獸醫學博士學位論文

Clinical evaluation of PCR diagnosis for detection of *Mycobacterium avium* subspecies *paratuberculosis* and epidemiological analysis of Korean isolates derived from cattle

Mycobacterium avium subspecies *paratuberculosis* 검출을 위한 PCR 진단법의 임상적 평가 및 소 유래 국내 분리주의 역학적 특성 분석

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Clinical evaluation of PCR diagnosis for detection of *Mycobacterium avium* subspecies *paratuberculosis* and epidemiological analysis of Korean isolates derived from cattle

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Abstract

Clinical evaluation of PCR diagnosis for detection of *Mycobacterium avium* subspecies *paratuberculosis* and epidemiological analysis of Korean isolates derived from cattle

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Johne's disease (JD) or paratuberculosis (PTB) is a chronic debilitating disease in ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The disease causes significant economic losses in livestock industries worldwide. Because MAP is slow growing bacteria and it has very long incubation period, the clinical signs of disease can be produced in more than two years after initial infection. The gold standard for MAP diagnosis is isolation and identification of the bacteria directly from feces or tissues. However, visible colonies cannot be seen for 6 to 16

weeks using this method, and its sensitivity is relatively low. Serological tests such as ELISA are also commonly used, but these also have low sensitivity. Fecal PCR is a diagnostic method that can complement the shortcomings of the culture method. Specifically, PCR can detect the DNA of pathogens present in very small numbers with greater sensitivity and speed than culture methods.

The purpose of this study was to investigate epidemiological situation of MAP infection in cattle based on fecal diagnosis to provide basic information, especially about subclinical infection which is essential for eradication of the disease.

In this study, we designed new cost-effective DNA extraction method (mGITC/SC) to improve detection sensitivity of fecal PCR and evaluated in terms of diagnostic efficiency compared with other DNA extraction methods. The detection limit of IS*900* real-time PCR was about 50 MAP (6 cfu) per g of MAP-spiked feces. The time taken per sample to extract DNA was about 60 minutes, and the cost was calculated to be $1 per sample.

For the fecal diagnosis of MAP, a PCR targeting MAP-specific elements, IS*900* and ISMap*02* was performed using fecal samples collected from Korean cattle herds. Of the 1,562 fecal samples obtained from 37 herds, regardless of diarrhea, 35 samples were positive in both IS*900* and ISMap*02*. At the herd level, 12 of the 37 herds were found to be positive for MAP. Thirty-five positive cows were considered to be in the subclinical stage because they did not show any clinical symptoms. In addition, the herd level prevalence investigated in this study was similar to those of the previous reports measured by ELISA-based methods.

Molecular typing was conducted to describe the genetic diversity of MAP in Korea using twelve MAP-positive fecal DNA samples and 19 MAP isolates obtained from 10 cattle herds located in 5 provinces. The most prevalent strains in Korea were the "bison-type" genotype (23 of the 31 samples) and distributed nationwide. Rest of 8 samples were cattle-type, and all the foreign isolates were also cattle-type. The bison-type strains which were discriminated only as INMV 68 in MIRU-VNTR were divided into 3 different subtypes by MLSSR typing. Cattle-type was divided into 3 subtypes by MIRU-VNTR and 8 subtypes by MLSSR. The allelic diversities of MIRU-VNTR and MLSSR were calculated as 0.567 and 0.866, respectively. To the best of our knowledge, this is the first epidemiological survey report about the MAP isolated from cattle in Korea using MIRU-VNTR and MLSSR typing methods.

In the present study, non-MAP mycobacteria were shown to be positive by ISMap*02* targeting PCR. Two bacterial isolates (Sample ID: BO-038 and BO-042) were cultured from bovine fecal samples that produced positive results in three of two ISMap*02* targeting PCR analyses with negative results in IS*900* real-time PCR. Species identification using 16S rRNA gene sequencing and *hsp65* gene partial sequencing revealed that strains BO-038 and BO-042 were *M. virginiense* and *M. nonchromogenicum*, respectively, which both belong to the *M. terrae* complex (MTC). Moreover, the two isolates shared a novel insertion sequence (IS) with high similarity to some parts of nucleotide sequences of ISMap*02*, and IS was presumed to be identical to that present in *M. heraklionense*. Both the novel IS and ISMap*02* were characterized as IS*1182* family members, and several sequences similar to

ISMap*02* were identified by BLAST analysis. The present study demonstrated the existence of ISMap*02*-like sequence for the first time. Moreover, this study found that two previously used ISMap*02* PCR targets were not suitable for MAP detection.

Based on the results, the fecal PCR diagnosis is an appropriate method to detect fecal shedder, which is important for understanding the MAP occurrence situation, and suggested that appropriate DNA extraction methods and MAP specific targets should be used to improve diagnostic efficiency. Moreover, the results of the molecular epidemiology survey of Korean MAP isolates can provide basic information for understanding of genetic diversity, evolutionary relationships, and inter and intra-species transmissions which are essential for establishing control strategy.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis,* fecal PCR diagnosis, Molecular typing, ISMap*02*, Insertion sequence, *Mycobacterium virginiense, Mycobacterium nonchromogenicum*

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List of abbreviations

AGID Agar gel immunodiffusion

BHI Brain heart infusion

BLAST Basic Local Alignment Search Tool

CD Cluster of differentiation

CFT Complement fixation test

cfu Colony forming unit

CMI Cellular mediated immune response

Ct Threshold cycle

DDA Dodecyl ammonium bromide

DI Discriminatory index

DIVA Differentiation of infected animals from vaccinated animals

ELISA Enzyme-linked immunosorbent assay

HEYM Herrold's egg yolk medium

HPC Hexadecylpyridinium chloride

IFN- γ	Interferon-γ
IL	Interleukin
ISs	Insertion sequences
JD	Johne's disease
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MDM	Monocyte derived macrophages
MIRU-VNTR.	variable-number tandem repeats of mycobacterial interspersed repetitive units
MLSSR	Multilocus short sequence repeat
MTC	*Mycobacterium terrae* complex
NAHIS	National Animal Health Monitoring System
NK cell	Natural killer cell
OD	Optical density
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCR-REA	Polymerase chain reaction-restriction endonuclease analysis
PPD	Purified protein derivatives
PTB	Paratuberculosis
SE	Standard error
SNPs	Single nucleotide polymorphisms

TNF- α	Tumor necrosis factor-α
USDA	United States Department of Agriculture
WGS	Whole genome sequencing
wgs	Whole genome shotgun contigs

General introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a causative agent of Johne's disease (JD) or paratuberculosis (PTB), which is a chronic debilitating disease in ruminants that is characterized by incurable enteritis and persistent diarrhea (Harris and Barletta, 2001). The disease is distributed worldwide and causes significant economic losses to the livestock industry because of premature culling and production losses (Sweeney, 1996; Whitlock and Buergelt, 1996). In the United States (US), MAP-positive herds experience economic losses of almost US$ 100 per cow and a disease cost of US$ 200 to 250 million annually (Ott et al., 1999). At the herd level, it has been estimated that more than 50% of dairy cattle farms were infected with MAP in most major dairy-producing countries (Nielsen and Toft, 2009; Ott et al., 1999). Moreover, the most recent herd level prevalence estimates are as high as 90% in the US dairy cattle industry (Lombard et al., 2013). These findings indicate that an infection rate of MAP is increasing and there is a need to establish an efficient program for control of this pathogen.

One of the most effective strategy to control of PTB is the elimination of fecal shedder. To detect subclinical fecal shedders, bacterial culture has always been used as the gold standard (Whitlock et al., 2000). However, the culture method has relatively low sensitivity and sometimes yields false negative results in cattle that are shedding low levels of bacteria (Collins, 1996). The PCR using fecal specimen

is an alternative diagnostic method that can detect low amount of MAP and has high sensitivity. However, there are several difficulties hinder effective detection. Because MAP has thick and lipid-rich cell wall, normal DNA extraction methods cannot yield enough amount DNA, and PCR inhibitors present in fecal materials lower the sensitivity of PCR (Amaro et al., 2008; Logar et al., 2012). Therefore, many attempts have been made to obtain high yield of DNA from MAP as well as high purity (Irenge et al., 2009; Leite et al., 2013; Logar et al., 2012; Zhang and Zhang, 2011).

In order to increase the reliability of PCR diagnosis, it is essential to use a target specific primer sets. IS*900* is generally used as a representative target in PCR diagnosis of MAP because it is a MAP specific marker with high sensitivity because there are 12–18 copies of it in the MAP genome (Englund et al., 1999; Millar et al., 1995; Moss et al., 1991; Vary et al., 1990). However, the IS*900*-like sequence has been found in non-MAP mycobacteria, posing a problem of specificity (Cousins et al., 1999; Englund et al., 2002; Godfroid et al., 2005; Motiwala et al., 2004; Rajeev et al., 2005). Several other targets have also been discovered, among which ISMap*02* is a putative insertion sequence (IS), known to be present in multi-copies (six copies) and specific to MAP, similar to IS*900* (Paustian et al., 2004). However, two isolates that were positive in two of the three commonly used ISMap*02* primer sets were identified in this study. Therefore, characterization of the isolates and analysis of the DNA sequences that showed positive in ISMap*02* PCR were needed to confirm the presence of ISMap*02*-like IS.

In Korea, national prevalence of paratuberculosis in individual cattle in Korea was

estimated as 3.3–7.1% until 2010 (Yoo and Shin, 2012). The prevalence estimates for paratuberculosis using Bayesian approach ranged 3.2–5.3% for conservative and 6.7–7.1% for liberal, depending on the prior two studies for paratuberculosis seroprevalence (Pak et al., 2003). Another study estimated the true national prevalence of paratuberculosis was 7.1% based on an analysis of serum ELISA (Park et al., 2006). National prevalence at herd level has not been estimated yet. A study in Gyeong-nam provinces of Korea, the prevalence rates at animal and herd level estimated to be 3.3 and 13.8%, respectively (Lee and Jung, 2009). The study revealed that seroprevalence of paratuberculosis in dairy herds were significantly higher than beef cattle herds.

Although the molecular detection methods for MAP have progressed rapidly, the application of these methods to milk, feces and environmental samples in Korea is now only beginning (Yoo and Shin, 2012). PCR-based diagnostics have been used in many countries and the methods have been improved constantly to overcome the limitations of other diagnostic methods such as the culture-based method (Alinovi et al., 2009; Bogli-Stuber et al., 2005). However, investigation of prevalence using a diagnostic approach such as fecal culture or fecal PCR that are efficient in detection of fecal shedder have not yet been reported in Korea.

Understanding the genetic diversity of MAP is necessary for establishing disease control strategies (Douarre et al., 2011). Molecular typing methods of MAP strains provide important information for genetic diversity, evolutionary relationships and assist in the understanding of inter and intra-species transmissions (Amonsin et al.,

2004; Leao et al., 2016; Stevenson et al., 2009). In Korea, however, there are no published reports on the molecular characteristics of MAP strains isolated from cattle.

The purpose of this study was to investigate epidemiological situation of MAP infection in cattle based on fecal diagnosis to provide basic information, especially about subclinical infection which is essential for eradication of the disease. This is organized into four chapters. For fecal PCR diagnosis, new DNA extraction method was designed and evaluated in chapter I. In chapter II, fecal PCR as a diagnostic method for paratuberculosis was evaluated using field samples and prevalence of MAP infection with fecal PCR was described. In chapter III, molecular characterization of Korean MAP strains isolated from cattle herds were described by IS*1311* PCR-REA, MIRU-VNTR, and MLSSR. Lastly in chapter IV, specificity of ISMap*02* was analyzed using two non-MAP mycobacterial strains which showed positive in ISMap*02* PCR.

Literature Review

Bovine paratuberculosis (Johne's disease)

Bovine paratuberculosis (PTB), also called Johne's disease (JD) is a chronic debilitating disease of cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Sweeney, 1996). MAP is an obligate intracellular pathogen of animals which can reproduce only within a host cells such as macrophages (Grant, 2005). The infected animals show a chronic diarrhea characterized by a chronic granulomatous enteropathy, which leads to progressive wasting, and eventually death (Coussens, 2001). The first report of PTB was in the early 1800s in Germany and was later described by Drs Johne and Frothingham (Chiodini et al., 1984). The disease has been observed primarily in ruminants (cattle, sheep, goats, deer, etc.), and various other non-ruminant animals worldwide (Harris and Barletta, 2001). In addition, the association of MAP with Crohn's disease, which is a type of chronic inflammatory bowel disease in humans has been mentioned in several studies (Singh et al., 2007). Bovine PTB is one of the most widespread and economically important diseases in cattle (Ott et al., 1999). Due to the loss of milk production and early culling of infected animals, the disease causes a serious economic damage to cattle industry, especially in dairy farms (Hasonova and Pavlik, 2006).

1. Epidemiology

1.1. Global prevalence of MAP infection

MAP is endemic in the bovine populations of many countries (Nielsen and Toft, 2009). Prevalence estimates have suggested to proper strategy in case of low, high prevalent, and absent of the infection (Nielsen and Toft, 2009). In 2007, the National Animal Health Monitoring System (NAHIS) in the US estimated that more than 68% of US dairy herds were infected with MAP (MAP was isolated from at least one environmental sample from 68% of herds) (USDA, 2008). In addition, the herd prevalence increased as herd size increased. For example, MAP was isolated in 95% of large (>500 cows) dairy herds. Within-herd prevalence among 106 US dairy herds estimated ranged from 0% to 27.3%, with a mean of 5.5% (USDA, 2005). In Canada, Seroprevalence at the animal level in dairy cattle ranged from 1.3% to 7.0%, while 9.8% to 40.0% at the herd level (Tiwari et al., 2006). In European countries, herd-level prevalence in dairy farms was estimated to be >50% (Nielsen and Toft, 2009). In the Netherlands, approximately 54% of cattle herds were considered to MAP infected (Muskens et al., 2000). In the Denmark, herd level prevalence of cattle was estimated to be 55%, and 85% especially in dairy herds (Bakker, 2010; Nielsen et al., 2000).

1.2. Transmission

Transmission of the disease is mainly occurred by the ingestion of MAP from the contaminated environment. Transmission may also occur by the ingestion of milk or colostrum from infected animals (Streeter et al., 1995). Young animals are most vulnerable to MAP infection, either by ingestion of colostrum and milk from an infected animal or through the teats contaminated with feces (Giese and Ahrens, 2000; Slana et al., 2008; Slana et al., 2009). Vertical transmission is the acquisition of infection from dams to new born calves, and is divided into prenatal infection and postnatal infection. MAP is known to be capable of prenatal infection, and many of the evidence supporting such a possibility (such as the presence of bacteria in the placenta or other sexual organs) have been found (Sweeney, 1996). Postnatal infection occurs mainly by exposure to bacteria present in colostrum or fecal material. In the calf, the highest susceptibility of the MAP is known as the first day of life (within 24 hours after birth) (Sweeney, 2011; Windsor and Whittington, 2010). Therefore, the key of the paratuberculosis eradication so far has been the periodic MAP screening of farms, isolation of calves from dam right after parturition, and supply of sterilized colostrum (Stabel, 2008; Sweeney, 1996).

1.3. Molecular epidemiology

Understanding the genetic diversity of MAP is necessary for establishing disease control strategies (de Kruijf et al., 2017). Several phenotypically distinct strains of MAP have been known for more than eight decades, but the genetic differentiation of MAP strain types has been challenging and only recent technologies have proven sufficiently discriminative for strain comparisons, tracing the sources of infection and epidemiological studies (Stevenson, 2015). There are two major group of strains known as 'Sheep type' or 'Type S' and 'Cattle type' or 'Type C' named after original source of the strains (Collins et al., 1990). Additional group of strains known as 'Bison type' or 'B type' were first differentiated based on the variation of 223bp of IS*1311* which were all had T at position 223bp, whereas all sheep type strains had C and cattle type strains had mixed with C and T (Whittington et al., 2001). Phylogeny of the MAP strain types clarified by whole genome sequencing (WGS) was shown in figure I (adapted from Stevenson et al., 2015).

Molecular typing methods of MAP strains provide important information for genetic diversity, evolutionary relationships, and assist in the understanding of inter and intra-species transmissions (Amonsin et al., 2004; Leao et al., 2016; Stevenson et al., 2009). Several methods for subtyping of MAP have been developed. Polymerase chain reaction and restriction endonuclease analysis (PCR-REA) of the IS*1311* locus has been used because it allows for the differentiation of MAP linages into three subtypes (cattle-, sheep-, and bison-type) according to host preferences

(Sevilla et al., 2005; Whittington et al., 2001). Typing techniques such as variable-number tandem repeats of mycobacterial interspersed repetitive units (MIRU-VNTR) and multilocus short sequence repeat (MLSSR) have been used to differentiate subtypes because the techniques using these targets provide high discriminatory power and are easy to use (Amonsin et al., 2004; Thibault et al., 2007). More recently, WGS of many epidemiologically linked isolates can determine the true degree of diversity and quantify relatedness (Ahlstrom et al., 2015). Single nucleotide polymorphisms (SNPs) identified through WGS are evolutionary stable and reliable to identify evolutionary relationships (Pearson et al., 2009).

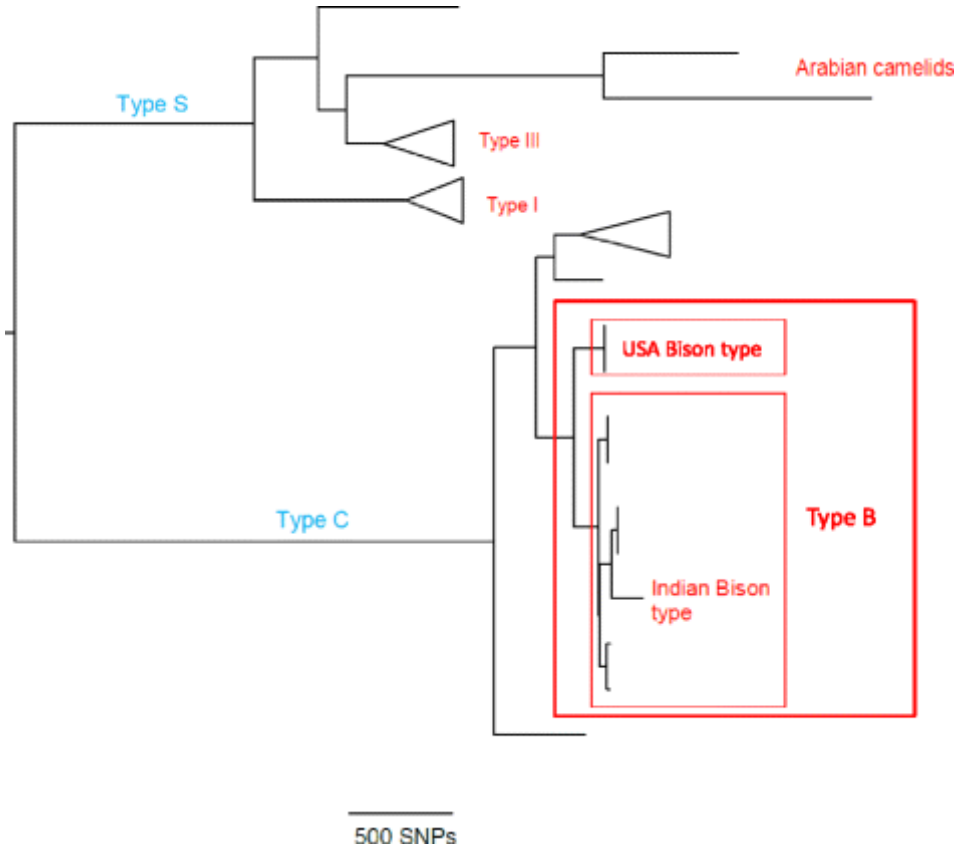

Figure I. The phylogeny of MAP strain types. Maximum likelihood tree of MAP strains based on whole genome SNPs. Adapted from Stevenson et al. (2015).

