Cytokine Gene Expression in Holstein-Friesian and Jersey Calves Infected with
*Mycobacterium avium* subsp. *paratuberculosis*

by
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ABSTRACT

CYTOKINE GENE EXPRESSION IN HOLSTEIN-FRIESIAN AND JERSEY CALVES INFECTED WITH MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS

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University of Guelph, 2013

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Dr. Niel A. Karrow

Breed susceptibility to bovine Johne’s disease has been implied suggesting that Jersey cattle are more susceptible to disease as compared to Holstein-Friesian. A sixty-day experimental in vivo infection study was carried out by surgically infecting Holstein-Friesian and Jersey calves with the etiological agent of Johne’s disease, Mycobacterium avium subsp. paratuberculosis (Map). Breed-specific comparison of expression of key cytokine transcripts in the ileocecal lymph nodes of Map-infected and uninfected calves by real-time PCR did not indicate any significant differences. The expression of IFN-γ transcript was found to be significantly different for the treatment groups in both breeds with a higher expression level in infected than in control animals but there was no indication this differed between breeds. These data indicate that there are no breed-specific differences in the expression of key cytokine transcripts at sixty days post Map-infection and warrants long-term infection studies to analyze breed-specific immune response and in defining breed susceptibility.
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DEDICATION

This thesis is dedicated to my parents Ambadevi and Mallikarjunappa and to my sister

Savitha for their love and sacrifices
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List of Abbreviations

CFU  Colony forming unit
EEA1  Early endosome auto antigen-1
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
γδ T cell  Gamma-Delta T cell
GWAS  Genome-wide association study
Il-1α  Interleukin 1 alpha
IL-6  Interleukin 6
IL-10  Interleukin 10
IL-12  Interleukin 12
IL-13  Interleukin 13
IL-17  Interleukin 17
IL-23  Interleukin 23
IFN-β  Interferon beta
IFN-γ  Interferon-gamma
IFN-γR1  Interferon gamma receptor chain 1
IFN-γ R2  Interferon gamma receptor chain 2
IgG1  Immunoglobulin G1
iNOS  inducible Nitric Oxide Synthase
ICLN  Ileocecal lymph node
JAK-STAT  Janus family kinase-signal transducer and activator of transcription
LAMP 1  Lysosomal-associated membrane protein 1
LAMP-2  Lysosomal-associated membrane protein 2
MAPK-38  p38-mitogen activated protein kinase
MDM  Monocyte-derived macrophages
Maa  *Mycobacterium avium* subsp. *avium*
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>MAP</td>
<td><em>Mycobacterium avium</em> subsp. <em>paratuberculosis</em></td>
</tr>
<tr>
<td>Mas</td>
<td><em>Mycobacterium avium</em> subsp. <em>sylvaticum</em></td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappaB</td>
</tr>
<tr>
<td>NOD2</td>
<td>Nucleotide oligomerization domain containing 2</td>
</tr>
<tr>
<td>NRAMP1</td>
<td>Natural Resistance Associated membrane Protein 1</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptors</td>
</tr>
<tr>
<td>PAMPS</td>
<td>Pathogen-associated membrane proteins</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative trait loci</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>SLC11A1</td>
<td>Solute carrier family member 1</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOCS1</td>
<td>Suppressor of cytokine signaling 1</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressor of cytokine signaling 2</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper 17 cells</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
</tbody>
</table>
CHAPTER 1

LITERATURE REVIEW

1.1. Johne’s disease: Introduction and occurrence

Johne’s disease is chronic granulomatous enteritis caused by the obligate intracellular bacteria *Mycobacterium avium* subsp. *paratuberculosis* (Map). The disease named after a German pathologist, Heinrich Albert Johne, is characterized by a latent sub-clinical phase that lasts for years, followed by clinical phase in which the affected animal exhibits chronic diarrhea, emaciation, decreased milk production, and reduced fertility (Whitlock *et al*., 1996). This mycobacterial infection of ruminants is highly contagious in nature and is present worldwide. Herd level prevalence of Johne’s disease based on seropositivity in dairy cattle varies across the globe with the maximum prevalence estimated to be in New Zealand (60%) and a minimum prevalence of 7% in Austria (Grant, 2005). In Europe, the prevalence of Johne’s disease or paratuberculosis in farmed animals is approximately 20% (Nielsen *et al*., 2009). According to the NAHMS Dairy 2007 survey, herd prevalence was estimated to be 68.1% in U.S. dairy operations. Recent study in Ontario indicated 26% of the total herds tested had at least 1 Johne’s ELISA test-positive cow (www.johnes.ca, 2013).

The extent of the occurrence of Johne’s disease has left a financial footprint on the dairy and beef industries as the disease is associated with decreased milk production, premature culling of infected animals, loss of slaughter value, loss of valuable animals, decreased marketing opportunities, and increased management and veterinary costs associated with its control. An annual herd-level economic loss associated with Johne’s disease in U.S. dairy operations is estimated to be $200-$250 million dollars (Ott *et al*., 1999).
Additional to the economic issues associated with Johne’s disease, there is a growing concern that Map plays a significant role in human medicine as a potential cause or exacerbating factor in Crohn’s disease (Scanu et al., 2007). Crohn’s disease is an inflammatory bowel disease of humans with intestinal pathology similar to paratuberculosis (Dalziel, 1913). Various studies have reported isolation of viable Map and detection of Map DNA from tissues including the peripheral blood of patients with Crohn’s disease and have implicated Map as a zoonotic pathogen (Sechi et al., 2005; Naser et al., 2004; Sanderson et al., 1992; Bull et al., 2003; Moss et al., 1992). Along the same line, remission of symptoms associated with Crohn’s disease has been reported following treatment with anti-mycobacterial antibiotics (Selby et al., 2000). As few reports indicate that Map can tolerate the milk pasteurization temperature (72 °C for 15 seconds), there is increased public health concern about exposure to Map (Donaghy et al., 2007). The above findings are suggestive that Map could be the etiological agent for Crohn’s disease, but they fail to explain a definitive cause-effective relationship between Map and Crohn’s disease as the Koch’s postulates have not been fulfilled (Nacy and Buckley, 2008). The issue of defining Map as a pathogen of public health concern continues to be debatable and warrants further research.

1.1.2. Etiology of Johne’s disease

Map is an obligate, intracellular, acid-fast, Gram-positive bacterium that belongs to the family of Mycobacteriaceae (Collins et al., 1990). Map is closely related with two other Mycobacterium avium subspecies - Mycobacterium avium subsp. avium (Maa), and Mycobacterium avium subsp. sylvaticum (Mas). Together, these three form the Mycobacterium avium complex. Map is classified into three strain types namely: Type 1, also called the ‘S’ strain, that infects sheep; Type ‘2’, also called the ‘C’ strain that infects cattle; and Type ‘3’ strain, known as intermediate strain (Collins et al., 1990). The ‘C’ strain,
compared to ‘S’ strain is fast growing with colonies visible 6 to 12 weeks post incubation and require supplementation of mycobactin J for their growth; whereas, the type ‘S’ strain is slow growing, with pigmented colonies visible after 16 weeks (Juste et al., 1991). Although very rare, sporadic isolation of ‘S’ strain in cattle is been reported (Muskens et al., 2001; Moloney et al., 2008).

1.1.3. Host range

Initially, it was perceived that Map primarily infects domestic ruminants like cattle, sheep, and goats. However, Map has been isolated from wild ruminants as well as wild non-ruminants suggesting their possible role as reservoir hosts. Wild ruminants such as bison (Buerkelt et al., 2000), white-tailed deer (Chiodini et al., 1983), elk (Jessup et al., 1981), and species such as wild rabbits, foxes, guanacos (Salgado et al., 2009), and primates such as mandrills and macaques (Mclure et al., 1986), have been shown to harbor Map. Isolation of Map from free-living amoebae has been reported suggesting their role as vectors in Map transmission (White et al., 2010) and as a potential vehicle for water-borne transmission. This broad range of hosts could account for spread of infection making Johne’s disease control difficult across the globe.

1.2 Development of disease: Modes of infection and modulation of host’s immune system

1.2.1. Modes of Infection

Neonatal calves and calves less than six months of age are highly susceptible to Map infection owing to their under-developed immune system (Windsor et al., 2010). The fecal-oral route is the primary mode of transmission wherein the calves’ contract infection by consumption of colostrum, milk, and feed contaminated with feces containing Map (Sweeney et al., 1996; Streeter et al., 1995). Intra uterine transmission of infection from the dam to its
offspring has also been reported (Whittington et al., 2009). Vertical transmission from sire to
calf may be possible as Map organisms have been identified in male genital organs and
semen and also found to persist in cryo-preserved semen (Larsen et al., 1981; Ayele et al.,
2004; Jorge et al., 1998). While animals develop some resistance with age, cattle of any age
can become infected when introduced into an environment with a high Map load (Rankin,
1962).

1.2.2. Entry of Map and its uptake by macrophages

Following their entry into the host via ingestion, Map translocates into gut-associated
lymphatic tissues (GALT) where it is phagocytozed by macrophages. Translocation across
the mucosal epithelium is facilitated by specialized intestinal absorptive cells called M cells
or enterocytes through fibronectin-dependent mechanisms (Momotani et al., 1988;
Siguroardottir et al., 2001; Secott et al., 2004; Ponnusamy et al., 2012) and also likely by
migratory dendritic cells that sample the pathogen from the intestinal lumen and migrate to
draining lymph nodes for antigen presentation (Coussens et al., 2010).

The uptake of Map by macrophages is mediated by various pattern recognition
receptors (PRRs) found on host macrophages that recognize Map-associated membrane
patterns (PAMPs). These PRRs include: complement receptors (Schlesinger et al., 1990);
mannose receptors (Astarie-Dequeker et al., 1999; Schlesinger et al., 1993); Toll-like
receptors 2 and 4 (TLRs); β-integrin receptors such as CD11a, CD18 (Souza et al., 2003);
and CD14 receptors (Peterson et al., 1995).

1.2.3. Map-macrophage interaction

Macrophages are one of the first types of immune cells that Map encounters in the
GALT, and the major host cell for the bacterium. The ability of Map to overcome the
microbicidal effects of the macrophage, to create a niche for itself within the macrophage and avert host’s immune response are intriguing subjects. Inside the macrophage, Map is internalized within an early phagosome that further undergoes a series of fusion and fission interactions with early and late recycling endosomes before transition into phagolysosome. This process called as ‘phagosome maturation’ involves transition of phagosome from early-, intermediate-, late-phagosome to phagolysosome along with acquisition of different endosomal markers. Rab 5, EEA1, transferrin receptors constitute the early phagosome markers, whereas the late phagosome acquires late endosomal markers such as Rab 7, V-ATPase, lysosomal hydrolases such as cathepsin D, and LAMP1 (Russell, 2011). The transition of phagosome is also coupled with a corresponding increase in acidification of each compartment by virtue of membranous V-ATPase complex that pump protons into the phagosome (Hackam et al., 1997).

