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Analysis of host gene expression profiles for identification of diagnostic biomarker in subclinical infection with *Mycobacterium avium* subspecies *paratuberculosis* in cattle

*Mycobacterium avium* subspecies *paratuberculosis*에 의한 소의 준임상형 감염 단계에서의 숙주 유전자 발현 분석을 통한 진단용 생물학적 표지자의 규명

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박현의
Analysis of host gene expression profiles for identification of diagnostic biomarker in subclinical infection with *Mycobacterium avium* subspecies *paratuberculosis* in cattle

A Dissertation
Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

To the Faculty of College of Veterinary Medicine
Department of Veterinary Medicine
The Graduate School
Seoul National University

By

Hyun Eui Park

2018
Abstract

Analysis of host gene expression profiles for identification of diagnostic biomarker in subclinical infection with *Mycobacterium avium* subspecies *paratuberculosis* in cattle

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Department of Veterinary Medicine
The Graduate School
Seoul National University

Johne’s disease (JD) is the chronic wasting disease of the ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) which cause major economic losses for dairy industry worldwide. JD is mainly distributed by fecal-oral route through contaminated materials such as feed, water, and milk with MAP. During the early stage of infection, infected cattle do not show clinical symptoms however, they shed low numbers of MAP into environment and MAP can be circulated in the herd and infect other cattle. Therefore, it is very important to detect infected cattle at early stage for successful eradication of the disease. However, current diagnostic methods, including fecal culture, fecal PCR, and ELISA are insufficient for diagnosis of subclinical stages of disease. Therefore, alternative diagnostic methods which enable
to detect subclinical cattle have been requested.

First, we described the gene expression profiles of MAP-infected cattle which were classified by the results of ELISA and fecal PCR. Six genes (LTF, HGF, HP, CXCR3, GBP6, and TFRC) were significantly up-regulated in subclinical cattle. These genes should be further evaluated to determine their suitability for diagnosis of subclinical infection of MAP. Various factors including infection dose, infected age, animal species, and coexistence of other disease might affect the accuracy of these prognostic biomarkers. Accordingly, field studies need to be conducted to determine the adequacy of these prognostic biomarkers for use as a diagnostic tool of subclinical stage of JD.

Second, we demonstrated that manipulation of host responses for the survival of MAP that occurs during the subclinical phases of JD in cattle. Downregulation of IL-17A, IL-17F, IL-22, IL-26, HMGB1, and IRF4 and upregulation of PIP5K1C indicate suppression of the Th1 response due to MAP infection and loss of granuloma integrity. In addition, increased expression of IRF5 and IRF7 suggest activation of IFN-α/β signaling during subclinical stages, which induced indoleamine 2,3-dioxygenase mediated depletion of tryptophan metabolism. Increased expression of CORO1A indicate modulation of calcium signaling, which enhanced the survival of MAP. Taken together, distinct host gene expression induced by MAP infection indicates enhanced survival of MAP during subclinical stages.

Third, we describes the response of eight host biomarkers (HP, TIMP1, MMP9, SERPINE1, TFRC, S100A8, DEFB1, and DEFB10) significantly discriminated
MAP-infected and non-infected cattle. Moreover, these eight biomarkers showed good accuracy (AUC ≥ 0.7) for diagnosis of subclinical animals. Additionally, four genes (TIMP1, S100A8, DEFB1, and DEFB10) showed sensitivity over 80% and specificity over 90%. Taken together, a real-time PCR method was developed based on eight biomarkers that can be used as a new diagnostic tool for JD with good diagnostic performance.

In conclusion, these results demonstrate the possibility of diagnosing subclinical cases of paratuberculosis using biomarker-based diagnostic techniques and expected to contribute to the development of biomarker based diagnostic methods to replace the currently used diagnostic methods.

**Keywords:** *Mycobacterium avium* subsp. *paratuberculosis*, biomarker, subclinical stage, diagnosis

**Student Number:** 2013-21548
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>CCL4</td>
<td>C-C motif chemokine 4</td>
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<tr>
<td>CCL5</td>
<td>C-C motif chemokine 5</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CFU</td>
<td>Colony forming unit</td>
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<tr>
<td>CHI3L1</td>
<td>Chitinase-3-like protein 1</td>
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<tr>
<td>CORO1A</td>
<td>Coronin 1A</td>
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<td>CXCL9</td>
<td>C-X-C motif chemokine 9</td>
</tr>
<tr>
<td>CXCL10</td>
<td>C-X-C motif chemokine 9</td>
</tr>
<tr>
<td>CXCR3</td>
<td>C-X-C chemokine receptor type 3</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
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<tr>
<td>ELANE</td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FAP</td>
<td>Fibronectin attached protein</td>
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<tr>
<td>GBP6</td>
<td>Guanylate-binding protein 6</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High mobility group box 1</td>
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<tr>
<td>HP</td>
<td>Haptoglobin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
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<tr>
<td>IL-17A</td>
<td>Interleukin 17A</td>
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<td>IL-17F</td>
<td>Interleukin 17F</td>
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<td>IL-26</td>
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<tr>
<td>IRF3</td>
<td>Interferon regulatory factor 3</td>
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<tr>
<td>IRF4</td>
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<tr>
<td>IRF5</td>
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<tr>
<td>IRF7</td>
<td>Interferon regulatory factor 7</td>
</tr>
<tr>
<td>IS900</td>
<td>Insertion sequence 900</td>
</tr>
<tr>
<td>JD</td>
<td>Johne’s disease</td>
</tr>
<tr>
<td>KLRB1</td>
<td>Killer cell lectin-like receptor subfamily B member 1</td>
</tr>
<tr>
<td>KLRC1</td>
<td>Killer cell lectin-like receptor subfamily C, member 1</td>
</tr>
<tr>
<td>LTF</td>
<td>Lactotransferrin</td>
</tr>
<tr>
<td>MAP</td>
<td>Mycobacterium avium subsp. paratuberculosis</td>
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<tr>
<td>M-cell</td>
<td>Microfold cell</td>
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<tr>
<td>MDM</td>
<td>Monocyte-derived macrophages</td>
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<tr>
<td>MMP9</td>
<td>Matrix metalloproteinase-9</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
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<tr>
<td><strong>NAHMS</strong></td>
<td>National Animal Health Monitoring System</td>
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<tr>
<td><strong>PCR</strong></td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td><strong>PBMC</strong></td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td><strong>PIGR</strong></td>
<td>Polymeric immunoglobulin receptor</td>
</tr>
<tr>
<td><strong>PIP5K1C</strong></td>
<td>Phosphatidylinositol 4-phosphate 5-kinase type-1 gamma</td>
</tr>
<tr>
<td><strong>ROC</strong></td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td><strong>S100A8</strong></td>
<td>Protein S100-A8</td>
</tr>
<tr>
<td><strong>S100A9</strong></td>
<td>Protein S100-A9</td>
</tr>
<tr>
<td><strong>SERPINE1</strong></td>
<td>Serine protease inhibitor 1</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>Standard deviation</td>
</tr>
<tr>
<td><strong>SPSS</strong></td>
<td>Statistical Package for Social Sciences software</td>
</tr>
<tr>
<td><strong>TACO</strong></td>
<td>Tryptophan-aspartate containing coat protein</td>
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<tr>
<td><strong>TFRC</strong></td>
<td>Transferrin receptor</td>
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<tr>
<td><strong>TNF-α</strong></td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td><strong>TLR</strong></td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td><strong>TRAIL</strong></td>
<td>Tumor necrosis factor related apoptosis-inducing ligand</td>
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</table>
General introduction

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a causative agent of bovine paratuberculosis which known as a chronic intestinal inflammatory disease of ruminants worldwide, inducing significant economic losses in the dairy industry (Garcia and Shalloo, 2015; Ott *et al.*, 1999; Wolf *et al.*, 2014). Bovine paratuberculosis is mainly transmitted by fecal-oral route with fecal-contaminated materials such as feed, water, and soil (Stabel, 1998; Whitlock and Buergelt, 1996). Paratuberculosis is characterized with long subclinical stages which have no clinical signs, but excrete MAP into the environment through feces (Arsenault *et al.*, 2014; Koets *et al.*, 2015). Progression of paratuberculosis divided into 4 stages based on immunological status and severity of clinical signs (Tiwari *et al.*, 2006; Whitlock and Buergelt, 1996). In the early stage of infection, there is no clinical symptom for a long time and the condition that does not shed the bacteria through feces persists and is called 'silent stage' (Tiwari *et al.*, 2006; Whitlock and Buergelt, 1996). Since the disease progresses, it still does not show clinical symptoms, but it progresses to intermittent excretion of MAP through the feces, which is called 'subclinical stage' (Tiwari *et al.*, 2006; Whitlock and Buergelt, 1996). After that, the clinical stage begins with initial clinical symptoms such as progressive weight loss with normal appetite (Tiwari *et al.*, 2006; Whitlock and Buergelt, 1996). With the progression of the disease, weight loss is getting worse and the feces gradually turns into diarrhea.
Diarrhea is intermittent at first, but gradually becomes constant, and a large amount of MAP is released through the feces. In an advanced clinical stage, animals become weak, lethargic, cachetic, and eventually dead (Tiwari et al., 2006; Whitlock and Buergelt, 1996). Currently, the diagnosis of paratuberculosis is based on clinical symptoms, histopathological examination, bacterial culture, interferon-gamma (IFN-γ) assay, and the PCR to detect the IS900 and ISMAP02 gene from feces (Britton et al., 2016). However, current diagnostic methods have a disadvantage that it is difficult to detect MAP-infected animals during the subclinical stage. Therefore, development of a new diagnostic method capable of detecting subclinically infected animals is required.

The biomarker is the material that measurable in the biological fluid such including nucleic acids (DNA, mRNA, microRNA, and small non-coding RNA), metabolites, and protein which reflect the physiological change of healthy or diseased individual (Strimbu and Tavel, 2010). Because biomarkers appear differently depending on the occurrence and progress of the specific diseases, it can be used for indicator substance in blood or body fluids that allows measurement of physiological change during the progress of the disease (Mayeux, 2004). Therefore, biomarkers have been used for various purposes such as early diagnosis of disease, evaluation of prognosis, and prediction of response to therapy (Chen et al., 2008). Especially, biomarkers considered as a non-invasive diagnostic tool for diverse diseases such as cancers, autoimmune diseases, and infectious diseases (Chen et al., 2008; Doecke et al., 2012; Keane et al., 2018; Stanescu et al., 2018; Unver and McAllister, 2018; Yan et al.,
The purpose of this study was to analyze host gene expression in whole blood of MAP-infected cattle during the subclinical stage to identify the diagnostic biomarker for paratuberculosis. This is organized into three chapters. The gene expression profiles of selected host biomarkers which have diagnostic potential were investigated in chapter I. Gene expression profiles of immune-regulatory genes which can enhance survival of MAP during subclinical stage was analyzed in chapter II. In chapter III, a real-time PCR method based on eight biomarkers was described that can be used as a new diagnostic tool for bovine paratuberculosis with good diagnostic performance.
Literature Review

Paratuberculosis

Bovine paratuberculosis is a chronic, wasting disease of wild and domestic ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis*. Paratuberculosis is characterized with a very long incubation period (Whitlock and Buergelt, 1996). In general, young calves within 4 months of age are most susceptible to infection, but clinical symptoms usually appear 2-4 years after infection. Due to the long incubation period, most of the infected animals maintain subclinical state which shows no apparent clinical symptoms but excretes MAP into the environment through feces (Whitlock and Buergelt, 1996). Transmission of the disease mainly occurs in the neonatal period by oral ingestion of MAP through the teat contaminated with feces (Whitlock and Buergelt, 1996). Also, contaminated feed, water, and soil can also be a source of transmission. Moreover, vertical transmission can occur through various ways such as placental infection in infected dams, mating with infected bulls (Adaska and Whitlock, 2012; Mercier *et al.*, 2016; Nielsen *et al.*, 2016; Thompson *et al.*, 2007). In the initial stage of infection, infected animals do not show clinical symptoms and do not shed MAP through the feces (Koets *et al.*, 2015). After that, as the disease progresses, infected animals begin to excrete MAP through the feces, but most animals do not show any clinical symptoms.
(Whitlock and Buergelt, 1996). At the beginning of the clinical stage, progressive weight loss and normal or increased appetite can be seen in infected animals (Whitlock and Buergelt, 1996). The clinical symptoms that are mainly observed are diarrhea, which is intermittent at first but gradually becomes constant. In clinical stage, animals excrete a large amount of MAP through the feces and show strong antibody response (Whitlock and Buergelt, 1996). Subsequent diarrhea leads to hypoproteinemia and dehydration. Infected animals become very weak and sometimes unable to stand, finally, cachexia and dehydration lead to the death of debilitated cattle (Whitlock and Buergelt, 1996).

Paratuberculosis causes huge economic loss to dairy industry by decreasing milk production, decreasing growth rate and culling of infected animals (Bates et al., 2018; Garcia and Shalloo, 2015). In the USA, approximately 68% of dairy herds are positive for paratuberculosis, and less than 10% of beef cattle herds are infected (Carter, 2012). According to the National Animal Health Monitoring System in the United States, economic losses from infected herds are estimated at $40 per year per infected cattle, with an overall estimated loss of $200 to $250 per year (Garcia and Shalloo, 2015).

1. Etiologic agent

*Mycobacterium avium* subsp. *paratuberculosis* is a small, gram-positive, acid-fast, rod that is facultative intracellular bacterium (Salem et al., 2013). The primary host
MAP is the ruminant such as cattle, goat, sheep, and deer (Stabel, 1998). However, non-ruminant animals can also be affected and act as a source of infection to herd (Florou et al., 2008; Münster et al., 2013). MAP contains a lipid component called mycolic acid in the cell wall, which is resistant to low pH, high temperature, drying and various chemical compounds (Minnikin et al., 1984). Because of these properties, MAP can survive for many years in the environment such as water and soil (Raizman et al., 2011; Rhodes et al., 2013). MAP is considered to be an important pathogen in public health because it can survive during the pasteurization of milk due to heat resistance (Donaghy et al., 2007; Stabel, 2008). Recently, MAP has been isolated from patients with Crohn's disease, an immune-mediated inflammatory bowel disease, and it has been argued that MAP is involved in the pathogenesis of Crohn's disease (Bull et al., 2003; Feller et al., 2007; Timms et al., 2016).

2. Epidemiology

*Mycobacterium avium* subsp. *paratuberculosis* classified into three groups based on genetic polymorphism of *IS900* and *IS1311* genes, characteristics of growth, and host species (Sevilla et al., 2005). Sheep types are mostly isolated from sheep and goats, whereas cattle types are isolated from relatively broad host species including domestic and wild ruminants, non-ruminants, and humans (Singh et al., 2009). The Bison type also has been reported to be isolated from livestock, wild ruminants, and
humans (Kaur et al., 2011; Sonawane et al., 2016). Recent studies have shown that the bison type is widely distributed in India and has also been isolated in Korea and Uganda (Kim et al., 2013; Okuni et al., 2012; Singh et al., 2009). Despite a lot of preventive measures, paratuberculosis occurs globally in North America, South America, Europe, Asia and Australia (Stabel, 1998). A national level of the test was conducted for detection of paratuberculosis by the National Animal Health Monitoring System (NAHMS) of USA in 1996. As a result, 21.6% of the cattle were confirmed as positive (Lombard, 2011). In 2007, 68.1% of the dairy herds were positive for paratuberculosis with the environment fecal sample (Lombard et al., 2013). Also, 95% of the dairy herd which breeds more than 500 cattle were positive for paratuberculosis (Carter, 2012). In the case of the beef cattle herd, nationwide surveys were conducted by NAHMS of the USA in 1997. In all 21 provinces, 10,372 beef cattle in 380 herds were selected for the survey, and only 0.4% of the beef cattle and 7.9% of the herd were positive (Dargatz et al., 2001). Between 2003 and 2010, more than 3,100,000 dairy cattle and over 692,000 beef cattle were tested for paratuberculosis by serum ELISA. As a result, 5 to 6.6% of dairy cattle and 2.1 to 3.6% of beef cattle were positive (Carter, 2012). Between 2003 and 2010, approximately 514,000 dairy cattle and 67,000 beef cattle were tested for fecal PCR and culture tests. The results showed that 7.2 to 14.1% of dairy cattle and 2.8 to 16.9% of beef cattle were positive (Carter, 2012). Also, ELISA test for 4,990 cattle in 158 dairy herds in Wisconsin showed that 50% of the herd and 7.29% of dairy cattle were positive (Collins et al., 1994). In the European countries, the prevalence of
Paratuberculosis was examined by ELISA. Prevalence of paratuberculosis was 14.7 to 24.1% in Germany, 19.0% in Austria, 7.7% in Norway, 2.4 to 6.8% in Italy suggesting that there is an endemic state of paratuberculosis in Europe, except Sweden (Nielsen and Toft, 2009). In Switzerland, 19.8% of dairy cattle were positive for fecal PCR and 5% of dairy cattle were positive by PCR with milk sample in Turkey (Corti and Stephan, 2002; Ozturk et al., 2010). In Japan, paratuberculosis was first confirmed in cattle which imported from the United States in 1959 (Momotani, 2012). However, until the 1980s, the outbreak of paratuberculosis was limited in imported animals, but since the 1980s, paratuberculosis occurred in Hokkaido, Toho, and Kanto regions and begin to spread entire Japan (Momotani, 2012). In the South Korea, prevalence of paratuberculosis was 0.96% in 1968, 6.7% in 1994 and 6.7% to 7.1% in 2003 (Kim et al., 1994; Pak et al., 2003). In 2009, 13.8% of herds and 3.3% of cattle were infected with MAP in the Gyeongnam area (Lee and Jung, 2009). Especially, the prevalence of paratuberculosis in black goats is estimated to be 4.6 to 15.3% according to animal level and 18.2 to 38.2% according to herd level (Lee et al., 2006).

