수의학박사학위논문

# Molecular biological characteristics and differential diagnostic investigation of *Brucella* strains

## 브루셀라균의 분자생물학적 특성 분석 및 감별진단법 개발

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김 지 연

# Molecular biological characteristics and differential diagnostic investigation of *Brucella* strains

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**Ji-Yeon Kim**

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# Molecular biological characteristics and differential diagnostic investigation of *Brucella* strains

Ji-Yeon Kim

**(Supervised by Prof. Yong Ho Park)**

## Abstract

Brucellosis is one of the major zoonotic diseases that cause abortion, infertility in various animals and humans throughout the world. The causative agent of *Brucella* consists of ten species; six species (*B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis* and *B. neotomae*) considered classical *Brucella* and four species (*B. ceti*, *B. pinnipedialis*, *B. microti* and *B. inopinata*) considered atypical *Brucella*. Besides, the genus expansion is still being processed, with the recent addition of *B. papionis* from baboons. Among them, three species are subdivided into biovars (bv.), i.e., *B. abortus* bv. 1-6 and 9, *B. melitensis* bv. 1-3, and *B. suis* bv. 1-5. In Korea, only *B. abortus* bv. 1 and 2, and *B. canis* have been isolated from livestock, wildlife and dogs.

Currently, brucellosis is managed as Category II disease in Livestock Epidemics Prevention and Control Act and Group III contagious disease in Infectious Disease Prevention Act in Korea. To eradicate brucellosis, South Korea as well as many other countries has employed a strict test-and-slaughter strategy. As a result, since bovine brucellosis reached its peak in 2006, its prevalence has been decreased less than 0.1% in recent years. Meanwhile, Mongolia is still brucellosis-outbreak country and human brucellosis has the second-highest incidence in the world. So, lots of *Brucella* strains

were obtained from various livestock, wildlife and humans. In this regard, it is a valuable work to characterize and apply them to evaluate new diagnostic methods.

For effective prevention and control of brucellosis, an accurate identification and epidemiological trace-back analysis are required. In particular, the classification of species and biovars in *Brucella* is mainly depends on morphological, biological and biochemical characteristics. However, some *B. abortus* strains showed the unique phenotypic features which didn't match those of any biovars in the current taxonomy. Besides, these untyped strains demonstrated that they did not belong to bv. 1, 2 or 4 by additional molecular detections, such as species-specific PCR, BaSS-PCR and *omp2a*-PCR. Also, comparative 16S rRNA sequencing analysis revealed that in comparison to *B. abortus* reference strains (bv. 1-6 and 9), the untyped strains harbored distinct genetic traits. Taking these phenotyping and molecular typing results altogether, the untyped strains were suspected to be *B. abortus* bv. 7, which was suspended from the taxonomy in 1986. Our findings might be helpful to prove the existence of *B. abortus* bv. 7 and support the re-introduction into the *Brucella* taxonomy.

For epidemiological analysis of Mongolian *Brucella* strains, the MLVA assay using 16 loci was performed. Mongolian *Brucella* strains were identified as *B. abortus* bv. 3 (9) and untype (6), and *B. melitensis* bv. 1 (67), bv. 3 (10) and Rev. 1 (17) by the classical biotyping. In the MLVA profiles, nine *B. abortus* bv.3 isolates were divided into four different genotypes, though six untype isolates were found to be in relation with only one genotype. The untyped *B. abortus* isolates predicted to be bv. 7, showed an identical MLVA profile with the Mongolian strains which were suggested as a reference bv. 7 strain in the recent report. In the MLVA assay including Web database, all sixty *B. abortus* strains were divided into two major clusters, and Mongolian *B. abortus* isolates were related with Chinese strains. In the MLVA-16 assay of *B. melitensis* strains, they were divided into 29 genotypes including four genotypes of *B. melitensis* Rev. 1 strains. As in the case of *B. abortus*,

Mongolian *B. melitensis* also showed a high genetic similarity with Chinese strains as compared to other countries.

To date, various diagnostic tools have been devloped to diagnose brucellosis, such as molecular detection and serologic tests using immunogenic antigens. For diagnosing of *B. abortus* infection with accuracy and rapidity, a new real-time PCR technique with a hybprobe from a specific SNP was developed. It could diagnose rapidly and exactly by an amplification curve monitoring and melting curve peak analysis. A total of 288 *Brucella* strains (22 reference strains, 156 Korean and 110 Mongolian isolates) and 8 non-*Brucella* were used to evaluate the diagnostic efficiency of the new PCR. With the regard to specificity, only *B. abortus* strains (bv. 1-6 and 9) revealed specific amplification curves from the 14th cycle, and *Tm* was 69 ºC in this PCR. In terms of sensitivity using *B. abortus* DNA and clinical samples, this new PCR was equal to or higher than that of 16S rRNA PCR. Therefore, it could be a useful molecular tool to diagnose *B. abortus* infection directly in animals and human due to its high sensitivity.

As part of the development of serological assays, selection of highly immunogenic antigens is required for differential diagnosis. The current brucellosis serologic diagnosis is mainly based on detecting anti-LPS antibody, but it occasionally shows cross-reaction due to similar O-polysaccharide structure. To minimize cross-reaction, the *B. abortus* RB51 strain which is LPS-lacking mutant of the virulent *B. abortus* 2308 strain was used to search the immuno-dominant proteins by 2-DE and MALDI-TOF using four types of antisera. As a result, the identified eleven candidate antigens could be useful as alternative or supplementeal antigens for brucellosis diagnosis.

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Keywords: *Brucella,* phenotypic and molecular characteristics, epidemiological analysis, MLVA-16, real-time PCR using hybprobe, SNP, immunogenic proteins

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# List of Abbreviation

| ACRONYM | FULL NAME |
|---|---|
| **BaSS-PCR** | *B. abortus* species-specific polymerase chain reaction |
| **MLVA** | Multilocus variable number of tandem repeat analysis |
| **HGDI** | Hunter-Gaston diversity index |
| **UPGMA** | Unweighted pair group method using arithmetic averages |
| **ISTB** | International subcommittee on the taxonomy of *Brucella* |
| **IS** | Insertion site |
| **AMOS-PCR** | *Brucella*-(*Abortus-Melitensis-Ovis-Suis*) PCR |
| **RAPD-PCR** | Randomly amplified polymorphic DNA PCR |
| **ERIC-PCR** | Enterobacterial repetitive intergenic consensus sequence PCR |
| **REP-PCR** | Repetitive intergenic palindromic sequence PCR |
| **RFLP-PCR** | Restriction fragment length polymorphism PCR |
| **HOOF-prints** | Hypervariable octameric oligonucleotide fingerprints |
| **MLST** | Multilocus sequence typing |
| **SNP** | Single nucleotide polymorphisms |
| **WGS** | Whole genome sequencing |
| **STT** | Standard tube agglutination test |
| **RBT** | Rose Bengal test |
| **BPAT** | Buffered plate agglutination test |
| **MRT** | Milk ring test |
| **CFT** | Complement fixation test |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **FPA** | Fluorescence polarization assay |
| **S-LPS** | Smooth-lipopolysaccharide |
| **IEF** | Isoelectric focusing |
| **2-DE** | Two-dimensional gel electrophoresis |
| **MALDI** | Matrix-assisted laser desorption/ionization |
| **TOF-MS** | Time-of-flight mass spectrometer |

# Literature Review

## Overview of *Brucella*

Brucellosis, is caused by members of the genus of *Brucella*, is a major zoonosis in both animals and humans [45]. This disease affects various livestock and wildlifes including marine mammals and humans [40, 56]. To date, all of 11 *Brucella* species are included in the *Brucella* taxonomy [137]), and the pathogenicity in humans are in order of *B. melitensis*, *B. suis* and *B. abortus*[95]. Currently, there are many taxonomical issues related to *Brucella* strains which result in the addtion of new species and/or biovars in the near future [45].

In 1887, David Bruce isolated the microorganism (*Micrococcus melitensis*) from a British soldier in Malta, and it was renmaed as *Brucella melitensis* in his honor. Since then, Sir. Themistocles Zammit discovered that contaminated goats' milk was a vector of transmission to humans of *B. melitensis* infection in 1905 [140].

Brucellosis in animals are mainly characterizsed by abortion, stillbirth, endometritis and retained placenta (female), and testis, orchitis and infertility (male). Main clinical symptoms in humans are undulant fever, anorexia, polyarthritis, meningitis, pneumonia, endocarditis, and so on [87]. Mostly, human brucellosis is associated with consumption of contaminated dairy products or occupational contact to infected animals or aborted materials. However, accidental infection can be occurred due to exposure to *Brucella* live vaccin strains or virulent *Brucella* organisms in the laboratory [22]. As brucellosis is one of important zoonotic bacterial diseases in animals and humans, the accurate diagnosis and understanding, and continuous control and prevention of brucellosis is crucial for eradicating the disease.

# I. Etiology of *Brucella*

## 1. History of the *Bruella* Genus

Since the first isolation of *B. melitensis* by David Bruce in 1887, a total of 11 *Brucella* species have been identified as the *Brucella* genus. In 1962, three species (*B. melitensis*, *B. abortus* and *B. suis*) were identified with specific biochemical characteristics [121]. In 1966, *B. neotomae* from a desert wood rat was recommended to constitute a new species [59]. Although it was isolated from atypical animal hosts, but it was in consistency with smooth colony morphology and criteria to identify *Brucella* species. Up to now, only about twenty-five *B. neotomae* were reported, but none of them have been recovered from domestic animals or humans [96].

In case of *B. ovis*, it was isolated firstly in 1952 from a ram in New Zealand, but it was accepted as a new species in 1970 [60]. It took almost two decades after the first report before acceptance of *B. ovis* as a new member of the *Brucella* genus. The main reasons were due to the fact that it differed from the existing standards for *Brucellae* differentiation and the clinical manifestation of infection in sheep also did not agree with previous pattern of brucellosis [25]. In addition, *B. ovis* was a rough-type strain, lacking expression of the O-side chain on its lipopolysaccharide (LPS) portion. Until then, only smooth *Brucella* strains which expressed the LPS O-side chain were virulent and could survive themselves within reservoir hosts [96].

Since 1966, a high incidence of abortion, epididymitis and reproductive disorders in dogs from the commercial breeding kennels was reported in the USA [13]. *Brucella*-like microorganisms were isolated from fetal tissues of aborted fetus. At that time, it was considered as a biotype of *B. suis* due to its biochemical characteristics, but it was renamed as *B. canis* [83]. Like *B. ovis*, *B. canis* was also rough type strain with lacking expression of O-side chain on its LPS.

Besides terrestrial animals, *Brucella* isolates from marine mammals have been reported from 1994. Namely, a gram-negative bacterium from an aborted bottlenose dolphin fetus showed typical biochemical and bacteriologic traits of *Brucella* genus [31]. Since then, numerous marine mammal brucellae have been isolated from the *Mysticeti* and *Odontoceti* suborders of cetaceans. These findings made it possible to taxonomic differentiation of them into *B. ceti* (porpoises and dolphins) and *B. pinnipedialis* (seals) [36].

In addition, two animal species were included as hosts for *B. microti*; common vole (*Microtus arvalis*) in Czech Republic [54] and wild red fox (*Vulpes vulpes*) in Austria [111]). Also, *B. microti* was known to survive long-time (about 6 months) in soil through the experimental data [108].

Another new strain, *B. inopinata*, was isolated from a woman patient with breast implant infection [112]. Besides, it was also identified in the wild Australian rodent [126] and lung lesion of a patient with chronic destructive pneumonia [127].

Recently, two gram-negative, non-motile coccoid bacteria isolated from clinical specimens of stillborn baboons (*Papio* spp.) showed typical phenotypic characteristics of the genus *Brucella*. As a result of ICSP Subcommittee on the Taxonomy of *Brucella*, they were named as *B. papionis* [137].

## 2. Taxonomic classification of *Brucella* species

Up to now, the genus of *Brucella* consists of eleven species, and their differentiation is depends on the bacteriologic, phenotyic characteristics and host preferences [40]. Among them, three species are subdivided into biovars (bv.), i.e., *B. abortus* bv. 1-6 and 9, *B. melitensis* bv. 1-3, and *B. suis* bv. 1-5. Of these, the history of *B. abortus* taxonomy has been changed repeatedly. Namely, *B. abortus* bv. 8 disappeared after 1978, and *B. abortus* bv. 7 was also deleted by the ISTB (International Subcommittee on the Taxonomy of *Brucella*; 1988) in 1986 because its

reference strain 63/75 consisted of a mixture of bv. 3 and bv. 5. However, the dispute about the presence of *B. abortus* bv. 7 is still occurring in some publications [5, 38, 45, 75, 136] Recently, Garin-Bastuji et al. [40] suggested the re-introduction of *B. abortus* bv. 7 into the *Brucella* classification and the establishment of the oldest Mongolian isolate (99-9971-135, 1988) as a potential reference strain for bv. 7. Besides, Whatmore et al. [137] reported that two novel Gram-negative and non-spore-forming coccoid bacteria (strains F8/08-60 (T) and F8/08-61)) from baboons were distinguished from the extant species of the genus *Brucella*. Thus, these two *Brucella* strains were named after *Brucella papionis* sp. nov. by ICSP Subcommittee on the Taxonomy of *Brucella* in 2014.

**Table 1.** Differential characteristics of species of the genus *Brucella*

| Species | Colony morphology[b] | Serum requirement | Lysis by phages[a] | | | | | Oxidase | Urease activity | Preferred host |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Tb | | Wb | $Iz_1$ | R/C | | | |
| | | | RTD[c] | $10^4$ RTD | RTD | RTD | RTD | | | |
| *B. abortus* | S | –[d] | + | + | + | + | – | (+)[e] | (+)[f] | Cattle and other *Bovidae* |
| *B. melitensis* | S | | – | – | (–)[g] | + | – | + | +[h] | Sheep and goats |
| *B. suis* | S | – | – | + | (+)[i] | (+)[i] | – | + | +[j] | Bv. 1: swine<br>Bv. 2: swine, hare<br>Bv. 3:swine<br>Bv. 4: reindeer<br>Bv. 5: rodents |
| *B. neotomae* | S | – | –[k] | + | + | + | – | – | +[j] | Desert wood rat[l] |
| *B. ovis* | R | + | – | – | – | – | + | – | – | Sheep |
| *B. canis* | R | – | – | – | – | – | + | + | +[j] | Dogs |
| *B. ceti* | S | ND | (–) | | (+) | (+) | – | (+) | + | Cetaceans |
| *B. pinipedialis* | S | ND | (–) | | (+) | (+) | – | (+) | +[h] | Pinnipeds |
| *B. microti* | S | – | – | + | + | + | ND | + | +[h] | Common voles |
| *B. inopinata* | S | ND | – | PL[m] | ND | ND | ND | ND | +[j] | Unknown |
| *B. papionis* | S | | PL[m] | PL[m] | + | ND | – | – | +[J] | Unknown |

(+)/(–) Most isolates positive/negative

a Phages: Tbilisi (Tb), Weybridge (Wb), Izatnagar1 ($Iz_1$) and R/C

b Normally occurring phase: S: smooth, R: rough

c RTD: routine test dilution

d *B. abortus* bv. 2 generally requires serum for growth on primary isolation

e Some African isolates of *B. abortus* bv. 3 are negative

f Intermediate rate, except strain 544 and some field strains that are negative

g Some isolates are lysed by Wb

h Slow rate, except some strains that are rapid

I Some isolates of *B. suis* bv. 2 are not or only partially lysed by phage Wb or $Iz_1$

j Rapid rate

k Minute plaques

l *Neotoma lepida*

m Partial lysis

ND Not determined

**Table 2.** Differential characteristics of species of the biovars of *Brucella* species

| Species | Biovar | $CO_2$ requirement | $H_2S$ production | Growth on dyes[a] | | Agglutination with monospecific sera | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Thionin | Basic fuchsin | A | M | R |
| *B. melitensis* | 1 | - | - | + | + | - | + | - |
| | 2 | - | - | + | + | + | - | - |
| | 3 | - | - | + | + | + | + | - |
| *B. abortus* | 1 | +[b] | + | - | + | + | - | - |
| | 2 | +[b] | + | - | - | + | - | - |
| | 3 | +[b] | + | + | + | + | - | - |
| | 4 | +[b] | + | - | +[c] | - | + | - |
| | 5 | - | - | + | + | - | + | - |
| | 6 | - | - | + | + | + | - | - |
| | 9 | + or - | + | + | + | - | + | - |
| *B. suis* | 1 | - | + | + | -[d] | + | - | - |
| | 2 | - | - | + | - | + | - | - |
| | 3 | - | - | + | + | + | - | - |
| | 4 | - | - | + | -[e] | + | + | - |
| | 5 | - | - | | - | - | + | - |
| *B. neotomae* | - | - | + | -[f] | - | + | - | - |
| *B. ovis* | - | + | - | + | -[e] | - | - | + |
| *B. canis* | - | - | - | + | -[e] | - | - | + |
| *B. ceti* | - | - | - | + | + | + | -[e] | - |
| *B. pinnipedialis* | - | + | - | + | + | + | -[e] | - |
| *B. microti* | - | - | - | + | + | - | + | - |
| *B. inopinata* | | - | + | + | + | - | + | |
| *B. papionis* | | - | - | - | - | + | - | - |

[a] Dye concentration in seum dextrose medium: 20 µg/mL
[b] Usually positive on primary isolation
[c] Some strains are inhibited by dyes
[d] Some basic fuchsin resistant strains have been isolated
[e] Negative for most strains
[f] Growth at a concentration of 10 µg/mL

# II. Molecular characterization of *Brucella* species

## 1. Classical *Brucella* species

Molecular approaches have found that most *Brucella* strains have two circular chromosome encoding 3.2 kb with two amplicons. With the developments of new molecular methods including DNA-DNA hybridization, G+C base ratio and DNA-ribosomal RNA hybridization revealed that the genus *Brucella* was closely related with plant pathogens, such as *Agrobacterium* and *Rhizobium*, suggesting a soil-associated common ancestor [101].

In 1968, the DNA agar method and a filter method at the molecular level were *Brucella* species [53]. As a result, three species (*B. abortus*, *B. melitensis* and *B. suis*) were considered as a single species. Moreover, *Brucella* species showed more than 80% DNA-binding ratio in the DNA-DNA hybridization method. In this study, the authors suggested that only one species, *B. melitensis*, should be accepted in the genus *Brucella* [130].

Also, the close genetic relationship among *Brucella* species was reported by PCR (polymerase chain reaction) and DNA sequencing methods. Namely, all known *Brucella* species possess identical 16S ribosomal rRNA [42] and *recA* gene sequences [109] and are almost same in the majority of housekeeping genes. On average, the genome sequences of six classcical *Brucella* species (*B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae*) show more than 94% homology at the nucleotide level. Among them, *B. melitensis* and *B. abortus* are the most highly related [131]. Also *B. canis* and *B. suis* also show very close relationship, but *B. ovis* and *B. neotomae* reveal a greater divergence from other *Brucella* species [135].

In 2002, the result of *Brucella* (*B. melitensis* 16M) whole genome sequencing analysis was reported firstly [27], followed by the genome sequencing of *B. suis* 1330

strain [101]. Their comparative whole genome sequencing results revealed >99% of nucleotide identity. In spite of these results, the ISTB (International Subcommittee on the Taxonomy of *Brucella*) agreed on a return to pre-1986 *Brucella* taxonomy of six *Brucella* species with biovars [97].

## 2. Novel *Brucella* species

### 2.1. *B. pinnipedialis* and *B. ceti*

Since the first isolation of atypical *Brucella* organisms from a bottlenose dolphin [31], another novel strain (*B. maris*) was confirmed from sea mammals in 1997 [57]. After 10 years of further studies, the atypical strains from marine mammals were subdivided into two novel species according to differential phenotypic and molecular traits [19]. These novel strains were named as *B. pinnipedialis* and *B. ceti* depends on preferred hosts [36]. The recent molecular typing methods such as MLVA (MultiLocus Variable Number Tandem Repeat Analysis) and IS*711*-based typing demonstrated that these two strains could be divided into discrete subclusters [74, 146].

### 2.2. *B. microti and B. inopinata*

These two strains are different from the classical *Brucella* strains phenotypically, with the characteristics of rapid growth and high metabolic activity. Due to their atypical biochemical traits, they were misidentified as *Ochrobactrum*, which is the closest genetic bacterium to *Brucella* [110, 112]. In the 16S rRNA and *recA* gene sequencing analysis, *B. microti* showed identical sequences with other *Brucella* species, but *B. inopinata* revealed much lower sequence similarity in several housekeeping genes [24]. Though *B. inopinata* is the most diverse species, it shares a DNA-DNA homology > 80% with *B. melitensis*. Thus, it still belongs to the genus of *Brucella*.

*2.3. B. papionis*

Two Gram-negative, non-motile coccid bacteria isolated from clinical specimes obtained from baboons (Papio spp.) that had delivered stillborn offspring were subjected to a polyphasic taxonomic study. These two isolates could be distinguished clearly from all known species of the genus *Brucella* and their biovars by both phenotypic and molecular properties. Therefore, by applying the species concept for the genus *Brucella* suggested by the ICSP Subcommittee of the Taxonomy of *Brucella*, they represent a novel species within the genus *Brucella*, for which the name *Brucella papionis* sp. nov. is proposed, with the type strain F8/08-60(T) [137].

## 3. Molecular typing methods of *Brucella* species

### 3.1. PCR-based assays

The first multiplex PCR to discriminate among *Brucella* species was AMOS (*Abortus-Melitensis-Ovis-Suis*)-PCR reported by Bricker and Halling [10]. This AMOS-PCR is composed of a single reverse primer for *Brucella*-specific insertion element IS*711* and four diffenrential forward primers for each species. However, this PCR assay could differentiate only four species among classical *Brucellae*, and some biovars within *B. abortus* and *B. suis* revealed negative results. Besides, BaSS (*B. abortus* species-specific)-PCR and enhanced AMOS-ERY PCR were known to differentiate *B. abortus* bv. 1, 2 and 4 in *B. abortus* biovars [11, 93].