1.2.4. MAP survival strategy

Pathogenesis of Johne’s disease is due to successful localization and multiplication of Map within the phagosome and Map has evolved strategies to escape microbicidal activity of macrophage. This favors its survival and leads to dissemination of the infection within the host. Numerous studies have been carried out to understand Map-macrophage interaction and to elucidate the strategies adopted by Map to survive within the host macrophage. Based on studies involving Mycobacterium tuberculosis and Map, the general consensus is that pathogenic mycobacteria are able to survive within macrophage by a number of mechanisms that include: inhibiting phagosomal maturation; interfering with macrophage apoptosis and phagosome acidification; down-regulating the expression of MHC molecules that allows them to evade antigen presentation by the macrophages; and by regulating different signal transduction pathways.
1.2.4.1. Inhibition of phagosomal maturation and acidification

Phagosomal acidification by macrophages is essential for processing and presentation of antigenic determinants via MHC molecules to initiate immune response. Significantly reduced co-localization of late endosomal marker, LAMP 1 has been reported in murine macrophage cell line J774 infected with live Map as compared to infection with live *Mycobacteria smegmatis* (Hostetter *et al.*, 2002). Similar findings are reported in infection of the J774 cell line with human-Map isolate (Rumsey *et al.*, 2006). Although these results are indicative of inhibition of phagosomal maturation by Map, they fail to explain the mechanisms associated with it. Higher pH (6.3) and lack of late-endosomal markers such as LAMP-2 in the Map-containing phagosome as compared to phagosome with dead Map and non-pathogenic mycobacteria like *Mycobacterium gordonae* and *Mycobacterium smegmatis* (pH 5.0) has been reported suggesting inhibition of phagosomal acidification and maturation by Map (Kuehnel *et al.*, 2001). Similarly, comparison of gene expression in Map and Maa-infected macrophages revealed decreased expression of endocytic pathway genes such as H-ATPases, LAMP-2 in Map-infected macrophages that correlated with reduced phagosome acidification as compared to Maa-infected macrophages (Weiss *et al.*, 2004), implying interference of Map with phagosome maturation.

1.2.4.2. Inhibition of Apoptosis

Apoptosis or programmed cell death of the infected macrophage is an important mechanism to deny intracellular bacteria like *Mycobacterium* the ability to establish a niche within the macrophage before the host immune response is activated (Keane *et al.*, 2000). Recent studies have demonstrated that intracellular *Mycobacterium* evades macrophage apoptosis, in order to induce chronic infection in the susceptible host. Macrophages from genetically resistant deer infected with Map have been shown to undergo apoptosis as
opposed to macrophages from the susceptible genotype (Dobson et al., 2013). Kabara et al (2012) have reported reduced caspase activity and apoptosis-signaling pathway activity in Map-infected macrophages and the resultant decrease in macrophage apoptosis. It has been reported that Map may evade apoptosis by affecting cytokine expression. Inhibition of apoptosis by *Mycobacterium tuberculosis* by inducing IL-10 secretion and by suppressing TNF-α secretion is been reported (Balcewicz-Sablinska et al., 1998). Also, the role of NF-κb, a transcription factor, in inhibiting apoptosis in bovine monocytes is documented. Weiss et al. (2008) have concluded that post infection of bovine monocytes with Map, NF-κb pathway activation takes place leading to increased TNF-α secretion and increased apoptosis.

1.2.4.3. Regulation of MHC molecules expression

It is hypothesized that inhibition of expression of antigen presenting MHC molecules on macrophages is one of the mechanisms by which *Mycobacterium* prevent antigen presentation leading to T cell activation. *In vitro* infection studies have reported downregulated expression of MHC class II molecules on macrophages infected with Map within 12 hours post-infection as compared to Maa (Weiss et al., 2002). In the same study, primed autologous lymphocytes lysed Maa-infected macrophages but not Map-infected macrophages. In an *in vivo* experimental infection study, microarray data from PBMCs from calves infected with Map indicated down-regulation of genes associated with antigen presentation and processing pathway (Purdie et al., 2012). All these findings support the hypothesis that by down-regulating expression of MHC molecules, Map prevents presentation of its’ antigenic determinants to T-cells to evade the host immune response. Although regulation of MHC molecules by *Mycobacterium spp.* is well documented, the exact mechanisms and the factors that mediate these changes are yet to be explored.
1.2.4.4. Regulation of signal transduction pathways

Activation and inhibition of different cell-signaling pathways by Map after its interaction with macrophages have been reported to have a bearing on the nature of cytokine production by macrophages and their effector function. Kinome analysis showed inhibition of JAK-STAT pathway in Map-infected monocytes in contrast to uninfected monocytes (Arsenault et al., 2012). Activation of JAK-STAT pathway is essential for Interferon-gamma signaling of Map-infected macrophages to stimulate CD4 T cells in order to control infection (Coussens et al., 2001). In this same study, the researchers also observed an increase in the expression of SOCS1, SOCS3 and decreased expression of IFN-γR1 and IFN-γR2 in Map-infected monocytes implying that Map inhibits IFN-γ responsiveness of macrophages. It is also been reported that Map initiates early activation of MAPK-p38 in monocytes that leads to increased expression of IL-10 thereby enhancing Map survival (Souza et al., 2006). Sommer et al. (2009) have reported that Map impairs CD40L (CD154) signaling of CD40 on MDMs leading to suppression of IL-12p40 and iNOS genes by means of activation of p38 pathway. IL-12 is a pro-inflammatory cytokine required for proliferation of CD4+ T cells.

1.3. Host immune response

In ruminants, progression of Johne’s disease occurs through different stages and is classified as early, sub-clinical, and late infection phase. Each phase is characterized by transition in the nature of immune response exhibited by the infected animal which, in turn, governs the pathological state of the affected host. The complex interplay between the host and the pathogen in Johne’s disease involves activation of different kinds of immune cells by numerous cytokines and co-stimulatory molecules.
1.3.1. Early infection

Early infection stage involves infection of macrophages by Map. As discussed earlier, post ingestion of Map, translocation of Map into ileocecal peyer’s patches via M cells takes place where Map get phagocytosed by macrophages. The events following phagocytosis by macrophages will largely influence the disease pathogenesis and its outcome and it includes activation of immune cells such as γδ T cells, antigen processing by macrophages, and activation of CD4+ T cell mediated immune response by cytokines.

In recent years, the role of γδ T cells during early infection stages of paratuberculosis has been extensively studied. γδ T cells constitute about 40% of circulating PBMC in calves and 10-15 % in adult cattle and are thought to be a link connecting the innate and adaptive immune response in mycobacterial infections of cattle and humans (Pollock et al., 2002). Early recruitment of γδ T cells and their presence in Map-induced lesions has been reported in experimental calves injected with live Map inoculum and Map-whole cell vaccine suggesting that they play a role in granuloma formation (Plattner et al., 2009). Given the relative abundance of the γδ T cells in the gut mucosa and their ability to present antigen to CD4+ T cells (Brown et al., 1998), and as a source of IFN-γ during the early stages of Map infection suggests they play an early role in controlling the infection.

The role of CD4+ T cells during early stages of Map infection has a great bearing on the outcome of infection in the host. CD4+ T cell subtypes are responsible for activating cell mediated immune response against intracellular Map. Activated macrophages secrete IL-12 and chemokines that recruit CD4+ T cells to the infection site. CD4+ T cells further recognize Map antigenic determinants presented by macrophages via MHC II molecules and in turn secrete IFN-γ, a pro-inflammatory cytokine. IFN-γ secreted further acts upon macrophages to induce IL-12 secretion and stimulate proliferation of CD4+ T cells leading to enhanced
inflammatory changes and polarizing Th1 cell mediated immune response (Coussens, 2001; Stabel, 2006). It is very well known that cell mediated immune response is essential in containment of intracellular mycobacterial infections such as Map. However, Map has evolved strategies to redirect early immune response to favor its survival. The early immunological responses induced will eventually predict the disease outcome in the infected animal (Hines et al., 2007; Mikkelsen et al., 2008).

1.3.2. Subclinical stage

The sub-clinical phase is the long latent phase of disease that may last for 2-5 years (Coussens et al., 2001). During this phase, the infected animal does not exhibit clinical signs, appears healthy but begins to intermittently shed Map in the feces during the late stages of subclinical phase. The lack of sensitive diagnostic tests to detect the disease during this stage and the risk associated with spreading of infection due to intermittent Map shedding may jeopardize control of Johne’s disease (Whitlock et al., 2000; McKenna et al., 2005; Collins et al., 2005). Predominance of a Th1-mediated immune response is observed during early subclinical stage as extensive proliferation of CD4 T cells with the resultant increased expression of pro-inflammatory cytokines like IFN-γ, TNF-α, IL-1α, IL-6 is detected in the ileal tissue of sub-clinically infected animals (Coussens et al., 2004; Sweeney et al., 1998; Khalifeh et al., 2004), in PBMCs (Coussens et al., 2004), and PBMCs stimulated with Map (Stabel, 2000). However, as the disease progresses Th1 mediated immune response wanes down coupled with an increased antibody mediated response (Coussens, 2001). The exact reason behind this transition and the time at which it takes place is not known. Detectable levels of IgG1 class of antibodies can be seen during the mid-to-late stage of subclinical infection. Understanding the nature of immune response and the factors associated with it during sub-clinical stage is critical in determining and implementing effective strategies to control Johne’s disease.
1.3.3. Clinical stage

By late sub-clinical phase and early clinical phase, Map-infected animal starts exhibiting clinical signs such as persistent diarrhea, reduced feed intake, debility, muscle wasting, and decreased milk production which eventually lead to death. With the onset of clinical signs, transition in the nature of immune response from Th1-to Th2-mediated immune response is noticed. CD4 T cell suppression (Bassey and Collins, 1997) coupled with increased proliferation of immune-regulatory and antibody-producing B cells is observed, which correlates with rise in readily detectable serum antibody levels. Pathological lesions such as thickened intestinal mucosa and granulomatous inflammation within the intestinal wall are evident leading to profuse protein losing enteropathy (Whitlock et al., 1996; Chiodini et al., 1984). The clinical stage is also characterized by constant shedding of Map in the feces, milk, and colostrum (Streeter et al., 1995; Sweeney et al., 1996), thereby increasing the risk of Map transmission within the herd. With a transition in the nature of immune response, predominant up-regulation of anti-inflammatory cytokines such as IL-10, TGF-β, IL-4 in PBMCs, ileum and associated lymph nodes is observed (Coussens et al., 2004; Sweeney et al., 1998; Stabel and Khalifeh 2004). Although a shift in the nature of immune response is well documented, the factors that govern this transition have yet to be elucidated (Coussens, 2001; Stabel, 2006).