Taken together, paratuberculosis is occurring in many countries around the world especially in nations where dairy industries have developed, and the number of infected animals and herds is steadily increasing.
3. Transmission

The transmission of paratuberculosis is mostly occurring through the fecal-oral route, and it is also possible to be spread with other pathways (Whitlock and Buergelt, 1996). Infected cattle release MAP through the feces during disease progression, which causes contamination of solid feed, water, grass and soil (Rhodes et al., 2013). MAP can survive for more than a year in the pasture and survive for longer periods of time in water which induces circulation of disease within the herd (Sweeney, 1996). The susceptibility to infections is the highest in young calves and the susceptibility to infection decreases with increases with age (Mortier et al., 2015). Although the relationship between the age of calves and susceptibility to infection has not been fully elucidated, several hypotheses have been suggested by various studies. In newborn calves, intestinal permeability is increased to accept large-sized substances such as antibodies in colostrum. Therefore, the calf is vulnerable to infection because MAP can easily entry the mucosa (Sweeney, 2011). High susceptibility in calf stage is can be related to the immunological changes that occur during the process of growing from calf to adult cattle. In the newborn period, innate and acquired immune mechanisms are not enough to protect the host from pathogens (Chase et al., 2008). With the growth of newborn calves, activation of innate and acquired immune mechanisms occurs and subsequently induces increase the resistance to disease (Chase et al., 2008). Due to the high susceptibility of calf stage, most of the infections occur when the calves are exposed to fecally contaminated
environment (Whitlock and Buergelt, 1996). It is also possible that direct intrauterine infection through the infected dam. For example, MAP was isolated in the uterine flush fluid of infected cattle which show clinical signs (Rohde and Shulaw, 1990). In addition, MAP can adhere to bovine ova was cultured in MAP-containing media (Rohde et al., 1990). Therefore, it is highly probable that infected calves born from the infected dam are infected. Also, semen of infected bulls can be the source of infection. In detail, MAP was detected in the semen, testes, epididymides and seminal vesicles of MAP-infected bulls including cattle with no clinical symptoms (Ayele et al., 2004).

4. Infection and Disease

Primary infection site of *Mycobacterium avium* subsp. *paratuberculosis* is the ileum and ileocecal valve (Siguréthardóttir et al., 2004). Transmission of the disease mainly occurs by fecal-oral route with contaminated materials such as feed, water, and teat (Sweeney, 1996). Paratuberculosis is characterized by weight loss and persistent diarrhea in the terminal stage of infection. In silent or subclinical stage, cattle may healthy for several months or years (Tiwari et al., 2006). In clinical stage, diarrhea may be constant or intermittent. With the progression of disease becomes more severe, show some of the following clinical signs: weight loss, normal or increased appetites, hypoproteinemia, intermandibular edema, chronic diarrhea and cachexia (Tiwari et al., 2006; Whitlock and Buergelt, 1996).
In general, intestinal lesions develop with severity and progression of the disease. Lesions are characterized by corrugated intestine walls, enlarged mesenteric lymph nodes, and formation of granulomas (Plattner et al., 2009; Tiwari et al., 2006; Whitlock and Buergelt, 1996). Sometimes, there is no correlate on between clinical symptoms and the severity of the lesion and mild intestinal lesions characterized by infiltration with a large number of lymphocytes, few macrophages, and no epithelioid macrophages in the submucosa (Sweeney, 2011). On the other hand, severe intestinal lesions consist of diffuse granulomatous lesions which contain epithelioid macrophages and giant cells in the mucosa and submucosa of the intestine (Sweeney, 2011). Infection of MAP induces the immunological change of host animal with the progression of the disease. In experimentally infected calves, proliferative response to MAP was observed in 6 months after infection (Koo et al., 2004). In detail, CD4+ memory T cell expressing CD25 and CD26 were predominant and CD8+ T cells proliferated due to MAP infection until 18 months after infection. Also, γδ T cells did not proliferate until 18 months after infection. However, after 18 months, γδ T cells which express CD25, ACT2, and CD26 were proliferated presence of the MAP antigens (Koo et al., 2004).

5. Pathogenesis

Macrophages and M cells are important in the pathogenesis and/or defense system of the gut against *Mycobacterium avium* subsp. *paratuberculosis* (Coussens, 2004).
Researches on the pathogenesis of paratuberculosis have been conducted through various in vivo and in vitro methods. In the beginning, many researchers started to study the invasion path of the MAP and classified the invasion path of the MAP into two categories based on infection dose (Buergelt et al., 1978; Gilmour and Gardiner, 1968; Sweeney et al., 2006). The identified invasion paths are as follows (Figure 1). When a large number of MAP enter the lymph node after infiltration through the tonsil and spread to the mesenteric lymph nodes and ileum through the blood vessels or the lymphatic vessels (Buergelt et al., 1978; Payne and Rankin, 1961). On the other hand, when a small amount of MAP invades, the microorganism invades through the intestinal mucosa of the ileum (Sweeney et al., 2006). When MAP is ingested and enters the ileum of the intestine, MAP is phagocytized by M cells and transported into the submucosal tissue where they survived and proliferated in macrophages after being phagocytosed (Arsenault et al., 2014; Koets et al., 2015). MAP can survive within macrophages after phagocytosed by manipulation of the host immune response in various ways (Arsenault et al., 2014). One of the surviving strategies is the inhibition of phagolysosomal maturation, which involved in the destruction of internalized pathogens (Arsenault et al., 2014). As shown in Figure 2, MAP can block responsiveness of macrophage through inhibition of toll-like receptor signaling and IFN-γ signaling which plays a central role in immune response to intracellular pathogens (Coussens et al., 2004). Previous studies using infection models for identification of invasion route have been conducted. Stabel et al., analyzed pathologic changes and fecal shedding of MAP when oral and
intraperitoneal infections occurred in newborn calves (Stabel et al., 2009). When oral infection occurs, colonization of MAP in intestinal tissues and associated lymph nodes is well established compared to intraperitoneal infection (Stabel et al., 2009). Primary colonization sites of MAP are the ileal and jejunal region of the small intestine and nearby lymphoid tissues (Sigurðardóttir et al., 2004). After ingestion of MAP through the fecal-oral route, it penetrates through the tonsil mucosa and then spreads through the bloodstream to the mesenteric lymph nodes and the ileum (Payne and Rankin, 1961; Sweeney et al., 2006). However, it is known that when a relatively small amount of oral infection occurs, it penetrates primarily into the mucosa of the ileum and spreads to adjacent lymph nodes (Sweeney et al., 2006). MAP-specific T cell proliferative responses were also observed in the gut surgical loop-infected model, confirming the increase of CD8+ and γδ-T cells in the lamina propria of infected areas and significant increase of TNF-α and IFN-γ was confirmed (Charavaryamath et al., 2013). Penetration of MAP in tonsil and the ileum occurs via microfold (M) cells of Peyer's patch (Bermudez et al., 2010; Momotani et al., 1988). As shown in Figure 1, activation of bacterial cell wall protein fibronectin attachment protein (FAP) occurs during the passing of the digestive tract of ruminants (Bannantine and Bermudez, 2013). This promotes opsonization by fibronectin and penetrates into the cell by the fibronectin receptor abundant on the surface of M cell. The penetration of MAP into M cells occurs very rapidly within 30 minutes of contact, and the MAP is then transferred to the submucosal layer (Lamont et al., 2012). In the submucosal layer, MAP is phagocytosed by the
macrophage and spread to nearby tissues and lymph nodes. Effective host defense against bacterial infections in the intestine depends on rapid clearance of bacteria from the intestinal tract. After infiltrating the intestinal mucosa, MAP phagocytosed by macrophages express several receptors (complement receptors (CR1, CR3, and CR4), immunoglobulin receptors (FcR), mannose receptors and scavenger receptors) which involved in the elimination of mycobacterial pathogens (Guirado et al., 2013). In particular, MAP invades to macrophages via complement receptors which induce inhibition of the activation of macrophages, thereby enhancing survival in macrophages by avoiding host immune response (Premanandan et al., 2009).

After the MAP enters the phagosome of the macrophage, the phagosome changes to a late endosome and binds to the lysosome for destroying of internalized pathogens (Koul et al., 2004). Thus, survival of internalized pathogen depends on its ability to inhibit the formation of mature phagolysosomes. As shown in Figure 2, MAP can survive inside the macrophage through inhibition of acidification and phagolysosome fusion inside the phagosome (Cheville et al., 2001). In the phagosome internalizing with MAP, the expression of transferrin receptor, which is an early phagosome marker, is increased and the expression of Lamp-1, which is a marker of the mature phagosome, is decreased (Hostetter et al., 2003). Rab GTPase, another late phagosome marker, was also decreased in the only living MAP containing phagosome. Thus, the living MAP is essential for inhibition of phagosomal maturation (Keown et al., 2012). Formation of the phagolysosome occurs with merging of the phagosome with the lysosome. MAP sulpholipids can
inhibit this mechanism and subsequently induce inhibition of maturation of phagolysosome (Arsenault et al., 2014). Also, membrane trafficking regulation of phagolysosome is inhibited by dephosphorylation of phosphatidylinositol phosphates with MAP SapM (Arsenault et al., 2014). Up-regulation of V-ATPase by MAP inhibits acidification of vacuole which subsequently induces phagolysosomal fusion (Weiss et al., 2002). Also, PtpA protein binds subunit of V-ATPase of macrophage and induce exclusion of V-ATPase from phagosome which subsequently inhibits phagolysosomal fusion (Wong et al., 2011). Fusion of lysosome and phagosome is induced by release of tryptophan–aspartate containing coat (TACO) from phagosome. However, as shown in Figure 2, MAP can inhibit releasing of TACO from phagosome. Also, MAP can manipulate MAPK-p38 pathway through LAM activation of TLR2 (Fratti et al., 2003). Activation of TLR2 also induces up-regulation of IL-10 which suppressing the Th1 immune response (Arsenault et al., 2014).
Figure 1. Invasion pathway of the orally ingested MAP.

After ingestion with oral route, (1) MAP pass through the GI tract. The ingested MAP also invades to the epithelium of tonsil and transferred to the ileum. Fibronectin attached protein of MAP is activated in the rumen (2) and opsonized by fibronectin during passing the lower digestive tract (3). In the ileum, M cell (4) uptake MAP and subsequently recognition of MAP with the fibronectin receptor occurs (5). Up-taken MAP moves from the epithelium to intra-epithelial macrophages, which recognize complement-coated MAP via complement receptors (6). Formation of granulomas (7) occurs with infected macrophages which internalize MAP but fail to remove. During the progression of JD (8), MAP can transfer to the unborn calf (9) and neonatal calf with milk (10) or fecal to oral route (11). (a) Mouth (b) Salivary Glands (c) Esophagus (d) Rumen (e) Reticulum (f) Omasum (g) Abomasum (h) Gallbladder (i) Pancreas (j) Duodenum (k) Jejunum (l) Ileum (m) Cecum (n) Large Intestine (o) Anus (p) Uterus (Arsenault et al., 2014).
Figure 2. Inhibition of phagolysosomal maturation by MAP.

Phagolysosome formation after ingestion of MAP is inhibited in various ways. First, sulpholipids in MAP inhibit the formation of the phagolysosome by inhibiting the fusion of the phagosome with the lysosome. SapM induces dephosphorylation of phosphatidylinositol phosphates which subsequently disrupting vesicle translocation between phagosome and lysosome. V-ATPase is an important factor in phagosome-lysosome fusion. PtpA induces exclusion of V-ATPase from the phagosome and subsequently induces failure of phagolysosome fusion. MAP inhibits Rab5 and subsequently induces insufficient recruitment of early endosome. TACO is the critical factor for phagolysosome fusion. MAP can interfere with releasing of TACO thereby inhibition of phagolysosome fusion occurs. MAP can affect MAPK-p38 signaling through the activation of TLR2. Activation of TLR2 induces suppression of the various innate immune responses (adapted from Arsenault et al., 2014).
6. Clinical manifestation

Paratuberculosis can be classified into four stages according to the degree of clinical symptoms, shedding level of MAP through the feces, and the degree of MAP-specific antibody in the serum (Whitlock and Buergelt, 1996).

6.1 Silent stage

In the silent infection state, it has the following characteristics; there are no clinical symptoms, and shedding of MAP through the feces is not observed. Also, there is no effective diagnostic method to detect infection. This stage can last for at least 2 to 10 years and animals in silent infections do not show any reduction in body weight or milk production. Animals in the silent stage do not have antibodies to MAP in the serum and may release MAP through the feces, but it cannot be detected by PCR. Therefore, it is very likely that the disease will be transmitted by animals in the silent stage.

6.2 Subclinical stage

In a subclinical infection, there is no clinical sign of infection yet, but the shedding of the MAP through the feces begins to be detected by PCR and may be confirmed through fecal culture. However, shedding of the MAP through the feces is mostly intermittent, and antibodies to MAP are not yet formed, so it is very difficult to make an accurate diagnosis with subclinically infected animals. Animals in the subclinical
stage are able to contaminate the environment and infect other animals in the herd because they release the MAP through the feces for a long time.

6.3 Clinical stage

Depending on the infection dose and the condition of the infected animal, initial clinical symptoms begin to appear after an incubation period of 2 to 10 years. The first clinical sign is a gradual loss of weight despite normal or increased appetite. Diarrhea starts with weight loss for about 3 to 6 months and occurs constantly or intermittently. Most of the animals in this stage have antibodies to MAP in the serum, which can be diagnosed by ELISA and the shedding of the MAP through the feces.

6.4 Advanced clinical stage

Animals entering the advanced clinical stage become more and more debilitated. Main clinical manifestations include chronic diarrhea, hypoproteinemia, and submandibular edema (bottle jaw). Animals at this stage can become dehydrated and lethargic due to chronic diarrhea. Also, infected animals excrete a large amount of MAP into the environment and have a strong MAP-specific antibody titer in the serum, but in many cases, it is often culled before this stage is reached.
7. Prevention and Control

Many countries have been developed about the programs for the prevention and control of paratuberculosis (Benedictus and Kalis, 2003; Groenendaal and Galligan, 2003; Rossiter and Burhans, 1996). Common point of control strategies for paratuberculosis is as follows; (1) regular screening for paratuberculosis in national level, (2) immediate separation between calf and dam after birth, (3) using safe colostrum obtained from cattle identified as negative for paratuberculosis to prevent vertical infection, (4) management of hygiene in the herd, especially the area of the neonatal calves. In particular, the equipment which contacts with the feces of infected animals should not be introduced to the calf area.

For successful eradication of paratuberculosis in the herd, animals should be quarantined immediately if they are suspected to be infected by fecal PCR and/or serum ELISA and should be culled after clinical symptoms such as diarrhea have developed. In addition, a diagnostic method for subclinical animals which show no clinical symptoms should be established, and these animals should be isolated from other animals. Also, when introducing a new animal from other herds, quarantine it in a separate area in the ranch for a certain period of time, and should confirm with the serum ELISA and fecal PCR that there is no infection. To prevent vertical infections, infected animals are encouraged to prohibit and cull breeding. Calves should be kept in a clean place and should always be careful not to be contaminated with feces and fluids from infected adult animals. After birth, the calf should be
isolated from the mother immediately and raised in a breeding facility separated from the other animals. Also, management of hygiene in the breeding facility is an important part for prevention of new infections. Therefore, all workers should be taken not to use contaminated clothing or equipment. In addition, colostrum and milk fed to the neonatal calf should be obtained from paratuberculosis free herd. In the herd, the number of livestock, the in and out of animals, and the results of the screening test should be recorded and stored. If there is an infected animal in the herd, the occurrence of clinical symptoms should be observed and recorded. In addition, the risk of disease transmission should be reduced by preventing contamination of feed, water, equipment, and vehicles by feces in the herd.

