as amenable to high throughput (de la Rua-Domenech et al., 2006; Schiller et al., 2010a).

The CCT (SICTT) is a comparative skin test where a biological balance of bPPD and aPPD are injected side by side at the mid cervical region of an animal. The swollen skin area at both injection sites is measured 72 hours post injection. Both measurements are used for comparative (bPPD minus aPPD) interpretation. An animal that is infected with bTB is expected to have a larger reaction to bPPD than aPPD, while an animal that is exposed or sensitized to *M. avium* or environmental non-pathogenic mycobacteria will have a larger reaction to aPPD (Buddle et al., 2009; de la Rua-Domenech et al., 2006; Francis et al., 1978; Monaghan et al., 1994; Schiller et al., 2010a). A comparative skin thickness measurement (bPPD minus aPPD) of more than 4 mm is considered positive. The SICCT is used as the primary routine test in the UK and Ireland, due to low specificity of CIT (6-12% false positive at time of evaluation in the 1940s) (Leslie, 1975). The CCT serves as a confirmatory test in most other countries with bTB control programs and is used serially with CFT or CIT in testing reactors or suspects from the initial screening test. Desensitization (failure to react) can occur when a second TST is applied within a short period of time after the first one. This leads to a reduced response or a false negative response; thus, CCT is usually applied immediately after reading of CFT/CIT, or after a resting period of 42-60 days (de la Rua-Domenech et al., 2006). The CCT in general have a sensitivity of 55.1-93.5% and specificity of 88.8-100%. The advantage of the CCT is the ability to differentiate *M. bovis* infection from sensitization with other mycobacteria resulting in higher

test specificity. An important disadvantage is that animals in an early stage of *M. bovis* infection, or animals co-infected with other mycobacteria, may not test as positive. The CCT (SICCT) also has all of the previously mentioned advantages and disadvantages of the CFT and the CIT (Monaghan et al., 1994; Schiller et al., 2010a).

The performance of TST is confounded by many factors. High exposure rates to *M. avium* and other environmental mycobacteria, along with infection and/or vaccination for Johne's disease are major causes of false positive tests and decreased specificity of TST. Immunosuppression caused by stress, parturition, poor nutrition, drug use (such as dextamethasone), and anergy in animals with generalized or advanced bTB, are major concerns for false negative results that affect the sensitivity of TST. Non-uniformity in preparations of PPD, differences in TST application sites, disparity in reading test result due to human factors and differences in interpretation schemes applied also contributed to the broad range of test sensitivity and specificity values reported in many studies (Gormley et al., 2006; Monaghan et al., 1994; Schiller et al., 2010b). Improvement of quality control during production and better post production standardization is needed for PPD to improve the performance of TST (Schiller et al., 2010a; Schiller et al., 2010b).

TST are used for testing domestic animals other than cattle and for testing wildlife species. Although the TST is an approved test for farmed deer in New Zealand (Cousins and Florisson, 2005), the specificity is less than desirable (de Lisle et al., 2001). The TST is unreliable in many species such as Eurasian badgers (Higgins, 1985), possums ((de Lisle et al., 2002), and camelids (Twomey et al., 2010). The need to handle an animal twice is not practical when testing free ranging wildlife species (Chambers, 2009).

Interferon gamma (IFN-γ) assay

The IFN-γ assay is an OIE approved alternative bTB test for international trade (Anon, 2008a). Similar to the TST, the IFN-γ assay was developed based on the memory CMI response. In this test, whole blood from bTB infected animals is stimulated with PPD which results in production and release of IFN-γ from activated CD4+ T cells, which is then measured in the plasma. This two-stage *in vitro* diagnostic test includes an initial step of overnight stimulation of heparinized whole blood with bPPD and aPPD as primary antigens, a mitogen or superantigen (such as Pokeweed or Staphylococcus enterotoxin B) as positive control for verification of lymphocyte viability (Coad, 2008; Waters et al., 2006b), and Phosphate buffered saline (PBS) as a non-stimulant to provide a baseline control. In the second step, plasma is harvested and IFN-γ is measured qualitatively using a sandwich enzyme linked immunosorbent assay (ELISA) (Rothel et al., 1990; Wood et al., 1990). The IFN-γ assay is similar to the CCT in that interpretation of the test is based on the differential measure of IFN-γ produced after stimulation with bPPD and aPPD. This enhances test specificity by allowing detection of animals that have been infected with members of the *M. avium* group.

The IFN-γ assay was developed in Australia in the late 1980s to supplement the CFT for Australia's bTB eradication program. The purpose of the test was to improve sensitivity over the CFT and to reduce labor, which was achieved in initial field trails with a test sensitivity of 93.6% (Wood et al., 1991). Data collected in many international studies document the sensitivity of the IFN-γ assay ranges from 73-100% and test specificity ranges from 85-99.6% (de la Rua-Domenech et al., 2006; Schiller et al., 2010a). The great disparity in reported test sensitivity and specificity likely are attributable to variations in assay protocols, technical parameters, and test interpretation criteria, as well as confounding factors of environmental mycobacteria

sensitization, disease prevalence, and cattle immunity and cattle stress. Similar to the TST, disparity in potency and performance of the PPDs used in the IFN-γ assay further contributes to the variation in test results reported in many studies (Cagiola et al., 2004; Schiller et al., 2010b). The PPDs from various sources vary significantly, even though OIE standards were followed during and after production. Decreased sensitivity over time is more pronounced in PPDs with lower potency (Schiller et al., 2010b).

The IFN-γ assay has been used alongside TST in parallel testing schemes in some countries to increase the overall diagnostic sensitivity. The parallel testing scheme is regularly applied in high disease prevalence herds or new bTB infected herds. The purpose is to allow early removal of infected cattle not detected by TST, which may be as high as 12-38 % (Gormley et al., 2006; Vordermeier et al., 2006). The IFN-γ assay also is used in serial testing schemes, serving as a confirmatory test for reactors from the TST. Serial testing schemes help improve overall test specificity, which is particularly useful for countries or regions with low bTB disease prevalence or high cross-reactivity from other mycobacteria. The UK, Ireland, and New Zealand deploy the test in both parallel and serial schemes to accommodate different circumstances in the field and regional differences in level of risk (Buddle et al., 2009).

When the IFN-γ assay is used after the TST, the influence of tuberculin injected for TST on IFN-γ responses has been questioned. In general, injection of bPPD for the CFT primes the immune cells for production of IFN-γ for 3-7 days after injection (Coad et al., 2010; Palmer et al., 2006; Rothel et al., 1992; Whipple et al., 2001). This effect was not noted after SICCT (Coad et al., 2010; Doherty et al., 1995; Gormley et al., 2004). Whipple et al. (2001) showed measurable priming effect in blood samples taken 3 to 28 days post CFT. The priming effect gradually decreased, but remained at a higher than pre-skin test level, throughout the study

period of 77 days. This study also showed that increased IFN-γ production due to the priming effect could compensate for decreased of IFN-γ levels due to overnight storage of a blood sample prior to antigen stimulation. These results provided the scientific facts for applying the IFN-γ test between 3-30 days post CFT in the USA, where the majority of tests are performed on overnight shipped blood (Anon, 2004).

The IFN-γ assay is more sensitive in detection of early infection than the TST, less labor intensive for sample collection (only one farm visit required); less operator bias in reading test results and, unlike TST, a lengthy delay is not required if repeat testing if needed. The IFN-γ assay is acknowledged for its ability to detect many of the infected cattle that escape the TST test (skin test negative) (Coad, 2008; Vordermeier et al., 2006; Vordermeier et al., 2008). A recent study showed that the IFN-γ test detected 13 out of 20 skin test-negative high risk animals that had confirmed bTB infection at postmortem (Coad, 2008).

The primary disadvantage of the IFN-γ test is that the blood samples used for testing are perishable and must be transport promptly to arrive at testing laboratory in less than 28 hours from the time of blood collection. This is not always possible when the testing facility is far from the farm (Gormley et al., 2004; Schiller et al., 2009b). An 'in-tube' antigen stimulation version of this assay like the 'QuantiFERON-TB Gold in Tube' for human TB testing would be a good option that would help overcome the problem of timely transport of blood to the laboratory for antigen stimulation. Other limitations are that the IFN-γ assay is an expensive laboratory test and it is susceptible to human error in labeling blood tubes during collection, or labeling and harvesting multiple samples during antigen stimulation. It is also unsuitable for testing young animals due to the non-specific release of IFN-γ by NK cells and potentially γδ T cells (de la Rua-Domenech et al., 2006; Schiller et al., 2010a).

The IFN-γ assay has been used for the testing of other domestic animals and wildlife species. The CERVIGAM and PRIMAGAM (Prionics, Switzerland) are two assay kits that were developed specifically for testing cervids and non-human primates. The use of the IFN-γ assay for other species like badgers and brush-tailed possums has also been explored. The requirement of species-specific antibodies for optimal performance of this assay has limited the application for many species. Development of a multispecies IFN-γ assay for non-bovine species such as camelids, cervids, dogs and cats would be a useful addition to current bTB tests (Schiller et al., 2010a).

Lymphocyte Proliferation Test (LPT)

Similar to the IFN-γ assay, the LPT is an *in-vitro* assay detecting the reactivity of peripheral blood lymphocytes to bPPD and aPPD. Purified lymphocytes or whole blood can be used for the LPT (Muscoplat et al., 1977; Thoen et al., 1980). The comparative blastogenic response to bPPD or aPPD stimulation is used; the result is considered positive when the comparative value is above a predetermined cut off. The use of aPPD to detect a response due to cross-reactive antigens associated with mycobacteria from the *M. avium* group increases the specificity of the assay. This assay is not used for routine diagnosis because the test is time-consuming and involves a complicated laboratory protocol that requires use of radioactive material, it is relatively expensive, and it must be performed shortly after blood is collected (Thoen et al., 1980; Wood et al., 1994).

Postmortem diagnostic tests for bTB

Examination of carcasses for gross lesions of bTB is an important component of the bTB surveillance program. Unfortunately, gross lesion examination is not very sensitive and will not

detect many infected animals, especially those animals in early stages of infection, or with latent infection. Gross lesions frequently seen in less advanced cases of bTB include tubercles in the bronchial, mediastinal, retropharyngeal and portal lymph nodes (Corner, 1994). In more advanced cases of bTB, the lung, liver, spleen and the surfaces of body cavities can be affected (Van Rhijn et al., 2008). Lesion size varies widely, and serial sectioning of organs and tissues may be required to detect smaller lesions contained within the tissue. In developed countries, tissues with gross lesions are subject to further confirmation by specific laboratory tests.

Direct smears made from clinical samples or from prepared tissues can be used for detection of *M. bovis* under the microscope. The classic Ziehl–Neelsen acid-fast staining technique is most commonly used for demonstration of acid-fast stained *M. bovis*. Staining methods such as fluorescent acid-fast stain and immunoperoxidase techniques may also give satisfactory results. As lesions are often paucibacillary, acid-fast organisms may be absent in histological sections. Detection of acid-fast stained bacilli is not specific for *M. bovis*; additional tests such as bacterial culture and polymerase chain reaction (PCR) are needed for confirmation of *M. bovis* infection. In the absence of special stains or specific tests, the presumptive diagnosis of mycobacteriosis can be made if the tissue has characteristic histological lesions consistent for bTB (Anon, 2008a).

Isolation of *M. bovis* in culture is considered the gold standard for diagnosis of bTB, even though it is not the most sensitive test. *M. bovis* has been cultured from tissues with or without visible lesions. Culture of *M. bovis* is highly dependent on sampling the right tissue, sample quality, and number of viable organisms. It is difficult to make an isolation from infected tissues that contain small numbers of organisms (Morrison et al., 2000). Depending on the media used, growth of *M. bovis* generally occurs within 3–6 weeks of incubation (Anon, 2008a). When

possible, every isolate of mycobacterium should be confirmed as being *M. bovis* by biochemical properties or by molecular techniques (Ayele et al., 2004).

PCR assays can be designed to be highly sensitive and specific, and PCR is now recognized as a definite test for *M. bovis*. Assays targeting 16S–23S rRNA, the insertion sequences IS6110 and IS1081, and genes coding for MTB complex-specific proteins, such as MPB70 and the 38 kDa antigen, have been used for identification of MTB complex. Specific identification of an isolate as *M. bovis* can be made using PCR assays that target single nucleotide polymorphisms (SNP) at nucleotide positions 285 in the oxyR gene, 169 in the pncA gene, 675/756/1311/1410 and 1450 of the gyrB gene. Also useful, are assays that detected the presence/absence of RDs (Regions of Difference) (Anon, 2008a; Parsons et al., 2002). Although direct PCR can produce a rapid result, it is recommended that culture be used in parallel to confirm a viable *M. bovis* infection.

Molecular typing techniques, such as spoligotyping (from 'spacer oligotyping') can identify *M. bovis* isolates and provide some molecular-typing information on the isolate that is of epidemiological value. Use of an international standard nomenclature for the spoligotypes allows worldwide comparison of *M. bovis* profiles. The mycobacterial interspersed repetitive units (MIRU) and variable number tandem repeat (VNTR) typing have also been used to increase the discrimination among strains of *M. bovis* and other species in *Mycobacterium tuberculosis* complex (Anon, 2008a; Ayele et al., 2004).

Emerging bTB diagnostic tests

It is generally accepted that CMI is the earliest and most robust immune response developed after infection with *M. bovis*, while the humoral (antibody) responses is weaker and develops later in bTB infection (Pollock et al., 2001; Welsh et al., 2005). Current antemortem

diagnostic tests such as TST and IFN-γ assay are based on measuring the CMI responses. At present, serological tests are not available for bTB diagnosis in cattle (Vordermeier et al., 2008). There are ongoing efforts for improving accuracy of existing tests, as well as development of new tests. The majority of the newly developed tests are serological tests for detection of antibodies against *M.bovis* proteins. Such tests might complement the CMI based tests, and especially helpful in detecting anergic cattle (Vordermeier et al., 2001).

There is a sizable demand for testing other species of domestic animals, zoo specimens, and wildlife species for bTB. This has been driven by the realization over the last few decades that bTB can infect and be maintained in several species. Alternative tests are very much needed for many species, where TST and IFN-γ assay have proven unreliable (Chambers, 2009; Fenton et al., 2010; Wernery et al., 2007). Due to the difficult nature of handling and testing of wildlife species, serological tests, especially rapid animal-side tests, are among the most attractive options (Buddle et al., 2000; Chambers, 2009; de Lisle et al., 2002; Lecu and Riquelme, 2008; Lyashchenko et al., 2008).

New antigens

The less than desirable specificity of TST and IFN-γ assay likely is due to use of the ill-defined and non-standardized antigen mixture in PPDs. Many antigens in the bPPD are shared among the various species of mycobacteria, which likely results in cross-reactivity in currently approved tests. One approach for improving specificity of current test has been to identify specific antigens of *M. bovis* that elicit a measurable immune response and do not show cross-reactivity with an immune response raised against other mycobacterium (Aagaard et al., 2003; Cockle et al., 2002; Ewer et al., 2006). The effort to find specific antigens of *M. bovis* started in the late 1990s and has yielded encouraging results. The complete genome sequences of *M.*

tuberculosis, M. bovis and M. bovis bacille-Calmette-Guerin (BCG) have provided important information for comparative genomic analyses and discovery of new antigens that are organism specific (Garnier et al., 2003; Hewinson et al., 2006). The antigenic properties of many *M. tuberculosis* or *M. bovis* proteins with potential for diagnostic use have been identified; including MPB70, MPB83, TB27.4, TB16.2, TB15.8, TB10.4, Rv3615c, OmpATb, Mb1961c and heparin binding haemogglutin (HBHA) (Aagaard et al., 2003; Borsuk et al., 2009; Cockle et al., 2002; Hewinson et al., 2006; Molicotti et al., 2008). CFP-10 and ESAT-6 are antigenic proteins that are deleted in *M. bovis* BCG genome. Those proteins may prove useful for development of DIVA (differentiate infected from vaccinated animal) tests for differentiating *M. bovis* infection from BCG vaccination (Vordermeier et al., 2009).

On going studies using purified synthetic proteins or fusion proteins made from defined antigens have produced promising results. Compared with bPPD, use of recombinant ESAT-6 for TST showed improved specificity but diminished test sensitivity (Pollock et al., 2003). A cocktail of recombinant proteins that included ESAT-6, CFP-10, Rv3615c and MPB83 at 10 μg each, showed comparable sensitivity with conventional TST (Whelan et al., 2009). Combinations of ESAT-6 and CFP-10 as antigenic stimulants in IFN-γ assay have been investigated by many researchers (Aagaard et al., 2006; Buddle et al., 2003; Denis et al., 2007; Pollock et al., 2000; Vordermeier et al., 2001; Waters et al., 2004), but the overall results indicate test sensitivity is reduced by about 10%. Additional antigens were investigated to supplement ESAT-6 and CFP-10 for improvement of sensitivity in IFN-γ assay, and were shown possible by the addition of Rv3615c (Sidders et al., 2008; Vordermeier et al., 2009), or OmpATb (Schiller et al., 2009a).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA appears to be the most suitable of the antibody-detection tests for bTB because of its simplicity, low cost, and high throughput. There have been numerous attempts to develop ELISA tests for bTB; however, the late and irregular humoral immune response in cattle against *M. bovis* has prevented success (Pollock et al., 2001; Welsh et al., 2005). The early versions of ELISA developed in the 1980s used complex mixtures of antigens such as tuberculin or crude *M. bovis* culture filtrates. These early tests generally had lower test sensitivity when compared to TST and suffered from poor specificity likely attributable to immunologic cross-reactivity (Auer, 1987; Plackett et al., 1989). More recently, use of defined antigens has been explored for improvement of ELISA. Native and recombinant proteins including MPB70, MPB83, ESAT-6 and CFP-10 have shown potential for use in an ELISA (Aagaard et al., 2003; Amadori et al., 2002; Lightbody et al., 1998). In general, use of individual antigens increases test specificity and decreases test sensitivity; use of antigens in combination tends to boost the test sensitivity (Amadori et al., 2002; Vordermeier et al., 2001).

Cattle infected with *M. bovis* show an anamnestic immune response, which occurs 2 to 8 weeks after a routine TST and might be exploited to improve the performance of an ELISA (Lightbody et al., 1998; Thom et al., 2004). Many studies document a variable humoral immune response in cattle infected with *M. bovis*, which hinders development of serologic tests. This has led to the conclusion that a panel of antigens will be required in any serologic test to maximize the antibody detection and improve test sensitivity (Amadori et al., 2002; Vordermeier et al., 2001). Although not currently adequate for use in diagnosis of bTB in cattle, the ELISA can overcome other problems associated with detection *M. bovis* infections in wildlife. Use of ELISA for detection of bTB has been explore in red deer (Griffin et al., 1994), white-tailed deer

(O'Brien et al., 2009) and wild boars (Boadella et al., 2011) with mixed results. Nevertheless, an ELISA has been approved as an ancillary parallel test for use in farmed deer in New Zealand.

Latex bead agglutination assay (LBAA)

Synthetic ESAT-6 peptide was conjugated to latex beads for development of a LBAA that shows promise by Koo et al. (2004). Ten randomly selected fields of $1mm^2$ in each well were used for calculation of the intensity of agglutination for the assay. The initial application of the assay included only 69 bTB positive cattle and 34 bTB negative cattle and showed a sensitivity of 95.7% and a specificity of 100%. No cross-reactivity was found with sera from cattle infected with *M. paratuberculosis* (Koo et al., 2004). A later version of LBAA was developed which used recombinant protein MPB70 (Koo et al., 2005). The sensitivity and specificity for both versions were evaluated in a side by side study where the later version with MPB70 was shown to be less sensitive but more specific than the version with ESAT-6 peptide (se: 86.7% vs 94.8%, sp: 97.8% vs 92.6%) (Koo et al., 2005).

Multi-antigen printed immunoassay (MAPIA)

The MAPIA (Chembio Diagnostic Systems, New York) is a cocktail-based serological assay, where a wide variety of *M. bovis* antigens are immobilized onto nitro-cellulose membranes by semi-automated micro-aerosolization. The testing procedure for MAPIA is similar to most standard membrane based blotting protocols. Nitro-cellulose membrane was chosen for its high protein binding capacity that would facilitate used of multiple antigens for detection of antibody. Use of a chromogenic substrate for detection of antibody binding allows visual detection of results which can be semi-quantitatively measured, using scanning densitometry (Lyashchenko et al., 2000). A panel of 12 mycobacterial antigens is used in

MAPIA; which includes 8 purified recombinant proteins of ESAT-6, CFP-10, Acr1, 38 kDa protein, MPB59, MPB64, MPB70 and MPB83, 2 fusion proteins of ESAT6/CFP10, and Acr1/MPB83, and 2 native antigens of bPPD and *M. bovis* culture filtrate (MBCF) (Lyashchenko et al., 2004; Lyashchenko et al., 2000; Waters et al., 2006a). MAPIA has been used for testing serum from experimentally infected cattle and antibody against MPB83 was detected in all infected animals. Antibody against CFP-10, ESAT-6 and MPB70 was detected less frequently. Further evaluation will be needed to determine the usefulness of MAPIA for cattle testing (Lyashchenko et al., 2004; Waters et al., 2006a),.

MAPIA has been evaluated in many captive and wildlife species. The antibody response against *M. bovis* varied among species, resulting in non-uniform antigen recognition patterns (Lyashchenko et al., 2008). As an example, antibody against MPB83 was commonly detected in Eurasian badgers, white-tailed deer, brush-tail possums (Lyashchenko et al., 2008), camels (Wernery et al., 2007), farmed red deer (Buddle et al., 2010) and cats (Fenton et al., 2010); antibody against ESAT-6 and/or CFP-10 was commonly detected in elephants (Greenwald et al., 2009) and wood bison (Himsworth et al., 2010); and antibody against Arc1/MPB83 was common in meerkats (Drewe et al., 2009). The MAPIA serves as a very useful tool for profiling the antigen recognition patterns of different animal species infected with bTB, which could provide valuable insight for understanding the different host responses to *M. bovis* infection.

Fluorescence polarization assay (FPA)

Fluorescein-labeled MPB70 has been used for detection of antibody against MPB70 in sera obtained from cattle. Reaction of antibody with fluorescein-labeled MPB70 will increase the polarization which is detected using an fluorescence polarization analyzer (Lin et al., 1996). A trial with 28 positive animals gave a sensitivity of 92.9% for FPA, while the CFT was only

53.6% when tested alongside. Specificity based on 5666 negative animals tested was 98.3% for FPA, with no cross-reactivity with antibody against *M. paratuberculosis* (Surujballi et al., 2002). Subsequently, in an international study, the FPA was shown to be useful for prediction of bTB infection status at the herd level (Jolley et al., 2007).

Rapid immunochromatographic assay/ Rapid test (RT)

The RT is a lateral flow-based rapid test employing a cocktail of selected *M. tuberculosis* and/or *M. bovis* antigens (including ESAT-6, CFP-10, and MPB83) and a blue latex-based signal detection system. RT is a ready to use disposable device available in plastic cassettes containing a strip of antigens. Serum and diluents added to an inoculation pad will flow laterally across the membrane through a conjugate pad that contains antigen-conjugated latex particles that bind antibody as the sample flows through. If antibody is present in the serum that is specific for one of the antigens, a colored immuno-complex is formed, which can be read visually. The results of the RT can be read within 20 minutes (Greenwald et al., 2003; Lyashchenko et al., 2006). The RT also is known as TB StatPak (Chembio Diagnostic Systems, New York), is available for many species, such as cattle (BovidTB STAT-PAK), non-human primates (PrimaTB STAT-PAK), white tail deer, reindeer, elk (CervidTB STAT-PAK), badgers (BrockTB STAT-PAK), camels, llamas, and alpacas (CamelidTB STAT-PAK) and exotic species like elephants (ElephantTB STAT-PAK). Only the PrimaTB STAT-PAK and ElephantTB STAT-PAK assays have been licensed for use in the USA.

Compared with other serologic assay using a multi-antigen format, the sensitivity of the RT generally is lower and non-specific cross-reactivity can affect specificity with sera from some species (Buddle et al., 2010; Chambers et al., 2009; Drewe et al., 2009; Fenton et al., 2010; Greenwald et al., 2009; Himsworth et al., 2010; Lyashchenko et al., 2008; O'Brien et al., 2009).

Despite less than desirable test accuracy, RT provides the convenience of an easy to run, point-of-care test that has its advantages in field applications. In particular, the RT is useful for screening wildlife species, zoo animals, and other species where the TST has been proven unsuitable.

Dual-path platform (DPP) VetTB test

DPP VetTB (Chembio Diagnostic Systems, New York) is a lateral flow-based test that uses 2 nitrocellulose strips that are connected in a 'T' shape inside a plastic cassette. Test serum added to a sample well migrates on the first nitrocellulose strip toward the second strip. Buffer is added to a conjugate well to release dried conjugate (protein A/G hybrid conjugated to colloidal gold particles) in the second strip, which then binds antibody in the test serum and that antibody-conjugate complex migrates through the second strip. The second strip is printed with a MPB83 test line, a CFP10/ESAT6 test line, and a control line. Binding of antibody-conjugate complex to the immobilized antigen(s) forms a visible line(s); absence of any visible lines in the area of the printed antigens indicates specific antibody was not present in the test sample. The result can be scored visually in 15-20 minutes, or by a DPP optical reader. There are only a few reports available for performance of DPP VetTB on elephant (Greenwald et al., 2009), farmed red deer (Buddle et al., 2010), and Eurasian wild boar (Boadella et al., 2011). In general, the available reports indicate the DPP VetTB is an improvement over the RT. Like the RT, the DPP VetTB is suitable for field application and is useful for screening wildlife species, zoo animals and other species.

Multiplex immunoassay (Enferplex)

Enferplex TB assay is a serum based multiplex chemiluminescent immunoassay. As many as 25 antigens are printed in a single well of a 96-well plate. Antibody detection is enhanced through chemiluminescent signal, captured with a digital imaging system and analyzed with software supplied by the manufacturer. The pattern of the antibody recognition profile for each serum sample is analyzed; a minimum of reactivity to 2 antigens in a test well is required for a positive reaction. The sensitivity and specificity of Enferplex was reported as 93.1% and 98.4%, respectively (Whelan et al., 2008). A recent study showed that Enferplex assay gave negative results sera from BCG-vaccinated calves, indicating the assay might be suitable as a DIVA test (Whelan et al., 2010). Extensive testing of this assay in field trials is needed to demonstrate its usefulness as a bTB test in cattle.

Single-antigen SeraLyte-Mbv system

Recently, a single antigen serology test (SeraLyte-Mbv system) based on MPB83 was developed that uses an advanced chemiluminescent based chemistry to achieve a high degree of analytical sensitivity (Green et al., 2009). Initial testing data showed a sensitivity of 89% and overall specificity of 98%. The assay shows cross-reactivity with antibody against *M. kansasii*, but not *M. avium*. Since *M. kansasii* is generally not a major problem to the bTB testing program, it was deemed not a critical issue by the authors. The system was able to detect all bTB positive sera (100% sensitivity) at 3 months post-infection (Green et al., 2009). The future of the test will depend upon more field testing data to support its test accuracy.

Other tests

Aside from the above mentioned tests, there are other proposed concepts for bTB testing. Western blot was evaluated as a tool for bTB testing (Chambers et al., 2002; O'Brien et al., 2009), but the results were not promising. Chemical sensors, also known as 'electronic nose', for detection of specific volatile compounds or biomarkers for bTB in serum provide a new approach, but this has not been evaluated in the field (Fend et al., 2005). Immunoassays that detect soluble interleukin-2 receptor α (sIL-2R- α) from peripheral blood T-lymphocytes (Onuallain et al., 1997) or nitric oxide production by peripheral blood mononuclear cells (PBMC) (Waters et al., 2002) are examples of CMI based tests capable of detecting diseased animals. A 7-plex cytokine/chemokine luminescence assay was shown to be useful for simultaneous detection of IFN-γ, IL-1β, IL-4, IL-10, IL-12, macrophage inflammatory protein-1β (MIP-1β) and TNF-α level in whole blood (Coad et al., 2010). This study demonstrated that a multiplex CMI based system could be a potential system for enhancement of test accuracy. Microarray and real-time quantitative PCR based techniques have been used for characterization of bovine cytokine, chemokine, transcription factors, and other responses to bTB infection; which provide valuable data for potential new test development (Almeida et al., 2006; MacHugh et al., 2009; McGuire and Glass, 2005; Thacker et al., 2007).

Vaccine development

M. bovis BCG is the only available vaccine against bTB. Efficacy trials for this vaccine have yielded variable results, which may be attributable to factors that include vaccine formulation, route of vaccination, and the extent of exposure of cattle to environmental mycobacteria (Hope and Villarreal-Ramos, 2008). Efficacy trials have been conducted on a number of other vaccines, such as a viral-vectored mycobacterial vaccine, an attenuated

mycobacterial vaccine, a DNA vaccine and a protein subunit vaccine; however, none of those vaccines were superior to BCG vaccine for protection (Buddle, 2010). Currently, the scientific data suggests that future vaccines will be based on enhancing, rather than replacing, BCG (Hewinson et al., 2006; Hope and Villarreal-Ramos, 2008). To date, the heterologous prime-boost strategy with combination of BCG-DNA, BCG-protein or BCG-viral-vectored vaccine has shown the most promising results (Buddle, 2010).

BCG based vaccination may be used to reduce the spreading of infection in cattle; however, it is recognized that the use of vaccine will compromise TST and other immunological tests. This obstacle can be overcome by the application of DIVA tests that allow differentiation of BCG vaccinated from *M. bovis* infected animals (Vordermeier et al., 2009). Significant progress has been made in development of DIVA tests, particularly those based on major antigenic targets of the RD1 region (i.e. CFP-10 and ESAT-6) that is deleted in *M. bovis* BCG genome (Hope and Villarreal-Ramos, 2008). BCG vaccination could be applied in combination with DIVA tests once those tests are fully validated and the regulatory requirements are amended accordingly. Vaccines would be useful to reduce bTB infection in wildlife reservoirs where other control strategies are difficult to implement. There remains some concern for impact of vaccine use on long-term reduction of disease prevalence, on the environmental, and on safety for human beings and other wildlife species.

Global bTB control efforts and challenges

Bovine TB eradication is an ongoing effort worldwide. Unfortunately, only a few countries with endemic bTB infection in livestock have active bTB control programs. The existing bTB control/eradication programs primarily are in developed countries and include systematic testing and removal of reactor animals, abattoir surveillance, and restricted movement

of cattle. After successful eradication of bTB, abattoir surveillance is usually continued to monitor for re-emergence of the disease (de la Rua-Domenech et al., 2006). While these programs have been successful in many countries, other countries that have wildlife reservoirs of bTB are continually plagued by outbreaks of disease. Countries or regions that have successfully eradicated bTB include Australia, Switzerland, many of the European Union (EU) countries, most of Canada and the USA, and Scotland.

Wildlife reservoirs for bTB hinder control/eradication efforts for bTB in New Zealand, the UK and Ireland, a few EU countries, and small regions of Canada and the USA. In New Zealand, the brush-tail possum is the most important wildlife reservoir for infection of cattle. In 1993, a pest management strategy for control of bTB in brush-tail possums was initiated. This strategy focused on eliminating brush-tail possums and resulted in reduction in bTB prevalence in cattle from 2.4% in 1993 to 0.34% in 2008. Control of the brushtail possum was achieved using poisoned oral bait (Ramsey et al., 2002). In addition to poison, oral vaccination with lipid-formulated *M. bovis* BCG has been tried (Ramsey et al., 2009). Continual management of bTB in the possum population is critical in New Zealand for control of bTB in cattle (Buddle et al., 2009). The UK and Ireland contend with the Eurasian badger as a wildlife reservoir of bTB (de la Rua-Domenech, 2006; Krebs, 1997). Efforts to reduce badger populations for control of bTB have been met by resistance from the general public (Donnelly et al., 2006; Jenkins et al., 2008). Consequently, the prevalence of bTB in cattle in the UK and Ireland has increased over the last 30 years. Vaccination of badgers for bTB likely will become an important part of the control program in the UK and Ireland (Delahay et al., 2000; Krebs, 1997).

In general, the EU has experienced increased numbers of bTB infected cattle in recent years with the overall herd prevalence reaching 0.53% in 2007 (Anon, 2009a). Animal trade and

spillover infection from various wildlife reservoirs have been important factors in the increase of bTB in some EU countries. Wild boar is a wildlife reservoir for bTB in Spain, France and Italy (de Mendoza et al., 2006; Naranjo et al., 2008). In those countries, wildlife disease management will be a critical part of the control effort for bTB. Canada faces a similar situation to the USA, as elk and bison are wildlife reservoirs of bTB in and around Riding Mountain National Park and Wood Buffalo National Park. Sporadic outbreaks of bTB occur in cattle herds adjacent to those areas. Control programs for bTB in cattle are in place for those areas; however, elimination of bTB from cattle is deemed impossible with the disease established in wildlife. Emphasis on disease management in the wildlife species is now a key component of the Canadian bTB control program (Schiller et al., 2010a).

Some countries in Latin America and the Caribbean have bTB control programs. Those programs include about 30% of the cattle in the region and have reduced the infection rate to <1%. The majority of cattle in Latin America and the Caribbean are located in areas without control programs (Lutze-Wallace et al., 2004; Lutze-Wallace and Turcotte, 2005, 2006; Nishi et al., 2006; Nishi et al., 2002). At least 80% of African cattle are raised in areas without bTB control (de Kantor and Ritacco, 2006; Michel et al., 2010; Thoen et al., 2009). The herd prevalence of bTB is estimated at 50% or more in some regions of Latin America and Africa (Cosivi et al., 1995; Michel et al., 2010). Asian and Eastern European countries also have bTB; however, the current status of bTB control in those countries is not known (de Kantor and Ritacco, 2006; Munyeme et al., 2008; Schiller et al., 2010a). Thus, large segments of the world's population are at risk of contracting bTB from their livestock at the animal-human interface.

Control of bTB in the USA

The USA bTB eradication program started in 1917, with bTB herd prevalence of 5% (Anon, 2009b). The test and slaughter program was based on the CFT as the primary screening test and the CCT as the secondary test. Between 1917 and 1940, roughly 232 million TST were administered and about 3.8 million cattle were destroyed (Olmstead and Rhode, 2004). By 1940, every state in the USA achieved the Modified-accredited State status (incidence rate <0.5%) and the program was considered a success (Essey and Koller, 1994). An abattoir surveillance program was started in the 1950s for continual monitoring and surveillance of bTB (Olmstead and Rhode, 2004). Sporadic outbreaks of bTB occurred, but were not considered a problem.

In 1994 *M. bovis* was detected in a hunter harvested white-tailed deer in the state of Michigan. Subsequently, additional bTB infected deer were found in ongoing surveillance efforts by Michigan Department of Natural Resources (DNR) (Kaneene et al., 2006). Michigan lost official TB free status in 1998 after 2 herds of cattle were found infected with bTB. White-tailed deer have been confirmed as the wildlife reservoir for *M. bovis* and are considered responsible for the re-emergence of bTB in cattle in Michigan (Schmitt et al., 1997). Control of bTB in the white-tailed deer population has been attempted through increased hunting and the ban of supplemental feeding and baiting of deer (O'Brien et al., 2006). Other control efforts included fencing off feed storage areas for cattle to reduce deer access; thereby reducing disease transmission from white-tailed deer to cattle (O'Brien et al., 2006). Disease outbreaks still occur periodically in the Michigan bTB zone, suggesting on going deer to cattle transmission of bTB. Changes in wildlife control strategies and cattle management in Michigan will be crucial for control of bTB, as eradication is considered unachievable in the short term (Hickling, 2002; O'Brien et al., 2006).

Sporadic outbreaks of bTB occur in Texas and California, mainly caused by introduction of cattle from Mexico (Anon, 2009b). Other outbreaks of bTB in cattle and/or deer have been reported in Minnesota, New Mexico, Ohio, Nebraska, Indiana, South Dakota and Colorado. Similar to Michigan, Minnesota has found bTB in deer; however, the deer population in the affected area is small and the prevalence of bTB in deer is much lower than in Michigan.

The current prevalence of bTB in cattle herds in the USA is estimated at 0.001 percent (Anon, 2009b). In 2009, the bTB control program in the USA was revamped to meet the current need for a better management schemes under conditions of decreased funding. Many infected herds in the USA have only a few bTB infected cattle and whole herd depopulation is not financially feasible. The 'test and remove' herd clean-up process is now preferred over depopulation. Because bTB infection in cattle is restricted to isolated foci in the USA, classification of entire states as bTB infected wastes valuable resources. Proposed zoning based on the geographical area around bTB foci would conserve resources by concentrating testing and movement control on only high risk areas. This also would allow fair trade for other geographical areas within the state that have minimal bTB risk (Anon, 2009b).

Future direction for bTB control

Decreased funding for bTB control program is the major factor that will influence the future direction for bTB control strategies. Animal welfare issues and the economic value of livestock and wildlife likely will restrict whole herd depopulations and prevent large population reductions in native wildlife. Management of wildlife reservoirs will be important for bTB control programs. Vaccination of wildlife will be a politically attractive, but likely unrealistic, option for disease management in the short term and culling of infected wildlife may be necessary. Future research should address the heterogeneity of the disease problem, further

understanding of animal behavior, ecology, transmission risk, and the potential effects of massive culling within an ecosystem. It will be crucial to create an approach that builds on a mechanistic understanding of all components of the problem. It also will be crucial to ensure that control strategies are implemented at all levels, from farm management, bTB testing of cattle, identification of cattle, movement controls for cattle, and reduction of wildlife reservoirs. Communication and education of the general public on the bTB control policies will be critical in setting eradication goals that must be supported by all the stakeholders (Dorn and Mertig, 2005; White et al., 2008).