2. Pathogenesis and immune responses to MAP

MAP infection is initiated by ingestion of fecal material orally contaminated by MAP (fecal-oral route). Following ingestion, MAP can pass through the M cell, which is specialized for uptake of particles that mainly bind to bacteria and transport them into the submucosal layer. After crossing the epithelial layer, MAP is phagocytosed by submucosal macrophages (Momotani et al., 1988). Like other mycobacteria, MAP is able to survive and replicate in non-activated macrophages by inhibiting phagosome-lysosome maturation (Kuehnel et al., 2001; Sweeney, 2011). MAP eventually causes the cell death of infected cells, after which liberated MAP can be phagocytosed by freshly accumulated macrophages and dendritic cells that are activated by cytokines such as tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ). IFN-γ, which plays an important role in activation of macrophages and T cells, is produced by infected cells, local γδ T cells, and natural killer (NK) cells (Boysen and Storset, 2009; Rogers et al., 2005). Activated macrophages and dendritic cells produce interleukin 12 (IL-12) and present antigen to naïve CD4+ T cells through MHC class II molecules. IL-12 triggers the differentiation of naïve CD4+ T cells into T helper 1 (Th1) cells. Th1 cells produce cytokines such as interleukin 2 (IL-2) and IFN-γ, which play roles in promotion of expansion of antigen specific Th1 cells and maturation of macrophages. These antigen specific responses change from innate immunity to cell mediated adaptive immunity.

In the later stages of infection, increasing antibodies are frequently observed with increasing bacterial shedding (Koets et al., 2002). Therefore, it has been accepted that switching from Th1 immune responses to Th2 responses is the cause of disease progression (Manning and Collins, 2001; Stabel, 2000; Sweeney et al., 1998). Many researchers investigated the immune regulatory mechanisms of host animals to identify causes of Th1-Th2 switches. An increase of IL-10 production by regulatory T cells (Tregs) or macrophages, which induces the down regulation of Th1 responses and stimulation of antibody production, is considered to cause this switch (Buza et al., 2004; Coussens et al., 2012; de Almeida et al., 2008). Along with Tregs, γδ T cells have been known to play a role in immune regulation. The progressive decrease of CD4+ T cell population in local immune response has been shown to be accompanied by increasing γδ T cell population (Koets et al., 2002). The cytokine mRNA profiles of γδ T cells demonstrated that subsets of bovine γδ T cells encode IL-10 and TGF-β, suggesting a potential regulatory role of γδ T cells (Hsieh et al., 1996; Park et al., 2000). Recently, however, a typical pattern of disease progression that can be explained by the Th1-Th2 switch was observed in only 40% of MAP-infected sheep with other cases showing simultaneous responses of both cellular and humoral immunity or cellular immunity only (Begg et al., 2011). A recent study using mathematical modeling suggested that Th1-Th2 switch may be a result of disease progression (increasing of extracellular bacteria) rather than cause (Magombedze et al., 2014). A similar study that used a mathematical model to analyze the correlation between Th1, Th2 expression and bacterial shedding revealed

a positive correlation between the amount of bacteria and humoral immune response was observed. However, there was no evidence of competition or synergy between Th1 and Th2 immunity (Ganusov et al., 2015). This study also suggested that MAP-specific cellular immune responses were predicted to increase shedding, whereas in some animals it was predicted to suppress the shedding. As a result, it can be inferred that adaptive immune responses play a limited role in disease protection. However, these mathematical modeling studies have some vulnerable points because they cannot consider all of the parameters. Therefore, these results or hypotheses should be confirmed by both *in vitro* and *in vivo* studies. Another modeling study suggested that long-term subclinical infection may related to innate immunity rather than adaptive immunity (Klinkenberg and Koets, 2015). The results using structural models (villus model, granulomatous model) of local infection showed that the long subclinical phase was due to structural organization of the granulomatous lesion. Moreover, the authors predicted that intermittent shedding was due to changes in the recruiting efficiency of macrophages influenced by external factors such as hormonal changes (ex. pregnancy, lactation).

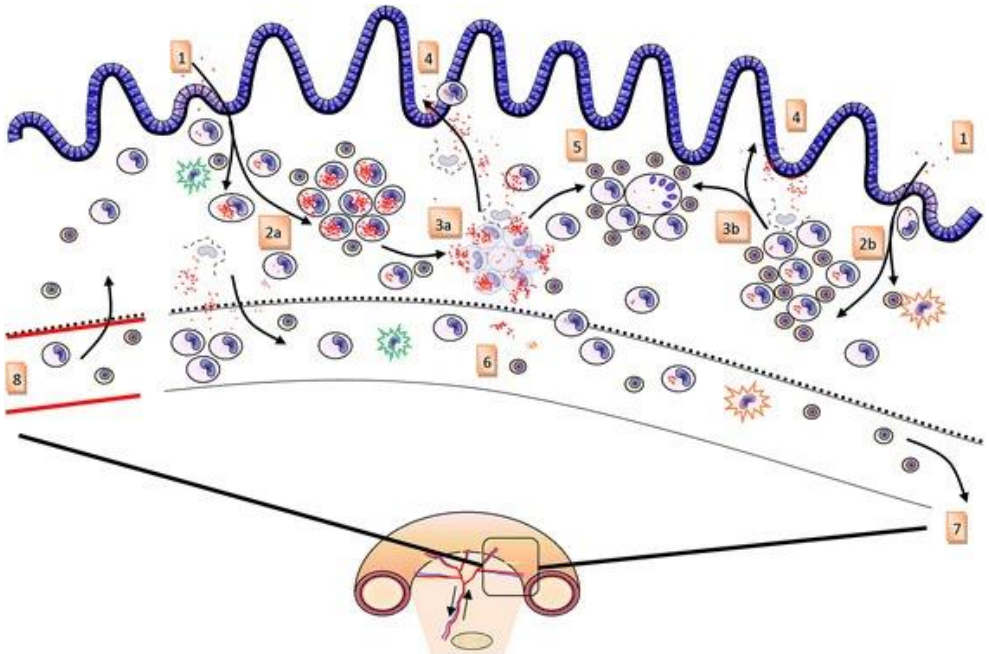

Figure II. A model for granuloma dynamics of bovine paratuberculosis. MAP enters into submucosal layer *via* M cell or enterocyte transcytosis (1); MAP taken up by macrophages and induces immune tolerance (2a) or inflammation (2b); MAP is released into lamina propria due to the bacterial replication (3a) or cell death of infected macrophages (3b); Released MAP is reintroduced into the intestinal lumen *via* fluid streams or released into the feces (4); Cell debris and released MAP antigens are removed by polynuclear giant cells (5); MAP and MAP antigen may spread to different sites in the intestine or enter the afferent lymph (6) and migrate to the draining lymph node activation of T and B cells (7); T cells and B cell derived antibodies as well as monocytes enters the intestine *via* the arterio-venous capillary bed (8). Adapted from Koets et al. (2015).

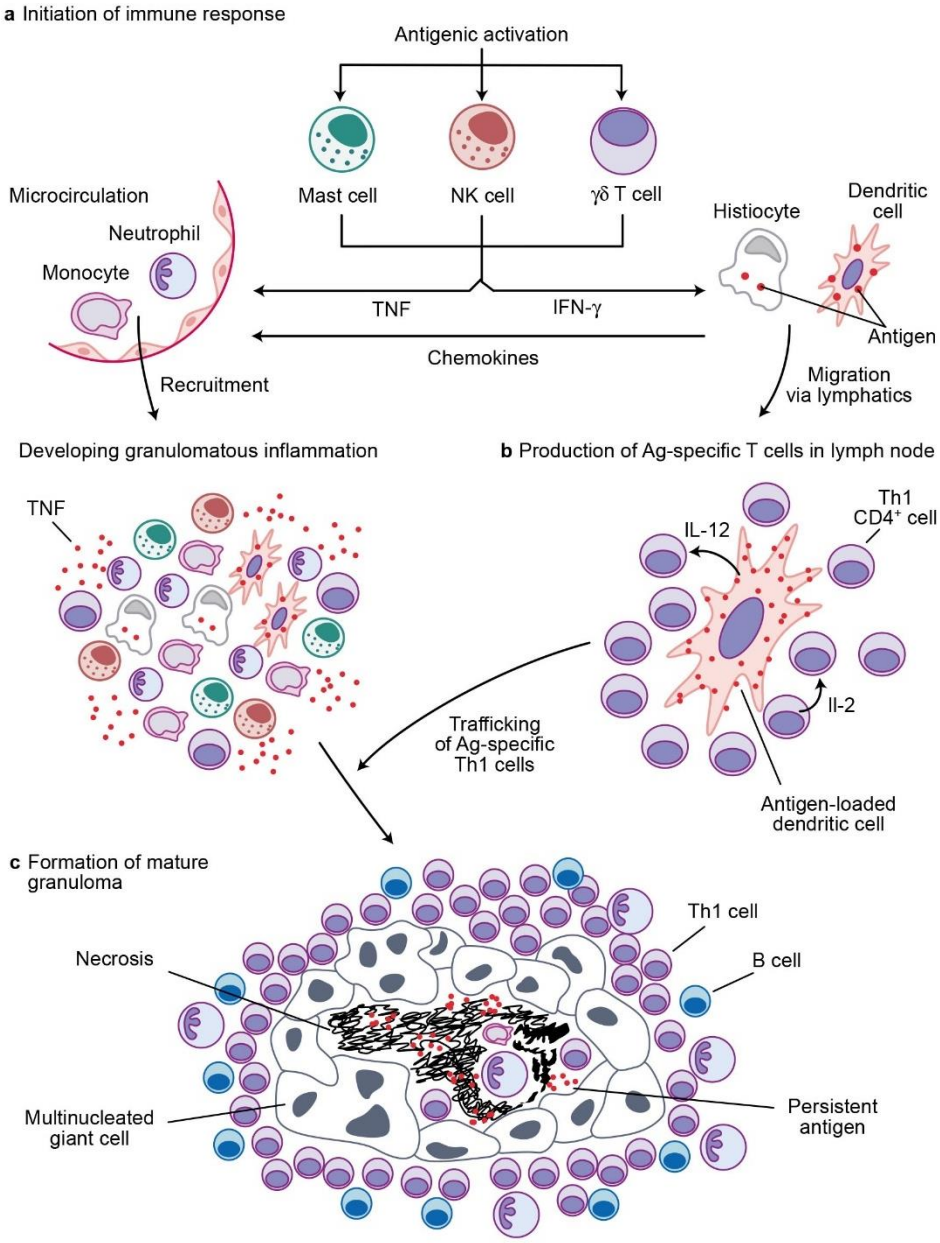

Figure III. Pathogenesis of granuloma formation. Adapted from Sarraf and Sneller. (2005).

3. Diagnostics

The situation of MAP infected herds can be properly explained by ‘iceberg phenomenon’. If a small number of animals show clinical signs of infection, there are more infected animals that do not show any clinical symptoms. Therefore, diagnosis of PTB only by a clinical sign is not sufficient to eradicate the disease. For the control and eradication of PTB within herd, detection of all infected animals is essential. The most effective way to eradicate the disease is to eliminate the fecal shedders thereby preventing the spread of MAP (Irenge et al., 2009).

3.1. Culture-based methods

Isolation and identification of etiological agent have been considered as the gold standard for diagnosis of PTB. One major distinguished feature between MAP and other mycobacteria is its inability to produce mycobactin, a siderophore that is responsible transport iron into cells, in laboratory culture (Li et al., 2005). Therefore, supplementation of Mycobactin J is essential to prepare a media. The most widely used media is herrold's egg yolk medium (HEYM) with modified Mycobactin J and modified Middlebrook 7H9 and 7H10 media. Because MAP requires incubation time from 8 to 16 weeks in these media, culture method has a disadvantage that it cannot be diagnosed rapidly (Stabel, 1997). In addition, there is another disadvantage of fecal culture that diagnostic sensitivity is lowered in individuals that are in early

stage of infection (Nielsen and Toft, 2008).

Liquid culture-based systems for MAP are widely used in clinical laboratories; the Bactec 460 radiometric system, the Bactec MGIT 960 fluorescence detection system (Grant et al., 2003; Gumber and Whittington, 2007; Shin et al., 2007), the Trek ESP pressure detection system (Ellingson et al., 2004), and the MB/BacT reflectance detection system (Stich et al., 2004). The liquid culture methods provide greater analytical and diagnostic sensitivity than culture based on solid media (Eamens et al., 2000). However, false-positive result could be produced because each of these systems reports non-discriminatory measures of metabolic activity (Whittington, 2009). Therefore, it is necessary to use additional laborious molecular-based methods to validate the growth of MAP (Yoo and Shin, 2012).

3.2. Serological tests

The serological tests have been developed for diagnosis of PTB in cattle such as agar gel immunodiffusion (AGID), complement fixation test (CFT), and ELISA (Sockett et al., 1992). Among them, the ELISA is the most widely used diagnostic method for PTB. ELISA technique has the advantage of developing simple and robust kits, inexpensive, and readily automated for high sample throughput (Weldingh et al., 2005). Commercial ELISA kit was developed using pre-absorption technique that incubation of sera with *M. phlei* to increase the detection specificity (Cox et al., 1991; Hill and Gill, 1989; Yokomizo et al., 1985). Although the ELISA

is at present the most sensitive and specific diagnostic method for PTB, diagnostic sensitivity of commercial kits was less than 30% in the fecal-culture positive cattle without any clinical signs (Collins et al., 2005). Therefore, it is necessary to identify MAP-specific antigens sensitized to the host immune system at the early stage of infection. In this context, many antigen candidates were characterized to improve the specificity and sensitivity of the antibody-based assays (Mikkelsen et al., 2011).

Many attempts have been made to discover highly reactive and MAP specific antigen candidates. MAP culture filtrate or whole cell lysates were resolved through two dimensional (2D)-gel electrophoresis and spots that showed high reactivity with sera, especially in early stage, were characterized by mass spectrometry, and then the antigenicity of the characterized proteins were evaluated (Bannantine and Paustian, 2006; Cho and Collins, 2006; Gumber and Whittington, 2009; Shin et al., 2008). In another study, MAP specific coding sequences were discovered by comparing the nucleotide sequences such as *M. avium* subsp. *avium* and *M. tuberculosis*, which are genetically similar to MAP (Paustian et al., 2004). Recently, analysis of the *M. tuberculosis* protein array probed with sera from MAP-infected cattle was conducted to discover reactive proteins in early stage of infection (Bannantine et al., 2017; Li et al., 2017). Since more than half of the orthologous proteins sharing >75% identity between MAP and *M. tuberculosis*, the *M. tuberculosis* array had utility as a screening tool for identifying candidate antigens for PTB diagnostics (Bannantine et al., 2017).

3.3. PCR-based methods

In the case of MAP diagnosis, a PCR method using DNA extracted from fecal specimens has the advantage of identifying infected cattle more rapidly than the bacterial culture method (Englund et al., 1999; Fang et al., 2002; Motiwala et al., 2004; Rajeev et al., 2005). The insertion sequence, IS*900* is the most widely used target of the PCR diagnosis because it presents as multicopies (12–20 copies) in MAP genome, thus could increase the sensitivity (Englund et al., 1999; Millar et al., 1995; Moss et al., 1991; Vary et al., 1990). However, the IS*900*-like sequence has been found in non-MAP mycobacteria, posing a problem of specificity (Cousins et al., 1999; Englund et al., 2002; Godfroid et al., 2005; Motiwala et al., 2004; Rajeev et al., 2005). Several other targets have also been discovered (F57, ISMav*2*, ISMap*02*, etc.). The ISMap*02* is a putative insertion sequence (IS), known to be present in multi-copies (six copies) and specific to MAP, similar to IS*900* (Paustian et al., 2004).

Although the PCR has great advantages in terms of time, cost, and sensitivity, this molecular tool is greatly influenced by the quality of DNA samples (Park et al., 2014b). Especially the fecal DNA extraction, it is important to recover highly pure nucleic acids because there are many PCR inhibitors in fecal specimen (Logar et al., 2012). Several methods for fecal DNA extraction were evaluated and compared in terms of yield, purity, and diagnostic sensitivity (Leite et al., 2013).

PCR-based diagnostics have been used in many countries and the methods have

been improved constantly to overcome the limitations of other diagnostic methods such as the culture-based method (Alinovi et al., 2009; Bogli-Stuber et al., 2005). Real-time based multiplex PCR methods have been developed to increase diagnostic specificity and sensitivity, and tested with various samples such as feces, milk, tissues, blood, and environmental samples (Irenge et al., 2009; Sevilla et al., 2014; Slana et al., 2009).

3.4. Development of novel diagnostic methods

One of the major attempts is to identify MAP specific antigens that can be used in the IFN-γ assay to measure Th1-mediated immune response elicited by animals in the early stage of infection (Mortier et al., 2014; Stabel, 2006). Another attempt is to identify biomarkers of the MAP infected animals by analysis of transcriptional changes that show early responses to infection. Accordingly, host transcriptional profiles during the early stage of infection in mouse RAW264.7 cells, MAP infected mouse models, and naturally infected cattle have been analyzed (Cha et al., 2013; Shin et al., 2015a; Shin et al., 2015b).

4. Prevention and control

Because there is no effective treatment for PTB, prevention of infection is the most effective control strategy. There are three major approaches to reduce or eradicate the PTB, efficient management to decrease transmission, testing and culling, and vaccination (Bastida and Juste, 2011). Management using testing and culling practices are used in most countries (Geraghty et al., 2014). Although the incidence of PTB can be reduced by efficient management, eradication can only be accomplished when all the infected animals are detected and culled (Gumber et al., 2009). Although diagnostic tests for PTB are improving, it is still not possible to detect all infected animals. For these reasons, testing and culling strategies using the present diagnostic methods are ineffective for eradication of the disease except when targeting only-high shedding animals (Bastida and Juste, 2011; Lu et al., 2008). Under these circumstances, vaccination can be the best control strategy unless animals can be detected during early infection. This is because vaccination can reduce the incidence of MAP shedding and manifestation of clinical signs, which is more cost-effective than testing and culling (Juste, 2012). However, vaccination is probably the least accepted strategy because of several drawbacks, which are discussed in the next section of this review.

5. Development of vaccines

Vaccination against PTB has been considered as an alternative strategy to control the disease when combined with management interventions. A variety of ideas for designing novel vaccines have emerged, and the tests of the efficacy of these vaccines are conducted constantly. However, no effective vaccines are commercially available. Studies of the development of vaccines for MAP were summarized.