Since 2006, more advanced conventional multiplex PCR assays have been introduced to identify *Brucella* organisms at species level simultaneously. At first, Bruce-ladder PCR assay using eight primer pairs was able to detect eight *Brucella* species and three vaccine strains (*B. abortus* S19 and RB51, and *B. melitensis* Rev. 1) at one time [39]. Later, more enhanced multiplex PCR techniques were developed to differentiate ten *Brucella* species in a single tube [61, 77]. Although these advanced

multiplex PCR assays allow accurate species discrimination of all *Brucella* species except the newest one (*B. papionis*), differentiation at biovar level is unattainable. However, a new Bruce-ladder multiplex PCR for the biovar typing in *B. suis* was developed by Lopez-Goni et al [73].

## 3.2. Non-specific high-resolution typing assays

Since 1990s, the PCR-based genotyping methods were applied to investigate genetic diversity about *Brucella* species and biovars. In chronogical order, randomly amplified polymorphic DNA PCR (RAPD-PCR) [32], enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) [80], repetitive intergenic palindromic sequences PCR (REP-PCR) [123], and restriction fragment length polymorphism PCR (RFLP-PCR) of the *omp*2 locus [19] were developed. However, these techniqes were not used commonly due to their complexcity and low reproducibility [113].

## 3.3. Specific high-resolution typing assays

### 3.3.1. Multilocus variable number of tandem repeats analysis (MLVA)

The advantages of the MLVA allows to array length variations in tandem repeats in bacterial DNAs. To date, almost every tandem repeats have been evaluated by several researchers. Especially, loci markers of three researchers (Bricker, Vergnaud and Whatmore) are used commonly [113].

### 3.3.2. HOOF-Prints

HOOF-Prints (hypervariable octameric oligonucleotide fingerprints) was the first MLVA assay made by Bricker et al [11]. The repeat unit 'AGGGCAGT' is present in most *Brucella* genome, so eight highly variable loci were selected for HOOF-Prints. Its

high discriminatory power enables us to investigate the trace-back analysis and epidemiological relatedness in brucellosis-outbreak. However, the identification of *Brucella* strains at a species level by HOOF-Prints is not recommended yet.

Currently, the MLVA-15 and MLVA-16 assays are used the most commonly for epidemiological analysis due to online access easily to MLVA-16 database in other *Brucella* isolates [4]. Especially, MLVA-16 assay is composed of two panels - low and high discriminatory index. The low index (panel 1) is able to reveal the major branch and species, and a high index (panel 2) with VNTR markers is appropriate for more detailed epidemiologic information.

3.3.3. Multilocus sequence typing (MLST)

The MLST method is based on sequence divergence in housekeeping genes. About 450 to 500 bp from each housekeeping genes are amplified by PCR, followed by DNA sequencing and comparative sequencing analysis. In 2007, an MLST assay was developed using nine loci (seven housekeeping genes, *omp*25 gene and an intergenic region) [135]. In this research, 160 *Brucella* strains containing all species and biovars were analyzed. As a result, four *Brucella* species (*B. abortus*, *B. melitensis*, *B. ovis* and *B. neotomae*) formed a respective cluster according to their species. In case of *B. suis*, biovars 1 to 4 grouped together, but *B. suis* bv. 5 formed a seperate cluster, and *B. suis* biovars 3 and 4 grouped closely with *B. canis* strains. Also, *Brucella* strains from marine mammals also formed as a separate cluster. Taken into consideration of the results, the MLST could be a useful tool to investigate the phylogenetic relationships in *Brucella* strains.

3.3.4. Real-time PCR of single nucleotide polymorphisms (SNP)

The MLST assay is available to amplify and analyze the corresponding target genes by PCR and DNA sequencing, but the entire procedure is complicated,

expensive and time-consuming. Accordingly, Gopaul et al. [46] developed a rapid and unambiguous assay based on SNP to discriminate *Brucella* at species level as a format of real-time PCR.

### 3.3.5. Whole genome sequencing (WGS)

Recently, the whole genome sequencing is indispensable for determination of bacterial taxonomy. It is being used gradually on behalf of MLST and large-scale SNP typing because it can provide incalculable value of data [113]. With the development of WGS, it can be applicable to design specific SNPs to complement MLVA typing and genetic cluster analysis efficiently [37].

# III. Serological diagnosis of brucellosis

## 1. Screening tests

### 1.1. Agglutination test

This test was the first serological diagnosis of brucellosis developed by Wright and Smith [139]. By adding the *Brucella* cells to the diluted serum, the pattern of cell pellets were observed after incubation. The procedure is almost identical with the standard tube agglutination test (STT). In this test, IgM isotype of antibody is the most active agglutinin at a neutral pH condition [21].

### 1.2. Rose-Bengal test (RBT)

The RBT and the buffered antigen plate agglutination test (BPAT) are based on the whole cell antigens of pH 3.65. The low pH inhibits some agglutination by IgM and induces agglutination by IgG1, thus reduces false-positive reaction. These two tests are used for screening test of individual animals, however, they occasionally induce false-negative reaction due to prozoning [125].

### 1.3. Milk-ring test (MRT)

The MRT uses the whole cell antigens with stained hematoxylin, and the MRT antigen is added to pools of bulk tank milk (BTM) sample in a farm. Compared to other tests, it shows relatively low sensitivity. It can cause false-positive result due to mastitis, colostrum and milk from the last lactating cycle. It is usually used for screening of brucellosis in dairy cattle farm [125].

## 2. Prescribed tests for international trade (OIE)

### 2.1. Complement fixation test (CFT)

The CFT is considered as a confirmatory test with high specificity in the OIE. The CFT is based on diluted serum of cattle, sheep or goats, antigen and a titrated amount of complement from are added each other. It is a confirmatory test with high specificity and recommended as international trade for brucellosis. However, it is very complicated and difficult to perform and required highly trained staff to maintain accurate titration and laboratory facilities [125].

### 2.2. Enzyme-linked immunosortent assay (ELISA)

The ELISA is a test that uses antibodies and color change to identify a substance. Antigens from the sample are attached to a surface. Then, a further specific antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate [89]. Among them, I-ELISA (indirect ELISA) is a very sensitive test, but it cannot differentiate sometiems between antibodies from vaccination or false-positive reaction and natural infection. In addition, the C-ELISA (competitive ELISA) is through competitive binding, it uses a MAb specific for one of the epitope of the *Brucella* OPS. Due to MAb, C-ELISA shows a higher specificity than I-ELISA, and its specificity is almost equivalent to or greater than the CFT [89].

### 2.3. Fluorescence polarization assay (FPA)

The application of fluorescence polarization offers unique advantages over conventional fluorescence imaging and quantitation. In fluorescence polarization studies, fluorophores tied to biological samples or chemical compounds help elucidate

the underlying mechanism of interest. If the molecule is labeled with a fluorescent marker and is examined by polarized light, then the smaller molecule will rotate faster than a large molecule. The time of rotation is measured by horizontal and vertical measurements [90]. The FPA had been developed as an alternative test to be able to perform outside rapidly. Its sensitivity and specificity for brucellosis are almost identical to those of C-ELISA. So, it is also a prescribed test by the OIE for international trade [125].

## IV. Current situation of brucellosis

Brucellosis is reported worldwide but it is adequately controlled in most developed countries. The geographical distribution of brucellosis is constantly changing, with new foci emerging or re-emerging [116]. This bacterial disase is still frequently occurred in the Middle East, Asia, Africa, South and Central America, the Mediterranean Basin and the *Brucella* species vary in their geograhpic distribution [99]. *B. abortus* is found worldwide in cattle-rasing countries except Japan, Canada, some of European countries, Australia, New Zealand and Israel, where it has been eradicated. But during the last two decades, the epidemiology of brucellosis has changed significantly, with the emergence of new global outbreaks in association with major political/historical events, successful control of the disease in many parts of the Mediterranean, and the referral of epidemiological data from countries where brucellosis was endemic but in an unknown proportion [99, 149]. Although estimates of the costs associated with brucellosis infections remain limited to specific countries, all data suggested that worldwide economic losses due to brucellosis are extensive not only in animal production (reduced milk, abortion and delayed conception), but also in public health (cost of treatment and productivity loss) [116].

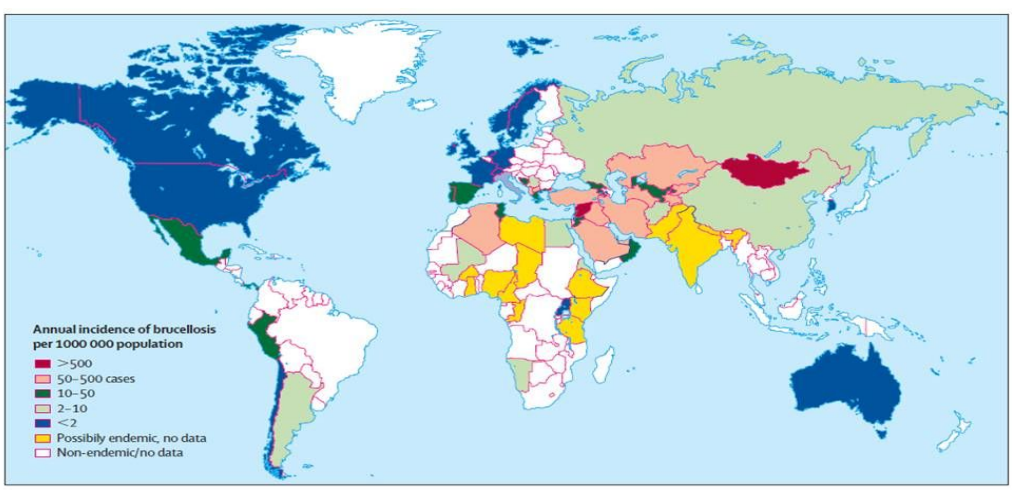


**Figure 1.** Worldwide incidence of human brucellosis [99]

In terms of human brucellosis, Syria is the highest incidence in the world, and Mongolia is the second-highest ranked country [99]. Mongolia is the highest brucellosis-outbreak country in Asia, and brucellosis remains one of the major veterinary and public health problems in this country. Due to Mongolian traditional nomadic system, the transmission of brucellosis has been occurred between animals and human, and livestock and wildlife. With diverse animal species and rearing style, various biovars and species in *Brucella* had been found [149]. However, the studies on brucellosis is lacking despite of its high incidence in Mongolia. In views of geographical distribution and variety of *Brucella* strains, Mongolia is very attractive country to investigate brucellosis and apply their isolates to new diagnostic tools.

## 1. South Korea

### 1.1. Outbreak of brucellosis

Outbreak of brucellosis in Korea was firstly reported in imported dariy cattle in 1955 [64]. After that, sporadic outbreaks had been occurred, and brucellosis in cattle has spread nationwide in 1990's. Especially, brucellosis had been increased dramatically in 2004, and the prevalence rate peaked in 2006, 2.02%. After that time, the prevalence had been decreased steadily less than 0.03% by 2015.

In 1956, Kim et al [64] had reported firstly to isolate *B. abortus* organisms from serologically-positive dairy cattle farm located in Gyeonggi province. Since then, there were reported to *B. abortus* in cattle, and Park and Lee reported to isolate *B. abortus* in aborted fetus of imported cows from USA in 1956. Besides cattle, Her et al [150] also reported *B. abortus* from domestic elk in 2008, and Kim et al [151] reported *B. abortus* from Chinese water deer in 2013 [151]. In Korea, *B. abortus* from livestock was mainly biovar 1 type, which was widely distributed worldwide [152]. Besides *B.*

*abortus*, *B. canis* at dogs also had been reported in Korea [153].

### 1.2. Prevention and control

The first regulation of a 'Control and prevention measures standard operation protocol (SOP) of Brucellosis and Tuberculosis' was established in 1964, a brucellosis control strategy based on 'test and slaughter' has been executed until now. For screening of brucellosis in cattle, cattle aged over one year should be examined regularly. Once brucellosis-positive cattle are confirmed, they should be slaughtered within 10 days from the diagnosis. Suspected cattle are to be re-tested within 1-2 months.

In terms of vaccination in Korea, RB 51 vaccine had been inoculated in cattle in 1997, but side effects such as abortion and early birth had been occurred in the pregnant cattle occasionally. Due to its side effects, most of the cattle farms are not vaccinated.

### 1.3. Human brucellosis in Korea

In humans, one case of *B. abortus* infection was officially recorded in 2003 [100]. Since tehn, human infections have continued to increase, which resulted from reinforcements to test brucellosis against farmers and employees in farms, laboratory personnels and epidemiologically related persons. Since the highest incidence of 215 cases in 2006, human brucellosis also had been decreased gradually [63].

Human brucellosis is usually prevented by controlling the infection in animals. Pasteurization of dairy products is a crucial safety measure where brucellosis is endemic area. Unpateurized dairy products and raw or undercooked animal products should not be consumed. Good hygiene and proective clothing/equipment are so important in preventing occupational exposure. In terms of treatment, antimicrobials are usually the manistay against brucellosis; long-term treatment may be required.

## 2. Mongolia

### 2.1. Outbreak of brucellosis

Mongolia is a large and developing country mainly based on the livestock industry and has the second-highest incidence of human brucellosis [117]. Mongolian society is based on a nomadic livestock system, and various animal species comingle while being raised, including sheep, goats, cattle, camels and yaks. In the 1950s, brucellosis was prevalent in Mongolia and seroprevalence rates were ranged from 2% to 17% in goats, sheep and cattle [145]. The *Brucella* seroprevalence rate among cattle in 1987 ranged from 3.8% to 35% before vaccination program, but appeared to be 5% - 10% with some focal areas close to 50% in 2003 [149]. Seroprevalence in sheep and goats is less, about 2%. Neverthless, *B. melitensis* appears to be the most common species of *Brucella* isolated from blood culture taken from acutely infected patients. In 2011, a national brucellosis serosurvey was conducted from 11,528 nomadic camps of 337 districts of 21 provinces in Mongolia. Livestock seroprevalence was found 0.7% of camels, 1.8% of cattle, 0.7% of sheep and 0.5% of goats using parellel interpretations of RBT, CFT and C-ELISA [85, 117].

### 2.2. Prevention and control

Attempts to control this enzootic infection have been unsucessful because of an inconsisten strategy varying between vaccination of livestock and the destruction of infected animals As of 2001, the Mongolian government implemented mass vaccination strategy applying *B. melitensis* Rev. 1 vaccine for sheep and goats, and *B. abortus* S19 vaccine for cattle and yaks from 1975 to 1985, and re-started in 2000 [145]. More than 33 million small ruminants and 9.5 million cattle/yaks were vaccinated during the mass vaccination campaign, making it one of the most successful

nationwide vaccination programmes in the world [145]. After 1990, Mongolia was no longer dependent on the former Soviet Union, brucellosis re-emerged as a preventable human disease in most countries which were part of the former Soivet Union and Mongolia. However, the mass livestock vaccination campaign which started in 2000 seemed to lose traction as reported cases of human brucellosis did not decline further after 2004 [145].

## 2.3. Human brucellosis in Mongolia

Approximately 23% of Mongolia's population lives in rural areas and leads a nomadic or seminomadic way of life. Their diet is heavily depends on meat and unpasteurized dairy products, reflecting the importance that large domesticated animals have played in the country's history. In Mongolia, transmission of brucellosis to humans occurs primarily from direct contract with animals through injury while handling them or during slaughtering and to a lesser extent, from drinking contaminated milk [128, 145]. As of 2001, about 8,000 human cases of chronic brucellosis were reported, and 1,000-1,500 new cases have been reported yearly since 1996. Treatment with doxycycline and gentamicin or rifampin is standard, but they are often administered for only 2 weeks rather than the minimal 6 weeks recommended in most recent reviews [154].

# General Introduction

*Brucellae* are Gram-negative, facultative intracelluar bacteria that are able to infect many mammalian species, including marine mammals and humans. To date, eleven species are recognized within the genus *Brucella* based on biochemical, molecular traits and host preferences. Of these, three species (*B. abortus*, *B. melitensis*, *B. suis*) have been divided into 7, 3 and 5 biovars. In Korea, infections with *B. abortus* bv. 1 and 2, and *B. canis* were previously reported only livestock and wild animals, as well as in dogs. However, *B. melitensis* infection was most recently confirmed in human. The source of recent human infection was possibly originated from other countries in which brucellosis is endemic. Therefore, comparison and characterization of *Brucella* spp. isolated from adjacent countries is important in epidemiological aspect.

Mongolia has shown a high-incidence of brucellosis in domestic and wild animals, and many foreign workers have been introduced from Mongolia in recent years. Those people could work as potential carriers for introducing new *Brucella* spp. from Mongolia to Korea. Therefore, characterization of *Brucella* strains isolated from Mongolia and comparison with those strains in Korea should be informative to analyze the epidemiology of brucellosis in Korea. In this research, a lot of *Brucella* strains had been obtained from diverse animals and humans in Mongolia through the collaborative project and used for phenotypic and genotypic characterizations.

Therefore, in Chapter I, we focused on the characterization and investigation of untyped *B. abortus* isolates by classical phenotyping. Their biochemical characteristics did not match those of any biovars of *B. abortus* in the current taxonomy. The untyped strains were identical with former *B. abortus* bv. 7 which was deleted from the taxonomy in 1986. To identify the existence of the former *B. abortus* bv. 7, the moleculr detection methods were additionally performed to characterize them.

In Chapter II, the MLVA assay with 16-loci markers were applied to investigate the epidemiological characteristics and relatedness of *Brucella* strains in Mongolia. Some publication on serologic surveillance for brucellosis and molecular typing of *Brucella* isolates in Mongolia had been reported earlier, but in-depth molecular genotyping research for understanding of Mongolian *Brucella* strains was very rare. Therefore, the molecular genotyping analysis might explain the epidemiological relationships of the isolates between animals and humans, and between Mongolia and other countries.

In Chapter III, we developed the new real-time PCR using hybprobe based on a SNP for differential diagnosis of *B. abortus* infection. The SNP-based real-time PCR using hybprobe has been recommended for bacteria with high DNA homology within species by amplification curves and melting peak analysis. To evaluate its dianostic specificity and sensitivity, a total of 288 *Brucella* strains (22 reference strains, 156 Korean isolates and 110 Mongolian isolates) and 8 non-*Brucella* strains known as cross-reactive bacteria were applied.

In Chapter IV, an immunoproteomic analysis was conducted to select the candidate proteins for brucellosis serologic assays. Though the current serological tests for brucellosis are chiefly based on whole cell or smooth LPS, they can occasionally induce false-positive reaction with cross-reactive bacteria haboring a similar LPS structure. Thus, 2-DE and MALDI-TOF analysis using *B. abortus* RB51 strain which is an LPS-lacking strain was applied to minimize the cross-reaction. In this research, four types of antisera - *B. abortus*-positive and -negative sera, and *E. coli* O157:H7 - and *Y. enterocolitica* O:9-positive sera were used to search immunogenic proteins. This proteomic analysis using *B. abortus* RB51 could be a promising strategy to find new differential antigens.

# Chapter I.

## Investigation of phenotypic and molecular characteristics of *Brucella abortus* strains from Mongolia

## Abstract

Recently, some *Brucella abortus* were isolated from human and various livestock in Mongolia, and the classical biotyping and molecular detection methods (PCR assays, 16s rRNA sequencing analysis and MLVA (Multi-locus variable number tandem repeat analysis) were applied to investigate them. Of the sixteen isolates, nine and seven were confirmed as biovar (bv.) 3 and untyped by the classical biotyping assay, respectively. The phenotypic characteristics of the untyped isolates were consistent with those of the former *B. abortus* bv. 7 which is not included in the current *Brucella* taxonomy. Additionally, genus and species-specific PCR supported that the untyped Mongolian isolates belonged to *B. abortus*. Also, the untyped isolates were clustered into a peculiar genotype as the results of 16s rRNA sequencing and MLVA patterns using 16 loci. Taken all together, the untyped *B. abortus* isolates, one of the most predominant *B. abortus* biovars in Mongolia were estimated to be bv. 7. Moreover, the Mongolian *B. abortus* isolates were closely associated with Chinese isolates according to MLVA. Therefore, the animal quarantine and control measures should be strengthened to prevent the spillover of *Brucella* species between adjacent countries.

**Keywords**: *Brucella abortus*, phenotyping, molecular detection, bv. 3 and 7, Mongolia

# I. Introduction

Brucellosis is a major zoonosis that occurs in domestic and wildlife as well as humans worldwide [29]. The genus *Brucella* consists of 11 species, and their classification are mainly based on biological and biochemical characteristics and host preferences [137].

In general, classical biotyping methods are used to identify and differentiate *Brucella* species and biovars. Based on them, three species of *Brucella* are currently divided into several biovars: *B. abortus* (bvs. 1-6 and 9), *B. melitensis* (bvs. 1-3), and *B. suis* (bvs. 1-5). *B. abortus* biovars have been changed. Specifically, *B. abortus* bv. 8 disappeared after 1978, and *B. abortus* bv. 7 was also deleted by the International Subcommittee on Taxonomy of *Brucella* (ISTB) in 1986 because its reference strain (63/75) consisted of a mixture of bv. 3 and 5 [40].. However, the debate about the existence of *B. abortus* bv. 7 is ongoing [5, 38]. Recently, Garin-Bastuji et al. [40] suggested the re-introduction of *B. abortus* bv. 7 into *Brucella* classification and the establishment of the oldest Mongolian isolate (99-9971-135, 1988) as a potential reference strain for bv. 7.

In addition, many molecular detection methods are also available for discriminating some *Brucella* species and biovars. For example, *B. abortus* species-specific-PCR (BaSS-PCR) and enhanced AMOS-ERY PCR were able to differentiate *B. abortus* bvs. 1, 2 and 4 from the other biovars [12, 93]**.** *Omp*2a-PCR is capable of distinguishing *B. abortus* biovars based on the size differences of the *omp*2a genes between bvs. 1, 2 and 4 and the other biovars. 16S rRNA sequencing analysis is also helpful for identifying *Brucella* at the species level [81]. Additionally, several multiplex PCR techniques that can discriminate all *Brucella* species, including vaccine strains that have been developed previously [61, 73, 77].