Many studies have been conducted and several vaccines have been tested but complete protection has not been obtained in the fields against paratuberculosis. Live vaccines which based on mutation of relA, lsr2, and pknG genes known as virulence factors of *Mycobacterium tuberculosis* and *Mycobacterium bovis* were developed using the allelic exchange technique. The ΔrelA mutant was superior to the ΔpknG mutant in attenuation of virulence and defense efficacy (Park et al., 2011; Park et al., 2008). Studies on subunit vaccines are also conducting. Several antigens have been evaluated as vaccine candidates such as heat shock protein 70 (Hsp70), antigen 85 complex proteins (Ag85A, Ag85B, Ag85C), lipoproteins (LprG, MAP0261c), and PPE family proteins (MAP1518, MAP3184) (Huntley et al., 2005; Koets et al., 1999; Shin et al., 2005). Until now, the Hsp70 protein among the vaccine candidates shows highest protection effect in experimentally infected animals (Vrieling et al., 2013).
8. Diagnosis

Various diagnostic methods are available for the detection of paratuberculosis, each has their own advantages and disadvantages (Britton et al., 2016). Diagnostic methods classified into three categories as follows: (1) assays to detect the pathogen in the specimen such as feces, blood, tissue, and milk, (2) assays to detect cellular immune response to MAP infection, (3) assays to detect humoral response to MAP infection. Combination of different diagnostic methods can ensure high sensitivity of the test.

8.1 Assays to detect the pathogen

Assays to detect the MAP in the biological specimen including feces, blood, tissue, and milk are widely used for detection of paratuberculosis. PCR methods have the advantage for detection of subclinically infected animals which shed MAP through feces but does not have antibody to MAP (Britton et al., 2016). The PCR has the advantage of being able to inspect a large number of samples in a short time. However, since PCR detects DNA of MAP, a positive result does not ensure the presence of live MAP bacteria. Also, PCR inhibitors in clinical specimens such as feces, blood, and milk can induce a false negative result. Therefore, various PCR techniques targeting MAP specific genes have been developed to improve the disadvantage of PCR assay (Bhide et al., 2006; Corti and Stephan, 2002). IS900 targeted PCR method can detect up to 10 to 100 CFU per ml in milk samples,
showing higher sensitivity than culture method. However, the specificity and sensitivity of the sample may be lowered due to the PCR-interfering substances in the sample. Recently, a triplex real-time PCR technique has been developed to increase the efficiency of diagnosis due to its high specificity and sensitivity, and it can detect up to $2.5 \times 10^3$ MAP per gram of feces (Irenge et al., 2009). In addition, optimization of the DNA extraction method has been conducted by many researchers (Mita et al., 2016; Park et al., 2014a; Timms et al., 2015).

### 8.2 Assays to detect cellular immune response to MAP infection

The representative diagnostic method based on cell-mediated immune response is the IFN-γ assay (Jungersen et al., 2012; Kalis et al., 2003). IFN-γ is an important mediator of cell-mediated immune response which activates macrophage after exposure of mycobacterial pathogens (Arsenault et al., 2014). Previous studies revealed that level of IFN-γ was up-regulated in MAP-infected cattle which show fecal shedding (Huda et al., 2004; Jungersen et al., 2012). Due to the decreased level of IFN-γ level with the progression of the disease, IFN-γ assay shows low sensitivity in the late stage of MAP infection (Nielsen and Toft, 2008). Therefore, researches focused on improvement of IFN-γ assay have been conducted. Hughes et al., suggest Map-specific proteins, MAP_3651c and MAP_0268c as a candidate for new antigens of IFN-γ assay which show high in sub-clinically infected sheep (Hughes et al., 2017). Also, Dernivoix et al., developed new IFN-γ assay based on three antigens (Ag6, MAP1637c, and MAP0586c) which showed the significantly higher
level of IFN-γ in MAP infected animal than M. bovis infected animals (Dernivoix et al., 2017).

8.3 Assays to detect humoral response to MAP infection

ELISA which detects specific antibodies to MAP has been developed as a serological diagnostic method. ELISA is a rapid, low-cost method for screening of MAP-infected animals in the herd (Collins, 1996). ELISA is the useful method for investigation of the infection prevalence in a large herd. ELISA methods show high sensitivity in cattle which have clinical symptoms (50 to 87 %) (Nielsen and Toft, 2008). However, the sensitivity of ELISA method in subclinically infected cattle is relatively low (7 to 22 %) (Nielsen and Toft, 2008). Therefore, many researches have been conducted for improvement of sensitivity and specificity of ELISA. Preabsorption of Mycobacterium phlei protein to sample can increase the specificity of the ELISA (Yokomizo et al., 1983). Modification of the ELISA method with formaldehyde-treated MAP antigen can improve the sensitivity and specificity of the test (95% sensitivity and 100% specificity) (Speer et al., 2006). Also, MAP antigen secreted during early to mid-log phase was used for development of ELISA for diagnosis of subclinically infected cattle (Shin et al., 2008).
Biomarkers

1. Definition of biomarkers

Biomarkers are measurable biological characteristics that reflect the severity or presence of various disease states (Strimbu and Tavel, 2010). Generally, a biomarker is the biological markers that can be used as an indicator of a particular disease state or other physiological states of an organism (Mayeux, 2004). Biomarkers include specific cell, enzyme, hormone, protein, metabolite, gene, and gene transcript in a variety of sources such as body fluids and tissues (Jimenez-Luna et al., 2018; Tsuyoshi and Yoshida, 2018; Yang et al., 2018). Because biomarkers appear differently depending on the occurrence and progress of the specific diseases, it can be used for indicator substance in body fluids that allows measurement of physiological change during the progress of the disease (Mayeux, 2004). Therefore, biomarkers have been used for various purposes such as diagnosis of disease, evaluation of prognosis, and prediction of response to therapy (Mayeux, 2004). Especially, biomarkers considered as a non-invasive diagnostic tool for diverse diseases such as cancers, autoimmune diseases, and infectious diseases (Manda et al., 2017; Moyer and Force, 2012; Schmidt et al., 2011; Shirmohammadi et al., 2018). For example, up-regulation of mutation in EGFRγIII protein was found in cerebrospinal fluid (CSF) of glioma patients and investigated for biomarker as an
indicator for glioma (Manda et al., 2017). In addition, the prostate-specific antigen has been used for the diagnosis of prostate cancer since the approval of US FDA in 1986, and it is known to be useful for the screening of prostate cancer though it is controversial issues about overtreatment (Moyer and Force, 2012). CXCL13 is known to increase in cerebrospinal fluid of patients with acute Lyme neuroborreliosis, and diagnosis using level of CXCL13 in CSF shows high sensitivity and specificity for the early diagnosis of atypical neuroborreliosis (Schmidt et al., 2011).

Currently, biomarkers have become widely used due to enhanced knowledge of the pathophysiological mechanism of disease and the development of next-generation sequencing technology.

2. Biomarkers for diagnosis of paratuberculosis

Paratuberculosis is a chronic wasting disease of ruminants which caused by Mycobacterium avium subsp. paratuberculosis (MAP) and characterized with persistent diarrhea and cachexia (Sweeney, 2011). Because the MAP can survive in the environment for a long time, the spread of the disease is mainly through the ingestion of contaminated feed or water (Whitlock and Buergelt, 1996). In general, infected cattle will undergo a subclinical phase that does not present symptoms for long periods of time. During the subclinical phase, the infected cattle emit MAP through the feces resulting in a continuous circulation of the MAP in the environment and the spread of the disease in the herd (Stabel, 1998). To date, there have been
developed PCR techniques to detect MAP in the feces and ELISA to detect antibodies against MAP in the body, but they show low sensitivity and specificity for detection of subclinically infected animals (Bögli-Stuber et al., 2005; Collins, 2011; Cousins et al., 1999; Englund et al., 2002; Sockett et al., 1992b).

In recent years, studies on host biomarker-based diagnostic methods have been conducted to overcome these problems. Diagnostic methods based on biomarkers can be classified into specific protein assay, transcriptomic analysis, and miRNA analysis depending on the type of the target indicator (Britton et al., 2016). Several studies were conducted for discovery of specific proteins which can discriminate MAP-infected animals and non-infected animals (De Buck et al., 2014; Seth et al., 2009; Stabel and Robbe-Austerman, 2011; You et al., 2012; Zhong et al., 2011). When sera from infected and uninfected individuals were analyzed through protein fingerprinting, sera from infected cattle showed more than two-fold upregulation of six proteins and two-fold downregulation of two proteins (You et al., 2012). They also found proteins with significantly altered expression in infected cattle were involved in iron regulation, leukocyte/lymphocyte directing, apoptosis, coagulation, and complement activation (You et al., 2012). The previous study analyzed serum of unexposed, MAP-vaccinated, and MAP-infected sheep for the discovery of specific biomarkers for ovine paratuberculosis. As a result, they found that transthyretin was significantly reduced in both of vaccinated and infected sheep while alpha hemoglobin was highly increased in the vaccinated sheep and moderately in the MAP-infected sheep compared to unexposed group (Zhong et al., 2011). Seth et al.,
found vitamin DBP increased in both *M. bovis* and MAP-infected cattle while cathelicidins increased significantly only in MAP-infected cattle (Seth *et al.*, 2009). These changes are believed to be related to the activation of innate immunity, which shares common changes that occur in humans when infected with *M. tuberculosis*. They also found an increase of transthyretin in MAP-infected cattle, which would increase the transport of vitamin A, resulting in increased differentiation of monocytes and activation of mucosal immunity to inhibit MAP proliferation (Seth *et al.*, 2009). Infection of MAP provokes specific host response in the early stage of infection. Stabel and Robbe-Austerman discovered early immune markers of MAP infection in the experimental infection model. All infected groups maintained a strong IFN-γ response during the study (Stabel and Robbe-Austerman, 2011). T cell activation markers such as CD25, CD26, CD45RO, and CD5 were significantly up-regulated in infected calves as compared to uninfected controls (Stabel and Robbe-Austerman, 2011). De Buck *et al.*, found that concentrations of acetone, citrate, glycerol, and iso-butyrate were significantly changed in infected cattle, indicating increased lipid metabolism in infected cattle (De Buck *et al.*, 2014). Significant changes in amino acid concentrations were also observed in infected cattle, indicating that protein turnover or deficiencies occurred in infected individuals (De Buck *et al.*, 2014).

Recently, studies on microRNA-based diagnostic methods have been carried out for early diagnosis of paratuberculosis. Seven miRNAs were down-regulated in MAP-infected animals compared to unexposed animals, and five and three miRNAs
decreased and increased in the exposed group, respectively, compared to the unexposed group. Six of the differentially expressed miRNAs are associated with an immune response and two are new miRNAs. This result suggests that miRNA expression is influenced by MAP infection and plays a key role in regulating host response to infection (Malvisi et al., 2016). Also, Farrell et al., identified a range of novel miRNA in bovine serum, and shown the utility of small RNA sequencing approaches to explore the potential of miRNA as novel biomarkers for paratuberculosis in cattle (Farrell et al., 2015). Summarized information on biomarker studies for diagnosis of bovine paratuberculosis was listed in Table 1.
Table 1. Biomarkers which identified in research of bovine paratuberculosis

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Type of biomarker</th>
<th>Identified biomarkers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Protein</td>
<td>Transferrin, gelsolin isoforms α &amp; β, thrombin</td>
<td>(You et al., 2012)</td>
</tr>
<tr>
<td>Serum</td>
<td>Protein</td>
<td>Alpha-haemoglobin , transthyretin</td>
<td>(Zhong et al., 2011)</td>
</tr>
<tr>
<td>Serum</td>
<td>Protein</td>
<td>Transthyretin, retinol binding proteins, and cathelicidin</td>
<td>(Seth et al., 2009)</td>
</tr>
<tr>
<td>Serum</td>
<td>Protein</td>
<td>CD25, CD26, CD45RO, and CD5</td>
<td>(Stabel and Robbe-Austerman, 2011)</td>
</tr>
<tr>
<td>Serum</td>
<td>Metabolite</td>
<td>Isobutyrate, acetone, and myo-inositol</td>
<td>(De Buck et al., 2014)</td>
</tr>
<tr>
<td>Macrophage</td>
<td>Gene expression</td>
<td>TFRC, CX3CR1, CCNE1, Cox6a2, IRF7, and Gdf15</td>
<td>(Cha et al., 2013)</td>
</tr>
<tr>
<td>Spleen</td>
<td>Gene expression</td>
<td>SAA3, complement factor D, and S100a8</td>
<td>(Shin et al., 2015a)</td>
</tr>
<tr>
<td>Whole blood</td>
<td>Gene expression</td>
<td>PRR19, IGFLR1, and T2 cadherin</td>
<td>(Shin et al., 2015b)</td>
</tr>
<tr>
<td>Whole blood</td>
<td>Gene expression</td>
<td>CD46, ICOS and CEP350</td>
<td>(David et al., 2014a)</td>
</tr>
<tr>
<td>Whole blood</td>
<td>Gene expression</td>
<td>BNBD9-Like, S100A9, GPR77, and C5a2</td>
<td>(David et al., 2014b)</td>
</tr>
<tr>
<td>Whole blood</td>
<td>Gene expression</td>
<td>TIMP1, HP, SERPINE1, TFRC, MMP9, DEFB1, DEFB10, and S100A8</td>
<td>(Park et al., 2017a)</td>
</tr>
<tr>
<td>Whole blood</td>
<td>Gene expression</td>
<td>TTYH3, LOC617313, ZNF467, and IDO1</td>
<td>(Malvisi et al., 2016)</td>
</tr>
<tr>
<td>Whole blood</td>
<td>MicroRNA</td>
<td>bta-mir-19b, bta-mir-19b2, bta-mir-301a and bta-mir-32</td>
<td>(Malvisi et al., 2016)</td>
</tr>
<tr>
<td>Serum</td>
<td>MicroRNA</td>
<td>miR-205, miR-432</td>
<td>(Farrell et al., 2015)</td>
</tr>
</tbody>
</table>
Transcriptomics

Transcriptomics is the quantitative study of all part of RNAs present in a sample which allow to understanding gene expression at genome-wide level (Mutz et al., 2013). Through whole-transcriptome analysis, the contribution of gene expression to the onset and progression of the disease can be understood in various diseases such as cancer, diabetes, autoimmune disease, and cardiovascular disease (Devaux, 2017; Guffanti et al., 2009; Li et al., 2018; Peck and Nguyen, 2012). Also, biological pathways and molecular mechanisms which regulate cell signaling, cell development, and immune response can be identified through the genome-wide RNA expression with next-generation sequencing technology (Casamassimi et al., 2017; Wang et al., 2009). The transcriptomic approach is widely used in biomarker research in various fields including cancer, autoimmune disease, and infectious disease (Hwang et al., 2018; Kamel and Al-Amodi, 2017; Peck and Nguyen, 2012; Sobhkhhez et al., 2017). Transcriptomic analysis of host gene expression analysis can be used to diagnose for specific diseases (Holcomb et al., 2017).

Recently, several studies have been carried out on biomarkers for the early diagnosis of paratuberculosis based on analysis of host transcriptome profile (Cha et al., 2013; David et al., 2014a; David et al., 2014b; Park et al., 2017a; Park et al., 2016; Shin et al., 2015a; Shin et al., 2015b). First, in vitro study showed the transcriptomic changes of mouse macrophages during the time of MAP infection,
and found that the types of genes that were changed during infection were different, and genes showing a steady increase throughout the infection period. Based on this, candidate genes that can be used as biomarkers during infection were selected (Cha et al., 2013). Second, many researchers attempted to identify host biomarkers through the animal models. Shin et al., reported that gene expression profiles that changed at 3 weeks and 6 weeks after MAP infection in mouse were related to metabolic process, cellular process, cell communication and immune system process (Shin et al., 2015a). Also, the gene expression profile of MAP-infected cattle shows that the production and metabolism of reactive oxygen species are decreased and IL-10 signaling, LXR/RXR signaling, and complement system is activated in the subclinical stage. These results demonstrate a balanced response that provides an immune-limiting mechanism during host-defense response (Shin et al., 2015b). Another study showed that increased expression of CD46, ICOS, and CEP350 in infected calves, but decreased expression of CTLA4, YARS, and PARVB. In addition, a comparison of seropositive and seronegative infected calves confirmed that IL6ST, gp130, and CD22 play an important role in inducing antibodies to MAP (David et al., 2014a). Also, the gene expression analysis at 6 months after infection, revealed that down-regulation of the neutrophil beta -diphenine-9 peptide (BNBD9-Like), S100 calcium binding protein A9 (s100A9) and G protein coupling receptor 77 (GPR77) or C5a anaphylatoxin chemotactic receptor (C5a2) and down-regulation of BOLA / MHC-I, BNBD9-like and upregulation of CD46 at 3, 6, 9, 12 and 15 months after inoculation was confirmed (David et al., 2014b). In addition, studies are being
conducted to develop a diagnostic method for paratuberculosis using the degree of specific gene expression in the host. Park et al., found that expression of eight genes (TIMP1, HP, SERPINE1, TFRC, MMP9, DEFB1, DEFB10, and S100A8) was significantly increased in the whole blood of MAP-infected cattle and investigate the diagnostic value of these biomarkers. As a result, four genes (TIMP1, S100A8, DEFB1, and DEFB10) showed the highest diagnostic accuracy in the subclinical group (Park et al., 2017a). Also, another study revealed that diagnostic potential of biomarkers for diagnosis of the early stage of MAP infection in cows and their offspring which show positive tissue MAP and negative fecal MAP (Park et al., 2017b). Taken together, many types of researches to identify biomarkers for diagnosis of paratuberculosis at the early stage were conducted.
Chapter I

Gene expression profiles of putative biomarker candidates in *Mycobacterium avium* subsp. *paratuberculosis* infected cattle

Abstract

This study was conducted to analyze gene expression of prognostic potential biomarker candidates using the whole blood of cattle naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). We conducted real-time PCR to evaluate 23 potential biomarker candidates. Experimental animals were divided into four groups based on fecal MAP PCR and serum ELISA. Seven (*KLRB1, HGF, MPO, LTF, SERPINE1, S100A8*, and *S100A9*) genes were up-regulated in fecal MAP positive cattle and three (*KLRB1, MPO*, and *S100A9*) were up-regulated in MAP seropositive cattle relative to uninfected cattle. In subclinically infected animals, 17 genes (*TFRC, S100A8, S100A9, MPO, GBP6, LTF, KLRB1, SERPINE1, PIGR, IL-10, CXCR3, CD14, MMP9, ELANE, CHI3L1, HP*, and *HGF*) were up-regulated
compared to the control group. Moreover, six genes (CXCR3, HP, HGF, LTF, TFRC, and GBP6) showed significant differences between experimental groups. Taken together, our data suggest that six genes (LTF, HGF, HP, CXCR3, GBP6, and TFRC) played essential roles in immune response to MAP during the subclinical stage and might be therefore be useful as prognostic biomarkers.