5.1. Commercially available vaccines

The first MAP vaccine, which was developed in 1926 by Vallee and Rinjard, consisted of a live non-virulent MAP and oil-based adjuvants. Since then, a number of whole-cell based vaccines, live attenuated vaccines and inactivated vaccines were developed to prevent bovine and ovine PTB. Currently, three commercial vaccines are all based on inactivated whole bacteria, Mycopar®, Gudair®, and Silirum®, of which only Mycopar® is approved for use in the US (Bastida and Juste, 2011). Meta-analysis of the efficacy of MAP vaccination, especially its production, epidemiological effects, and pathogenic effects, was conducted by Bastida and Juste in 2011 using previously published papers (Bastida and Juste, 2011). From this meta-analysis, it was concluded that vaccination against MAP is a useful strategy for reducing contamination by this pathogen, production losses and pathologic effects. Despite the many advantages of vaccination, it has not been encouraged in cattle in

most of countries because of several drawbacks. One major drawback of whole-cell based vaccination is interference with diagnostic tests currently used in bovine tuberculosis and PTB (Kohler et al., 2001; Muskens et al., 2002). These vaccines have the potential to produce false positive animals in serological tests for PTB such as ELISA because the commercial ELISA kit consisted of crude MAP antigens, which hinder differentiation of infected animals from vaccinated animals (DIVA) (Santema et al., 2011). The caudal fold skin test using *M. bovis* purified protein derivatives (PPD-B) is most widely used field screening tool for diagnosis of bovine tuberculosis (Good and Duignan, 2011). However, in the IFN-γ assay, stimulation with PPD-B produced robust responses similar to PPD-J (MAP purified protein derivatives) in MAP vaccinated animals (Muskens et al., 2002; Stabel et al., 2011). Because of this cross-reaction with other mycobacteria such as *M. avium* subspecies, comparative cervical test has been used as a complementary test to discriminate *M. bovis* infection from other mycobacterial infections by comparing the reactivity of each antigen using PPD-B and PPD-A (*M. avium* purified protein derivatives). However, this strategy may also cause problems with diagnostic sensitivity owing to the higher PPD-A reactivity because MAP vaccination can reduce the differences between PPD-B and PPD-A in *M. bovis* infected animals (Santema et al., 2011). Therefore, many countries that are running *M. bovis* eradication programs do not use vaccination policies. However, these problems can be overcome by development of new diagnostic methods or vaccines. Emerging serologic tests using *M. bovis* specific antigens such as ESAT-6, CFP-10, and MPB83 did not produce positive

results in MAP vaccinated animals (Stabel et al., 2011). Another drawback of whole cell-based vaccines is the substantial tissue damage at the injection site and accidental self-inoculation, which may cause serious side-effects (Patterson et al., 1988). However, there is a vaccine adjuvanted with highly refined mineral oils such as Silirum® to reduce the formation of granuloma at the site of injection (Rosseels and Huygen, 2008).

5.2. Live attenuated vaccines

Many researchers have been interested in development of live attenuated vaccines against MAP. These types of vaccines can elicit protective mucosal and systemic immune responses because the diverse antigens included in this vaccine can stimulate both innate and adaptive immunity (Faisal et al., 2013; Ghosh et al., 2014). Another advantage of this vaccine is that manufacture of live attenuated vaccine is cost effective and easies than that of other vaccines such as subunit vaccines (Ghosh et al., 2015). Many vaccine candidates have been produced by mutagenesis to attenuate the virulence of MAP. Mutants of MAP have been made by phage-mediated techniques, transposon mutagenesis and allelic exchange mutagenesis (Foley-Thomas et al., 1995; Harris et al., 1999; Park et al., 2008). Many transposon mutant libraries have been created to identify virulence mechanisms thereby finding vaccine candidates (Harris et al., 1999; Scandurra et al., 2010; Shin et al., 2006). Direct mutagenesis using allelic exchange techniques has also been tried by deletion

of genes already known to be pathogenic or essential for intracellular survival in *M. tuberculosis* or *M. bovis* (Chen et al., 2012; Ghosh et al., 2015; Ghosh et al., 2014; Park et al., 2008; Scandurra et al., 2010). The ΔrelA, Δlsr2, and ΔpknG mutants were generated by Park et al. and each gene was known to be related to virulence factors in *M. tuberculosis* and *M. bovis* (Colangeli et al., 2007; Dahl et al., 2003; Walburger et al., 2004). Two of these candidates, ΔrelA and ΔpknG were evaluated for virulence attenuation and efficacy as vaccine candidates using macrophages and ileal cannulation models of natural hosts (cattle), and goats (Park et al., 2011). The ΔsigL and ΔsigH mutants that are knocked out of the sigma factor gene were selected as live attenuated vaccine candidates because the sigma factors involved in part of the global virulence regulation provide resistance to the host bactericidal activities (Ghosh et al., 2014; Ghosh et al., 2013). The WAg906 (ΔMAP1566), WAg913, and WAg915 (ΔppiA) mutants were evaluated by Scandurra et al. (2010). WAg906 and WAg913 were made by transposon mutagenesis using MAP 989 strain, and WAg915 was made by allelic exchange of the ppiA gene (Cavaignac et al., 2000; Scandurra et al., 2010).

Recently, a three phase vaccine candidate evaluation strategy was established by Johne's Disease Integrated Program (JDIP) research consortium to improve the efficiency of the efficacy test on the MAP live attenuated vaccines (Bannantine et al., 2014). Phase I is a screening test using the MDM model, phase II is a challenge test using the mouse model, and phase III is an evaluation of protective effects using a goat model. The phase I test was conducted by Lamont et al. to evaluate many live

attenuated vaccine candidates constructed until 2014 (Lamont et al., 2014).

5.3. Subunit vaccines

Subunit vaccines have been developed to overcome the drawbacks of whole-cell based vaccines. Whole-cell based vaccines interfere with the diagnosis of both tuberculosis and paratuberculosis in vaccinated animals. However, subunit vaccines using well defined recombinant MAP proteins or DNA encoding immunogenic antigens can overcome the interference issues (Rosseels and Huygen, 2008). Many attempts have been made to identify MAP antigens to develop subunit vaccines using genomic or proteomic analysis. Because the production of IFN-ɣ induced by Th1 mediated immune responses is crucial to reducing the number of bacteria in the early stages of MAP infection, identifying antigens that induce strong Th1 responses is essential to the development of subunit vaccines (Rosseels and Huygen, 2008). Finding an antigen is also related to development of immunodiagnostic method as well as development of subunit vaccines. Several proteins have been identified as vaccine candidates. Several antigens were tested for their potential for use as a vaccine candidates: heat shock protein 70 (Hsp70) (Koets et al., 1999), antigen 85 complex proteins (Ag85A, Ag85B, Ag85C) (Shin et al., 2005), lipoproteins (LprG, MAP0261c) (Huntley et al., 2005; Rigden et al., 2006), PPE family proteins (MAP1518, MAP3184) (Nagata et al., 2005), superoxide dismutase (SOD) (Shin et al., 2005), and alkyl hydroperoxide reductases (AhpC, AhpD) (Olsen et al., 2000).

DNA vaccination against mycobacteria showed very effective protective immune responses in small rodents (Huygen, 2005, 2006). Moreover, DNA vaccines have advantages of storage and delivery because they are very stable. Several candidates were evaluated for their ability to induce protective immune responses; however, they were only evaluated in mouse models. Recently, combination of MAP-specific antigens and viral vectors was attempted to increase the ability of antigenic effects of DNA vaccines (Bull et al., 2007; Bull et al., 2014). The advantage of viral vectored vaccines is to provide high delivery of antigens to antigen presenting cells, thereby increasing antigen specific CD4+ and CD8+ immune responses (Guzman et al., 2012; Norbury et al., 2002; Reyes-Sandoval et al., 2012).

Insertion sequences

Insertion sequences (ISs) is a short DNA sequence that acts as a simple transposable element. Transposable element is a DNA sequence that can change its position within or between genomes. Transposable elements include transposons (Tn) and ISs (Craig et al., 2002). IS encodes only the enzymes necessary for its transposition and can be repeatedly inserted into many other sites in the genome using mechanisms independent of the large regions of DNA homology between the IS and the target (Berg and Howe, 1989; Craig et al., 2002). The size of the IS is relatively small (0.7 kb to 2.5 kb), and has one or two open reading frames (ORFs) over the entire length of the IS. The ORF encodes transposases (TPases), which catalyzes the cleavage of DNA and the movement of sequences leading to IS transposition (Craig et al., 2002). The coding region in an IS is usually flanked by imperfect terminal repeat sequences (IR). The length of IR varies from 10 to 40 bp. The IRs have two functional domains (Mahillon and Chandler, 1998). One (II) is located within the IR and is involved in TPase binding. The other (I) comprising the terminal 2 or 3 bp participates in cleavage and strand transfer reactions to induce the transposition of the element (Derbyshire et al., 1987; Derbyshire et al., 1990; Huisman et al., 1989; Johnson and Reznikoff, 1983; Makris et al., 1988; Zerbib et al., 1990) (Figure IV). A short flanking directly repeated duplication (DR) is often generated in the target DNA as a consequence of insertion. The length of DR varies from 2 to 14 bp, which characterized by a given element (Mahillon and Chandler,

1998).

ISs are grouped into different families according to their various properties (Mahillon and Chandler, 1998): (i) similarities in genetic organization (arrangement of ORFs); (ii) similarities in the recombinases/transposases (TPases) which mediate the transposition reactions; (iii) features of their IRs (the length and sequence of the IRs); and (iv) fate of the nucleotide sequence of their target sites (the length and sequence of the short flanking DRs). More than 4,000 ISs have been described and 26 families were identified in the prokaryotes to date (www-is.biotoul.fr; information section).

The family of ISs are mainly distinguished by the type of TPases based on the catalytic chemistry (Siguier et al., 2014). ISs include DDE, DEDD, HUH and Ser transposases. The majority of these are classical ISs and encode TPases of the DDE superfamily (Siguier et al., 2014). The DDE transposase have an RNaseH-like fold containing the motif of three catalytically active residues, DDE motif, which harbor the aspartate (D), aspartate (D) and glutamate (E) in the primary sequence, namely regions N2, N3 and C1, respectively (De Palmenaer et al., 2008; Nesmelova and Hackett, 2010). Although the DDE motif is highly conserved, the sequences and structures of DDE transposases show great variability (Nesmelova and Hackett, 2010).

The ISs are involved in a wide variety of biological transactions, which caused by various kinds of genome rearrangements, and are leading to genome reshuffling and evolution (De Palmenaer et al., 2008). The genome rearrangements such as deletion,

inversions or duplication/amplification events within a bacterial genome can be induced by proliferation of ISs (Alokam et al., 2002; Reif and Saedler, 1975; Riehle et al., 2001; Sun and Dennis, 2009). Transposition of ISs also can cause diverse modification of gene expressions associated with the presence of outwardly directed promoters or transcriptional regulatory signals at their extremities (Gaffe et al., 2011). These IS-mediated gene sets may ultimately cross barriers between strains and species through the horizontal gene transfer mechanisms such as conjugation (Ochman et al., 2000).

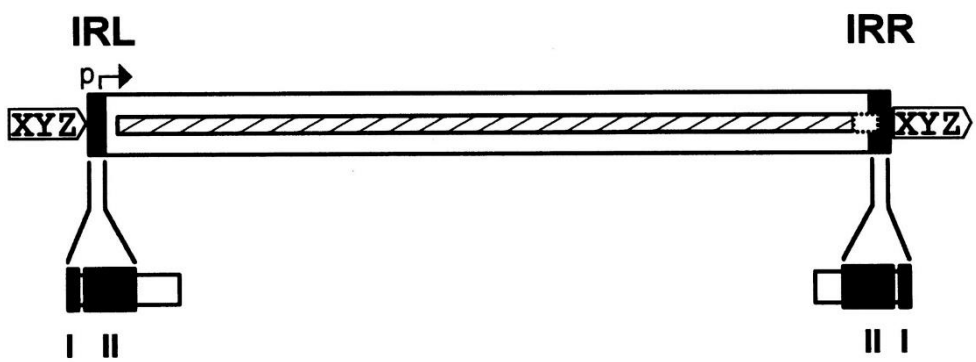

Figure IV. Organization of a typical IS. The IS is represented as an open box in which the terminal IRs are shown as grey boxes labelled IRL (left inverted repeat) and IRR (right inverted repeat). Adapted from Mahillon and Chandler (1998).

Mycobacterium terrae complex

Mycobacterium terrae complex (MTC) was characterized firstly in 1981 by the International Working Group in Mycobacterial Taxonomy (IWGMT). MTC initially consisted of two species of bacteria, *M. terrae* and *M. nonchromogenicum* which are slow-growing nonchromogenic species (Tortoli et al., 2013; Wayne et al., 1996). The bacteria belong to MTC could not be differentiated by phenotypic methods such as biochemical tests or cultural methods, making description of pathogenicity in species unclear (Ridderhof et al., 1991). The consistency of the MTC was confirmed by the presence of the unique genetic signature, the two-nucleotide insertion in helix 18 of 16S rRNA gene, in comparison with slow-growing mycobacteria in the early 1990s. This genetic signature is still considered as the most reliable marker of mycobacteria in the MTC (Kirschner et al., 1993; Springer et al., 1996). Recently, as the accessibility of DNA sequencing technology has been greatly increased, many mycobacteria which were difficult to classify by phenotypic methods have been identified by 16S rRNA gene sequencing, rpoB sequencing, and *hsp65* sequencing (Kim et al., 2001; Kim et al., 2006; Stahl and Urbance, 1990; Stone et al., 1995). As a result, many new MTCs have been reported: *M. hiberniae* (Kazda et al., 1993), *M. arupense* (Cloud et al., 2006), *M. heraklionense* (Tortoli et al., 2013), *M. senuense* (Mun et al., 2008), *M. minnesotense* (Hannigan et al., 2013), *M. longobardum* (Tortoli et al., 2013), *M. algericum* (Sahraoui et al., 2011), *M. engbaekii* (Tortoli et al., 2013), *M. virginiense* (Vasireddy et al., 2016), etc.

Most MTCs are environmental bacteria, sometimes isolated from animal host and human patient (Smith et al., 2000; Tasler and Hartley, 1981). MTC was isolated from tenosynovitis patients (Vasireddy et al., 2016). In addition, *M. nonchromogenicum* was isolated from cavitary pulmonary and extrapulmonary infections in a immunocompetent patient (Halstrom et al., 2015).

In the veterinary field, the isolation of MTC in cow milk was reported in Brazil, and there is also a report on the isolation of *M. nonchromogenicum* in bovine nasal mucus in Northern Ireland (Bolanos et al., 2018; McCorry et al., 2004). In Korea, the isolation of *M. nonchromogenicum* from bronchial lymph node and lung in Hanwoo was reported (Kim et al., 2014).

Chapter I

Effective DNA extraction method to improve detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces

Abstract

Paratuberculosis (PTB) caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has extended latent periods of infection. Due to this property, difficulties in the detection of fecal shedder have been raised, especially when using methods involving fecal culture. Alternatively, PCR-based method using DNA directly extracted from fecal materials has been used to diagnose MAP. However, normal DNA extraction methods are unsuitable for recovering sufficient amounts of DNA for MAP diagnosis due to MAP's thick and lipid-rich cell wall. Also, an abundance of PCR inhibitors in bovine feces decrease the reliability of PCR as a diagnostic tool. A newly designed method for DNA extraction from fecal specimens, mGITC/SC was evaluated in terms of diagnostic efficiency. The detection limit of IS*900* real-time PCR was approximately 50 MAP (1.5 cfu) in 250mg of feces (6 cfu per g). Also,

this DNA extraction method was faster and cheaper than that using commercial kit or other methods. Consequently, the mGITC/SC is an economical DNA extraction method that could be a useful tool for detecting MAP from fecal specimens.

Key words: *Mycobacterium avium* subsp. *paratuberculosis,* DNA extraction, feces, real-time PCR

Introduction

Paratuberculosis (PTB), or Johne's disease (JD), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is one of the most widespread and economically significant diseases in cattle (Ott et al., 1999). Because PTB has long incubation period, infected cows without clinical signs of the disease might excrete feces containing MAP for months and years, contaminating their environment (Whittington et al., 2004). Therefore, finding a fecal shedder in the subclinical stage is the most important and efficient strategy for controlling PTB.

To detect subclinical fecal shedders, bacterial culture has always been used as the gold standard (Whitlock et al., 2000). However, MAP is a slow-growing bacteria and identifying the colony would take a long time (up to 16 weeks) (Collins, 1996). Indeed, the culture method has relatively low sensitivity and sometimes yields false negative results in cattle that are shedding low levels of bacteria (Collins, 1996).

In the case of MAP diagnosis, a PCR method using DNA extracted from fecal specimens has the advantage of identifying infected cattle more rapidly than the bacterial culture method. The insertion sequence IS*900* has been used to detect MAP because of presence of multicopy (12-20) of the gene (Bull et al., 2000).

Because MAP has a thick and lipid-rich cell wall, bacterial cell lysis is more difficult when using lysing methods typically used for other bacteria (Amaro et al., 2008). Additionally, many PCR inhibitors are inherently present in feces (Logar et al., 2012). Therefore, many studies have also focused on obtaining high yields of

DNA from fecal specimens, as well as on its purity, in order to diagnose MAP effectively (Amaro et al., 2008; Logar et al., 2012; Zhang and Zhang, 2011).

In the present study, we designed a new method for extracting MAP DNA from fecal specimens. The diagnostic efficiency of this new DNA extraction method was compared with commercial kits and other published DNA extraction methods in terms of purity of fecal DNA, diagnostic sensitivity and cost-effectiveness using MAP spiked fecal samples (Irenge et al., 2009; Leite et al., 2013; Logar et al., 2012; Zhang and Zhang, 2011).

Materials and Methods

Bacterial culture and sample preparation

Mycobacterium avium subsp. *paratuberculosis* ATCC19698 was cultured in modified Herrold's egg yolk medium (HEYM) supplemented with 2 mg/L of Mycobactin J (ID-Vet, Montpellier, France) and three antibiotics (nalidixic acid, vancomycin, amphotericin B) conditioned at 37°C for 8 weeks. Colonies were harvested by swab and transferred into 5 mL of phosphate buffered saline (PBS) supplemented with 0.25% Tween 80, and then vortexed for 1 min to minimize the clumping of cells (Zhang and Zhang, 2011). Optical density (OD_{600}) of suspended

cells was measured by GeneQuant™ pro spectrophotometer (GE healthcare, United Kingdom). The number of cells of OD_{600} = 1.0 were counted by direct microscopic count and confirmed by colony forming unit (cfu) enumeration on HEYM slant. Spiked fecal samples were prepared by 10-fold dilution of MAP ranged from 1.5×10^6 to 0.15 cfu into 250 mg of feces. Negative control of feces was treated with PBS supplemented with 0.25% Tween 80. Three sets of spiked samples were prepared using feces from three different healthy cows.

DNA extraction using the mGITC/SC method

DNA was extracted from pure cultured MAP and MAP-spiked fecal samples. DNA extraction was composed of two major steps, bacterial cell lysis and DNA purification. The guanidinium thiocyanate (GITC) buffer was used in lysis step described by Boom et al. (1990) and spin column (SC) was used for purification of DNA. This method was named as mGITC/SC. First, one mL of GITC L6 lysis buffer (5.25 M GuSCN, 50 mM Tris-HCl (pH 6.4), 20 mM EDTA, 1.3% Triton X-100, distilled water) was added to the MAP suspensions (100 µL of pure culture and 250 mg of MAP-spiked feces) in a 2 mL tube, vortexed for 30 sec, and then incubated at 95°C for 15 min. The tube was vortexed again and centrifuged at 13,000 × g for 2 min. The supernatant (300 µL) was transferred into a new 1.5 mL tube containing 700 µL of L6 lysis buffer and incubated at 70°C for 5 min, after which 250 µL of 100% ethanol were added; the mixture was then incubated at 56°C for 5 min. After

incubation, the mixture was passed through a mini spin column fitted with a silica membrane (Epoch Biolab, USA), washed with 700 µL of L2 washing buffer (5.25 M GuSCN, 50 mM Tris-HCl, distilled water) and washed again with 700 µL of 70% ethanol twice. Finally, DNA was eluted with 40 µL of nuclease-free water. The yield and purity of the extracted DNA was checked by spectrophotometer (ND-1000, Thermo Fisher Scientific, USA).