Increased movement of animals within a country, and more frequent international livestock trade, increase the risk for spreading bTB and thus complicate the bTB control/eradication programs. Regionalization and zoning become essential for disease control and effective pre-movement testing will be crucial to monitor and control spread of disease (Livingstone et al., 2006; White et al., 2008). The current screening tests (TST and IFN-γ) are regarded as good herd tests, but are not optimal for individual animal tests. Availability of highly accurate individual animal tests will be essential for effective pre-movement testing in an era of active global livestock trading.

Application of Microarray Technologies in bTB research

Microarray technologies have been applied successively in cancer research and disease pathogenesis studies. In infectious disease studies, microarray provides insight into the host-pathogen interplay. This enhances understanding of host defense mechanisms and the tactics pathogens employ to evade the host immune system. Microarray based studies facilitate rapid and large scale examination of global gene expression profiles, without prior knowledge of target genes to be tested. Host genomic-based microarray libraries are very useful for studies of host

response to various infections (McGuire and Glass, 2005), and provide a means for mining differential host transcriptome response. This facilitates identification of cellular pathways important in pathogenesis, and offers an opportunity for the discovery of diagnostic molecular markers predictive for specific infectious, metabolic, or genetic diseases (Boldrick et al., 2002; Jenner and Young, 2005; Ramilo et al., 2007; Sarmento et al., 2008).

In microarray studies, the altered gene expression profile, as measured by changes in host cell transcriptional activity can show common patterns of the host's response to pathogens across different host cell types, or common patterns among various pathogens (Bejjani and Shaffer, 2008; Coussens et al., 2005; Meade et al., 2008; Stern et al., 2009). Altered gene expression profiles also reveal unique patterns that are host cell type specific or pathogen specific (Boldrick et al., 2002; Jenner and Young, 2005). Microarray studies examine alterations in expression levels between infected and non-infected individuals and are very useful for identifying a small number of gene targets that consistently show substantial altered expression due to the infection (Chaussabel et al., 2005; Paranavitana et al., 2008; Ramilo et al., 2007; Sarmento et al., 2008). Similarly useful are microbial genomic based microarrays that allow study of microbial gene expression during the course of infection and have great potential for applications in clinical microbiology (Blanco et al., 2009a; Kendall et al., 2004; Miller and Tang, 2009).

Microarray technologies have been used extensively to gain an understanding of human TB infection caused by *M. tuberculosis;* data from over 5,700 *M. tuberculosis* microarray studies are currently available in a web-based TB Database (http://www.tbdb.org) (Galagan et al., 2010). Microarrays have been used to study *M. bovis* infection in several animal species including cattle, European wild boar, and Iberian red deer. Studies of bTB in cattle have shown differences in gene expression profiles between antigen-stimulated and non-stimulated PBMC

from cattle with bTB, and differences in gene expression profiles in antigen-stimulated and non-stimulated PBMC between cattle with bTB and healthy cattle (MacHugh et al., 2009; Meade et al., 2007; Meade et al., 2008; Meade et al., 2006). Wedlock et al. (2006) and Widdison et al. (2008) have examined the differential gene expression in bovine alveolar macrophages infected with virulent versus attenuated strains of *M. bovis*, and *M. tuberculosis* versus *M. bovis*. Those studies showed unique chemokine expression profiles in macrophages infected with the virulent strain of *M. bovis* compared with macrophages infected with an avirulent strain of *M. bovis* or with *M. tuberculosis*, which generally is less virulent in cattle than *M. bovis* (Wedlock et al., 2006; Widdison et al., 2008). Altered gene transcription profiles associated with natural *M. bovis* infection in European wild boar in Spain have provided information for understanding the pathogenesis of bTB in this species (Galindo et al., 2009; Naranjo et al., 2006a; Naranjo et al., 2006b). Similarly, microarrays were used to study the gene expression profile of bTB infected Iberian red deer, providing the first large scale analyses of the infected cervine gene expression profiles (de Mera et al., 2008).

Application of quantitative real-time polymerase chain reaction assays in bTB research

Quantitative real-time polymerase chain reaction (qPCR) is used for validation of altered gene expression discovered through microarray hybridizations (de Mera et al., 2008; Galindo et al., 2009; Meade et al., 2007; Meade et al., 2008; Meade et al., 2006; Naranjo et al., 2006b). Quantitative detection of mRNA can be performed on the same sample analyzed with microarray hybridization (Chuaqui et al., 2002; Dallas et al., 2005; Skrzypski, 2008). The qPCR procedures are practical for use on patient samples; therefore, the validation assay can be easily converted to a diagnostic assay. In addition to use as a validation tool, qPCR has served as the primary tool

in several studies that characterized the expression profiles of cytokine genes associated with bTB infection in cattle (Blanco et al., 2009b; Thacker et al., 2007; Widdison et al., 2006; Witchell et al., 2010), cervine (Harrington et al., 2006; Thacker et al., 2006, 2009) and European wild boar (de la Lastra et al., 2009).

CHAPTER TWO

Gene Expression Profiling of Cattle Infected or Not Infected with Bovine Tuberculosis: Four Hour Stimulation of Whole Blood with Tuberculin

Introduction

The bTB control program in the USA has reduced the prevalence of bTB infected cattle herds from an estimated 5% of all herds in 1917 to <0.001% (Anon, 2009b). The antemortem diagnostic tests currently approved for detection of bTB in the field measure cell mediated immune responses. Those approved tests include the intradermal caudal fold tuberculin test (CFT), as the primary test, and either the intradermal comparative cervical tuberculin test (CCT) or the whole blood Interferon gamma (IFN-γ) assay as secondary tests. Cattle that show positive reactions in successive primary and secondary tests are culled for postmortem examination. Predictably, as prevalence of bTB infected cattle decreases, the proportion of test-false positive cattle culled for postmortem examination increases (Monaghan et al., 1994). The prevalence of bTB in the state of Michigan is low, and only 1-2 % of cattle that test as positive reactors on two successive antemortem tests for bTB are confirmed as positive for bTB on postmortem examination (Anon, 2008c). The process of postmortem examination is not perfect and an early infection with *M. bovis*, prior to development of lesions, may not be detected. There is a need for ancillary tests for bTB that improve the positive predictive value of the testing process. Hopefully, this would decrease the number of healthy cattle slaughtered as bTB suspects.

DNA microarray technologies facilitate rapid and large scale examination of global gene expression profiles, and have been particularly useful in studies of host response to various infections (Boldrick et al., 2002; Jenner and Young, 2005; Ramilo et al., 2007; Sarmento et al.,

2008). Use of peripheral blood transcriptome profiles has proven valuable for identification of pathogen-associated immune response signatures, which could be utilized for development of diagnostic assays (Boldrick et al., 2002; MacHugh et al., 2009; Paranavitana et al., 2008; Ramilo et al., 2007). Recently, microarray platforms of bovine genes have been used to study pathogenic processes, and to identify molecular markers of infection for two mycobacterial pathogens of cattle; *M. avium* subspecies *paratuberculosis* and *M. bovis* (Coussens et al., 2003; Coussens et al., 2002; Coussens et al., 2005; MacHugh et al., 2009; Meade et al., 2007; Meade et al., 2008; Meade et al., 2006; Skovgaard et al., 2006). Comparison of gene expression profiles of peripheral blood mononuclear cells (PBMC) from bTB infected cattle with PBMC from healthy cattle that were bTB test negative showed distinct differences in gene regulation (Meade et al., 2007; Meade et al., 2008; Meade et al., 2006).

In the current study, gene expression profiles were examined for 3 groups of cattle after a 4 hour stimulation of whole blood with tuberculin. Those groups included cattle that were single or double test-false positive (SFP or DFP) reactors for bTB in antemortem diagnostic tests and cattle that were truly infected (bTB). This study differs from previous studies in that gene expression profiles from cattle that had bTB were compared with cattle confirmed bTB free at postmortem examination, but were false positive reactors for bTB on antemortem tests. This difference is critical because cattle that test-false positive for bTB are difficult to differentiate from cattle that truly have bTB in currently approved diagnostic assays. Hence, the purpose of this study was to identify differential gene expression profiles among cattle that were similar in response to bTB tests. The hypothesis was that the gene expression profiles of the 3 groups of cattle would differ from each other and that altered expression of select genes would allow differentiation of test-false positive cattle from cattle truly infected with bTB.

Materials and Methods

Experimental animals and bTB infection status

Experimental animals in this study consisted of cattle culled from herds because they showed positive reactions in antemortem diagnostic tests for bTB. The cattle were transported to the Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University (MSU) the day before a regulatory bTB postmortem examination was done. A presumptive positive or negative diagnosis was made for each animal at the DCPAH based on the presence of gross and/or microscopic lesions consistent with bTB. Regardless of the presumptive diagnosis, fresh and formalin fixed tissues from all cattle examined postmortem were submitted to United States Department of Agriculture's National Veterinary Services Laboratories (NVSL) for microscopic examination, PCR assay, and mycobacterial culture. Final diagnosis was made by the NVSL based on results of PCR assays and mycobacterial culture.

Three study groups of cattle were used for microarray analysis. The study groups included bTB positive cattle (bTB, n=4) that were positive reactors in antemortem diagnostic tests for bTB and confirmed positive for bTB by postmortem examinations; double test-false positive reactor cattle (DFP, n=4) that were positive reactors on primary and secondary antemortem diagnostic tests and were negative for bTB on postmortem examination; and single test-false positive cattle (SFP, n=7) that originated from bTB positive farms. This last group of cattle was positive reactors in the CFT and negative reactors in the CCT or IFN-γ assay, and was negative for bTB on postmortem examination. The number of cattle in each group was expanded to 10 for validation of altered gene expression, using qPCR assays. Finally, healthy cattle from Michigan State University's cattle herds (n=12) that had recent negative antemortem test records

for bTB, *M. avium* subspecies *paratuberculosis*, bovine leukosis virus, and bovine viral diarrhea virus were used as blood donors to obtain a reference pool of control RNA for the study.

Blood collection and antigen stimulation

Blood (~45 ml total) was collected from each animal in the bTB, DFP, and SFP groups into 10 ml heparinized tubes (Vacutainer®, BD Diagnostics, Franklin Lakes, NJ) immediately before euthanasia for postmortem examination. Within 3 hours of collection, the blood from each animal was pooled into individual sterile 50ml conical tubes and stimulated with purified protein derivative prepared from the filtrate of a heat-killed *M. bovis* (bPPD) (Prionics AG, Switzerland) at 20µg bPPD/ml of blood. The blood was incubated at 38 ± 1°C for 4 hours prior to harvest. Blood samples from the 12 healthy cattle were similarly collected, processed, and stimulated.

Isolation of peripheral blood mononuclear cells (PBMC), and purification of RNA

After stimulation, the blood was centrifuged at 1200 x g for 15 minutes at 18°C to form layers of plasma, buffy coat cells, and red blood cells. Buffy coat cells and 2 ml of red blood cells immediately below the buffy coat cell layer were harvested and transferred to new 50 ml conical tubes. Two rounds of hypotonic lysis of red blood cells were performed by addition of ice-cold diethylpyrocarbonate (DEPC) treated-sterile de-ionized water for 2 minutes, followed by addition of an equal volume of ice-cold DEPC-treated sterile 2X saline (1.7% w/v NaCl). Intact cells were pelleted by centrifugation at 1200xg for 15 minute at 18°C after the first round of hypotonic lyses, then at 190xg for 10 min at 4°C after the second round. After the second round of hypotonic lyses, the supernatant was decanted and 1 ml TRIzol Reagent (Invitrogen, Carlsbad, CA) was added to the loose cell pellet for each 9 ml beginning volume of whole blood.

This mixture was frozen at -84°C until use. For isolation of RNA, the mixture was thawed on ice, and subjected to 10 passages through a 20 gauge needle. The resulting homogenate was divided into 1 ml aliquots and the remainder of the RNA extraction procedure was performed according to the manufacturer's recommendations. The total cellular RNA from each animal was then pooled into a single tube and treated with RQ1 RNase-Free DNase (Promega, Madison, WI) according to manufacturer's recommendations. The treated RNA was extracted again using equal volumes of phenol-chloroform, followed by purification using MEGAclear Purification Kit (Ambion, Austin, TX). The purified RNA was immediately stored at -84°C until use.

Before use, the RNA from each of study cattle was thawed on ice and the integrity and concentration of the RNA was determined using an Agilent 2100 Bioanalyzer and RNA Nano 6000 Kit (Agilent Technologies, Santa Clara, CA). The RNA from the 12 healthy cattle was mixed to form a homogenous control reference pool and the integrity and concentration of that pooled RNA was similarly determined.

Microarray content and experimental design

The BOTL-5 cDNA microarray used in this study was the 5th generation of a previously described bovine total leukocyte immunogenetic microarray (Coussens and Nobis, 2002; Yao et al., 2001). An extensive list of studies utilizing the BOTL microarray series can be found at the MSU Center for Animal Functional Genomics website (http://cafg.msu.edu). Specific gene content and sequence information for the BOTL-5 microarray is available at the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO, platform number GPL5751). Briefly, BOTL-5 contains 3,888 features including 1,391 genes or expressed sequence tags (ESTs) spotted in duplicate along with multiple replicates of microarray specific control features. A common reference design was used for microarray hybridization in this study.

RNA from the groups of study cattle was labeled with the Cy3 and co-hybridized with the control reference pool of RNA labeled with Cy5.

Synthesis of cDNA and microarray hybridization

The complementary single-stranded deoxyribonucleic acid (cDNA) synthesis and dye labeling were performed with 15 µg aliquots of total RNA using the SuperScript III Fluorescent Labeling Kit containing the Cy5 and Cy3 dyes (Cat# L101401; Invitrogen Corp., Carlsbad, CA) following the manufacturer's recommendations. For each microarray experiment, the Cy3-labeled sample and Cy5-labeled reference pool were combined and concentrated, using a Microcon 30 centrifugal filter unit (Millipore, Billerica, MA). The labeled cDNA mixture was eluted in 110 µl of SlideHyb buffer #3 (Ambion, Austin, TX) and heated for 5 minutes at 70°C prior to hybridization.

The hybridization was done using a GeneTAC HybStation (Genomic Solutions Inc., Ann Arbor, MI) and an 18-hour step-down protocol (3 hours at 60°C, 3 hours at 55 °C, 12 hours at 50 °C). Immediately following hybridizations, the slides were subjected to 5 washes of 30 sec each at 50° C with a mixture of 2x SSC and 0.1% SDS, 5 washes of 30 sec each at 42° C with 0.2x SSC and 0.1% SDS, and 5 washes of 30 sec each 42° C with 0.2% SSC. After removal from the hybridization unit, the microarray slides were rinsed once in 0.2x SSC and once in double distilled water, and then dried by centrifugation for two minutes at 1,200 x g. The hybridized cDNA microarrays were scanned immediately using a GeneTAC LS IV microarray scanner and GeneTAC LS software (Genomic Solutions Inc., Ann Arbor, MI).

Microarray data processing, normalization, and analysis

Microarray images were processed using GenePix Pro 6.0 software (Molecular Devices, Downingtown, PA) to generate spot intensity files. The output files were analyzed using the LIMMA (LInear Models for MicroArray) software package (Smyth, 2005) implemented in the R language and environment (http://www.r-project.org)(R Development Core Team, 2011). Briefly, background correction (Ritchie et al., 2007) and normalization within the microarray (Smyth and Speed, 2003) was performed prior to linear regression analysis. Prior to and after normalization, MA plots of data were generated for each microarray for visualization of the normalization effect. The median fluorescence intensity was used for data analysis. The relative fold change of each gene feature for each animal was determined by comparison with the control reference pool of RNA. Use of a common reference pool of RNA for all microarrays enabled comparison of data across different microarrays. Thus, altered expression of gene features was determined for each group of cattle and comparisons were also made of altered gene expression data between groups of cattle (bTB vs SFP, bTB vs DFP and DFP vs SFP). The empirical Bayes moderated T statistic was used for data analyses (Smyth, 2004).

Gene functional annotation and classification of microarray data

The database for annotation, visualization, and integrated discovery (DAVID) (Huang et al., 2009a; Huang et al., 2009b) was used to annotate the differentially expressed genes identified in the microarrays. Identification of enriched functionally related gene groups within each of the study groups was done using the Functional Annotation Clustering tool in DAVID. Classification of genes into major terms based on functional and biological processes was complied using information from the BOTL5 microarray annotation database (available at http://cafg.msu.edu) and the Gene Ontology Classification tool in DAVID.

Visualization of result by cluster analysis

Cluster analysis of highly differentially expressed genes ($p \leq 0.01$) among cattle in all study groups was performed using Genesis software (Institute for Genomics and Bioinformatics, Graz University of Technology, Austria). Hierarchical clustering based on the complete linkage algorithm was performed to cluster the cattle using microarray expression data for comparison with original group assignments of the cattle, which was made using results of antemortem and postmortem diagnostic tests.

Quantitative real-time PCR validation of differential gene expression

Twelve potential reference genes were evaluated for stability of expression level within and between the study groups of cattle (Appendix A). Succinate dehydrogenase complex subunit A (SDHA) was considered the optimum reference gene for this study. The list of genes selected for validation of expression using qPCR assay, PCR primer sequences, primer concentration, PCR efficiency, and amplicon size are given in Table 2.3. The PCR primers for the gene targets were designed in Clone Manager Suite 7.0 (Sci-Ed Software, Cary, NC) or Primer Express 3.0 software (Applied Biosystem, Foster City, CA), and were synthesized by Integrated DNA Technologies (Coralville, IA). All primers were tested for amplification with the control reference pool of RNA and with a no template control (NTC). Optimal primer concentration for qPCR was determined empirically (data not shown).

Samples of RNA from 10 cattle for each study group were used in qPCR to validate altered gene expression revealed after analysis of data from the microarray experiments. The samples of RNA subjected to qPCR included some of the original samples used in the microarray experiments plus new samples of RNA from additional cattle that met the criteria for each study group. Synthesis of cDNA was performed with 2 µg of total RNA from each study

animal and from the common reference pool of RNA, according to reagent manufacturer's recommendations (Superscript™ II Reverse Transcriptase and Oligo (dT)12-18 Primer, Invitrogen, Carlsbad, CA). Upon completion of cDNA synthesis, the RNA template in each reaction was removed with 1U of RNase H (Invitrogen, Carlsbad, CA). The cDNA was purified using QuickClean enzyme Removal Resin (Clontech Laboratories, Mountain View, CA) according to the manufacturer's recommendations. The concentration of purified cDNA was measured by spectrometry (ND-1000, NanoDrop Technologies, Wilmington, DE) and diluted to final concentration of 10 ng/µl. All cDNA were stored at -20°C until use in qPCR assays.

The qPCR assays were performed in triplicate using SYBR Green PCR Master Mix and an ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA). Each 20 µl reaction consisted of 1x SYBR Green PCR master mix, 30 ng of cDNA and a pair of primers at pre-determined optimal concentrations (Table 2.3). The reaction conditions were 95 °C for 10 minutes, then 40 cycles of 95 °C for 15 seconds and 58°C for 1 minute. Dissociation curve analysis was done for each reaction.

PCR efficiency determination and qPCR data analysis

The delta Rn data and the cycle threshold (Ct) data exported from the ABI 7500 SDS software were used to verify that acceptable PCR efficiency was achieved for each reaction and for calculation of the relative expression level of the targeted genes, respectively. The efficiency of each qPCR reaction was based on the slope of the exponential phase of the PCR amplification plot, as shown in the formula,

PCR efficiency = 10^(-1/slope)

The mean value of PCR efficiency was calculated for each gene target using the LinRegPCR program (Ramakers et al., 2003; Ruijter et al., 2009). In that program, a PCR reaction of 100%

efficiency received a score of 2.0 and possible scores descend to 1.0, which represents no detectable PCR reaction occurred (Table 2.3).

The efficiency corrected ΔΔCT algorithm (as shown in the formula below) for calculation of differential gene expression was used for qPCR analysis (Livak and Schmittgen, 2001; Pfaffl, 2001).

$$\textbf{relative expression } (\Delta\Delta CT) = \frac{E_{target}^{\Delta Ct\ target\ (control\text{-}sample)}}{E_{reference}^{\Delta Ct\ reference\ (control\text{-}sample)}}$$

The mean Ct value from triplicate PCR reactions for each gene target in each sample of RNA was used for calculation of ΔΔCT values, and the overall mean ΔΔCT value for each gene target was derived from all animals in the group. The SDHA gene was used as the reference/normalizer gene, and the common reference pool (as in the microarray experiments) was used as the calibrator. Thus, the calculated differential expression reflected altered expression of a gene target among cattle in a study group relative to the common reference pool of RNA. The $\log_2$ ΔΔCT values were used for statistical analyses. The T-test was used to determine the statistical significance of altered gene expression of gene targets at the group level. The statistical significance of differential expression of a gene target among groups of cattle was determined with the ANOVA test based on linear fixed effect models (Steibel et al., 2009) performed in the MAANOVA (MicroArray ANalysis of VAriance) software package (Wu, 2008). Simultaneous fitting of multiple linear models for multiple genes was done with 5000 permutation tests and with the jsFDR method for false discovery rate (FDR) adjustment (Storey, 2002). Finally, Genesis software was used to generate heat maps for graphical presentation of the gene expression data.

Results

Comparison of PBMC gene expression profiles from SFP, DFP, and bTB cattle

A total of 1,391 gene features on the BOTL-5 microarray were analyzed, of which, 415 gene features were differentially expressed ($p \leq 0.05$) in one or more of the study groups of cattle. Overall, more genes with altered expression were found in the bTB test-false positive cattle than in the true bTB positive cattle. The SFP group of cattle showed 192 differentially expressed gene features, the DFP group of cattle showed 174, and the bTB group of cattle showed 119 (Figure 2.1a). The majority of the 415 gene features that had altered expression were unique to the individual study groups; however, 52 gene features were shared between 2 groups of cattle, and 9 gene features, all upregulated, were common to all 3 groups of cattle (Figure 2.1b). Those 9 gene features included platelet-derived growth factor, ribosomal protein, ATPase, methionyl-tRNA synthetase, brain protein I3, OTU domain and signal-induced proliferation-associated-1 gene. Overall, the number of gene features that showed increased expression was almost twice that of gene features showing decreased expression (Figure 2.1a).

DAVID analysis and classification of altered gene expression profiles

The Functional Annotation Clustering tool in the DAVID software was used to identify enriched functionally-related gene groups within the differentially expressed genes for each group of cattle. In the SFP group, 138 of 192 differentially expressed gene features mapped to known biological functions and 13 significantly enriched ($p \leq 0.01$) biological processes were identified. The 3 most highly enriched processes were negative regulation of apoptosis (p =1.1E -6), positive regulation of RNA transcription (p = 4.3E-6), and cellular biosynthesis (p = 6.1E-6). In the DFP group, 138 of 174 differentially expressed gene features mapped to known biological functions, and the only 2 significantly enriched biological processes identified were ribosomal

protein (p = 2.1E-5) and regulation of phosphorylation (p = 2.4E-3). In bTB group, 97 of 119 differentially expressed gene features mapped to known biological functions and 4 significantly enriched biological processes were identified. The enriched processes were regulation of cell division (p = 4.2E-3), regulation of apoptosis (p = 4.5E-3), phosphate metabolic process (p = 7.3E-3) and regulation of protein modification process (p = 8.7E-3).

Classification of differentially expressed genes in each study group into major terms of functional and biological processes is summarized in Table 2.1. In the SFP and the DFP groups of cattle, genes involved in immune response was the most represented term at 14% and 13%, respectively. The next two most represented terms for both the SFP and DFP groups were cell growth and biosynthesis (13.0% and 12.6%, respectively), and transcription and nuclear factors (8.3% and 10.9%, respectively). In contrast, the 3 most represented terms for bTB group were cell growth and biosynthesis (14.3%), transcription and nuclear factors (12.6%) and apoptosis (9.2%). The immune response term for bTB group was fourth at 8.4%.

Identification and cluster analysis of gene features with power for group differentiation from microarray data

The objective of this study was to find molecular markers that can differentiate test-false positive cattle from the true bTB infected cattle. Thus, the microarray data was analyzed at a more stringent level ($p \leq 0.01$) to find gene features that had highly significant altered expression. At $p \leq 0.01$, the number of differentially expressed gene features was reduced to 122. In both the SFP and DFP groups of cattle, the ratio of gene features showing increased expression levels to those showing decreased expression levels was at least 2:1. In contrast, the ratio of increased expression to decreased expression was 1:1 in the bTB group of cattle (Figure 2.2a). The fold change ranged from -1.86 to 2.93 for all 122 genes showing altered expression.

Only 9 of the 122 gene features were shared by two groups of cattle, the remaining 113 genes were uniquely regulated within a group of cattle (Figure 2.2b).

When a comparison of gene expression data was done between groups of cattle, only 55 gene features showed significant statistical power to differentiate the 3 study groups. Cluster analysis based on those 55 gene features was performed using Genesis software. The results of hierarchical clustering of the cattle based on complete linkage clustering (Figure 2.3) shows that the 3 groups of cattle can be differentiated, confirming that each group of cattle had a unique expression profile. The altered expression levels of 17 and 23 gene features (Table 2.2) were useful for differentiation of the bTB group from either the DFP group (bTB vs DFP) or the SFP group (bTB vs SFP), respectively. Altered expression levels of 22 gene features were useful for differentiation of the DFP group from the SFP group (DFP vs SFP).

Differentiation of a particular group of cattle from each of the other two groups was possible, but only a few gene features were useful for that purpose. The DFP group of cattle could be differentiated from the bTB and the SFP groups using the altered expression levels of 5 gene features. Those genes were thioredoxin-related transmembrane protein 4 (TMX4); transmembrane protein; adipocyte associated 1 (TPRA1); major histocompatibility complex, class II, DM alpha-chain (BOLA-DMA); Fc fragment of IgG, receptor transporter alpha (FCGRT); and ribosomal protein L19 (RPL19). The altered expression level of only one gene feature, tripartite motif-containing 13 (TRIM13), was useful for differentiating the SFP group of cattle from the bTB and DFP groups. Similarly, altered expression of only one gene feature, clone BOTL0100011_A05 (a gene feature of unknown function) was useful for differentiation of the bTB group of cattle from the SFP and DFP groups.

Group level gene expression profiling with quantitative real-time PCR (qPCR)

Validation of altered gene expression levels using qPCR was done for 17 gene features selected from the microarray data as showing substantial altered expression among cattle or as showing unique regulation within a group of cattle (Table 2.3). An additional 16 genes that code for cytokines or chemokines were selected from the literature for qPCR analysis (Table 2.3). The cytokines or chemokines produce by those genes are reported to be important mediators in bovine and human TB infections. Samples of RNA from 30 cattle (10 cattle per study group) were used to assess altered expression of the 33 selected genes. Information on PCR primers used for qPCR and on the PCR efficiency for each set of primers is given in Table 2.3.

With the extended panel of 10 cattle per study group, the statistical significance of the altered gene expression within each group of cattle could be assessed with greater accuracy. The qPCR assays identified two genes (BOLA-DRA and IL-1β) that showed considerable variation in expression level among cattle within a study group; therefore, those genes were excluded from further analyses. Seven genes showed increased expression in all 3 study groups and 12 genes showed decreased expression in all 3 study groups (Figure 2.4 a & b). The gamma interferon (IFN-γ) gene showed the greatest increase in expression in all study groups (2.92 to 7.42 fold). Interleukin-2 (IL-2) also showed a marked increase in expression (1.84 to 2.74 fold). Other genes that showed increased expression in all groups of cattle were serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit beta isoform (PPP2R5B), lymphotoxin beta receptor (LTBR), ADP-ribosylation factor 3 (ARF3) and 2 clones with unknown function (BOTL0100008_C07 and BOTL0100011_A05).

Pro-inflammatory cytokines were among the down regulated genes, including interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and interleukin-1 alpha (IL-1α). Also

down regulated were anti-inflammatory cytokines, including interleukin-10 (IL-10) and transforming growth factor beta (TGF-β), along with several chemokines, including interleukin-8 (IL-8), chemokine (C-X-C motif) ligand 2 (CXCL-2) and chemokine (C-X-C motif) ligand 6 (GCP2). The decreased expression for many of those genes was especially evident in cattle from the DFP group. Other genes that showed decreased expression included the major histocompatibility complex Class II molecule (BOLA-DMA), prostaglandin-endoperoxide synthase 2 (PTGS2), tripartite motif-containing 13 (TRIM13) and transmembrane protein, adipocyte asscociated 1 (TPRA1). Among the genes analyzed, interleukin-4 (IL-4) was unique in showing marked increase in expression in both the SFP and DFP groups of cattle while the expression level remained unchanged for the bTB group (Figure 2.4c).

Analysis of altered gene expression in individual cattle

The expression levels of many genes were highly variable among cattle within each study group, which lead to overlapping expression profiles among the study groups as illustrated for IFN-γ in Figure 2.5. Thus, data from qPCR assays were analyzed at the individual animal level using the ANOVA test to identify genes that could significantly differentiate individual cattle within a group from cattle in the other groups. Of the 33 genes selected for qPCR assay, 20 were found to have differential power at $p \leq 0.05$; however, after FDR adjustment ($adj\ p \leq 0.05$), the number of genes with significant differential power was reduced to 16. The expression levels of 15 of those genes could be used to differentiate DFP cattle from SFP cattle (Figure 2.6a). Only 6 genes differentiated bTB cattle from DFP cattle and only 1 gene significantly differentiated bTB cattle from SFP cattle. These results suggest that the gene expression profile of the SFP cattle was more similar to that of the bTB cattle than the DFP cattle. This finding was

unexpected, because it was anticipated that the SFP and the DFP groups of cattle would be closer in expression profile to each other than to the bTB cattle.

The SFP cattle originated from bTB positive herds and exposure of some SFP cattle with *M. bovis* infected cattle might explain the gene expression profiles observed. Thus, a re-grouping of the 20 SFP and DFP cattle was done based on bTB exposure history (see Appendix B). Four cattle in the DFP group had originated from bTB positive herds; therefore, they were grouped together with the 10 cattle in SFP group to form a new group designated as test-false positive-bTB exposed (FP-ex, n=14). The remaining 6 cattle in DFP group did not have a history of potential bTB exposure and were designated as double test-false positive- non bTB exposed (DFP-non-ex, n=6). Data obtained from qPCR assays were re-analyzed based on the new groups of cattle. Overall, the allotment of cattle into new groups had little effect on the mean values of gene expression using the T test statistic. However, the mean expression levels of 7 down regulated genes in the DFP-non-ex group were changed by >2 fold when the 4 bTB-exposed DFP cattle were removed (Figure 2.7). A more pronounced effect was seen at the individual animal level, using the ANOVA test. After the cattle were allotted into new groups, the differential power of 2 genes (PRKCI and TMX4) became not significant, but 7 additional genes were found to have differential power among 2 or more groups of cattle. Thus, 21 genes were deemed significant ($adj\ p \leq 0.05$) for differentiation of the new groups of cattle (Figure 2.6b). Cattle in DFP-non-ex group could be differentiated from the bTB using 13 genes (Table 2.4). Seventeen genes could be used to differentiate the FP-ex and the DFP-non-ex cattle, but none of the genes could be used to differentiate the FP-ex cattle from the bTB cattle.

Based on expression data for the aforementioned 16 and 21 genes that had significant differential power among cattle, heat maps were made to segregate cattle from the original SFP,

DFP, and bTB groups and from the new FP-ex, DFP-non-ex, bTB groups (Figures 2.8a & b). The results confirmed that the gene expression profiles for cattle in the DFP-non-ex group were distinctly different from gene expression profiles for cattle in the FP-ex and bTB groups. The allotment of all bTB-exposed cattle into the FP-ex group also appeared justified as showed with the result of cluster analyses.

Discussion

Currently, the OIE-approved bTB tests for international trade of cattle are the tuberculin skin tests (TST), which are based on a physically measurable cell mediated inflammatory response against tuberculin antigen injected into either the skin of the neck or the caudal fold of the tail, and the IFN-γ assay, which measures IFN-γ secreted into plasma after stimulation of whole blood with tuberculin antigen (Anon, 2008a; Schiller et al., 2010a). The TSTs are most commonly used to screen for bTB and normally are effective for control of bTB. However, limitations in sensitivity and specificity of TST are well recognized (de la Rua-Domenech et al., 2006; Schiller et al., 2010a). To increase diagnostic sensitivity, the IFN-γ assay is used in some countries in parallel with, or sequential to, various applications of the TST (Coad, 2008; Schiller et al., 2010c). Regardless of testing schemes employed, false positive and false negative test results remain an issue for bTB control programs. In Michigan, the current rate of bTB infection is extremely low, which leads to far more test-false positive cattle being culled as bTB suspects than the number of bTB infected cattle identified at postmortem examination. This has driven an interest in comparing the gene expression profiles of bTB positive and bTB test-false positive cattle. The working hypothesis was altered transcription levels of select genes could be used to discriminate between cattle infected with bTB and cattle that test-false positive in currently approved antemortem diagnostic assays.

To test this hypothesis, mRNA expression levels were evaluated by microarray analysis that made use of a common reference design. The use of a common reference on each microarray allows comparison of gene expression across various study groups (Churchill, 2002; Dobbin et al., 2003; Steibel and Rosa, 2005). The common reference used in the current study was a pool of RNA extracted from the PBMC of healthy cattle after samples of whole blood from those cattle were stimulated for 4 hours with bPPD. The methods for antigen stimulation and for RNA extraction from PBMC of healthy cattle were identical to those used for the 3 study groups of SFP, DFP, and bTB infected cattle. Previous studies have shown that bPPD stimulation of PBMC from healthy cattle will induce altered gene expression (Meade et al., 2008; Meade et al., 2006). By normalizing each microarray with a pool of RNA from bPPD stimulated cells obtained from healthy cattle, it was hoped to that any changes in gene expression that were due to non-specific stimulation caused by bPPD would be filtered out.

After 2-4 hours of antigen stimulation, comparable microarray studies on cattle infected with *M. avium* subspecies *paratuberculosis* (Coussens et al., 2004) or bTB (Meade et al., 2008; Meade et al., 2006) have shown rapid changes in gene expression profiles of PBMC. Importantly, a marked increase was reported in the number of differentially expressed genes in bTB positive cattle compared with TST negative cattle following a 3 hour stimulation of whole blood with bPPD (Meade et al., 2008). In the current study, differences in gene expression profiles were found between bTB positive cattle (bTB group), and bTB test-false positive cattle (SFP and DFP groups) after stimulation of whole blood with bPPD for 4 hours. When the arithmetic means for fold change of gene expression were compared at 0.05 and 0.01 levels of significance, the numbers of genes showing altered expression were similar among the SFP ($n=192$ at $p\leq0.05$ and $n=51$ at $p\leq0.01$) and DFP groups of cattle ($n= 174$ at $p\leq0.05$and $n=60$ at

$p \leq 0.01$). In comparison, the numbers of genes showing altered expression in the bTB group were substantially less (n=119 at $p \leq 0.05$ and n=20 at $p \leq 0.01$). Most genes that showed altered expression were unique to the individual groups of cattle and only a few genes were shared among 2 or more groups of cattle (Figure 2.1 & 2.2). Using the fold change in gene expression for individual animals, the SFP and DFP cattle were differentiated from the bTB cattle using cluster analysis (Figures 2.3).

The lists of genes showing altered expression ($p \leq 0.05$) in microarray analysis were imported into DAVID software to determine the enriched functionally-related gene groups and to perform functional annotation for clustering of genes showing altered expression for each group of cattle. The enriched functionally-related gene groups differed for the SFP, DFP, and bTB cattle as determined by DAVID software, further suggesting the transcriptional response to antigen stimulation was dissimilar among the cattle and that grouping of cattle based on altered gene expression was feasible. Classification of differentially expressed genes into major terms of functional and biological processes (Table 2.1) showed similar profiles of altered gene terms for the SFP and DFP groups of cattle. The altered gene terms for the bTB group of cattle were clearly different from the SFP and DFP groups of cattle. Again, those results were consistent with the hypothesis that cattle infected with bTB can be differentiated from non-infected cattle based on gene expression profiles.

Although microarray hybridization analyses are useful as a general screening tool for identifying genes that show altered expression (MacHugh et al., 2009; Sarmento et al., 2008), qPCR is accepted as the more sensitive and accurate assay for quantifying differential gene expression (Bustin, 2000; Hendriks-Balk, 2007). Thus, qPCR was used to validate altered expression levels for select genes. The qPCR assays conducted in the current study confirmed

that there were differences in gene expression between the SFP, DFP and bTB groups of cattle (Figure 3.4). Compared with the reference pool of RNA, the expression of many genes was decreased at 4 hours post stimulation; especially in the DFP group of cattle. This finding was consistent with other studies that report a temporal decrease in level of gene expression following antigen stimulation of PBMC (Coussens et al., 2004; Meade et al., 2006). The greatest increase in gene expression was observed for the cytokine IFN-γ, an essential event for the whole blood IFN-γ assay for bTB (Wood et al., 1990). However, the IFN-γ gene was useless for separating infected from non-infected cattle because the expression levels for that gene varied considerably among animals both within and between groups (Figure 2.5).

The gene expression data from qPCR assays were analyzed to identify gene targets that might differentiate antemortem test-false positive cattle from the true bTB infected cattle. That process identified 16 genes that showed promise for being able to differentiate the DFP cattle from the bTB and SFP groups of cattle (Figure 2.6a). However, none of the genes subjected to qPCR assay could differentiate the bTB group of cattle from the SFP group. The origin of the cattle in the SFP group suggested a possible explanation for that finding. All of the cattle in the SFP group were exposed to *M. bovis* infected herdmates; thus, it was possible that some of the cattle in the SFP groups were infected with *M. bovis*. Similarly, a few of the cattle in the DFP group were exposed to *M. bovis* infected herdmates and may have been infected with *M. bovis*.