To date, studies examining brucellosis in Mongolia have mainly focused on serological monitoring and prevalence in humans and domestic animals [29, 117, 145].

Mongolia shows the second highest incidence of human brucellosis, especially since a rapid increase in notified cases of human brucellosis has been observed in 2000. Due to its traditional rearing style and eating habits, the transmission of brucellosis between humans and animals can be occurred continuosuly [128].

Therefore, the aims of the study were to characterize and investigate the strains by using the classical biotyping assay and molecular detection methods.

# II. Materials and methods

## 1. Bacterial strains and classical biotyping

From 2012 to 2014, the Institute of Veterinary Medicine (IVM) in Mongolia provided *Brucella* strains isolated from aborted domestic animals and human with epidemiological data, from 2 hospitals and 10 farms. After primary bacterial growth, pure colonies obtained after more than 3 serial passages. A total of 16 *B. abortus* isolates and 16 *Brucella* reference strains (*B. abortus* bvs. 1-6, and 9, and other nine *Brucella* species) were tested according to classical typing (Table I-1). The pure colonies were tested according to classical *Brucella* typing methods such as the oxidase test, urea hydrolysis, $CO_2$ requirements, $H_2S$ production, an agglutination test with A-, M-, and R-monospecific sera (AHVLA; Animal Health and Veterinary Laboratories Agency, UK), growth on thionin and basic fuchsin dyes, and lysis of Tb, Wb, Iz, and R/C phages (AHVLA, UK), provided by the OIE manual 7$^{th}$ edition, 2012 [125].

## 2. DNA extraction for PCR assays

The genomic DNAs of 16 Mongolian *B. abortus* isolates and 16 reference *Brucella* strains were prepared using the QIAamp DNA extraction kit (Qiagen Korea Ltd., #2012, Seoul, Korea) according to the manufacturer's protocols. The 16 reference strains were same as follows: *B. abortus* bv. 1 (544), *B. abortus* bv. 2 (86/8/59), *B. abortus* bv. 3 (Tulya), *B. abortus* bv. 4 (292), *B. abortus* bv. 5 (B3196), *B. abortus* bv. 6 (870), *B. abortus* bv. 9 (C68), *B. canis* (RM6/66), *B. suis* bv. 1 (1330), *B. ovis* (63/290), *B. neotomae* (5K33), *B. melitensis* bv. 1 (16M), *B. ceti* (B1/94), *B. pinnipedialis* (B2/94), *B. microti* (CCM4915) and *B. inopinata* (B01). BaSS PCR assay and differential multiplex PCR were performed using the extracted *Brucella* DNA samples [12, 61]. In addition, *omp*2a-PCR was used as a complementary method for re-confirmation of the *Brucella* growth on thionin dye [40].

### 3. Multiplex PCR

To determine the species of each *Brucella* isolate at one time, the extracted DNA samples were applied to the advanced multiplex PCR as reported by Kang et al [61]. The PCR products of the *B. abortus* isolates were analyzed by electrophoresis using 1.5% agar gel with a 100-bp ladder matched to those of the 10 *Brucella* reference species listed in Table I-1. The identification results of the PCR were also compared to those of the classical biotyping assay.

### 4. BaSS-PCR and *omp*2a-PCR

To discriminate whether the Mongolian *B. abortus* isolates were bv. 1, 2 and 4 or not, *omp2a*-PCR and BaSS-PCR were conducted. In particular, *omp*2a PCR was used concurrently for re-confirmation of the *Brucella* growth test on thionin dye as a complementary method. The PCR assays followed the instructions of Cloeckaert et al [16] and Bricker et al [12], respectively.

### 5. 16S rRNA sequencing analysis

The genomic DNAs of *Brucella* strains were extracted using InstaGene$^{TM}$ Matrix (Bio-Rad; Hercules, CA 94547, USA). The 16S rRNA genes were amplified and sequenced using universal primers (27F: 5' - AGAGTTTGAT CMTGGCTCAG - 3', 1492R: 5' - TACGGYTACCTTGTTACGACTT - 3', 518F: 5' - CCAGCAGCCGCGGTAATACG - 3' and 800R: 5' - TACCAGGGTATCTAATCC - 3') [92]. This sequence alignment was analyzed using CLC Main Workbench software version 6.0 (Qiagen Corp., Denmark).

**Table I-1**. Bacterial strains used in biotyping and molecular typing

| Species and strain | Biovars | Strain ID | Animals | Province |
|---|---|---|---|---|
| **Reference strains** | | | | |
| *B. abortus* | 1 | 23448(544) | | |
| *B. abortus* | 2 | 23449(86/8/59) | | |
| *B. abortus* | 3 | 23450(Tulya) | | |
| *B. abortus* | 4 | 23451(292) | Cattle | |
| *B. abortus* | 5 | 23452(B3196) | | |
| *B. abortus* | 6 | 23453(870) | | |
| *B. abortus* | 9 | 23455(C68) | | |
| *B. melitensis* | 1 | 23456(M16) | Sheep, goats | |
| *B. ovis* | | 25840(63/290) | Sheep | |
| *B. suis* | 1 | 23444(1330) | Pigs | |
| *B. canis* | | 23365(RM6/66) | Dogs | |
| *B. ceti* | | B1/94 | Dolphins | |
| *B. pinnipedialis* | | B2/94 | Seals | |
| *B. microti* | | CCM4915 | Voles | |
| *B. neotomae* | | 23459(5K33) | Rodents | |
| *B. inopinata* | | BO1 | Unknown | |
| **Mongolian isolates** | | | | |
| *B. abortus* | untype | A1-3452 | Sheep | Khentii |
| *B. abortus* | untype | A1-3453 | Sheep | Khentii |
| *B. abortus* | untype | A1-3470 | Human | - |
| *B. abortus* | 3 | A1-3476 | Sheep | Sukhbaatar |
| *B. abortus* | 3 | A1-3480 | Camel | Khentii |
| *B. abortus* | untype | A1-4111 | Human | - |
| *B. abortus* | untype | A1-4113 | Human | - |
| *B. abortus* | untype | A1-4141 | Cattle | Bulgan |
| *B. abortus* | untype | A1-4142 | Cattle | Bulgan |
| *B. abortus* | 3 | A1-4164 | Cattle | Sukhbaatar |
| *B. abortus* | 3 | A1-4165 | Cattle | Sukhbaatar |
| *B. abortus* | 3 | A1-4166 | Cattle | Sukhbaatar |
| *B. abortus* | 3 | A1-4167 | Cattle | Khentii |
| *B. abortus* | 3 | A1-4168 | Cattle | Sukhbaatar |
| *B. abortus* | 3 | A1-4169 | Cattle | Sukhbaatar |
| *B. abortus* | 3 | A1-4170 | Cattle | Sukhbaatar |

# III. Results

## 1. Biotyping assays

Of the 16 Mongolian *B. abortus* isolates, 9 isolates showed characteristics of *B. abortus* bv. 3 (Table I-2). Briefly, they were agglutinated only with the A monospecific sera, not with the M or R sera; they also grew on the media with thionin and basic fuchsin and were lysed by all the phages except R/C. One of the isolates was from camel milk in Khentii province, one isolate was from the placenta of sheep in Sukhbaatar and seven isolates were from cattle in Khentii and Sukhbaatar, Mongolia.

In addition to those isolates, seven isolates from humans and sheep in Khentii and cattle in Bulgan did not agree with any of the biotyping profiles in the current *B. abortus* taxonomy. The seven strains showed characteristics typical of *B. abortus* species: smooth colonies, oxidase +, urease +, and phage typing by Tb, Wb, Iz and R/C. However, their biotyping results for the differentiation of the biovars did not correspond to any current biovars. Their unique characteristics were identical with the former *B. abortus* bv. 7 in the agglutination test (A +, M +, R -) with regards to the monospecific sera and growth (thionin +, basic fuschin +) on dyes (Table I-2).

## 2. Molecuar typing assays

To discriminate and support the classical biotyping assays of the Mongolian isolates, the advanced multiplex PCR assay was performed to differentiate the *Brucella* species. This multiplex PCR is a simple and time-saving molecular technique that is able to distinguish between the 10 *Brucella* species, including vaccine strains. All 16 Mongolian isolates were confirmed to belong to *B. abortus*, which had four amplicons; 152, 450, 587 and 1,682 bp (Fig. I-1).

In addition, BaSS and *omp*2a-PCR were carried out in parallel to support the biotyping results. All the isolates showed only two amplicons of 180- and 800 bp in the BaSS PCR (Fig. I-2). They did not have a 500 bp amplicon to differentiate *B. abortus*

bv. 1, 2 and 4 (Fig. I-2). The *omp2a*-PCR also showed the same PCR products of 1,216 bp from all the Mongolian isolates (Fig. I-2), demonstrating that they did not belong to bv. 1, 2 or 4. These results concurrently demonstrated thionin resistance characteristics [40].

According to these three PCR assays, the Mongolian *B. abortus* isolates were considered to belong to other biovars, not bv. 1, 2 or 4. These results showed that the isolates were similar to *B. abortus* bv. 3 and the former bv. 7 based on the biotyping methods.

**3. 16S rRNA sequencing analysis**

A comparative sequence analysis among the suspected *B. abortus* bv. 7 isolates and other *Brucella* species, for which the entire genome has been sequenced, revealed a 1-bp difference in the 16S rRNA region. This SNP is a C to T transition at position 926 of the partial 16S rRNA sequence (1,454 bp) of the reference *B. abortus* (NC_006932.1 and 006933.1). This finding suggests that these untyped Mongolian *B. abortus* strains could represent a genetic source that is different from the current *B. abortus* biovars.

**Table I-2.** *B. abortus* isolates from Mongolia and reference strains, and their biochemical characteristics

| Strain | ID. | Biovar | $CO_2$ | $H_2S$ | Oxidase | Catalase | Urease | Agglutination with monospecific sera | | | Growth on dyes | | Lysis by phages at RTD | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | A | M | R | Thionin | Basic fuchsin | Tb | Tb $10^4$ | Wb | Iz | R/C |
| Isolates | A1-3452 | untype | - | + | + | + | + | + | + | - | + | + | + | + | + | + | - |
| | A1-3453 | untype | - | + | + | + | + | + | + | - | + | + | + | + | + | + | - |
| | A1-3470 | untype | - | + | + | + | + | + | + | - | + | + | + | + | + | + | - |
| | A1-3476 | 3 | + | + | + | + | + | + | - | - | + | + | + | + | + | + | - |
| | A1-3480 | 3 | + | + | + | + | + | + | - | - | + | + | + | + | + | + | - |
| | A1-4111 | untype | - | + | + | + | + | + | + | - | + | + | + | + | + | + | - |
| | A1-4113 | untype | - | + | + | + | + | + | + | - | + | + | + | + | + | + | - |
| | A1-4141 | untype | - | + | + | + | + | + | + | - | + | + | + | + | + | + | - |
| | A1-4142 | untype | - | + | + | + | + | + | + | - | + | + | + | + | + | + | - |
| | A1-4164 | 3 | + | + | + | + | + | + | - | - | + | + | + | + | + | + | - |
| | A1-4165 | 3 | + | + | + | + | + | + | - | - | + | + | + | + | + | + | - |
| | A1-4166 | 3 | + | + | + | + | + | + | - | - | + | + | + | + | + | + | - |
| | A1-4167 | 3 | + | + | + | + | + | + | - | - | + | + | + | + | + | + | - |
| | A1-4168 | 3 | + | + | + | + | + | + | - | - | + | + | + | + | + | + | - |
| | A1-4169 | 3 | + | + | + | + | + | + | - | - | + | + | + | + | + | + | - |
| | A1-4170 | 3 | + | + | + | + | + | + | - | - | + | + | + | + | + | + | - |
| Reference strains | 544 | 1 | (+)[1] | + | + | + | (+)[1] | + | - | - | - | + | + | + | + | + | - |
| | 86/8/59 | 2 | (+)[1] | + | + | + | + | + | - | - | - | - | + | + | + | + | - |
| | Tulya | 3 | (+)[1] | + | (+)[1] | + | + | + | - | - | + | + | + | + | + | + | - |
| | 292 | 4 | (+)[1] | + | + | + | + | - | + | - | - | (+)[1] | + | + | + | + | - |
| | B3196 | 5 | - | - | + | + | + | - | + | - | + | + | + | + | + | + | - |
| | 870 | 6 | - | - | + | + | + | + | - | - | + | + | + | + | + | + | - |
| | 99-9971-135[2] | 7 | - | + | + | + | + | + | + | - | + | + | + | + | + | + | - |
| | C68 | 9 | +/- | + | + | + | + | - | + | - | + | + | + | + | + | + | - |

[1] Most strains were positive.

[2] Garin-Bastuji et al., 2014.

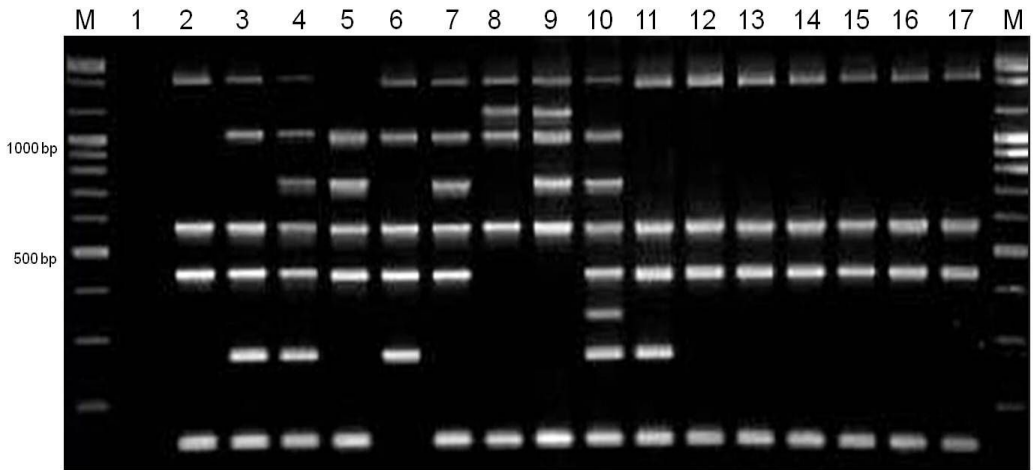


**Figure I-1.** Advanced differential multiplex PCR assay for 10 *Brucella* reference species and Mongolian *B. abortus* isolates from each animal species. Lane M: 100 bp DNA marker, lane 1: Negative control (D. W.), lane 2: *B. abortus* bv. 1 (544), lane 3: *B. canis* (RM6/66), lane 4: *B. suis* bv. 1 (1330), lane 5: *B. ovis* (63/290), lane 6: *B. neotomae* (5K33), lane 7: *B. melitensis* bv. 1 (M16), lane 8: *B. ceti* (B1/94), lane 9: *B. pinnipedialis* (B2/94), lane 10: *B. microti* (CCM4915), lane 11: *B. inopinata* (BO1), lane 12: A1-3476 (from sheep), lane 13: A1-3480 (from camel), lane 14: A1-4164 (from cattle), lane 15: A1-3453 (from sheep), lane 16: A1-3470 (from human), lane 17: A1-4141 (from cattle).

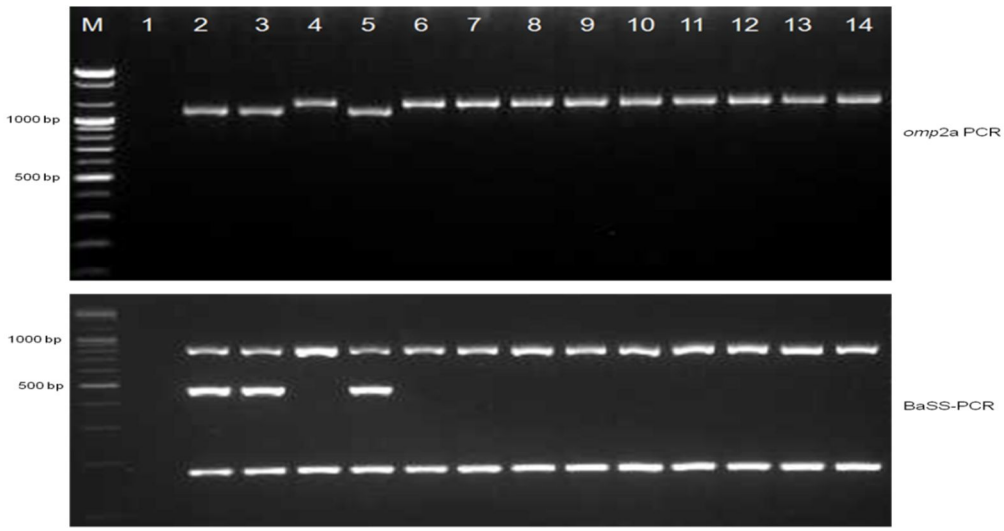


**Figure I-2.** PCR results for *B. abortus* reference strains and Mongolian *B. abortus* isolates from each animal species. Lane M: 100 bp DNA marker, lane 1: Negative control, lane 2: *B. abortus* bv.1 (544), lane 3: *B. abortus* bv.2 (86/8/59), lane 4: *B. abortus* bv.3 (Tyula), lane 5: *B. abortus* bv.4 (292), lane 6: *B. abortus* bv.5 (B3196), lane 7: *B. abortus* bv. 6 (870), lane 8: *B. abortus* bv.9 (C68), lane 9: A1-3476 (from sheep), lane 10: A1-3480 (from camel), lane 11: A1-4164 (from cattle), lane 12: A1-3453 (from sheep), lane 13: A1-3470 (from human), lane 14: A1-4141 (from cattle).

# IV. Discussion

Brucellosis is known to be an important zoonosis that causes major reproductive disorders such as abortion and infertility in livestock; this disease can spread between humans and animals through contact with infected animals or contaminated products [29]. Mongolia is a large and developing country with an economy that is based on the livestock industry and has the second-highest incidence of human brucellosis next to the former Soviet Union [117]. Because the rearing system is mainly based on traditional nomadic livestock rearing, brucellosis can be easily transmitted between animals and humans [145]. Therefore, it is important to identify the characteristics of Mongolian *Brucella* strains accurately using phenotypic and molecular typing techniques.

In our present research, many *Brucella* strains were isolated from diverse animal species in Mongolia over the course of 3 years. Among these strains, 16 *B. abortus* isolates were identified by classical biotyping and molecular detection methods. In the classical biotyping analysis, the 16 Mongolian isolates showed the same results, except in the agglutination test with M and the $CO_2$ requirement. *B. abortus* bv. 3 isolates require $CO_2$ for growth and do not agglutinate with M serum. As some of the isolates demonstrated only weak growth on thionin dye medium, a complementary molecular tool was implemented to confirm these results. In particular, *omp2a*-PCR can be used to determine thionin resistance [12], and biovar discrimination is based on size differences in the *omp2a* gene in *Brucella* species; biovars 1, 2 and 4 produce *omp2a* amplicons that are 120 bp shorter than those of the other biovars [16, 34]. This molecular technique was very helpful for confirming *Brucella* growth on thionin dye medium, reducing risky, laborious and subjective analyses.

In contrast, the characteristics of the untyped strains did not match those of any biovars of *B. abortus* in the current taxonomy. Thus, additional molecular detection methods, such as species-specific multiplex PCR, BaSS-PCR and *omp2a*-PCR, were

performed to evaluate the biovars in greater detail. In agreement with the biotyping results, the molecular methods also demonstrated that the untyped isolates did not belong to bv. 1, 2 or 4. Moreover, comparative 16S rRNA sequencing revealed that in comparison to *B. abortus* reference strains (bvs. 1-6 and 9), the untyped strains harbor an SNP, a C to T transition, in a region of chromosome II (NC_006933.1). This finding also indicates that the untyped Mongolian *B. abortus* isolates possess a distinct genetic feature.

Taking the phenotypic and molecular typing results together, the untyped strains were suspected to be *B. abortus* bv. 7, which was deleted from the *Brucella* taxonomy in 1988. Garin-Bastuji et al [40] also verified the presence of *B. abortus* bv. 7, demonstrating that the four *B. abortus* isolates from the AHVLA and ANSES collections harbor the same phenotypic and molecular characteristics as the former bv. 7 strain. Therefore, these authors proposed the re-introduction of bv. 7 into the *Brucella* taxonomy. The *B. abortus* isolates in our study showing the same polyphasic traits as the former bv. 7 strain might be helpful for proving the existence of *B. abortus* bv. 7. Therefore, our study suggests the possibility of reinstating *B. abortus* bv. 7 into the *Brucella* taxonomy, and the oldest Mongolian strain (99-9971-135) would serve as a suitable reference for *B. abortus* bv. 7 strain. Also, the antigenicity of *Brucella* species has been known that there is no difference depends on biovars [15].

As described above, Mongolian society is based on a nomadic livestock system, and various animal species comingle while being raised, including sheep, goats, cattle, camels and yaks. In fact, the positive rates of sero-surveillance for brucellosis by ELISA were reported to be 0.7% (camels), 1.6% (cattle), 0.4% (sheep) and 0.3% (goats) in Mongolia in 2011 [85]. To prevent the spread of brucellosis among animals, the Mongolian government has managed a national vaccination program for all domestic animals, except camels, since 2000 [29]. However, despite its economic importance in nomadic herds, camel brucellosis appears to be overlooked [120]. Camels are known to be primarily infected by *B. abortus* or *B. melitensis* and to act as silent carriers of

brucellosis [49]. Although the camel population is low (0.7%) in Mongolia [85], camels can spread brucellosis through contact with other domestic animals. Additionally, certain *B. abortus* strains were obtained from various livestock other than cattle, and more strict control and prevention programs for livestock, including camels, are required to minimize economic losses and improve productivity in Mongolia..