Key words: Biomarkers, Cattle, MAP, Paratuberculosis, Subclinical stage
Introduction

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the etiologic agent of bovine paratuberculosis, a chronic wasting disease that affects domestic and wild ruminants (Whitlock and Buergelt, 1996). During the subclinical stage, animals still do not have clinical symptoms and; however, they shed low numbers of MAP into environment and MAP can be circulated in the herd and infect other animals (Tiwari et al., 2006). Therefore, it is very important to remove subclinical stage animals to control the disease. However, current diagnostic methods including fecal culture, fecal PCR, and ELISA are insufficient for diagnosis of subclinical stages of disease (Al Hajri and Alluwaimi, 2007; Bögli-Stuber et al., 2005; Englund et al., 2002).

Host biomarkers have been proposed as a diagnostic tool for chronic disease (Li et al., 2008; Pomorska-Mól et al., 2012; Walzl et al., 2011). Many potential biomarkers have been proposed for the detection of MAP infection in previous studies (David et al., 2014a; David et al., 2014b; Seth et al., 2009; Shin et al., 2015a; Shin et al., 2015b; Verschoor et al., 2010). Although several prognostic markers of bovine paratuberculosis have been suggested, there are still barriers preventing the use of effective diagnostic markers from the biomarkers suggested in previous studies. These studies were designed to analyze gene expression in specific type of cells or organ. Whole blood sample is easy to take and can be represent prognosis and severity of disease (Dickinson et al., 2015; Mejias et al., 2013). Therefore, the present study was conducted to identify gene expression of whole blood in MAP.
infected cattle in different stages of infection based on the presence of MAP in feces and MAP antibody.

**Materials and Methods**

**Experimental animals**

Twenty seven Holstein cows were selected from national farm in the mid-west region of South Korea by detection of MAP-specific antibodies using a commercial ELISA kit (IDEXX Laboratories, Inc., Westbrook, ME, USA) and MAP in feces by PCR (Park *et al.*, 2014a) in 2014. The detection was performed two times with 6 month interval for precise classification of infection status. The animals were divided by based on the results of PCR and ELISA; a control group \((n = 5)\) that was ELISA and PCR negative, Group 1 \((n = 7)\), which was ELISA and PCR positive, Group 2 \((n = 6)\), which was ELISA negative and PCR positive, and Group 3 \((n = 9)\), which was ELISA positive and PCR negative. Also, infected animals (Group 1 to 3; \(n = 22\)) were divided into four groups according to the ELISA S/P ratio; EL200 group \((n = 3)\) which was showed S/P ratio \(\geq 200\), EL100 group \((n = 6)\) which was showed S/P ratio \(< 200\) and \(\geq 100\), EL45 group \((n = 7)\) which was showed S/P ratio \(< 100\) and \(\geq 45\), ELneg group (Same as Group 2; \(n = 6\)) which was showed S/P ratio <45. Subclinically infected animals defined as MAP antibody negative and MAP positive
in feces (Group 2 and ELneg group). All animal procedures were performed with the permission by the National Institute of Animal Science (2013-046).

**Selection of biomarker candidates**

Twenty three genes that differed by more than absolute log 2 fold change value ≥ 1.5 and therefore had the potential for use as prognostic markers were selected based on our previous studies (Cha et al., 2013; Shin et al., 2015a; Shin et al., 2015b). The selected prognostic marker candidates are listed in Table 2.

**Extraction of total RNA from blood**

Peripheral blood from cattle was collected from the tail vein using a PAX gene Blood RNA tube (PreAnalytiX/Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA was extracted using a PAX gene Blood RNA kit (PreAnalytiX/Qiagen, Hilden, Germany) according to the manufacturer’s instruction.

**Real-time PCR**

Total RNA was used to prepare cDNA with random primers using a QuantiTect®
Reverse Transcription Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions. The expression of 23 genes was analyzed by quantitative real time RT-PCR. Real time PCR reactions were conducted with using the Rotor-Gene SYBR Green PCR kit (Qiagen Inc., Valencia, CA, USA). The primers used in this study are listed in Table 3. Each reaction was initiated with 95°C at 10 min, followed by 40 cycles of 95°C for 15 s, 60°C at 45 s. The expression level was determined by the $2^{-\Delta\Delta C_T}$ method using a housekeeping gene, β-actin, as a reference. Up-regulated genes were as defined as log 2 fold change value $\geq 1.0$ and down-regulated genes were as defined as log 2 fold change value $< 1.0$. Differentially expressed genes were as defined as absolute log 2 fold change value $\geq 1.5$ according to previous study (Shin et al. 2015a).

**Statistical analysis**

Data were reported as the mean ± standard error of the mean (S.E.M.) of three or more independent experiments. Statistical significance was determined by ANOVA ($p < 0.05$) with post hoc $t$-tests done using the statistical package for social science (SPSS) software version 21. Differences were considered significant at a $p <0.05$. 
Results

Gene expression profile of the experimental groups

In group 1, two genes (KLRB1 and MPO) were up-regulated, while remaining 21 genes were down-regulated. In group 2, 17 genes (TFRC, S100A8, S100A9, MPO, GBP6, LTF, KLRB1, SERPINE1, PIGR, IL-10, CXCR3, CD14, MMP9, ELANE, CHI3L1, HP, and HGF) were up-regulated, while remaining six genes were down-regulated. Of the up-regulated genes, six genes (CXCR3, HP, HGF, LTF, TFRC, and GBP6) were showed a significant difference between group 1 and group 2 (p<0.05) (Figure 3). Also, two genes (CXCR3 and TFRC) were showed a significant difference between group 1 and group 3 (p<0.05) and four genes (HP, HGF, LTF, and GBP6) were showed a significant difference between group 2 and group 3 (Table 2 and Figure 3). In group 3, eight genes (CXCL9, KLRB1, PIGR, MPO, GBP6, S100A8, HGF, and S100A9) were up-regulated, while 15 genes were down-regulated. In EL45 group, six genes (SERPINE1, S100A9, MPO, PIGR, CXCL9, and HGF) were up-regulated while remaining 17 genes were down-regulated. In EL100 group, only two genes (KLRB1 and MPO) were up-regulated while remaining 21 genes were down-regulated. In EL200 group, five genes (KLRB1, MPO, S100A9, HGF, and CXCL9) were up-regulated while remaining 18 genes were down-regulated. Of the up-regulated genes, only three genes (GBP6, HGF, and LTF) showed a significant
change between groups (Figure 3). In fecal MAP positive groups, seven genes (KLRB1, MPO, HGF, LTF, SERPINE1, S100A8, and S100A9) were up-regulated. In MAP seropositive group, three genes (KLRB1, MPO, and S100A9) were up-regulated (Table 2).

**Discussion**

Gene expression of whole blood can reflect infection status without reference to primary infection site. For example, distinct whole blood gene expression signature was observed in Crohn’s disease and ulcerative colitis patients (Barnes et al., 2015). Likewise, although the MAP is the enteric pathogen, distinct gene expression signature of whole blood was found in previous study (Shin et al., 2015b). In current study, we examined gene expression profiles of MAP-infected cattle which were classified by the results of ELISA and fecal PCR. We found that there were many genes which were expressed differentially between the infected cattle and healthy cattle.

Among the up-regulated genes, 12 genes (SERPINE1, HGF, LTF, KLRB1, S100A8, S100A9, HP, MPO, TFRC, GBP6, IL10, and PIGR) were differentially expressed in group 2. Of the differentially expressed genes, six genes (HGF, LTF, HP, TFRC, CXCR3 and GBP6) were showed statistically significant change (Figure 3). HGF is known to be a multifunctional protein that includes tissue protection, regeneration, and suppression of chronic inflammation (Nakamura et al., 2011). Significant up-
regulation of the *HGF* in subclinical stage seems to be a response to chronic inflammation due to MAP infection. Lactoferrin inhibit bacterial invasion to epithelial cell by binding to bacterial surface proteins (Valenti and Antonini, 2005). Significant up-regulation of *LTF* in subclinical stage might be associated with bacterial invasion. Haptoglobin was known to inhibit several function of neutrophil such as phagocytosis and respiratory burst (Rossbacher et al., 1999). Also, HP was known to have protective functions in inflammatory bowel disease via inhibition of several cytokine such as IL-17, IFN-γ, TNF, and IL-6 (Vanuytsel et al., 2013). Up-regulation of the *HP* in subclinical stage could be related to anti-inflammatory activity against immune response due to MAP infection. *GBP6* is a member of the IFN-γ-inducible GTPase super family, which is involved in host immune response (Kim et al., 2011). A previous study showed that IFN-γ-inducible GTPase provide protection against intracellular bacteria via oxidative killing and transfer of antimicrobial peptides to autophagolysosomes (Kim et al., 2011). *TFRC* encodes transferring receptors known as surface markers of unmatured phagosome infected with *M. tuberculosis* (Chua et al., 2004; Clemens and Horwitz). Up-regulation of the *TFRC* gene was observed at 6 h p.i. and maintained at 24 and 48 h p.i. in THP cells infected with MAP (Shin et al., 2015c). *TFRC* was up-regulated in Raw 264.7 cells infected with MAP at 6 h p.i. (Cha et al., 2013). Significant up-regulation of *GBP6* and *TFRC* between groups could be related to intracellular killing in early stage of MAP infection.

Although the gene expression of whole blood can reflect biological changes due to
disease, whole blood samples were collected only single time and analyzed in this study. More distinct changes might be observed if we had sampling at the multiple times. Subclinical animals which cannot detected by ELISA excrete low numbers of MAP into feces and subsequently infect other animals in the herd. Six genes (LTФ, HGF, HP, CXCR3, GBP6, and TFRC) which were significantly up-regulated in group 2 might be useful prognostic biomarkers of subclinical stages of JD. These genes should be further evaluated to determine their suitability for diagnosis of subclinical stage JD. Various factors including infection dose, infected age, animal species, and coexistence of other disease might affect the accuracy of these prognostic biomarkers. Accordingly, field studies need to be conducted to determine the adequacy of these prognostic biomarkers for use as a diagnostic tool of subclinical stage of JD.
Table 2. Fold change of selected biomarker candidate genes between experimental groups and control group

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<th>Gene group</th>
<th>Accession No.</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Location</th>
<th>Fold change</th>
<th>Group 1 vs. control</th>
<th>Group 2 vs. control</th>
<th>Group 3 vs. control</th>
<th>Fecal MAP positive group vs. control</th>
<th>MAP sero-positive group vs. control</th>
<th>ELneg vs control</th>
<th>EL45 vs control</th>
<th>EL100 vs control</th>
<th>EL200 vs control</th>
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Table 3. Nucleotide sequence of primers used for real-time PCR

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Figure 3. Gene expression profiles of biomarker candidates.

(A) Differentially expressed biomarker genes of cattle infected with *Mycobacterium avium* subspecies *paratuberculosis* in group 2 compared to non-infected cattle. The expression level was normalized by the $2^{\Delta\Delta CT}$ method in terms of the beta-actin expression level relative to the control group (*, $p<0.05$). (B) Expression of Th1 and Th2 type immune-related genes of cattle infected with *Mycobacterium avium* subspecies *paratuberculosis* compared to non-infected cattle. The expression level was normalized by the $2^{\Delta\Delta CT}$ method in terms of the beta-actin expression level relative to the control group (*, $p<0.05$). (C) Differentially expressed biomarker genes of cattle infected with *Mycobacterium avium* subspecies *paratuberculosis* in ELneg group compared to non-infected cattle. The expression level was normalized by the $2^{\Delta\Delta CT}$ method in terms of the beta-actin expression level relative to the control group (*, $p<0.05$ **, $p<0.01$).
Chapter II

Gene expression profiles of immune-regulatory genes in whole blood of cattle with a subclinical infection of *Mycobacterium avium subsp. paratuberculosis*

Abstract

Johne’s disease (JD) is a chronic wasting disease of ruminants caused by *Mycobacterium avium subsp. paratuberculosis* (MAP), resulting in inflammation of intestines and persistent diarrhea. The initial host response against MAP infections is mainly regulated by the Th1 response, which is characterized by the production of IFN-γ. With the progression of disease, MAP can survive in the host through the evasion of the host’s immune response by manipulating the host immune response. However, the host response during subclinical phases has not been fully understood. Immune regulatory genes, including Th17-derived cytokines, interferon regulatory factors, and calcium signaling-associated genes, are hypothesized to play an important role during subclinical phases of JD. Therefore, the present study was
conducted to analyze the expression profiles of immune regulatory genes during MAP infection in whole blood. Different expression patterns of genes were identified depending on the infection stages. Downregulation of *IL-17A, IL-17F, IL-22, IL-26, HMGB1*, and *IRF4* and upregulation of *PIP5K1C* indicate suppression of the Th1 response due to MAP infection and loss of granuloma integrity. In addition, increased expression of *IRF5* and *IRF7* suggest activation of IFN-α/β signaling during subclinical stages, which induced indoleamine 2, 3-dioxygenase mediated depletion of tryptophan metabolism. Increased expression of *CORO1A* indicate modulation of calcium signaling, which enhanced the survival of MAP. Taken together, distinct host gene expression induced by MAP infection indicates enhanced survival of MAP during subclinical stages.

Keywords: Paratuberculosis, *Mycobacterium avium* subsp. *paratuberculosis*, Johne’s disease, Subclinical, Host response, Whole blood
Introduction

Paratuberculosis (PTB) or Johne’s disease (JD) is a chronic infectious disease leading to persistent diarrhea, progressive wasting, and cachexia, all of which are caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Whitlock and Buergelt, 1996). MAP can affect a range of ruminants, including cattle, goats, lamb, and deer (Whitlock and Buergelt, 1996) as well as non-ruminants such as parrots, baboons, tamarins, cavies, lemurs, and wallabies (Münster et al., 2013). The transmission of PTB usually occurs through the ingestion of contaminated materials such as feed, colostrum, water, and soil (Fecteau et al., 2013; Tiwari et al., 2006). After ingesting contaminated materials, intestinal M cells, which are located in Peyer’s patches in the ileum, uptake and transfer MAP to macrophages that are distributed in the mesenteric lymph nodes (Rathnaiah et al., 2017). Generally, ingested pathogens are eliminated within the macrophage. However, MAPs can survive in host macrophages by interfering with phagosome maturation (Rumsey et al., 2006). Previous studies have suggested that MAP can inhibit host Rab proteins, which are essential for the phagosome–lysosome fusion following phagocytosis in human and mouse macrophages (Hostetter et al., 2003).

Due to the persistence of MAPs, macrophages form granulomas during subclinical phases of infection in the intestinal lymphoid tissue (Buergelt et al., 1978). Previous studies have suggested immunological changes related to fecal shedding during the progression of disease (Ganusov et al., 2015; Magombedze et al., 2016). Fecal
shedding has been shown to occur with the downregulation of cell-mediated immune response and upregulation of the humoral immune response in vivo (Magombedze et al., 2016). In contrast, some infected animals show increased fecal shedding with the activation of cellular immunity (Ganusov et al., 2015). However, specific mechanisms of the immune response that induces fecal shedding are not clear.

In the subclinical stages, infected macrophages with MAP upregulate expression of CD29, CD56, IL-1α, and TRAF1, resulting in the recruitment of immune cells to the sites of infection (Arsenault et al., 2014). Activated macrophages with MAP move to the local lymph nodes and present an antigen for stimulating naive T cells to induce the Th1 response, which involves the production of interferon gamma and pro-inflammatory cytokines such as IL-6, IL-1α, and IL-2 (Coussens, 2001). Dominance of the Th1 response continue during the subclinical stages, and activated Th1 lymphocytes induce the cell-mediated immune response by producing IL-2, TNF-β, and IFN-γ (Coussens, 2001).