IS*900* real-time PCR assay

The extracted DNA samples were analyzed by real-time PCR targeting the IS*900* element specific to MAP. The primer sequences were SF214; 5'-ATGACGGTTACGGAGGTGGTT-3' (forward primer), SR289; 5'-TGCAGTAATGGTCGGCCTTAC-3' (reverse primer), and PR265; 5'-FAMCGACCACGCCCGCCCAGATAMRA-3' (probe) as described previously (Ravva and Stanker, 2005). The real-time PCR reaction was conducted using a Rotor-Gene Q real-time PCR cycler (Qiagen Inc., Germany) and a reaction mixture consisting of 1× Rotor-Gene Probe PCR master mix (Qiagen Inc., Germany), 400 nM primers, 100 nM probe, 4 µL of template DNA, and nuclease-free water to give a total volume of 20 µL. Samples were amplified according to the following conditions: 1 cycle at 95°C for 5 min, 45 cycles at 95°C for 15 sec, 60°C for 1 min. The positive control consisted of 10 ng of MAP (ATCC 19698) genomic DNA, and nuclease-free water was used as a no template control for this reaction.

Results

Efficiency of DNA extraction with the mGITC/SC method

Concentration of pure cultured MAP at OD_{600} 1.012 was approximately 1.02×10^9 cells/mL, and HEYM enumeration resulted in 3.03×10^7 cfu/mL. The concentration of the DNA harvested from 3.03×10^7 cfu of MAP was 2.3 ± 0.15 ng/uL (SE), corresponds to 3.04 fg of DNA per cfu. In the spiked fecal samples, the average concentration of extracted DNA was 7.22 ± 0.38 ng/μL from the 250 mg of feces, corresponds to 116 ± 6.0 ng per 100mg of feces. The average purity of DNA (Abs260/Abs280) from fecal samples was 1.67 ± 0.03 (Table 1.1).

Evaluation of diagnostic sensitivity of the mGITC/SC method

The IS*900* real-time PCR was conducted to evaluate the diagnostic sensitivity of the mGITC/SC method. Serial dilutions of DNA extracted by mGITC/SC were assayed to determine the detection limit of IS*900* real-time PCR. Minimum detectable limit was 4.6 fg of DNA, corresponds to *C*t value of 37.03 (Figure 1.1). In the spiked fecal samples, *C*t values for the real-time PCR ranged from 18.9 ± 0.43 to 38.3 ± 0.1 and linear correlation between the Ct values and MAP concentration was analyzed using standard curve analysis (Figure 1.2). Experimental detection limit was 1.5 cfu in 250 mg of feces (6 cfu/g). No amplification signals were obtained

with the all negative controls (no template controls, negative fecal samples).

Discussion

One of the MAP diagnostic method, fecal PCR has been used for detection of MAP infection. However, fecal PCR has some challenges to use as a definitive diagnostic method for MAP. Therefore, based on a modification of the conventional method, a new DNA extraction method was designed and its diagnostic efficiency evaluated against a commercial kit. In this study, DNA extraction method for the detection of MAP in bovine feces was described. To lyse MAP's thick and lipid-rich cell wall, the bead beating method has been used in MAP DNA extraction (Odumeru et al., 2001). However, heating method combined with guanidine lysis was used in mGITC/SC and this could also provide high yield and PCR sensitivity. Diagnostic efficiency of this new method was evaluated in terms of purity, yield and real-time PCR sensitivity. DNA purity is major consideration in fecal DNA extraction because of many inhibitors in feces affecting PCR reaction (Monteiro et al., 1997; Thornton and Passen, 2004). Purity of DNA with mGITC/SC recorded the fourth highest value compared to other methods described in Leite et al. (2013). According to the study, there were no significant correlation between DNA purity and PCR sensitivity due to the inaccuracy of measuring DNA purity by spectrophotometry. In quantitative analysis, minimum detectable amount of DNA extracted from pure cultured MAP

was about 4.6 fg, corresponds to 1.5 cfu (3.04 fg per cfu). Detection limit of real-time PCR on MAP-spiked fecal samples was also appeared similar level to pure cultured MAP. This result suggests that mGITC/SC can detect the lowest level of MAP in the MAP-spiked feces.

Major advantageous feature of the mGITC/SC method is that there is no need for pre-treatment of the fecal specimen. The mGITC/SC method uses 250 mg of feces mixed directly with 1 mL of GITC buffer to lyse the MAP cells. Other in-house DNA extraction methods use about 1 g of feces mixed with large amounts of water or buffers followed by a settle down and washing step; the time required for this step is more than 1 hour (Irenge et al., 2009; Zhang and Zhang, 2011). Because of differences between the initial extraction steps in the mGITC/SC method and the other methods, mGITC/SC has the advantage of extraction time. Another feature of mGITC/SC method is relatively low cost compared to other in-house methods or commercial kits. These two features allow to extraction of MAP DNA from bovine feces more quickly and economically.

Table 1.1. Comparison of the detection limits and amplification efficiencies of the two DNA extraction methods. Each data was provided by the studies describing the corresponding methods.

Methods	Minimum detectable MAP cells (cfu/g)	A260/280	Time	Cost ($)	References
mGITC/SC	50 (6)	1.67 ± 0.03	60 min	1	This study
Kit A	50 (6)	1.87 ± 0.08	50 min	4	This study
Kit B	90 (2.4)	Data not shown	60 min	6.8	(Logar et al., 2012)
Protocol A	- (3)	2.0	3 hr	3	(Zhang and Zhang, 2011)
Protocol B	250 (12.5)	Data not shown	>3 hr	Data not shown	(Irenge et al., 2009)

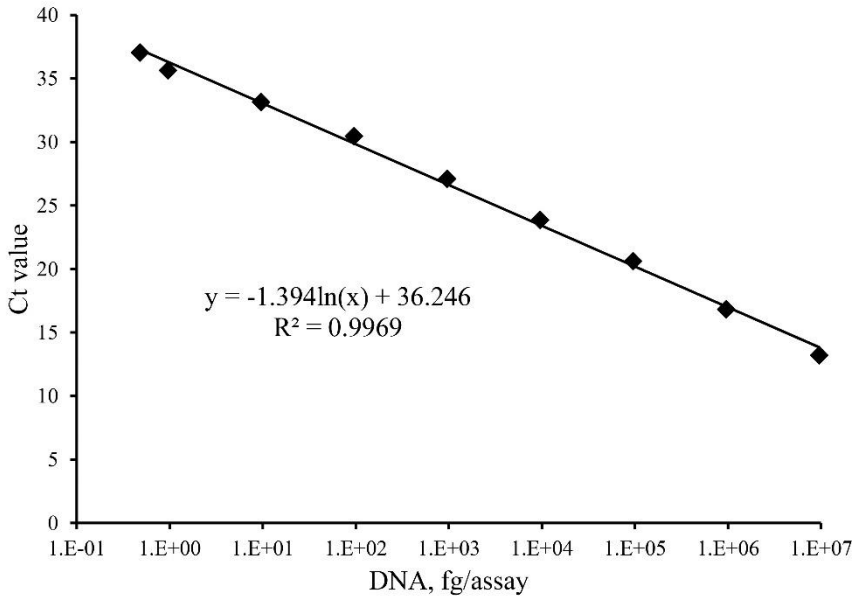

Figure 1.1. Real-time PCR assay of serial dilutions of DNA extracted from pure cultured MAP ATCC19698. R^2 (the coefficient of determination) are indicated on the graph.

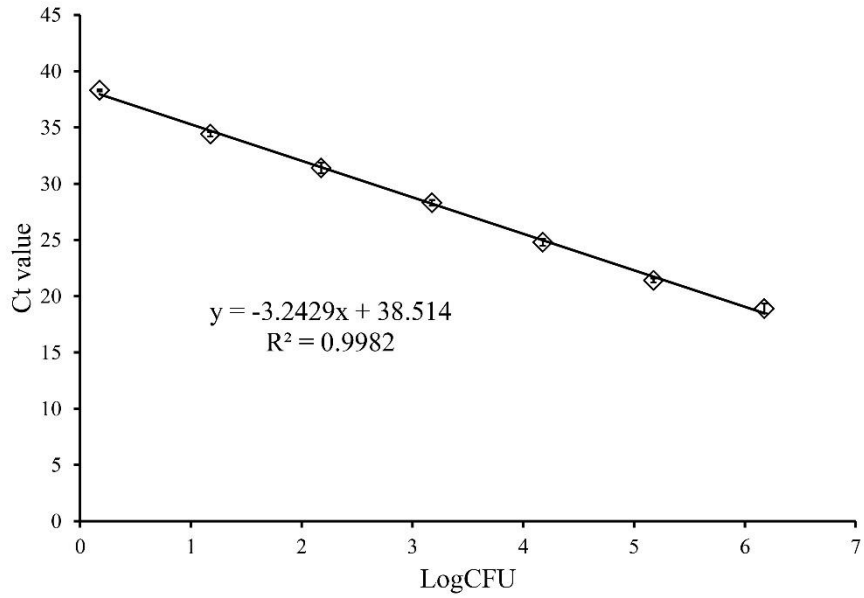

Figure 1.2. The detection limit and amplification efficiency of mGITC/SC in MAP-spiked fecal samples. *C*t values are the average of 3 independent experiments. R^2 (the coefficient of determination) are indicated on the graph.

Chapter II

PCR-based detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle in Korea using fecal samples

Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of bovine paratuberculosis (PTB). The first step in the control of PTB is the identification and isolation of sub-clinical fecal shedders from the herd. In the current study, real-time and nested PCR targeting MAP-specific genetic elements (IS*900*, ISMap*02*) DNA isolated from fecal samples were used to detect MAP infection in cattle. Of the 1,562 fecal samples obtained from 37 herds, regardless of diarrhea, 35 samples tested positive in both IS*900*-targeted real-time and ISMap*02*-targeted nested PCR. At the herd level, 12 of the 37 herds were found to be positive for MAP. Thirty-five positive cows were considered to be in the subclinical stage because they did not show any clinical symptoms. In addition, the herd level prevalence investigated in this study was similar to those of the previous reports measured by

ELISA-based methods. These suggests that PCR diagnosis might be a useful tool for investigation of the prevalence of MAP infection, especially a herd level.

Keywords: Diagnosis, Korea, *Mycobacterium avium* subsp. *paratuberculosis*, Nested PCR, Real-time PCR

Introduction

Mycoabcterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis (PTB) or Johne's disease (JD), which is characterized by chronic and debilitating enteritis in ruminants. MAP have a global distribution and cause serious economic losses to the cattle industry (Ott et al., 1999). Clinical onset of PTB is noted after 2 or more years of the initial infection, which usually occurs shortly after birth (Irenge et al., 2009). Additionally, the bacteria are shed in the feces of infected animals that show no clinical signs. These 'sub-clinical' fecal shedders contaminate various components of the cattle sheds, such as feed, water, bedding, and other materials. Contamination of cattle shed components results in spreading of the infection to other animals through the fecal–oral route. Specifically, young animals that are highly susceptible to MAP are easily infected as they ingest the bacteria via milk, colostrum, and the surface of the teats, which get contaminated through the cattle shed components (Giese and Ahrens, 2000; Slana et al., 2009). Therefore, identification and isolation of sub-clinical fecal shedders is the most effective strategy to control PTB.

Culture-based methods are the 'gold standard' for diagnosis of MAP infection. However, these methods are time-consuming and have relatively low sensitivity. Recently, molecular biological methods such as PCR have been developed for the detection of MAP infection in animals (Fang et al., 2002). Several target sequences unique to MAP have been identified for PCR-based detection. The insertion

sequence IS*900* is the most selected target element because it is present in multiple copies and thought to be unique to MAP (Bull et al., 2000). However, recent studies have reported the presence of IS*900*-like sequences in non-MAP mycobacteria, which could lower the specificity of IS*900*-based assays. Therefore, a new target sequence, ISMap*02*, which is also present in multiple copies and is specific to MAP, has been identified for real-time or nested PCR-based assays. ISMap*02* nested PCR is usually combined with IS*900* real-time PCR to improve detection rates (Douarre et al., 2010; Irenge et al., 2009; Stabel and Bannantine, 2005).

PCR-based diagnostics have been used in many countries and the methods have been improved constantly to overcome the limitations of other diagnostic methods such as the culture-based method (Alinovi et al., 2009; Bogli-Stuber et al., 2005). In Korea, the diagnosis of PTB has been attempted mainly using a serum ELISA with commercial kits. National prevalence of PTB in Korea was investigated based on serum ELISA test in 2006 (Park et al., 2006). However, investigation of prevalence using a diagnostic approach such as fecal culture or fecal PCR that are efficient in detection of fecal shedder have not yet been reported in Korea.

The aim of this study was to evaluate the diagnostic value of the fecal PCR in the diagnosis of PTB in Korea, especially for detection of sub-clinical fecal shedders. In the present study, duplex PCR assay targeting the IS*900* and ISMap*02* elements was conducted for detection of MAP infection from the fecal DNA individually extracted from cows in Korean cattle herds. In addition, approximate rate of infection by PTB in Korean cattle herds was estimated by the fecal PCR diagnosis.

Materials and Methods

Sample collection and preparation of fecal DNA

Fecal samples from 982 beef cattle (Hanwoo) in 25 herds and 580 dairy cattle (Holstein) in 12 herds, distributed in 6 provinces (Gyeong-gi, Gang-won, Chung-nam, Chung-buk, Gyeong-nam, and Jeju) regardless of diarrhea, were collected between September 2013 and August 2014 (Table 2.1). The samples were collected individually from the rectum and transported immediately to the laboratory in a container maintained at 4°C. DNA was extracted using the mGITC/SC method (Park et al., 2014a). First, one mL of GITC L6 lysis buffer (5.25 M GuSCN, 50 mM Tris-HCl (pH 6.4), 20 mM EDTA, 1.3% Triton X-100, distilled water) was added to the MAP suspensions (100 µL of pure culture and 250 mg of MAP-spiked feces) in a 2 mL tube, vortexed for 30 sec, and then incubated at 95°C for 15 min. The tube was vortexed again and centrifuged at 13,000 × g for 2 min. The supernatant (300 µL) was transferred into a new 1.5 mL tube containing 700 µL of L6 lysis buffer and incubated at 70°C for 5 min, after which 250 µL of 100% ethanol were added; the mixture was then incubated at 56°C for 5 min. After incubation, the mixture was passed through a mini spin column fitted with a silica membrane (Epoch Biolab, USA), washed with 700 µL of L2 washing buffer (5.25 M GuSCN, 50 mM Tris-HCl, distilled water) and washed again with 700 µL of 70% ethanol twice. Finally, DNA

was eluted with 40 μL of nuclease-free water.

IS*900* real-time PCR with fecal DNA

The real-time PCR was performed using the reaction conditions described previously (Park et al., 2014a). The primer sequences for the IS*900* real-time PCR were described in Table 2.2. The real-time PCR reaction was conducted using a Rotor-Gene Q real-time PCR cycler (Qiagen Inc., Germany) and a reaction mixture consisting of 1× Rotor-Gene Probe PCR master mix (Qiagen Inc., Germany), 400 nM primers, 100 nM probe, 4 μL of template DNA, and nuclease-free water to give a total volume of 20 μL. Samples were amplified according to the following conditions: 1 cycle at 95°C for 5 min, 45 cycles at 95°C for 15 sec, 60°C for 1 min.

ISMap*02* nested PCR with fecal DNA

Nested PCR was performed using primer sequences described previously with modifications (Stabel and Bannantine, 2005). The primer sequences for the ISMap*02* nested PCR were described in Table 1. Briefly, the 20-μL PCR mixture comprised 1 μL of template DNA, 10.5 μL of nuclease-free water, 2 μL of i-Taq™ 10× PCR buffer (Intron biotechnology, Korea), 2.5 mM of $MgCl_2$, 0.25 mM of deoxyribonucleotide triphosphates, 500 nM of each primer, and 2.5 U of i-Taq™ DNA polymerase. The PCR conditions were as follows: initial denaturation at 94°C

for 5 min, 35 cycles at 94°C for 15 sec, 58°C for 15 sec, and 72°C for 20 sec, followed by a final extension at 72°C for 7 min. The amplicons (1 μL) from the first PCR were used as the template in the nested PCR, which was performed using similar reaction conditions (30 cycles). After electrophoresis on a 2% agarose gel, presence of 117 bp size bands was considered as a positive result. To minimize the risk of carry-over contamination in the nested PCR, reagent preparation at each step and PCR amplification were performed using different sets of the pipetting system and on different work benches with a separate air-conditioning. Tips with filters were used to protect the PCR samples from aerosol contamination.

Results

Detection of MAP infection using fecal PCR

The PCR results are summarized in Table 2.3. IS*900* was detected in 73 samples, accounting for 4.7% of the total samples, whereas ISMap*02* was detected in 53 samples, accounting for 3.4% of the total samples. Samples that showed positive signals in both IS*900* real-time PCR and ISMap*02* nested PCR were considered as positives. Thirty-five samples that showed a positive result for both target sequences were considered as definite MAP positives (2.2%). Thirty-eight (2.4%) samples were

detected positive only for IS*900*; whereas 18 (1.2%) samples were detected positive only for ISMap*02*. Infection rate of MAP at each herd varied from 0% to 30.8%. At the herd level, 12 herds (32.4%) were detected positive for MAP infection among the 37 herds.

Age distribution of positive resulted cows

Age distribution of the positive samples was described in Table 2.4. In both Hanwoo and Holstein cattle, the number of positive samples showed no notable difference with respect to age. However, the number of positive samples (3.2%) noted in Hanwoo cattle aged more than 7 years was higher than that in other age groups. These samples were obtained from 2 herds, which had a higher proportion of old cattle than the other herds (23.1% and 23.7% of collected samples). Population of cattle aged more than 7 years was 9.5% in other herds.

Discussion

In a previous study, seroprevalence of MAP in Korean cattle was reported in about 7.1% samples analyzed by ELISA followed by the Bayesian approach (Pak et al., 2003; Park et al., 2006). In addition, the seroprevalence of MAP in the Gyeong-nam province in Korea was estimated to be 3.3% (Lee and Jung, 2009). The present

investigation also revealed a similar result at the individual level (2.2%). Although the prevalence of MAP at the cattle herd level has not been reported earlier in Korea, the prevalence found in the present study was similar to that in the Korean black goat (Lee et al., 2006). Cattle type-specific statistical analysis showed 41.7% positive samples in dairy herd, which was similar to that reported in a previous study conducted in Gyeong-nam, whereas 28.0% positive samples were noted in beef herd, which was much higher than a previous report (Lee and Jung, 2009).

A weak correlation was noted between the results of two PCR assays. The number of single positives for IS*900* noted in real-time PCR was similar to the number of positives for both the sequences. This could be because the sensitivity of IS*900* real-time PCR was higher than the ISMap*02* nested PCR. Theoretical sensitivity of IS*900* is higher than that of ISMap*02* because 12–20 copies of the IS*900* elements and only 6 copies of the ISMap*02* elements are present in MAP (Stabel and Bannantine, 2005; Whipple et al., 1990). Experimental sensitivity of IS*900* real-time PCR and ISMap*02* nested PCR can be correlated to their theoretical sensitivity. Detection limit of IS*900* real-time PCR is 10 fg of DNA extracted from pure cultured cells, whereas that of the ISMap*02* nested PCR is 100 fg (Stabel and Bannantine, 2005). Our previous study reported that the detection limit of IS*900* real-time PCR was 4.6 fg of DNA extracted from pure cultured cells by mGITC/SC method (Park et al., 2014a). Therefore, single positive of IS*900* real-time PCR may indicate the presence of MAP at a very low level. Although IS*900* PCR has relatively higher sensitivity, it lacks specificity, which is a notable problem because of the presence of IS*900*-like

sequences (Kim et al., 2002; Rajeev et al., 2005; Tasara et al., 2005). This problem can be solved by designing primers that are not complementary to all the known IS*900*-like sequences. However, confirming MAP-specificity in every IS*900* single positive would be time-consuming and cumbersome (Irenge et al., 2009).