# Chapter II.

## Molecular epidemiological characteristic analysis of *Brucella* strains in Mongolia

## Abstract

Mongolia has a high incidence of brucellosis in human and animals due to livestock husbandry. To investigate the genetic characteristics of Mongolian *B. melitensis* isolates, the multi-locus variable-number of tandem-repeat analysis (MLVA)-16 assay was performed with 94 *B. melitensis* isolates. Isolates were identified as *B. melitensis* biovar (bv.) 1 (67), bv. 3 (10) and *B. melitensis* Rev. 1 vaccine strains (17) using a classical biotyping assay and differential multiplex PCR. Of 16 loci, bruce30 had the highest diversity index of 0.685 for *B. melitensis* isoates, followed by bruce18 and bruce16 at 0.506 and 0.503, respectively. MLVA-16 assay indicated that the *B. melitensis* isolates were divided into 29 genotypes, including 4 genotypes of *B. melitensis* Rev. 1. Three human isolates were grouped at 2 genotypes with sheep isolates. These results demonstrate that *B. melitensis* isolates are cross-infected between human and domestic animals and between animal species. Moreover, Mongolian *B. melitensis* isolates had high genetic similarity with Chinese strains compared with other countries, likely due to the proximity between Mongolia and China. The accurate and continuous epidemiological analysis between adjacent countries is required to prevent the spread of brucellosis in these countries.

**Keywords**: *Brucella melitensis*, Epidemiology, MLVA-16, Mongolia, Zoonoses

# I. Introduction

Brucellosis is caused by the genus *Brucella* and is recognized as a zoonotic disease of global importance. Brucellosis has resulted in substantial economic losses due to abortion and slaughtering in animal husbandry of developing and underdeveloped countries [99, 116]. Brucellosis has occurred sporadically in some countries officially declared brucellosis-free [41, 44]. Among *Brucella* species, *Brucella melitensis*, *B. abortus, B. suis* and *B. canis* are responsible for more than 500,000 human infections per year worldwide, and these infections are accompanied early by undulant fever [99]. *B. melitensis* is considered the most pathogenic strain within the species. It has been isolated from various hosts, such as goat, sheep, camel and fish, and human infection by *B. melitensis* has been frequently reported [28, 49, 144].

Mongolia is a developing country, and because Mongolia has a nomadic livestock rearing system, brucellosis is easily to spread between different animal species and humans [128]. In the 1950s, brucellosis was prevalent in Mongolia, and seroprevalence rates were ranged from 2% to 17% in goats, sheep and cattle [145]. Consequently, the Mongolian government implemented a mass vaccination strategy by applying *B. melitensis* Rev. 1 vaccine for sheep and goats and *B. abortus* S19 vaccine for cattle and yaks from 1975 to 1985, and this programme re-started in 2000 [145]. Nonetheless, human brucellosis has been increased rapidly during 1990-2000 [128]. Incidence (annual cases per million of population) of human brucellosis in Mongolia was over 605.9 [99].

There have been some publications on the serological surveillance of brucellosis and molecular typing of *Brucella* isolates in Mongolia [68, 145]. In the previous analysis of semi-nomadic herders in central Mongolia, it also demonstrated that human brucellosis was related with animal brucellosis [35]. However, in-depth molecular genotyping studies to understand the characteristics of Mongolian *B. melitensis* have

been rare. Accurate identification and epidemiological analysis are considered crucial for the control and prevention of brucellosis in Mongolia.

Several molecular techniques have been used to investigate the epidemiological characteristics of *Brucella* strains. Pulse-field gel electrophoresis (PFGE) and polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) have been developed but have shown limited discriminative power [131, 134], Recently, multiple-locus variable-number of tandem repeat analysis (MVLA) had been useful for trace-back and the analysis of genetic characteristics of *Brucella* isolates [70].

Therefore, this study aimed to investigate the genetic characteristics and relatedness of *B. abortus* and *B. melitensis* isolates from animals and human in Mongolia and compare epidemiological relationships with other counties using an MLVA assay. The accurate epidemiological analysis of *Brucella isolates* might be helpful to control and prevent brucellosis in Mongolia. To our knowledge, this is the first report on molecular genotyping of *B. melitensis* from animals and human in Mongolia.

## II. Materials and methods

### 1. Bacterial strains

Twenty-two *Brucella* reference strains, 16 *B. abortus* and 94 *B. mellitensis* were used (Table S1). Each *Brucella* strains originated from sheep (3), cattle (9), camel (1) and human (3) in *B. abortus*, and sheep (62), goats (24) and human (8) in *B. melitensis* from 10 provinces of Mongolia from 2010 to 2014. Animal-derived strains were isolated from aborted-materials such as placenta and stomach contents or spleen of aborted fetus in sheep and goats. Human isolates were obtained from blood samples of brucellosis-infected patients.

*Brucella* strains were cultivated on tryptic soy agar (BD, Franklin Lakes, NJ) and modified *Brucella* selective medium supplemented with 5% bovine serum (GIBCO, Grand Island, NY, USA) for 3-4 days at 37 ˚C (14). All isolates were identified using classical biotyping assays based on $CO_2$ requirement, urea hydrolysis, oxidase production, dye sensitivity (thionin and basic fuchsin), agglutination with monospecific sera (anti-A, anti-M, anti-R), and phage typing (Tbilisi, Weybridge, $Izatnagar_1$, and R/C) (15). DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen Korea, Ltd., Seoul, Korea). Differential multiplex PCR was used to confirm the species of the isolates [61].

### 2. MLVA assay

MLVA assay using 16 loci was performed according to a previously described method [70]. The PCR conditions were modified as follows: an initial step of 96 ˚C for 5 min; followed by 35 cycles of 96 ˚C for 30 s, 60 ˚C for 30 s, and 70 ˚C for 1 min; the PCR ended with a final extension step at 70 ˚C for 5 min. Amplification was performed using a T3000 thermocycler (Biometra, Gottingen, Germany), and the amplification products were developed by electrophoresis on 2.5% agarose gels. The PCR product size was determined by using 25/100-bp DNA ladder (Bioneer, Daejeon, South Korea)

as a molecular size marker or the DNA chips kit in the Agilent 2100 Bioanalyzer (Agilent Technologies, Deutschland GmbH, Germany). The number of repeat copies was estimated by comparing amplified PCR products with those of reference *B. abortus* and *B. melitensis*.

**3. Data analysis**

Fragment sizes of 16 loci were converted to copy numbers of tandem repeat unit (U) and analysed by Bionumerics, ver. 5.1 (Applied Maths, St-Martens-Latem, Belgium). Genetic diversity was calculated using the Hunter Gaston Diversity Index (HGDI) [55] and the online V-DICE bioinformatics tool available at the HPA website (http://www.hpa.org.uk/srmd/bioinformatics/tools/tools.htm). HGDI values range from 0 (no variation) to 1 (high variation). Genotype profiles of MLVA-16 for *B. melitensis* isolates were listed in Table S1. Cluster and minimum-parsimony analysis were conducted using the categorical coefficient and UPGMA (unweighted pair group method using arithmetic averages) algorithm. Comparisons with other countries' isolates were performed using the *Brucella*2012/2013 MLVA database (http://mlva.u-psud.fr) [70]. Comparisons were restricted to *Brucella* isolates containing detailed information on biovar, host and country.

# III. Results

## 1. Identification of *Brucella* isolates using classical biotyping and multiplex PCR

Sixteen *B. abortus* were identified as *B. abortus* bv. 3 (9) and untype (7), and ninety-four *B. melitensis* strains were identified as bv. 1 (67), bv. 3 (10) and untype (17) using conventional biotyping methods [62, 94]. The classical biotyping patterns of *B. abortus* untype strains were identical with those of former *B. abort*us bv. 7, and the remaining 17 *B. melitensis* untype isolates showed no growth in thionin dye medium. These each untyped *Brucella* strains were confirmed as *B. abortus* and *B. melitensis* Rev. 1 vaccine strains using the differential multiplex PCR assay [61].

## 2. Genotyping of *Brucella* strains by MLVA-16

In the MLVA-16 assay including panel 1, 2A and 2B loci, all of the *B. abortus* strains showed the same profile patterns for 8 markers of panel 1 (4-5-3-12-2-2-3-1) and 3 markers of 2A (6-42-8). Additionally, of the 5 markers of panel 2B, bruce 16 and 30 displayed only one allelic type, whereas bruce 04, 07 and 09 markers had two, three and five allelic types, respectively (Table II-1). The nine *B. abortus* bv. 3 isolates were divided into four different genotypes, though seven untyped isolates were found to be in relation with only one genotype (Table II-1 and Fig. II-1). The untyped *B. abortus* isolates predicted to be bv. 7, showed an identical MLVA profile with the Mongolian strains (99-9971-135 and 99-9971-159) in a recent report [40] (Table II-1). Also, Korean *B. abortus* bv. 1 strains were grouped in a cluster G, with Chinese, European and so on.

In case of *B. melitensis* strains, 77 of *B. melitensis* bv. 1 and bv. 3 isolates were divided into 25 genotypes with genetic similarity coefficients ranging from 76.2-100 % (Fig. II-2). In the 25 genotypes, two genetic patterns (1-5-3-13-2-3-3-2-4-40-8-5-4-3-4-6 and 1-5-3-13-2-3-3-2-5-40-8-5-4-3-3-7) were observed to be major profiles.

Regarding markers, 7 loci (bruce06, bruce08, bruce11, bruce12, bruce42, bruce45 and bruce55) in panel 1, 2 loci (bruce19 and bruce21) in panel 2A, and 1 locus (bruce30) in panel 2B were homogeneous in Mongolian *B. melitensis* isolates. The 17 *B. melitensis* Rev. 1 vaccine strains were divided into 4 genotypes with genetic similarity coefficients ranging from 90.8-100 % (Fig. II-2). The main profile of *B. melitensis* Rev. 1 strains was 3-4-2-13-4-2-3-3-8-36-6-2-4-7-3-6 (Table II-2).

The diversity indexes were 0.685 in bruce30 (panel 2B), 0.506 in bruce18 (panel 2A), 0.503 in bruce16 (panel 2B) and 0.377 in bruce43 (panel 1). In *B. melitensis* Rev. 1 strains, two loci, bruce09 and bruce30 of panel 2B, had diversity indexes of 0.382 and 0.228, habouring two and three allelic types, respectively (Table II-3). In addition, *B. melitensis* and Rev. 1 isolates were differentiated at 8 loci (bruce06, bruce08, bruce11, bruce42, bruce55, bruce19, bruce21, and bruce09) (Table II-2).

### 3. Epidemiological relationships between *Brucella* strains

In the MLVA data, a total of 60 *B. abortus* strains including Web database were divided into 2 major clusters (I and II), and cluster II was split into 5 subgroups (II-1~II-5) (Figure II-1). Group I included *B. abortus* isolates from Africa, and the II-1 subgroup mainly consisted of European strains. All the Mongolian *B. abortus* isolates were included in two subgroups: II-2 and II-3. Of a total of 46 genotypes, the untyped isolates clustered to one genotype, II-2, with representative of *B. abortus* bv. 7 strain in a previous study [40]. One strain (A1-4170) out of the nine *B. abortus* bv. 3 isolates was belonged to the II-2 and closely related to the Chinese strains from cattle. Most of the bv. 3 isolates, including those obtained from camels, were located in the II -3 and these were also grouped with the Chinese *B. abortus* bv. 3.

Ninety-four *B. melitensis* isolates, including 17 Rev. 1 strains were clustered into six groups corresponding to 29 genotypes. Five groups (Ⅰ-Ⅴ) were closely related to *B. melitensis*, whereas group Ⅵ consisted of Rev. 1 isolates (Fig. II-2). Human-derived

isolates were located in groups Ⅰ, Ⅲ and Ⅴ. In particular, 3 human strains (HU-4, 6, and 7) in groups Ⅰ and Ⅲ had the same genotype as sheep isolates. Of the eight human isolates, these three isolates were linked to animal strains based on molecular analysis. And, *B. melitensis* isolated from sheep in the Khentii province were primarily in groups Ⅰ to Ⅲ except for three isolates (Go-5, N-1 and N-6). Mongolian *B. melitensis* in Sukhbaatar were evenly distributed between groups Ⅰ to Ⅴ. In contrast, *B. melitensis* strains from sheep in Dornod were only in group Ⅳ; thus, they seemed to be localized in this area. *B. melitensis* isolates from sheep and goats in these three provinces appeared to be correlated with each other due to mixed breeding among farms.

*B. melitensis* Rev. 1 isolates in group Ⅵ had 4 genotypes, including 2 types habouring only 1 strain. They were all isolated from aborted materials, and the genotype changes might be due to quality control of the vaccine strain or their pathogenicity in pregnant animals.

Mongolian domestic isolates were compared with foreign strains using an MLVA-16 assay. In the minimum parsimony analysis, *B. melitensis* isolates were shown to be distributed worldwide and clustered into three major clades composed of clusters Ⅰ, Ⅱ and Ⅲ (Fig. II-3). Mongolian isolates belonged to cluster Ⅰ, and a significant association with Chinese strains was observed. Chinese strains were related to Turkey strains, linking Asian and some European strains. Clusters Ⅱ and Ⅲ were divided into east and west Mediterranean groups.

**Table II-1.** MLVA-16 profile patterns for the 16 Mongolian *B. abortus* isolates.

| Strain | Biovar | Animals | Panel 1 | | | | | | | | Panel 2A | | | Panel 2B | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | bruce 06 | bruce 08 | bruce 11 | bruce 12 | bruce 42 | bruce 43 | bruce 45 | bruce 55 | bruce 18 | bruce 19 | bruce 21 | bruce 04 | bruce 07 | bruce 09 | bruce 16 | bruce 30 |
| A1-3452 | untype | Sheep | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 5 | 6 | 4 | 3 | 3 |
| A1-3453 | untype | Sheep | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 5 | 6 | 4 | 3 | 3 |
| A1-3470 | untype | Human | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 5 | 6 | 4 | 3 | 3 |
| A1-3476 | 3 | Sheep | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 5 | 5 | 9 | 3 | 3 |
| A1-3480 | 3 | Camel | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 5 | 5 | 9 | 3 | 3 |
| A1-4111 | untype | Human | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 5 | 6 | 4 | 3 | 3 |
| A1-4113 | untype | Human | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 5 | 6 | 4 | 3 | 3 |
| A1-4141 | untype | Cattle | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 5 | 6 | 4 | 3 | 3 |
| A1-4142 | untype | Cattle | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 5 | 6 | 4 | 3 | 3 |
| A1-4164 | 3 | Cattle | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 4 | 5 | 8 | 3 | 3 |
| A1-4165 | 3 | Cattle | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 4 | 5 | 8 | 3 | 3 |
| A1-4166 | 3 | Cattle | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 4 | 5 | 8 | 3 | 3 |
| A1-4167 | 3 | Cattle | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 4 | 5 | 8 | 3 | 3 |
| A1-4168 | 3 | Cattle | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 4 | 5 | 8 | 3 | 3 |
| A1-4169 | 3 | Cattle | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 4 | 5 | 7 | 3 | 3 |
| A1-4170 | 3 | Cattle | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 4 | 7 | 6 | 3 | 3 |
| 99-9971-135[1] | 7 | Cattle | 4 | 5 | 3 | 12 | 2 | 2 | 3 | 1 | 6 | 42 | 8 | 5 | 6 | 4 | 3 | 3 |

[1] Garin-Bastuji et al., 2014.

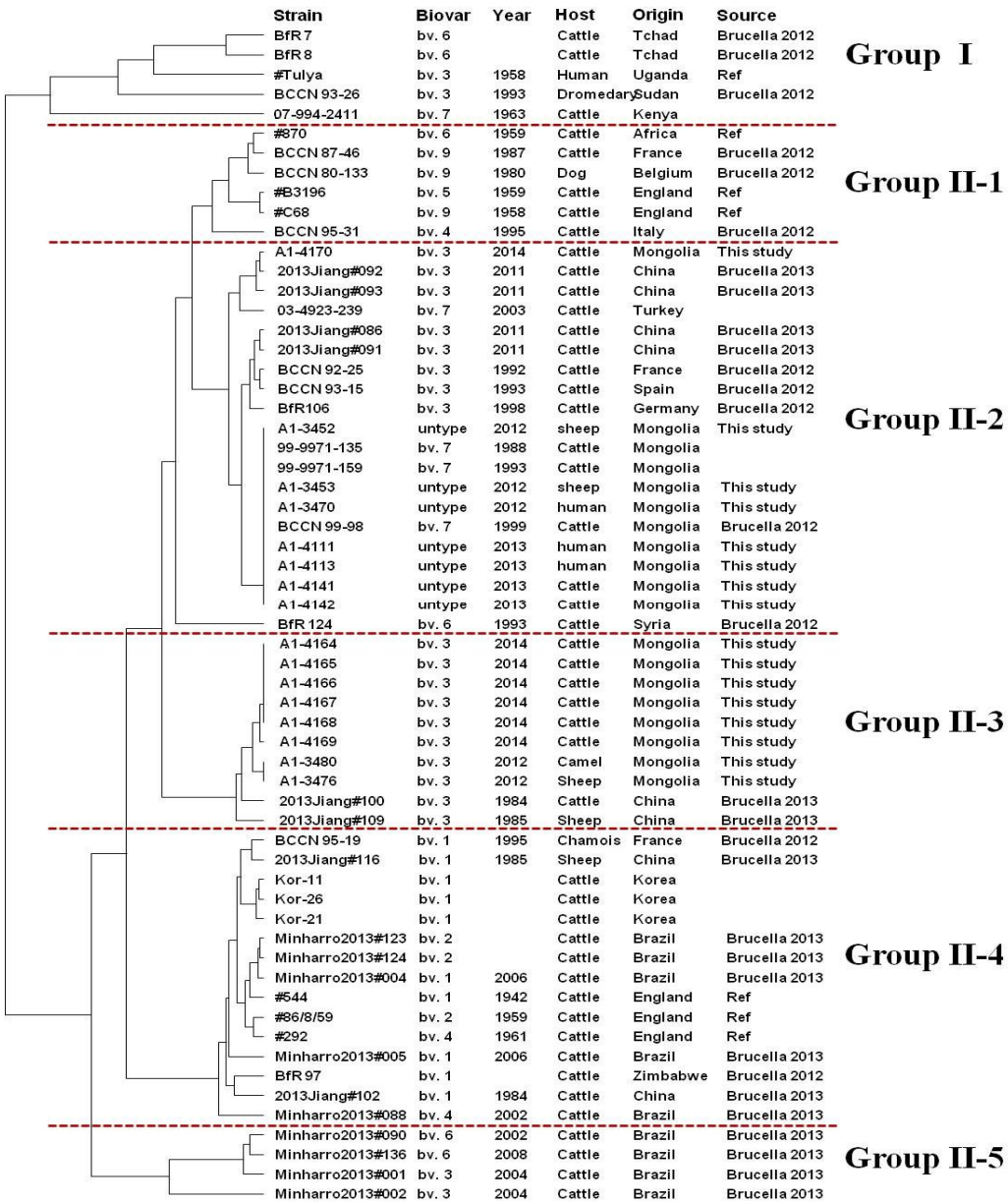


**Figure II-1.** Clustering analysis of 60 *B. abortus* strains including 16 Mongolian strains. The MLVA data of these strains were downloaded from an MLVA database (*Brucella*2012 and *Brucella*2013). The strain name, biovar, isolation year, host, origin and source of each strain are shown.