During the late subclinical stages of JD, the Th1 response is gradually diminished and the Th2 response is enhanced, which induces the humoral immune response (Stabel, 2006). With the progression of disease from the subclinical phase to the clinical phase, the cell-mediated immune response totally diminishes, and the humoral immune response, which is characterized by the production of IL-4 and IL-10, becomes prominent (Stabel, 2006). Furthermore, with the shift from the Th1 to Th2 response, clinical signs and lesions become more severe. Therefore, understanding the host response during subclinical phases is critical in identifying
the pathogenesis of JD. We hypothesized that immune regulatory genes play an important role in the subclinical phases of JD during the immunological shift from the Th1 to Th2 response.

The Th17-derived cytokine is known to protect hosts from extracellular bacteria causing respiratory and intestinal tract infections (Dubin and Kolls, 2008). However, several studies have suggested that Th17-derived cytokines may have immune regulatory roles against the infection of intracellular bacteria such as *Salmonella* and *Listeria monocytogenes* (Hamada et al., 2008; Schulz et al., 2008). IL-17 deficient mice show enhanced bacterial numbers in the spleen and liver after an infection by *Salmonella enterica* (Schulz et al., 2008). In addition, with the infection of *Listeria monocytogenes*, bacterial numbers and granuloma formation were increased in the liver (Hamada et al., 2008).

Interferon regulatory factor (IRF) is a transcription factor that has regulatory roles in the immune system (Honda and Taniguchi, 2006). In particular, IRFs regulate the innate immune response via pattern recognition receptor signaling such as TLRs, CLR, RLRs, and NLRs (Zhao et al., 2015). High mobility group box 1 (HMGB1) is a non-histone nuclear protein that is involved in the regulation of the immune response (Lotze and Tracey, 2005; Scaffidi et al., 2002). PIP5K1C has been known to have an immune-regulatory function via the modulation of neutrophil polarization and infiltration (Xu et al., 2010). In addition, CORO1A have diverse functions, including calcium homeostasis, cytoskeletal dynamics, and maintenance of immune cell diversity and function (Jayachandran and Pieters, 2015).
However, the role of these immune-regulatory genes in JD has not been yet fully understood. Therefore, we analyzed the expression of immune regulatory genes among cattle groups that have different levels of antibodies to MAP and fecal shedding in order to determine the role of immune-regulatory genes during subclinical phases of JD.
Materials and Methods

Animals

The animals were selected from a national farm in the mid-west region of South Korea during 2016 to 2017. In this farm, the presence of infectious diseases, including paratuberculosis, bovine tuberculosis, brucellosis, infectious bovine rhinotracheitis, and bovine viral diarrhea, were investigated two times per year in spring and autumn. In total, 79 Holstein cows were selected for the experiments, according to the results of ELISA performed using a commercial ELISA kit (IDEXX Laboratories, Inc., Westbrook, ME, USA) and fecal detection of MAP by PCR. In brief, fecal DNA was extracted using the mGITC/SC method (Park et al., 2014a) and amplification of IS900 and ISMAP02-targeted real-time PCR was conducted as previously described with slight modification. (Sevilla et al., 2014; Zhang and Zhang, 2011). In total of 20 μl of reaction mixture consisted with 10 μl of 2 × Rotor-Gene Probe PCR master mix (Qiagen, Hilden, Germany), 500 nM primers, 200 nM probes, 4 μl fecal DNA, and 4 μl nuclease free water. Real-time PCR reaction was performed under the following conditions: 1 cycle at 95°C for 5 min, followed by 45 cycles at 95°C for 15 s, and 60°C for 1 min. The fecal sample was regarded to be positive when both IS900 and ISMAP02 real-time PCR results were positive. Primers used in real-time PCR were listed in Table 4. For the selection of animals, ELISA and fecal PCR were performed 4 times within a 6-month interval to ensure precise
classification of animals. For evaluation of clinical status of animals, all animals were monitored for two years for the presence of chronic diarrhea and cachexia. This study was carried out in strict accordance with the guidelines of the Institutional Animal Use and Care Committee of the National Institute of Animal Science. The protocol was approved by the Institutional Animal Use and Care Committee of the National Institute of Animal Science (Permit number 2013-046).

**Sampling and extraction of total RNA from whole blood**

Peripheral blood samples (3 ml) were collected from the tail vein of cattle with the BD Vacutainer® Plus Plastic K2EDTA Tubes and BD Vacutainer® Plus Plastic Serum Tubes. The extraction of total RNA from whole blood was performed as previously described (Park et al., 2016). In brief, 125 µl of whole blood was mixed with the same volume of RNase-free water and 750 µl of Trizol LS reagent (Ambion, Foster City, CA, USA) and incubated at room temperature for 5 min. Thereafter, 200 µl of chloroform (Sigma-Aldrich, Castle Hill, Australia) was mixed and centrifuged at 13,523 ×g and 4°C for 15 min. The supernatant was collected into a 1.5 ml tube, mixed with the same volume of 70% ethanol, and then transferred to an RNAeasy column (Qiagen, Hilden, Germany) and centrifuged at 8,500 ×g for 15 sec. After the wash steps, 30 µl of RNase-free water was added and centrifuged at 8,500 ×g for 1 min. Eluted RNA was stored at -80°C until use. For the separation of serum, 3 ml of blood samples were centrifuged at 1,500 ×g for 10 min. Separated serum was
transferred to 1.5 ml tube and analyzed for the presence of MAP-specific antibodies using a commercial ELISA kit.

**Selection of immune regulatory genes**

Eleven genes that are related to immune regulatory function were selected based on previous studies (Khare *et al.*, 2016; Shin *et al.*, 2015a) and classified to three categories as follows; Th17-derived cytokines (*IL-17A, IL-17F, IL-22,* and *IL-26*), calcium signaling (*HMGB1, CORO1A, and PIP5K1C*), and interferon regulatory factors (*IRF3, IRF4, IRF5,* and *IRF7*) (Table 4).

**Optimization of real-time PCR conditions**

Real-time PCR conditions were optimized with an identical cDNA template for each gene. Five concentrations of both forward and reverse primers ranging from 0.25 μM to 1.25 μM, with a 0.25 μM interval, were tested. In addition, five annealing temperatures from 56°C to 64°C with a two-degree interval were tested. For further experiments, optimal primer concentrations and annealing temperatures that showed the highest fluorescence value were selected for further analysis.

**Real-time PCR**
The cDNA was synthesized with random primers using a QuantiTect® Reverse Transcription Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions. The expression of eleven immune regulatory genes was identified by quantitative real-time RT-PCR with a Rotor-Gene multiplex PCR kit (Qiagen Inc). Briefly, a total of 18 μl reaction mixture included 10 μl of SYBR master mix, RNase-free water, and 0.5 μM forward and reverse primers. Finally, 2 μl of cDNA template was added to the mixture to a final volume of 20 μl. Specific amplification with primers for each target was identified by a homology search (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and agarose gel electrophoresis. The primers used in this study are shown in Table 4. Real-time PCR was performed with triplicate samples at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 45 s. A no-template sample was used for the negative control. The gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method with β-actin for the housekeeping gene.

**Statistical analysis**

Statistical significance was confirmed by ANOVA with Tukey’s post hoc test among the experimental groups using the GraphPad Prism software version 7.00 (GraphPad Software, Inc., La Jolla, CA, USA). A $P$ value of less than 0.05 ($p < 0.05$) was considered as statistically significant, and all experiments were recorded as the means of biological triplicates.
Results

Animals

The study subject included 79 heifers that were classified into five groups based on the results of the PCR and ELISA. Non-infected group \((n = 27)\) was defined as those that were ELISA- and PCR-negative during the entire examination. Infected animals were classified into four groups according to the ELISA sample to positive (S/P) ratio. The EL Neg group \((n = 23)\) was defined as those with a S/P ratio < 45 and PCR-positive. The EL Low group \((n = 9)\) was defined as those with a S/P ratio <100 and ≥ 45. The EL Mid group \((n = 8)\) was defined as those with a S/P ratio <150 and ≥ 100. The EL High group \((n = 12)\) was defined as those with S/P ratio ≥ 150. All animals did not show chronic diarrhea and cachexia for two years. Furthermore, infected animals were classified into two groups based on the presence of fecal shedding. The FP group \((n = 41)\) was defined as fecal PCR-positive, and the FN group \((n = 11)\) was defined as fecal PCR-negative. The age of animals ranged from 2 to 10 years, and the mean ± SD for the age of the animals for each group was as follows: Non-infected group, 4.92 ± 2.09 years; EL Neg group, 4.69 ± 1.74 years; EL Low group 6.11 ± 1.45 years; EL Mid group 4.87 ± 1.35 years; EL High group 5.16 ± 1.02 years; FP group, 5 year ± 1.61 years; FN group, 5.36 ± 1.28 years. Among these groups, age did not show any significant differences \((p > 0.05)\) (Figure 4).
Detailed characteristics of animals were presented in Table 5.

**Optimization of real-time PCR conditions**

The optimal concentration of primers and annealing temperature was determined by conducting real-time PCR with three primer concentrations and annealing temperatures. The combination of forward and reverse primers at 0.5 μM and an annealing temperature of 60°C showed the highest fluorescence and lowest C_T value. The combination of 0.5 μM forward and reverse primers and 60°C of an annealing temperature of were used in further analysis.

**Gene expression profiles between the infected groups**

The expression of Th17-derived cytokine genes is presented in Figure 5 and 6. IL-17A was downregulated in the EL Low, Mid, and High groups compared to the non-infected and EL Neg groups. In addition, IL-17A was downregulated in the EL Mid group compared to the EL Low group and upregulated in EL High group compared to the EL Mid group. IL-22 was downregulated in the EL Mid and High groups compared to the EL Neg group. IL-26 was downregulated in EL Neg and High groups compared to the non-infected group. Furthermore, IL-26 was upregulated in the EL Mid group compared to the EL Neg group. When compared with the presence of fecal shedding, 3 genes (IL-17A, IL-17F, and IL-26) were downregulated in the
FP group compared to the non-infected group. In addition, two genes (IL-17F and IL-26) were downregulated in the FN group compared to the non-infected group. Furthermore, the expression of IL-17A was increased in the FN group compared to the FP group.

The expressions of interferon regulatory factors are presented in Figure 7 and 8. IRF3 was downregulated in the EL Mid and High groups compared to the EL Neg group. On the other hand, IRF5 was upregulated in the EL Neg, Low, and High groups compared to the non-infected group. The expression of IRF7 was increased in the EL Neg group compared to the non-infected group. In addition, IRF7 was downregulated in the EL Mid group compared to the EL Neg group. According to the fecal shedding-based classification, IRF5 was upregulated in the FP group compared to the non-infected group. In contrast, IRF4 was downregulated in the FP and FN groups compared to the non-infected group.

The expressions of calcium signaling-associated genes are presented in Figure 9 and 10. HMGB1 was downregulated in the EL Neg, Low, Mid, and High groups compared to the non-infected group. In addition, the expression of HMGB1 was decreased in the FP and FN groups compared to the non-infected group. The expression of PIP5K1C was increased in the FP group compared to the non-infected group and decreased in the FN group compared to the FP group. CORO1A was upregulated only in the FP group compared to the non-infected group.
Discussion

Gene expression profiling of whole blood is a useful indicator of the progression of diseases and immune responses in chronic infectious diseases (Mejias et al., 2013; Seok et al., 2013). Although gene expression in whole blood does not completely reflect the immunological changes at the site of infection, alteration of gene expression in peripheral blood may be specific to disease progression and provide useful information for identification of pathophysiology (Andrés-Benito et al., 2017; Li et al., 2017; Smith et al., 2017). Several studies have investigated the host response to MAP infection in whole blood, peripheral blood mononuclear cells (PBMC), and monocyte-derived macrophages (MDM) (Casey et al., 2015; David et al., 2014a; David et al., 2014b; Marino et al., 2017; Park et al., 2016; Shin et al., 2015b; Verschoor et al., 2010). However, the host responses during the immunological shift period between early subclinical to late subclinical stages were not fully understood. Therefore, the present study was conducted to identify the host response during the subclinical stages that revealed different levels of antibodies and the presence of MAP shedding in feces.

The present study suggests a difference in the gene expression profile of non-infected animals and infected animals with subclinical phases with different levels of fecal shedding and antibodies to MAP. The expression of Th17-derived cytokine genes differed between the experimental groups. Although the expression of all
genes was not perfectly matched, Th17-derived cytokine genes were downregulated during the progression of disease. Th17-derived cytokines play an important role in the early stage of mycobacterial infection (Torrado and Cooper, 2010). IL-26 and IL-17F are the main effector cytokines of the Th17 response and are associated with host defense against intracellular bacteria (Coulter et al., 2017; Tengvall et al., 2016). IL-26 induces priming of immune cells and direct killing of pathogens via membrane pore formation (Stephen-Victor et al., 2016). Furthermore, previous studies have revealed that IL-26 upregulates tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) expression in human NK cells, which induces the elimination of hepatitis C-infected hepatocytes (Miot et al., 2015). Moreover, IL-17 provides protective immunity against intracellular pathogens by modulation of Th1 response and neutrophilic recruitment (Raffatellu et al., 2008; Umemura et al., 2007). In addition, Robinson et al. analyzed the expression of Th17 cytokines in tissue samples pooled with jejunum, ileocecal valve, and adjacent lymph node of MAP-infected red deer (Robinson et al., 2011). Expression of IL-17 was not significantly changed in early stage of infection, but was significantly increased at late stage. On the other hand, the expression of IL-21, IL-22, and IL-23 were not significantly changed depending on the stage of infection (Robinson et al., 2011). In this study, the expression of IL-17A and IL-17F tended to decrease with the progression of disease. This difference may be due to the difference between the host animal (red deer vs cattle) and the sample (jejunum vs whole blood). Park et al., found that the expression of IL-17, IL-22, IL-23 and RORC was upregulated after the stimulation
of MAP in PBMC isolated from cattle infected with MAP (Park et al., 2014b). This suggests that Th17-derived cytokines play an important role in the early stages of JD. Therefore, downregulation of Th17-derived cytokine genes reflects insufficient immune response for eliminating intracellular MAP during all subclinical stages in this study.

Formation of granuloma is an important factor in the pathogenesis of JD (Koets et al., 2015). MAP crosses the intestinal barrier through M cells or enterocytes and subsequently up-taken by macrophages located in the lamina propria. Thereafter, cytokine production by activated macrophages and MAP antigens attracts new monocytes. Finally, multi-nucleated giant cells and epithelioid cells are formed, resulting in the formation of new granuloma (Koets et al., 2015). Formation of granulomas is tightly regulated by immune responses such as TNF-signaling and neutrophil trafficking (Bernut et al., 2016a). Neutrophil recruitment regulated by the TNF/IL-8 axis is essential for the establishment and maintenance of granulomas, which provides protective immunity to the host during mycobacterial infections (Bernut et al., 2016b; Smith et al., 2009). Therefore, impaired recruitment of neutrophil can induce unstable maintenance of granulomas. Th17-derived cytokines, including IL-17, IL-23, and IL-26, enhance granuloma integrity through the modulation of neutrophil recruitment via CXCR3 signaling (Seiler et al., 2003). In addition, PIP5K1C encodes protein phosphatidylinositol phosphate kinases, which regulate E-cadherin sorting for degradation, and increased activity of PIP5K1C induced the downregulation of E-cadherin (Schill et al., 2014). A previous study
suggested that E-cadherin is expressed in macrophages if the macrophages fail to eliminate intracellular pathogens, which subsequently contributes to the formation of granulomas (Cronan et al., 2016). Therefore, upregulation of PIP5K1C and downregulation of IL-17A and IL-17F in the whole blood can reflect loss of granuloma integrity, which induces bacterial shedding through feces.

Maturation of phagosomes is an important process in the defense against microbial pathogen (Uribe-Querol and Rosales, 2017). However, intracellular pathogens can survive and replicate in the phagocytes by disrupting phagosome maturation (Queval et al., 2017). CORO1A encodes protein coronin 1 in mammalian cells, and is involved in actin dynamics (Rybakin and Clemen, 2005). A recent study revealed that coronin 1 is an essential factor for modulating calcium signaling after the invasion of pathogenic mycobacteria (Jayachandran et al., 2007). Coronin 1 modulates physiological Ca\(^{2+}\) fluxes and induces the activation of calcineurin, subsequently blocking phagosome–lysosome fusion (Jayachandran et al., 2007). Moreover, IL-22 activates phagosome maturation via enhancing calgranulin A expression in MDMs infected with Mycobacterium tuberculosis (Dhiman et al., 2014). Calgranulin A was upregulated in MAP-infected animals and has been proposed as a diagnostic biomarker for subclinical MAP infections (Park et al., 2016; Verschoor et al., 2010). Thus, upregulation of CORO1A and downregulation of IL-22 indicate enhanced intracellular survival of MAP during subclinical stages.