1.3.4. Cytokines and their role in Johne’s disease

The role of different cytokines during the host immune response to MAP infection is been extensively studied. Immune and pathological aspects of Johne’s disease can be attributed to the various cytokines produced from MAP-infected macrophages, and T cells both at the tissue level and in the peripheral blood. The nature of the immune response and the cytokines produced during the pathogenesis of Johne’s disease can be classified into pro-
inflammatory, anti-inflammatory and regulatory. IFN-γ, IL12 constitute the protective pro-inflammatory cytokines whereas IL-10, TGF-β induces anti-inflammatory and immunoregulatory function.

IL-12 is a pro-inflammatory cytokine produced from DCs responsible for priming CD4+ T cells. It induces IFN-γ secretion by CD4+ T cells and initiates development of Th1 cell response against intracellular pathogens (Damenga et al., 2000).

IFN-γ is a Th1 polarizing cytokine implicated in the containment of mycobacterial infections during the early stages (Coussens, 2001; Stabel, 2006). IFN-γ assay, a diagnostic tool to detect sub-clinical stages of Johne’s disease is based on measurement of production of IFN-γ by Map-stimulated PBMCs (Stabel, 1995; Jungersen et al., 2002). Upregulation of gene expression of IFN-γ is noticed in ileum, mesenteric lymph nodes and in Map-stimulated PBMCs of sub-clinically infected animals (Sweeney et al., 1998; Coussens et al., 2004); however, as the disease progresses its expression is downregulated. The ability of IFNγ to enhance the anti-mycobacterial activity of macrophages against intracellular Map is well documented. It has been reported that IFN-γ induces nitric oxide synthesis and phagosome maturation in Map-infected macrophages, which negatively affects the intracellular survival of Map (Zhao et al., 1997; Hostetter et al., 2003; Khalifeh et al., 2009). However protective in nature, prolonged production of IFNγ at the infection site leads to chronic inflammation and contributes to immunopathology associated with Johne’s disease (Clarke, 1997; Coussens, 2001; Stabel, 2006).

IL-10 is an anti-inflammatory cytokine produced by macrophages and Tr1 (Treg cells producing IL-10) cells known for their immuno-regulatory role in the pathogenesis of Johne’s disease. Upregulation of IL-10 transcripts has been measured in the tissues of Johne’s infected animals during the clinical stage which further results in decrease in CD4+ T cell
activity and IFN-γ production (Khalifeh et al., 2004; Coussens, 2002). The immunosuppressive activity of IL-10 and its role in enhancing Map survival is well documented. Addition of exogenous IL-10 into Map-infected PBMC cultures, for example, resulted in increased Map viability (Khalifeh et al., 2002) and in MDMs-infected with Map, increased production of IL-10 and in decreased expression of IFN-γ, TNF-α, IL-12, and MHC molecules was detected. Additionally, increased production of nitric oxide, acidification, apoptosis that correlated with decreased Map viability was also observed (Weiss et al., 2005). Similarly, decreased levels of INF-γ and upregulation of IL-10 were noticed in Map-stimulated PBMCs harvested from sub-clinically infected animals (de Almeida et al., 2008). The increased production of IL-10 at the infection site is largely attributed to antigen-specific regulatory Treg cells (CD4+ CD25+) that proliferate at the infection site over time to regulate the immunopathology associated with protracted pro-inflammatory response in the infected host (Coussens et al., 2012). Following neutralization of IL-10, Buza et al. (2004) observed enhanced levels of IFN-γ expression in PBMCs of cattle infected with Map and stimulated ex vivo with Johnin purified protein derivative. The above findings are suggestive that IL-10 production suppresses IFN-γ production and further promotes Map survival and dissemination of the infection as the host enters clinical stage.

TGF-β is produced from Th3 cells known for its immunoregulatory role during clinical stages of Johne’s disease (de Almeida et al., 2008). Upregulation of TGF-β along with IL-10, and is evident in ileum, ileo-cecal lymph node, mesenteric lymph node is observed in cattle that are in clinical stages of infection that correlated with decreased expression of IFN-γ (Khalifeh et al., 2004). Similar changes were observed after addition of exogenous IL-10 and TGF-β into PBMC cultures infected with Map (Khalifeh et al., 2004).
IL-4 is a Th2 polarizing cytokine whose levels are upregulated during the transition from a protective cell-mediated immune response to ineffective antibody mediated immune response. Upregulation of IL-4 during clinical phase correlates with increased levels of detectable IgG1 antibody levels.

IL-17: Although less studied in the context of Johne’s disease, the role of Th17 cells in *Mycobacterium tuberculosis* (Mtb) infection has become apparent. It has been reviewed that, along with differentiation of Th1 cells, another subset of T cells called Th17 cells also undergo differentiation during the early stages of the immune response to Mtb infection (Khader et al., 2008). Th17 cells produce the cytokine IL-17, that is pro-inflammatory in nature and is thought to be responsible for early control and containment of Mtb within the granulomas; however, it is also reported that continuous production of IL-17 results in increased neutrophil recruitment and leads to extensive tissue damage at the site of infection. This suggests that IL-17, like IFN-γ, can be both beneficial and detrimental to the host (Torrado et al., 2010). It will be interesting to determine the role of Th17 cells and IL-17 in Map infection and its impact on the immune response in the infected animal.

IFN-β: It is been reported that IFN-β, a type 1 interferon, is preferentially expressed in humans during lepromatous (L-lep) form of *Mycobacterium leprae* infection, whereas IFN-γ expression was higher in self-healing tuberculoid-form of leprosy (Teles et al., 2013). The authors have concluded that IFN-β induces downstream IL-10 secretion that antagonizes expression of pro-inflammatory cytokines such as IFN-γ that is responsible for controlling mycobacterial infections. It would be of great interest to know if similar mechanisms also exist in Map infection that might play a role in disease outcome.
1.4. Genetics and Johne’s disease

1.4.1. Heritability to Map infection

In genetics, a heritability estimate is the measure of inheritance of a particular trait. The heritability estimates to Map infection in cattle range from 0.06 to 0.228 (Koets et al., 2000; Mortensen et al., 2004; Gonda et al., 2006; Kupper et al., 2012). This wide range of heritability estimates can be attributed to different statistical models used to derive estimates, and diagnostic tests (i.e. fecal culture, milk or serum Map-specific immunoglobulins, histopathology) utilized by researchers to classify the disease phenotype. However, the non-zero value of heritability estimate of these studies is an apparent indicator of the potential role of genetic determinants that render the animal vulnerable to Map infection. Similar to cattle, a heritability estimate of lifetime incidence in Romney and Merino sheep breeds for Johne’s disease is estimated to be 0.07 and 0.18 respectively (Hickey et al., 2003). Even though heritability estimates appear to be low-to-moderate, employing genetic selection strategies in breeding could prove constructive for producing off-spring that are more resistant to Map infection. The need to adopt genetic selection in breeding becomes even more apparent considering the lack of treatments and vaccines with high efficacy for Map infection.

1.4.2. Breed Susceptibility to Map infection

The heritable nature of Johne’s disease, its high incidence rate in cattle and sheep, and the economic implications associated with Johne’s disease have prompted researchers to investigate host genetic aspects associated with disease occurrence. Although the genetic basis of breed susceptibility to Map infection has not been clearly defined, it has been suggested that resistance may vary across breeds. A survey conducted by Cetinkya et al. (1997) reported on the high incidence rate of Johne’s disease in Channel Island breeds of cattle like Jersey and Guernsey (odds ratio 10.9-12.9) compared to Holstein Friesian breed.
Similarly Sorge et al. (2011) reported a higher odds ratio (1.4 to 8.3) of Jersey cattle being tested milk ELISA positive for Map antibodies in comparison with other breeds. After screening 4579 purebred cattle of 14 different breeds, Roussel et al. (2005) reported a greater odds ratio for *Bos indicus* purebreds (17-fold) and crosses (3.5-fold) as opposed to *Bos taurus* for being sero-positive for Map-specific immunoglobulins. For sheep, necropsy records have also shown significant differences in the mean lifetime incidence of ovine Johne’s disease in the Merino breed (4.78%) compared to the Romney breed (3.49%) of sheep (Hickey et al., 2003). These studies support the role of breed genetic characteristics that factor in defining susceptibility to Map infection.

### 1.4.3. Selection of phenotype

In order to selectively breed for disease resistance, a reliable diagnostic phenotype must be available to livestock breeders. With reference to Johne’s disease, the phenotype in question is resistance to Map infection. However, resistance to Map infection is a broad term that encompasses many factors. Given the complex nature of Johne’s disease and its development, indicators of Johne’s disease that can be selected as phenotypes include: direct indicators such as Map load in feces and tissues, and indirect indicators that assess the host immune response during infection.

#### 1.4.3.1. Direct indicators of phenotype:

a) Map load in feces – The shedding of Map in feces usually starts during late subclinical phase, is more frequent as the animal reaches clinical phase of infection and coincides with the inability of the host’s immune system to control Map. Detection of Map in feces can be achieved by Map culture of fecal samples or by molecular detection of Map DNA in the fecal samples. These methods have their own advantages and disadvantages. Detection of Map by culturing indicates presence of viable Map, their enumeration, and
potential for transmission. The specificity of culture technique is very high (99%) but it lacks sensitivity (~60%) (Collins et al., 2006) Another disadvantage of detection by culture is the longer incubation period (8-16 weeks) required to confirm the presence of Map.

b) Detection of Map DNA in feces – This method is highly sensitive with the quick turnaround time in comparison to detection by culture. However, the disadvantage with fecal PCR is its inability to confirm the presence of viable Map in the feces and may be affected by the presence of PCR inhibitors

b) Map load in tissues – Similar to Map load in feces, Map load in feces could be employed as direct phenotypic measure of Map infection but this is not feasible since it would require slaughtering the animal or surgical biopsy for sample collection which would be expensive and laborious.