**Table II-2.** Genetic profiles of Mongolian *B. melitensis* and Rev. 1 strains

| Species | MLVA profiles (16 loci) | | | | | | | | | | | | | | | | No. of strains | Animal hosts (No. of strains) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 06 | 08 | 11 | 12 | 42 | 43 | 45 | 55 | 18 | 19 | 21 | 04 | 07 | 09 | 16 | 30 | | |
| *B. melitensis* (n=77) | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 4 | 40 | 8 | 5 | 4 | 3 | 4 | 6 | 13 | Sheep (13) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 5 | 40 | 8 | 5 | 4 | 3 | 3 | 7 | 13 | Sheep (10), goat (3) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 4 | 40 | 8 | 5 | 4 | 3 | 3 | 7 | 8 | Sheep (6), goat (2) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 5 | 40 | 8 | 5 | 3 | 3 | 3 | 5 | 6 | Sheep (5), human (1) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 4 | 40 | 8 | 5 | 3 | 3 | 3 | 5 | 5 | Sheep (4), goat (1) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 4 | 40 | 8 | 5 | 4 | 3 | 3 | 6 | 4 | Sheep (3), goat (1) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 5 | 40 | 8 | 5 | 4 | 3 | 3 | 6 | 3 | Sheep (1), human (2) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 5 | 40 | 8 | 5 | 4 | 3 | 3 | 7 | 3 | Goat (3) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 4 | 40 | 8 | 5 | 4 | 3 | 4 | 6 | 2 | Sheep (2) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 4 | 40 | 8 | 5 | 4 | 3 | 4 | 7 | 2 | Goat (2) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 5 | 40 | 8 | 4 | 4 | 3 | 3 | 6 | 2 | Human (2) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 5 | 40 | 8 | 6 | 4 | 3 | 5 | 4 | 2 | Sheep (2) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 5 | 40 | 8 | 5 | 4 | 3 | 4 | 7 | 2 | Sheep (1), goat (1) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 4 | 40 | 8 | 4 | 4 | 3 | 4 | 6 | 1 | Sheep (1) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 4 | 40 | 8 | 4 | 4 | 3 | 4 | 5 | 1 | Sheep (1) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 4 | 40 | 8 | 5 | 4 | 3 | 6 | 6 | 1 | Sheep (1) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 5 | 40 | 8 | 4 | 4 | 3 | 3 | 5 | 1 | Human (1) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 5 | 40 | 8 | 6 | 4 | 3 | 3 | 7 | 1 | Human (1) |
| | 1 | 5 | 3 | 13 | 2 | 2 | 3 | 2 | 5 | 40 | 8 | 6 | 4 | 3 | 9 | 7 | 1 | Human (1) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 4 | 40 | 8 | 5 | 4 | 3 | 4 | 5 | 1 | Sheep (1) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 4 | 40 | 8 | 6 | 4 | 3 | 4 | 6 | 1 | Goat (1) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 5 | 40 | 8 | 5 | 4 | 3 | 4 | 6 | 1 | Sheep (1) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 5 | 40 | 8 | 5 | 4 | 3 | 3 | 5 | 1 | Sheep (1) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 5 | 40 | 8 | 5 | 4 | 3 | 3 | 4 | 1 | Sheep (1) |
| | 1 | 5 | 3 | 13 | 2 | 3 | 3 | 2 | 5 | 40 | 8 | 5 | 4 | 3 | 3 | 8 | 1 | Sheep (1) |
| *B. melitensis* Rev. 1 (n=17) | 3 | 4 | 2 | 13 | 4 | 2 | 3 | 3 | 8 | 36 | 6 | 2 | 4 | 7 | 3 | 6 | 11 | Sheep (4), goat (7) |
| | 3 | 4 | 2 | 13 | 4 | 2 | 3 | 3 | 8 | 36 | 6 | 2 | 4 | 6 | 3 | 6 | 4 | Sheep (2), goat (2) |
| | 3 | 4 | 2 | 13 | 4 | 2 | 3 | 3 | 8 | 36 | 6 | 2 | 4 | 7 | 3 | 5 | 1 | Goat (1) |
| | 3 | 4 | 2 | 13 | 4 | 2 | 3 | 3 | 8 | 36 | 6 | 2 | 4 | 7 | 3 | 7 | 1 | Sheep (1) |

**Table II-3.** HGDI values obtained from *B. melitensis* and Rev. 1 strains

| | Locus | HGDI (*B. melitensis*) | | | | HGDI (*B. melitensis* Rev.1) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | DI | CI | K | max (pi) | DI | CI | K | max (pi) |
| **Panel 2B** | bruce04 | 0.238 | 0.117-0.358 | 3 | 0.870 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce07 | 0.248 | 0.136-0.360 | 2 | 0.857 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce09 | 0.000 | 0.000-0.089 | 1 | 1.000 | 0.382 | 0.169-0.596 | 2 | 0.765 |
| | bruce16 | 0.503 | 0.420-0.587 | 5 | 0.636 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce30 | 0.685 | 0.641-0.730 | 5 | 0.390 | 0.228 | 0.000-0.480 | 3 | 0.882 |
| **Panel 2A** | bruce18 | 0.506 | 0.504-0.509 | 2 | 0.506 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce19 | 0.000 | 0.000-0.089 | 1 | 1.000 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce21 | 0.000 | 0.000-0.089 | 1 | 1.000 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| **Panel 1** | bruce06 | 0.000 | 0.000-0.089 | 1 | 1.000 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce08 | 0.000 | 0.000-0.089 | 1 | 1.000 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce11 | 0.000 | 0.000-0.089 | 1 | 1.000 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce12 | 0.000 | 0.000-0.089 | 1 | 1.000 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce42 | 0.000 | 0.000-0.089 | 1 | 1.000 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce43 | 0.377 | 0.279-0.474 | 2 | 0.753 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce45 | 0.000 | 0.000-0.089 | 1 | 1.000 | 0.000 | 0.000-0.314 | 1 | 1.000 |
| | bruce55 | 0.000 | 0.000-0.089 | 1 | 1.000 | 0.000 | 0.000-0.314 | 1 | 1.000 |

HGDI: Hunter-Gaston diversity index, which measures the variation of the number of repeats at each locus, and ranges from 0.0 (no diversity) to 1.0 (complete diversity); DI: diversity index, CI: confidence interval; K: Number of different repeats present at this locus in this sample set; max (pi): Fraction of samples that have most frequent repeat number in this locus (range 0.0 to 1.0).

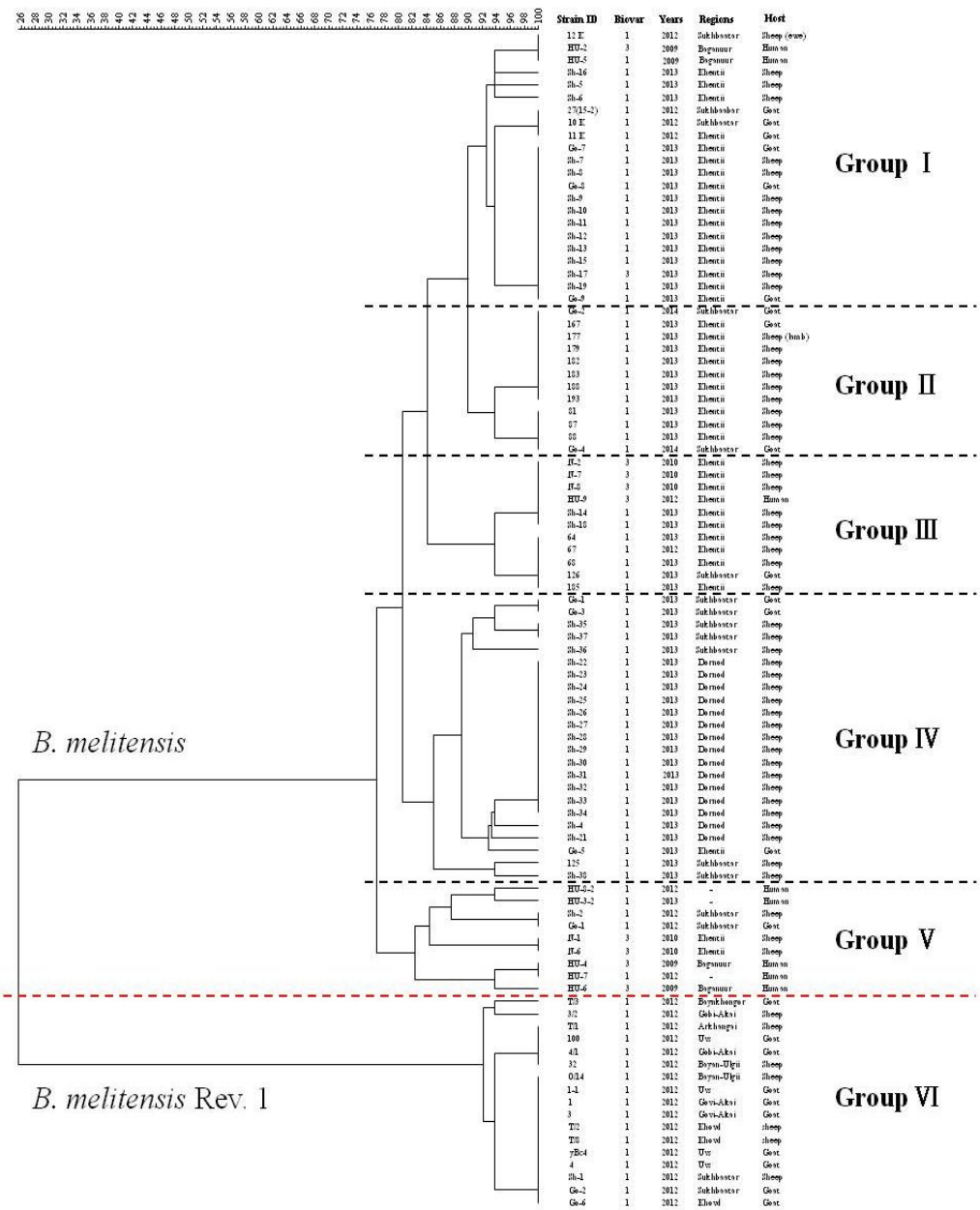


**Figure II-2.** Dendrogram for 94 *B. melitensis* and Rev. 1 vaccine isolates based on an MLVA-16 assay. The following data for isolates is included: strain identification (ID), biovar, year, geographic region and host.

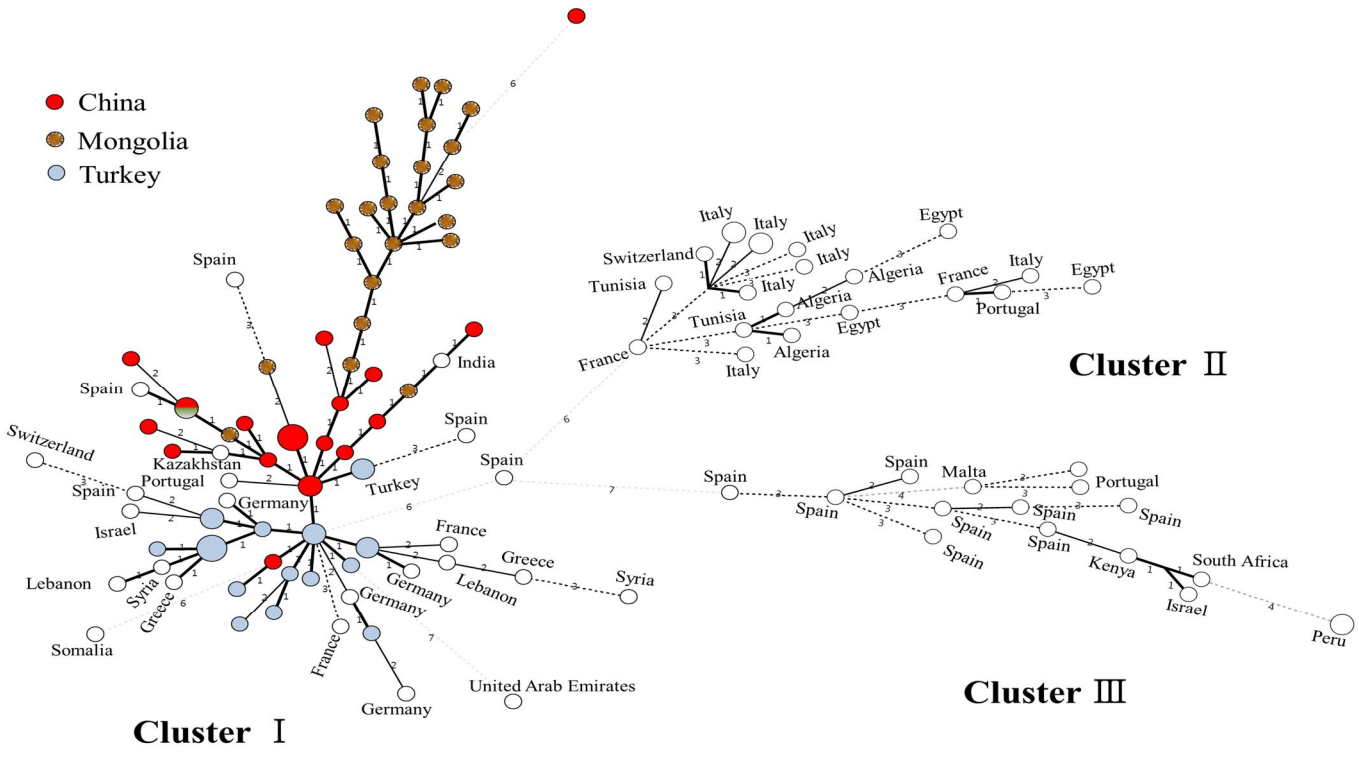


**Figure II-3.** Minimum parsimony analysis of Mongolian isolates with other foreign *B. melitensis* strains. Mongolia, China and Turkey strains are indicated with a different colour.

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# IV. Discussion

Brucellosis a major zoonosis; however, it is also considered a food-borne disease in some countries, such as Mongolia, that use unpasteurized milk and raw meat from sheep and goats. Brucellosis in Mongolia is a prevalent disease in animals and humans; livestock seroprevalence ranged from 0.5-1.8 %, depending on the species, in a national brucellosis serosurvey of Mongolia in 2011, and the human brucellosis incidence was the second highest in the world [99, 128]. Another study from 2010 reported that the seroprevalence of brucellosis in Mongolia was 27.3 % in humans, followed by sheep (6.2 %), goats (5.2 %), cattle (16.0 %), horses (8.3 %) and dogs (36.4 %) [145]. This difference was significantly associated with provincial level [128].

Based on serological surveys, MLVA-16 assays must be used to assess the correlation for risk factors of brucellosis within Mongolia. Some molecular genotyping of *B. abortus* (n=7) and *B. melitensis* (n=14) from seven provinces in Mongolia had been reported previously [68]. In this first report of genotyping of *Brucella* in Mongolia, the *Brucella* isolates (from sheep and cows) circulated at three provinces (Khentii, Bulgan and Khubsgul) bordering with Russia.

A previous study compared the genetic characterization of *B. melitensis* using an MLVA-12 assay from three adjacent countries: Mongolia (n=18), Russia (n=6) and Azerbaijan (n=4) [69]. This study also demonstrated that *B. melitensis* isolates from these contiguous countries were very similar and thus appeared to originate from a common ancestor. These previous Mongolian *B. melitensis* strains were all bv. 2, but our study found two biovars (bv. 1 and 3) and Rev. 1 vaccine strains. The difference of these biovars depends on agglutination with anti-rabbit monospecific AMR sera. These differences in results might be due to misreading of agglutination.

In the case of *B. abortus* strains, consistently with the classical biotyping and molecular detection results, the MLVA profile also indicated that all Mongolian isolates belonged to *B. abortus*. The Mongolian *B. abortus* bv. 3 and untyped were

located very close to each other in the II-2 and II-3 subgroups (Fig. II-1). The untyped strains were identical with previous bv. 7 [40] and exhibited a consistent genotype, regardless of the animal species and province in Mongolia. These isolates might represent a linkage with the past, as they have spread to a variety of animals and humans through contact since at least the 1980s. Therefore, our study suggests the possibility of reinstating *B. abortus* bv. 7 into the *Brucella* taxonomy, and the oldest Mongolian strain (99-9971-135) would serve as a suitable reference for *B. abortus* bv. 7 strain. Furthermore, eight biovar 3 strains grouped into the II-3 clustered with two *B. abortus* bv. 3 strains from sheep and cattle in China (Fig. II-1). Accordingly, the Mongolian isolates appear to, until recently, have had close genetic relationships with the strains from China. Kulakov et al. [68] performed MLVA typing for *B. abortus* bv. 3 isolates from 7 provinces in Mongolia, and Chen et al. [15] reported that *B. abortus* bv. 3 is the most prevalent type in Inner Mongolia. Because Inner Mongolia showed the highest incidence of brucellosis in China, its prevalent biovars might be related to those of Mongolia due to the geographical proximity. Such knowledge about the distribution of predominant biovars could provide key information regarding the source of infections [6].

Meanwhile, *B. melitensis* in our study were classified as bv. 1, 3 and Rev. 1 strains. *B. melitensis* bv. 3 has been isolated in several countries but is particularly prevalent in Mediterranean and Middle East countries. In Asia, the frequency of *B. melitensis* bv. 3 outbreak has increased significantly from inner Mongolia to other areas in China since 2005 [58, 141]. Biovar 1 has predominantly been observed in Latin America, and biovar 2 had been observed in some southern European countries [7].

Compared with previous published genotyping data from other countries, Mongolian *B. melitensis* strains were closely associated with Chinese strains. Similar to the recent genotyping studies of *B. melitensis* from animals and humans in geographically adjacent countries, such as China [58, 141], *B. melitensis* isolates were grouped with each other regardless of livestock species and/or human. *Brucella* strains from eastern

and southern parts in Mongolia were closely related to Inner Mongolia in China; thus, many *B. melitensis* bv. 1 and bv. 3 from these areas were very similar to *Brucella* isolates from Chinese strains. According to a recent report on Inner Mongolia, three stages of incidence occurred: high incidence (1950-1960s), decline (1970-1980s) and re-emergence (1990-2000s), which were predominated by *B. melitensis* bv. 2 and bv. 3, *B. abortus* bv. 3, and *B. melitensis* bv. 1, respectively, indicating changes in the predominant biovars [15]. The *B. melitensis* strain has remained endemic in many parts of the world, including Italy, Spain, Latin America, the Middle East, parts of Africa, and Asia, despite strong efforts for control and prevention.

Mongolia maintains the traditional nomadic rearing system for several types of livestock. These nomadic camps move along pasture, especially in warm spring and summer [145]. In the nomadic process, the possibility of contact with other nomadic livestock or wildlife is increased. Thus, *Brucella* strains from animal species of different areas might be included in the same group and have high similarity. In addition, individuals in the nomadic herds are more likely to transmit brucellosis through injury during handling and/or consumption of half-cooked liver from slaughtering livestock and from consumption of contaminated raw milk and cheese [145]. Thus, herder families can also be infected with brucellosis via direct or indirect contact. Although only eight human *B. melitensis* and three *B. abortus* isolates were included here, most were obtained from traditional nomadic herds. Three isolates were obtained from children under 10 years of age, suggesting that continuous exposure and transmission of brucellosis occurs within traditional Mongolian nomadic camps, as described previously [128]. Other human *B. melitensis* strains were obtained from veterinarians or farm workers.

Also, camels are known to be primarily infected by *B. abortus* or *B. melitensis* and act as silent carriers of brucellosis [49]. However, despite its economic importance in nomadic herds, camel brucellosis appears to be overlooked [120].. Therefore, more

strict control and prevention programs for livestock, including camels, are required to minimize economic loss in Mongolia.

Meanwhile, *B. melitensis* Rev. 1 vaccine strains showed high similarity to each other at 92%, despite different geographical distributions. This finding is likely due to the nationwide vaccination programme in Mongolia. As reported previously, the *B. melitensis* Rev. 1 vaccine strain had good efficacy for preventing brucellosis in small ruminants but induced abortion in pregnant animals depending on the administration dose, time and route [8]. Because the recovery of the *B. melitensis* Rev. 1 vaccine strain might be hazardous to animals and humans, a more careful vaccination programme is required for small ruminants considering condition of individuals [58]..

Our results demonstrate the need for accurate identification and molecular typing of *Brucella* strains to improve epidemiological surveillance efficiency and determine pathogenic relationships and trace-back in brucellosis outbreak regions. These studies will be helpful in determining the proper countermeasures for the prevention, surveillance, and management of brucellosis in Mongolia, neighbouring countries, and countries involved in the trade and distribution at-risk brucellosis species worldwide.

# Chapter III.

## Differential diagnosis of *Brucella abortus* by real-time PCR based on a single-nucleotide polymorphisms

## Abstract

To diagnose brucellosis effectively, many genus- and species-specific detection methods based on PCR have been developed. With conventional PCR assays, real-time PCR techniques have been developed as rapid diagnostic tools. Among them, real-time PCR using hybridization probe (hybprobe) has been recommended for bacteria with high DNA homology among species, with which it is possible to make an accurate diagnosis by means of an amplification curve and melting peak analysis. A hybprobe for *B. abortus* was designed from a specific single-nucleotide polymorphism (SNP) on the *fbaA* gene. This probe only showed specific amplification of *B. abortus* from approximately the 14th cycle, given a melting peak at 69ºC. The sensitivity of real-time PCR was revealed to be 20 fg/*μl* by 10-fold DNA dilution, and the detection limit was 4 CFU in clinical samples. This real-time PCR showed greater sensitivity than that of conventional PCR and previous real-time PCR based on Taqman probe. Therefore, this new real-time PCR assay could be helpful for differentiating *B. abortus* infection with rapidity and accuracy.



**Keywords**: *Brucella abortus*, *fbaA* gene, hybprobe, real-time PCR, SNP.

# I. Introduction

Brucellosis is known as a major zoonotic disease that can cause reproductive problems, such as abortion, stillbirth or infertility in livestock and wild animals [78, 138]. The genus *Brucella* consists of ten species; six species (*Brucella abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis* and *B. neotomae*) considered classic members and four species (*B. ceti*, *B. pinnipedialis*, *B. microti* and *B. inopinata*) considered atypical types of *Brucella.* So far, classification of *Brucella* species has been mainly based on host preferences and classical phenotypic biotyping [78, 137]. Moreover, the genus expansion is still being processed, with the recent addition of *B. papionis* [137].

In terms of the diagnosis of brucellosis, serological assays and bacterial cultivation have mainly been used. Serologic methods are very sensitive and rapid methods to perform, but sometimes false-positive reactions occur with cross-reactive bacteria, such as *Yersinia enterocolitica* O:9, due to the similar structure of the O-chain in the smooth lipopolysaccharide portion [9, 51]. In contrast, bacterial culture is considered a 'gold standard' with high specificity, but it is time-consuming and also requires a highly trained workforce and a well-equipped laboratory due to the biohazard risks with *Brucella* [133]. Also, the mean sensitivity of bacterial culture was about 46%, and it may be different depends on the type of sample, sample site, sample numbers, volume of inoculums, culture technique and expertise. Properly performed bacterial isolation and biotyping can be more sensitive than serology in identifying infected cattle in herds. However, the amount of effort required and the expense incurred makes this test impractical for use in large scale surveillance program [147].

To overcome these disadvantages, molecular detection methods have been introduced as an alternative for diagnosing brucellosis. Many genus- or species-specific PCR assays, using 16S rRNA and the *bcsp31*, IS*711* and *omp2* genes, have been developed [11, 16, 34, 47, 51]. Additionally, various multiplex PCRs that can differentiate at the species level have been established [61, 73, 77]. All of these

molecular detection methods are very effective for detecting *Brucella* strains [106]. Since the development of conventional PCR assays, real-time PCR and loop-mediated isothermal amplification assay (LAMP)-PCR has been introduced as rapid diagnostic tools. Recently, the application of single nucleotide polymorphisms (SNPs) in the microbiological field has shown some merits for diagnosing bacteria with high homology of their DNA [115]. SNP-based PCR assays have been introduced for differentiating bacterial strains and species [78].

Here, we developed a new real-time PCR assay with a hybprobe from a specific SNP to distinguish *B. abortus* from other *Brucella* species. Real-time PCR assay using this hybprobe could diagnose rapidly, using an amplification curve with real-time monitoring, and exactly, using melting peak analysis [47], so it is expected to provide more sensitive, rapid and accurate diagnostic efficiency in detecting *B. abortus* infection.