The cattle from both the SFP and DFP groups that had been exposed to *M. bovis* infected herdmates were allotted into a new group of bTB-exposed cattle (FP-ex). The cattle in the original DFP group with no known exposure to bTB were allotted into a second new group (DFP-non-ex). When the data from the qPCR assays were re-analyzed using the new groups of cattle, 21 genes showed promise for being able to differentiate the DFP-non-ex group of cattle

from the bTB and FP-ex groups of cattle, but none of those 21 genes could differentiate the FP-ex cattle from the bTB cattle (Figure 2.6b). Heat maps of gene expression profiles of the cattle as originally allotted and after being allotted based on bTB exposure history (Figure 2.8 a & b) show that the gene expression profiles of the DFP-non-ex cattle differ from the other groups of cattle. However, the gene expression profiles for all but 2 cattle in the FP-ex group (FP-ex-9 and FP-ex-14) are similar to those of the bTB group.

It is thought that that up to 30% of cattle in an infected herd can become infected with bTB (Philips et al. (2003). Cattle with an effective innate immune response may clear an infection with *M. bovis*. In that case, those cattle can test positive by TST and/or IFN-γ assay but lack lesions at postmortem examination and be negative on cultures for *M. bovis* (Pollock et al., 2000). Similarly, cattle in an early stage of infection with *M. bovis* may test positive by TST or IFN-γ assay but lack lesions at postmortem examination and be negative on cultures for *M. bovis* (Corner, 1994; Morrison et al., 2000). Latent infection with *M. tuberculosis* occurs in humans and is believed to occur in cattle (Morrison et al., 2005; Pollock and Neill, 2002; Van Rhijn et al., 2008; Vordermeier et al., 2008). It is likely that some latently infected cattle would test positive by TST and/or IFN-γ assay, but lack lesions at postmortem examination and be negative on cultures for *M. bovis* (Pollock and Neill, 2002; Vordermeier et al., 2008). Thus, failure to identify genes with altered expression that can differentiate bTB infected cattle from bTB exposed, test-false positive cattle may have been due to use of "non-infected" cattle in this study that actually were infected with bTB.

The current study examined altered expression of genes in PBMC at 4 hours post stimulation with bPPD. The gene expression profiles of the DFP-non-ex group of cattle were clearly different than those of the bTB group of cattle. That finding provides support to the

hypothesis that detection of altered expression of a few genes could be used to differentiatie bTB infected cattle and test-false positive cattle. However, it was not possible to differentiate the bTB infected cattle from the antemortem test-false positive cattle that had been exposed to bTB infected cattle in the field, using gene expression profiles. Temporal studies that used antigen stimulation of PBMC from cattle infected with bTB have shown that there is a rapid and transient burst of gene expression that occurs with hours of antigen stimulation. A second burst of altered gene expression occurs at 12 to 24 hours post stimulation (MacHugh et al., 2009; Meade et al., 2008; Meade et al., 2006). The current study tried to capitalize on the early burst of altered gene expression. The results of the current study indicate that the later burst of altered gene expression should be investigated to determine if altered expression of genes at 12 to 24 hours post stimulation can be used to identify gene with differential power to clearly separate bTB infected cattle from antemortem test-false positive cattle.

Table 2. 1 Classification of statistically significant ($p \leq 0.05$) differentially expressed genes from microarray analysis into major terms based on their functional and biological processes using the Gene Ontology Classification tool. The groups of cattle being compared either tested negative for infection with *M. bovis* at postmortem examination and tested as positive reactors in the CFT (SFP), the CFT and CCT or INF-γ assay (DFP), or were positive for infection with *M. bovis* on postmortem examination (bTB).

Major terms	SFP	DFP	bTB
Immune response	13.5%	12.6%	8.4%
Cell growth and biosynthesis	13.0%	12.6%	14.3%
Transcription and nuclear factors	8.3%	10.9%	12.6%
Apoptosis	6.3%	10.9%	9.2%
Signal transduction	5.2%	8.0%	5.9%
Ribosomal protein	2.1%	5.7%	3.4%
Cell division & proliferation	7.3%	4.6%	5.9%
Protein metabolism	5.2%	5.2%	6.7%
Neurogenesis	4.2%	2.3%	1.7%
Cell organization	3.6%	5.7%	1.7%
Transport	3.1%	1.7%	4.2%
Energy pathways	2.6%	2.3%	3.4%
Cell adhesion	1.6%	1.1%	3.4%
Regulation of translation	0.5%	1.1%	1.7%
Unknown functions	23.4%	14.9%	17.6%

Table 2. 2 Genes from microarray analysis that showed statistically significant ($p \leq 0.01$) differential power between the single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB) groups of cattle after a 4 hour stimulation of whole blood with tuberculin. Differential expression in relative fold change (Δ FC) was computed between the bTB and the DFP groups (bTB - DFP), the bTB and the SFP groups (bTB - SFP), and the DFP and the SFP groups (DFP - SFP).

Microarray Gene ID	Gene Description	Gene Symbol	bTB - DFP		bTB - SFP		DFP - SFP	
			Δ FC	*p*	Δ FC	*p*	ΔFC	*p*
BOTL0400543_PCR	pleiotrophin	PTN	1.96	0.0088				
BOTL0100010_G07	ADP-ribosylation factor 3	ARF3	1.96	0.0010				
BOTL0100002XC05R	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	DDX5	1.78	0.0030				
BOTL0100002XB09R	major histocompatibility complex, class II, DR alpha	BOLA-DRA	1.63	0.0038				
BOTL0100003XD08R	unknown	-	1.49	0.0083				
BOTL0100006XB10R	Wiskott-Aldrich syndrome-like	WAS	1.42	0.0064				
BOTL0100002XH05R	unknown	-	-1.41	0.0063				
BOTL0400610_PCR	similar to subtilisin-like proprotein convertase	PCSK6	-1.61	0.0094				
BOTL0100012_C01	Bos taurus zinc finger protein 701-like (LOC100140226)		-1.70	0.0056				
BOTL0400491_PCR	similar to G protein-coupled receptor 98	GPR98	-1.79	0.0030				

Table 2.2 (cont'd)

BOTL0400247_PCR	similar to GDNF family receptor alpha 2 preproprotein	GFRA2	-2.21	0.0086				
BOTL0100011_A05	unknown	-	-1.57	0.0078	-1.58	0.0030		
BOTL0100008_F05	Fc fragment of IgG, receptor, transporter, alpha	FCGRT	2.47	0.0019			-1.95	0.0071
BOTL0100010_B02	major histocompatibility complex, class II, DM alpha-chain	BOLA-DMA	1.77	0.0009			-1.59	0.0018
BOTL0100010_H10	thioredoxin-related transmembrane protein 4	TMX4	1.61	0.0039			-1.76	0.0004
BOTL0100003XG06R	transmembrane protein, adipocyte asscociated 1	TRRA1	-2.20	0.0094			2.10	0.0063
BOTL0400407_PCR	ribosomal protein L19	RPL19	-2.23	0.0024			2.44	0.0003
BOTL0400258_PCR	fms-related tyrosine kinase 3-like	FLT3			2.35	0.0057		
BOTL0400511_PCR	protein kinase C, iota	PRKCI			2.02	0.0045		
BOTL0400035_PCR	similar to beta isoform of regulatory subunit B56, protein	PPP2R5B			1.89	0.0008		
BOTL0400047_PCR	mitogen-activated protein kinase kinase kinase 4	MAP3K4			1.76	0.0069		
BOTL0400403_PCR	similar to double minute 2 protein	MDM2			1.72	0.0095		

Table 2.2 (cont'd)

BOTL0400328_PCR	Lutheran blood group (Auberger b antigen included)	BCAM	1.66	0.0068
BOTL0400560_PCR	similar to retinoid X receptor beta	RXRB	1.54	0.0097
BOTL0100010_E06	ribosomal protein L23a pseudogene 12	RPL23AP12	-1.38	0.0100
BOTL0100013_C02	KIAA0240	KIAA0240	-1.42	0.0036
BOTL0100013_B11	Dicer1, Dcr-1 homolog (Drosophila)	DICER1	-1.43	0.0059
BOTL0100013_E06	protein tyrosine phosphatase, receptor type, C-associated protein	PTPRCAP	-1.46	0.0062
BOTL0400443_PCR	cullin 1	CUL1	-1.47	0.0023
BOTL0400216_PCR	ephrin-B1	EFNB1	-1.47	0.0040
BOTL0100008_C07	notch homolog 2 (Drosophila)	NOTCH2	-1.58	0.0047
BOTL0100013_D01	nucleoporin 210kDa	NUP210L	-1.59	0.0050
BOTL0100012_H08	vacuolar proton-ATPase, subunit D; V-ATPase, subunit D	ATP6V0D1	-1.60	0.0021
BOTL0100001XE12R	insulin-like growth factor binding protein 2, 36kDa	IGFBP2	-1.60	0.0059

Table 2.2 (cont'd)

BOTL0100011_G07	armadillo repeat containing 3	ARMC3	-1.66	0.0041		
BOTL0400029_PCR	activating transcription factor 1	ATF1	-1.66	0.0019		
BOTL0100003XH04R	similar to Mitochondrial ribosomal protein L41	MRPL41	-1.75	0.0099		
BOTL0400120_PCR	prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2)	PTGS2	-1.83	0.0060		
BOTL0100004XH06R	tripartite motif-containing 13	TRIM13	1.53	0.0025	1.45	0.0063
BOTL0100006XH11R	unknown	-			2.45	0.0037
BOTL0400249_PCR	similar to glucagon receptor	GCGR			2.04	0.0004
BOTL0400592_PCR	similar to Granzyme H precursor (Cytotoxic T-lymphocyte protein	GZMH			1.75	0.0064
BOTL0400426_PCR	caspase 8 associated protein 2	CASP8AP2			1.66	0.0007
BOTL0400140_PCR	chemokine (C-C motif) receptor 5	CCR5			1.63	0.0017
BOTL0400024_PCR	Sin3A-associated protein, 18kDa	SAP18			1.59	0.0037
BOTL0400190_PCR	similar to DNA fragmentation factor alpha subunit	DFFA			1.54	0.0042

Table 2.2 (cont'd)

BOTL0100013_B01	adaptor-related protein complex 1, sigma 1 subunit	AP1S2	1.44	0.0065
BOTL0100008_B08	unknown	-	-1.41	0.0054
BOTL0100010_B10	probable E3 ubiquitin-protein ligase HERC1	HERC1	-1.54	0.0059
BOTL0100002XG05R	H3 histone, family 3B (H3.3B)	H3F3B	-1.55	0.0038
BOTL0100010_F10	polymerase (RNA) II (DNA directed) polypeptide G	POLR2G	-1.75	0.0035
BOTL0400072_PCR	hypothetical protein LOC100133104	-	-1.78	0.0099
BOTL0100013_F05	polycystic kidney and hepatic disease 1 protein	PKHD1	-1.89	0.0037
BOTL0400607_PCR	secreted protein, acidic, cysteine-rich	SPARC	-2.12	0.0040
BOTL0100010_A04	adenylate kinase domain containing 2	AKD2	-2.13	0.0043

Table 2. 3 Genes selected for qPCR analysis, nucleic acid sequence and concentration (nM) of PCR primers for those genes [forward primer (F) and reverse primer (R)], PCR efficiency (E), and PCR amplicon sizes (bp).

	gene symbol	gene name	primer (5' - 3')	Primer conc. (nM)	PCR eff. (E)	Amplicon size (bp)
#	ARF3	ADP-ribosylation factor 3	F: TTGCCTAATGCCATGAATGC R: CACAGGTGGCCTGAATGTA	300	1.817	91
#	BOLA-DMA	major histocompatibility complex, class II, DM α-chain	F: TTGTTGGCTTGGTCCTCTTC R: ACACCTCCTGCTTGGATGG	300	1.975	105
#	BOTL08_C07	unknown	F: ATCACTTCCCGCCTCCTTAG R: AGGCAGGTGACCAAGGAAAC	600	1.925	92
#	CXCL2	C-X-C ligand 2 (GRO-alpha)	F: AACAAGGCTAGTGCCAACTG R: CCACTGAGGCTGCTGGAG	300	1.912	68
#	DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	F: AGAGATCTGGTGGGTAGCTTTA R: ACCCTATCCTCTCCTTGCAAAC	300	1.917	79
#	IL-4	interleukin-4	F: GCCACACGTGCTTGAACAAA R: TGCTTGCCAAGCTGTTGAGA	450	1.910	63
#	LTBR	lymphotoxin beta receptor (TNFR superfamily member3)	F: CCGGAGTGACGAGGAAGAC R: CAAAACTCGCCCTTATACCTTG	450	1.859	104
#	PPP2R5B	protein phosphatase 2, regulatory subunit B', beta isoform	F: GTGGTCCTGGCAACAGAAC R: CTGGAGCCCAGCTTTGTG	300	1.895	110

Table 2.3 (cont'd)

#	PRKCI	protein kinase C, iota	F: CAAGGACCCAAAGGAACGATT R: ACCACCTGCTTTTGCTCCAT	300	1.897	114
#	PTGS2	prostaglandin-endoperoxide synthase 2(cyclooxygenase-2)	F: CGACACCAAGAACGTATTCCTA R: GAGATGTGGAAAAGAAGCATTG	300	1.930	105
*	BOLA-DRA	MHC class II DR alpha	F: GCTCTGGTGGGCATCATTG R: CCTCGGCGTTCAACGGTG	300	1.910	77
*	TPRA1	Bos taurus transmembrane protein, adipocyte asscociated 1	F: GTGCGCAGACATCATTGAG R: GGCGCAAAGAAGCTGAAG	450	1.974	72
*	TRIM13	tripartite motif-containing 13	F: CTGGCACGTTCATTAGCAAG R: GGCCAAGCAGAATGACCAC	300	1.962	69
*	FCGRT	Fc fragment of IgG, receptor, transporter, alpha	F: GGCCCGAATCGTTGTGTT R: GAAGCCCAAGGCTTACACC	450	1.822	81
*	TMX4	thioredoxin-related transmembrane protein 4	F: ACCTTGACTTGTGCTCACTT R: TGGAGGTACCACTGGAACTG	300	1.993	85
*	BOTL11_A05	unkown	F: CACACTCTATGGCGCAAATC R: CCCTGGACCACCACCTCTA	300	1.903	75
*	RPL19	ribosomal protein L19	F: GGCTCCAGGCCAAGAAAG R: AATTGCCGAGGCCACTATG	300	1.972	106
§	CSF3	colony stimulating factor 3 (granulocyte)	F: CTGGGTGAGACTGGGAAATG R: TCTCTCACACCCCGTCACA	300	1.959	62

Table 2.3 (cont'd)

§ GCP2	granulocyte chemotactic protein 2 (CXCL6)	F: CATTGGAATGCTGTATATGGAGAT R: TCTTCCAAAGGTCAAGAGTAAGA	300	1.874	122
§ IL-10	interleukin-10	F: CTTGTCGGAAATGATCCAGTTTT R: TCAGGC CCGTGG TTCTCA	300	1.948	66
§ IL-10RA	interleukin-10 receptor A	F: GTCACCCTGCCACTGATCAC R: GGCAGCGTGCAGCTGAAATC	300	1.828	84
§ IL-6	interleukin-6	F: GGCTCCCATGATTGTGGTAGTT R: GCCCAGTGGACAGGTTTCTG	300	1.873	64
§ IL-12p40	interleukin-12, p40 subunit	F: CAAACCAGACCCACCCAAGA R: GACCTCCACCTGCCGAGAA	300	1.896	64
§ IL-15	interleukin-15	F: GGCTGGCATTCATGTCTTCA R: CATACT GCCAGT TTGCTTCTGTTT	300	1.850	74
§ IL-18	interleukin-18	F: GAAAATGATGAAGACCTGGAATCA R: ACTTGGTCATTCAAATTTCGTATGA	300	1.896	84
§ IL-1b	interleukin-1 beta	F: AAGCAGGCGCATCTGTGAA R: ATGGCACTCTAACCCGGAAA	450	1.915	70
§ IL1R2	interleukin-1 receptor 2	F: ATACCTGTGCCATGACGTATGC R: CGGAGTTTGATATTCCTGGTGAT	300	1.923	67
§ IL2	interleukin-2	F: TGATGCAACAGTAAACGCTGTAG R: GAGAGGCACTTAGTGATCAAGTC	450	1.928	95

Table 2.3 (cont'd)					
§ IL-1α	interleukin-1 alpha	F: TTGGTGCACATGGCAAGTG R: GCACAGTCAAGGCTATTTTTCCA	450	1.948	72
§ IL-8	interleukin-8	F: GGAAAAGTGGGTGCAGAAGGT R: GGTGGTTTTTTCTTTTTCATGGA	100	1.888	80
§ INF-γ	interferon, gamma	F: TGGCATGTCAGACAGCACTTG R: CCTGAAGCGCCAGGTATAAGG	450	1.932	96
§ TGFβ	transforming growth factor, beta	F: CTGAGCCAGAGGCGGACTAC R: TGCCGTATTCCACCATTAGCA	300	1.897	63
§ TNFα	tumor necrosis factor, alpha	F: TCTACCAGGGAGGAGTCTTCCA R: GTCCGGCAGGTTGATCTCA	300	1.871	68
SDHA	succinate dehydrogenase complex subunit A	F: CCACGCCAGGGAGGACTTC R: CGTAGGAGAGCGTGTGCTTC	300	1.879	116

 # genes that showed substantial altered expression within a group of cattle in microarray studies

*** genes that had differential power between groups of cattle using microarray expression data analyzed with MANOVA**

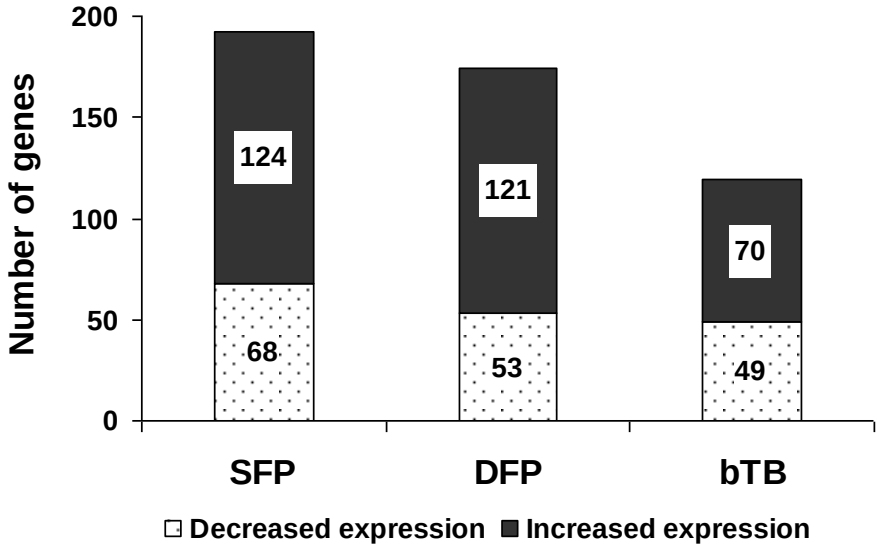
§ genes that were selected from the literature as being relevant to the bTB infection

Table 2. 4 Genes that showed differential power (*adj p* ≤ 0.05) between cattle as determined by qPCR analysis. Cattle that could be differentiated were double test-false positive with no bTB-exposure history (DFP-non-ex) and cattle that were bTB infected (bTB).

The relative expression level of each gene for each animal was calibrated with the reference pool of RNA using the PCR efficiency corrected-ΔΔCT algorithm; differential expression level (Δ $\log_2$ FC) of the bTB and DFP-non-ex groups of cattle (bTB minus DFP-non-ex) was determined using ANOVA analysis.

Gene	Δ $\log_2$ FC (bTB vs DFP-non-ex)	*adj p*
IL-1a	3.59	0.0023
IL-6	3.24	0.0044
IL-10	2.87	0.0000
TNFa	2.36	0.0005
CSF3	2.22	0.0024
IL1R2	2.11	0.0307
IL12-p40	2.08	0.0039
IL-15	1.59	0.0031
BOLA_DMA	1.57	0.0118
IL10RA	0.87	0.0437
IL-18	0.83	0.0179
RPL19	0.72	0.0350
ARF3	0.54	0.0392

(a)



(b)

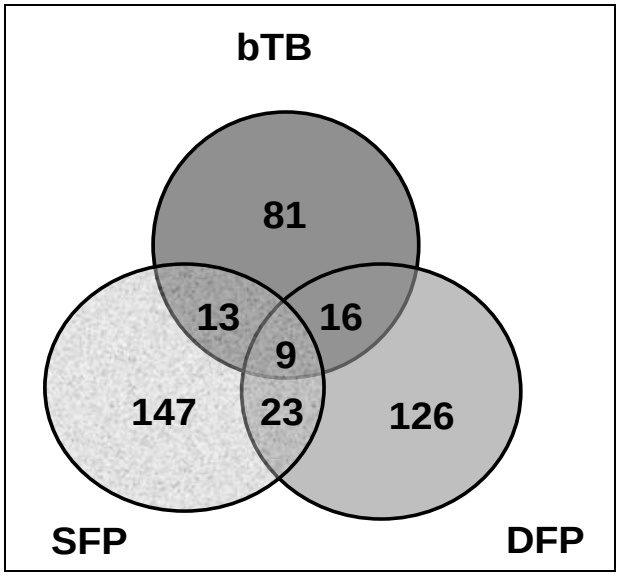
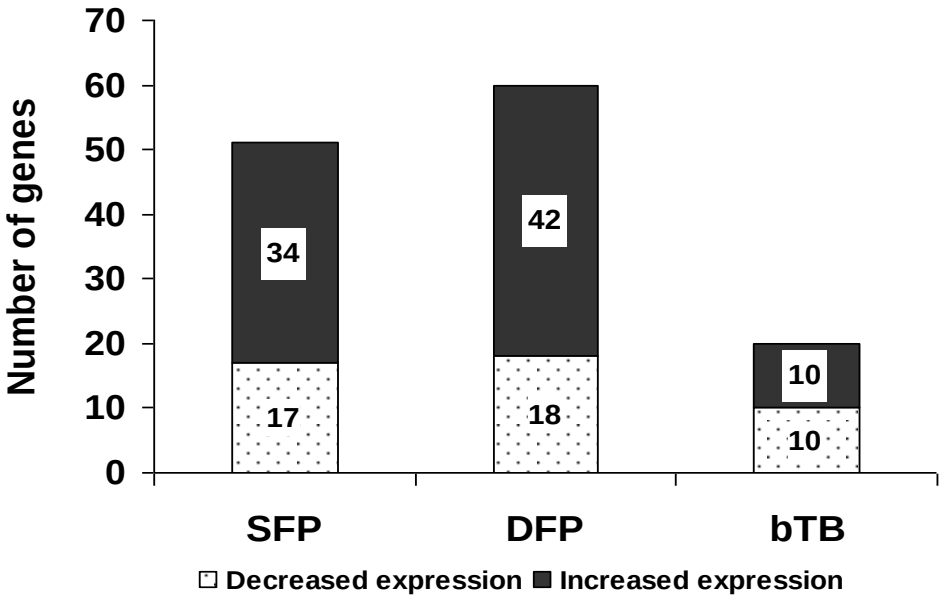


Figure 2. 1 Number of genes from microarray analysis that were differentially expressed ($p \leq 0.05$) within single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB) groups of cattle. The RNA used for microarray anlaysis was harvested after a 4 hour stimulation of whole blood with tuberculin and the comparison was with a reference pool of mRNA harvested from the blood of healthy cattle after a 4 hour stimulation with tuberculin.

(a) The number of genes for each group of cattle that showed increased expression (solid box) or decreased expression (shaded box) relative to the reference pool of RNA is indicated by the figure in the boxes.

(b) A venn diagram showing the number of differentially expressed genes that was found within a single group or within multiple groups of cattle relative to the pool of healthy control cattle.

(a)



(b)

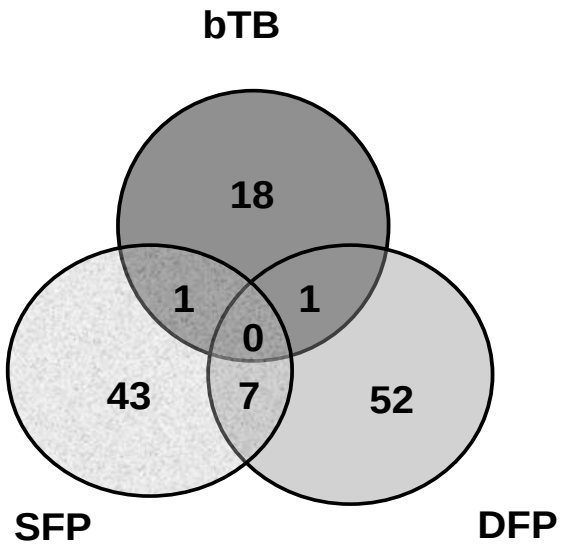


Figure 2. 2 The number of genes from microarray analysis that were differentially expressed ($p \leq 0.01$) within single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB) groups of cattle. The RNA used for microarray anlaysis was harvested after a 4 hour stimulation of whole blood with tuberculin and the comparison was with a reference pool of mRNA harvested from the blood of healthy cattle after a 4 hour stimulation with tuberculin.

(a) The number of genes that showed increased expression (solid box) or decreased expression (shaded box) for each group of cattle relative to the reference pool of RNA is indicated by the figure in the boxes.

(b) A venn diagram showing the number of differentially expressed genes that was found within a group or multiple groups of cattle relative to the pool of healthy control cattle.

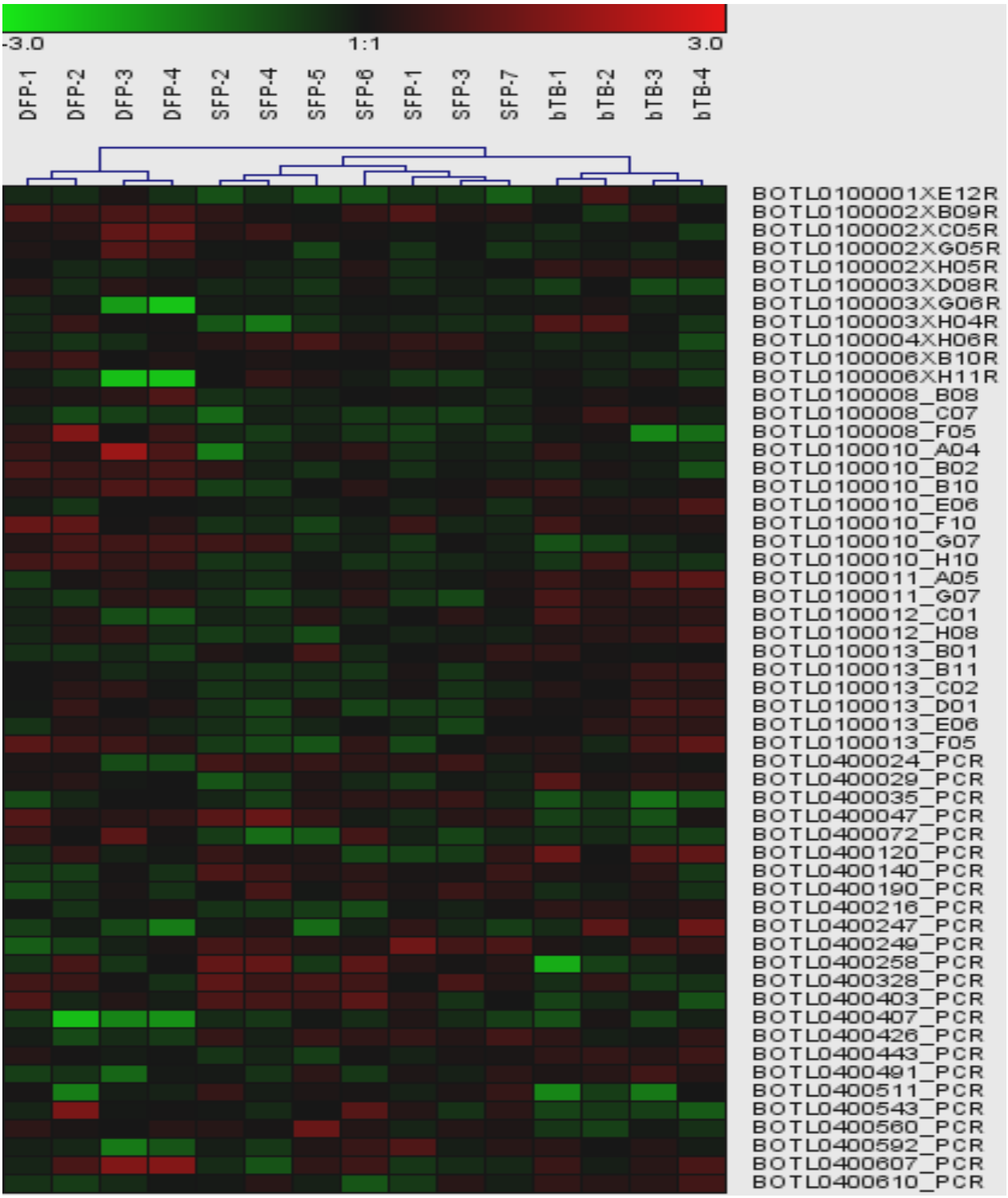


Figure 2.3 A heat map generated from cluster analysis using hierarchical clustering of cattle based on the complete linkage algorithm. The statistically significant ($p \leq 0.01$) differentially expressed genes identifed in microarray analysis were used. The heat map shows that gene expression profiles segregate individual cattle into their respective groups. "For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation."

(a)

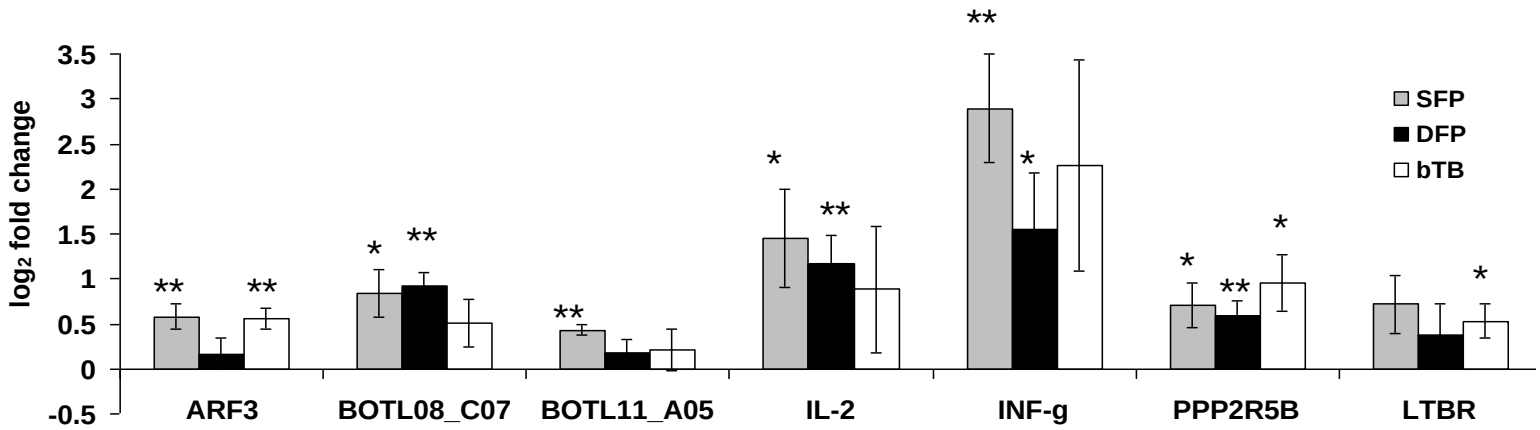


Figure 2. 4 The relative gene expression levels compared with the reference pool of RNA from healthy cattle as determined by qPCR assays for the single test-false positive (SFP)(shaded box), double test-false positive (DFP)(solid box) and bTB infected (bTB)(clear box) groups of cattle. Gene expression levels (in $\log_2$ fold change) were calculated using the PCR efficiency corrected-ΔΔCT algorithm, in which the reference pool of RNA was set as baseline (0 value at Y-axis), and used as the calibrator. Statistically significant differences are shown at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***) and the error bars represent the standard error of the mean expression level for a group of cattle.

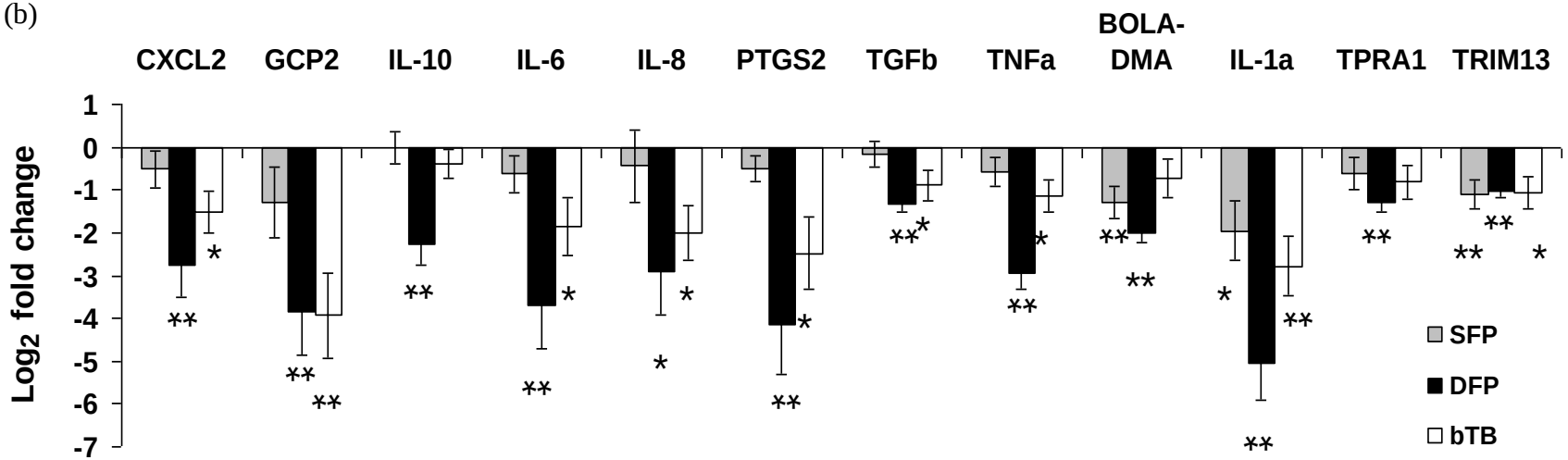
(a) Genes with increased expression in all groups of cattle.

(b) Genes with decreased expression in all sgroups of cattle.

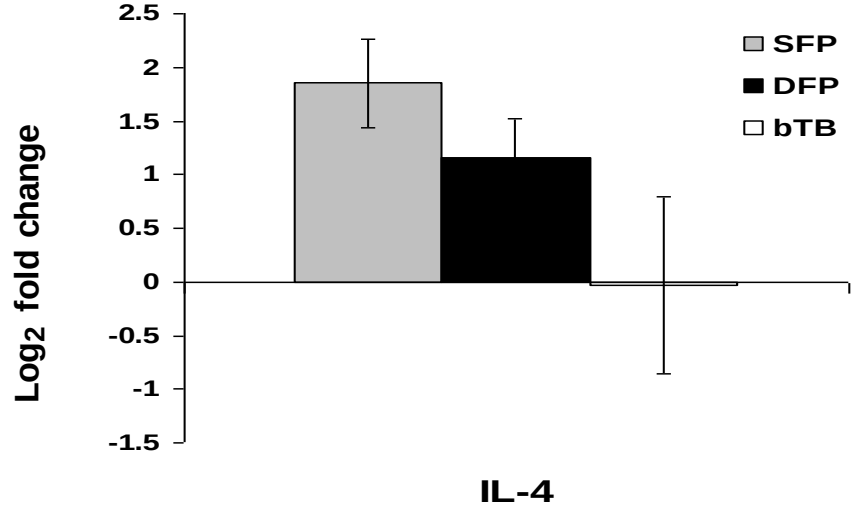
(c) Altered expression levels of IL-4 for all groups of cattle.

Figure 2.4 (cont'd)

(b)



(c)



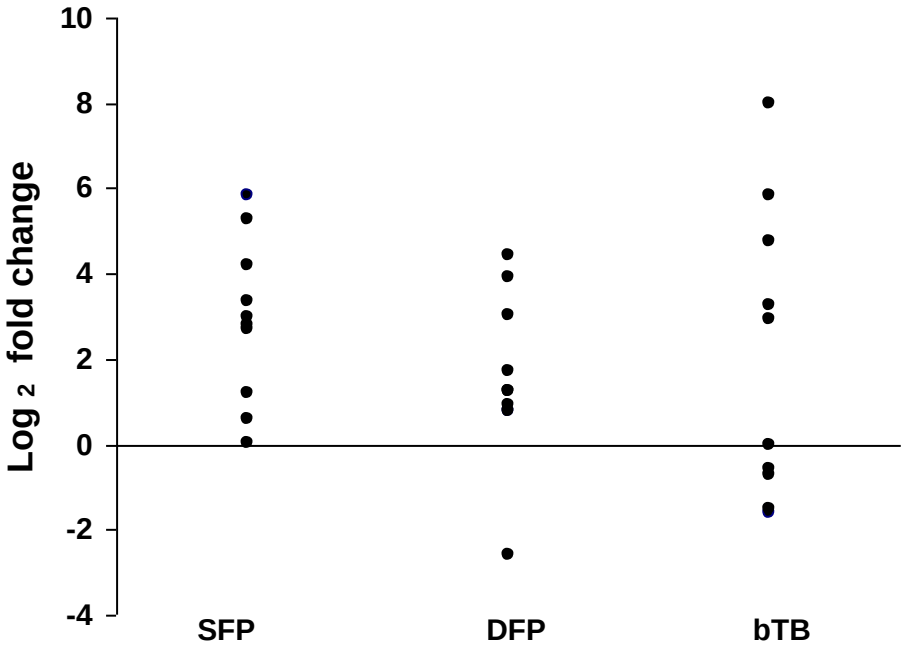


Figure 2. 5 A scatter plot showing the expression levels of the IFN-γ gene determined by qPCR assay for the single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB) groups of cattle as compared with the reference pool of RNA from healthy cattle.