Some samples were positive only for ISMap*02* and can be assumed as true positives because ISMap*02* is known to be MAP-specific (Stabel and Bannantine, 2005). Moreover, various PCR inhibitors obtained from fecal samples may lower the sensitivity of IS*900* real-time PCR when low amounts of target DNA is used for the assay (Thornton and Passen, 2004). Even though a large amount of bacteria are present in feces, PCR inhibitors sometimes can lead to false-negative results (Stabel and Bannantine, 2005). However, the possibility of non-specific bands similar in size should be considered during electrophoresis of samples after nested PCR. Additionally, there may be a potential risk in resulting false-positive data in attempting to increase the sensitivity via the inclusion of nested PCR (Douarre et al., 2010). Consequently, the samples showing a positive result for both the sequences could be confirmed as definite positive, but samples showing a positive result for a single sequence should be investigated further. Therefore, developing a comprehensive system that allows further investigation of the false-positive results is crucial. Such a system may utilize repeated analysis of samples for validation by PCR or may include other types of tests such as ELISA.

Analysis of the age-wise distribution of MAP-infection showed that infection rates were similar in all age groups. However, in the group with age less than 2 y, no calves

or heifers less than one-year-old showed a positive result. This phenomenon is similar to the pathogenesis of MAP infection indicating fecal shedding without clinical signs. In fact, all the cattle diagnosed as positive for MAP had no clinical signs such as diarrhea. This suggests that, the PCR method is considerable when the sub-clinical fecal shedders of infection are to be diagnosed.

Molecular biological diagnostic methods, such as PCR, are used as supplementary detection methods because they cannot determine the number of viable bacteria (Kralik et al., 2010). In case of MAP, however, the PCR-based method is worth considering because it provides higher sensitivity and specificity than other detection methods. In this study, we detected MAP from fecal samples of cattle showing no clinical signs using an improved DNA extraction method and PCR analysis. Our findings suggest that detection of MAP-specific genetic elements using PCR can be used for early diagnosis, which will in turn help control MAP infection because identification and isolation of fecal shedders from the herd is crucial for preventing the spread of infection.

Table 2.1. Number of fecal samples collected from beef and dairy cattle herds in Korea

Classification	Species	Region	Herd ID	Sampling date	No. of samples
Beef	Hanwoo	Gyeong-gi	GY2	09/2013	6
			GY3	09/2013	16
		Gang-won	GW2	09/2013	52
			GW3	03/2014	643
		Jeju	JJ1	11/2013	13
			JJ2	11/2013	11
			JJ3	11/2013	13
			JJ4	11/2013	13
			JJ5	11/2013	7
			JJ6	11/2013	7
			JJ7	11/2013	13
			JJ8	11/2013	16
			JJ9	11/2013	11
		Gyeong-nam	GN1	03/2014	21
			GN2	03/2014	25
		Chung-buk	CB1	03/2014	25
			CB2	03/2014	18
			CB3	03/2014	20
			CB4	03/2014	10
			CB5	03/2014	10
			CB6	03/2014	5
			CB7	03/2014	5
			CB8	03/2014	8
			CB9	03/2014	7
			CB10	03/2014	7
			Subtotal		**982**
Dairy	Holstein	Gyeong-gi	GY1	09/2013	18
			GY4	09/2013	10
			GY5	09/2013	24
			GY6	09/2013	15
			GY7	09/2013	29
			GY8	03/2014	40
			GY9	03/2014	20
			GY10	03/2014	20
			GY11	03/2014	20
		Chung-nam	CN1	04/2014	284
		Gang-won	GW1	09/2013	60
			GW4	08/2014	40
			Subtotal		**580**
	Total				**1,562**

Table 2.2. Primer sequences used for IS*900* real-time PCR and ISMap*02* nested PCR

Target	**Purposes**	**Primer name**	**Primer sequences (5'-3')**	**Reference**
IS*900* element	Real-time PCR	SF214 SR289 PR265	F: ATGACGGTTACGGAGGTGGTT R: TGCAGTAATGGTCGGCCTTAC Probe: FAM-CGACCACGCCCGCCCAGA-TAMRA	(Zhang and Zhang, 2011)
ISMap*02* element	Nested PCR	ISMap*02* (first round) ISMap*02* (second round)	F: GCACGGTTTTTCGGATAACGAG R: TCAACTGCGTCACGGTGTCCTG F: GGATAACGAGACCGTGGATGC R: AACCGACGCCGCCAATACG	Stabel and Bannantine (2005)

Table 2.3. Number of samples infected with *Mycobacterium avium* subsp. *paratuberculosis* in Hanwoo and Holstein cattle herds

Species	Herd ID	No. of samples	No. of samples positive (percentage)		
			PCR		
			IS*900*	ISMap*02*	Both
Hanwoo	GY2	6	1(16.7)	0(0)	0(0)
	GY3	16	0(0)	0(0)	0(0)
	GW2	52	4(7.7)	1(1.9)	1(1.9)
	GW3	643	36(5.6)	22(3.4)	13(2.0)
	JJ1	13	0(0)	0(0)	0(0)
	JJ2	11	2(18.2)	0(0)	0(0)
	JJ3	13	0(0)	0(0)	0(0)
	JJ4	13	6(46.2)	4(30.8)	4(30.8)
	JJ5	7	0(0)	0(0)	0(0)
	JJ6	7	2(28.6)	1(14.3)	1(14.3)
	JJ7	13	0(0)	0(0)	0(0)
	JJ8	16	0(0)	0(0)	0(0)
	JJ9	11	0(0)	0(0)	0(0)
	GN1	21	0(0)	0(0)	0(0)
	GN2	25	0(0)	0(0)	0(0)
	CB1	25	3(12.0)	4(16.0)	3(12.0)
	CB2	18	1(5.6)	0(0)	0(0)
	CB3	20	1(5.0)	2(10.0)	1(5.0)
	CB4	10	2(20.0)	1(10.0)	0(0)
	CB5	10	0(0)	0(0)	0(0)
	CB6	5	1(20.0)	1(20.0)	1(20.0)
	CB7	5	1(20.0)	0(0)	0(0)
	CB8	8	0(0)	0(0)	0(0)
	CB9	7	0(0)	0(0)	0(0)
	CB10	7	0(0)	0(0)	0(0)
	Subtotal	**982**	**60(6.1)**	**36(3.7)**	**24(2.4)**
Holstein	GY1	18	0(0)	0(0)	0(0)
	GY4	10	1(10.0)	1(10.0)	1(10.0)
	GY5	24	0(0)	0(0)	0(0)
	GY6	15	0(0)	0(0)	0(0)
	GY7	29	0(0)	0(0)	0(0)
	GW1	60	3(5.0)	3(5.0)	3(5.0)
	GW4	40	0(0)	0(0)	0(0)
	GY8	40	2(5.0)	1(2.5)	1(2.5)
	GY9	20	0(0)	0(0)	0(0)
	GY10	20	0(0)	0(0)	0(0)
	GY11	20	1(5.0)	2(10.0)	1(5.0)
	CN1	284	6(2.1)	10(3.5)	5(1.8)
	Subtotal	**580**	**13(2.9)**	**17(2.9)**	**11(1.9)**
Total		**1,562**	**73(4.7)**	**53(3.4)**	**35(2.2)**

Table 2.4. Age distribution of the number of Hanwoo and Holstein cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*

Species	**No. of positive samples/tested (%)**				
	Age distribution				
	1–2	3–4	5–6	≥7	unknown
Hanwoo	6/326(1.8)	7/269(2.6)	2/113(1.8)	6/187(3.2)	3/87(3.4)
Holstein	2/87(2.3)	2/154(1.3)	2/105(1.9)	1/64(1.6)	4/170(2.4)
Total	8/413(1.9)	9/423(2.1)	4/218(1.8)	7/251(2.8)	7/257(2.7)

Chapter III

Genetic diversity of bovine *Mycobacterium avium* subsp. *paratuberculosis* in Korea discriminated by IS*1311* PCR-REA, MIRU-VNTR, and MLSSR genotyping

Abstract

The aim of this study was to describe the genetic diversity of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) obtained from individual cows in Korea. Twelve MAP-positive fecal DNA samples and 19 MAP isolates were obtained from 10 cattle herds located in 5 provinces in Korea. In addition, 5 MAP isolates obtained from the Czech Republic and Slovakia and 3 isolates from Australia were genotyped for comparison with the domestic isolates. The most prevalent strains in Korea were of the "bison-type" genotype (23 of 31 fecal DNA/isolates) and were distributed nationwide. The remaining MAP isolates (8) and all of the foreign isolates were identified as "cattle-type". The bison-type strains which were discriminated only as

INMV 68 in MIRU-VNTR typing. MLSSR typing differentiated the bison-type strains into 3 different subtypes. The cattle-type strains were divided into 3 subtypes by MIRU-VNTR and 8 subtypes by MLSSR. The allelic diversities in the MIRU-VNTR and MLSSR results were calculated as 0.567 and 0.866, respectively. These results suggest that MIRU-VNTR typing cannot provide a sufficient description of the epidemiological situation of MAP. Therefore, an alternative method, such as MLSSR, is needed for typing of MAP strains to elucidate the molecular epidemiology of MAP infections. Overall, this study is the first epidemiological survey report in Korea using both MIRU-VNTR and MLSSR typing methods, and it has provided basic data necessary to elucidate the characteristics of MAP infections in Korea.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis,* IS*1311* PCR-REA, MIRU-VNTR, MLSSR, Korea

Introduction

Paratuberculosis (PTB) or Johne's disease is a chronic and debilitating disease in ruminants that is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The disease is characterized by intermittent to persistent diarrhea, progressive wasting, and eventually leads to death. Currently, there are no treatments available to cure the infection (Coussens, 2001). The disease occurs worldwide and causes serious economic losses to the cattle industry due to premature culling and production losses (Sweeney, 1996). In most of the major dairy-producing countries, it has been estimated that more than 50% of dairy cattle herds were infected with MAP (Nielsen and Toft, 2009; Ott et al., 1999). In Korea, the national prevalence of PTB in individual cattle was estimated at 3.3%–7.1% until 2010 (Yoo and Shin, 2012). Recently, 12 of 37 herds (32.4%) tested in Korea were found to be MAP-positive, as determined by performing PCR of fecal DNA samples (Park et al., 2016). Despite the high herd-level prevalence, there have been no reports on the molecular characterization of MAP strains isolated from cattle in Korea. Understanding the genetic diversity of MAP is necessary for establishing disease control strategies (Douarre et al., 2011). Molecular typing methods for MAP strains provide important information for genetic diversity and evolutionary relationships and can assist in elucidating inter- and intra-species transmissions (Amonsin et al., 2004; Leao et al., 2016; Stevenson et al., 2009). Several methods for subtyping of MAP have been developed. Polymerase chain reaction and restriction endonuclease analysis (PCR-

REA) of the IS*1311* locus of MAP has been used, and it allows for the differentiation of MAP linages into three subtypes (cattle-, sheep- and bison-type) according to host preferences (Sevilla et al., 2005; Whittington et al., 2001). Recently, typing techniques such as variable-number tandem repeats of mycobacterial interspersed repetitive units (MIRU-VNTR) and multilocus short sequence repeat (MLSSR) have been used to differentiate subtypes (Ahlstrom et al., 2015; de Kruijf et al., 2017; Douarre et al., 2011; Fernandez-Silva et al., 2012; Imperiale et al., 2017; Sevilla et al., 2005; Stevenson et al., 2009; Thibault et al., 2007, Thibault et al., 2008). The techniques using these targets provide high discriminatory power and are easy to use (Amonsin et al., 2004; Thibault et al., 2007). The aim of this study was to describe the genetic diversity of MAP isolated from individual cows in Korea by using IS*1311* PCR-REA, MIRU-VNTR, and MLSSR typing methods.

Materials and Methods

Collection of fecal DNA positive in MAP

During 2013 and 2014, detection of MAP by PCR was conducted by using fecal material collected from 37 herds across 6 provinces of Korea (Table 2.1). From a total of 1,562 fecal DNA samples, 35 were shown to be positive for MAP IS*900* and ISMap*02*. The IS*1311* targeting PCR was conducted using fecal DNA, and the PCR-

positive fecal DNA samples were subjected to subsequent REA analysis.

Bacterial isolation

Nineteen Korean MAP isolates and 8 foreign isolates were used for genotyping. Korean isolates were obtained from feces or tissues that were collected from 5 herds in 3 provinces (Chung-nam, Jeon-buk, and Jeju). Among them, 6 isolates were obtained from 35 of fecal PCR-positive fecal samples. Along with the Korean isolates, three foreign MAP strains kindly provided by Dr. Jacek Gwozdz (Johne's Disease Laboratory, Department of Primary Industries, Australia) and 5 DNA samples kindly provided by Dr. Iva Slana (Veterinary Research Institute, Czech Republic) were used in this study to compare their epidemiological characteristics with those of the Korean isolates. The culture of MAP from feces and tissues was conducted by using the VersaTREK® system. Briefly, fecal samples were processed for decontamination by using a modified Cornell method as described previously (Corbett et al., 2017). Tissue decontamination was conducted as described previously (Mortier et al., 2013). After decontamination, the suspension was inoculated into VersaTREK® Para-JEM broth (Thermo Scientific, USA) containing para-JEM supplements (para-JEM GS, para-JEM AS, para-JEM EYS, and para-JEM blue (Thermo Scientific, USA)) and then incubated at 37°C in the VersaTREK® instrument (Trek Diagnostic System, Thermo Scientific, USA). After incubation for six weeks, tubes that were shown by the instrument to have a positive result were

inoculated into modified Herrold's egg yolk medium with Mycobactin J (IDvet, France) and cultured for six weeks. The sub-cultured colonies were confirmed positive by using MAP species-specific primers such as IS*900* and ISMap*02* in duplex real-time PCR.

DNA preparation of MAP isolates

A colony of each isolate was cultured in 5 mL of Middlebrook 7H9 broth supplemented with Middlebrook OADC and Mycobactin J (2 mg/L). After incubation at 37°C for 6 weeks, the bacterial suspension was centrifuged at 3,000 × g for 20 min, and the pellet was lysed in 1 mL of L6 lysis buffer (5.25 M GuSCN, 50 mM Tris-HCl at pH 6.4, 20 mM EDTA, 1.3% Triton X-100, distilled water). Subsequent processes were carried out as previously described (Park et al., 2014a).

Characterization of MAP isolates/fecal DNA with IS*1311* PCR-REA

Polymorphism present in 223 bp of IS*1311* was analyzed by performing IS*1311* PCR-REA to characterize the three types (cattle-, bison-, and sheep-type) of MAP isolates and fecal DNA samples. The IS*1311* PCR was conducted by using M56 and M119 primers following the method of Marsh et al. (1999) with some modifications. The PCR products that were 608 bp were then digested by *Hinf*I (Sevilla et al., 2005). The band patterns were visualized after electrophoresis on a 2% agarose gel.

Genotyping of MAP isolates with MIRU-VNTR

Genotyping of the 27 MAP isolates was conducted by using eight MIRU-VNTR targets (292, X3, 25, 47, 3, 7, 10, and 32) that were established previously (Thibault et al., 2007). PCR amplification of each locus was conducted following amplification conditions described previously (Thibault et al., 2007). The repeat numbers of the PCR products were analyzed after electrophoresis on 2% agarose gel. The PCR results and MAP strains were identified according to information in the INMV database (http://mac-inmv.tours.inra.fr/index.php).

MLSSR analysis with MAP isolates

The MLSSR analysis was carried out by performing PCR amplification and sequencing. Eleven short sequence repeat loci were analyzed as described previously (Amonsin et al., 2004). The PCR amplicons were then sequenced by the Macrogen Inc. DNA sequencing service (http://dna.macrogen.com), and the number of short sequence repeats in each locus was identified.

Calculation of discriminatory power

The discriminatory index (DI) described by Hunter and Gaston (1988) was used

as a numerical indicator of the discriminatory power of the MIRU-VNTR and MLSSR typing methods. The DI was calculated using the following equation:

$$DI = 1 - \left[\frac{1}{N(N-1)}\sum_{j=1}^{s} n_j(n_j - 1)\right]$$

where N is the total number of isolates in the typing scheme, s is the total number of distinct types discriminated by each typing method, and n_j is the number of isolates belonging to the *j*th type.

Genetic relationship analysis

The genetic relationships among the 27 strains were investigated by creating minimum spanning trees (MST) using 11 SSR markers. The MST was created by using BioNumerics software (Applied Maths, USA). Through the MST, the shortest possible distance between nodes was linked.

Results

IS*1311* PCR-REA typing

Eighteen of 35 fecal DNA samples showed the expected sizes in the IS*1311* PCR results. Among the 18, six were identical to MAP isolates cultured from the fecal samples. The remaining 12 fecal DNA samples were analyzed by IS*1311* PCR-REA typing. Among the 31 samples (12 fecal DNA and 19 MAP isolate samples) obtained from Korea, 8 samples had band patterns representative of cattle-type genotypes, while 23 had bison-type genotype patterns (Figure 3.1, Table 3.1). All 8 foreign MAP isolates had cattle-type genotype patterns. Two herds each located in Korean regions 3 and 6 showed both genotypes, while other herds only showed one genotype. The cattle-type strain was detected in 6 herds located in 5 regions in 3 Korean provinces, while the bison-type strain was detected in 7 herds located in 5 regions in 4 Korean provinces (Table 3.1).

MIRU-VNTR typing

Genotyping using the MIRU-VNTR method differentiated 4 subtypes among the 27 MAP isolates (Table 3.2). The most prevalent type was INMV 68, which was present in 62.9% (n = 17) of the isolates. The INMV 2, INMV 1, and INMV 5 types were present in 18.5%, 14.8%, and 3.7% of the isolates, respectively. Three MIRU-

VNTR loci (MIRU 292, VNTR 25, and VNTR 10) were polymorphic. Three different alleles were observed in MIRU 292, whereas only two variable alleles were observed in VNTR 25 and VNTR 10. Among the domestic isolates, two MIRU-VNTR types (INMV 2 and INMV 68) were observed (Table 3.3). Two cattle-type strains isolated from a herd were identified as INMV 2, whereas all isolates identified as bison-type were from five herds in three regions belonged to the INMV 68 type. Foreign isolates identified as cattle-type were differentiated into three INMV types according to the country in which they were isolated. All three isolates from Australia were identified as INMV 2 subtypes, whereas four isolates from the Czech Republic were identified as INMV 1, and an isolate from Slovakia was INMV 5. Allelic diversity was calculated as 0.567 when both domestic and foreign isolates were included in the calculation. The DI value for domestic isolates only was 0.199. The MIRU 292 showed the highest allele diversity observed in this study. The allelic diversities for each locus were 0.556 for MIRU 292, 0.484 for VNTR 25, and 0.074 for VNTR 10.

MLSSR typing

Eleven SSR loci were characterized in 27 MAP isolates. The MLSSR analysis identified 11 subtypes among the 27 isolates (Table 3.4). The most prevalent type was MLSSR type 1, which was identified in 8 isolates (29.6%). Of the 11 MLSSR types detected in this study, 7 were unique to one isolate (3.7%). Among the 11

MLSSR loci, loci 1, 2, 6, 7, 8, and 9 were polymorphic with two to four different alleles, whereas the others did not show any polymorphisms. Locus 2 had the highest allelic diversity with a DI value of 0.707. The allelic diversity of loci 1, 6, 7, 8, and 9 were 0.074, 0.313, 0.544, 0.313, and 0.262, respectively. The overall DI value of the MLSSR typing was calculated to be 0.866. Among the domestic isolates, five MLSSR types were detected and the DI for those isolates was 0.743 (Table 3.3). Five isolates from the Czech Republic and Slovakia showed distinctive MLSSR types, whereas three Australian isolates had the same MLSSR type (Table 3.3). There were no MAP strains that had similar MLSSR types among the three different countries assessed in this study.