1.4.3.2. Indirect indicators of phenotype:

a) Map specific antibodies in serum or milk - The presence of Map-specific antibodies in the animal is an indicator of exposure of the animal and a potential shift from cell-mediated immune response to antibody-mediated immune response. Commercially available ELISA diagnostic kits are employed to detect the presence of Map-specific antibodies. Optical density value obtained from the kit assay is transformed to express the results as sample-to-positive ratios (S/P) expressed in percentages. Sample-to-positive ratio is defined as the ratio of difference between optical density of sample and negative control to the difference between the optical density of sample and positive control. Heritable estimates for infection status, as determined by antibody detection in milk samples have been found to range from 0.031-0.097 (van Hulzen et al., 2012). Detectable levels of IgG1 antibody subtypes appear in serum and milk as the animal reaches the clinical phase of infection after fecal shedding is seen. Milk/serum ELISA is the commonly used method employed for
detection of Map-specific antibodies as it is cost-effective, simple to perform with a quick turnaround time as compared to detection by culture. However, the drawback of this method is its low sensitivity (30%) that may not allow for detection during the early phase of infection and could yield erroneous false negative results.

b) Cell-mediated immune response detection – This involves measuring interferon-gamma (IFN-γ) that drives the cell-mediated immune response in the infected animal. Interferon-gamma released from the lymphocytes after in vitro challenge with Map antigen is measured by ELISA and is used as an indicator of exposure or disease status of the animal to detect the disease status. However, this method can only detect early phase of infection and is highly variable.

As discussed above, all of the assays that can be used to define the disease phenotype lack reliability and can be variable, therefore the best approach to define the Johne’s disease phenotype should ideally include a direct and indirect indicator of disease resistance.

1.4.4. Genetic studies concerning Map infection

In addition to breed-specific studies, evidence for the genetic basis for susceptibility to Johne’s disease has also been found through candidate gene and genome-wide association studies. Both types of association studies use genetic markers that can be used in selective breeding program to breed for resistance to Johne’s disease. Marker-assisted genomic selection have the potential to reduce the generation interval thereby speeding up the breeding program because animals can be selected based on genotype as opposed to phenotype. Unfortunately different phenotypes have been used in association studies and this adds to uncertain contribution of each gene as discussed below.
1.4.4.1. Candidate gene studies

Candidate gene studies involve studying the association between DNA polymorphism in a specific gene and a phenotype. The candidate genes are selected based on information available in scientific literature, and based on their functional role in the pathogenesis of Johne’s disease, or a similar disease, such as human Crohn’s disease. Candidate gene studies usually involve a case-control experimental design where gene polymorphisms in the candidate gene serve as genetic markers that can be used to investigate statistical associations with the disease phenotype. Polymorphisms in the candidate genes are typically either SNPs or micro-satellite markers. Genes coding the proteins of the immune system are some of the candidate genes that have been studied in the context of Johne’s disease. It has been reported that polymorphism in the genes: Toll-like receptors 1, 2, 4 (Mucha et al., 2009, Koets et al., 2010); Nucleotide binding oligomerization domain containing 2 (NOD2) (Pinedo et al., 2009; Ruiz-Larranaga et al., 2010); Solute carrier family 11 member 1 (SLC11A1); (Reddacliff et al., 2005, Ruiz-Larranaga et al., 2010); Major Histocompatibility complex (Reddacliff et al., 2005); IL-10Rα (Verschoor et al., 2010); SP110 (Ruiz-Larranaga et al., 2010); and IFN-γR2, IL-12Rβ1, IL-12Rβ2, IL-23R (Pant et al., 2011) have been associated with Map infection status in different cattle populations.

a) Toll-like receptors - 1, 2, and 4 are pattern-recognition receptors that recognize Map-associated membrane patterns and initiate the host innate and adaptive immune responses in the infected host; b) NOD2 (CARD 15) is a PRR implicated in recognition of the mycobacterial cell wall constituent, muramyl dipeptide (Girardin et al., 2003). NOD2 further stimulates the transcription factor NF-κB that regulates pro-inflammatory cytokine expression (Abbott et al., 2004).
b) Cytokines:

INF-γ, IL-12 and IL-10 are the cytokine genes that are studied for their associations with Johne’s disease. IFNγ and IL-12 are pro-inflammatory protective Th1 cytokines that control the infection in the early stages and play a major role in the early cell-mediated immune response driving Th1 response in the infected host (Coussens et al., 2004). IL-10 is an anti-inflammatory, immunoregulatory cytokine that is involved in regulating the host inflammatory response to Map infection and its levels are high during the clinical phase (Khalifeh et al., 2004).

c) Antigen presenting molecules and metal binding proteins: In Merino sheep, Reddacliff et al. (2005) identified associations between MHC alleles and Map infection; MHC molecules are responsible for presenting processed antigen from antigen presenting cells to T helper cells and in initiating adaptive immunity in the infected host. SLC11A1, formerly called as NRAMP1, is a divalent phagosomal metal ion (Mn$^{+2}$, Fe$^{+2}$) transporter that controls intracellular bacterial replication by regulating inducible nitric oxide synthesis (Soe-Lin et al., 2008).

1.4.4.2. Genome-wide association studies

Genome-wide association studies aim to identify Quantitative trait loci (QTLS) on chromosomal regions associated with phenotype of interest. The identified regions can be further investigated for presence of candidate genes SNPs. With respect to Map infection in cattle, four genome-wide association studies (GWAS) and two genome-wide linkage analyses studies have been carried out till date.

Gonda et al. performed the first genome-wide linkage analysis study involving 12 paternal half-sib families, using micro-satellite markers and identified a QTL on bovine
chromosome (BTA) 20 that was found to be significantly associated with Map infection based on samples testing positive for either for serum ELISA, fecal culture, or both (Gonda et al., 2007). In another sire-maternal grand sire genome linkage analysis study, Van Hulzen et al. (2012) identified 5 SNPs in chromosomes 4, 15, 18, and 28 and 13 other putative SNPs associated with Map-specific antibody response in milk; this finding implicates polygenic effect on this disease phenotype.

Settles et al. (2009) performed the genome-wide association study (GWAS) in 245 Holstein cows from three different herds in a case-control study design. In this study, infection status was based on presence of Map in tissue, presence of Map in feces, presence of Map in both tissue and feces and Map culture positivity in tissue, feces, and both. Genotyping was carried out using Bovine SNP50 BeadChip. The authors identified two QTLs, one on chromosome 3 associated with tissue culture positivity, and another on chromosome 9 to be significantly associated Map culture positivity in both tissues and feces.

In another GWAS, Pant et al. (2010) identified 12 putative QTLs for resistance to Map infection in a resource Holstein population (n=232) obtained from 6 different commercial herds in Southern Ontario. The disease phenotype used in this case-control study included either milk or serum ELISA testing. The authors identified 4 SNPs (ss61491930, ss61558503, rs43505295 and ss86310793) on chromosome 7 that were significantly associated with resistance to Map infection after accounting for genome-wide correction for multiple testing. Candidate genes such as IL-4, IL-13, IL-5, IRF1, SLC39A3 (Solute carrier family 39, member 3), TNFIP8L1 (Tumor necrosis factor, alpha-induced protein like 1) and TICAM1 (Toll-interleukin 1 receptor domain containing adaptor molecule) were found within or adjacent to these surrounding QTL regions. Minozzi et al. (2010) also identified QTL on chromosomes 9, 11 and 12 that were associated with serum antibody response.
Lastly, Kirkpatrick et al. (2010) performed a case-reference study involving two independent populations comprising cases (tested by ELISA and fecal culture) were compared with genotypes from sires that are used for AI in US dairy industry. Data from the two populations were analyzed separately and combined and the authors identified 51 SNPs associated with susceptibility to Map infection.

1.4.5. Conclusion

Given the heritable nature of Johne’s disease, the issues associated with its control, its detection, and the existence of genetic variants, it may be reasonable to employ genetic selection in breeding programs to produce off-springs with enhanced resistance to Johne’s disease. Genetic association studies have helped identify genetic variants that are associated with susceptibility or resistance to Map infection. However, there are a number of uncertainties associated with these studies. a) The uncertainty of diagnosing Johne’s disease, for example, may introduce disease classification bias. Additionally, since Johne’s disease is a complex chronic disease with multiple stages seen during its pathogenesis, the genetic associations seen during one stage of the disease may differ from the other disease stages and drawing a conclusive inference can therefore be not possible. b) Phenotype selection: As mentioned above, in particular reference to case-control study designs, methods employed to define the disease phenotype vary, and this could influence the conclusions that are derived. Also, GWAS indicate that genetic susceptibility/resistance to Map infection is polygenic in nature which suggests that genetic selection could be challenging without government incentives for large scale genotyping programs. All these reasons help to explain why results from each GWAS conducted so far are inconsistent (Kirkpatrick and Shook, 2011). It is also possible that genetic variation may differ between different populations and there is a need for more validation studies.
Table 2. Candidate gene polymorphisms significantly associated with susceptibility to Map infection in cattle and sheep.

Source: Kirkpatrick and Shook (2011); Purdie et al. 2011 with some modification

<table>
<thead>
<tr>
<th>GENE</th>
<th>Species</th>
<th>Polymorphism (Location)</th>
<th>Location</th>
<th>Test used</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD2</td>
<td>Cattle</td>
<td>c.2197 T&gt;C (LRR)</td>
<td></td>
<td>Fecal culture, PCR, ELISA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.1908 C&gt;T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC11A1</td>
<td>Cattle</td>
<td>Microsatellite 1067 C&gt;G</td>
<td>Transmembrane domain 8</td>
<td>Fecal culture, PCR, ELISA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1157-91A&gt;T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP110</td>
<td>Cattle</td>
<td>C.587 A&gt;G</td>
<td></td>
<td>Fecal culture, ELISA</td>
</tr>
<tr>
<td>IL10RA</td>
<td>Cattle</td>
<td>984G &gt; A, 1098C &gt; T, 1269T &gt; C, and 1302A &gt; G All coding regions</td>
<td>All polymorphisms in the coding regions</td>
<td>Milk ELISA</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR1</td>
<td>Cattle</td>
<td>G658A (Ectodomain)</td>
<td>Ectodomain</td>
<td>Clinical signs</td>
</tr>
<tr>
<td>TLR4</td>
<td>Cattle</td>
<td>892G&gt;A;895G&gt;A;1165G&gt;A</td>
<td>Ectodomain</td>
<td></td>
</tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td>TLR2</td>
<td>Cattle</td>
<td>2038A&gt;G)</td>
<td>Toll/IL-1R domain</td>
<td>Clinical signs</td>
</tr>
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<td></td>
<td></td>
<td>1903T/C</td>
<td>Putative LRR</td>
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</tr>
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<td>Sheep</td>
<td>448A&gt;G</td>
<td>Ectodomain</td>
<td>Clinical signs, ELISA, PCR</td>
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<td>517G&gt;Y</td>
<td>Coding</td>
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<td>658A&gt;G</td>
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<td>Sheep</td>
<td>2008A&gt;Y; 2037T&gt;C</td>
<td>Toll/IL-1R domain</td>
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<tr>
<td>TLR4</td>
<td>Sheep</td>
<td>1066T&gt;C</td>
<td>Ectodomain</td>
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</tr>
</tbody>
</table>
CHAPTER 2
Experimental Rationale, Hypothesis and Objectives

2.1. Rationale

Johne’s disease is chronic, granulomatous enteritis of domestic and wild ruminants caused by the intracellular bacteria Map. There have been two major concerns about the disease in recent years: first, the disease has severe economic implications for the dairy and beef industries; second, Johne’s disease is attracting attention because of a possible association between Map and Crohn’s disease, an inflammatory bowel disease in humans.