Gene expression levels (in $\log_2$ fold change) were calculated using the PCR efficiency corrected-ΔΔCT algorithm, where expression level of the reference pool of RNA was set as baseline (0 value at Y-axis), and used as the calibrator.

(a)

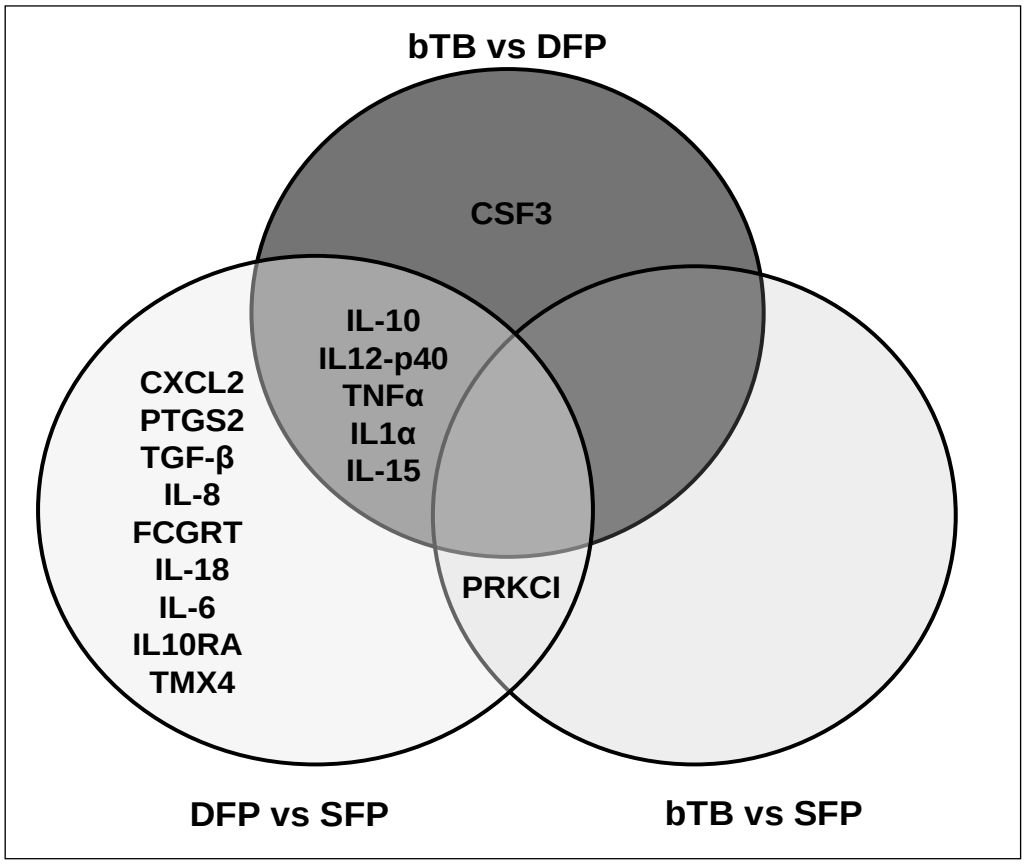
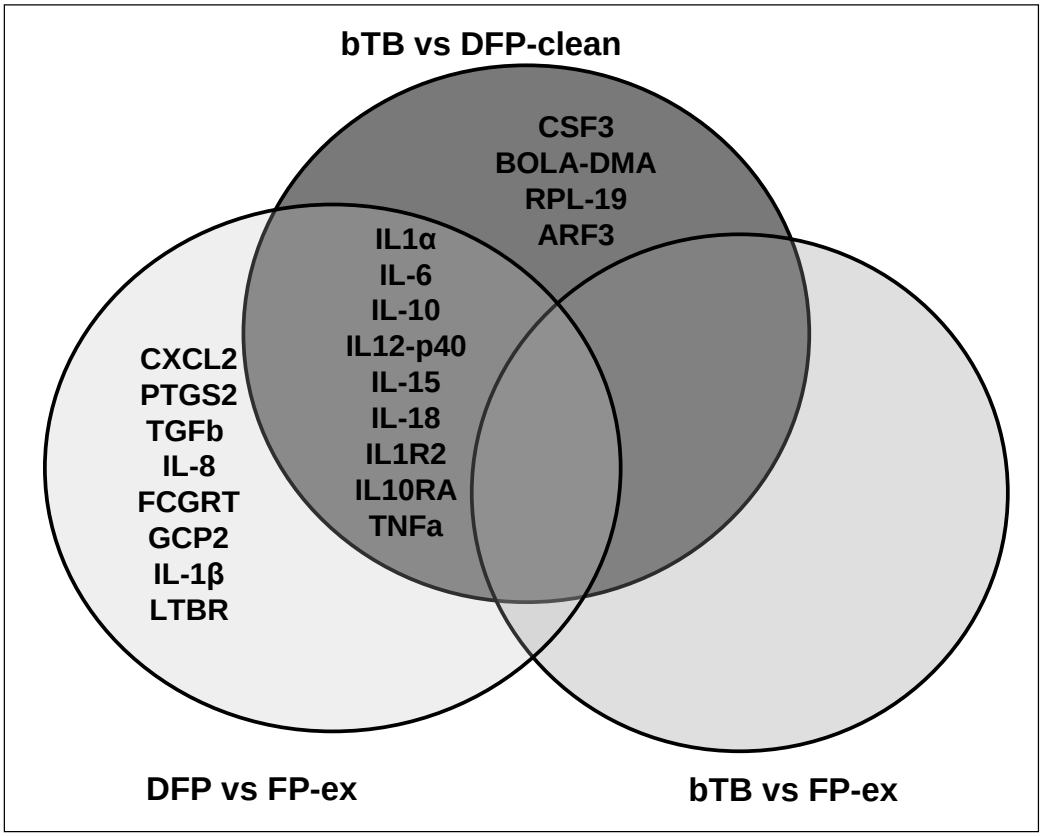


Figure 2. 6 Venn diagrams showing the statistically significant *(adj* $p \leq 0.05$) differentially expressed genes that were unique to or common among (a) single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB) groups of cattle and (b) test-false positive-exposed (FP-ex), double test-false positive-non exposed (DFP-non-ex) and bTB infected (bTB) groups of cattle as determined by qPCR assay.

Gene expression level for each animal was calibrated relative to the reference pool of RNA from healthy cattle, using the PCR efficiency corrected-ΔΔCT algorithm; differential expression between 2 groups of cattle (i.e. X vs Y) was determined using ANOVA analysis.

Figure 2.6 (cont'd)

(b)



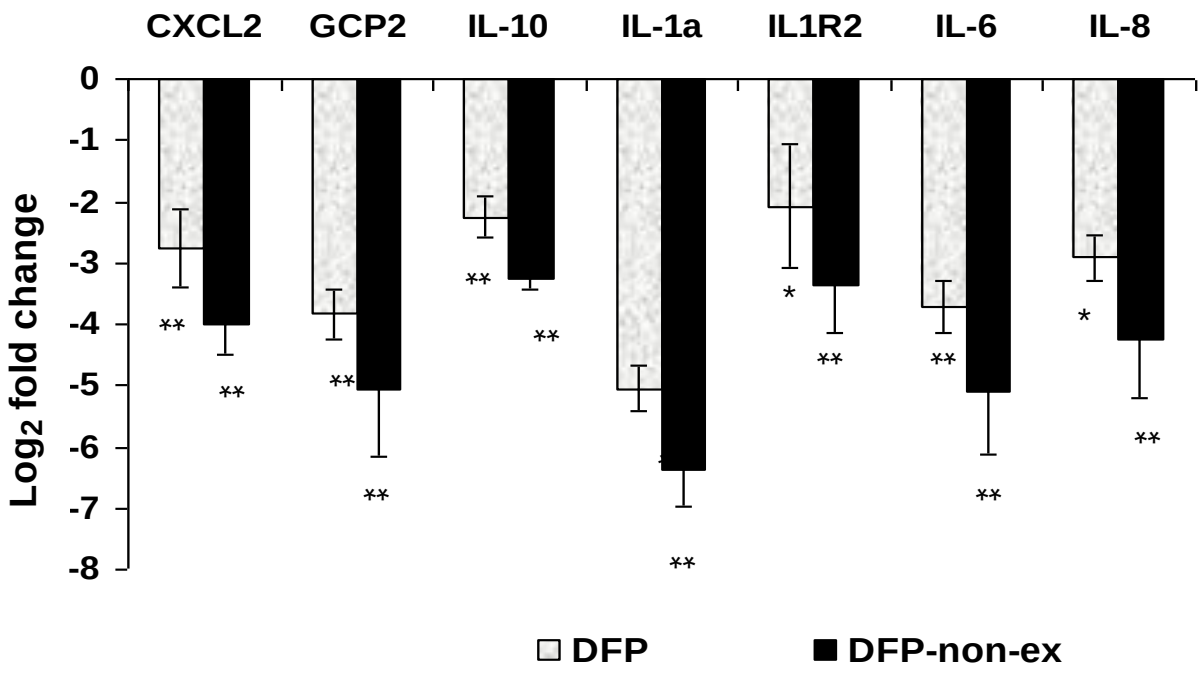


Figure 2. 7 The statistically significant ($p \leq 0.05$* and $p \leq 0.01$) mean expression level of genes after the double test-false positive (DFP) cattle (shaded box) were separated into groups of double test-false positive cattle with no history of bTB exposure (DFP-non-ex) (solid box) and double test-false positive cattle exposed to bTB infected cattle (DFP).**

Gene expression levels (in $\log_2$ fold change) were calculated using PCR efficiency corrected-ΔΔCT algorithm, where expression level of the reference pool of RNA from healthy cattle was set as baseline (0 value on Y-axis), and used as the calibrator. The error bars represent the standard error of the mean level of expression for each gene.

(a)

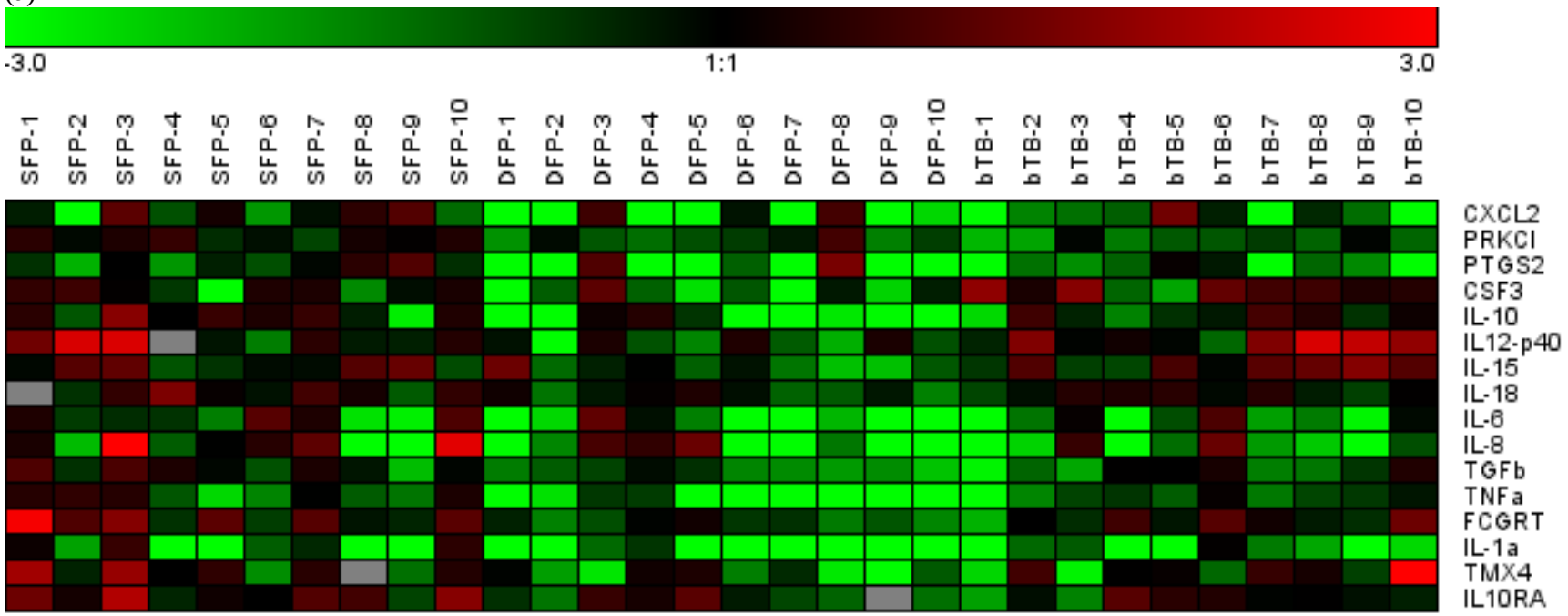
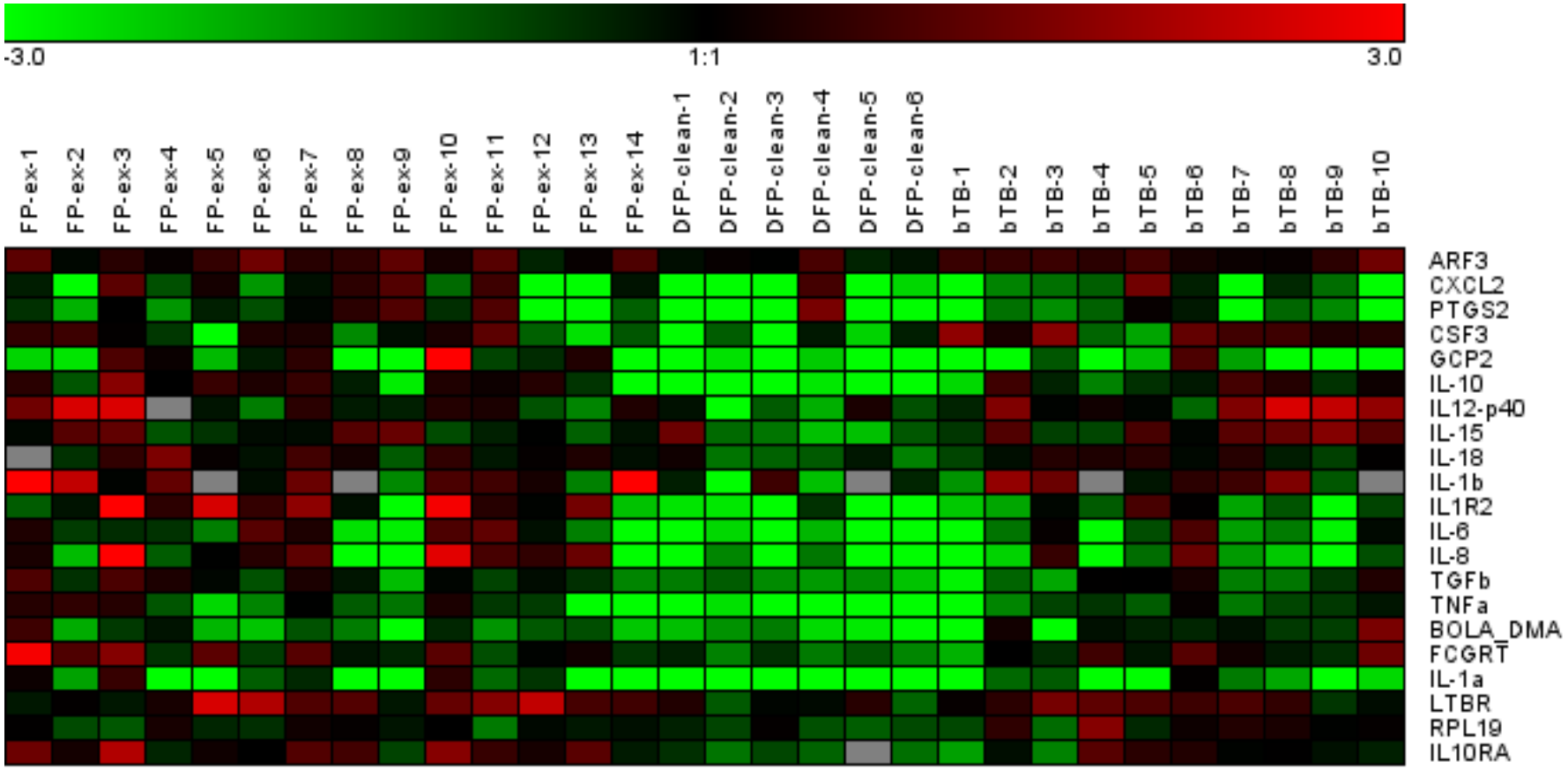


Figure 2. 8 Heat maps of gene expression profiles for individual animals based on statistically significant (*adj* $p \leq 0.05$) differential expression detected in qPCR assays. The heat maps show the (a) initial groups of single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB) cattle and (b) double test-false positive-bTB exposed (FP-ex) cattle, double test-false positive-non bTB exposed (DFP-non-ex) and bTB infected (bTB) groups of cattle.

Figure 2.8 (cont'd)

(b)



CHAPTER THREE

Gene Expression Profiling of Cattle Infected or Not Infected with Bovine Tuberculosis: Overnight Stimulation of Whole Blood with Tuberculin

Introduction

The altered gene expression profiles of peripheral blood mononuclear cells (PBMC) at 4 hours post-stimulation with purified protein derivative (bPPD) made from cultures of *M. bovis*, as presented in Chapter 2, showed differences among the single antemortem test-false positive (SFP), double antemortem test-false positive (DFP), and bovine tuberculosis (bTB) infected groups of cattle. The data presented in Chapter 2 also showed there was potential for using molecular markers as the basis for a diagnostic test for detection of bTB. However, altered gene expression obtained after a 4 hour stimulation of PBMC with antigen proved less than optimal for differentiation of bTB cattle from all "non-infected" cattle that showed positive reactions on antemortem tests for bTB. While the gene expression profiles for the DFP group of cattle were clearly distinctive, the gene expression profiles of the bTB and the SFP groups of cattle appeared similar to each other.

Gene expression studies for cattle afflicted with Johne's disease (Coussens et al., 2004) or bTB cattle (Meade et al., 2006) showed rapid onset and transient duration of altered expression (increased or decreased) for many genes. In particular, altered expression of many immunogenic genes was noted between 2-4 hours post antigen stimulation, with expression levels returning to baseline values by 6 to 8 hours post stimulation. A later perturbation of gene expression at 12 to 24 hours post stimulation also has been reported (MacHugh et al., 2009;

Meade et al., 2008; Meade et al., 2006). In a diagnostic laboratory setting, overnight antigen stimulation would be preferred over a 4 hour antigen stimulation, because the workflow can be better managed within a normal 10 hours work day. The overnight scheme allows for antigen stimulation to be started the day samples arrive at a laboratory and for harvest of cellular RNA the following workday. A 4 hour antigen stimulation scheme would force the entire procedure to be completed within one day. This would be difficult to accomplish unless either the blood samples arrived at the laboratory early in the workday or the length of the workday was extended. Therefore, identification of molecular markers that could distinguish infected from non-infected cattle after an overnight antigen stimulation of blood would be more convenient for use as a diagnostic test for bTB.

The current study examined altered gene expression profiles from SFP, DFP, and bTB groups of cattle after overnight stimulation of whole blood with bPPD. The BLOPlus (bovine long oligo plus) bovine microarray library, which covered over 10,800 bovine gene features (including those gene features in the BOTL5 library) was used for this study. This library was larger than the BOTL5 library used in Chapter 2, and offered better coverage for profiling more gene features. Use of the BLOPlus library allows some comparisons to be made with the 4 hour antigen stimulation study presented in Chapter 2 because BLOPlus library includes the BOTL5 library.

The working hypothesis for the current study was gene expression profiles of the SFP, DFP and bTB groups of cattle would differ from each other after overnight stimulation of PBMC with bPPD. The objective of the study was to analyze data from microarray hybridizations and generate gene expression profiles for each of the 3 groups of cattle. Comparison of the gene

expression profiles should lead to identification of genes with altered expression that can be tested to determine their predictive value for diagnosis of bTB.

Materials and Methods

Experimental animals and their bTB infection status

Selection of cattle for this study was done as described in Chapter 2. The study cattle were grouped based on their response in antemortem diagnostic tests for detection of bTB and on postmortem assays used to confirm or refute the results from the antemortem tests. For microarray analysis, the study groups included bTB positive cattle (n=4), DFP cattle (n=9) and SFP cattle (n=9). Healthy cattle from the MSU Dairy Farm (n=9) with recent negative test records for bTB, *M. avium* subspecies *paratuberculosis,* bovine leukosis virus, and bovine viral diarrhea virus were used to obtain a reference pool of control RNA.

Blood collection and antigen stimulation

Blood samples from the study animals were collected into multiple acid citrate dextrose (ACD)-containing tubes and/or heparin-containing tubes (Vacutainer®, BD Diagnostics, Franklin Lakes, NJ) prior to euthanasia for postmortem examination. Within 3 hours of collection, the blood from each animal was pooled into individual sterile 50ml conical tubes and stimulated with bPPD (Prionics AG, Switzerland) at 20µg of bPPD/ml of blood. The blood was incubated at 38 ± 1°C for 20-22 hours prior to harvest. Blood samples from 9 healthy cattle housed in MSU Dairy Farm were collected into ACD-containing tubes and transported immediately to the laboratory. At the laboratory, the blood from each of those cattle was pooled into individual sterile 50ml conical tubes. Stimulation with bPPD was not done, instead the

blood was processed immediately to obtain cellular RNA from the peripheral blood mononuclear cells (PBMC).

Isolation of PBMC, and purification of RNA

Isolation of PBMC was done using several rounds of centrifugation and flash lysis of red blood cells as described in Chapter 2. The RNA extraction and purification protocols were done as described in Chapter 2. The purified RNA was stored at -84° C. Immediately before use, an aliquot of RNA from each study animal was thawed on ice and the integrity and concentration of the RNA was determined using the Agilent 2100 Bioanalyzer and RNA Nano 6000 Kit (Agilent Technologies, Santa Clara, CA). The RNA from the 9 healthy cattle was pooled and the integrity and concentration of that pooled RNA was similarly determined.

Microarray content

The BLOPlus microarray was used for this study. It is a second generation of the bovine long oligo microarray. The microarray contains 10,219 spots of 70-mer oligos derived from genes and ESTs included in several other cDNA microarrays available at the MSU Center for Animal Functional Genomics, and 581 spots of various positive and negative controls. The 10,219 spots of 70-mer oligo were derived from 7,449 ESTs in the National Bovine Functional Genomics Consortium (NBFGC) cDNA microarray (Suchyta et al., 2003), 1,865 bovine genes and ESTs derived from the highly published BOTL cDNA microarray library (Coussens and Nobis, 2002; Yao et al., 2001) and an additional 905 genes considered important in the study of bovine immunobiology. The control spots for the BLOPlus microarray include ten bovine control genes and ten Stratagene Alien Genes, spotted multiple times on the array. The gene content and sequence information for BLOPlus microarray can be found at the National Center

for Biotechnology Information Gene Expression Omnibus (NCBI GEO, platform number GPL9176), or at the MSU Center for Animal Functional Genomics website (http://cafg.msu.edu). The BLOPlus microarray was printed at the Research Technology Support Facility (www.rtsf.msu) at MSU. The platform for this microarray is the SuperAmine slide from ArrayIt Microarray Technologies (www.arrayit.com). The slide has positively charged, linear primary amines attached covalently to the glass surface facilitate attachment of the negatively charged DNA backbone.

Experimental design

A common reference with dye-swap design was used for microarray hybridization in this study. Each microarray hybridization reaction used cDNA from a single test animal that was co-hybridized with the common reference pool of cDNA from the healthy control cattle. The samples of cDNA from the study animals and aliquots of cDNA from the common reference pool were labeled with the Alexa Fluor 555 or the Alexa Fluor 647 dyes. The two dyes were used to alternately label cDNA from the study animals and aliquots of cDNA from the common reference pool, as illustrated in the diagram in Figure 3.1. Thus, for each study group, the cDNA from half of the study animals within the group was labeled with one dye and the cDNA from the other half was labeled with the second dye.

The cDNA from each animal in the DFP group and from the SFP group was used once for microarray hybridization. The cDNA from 3 of the 4 cattle in the bTB group was divided into 2 aliquots and each aliquot was labeled with a different dye. The two differently labeled aliquots of cDNA were co-hybridized on the microarray slides with oppositely labeled cDNA from the common reference pool. This resulted in 2 microarray hybridization reactions for those 3 cattle. There was insufficient RNA from the fourth bTB animal to alternate dye labels. The

two microarray slides from a single animal were treated as technical replicates for downstream data analysis. Each animal within a study group was considered as a biological replicate. In total, 25 microarray slides were generated; 9 slides each from the DFP and SFP groups, and 7 slides from the bTB group.

Synthesis of cDNA and microarray hybridization

The synthesis of cDNA and dye labeling were performed with 10 µg aliquots of total RNA using Superscript *Plus* Indirect cDNA Labeling System containing Alexa Fluor 555 or Alexa Fluor 647 (Cat# L1014-06, Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. For each microarray experiment, cDNA from a test animal and from the common reference pool were labeled with different dyes (Alexa Fluor 555 or the Alexa Fluor 647), then the labeled cDNAs were combined and concentrated through a Microcon 30 Centrifugal Filter Unit (Millipore, Billerica, MA). The labeled mixture of cDNAs was eluted in 110 µl of SlideHyb buffer #1 (Ambion, Austin, TX) and heated for 5 minutes at 70°C prior to hybridization on a microarray.

The hybridization step was preformed in a GeneTAC HybStation (Genomic Solutions Inc., Ann Arbor, MI). An 18-hour step-down protocol (6 hours at 42°C, 6 hours at 35°C, 6 hours at 30 °C) was used. Immediately following hybridizations, the slides were subjected to 5 washes of 30 sec each at 50° C with 2x SSC + 0.1% SDS, 5 washes of 30 sec each at 42° C with 0.2x SSC + 0.1% SDS, and 5 washes of 30 sec each 42° C with 0.2% SSC. After removal from the hybridization unit, the microarray slides were rinsed once in 0.2x SSC and once in double distilled water, and then dried by centrifugation for two minutes at 1,200 x g. The hybridized cDNA microarrays were scanned immediately using a GenePix 4000B two-laser scanner

(Molecular Devices, Downingtown, PA), and the GenePix Pro 6.0 software (Molecular Devices, Downingtown, PA).

Microarray data processing, normalization, and analysis

Microarray images were processed using GenePix Pro 6.0 software to generate spot intensity files for downstream data analysis. The data analysis was performed using the LIMMA (LInear Models for MicroArray) software package (Smyth, 2005), and the MAANOVA (MicroArray ANalysis of VAriance) software (Wu, 2008) implemented in the R language and environment (http://www.r-project.org) (R Development Core Team, 2011).

In LIMMA analysis, background correction (Ritchie et al., 2007) and normalization within the microarray (Smyth and Speed, 2003) was performed prior to linear regression analysis for determination of differential expressed gene spots and their relative fold change (Smyth, 2004). MA plots of data prior to and after normalization for each microarray were generated for visualization of the normalization effect (Figure 3.2). Following normalization, the microarray data were analyzed, based on the linear regression model of fixed effects, as shown in the formula below,

Model = fixed effect of (Dye + Array + Sample + Breed + Group + Age) + random error

For each study group of cattle, an empirical Bayes moderated T test was performed to identify genes showing altered expression that was significantly different from the common reference pool. The *p* values were further adjusted for false discovery rate (FDR) using Benjamini-Hochberg (BH) correction method (Benjamin, 2001). Differential gene expression between the study groups (bTB vs SFP, bTB vs DFP, and DFP vs SFP) was calculated using the fixed effect model.

A mixed effects model analysis was performed using MAANOVA. Confounding factors (breed and age) that could contribute to variation in gene expression of individual cattle and microarray hybridization (array and sample) were evaluated to determine the model best fit for data analysis. All cattle used in this study were female, and thus, gender was not included as an effect in the model. The square root of variance of each random effect in the mixed model used in data analysis (Figure 3.3) showed that the variance for array, sample, and breed were large enough these factors should be taken into account as random effects in the analysis model. Age of the cattle (in months) was used as a covariate in the mixed model. Taking all the factors evaluated into account; a mixed model was used that had fixed effects of dyes and study groups; random effects of microarray platform, cDNA sample, and breed of animal; and the age of cattle as a covariate. The final model for this study is illustrated as the formula below;

Model = fixed effect of (dye + group) + random effect of (array + sample + breed)

+ covariate (age) + random error

The defined mixed model was used in subsequent analyses for identification of differentially expressed gene features. Differential gene expression between the study groups (bTB vs SFP, bTB vs DFP, and DFP vs SFP) was re-calculated using the mixed model analysis performed with 500 permutation tests and the jsFDR method for false discovery rate (FDR) adjustment (Storey, 2002).

Gene functional annotation and gene ontology classification of microarray data

The differentially expressed genes identified from microarray hybridization reactions were analyzed with the database for annotation, visualization and integrated discovery (DAVID) (Huang et al., 2009a; Huang et al., 2009b) to determine the gene ontology (GO) term enrichment. The Functional Annotation Clustering tool from DAVID was used to aid identification of the

most relevant GO terms, KEGG (Kyoto Encyclopedia of Genes and Genomes) or BioCarta pathways, associated with the differentially expressed genes. Gene information of *Bos taurus* was used as the background for the analysis.

Quantitative real-time polymerase chain reaction (qPCR) assay

Validation of altered expression using qPCR was attempted for 48 genes. Of those, 33 genes were selected from data generated during analysis of the microarray hybridizations performed in the current study. An additional 15 genes were either identified in the previous chapter as potentially having power to segregate cattle into groups of infected and non-infected animals or were genes for cytokines and chemokines reported as important mediators in bovine and human TB infections. After evaluation of 12 potential reference genes, succinate dehydrogenase complex subunit A (SDHA) was determined to be the most suitable reference gene for this study (data presented in Appendix A).

Nucleic acid sequences for the 70-mer oligos representing the selected gene feature from the BLOPlus microarray were entered into the Basic Local Alignment Search Tool (BLAST) to verify the gene identity and to obtain additional sequence information to facilitate design of PCR primers. The PCR primers for the gene targets were designed in Clone Manager Suite 7.0 (Sci-Ed Software, Cary, NC) or Primer Express 3.0 software (Applied Biosystem, Foster City, CA), and were synthesized by Integrated DNA Technologies (Coralville, IA). All primers were tested for amplification with the control reference pool of RNA and with a no template control (NTC). The optimal concentration for each primer was determined empirically (data not shown). The primer sequences and primer concentration used are listed in Table 3.4.

Samples of RNA from 10 cattle for each study group were used in qPCR assays for quantification of gene expression. Some of the 10 cattle in each group were included in the

microarray experiments. The additional cattle needed to bring each group to 10 animals met the criteria for the study group to which they were assigned. The cDNA synthesis and qPCR assays were carried out as described in Chapter 2.

PCR efficiency determination and qPCR data analysis

The delta Rn data and the cycle threshold (Ct) data exported from the ABI 7500 SDS software were used to verify that acceptable PCR efficiency was achieved and for calculation of the relative expression level of the targeted genes. The efficiency of each qPCR reaction was determined using LinRegPCR program (Ramakers et al., 2003; Ruijter et al., 2009). For this study, the mean values for PCR efficiency from 10 and 25 study cattle were calculated and compared as shown in Table 3.1. There was essentially no difference in PCR efficiency between the means of 10 and 25 cattle, hence, the PCR efficiency for all gene targets used in this study was calculated from average values for the 25 samples available at time of initial analysis (Table 3.4). The efficiency corrected ΔΔCT algorithm for calculation of differential gene expression was used for qPCR analysis (Livak and Schmittgen, 2001; Pfaffl, 2001), as detailed in Chapter 2.

Visualization of results using cluster analysis

Cluster analysis was performed using the Genesis software (Institute for Genomics and Bioinformatics, Graz University of Technology, Austria) (Sturn et al., 2002). Hierarchical clustering based on the complete linkage algorithm was performed to cluster the cattle using data obtained for individual animals in the qPCR assays, for comparison with original group assignments of the cattle, which was made using results of antemortem and postmortem diagnostic tests.

Results

Microarray data analysis using LIMMA

Analysis of data from the 25 microarray hybridizations completed in this study was first done with a fixed model using LIMMA software. MA plots were generated from the data prior to and after normalization of each microarray, the skewed dye effect on gene features that showed extremely low or high in expression levels (on both ends of the dot plots) was effectively corrected by the normalization procedure (Figure 3.2). When compared with the reference pool of RNA from healthy cattle, 1,058 gene features (10.35% of all gene features) were differentially expressed ($p \leq 0.01$) in one or more of the groups of cattle. Following BH correction for FDR adjustment, 281 gene features were differentially expressed at an *adjusted (adj)* $p \leq 0.01$. Overnight antigen stimulation caused altered expression of a similar number of gene features in each group of cattle; 146 in the SFP group, 154 in the DFP group, and 151 in the bTB group. Of the 281 gene features showing altered expression, 68 were shared among two groups of cattle and 51 gene features were shared among all three groups of cattle (Appendix C and Figure 3.4).

The SFP and DFP groups of cattle were similar in that they had about twice as many gene features that were up-regulated as were down-regulated (Figure 3.5). In contrast, the bTB group of cattle showed about twice as many gene features that were down-regulated as were up-regulated. The range in fold change of gene expression was similar for all three groups; -2.11 to +5.51 for the SFP group, -1.88 to +4.63 for the DFP group, and -2.45 to +5.35 for the bTB group. Unique gene features with high levels of altered expression in each group were selected for validation using qPCR assays. Comparisons of altered gene expression data between groups of cattle (bTB vs SFP, bTB vs DFP and DFP vs SFP) were made using a fixed effects model. At $p \leq 0.01$, 127 gene features showed differential expression levels between the bTB and the SFP

group, 105 between the bTB and the DFP group, and 46 between the DFP and the SFP group (Figure 3.6).

MAANOVA analysis of microarray data with mixed effect model

The differential gene expression profiles were compared between groups of cattle (bTB vs SFP, bTB vs DFP and DFP vs SFP) in a mixed effects model, using the MAANOVA software. At $p \leq 0.01$, 184 gene features showed significant difference in expression levels between the bTB and the SFP group, 155 between the bTB and the DFP group, and 119 between the DFP and the SFP group (Figure 3.6). Comparison of the results from the fixed effect model analysis (LIMMA) with those from the mixed effects model analysis (MAANOVA) revealed that more gene features showed statistically significant altered expression using the mixed model analysis. This likely was due to the correction made in adjustment for random effects in the mixed effects model.

The data generated using the mixed effect model was further corrected for false discovery rate (FDR) using the jsFDR adjustment. The data was then filtered to identify gene features that showed a fold change for gene expression that was greater than 1.5. This selection criterion was used to identify gene features that likely would have value for group differentiation. After this process, the number of gene features with altered expression that might differentiate groups of cattle was reduced from 184 to 75 for the (bTB vs SFP), from 155 to 20 for the (bTB vs DFP), from 119 to 10 for the (SFP vs DFP).

The differential gene expression profiles also were compared based on bTB status (bTB vs SFP and DFP groups combined). At $p \leq 0.01$, a total of 200 gene features showed differential expression based on bTB status. This gene list also was adjusted for FDR and filtered for gene features that showed a fold change for gene expression that was greater than 1.5. This resulted in

42 genes meeting the selection criteria. Of those 42 gene features, 22 were identified as differentially expressed in the separate analyses of bTB vs SFP and bTB vs DFP. Those 22 genes were selected for validation using qPCR assays.

Enrichment analysis of microarray data

The DAVID program was used for enrichment analysis of differentially expressed genes (Figure 3.5) from each of the SFP, DFP and bTB groups of cattle. Enrichment categories for each group ($p \leq 0.01$) are presented in Table 3.2. The gene ontology (GO) terms for small chemokines, pattern and polysaccharide binding, cell migration, and angiogenesis were the four most highly enriched terms for both the SFP and DFP groups; with exception for the small chemokines, those terms were less enriched in the bTB group. The GO terms for antigen processing and presentation, adaptive immune response, and major histo-compatibility (MHC) and autoimmune response were highly enriched terms for the bTB group; those terms were considerably less important in the other 2 study groups. Overall, the most enriched terms for bTB group were involved with immunological responses, while the most enriched terms for the SFP and DFP groups were involved in cell growth and activation.

Group level gene expression profiling with quantitative real-time PCR (qPCR)

The expression levels of 48 genes selected from the microarray hybridization data or from the literature were quantified using qPCR assays. Information for these genes is listed in Table 3.4. The mean expression levels were calculated for each of the study groups using 10 cattle per group. Gene expression levels of 13 genes calculated from qPCR assays were compared with values for gene expression obtained from microarray hybridizations (Table 3.3). The gene expression levels obtained from qPCR assays correlated with the gene expression

levels derived from the microarray hybridizations, supporting the validity of microarray results. Overall, the altered gene expression levels detected in the qPCR assays showed a greater distance, positive or negative, from the reference pool than comparable values obtained from the microarray analyses.

The T test was used to determine the statistical significance of the mean expression value for altered gene expression at the cattle group level. When that was done, 8 genes showed considerable variation in fold changes for expression values among cattle within each study group; therefore, those genes were excluded from further analysis. The 8 genes were IFN-γ, TNF-α, TGF-β, DDX-5, IGBP1, RPL13, RPL7A and RPS5 (data not shown). For the remaining 40 genes, the mean expression values within groups of animals were ranked based on their range of fold change in expression compared with the reference pool of RNA (Figure 3.7a to f). The highest rankings included 6 genes with altered expression values greater than 100 fold in at least one group of cattle (Figure 3.7a), followed by 7 genes with altered expression values ranging from 10 to 100 fold (Figure 3.7b), and then 6 genes with altered expression ranging from 3 to 10 fold (Figure 3.7c). The remaining 21 genes showed altered expression values of less than 3 fold (Figure 3.7d, e & f); however, differences in the mean expression levels of those genes between groups of cattle suggested that they might be useful for prediction of disease status. In general, genes that had altered expression at > 3 fold were up-regulated and genes that had altered expression at < 3 fold were down-regulated.