# II. Materials and methods

## 1. Bacterial strains and DNA samples

A total of 296 *Brucella* strains were included: 22 *Brucella* reference strains; 110 Mongolian isolates (16 *B. abortus* and 94 *B. melitensis*); 156 Korean isolates (84 *B. abortus* and 72 *B. canis*); and 8 non-*Brucella* strains reported to be serologically cross-reactive or phylogenetically similar bacteria (Table III-2). *B. abortus* and *B. melitensis* from Mongolia were provided by Institute of Veterinary Medicine (IVM), through a collaborative project conducted from 2012 to 2014. The isolates from Mongolia were *B. abortus* bv. 3 (9 strains) and untype (7) and *B. melitensis* bv. 1 (67), bv. 3 (10) and Rev. 1 (17). In addition, all of the Korean *B. abortus* bv. 1 was obtained from slaughtered cattle with brucellosis beginning in 2008, and the *B. canis* was from dog-breeding farms during 2002-2011. All of the *Brucella* isolates were identified by the classical biotyping assay including colony morphology, lysis by phages, oxidase, urease activity, growth on dyes and agglutination with monospecific sera (anti-A, -M and -R) [125] and also confirmed specific bands for *Brucella* species by the differential multiplex PCR [61]. Genomic DNA for real-time PCR was extracted using a Blood & Tissue kit (Qiagen Ltd., Seoul, South Korea) per the manufacturer's instructions.

## 2. Hybprobe design and real-time PCR conditions

To develop *B. abortus*-specific real-time PCR assays, comparative sequence analysis, using *fbaA* gene region in whole genome sequences and/or partial sequences of 22 *Brucella* reference strains, was performed with the CLC Main Workbench software program version 6.0 (Insillicogen Inc., Aarhus N, Denmark). Based on the new *B. abortus*-specific SNP sites, the primer and probe sets were designed and developed using BEACON designer (Sigma-Aldrich, St. Louis, MO, USA).

Real-time PCR with hybprobe was performed using 4.0 μL of 5 × genotyping master mix, 0.5 μL of each primer, 0.3 μL of each hybprobe, 13.4 μL of D.W. and 1.0 μL of

DNA in a 20 μL total volume. After centrifugation for the removal of bubbles from the PCR plate, amplification and melting curve analysis were conducted using a LightCycler® 480 Ⅱ (Roche Diagnostic, Mannheim, Germany). The real-time PCR amplification was performed with an initial denaturation step of 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 sec, 65 °C for 15 sec and 68 °C for 15 sec. After amplification, melting analysis was performed at between 40 °C and 80 °C at a rate of increase of 0.1 °C.

**Table III-1 . Primers and probes for detection of *B. abortus* using specific SNPs**

| | | Sequence (5'- 3') | Comment |
|---|---|---|---|
| Primer | Forward | GATGCGCCGGTTATCCTG | 176 bp size |
| | Reverse | GTGAAGCCCGCCTGGATG | |
| Probe | Anchor | ACGGAGCATGATGTCATTGGCATAGGAACG | SNP site[a)] |
| | Sensor | ATAGATTTCCG**C***CACGGCATCCATC | |

a) Single nucleotide polymorphism.

### 3. Specificity and sensitivity of real-time PCR assay:

The specificity of real-time PCR assay using 22 *Brucella* reference strains, *Brucella* isolates and non-*Brucella* bacteria was assessed (Table III-2). Its sensitivity was determined from a DNA concentration of 1 ng/μL to 1 fg/μL by serial 10-fold dilution of *B. abortus* 544 reference strain. DNA concentration was measured using a Nanodrop ND-1000 UV/UVS spectrophotometer (Nanodrop Tech., Wilmington, DE, USA). These results were compared with those of a 16S rRNA [106] and BaSS-PCR assays [11], which were used to identify *Brucella* species and *B. abortus* biovars 1, 2 and 4.

## 4. Detection limits of real-time PCR assay

To compare the analytical sensitivity of real-time PCR assay in the clinical specimens, artificial inoculation using a *B. abortus* strain in the clinical samples was conducted. Briefly, ten-fold serial dilutions of the *B. abortus* strain with 0.85% saline were processed into the macerated lymphoid tissue, and then, each spiked sample was cultivated on three tryptic soy agars and was calculated in colony-forming units (CFU). The DNA of the spiked samples was extracted using a commercial blood and tissue kit (Qiagen Ltd.) according to the manufacturer's protocols.

## 5. Evaluation of real-time PCR assay

To apply real-time PCR assay to the clinical specimens, twelve samples (supramammary, submandibular, inguinal and parotid lymph nodes, testicle and buffy coat) were acquired from seropositive Korean native cattle on a breeding farm (Table III-3). These specimens were ground in 1 mL of PBS buffer and spread onto tryptic soy agar supplemented with 5% bovine serum (GIBCO, Grand Island, NY, USA) and 5% sheep blood agar for 3-4 days at 37 $^{\circ}$C and 5% $CO_2$. Genomic DNA from 200 μL of ground sample was extracted using a Blood & Tissue kit (Qiagen Ltd.) per the manufacturer's instructions and was submitted to real-time PCR assay.

**Table III-2.** Bacterial strains and comparison of the two conventional PCRs

| Species | Strains | PCR results | | |
|---|---|---|---|---|
| | | 16S rRNA | BaSS | Realtime PCR |
| ***Brucella* species** | | | | |
| *B. abortus* bv. 1 (544) | ATCC 23448 | + | + | + |
| *B. abortus* bv. 2 (86/8/59) | ATCC 23449 | + | + | + |
| *B. abortus* bv. 3 (Tulya) | ATCC 23450 | + | ±[a)] | + |
| *B. abortus* bv. 4 (292) | ATCC 23451 | + | + | + |
| *B. abortus* bv. 5 (B3196) | ATCC 23452 | + | ±[a)] | + |
| *B. abortus* bv. 6 (870) | ATCC 23453 | + | ±[a)] | + |
| *B. abortus* bv. 9 (C68) | ATCC 23455 | + | ±[a)] | + |
| *B. canis* (RM6/66) | ATCC 23365 | + | ±[a)] | - |
| *B. suis* bv. 1 (1330) | ATCC 23444 | + | ±[a)] | - |
| *B. suis* bv. 2 (Thomsen) | ATCC 23445 | + | ±[a)] | - |
| *B. suis* bv. 3 (686) | ATCC 23446 | + | ±[a)] | - |
| *B. suis* bv. 4 (40) | ATCC 23447 | + | ±[a)] | - |
| *B. suis* bv. 5 (513) | NCTC 11996 | + | ±[a)] | - |
| *B. ovis* (63/290) | ATCC 25840 | + | ±[a)] | - |
| *B. neotomae* (5K33) | ATCC 23459 | + | ±[a)] | - |
| *B. melitensis* bv. 1 (16M) | ATCC 23456 | + | ±[a)] | - |
| *B. melitensis* bv. 2 (63/9) | ATCC 23457 | + | ±[a)] | - |
| *B. melitensis* bv. 3 (Ether) | ATCC 23458 | + | ±[a)] | - |
| *B. ceti* (B1/94) | NCTC 12891 | + | ±[a)] | - |
| *B. pinnipedialis* (B2/94) | NCTC 12890 | + | ±[a)] | - |
| *B. microti* (CCM 4915) | BCCN 07-01 | + | ±[a)] | - |
| *B. inopinata* (B01) | BCCN 09-01 | + | ±[a)] | - |
| Mongolian *B. abortus* | 16 isolates | + | ±[a)] | + |
| Mongolian *B. melitensis* | 94 isolates | + | ±[a)] | - |
| Korean *B. abortus* | 84 isolates | + | + | + |
| Korean *B. canis* | 72 isolates | + | ±[a)] | - |
| **Non-*Brucella* organisms** | | | | |
| *Ochrabactrum anthropi* | Field strain | + | - | - |
| *Escherichia coli* O157:H7 | Field strain | - | - | - |
| *Pasteurella multocida* | ATCC 43017 | - | - | - |
| *Salmonella* Typhimurium | ATCC 14028 | - | - | - |
| *Campylobacter jejuni* | ATCC 33560 | - | - | - |
| *Yersinia enterocolitica* O:9 | NCTC 11174 | - | - | - |
| *Staphylococcus aureus* | Field strain | + | - | - |
| *Clostridium perfringens* type A | ATCC 13124 | - | - | - |

±[a)]: They were identified with *Brucella* spp. and showed two amplified products (180 bp and 800 bp).

## III. Results

*B. abortus*-specific SNPs were detected at the *fbaA* gene of *B. abortus* chromosome II (Genbank accession No. AE 017224), with cytosine changed to thymine at 360432 on *B. abortus* chromosome II. Based on the sequence of the *fbaA* gene, a primer set 176 bp in size and a hybprobe with a specific SNP were designed (Table III-1).

Real-time PCR assay showed a positive reaction only to *B. abortus* reference strains (biovars 1-6 and 9) and *B. abortus* organisms from clinical specimens, whereas it yielded negative reactions to other *Brucella* species and non-*Brucella* bacterial strains (Table III-2 and Fig. III-1). Only *B. abortus* species showed specific amplification from approximately the 14th cycle (Fig. III-1a). Additionally, the specific amplification was also confirmed by melting curve analysis. The *Tm* calling value of *B. abortus* reference strains and isolates was generated at 69 ºC. In contrast with this finding, other *Brucella* species and non-*Brucella* strains showed less than a low melting peak at a temperature of 62 ºC (Fig. III-1b). In the 16S rRNA PCR, some bacterial strains, such as *Ochrabactrum anthropi* and *Staphlyococcus aureus*, were diagnosed as positive reactions with the amplified product of 905 bp. BaSS-PCR showed different results depending on the biovar of *B. abortus*. In case of *B. abortus* bv. 1, 2 and 4, three specific bands (180 bp, 500 bp and 800 bp) appeared, but other bvs. of *B. abortus* and other *Brucella* species showed only two bands (180 bp and 800 bp), and non-*Brucella* bacteria revealed only an 800 bp-band product as an internal control. Therefore, BaSS-PCR has the limitation of detecting *B. abortus* bv. 1, 2 and 4 only, so *B. abortus* infection caused by other biovars could not be differentiated (Table III-2).

The sensitivity of real-time PCR was assessed by means of successive 10-fold serial dilution of the genomic DNA of the *B. abortus* 544 reference strain, and it was revealed to be 20 fg/μL (data not shown). In addition, the detection limit for *B. abortus* in the clinical samples was 4 CFU/μL. In contrast, the detection limit of conventional

BaSS-PCR showed 80 CFU/μL. Our new real-time PCR showed 20 times higher sensitivity than those with BaSS-PCR (Fig. III-2), but equal sensitivity to 16S rRNA PCR (data not shown).

In addition, the application of the real-time PCR to the clinical specimens was conducted using brucellosis-positive Korean native cattle. Here, *B. abortus* was isolated from tissue samples, such as various lymph nodes and buffy coats. All of the samples were confirmed as positive by generating a fluorescent signal during real-time PCR (Table III-3). The range of mean Ct ranged between 28 and 30, except for buffy coat, and the *Tm* values were almost identical to the reference *B. abortus* 544 strain.

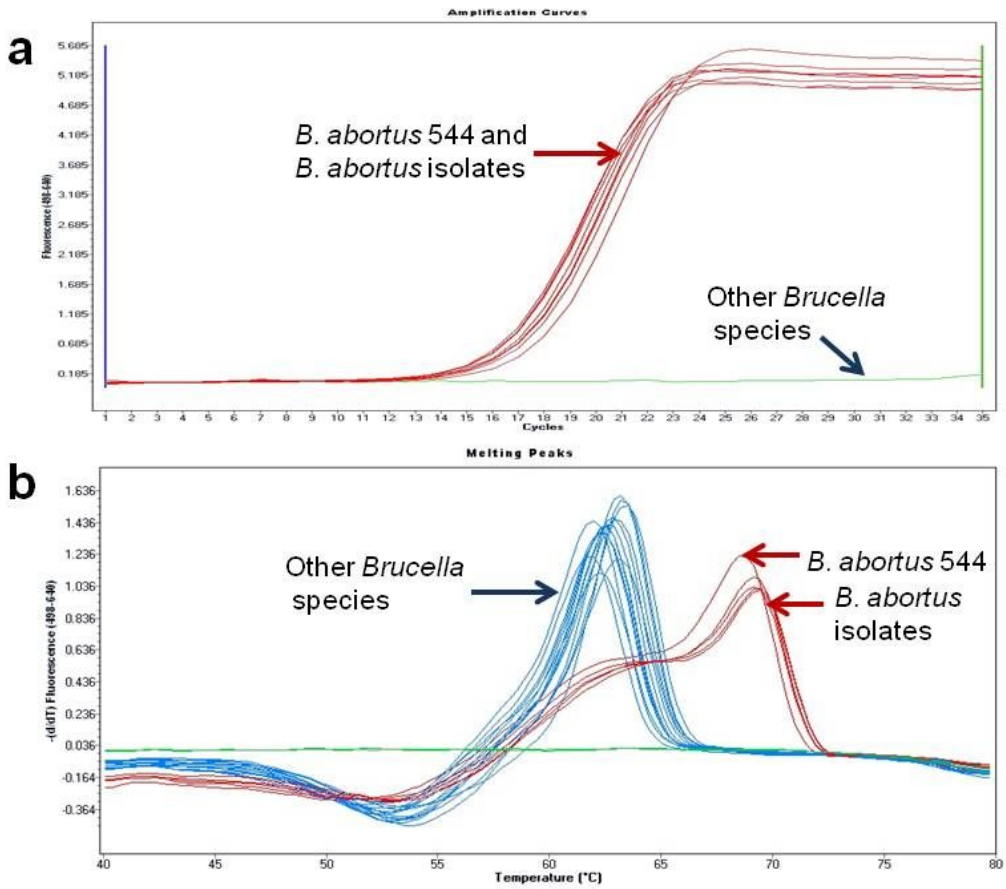


**Figure III-1.** Amplification curves (a) and melting peak analysis (b) in *B. abortus* 544 reference strain and Korean *B. abortus* isolates.

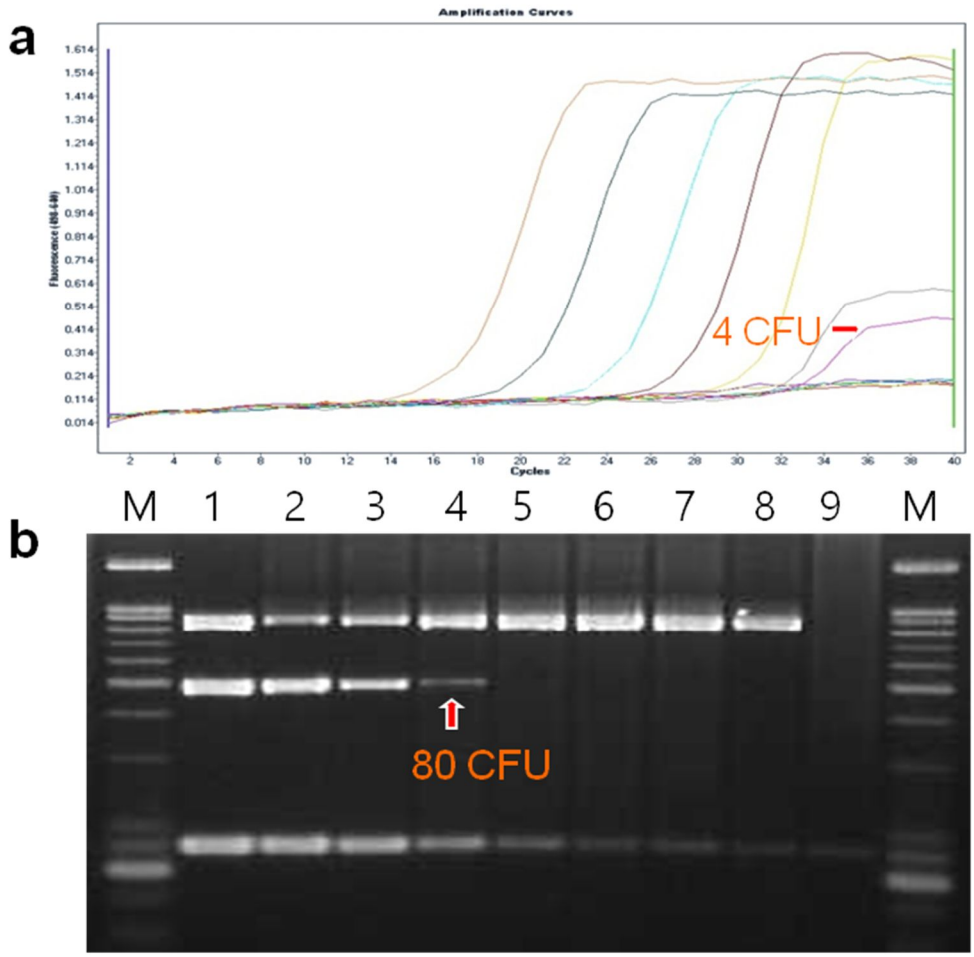


**Figure III-2.** Detection limits of the hybridization probe-based real-time PCR (a) and BaSS-PCR (b) determined by DNA extracted from lymphoid tissue inoculated with 10-fold diluted *B. abortus* strains serially. (a) There were ranged from $4 \times 10^{6}$ to $4 \times 10^{-2}$ CFU/μL. (b) M: 100-bp DNA ladder, lanes 1 to 7: $8 \times 10^{4}$ to $8 \times 10^{-2}$ CFU/μL, lane 8 and 9: internal and negative controls (D. W.).

**Table III-3.** Direct detection from clinical specimens of brucellosis-positive Korean native cattle by real-time PCR

| Sample No. | Specimen | Isolation[a)] | RBT[b)] | Real-time PCR | |
|---|---|---|---|---|---|
| | | | | Mean Ct | *Tm* |
| 1 | Buffy coat | + | + | 16.89 | 69.43 |
| 2 | Buffy coat | + | + | 16.88 | 69.41 |
| 3 | Calf submandibular LN[c)] | + | NT[d)] | 29.24 | 69.23 |
| 4 | Submandibular LN-1 | + | + | 28.45 | 69.46 |
| 5 | Submandibular LN-2 | + | + | 29.28 | 69.15 |
| 6 | Submandibular LN-3 | + | + | 29.74 | 69.42 |
| 7 | Submandibular LN-4 | + | + | 28.79 | 69.38 |
| 8 | Submandibular LN-5 | + | + | 29.13 | 69.08 |
| 9 | Supramammary LN | + | + | 28.89 | 69.41 |
| 10 | Calf inguinal LN | + | NT | 29.29 | 69.06 |
| 11 | Parotid LN | + | - | 29.27 | 69.13 |
| 12 | Testicle | + | + | 28.65 | 69.34 |

[a)]Isolation: The bacterial strains were identified by classical phenotyping and differential multiplex PCR (Kang et al., 2011).

[b)] RBT: Rose-Bengal test.

[c)] LN: Lymph node.

[d)] NT: Not tested.

# IV. Discussion

For decades, PCR-based assays have been developed continually as a form of real-time PCR. It is able to detect target microorganisms more sensitively, specifically and rapidly than conventional PCRs [51, 122]. Unlike endpoint detection methods, such as agarose gel electrophoresis, real-time PCR is used for the quantitative measurement of amplified products using fluorescence during each PCR cycle. These reactions can be classified into two main types according to the fluorescent dye and the specificity of the PCR [86]. The former uses double-stranded DNA-intercalating dye (e.g., SYBR Green I), and the latter uses fluoroephores that bind to oligonucleotides. This type can be divided into three subtypes depending on the fluorescent molecules: (i) primer-probes; (ii) hydrolysis and hybridization probes; and (iii) analogs of nucleic acids [86]. First, SYBR Green I, as one of the most commonly used DNA-binding dyes, binds to total amounts of DNA generated during PCR, so it can induce specific and non-specific amplification [86, 119]. At the same time, Taqman probe is a representative hydrolysis type, and it is designed to bind to a specific site of the target DNA, so it shows greater specificity than SYBR Green I. However, it has the disadvantage that the primer-dimer can be generated even if the primer design is not appropriate. Contrarily, hybprobe-based real-time PCR offers two main advantages: first, it requires two additional probes for binding, so it can show improved specificity to distinguish between closely related strains; second, hybprobe does not rely on the hydrolysis reaction, so melting curve analysis can be applied to differentiate based on the probe *Tm* [88]. Thus, its application has increased in diverse fields including pathogen and SNP detection[71, 86].

In particular, the application of SNPs in microbial molecular typing has been increasing in the diagnostic field. SNPs in the conserved region can be very strong markers for detecting and differentiating etiological agents specifically. Therefore, we

designed a hybprobe from another *B. abortus*-specific SNP in the conserved *fbaA* gene, although this gene had been already used in real-time PCR with Taqman probe [46].

With regard to specificity, only *B. abortus* strains revealed specific amplification curves from the 14th cycle, and *Tm* was 69 ºC in our new real-time PCR. Not only other *Brucella* species but also highly genetically and serologically related bacteria were not amplified. Especially, it yielded a negative reaction from two cross-reactive bacteria by 16S rRNA PCR - *O. anthropi* and *S. aureus* [2, 52]. In terms of sensitivity using *B. abortus* DNA, our real time PCR assay was equal to or higher than that of 16S rRNA PCR [106]. In addition, our new real-time PCR showed 20 times higher sensitivity and detected all biovars of *B. abortus* as compared with BaSS-PCR (Table 1 and Fig. III-2). Therefore, this new real-time PCR could be valuable for diagnosing *B. abortus* infection in terms of its accuracy, specificity and sensitivity.

Besides two conventional PCRs, our new PCR showed improved analytical sensitivity, compared to other real-time PCRs. Using serially diluted DNA samples, our assay revealed 20 fg, but the sensitivity of the two previous methods using 5'-nuclease IS*711* were reported as 150 fg and 250 fg, respectively [103, 105]. Additionally, a previous real-time PCR detecting the same target gene (*fba*) reported sensitivity of 50 fg or 15 cells [46], which was lower than in our current study (20 fg or 4 CFU). Bounaadja et al. (2009) [9] reported a 10 times higher sensitivity compared to our assay of 2 fg by targeting three genes (*IS711*, *bcsp31* and *per* gene). However, it was only evaluated by using DNA samples extracted from pure bacterial culture, not using DNA samples extracted from artificially infected clinical samples such as in the current study. Therefore, it is hard to make a direct comparison between Bounaadja's and our methods. Clinical specimens such as tissue and blood samples (buffy coat) are applicable type to our new PCR. However, the application trial of milk samples is required in further research. Using those types of samples, our PCR showed identical results with those by bacterial culture methods. In addition, it was successfully demonstrated that our new real-time PCR can differentiate even single nucleotide

difference in a target gene. Threfore, our new assay may be applicable for differential diagnosis for vaccinated and infected animals with identification of SNP on a specific target gene in vaccine and wild type *B. abortus* strains.