Although the genetic susceptibility to Map infection in cattle has been widely reviewed, there is little conclusive evidence of breed susceptibility. A few reports suggest that Channel Island breeds of cattle such as the Guernsey and Jersey breeds are more susceptible than the Holstein Friesians (Cetinkya et al., 1997; Mcnab et al., 1991; Jakobsen et al., 2000; Sorge et al., 2011). Albeit, these findings point towards genetic component to susceptibility, they fail to define the underlying genetic basis for the existence of such breed susceptibility in cattle.

Immune response is a critical defense mechanism that determines the outcome of any infection. As discussed earlier, the nature of immune response elicited in the host against Map infection has a greater influence in the fight against infection and its eventual outcome. Previous studies concerning immune response have determined systemic and local tissue response in cattle classified as sub-clinical and clinically ill, respectively (Coussens et al., 2004; Sweeney et al., 1998; Khalifeh et al., 2004; Stabel, 2000). Although these studies provide insights about nature of immune response during a particular stage of infection, it must be noted that classifying animals as sub-clinical and clinically is not straightforward as
reviewed before. Also, it should be considered that calves less than 6 months of age are highly susceptible to Map infection and it is the early immune response mounted post-infection is the critical factor that determines disease pathogenesis (Hines et al., 2007)

As there are evidences for association between SNPs in immune candidate genes and Johne’s disease and with the above rationale behind i.e. the influence of age, early immune response, one way of addressing the knowledge-gap concerning breed-susceptibility is by experimentally infecting Holstein-Friesian and Jersey calves with Map followed by comparing the nature of early immune response elicited in them. The differentially expressed genes, if any observed, could be further investigated for genetic variants that could be used in candidate gene studies to assess breed susceptibility to Map infection.

Therefore, the objective of this thesis is to determine and compare the cytokine gene expression profiles in ileocecal lymph nodes of experimentally Map-infected Holstein Friesian and Jersey calves. The cytokines selected for analysis represent different arms of immune response and includes: IFN-γ and IL-12 promotes a Th1 cell-mediated immune response against intracellular pathogens response; IL-13 is associated with Th2 response and antibody-mediated immune response against intracellular pathogens; IL-10 and TGF-β associated with T-regulatory immunoregulatory response; IL-17 and IL-23 associated with inflammatory response; and IFN-β based on its role in Mycobacterial infections (Coussens, 2001; Teles et al., 2013).

The results of this study could help determine breed-specific immune factors associated with Map infection.
2.2. Experimental Hypothesis

Our hypothesis is that the expression of pro-inflammatory cytokines such as IFN-\(\gamma\), IL-12, IL-17, IL-23 will be lower in Jersey breed in comparison to Holstein-Friesian and this will probably influence breed-susceptibility. This is based on the role of pro-inflammatory cytokines in early control and containment of Map infection.

2.3. Objectives

a) To measure early changes in the transcript levels of cytokines (IFN-\(\gamma\), IL-10, TGF-\(\beta\), IL-12, IL-17, IL-23, IL-13, IFN-\(\beta\)) in the ileocecal lymph node from Holstein-Friesian and Jersey calves infected with Map; and b) Compare the measured cytokine transcripts across two breeds to further the understanding of breed susceptibility to Map infection.
CHAPTER 3

Cytokine gene expression in Holstein-Friesian and Jersey calves infected with

*Mycobacterium avium* subsp. *paratuberculosis* (Map)

3.1. Abstract

Genetic susceptibility to Johne’s disease is well studied. Few reports have suggested breed susceptibility indicating that Channel Island breeds of cattle such as Jersey are more susceptible as compared to Holstein-Friesian breed to Map infection. However, the genetic basis to define influence of breed on susceptibility is yet to be explored. In this study, experimental infection trials were conducted by surgically injecting Map into ileocecal peyers patches of Holstein-Friesian and Jersey calves to determine and compare the expression of key cytokine transcripts (IFN-γ, IL-10, IL-12, IL-23, TGF-β, IL-13, IL-17, IFN-β) during the first 60 days after infection. Real-time PCR analysis of gene expression in ileocecal lymph node did not reveal any breed-specific differences in the cytokines investigated. The expression of IFN-γ was found to be significant between the treatment groups in both breeds as its expression was found to be higher in infected calves compared to controls. Based on these findings, it can be concluded that breed-specific differences do not exist at sixty days post-infection. It further warrants long-term experimental infection studies to understand and define breed susceptibility given the chronic nature of Johne’s disease.

3.2. Introduction

Johne’s disease is a chronic debilitating disease of ruminants caused by the bacteria *Mycobacterium avium* subsp. *paratuberculosis* (Map). This mycobacterial disease, with a worldwide prevalence, is responsible for severe economic losses to the dairy and beef industries. The chronic nature of the disease and the lack of reliable diagnostic techniques
that can detect the disease at early stages complicate its control (Tiwari et al., 2006). Additionally, the available vaccines lack efficacy in that they can only prolong the onset of disease without eliminating the infection (Koets et al., 2006; Sweeney et al., 2009). Genetic studies have shown that Johne’s disease is heritable, and numerous genetic variants that are associated with susceptibility and resistance of animals to Map infection have been identified (Koets et al., 2000; Mortensen et al., 2004; Gonda et al., 2006; Kupper et al., 2012). Additionally, a few studies have reported that the Jersey breed of cattle is more susceptible to Map infection as compared to Holstein-Friesian, thereby implicating breed susceptibility (Mcnab et al., 1991; Cetinkaya et al., 1997; Jakobsen et al., 2000; Sorge et al., 2011).

The immune response to Map infection within the host is complex. For example, a transition in the nature of the immune response has been described as the disease progresses, with an initial strong Th1 cell-mediated response during early subclinical infection being essential for activation of macrophages and subsequent control of infection (Coussens, 2001). In order to favour its survival within the host, Map has evolved strategies to regulate the immune response (Sohal SJ et al., 2008). As the disease progresses, a gradual shift in the immune response from a protective Th1-to-Th2 antibody-mediated response possibly takes place in response to excessive tissue damage or increased antigen load. Since the Th2 mediated antibody response is apparently not capable of controlling infection, it results in dissemination of infection within the host further leading to spread of the disease within the herd. Different immune factors that play a role during the host immune response and pathogenesis involve pro-inflammatory, anti-inflammatory and immuno-regulatory cytokines. It has been shown that the expression of different cytokines varies at different stages of infection and is likely responsible for polarizing the immune response in the host. The expression of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1α aimed at controlling
bacterial replication is higher during subclinical stages of infection (Coussens et al., 2004; Sweeney et al., 1998; Khalifeh et al., 2004). Anti-inflammatory and immunoregulatory cytokines such as IL-10, TGFβ, IL-4 expression increases as the disease progresses from late subclinical to clinical stages (Coussens et al., 2004; Sweeney et al., 1998; Stabel and Khalifeh 2004). These cytokines are produced in response to excess inflammatory changes at the infected site and are also thought to be induced by Map to escape intracellular killing.

There is little evidence to define the genetic basis of breed susceptibility. As there is evidence for genetic susceptibility and association of immune factors as candidate genes with Johne’s disease, one way of defining the genetic basis of breed susceptibility is by understanding and comparing the immune response in different breeds during Map infection. With this rationale behind, the objectives of this study were to measure: a) early changes in the transcript levels of important cytokines at the site of infection (ileocecal lymph node) in Holstein Friesian and Jersey calves infected with Mycobacterium avium subsp. paratuberculosis; and b) compare the same across two breeds to further the understanding of breed susceptibility to Map infection.

3.3 Materials and Methods

3.3.1. Experimental design and housing

A total of 29 calves comprising of Holstein-Friesian (n=14) and Jersey (n=15) bull calves were used for this study. The infection trials were carried out in 3 different blocks as outlined in table 2. The Holstein-Friesian bull calves were acquired from the University of Guelph Elora and Ponsonby research stations and the Jersey calves were purchased from local Jersey dairy farms of Southern Ontario. These farms actively participated in routine Johne’s testing and only calves from Johne’s-free dams were used for this study. Immediately
after birth, the calves were separated from their mother and were fed commercially available artificial colostrum (Calf’s Choice Total™, the Saskatoon Colostrum Company Limited provided by Grober Inc.,) as per manufacturer’s instructions. The entire experimental trial was carried out in University of Guelph Central Animal Facility Biosafety Level 2 research isolation unit, where the calves were housed in pairs according to breed, and the uninfected control and infected calves were kept separate for the entire study duration. The study was reviewed and approved by the University of Guelph Animal Care Committee. All the procedures and management of calves was in compliance with the rules and regulations of the Canadian Council of Animal Care.

3.3.2. Inoculum preparation

GC86mCherry strain of *Mycobacterium avium* subsp. *paratuberculosis* (Map) was used for this experimental study. GC86 is a field strain of Map isolated from the feces of infected cows in Southern Ontario by members of Dr. Mutharia’s laboratory. This strain was transformed to express a fluorescent marker mCherry by a graduate student from our laboratory, Philip Mead, and used for the infection of calves in this study. The bacterium was cultured in 7H9 Middlebrook broth (Sigma-Aldrich) supplemented with 10% (v/v) of Oleic acid-albumin-dextrose-catalase (OADC), 5 g/L glycerol, 1 g/L casitone, 2 mg/L mycobactin J (Allied Monitor, Fayette, MO, USA) and 0.025 % (v/v) tyloxapol. Upon reaching the log phase (OD: 0.8-1.0) of growth, the bacterial culture was harvested, and 1 mL aliquots of the culture to be used as inoculum were made and were frozen until further use. Seventy-two hours preceding infection of calves, the frozen inoculum was thawed and incubated at 37°C. On the day of infection, each aliquot was centrifuged, the supernatant discarded, and the pellet suspended in 250 µL normal saline solution; this was used as the challenge inoculum for each calf. The number of colony forming units (CFUs) in each inoculum was determined to be approximately $10^8$ CFU after serial dilution and plating on 7H11 agar plates. The purity
of inoculum was tested for contamination by streaking on Luria-Bertani (LB) and Brain heart infusion (BHI) agar plates and IS900 positivity of Map was confirmed by PCR.