Analysis of altered gene expression in individual cattle

The qPCR data were analyzed at the individual animal level using the ANOVA test to identify genes that could significantly differentiate individual cattle within a group from cattle in the other 2 groups. Of the 40 genes analyzed, 24 genes were judged significant at $p \leq 0.05$ level

(see Table 3.5). Upon jsFDR adjustment ($adj\ p \leq 0.05$), only 20 of the 24 were judged significant. The 4 genes judged not significant at $adj\ p \leq 0.05$ were ATPIF1, BOLA-DRB4, CCNG1 and TLR4. Differentiation of the bTB group from the DFP group was possible using 15 genes, differentiation of the SFP group from the DFP group was possible using 10 genes, and differentiation of the bTB group from the SFP group was possible using 5 genes (Figure 3.8). The only gene able to differentiate cattle among all 3 groups was CXCL2. In general, the 15 genes that could differentiate bTB cattle from DFP cattle showed the highest statistical significance (Table 3.5).

Visualization of qPCR results by cluster analysis

The 20 genes that remained statistically significant ($adj\ p \leq 0.05$) after FDR adjustment (listed in Figure 3.8) were evaluated by cluster analysis, using their log fold change in expression values for individual cattle. When this was done, the individual cattle formed two main clusters. One cluster contained all but one of the bTB positive animals, one DFP animal, and 5 of the SFP animals. The other cluster consisted of all but one of the DFP animals, one of the bTB positive animals, and 5 of the SFP animals (Figure 3.9). Thus, after overnight stimulation of blood with bPPD, cattle from the SFP group were equally split into both clusters; similar to the cluster analysis done in Chapter 2 using blood stimulated with bPPD for 4 hours.

Discussion

In this study, gene expression profiles for three groups of cattle (SFP, DFP and bTB) were analyzed after overnight stimulation of PBMC with bPPD. The gene expression profiles were derived from a series of microarray hybridizations using the BLOPlus platform, which contained 10,219 gene features. Previous studies reported that a rapid and transient change in

expression levels of certain genes occurs in response to brief (2 to 4 hours) antigen stimulation of cattle PBMC, and that a second wave of altered gene expression occurs after 12 to 24 hours of antigen stimulation (Coussens et al., 2004; MacHugh et al., 2009; Meade et al., 2008; Meade et al., 2006). Meade et al. (2006) reported that the number of genes showing altered expression was greater after 12 hours of stimulation of PBMC with bPPD than after 24 hours of stimulation. The practicality of both the 12 and 24 hours time points were assessed for use in a diagnostic setting that had a 10 hours work day. The 12 hours period of antigen stimulation would necessitate use of overtime labor or employing a second shift of laboratory technicians. The most practical duration of stimulation was considered to be between 16 and 22 hours (overnight); hence, overnight stimulation of PBMC was done in the current study.

After overnight stimulation of PBMC with bPPD, the ratio of genes showing increased expression to those showing decreased expression among the SFP and DFP groups of cattle was about 2:1; which was just the opposite of the 1:2 ratio found for the bTB group of cattle. Over representation of genes showing decreased expression is consistent with previous reports of gene expression studies of cattle infected with mycobacteria, specifically *M. bovis* or *Mycobacterium avium* subspecies *paratuberculosis* (Meade et al., 2006, Coussens et al., 2004). The effect of bPPD stimulation on primed memory T cells *in vitro* may be similar to mycobacterial infection *in vivo*, where re-programming of the host response system by pathogenic mycobacterium leads to suppression of the host defensive cellular responses, thus favoring pathogen invasion and survival in the host (Cosma et al., 2003; Jenner and Young, 2005; Koul, 2004).

The genes that showed significant altered expression in the microarray studies were entered into the Functional Annotation Clustering tool in the DAVID software to identify the major gene enrichment profiles for each group of cattle. When this was done, the gene

enrichment profiles were similar for the SFP and the DFP groups. Except for the enrichment of genes associated with small chemokines, which was observed in all groups of cattle, the gene enrichment profile of the bTB group clearly differed from the SFP and DFP groups. The gene enrichment profile of the bTB group reflected an adaptive immunogenic response, while gene enrichment profiles of the SFP and DFP groups were suggestive of a more general response reflecting cell activation.

The data from the qPCR assays for validation of altered expression for 48 selected genes was analyzed to determine statistical significance for differentiation between groups of cattle, then for differentiation of individual cattle within each group. When this was done, the list of genes with the potential power to differentiate infected from non-infected cattle was reduced from 48 to 20. Many of those genes have been reported previously as being important in animal or human infections with mycobacteria. The expression levels of several small chemokines, especially the C-X-C motif chemokines; including chemokine C-X-C motif ligand 2 (CXCL2), granulocyte chemotactic protein 2 (GCP2, also known as CXCL6), and interleukin-8 (IL-8, also known as CXCL8), were highly increased in all groups of cattle after overnight stimulation of PBMC with bPPD. CXCL2, which differentiated all 3 groups of cattle, is secreted by monocytes and macrophages and is chemotactic for polymorphonuclear leukocytes (Wolpe et al., 1989). Both GCP-2 and IL-8 are secreted by macrophages and are chemotactic for granulocytes (Linge, 2008; Proost et al., 1993). Additionally, GCP-2 has antibacterial properties (Linge, 2008; Proost et al., 1993), and IL-8 can be secreted by any cell possessing toll-like receptors and served as an important mediator for the innate immune response (Matsushima, 1989). Increased expression of IL-8 and CXCL2 was observed in microarray hybridization experiments that used *M. bovis* infected bovine alveolar macrophages(Wedlock et al., 2006). Interestingly, macrophages

infected with virulent *M. bovis* show significantly higher expression of both genes compared with macrophages infected with an attenuated isogenic strain of *M. bovis*. Elevated level of IL-8 has also been reported in human TB clinical samples, and alveolar macrophages derived from human TB patients (Zhang et al., 1995).

Other highly expressed genes encoded the colony-stimulating factor 3 (CSF3) and the prostaglandin-endoperoxide synthase 2 (PTGS2). CSF3, also known as granulocyte colony-stimulating factor (G-CSF), is a cytokine produced by a number of different tissues. It is important for stimulation of survival, proliferation, differentiation and function of neutrophil precursors and mature neutrophils (Rapoport et al., 1992). PTGS2, also known as cyclooxygenase-2 (cox2), is a key enzyme in the biosynthesis of prostanoids, prostaglandins, prostacyclin, and thromboxanes. In humans, PTGS2 induced prostanoid biosynthesis is involved in inflammation and mitogenesis (Maglott et al., 2011). Interestingly, gene expression level of PTGS2 was much lower in the DFP group of cattle when compared with the SFP and the bTB groups. To date, no association of CSF3 or PTGS2 with human TB or bTB has been reported.

Several of the Th1 cytokine genes had statistically significant differential power, including the interleukin-1 beta (IL-1β), interleukin-1 receptor 2 (IL1R2), and interleukin-6 (IL-6). Production of IL-1β, IL-6, interleukin-12 (IL-12), and tumor necrosis factor alpha (TNF-α) in *M. tuberculosis* infected monocytes has been documented (Fulton et al., 1998; Fulton et al., 1996; Toossi, 1996). Th1 response plays an important role in the adaptive response to mycobacterial infection (van Crevel et al., 2002). IL-1β is produced by activated macrophages, is an important mediator of the inflammatory response, and is involved in cell proliferation, differentiation, and apoptosis (Maglott et al., 2011). Increased levels of IL-1β and other pro-

inflammatory factors were observed following *in-vitro* infection of macrophages with *M. bovis* (Denis and Buddle, 2008). IL-6 is secreted by T cells and macrophages; it acts as both a pro-inflammatory and anti-inflammatory cytokine. IL-6 is an important factor in the response to trauma and tissue damage, has a role in inflammation and response to microbial molecules, and is a mediator of fever and the acute phase response. IL-6 produced by macrophages infected with mycobacteria reportedly has a suppressive effect on T cell responses and IFN-γ production (Nagabhushanam et al., 2003; VanHeyningen et al., 1997).

Interleukin-10 (IL-10) and interleukin-4 (IL-4) were up-regulated in all groups of cattle. IL-10 showed potential to differentiate the bTB group from the DFP and the SFP groups of cattle, and IL-4 showed potential to differentiate the SFP group from the DFP group of cattle. IL-10 is produced primarily by monocytes and lymphocytes, and interacts with the IL10RA. IL-10 plays an important role in immuno-regulation and inflammation; it suppressed the antigen presentation capacity of antigen presenting cells by down-regulating expression of Th1 cytokines, MHC class II antigens, and costimulatory molecules on macrophages. IL-10 also enhances B cell survival, proliferation, and antibody production (Maglott et al., 2011). Increased levels of IL-10 have been reported in bovine lymph nodes and macrophages, and in human monocytes infected with *M. bovis* or *M. tuberculosis* (Denis and Buddle, 2008; Fulton et al., 1998; Witchell et al., 2010). Increased levels of IL-10 coupled with an enhanced humoral response is associated with lesion severity in bTB, and could be an important marker for disease progression when measured as an IFN/IL-10 ratio (Welsh et al., 2005; Witchell et al., 2010). IL-4 is a key cytokine regulating differentiation of naive helper T cells to Th2 cells. IL-4 stimulates activated B-cell and T-cell proliferation, induces B-cell class switching to IgE, and

up-regulates MHC class II production. It serves as a key regulator in humoral immunity (Maglott et al., 2011).

Because of excessive animal to animal variation in gene expression levels, IFN-γ and TNF-α did not have statistically significant power to differentiate groups of cattle. Similarly, substantial animal to animal variation was reported for those genes in a study of cytokine profiling of bTB infected cattle (Blanco et al., 2009b). However, those genes encode pro-inflammatory cytokines that are important in human TB and bTB infections (Thacker et al., 2007; van Crevel et al., 2002; Waters et al., 2003). Increased expression of IFN-γ and TNF-α has been reported in many studies of PBMC (Blanco et al., 2009b; Thacker et al., 2007; Waters et al., 2003) or lymph nodes (Witchell et al., 2010) harvested from bTB infected cattle. In the current study, a substantial increase in mRNA transcription for both the IFN-γ and TNF-α was observed in the bTB group of cattle, while only marginal changes in transcription levels were observed for the SFP and DFP groups of cattle (data not shown).

Cluster analysis is an effective way to visualize gene expression profiles and to group study members based on the gene expression data (Tan, 2006). The 20 genes that showed statistically significant power for differentiation of groups of cattle were used for cluster analysis. When this was done, distinct gene expression profiles of cattle that were generated that differentiated the DFP group of cattle from the bTB group. All of the cattle in the DFP group originated from bTB free herds. On the other hand, cattle in the SFP group showed disparate gene expression profiles resulting in half (n=5) of the SFP cattle clustering with the bTB group and the other half clustering with the DFP group. This result might suggest potential bTB infection within the SFP group. Since cattle in the SFP group originated from bTB infected herds, the risk of bTB infection can not be ruled out. The history of exposure to bTB infected

herdmates, along with the gene expression profiles similar to confirmed bTB infected cattle, is suggestive of potential bTB infection in some of the SFP cattle. This is similar to what was found after a 4 hour stimulation of PBMC with bPPD (Chapter 2). As discussed in Chapter 2, some cattle in the SFP group may have been at an early stage of bTB infection, or may have been infected with and cleared *M. bovis*, or may have been latently infected with bTB; any of those events may result in an animal that is negative for lesions consistent with bTB and is negative on culture of tissues for *M. bovis* (Pollock et al., 2000; Pollock and Neill, 2002; Vordermeier et al., 2008).

In conclusion, the SFP group of cattle could not be separated from the bTB cattle using altered gene expression that occurred after 4 hours (Chapter 2) or after 16 to 20 hours (Chapter 3) of stimulation of PBMC with bPPD. For both of those periods of antigen stimulation, the SFP cattle originated from bTB positive farms and the cattle had been exposed to bTB infected herdmates. Clearly, a new group of single antemortem test-false positive cattle that have not been exposed to bTB infected cattle should be evaluated to determine if this is a universal problem or if this might be a problem restricted to bTB positive herds. The overall results of the current study indicated that overnight stimulation with antigen induces gene expression profiles that are useful for differentiation of double test-false positive, non-bTB exposed cattle from the true bTB infected cattle. Further, 24 gene candidates were identified that potentially can be used as diagnostic molecular markers to identify bTB infected cattle and aide in reduction of unnecessary culling of test-false positive animals.

Table 3. 1 Comparison of mean PCR efficiencies computed by the LinRegPCR program using different numbers of samples. The mean PCR efficiencies being compared were computed from a set of 10 samples and a set of 25 samples.

gene name	gene symbol	mean PCR_efficiency (n=10)	mean PCR_efficiency (n=25)
ataxia telangiectasia and Rad3-related protein	ATR	1.927	1.925
MHC class II DR, alpha chain	BOLA-DRA	1.912	1.910
MHC class II DR, beta chain 4	BOLA-DRB4	1.945	1.942
CD53 antigen	CD53	1.816	1.816
CD79a antigen	CD79A	1.920	1.924
colony-stimulating factor 3	CSF3	1.969	1.959
C-X-C ligand 2 (Growth-regulated protein homolog alpha)	CXCL2	1.907	1.912
Eukaryotic translation elongation factor 1 beta 2	EEFIB2	1.925	1.925
H3 Histone, family 3A	H3F3A	1.894	1.895
inteferon-induced membrane protein Leu-13/9-27	IFITM	1.931	1.931
interleukin 8	IL-8	1.894	1.888
legumain	LGMN	1.944	1.944
Poly(A)-binding protein-interacting protein 2	PAIP2	1.935	1.933
S100 calcium-binding protein A4	S100A4	1.908	1.907
succinate dehydrogenase complex subunit A	SDHA	1.885	1.883
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	YWHAZ	1.898	1.896

Table 3. 2 Application of DAVID analysis for segregation of the statistically significant ($p \leq 0.05$) differentially expressed genes in microarray analysis into major Gene Ontology (GO) terms. The genes being compared were expressed after overnight stimulation of whole blood, which was obtained immeditately before postmortem examination of the single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB) cattle.

	SFP		DFP		bTB	
	GO terms	***p* value**	**GO terms**	***p* value**	**GO terms**	***p* value**
1	small chemokines	1.4E-5	small chemokines	1.8E-5	antigen processing & presentation	7.2E-6
2	pattern & polysaccharide binding	1.0E-4	pattern & polysaccharide binding	1.6E-4	small chemokines	1.5E-5
3	cell migration & localization	2.3E-4	cell migration & localization	2.4E-4	adaptive immune response	4.0E-4
4	angiogenesis	1.0E-3	angiogenesis	3.1E-4	MHC & autoimmune	1.0E-4
5	Chemotaxis	2.0E-3	MHC-class II	2.3E-3	cell migration & localization	1.7E-3
6	MHC-class II	2.2E-3	antigen processing & presentation	7.8E-3	chemotaxis	1.7E-3
7	immune response-activating signal transduction	6.6E-3			pattern & polysaccharide binding	1.8E-3
8	locomotory behavior	1.0E-2			regulation of leukocytes activation	3.2E-3
9					angiogenesis	3.3E-3

Table 3. 3 Validation of altered expression of genes selected from microarray experiments, using qPCR assays. The mean expression levels (in log_2 fold change) for each group were calculated as relative to expression level of the reference pool of RNA from healthy cattle.

		Microarray (log_2 fold change)			qPCR (log_2 fold change ± standard error)		
clone ID	**gene symbol**	**SFP**	**DFP**	**bTB**	**SFP**	**DFP**	**bTB**
Bt00006937	**BOLA-DRA**	-1.93	-1.14	-2.26	-1.56 ± 0.25	-0.77 ± 0.11	-0.89 ± 0.30
Bt00006663	**BOLA-DRB4**	-1.26	-0.95	-1.88	-1.56 ± 0.24	-0.99 ± 0.11	-0.79 ± 0.31
Bt00003921	**CXCL2**	+3.42	+2.95	+4.16	+7.78 ± 0.49	+ 5.98 ± 0.43	+9.64 ± 0.29
Bt00000692	**GCP2**	+5.51	+4.63	+4.98	+13.80 ± 0.45	+11.84 ± 0.90	+14.55 ± 0.16
BLO_ext_00786	**IL-8**	+4.97	+4.58	+5.35	+ 9.55 ± 0.75	+ 7.16 ± 0.51	+10.14 ± 0.22
BLO_ext_01355	**SPP1**	+1.87	+1.44		+10.50 ± 0.59	+10.11 ± 1.11	+8.61 ± 0.53
BLO_ext_00656	**LGMN**	+2.25	+2.33		+2.98 ± 0.46	+2.79 ± 0.36	+2.88 ± 0.21
BLO_ext_01671	**TLR4**	+1.61	+1.98		+3.54 ± 0.47	+2.48 ± 0.34	+3.55 ± 0.19
BLO_ext_01386	**MSR1**	+2.25	+1.54		+5.65 ± 0.33	+5.72 ± 0.48	+5.53 ± 0.56
Bt00006972	**CD53**			-1.45		-0.56 ± 0.24	-0.47 ± 0.18
Bt00007822	**EEF1B2**			-1.08	-0.60 ± 0.13		-0.97 ± 0.21
BLO_ext_00170	**H3F3A**			-1.2	-0.83 ± 0.07	-1.12 ± 0.13	-1.44 ± 0.11
Bt00006655	**CSF3**			+1.1	+5.26 ± 0.66	+2.80 ± 0.61	+8.85 ± 1.07

Table 3. 4 Genes selected for qPCR analysis, nucleic acid sequence and concentration (nM) of PCR primers [forward primer (F) and reverse primer (R)] for those genes, PCR efficiency (E), and amplicon sizes (bp).

	gene symbol	gene name	primer (5' - 3')	Primer conc (nM)	PCR efficiency (E)	Amplicon size
#	ATR	ataxia telangiectasia and Rad3 related protein	F: GACATCGAGCAGCGACTAC R: GCACGTGTCCTTCGATAGA	300	1.925	82
#	BOLA-DRA	MHC class II DR alpha	F: GCTCTGGTGGGCATCATTG R: CCTCGGCGTTCAACGGTG	300	1.910	77
#	BOLA-DRB4	MHC class II DR beta4	F: AGGGCTCCTGAGCTGAAGTG R: GTGAAGAAGCTGGGACAGAAG	300	1.942	66
#	CD53	CD 53 antigen	F: GGTGTTGGTCAGGCACTTCC R: AGGCTACCGCCATGAATGAG	300	1.816	66
#	CD79A	CD 79A antigen	F: CCTGGGAGTGTCCTGACTCAA R: GATGAGGACGAGGATGACCTAC	300	1.924	75
#	CXCL2	C-X-C ligand 2 (GRO-alpha)	F: AACAAGGCTAGTGCCAACTG R: CCACTGAGGCTGCTGGAG	300	1.912	68
#	GCP2	granulocyte chemotactic protein 2 (CXCL6)	F: CATTGGAATGCTGTATATGGAGAT R: TCTTCCAAAGGTCAAGAGTAAGA	300	1.874	122
#	IFITM	interferon-induced membrane protein Leu-13/9-27	F: CTACCGCCAAGTGCCTGAAC R: TCGTAGGCTGCCATGTAGAC	300	1.931	100
#	IL1R2	interleukin-1 receptor 2	F: ATACCTGTGCCATGACGTATGC R: CGGAGTTTGATATTCCTGGTGAT	300	1.923	67
#	LGMN	legumain	F: TGCCTCCTTGAAGCTTTACC R: GCAGCTCTCCAGTCTCTGA	300	1.944	73

Table 3.4 (cont'd)

# MSR1	macrophage scavenger receptor 1	F: ATCTCCAACCCATACACATTTCT R: CATGGCCCAAACATATCATGAAC	300	1.900	102
# SPP1	secreted phosphoprotein 1 (Osteopontin)	F: AACCAACAGTAGCGAGCTTT R: AGGCTATGGAATTCTTGGCTGAG	300	1.908	117
# TGFB-R2	transforming growth factor beta receptor 2	F: TTGGAGAGGGACTGGCAATG R: CGTGTCTGCTAAAACCCCAATG	300	1.946	80
# TLR4	toll-like receptor 4	F: GAGGAGAATCCCCTGATGTG R: TGGTACATGGCGGCATTTA	300	1.980	80
* ATPIF1	ATPase inhibitory factor 1	F: CCGCCTTGAAGAAACACCAT R: TTTCTGCAGGCGCTCAATC	300	1.950	68
* B2M	beta-2-microglobulin	F: AGTAAGCCGCAGTGGAGGTG R: GCGCAAAACACCCTGAAGAC	100	1.878	110
* BLO_79	unknown	F: TGCTCATTCACCCTCTACAAA R: CACCAACTGGAGACCAGAT	300	1.936	80
* BOTL 09_D04	unknown	F: GTGGTGGACCCAGGAATC R: CAGACGGCAACAGAACAAC	300	1.882	81
* CCNG1	cyclin G1	F: TGTGTAGCCTGAATCCATCC R: ACTGTGGGTCTTTGGTTCAT	300	1.935	94
* CPA3	carboxypeptidase A3	F: TTGCCCTCTGTTTGGAATAAGC R: AGGATCTGTTCAGGTGGTATGG	450	1.912	107
* CSF3	colony stimulating factor 3 (granulocyte)	F: CTGGGTGAGACTGGGAAATG R: TCTCTCACACCCCGTCACA	300	1.959	62

Table 3.4 (cont'd)

* CTSS	cathepsin S	F: CAGCTGAGACCATCTGTCAT R: CCATCAAGTTTCAGCAGCATA	300	1.901	79
* DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	F: AGAGATCTGGTGGGTAGCTTTA R: ACCCTATCCTCTCCTTGCAAAC	300	1.914	79
* EEF1B2	eukaryotic translation elongation factor 1 beta 2	F: CTTGCCCAGTATGAGTCAAAG R: CTCATCATCCCAAGGTTTCAC	300	1.925	90
* H3F3A	H3 histone, family 3A	F: ATGGCTCGTACAAAGCAGAC R: ACCAGGCCTGTAACGATGAG	100	1.895	135
* IGBP1	immunoglobulin (CD79A) binding protein 1	F: CTACGGCAACCGGCAGAA R: AAGCCCCTGTTGTCCTGTC	300	1.905	61
* PAIP2	poly(A)-binding protein-interacting protein 2	F: CTACAATAGTCTGCAGCACAAC R: GGCAGCTTAACCTACCCAAA	300	1.933	89
* RPL13	ribosomal protein large 13	F: GAAGCCGCAGAACAGGATG R: GCCCTGGCTCCTTACACAAC	300	1.894	104
* RPL4	ribosomal protein large 4	F: AGACCATGCGCAGGAACAC R: GCTGCTGCTGCCTTATCCA	100	1.887	73
* RPL7A	ribosomal protein large 7A	F: CCTGTGCCGCAAGATGGG R: GTCCTCCGAGTTGACTTGTG	100	1.835	114
* RPS5	ribosomal protein S5	F: GGCCATCTGGCTGCTGTG R: GGAGCCCTTAGCTGCGTTG	100	1.869	106
* S100A4	S100 calcium-binding protein A4	F: ATCGCCATGATGTGCAATGAG R: CCCACACCTGAGGAGCCTTC	100	1.907	84

Table 3.4 (cont'd)

* TARS	threonyl-tRNA synthetase	F: CACGGAGAGCGCACCATC R: GCCAAGCTGGGCAAAGTTC	300	1.912	110
§ IL-10	interleukin-10	F: CTTGTCGGAAATGATCCAGTTTT R: TCAGGC CCGTGG TTCTCA	300	1.948	66
§ IL12p40	interleukin-12, p40 subunit	F: CAAACCAGACCCACCCAAGA R: GACCTCCACCTGCCGAGAA	300	1.896	64
§ IL-15	interleukin-15	F: GGCTGGCATTCATGTCTTCA R: CATACT GCCAGT TTGCTTCTGTTT	300	1.850	74
§ IL-18	interleukin-18	F: GAAAATGATGAAGACCTGGAATCA R: ACTTGGTCATTCAAATTTCGTATGA	300	1.896	84
§ IL1b	interleukin-1 beta	F: AAGCAGGCGCATCTGTGAA R: ATGGCACTCTAACCCGGAAA	450	1.915	70
§ IL2	interleukin-2	F: TGATGCAACAGTAAACGCTGTAG R: GAGAGGCACTTAGTGATCAAGTC	450	1.928	95
§ IL-4	interleukin-4	F: GCCACACGTGCTTGAACAAA R: TGCTTGCCAAGCTGTTGAGA	450	1.910	63
§ IL-6	interleukin-6	F: GGCTCCCATGATTGTGGTAGTT R: GCCCAGTGGACAGGTTTCTG	300	1.873	64
§ IL-8	interleukin-8	F: GGAAAAGTGGGTGCAGAAGGT R: GGTGGTTTTTTCTTTTTCATGGA	100	1.888	80
§ INF-γ	interferon, gamma	F: TGGCATGTCAGACAGCACTTG R: CCTGAAGCGCCAGGTATAAGG	450	1.932	96

Table 3.4 (cont'd)

§ TGFβ	transforming growth factor, beta	F: CTGAGCCAGAGGCGGACTAC R: TGCCGTATTCCACCATTAGCA	300	1.897	63
§ TNFα	tumor necrosis factor, alpha	F: TCTACCAGGGAGGAGTCTTCCA R: GTCCGGCAGGTTGATCTCA	300	1.871	68
§ INFGR1	interferon gamma receptor 1	F: ACATGTGAACCTTTGCATCTAC R: GTTAGAAAGGACCCTGGAGTTA	300	1.925	82
§ IL-10RA	interleukin-10 receptor A	F: GTCACCCTGCCACTGATCAC R: GGCAGCGTGCAGCTGAAATC	300	1.828	84
§ PTGS2	prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2)	F: CGACACCAAGAACGTATTCCTA R: GAGATGTGGAAAAGAAGCATTG	300	1.930	105
SDHA	succinate dehydrogenase complex subunit A	F: CCACGCCAGGGAGGACTTC R: CGTAGGAGAGCGTGTGCTTC	300	1.879	116

 # genes that showed substantial altered expression within a group of cattle

*** genes that showed differential power between groups of cattle when analyzed using MANOVA**

§ genes that were select from the literature as being relevant to bTB infection

Table 3. 5 The relative expression level of genes that showed differential power between the single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB) groups of cattle.

The relative gene expression levels were derived after whole blood was stimulated over night with tuberculin. The calibrator was derived from non-stimulated whole blood from healthy cattle and differences in expression levels were calculated using the PCR efficiency corrected-ΔΔCT algorithm. Differential expression (Δ $\log_2$ fold change) between 2 groups of cattle (i.e. X vs Y) was determined using ANOVA analysis.

	bTB vs DFP		bTB vs SFP		SFP vs DFP	
	Δ $\log_2$ FC	*adj p*	Δ $\log_2$ FC	*adj p*	Δ $\log_2$ FC	*adj p*
CXCL2	**3.66**	**0.0001**	**1.86**	**0.0200**	**1.80**	**0.0173**
CSF3	**6.05**	**0.0002**	**3.60**	**0.0200**	2.46	0.0536
IL-10	**1.83**	**0.0007**	**1.63**	**0.0080**	0.20	0.3527
IL1R2	**3.81**	**0.0012**	**2.92**	**0.0200**	0.89	0.2574
ATR	**-0.63**	**0.0110**	**-0.56**	**0.0498**	-0.07	0.3527
PTGS2	**3.92**	**0.0007**	0.98	0.2264	**2.94**	**0.0142**
IL-8	**2.98**	**0.0015**	0.59	0.3315	**2.39**	**0.0173**
GCP2	**2.71**	**0.0074**	0.75	0.2919	**1.96**	**0.0466**
CTSS	**0.92**	**0.0187**	0.12	0.4444	**0.79**	**0.0466**
IL-6	**2.93**	**0.0012**	1.45	0.1030	1.48	0.0647
CPA3	**-2.60**	**0.0099**	-0.98	0.2207	-1.62	0.0780
S100A4	**1.31**	**0.0110**	0.61	0.1800	0.70	0.1182
EEFIB2	**-0.67**	**0.0187**	-0.37	0.1800	-0.30	0.1821
IL-1b	**2.02**	**0.0309**	1.86	0.0786	0.17	0.3628
RPS5	**-0.59**	**0.0320**	-0.33	0.1800	-0.26	0.2310
IL-4	2.63	0.1703	-4.74	0.0517	**7.37**	**0.0068**
TARS	0.68	0.0691	-0.21	0.3823	**0.90**	**0.0368**
B2M	0.26	0.1980	-0.29	0.1800	**0.55**	**0.0368**
IL-18	0.00	0.5329	-1.13	0.0517	**1.14**	**0.0368**
BOLA-DRA	-0.12	0.4429	0.67	0.1030	**-0.79**	**0.0466**
ATPIF1 *	-0.07	0.4429	**-0.42**	**0.0826**	0.35	0.0911
BOLA-DRB4 *	0.20	0.3919	**0.77**	**0.0786**	-0.57	0.1088
CCNG1 *	**-0.42**	**0.0691**	-0.29	0.1800	-0.14	0.3215
TLR4 *	**1.08**	**0.0560**	0.01	0.5366	**1.07**	**0.0614**

*** statistically significant at $p \leq 0.05$, but not statistically significant after FDR adjustment.**

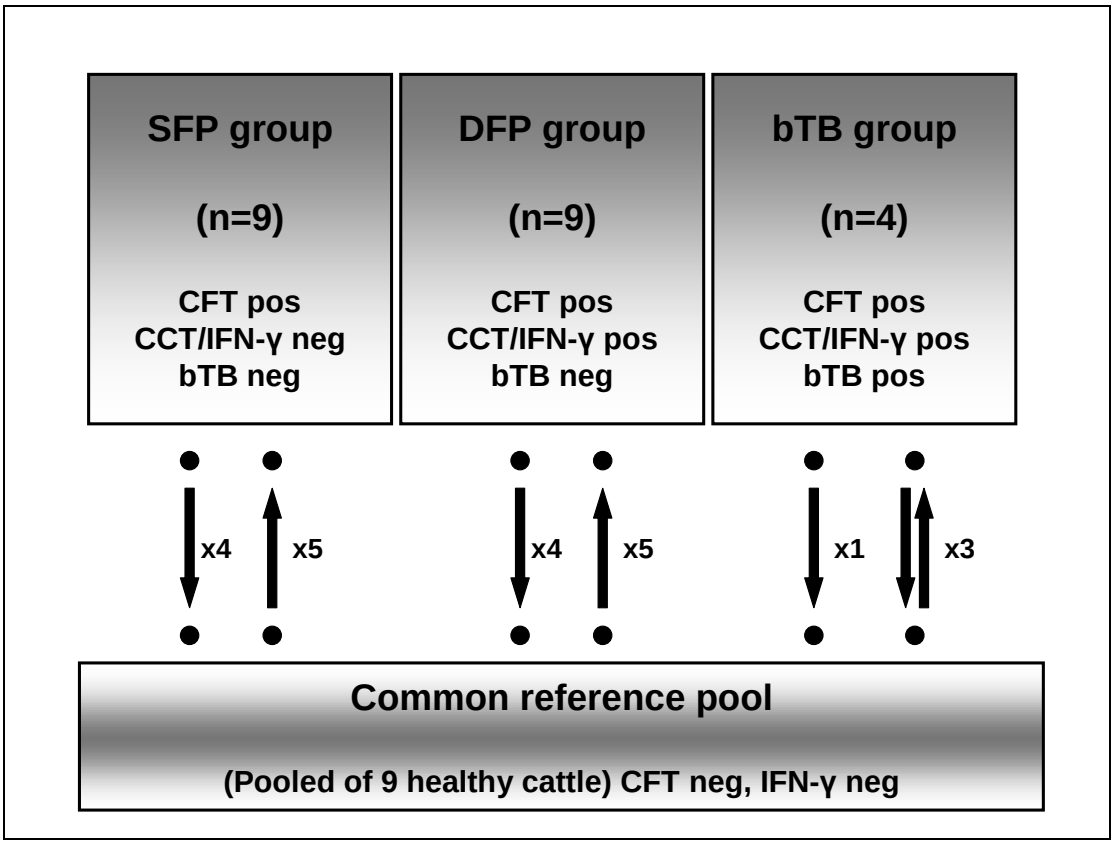


Figure 3. 1 A common reference- balanced dye swap design used for comparison of the single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB) groups of cattle to a common reference pool of RNA from healthy cattle.

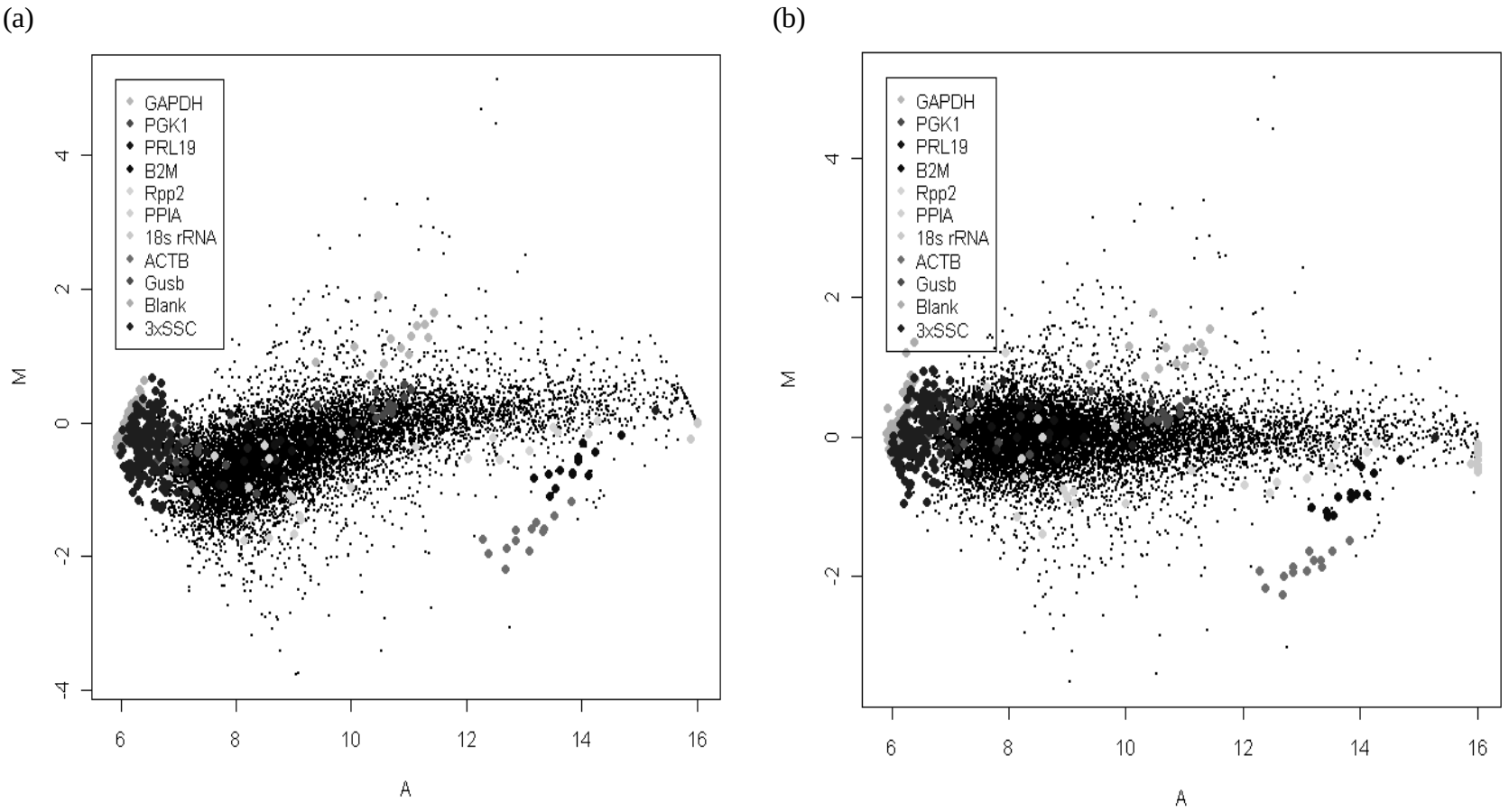


Figure 3. 2 Repreasenatative *M-A* plots of microarray data obtained when RNA obtained from whole blood of bTB infected animal after overnight stimulation with tuberculin was compared with pooled RNA from non-stimulated whole blood from healthy cattle. For each spot on microarray, *M* is calculated as difference in log intensities of the 2 dyes used to label the RNAs, and *A* is calculated as average log intensitiy of the 2 dyes. Skewed dye effect on the microarray is represented by spots that are located away from 0 on the Y-axis of the *M-A* plot. The skewed dye effect for gene features with extremely low or high expression (on both ends of the dot plots) in (a) was evident prior to normalization. This was effectively corrected (b) after norm exp normalization.