Expression of interferon regulatory genes was different between experimental groups. First, the expression of IRF4 was decreased in all infected groups compared
to the non-infected group. IRF4 is an essential factor for the differentiation of T and B cells as well as the generation of plasma cells (Nam and Lim, 2016). In a previous study, *IRF4* knockout mice failed to provoke Th1 immune response against *Listeria monocytogenes* infections (Mahnke *et al.*, 2016). Decreased expression of *IRF4* can induce downregulation of the Th1 immune response, which can enhance the persistent survival of MAP. *HMGB1* expression is upregulated by the IFN-γ that is secreted in activated macrophages (Rendon-Mitchell *et al.*, 2003). During early infections, mycobacterial pathogen can inhibit the activation of macrophages via the induction of anti-apoptotic and anti-inflammatory response (Abendaño *et al.*, 2014; Awuh *et al.*, 2015). This result coincided with a previous study that showed downregulation of a complement immune pathway induced by MAP and consequently enhanced intracellular survival in macrophages (Shin *et al.*, 2015b).

Interferon regulatory factor (IRF) is the transcriptional regulator of IFN genes that regulates the immune response to intracellular pathogen. *IRF5* and *IRF7* have been shown to activate type I interferons including IFN-α, -β, -ω, -ε, -κ, and pro-inflammatory cytokines (Honda and Taniguchi, 2006; McNab *et al.*, 2015). IFN-α/β is the most widely expressed type I IFN, which has diverse effects on innate and adaptive immunity (McNab *et al.*, 2015). In general, IFN-α/β have been shown to protect the host from intracellular pathogens, including *Chlamydia trachomatis*, *Legionella pneumophila*, and *Salmonella Typhimurium* (Bukholm *et al.*, 1984; Ishihara *et al.*, 2005; Schiavoni *et al.*, 2004). More specifically, IFN-α/β inhibit intracellular replication of *Chlamydia trachomatis* through indoleamine 2,3-
dioxygenase (IDO) mediated depletion of L-tryptophan (Ishihara et al., 2005). A previous study showed upregulation of IDO in THP-1 monocytes, PBMCs, and intestinal tissues of MAP-infected animals (Plain et al., 2011). However, IDO-mediated tryptophan depletion also has a detrimental effect on the host. For example, low concentrations of tryptophan inhibit T cell proliferation (Munn et al., 2005). In addition, metabolites of IDO-mediated tryptophan metabolism such as kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid can inhibit T cell proliferation via apoptosis and arrest of the cell cycle (Fallarino et al., 2003; Frumento et al., 2002; Terness et al., 2002). Therefore, upregulation of IRF5 and IRF7 can result in the inhibition of T cell proliferation via IDO-mediated tryptophan depletion. In another study, expression of IRF5 was downregulated after 3 weeks of infection with MAP in the spleen of mice (Shin et al., 2015a). These differences are possibly related to different host species or differences in the first infection time and dose. Taken together, whether IFN-α/β is beneficial or detrimental for the host is not clear in MAP infections. Therefore, the specific role of IFN-α/β during subclinical stages of JD should be identified in further studies.

Several studies revealed molecular mimicry between MAP protein and host IRF5 protein (Bo et al., 2018; Cossu et al., 2015; Mameli et al., 2016). Mameli et al., identified that molecular mimicry between MAP_4027_18-32 and IRF5_424-434 peptide (Mameli et al., 2016). Also, they found that antibodies to both MAP_4027_18-32 and IRF5_424-434 peptides were significantly elevated in sera and cerebrospinal fluid of multiple sclerosis patients when compared to healthy individuals (Mameli et al.,
2016). Cossu et al., found similar result in sera of multiple sclerosis patients and these two peptides induce activation of the Th1 response in the whole blood while suppressing the Th2 response (Cossu et al., 2015). Recently, Bo et al., analyzed the serum of rheumatoid arthritis patients and suggests exposure to MAP can trigger specific humoral immune response against host IRF5 protein due to molecular mimicry between MAP_402718-32 and IRF5_424-434 peptides in rheumatoid arthritis patients (Bo et al., 2018). Taken together, humoral immune response to IRF5_424-434 peptide which induced by exposure to MAP may weaken the Th1 response and activate the Th2 response during subclinical stage of JD.

In conclusion, we propose a novel model for the host response, which enhances the survival of MAP (Figure 11). Downregulation of IL-17A, IL-17F, IL-26, and upregulation of PIP5K1C induce loss of granuloma integrity results in fecal shedding and dissemination of the pathogen. Downregulation of IRF4 resulted in impaired Th1 immune response, which decreased expression of HMGB1 and enhanced the downregulation of the Th1 immune response. In addition, increased expression of IRF5 and IRF7 suggest that activation of IFN-α/β signaling during subclinical stages induce IDO-mediated tryptophan metabolism. IDO-mediated depletion of tryptophan indicates an inhibition of T cell proliferation, subsequently leading to an immunosuppressive state. Upregulation of CORO1A suggest the possibility of the failure to intracellularly eliminate MAP. Taken together, this model suggests manipulation of host responses for the survival of MAP that occurs during the subclinical phases of JD. However, this model was established based on the gene
expressions of whole blood, which is not specific to individual immune cell subsets. Therefore, more specific roles of the immune regulatory genes during subclinical phases should be identified via interactions between different immune cells in co-culture systems or in vivo in further studies.
Table 4. Oligonucleotide sequences of primers and probes used for real-time PCR

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<th>Target gene</th>
<th>Primer sequence (5′→3′)</th>
<th>PCR product size (base pair)</th>
<th>Reference</th>
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| β-actin     | F: GCA AGC AGG AGT ACG ATG AG  
              R: GCC ATG CCA ATC TCA TCT CG | 134 | (Park et al., 2017a) |
| IL-17A      | F: CAC AGC ATG TGA GGG TCA AC  
              R: GTG GAG AGT CCA AGG TGA GG | 101 | In this study |
| IL-17F      | F: GAG GAA GCA AAA CGG CTG TC  
              R: CTG ATC TGC CAT CGG GTC AT | 115 | In this study |
| IL-22       | F: CTG TAG GCT CAA CGA GTC CG  
              R: CGC TTC GTC ACC TGA TGG AT | 150 | In this study |
| IL-26       | F: AAC GAT TCC AGA AGA TCG CA  
              R: CCA CAA AGT GCA TTT CCT TGC | 164 | In this study |
| HMGB1       | F: CGA ACA TCC TGG CCT GTC TA  
              R: TTA GCT CGG TAT GCG GCA AT | 150 | In this study |
| CORO1A      | F: ACC CTG ACA CCA ACA TCG TC  
              R: TTC ACC TCC AGA CCA CG | 166 | In this study |
| PIP5K1C     | F: GAG ATT GTG GTC CCC AAG GA  
              R: CTC CTC TCA TCG GTG GGA AA | 191 | In this study |
| IRF3        | F: GAA CCC AAA AGC CTC GGA TAC  
              R: CTC TCT CCA TCG GCC AAC TGG GT | 162 | In this study |
| IRF4        | F: GCA GAG ATC CCG TAC CAG TG  
              R: TCG GCA GAC CTT ATG CTT GG | 167 | In this study |
| IRF5        | F: AGA CCT CAA AGA CCG CAT GG  
              R: TTA CTG CAT GCC AAC TGG GT | 154 | In this study |
| IRF7        | F: CGC AAC GCT TTT TGA TGT TG  
              R: TGG AGG TGG GCC ATC TTC TA | 146 | In this study |
| IS900       | F: ATG ACG GTT AGC GAG GTG GTT  
              R: TGC AGT AAT GAT CGG CCT TAC  
              Probe: FAM-CGA CCA CGC CCC AGA-TAMRA | 76 | (Zhang and Zhang, 2011) |
| ISMAP02     | F: CGG CTG GAC AGC GAA TG  
              R: CAT GAG CAG TAT CTT TCG  
              Probe: JOE-ATC CGT CCC AGT GGC GGA GTC AC-BHQ-1 | 67 | (Sevilla et al., 2014) |
Table 5. Basic characteristics of study subjects

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Figure 4. Comparison of mean age among the experimental animals.

The mean age of the experimental animals among the experimental groups was represented with a bar graph. Statistical significance was calculated by ANOVA with Tukey’s post hoc test among the experimental groups. A $P$ value of less than 0.05 ($p < 0.05$) was considered as statistically significant.
Figure 5. Differences in gene expression levels of Th17-derived cytokine genes of the EL Neg, EL Low, EL Mid, and EL High groups compared to control group. Scatter plots for each gene represent for each individual animal. Values of relative gene expression were normalized to the reference gene β-actin. * indicates a p-value <0.05; ** indicates a p-value <0.01; *** indicates a p-value <0.001; **** indicates a p-value <0.0001. The EL Neg group (n = 23) was defined as those with a S/P ratio < 45 and PCR-positive. The EL Low group (n = 9) was defined as those with a S/P ratio <100 and ≥45. The EL Mid group (n = 8) was defined as those with a S/P ratio <150 and ≥100. The EL High group (n = 12) was defined as those with S/P ratio ≥150. Gene expression value of EL Neg, Low, Mid, and High groups were compared to Control group and statistical significance was calculated by ANOVA with Tukey’s post hoc test.
Figure 6. Differences in gene expression levels of Th17-derived cytokine genes of the FP, and FN groups compared to control group.

Scatter plots for each gene represent for each individual animal. Values of relative gene expression were normalized to the reference gene β-actin. * indicates a p-value <0.05; ** indicates a p-value <0.01; *** indicates a p-value <0.001; **** indicates a p-value <0.0001. The FP group (n = 41) was defined as fecal PCR positive, and the FN group (n = 11) was defined as fecal PCR- negative. Gene expression value of FP and FN groups were compared to Control group and statistical significance was calculated by ANOVA with Tukey’s post hoc test.
Figure 7. Differences in gene expression levels of interferon regulatory factors of the EL Neg, EL Low, EL Mid, and EL High groups compared to control group. Scatter plots for each gene represent for each individual animal. Values of relative gene expression were normalized to the reference gene β-actin. * indicates a $p$-value $<0.05$; ** indicates a $p$-value $<0.01$; *** indicates a $p$-value $<0.001$; **** indicates a $p$-value $<0.0001$. The EL Neg group ($n = 23$) was defined as those with a S/P ratio $< 45$ and PCR-positive. The EL Low group ($n = 9$) was defined as those with a S/P ratio $<100$ and $\geq 45$. The EL Mid group ($n = 8$) was defined as those with a S/P ratio $<150$ and $\geq 100$. The EL High group ($n = 12$) was defined as those with S/P ratio $\geq 150$. Gene expression value of EL Neg, Low, Mid, and High groups were compared to Control group and statistical significance was calculated by ANOVA with Tukey’s post hoc test.
Figure 8. Differences in gene expression levels of interferon regulatory factors of the FP, and FN groups compared to control group.

Scatter plots for each gene represent for each individual animal. Values of relative gene expression were normalized to the reference gene β-actin. * indicates a p-value <0.05; ** indicates a p-value <0.01; *** indicates a p-value <0.001; **** indicates a p-value <0.0001. The FP group (n = 41) was defined as fecal PCR positive, and the FN group (n=11) was defined as fecal PCR- negative. Gene expression value of FP and FN groups were compared to Control group and statistical significance was calculated by ANOVA with Tukey’s post hoc test.
Figure 9. Differences in gene expression levels of calcium signaling-associated genes of the EL Neg, EL Low, EL Mid, and EL High groups compared to control group.

Scatter plots for each gene represent for each individual animal. Values of relative gene expression were normalized to the reference gene β-actin. * indicates a $p$-value <0.05; ** indicates a $p$-value <0.01; *** indicates a $p$-value <0.001; **** indicates a $p$-value <0.0001. The EL Neg group ($n = 23$) was defined as those with a S/P ratio < 45 and PCR-positive. The EL Low group ($n = 9$) was defined as those with a S/P ratio <100 and $\geq$ 45. The EL Mid group ($n = 8$) was defined as those with a S/P ratio <150 and $\geq$ 100. The EL High group ($n = 12$) was defined as those with S/P ratio $\geq$ 150. Gene expression value of EL Neg, Low, Mid, and High groups were compared to Control group and statistical significance was calculated by ANOVA with Tukey’s post hoc test.
Figure 10. Differences in gene expression levels of calcium signaling-associated genes of the FP, and FN groups compared to control group.

Scatter plots for each gene represent for each individual animal. Values of relative gene expression were normalized to the reference gene β-actin. * indicates a p-value <0.05; ** indicates a p-value <0.01; *** indicates a p-value <0.001; **** indicates a p-value <0.0001. The FP group (n = 41) was defined as fecal PCR positive, and the FN group (n = 11) was defined as fecal PCR-negative. Gene expression value of FP and FN groups were compared to Control group and statistical significance was calculated by ANOVA with Tukey’s post hoc test.
Figure 11. Novel model for the manipulation of host responses by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) for its survival during subclinical stages of Johne’s disease.

MAP can manipulate host responses to enhance its survival. Upregulation of *PIP5K1C* and downregulation of *IL-17A, IL-17F, and IL-26* induces a loss of granuloma integrity, which can result in fecal shedding and dissemination of MAP. In addition, downregulation of *IRF4* and *HMGB1* can impair intracellular elimination of MAP. Upregulation of *CORO1A* modulates intracellular Ca\(^{2+}\), which can block phagosome–lysosome fusion. Moreover, decreased expression of *IL-22* indicates an inhibition of phagosome maturation. Upregulation of *IRF5* and *IRF7* activates IFN-α/β signaling, which upregulate IDO-mediated tryptophan depletion and subsequently induce the inhibition of T cell proliferation. Taken together, a manipulated host response enhances the survival of MAP during the subclinical phases of JD.
Chapter III

Establishment a real-time reverse transcription PCR based on host biomarkers for the detection of the subclinical cases of *Mycobacterium avium* subsp. *paratuberculosis*.

Abstract

Johne’s disease (JD) is a chronic enteric inflammatory disease of cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) that causes large economic losses in the dairy industry. Spread of JD is mainly provoked by a long subclinical stage during which MAP is shed into the environment with feces; accordingly, detection of subclinical animals is very important to its control. However, current diagnostic methods are not suitable for detection of subclinical animals. Therefore, the current study was conducted to develop a diagnostic method for analysis of the expression of genes of prognostic potential biomarker candidates in the whole blood
of Holstein cattle naturally infected with MAP. Real-time PCR with nine potential biomarker candidates was developed for the diagnosis of MAP subclinical infection. Animals were divided into four groups based on fecal MAP PCR and serum ELISA. Eight genes (TIMP1, HP, SERPINE1, TFRC, MMP9, DEFB1, DEFB10, and S100A8) were up-regulated in MAP-infected cattle (p <0.05). Moreover, ROC analysis revealed that eight genes (TIMP1, HP, SERPINE1, TFRC, MMP9, DEFB1, DEFB10, and S100A8) showed fair diagnostic performance (AUC≥0.8). Four biomarkers (TIMP1, S100A8, DEFB1, and DEFB10) showed the highest diagnostic accuracy in the PCR positive and ELISA negative group (PN group) and three biomarkers (TFRC, HP, and SERPINE1) showed the highest diagnostic accuracy in the PCR negative and ELISA positive group (NP group). Moreover, three biomarkers (S100A8, HP, and DEFB10) were considered the most reliable for the PCR positive and ELISA positive group (PP group). Taken together, our data suggest that real-time PCR based on eight biomarkers (TIMP1, HP, SERPINE1, TFRC, MMP9, DEFB1, DEFB10, and S100A8) might be useful for diagnosis of JD, including subclinical stage cases.

Keywords: Paratuberculosis, Mycobacterium avium subsp. paratuberculosis, Johne’s disease, Biomarker, Real-time PCR, Diagnosis, Subclinical stage
Introduction

Johne’s disease (JD) is a chronic inflammatory disease of the gastrointestinal tract of ruminants with granulomatous lesions that is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Whitlock and Buergelt, 1996). Especially, JD causes huge economic losses to dairy industry due to decreased milk production and weight loss (Garcia and Shalloo, 2015). MAP infection in cattle can be divided into four stages depending on the clinical signs and MAP shedding levels including the silent, subclinical, clinical, and advanced clinical stage (Whitlock and Buergelt, 1996). In the silent stage, the infected cattle do not show any clinical sign or excrete MAP into the environment (Tiwari et al., 2006). During the subclinical stage, infected cattle still do not have clinical symptoms; however, they shed low numbers of MAP into environment, which can be circulated in the herd and infect other animals (Tiwari et al., 2006). After subclinical stage, infected cattle enter clinical stage and start to show clinical signs such as gradual weight loss, diarrhea, and decreased milk production (Tiwari et al., 2006). Finally, infected cattle become cachectic and lethargic in advanced clinical stage (Tiwari et al., 2006). Accordingly, it is very important to remove infected cattle in the subclinical stage to control the disease. However, current diagnostic methods are insufficient for diagnosis of subclinical stages of disease (Mortier et al., 2015). Although fecal culture has been considered a gold standard for the diagnosis of MAP (Whittington et al., 2013), this method is time-consuming and shows low sensitivity, especially in subclinical stages
of the disease (Bögli-Stuber et al., 2005; Sockett et al., 1992a). PCR allows rapid detection of MAP in clinical samples such as feces, milk and blood (Sevilla et al., 2014); however, PCR-based methods are also limited in their usefulness for diagnosis of subclinical stages of disease because of the low sensitivity (Wells et al., 2006) and low specificity caused by genetic similarities with other mycobacteria (Cousins et al., 1999; Englund et al., 2002). Although ELISA has been used for detection of antibodies to MAP in clinical samples such as serum and milk, this method is also inadequate for diagnosis of fecal shedders in the subclinical stage, especially in 1–2 year old cattle (Al Hajri and Alluwaimi, 2007). Therefore, new diagnostic tools have been requested to detect MAP-infected animals at early stage of infection.