Because the genus *Brucella* is an intracellular bacterium, and the number of bacteria in specimens is usually small [122], a highly sensitive diagnostic technique is required for accurate differential diagnosis. This new real-time PCR could be very useful for directly diagnosing brucellosis caused by *B. abortus* in infected animals due to the high detection limit. In conclusion, our new real-time PCR based on hybprobe could be an efficient diagnostic technique with high sensitivity and rapidity for *B. abortus*-infected animals in the field, and it could also be applicable in public health.

# Chapter IV.

## Immunoproteomics of *Brucella abortus* RB51 as candidate antigens in serological diagnosis of brucellosis

## Abstract

The current diagnosis of brucellosis is chiefly based on testing for antibodies that react with lipopolysaccharide (LPS). However, such antibodies occasionally cross-react with some of the gram-negative bacteria that have an O-polysaccharide (OPS) similar in structure to that of *Brucella abortus*. Therefore, the aim of the present study was to identify new candidate antigens from *B. abortus* RB51, a mutant strain lacking the LPS portion, which might prove valuable in minimizing cross-reactions in brucellosis serological assays. To detect potential diagnostic antigens, immobilized pH gradients (IPG) strips with three ranges (pH 3-5.6, 4-7 and 6-11) were used in this study. After separating the insoluble proteins of *B. abortus* RB51 strain using two-dimensional electrophoresis (2-DE), their immunogenicity was evaluated by western blotting using four types of antisera – *B. abortus*-positive, *B. abortus*-negative, *Yersinia enterocolitica* O:9-positive, and *Escherichia coli* O157:H7-positive bovine sera. Among the several immunogenic spots, the spots showing specific reactivity with only the *B. abortus*-positive antisera, were considered as candidate antigens. Overall, a total of 11 immuno-reactive proteins were identified, as follows : Cu/Zn superoxide dismutase (SOD), histidinol dehydrogenase (*his*D), chaperonin DnaK, chaperonin GroES, beta-ketoadipyl CoA thiolase, two-component response regulator, the cell-division protein FtsZ, aldehyde dehydrogenase (ALDH), 50s ribosomal protein L10 and invasion protein B. The selected highly immunogenic protein spots might be useful as alternative diagnostic antigens for brucellosis and helpful in reducing the cross-reactivity due to the structural similarity of the S-LPS molecule of *B. abortus* and other gram-negative bacteria.

**Keywords**: *Brucella abortus* RB51, 2-DE, western blotting, immunogenic protein

# I. Introduction

Brucellosis is one of the major zoonotic bacterial diseases that cause abortion and infertility in animals in many countries [3, 30, 98]. Bacterial culture and identification are very reliable methods to confirm brucellosis, but they are not effective methods for conventional screening due to their costs, and time-consuming nature, among other reasons. In addition, depending on the type of samples, DNA detection methods are not fully reliable [79].

To supplement brucellosis diagnostic techniques, several serological assays, such as the standard tube agglutination test (STAT), the fluorescence polarization assay (FPA), the complement fixation test (CFT) and enzyme-linked immunosorbent assays (ELISAs), have been applied. The diagnostic antigens for these tests are mainly based on whole cell or on smooth lipopolysaccharide (S-LPS) fractions [3, 66, 84]. Although S-LPS can induce a very powerful antibody response, the antibodies have the critical drawback of cross-reacting with various bacteria, including *Yersinia enterocolitica* O:9, *Escherichia coli* O157, *Salmonella typhimurium*, and *Vibrio cholera*, among others. The cross-reactivity was attributed to the high structural similarity of the OPS epitope of the S-LPS molecule [3, 66, 84, 91, 98]. To discriminate brucellosis from diseases caused by the cross-reactive bacteria, many attempts have been made to detect immunogenic protein antigens devoid of the LPS portion [1, 3, 17, 96, 114]. Because the RB51 strain is an LPS-lacking mutant of the virulent *Brucella abortus* 2308 strain [1, 114], it has been reported to not induce the production of antibodies that confound the conventional serological diagnosis of brucellosis.

Currently, 2-DE and MALDI-TOF are considered to be basic, sensitive, and accurate proteomic approaches to seek specific proteins [48]. Therefore, selecting new immunogenic antigens of *B. abortus* RB51 using these techniques could be a promising strategy for minimizing cross reactivity in the brucellosis serological tests.

In the present study, we utilized proteomic techniques, such as 2-DE, western blotting, and MALDI-TOF/TOF analysis to identify strong immuno-dominant protein

spots efficiently. To improve the differential diagnosis of brucellosis, we applied four types of antisera – *B. abortus*-positive and -negative sera, and *E. coli* O157:H7- and *Y. enterocolitica* O:9-positive sera as representative sera that cross-react with *Brucella*. We hypothesized that the discovery of new candidate antigens of *B. abortus* RB51 could facilitate diagnosis by minimizing the cross-reactivity in brucellosis serological assays.

## II. Materials and methods

### 1. Bacterial strains

Four types of bacteria, including *B. abortus* RB51, *B. abortus* 2308, *Y. enterocoitica* O:9 and *E. coli* O157:H7, were acquired from the OIE Reference Lab. for brucellosis of the Animal and Plant Quarantine Agency (QIA) in the Republic of Korea. The bacterial strains were stored at -80°C until use and were cultured in tryptic soy broth (TSB; Difco, BD, USA) and incubated at 37°C with shaking for 24-48 h.

### 2. Antisera

To obtain antisera directed against *B. abortus* 2308, seven adult cattle aged 23-27 months were inoculated with a dose of 4 x $10^7$ CFU/head of *B. abortus* 2308 via the conjunctival route. After the challenge, weekly or biweekly blood sampling was performed for 12-14 weeks. The antibody titers of the sera were determined using a commercial ELISA (competitive ELISA) kit (Svanovir, Sweden) as described by manufacturer, and the sera were pooled for use as *B. abortus*-positive sera. Moreover, to test the cross-reactivity with antisera against *Y. enterocolitica* O:9 and *E. coli* O157:H7, five and two cattle, respectively, were challenged orally with a dose of $10^{10}$ heat-killed microorganisms per head. Their sera were also collected weekly for three months to determine the titer of antibodies directed against brucellosis, using the STAT. Among the antisera from the cattle challenged with *Y. enterocolitica* O:9 or *E. coli* O157:H7, only the antisera showing a false-positive reaction in the STAT were selected for use as cross-reactive antisera for western blotting. The two challenge experiments were approved by the ethical committee as NVRQS-AEC-2008-12 (former) and C-AD13-2011-13-01 (latter). The seven cattle that received the *B. abortus* 2308 challenge were euthanized according to the protocol of the Institutional Animal Care and Use Committee of the Republic of Korea.

## 3. Extraction of insoluble proteins from the *B. abortus* RB51 strain

The insoluble proteins of the *B. abortus* RB51 strain were extracted as previously reported [66]. Briefly, *B. abortus* RB51 was cultured in TSB in a shaking incubator (at 150 rpm) at 37°C for 48 h. The bacteria were collected by centrifugation at 8,000 x g for 20 min at 4°C, washed twice with chilled PBS (pH 7.6), and re-suspended in distilled water (D. W.). Then, the bacterial cells were disrupted using a French press (Thermo Electron Corp. MA, USA) in a 1" DIA cell for 30 min at 18,000 psi. After centrifugation for 30 min at 20,000 x g, the pellets were washed twice with 2 mL of D. W. and dissolved in 2 mL of lysis buffer (2 M thiourea, 5 M urea, 2% Chaps, 2% SB3-10, and 40 mM Tris base) by stirring. Twenty microliters of protease inhibitor (GE Healthcare, UK) was added to the solution, and it was placed on ice for 5 min. Following the addition of 20 μL of Nuclease Mix (GE Healthcare), the mixture was placed at room temperature for 40 min. After centrifugation at 20,000 x g for 30 min, the supernatant obtained was treated with TCA (trichloroethanoic acid)-acetone, following the modified method of Maserti et al. [76] and Ko et al. [66] to remove lipids and salts. After collecting the pellet following TCA-acetone treatment, it was re-suspended in lysis buffer according to a previous report [82] and applied to 2-DE as the insoluble proteins.

## 4. Two-dimensional electrophoresis (2-DE) and western blotting

### 4.1 Isoelectric focusing (IEF)

IEF, the first dimension of 2-DE, was conducted as described by Görg et al. [48] and Ko et al. [66], with modifications. In brief, IPG strips with three different pH ranges (3-5.6, 4-7, and 6-11; 13 cm) (Immobiline Drystrip, GE Healthcare) were rehydrated overnight at room temperature in the IPG strip holder with 250 μL of the total protein solution containing rehydration buffer (GE Healthcare), 2% IPG buffer (pH 4-7 and pH 6-11; GE Healthcare, Sweden), 1% of TBP-reducing agent (Bio-Rad, USA), and the lysed protein at a final concentration of 100 μg. IEF was performed using a Protean

IEF system (Ettan IPGphor II, Amersham Biosciences, USA) at 22°C for 24 h with a linear increase from 30 V to 8,000 V and was terminated at 80,000 voltage hours.

## 4.2 2-DE and western blotting

After IEF was accomplished, the IPG strips were incubated with 10 mL of equilibration buffer I followed by 10 mL of equilibration buffer II [142] and then were simultaneously applied to SDS-PAGE gels (5% stacking gel and 10% running gel). The twin gels were electrophoresed at the same time for 1.5 h at 10 mA and for 2.5 h at 80 mA for the stacking gel and the running gel, respectively, using an electrophoresis device (Hoefer SE 600 Ruby, Amersham Bioscience, USA). One of the twin gels was stained with Coomassie brilliant blue (CBB), and the other gel was used for immunoblotting.

For western blotting, the individual gels were transferred onto PVDF membrane for 2 h at 500 mA using transfer buffer by transfer buffer and a transfer instrument (TE62, Hoefer, USA), with cooling [66]. Next, the PVDF membranes were rinsed with methanol and dried at room temperature, then blocked using 2% skim milk (Difco, BD, USA) in Tris-buffered saline (TBS) for 3 h at RT. After washing three times with TBS containing 0.05% Tween-20 (TBS-T), the PVDF membrane were immersed in a solution containing a primary antiserum (four types of bovine sera – *B. abortus*-positive serum, *B. abortus*-negative serum, *Y. enterocolitica* O:9-positive serum or *E. coli* O157:H7 positive serum) at 1:10,000 dilution and incubated at RT for 2 h. The secondary antibody, peroxidase-labeled goat anti-bovine IgG (H+L) (KPL, USA) was applied at 1:20,000 dilution and incubated at RT for 1 h. After washing the PVDF membranes with TBS-T repeatedly, then they were developed for 10 min using a substrate kit (West Q-chemiluminescent substrate kit, Genedepot, USA), following the manufacturer's protocol.

## 5. Image analysis

Quantitative imaging and analysis of the western blots was performed using a Fluorchem Q system (Bucher, Switzerland) according to the manufacturer's instructions. The device allowed quantitative detection, so that intensity values could be easily normalized to a loading control to correct for inaccuracies. The immuno-dominant protein spots were selected based on the intensity of their labeling with *B. abortus*-positive antiserum relative to their labeling with the other three types of antisera.

## 6. In-gel digestion

Enzymatic digestion within the 2-DE gels was performed using porcine trypsin, as described previously [33, 66]. The spots showing significant immunoreactivity only with the antisera from the *B. abortus*-infected cattle were excised from the 2-DE gels manually. The gel plugs were washed with 50% acetonitrile to eliminate interfering substances, such as SDS, salts, and stain. After drying and rehydration with a solution of trypsin (8-10 ng/μL), incubated for 10 h at 37ºC, and then 5 μL of 0.5% trifluoroacetic acid was added to complete the proteolytic reaction. After recovering the tryptic peptides using 50% acetonitrile, the peptide mixture were desalted using $C_{18}$ZipTips (Millipore, MA, USA) and eluted using 1-5 μL of acetonitrile. An aliquot of the solution was then blended with the same volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, and then 1 μL of each mixture was dropped onto a MALDI target plate.

## 7. MALDI-TOF/TOF

The samples were analyzed using an Applied Biosystems 4700 proteomics analyzer with TOF/TOF$^{TM}$ ion optics [72]. The MS/MS data were acquired using a Nd:YAG laser with a 200-Hz repetition rate, and up to 4,000 scans were accumulated for each spectrum. The MS/MS mode utilized 2 keV collision energy, using air as the collision

gas so that nominally single collision conditions were achieved. Although the precursor selection had a possible resolution of 200, in these studies of known single component analytes, a resolution of 100 was utilized. The MS data were acquired using the instrument default calibration, without applying internal or external calibration. The sequence tag searches were conducted using the MASCOT program (http://www.matrixscience.com).

# III. Results

## 1. Protein patterns in the 2-DE gel and western blots

To analyze the immunogenic proteins of *B. abortus* RB51, the 2-DE gels and western blots of the insoluble proteins of *B. abortus* RB51 were obtained using three overlapping pH ranges (3-5.6, 4-7, and 6-11) and four types of antisera (sera from cattle infected with *B. abortus*, *Y. enterocolitica* O:9 or *E. coli* O157:H7 and brucellosis-negative cattle). As a result, the protein patterns in the 2-DE gels were very similar between pH 3-5.6 and pH 4-7, but not between pH 6 and 11. Therefore, gels representing only two ranges of pH (pH 3-5.6 and 6-11) are presented in Fig. IV-1 to show the proteins separated across a wider range. The number of spots observed was almost same in the ranges of pH 3-5.6 and 4-7, but far fewer spots were detected in the range of pH 6-11. Most of the detected immunogenic antigens were located in the range of pH 3-7, and their molecular weights ranged from 15 kDa to 100 kDa (Fig. IV-1A). However, the most strongly immunogenic spots by the naked eye and the quantity value were observed in the region of 6-11 and had molecular weights of approximately 15-30 kDa. In particular, three adjacent spots (7104, 7018 and 8505) in the range of pH 6-11 with similar molecular weights close to 20 kDa displayed very powerful immuno-reactivity with the *B. abortus*-positive antiserum (Fig. IV-2B). Overall, many immunogenic spots were found to react with the negative, *Y. enterocolitica* O:9 and *E. coli* O157:H7-positive antisera, but the intensity of the labeling was relatively weak.

In the range of pH 3-5.6, the protein antigens showing the strongest immuno-reactivity with the *B. abortus*-positive serum had apparent molecular weights of approximately 40kDa, but these spots overlapped one another, forming a horizontal streak (Fig. IV-1A). Because the streak pattern was also obtained using the other three types of antisera, these proteins appeared to be common antigens.

## 2. Selection and identification of candidate antigens of *B. abortus* RB51

Several immunogenic protein spots were detected among the *B. abortus* RB51 insoluble proteins using four types of antisera. However, some of the spots were excluded from the set of candidate antigens due to overlapping reactivity with the negative serum and/or *Y. enterocolitica* O:9 and *E. coli* O157:H7 antisera. As a result, only 11 protein antigens were selected and identified in the present study (Table IV-1). The following 8 antigens were identified among the protein spots that displayed the strongest immunoreactivity with the *B. abortus*-positive antiserum, but did not show any reactivity with the *Y. enterocolitica* or *E. coli* O157:H7-positive antisera or with the *B. abortus*-negative serum: Cu/Zn-SOD (spot 1203), *his*D (spot 1506), chaperonin DnaK (spot 2811), chaperonin GroES (spot 3030), two-component response regulator (spot 4303), cell-division protein FtsZ (spot 1506), ALDH (spot 6604), and a hypothetical protein (spot 1607). Of these, spot 3030 appeared to be the most immunogenic *B. abortus* protein. It was identified as chaperonin GroES, with a molecular weight of 21.1 kDa and a pI of 4.2 experimentally and a quantity value of 9,856 (Table IV-1). Another chaperone protein, DnaK (spot 2811) exhibited the second highest immunoreactivity, and had an experimental molecular weight of 71.2 kDa, a pI of 4.6 and a quantity value of 4,269. In addition, Cu/Zn-SOD (spot 1203) and ALDH (spot 6604) appeared to have strong immunogenicity. *his*D (spot 1506), two-component response regulator (spot 4303), cell-division protein FtsZ (spot 5702) and a hypothetical protein (spot 1607) showed immunoreactivity to the *B. abortus*-positive antiserum. They were classfied as specific immunogens of *B. abortus*. Moreover, beta-ketoadipyl CoA thiolase (spot 4101), invasion protein B (spot 8505) and 50s ribosomal proteins L10 (spot 7104 and 7108) revealed much stronger immunoreactivity with the *B. abortus* antisera than with the other three types of antisera as shown as Table IV-1. As a result, these spots were grouped as relatively strong immunogenic proteins.

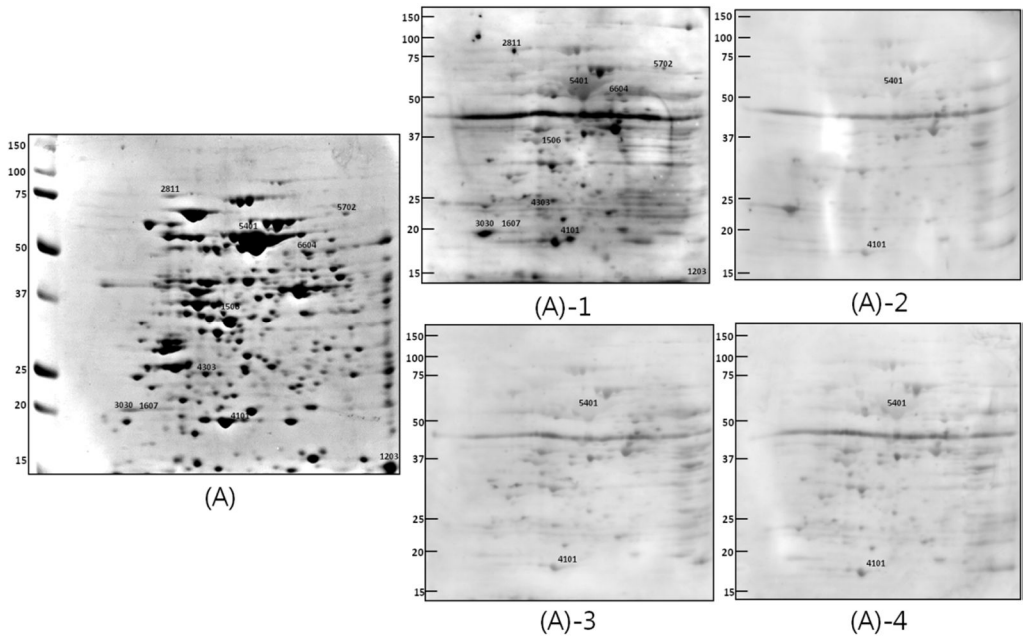


**Figure IV-1.** (A) Coomassie-stained insoluble proteins of *B. abortus* RB51 on a SDS-PAGE gel prepared using IPG strips encompassing pH 3–5.6: (A-1) the antisera of cattle after challenge with *B. abortus* 2308, (A-2) the antisera from non-infected cattle, (A-3) the antisera of cattle infected experimentally with *Y.enterocolitica* O:9, (A-4) the antisera of cattle infected experimentally with *E. coli* O157:H7.

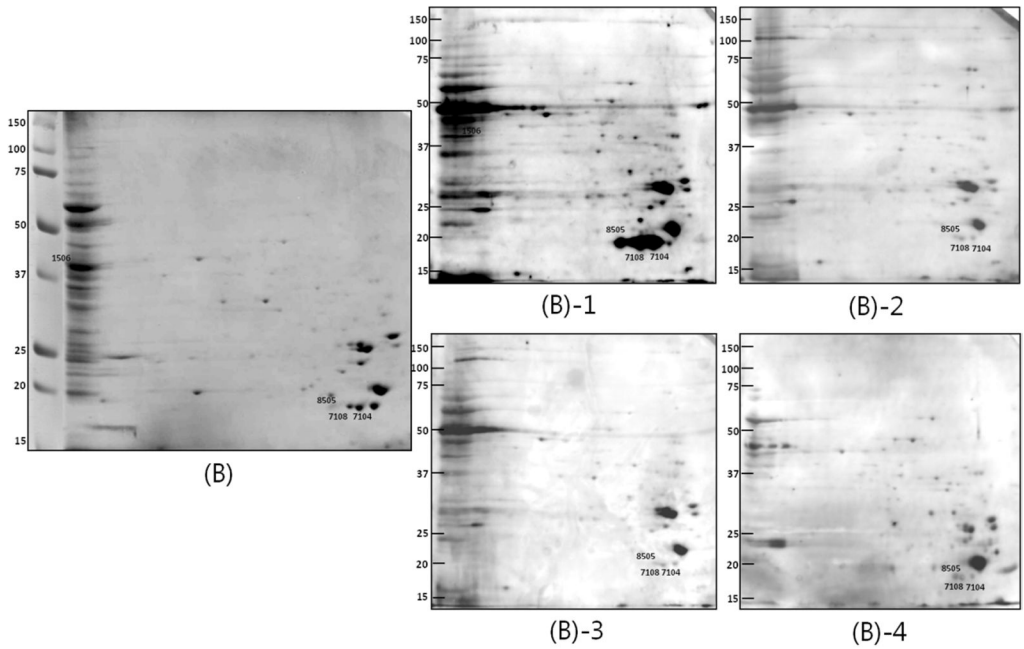


**Figure IV-2.** (B) Coomassie-stained insoluble proteins of *B. abortus* RB51 on a SDS-PAGE gel prepared using IPG strips encompassing pH 6–11: (B-1) the antiseraof cattle after challenge with *B. abortus* 2308, (B-2) the antisera from non-infected cattle, (B-3) the antisera of cattle infected experimentally with *Y.enterocolitica* O:9, (B-4) the antisera of cattle infected experimentally with *E. coli* O157:H7.