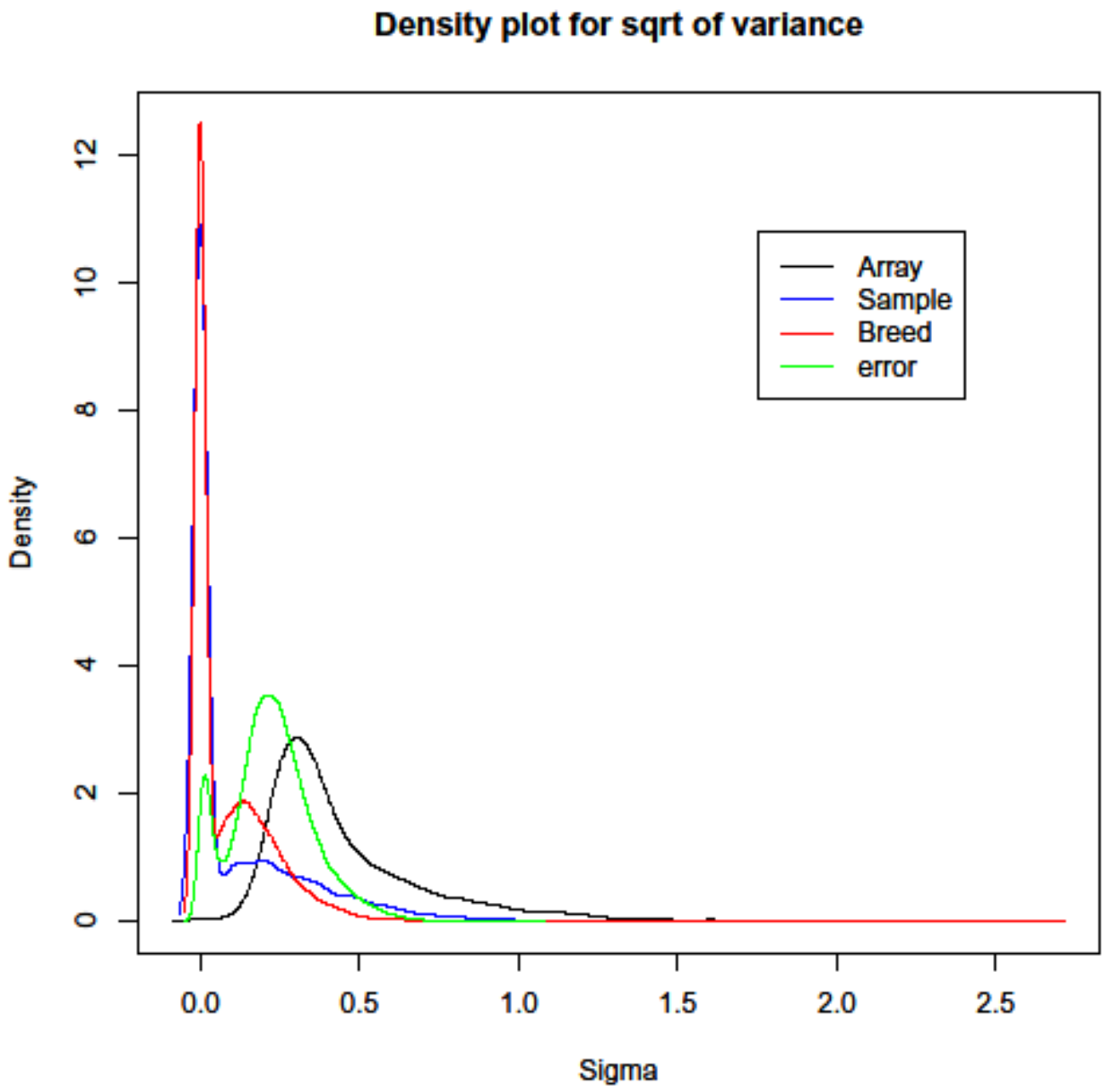


Figure 3. 3 The density plot of the square root of variance for each random effect examined in the mixed model for microarray data analysis. The large variance for array, sample, and breed illustrate the need to account for those factors as random effects in the analysis model.

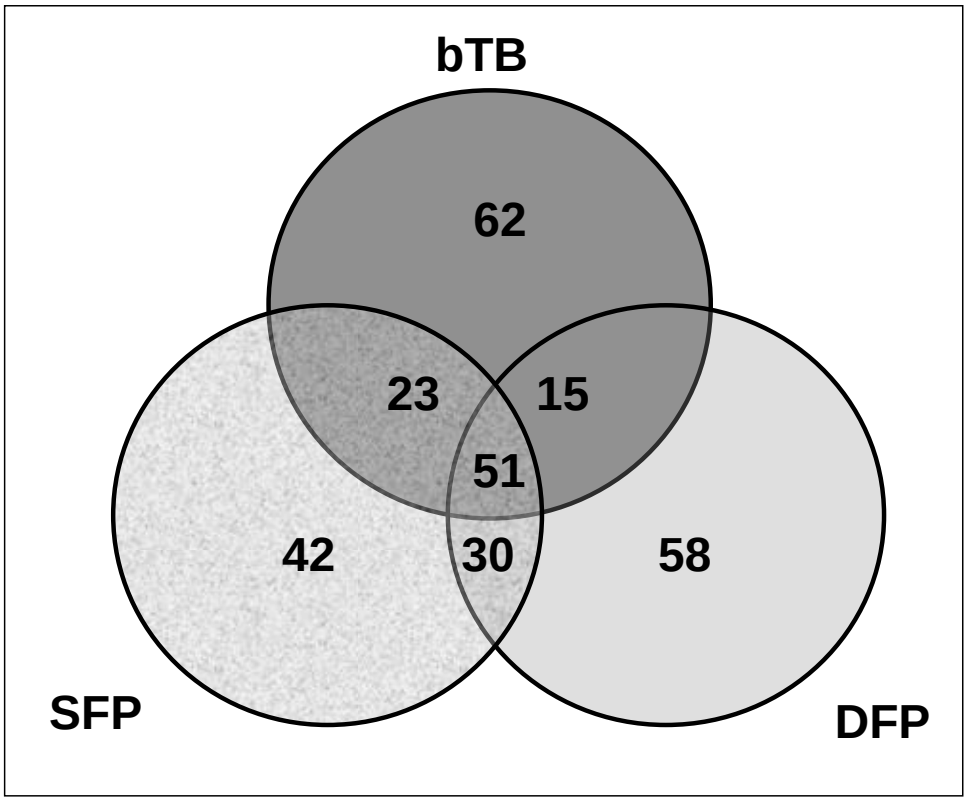


Figure 3. 4 The number of genes from microarray analysis that were differentially expressed (*adj p* $\leq$ 0.01) within single test-false positive (SFP), double test-false positive (DFP), and bTB infected (bTB) groups of cattle after an overnight stimulation of whole blood with tuberculin, as compared with non-stimulated whole blood from the pool of healthy cattle. Overlapping areas indicate genes that showed altered expression in multiple groups of cattle.

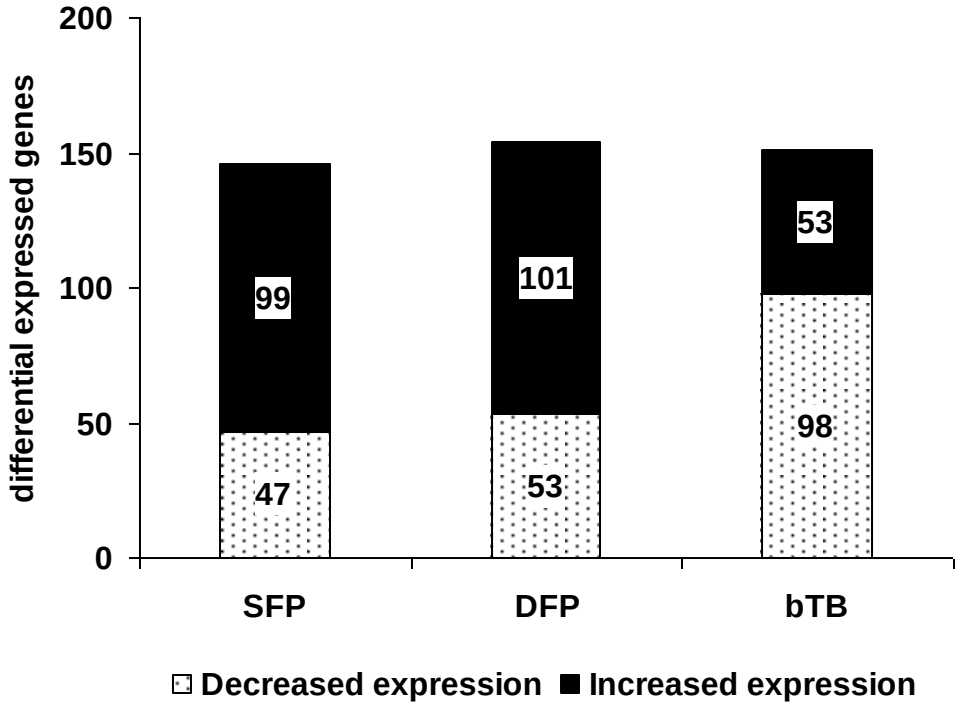


Figure 3. 5 Differentially expressed genes (at *adj p* ≤ 0.01) identified from microarray analysis of single test-false positive (SFP), double test-false positive (DFP), and bTB infected (bTB) groups of cattle after an overnight stimulation of whole blood with tuberculin, as compared with non-stimulated whole blood of the pool of healthy cattle. The number of genes with increased expression (solid box) and decreased expression (shaded box) is indicated with the figure in the boxes.

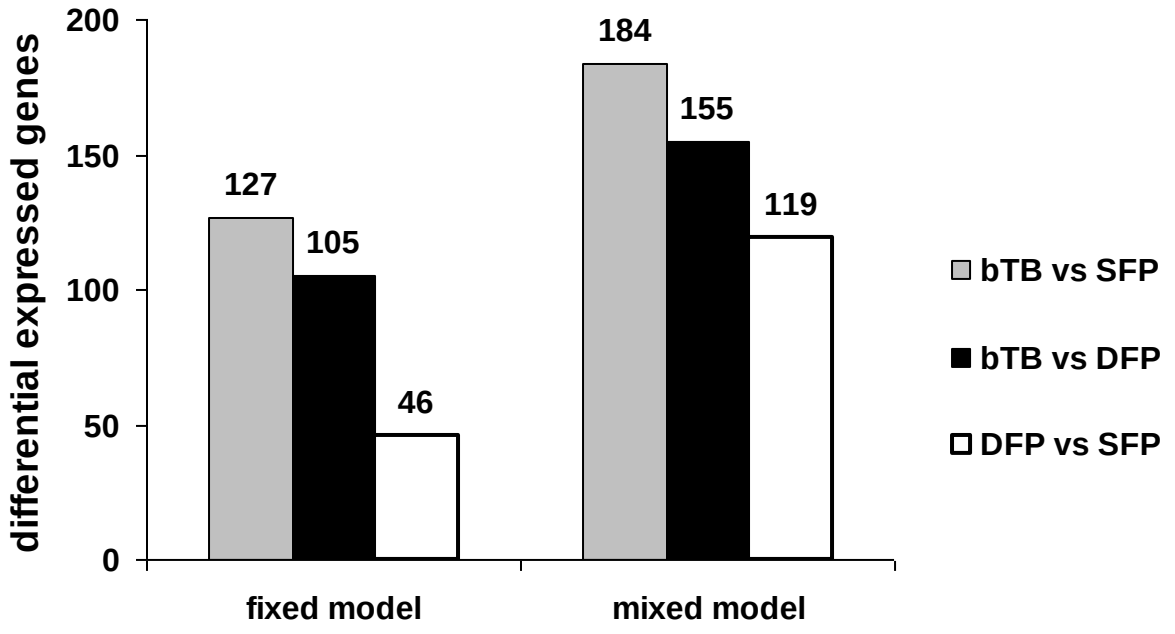


Figure 3. 6 Comparisons of differentially altered gene expression between single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB) groups of cattle in the number of genes identified as differentially expressed ($p \leq 0.01$) after analysis of data from microarray hybridizations, using the fixed effect model (LIMMA) or the mixed effect model (MAANOVA).

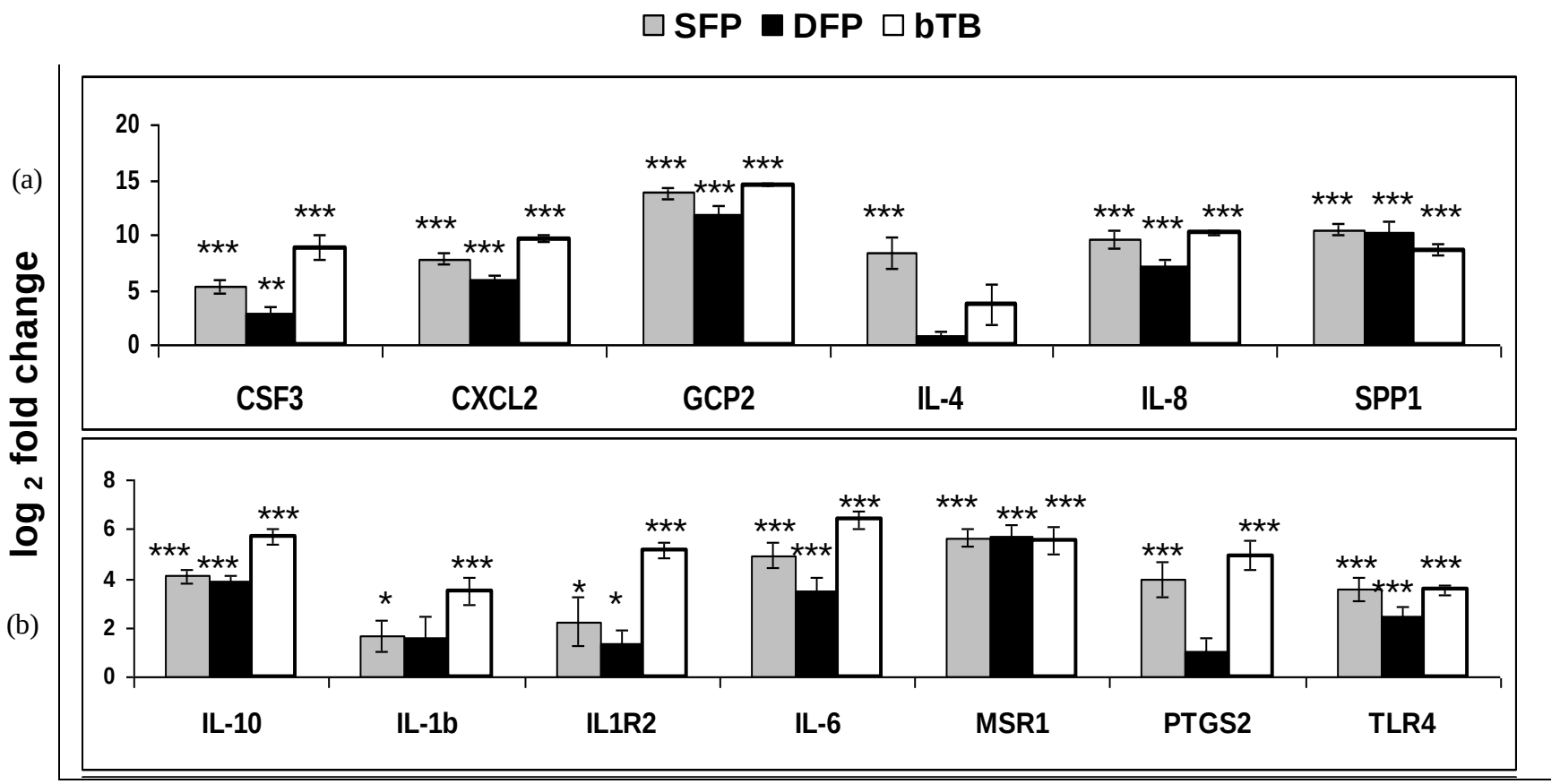


Figure 3. 7 The relative gene expression levels compared with the reference pool of RNA from healthy cattle as determined by qPCR assays for the single test-false positive (SFP)(shaded box), double test-false positive (DFP)(solid box) and bTB infected (bTB)(clear box) groups of cattle. Gene expression levels (in $\log_2$ fold change) were calculated using the PCR efficiency corrected-ΔΔCT algorithm, in which the reference pool of RNA was set as baseline (0 value at Y-axis), and used as the calibrator. Statistically significant differences are shown at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***) and the error bars represent the standard error of the mean expression level for a group of cattle.

Figure 3.7 (cont'd)

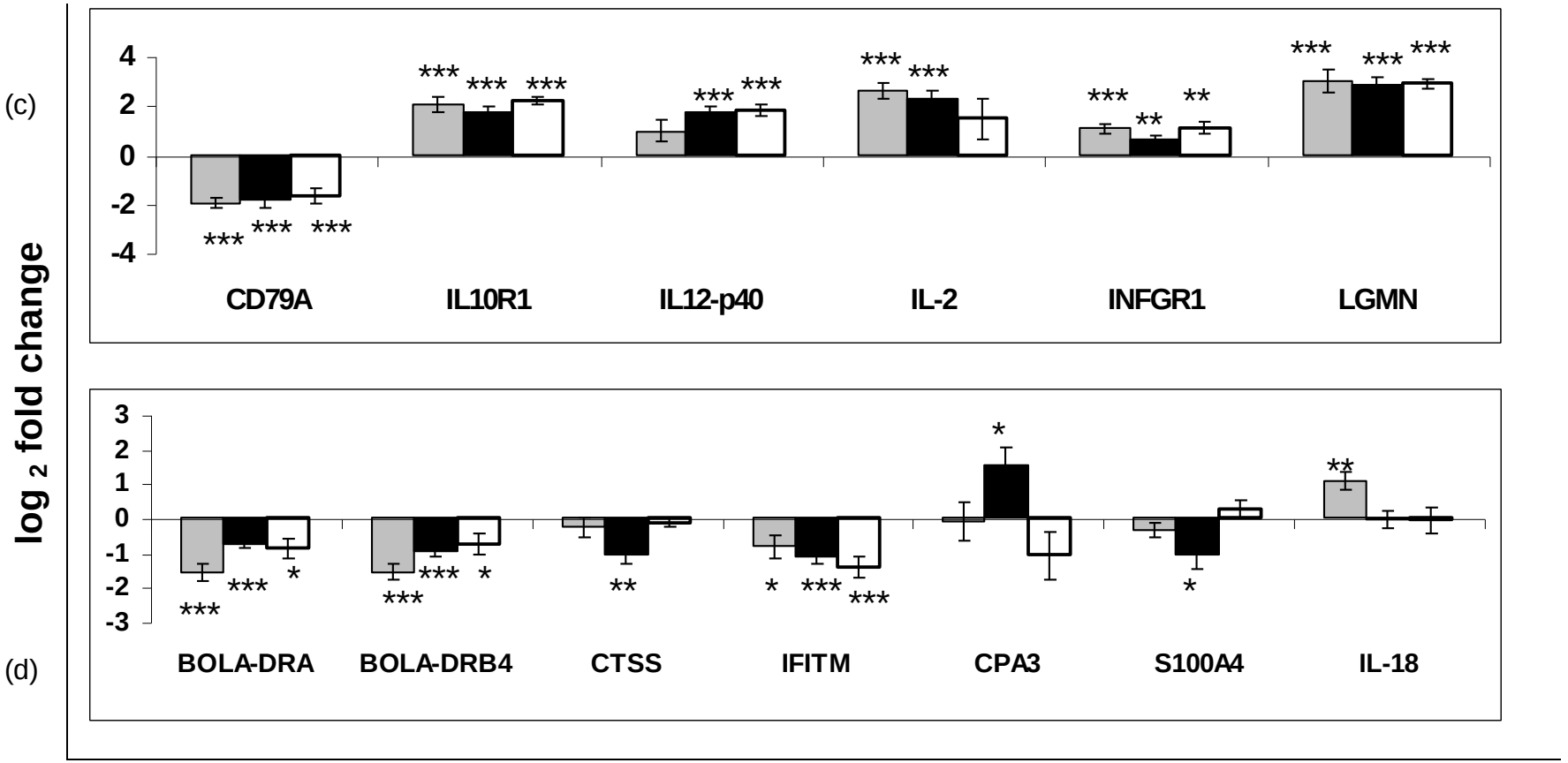
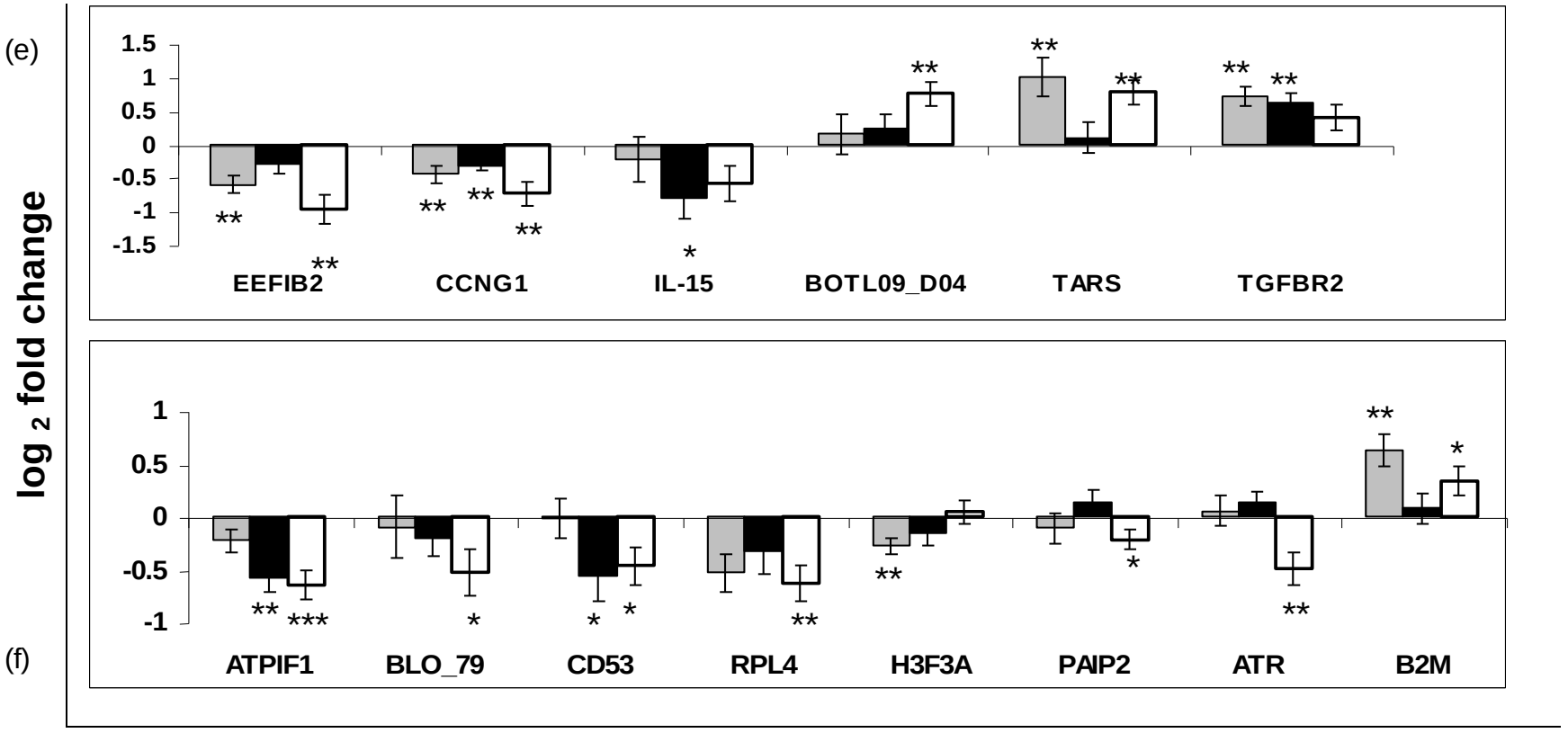


Figure 3.7 (cont'd)



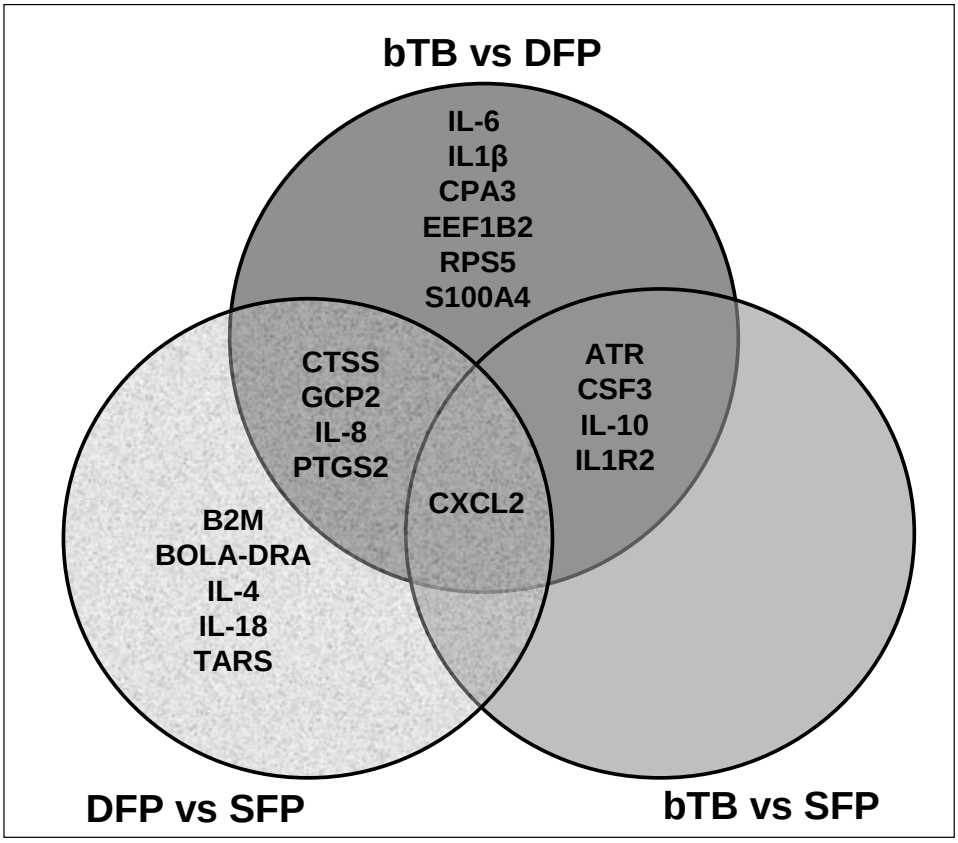


Figure 3. 8 A Venn diagram showing the statistically significant *(adj p* ≤ 0.05) differentially expressed genes unique to or common among the single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB) groups of cattle, as determined by the qPCR assay.

Gene expression level for each animal was **calibrated as relative to** the reference pool of RNA from healthy cattle **using the PCR efficiency corrected-ΔΔCT algorithm; differential expression between 2 groups of cattle (i.e. X vs Y) was determined using ANOVA analysis.**

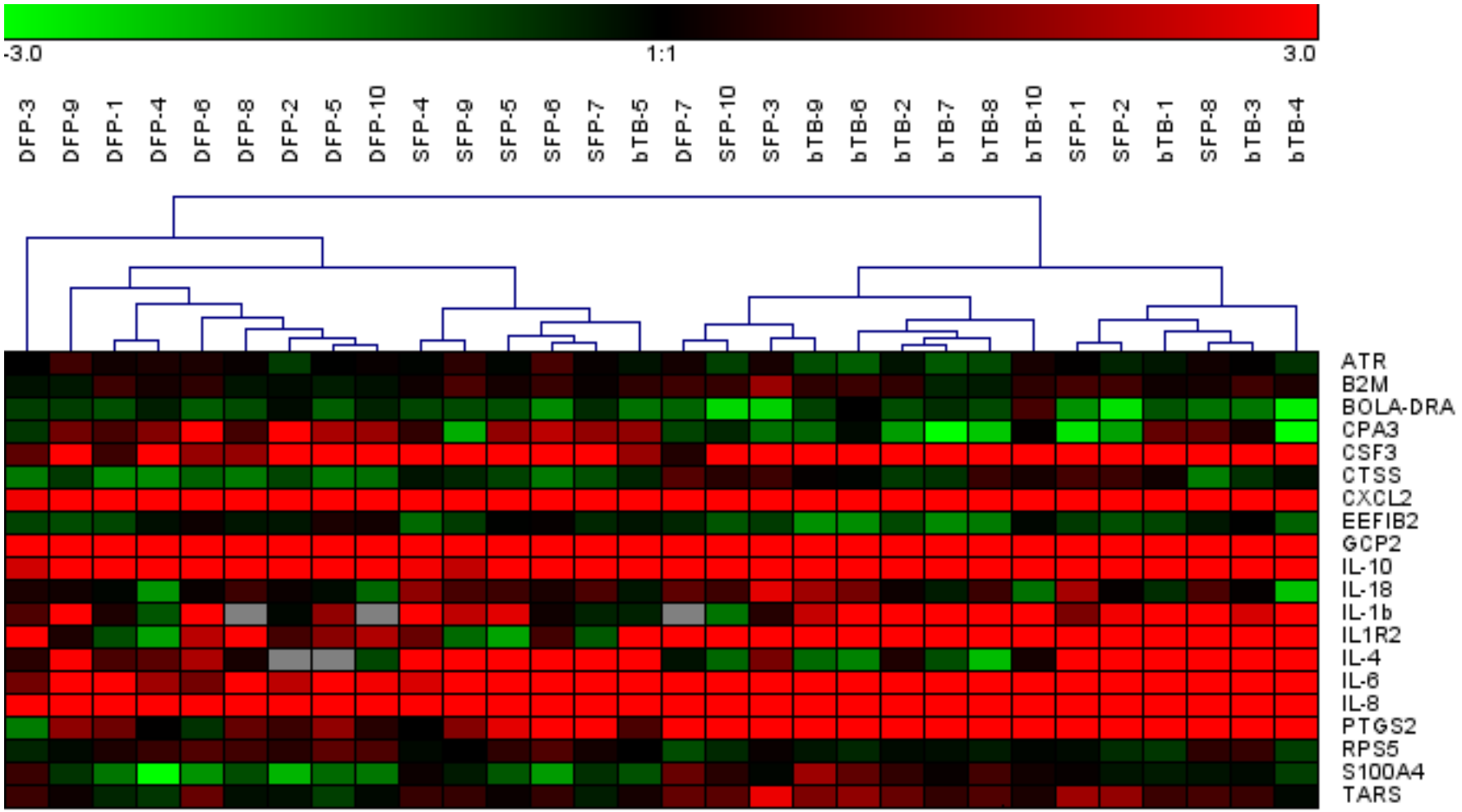


Figure 3. 9 Hierarchical clustering of individual cattle based on the complete linkage algorithm using statistically significant *(adj p* ≤ 0.05) differentially expressed genes (from qPCR data) shows that gene expression profiles segregate individual cattle in the double test-false positive (DFP) and bTB infected (bTB) groups into two main clusters, while cattle from the single test-false positive (SFP) group were split among those clusters.

CHAPTER FOUR

Validation of Molecular Markers for Diagnosis of Bovine Tuberculosis

Introduction

The studies presented in Chapters 2 and 3 were designed initially to include only cattle that underwent postmortem examination for bovine tuberculosis (bTB) and were confirmed as positive or as negative for bTB. The cattle confirmed positive for bTB formed the bTB group. All of the cattle declared negative for bTB had reacted positively on antemortem tests for bTB and were divided into 2 groups. One group of cattle had reacted positively on only the caudal fold skin test (CFT) and were termed single test-false positive (SFP). The other group of cattle, termed double test-false positive (DFP), reacted positively on the CFT and on either the comparative cervical skin test (CCT) or the interferon gamma (IFN-γ) assay. The results from studies presented in Chapters 2 and 3 were highly suggestive that some of the single test-false positive (SFP) cattle may have been infected with bTB and that infection was not identified on postmortem examination. Because all of the cattle in the previously studied SFP group originated from bTB infected farms, an early and undetectable infection with *M. bovis* was possible. Thus, the gene expression profiles created in previous microarray hybridization and qPCR assays may have been distorted by inclusion of bTB infected cattle in what were believed to be groups of non-infected cattle. To create a group of CFT reactors that had very low risk for exposure with *M. bovis*, blood samples were obtained from 6 CFT reactors in a bTB free herd. Cattle in the herd underwent yearly testing for bTB and bTB had never been detected in the herd. Further, the source herd was located in an area of Michigan considered free of bTB and was well

removed from areas in Michigan known to have bTB. Those 6 cattle were designated as single reactors (SR), to differentiate them from the SFP group, and were not examined postmortem for bTB.

The studies reported in Chapters 2 and 3 were class comparison studies, where gene expression profiles of different groups of cattle were defined and compared to identify differentially expressed genes at the group level and at the individual animal level. A gene expression profile that consisted of 26 genes selected from the study presented in Chapter 3 was generated for the SR group. This profile was combined with similar gene expression profiles generated (in Chapter 3) for SFP, DFP and bTB groups of cattle for comparison. The first objective of this study was to evaluate the differential power of the 26 genes by comparing results of gene expression profiles from the SFP, DFP, bTB and SR groups of cattle.

Cluster analysis is based on simple statistical analysis of fold change of gene expression for class comparison and prediction. In this type of analysis, tested samples are segregated into groups based on the similarities and differences (referred to as distances) among their expression profiles. Cluster analysis is considered an unsupervised method of analysis, where no information from the predefined grouping criteria is used in the analysis. Because there are various ways of measuring distances and partitioning, this method is regarded as subjective (Bair and Tibshirani, 2004; Simon et al., 2003). Cluster analysis was used in the first part of the current study for presentation of class comparison results.

The second part of this study is a class prediction study; with emphasis on developing a predictor model that can be used to accurately predict the infection status of cattle on the basis of the expression levels of a few selected genes. Use of a supervised method is more accurate for class prediction studies, where predefined grouping criteria are used in differentiation of samples

for segregation into groups. Construction of a class predictor requires selection of a subset of informative genes, establishing a weight assignment for each gene, and defining the prediction rule. Linear discriminate analysis (LDA) is a powerful supervised method that has been used for class prediction studies. This method of analysis allows identification of a minimum number of informative genes/biomarkers that can be used for group distinction when starting with large gene expression profiles generated from microarray data (Guillot et al., 2007; Simon et al., 2003). In this study, LDA was used for construction of predictor models for differentiation of bTB positive cattle from the test-false positive cattle.

Over-fitting of a predictor model is one of the major limitations of most supervised methods. Over-fitting occurs when the model features are optimized to overcome random variations in the original data. This creates a model that will fit well for the original data set, but the model may not hold for an independent data set (Simon et al., 2003). Ideally, a class prediction study in which a predictor model is constructed using the original data as a training data set, would include validation of the predictor model using an independent data set. To form an independent data set, some samples that could be used to form the training data set may be set aside. When this is done, the number of samples available for the training data set may become too small to permit construction of a prediction model that performs well. This is a problem often encountered when a limited number of samples are available, as was the case for the current study.

The leave one out cross validation (LOOCV) statistical method is a powerful method used to overcome the limitation of LDA. The LOOCV is used to estimate the error rate of a predictor model, and to cross validate the prediction result to avoid the over-fitting problem (Guillot et al., 2007). This method uses data efficiently and allows most, if not all, of the data to

be used for construction of the predictor model. The cross validation is accomplished by excluding each sample, one sample at a time, from the training set. The excluded sample is then classified with the predictor model that was built from all other data points. LOOCV increases the performance of a predictor model without over-fitting, and provides an unbiased true error rate for the predictor model (Guillot et al., 2007; Simon et al., 2003).

The second objective of this study was to evaluate the performance of biomarkers that might be useful for prediction of disease status. The goal was to differentiate test-false positive from truly bTB infected cattle. To accomplish this objective, LDA was used for identification of biomarkers that might differentiate test-false positive cattle from the truly bTB infected cattle. The LOOCV method was then used to evaluate a predictor model that was based on select biomarkers. The performance of the predictor model was then validated using a set of 48 samples of known bTB status. Finally, the predictor model was used to reclassify the bTB exposed cattle in the SFP group.

Materials and Methods

Gene target selection

Based on analyses of differential gene expression done in Chapter 3, a subset of 26 genes was selected to create gene expression profiles for the groups of animals used in the current study. Of those 26 genes, 20 genes showed significant ($adj\ p \leq 0.05$) differential expression among the SFP, DFP, and bTB groups of cattle (Figure 3.8). Four genes were included that showed significant differential expression at $p \leq 0.05$, but lost statistical significance after the false discovery rate (FDR) adjustment (listed in Table 3.5). Also included were the genes for 2 pro-inflammatory cytokines, TNF-α and IFN-γ. Those genes did not show significant differential expression for the SFP, DFP, and bTB groups used in Chapter 3, but were

significantly up-regulated in all 3 groups of cattle. The complete list of genes used in the current study is shown in Table 4.1.

Groups of cattle

An additional 33 cattle were added to the original study groups used in Chapter 3 so that the SFP group was increased from 10 to 21 cattle, the DFP group was increased from 10 to 22 cattle, and the bTB group was increased from 10 to 20 cattle. Six cattle from the Michigan State University (MSU) dairy farm that tested positive on the caudal fold test (CFT) and were negative on the IFN-γ assay formed a new study group; which was designated as single reactor (SR) to differentiate it from the SFP group. The bTB testing results for all 69 cattle used in the current study is given in Appendix D.

Sample preparation and qPCR-protocol

Stimulation of peripheral blood mononuclear cells, sample preparation, and methods for qPCR were as described in Chapter 3.

qPCR analysis for the SR group

After completion of the qPCR assays, the mean values for altered gene expression for the SR group were computed as described in Chapter 3. Using those values, the statistical significance of altered gene expression was determined using the T test statistic. The data from the qPCR assays for the 26 genes from each of the 30 cattle included in the SFP, DFP and bTB groups from Chapter 3 were imported and used for comparative analysis. Statistical significance of differentially expressed genes between the bTB group and each of the 3 groups of test-false positive cattle, i.e. (bTB vs SR), (bTB vs SFP) and (bTB vs DFP) was computed using multiple ANOVA tests implemented in MAANOVA software as described in Chapter 2.