Biomarkers, which are considered indicators of specific pathogenic conditions or therapeutic responses to treatment (Strimbu and Tavel, 2010), are commonly used as diagnostic tools for various diseases (Dehnad et al., 2016; Goyal et al., 2017; Walker et al., 2016; Waters et al., 2016). Recently, host biomarkers discovered using transcriptomics, metabolomics, and proteomics have been proposed as alternative diagnostic methods for JD (David et al., 2014a; David et al., 2014b; De Buck et al., 2014; You et al., 2012). Biomarkers indicating early stages of MAP-infection were proposed by analyzing gene expression profiles of blood in cattle with experimental MAP infection (David et al., 2014a; David et al., 2014b). A metabolic profiling in cattle with experimental infection of MAP revealed that four metabolites (iso-butyrate, branched chain amino acids, leucine, and isoleucine) were increased in
serum of the MAP-infected cattle while citrate was decreased (De Buck et al., 2014). Moreover, six proteins (transferrin, gelsolin isoforms α & β, complement subcomponent C1r, complement component C3, amine oxidase-copper containing 3, and coagulation factor II) were proposed as biomarkers after they were found to increase by at least 2-fold in MAP-infected cattle, as were two proteins (coagulation factor XIII-B polypeptide, and fibrinogen γ chain and its precursor) that were reduced by nearly two fold in MAP-infected cattle (You et al., 2012). Our previous studies also proposed several biomarkers that were up-regulated in MAP infected macrophages, mice, and cattle (Cha et al., 2013; Shin et al., 2015a; Shin et al., 2015b). Transcriptional profiles of MAP-infected macrophage RAW 264.7 cells and a mouse model suggested five and three genes as prognostic biomarkers, respectively (Cha et al., 2013; Shin et al., 2015b). β-defensins were also suggested as prognostic biomarkers in subclinical animals of MAP-naturally infected cattle (Shin et al., 2015a). However, application of those biomarkers for diagnosis of JD has yet to be investigated. Therefore, we developed a real-time PCR method using the biomarkers for diagnosis of JD by measuring the gene expression level of several biomarkers in whole blood.
Materials and Methods

Experimental design and animals

About 300 Holstein cattle were raised on the national farm in Cheonan city which located in mid-west region of the South Korea. The cattle were regularly tested for absence of JD two times per year using fecal PCR and serum ELISA. A total of three to eight year old forty-four cows were selected for further analysis after detection of MAP-specific antibodies using a commercial ELISA kit (IDEXX Laboratories, Inc., Westbrook, ME, USA) and MAP in the feces by PCR (Park et al., 2016). The detection was performed four times with a 6-month interval to enable accurate classification of infection status. The animals were divided into the following groups based on the results of PCR and ELISA: NN, PCR and ELISA negative; PN, PCR positive and ELISA negative; NP, PCR negative and ELISA positive; PP, PCR positive and ELISA positive. All animal procedures were approved by the National Institute of Animal Science (2013-046). Detailed characteristics of study subjects are shown in Table 6.

Selection of biomarker candidates

Nine genes that were significantly up-regulated in MAP infected macrophages,
mice, and cattle were selected for use as diagnostic biomarkers based on our previous studies (Table 7) (Cha et al., 2013; Park et al., 2016; Shin et al., 2015a; Shin et al., 2015b). All datasets used in selection of the biomarkers are available at Gene Expression Omnibus (GEO) under accession number GSE62836, http://dx.doi.org/10.4014/jmb.1302.02021, and http://dx.doi.org/10.4014/jmb.1408.08059

**Extraction of total RNA from blood**

Peripheral blood was collected from the tail vein of cattle using a BD Vacutainer® Plus Plastic K$_2$EDTA Tubes. A total of 125 µl of whole blood was then mixed with 125 µl of RNase-free water and 750 µl of Trizol LS reagent (Ambion, Foster City, CA, USA) and incubated at room temperature for 5 min. Next, 200 µl of chloroform was added to the mixture and it was centrifuged at 13,523 g and 4°C for 15 min. The supernatant was subsequently transferred to an RNAeasy column (Qiagen, Hilden, Germany) and centrifuged at 8,500 g for 15 sec. After washing, RNA was eluted in 30 µl of RNase-free water and immediately stored at -80°C until use.

**Optimization of primer and probe concentrations**

The optimal concentration of primer and probe concentration was determined with an identical cDNA template for each biomarker gene. Three concentrations (0.5 µM,
0.75 μM, and 1 μM) of both forward and reverse primers with a constant probe concentration were tested. The combination showing the highest fluorescence value was tested at three different concentrations of the probe (0.1 μM, 0.2 μM, and 0.3 μM). For further experiment, primer and probe concentration that showing the highest fluorescence value was selected.

Real-time PCR

Total RNA was employed to prepare cDNA with random primers using a QuantiTect® Reverse Transcription Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions. The expression of nine biomarker genes was measured by quantitative real time RT-PCR, which was conducted using a Rotor-Gene multiplex PCR kit (Qiagen Inc., Valencia, CA, USA). In brief, total of 18 μl reaction mixture was prepared consists of 10 μl Master mix, RNase-free water, 0.5 μM forward and reverse primers, and 0.1 μM probe for each of the biomarker genes. After that, 2 μl of cDNA template was added to a final volume of 20 μl. The specificity of the primers and probes for each biomarker genes was confirmed by homology search (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and agarose gel electrophoresis. The primers and probe used in this study are shown in Table 7. Sensitivity of real-time PCR reactions was confirmed by real-time PCR reaction using the known copy numbers calculated from purified PCR products which serially
diluted from $10^9$ to $10^2$ copies of the templates. The real-time PCR was conducted for 45 cycles and $C_T$ values were obtained. Negative control was included with no template. Real-time PCR was conducted by subjecting the samples to 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 45 s. The expression level was determined by the $2^{-\Delta\Delta C_{t}}$ method using the housekeeping gene, β-actin, as a reference.

**Statistical analysis**

Data were reported as the means ± the standard error of the mean (S.E.M.) of three independent experiments. Statistical significance was determined by ANOVA ($p \leq 0.05$) with Dunnett’s post hoc test using the GraphPad Prism software version 7.00 (GraphPad Software, Inc., La Jolla, CA, USA). Receiver operator characteristics (ROC) curve analysis was conducted using the statistical package for social science (SPSS) software version 21.0 (SPSS Inc., Chicago, IL, USA) and the MedCalc Statistical Software version 13.3.3 (MedCalc Software, Ostend, Belgium). Higher AUC scores were considered to show better discriminatory powers as follows: excellent discriminatory power, AUC ≥ 0.9; good discriminatory power, 0.8 ≤ AUC < 0.9; fair discriminatory power, 0.7 ≤ AUC < 0.8; poor discriminatory power, AUC < 0.7 (Muller et al., 2005). The optimal cutoff values were calculated for each ROC curve while maximizing the Youden Index. Sensitivity and specificity were calculated based on cut-off value which showed highest AUC value in the ROC
curve for each biomarker gene. A $p < 0.05$ was considered to indicate statistical significance.

**Results**

**Specificity of probe and primers**

Specificity of primers and probes were confirmed by homology search. Also, to confirm the specificity for each biomarker genes, RT-PCR and agarose gel electrophoresis was performed. Single PCR band were confirmed for each biomarker gene and the β-actin gene and non-specific PCR product was not observed confirmed in the negative control with no cDNA sample (Figure 12).

**Sensitivity of real-time PCR reactions**

Real-time PCR for the each biomarker gene was performed using the specific primers, probes and the purified PCR products. Amplification plots were presented for biomarker genes with increased template copy numbers from $10^2$ to $10^9$. Amplification plot shows that fluorescence increase with increased template copy numbers (Figure 13). Also, real-time PCR was highly sensitive to detect low level of gene expression of biomarker genes (about $10^2$ copies of the template cDNA) and negative control sample with no template DNA showed no increasing of fluorescence
Optimization of primer and probe concentrations

The optimal concentration of primer and probe concentration was determined by conducting real-time PCR with three primer and probe concentrations. The combination of forward and reverse primer at 0.5 µM for biomarker genes and β-actin gene revealed highest florescence and lowest $C_T$ value. With this primer concentration, 0.1 µM of probe showed highest florescence and lowest $C_T$ value. Combination of 0.5 µM forward and reverse primers and 0.1µM probe concentration was used in further analysis.

Gene expression level of biomarkers in MAP infected cattle

Experimental animals were divided into four groups based on the results of fecal PCR and serum ELISA conducted three times with a 6 month interval. When compared with the non-infected NN group, expression of eight genes (S100A8, DEFB1, DEFB10, MMP9, TIMP1, HP, SERPINE1, and TFRC) showed higher expression in the PN group ($p<0.05$), while higher expression of seven other genes (TIMP1, HP, SERPINE1, TFRC, DEFB1, DEFB10, and MMP9) was observed in the NP group ($p<0.05$). Moreover, in the PP group, five genes (S100A8, MMP9, HP, SERPINE1, and TFRC) showed significantly higher expression in the PP group ($p$
<0.05). Four genes (TFRC, HP, SERPINE1, and MMP9) were up-regulated in all infected groups, while three genes (TIMP1, DEFB1, and DEFB10) were up-regulated in the PN group and the NP group, and S100A8 was up-regulated in the PN group and the PP group (Figure 14 and Table 8). The mean fold changes of each biomarker are shown in Table 8.

**Discrimination between infected and non-infected animals**

The AUC score of biomarkers was calculated during ROC analysis. In the PN group, the AUC scores of eight genes (TIMP1, DEFB1, TFRC, DEFB10, S100A8, SERPINE1, MMP9, and HP) were ≥0.8. In the NP group, four genes (HP, TIMP1, TFRC, and SERPINE1) had AUC scores ≥0.8, while six genes (S100A8, HP, SERPINE1, TFRC, MMP9, and DEFB10) in the PP group had AUC scores ≥0.8 (Figure 15). When the diagnostic accuracies of individual biomarkers were calculated by ROC curve analysis, the most accurate biomarker in the PN group was Timp1, with an AUC value of 0.985, while the most accurate biomarker in the NP group was HP, with an AUC value of 0.942. Additionally, the most accurate biomarker in the PP group was S100A8, with an AUC value of 0.896 (Figure 15). Similarly, in the PN group, TIMP1 showed the most accurate diagnostic performance, with a sensitivity of 100% and a specificity of 90.9%. In the NP group, HP showed the most accurate diagnostic performance, with a sensitivity of 92.9% and a specificity of 90.9%. Moreover, S100A8 showed the most accurate diagnostic
performance in the PP group, with a sensitivity of 85.7% and a specificity of 90%. Other details pertaining to the diagnostic performance of biomarkers are shown in Table 8.

**Discussion**

Early diagnosis of JD is the most important requirement to eradicate it from MAP-infected herds. However, current diagnostic methods are not sufficient for the diagnosis of subclinical stage animals that are actively dispersing MAP into the environment via fecal shedding (Tiwari *et al.*, 2006). Recently, several studies have attempted to diagnose subclinical stages of JD by analyzing host-pathogen interactions, including gene expression, miRNA, protein, and metabolites to MAP infection (David *et al.*, 2014a; David *et al.*, 2014b; De Buck *et al.*, 2014; Malvisi *et al.*, 2016; You *et al.*, 2012). Some of the studies have been conducted to identify prognostic biomarkers of JD by understanding host response to infection during the progression of JD (Cha *et al.*, 2013; Plain *et al.*, 2011; Seth *et al.*, 2009; Verschoor *et al.*, 2010; Wang *et al.*, 2011). However, no attempt has been made to apply biomarkers as diagnostic tools. Therefore, the present study was conducted to diagnose MAP infection using a real-time PCR method based on potential prognostic biomarkers.

In the present study, several biomarkers showed good discriminatory ability
(AUC ≥ 0.8) between MAP-infected cattle and non-infected cattle. Three genes (HP, SERPINE1, and TFRC) showed good discriminatory ability (AUC ≥ 0.8) in fecal PCR-positive and/or serum ELISA-positive groups (PN, NP, PP). Acute phase proteins are blood proteins that respond to infection and inflammation and have been used as diagnostic and prognostic biomarkers in veterinary medicine (Eckersall and Bell, 2010). HP is the major acute phase protein of cattle that responds to infection (Eckersall and Bell, 2010; El-Deeb and Elmoslemany, 2016). Moreover, HP is known to exert anti-inflammatory activity by down-regulating neutrophil activity via inhibition of both lipoxygenase and cyclooxygenase (Saeed et al., 2007) and to inhibit bacterial growth by interfering with iron acquisition by the host cell (Eaton et al., 1982). Moreover, HP inhibits phagocytosis and intracellular killing of pathogens (Rossbacher et al., 1999). This anti-inflammatory response induced by HP might reduce the harmful aspects of inflammation that could be destructive to the host itself. In that regard, up-regulation of HP in MAP-infected animals might be a host response to early infection of MAP. HP showed highest diagnostic accuracy for the NP group and whole infected animals, with AUC values of 0.942 and 0.901, respectively.

The initial response to MAP infection is dominant cell-mediated immunity, which is characterized by increasing interferon gamma release (Stabel, 2000). SERPINE1 is known to be an essential element of the fibrinolytic system that is related to blood coagulation (Furie and Furie, 1988). SERPINE1 also acts as an inflammatory mediator by increasing the level of interferon gamma in blood to eliminate the
pathogen in the early phase of an infectious disease (Lim et al., 2011; Wang et al., 2013). Therefore, increasing gene expression levels of Serpine1 might be related to interferon gamma release due to MAP infection. In addition, expression of MAP0403 in MAP was increased in infected macrophages and MAC-T cells in recent study (Kugadas et al., 2016). MAP0403 is kind of serine protease which served as a key element of the stress response network in intraphagosomal survival of MAP (Kugadas et al., 2016). Up-regulation of SERPINE1 might be a counter response to intraphagosomal survival of MAP in host cells. The diagnostic accuracy of SERPINE1 was good (AUC≥0.8) in all infected animals (PN, NP, PP group).

Iron is an important nutrient in innate immune response to bacterial pathogen (Johnson and Wessling-Resnick, 2012). TFRC is the one of the key elements of the iron metabolism, transfers iron to cells from transferrin protein (Johnson and Wessling-Resnick, 2012). TFRC is known to down-regulated in response to intracellular pathogen infection (Cha et al., 2013); however, its expression was significantly increased in all infected animals in the present study. This phenomenon might be related to the alternative iron acquisition system of MAP, which acts in a host-independent manner using mycobactin (Wang et al., 2015), however, further studies are needed to confirm this.

MMP9 is a matrix metalloproteinase related to leukocyte migration to infection sites and tissue destruction if it is secreted in excess amounts (Goetzl et al., 1996). The level of MMP9 was regulated by TIMP1, which inhibits the activity of MMP9 (Goetzl et al., 1996). MMP9 and TIMP1 are known to be up-regulated in tuberculosis
infection and have therefore been proposed as biomarkers for diagnosis of tuberculosis (Chen et al., 2017). The simultaneous up-regulation of MMP9 and TIMP1 in infected animals might be caused by inflammatory conditions due to the early stages of MAP infection. Two genes (MMP9 and TIMP1) showed good discriminatory ability (AUC ≥ 0.8) in the PN group.

β-defensins exhibit antimicrobial functions, providing first protection against pathogens while playing an immune-modulation role (Meade et al., 2014). Moreover, β-defensins interplay between innate and adaptive immune responses by down-regulating pro-inflammatory cytokines (Allaker, 2008). In the present study, DEFB1 and DEFB10 were significantly up-regulated in both the PN group and the NP group. Moreover, DEFB1 and DEFB10 showed excellent discriminatory ability (AUC ≥ 0.9) in the PN group.

S100A8 and S100A9 are members of a calcium-binding cytosolic protein family that are located in the cytoplasm (Schiopu and Cotoi, 2013). S100A8 and S100A9 form a heterodimer known as calprotectin that induces an inflammatory response via activation of TLR4 signaling (Vogl et al., 2007). Moreover, calprotectin is known to induce leukocyte migration in the early phase of bacterial infection (Achouiti et al., 2012). In previous studies, serum S100A8/A9 have been proposed as prognostic biomarkers for disease progression and therapeutic response in inflammatory bowel diseases (IBD) (Cayatte et al., 2012; Leach et al., 2007). In the present study, S100A8 showed good discriminatory ability (AUC ≥ 0.8) in the PN and PP groups. However, gene expression of S100A9 was not significant in all infected animals. Generally,
S100A8 and S100A9 exist as heterodimers, but they also exist as homodimers (Vogl et al., 2004). The inconsistent gene expression levels between S100A8 and S100A9 might be related to the presence of the homodimer form.