Genetic relationship

The genetic relationship between the various strains was constructed by creating MST (Figure 3.2). The 11 SSR loci were used for MST construction among the 27 isolates. In Figure 3.2, the size of each node indicates the population size of the sample, and the country of origin is labeled with their respective color. The predominant genotypes were types 1, 2 and 3, which all belong to the bison-type genotype. In addition, the strains corresponding to INMV 1 and INMV 5, which were isolated in the Czech Republic and Slovakia, were clustered in the same group with the bison-type strains. MLSSR types 4, 5, and 6, which belonged to INMV 2, were not clustered in the same group because the clonal distance between these strains

was calculated as 2.0.

Discussion

In the present study, three molecular typing methods, IS*1311* PCR-REA, MIRU-VNTR, and MLSSR were applied in an epidemiological analysis of MAP isolated in Korea. The IS*1311* PCR-REA results showed that cattle-type and bison-type genotypes co-existed in Korean cattle farms, and especially, the bison-type strains were distributed nationwide. Because bison-type strains are not common in cattle in other countries (Sohal et al., 2014it is thought that the wide distribution of the bison-type in Korea has an important meaning in an epidemiological aspect. Bison-type strains of MAP have been reported in several countries other than Korea (Leao et al., 2016; Singh et al., 2010; Sohal et al., 2014; Sonawane et al., 2016). On the American continent, Sohal et al. (2014) reported bison-type strains isolated from domestic species in Quebec, Canada. In India, the bison-type strain was the predominant strain identified in most domestic ruminant species, and that type was also found in some wild animals (Singh et al., 2010; Sonawane et al., 2016). In Korea, bison-type strains were reported in wild boars and domestic species such as cattle, goat, and elk (Kim et al., 2013). Because the bison-type genotype was discovered in both wildlife and domestic animals in Korea, it could be suggested that wildlife species have the

potential to spread MAP between wildlife and domestic cattle. However, detailed epidemiological studies are needed to clarify the relationship between two host species.

Through the MIRU-VNTR analysis, four INMV types were observed in domestic and foreign isolates. The characteristic feature of the MIRU-VNTR genotyping was that all 17 bison-type isolates appeared as the INMV 68 type. The reason for the low DI of the MIRU-VNTR value may be because many isolates belonged to one group. The INMV 68 type has been reported previously in other bison-type strains in Canada (Ahlstrom et al., 2015; Sohal et al., 2014). Therefore, the results may suggest that MIRU-VNTR loci are highly conserved in bison-type MAP strains. Two cattle-type domestic isolates were identified as INMV 2, while other foreign isolates were identified as INMV 1 or INMV 2, except for 1 isolate identified as INMV 5 from Slovakia. The INMV 1 and INMV 2 are the most prevalent types observed worldwide (Ahlstrom et al., 2015; de Kruijf et al., 2017; Gioffre et al., 2015; Mobius et al., 2008; Sonawane et al., 2016), and the INMV 5 type has been reported in Argentina and Germany (Fernandez-Silva et al., 2012; Imperiale et al., 2017). In this study, no unique INMV types were observed in the MIRU-VNTR genotyping results. Although two common INMV types were observed in Korea, that result does not mean that there were only two introductory events in Korea because MIRU-VNTR may overestimate relatedness (Ahlstrom et al., 2015). Ahlstrom et al. (2015) pointed out the limitations of the MIRU-VNTR method by identifying that several isolates belonging to three common INMV types were highly unrelated to each other through

a single nucleotide polymorphism analysis. Therefore, an additional typing method with a high discriminatory power was needed to obtain more detailed information about the epidemiological status of MAP in Korea.

The MLSSR molecular typing discriminated 27 isolates into 11 subtypes, especially as bison-type isolates, that were not discriminated by MIRU-VNTR; those subtypes were divided into three different MLSSR types. The SSR loci 3, 4, 5, 10, and 11 were not variable for MAP isolates as has been reported in other studies (Amonsin et al., 2004; Douarre et al., 2011; Thibault et al., 2008). The most variable SSR marker for the isolates was locus 2, which revealed four different alleles. Several previous studies have reported that the combined results of MIRU-VNTR and MLSSR typing produce the highest DI values (Douarre et al., 2011; Fernandez-Silva et al., 2012; Sohal et al., 2014). In this study, however, there were no MLSSR types that were divided into different INMV types, suggesting that the MLSSR alone has sufficient discriminatory power in cattle-derived MAP strains. However, there is a potential disadvantage of MLSSR because a high number of G repeats at loci 1 and 2 may not be accurately read due to a sequencing technology limitation (Douarre et al., 2011). Therefore, sequencing of loci 1 and 2 was performed repeatedly in both directions to increase the reliability of the sequencing results. In addition, all alleles >11 were classified into the same type in this study, as also described in previous studies, thereby limiting the interpretation (Douarre et al., 2011; Thibault et al., 2008). Another potential disadvantage of the MLSSR typing is that the SSR locus 2 has questionable stability. There is a claim that the SSR locus 2 is unstable for

epidemiological study because strand slippage events could occur during chromosomal duplication in the course of bacterial spreading (Kasnitz et al., 2013). In that study, different G repeat numbers at SSR locus 2 were observed in multiple isolates from individual animals or from a single cattle herd. This instability argument could be supported indirectly by the present study because the bison-type strains were divided into three subtypes only by the SSR locus 2, and all of the subtypes were detected in one herd. However, the study reported by Kasnitz et al. (2013) showed that the SSR locus 2 was stable after 12-fold subculture *in vitro*. Therefore, the three different bison-type strains identified in this study need to be further validated through detailed genotyping methods such as whole genome sequencing (WGS)-based analysis (Bryant et al., 2016; Leao et al., 2016).

The clonal distance of each strain obtained by using the MLSSR typing results are shown as an MST. The characteristic features of the MST were that the cattle-type and the bison-type did not appear as two distinct clusters. Instead, the INMV 1 and 5 types were clustered to the same group as the bison-type strains. It has been reported that INMV 1 strains are closely related to the bison-type based on an MST analysis using the combination of MIRU-VNTR and MLSSR markers (Sohal et al., 2014); however, the relationship between INMV 5 and the bison-type genotype has not yet been clarified. Based on these results, it can be inferred that the bison-type is not phylogenetically distinguished from the cattle-type. Recently, type B (bison-type) strains were classified as a subgroup of type C (cattle-type) through WGS-based genotyping (Bryant et al., 2016).

A limitation of this study is that the results of the analysis cannot represent the whole Korean epidemiological situation because the number of MAP isolates/DNA samples was low. However, the samples used in this study, especially the MAP-positive fecal DNA samples that were obtained from several herds nationwide and used in the prevalence test, could reflect the present situation in Korea to a certain extent. To the best of our knowledge, this is the first epidemiological survey report about MAP strains isolated from cattle in Korea that used both MIRU-VNTR and MLSSR typing methods.

In conclusion, MLSSR typing is a highly discriminatory genotyping method that can be used as an alternative to MIRU-VNTR genotyping. Moreover, the genotyping results in this study indicate that the prevalent MAP strains in cattle in Korea may be bison-type strains, and this finding has important epidemiological meaning for the control and prevention of PTB in Korea.

Table 3.1. Geographic distribution of IS*1311* polymerase chain reaction-restriction endonuclease analysis genotypes identified in MAP fecal DNA and MAP isolates obtained from cattle in Korea, Czech Republic, Slovakia, and Australia.

Country	**Province**	**Herd location**	**Herd ID**	**Isolates/DNA**	**No. of isolates/DNA**	**Genotype (IS*1311* PCR-REA)**
Korea	Gyeong-gi	Region 1	A	DNA	1	Cattle
		Region 2	B	DNA	1	
	Chung-nam	Region 3	C	Isolates	2	Cattle
				Isolates	11	Bison
	Jeon-buk	Region 4	D	Isolates	3	Bison
	Gang-won	Region 5	E	DNA	1	Cattle
				DNA	1	Bison
			F	DNA	2	Cattle
		Region 6	G	DNA	1	Cattle
				DNA	4	Bison
	Jeju	Region 7	H	Isolate	1	
			I	Isolate	1	Bison
				DNA	1	
			J	Isolate	1	
Australia	Unknown	Region 8	Unknown	Isolate	2	
Czech Republic	Unknown	Region 9	Unknown	Isolate	1	
		Region 10	Unknown	Isolate	1	
			Unknown	Isolate	1	Cattle
			Unknown	Isolate	1	
			Unknown	Isolate	1	
Slovakia	Unknown	Region 11	Unknown	Isolate	1	

Table 3.2. variable-number tandem repeats of mycobacterial interspersed repetitive units profiles of 27 bovine MAP isolates.

INMV group	No. of isolates (%)	No. of copies of MIRU-VNTR								Numerical code
		292	X3	25	47	3	7	10	32	
INMV 1	4 (14.8)	4	2	3	3	2	2	2	8	42332228
INMV 2	5 (18.5)	3	2	3	3	2	2	2	8	32332228
INMV 5	1 (3.7)	4	2	3	3	2	2	1	8	42332218
INMV 68	17 (62.9)	2	2	5	3	2	2	2	8	22532228

Table 3.3. Geographic distribution of variable-number tandem repeats of mycobacterial interspersed repetitive units and multilocus short sequence repeat types identified for 27 MAP isolates.

Country	Herd location	Herd ID	MIRU-VNTR	MLSSR	No. of isolates
Korea	Region 3	C	INMV 68	Type 1	5
			INMV 68	Type 2	3
			INMV 68	Type 3	3
			INMV 2	Type 4	1
			INMV 2	Type 5	1
	Region 4	D	INMV 68	Type 1	1
			INMV 68	Type 2	1
			INMV 68	Type 3	1
	Region 7	H	INMV 68	Type 1	1
		I	INMV 68	Type 1	1
		J	INMV 68	Type 2	1
Australia	Region 8	Unknown	INMV 2	Type 6	2
	Region 9	Unknown	INMV 2	Type 6	1
Czech Republic	Region 10	Unknown	INMV 1	Type 7	1
		Unknown	INMV 1	Type 8	1
		Unknown	INMV 1	Type 9	1
		Unknown	INMV 1	Type 10	1
Slovakia	Region 11	Unknown	INMV 5	Type 11	1

Table 3.4. Multilocus short sequence repeat profiles of 27 bovine MAP isolates.

MLSSR types	No. of isolates (%)	MLSSR locus										
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	8 (29.6)	7	>11	5	5	5	4	4	4	4	5	5
2	5 (18.5)	7	11	5	5	5	4	4	4	4	5	5
3	4 (14.8)	7	10	5	5	5	4	4	4	4	5	5
4	1 (3.7)	7	11	5	5	5	5	5	5	5	5	5
5	1 (3.7)	>11	9	5	5	5	5	5	5	5	5	5
6	3 (11.1)	7	>11	5	5	5	5	5	5	4	5	5
7	1 (3.7)	7	9	5	5	5	4	5	4	4	5	5
8	1 (3.7)	7	10	5	5	5	4	5	4	4	5	5
9	1 (3.7)	7	>11	5	5	5	4	6	4	5	5	5
10	1 (3.7)	7	11	5	5	5	4	6	4	5	5	5
11	1 (3.7)	7	10	5	5	5	4	6	4	4	5	5

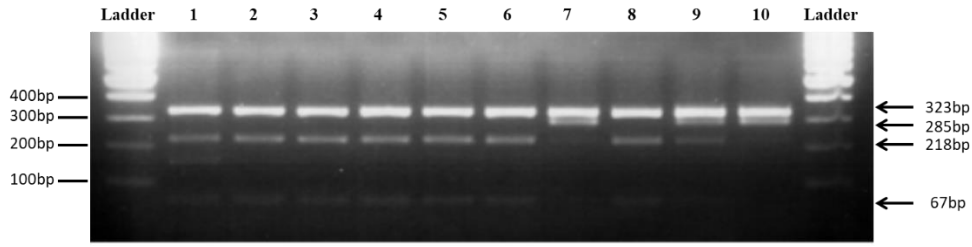

Figure 3.1. Representative results of IS*1311* Polymerase chain reaction and restriction endonuclease analysis strain typing. Ladder: 100 bp size marker; 1-6, 8: Bison type; 7, 9: Cattle type; 10: Cattle type (MAP ATCC19698)

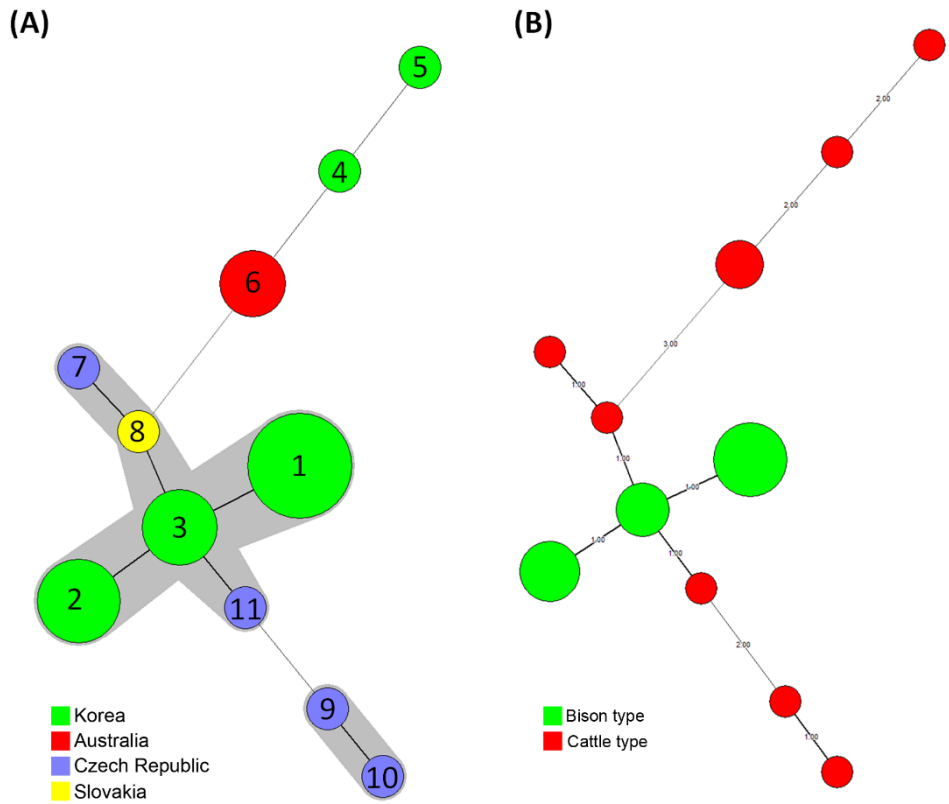

Figure 3.2. Minimum spanning tree based on multilocus short sequence repeat genotypes among 27 MAP isolates. Each genotype is displayed as a pie chart, the size of which is proportional to the number of strains; color-coding shows strain distribution by country (A) or by IS*1311* PCR-REA type (B). The numbers on the pie chart indicate the MLSSR type, and genotypes within a clonal distance of 1.0 were clustered by using a gray background.

Chapter IV

An ISMap*02*-like insertion sequence in *Mycobacterium* spp. interferes with specific detection of *Mycobacterium avium* subsp. *paratuberculosis*

Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a causative agent of Johne's disease or paratuberculosis (PTB), which is a chronic debilitating disease in ruminants, that is characterized by incurable enteritis and persistent diarrhea. ISMap*02* is one of the major targets of PCR because it is present in multicopies (six copies) and known to be specific to MAP. However, in the present study, non-MAP mycobacteria were shown to be positive by ISMap*02* targeting PCR. Two bacterial isolates (Sample ID: BO-038 and BO-042) were cultured from bovine fecal samples that produced positive results in three of two ISMap*02* targeting PCR analyses with negative results in IS*900* real-time PCR. Species identification using 16S rRNA gene sequencing and *hsp65* gene partial sequencing revealed that strains BO-038 and BO-042 were *M. virginiense* and *M. nonchromogenicum*, respectively, which both belong

to the *M. terrae* complex (MTC). Moreover, the two isolates shared a novel insertion sequence (IS) with high similarity to some parts of nucleotide sequences of ISMap*02*, and IS was presumed to be identical to that present in *M. heraklionense*. Both the novel IS and ISMap*02* were characterized as IS*1182* family members, and several sequences similar to ISMap*02* were identified by BLAST analysis. In addition, the DDE transposase of the novel IS showed great similarity in the N-terminal portion with the IS*5*/*1182* DDE transposase of other mycobacteria. These results suggest that ISMap*02* has a conserved region with similarity to other ISs, and that the diagnostic value of the primer sets targeting that region are questionable.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*, ISMap*02*, Diagnosis, Polymerase chain reaction, *Mycobacterium terrae* complex

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a causative agent of Johne's disease or paratuberculosis (PTB), which is a chronic debilitating disease in ruminants, that is characterized by incurable enteritis and persistent diarrhea (Harris and Barletta, 2001). The gold standard for MAP diagnosis is isolation and identification of the bacteria directly from feces or tissues (Whitlock et al., 2000). However, visible colonies cannot be seen for 6 to 16 weeks using this method, and its sensitivity is relatively low (Collins, 1996). Serological tests such as ELISA are also commonly used, but these also have low sensitivity (Collins et al., 2005). Because humoral immune response increases relatively late in disease progression, ELISA does not effectively detect individuals in the subclinical stage of infection, which is important for PTB eradication (Kennedy and Benedictus, 2001). Fecal PCR is a diagnostic method that can complement the shortcomings of the culture method. Specifically, PCR can detect the DNA of pathogens present in very small numbers with greater sensitivity and speed than culture methods (Alinovi et al., 2009; Bogli-Stuber et al., 2005). Several targets have been found to increase the specificity and sensitivity of PCR. IS*900* is generally used as a representative target in PCR diagnosis of MAP because it is a MAP specific marker with high sensitivity because there are 12–18 copies of it in the MAP genome (Englund et al., 1999; Millar et al., 1995; Moss et al., 1991; Vary et al., 1990). However, the IS*900*-like sequence has

been found in non-MAP mycobacteria, posing a problem of specificity (Cousins et al., 1999; Englund et al., 2002; Godfroid et al., 2005; Motiwala et al., 2004; Rajeev et al., 2005). Several other targets have also been discovered, among which ISMap*02* is a putative insertion sequence (IS), known to be present in multi-copies (six copies) and specific to MAP, similar to IS*900* (Paustian et al., 2004).

Several PCR primer sets targeting ISMap*02* have been identified and used for diagnosis (Irenge et al., 2009; Sevilla et al., 2014; Stabel and Bannantine, 2005). These primer sets have been evaluated using nested PCR or real-time PCR techniques (Douarre et al., 2010; Irenge et al., 2009; Park et al., 2016; Sevilla et al., 2014; Stabel and Bannantine, 2005). Evaluation by nested PCR showed comparable sensitivity when compared with IS*900* real-time PCR, and several tests of feces that were negative based on IS*900* were found to be positive by nested PCR (Douarre et al., 2010; Park et al., 2016; Stabel and Bannantine, 2005). In addition, triplex real-time PCR using ISMap*02*, IS*900* and internal amplification control (IAC) was more sensitive than other real-time PCR analyses targeting F57 and ISMav*2* elements, which were present as a single copy and three copies, respectively (Schonenbrucher et al., 2008; Sevilla et al., 2014). Similarly, the ISMap*02* target has been used together with IS*900* to increase the sensitivity and specificity of PCR diagnosis.