Visualization of result by Cluster Analysis

Cluster analysis was performed using the Genesis software as described in Chapter 3. The gene expression data determined in qPCR assays on RNA from the SR group (n=6) was combined with data from the 30 cattle (SFP, DFP and bTB groups) in Chapter 3 to generate new heat map that included gene expression data for 36 cattle.

Linear Discriminant Analysis (LDA)

Linear discriminant analysis (Ripley, 1996) was performed using the klaR library implemented in the R language and environment (R Development Core Team, 2011). Log_2 fold change of altered gene expression data for cattle in the SR, DFP and bTB groups (n=26) were analyzed at the animal level to select the minimum number of genes (classifiers) that provided the best separation between the 3 study groups of cattle. Selection of classifier genes was performed using a step-wise selection model where an additional gene was added to the classifier set only when that addition increased the prediction accuracy by 0.1%. Conditional on the selected classifier, LDA was used to compute the posterior probability that cattle in the SFP group belonged in one of the SR, DFP or bTB groups.

Construction and testing of prediction model

Leave one out cross validation (LOOCV) analysis was used for validation of identified classifiers and for construction and testing of predictor models. This analysis was performed with the klaR library implemented in R language and environment (R Development Core Team, 2011). In brief, an animal was left out of the training dataset and the LDA was used to fit the model to the other (n-1) animals. The fitted model was used to predict the disease status of the animal left out of the analysis, conditional on the gene expression information only. The

operation was repeated for all samples, leaving each one out, one at a time. The misclassification error was obtained by comparing predicted versus actual disease status.

The LOOCV analysis used 69 cattle that included the original 36 cattle studied in Chapter 3 and 33 newly added cattle. The cattle were divided into 4 defined study groups of SFP (n=21), DFP (n=22), bTB (n=20) and SR (n=6). Data from the cattle in the SR, DFP, and bTB groups was used as the training data. Three training data sets were built by inclusion of a different number of cattle in each set. The first data set contained the original 26 cattle evaluated in the LDA analysis. The second data set contained the original 26 cattle plus10 extra cattle (5 of DFP and 5 of bTB) for 36 cattle, total. Finally, an addition of 10 more cattle (5 of DFP and 5 of bTB) was done to form the third data set that included 46 cattle. Three predictor models were generated from the LDA analyses of the different training data sets. The predictor models included classifiers for the best separation of study groups. Those predictor models were validated and used in LOOCV for prediction of disease status for 69 cattle. Specificity of each predictor model was calculated based on the predicted disease statuss for cattle in the SR (n=6) and DFP (n=22) groups, and sensitivity was calculated based on the cattle in bTB (n=20) group. Finally, prediction of disease status for cattle in the SFP group (n= 21) was performed, but that data was not included in the calculation of specificity due to the uncertainty of bTB status for this group of cattle.

Results

qPCR analyses

When compared with the qPCR assay data generated with the reference pool of RNA from healthy cattle, the 6 cattle in SR group had altered gene expression levels that were statistically significant at the cattle group level for 16 of the 26 genes tested (Table 4.1). Ten of

those 16 genes showed a significant increase in expression and 6 genes showed a significant decrease in expression. Differential expression of the 26 genes between the bTB and the SR groups of cattle at individual animal level was computed using ANOVA test. Fifteen genes showed statistically significant ($adj\ p \leq 0.05$) differential expression between the bTB and SR groups (Table 4.1).

A comparative analysis was done using data generated in qPCR assays for the cattle in the SR group and data generated in qPCR assays for the SFP, DFP and bTB groups of cattle in Chapter 3. ANOVA analysis was performed to assess the potential for differentiation of the bTB group from each one of the test-false positive groups [(bTB vs SFP), (bTB vs DFP) and (bTB vs SR)]. Twenty four out of the 26 genes analyzed showed potential for differentiation of the bTB group from one or more of the other groups (Figure 4.1). The altered expression levels for 6 genes (CSF3, CXCL2, IL-10, IL1β, IL1R2, and IL-6) differentiated the bTB group from all other groups of cattle.

Cluster analysis

Cluster analysis was performed using the individual gene expression data generated in qPCR assays for the 24 genes that showed potential for differentiation of the bTB group of cattle from one or more of the test-false positive groups. Figure 4.2 shows the results from the cluster analysis for 36 cattle in the SFP, DFP, bTB and SR groups. The clusters formed by the SR and the DFP groups were well defined. Similarly, the bTB group of cattle clustered together with the exception of one animal (bTB-5). The cattle in the SFP group were split into the clusters formed by the bTB group and the SR/DFP groups, similar to what was found in Chapter 3. Although cattle in the SFP and the SR groups were test-false positive on only the CFT, they did not cluster

together. The SR group demonstrated a gene expression profile that was relatively close to that of the DFP group and, together, the SR and DFP groups formed a core bTB negative cluster.

Linear Discriminant Analysis (LDA)

Linear discriminant analysis was performed on the data used for cluster analysis. Altered gene expression levels for the SR, DFP and bTB groups were used for classifier determination. Based on the input data, IL-1R2, BOLA-DRB4, and ATR were selected as classifiers that best separated the SR, DFP, and bTB groups of cattle. Those classifiers were used to calculate the probability that cattle in the SFP group belonged in one of the SR, DFP, or bTB groups of cattle. Table 4.2 shows the posterior probability of the likelihood that an individual animal would segregate into the SR, DFP, or bTB groups based on the expression data of the classifier genes. The results from this analysis were promising for differentiation of the test-false positive cattle in the SR and DFP groups from the bTB positive cattle. However, the results from classification of cattle in the SFP group were variable. It was predicted that 4 of the 10 cattle in the SFP group would best fit with the bTB group (probability > 0.84), while with the other six cattle in the SFP group best fit with the DFP group. Surprisingly none of the SFP cattle best fit with the SR group. Those results raised further question for potential bTB infection in some of the cattle in the SFP group. Considering that the herds of origin for all cattle in the SFP group were infected with bTB, exposure of some cattle in the SFP group with *M. bovis* was likely.

Construction and performance of prediction models

Construction of a prediction model was carried out using expression data of 21 genes, with IL-1β, IL-4 and CPA3 being removed due to missing data points from several cattle. To predict disease status in LOOCV, only differential gene expression data from the SR, DFP and

bTB groups were used to build the training data set. Also, calculations of sensitivity of the predictor model was based on data from bTB groups of cattle (n=20), and calculation of specificity is based on the SR and DFP groups of cattle (n=28). The results of the LOOCV analyses are shown in Table 4.3.

The initial predictor model built based on the training set of 26 cattle identified IL-1R2, BOLA-DRB4 and ATR genes as classifiers. This predictor model was then used for cross validation and for prediction of group identity for 69 cattle. The sensitivity and specificity of the predictor model was calculated as 95% and 82.1% respectively. The second predictor model was built based on data from a training set of 36 cattle which identified the BOLA-DRB4 and IL-10 genes as classifiers; thus, these 2 genes were used to classify disease status. The sensitivity and specificity for this predictor model was 85% and 92.9% respectively. The second predictor model showed an increased specificity over predictor model 1, but sensitivity was decreased. A third predictor model was built based on data from 46 cattle and the classifier genes used in that model were BOLA-DRB4, CCNG1, CXCL2, IL-10 and TARS. The third predictor model had a sensitivity of 90% and a specificity of 92.9%. Finally, a predictor model was built that included all of the classifier genes used in the other predictor models, namely IL-1R2, ATR, BOLA-DRB4, CCNG1, CXCL2, IL-10 and TARS. This fourth predictor model had a sensitivity of 95% and a specificity of 92.9%. The fourth predictor was selected for use in subsequent analyses.

The cross validation and prediction results of all cattle (n=69) based on the fourth predictor model is given in Table 4.4. That predictor model falsely identified one confirmed bTB positive animal (bTB-5) as not infected and two DFP cattle from outside of the bTB endemic area as infected, the calculated sensitivity (se), specificity (sp), positive predictive value

(PPV) and negative predictive value (NPV) for that model was 95%, 92.9%, 90.5 and 96.3 respectively (Table 4.5). Using that model to analyze data from the expanded SFP group (n=21), six cattle were identified as infected. It is worth mentioning that five of those 6 cattle had been predicted as infected by all four predictor models.

Discussion

Comparative analysis of gene expression profiles from the SFP, DFP, bTB and SR groups of cattle clearly showed that the expression profile of the SR group was different from the SFP, DFP, and bTB groups. Most of the selected genes (24 of the 26) remained useful for differentiation of the study groups. Six genes (CSF3, CXCL2, IL-10, IL1β, IL1R2, and IL-6) differentiated the bTB group from the other groups of cattle. Those genes are the highly expressed chemokines and cytokines (as shown in Chapter 3, Figure 3.7). The unique profiles and distinct clustering of the SR, DFP and bTB cattle, as shown in Figure 4.2, affirmed the potential use of biomarkers for differentiation of the test-false positive cattle from bTB positive cattle. The addition of the SR group into the comparative study had little effect on the grouping of the SFP cattle, as shown in cluster analysis. Although all cattle in the SFP and SR groups tested similarly in antemortem diagnostic tests, their gene expression profiles were not similar to each other, and those groups separated from each other in cluster analysis.

LDA can be an effective analytical tool for identification of classifiers that are useful for group differentiation. The advantage of LDA is that it will select a minimum number of classifiers for the best separation of groups (Simon et al., 2003). A common problem associated with use of LDA for small data sets is the classifiers selected can be prone to "noise" when used with new samples, which impairs test performance and reduces the predictive value. Classifiers selected from large data sets are more likely to perform well as predictors for new samples

(Grate, 2005). In this study, the LOOCV method was used to compensate for the small number of samples available and improve the selection of models for prediction of status of new samples.

The various analytical methods used on gene expression profiles in this study proved accurate for identification of bTB cattle, with the exception of one bTB positive cow (bTB-5). As shown in Appendix D, that cow was among the 7 bTB infected cattle that tested positive in the CFT, but tested negative in the confirmatory IFN-γ assay. That cow was culled from a TB positive farm after one herd-mate was identified as bTB infected. The postmortem examination for cow bTB-5 reported that there 2 small caseous abscesses (2-4mm in diameter) in the caudal mediastinal lymph node, and only rare acid-fast bacilli were observed microscopically in the cytoplasm of macrophages and multinucleated giant cells. Significant lesions were not observed in other lymph nodes. Using cluster analysis (Figure 4.2), the gene expression profile for cow bTB-5 appeared closer to that of the DFP group than the bTB group. The other 6 CFT positive, IFN-γ negative and bTB positive cattle all originated from a highly infected farm. The gene expression profiles for those 6 cattle were consistent with other bTB infected cattle using cluster analysis and the predictor model identified all those 6 cattle as infected. The necropsy report indicated advanced systemic bTB infection in those cattle, which might suggest were immunologically anergic and not capable of producing sufficient IFN-γ to allow for a positive test.

The sensitivity and specificity of the predictor model based on expression levels of 7 genes were 95% and 92.9%, respectively. Those values were determined using confirmed bTB positive cattle and antemortem test-false positive cattle that originated outside of the bTB endemic area of Michigan. The predictor model out performed the current antemortem tests for bTB by correctly identifying most of the test-false positive cattle as not infected with bTB.

When the predictor model was applied to all 21 cattle in the SFP group, 6 cattle were predicted as bTB infected. Those 6 cattle originated from four different bTB infected farms. Additional SFP cattle from those farms were included in the current study and were predicted as not infected. It should be mentioned that 5 of those 6 SFP cattle had been predicted as infected by all of the four predictor models tested. Further, while many cattle on a positive farm may remain uninfected, up to 30% of cattle on a positive farm are at risk for being infected (Phillips et al., 2003). Unless prediction models developed in the current study were biased, it is likely those 6 cattle were in an early stage of infection or had cleared a recent infection.

In conclusion, this study showed that altered expression of select genes can be used for discrimination of bTB test-false positive cattle from bTB infected cattle. Further, a predictor model based on altered expression of 7 genes was developed that showed potential as a differential diagnostic test for bTB. A limitation of this study was that the training data set included only 46 cattle, and more importantly, only 6 cattle were in the SR group. Additional cattle should be added to the training data set, especially to the SR group, to improve the training data set and increase the accuracy of the predictor model.

Table 4. 1 The relative gene expression levels (log_2 fold change [FC]) for the single reactor (SR) group of cattle after an overnight stimulation of whole blood with tuberculin, as compared with non-stimulated whole blood from a pool of healthy cattle. The differential gene expression (Δ log_2 FC) between the single reactor and bTB infected groups (bTB vs SR) was determined from qPCR assays using ANOVA analysis.

Gene	SR			bTB vs SR	
	log_2 FC	Std error	*p* value	Δ log_2FC	*adj p* value
GCP2	**11.30**	**0.47**	**0.0000**	**3.25**	**0.0010**
IL-8	**7.80**	**0.37**	**0.0000**	**2.34**	**0.0048**
CXCL2	**7.29**	**0.46**	**0.0000**	**2.36**	**0.0010**
CSF3	**5.48**	**1.14**	**0.0048**	**3.38**	**0.0069**
IL-10	**4.52**	**0.25**	**0.0000**	**1.20**	**0.0063**
PTGS2	**3.37**	**0.40**	**0.0004**	1.55	**0.0435**
IL-6	**3.20**	**0.54**	**0.0019**	**3.18**	**0.0006**
TLR4	**2.51**	**0.34**	**0.0007**	1.03	**0.0315**
BOLA-DRB4	**0.71**	**0.16**	**0.0061**	**-1.50**	**0.0006**
BOLA-DRA	**0.23**	**0.10**	0.0725	**-1.12**	**0.0033**
ATR	**-0.39**	**0.12**	**0.0251**	-0.11	0.1863
CCNG1	**-0.44**	**0.14**	**0.0242**	-0.29	0.0788
TNFα	**-0.83**	**0.23**	**0.0144**	**1.55**	**0.0033**
S100A4	**-1.04**	**0.36**	**0.0334**	**1.28**	**0.0069**
IL-4	**-2.10**	**0.40**	**0.0033**	**5.79**	**0.0047**
INF-γ	**-2.73**	**0.67**	**0.0098**	**3.62**	**0.0006**
B2M	0.26	0.21	0.2629	0.07	0.2196
IL-1β	0.05	0.44	0.9073	**3.44**	**0.0007**
TARS	-0.01	0.21	0.9753	**0.78**	**0.0217**
IL1R2	-0.02	0.74	0.9835	**5.17**	**0.0002**
ATPIF1	-0.11	0.56	0.8487	-0.53	0.0530
RPS5	-0.19	0.37	0.6322	-0.03	0.2505
IL-18	-0.31	0.21	0.1924	0.25	0.1863
CPA3	-0.33	0.68	0.6483	-0.76	0.1447
CTSS	-0.46	0.26	0.1337	0.32	0.1422
EEF1B2	-1.20	0.51	0.0634	0.23	0.1705

Table 4. 2 The posterior probability value for an individual animal to be segregate into the single reactor (SR), double test-false positive (DFP) or bTB infected (bTB) groups of cattle, as computed using the expression data of classifier genes, IL-1R2, BOLA-DRB4 and ATR.

input information		posterior probability			posterior prediction
Animal ID	Study group	as DFP	as SFP	as bTB	as group
0003H	SFP	0.1509	0.0000	0.8491	bTB
0005H	SFP	0.0471	0.0000	0.9529	bTB
0566H	SFP	0.0276	0.0000	0.9724	bTB
2007	SFP	0.9535	0.0216	0.0249	DFP
4685	SFP	0.9073	0.0925	0.0001	DFP
7342	SFP	0.9993	0.0001	0.0006	DFP
7763	SFP	0.7254	0.2742	0.0005	DFP
9634	SFP	0.8013	0.0006	0.1981	DFP
9703	SFP	0.9895	0.0104	0.0001	DFP
9979	SFP	0.1084	0.0000	0.8916	bTB
0420	DFP	0.9703	0.0294	0.0004	DFP
20085	DFP	0.6644	0.2721	0.0635	DFP
30911	DFP	0.6646	0.0040	0.3313	DFP
3232	DFP	0.8597	0.1403	0.0001	DFP
6541	DFP	0.9659	0.0027	0.0314	DFP
7004	DFP	0.9650	0.0015	0.0335	DFP
7030	DFP	0.8707	0.0003	0.1290	DFP
7145	DFP	0.8018	0.0018	0.1964	DFP

Table 4.2 (cont'd)

7622	DFP	0.9938	0.0055	0.0007	DFP
8753	DFP	0.9052	0.0238	0.0710	DFP
0912	bTB	0.0152	0.0000	0.9847	bTB
3835	bTB	0.0066	0.0000	0.9933	bTB
3914	bTB	0.1027	0.0000	0.8973	bTB
4330	bTB	0.0659	0.0000	0.9341	bTB
4690	bTB	0.2040	0.0000	0.7960	bTB
6798	bTB	0.0002	0.0001	0.9998	bTB
6814	bTB	0.0014	0.0007	0.9979	bTB
6817	bTB	0.0062	0.0007	0.9931	bTB
6834	bTB	0.0354	0.0936	0.8710	bTB
8031	bTB	0.1737	0.0681	0.7582	bTB
4300	SR	0.0257	0.9565	0.0178	SR
4312	SR	0.0666	0.9333	0.0000	SR
4371	SR	0.0335	0.9642	0.0023	SR
4387	SR	0.0721	0.8372	0.0907	SR
4404	SR	0.0087	0.9912	0.0000	SR
4453	SR	0.0067	0.9933	0.0000	SR

Table 4. 3 Leave one out cross validation (LOOCV) analysis for four predictor models. Performance of the predictor models were evaluated using gene expression data from 68 cattle; where sensitivity was determined using 20 bTB infected cattle and specificity was determined using 28 non-bTB infected cattle in the single reactor (SR) (n=6) and double test-false positive (DFP) (n=22) groups.

predictor model	Classifiers used in the predictor model	sensitivity	specificity
1	ATR + BOLA-DRB4 + IL1R2	95.0%	82.1%
2	BOLA-DRB4 + IL-10	85.0%	92.9%
3	BOLA-DRB4 + CCNG1 + CXCL2 + IL-10 + TARS	90.0%	92.9%
4	BOLA-DRB4 + CCNG1 + CXCL2 + IL-10 + TARS + ATR + IL1R2	95.0%	92.9%

Table 4. 4 The cross validation and disease status prediction results using leave one out cross validation (LOOCV) analysis. The posterior probability for an individual animal (n=69) to be segregate into the single reactor (SR), double test-false positive (DFP) or bTB infected (bTB) groups of cattle, as determined by the expression levels of defined classifiers in predictor model 4 (Model = BOLA-DRB4 + CCNG1 + CXCL2 + IL-10 + TARS + ATR + IL1R2).

input information		posterior probability			posterior prediction
animal ID	study group	as DFP	as SR	as bTB	as group
0420	DFP	0.9860	0.0137	0.0003	DFP
20085	DFP	0.9181	0.0561	0.0259	DFP
30911	DFP	0.9998	0.0001	0.0000	DFP
3232	DFP	0.9582	0.0418	0.0000	DFP
6541	DFP	0.9927	0.0011	0.0061	DFP
7004	DFP	0.9999	0.0000	0.0001	DFP
7030	DFP	0.9789	0.0000	0.0210	DFP
7145	DFP	0.9777	0.0009	0.0214	DFP
7622	DFP	0.9995	0.0005	0.0000	DFP
8753	DFP	0.8971	0.0126	0.0903	DFP
6185	DFP	0.0165	0.0003	0.9832	bTB
9416	DFP	0.1311	0.0000	0.8689	bTB
4150	DFP	0.9974	0.0024	0.0002	DFP
5558	DFP	0.6687	0.0003	0.3310	DFP
7572	DFP	0.9993	0.0000	0.0007	DFP
7577	DFP	0.8094	0.0000	0.1905	DFP
7641	DFP	0.9905	0.0000	0.0094	DFP
8005	DFP	0.9919	0.0079	0.0001	DFP
OH-1378	DFP	0.9979	0.0004	0.0017	DFP
OH-1437	DFP	0.9998	0.0000	0.0002	DFP

Table 4.4 (cont'd)

OH-1666	DFP	0.9995	0.0000	0.0005	DFP
OH-3503	DFP	0.9988	0.0005	0.0008	DFP
4300	SR	0.0034	0.9807	0.0159	SR
4312	SR	0.0014	0.9984	0.0001	SR
4371	SR	0.0095	0.9691	0.0215	SR
4387	SR	0.0436	0.8764	0.0800	SR
4404	SR	0.0013	0.9986	0.0000	SR
4453	SR	0.0014	0.9974	0.0011	SR
0912	bTB	0.0001	0.0000	0.9999	bTB
3835	bTB	0.0011	0.0000	0.9989	bTB
3914	bTB	0.3081	0.0000	0.6919	bTB
4330	bTB	0.2772	0.0003	0.7225	bTB
4690	bTB	0.9665	0.0000	0.0335	DFP
6798	bTB	0.0000	0.0001	0.9999	bTB
6814	bTB	0.0001	0.0032	0.9967	bTB
6817	bTB	0.0000	0.0003	0.9997	bTB
6834	bTB	0.0142	0.1426	0.8432	bTB
8031	bTB	0.0037	0.0127	0.9836	bTB
1409	bTB	0.0028	0.0011	0.9961	bTB
1413	bTB	0.0000	0.0000	1.0000	bTB
1419	bTB	0.0007	0.0001	0.9992	bTB
2835	bTB	0.0004	0.0000	0.9996	bTB
6845	bTB	0.0000	0.0000	0.9999	bTB
6857	bTB	0.0035	0.0000	0.9965	bTB
6860	bTB	0.0017	0.0011	0.9972	bTB

Table 4.4 (cont'd)

9401	bTB	0.0588	0.0709	0.8703	bTB
1673	bTB	0.0213	0.0093	0.9694	bTB
6861	bTB	0.0008	0.0000	0.9992	bTB
0003H	SFP	0.6882	0.0000	0.3118	DFP
0005H	SFP	0.3290	0.0000	0.6710	bTB
0566H	SFP	0.9787	0.0000	0.0213	DFP
2007	SFP	0.9981	0.0017	0.0002	DFP
4685	SFP	0.9927	0.0070	0.0003	DFP
7342	SFP	1.0000	0.0000	0.0000	DFP
7763	SFP	0.7599	0.2370	0.0032	DFP
9634	SFP	0.9927	0.0000	0.0073	DFP
9703	SFP	0.9997	0.0003	0.0000	DFP
9979	SFP	0.9899	0.0000	0.0101	DFP
2140	SFP	0.0000	0.0000	1.0000	bTB
4490	SFP	0.0000	0.0000	1.0000	bTB
7709	SFP	0.6168	0.0000	0.3832	DFP
7792	SFP	0.7420	0.0000	0.2580	DFP
0012H	SFP	0.0005	0.0000	0.9995	bTB
2361	SFP	0.5740	0.0131	0.4129	DFP
2509	SFP	0.0163	0.0013	0.9824	bTB
2642	SFP	0.9787	0.0000	0.0213	DFP
3305	SFP	0.0001	0.0000	0.9999	bTB
3306	SFP	0.9533	0.0001	0.0466	DFP
6178	SFP	0.9632	0.0366	0.0002	DFP

Table 4. 5 Performance of Predictor Model 4 for specificity, sensitivity, positive predicted value and negative predicted value when tested using 48 cattle in the single reactor group (n=6), double test-false positive group (n=28) and bTB infected group (n=20). Antemortem test results for SR group (no postmortem performed) and postmortem tests results for DFP and bTB groups were used to determine bTB infection status.

		bTB status (as confirmed with postmortem test results)		
		POSITIVE	**NEGATIVE**	
Predictor Model 4 outcome	**POSITIVE**	19	2	Positive predictive value = 19 / 21 = 90.5 %
	NEGATIVE	1	26	Negative predictive value = 26 / 27 = 96.3 %
		Sensitivity = 19 / 20 = 95.0 %	Specificity = 26 / 28 = 92.9%	

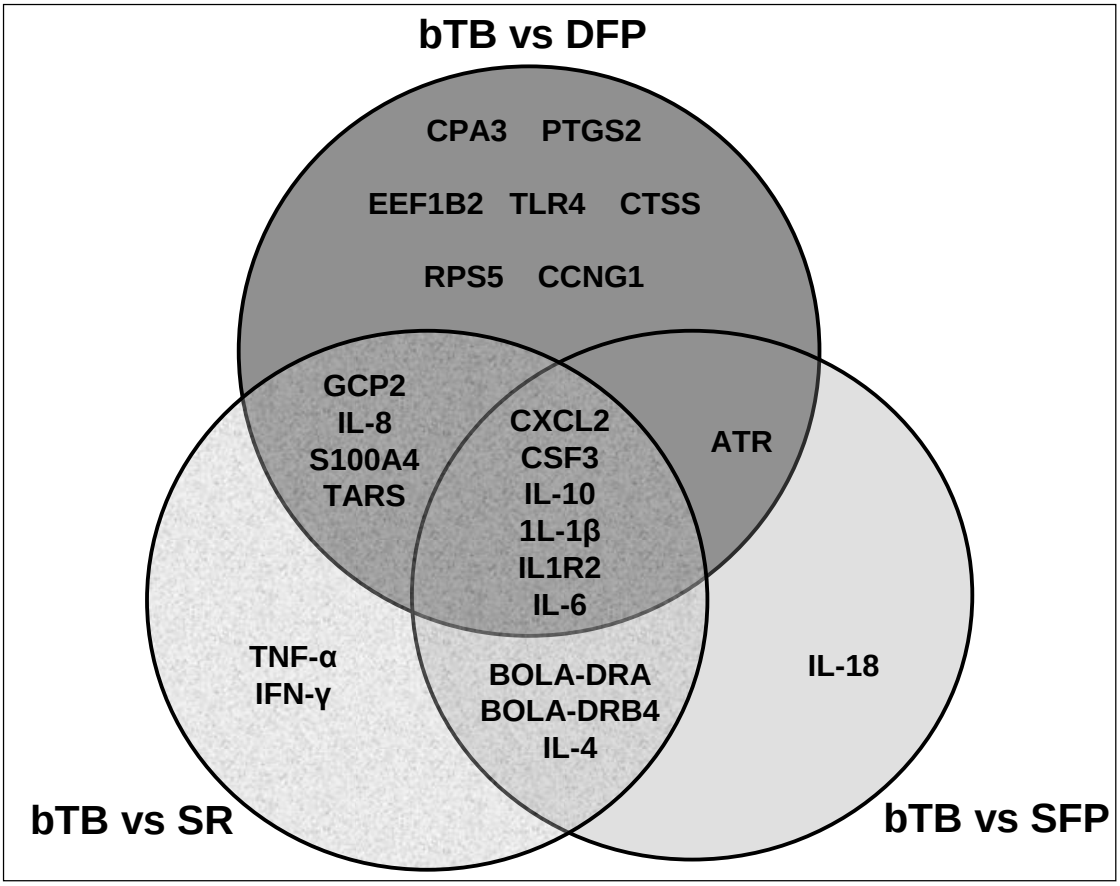


Figure 4. 1 Genes with that showed significant power for differentiation ($p \leq 0.01$) of the bTB infected (bTB) group of cattle from one or more of the test-false positive groups of cattle. The test-false positive groups of cattle were single reactor (SR), single test-false positive (SFP) and double test-false positive (DFP).

Gene expression level for each animal was **calibrated relative to** the reference pool of RNA from healthy cattle **using the PCR efficiency corrected-ΔΔCT algorithm; differential expression between 2 groups of cattle (i.e. X vs Y) was determined using ANOVA.**

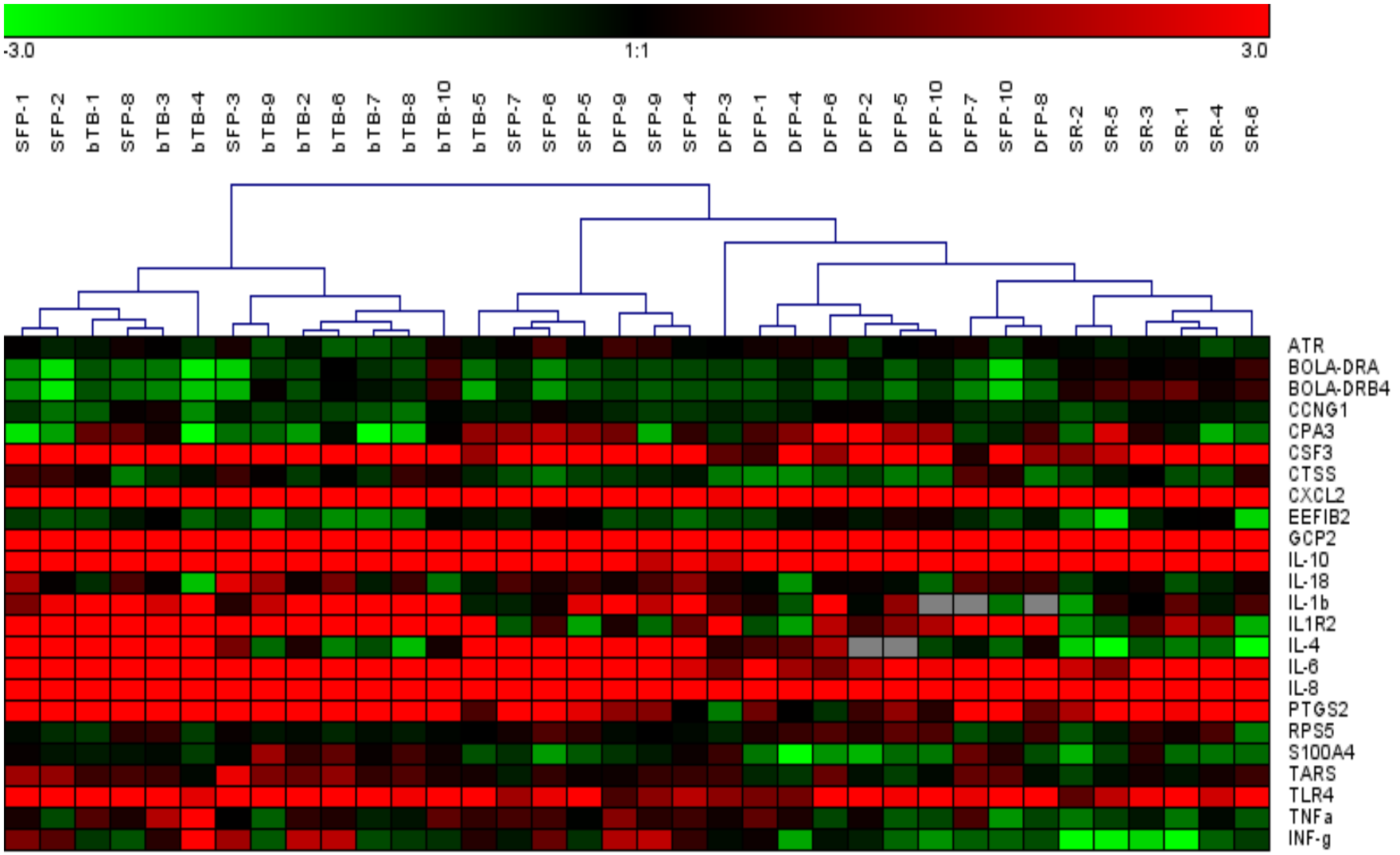


Figure 4. 2 Hierarchical clustering of individual cattle based on the complete linkage algorithm using statistically significant *(adj p* ≤ 0.05) differentially expressed genes (from qPCR data) for the single test-false positive (SFP), double test-false positive (DFP), single reactor (SR), and bTB infected (bTB) groups of cattle.

CHAPTER FIVE

Conclusions & Future Studies

General Conclusions

Transcriptional profiling using the microarray platform has been a valuable tool for studying bTB in various species, including cattle (Meade et al., 2007; Meade et al., 2008; Meade et al., 2006), European wild boar (Galindo et al., 2009; Naranjo et al., 2006b) and Iberian red deer (de Mera et al., 2008). Similarly, qPCR assays for detection of altered gene expression have provided significant information in studies of cattle infected with bTB (Blanco et al., 2009b; Thacker et al., 2007), European wild boar (de la Lastra et al., 2009) and cervids (Harrington et al., 2006; Thacker et al., 2006, 2009). In all published reports, diseased animals were compared with healthy counterparts. The bTB status of animals was confirmed by pathologic finding or laboratory methods in some studies. Other studies used results of antemortem tests to define bTB infection status and did not include postmortem confirmation of disease status. As reviewed in Chapter 1, the limitations of current bTB tests are well documented, with test-false positive results being an important issue in the bTB control programs when disease prevalence is low. In Michigan, the current rate of bTB infection is extremely low. Cumulative data from antemortem bTB testing and postmortem examinations over the past 12 years indicate that > 98% of antemortem test positive cattle did not have bTB.

The series of studies in this dessertation is the first attempt to use gene expression profiling of bTB test-false positive cattle in comparison to bTB infected and healthy cattle. The working hypothesis was that gene expression profiles of the test-false positive cattle would differ from the bTB infected cattle despite similarities in response to current bTB testing methods. It

also was hypothesized that altered transcription levels of select genes could discriminate between cattle infected with bTB and cattle that test-false positive by current antemortem bTB tests.

To test these hypotheses, microarray technologies and qPCR assays were used to examine gene expression profiles of PBMC from 5 groups of cattle. One group of cattle had confirmed infection with bTB (bTB group). Four groups of cattle were considered not infected with bTB; one of those groups consisted of healthy cattle used to provide a reference pool of RNA. There was a group of double antemortem test-false positive cattle (DFP group). Finally, there was a group of single antemortem test-false positive cattle (SFP) that had been exposed to cattle that had bTB, and a group of single antemortem test-false positive cattle from a bTB free herd (SR group). Each group of cattle showed distinct gene expression profiles. Further, the gene expression profiles readily differentiated true bTB infected cattle from DFP and SR groups of cattle.

An unanticipated finding made during these studies was that gene expression profiles failed to clearly distinguish the bTB-exposed SFP group of cattle from the bTB infected cattle. Instead, the gene expression profiles of some bTB-exposed SFP cattle closely resembled those of bTB infected cattle, which could be explained if those SFP cattle were infected with bTB and the infection was not detected during postmortem examination or in subsequent laboratory testing.

The goal of these studies was to identify molecular markers that could be used to detect bTB infected cattle, and to differentiate infected cattle from non-infected cattle. The gene expression profiles generated after overnight antigen stimulation of whole blood with bPPD were mined to identify 24 gene candidates that might accomplish that goal. Using a small data set (n=69 cattle), a predictor model was built that used seven genes (IL-1R2, ATR, BOLA-DRB4, CCNG1, CXCL2, IL-10 and TARS) as a biosignature for prediction of bTB status. That

predictor model was shown to have high sensitivity (95%), specificity (92.9%), positive predictive value (90.5%) and negative predictive value (96.3%) for prediction of bTB status in cattle that were positive on antemortem diagnostic tests. The overall results supported the main hypothesis of this study, which was gene expression levels can be used to differentiate antemortem test-false positive cattle from true bTB positive cattle. At this point, the predictor model is not fully validated and will require refinement before it is used for diagnostic purposes. However, it can be concluded from these studies that there is great potential for using altered gene expression profiling to develop an ancillary test that can reduce the unnecessary culling of test-false positive cattle.

Future Studies

This research has demonstrated the effectiveness of using altered expression of select genes for prediction of bTB status. The studies presented focused on a very small subset of genes in the bovine genome which were included in the microarrays used and then on only a small number of genes selected from the microarray data. Additional candidate genes with greater differential power likely exist which would be superior to those genes used in the current studies. The data from the microarray studies should be evaluated again to determine if there are additional genes that should be tested.

Microarray based studies generate massive pools of data. In this study, the microarray data was only used for examining differential expression profiles and finding biomarkers for group differentiation. There is a wealth of information in the microarray data generated here that has yet to be explored. The data generated in the studies presented here may prove valuable for elucidating functional and regulatory pathways activated in bTB infected cattle. Pathway related

studies will provide a better understanding of natural bTB infection in cattle, and should result in discovery of new directions for improvement of bTB tests.

Gene enrichment analyses of the altered gene expression using DAVID software (in Chapters 2 and 3) demonstrated that different functional and biological processes were enriched in each of the study groups. The multi-GOEAST tool in the GOEAST (Gene Ontology Enrichment Analysis Software Toolkit) software provides a means to compare the results of multiple enrichment analyses from different experiments (Zheng and Wang, 2008). Differential expression of genes after overnight stimulation of peripheral blood mononuclear cells with bPPD (Chapter 3) was analyzed using the Multi-GOEAST program to map the shared and unique altered biological or functional pathways for each group of cattle. The results showed that translation regulator, ATPase regulator, and transmembrane transporter activity were uniquely regulated by the bTB group of cattle. Altered expression of several genes associated with metallopeptidase activity was unique for the DFP group of cattle (data not shown). Future study of the genes involved in identified pathways may be very important for understanding the disease associated gene regulation in the test-false positive or bTB infected cattle.

In order to bridge identification of biomarkers with potential differential power for prediction of disease status to development of a diagnostic assay, more work in several areas must be done. First and foremost, more data from cattle that are classified as (non-exposed) single test-false positive reactors (SR) should be added to the training data set. Similar with previous studies of animals with natural infection with bTB (Naranjo et al., 2006a), this study was disadvantaged by being unable to control animal selection and infection conditions. As a result, high animal to animal variation within the defined study groups occurred, and this variation directly introduced noise to the data set. Addition of more cattle for all study groups

into the training data set would improve the validity of the data and performance of the predictor model. The continual testing for bTB in Michigan should allow the addition of antemortem test-false positive cattle, but the low prevalence of bTB in Michigan will limit the addition of more bTB infected cattle.