3.3.3. Animal Infection

Under aseptic conditions, infection of each calf was carried out by direct inoculation of 250 µL challenge inoculum containing \(10^8\) CFUs of Map into ileo-cecal peyer’s patches as described by Plattner et al. (2011). Under local anesthesia by lidocaine, a 3- to -4 cm vertical incision was made in the paralumbar fossa. After gaining access through peritoneum into abdominal cavity, the distal ileocecal junction was isolated and exteriorized. This was followed by injecting 250 µL of challenge inoculum containing \(10^8\) CFU of Map per calf inoculum into antimesenteric peyer’s patches using a 26G needle attached to a 1mL tuberculin syringe. The exteriorized intestine was replaced into the abdominal cavity and the wound was closed in a standard 3 layer suture pattern. Similarly, the control groups of calves were surgically injected with 250 µL normal saline into their peyer’s patches. To mark the site of infection, sterile diluted India ink (1:100 in saline) dye was injected into sub-serosa.

3.3.4. Tissue sample collection

Sixty-days post-infection, the calves were humanely euthanatized by intravenous overdosing of pentobarbital sodium (Euthansol\textsuperscript{TM} (102 mg/kg); Schering-Plough Canada Inc.,) Tissues for Map bacterial culture, histopathology and gene expression studies were aseptically collected: for Map culture, the distal ileum and ileocecal lymph nodes were collected in sterile bags (Nasco Whirl Pack\textsuperscript{®}). For histopathology, ileum, ileocecal valve were collected in 10% neutral buffer formalin, and for gene expression studies ileum, ileocecal lymph node, mesenteric lymph node, liver, and spleen were collected in 2 mL cryo-vials (Corning) and immediately snap-frozen in liquid nitrogen and stored at -80\textdegree C until they were used.
3.3.5. Tissue culture

Pairs of the distal ileum and ileocecal lymph nodes collected from each calf were submitted to University of Guelph Laboratory Services Division (Guelph, ON) for BACTEC culture. Tissues samples that were BACTEC culture positive were also subjected to IS900 PCR and acid-fast staining to confirm the presence of Map.

3.3.6. Experimental parameters studied

The objective of this thesis was to quantify the gene expression of immunoregulatory cytokines in the ileocecal lymph nodes of Jersey and Holstein-Friesian calves infected with Map by real time PCR. Accordingly, key cytokines that play important role during the immune response of Johne’s disease and that represent different arms of immune response were selected. The genes included: interferon-γ (IFN-γ), interleukin-13 (IL13), interleukin-10 (IL-10), transforming growth factor-β (TGF-β), interleukin-12 (IL-12), interleukin-23 (IL-23), interleukin-17 (IL-17), and interferon-β (IFN-β). Since we used florescent marker expressing strain in this study, we were also interested in determining if there was still stable expression of florescent marker, mCherry, from calf passaged GC86mCherry Map strain.

3.3.7. Total RNA extraction

Approximately 50-100 mg of ileocecal lymph node archived at 80°C was homogenized using tissue homogenizer (Pellet Pestle mortar, Kontes) and total RNA extraction was done using Trizol® reagent (Invitrogen) as per manufacturer’s instructions. Total RNA concentration was determined using NanoDrop (ND-1000, NanoDrop Technologies, Wilmington DE) and the 260:280 ratio of all the samples was determined to be >1.7. RNA quality was further assessed using Agilent Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and samples with RIN above 5 were used for cDNA synthesis.
3.3.8. cDNA conversion

For cDNA synthesis, a total of 1 µg of total RNA from each sample was used for reverse transcription. A reaction mix containing 1 µg of RNA, 1 µL of oligo(dT)_{12-18} (Invitrogen) and 1 µL of 25 mmol dNTP was prepared and diluted to 13 µL by adding nuclease-free water. This reaction mix was incubated at 65 °C for 5 minutes, and quickly chilled on ice. Then 4 µL of 5X first strand buffer, 1 µL of 0.1 mM DTT and 1 µL of reverse transcriptase (Superscript III, Invitrogen) was added for cDNA synthesis at 50 °C for 60 minutes in a thermocycler.

3.3.9. RT-PCR

Semi-quantitative expression of target genes was determined using the ABI Prism Sequence Detection System and ABI prism 7000 SDS software (Applied Biosystems, Burlington, ON, Canada). Real-time PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) and the PCR conditions consisted of 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. Dissociation curves were generated at the end of amplification to ensure presence of single amplified product. Differential expression analysis was performed by standard curve method by two-fold dilution of pooled cDNA ranging from 1:40 to 1:1.25. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene for this study because its expression was found to be constant across treatments.

Primers of each gene were designed using online software Primer3 software and purity and size of gene products was confirmed on a 2% agarose gel. The primer sequences, annealing temperature and the amplicon length are summarized in Table 3.
Average Ct value of each sample was obtained from auto Ct function of ABI prism 7000 SDS software. Input values for target genes and housekeeping gene was derived by standard curve developed after serial dilution of pooled cDNA from 40 ng to 1.25 ng. Relative expression of target genes was determined after standardizing their expression to housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression by dividing the input values of target gene by the input value of housekeeping gene. The expression of GAPDH was constant across all treatments.

3.3.10. Statistical Analyses

The gene expression data obtained were log transformed as residual plots indicated skewed distribution with variance increasing with the mean. The data was further subjected to Brown and Forsythe test for homogeneity of variance between breeds and between treatment groups using Proc GLM procedure of SAS. The Brown and Forsythe test indicated no breed differences between the variances of the transformed data, but indicated two genes; IL-17 and IFN-β had different variance between treatment groups. A simple contrast test was done to compare the expression between control calves from both breeds.

The real-time expression data was analyzed by ANOVA using Proc. Mixed procedure of SAS as randomized complete block design where the effects of breed (Holstein-Friesian and Jersey), treatment (control and infected), and their interaction were considered as fixed effects, and different blocks as a random effect. Models for IL-17 and IFN-β also accounted for different variance in each treatment groups. Least square means were determined and compared to assess the treatment effects in two breeds. Statements of statistical significance were based on P<0.05.
3.4. Results

3.4.1. Post surgical changes

No calves developed clinical signs pertinent to Johne’s disease post surgical infection. Normal healing was observed at the incision site; however, 5 calves had swelling at the surgical site. Due to persistent purulent inflammation, such calves were treated accordingly as per a veterinarian’s advice that included irrigating the purulent site with dilute betadine solution and injecting antibiotic ceftiofur sodium (0.5 mg/kg body weight, subcutaneous injection for 3 days) which has been previously reported (Hines et al., 2007)

3.4.2. Necropsy lesions

Except for peritoneal adhesions of omentum at the surgical site, no gross pathology related to Map infection was observed during post mortem examination of calves.

3.4.3. Histopathology

Ileocecal valve, ileum and ileocecal lymph node from each calf was examined for histopathological changes. No lesions were observed in control treatment calves. The histopathological changes in infected group of calves were variable and the observed lesions included presence of aggregates of macrophages in the lamina propria of 15 of the 17 infected calves (Fig. 3.1A). Additionally, sporadic presence of Langhans-type multinucleated giant cells was observed in 4 of the 17 calves. Ziehl-Nielssen staining showed the presence of acid-fast bacteria within macrophage aggregates in 4 calves (calf No. 2, 5, 10 and 11; Fig 3.1B). No lesions were identified in 2 infected calves.
3.4.4. Bacterial Culture

Ileocecal valve and ileocecal lymph node from each calf was submitted for Map BACTEC culture. Tissue samples from control group of calves were all culture negative. Except for four calves in the infected group, all other calves had at least one tissue being BACTEC culture positive and confirmation was done by PCR and acid-fast staining for Map. Ileal tissue was found to be culture positive in majority of infected calves (10/17). In three calves, both ileum and ileocecal lymph node were found to be Map-culture positive. The two calves with no lesions detected were culture positive.

3.4.5. Real-time PCR

Relative expression of key cytokine transcripts in ileocecal lymph node was determined using GAPDH as housekeeping gene. A significant overall difference in the expression of IFN-γ between control and infected animals (p<0.02) with a higher expression level in infected than in control animals was observed in both Holstein and Jersey breeds, but there was no indication that this differed between the breeds. The expression of IL-23 transcript showed significant interaction effect between breed and treatment (p<0.05). Least-squares means indicated the average expression of IL-23 was lower in infected Holsteins than in control Holsteins, but was higher in infected Jerseys than in control Jerseys. Comparison of IL-23 expression between the control groups of both breeds indicated a significant difference (p<0.05) in the expression of IL-23, wherein the expression was found to be higher in Holstein control calves in comparison to Jersey controls. No significant breed or treatment differences were observed in the expression of IL-10, IL-12, IL-17, IL-23, TGF-β, and IFN-β.
3.5. Discussion

The goal of this study was to determine and compare early immune response in Holstein-Friesian and Jersey calves infected with Map and to further the understanding of breed susceptibility in Johne’s disease. There are reports suggesting higher incidence rates of Johne’s disease in Channel Island breeds like Jersey compared to Holstein-Friesian (Witters, 1959; Jorgensen, 1972; Cetinkaya et al., 1997; Jakobsen et al., 2000; Sorge et al., 2007). These reports are based on incidental occurrence of Johne’s disease based on survey analysis, milk ELISA test results and point towards a possible genetic influence in conferring breed susceptibility.