**Table IV-1.** Identification of immunoreactive proteins of *B. abortus* RB51 as determined by 2DE and western blot analysis

| No. of spot | Quantity[a] | | | | | Protein identification | Locus tag | Score | Mw (kDa) | | pI (experi-mental/ theoretical) | Sequence coverage |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2D gel | Sera | | | | | | | | | | |
| | | *B. abortus* | | YE O:9 | EC 157 | | | | Experi-mental | Theo-rectical | | |
| | | (+)ve | (-)ve | (+)ve | (+)ve | | | | | | | |
| 1203 | 16,565.1 | 2698.2 | ND[b] | ND | ND | Cu/Zn superoxide dismutase (SOD) | BAB2_0535 | 149 | 18.1 | 18.2 | 5.9/6.1 | 74 |
| 1506 | 1949.9 | 381.9 | ND | ND | ND | Histidinol dehydrogenase (*his*D) | BAbS19_I18660 | 97 | 36.2 | 46.0 | 6.0/5.1 | 49 |
| 1607 | 5156.9 | 299.4 | ND | ND | ND | Hypothetical protein | BAB1_1885 | 114 | 21.5 | 20.4 | 4.7/4.6 | 56 |
| 2811 | 8873.4 | 4269.7 | ND | ND | ND | Chaperonin DnaK | BAbS19_I19910 | 179 | 70.2 | 68.3 | 4.8/4.9 | 35 |
| 3030 | 1582.7 | 9856.5 | ND | ND | ND | Chaperonin GroES | M1M_01897 | 63 | 28.1 | 26.7 | 4.0/5.1 | 56 |
| 4101 | 12164.8 | 5864.2 | 174.4 | 123.7 | 130.2 | Beta-ketoadipyl CoA thiolase | BAB1_1639 | 64 | 19.8 | 41.8 | 4.9/6.3 | 44 |
| 4303 | 1117.2 | 881.3 | ND | ND | ND | Two-component response regulator | BAbS19_I15640 | 63 | 24.4 | 29.1 | 4.8/5.3 | 37 |
| 5702 | 8851.8 | 902.4 | ND | ND | ND | Cell division FtsZ | BAB2_ 0189 | 208 | 62.5 | 60.8 | 5.4/5.4 | 66 |
| 6604 | 9927.5 | 2678.3 | ND | ND | ND | Aldehyde dehydrogenase (ALDH) | BAbS19_II05330 | 159 | 51.2 | 51.6 | 5.2/5.8 | |
| 7104 | 38721.8 | 42701.1 | 1921.7 | 2821.7 | 1883.3 | 50S ribosomal protein L10 | AAL51928 | 141 | 22.2 | 21.8 | 9.9/10.1 | 61 |
| 7108 | 77490.4 | 48267.2 | 1328.6 | 1697.5 | 1775.5 | | | | | | | |
| 8505 | 14367.6 | 32221 | 1481.5 | 2017.5 | 3597.7 | Invasion protein B | YP_005114253 | 136 | 26.4 | 22.5 | 9.1/10.2 | 25 |

[a] Quantity of each spot was normalized by total valid spot intensity
[b] ND: not detected

# IV. Discussion

To eradicate bovine brucellosis, South Korea as well as many other countries has employed a strict test-and-slaughter strategy. Various diagnostic approaches have been developed, such as bacterial culturing, DNA detection methods and serological assays. Among them, serological assays are the mainstay for brucellosis diagnosis because of their advantageous low cost, availability and diagnostic sensitivity [79]. Several serological methods have been applied to diagnosing bovine brucellosis, but most of them are based on the S-LPS antigen. Although S-LPS is very powerful immunogen for detecting brucellosis, it has the disadvantage of occasionally cross-reacting with sera directed against other gram-negative bacteria. Therefore, many trials have been conducted to develop LPS-free diagnostic antigens to compensate for this limitation by detecting immunogenic proteins [3, 66, 79, 84, 91, 96]. Besides, several articles about the evaluation of RB51 antigen or rough-LPS of *B. abortus* RB51 strain had been reported earlier [155-157]. In these previous reports, they were applied as diagnostic antigens in CFT [155, 156] and indirect ELISA [157]. However, there were no preoteomic approaches to detect differential immunogenic proteins in *B. abortus* RB51 strain which was known as mutant strain lacking of S-LPS portion.

Therefore, to select potential immunogenic proteins for diagnosing brucellosis effectively in *B. abortus* RB51 strain, we used 2-DE and western blotting to separate and analyze the insoluble proteins of *B. abortus* RB51. Because the MALDI mass spectrometry technique is one of the basic proteomic approaches, it has been extensively used in searching for proteins for vaccines and diagnostics for a variety of infectious disease [48].

In our study, eleven immunogenic protein spots were found, and they were classified as useful candidate antigens for detecting *B. abortus* infections. Of these, chaperonin DnaK, GroES, Cu/Zn-SOD, ALDH, *his*D, two-component response regulator, cell-division protein, FtsZ, and a hypothetical protein were classified as

specific immunogens of *B. abortus*. Three other proteins, including invasion protein B, beta-ketoadipyl CoA thiolase, and 50S ribosomal protein L10, were grouped as relatively strong immuogenic proteins. The identification of some of the spots (chaperonin GroES, DnaK, Cu/Zn-SOD and *his*D) as effective immunogens is in agreement with previous reports [3, 20, 27, 66, 124]. .

The most prominently immunogenic protein in this study was GroES, which had been identified as a potential immunoreactive protein in previous studies [3, 124]. GroES is a member of the heat shock protein (*hsp*) family, and is classified as a chaperone protein. Chaperonin is one of the more prominently immunogenic antigens of *B. abortus* and is known to be closely associated with *Brucella* virulence via protein folding and degradation. In our study, two types of chaperone proteins were present experimentally at approximately 28.1 kDa (spot 3030) and 58 kDa (spot 5401) on a 2-DE gel (Fig. 1A). GroEL has been identified as an immunogen in a previous report [3, 66]. However, this protein showed immunoreactivity with all four types of sera in this study and was accordingly excluded from our selection.

Moreover, because DnaK is a chaperonin, it is affiliated with the hsp70 family, which has a highly conserved region, and is known to be highly immunogenic [14, 17, 18], Consistent with that finding, recombinant DnaK was reported to induce a statistically significant of protection against *B. abortus* [26, 43]. Moreover, Rafie-Koplin et al. [104] and Köhler et al. [67] revealed that DnaK expression increased the intracellular survival of *Brucella*. Al Dahouk et al. [3] noted that DnaK did not show immunoreactivity with *Y. enterocolitica* O:9, which is consistent with our results. Accordingly, DnaK might be a candidate antigen for the improvement of brucellosis diagnosis.

The immunogenic protein Cu/Zn-SOD is known to be part of an antioxidant defense system that protects microorganisms against ROS (reactive oxygen species) and contributes to the survival of *Bruclla* in intracellular environments [65, 98].

Not only is Cu/Zn-SOD known to be closely related with the virulence of many microbes [65, 129, 143], but it is also found to be differential antigenic protein for brucellosis detection by immunoproteomic analysis [3, 98]. Like Cu/Zn-SOD, ALDH is involved in defense against oxidative stress [118]. The levels of ALDH increase when bacteria encounter various stressful conditions. In addition to the proteins mentioned above, *his*D, two-component response regulator, and cell-division protein FtsZ were all reported as immunogenic proteins in various publications [20, 23, 132].

To date, several trials in detecting the immunogenic proteins of *Brucella* have focused mainly on the pI range of 4-7. Thus, strongly alkaline proteins, such as the ribosomal proteins with pIs between 9- and 12 received relatively little attention as immunogenic proteins. As shown in Fig. IV-2, there were very predominant protein spots with molecular weights of approximately 22.2 kDa. In our analysis, they were identified as 50s ribosomal protein L10 and invasion protein B. Although they showed immunoreactivity with all four types of sera, they had 15-35 times stronger reactivity with the *B. abortus* antiserum than with the other sera. The 50s ribosomal protein L10 is not considered generally as a specific antigen for detecting a *B. abortus* infection, but it is deeply involved in the function of ribosomal protein L7/12. These two ribosomal proteins are located near the 50s ribosomal subunit and forms an L7/12-L10 complex [102]. L7/L12 have been reported to be immunogenic proteins in several articles [3, 124]. A ribosomal protein that interacts with the 50s ribosomal protein L7/12, L10 was found to be very strong immunogen in our study.

In our 2-DE analysis, relatively few (eleven) immunogenic protein spots were identified compared with previous studies. Most 2-DE studies [20, 104, 132] characterized and analyzed the entire proteomes of *Brucella* species under specific conditions, such as pH, stress, and laboratory culture condition. In contrast, in this study, we used the insoluble proteins to select *B. abortus*-specific immunogens

using four types of antisera. Because *Y. enterocolitica* O:9 and *E. coli* O157:H7 are known to be representative cross-reactive bacteria with *B. abortus*, many proteins were exempted from our analysis due to their having common antigens. Consequently, many common antigens were ruled out as candidate antigens. In a previous 2-DE study using five types of antisera, the authors also found only six immunogenic proteins of *B. abortus* 1119-3 [3]. Also, two major potential proteins (BP26 and BCSP31) were not included in this study. The reasons might be from that BP26 was shown to only reacti with acute-brucellosis antisera [107]. Because experimentally infected cattle with living *B. abortus* might be experience same infection course with naturally infected cattle [67], so the kinetics of bovine serum antibody response to *B. abortus* RB51 proteins may be affected by infection course of brucellosis. Futhermore, BCSP31 did not appear as a discrete spot in our study. Considering its molecular size, it might be included in the horizontal streak on the western blots due to powerful immunogenicity as shown in Fig. IV-1.

In conclusion, our results indicated that 11 specific proteins of *B. abortus* RB51 have immunoreactivity with antisera from cattle experimentally infected with *B. abortus* and not with *Y. enterocolitica* O:9- and *E. coli* O157:H7-positive or with brucellosis-negative sera. Although the reactivity against antisera from naturally infected cattle should be further investigated, our findings suggest that the immunogenic proteins of *B. abortus* RB51 could be useful alternative or supplemental antigens for serological diagnosis of brucellosis.

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# General Conclusion

Brucellosis still remains a major zoonotic disease worldwide, and it causes economic loss by reproductive problems. Mongolia is one of the high-prevalence of brucellosis-outbreak countries, its molecular and epidemiological investigation is rare. Despite of its high-outbreak of brucellosis in Mongolia, the studies on brucellosis is lacking compared to adjacent country, China. Also, various biovars and species in the genus *Brucella* had been reported in Mongolia due to its unique nomadic or semi-nomadic rearing system and various animal species.

For those reasons, Mongolia is very attactive country to investigate the *Brucella* strains' characteristisc depends on animals hosts, geographical distribution, and evaluate newly developed diagnostic methods using their diverse *Brucella* isolates.

Therefore, we focused on the characterization and investigation of *Brucella* strains in Mongolia by phenotyping and molecular detection methods, and the evaluation of new differential diagnosis of *Brucella* infection efficiently.

A total of 16 *B. abortus* strains were obtained from humans and various animal species including camel in Mongolia. They were identified as *B. abortus* by differential multiplex PCR assay, but seven of them didn't belong to any biovars in *B. abortus* using classical biotyping assay in the current taxonomy. Their biological and biochemical characteristics were identical with those of the former bv. 7 which was suspended from *Brucella* taxonomy in 1986 because its reference strain 63/75 consisted of a mixture of bv. 3 and bv. 5. To investigate the existence of the former *B. abortus* bv. 7, additional molecular techniques were applied. According to representative biovar-differential PCR assays such as BaSS (*B. abortus* species-specific) PCR and *omp*2a-PCR, the untyped *Brucella* strains revealed that they did not belong to bvs. 1, 2 or 4. Moreover, comparative 16s rRNA sequencing analysis demonstrated that the untyped strains harbored a specific SNP site that

distinguishes from other biovars in *B. abortus*. Taking the phenotypic and molecular typing results together, the untyped strains were suspected to be *B. abortus* bv. 7. The *B. abortus* isolates in our study showing the same polyphasic traits as the former bv. 7 strain might be helpful for proving the existence of *B. abortus* bv. 7. Therefore, our finding suggests the possibility of reinstating *B. abortus* bv. 7 into the *Brucella* taxonomy.

Besides *B. abortus*, 94 *B. melitensis* (bv. 1, 3 and Rev. 1) from Mongolia were obtained in this research. *B. melitensis* is considered as the most pathogenic strain within *Brucella* species, and its main host is goat and sheep. To date, some publications on serological surveillance for brucellosis and molecular typing of *Brucella* isolates in Mongolia had been reported earlier, but in-depth epidemiological analysis based on molecular typing was rare. Therefore, accurate identification of the epidemiological characteristics is crucial to prevent and control of brucellosis in Mongolia. In this study, we applied MLVA-16 assay to investigate Mongolian *Brucella* strains and compare to other countries. In. *B. abortus*, Mongolian *B. abortus* bv. 3 and untyped strains were located very close to each other, and untyped strains were identical with previous bv. 7 strain and exhibited a consistent genotype, regardless of the animal species, location and isolation year. Furthermore, Mongolian *B. abortus* bv. 3 strains clustered with Chinese strains. Inner Mongolia showed the highest incidence of brucellosis in China, its prevalent biovars might be related to those of Mongolia due to geographical proximity. This result was also found in the MLVA typing and minimum parsimony analysis of *B. melitensis*, Mongolian *B. melitensis* was closely related with Chinese strains compared with other countries. Also, *Brucella* strains from animals and humans were located in the same group, so transmission of brucellosis between them occurred frequently. Therefore, more strict animal quarantine and control

measures are required to prevent the spillover of *Brucella* species among the border or the transfer between animals and humans.

To complement the disadvantages of the bacterial culture and the current serological assays to diagnose brucellosis, various molecular detection tools such as PCR assays had been developed. With the development of rapid PCR diagnosis such as a real-time PCR, we developed the new *B. abortus*-specific real-time PCR based on SNP using hybprobe to diagnose *B. abortus* infection. The *B. abortus*-specific SNPs were detected at the *fba*A gene of *B. abortus* chromosome II, with cytosein changed to thymine at 360432 of the *fba* gene. In terms of specificity and sensitivity, our new real-time PCR showed a superior diagnostic efficiency for differential diagnosis of *B. abortus* infection with accuracy and rapidity even though it has a limitation to discriminate *B. abortus* vaccine strains. Because the genus *Brucella* is an intracellular bacterium and the number of bacteria in specimens is usually small, a highly sensitive diagnostic technique is required for accurate differential diagnosis. This new real-time PCR could be very useful for directly diagnosing brucellosis caused by *B. abortus* in infected animals due to the high detection limit. In conclusion, our new real-time PCR based on hybprobe could be an efficient diagnostic technique with high sensitivity and rapidity for *B. abortus*-infected animals in the field, and it could also be applicable in public health.

The current brucellosis serodiagnoses are chiefly based on detecting anti-LPS antibodies. The LPS antigen elicits so strong antibody response, but it also induce the cross-reaction with some gram-negative bacteria haboring similar OPS structure. To overcome this disadvantage, we investigated the new potential proteins to distinguish brucellosis using *B. abortus* RB51 strains by 2-DE and MALDI-TOF. To detect immunogenic proteins in the whole pH ranges, three

ranges (pH 3-5.6, 4-7 and 6-11) were applied using 3 types of IPG strips. After separating the insoluble proteins of *B. abortus* RB51 using 2-DE, their immunogenicity was evaluated by western blotting using four types of antisera (*B. abortus*-positive and -negative sera, *E. coli* O157:H7- and *Y. enterocolitica* O:9-positive sera). Due to applying four types of antisera, many overlapped proteins were ruled out, so only eleven immunogenic proteins were found in this study. The candidate proteins ranged from pH 3-7 were consistent with the previous proteomic research, and the selected antigens in alkaline ranges were closely related with the function of immunogenic proteins, ribosomal protein L7/12. These identified highly immunogenic proteins might be useful as alternative antigens for brucellosis and helpful in reducing the cross-reaction.

In conclusion, our molecular biological and epidemiological investigation of Mongolian *Brucella* strains might provide valuable information about the characteristics of *Brucella* strains in Asia area and epidemiological trace-back analysis. It may play a cruial role in preventing and controlling brucellosis. Also, our new developed real-time PCR and proteomic analysis results are expected to contribute to diagnose *B. abortus* infection more efficiently, with obtaining brucellosis-free status in Korea in the near future.

국 문 초 록

# 브루셀라균의 분자생물학적 특성 분석 및 감별진단법 개발

서울대학교 대학원 수의미생물학 전공

김 지 연

(지도교수: 박 용 호)

브루셀라병은 가축을 비롯한 다양한 동물종에서 유산 등의 질병을 일으켜 축산업계에 큰 피해를 일으키는 질병으로, 사람에게도 감염되는 주요 세균성 인수공통전염병이다. 현재 국내를 비롯한 아시아 및 유럽 등 전세계적으로 발생하고 있으며, 브루셀라균 제거 및 전파 방지를 위하여 국가별로 강력한 살처분 정책 또는 백신 정책을 시행하고 있다. 이처럼, 브루셀라병의 효과적인 근절을 위하여는 강력한 방역 정책 실시와 함께 원인체의 특성을 명확히 파악하고, 질병의 유래 및 전파 원인을 알아내기 위한 역학적 추적 기법 개발 및 분석 역시 병행되어야 한다. 이와 함께 브루셀라병을 신속하게 감별 진단하는 항원 및 항체 진단법 개발 역시 꾸준히 진행되어야 할 과제이다.

본 연구에서는, 우리나라보다 다양한 축종의 동물 유래 및 생태형(biovar) 브루셀라 분리주를 확보 가능한 몽골 유래 브루셀라균주를 이용하여 분자생물학적 특성 분석 및 이들에 대한 역학적 특성 분석을 실시하였다. *B. abortus* 16 균주 중 9 주는 *B. abortus* bv. 3 이었으며, 나머지 7 균주는 현재 브루셀라균 분류 체계에 속하지 않는 untype 이었다. 이 untype 균주를 대상으로 생화학적 및 분자학적 진단 기법을 적용한 결과, 1986 년 브루셀라균 분류 체계에서 삭제된 *B. abortus* bv. 7 과 동일한 특성을 지니고 있음이 확인되었다. 이 결과를 통해 현재는 삭제되어 있는 *B. abortus* bv. 7 이 다시 공식적인 *B. abortus* 의 생태형의 하나로 재등록되어야 한다는 의견을 뒷받침하고 있다.

또한 본 연구에서 확보된 *B. abortus* 16 주와 *B. melitensis* 94 균주를 이용하여 실시한 역학적 상관관계를 비교 분석하였다. *B. abortus* 균주의 경우 *B. abortus* bv. 3 분리주는 4 개, untype 분리주는 단일 유전형으로 확인되었다. 몽골 포함 국외 분리주 총 60 균주를 대상으로 16 부위 마커 유전자를 분석하여 균주 간 유전적 상관성을 도식화한 결과 크게 2 개의 그룹(A, B)으로 분류되었고, 이 중 B 그룹은 5 개의 subgroup 으로 분류되었다. 한편 *B. melitensis* 분리주의 경우 백신균주인 Rev. 1 strain 을 포함 총 29 유전형을 보이며, 클러스터 분석 결과 총 6 개의 그룹으로 분류되었다. 몽골 유래 브루셀라균주의 경우 2 종의 브루셀라균종 모두 중국 분리주와 가장 역학적 상관성이 높은 것으로 확인되었다.

한편 국내에서 발생하는 소 브루셀라병을 신속하게 감별진단하기 위하여 *B. abortus* 균 내 *fbaA* 유전자 중 특이 SNP 부위를 선별, primer 와 probe 를 디자인 하였다. 기존 real-time PCR 기법을 보완하고 진단 효율성 증대를 위하여 amplification curve 와 melting peak 분석을 함께 실시하는 hybprobe 를 적용하였다. 브루셀라균주 DNA 샘플 및 가검 샘플을 적용한 결과, 기존의

PCR 및 real-time PCR 진단법보다 동등하거나 향상된 수준의 진단 효율성을 보여 추후 *B. abortus* 로 인한 브루셀라병 진단에 있어 유용하게 쓰일 것으로 기대된다.

또한 항체 진단법 개발의 일환으로 브루셀라균 내 면역원성이 높은 단백질 항원을 검색하고자 하였다. 기존 연구를 보완하는 방안으로 강한 면역원성을 지니는 LPS 부위가 결여된 *B. abortus* RB51 균주에서 insoluble protein 을 추출, 4 종의 antiserum 을 이용하여 2-DE 와 western blot 기법을 이용하여 면역원성이 우수한 단백질 항원을 검색하였다. 그 결과 교차반응을 유발하는 여시니아나 대장균 O157:H7 에는 반응하지 않고 브루셀라균에만 면역원성을 보이는 11 종의 단백질 항원을 선발하였고, 이들 항원은 추후 브루셀라병 진단 시 보완 또는 대체할 수 있는 진단용 항원으로서 적용 가능할 것으로 예상된다.

결론적으로, 분리된 브루셀라균에 대한 명확한 특성 조사 및 분석은 브루셀라병 발생국에서는 병의 근절을 위하여 가장 기본적으로 시행해야 할 과제이며, 브루셀라병의 유래 및 동물 축종 및 사람 간 전파 경로를 파악하기 위한 MLVA typing 기법 적용은 필수적이라 할 수 있다. 덧붙여, 개선된 브루셀라병 감별진단법 개발은 추후 인의 및 동물용 브루셀라병 진단에 있어 효과적으로 적용할 수 있을 것으로 사료된다.

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주요어: *Brucella,* phenotypic and molecular characteristics, epidemiological analysis, MLVA-16, real-time PCR using hybprobe, SNP, immunogenic proteins

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