Comparative studies have shown that the immune response in the peripheral blood (PBMC) highly reflects the immune response at sites of active disease, i.e. lymph nodes (Rhodes et al., 2000). Several studies using various tissue types, including the formalin fixed paraffin embedded samples, have demonstrated altered gene expression in bTB infected animals (Naranjo et al., 2007a; Naranjo et al., 2006b; Witchell et al., 2010). The availability of archived lymph node specimens from all bTB postmortem cattle in Michigan (from 1998 to present) would be a valuable asset for a retrospective study. Those archived specimens might be used to greatly expand the number of bTB infected samples used in the current studies and to provide material for development of an ancillary postmortem test that might detect additional bTB positive cattle.

A statistically-based predictor model, as developed and used in the current studies, is a research tool for determination of the performance of selected genes (classifiers). Figure 5.1 shows the expression data of study samples for the seven classifiers in the predictor model selected for use in Chapter 4. The difference in individual gene expression levels of 3 genes (IL-10, CXCL2 and IL1R2) show promise for differentiation of test-false positive cattle from true bTB infected. However, the individual gene expression levels of ATR, BOLA-DRB4, CCNG1, and TARS do not appear useful for differentiation of the groups of cattle. Clearly, other gene candidates should be explored to replace those 4 genes that fail to show a clear distinction among groups of cattle. This might be done by evaluating genes that have already been identified in the microarray studies.

Instead of using quantification of mRNA expression as a diagnostic assay, quantification of the gene product in cells or serum might be used, provided that the altered mRNA transcription observed in this study correlates with measurable altered protein expression. A protein based test is certainly more cost effective and generally easier to perform than a PCR based quantification assay. Proteomic analyses have shown that differentially expressed peptides/proteins can be useful as biomarkers in diagnostic applications (Agranoff et al., 2006; Naranjo et al., 2007b; Seth et al., 2009). Limited studies have examined the correlation of mRNA and protein expression. Most of the studies in human and yeast have found an overall positive, but far from perfect correlation of mRNA level and final protein abundance. Unfortunately, most studies have concluded that, at the genomic level, mRNA expression poorly predicts the corresponding protein expression level (Greenbaum et al., 2003; Guo et al., 2008; Gygi et al., 1999; Maier et al., 2009).

Finally, the current study was carried out using bPPD for stimulation of PBMC because it was readily available and it was the antigen stimulant used in field tests for bTB. As reviewed in Chapter 1, bPPD is a crude extract of *M. bovis* antigens that are cross-reactive with antigens from other mycobacteria. Many purified proteins such as CFP-10, ESAT-6, or ESAT-6/ CFP10 fusion protein etc. elicit a more specific response in *M. bovis* sensitized and infected animals. Alternatively, use of a more potent antigenic stimulant might be explored. Whole mycobacterial lysate (such as *M. avium* subspecies *paratuberculosis* lysate) has been found to be a more potent stimulant of PBMC than PPD (Coussens personal communication). Refinement of the current experiments using either a pure antigen, or whole mycobacterial lysate (such as *M. bovis* BCG lysate) may provide better differentiation of infected from non-infected cattle.

In conclusion, the current studies have provided a wealth of preliminary data, and may serve as the first of a series of research studies to further explore and better define a very complicated and important disease affecting cattle and many other species.

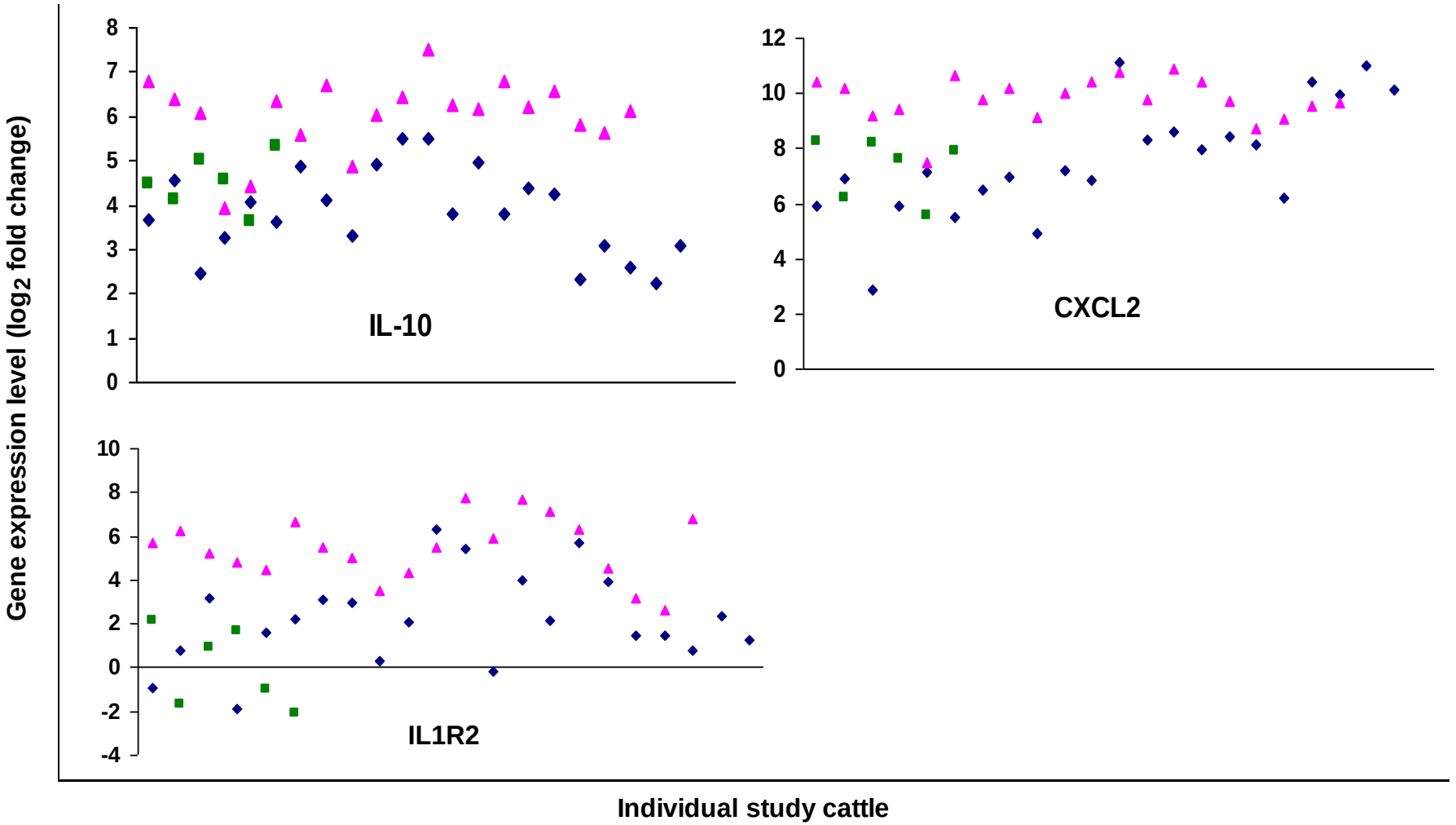
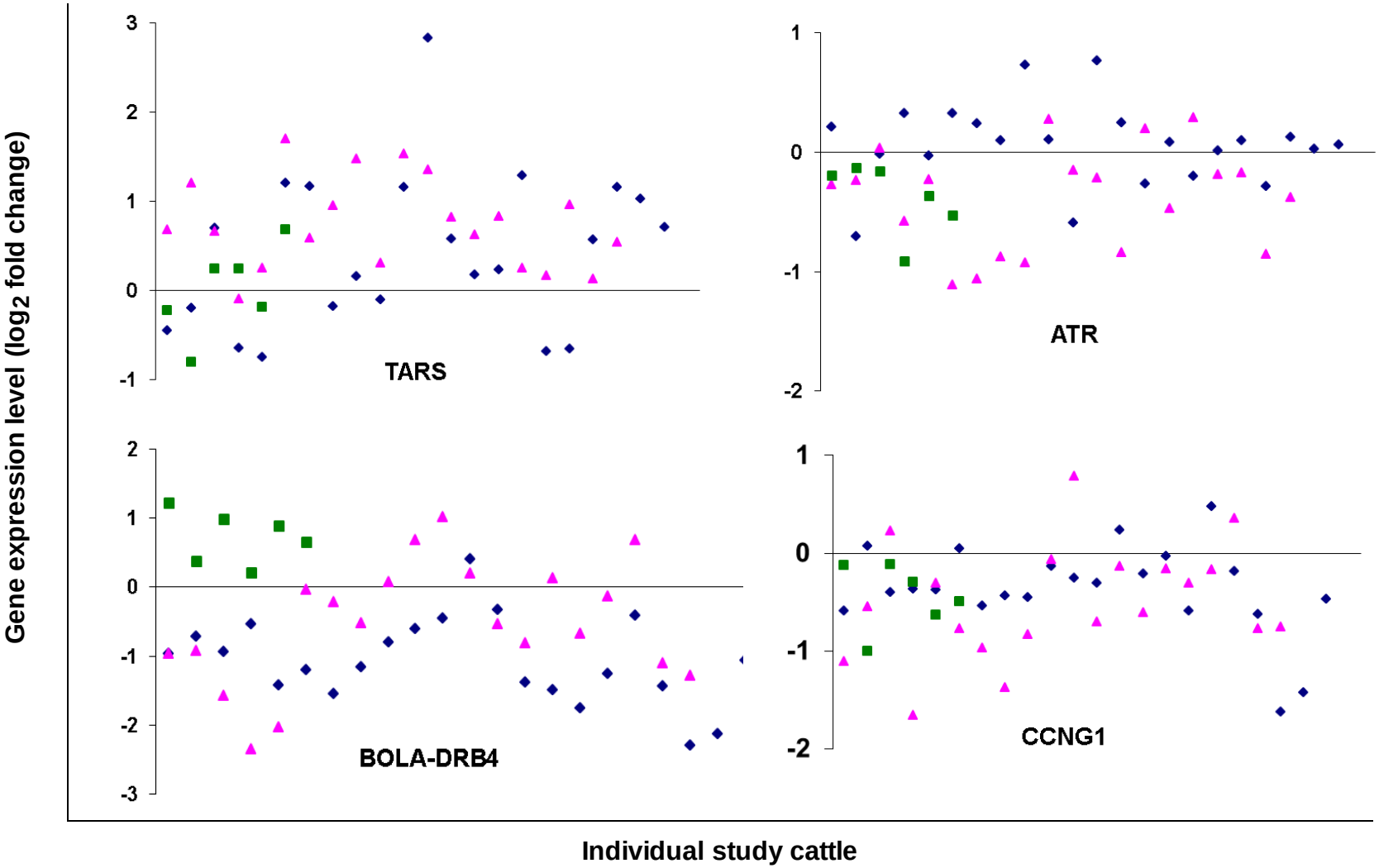


Figure 5.1 Altered gene expression levels (log_2 fold change of qPCR data) of the seven selected classifiers for the predictor model. The altered gene expression levels of individual cattle in the bTB infected group (bTB) (▲), double test-false positive (DFP) (◆) and the single reactor (SR) (■) groups of cattle were determined by comparison of gene expression values from a pool of healthy cattle (represent by the 0 line in each graph).

Figure 5.1 (cont'd)



APPENDICES

Appendix A

Analysis of reference genes for qPCR

Introduction

Quantitative real-time PCR (qPCR) has become the method of choice for quantification of gene expression levels. It is very sensitive and accurate method for quantification of mRNA transcripts, allowing a direct measure of differential transcription of mRNA for genes of interest, and an indirect measure of regulation of gene expression in biological process (Bustin, 2000; Hendriks-Balk, 2007). Relative qPCR is a rapid and robust method for quantification and is preferred over the absolute qPCR when the absolute copy number of mRNA is not required (Bustin, 2000). In relative qPCR, a reference gene is used to normalize disparity in RNA recovery and cDNA synthesis efficiency. This permits true comparisons of gene regulation between samples from within a group and between samples among different groups (Bustin, 2000; Huggett et al., 2005; Pfaffl, 2001). The reference gene is subjected to the same experimental condition as the genes of interest, and thus serves as a normalizer for correction of experimental variability. The underlying assumption is that the reference gene is expressed at a constant level, and that level of expression remains unchanged across sample types and experimental treatments. Thus, the detected level of expression of the reference gene will correlate with experimental error, which can be normalized directly. Use of a reference gene with an expression level that fluctuates randomly can lead to increased non-specific variation, and use of a reference gene with an expression level that changes with the sample type or with experimental treatments can lead to erroneous interpretation of data (Bustin, 2000; Dheda et al., 2004).

Conventional housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta actin (ACTB) and 18S rRNA were widely used as reference genes in early gene expression studies because it was assumed that their expression levels remained constant. However, many studies have shown that expression of reference genes can be influenced by sample type and experimental treatments (Bustin, 2002; Goossens et al., 2005; Schmittgen and Zakrajsek, 2000; Vandesompele et al., 2002). Use of the18S rRNA as a reference gene was based on the assumption that the rRNA:mRNA ratio would be the same in all samples and would remain unchanged after treatment; however, that assumption is not always valid (Solanas et al., 2001). Moreover, the abundance of rRNA, as compared with mRNA, in the total RNA sample could introduce technical issues in the qPCR assay. The disproportionate rRNA:mRNA ratio can complicate optimization of the PCR reaction and performance of the qPCR assay when the preferred fixed sample concentration for each reaction is used. The outcome can be a wide range of qPCR amplification plots for rRNA target that affects the baseline subtraction step in qPCR analysis (Hendriks-Balk et al., 2007). Proper validation of reference genes under specific experimental conditions and sample types is critical for accurate gene quantification by the relative qPCR method.

Several programs such as BestKeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) have been developed to evaluate the stability of candidate reference genes. Those programs employ different algorithms for calculation of stability values, which may result in different estimates for a stability value. Side by side evaluations using 2 or all 3 of these programs have shown that the best agreement is between geNorm and NormFinder for ranking the most and least stable genes (Perez et al., 2008; Skovgaard et al., 2007; Wood et al., 2008). To date, there is no single best program for ranking

of suboptimal reference genes. The ranking of the candidate genes by 2 or all 3 programs may be necessary before deciding on the best choice for a reference gene (Robinson et al., 2007). Alternatively, a normalization factor based on 2 to 3 reference genes has been proposed for use as the normalizer. Use of multiple reference genes for a normalization factor can improve the accuracy of quantification (Vandesompele et al., 2002). However, this method is costly, labor intensive, and not practical for use with a large number of samples or when resources are limited. This is because all of the normalizer genes must be included on every qPCR plate for every sample (Dheda et al., 2005; Vandesompele et al., 2002).

In the current study, gene expression profiles were determined for bTB free and bTB infected cattle using mRNA harvested from PBMC after a 4 hour or after an overnight stimulation with tuberculin antigen (bPPD). Before the initiation of the study, a literature search was performed to identify reference genes previously used in gene expression studies employing 1) various bovine sample types, 2) leukocytes or PBMC of species other than bovine, or 3) antigen stimulation studies of human tuberculosis patients. Twelve commonly used and previously validated reference genes were chosen for evaluation using the BestKeeper, geNorm and NormFinder programs. An in-house evaluation was done to determine the most suitable reference gene (with minimal variability) for use in qPCR study of gene expression under the defined experimental conditions as described in Chapters 2, 3, and 4.

Materials and Methods

A total of 12 commonly used reference genes selected from the literature were considered for use in this study (Table A.1). Published primer sequences for those genes were evaluated in the Clone Manager Suite 7 software package. Primers that met the study criteria were used as published, otherwise new primers were designed in Clone Manager Suite 7 and Primer Express

3.0 software. All primers (as listed in Table A.1) were synthesized by Integrated DNA Technologies.

Blood samples were collected from cattle as described in Chapter 2. Aliquots from each blood sample were subjected to 1 of 3 treatments; one aliquot was processed for RNA extraction without antigen stimulation, a second aliquot was stimulated with bPPD for 4 hours and then processed for RNA extraction, and the third aliquot was stimulated with bPPD overnight before being processed for RNA extraction. The 2 different stimulation time-points were evaluated simultaneously, to identify a reference gene that could be used for both the 4 hour stimulation study (see Chapter 2) and the overnight stimulation study (see Chapter 3). The RNA extraction, cDNA synthesis and qPCR assays were performed as described in Chapter 2. A constant amount of cDNA (20 µg/reaction) was used in duplicate qPCR reactions for each reference gene. The raw cycle threshold (Ct) value for each reaction was exported into an Excel spreadsheet; the ΔCt value was calculated as the difference in Ct of a stimulated sample (4 hours/overnight) from the Ct of the non-stimulated sample from a given animal [Ct(stimulated) minus Ct(unstimulated)].

The stability of each reference gene was evaluated and compared in BestKeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) software. The most stable gene, as determined by use of all programs, was selected and used as reference gene for subsequence qPCR assay.

Results

Initially, blood samples from 6 cattle were used to test the 12 selected reference genes using qPCR. The BestKeeper program used the raw Ct value, while both the geNorm and the NormFinder used the ΔCt value for calculation of the stability value. The ΔCt value was calculated as [Ct(4 hours/overnight stimulated sample)] minus [Ct (no antigen stimulation

sample)], and the $2^{\Lambda-\Delta Ct}$ value was used to compute the stability value in the NormFinder or the geNorm software programs.

In the BestKeeper program, the raw Ct value of all data points were used to compute the geometric means, arithmetic means and standard deviation for each reference gene. Stability of the reference gene was determined based on repeated pair-wise correlation analysis and standard deviation of geometric means. In the geNorm program, the gene expression stability measure (M) for a reference gene was calculated as the average pairwise variation (V) for that gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value then allows ranking of the tested genes according to their expression stability. The NormFinder program utilized a mathematical model to estimate the reference gene's stability based on direct estimation of expression variation and the taking into account of sample subgroups within the data; no sample subgroup was defined in the current study.

The ranking of the stability values for all reference genes by all three software programs is listed in Table A.2. The stability ranking for the reference genes varied among the 3 programs, especially with the BestKeeper program that was not based on a ΔCt value. Significant discrepancies were observed for a few genes, such as SDHA, B2M and ACTB. Some consistency in ranking was observed for very unstable gene candidates. H2A was determined as the least stable gene, with GAPDH, TBP and HPRTI also considered as unstable genes. The most stable gene was not clearly identified.

Based on the initial result, 5 of the most stable genes ranked by all 3 programs were selected for further evaluation; these genes are SDHA, H3F3A, YWHAZ, B2M and UBC. Despite the lower stability ranking, ACTB and GAPDH were also selected for further evaluation because these were the most commonly used reference genes in the published literature.

Samples of blood were obtained from an additional 12 cattle that represented all 3 study groups used throughout this dissertation, so that number of sample was increased from 6 to 18 cattle. The qPCR assays and data analysis were performed as above. The stability rankings of the 7 selected genes by the 3 different software programs are listed in Table A.3. Discrepancy was again observed in stability rankings among the 3 programs. The GAPDH gene remained the most unstable among the 7 selected genes. Interestingly, ACTB was ranked the third most stable gene by the BestKeeper program and was ranked as second least stable gene by the other 2 programs. SDHA was shown to be the most stable gene by all 3 programs. Based on this result, SDHA was selected the reference gene for this study.

Discussion

It is clear from the literature that expression of many housekeeping genes can be influenced by different experimental conditions, which should prevent their use in qPCR assays when those conditions are encountered (Bustin, 2002). Under the conditions of this study, expression of the GAPDH and ACTB were not stable. Wedlock et al. (2006) reported increased expression of housekeeping molecules such as gamma-actin, ACTB, and B2M in *M. bovis* infected macrophages. In a similar gene expression study of human TB; GAPDH, ACTB, and B2M were found unstable when tuberculin antigen stimulation was used (Dheda et al., 2004). Furthermore, the use of GAPDH as a reference gene resulted in erroneous interpretation of the IL-4 gene expression in TB patients (Dheda et al., 2005). The influence of a stimulant on the expression of housekeeping genes in various cell cultures has been reported (Roge et al., 2007; Schmittgen and Zakrajsek, 2000). In the current study, SDHA was found to be the most stable reference gene. SDHA has been used in gene expression studies involving bovine

polymorphonuclear leukocytes (De Ketelaere et al., 2006), the developing bovine embryo (Goossens et al., 2005), and bovine liver and pituitary tissues (Lisowski et al., 2008).

In this study, the ranking of gene stability by the geNorm and NormFinder programs was similar, and seldom in agreement the BestKeeper program. Overall, the programs agreed on the least stable genes and, to a lesser extent, on the most stable genes. A similar observation was reported by Perez et al. (2008) using the programs to evaluate reference genes for the study of gene expression in bovine muscle tissue (Perez et al., 2008). Wood et al. (2008) reported good agreement when evaluating reference genes with all three programs, and Skovgaard et al. (2007) found good agreement between geNorm and NormFinder for ranking of reference genes (Skovgaard et al., 2007; Wood et al., 2008).

Table A. 1 Reference genes selected for qPCR evaluation (listed in alphabetical order), sequence and concentration (nM) of PCR primers [forward primer (F) and reverse primer (R)] and amplicon sizes (bp).

gene name	gene symbol	sequences (5' -3')	amplicon size (bp)	concentration (nM)
actin, beta	ACTB	F: TGTCCACCTTCCAGCAGATG R: GGGTGTAACGCAGCTAACAG	108	50
beta-2-microglobulin	B2M	F: AGTAAGCCGCAGTGGAGGTG R: GCGCAAAACACCCTGAAGAC	110	50
glyceraldehyde-3-phosphate dehydrogenase	GAPDH	F: GCATCG TGGAGG GACTTA TGA R: GGGCCA TCCACA GTCTTC TG	67	50
histone H2A.l	H2A	F: GTCGTGGCAAGCAAGGAG R: AGTTACCCTTGCGGAGCAG	111	900
H3 Histone, family 3A	H3F3A	F: ATGGCTCGTACAAAGCAGAC R: ACCAGGCCTGTAACGATGAG	135	50
hydroxymethylbilane synthase	HMBS	F: GCATGCTTTGGAGAGGAATGA R: AATGGTGAAGCCAGGAGGAA	85	300
hypoxanthine phosphoribosyltransferase 1	HPRTI	F: CGGACTCTCATCTTAGGCTTTG R: TGTTGTGGGATATGCCCTTGAC	109	100
regulator of chromosome condensation 1	RPII	F: TCTACTTACTCGCCCACCTC R: GCATAGCTCACCCTCAGTTC	115	100

Table A.1 (cont'd)

succinate dehydrogenase complex subunit A	SDHA	F: CCACGCCAGGGAGGACTTC R: CGTAGGAGAGCGTGTGCTTC	116	50
TATA box binding protein	TBP	F: ACAACAGCCTCCCACCCTATGC R: GTGGAGTCAGTCCTGTGCCGTAA	111	900
tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein, zeta polypeptide	YWHAZ	F: GCATCCCACAGACTATTTCC R: AGGCAAAGACAATGACAGAC	122	50
ubiquitin carboxyl-terminal hydrolase L5	UBC	F: CTCCCTACCTGCATCATGTG R: GGAATTTGGGCCAGTGCTC	72	50

Table A. 2 Stability ranking of the 12 selected reference genes as analyzed by the geNorm, Normfinder and BestKeeper software. 1 represents the most stable gene, while 12 represents least stable gene determine by the program.

gene symbol	geNorm	Normfinder	BestKeeper
SDHA	1	1	6
H3F3A	2	2	4
YWHAZ	3	4	1
B2M	4	8	2
UBC	5	5	5
HMBS	6	3	9
RPII	7	7	7
ACTB	8	6	3
HPRTI	9	9	8
TBP	10	10	10
GAPDH	11	11	11
H2A	12	12	12

Table A. 3 Stability ranking of the final 6 selected reference genes as analyzed by the geNorm, Normfinder and BestKeeper software. 1 represents the most stable gene, while 12 represents least stable gene determine by the program.

gene symbol	geNorm	Normfinder	BestKeeper
SDHA	1	1	1
YWHAZ	2	4	5
UBC	3	5	6
H3F3A	4	2	2
B2M	5	3	4
ACTB	6	6	3
GAPDH	7	7	7

Appendix B

Table B. 1 Group designations for the cattle used in Chapter 2 and results of the IFN-γ ELISA and qPCR for IFN-γ mRNA after stimulation of blood obtained immediately before euthanasia. Cattle in the SFP group would have been negative in the IFN-γ ELISA using blood obtained on the farm. Cattle in the DFP group would have been positive in the IFN-γ ELISA using blood obtained on the farm. Cattle in the bTB group may have been positive or negative in the IFN-γ ELISA using blood obtained on the farm.

Sample ID	Initail study group ID	Re-grouping ID	Postmortem IFN-γ assay results	IFN-γ mRNA qPCR results (log_2 FC)
0915	SFP-1	FP-ex-1	neg	5.84
2007	SFP-2	FP-ex-2	neg	2.77
2232	SFP-3	FP-ex-3	neg	3.34
2237	SFP-4	FP-ex-4	neg	4.18
2240	SFP-5	FP-ex-5	neg	2.68
2255	SFP-6	FP-ex-6	neg	0.60
8052	SFP-7	FP-ex-7	neg	1.20
8066	SFP-8	FP-ex-8	neg	5.26
9703	SFP-9	FP-ex-9	neg	3.00
9764	SFP-10	FP-ex-10	neg	0.03
0420	DFP-1	DFP-non-ex-1	pos	0.76
1693	DFP-2	DFP-non-ex-2	pos	1.72
2229	DFP-3	FP-ex-11	pos	4.43
2238	DFP-4	FP-ex-12	neg	1.26
2445	DFP-5	FP-ex-13	neg	3.93
0911	DFP-6	FP-ex-14	pos	1.23
3232	DFP-7	DFP-non-ex-3	neg	0.79
4648	DFP-8	DFP-non-ex-4	neg	3.02
6297	DFP-9	DFP-non-ex-5	neg	-2.58
7622	DFP-10	DFP-non-ex-6	pos	0.91

Table B.1 (cont'd)

0912	bTB-1	bTB-1	neg	-1.60
3835	bTB-2	bTB-2	pos	-0.73
3838	bTB-3	bTB-3	neg	7.99
4330	bTB-4	bTB-4	pos	3.25
4421	bTB-5	bTB-5	neg	4.75
4440	bTB-6	bTB-6	pos	5.81
6798	bTB-7	bTB-7	pos	-0.57
6834	bTB-8	bTB-8	pos	-1.51
8031	bTB-9	bTB-9	pos	no data
8059	bTB-10	bTB-10	pos	2.92

Appendix C

Table C. 1 Genes from microarray analysis that showed statistically significant (*adj p* ≤ 0.01) differential expression in the overnight tuberculin stimulated whole blood of all 3 study groups of cattle [the single test-false positive (SFP), double test-false positive (DFP) and bTB infected (bTB)], as compared with the pool of unstimulated healthy control cattle.

Microarray gene ID	Gene Description	SFP		DFP		bTB	
		log_2 FC	*adj p*	log_2 FC	*adj p*	log_2 FC	*adj p*
BLO_ext_00102	HUMLYN lyn tyrosine kinase	-1.10	0.0000	-0.87	0.0006	-1.57	0.0000
BLO_ext_00110	Growth-regulated protein homolog gamma precursor (GRO-gamma)	3.07	0.0000	2.98	0.0000	3.68	0.0000
BLO_ext_00260	unkown	2.09	0.0000	2.07	0.0000	1.44	0.0006
BLO_ext_00279	Tropomyosin 3	-1.14	0.0001	-0.88	0.0016	-1.44	0.0000
BLO_ext_00297	Thrombospondin-1 precursor	1.77	0.0009	1.46	0.0049	1.62	0.0088
BLO_ext_00308	Monocyte differentiation antigen CD14 precursor (Myeloid cell-specific leucine-rich glycoprotein)	1.52	0.0004	1.31	0.0018	1.59	0.0013
BLO_ext_00503	S85192 vascular endothelial growth factor	1.40	0.0002	1.03	0.0044	1.39	0.0012
BLO_ext_00541	Growth-regulated protein homolog alpha precursor (GRO-alpha)	3.72	0.0000	2.90	0.0009	3.82	0.0002
BLO_ext_00596	C-X-C chemokine receptor type 4 (CXC-R4) (CD184 antigen)	1.82	0.0001	2.27	0.0000	2.01	0.0002
BLO_ext_00608	NF kappa B inhibitor alpha	1.63	0.0014	1.84	0.0004	1.72	0.0034

Table C.1 (cont'd)

BLO_ext_00756	CXXC5 protein	-0.95	0.0013	-0.78	0.0071	-0.92	0.0069
BLO_ext_00786	Interleukin-8 precursor (IL-8) (CXCL8)	4.97	0.0000	4.58	0.0000	5.35	0.0000
BLO_ext_01111	Prolactin regulatory element-binding protein (Mammalian guanine nucleotide exchange factor mSec12)	-0.96	0.0042	-0.90	0.0074	-1.37	0.0007
BLO_ext_01145	Thrombomodulin (TM) (Fetomodulin) (CD141 antigen)	3.45	0.0000	3.21	0.0000	4.15	0.0000
BLO_ext_01193	BOSIN (O19197) MHC class II alpha subunit precursor (Fragment), complete	-2.11	0.0000	-1.42	0.0002	-2.35	0.0000
BLO_ext_01345	VEGFB vascular endothelial growth factor	2.32	0.0000	2.23	0.0001	2.61	0.0001
BLO_ext_01516	unknown	4.51	0.0000	3.75	0.0000	4.72	0.0000
BLO_ext_01622	HSPA12B protein	-0.67	0.0053	-0.87	0.0005	-0.77	0.0065
BLO_ext_01694	Growth-regulated protein homolog alpha precursor (GRO-alpha)	2.99	0.0002	2.58	0.0011	3.27	0.0005
Bt00000296	Phosphoglycerate mutase 1 (Brain)	0.93	0.0094	1.17	0.0012	1.14	0.0069
Bt00000391	lymphotoxin-beta	-1.03	0.0034	-1.05	0.0027	-1.27	0.0020
Bt00000692	Small inducible cytokine B6 precursor (CXCL6) (Granulocyte chemotactic protein 2) (GCP-2)	5.51	0.0000	4.63	0.0000	4.98	0.0000
Bt00000725	unknown	1.79	0.0000	1.93	0.0000	1.66	0.0001
Bt00001134	Mannosidase, alpha, class 1C, member 1	0.92	0.0027	1.05	0.0007	1.59	0.0000

Table C.1 (cont'd)

Bt00001318	HUMLYN lyn tyrosine kinase	-0.95	0.0004	-0.87	0.0009	-1.72	0.0000
Bt00001530	Dual specificity protein phosphatase 1 (MAP kinase phosphatase 1) (MKP-1)	1.79	0.0035	2.09	0.0008	1.92	0.0077
Bt00001597	Thrombomodulin (TM) (Fetomodulin) (CD141 antigen)	1.74	0.0004	2.05	0.0001	2.88	0.0000
Bt00001603	EP4 receptor	0.64	0.0083	0.81	0.0009	0.88	0.0019
Bt00001795	para-hydroxybenzoate--polyprenyltransferase, mitochondrial precursor	-0.76	0.0041	-0.96	0.0005	-1.03	0.0010
Bt00001908	Rhombotin-2 (Cysteine-rich protein TTG-2) (T-cell translocation protein 2) (LIM-only protein 2)	-1.72	0.0000	-1.42	0.0000	-1.56	0.0000
Bt00001967	Thrombospondin-1 precursor	1.53	0.0030	1.54	0.0026	2.01	0.0010
Bt00002360	MSTP014	-1.52	0.0001	-1.37	0.0002	-2.28	0.0000
Bt00003194	Mtm (Myotubularin) family protein 9, isoform a (Myotubularin-related protein MTM-9)	1.32	0.0006	1.01	0.0067	1.23	0.0053
Bt00003589	Tropomyosin 4	1.60	0.0000	1.46	0.0000	1.49	0.0001
Bt00003606	Protein LRP16	1.40	0.0017	1.28	0.0035	1.37	0.0088
Bt00003921	Growth-regulated protein homolog alpha precursor (GRO-alpha)	3.42	0.0000	2.95	0.0000	4.16	0.0000
Bt00004631	protein kinase C, beta 1 polypeptide	-0.89	0.0032	-0.82	0.0063	-1.31	0.0003

Table C.1 (cont'd)

Bt00005118	natural resistance associated macrophage protein	1.14	0.0002	0.96	0.0013	1.38	0.0001
Bt00005241	unknown	1.14	0.0005	1.07	0.0010	1.72	0.0000
Bt00006457	Phospholipase D family, member 4 (PPRR2488)	-0.98	0.0012	-0.86	0.0035	-1.48	0.0000
Bt00006663	MHC class II antigen	-1.26	0.0002	-0.95	0.0032	-1.88	0.0000
Bt00006937	MHC class II DR-ALPHA	-1.93	0.0000	-1.14	0.0029	-2.26	0.0000
Bt00007151	X3 1,2-cyclic-inositol-phosphate phosphodiesterase	-1.03	0.0004	-0.96	0.0009	-1.06	0.0014
Bt00007209	Butyrate response factor 1 (TIS11B protein) (EGF-response factor 1) (ERF-1)	1.08	0.0002	1.06	0.0002	0.92	0.0048
Bt00007220	MHC class II antigen	-1.53	0.0033	-1.40	0.0067	-2.20	0.0004
Bt00007282	Bos taurus isolate Rom478 mitochondrion,	-1.21	0.0038	-1.88	0.0000	-1.88	0.0002
Bt00007525	myotrophin	-0.72	0.0023	-0.76	0.0012	-1.38	0.0000
Bt00007545	Ogt protein, partial	-0.60	0.0092	-0.77	0.0010	-0.85	0.0016
Bt00007670	ATP synthase beta chain, mitochondrial precursor	-0.77	0.0013	-0.68	0.0037	-1.02	0.0003
Bt00007963	Thrombomodulin (TM) (Fetomodulin) (CD141 antigen)	3.07	0.0000	3.39	0.0000	3.98	0.0000
Bt00008223	Phosphoglycerate mutase 1 (Brain)	1.23	0.0002	1.28	0.0001	1.47	0.0001

Appendix D

Table D. 1 Group designations for the cattle used in Chapters 3 and 4; results of the CFT, CCT, or IFN-γ ELISA on the farm; and results of the IFN-γ ELISA after stimulation of blood obtained immediately before euthanasia.

Sample ID	Study group ID	Antemortem testing			Postmortem
		CFT	CCT	IFN-γ	IFN-γ results
0420	DFP-1	pos	NP	pos	pos
20085	DFP-2	pos	NP	pos	NP
30911	DFP-3	pos	NP	pos	pos
3232	DFP-4	pos	NP	pos	neg
6541	DFP-5	pos	pos	NP	pos
7004	DFP-6	pos	pos	NP	neg
7030	DFP-7	pos	pos	NP	NP
7145	DFP-8	pos	pos	NP	neg
7622	DFP-9	pos	NP	pos	pos
8753	DFP-10	pos	NP	pos	neg
6185	DFP-11	pos	NP	pos	neg
9416	DFP-12	pos	NP	pos	neg
4150	DFP-13	pos	pos	NP	neg
5558	DFP-14	pos	NP	pos	neg
7572	DFP-15	pos	NP	pos	neg
7577	DFP-16	pos	NP	pos	neg
7641	DFP-17	pos	NP	pos	neg
8005	DFP-18	pos	NP	pos	neg
OH-1378	DFP-19	pos	NP	pos	NP
OH-1437	DFP-20	pos	NP	pos	NP
OH-1666	DFP-21	pos	NP	pos	NP
OH-3503	DFP-22	pos	NP	pos	NP

Table D.1 (cont'd)					
4300	SR-1	pos	NP	neg	NP
4312	SR-2	pos	NP	neg	NP
4371	SR-3	pos	NP	neg	NP
4387	SR-4	pos	NP	neg	NP
4404	SR-5	pos	NP	neg	NP
4453	SR-6	pos	NP	neg	NP
912	bTB-1	pos	pos	NP	neg
3835	bTB-2	pos	NP	pos	pos
3914	bTB-3	pos	NP	pos	neg
4330	bTB-4	pos	NP	pos	pos
4690	bTB-5	pos	NP	neg	pos
6798	bTB-6	pos	NP	pos	pos
6814	bTB-7	pos	NP	pos	neg
6817	bTB-8	pos	NP	pos	neg
6834	bTB-9	pos	NP	pos	pos
8031	bTB-10	pos	NP	pos	pos
1409	bTB-11	pos	NP	pos	pos
1413	bTB-12	pos	NP	pos	neg
1419	bTB-13	pos	NP	neg	neg
2835	bTB-14	pos	NP	pos	pos
6845	bTB-15	pos	NP	neg	neg
6857	bTB-16	pos	NP	pos	pos
6860	bTB-17	pos	NP	neg	neg
9401	bTB-18	pos	NP	neg	pos
1673	bTB-19	pos	NP	neg	neg
6861	bTB-20	pos	NP	neg	neg
0003H	SFP-1	pos	NP	neg	neg
0005H	SFP-2	pos	NP	neg	neg

Table D.1 (cont'd)					
0566H	SFP-3	pos	NP	neg	neg
2007	SFP-4	pos	neg	NP	neg
4685	SFP-5	pos	neg	NP	neg
7342	SFP-6	pos	NP	neg	neg
7763	SFP-7	pos	NP	neg	neg
9634	SFP-8	pos	neg	NP	neg
9703	SFP-9	pos	neg	NP	neg
9979	SFP-10	pos	NP	neg	neg
2140	SFP-11	pos	NP	neg	neg
4490	SFP-12	pos	neg	NP	NP
7709	SFP-13	pos	NP	neg	neg
7792	SFP-14	pos	NP	neg	neg
0012H	SFP-15	pos	NP	neg	neg
2361	SFP-16	pos	NP	neg	neg
2509	SFP-17	pos	NP	neg	neg
2642	SFP-18	pos	NP	neg	neg
3305	SFP-19	pos	NP	neg	neg
3306	SFP-20	pos	NP	neg	neg
6178	SFP-21	pos	neg	NP	pos

pos = positive, neg = negative, NP = not performed.

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