To address this further, we experimentally infected Holstein-Friesian and Jersey calves by direct inoculation of Map into ileocecal peyer’s patches and analyzed the expression of key cytokines in ileocecal lymph node to determine early immune response. Ileocecal lymph node was selected because it is the closest draining lymph node to the site of infection. The lymph node is an important secondary lymphoid tissue that acts as a central hub for antigen presentation and initiation of the host immune response. The sixty-day experimental exposure period was based on the exposure period that was used to develop the infection model (Plattner et al., 2011). It was observed that at sixty days post-infection, for example, there was significantly higher expression of Map-specific IFN-γ in the ICLN as compared to peripheral blood mononuclear cells (Plattner et al., 2011). Our hypothesis is that the expression of pro-inflammatory cytokines such as IFN-γ, IL-12, IL-17, IL-23 will be lower in Jersey breed in comparison to Holstein-Friesian and this will probably influence breed-susceptibility. This is based on the role of pro-inflammatory cytokines in early control and containment of Map infection.
Considering the Th1/Th2 paradigm associated with immune response in Johne’s disease, eight cytokines that represent different immune functions were selected for real-time analysis: IFN-γ and IL-12 promote a Th1 cell-mediated immune response against intracellular pathogens response; IL-13 is associated with Th2 response and antibody-mediated immune response against intracellular pathogens; IL-10 and TGF-β are associated with the regulatory T cell immunoregulatory response; IL-17 and IL-23 are associated with the host inflammatory response; and IFN-β has been reported to regulate IFN-γ during Mycobacterial infections (Coussens, 2001; Teles et al., 2013).

The expression of cytokine transcripts in ileocecal lymph node was determined by real-time PCR. Following analysis of real-time expression data, one of the observed findings was the significant interaction between breed and treatment in the expression of IL-23. Least square means of IL-23 expression indicated cross-over interaction as its expression was found to be lower in infected Holsteins compared to control Holsteins and higher in infected Jerseys compared to control Jerseys. A significant difference in the expression of IL-23 was also observed between Holstein and Jersey controls, with higher expression seen in Holsteins (1.194 fold increase). Since there was interaction effect between breed and treatment, the main effect of breed and treatment could not be considered independent and their effects were non-testable. To our knowledge, the role of IL-23 in immune response against Map in cattle has not been investigated. IL-23 is a pro-inflammatory macrophage cytokine that mediates proliferation of a subset of IL-17-producing CD4+ T cells called Th-17 cells (McKenzie et al., 2007). In sheep, it was shown that there was significantly higher expression of IL23A expression in ileal mucosa in paucibacillary form of paratuberculosis in comparison to multibacillary form aimed at increased lymphocytic infiltration and inflammatory changes to inhibit bacterial replication a (Gossner et al., 2012). Since the paucibacillary form is associated with predominance of Th1 response aimed at inducing inflammatory changes, and
the multibacillary form is predominated by antibody response, the above finding further validate the inflammatory role of IL-23. Khader et al. (2007) have reported increased IFN-γ response, chemokine secretion with accumulation of CD4+ T cells coupled with decreased bacterial growth in lungs after treatment with exogenous IL-17 in IL-23 deficient mice that were vaccinated and subsequently challenged with *Mycobacterium tuberculosis*.

Furthermore, within breed comparison revealed a significant difference in the expression of IFN-γ between the treatment groups. In both breeds, the expression of IFN-γ in infected calves was significantly higher (1.45 fold in Holstein and 1.43 fold in Jerseys) than control animals (Figure 3.1). This further reiterates the role of IFN-γ during early immune response against Map and is also a reflection of active immune status of the infected animal against Map. IFN-γ is a pro-inflammatory cytokine that mediates cell-mediated Th1 immune response aimed at enhancing anti-mycobacterial activity of macrophages against Map. The significant treatment difference observed in its expression is a reflection of active immune status in the Map-infected animal trying to combat the effects of Map. This result further reiterates the role of IFN-γ post-infection and validates the use of this infection model and exposure period for studying subclinical stages of MAP infection. Since there was no breed-specific difference in the expression of IFN-γ was found, it can be concluded that IFN-γ expression is not different between breeds at 60 days post-infection. It may be possible that differences might be observed as the disease progresses and might possibly influence breed-susceptibility in the longer run.

It is of no surprise that the expression of Th2 polarizing cytokines and immunoregulatory cytokines did not show any differences between the two breeds. These cytokines are induced during the late subclinical and clinical stages of infection (Coussens, 2001) and they might play a more significant role as the disease progression takes place.
It should be noticed that not all the infected calves were shown to be culture-positive, and in two calves we were unable to identify any histopathological changes. One of the calves (calf number 10) for example, had very distinct histopathological lesions with presence of acid fast bacteria, but was culture negative. The reason for this lays in the apparent difficulty in sampling of tissue and low sensitivity (~60%) of detection of Map by culturing (Collins et al., 2006). Another explanation for culture negativity is that only a small amount of tissue was processed for Map culture and it is likely that MAP is not uniformly distributed in the tissue.

One of the other objectives of this study was also to assess the stability of expression of florescent marker, mCherry, incorporated in GC86 Map strain used in this study. One of the graduate students in our lab, Philip Mead, cultured the calf passaged GC86 bacteria and examined for its florescence. Microscopic images captured indicated the presence of mCherry marker and its stable expression 60-days post infection in the GC86 strain (Figure 3.3 A and B). This finding opens up possible avenues that may help in understanding pathogenesis and host-pathogen interaction in the future. Although much work needs to be done in this area, we hope that the expression of this florescent marker might allow us to differentiate infected cells versus non-infected cells and in the process develop a better understanding of host cell dynamics against the invading pathogen.

3.6. Conclusion

In conclusion, to our knowledge, this is a first of such study that compared breed-specific gene expression changes in Holstein-Friesian and Jersey calves experimentally infected with Map. Our findings did not indicate any breed-specific differences in the cytokines investigated at least during first sixty days post-infection that might have played a putative role in defining breed-susceptibility to Map infection. It is well defined that calves
less than six months of age are susceptible to infection and further development of disease is largely dependent on its ability to evoke protective immune response post-infection. Since there is a knowledge-gap concerning breed susceptibility to Map infection, we believed that one way of addressing it would be by measuring and comparing early immune responses in Holstein-Friesian and Jersey calves infected with Map. One of the limitations of this study is the limited duration of time (60 days post-infection) for which the infection trials were carried out for. It only gives an understanding about immune response at one time point post-infection. As Johne’s disease is a chronic disease with a variable incubation period (2-5 years) and since there were no breed-specific differences observed at sixty days post-infection, it would be beneficial in future to conduct long term experimental infection study. It could be possible that breed-specific differences might be observed as the disease progresses and it may serve well in defining breed susceptibility to Johne’s disease. This could be further addressed by carrying out longitudinal infection studies with different end points to conclusively define breed susceptibility in cattle to Johne’s disease. Complete gene-expression profiling of tissues can also be considered to identify factors other than immune that could be breed-specific.
### Table 3.1: Block experimental design of Map infection study using Holstein-Friesian and Jersey calves

<table>
<thead>
<tr>
<th>Block</th>
<th>Treatment</th>
<th></th>
<th>Breed</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Holstein-Friesian</td>
<td>Jersey</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2: Summary of the designed oligo primer sequences (L left primer, R right primer)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INF-γ L</td>
<td>GCTGATTCAAATTCGGTGGGA</td>
<td>123</td>
</tr>
<tr>
<td>INF-γ R</td>
<td>AGATTCTGACTTCTCTTCGCT</td>
<td></td>
</tr>
<tr>
<td>IL-13 L</td>
<td>CATGTACTGTGCAGCCCTG</td>
<td>104</td>
</tr>
<tr>
<td>IL-13 R</td>
<td>CTGAGGGGCTTGTGAGGACA</td>
<td></td>
</tr>
<tr>
<td>IL-17 R</td>
<td>GCAGGAGTCATCATCCCACA</td>
<td>142</td>
</tr>
<tr>
<td>IL-17 L</td>
<td>AGGTGGAGCGCTGTTGATAA</td>
<td></td>
</tr>
<tr>
<td>INFβ1 R</td>
<td>GTCTGAGCCAAATCCAGAAG</td>
<td>131</td>
</tr>
<tr>
<td>INFβ1 L</td>
<td>ACACCTGTCGTACTCTTTGG</td>
<td></td>
</tr>
<tr>
<td>IL10 L</td>
<td>AAAGCCATGAGTGAGTTTGACA</td>
<td>155</td>
</tr>
<tr>
<td>IL 10 R</td>
<td>TGGATTGGATTTCAGAGGCTTT</td>
<td></td>
</tr>
<tr>
<td>IL 12 A L</td>
<td>TGATGGATCTAAGAGGCAAAT</td>
<td>159</td>
</tr>
<tr>
<td>IL12 A R</td>
<td>GAAGGATGCAGAGCTTGACTTT</td>
<td></td>
</tr>
<tr>
<td>IL-23 L</td>
<td>AGCAACTCTTGAGCCCCCTAAAG</td>
<td>196</td>
</tr>
<tr>
<td>IL-23 R</td>
<td>TCAGCCTCTCTAGTGCAACA</td>
<td></td>
</tr>
<tr>
<td>TGF-β L</td>
<td>CTGTGTTCGTAGCTCTACATTG</td>
<td>129</td>
</tr>
<tr>
<td>TGF-β R</td>
<td>TACTGTGTATCCAGGTCCAGAT</td>
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</tr>
<tr>
<td>GAPDH L</td>
<td>ACGTGCTGTTGTGGATCTGAC</td>
<td>112</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>TAGCCTAGAATGCCCCTTGAGAG</td>
<td></td>
</tr>
</tbody>
</table>
3.7. Figures

A.

Figure 3.1: A) Macrophage aggregates in the lamina propria of small intestine (arrowhead); B) Ziehl-Nielsen staining showing acidfast bacteria within the granuloma (arrowheads)
Figure 3.2: Relative mRNA transcript expression of different cytokines in Holstein-Friesian and Jersey calves. (A) IFN-γ; (B) IL-23; (C) IL-10; (D) IL-13; (E) TGF-β; (F) IL-12; (G) IL-17; (H) IFN-β. Relative expression is based on least square mean values after the expression was normalized to GAPDH housekeeping gene.

* denotes significant differences at p<0.05

** denotes significant difference between control animals from both breeds at p<0.05
Figure 3.3: Microscopic visualization of calf passaged GC86 mCherry MAP (400X)
A. DIC image of GC86 mCherry MAP B. Fluorescent image of GC86 mCherry MAP.
(Picture courtesy: Philip Mead)
CHAPTER 4
GENERAL CONCLUSIONS

4.1. General Discussion

Johne’s disease is an issue of global concern. With its characteristic “not easy to diagnose” presence in the herd tag coupled with the absence of accurate treatment and effective vaccination has led to economic losses of millions of dollars. However, what is alarming is that the reported losses are an underestimate considering the obscure, lurking nature of the disease that can go unnoticed while constantly affecting farm profit. The disease is not any more only the producers’ problem but is also garnering significant public health interest owing to the suspected zoonotic nature of its etiological agent, *Mycobacterium avium* subsp. *paratuberculosis*.