However, two isolates that were positive in two of the three commonly used ISMap*02* primer sets were identified in this study. Therefore, morphology, growth rate and IS*900* real-time PCR were used to determine if these two isolates belonged to MAP. The isolates were then confirmed as *M. virginiense* and *M.*

nonchromogenicum belonging to the *M. terrae* complex (MTC) through 16S rRNA gene sequencing and *hsp65* gene sequencing. Finally, a unique IS similar to ISMap*02* was discovered from these two isolates by PCR using a primer set that targeted the entire sequence of the ISMap*02*.

Materials and Methods

Collection of bacterial isolates

During September 2013 to August 2014, PCR screening for paratuberculosis was conducted at Korean cattle farms (Table 2.1). Specifically, IS*900* real-time PCR and ISMap*02* nested PCR were used to detect MAP from DNA extracted directly from individual fecal samples. Of the 1,562 samples tested, 35 were positive (Park et al., 2016) and were subsequently cultured to confirm the infection. Pre-treatment of fecal samples was performed using the Cornell method with some modifications (Stabel, 1997). Briefly, 2 g of feces were suspended in 35 ml of distilled water, shaken for 30 minutes and allowed to settle for 30 minutes. Next, 5 mL of supernatant were transferred to 25 mL of 0.9% hexadecylpyridinium chloride (HPC) in half strength brain heart infusion (BHI) broth and incubated at 37 °C for 24 hours. Following centrifugation at 1,700×g for 20 min, the supernatant was discarded and the

remaining pellet was suspended in 1 mL of antibiotic brew (Vancomycin and Nalidixic acid: 100 μg/mL, Amphotericin B: 50 μg/mL in 1/2 strength BHI), then incubated at 37 °C for 24 hours. Next, 200 μL of the suspension was inoculated into a modified Herrold's egg yolk medium (HEYM) slants with or without 2 mg of Mycobactin J (MJ) and observed for 8–16 weeks.

Identification of bacteria

The isolated bacteria were identified to determine whether they were *Mycobacterium* spp. and if so, which species they were. Species identification was conducted using 16S rRNA gene sequencing and *hsp65* partial gene sequencing using cultured bacterial DNA. To extract the genomic DNA from cultured bacteria, cells were first harvested and lysed in L6 lysis buffer (5.25 M GuSCN, 50 mM Tris-HCl (pH 6.4), 20 mM EDTA, 1.3% Triton X-100, distilled water), then processed as previously described (Park et al., 2014a). PCR for 16S rRNA gene sequencing was subsequently conducted using the universal primers, 27F and 1492R, which were described in a previous study (Jiang et al., 2006). Briefly, a 50 μL PCR mixture composed of 2 μL of template DNA, 34 μL of nuclease-free water, 5 μL of i-Taq™ 10× PCR buffer (Intron Biotechnology, Korea), 0.2 mM of deoxyribonucleotide triphosphates, 400 nM of each primer, and 2 U of i-Taq™ DNA polymerase was prepared. The sample was then subjected to the following PCR conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for

30 sec, and 72°C for 2 min, and then final extension at 72°C for 7 min. PCR analysis for the *hsp65* partial gene was conducted using the primers developed by Telenti et al. (1993). PCR amplicons were purified by gel-extraction using a MEGAquick-spin™ Total Fragment DNA Purification Kit (Intron Biotechnology, Korea) according to the manufacturer's protocols, after which they were sequenced. The obtained sequences of the 16S rRNA gene and the *hsp65* gene sequences were then compared with those of known sequences available in the GenBank database. Sequencing alignments of the two isolated strains and several sequences from GenBank selected by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were generated and phylogenetic analysis was conducted using MEGA version 7 (Kumar et al., 2016).

ISMap*02* targeting PCR

A total of three previously published primer sets targeting ISMap*02* elements were tested against the isolated bacteria to determine if they produced positive results or not (Irenge et al., 2009; Sevilla et al., 2014; Stabel and Bannantine, 2005). Briefly, DNA samples of the isolated bacteria were analyzed by nested PCR using the ISMap*02*-1 primer set and by real-time PCR using the ISMap*02*-2 and ISMap*02*-3 primer sets (Table 4.1). The reaction mixture of nested PCR consisted of 1 µL template DNA, 12.5 µL nuclease-free water, 2 µL i-Taq™ 10× PCR buffer (Intron Biotechnology, Korea), 0.25 mM deoxyribonucleotide triphosphates, 500 nM each

primer, and 2.5 U of i-Taq^{TM} DNA polymerase to give a total volume of 20 µL. The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 15 sec, 58°C for 15 sec, and 72°C for 20 sec, and then final extension at 72°C for 7 min. The amplicons (1 µL) from the first round of PCR were used as the template in nested PCR, which was conducted using similar reaction conditions (30 cycles). Real-time PCR was performed using a Rotor-Gene Q real-time PCR cycler (Qiagen, Hilden, Germany) and a reaction mixture consisting of 1× Rotor-Gene Probe PCR master mix (Qiagen, Germany), 400 nM primers, 200 nM probes, 4 µL template DNA, and nuclease-free water to give a total volume of 20 µL. Samples were amplified under the following conditions: 1 cycle at 95°C for 5 min, followed by 45 cycles at 95°C for 15 sec, and 60°C for 1 min.

Analysis of ISMap*02* and the novel ISs

Sequence alignment was performed to confirm the presence of other bacteria that had the same sequence as ISMap*02*. The IS of ISMap*02* corresponding to a total of 1,782 bp was used for analysis. In addition, PCR primers were constructed using the entire sequence of the ISMap*02* element as a target to confirm whether ISMap*02*-like ISs exist in the two isolates. Because the 5' and 3' end of this element was reverse complementary identical, one primer covering this sequence was used to amplify the entire element (Table 4.1). The PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55°C for

30 sec, and 72°C for 2 min, and then final extension at 72°C for 7 min. PCR amplicons of about 1.8 kb were sequenced by the primer walking technique. Comparison of ISMap*02* positions that were retrieved by Paustian et al. (2004) and the ISs newly identified from the isolates was performed using the BLAST tool provided by NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition, the basic characteristics of the ISMap*02* and the novel IS were analyzed by several web-based tools, including ISFinder (https://www-is.biotoul.fr/blast.php) and ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/).

Results

Isolation of bacteria

Among 35 cultured samples, MAP was cultured from six samples. During cultivation, colonies were found in HEYM without Mycobactin J in two samples (Sample ID: BO-038 and BO-042). Because there was a difference in the growth rates of a typical MAP, these two isolated colonies were inoculated into HEYM media with and without MJ to observe the growth patterns. Two weeks after inoculation, visible colonies were observed in both samples in both types of media. Both strains formed colonies that were small and round, which was different from

the typical colony shape of MAP. Ziehl-Neelsen staining of the colony revealed that the bacteria were red-stained acid-fast rods. Taken together, these results indicated that the isolates were not MAP. Subsequently, PCR targeting IS*900* and ISMap*02* was performed using colony PCR and both strains were negative upon IS*900* and positive upon ISMap*02* nested PCR.

Species identification

Species identification using 16S rRNA gene sequencing and *hsp65* partial gene sequencing revealed that strain BO-038 and BO-042 belong to the *Mycobacterium terrae* complex (MTC). The partial sequence (1,457 bp) of the 16S rRNA gene of strain BO-038 was compared with that of other bacteria in the GenBank database found to be 99.9% identical (1 bp mismatch) to that of *M. virginiense* strain MO-233, which was proposed by Vasireddy et al. (2016) as a new strain belonging to the MTC. Among type strains, *M. arupense* (4 bp mismatch) was the most similar to strain BO-038, followed by *M. engbaekii* (12 bp mismatch). The partial sequence (1,457 bp) of the 16S rRNA gene of strain BO-042 did not match any sequences in the GenBank database with 100% homology, but the most similar type strain was *M. nonchromogenicum* (3 bp mismatch), followed by *M. arupense* (6 bp mismatch). The phylogenetic relationship based on the 16S rRNA gene sequence between strains isolated in this study and several mycobacterial strains is described in Figure 4.1.

Through *hsp65* gene sequencing, a 401 bp sequence (excluding the primer region)

was obtained from isolates BO-038 and BO-042. The closest match for strain BO-038 was the *M. terrae* strain variant MS267 (accession No. AY550213), which showed 99% (1 bp mismatch) similarity. Among the type strains, *M. engbaekii* was most similar to strain BO-038 (98% similarity, 9 bp mismatch). Direct comparison of BO-038 with that of *M. virginiense* MO-233 (Kindly provided by Dr. Ravikiran Vasireddy, University of Texas Health Science Center) revealed a 3 bp mismatch. Additionally, strain BO-042 showed 99% similarity (1 bp mismatch) to *M. nonchromogenicum* type strain. The phylogenetic relationship based on the *hsp65* partial gene sequence between strains isolated in this study and several mycobacterial strains is shown in Figure 4.2. Taken together, the results revealed that both isolates belonged to MTC but were different from each other, with BO-038 being most similar to *M. virginiense* and strain BO-042 most similar to *M. nonchromogenicum.*

ISMap*02* targeting PCR of the isolates

Three primer sets targeting ISMap*02* elements were tested and positive results were obtained using two of the sets (ISMap*02*-1 and ISMap*02*-2). The PCR amplicons for the ISMap*02*-1 and ISMap*02*-2 primers of strain BO-038 and BO-042 were sequenced and compared with the original ISMap*02* sequences of MAP. Sequences of strains BO-038 and BO-042 of ISMap*02*-1 nested PCR amplicons revealed a 16 nucleotide difference and 79% sequence identity in both strains when

compared to the corresponding original sequences. Additionally, sequence comparison of the two strains revealed that they were completely consistent. Moreover, comparison between the original sequence and PCR amplicon of ISMap*02*-2 revealed five nucleotide differences (sequence identity: 92%), while the sequence identity between the two strains was 100%.

Analysis of the ISMap*02* and the novel IS

The known size of ISMap*02* was 1,674 bp, but this was not the complete sequence of this IS. Specifically, there was a direct repeats (DR) site starting with CTAG in the left and right extended region of 1,674 bp, and inverted repeats (IR) were identified next to DR. Therefore, the size of the entire ISMap*02*, including DR, was 1,782 bp, and it had the same sequence in all six copies of ISMap*02*. BLAST analysis using this 1,782 bp sequence was conducted to identify similar sequences. The most similar sequence was found in *M. hominissuis* TH135, with 99% homology. The only difference between ISMap*02* and the sequence of *M. hominissuis* TH135 was that C repeats in 1073–1077 bp of ISMap*02* contained one additional cytosine. In addition to *M. hominissuis*, several sequences similar to ISMap*02* were found in other mycobacterial strains (Table 4.2). Because strains BO-038 and BO-042 were considered to belong to the MTC, additional BLAST analysis was conducted using a whole genome shotgun contig (wgs) database of MTC. The analysis revealed that *M. kumamotonense* and *M. heraklionense* had similar sequences to ISMap*02* (Table

4.2). In the case of *M. heraklionense*, the query cover was low (31%), but the aligned part was the end of the shotgun sequence, making complete sequence comparison impossible.

Using PCR targeting the entire ISMap*02*, approximately 1.8 kb bands similar to ISMap*02* were obtained from the two isolates. Subsequent purification and sequencing using the primer walking technique revealed identical 1,780 bp sequences between both isolates. BLAST analysis of the 1,742 bp sequence (excluding the primer region) showed 100% homology with *M. heraklionense* strain 1211594.5 in contig 30 region from 551 bp to 1,742 bp and contig 6, 29, 32, 74, 88 and 120 from 1 bp to 542 bp (Figure 4.3). Although these findings suggested that the IS of the isolates may be identical to that of the *M. heraklionense* strain 1211594.5, this could not be confirmed because the entire sequences of the isolates were not covered by wgs of *M. heraklionense*. Comparison between ISMap*02* and the IS from isolates showed that the query cover was 99% and the similarity was 75% (Figure 4.4).

BLAST analysis of the Novel IS and the ISMap*02* using ISFinder showed that the most similar ISs were ISMgi*4* and ISMgi*2*, respectively, and these to the IS*1182* family. The ORF site encodes the DDE transposase of the novel IS was confirmed to be a nucleotide sequences from 70 bp to 1,617 bp with reference to the ORF site of ISMgi*2* and the prediction result with ORF finder. This ORF site encoded a 515 amino acids (a.a.) protein, and BLAST analysis of this protein showed high similarity to the N-terminal portion of IS*5*/*1182* DDE transposase of other

mycobacteria, although the 288–496 a.a. portion corresponding to DDE_Tnp_1 domain was variable. The IS of the MAH, which was found to have the highest similarity with ISMap*02*, was not registered in the ISFinder server and was not directly comparable.

Discussion

Polymerase Chain Reaction is used as a diagnostic method for many pathogens because it requires less time and has higher sensitivity than other diagnostic methods. However, it is important to identify specific PCR targets that do not exhibit non-specific responses that can result from the high sensitivity of the PCR. In this regard, several MAP-specific targets for the diagnosis of MAP have been found. Among them, IS*900* and ISMap*02* are widely used in this diagnosis because they are the insertion sequences that exist in several copies in the genome of MAP that are also regarded as MAP-specific.

In the present study, we isolated two non-MAP mycobacteria that were positive for ISMap*02* nested PCR. Sequencing of the 16S rRNA and *hsp65* genes revealed that the two isolates were likely to be *M. virginiense* and *M. nonchromogenicum* belonging to MTC. These findings suggest that there was a relationship between isolation of the ISMap*02*-positive strains other than MAP and the phenomenon of only one positive ISMap*02* observed upon fecal PCR diagnosis in previous studies

(Douarre et al., 2010; Park et al., 2016). Previous studies have suggested two possibilities for the cause of ISMap*02* single positivity. One was the that the samples that were positive for ISMap*02* only were true positives because that PCR target was known to be MAP specific. The other was the technical problems associated with gel-based PCR such as similar sized bands. However, this study showed that ISMap*02* single positivity can also occur in *M. nonchromogenicum* or *M. virginiense*-infected samples. In fact, both strains were isolated from two of the four farms where ISMap*02* only positive samples were found (Park et al., 2016). Furthermore, several studies have reported that *M. nonchromogenicum* has been isolated from cows (Gcebe et al., 2013; McCorry et al., 2004; Rónai et al., 2016). However, the ISMap*02* single positives were not tested because the only samples that were positive for both were cultured.

The positive reaction during PCR analysis of the isolates using two of the three primer sets targeting ISMap*02* was due to the presence of a sequence similar to ISMap*02*, and this ISMap*02*-like IS was expected to exactly match that of *M. heraklionense* strain 1211594.5. In fact, the sequence of *M. heraklionense* corresponding to the primer region of ISMap*02*-1 (second round) revealed a 4 bp difference in the forward and reverse primers, respectively. In the case of ISMap*02*-2, a 2 bp mismatch was observed in the forward primer, no mismatch was observed in the reverse primer, and there was a 2 bp mismatch in the probe sequence. Furthermore, sequences similar to ISMap*02* were found in other mycobacteria such as *M. gilvum* upon BLAST analysis. Because PCR amplification was possible even

though the template did not have exactly the same sequence as the primers, these bacteria are considered to also have the potential to produce false-positives in the ISMap*02* targeting PCR.

As in the BLAST analysis using ISFinder, ISMap*02* was presumed to be an IS similar to the ISMgi*2* of *M. gilvum*. Because ISMgi*2* is an IS belonging to the IS*1182* family, ISMap*02* was also assumed to have the characteristics of the IS*1182* family. One of the characteristics of the IS*1182* family is that it has DDE transposase. The protein BLAST revealed that the DDE transposase of the novel IS had a conserved region in the N-terminal portion, whereas the DDE_Tnp_1 domain region was variable. Therefore, it can be hypothesized that the nucleotide sequence of ISMap*02* is also conserved in the N-terminal portion of DDE transposase, while it is variable in the DDE_Tnp_1 domain region. This hypothesis could be supported by the ISMap*02* PCR results of two isolates, which were positive based on the two primer sets targeting the N-terminal region, while one primer set targeting the portion of DDE_Tnp_1 domain produced a negative result. However, the same ORF that was found in the novel IS was not found in ISMap*02* because a frameshift occurred by addition of one cytosine residue at 1,073 bp.

Because the same IS was identified in different species, the possibility of IS present in the plasmid was considered. However, plasmids were not discovered in both isolates; therefore, it was assumed that the ISs exist in the chromosome, although this was not confirmed due to the absence of a complete genome sequence database for both *M. nonchromogenicum* and *M. virginiense*. The presence of the

novel IS is not considered to be a general characteristic of the MTC because the type strain of *M. terrae* (KCTC9614) did not produce a positive reaction upon PCR amplification of the ISMap*02* primer sets used in the study (data not shown). In mycobacteria, genetic rearrangement by IS insertion is known to be limited mainly to species or subspecies levels (Dale, 1995; Kunze et al., 1992; Wall et al., 1999; Whittington et al., 1998). Therefore, it is worthwhile to use the novel IS as a candidate that allows *M. nonchromogenicum*, *M. virginiense*, and *M. heraklionense* to be distinguished from other species belonging to MTC.

Overall, the results indicated that ISMap*02* has a conserved region that may be similar in other ISs. Therefore, neither the ISMap*02*-1 nor the ISMap*02*-2 primer sets targeting the conserved region of ISMap*02* are suitable for PCR diagnosis. However, the DDE_Tnp_1 domain region of ISMap*02* is thought to have value for MAP diagnosis because this region has a variable characteristic, even within the same IS*5*/*1182* family. In conclusion, when selecting the PCR primer for diagnosis using IS, it is necessary to first identify the characteristics of the IS, select a region known to be variable, then design a primer to reduce the possibility of potential positives.