An ideal biomarker for diagnosis of JD should be able to discriminate between infected and non-infected animals with high sensitivity and specificity. Our data showed that the response of eight biomarkers (HP, TIMP1, MMP9, SERPINE1, TFRC, S100A8, DEFB1, and DEFB10) significantly discriminated MAP-infected and non-infected animals. Moreover, eight biomarkers (HP, TIMP1, MMP9, SERPINE1, TFRC, S100A8, DEFB1, and DEFB10) showed good accuracy (AUC ≥ 0.7) for diagnosis of subclinical animals. Additionally, four genes (TIMP1, S100A8, DEFB1, and DEFB10) showed sensitivity over 80% and specificity over 90%. It is generally very difficult to detect subclinical stages of JD using currently available diagnostic methods such as bacterial culture, fecal PCR and serum ELISA (Mortier et al., 2015). Fecal PCR is a reliable method for diagnosis of MAP infection; however, intermittent shedding of MAP into feces because of immunological changes during the progress of disease can interfere with accurate diagnosis (Mitchell et al., 2015). Moreover, although serum ELISA is a simple, fast and cost-effective method for diagnosis of JD, it is known to have low sensitivity for MAP-infected animals that do not show clinical signs (Nielsen and Toft, 2008). However, our real-time PCR method based on biomarkers showed relatively precise diagnostic results. In that regard, combination of eight biomarker genes (HP, TIMP1, MMP9, SERPINE1, TFRC, S100A8, DEFB1, and DEFB10) might be used for diagnosis of
JD, including in subclinical stage animals.

In conclusion, a real-time PCR method was developed based on eight biomarkers that can be used as a new diagnostic tool for JD with good diagnostic performance. Moreover, this real-time PCR based on biomarkers might be used for diagnosis of JD, especially in subclinical stage animals that cannot be detected by current diagnostic methods. Although our developed diagnostic method might be applied to field test, this method will be more concreted if possible limitations in our study such as the low number of samples and sampling times would be addressed in future studies by including large scale field investigations.
Table 6. Characteristics of study subjects

<table>
<thead>
<tr>
<th>Number of subjects</th>
<th>All (n=44)</th>
<th>NN group (n=11)</th>
<th>PN group (n=12)</th>
<th>NP group (n=14)</th>
<th>PP group (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heifers, n (%)</td>
<td>44 (100)</td>
<td>11 (100)</td>
<td>12 (100)</td>
<td>14 (100)</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Median age (Years)</td>
<td>6 (4 to 9)</td>
<td>4 (4 to 7)</td>
<td>6 (4 to 9)</td>
<td>6.5 (4 to 8)</td>
<td>6 (5 to 8)</td>
</tr>
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<td>Serum ELISA</td>
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<td></td>
<td></td>
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<tr>
<td>Positive, n (%)</td>
<td>21 (47.7)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>14 (100)</td>
<td>7 (100)</td>
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<tr>
<td>Negative, n (%)</td>
<td>23 (52.3)</td>
<td>11 (100)</td>
<td>12 (100)</td>
<td>0 (0)</td>
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<tr>
<td>Fecal PCR</td>
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<tr>
<td>Positive, n (%)</td>
<td>19 (43.2)</td>
<td>0 (0)</td>
<td>12 (100)</td>
<td>0 (0)</td>
<td>7 (100)</td>
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<td>Negative, n (%)</td>
<td>25 (56.8)</td>
<td>11 (100)</td>
<td>0 (0)</td>
<td>14 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Target gene</td>
<td>Primer sequence (5’→3’)</td>
<td>Product size (base pair)</td>
<td>Reference</td>
<td></td>
<td></td>
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<td>-------------</td>
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</tr>
<tr>
<td>β-ACTIN</td>
<td>F: GCAAGCAGGAGTACGATGAG</td>
<td>134</td>
<td>Park et al., 2017</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GCCATGCCCAATCTCATCTCG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: FAM-TTCTAGGGCGACTGTTAGCTGCGTAC- BHQ1</td>
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<td></td>
</tr>
<tr>
<td>MMP9</td>
<td>F: CCCGGATCAAGGATACGCC</td>
<td>177</td>
<td>Park et al., 2016</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>R: GGGCGAGGACCATACAGATG</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Probe: HEX-AGTTTGCCACGCGCTGGCCTAGAT- BHQ1</td>
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<tr>
<td>SERPINE1</td>
<td>F: TGCGAAATTCAGGATGCGG</td>
<td>191</td>
<td>Park et al., 2016</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GGGTGAGAAAACACGTTGC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Probe: FAM-AGACTTTTGGAGTGAAGGTGTTACAC- BHQ1</td>
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<tr>
<td>TIMP1</td>
<td>F: TCTCGAATCTCCAGATGTC</td>
<td>125</td>
<td>Park et al., 2017</td>
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<tr>
<td></td>
<td>R: CCTCAAGGCACTGAAACCTT</td>
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<tr>
<td></td>
<td>Probe: HEX-GTTCGTGGGAGCCACGAGATCAGAATG- BHQ1</td>
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<tr>
<td>HP</td>
<td>F: CCAAGTACCAGGACGACACC</td>
<td>131</td>
<td>Park et al., 2017</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: ACCATACTCAGCCACAGCAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td>Probe: FAM-ACGACAAAGGAAGACGACACCTGGGTATG- BHQ1</td>
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<tr>
<td>S100A8</td>
<td>F: ATTTTGGGGAGACCTGGTGG</td>
<td>124</td>
<td>Park et al., 2017</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: ACGGCGTGTGAAATTCCCTT</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Probe: FAM-TAACTCTCCCTGATGACCCTACACACACG- BHQ1</td>
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<tr>
<td>S100A9</td>
<td>F: AGGGCTACGGGAGGAGGACAG</td>
<td>134</td>
<td>Park et al., 2016</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>R: GCTGGCCCTCCTGATGAGTGG</td>
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<tr>
<td></td>
<td>Probe: HEX-ATGGAGGTACGGCCACAGCAC-BHQ1</td>
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</tr>
<tr>
<td>TFRC</td>
<td>F: CAAAGTTCTCGCCAGCCAC</td>
<td>188</td>
<td>Park et al., 2016</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: AACAGAAAGGAAGCCGCTGGG</td>
<td></td>
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<td></td>
<td>Probe: HEX-TATCGGGACAGCAGACTGGATGACGGAAG- BHQ1</td>
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</tr>
<tr>
<td>DEFB1</td>
<td>F: CGAATGGAGGCATCTGTGTTG</td>
<td>110</td>
<td>Park et al., 2017</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CTTGCCTCTTTTACCACCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: FAM-TGCTGGGCACACATGACGATGATGACG- BHQ1</td>
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<tr>
<td>DEFB10</td>
<td>F: ATCTAAGCTGCTGAGGGAAT</td>
<td>97</td>
<td>Park et al., 2017</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CATTCTGGCCGCTGCTAA</td>
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<tr>
<td></td>
<td>Probe: HEX-GTTCGTACAGGTGCCCGCCAGCA-BHQ1</td>
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</tr>
</tbody>
</table>
Table 8. Mean fold change of selected biomarker genes between infected animals and non-infected animals

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Location</th>
<th>Mean fold change (log2 value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PN vs. NN</td>
</tr>
<tr>
<td>NM_003234.2</td>
<td>TFRC</td>
<td>Transferrin receptor (p90, CD71)</td>
<td>Plasma membrane</td>
<td>1.6</td>
</tr>
<tr>
<td>NM_174744</td>
<td>MMP9</td>
<td>Matrix metalloproteinase 9</td>
<td>Extracellular Space</td>
<td>2.9</td>
</tr>
<tr>
<td>NM_002964.4</td>
<td>S100A8</td>
<td>S100 calcium binding protein A8</td>
<td>Cytoplasm</td>
<td>1.6</td>
</tr>
<tr>
<td>NM_002965.3</td>
<td>S100A9</td>
<td>S100 calcium binding protein A9</td>
<td>Cytoplasm</td>
<td>0.4</td>
</tr>
<tr>
<td>NM_174137</td>
<td>SERPINE1</td>
<td>Serpin peptidase inhibitor</td>
<td>Extracellular Space</td>
<td>1.9</td>
</tr>
<tr>
<td>NM_005143.3</td>
<td>HP</td>
<td>Haptoglobin</td>
<td>Extracellular Space</td>
<td>2.3</td>
</tr>
<tr>
<td>NM_174471.3</td>
<td>TIMP1</td>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>Extracellular Space</td>
<td>1.7</td>
</tr>
<tr>
<td>NM_00132454</td>
<td>DEFB1</td>
<td>Defensin beta 1</td>
<td>Extracellular Space</td>
<td>5.2</td>
</tr>
<tr>
<td>NM_00111508</td>
<td>DEFB10</td>
<td>Defensin beta 10</td>
<td>Extracellular Space</td>
<td>2.3</td>
</tr>
</tbody>
</table>
Figure 12. Gel electrophoresis of PCR products of biomarkers genes and β-actin gene. The biomarker genes and β-actin gene expression from bovine whole blood cDNA were confirmed by RT-PCR. A single PCR product was observed with expected size for each biomarker and β-actin gene. No band was observed in the PCR products of negative control without template DNA sample. In the figure (L) indicates 100 bp DNA size marker, (+) indicates PCR product with template cDNA, (-) indicates PCR product without cDNA.
Figure 13. Amplification plots of the biomarker genes in the real-time PCR.

Real-time PCR was conducted with PCR product that serially diluted 10-fold from 10⁹ to 10² copy numbers. The emission of fluorescence was measured at each cycle numbers and negative control sample with no template DNA showed no increasing of fluorescence. (A) *DEFB1*; (B) *DEFB10*; (C) *HP*; (D) *MMP9*; (E) *S100A8*; (F) *S100A9*; (G) *SERPINE1*; (H) *TFRC*; (I) *TIMP1*
Figure 14. Gene expression level of biomarkers in MAP-infected cattle.

The gene expression level of biomarker genes in cattle infected with *Mycobacterium avium* subspecies *paratuberculosis* compared to non-infected cattle. The data are shown as scatter plots with each dot representing a single animal. (*, p<0.05 **, p<0.01 ***, p<0.001 ****, p<0.0001).
Figure 15. Discriminatory ability of biomarkers between infected animals and control animals.

Receiver operator characteristics curves of biomarker genes in cattle infected with *Mycobacterium avium* subspecies *paratuberculosis* compared to non-infected cattle.
General conclusions

Up to date, it is still far from complete understanding pathogenesis and efficient diagnostic method for the subclinical stage of MAP infection. To development effective diagnostic methods, it is necessary to understand host gene expression as which are involved in the pathogenesis of paratuberculosis. Several biomarkers were identified by gene expression profiling of whole blood was analyzed and the new real-time method was developed using host biomarker. Moreover, expression of immune-regulatory genes in whole blood of the subclinical animal was analyzed. Expression of immune-regulatory genes indicates that manipulation of host response for enhancing the survival of MAP.

1. Six genes (CXCR3, HP, HGF, LTF, TFRC, and GBP6) showed significant differences between experimental groups based on fecal shedding and ELISA S/P ratio. Taken together, our data suggest that six genes (LTF, HGF, HP, CXCR3, GBP6, and TFRC) played essential roles in immune response to the MAP during the subclinical stage and might be useful as prognostic biomarkers.

2. Downregulation of Th17 cytokine genes and upregulation of PIP5K1C induce loss of granuloma integrity results in fecal shedding and dissemination of the pathogen. Downregulation of IRF4 resulted in the impaired Th1 immune
response, which decreased expression of HMGB1 and enhanced the downregulation of the Th1 immune response. In addition, increased expression of IRF5 and IRF7 suggest that activation of IFN-α/β signaling during subclinical stages induce IDO-mediated tryptophan metabolism. IDO-mediated depletion of tryptophan indicates an inhibition of T cell proliferation, subsequently leading to an immunosuppressive state. Upregulation of CORO1A suggests the possibility of the failure to intracellularly eliminate MAP. Taken together, this model suggests manipulation of host responses for the survival of MAP that occurs during the subclinical phases of JD.

3. A real-time PCR method was developed based on eight biomarkers (HP, TIMP1, MMP9, SERPINE1, TFRC, S100A8, DEFB1, and DEFB10) that can be used as a new diagnostic tool for JD with good diagnostic performance. Moreover, this real-time PCR based on biomarkers might be used for diagnosis of JD, especially in subclinical stage animals that cannot be detected by current diagnostic methods.

On the basis of these results, selected biomarkers and immune-regulatory genes seem to play important role in the pathogenesis of MAP during the subclinical stage. This study provided a deeper understanding of host responses to the MAP during the subclinical stage for the development of alternative diagnostic methods based on biomarkers.
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국문초록

*Mycobacterium avium* subspecies *paratuberculosis*에 의한 소의 준임상형 감염

단계에서의 숙주 유전자 발현 분석을 통한 진단용 생물학적 표지자의 규명

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요네병은 *Mycobacterium avium* subsp. *paratuberculosis* (MAP)에 의해 야기되는 반추 동물의 만성 소모 질환이다. 요네병은 전세계 낙농 산업에 심각한 경제적 손실을 초래하며 주로 분변에 오염된 사료, 물, 우유와 같은 물질의 섭취를 통해 감염된다. 감염이 일어난 이후, 초기 단계에서 동물은 임상 증상을 나타내지 않으나 분변을 통해 요네병균을 체외로 배출하여 환경을 오염시키고 요네병 전파의 근원이 된다. 따라서 질병을 근절하기 위해 감염된 동물을 감염 초기에 진단하여 제거하는 것이 필수적이다.
의 근절에 매우 중요하다고 할 수 있다. 그러나 분변 내의 균을 검출하는 PCR이나 배양법, 혈청 내의 요네병균에 특이적인 항체를 검출하는 ELISA 등과 같은 현재의 진단 방법은 임상증상을 나타내지 않는 준임상형 개체의 진단에 적합하지 못하다. 따라서 준임상형 개체를 탐지할 수 있는 진단 기법이 필요한 실정이다.

본 연구에서는 먼저 요네병에 감염된 소의 전혈에서의 유전자 발현 양상을 분석하여 바이오마커 후보들을 평가하였다. 준임상 소의 집단에서 다음 6개의 유전자 (LTF, HGF, HP, CXCR3, GBP6, 및 TFRC)의 발현이 유의적으로 증가하였으며, 이들 유전자는 준임상형 단계에서 요네병균의 범인기전과 관련이 있는 것으로 생각되며, 준임상형 소를 진단하기 위한 바이오마커로 사용될 수 있을 것으로 여겨진다.

둘째로 본 연구에서는 준임상형 소의 전혈 내에서 면역 조절 관련 기능을 가진 유전자들의 발현을 분석하였다. IL-17A, IL-17F, IL-22, IL-26, HMGB1 및 IRF4 유전자들의 발현의 감소 및 PIP5K1C의 발현의 증가는 Th1 반응의 억제를 유도하는 것으로 생각된다. 또한, IRF5 및 IRF7 유전자의 발현 증가는 준임상형 단계에서 인터페론 알파/베타 신호체계의 활성화를 암시하며, 이는 indoleamine 2,3-dioxygenase에 의한 트립토판 대사의 감소를 유도하여 T cell의 증식을 억제하는 것으로 여겨진다. CORO1A 유전자의 증가 된 발현은 칼슘 신호 전달의 조절
을 통하여 MAP의 숙주 내에서의 생존율 강화시킬 것으로 생각된다. 종합해보면, MAP 감염에 의해 유도된 면역조절 관련 유전자 발현이 준임상형 단계에서 MAP의 생존을 증가 시킨다는 것으로 예상된다.

세번째로 8종류의 숙주 바이오마커 (HP, TIMP1, MMP9, SERPINE1, TFRC, S100A8, DEFB1, DEFB10)를 이용하여 준임상형 소를 진단하기 위한 Real-time PCR 기법을 개발하였다. 이들 8종의 바이오마커는 준임상형 개체의 진단에 좋은 정확도 (AUC≥0.7)를 보였다. 또한 그 중 4종의 유전자 (TIMP1, S100A8, DEFB1, DEFB10)는 80 % 이상의 민감도와 90 % 이상의 특이도를 보였다.

결론적으로 이러한 결과들은 바이오마커 기반의 진단기법을 통해 요네병 준임상형 개체를 진단할 수 있는 가능성을 보여주었다. 그러므로 본연구 결과는 현재 사용되고 있는 진단법을 대체할 만한 바이오마커 기반의 진단법의 개발에 공헌할 것으로 생각된다.

핵심어: 요네병 원인균, 바이오마커, 준임상형, 진단