Genetic susceptibility to Johne’s disease is well reviewed (Koets *et al.*, 2000; Mortensen *et al.*, 2004; Gonda *et al.*, 2006; Kupper *et al.*, 2012). Estimates of heritability have indicated genetic influence in defining susceptibility of population to Map infection. The involvement of genetic factors points towards a potential for adapting selective breeding to produce resistant off-springs to Map infection. The advantage of genetic selection is that the changes it brings about can be passed on from generation-to-generation and the changes induced will be permanent. The inherent nature of Johne’s disease and the difficulty associated with its control further favors employing genetic selection in breeding.

Alongside age and genetic susceptibility, another risk factor associated with susceptibility to Map infection is breed (Cetinkaya *et al.*, 1997; Mcnab *et al.*, 1991; Jakobsen *et al.*, 2000; Sorge *et al.*, 2011). For long, it has been reported that Channel Island breeds of cattle such as Jerseys have greater risk of being positive for Johne’s disease as compared to their Friesian counterparts. Although this implies breed susceptibility, the studies are lacking that explains the role of a particular breed’s genetic makeup that influences its
susceptibility/resistance to Map infection. The ability of the host to fight against Map is a true reflection of its immune potential to overcome infection. Different immune factors have been shown to be associated with Johne’s disease through candidate gene and genome-wide association studies.

One way of addressing the knowledge-gap concerning breed susceptibility is by understanding the immune response in both breeds to Map infection and this was the rationale behind the current research project. Our hypothesis was that breed-specific immune differences exist in Jerseys compared to Holstein-Friesians during early stages of infection and this could probably influence its susceptibility. To test our hypothesis, we carried out experimental infection trials in three different blocks where Jersey and Holstein-Friesian calves were infected with Map. Each study was carried out for 60 days and the immune response was measured by analyzing expression of key cytokines that are involved in immune response against Map by RT-PCR.

Eight cytokines that represent different arms of immune response were analyzed for their expression and that included: IFN-γ and IL-12 associated with Th1 response; IL-13 associated with Th2 response; IL-10, TGF-β associated with immunoregulatory response; IL-17 and IL-23 associated with inflammatory response; and IFN-β based on its role in Mycobacterial infections. Breed-specific comparison did not indicate any significant differences in the expression of cytokines investigated between Holstein-Friesian and Jersey calves.

Immune response to Map infection is complex and corresponds to different stages of infection. An early induction of Th1 response in the infected host is crucial to promote antibacterial and phagocytic activity of macrophages for defense against Map. The macrophage-effector function is influenced by IFN-γ, a pro-inflammatory cytokine and its
higher expression is shown to be higher during early stages of infection. However, as the
disease progresses transition from Th1-to-Th2 response is observed. This can lead to the
inability of host to control the infection ultimately ending up in disease spread. Although it is
clear that pro-inflammatory cytokines induce protective immune function against Map, our
study did not reveal any significant differences between these two breeds in the gene
expression of IFN-γ, IL-12, and IL-23 that might influence breed-susceptibility to Johne’s
disease.

It is clear that pro-inflammatory cytokines induce protective immune function that is
essential to limit bacterial replication. In our study, within breed comparison indicated
significant difference in the treatment groups in both breeds for IFN-γ wherein, IFN-γ
expression was found to be significantly higher in infected calves. This further emphasizes
the role of IFN-γ in immune response against Map and is a reflection of active immune
status.

Our hypothesis was that breed-specific differences in the expression of pro-
inflammatory cytokines might exist post-infection and this could probably be a driving factor
in conferring breed-susceptibility. However, the findings of our study did not indicate any
such differences and the reasons could be as follows:

a) Our study employed single time-point (60 days post-infection) to measure cytokine
levels and it may be possible that changes may be observed as the disease progression take
place;

b) Also, our study only looked at transcript levels of cytokines and it can be argued
that the same differences may not be observed at the protein level. This could probably be
due to epigenetic changes, presence of SNPs that might further influence the protein
expression. However, looking at such changes was beyond the scope of this study.
c) Our study only included few key cytokines that have a role during immune response against Map. Given the complex nature of Johne’s disease and its pathogenesis, it might be possible that breed-specific changes may not be limited to genes we investigated and probably other unidentified factors might have a role. This could be addressed by employing complete gene-expression profiling of the tissues and then determining breed-specific differences.

d) Also, the sample size used in this study may not have enough to power to detect any significant difference.

4.2. Future directions

Defining breed-susceptibility and identifying the putative factors that influence it helps in determining genetic markers that could potentially be employed in future marker-assisted selection and vaccine development. Identifying resistant factors could potentially go a long way in employing genetic selection in breeding to produce resistant-offsprings. In this study, our objective to identify any breed-specific changes in immune response that are predictive of disease outcome did not indicate any changes sixty days post-infection. As Johne’s disease is a chronic inflammatory disease, an ideal experimental study would require infecting calves with Map and observing changes over a longer duration of time might improve our understanding of breed-susceptibility to Johne’s disease. One of the challenges associated with Johne’s disease is defining experimental endpoints. Further studies looking at breed-susceptibility should consider the above factors in mind and it would be beneficial to employ longitudinal experimental trials with different endpoints to have a broader knowledge concerning breed-susceptibility. Furthermore, advancements in molecular science such as microarray technology, next-generation sequencing that enables complete profiling of tissues
could be considered in screening infected tissues. Complete profiling enables identification of many factors, not just limited to immune response that may influence breed-susceptibility.
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www.johnes.ca (Johne’s education and management assistance program)

## Appendix A: Summary of culture results and histopathology findings in infected calves

<table>
<thead>
<tr>
<th>Calf number (infected)</th>
<th>Breed</th>
<th>Culture positive tissue</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>834</td>
<td>Holstein-Friesian</td>
<td>ileum, ICLN</td>
<td>macrophage cluster, neutrophils infiltration in lamina propria</td>
</tr>
<tr>
<td>877</td>
<td>Holstein-Friesian</td>
<td>ileum</td>
<td>No lesions detected</td>
</tr>
<tr>
<td>909</td>
<td>Holstein-Friesian</td>
<td>Ileum</td>
<td>Granulomas in the lamina propria</td>
</tr>
<tr>
<td>910</td>
<td>Holstein-Friesian</td>
<td>Ileum</td>
<td>Multifocal granulomas and presence of giant cells</td>
</tr>
<tr>
<td>912</td>
<td>Holstein-Friesian</td>
<td>Ileum</td>
<td>Few areas of macrophage aggregates</td>
</tr>
<tr>
<td>913</td>
<td>Holstein-Friesian</td>
<td>Negative</td>
<td>Presence of few macrophages</td>
</tr>
<tr>
<td>961</td>
<td>Holstein-Friesian</td>
<td>Negative</td>
<td>Granulomas and macrophages in Lamina propria</td>
</tr>
<tr>
<td>963</td>
<td>Holstein-Friesian</td>
<td>Ileum</td>
<td>MNGCs and clusters of macrophages in Lamina propria; increased IEL</td>
</tr>
<tr>
<td>1</td>
<td>Jersey</td>
<td>Ileum, ICLN</td>
<td>Aggregates of macrophages in lamina propria</td>
</tr>
<tr>
<td>2</td>
<td>Jersey</td>
<td>Ileum, ICLN</td>
<td>Aggregates of macrophages in lamina propria</td>
</tr>
<tr>
<td>5</td>
<td>Jersey</td>
<td>ICLN</td>
<td>Aggregates of macrophages in lamina propria</td>
</tr>
<tr>
<td>8</td>
<td>Jersey</td>
<td>Ileum</td>
<td>Aggregates of macrophages in lamina propria</td>
</tr>
<tr>
<td>9</td>
<td>Jersey</td>
<td>Ileum</td>
<td>No lesions detected</td>
</tr>
<tr>
<td>10</td>
<td>Jersey</td>
<td>Negative</td>
<td>Presence of aggregates of macrophages, multinucleated giant cells in lamina propria</td>
</tr>
<tr>
<td>11</td>
<td>Jersey</td>
<td>Ileum</td>
<td>Presence of aggregates of macrophages, multinucleated giant cells in lamina propria</td>
</tr>
<tr>
<td>13</td>
<td>Jersey</td>
<td>Negative</td>
<td>macrophage clusters, increased IEL, increased blunting and fusion of villi</td>
</tr>
<tr>
<td>15</td>
<td>Jersey</td>
<td>ICLN</td>
<td>multinucleated giant cells in lamina propria</td>
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APPENDIX B: Sire details of each calf used in the study

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<th>Calf Number</th>
<th>Treatment</th>
<th>Sire name (Herdbook #)</th>
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<tbody>
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<td>Sid USA000062175895</td>
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<td>909</td>
<td>Infected</td>
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<tr>
<td>910</td>
<td>Infected</td>
<td>Subtil - CANM106223817</td>
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<tr>
<td>912</td>
<td>Infected</td>
<td>Windbook CANM7816429</td>
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<tr>
<td>913</td>
<td>Infected</td>
<td>Lauthority CANM103455217</td>
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<td>Infected</td>
<td>Legitimate CANM9684611</td>
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<tr>
<td>963</td>
<td>Infected</td>
<td>Dakota CANM106387935</td>
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<td>835</td>
<td>Control</td>
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<td>Yaakov USA000140334638</td>
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<td>Control</td>
<td>Rescue CANM106235291</td>
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<td>Control</td>
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<td>Control</td>
<td>Dakota CANM106387935</td>
</tr>
<tr>
<td>969</td>
<td>Control</td>
<td>Windbrook CANM7816429</td>
</tr>
<tr>
<td>Calf Number</td>
<td>Treatment</td>
<td>Sire name</td>
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<td>-----------</td>
<td>-----------</td>
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<td>2</td>
<td>Infected</td>
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<tr>
<td>5</td>
<td>Infected</td>
<td>Bridon Remake Comerica 8422994</td>
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<tr>
<td>8</td>
<td>Infected</td>
<td>All Lynn’s Louie Valentino116279413</td>
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<td>Infected</td>
<td>ActionJEUSAM11023978</td>
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<td>Infected</td>
<td>Sunset Canyon Belvedere 114495974</td>
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<td>Infected</td>
<td>Bridon Swat 7845900</td>
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<tr>
<td>13</td>
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<td>Arethusa Verbatim Response 116023706</td>
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<td>Heartland Merchant Topeka 67332021</td>
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<td>3</td>
<td>Control</td>
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<td>Control</td>
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<td>7</td>
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<td>GROVE GEMINI 137756</td>
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<td>Control</td>
<td>Rockella Impression 11227649</td>
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<tr>
<td>14</td>
<td>Control</td>
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