Table 4.1. Primer sequences used in this study

Target	Purposes	Primer name	Primer sequences (5'-3')	Reference
16S rRNA gene	Species identification: sequencing	27F/1492R	F: AGAGTTTGATCMTGGCTCAG R: CGGTTACCTTGTTACGACTT	Jiang et al. (2006)
Hsp65 gene		Tb11/Tb12	F: ACCAACGATGGTGTGTCCAT R: CTTGTCGAACCGCATACCCT	Telenti et al. (1993)
IS*900* element	Real-time PCR	IS900	F: ATGACGGTTACGGAGGTGGTT R: TGCAGTAATGGTCGGCCTTAC Probe: FAM-CGACCACGCCCGCCCAGA-TAMRA	Zhang et al. (2011)
ISMap*02* element	Nested PCR	ISMap02-1 (first round)	F: GCACGGTTTTTCGGATAACGAG R: TCAACTGCGTCACGGTGTCCTG	Stabel and Bannantine (2005)
		ISMap02-1 (second round)	F: GGATAACGAGACCGTGGATGC R: AACCGACGCCGCCAATACG	
	Real-time PCR	ISMap02-2	F: CGCCAGGAACGCAAACAT R: GTGCAGGGTCGCTCTGATG Probe: DragonflyOrange-ACTCCGCATCCAACAACTCACGCTG-BHQ-2	Irenge et al. (2009)
		ISMap02-3	F: CGGCTGGACACGGAATG R: CATGAGCGACAGTATCTTTCGAA Probe: JOE-ATCCGTCCCAGTGGCGGAGTCAC-BHQ-1	Sevilla et al. (2014)
	Sequencing	ISMap02-1782	CTAGGAGCCTGCTGAATTA	This study

Table 4.2. Sequences producing significant alignments with ISMap*02* elements in BLAST analysis

Description	**Query cover**	**Identity**	**Accession**
Mycobacterium avium subsp. *hominissuis* TH135 chromosomal DNA, complete genome	100%	99%	AP012555.1
Mycobacterium vanbaalenii PYR-1, complete genome	100%	79%	CP000511.1
Mycobacterium gilvum PYR-GCK plasmid pMFLV01, complete sequence	99%	79%	CP000657.1
Mycobacterium gilvum Spyr1 plasmid pMSPYR101, complete sequence	58%	83%	CP002836.1
Mycobacterium gilvum Spyr1, complete genome	58%	83%	CP002835.1
Mycobacterium gilvum PYR-GCK, complete genome	62%	83%	CP000656.1
Mycobacterium kumamotonense strain DSM 45093	100%	83%	MVHU01000033.1
Mycobacterium heraklionense strain 1211594.5	31%	85%	LZME01000149.1 LZME01000134.1 LZME01000118.1 LZME01000088.1 LZME01000084.1 LZME01000025.1

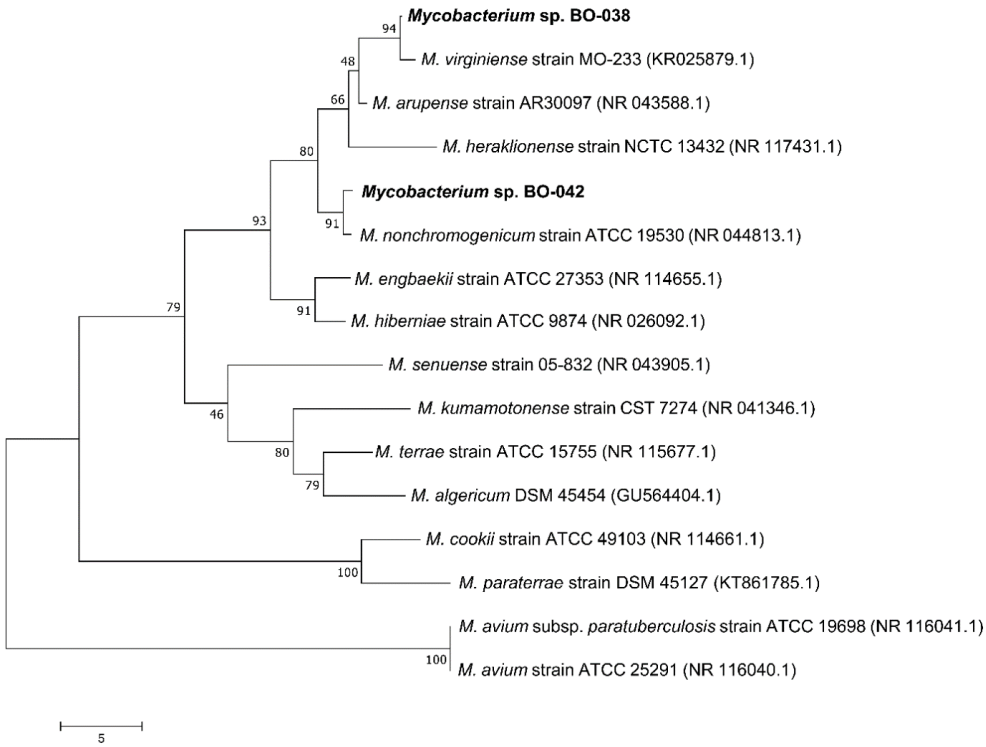

Figure 4.1. Phylogenetic relationships of strain BO-038 and BO-042 with other *Mycobacterium* species based on 16S rRNA gene partial sequences (1,457 bp). Neighbor-joining method was used to build this tree and the bootstrap percentage values from 1,000 re-samplings of the datasets were shown at each node. Length of the bar indicated the number of nucleotide differences.

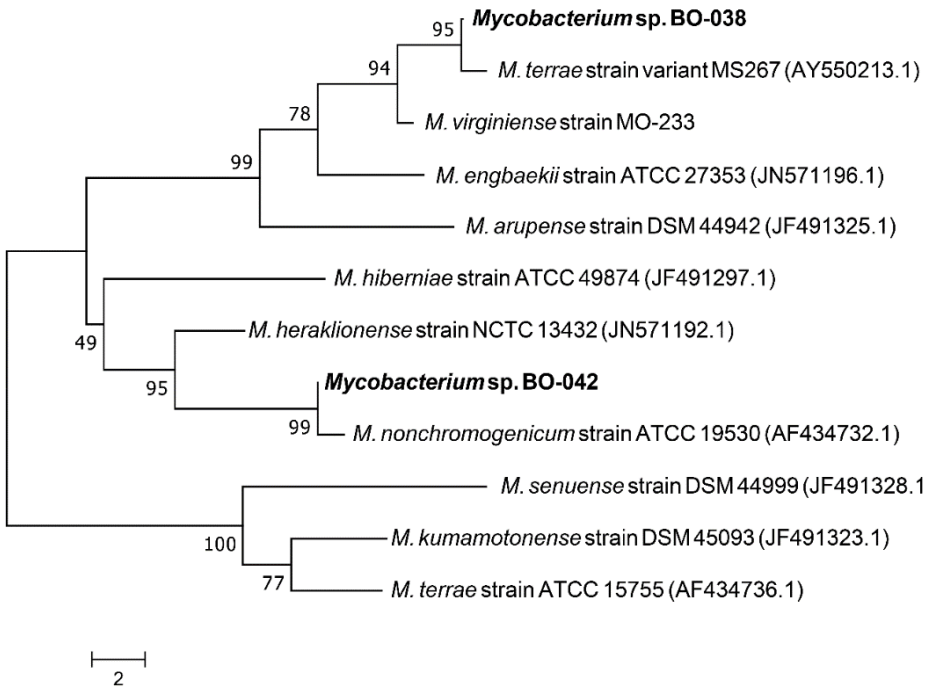

Figure 4.2. Phylogenetic relationships of strain BO-038 and BO-042 with other *Mycobacterium* species based on *hsp65* gene partial sequences (401 bp). Neighbor-joining method was used to build this tree and the bootstrap percentage values from 1,000 re-samplings of the datasets were shown at each node. Length of the bar indicated the number of nucleotide differences.

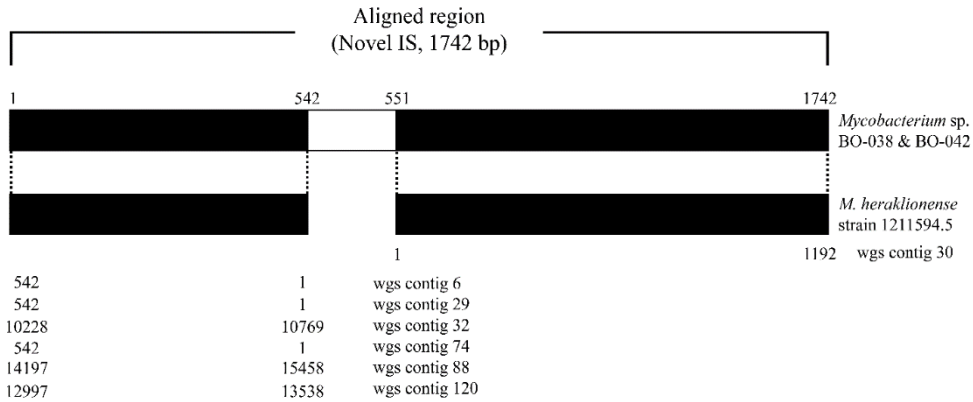

Figure 4.3. Sequence alignment between the novel IS and the whole genome shotgun sequences (wgs) of *M. heraklionense* strain 1211594.5. The regions of nucleotide sequence spanning from 1 bp to 542 bp and 551 bp to 1,742 bp were covered in wgs of *M. heraklionense* with 100% identity. Alignments of the relevant contigs to novel IS are listed with respective ranges of nucleotide residues.

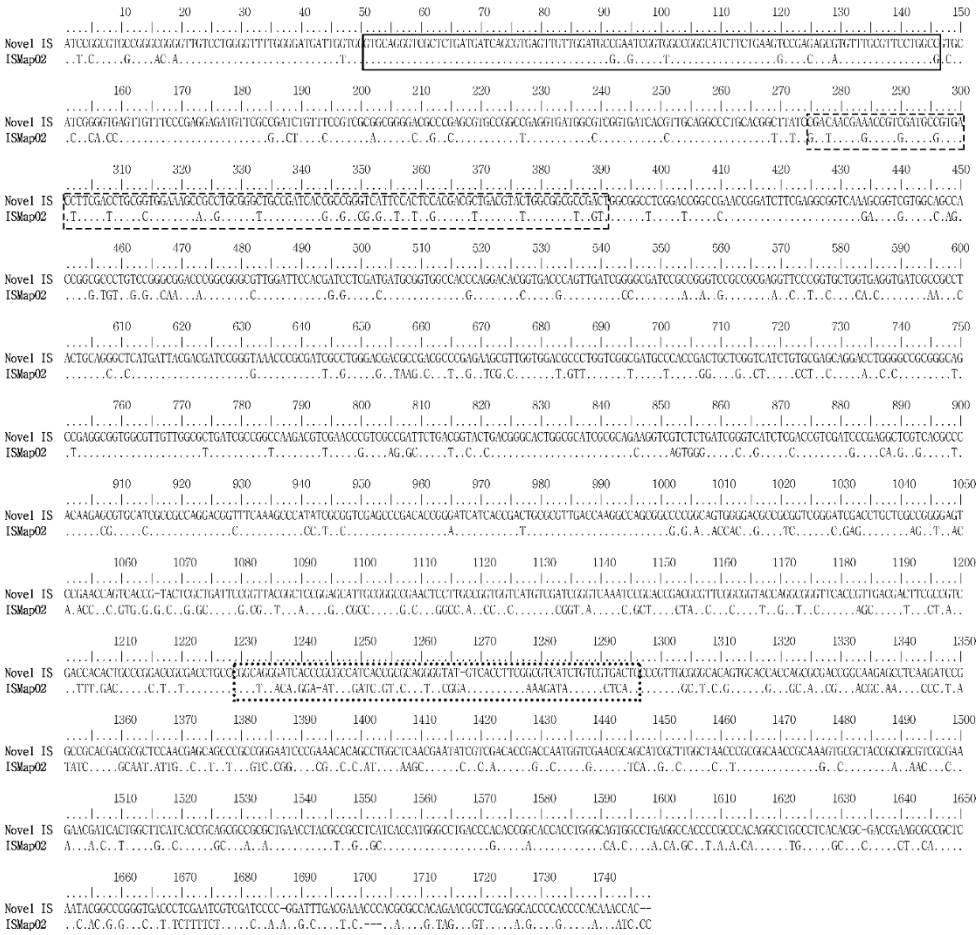

Figure 4.4. Sequence alignment between the novel IS and ISMap*02*. Dots represent same base pairs, while letters show different base pairs. Total of 1,742 bp of the novel IS nucleotide sequences excluding primer region were subjected to the comparison. The region targeted by ISMap*02*-2 PCR was shown with the solid-lined box, the ISMap*02*-1 was shown with the dashed-lined box, and the ISMap*02*-3 was shown with the dotted-lined box.

General conclusions

Because of the difficulty in isolating the causative agent and the convenience of diagnosis, the prevalence of PTB in Korea has been mainly reported only by antibody test using commercial ELISA kit. In this study, a PCR diagnosis using fecal DNA was performed to detect fecal shedder, which is important for eradication of PTB, and a molecular epidemiological analysis was carried out using isolates obtained from positive cattle. In addition, non-MAP mycobacteria that have similar sequences with ISMap*02*, a widely used target in PCR diagnosis, have been isolated and identified, and the ISMap02 has been analyzed to characterize IS as a diagnostic target. The characteristics of the ISMap*02* were also analyzed to provide a cautionary point when ISs are used as a diagnostic target.

1. The minimum detectable limit of the MAP by newly designed fecal DNA extraction method, mGITC/SC was approximately 50 MAP (1.5 cfu) in 250mg of feces (6 cfu per g). When compared with other DNA extraction methods, the mGITC/SC showed sufficient detection sensitivity and had advantages in terms of cost and time.

2. Of the 1,562 fecal samples obtained from 37 herds, regardless of diarrhea, 35 samples were positive in both IS*900* and ISMap*02*. At the herd level, 12 of the 37

herds were found to be positive for MAP. Through the fecal PCR, fecal shedders which does not show any clinical signs were detected. The detection rate was similar to the MAP prevalence reported previously using the ELISA method. These results suggested that PCR diagnosis might be a useful tool for investigation of the prevalence of MAP infection, especially a herd level.

3. The most prevalent strains in Korea were the “bison-type” genotype (23 of the 31 samples) and distributed nationwide. Rest of 8 samples were cattle-type. The bison-type strains which were discriminated only as INMV 68 in MIRU-VNTR were divided into 3 different subtypes by MLSSR typing. Cattle-type was divided into 3 subtypes by MIRU-VNTR and 8 subtypes by MLSSR. Among the tested typing methods, MLSSR was the most discriminatory. These results could provide basic data necessary to understand the situation of MAP infections in Korea.

4. The novel IS which showing false-positive in ISMap*02*-specific PCR was found in *M. virginiense* and *M. nonchromogenicum*, that were isolated from this study. Both the novel IS and ISMap*02* were characterized as IS*1182* family members, and several sequences similar to ISMap*02* were identified by BLAST analysis. The results indicated that ISMap*02* has a conserved region with similarity to other ISs, and that the diagnostic value of the primer sets targeting that region are questionable.

On the basis of these results, the fecal PCR diagnosis is an appropriate method to

detect fecal shedder, which is important for understanding the MAP occurrence situation, and suggested that appropriate DNA extraction methods and MAP specific targets should be used to improve diagnostic efficiency. Moreover, the results of the molecular epidemiology survey of Korean MAP isolates can provide basic information for understanding of genetic diversity, evolutionary relationships, and inter and intra-species transmissions which are essential for establishing control strategy.

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국문초록

Mycobacterium avium subspecies *paratuberculosis* 검출을 위한 PCR 진단법의 임상적 평가 및 소 유래 국내 분리주의 역학적 특성 분석

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요네병은 반추류의 만성 소모성 질병으로, *Mycobacterium avium* subsp. *paratuberculosis* (MAP)의 감염에 의해 발병한다. MAP는 전 세계적으로 퍼져 있으며 각국의 축산업에 막대한 경제적 손실을 초래한다. MAP는 느리게 자라는 세균으로서 긴 잠복기를 가지기 때문에 최초 감염 후 임상증상이 발현되기까지는 2년 이상이 걸린다. 요네병 진단의 gold standard는 원인체를 분리 및 동정하는 것이다. 그러나 인공배지에서 배양이 되기까지에는 6주에서 16주까지의 긴 시간이 걸리고 진단 민감도도 상대적으로 낮다. ELISA와 같은 혈청학적 검사법 또한 일반적으

로 사용되는 진단법이나, 마찬가지로 민감도가 낮은 단점이 존재한다. 분변 PCR은 배양법의 단점을 보완할 수 있는 진단법이다. PCR은 매우 적은 양의 병원체를 검출 가능하여 배양법에 비해 높은 민감도를 가지고 빠르게 진단 가능하다.

본 연구의 목적은 분변 진단을 기반으로하여 MAP 감염, 특히 준임상형 감염에 대한 역학적 상황을 조사함으로써 이 질병의 청정화에 중요한 기초적인 정보를 제공하는 것이었다.

본 연구에서는 진단의 민감도를 높이고자 새로운 비용 효율적인 DNA 추출방법을 고안하였고(mGITC/SC) 효율성의 측면에서 고안된 DNA 추출법을 다른 DNA 추출법들과 비교하였다. IS*900*을 이용한 real-time PCR법의 진단 한계는 MAP가 심어져 있는 분변에서 약 50개의 MAP 균 수(그람 당 6 cfu) 정도였다. 한 개의 시료로부터 DNA를 추출하는 데 걸린 시간은 약 60분 정도였으며 필요한 비용은 시료 당 1달러 수준이었다.

본 연구에서는 MAP에 특이적인 요소인 IS*900*과 ISMap*02*의 두 가지를 타겟으로 하는 PCR 기법을 통해 국내 소 사육 농장에서부터 채취된 분변으로부터 MAP 검출이 실시되었다. 총 37개의 농장으로부터 얻은 1,562개의 분변 시료 중 35개의 시료에서 IS*900*과 ISMap*02* 모두에 양성반응을 나타내었다. 농장단위로는 37개의 농장 중 12개의 농장

이 요네병에 양성으로 나타났다. 35마리의 양성개체 모두 임상증상을 나타내지 않았기 때문에 이들 개체들은 모두 준임상형 단계에 있는 것으로 판단되었다. 또한 농장단위의 발생율은 이전 연구에서의 ELISA법을 이용한 발생율 보고 당시 농장단위 발생율과 유사했다.

본 연구에서는 5개 지방의 10개 농장으로부터 얻어진 12개의 MAP 양성 분변유래 DNA 시료와 19개의 MAP 분리주가 분자역학적으로 분류되었다. 국내에서 가장 만연한 유전형은 'bison-type'이었으며(31개 시료 중 23개) 국내 전역에 분포했다. 나머지 8개의 시료는 cattle type 이었으며, 모든 외래균주 또한 cattle type이었다. Bison type 균주는 MIRU-VNTR 에서 모두 INMV 68형으로 나타났고 MLSSR에서는 세 가지 다른 아형으로 분류되었다. Cattle type 균주들은 MIRU-VNTR에서 3가지 아형으로 구분되었고, MLSSR에 의해서는 8 가지 다른 아형으로 구분되었다. MIRU-VNTR과 MLSSR의 형질다양성은 각각 0.567과 0.866으로 나타났다. 본 연구는 국내에서 소 유래 분리주들에 대한 MIRU-VNTR과 MLSSR을 이용한 최초의 분자역학조사로 국내 분리주들의 다양성에 대한 기초 정보를 제공하였다.

본 연구에서는 MAP가 아닌 다른 마이코박테리아에서 ISMap*02* PCR에 대한 양성반응을 관찰하였다. ISMap*02* PCR에 양성반응을 나타내면서 IS*900* real-time PCR에서는 음성을 나타낸 두 가지 분리주(샘플 ID:

BO-038, BO-042)가 소 분변시료로부터 배양되었다. 16S rRNA 유전자 전체의 유전자염기서열분석과 *hsp65* 유전자의 부분 유전자염기서열분석을 통한 동정 결과 BO-038은 *M. virginiense*로 동정되었으며, BO-042는 *M. nonchromogenicum*으로 동정되었다. 두 가지 균주는 모두 *M. terrae* 복합체(MTC)에 속하는 균이었다. 두 분리주들은 모두 ISMap*02*와 일부 서열이 유사한 새로운 삽입서열(IS)을 가지고 있음이 확인되었고, 이 IS들은 *M. heraklionense* 균주가 가진 IS와 완전히 동일한 것으로 추측되었다. 새로운 IS와 ISMap*02* 모두 IS*1182* family로 특징지어졌고 ISMap*02*와 유사한 다른 몇 가지 IS들이 BLAST 분석을 통해 확인되었다. 본 연구를 통해 ISMap*02*에 대한 유사 서열의 존재가 최초로 실험적으로 밝혀졌으며, 기존에 사용되는 두 가지 ISMap*02* PCR 타겟이 MAP 검출용으로는 부적합하다는 사실을 밝혔다.

이 결과들로부터, PCR 진단이 MAP 발생상황을 이해하는 데 중요한 분변 배설 개체들을 검색하는데 적절한 방법이며, 진단의 효율성을 증대시키기 위해서는 적절한 DNA 추출방법과 MAP에 특이적인 타겟이 사용되어야 함을 보였다. 또한 한국의 MAP 분리 균주에 대한 분자 역학 조사의 결과는 이 질병에 대한 제어 전략의 수립에 필수적인 유전적 다양성, 진화적 관계, 종간 및 종내 전이에 대한 이해를 위한 기초 정보를 제공할 수 있다.

핵심어: 요네병균, 분변 PCR 진단, 분자 타이핑, ISMap*02*, 삽입서열, *Mycobacterium virginiense*, *Mycobacterium nonchromogenicum*