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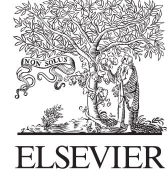

ORIGINAL ARTICLE

Evaluation and Selection of Multilocus Variable-Number Tandem-Repeat Analysis Primers for Genotyping *Brucella abortus* Biovar 1 Isolated from Human Patients

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Abstract

Objectives: Brucellosis is the most common bacterial zoonosis in the world. Multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) is a molecular method for genotyping bacterial species. *Brucella abortus* biovar I was isolated from most of the brucellosis-suspected patients in Korea. This study was conducted to investigate the ability of various MLVA primers that are used for molecular typing *B. abortus* isolates and for analyzing their epidemiological data.
Methods: A total of 80 human isolates of *B. abortus* biovar I isolated from human patients and the reference strain were used for MLVA. Genetic diversity was determined by calculating the Simpson's diversity index (DI) of each VNTR locus. The *Brucella* strains were subcultured 30 times to determine the stability of each locus. The DNA of the strains cultivated in each passage was extracted and subjected to MLVA for further investigation.
Results: The 15 VNTR loci were selected based on high DI values. The DIs of the 15 VNTR loci showed considerable discrimination power ranging from 59% for Bruce 43 to 87% for Bruce 22. Bruce 09, Bruce 11, Bruce 16, Bruce 42, and Bruce 43 were confirmed to remain stable *in vitro* among the 15 VNTR loci selected.
Conclusion: The results of this study suggest that the five loci subsets may be a useful epidemiological tool for investigating *B. abortus* biovar 1 outbreak.

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1. Introduction

Brucellosis, caused by species of the Gram-negative bacterium *Brucella*, continues to be a problem in humans and animals throughout the world [1]. In Korea, brucellosis is an endemic disease, and *Brucella abortus* is the prevailing strain in human infections [2]. Human brucellosis occurs in livestock workers and veterinarians who live and work with cattle farms in rural areas [3].

Brucellosis spreads through foods contaminated with the bacterium *Brucella*, such as untreated milk products, cheese, or meat, as well as during contact with infected animals [4] and exposure to air containing the pathogen. Laboratory technicians and butchers usually acquire the infection when exposed to an infectious environment. It has been reported that more than 90% of brucellosis cases worldwide are attributed to *Brucella melitensis* and that *B. abortus* has been identified as the main pathogen causing the infection in Korea [5].

Species identification and subtyping are important factors for epidemic surveillance and for investigating regions with a high incidence of brucellosis [6,7]. In general, microbiological methods and molecular typing are used to identify *Brucella* pathogens and to classify their species. *B. abortus* has been known to have at least seven biovars, and biovar 1 was isolated from most brucellosis-suspected patients in Korea. Phenotypic characterization does not provide sufficient data to trace the origin of the source of infection for epidemiological investigation. Recently, molecular biological methods have been developed and used for *Brucella* molecular typing purposes and epidemiological applications. This method is fast and easily standardized, and allows for strain clustering based on variable-number tandem-repeat (VNTR) similarities [8].

In addition, because brucellosis has more than 90% DNA homology among species [8,9], epidemic studies have been mainly conducted using molecular genetic analysis [10]. Recent proactive studies on the genomic sequence of brucellosis and multilocus VNTR analysis (MLVA) have been mainly conducted for the identification of various *Brucella* species involved in causing the infection [9,11−14]. The MLVA has been used not only for *Brucella* identification but also for the identification of other pathogens [15]. Furthermore, it is useful for investigating molecular genetic pathogenesis.

In this study, 78 MLVA primers were applied to 80 strains of *B. abortus* isolated from humans between 2003 and 2007. This study was conducted to identify those MLVA primers that are appropriate for the Korean isolates, to establish a method that rapidly identifies species using the selected primers, and to present epidemiological data for the molecular typing of *B. abortus* biovar 1 isolates.

2. Materials and Methods

2.1. Bacterial isolates

A total of 80 human brucellosis isolates were submitted to our laboratory in Korea during the period between 2003 and 2007 from the following ten provinces: Gyeonggi-do (GG), Gyeongbuk (GB), Gyeongnam (GN), Chungbuk (CB), Chungnam (CN), Daegu (DG), Jeonbuk (JB), Gwangju (GJ), Jeju (JJ), and Gangwon (GW). The reference strain, *B. abortus* 2308, was also included in the study. Genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) by following the manufacturer's instructions.

2.2. In vitro *stability test*

The stability of MLVA loci was evaluated *in vitro* using culture-passage methods. A single colony was taken from each of the *B. abortus* 2308 strain and human isolates 1/3, and inoculated on the blood agar plate. The strains were incubated for 3−4 days at 5% CO_2, 37 °C for 30 passages. The DNA of the strains cultivated in each passage was extracted and subjected to MLVA.

2.3. Polymerase chain reaction amplification and electrophoresis

The MLVA primers used in our assay were previously reported by Al Dahouk et al [8]. The DNA amplification was performed using 15 μL of solution containing 10 ng of genomic DNA, 1 U of i-Max II DNA polymerase (iNtRON Biotechnology, Gyeonggi-do, Korea), 2.5 mM deoxyribonucleotide triphosphate mixture, 10× polymerase chain reaction (PCR) buffer, 10 pmol of each primer (Bioneer, Daejeon, Korea), 5× betaine (Sigma Aldrich, St. Louis, MO, USA), and distilled sterilized water. Each reaction mixture was run in the Gene Amp PCR system 9700 (Applied Biosystems). After initial denaturation for 5 minutes at 96 °C, 30 cycles of multiple passages were ran: first, for a 30-second duration at 96 °C, followed by another 30-second run at 60 °C and a 1-minute run at 70 °C, with a final extension step at 70 °C for 5 minutes. The PCR product was loaded onto 2% or 3% agarose gel (GenDEPOT, Barker, NY, USA) with a size marker (100-bp ladder; Bioneer, Daejeon, Korea). Electrophoretic separation was run for 45 minutes.

2.4. Genotyping assay

Brucella DNA was prepared using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The fluorescent primers were synthesized with one of the following three dyes (ABI, London, UK): NED (yellow), 6-FAM (blue), and HEX (green; Table 1). The PCR was performed using i-Max II polymerase (iNtRON Biotechnology) for DNA amplification. The PCR product was analyzed with an ABI PRISM 3730xl Analyzer using

Table 1. Primers used for multiplex PCR

Primer name	5′→3′ sequence*
Bruce 01	HEXGGTCTGGGAAAACATGAAAAGC
Bruce 04	6-FAMCTGACGAAGGGAAGGCAATAAG
Bruce 25	NEDGGGAGTATGTTTTGGTTGCACA
Bruce 11	6-FAMCTGTTGATCTGACCTTGCAACC
Bruce 30	NEDTGACCGCAAAACCATATCCTTC
Bruce 07	HEXGCTGACGGGGAAGAACATCTAT
Bruce 42	6-FAMCATCGCCTCAACTATACCGTCA
Bruce 72	NEDGAAGACGGCTATCGACTGGTCT
Bruce 09	HEXGCGGATTCGTTCTTCAGTTATC
Bruce 16	6-FAMACGGGAGTTTTTGTTGCTCAAT
Bruce 71	NEDGAAGACGGCTATCGACTGGTCT
Bruce 14	HEXTTGCTTTATCTTATCTGATTCTTTCAA
Bruce 22	6-FAMGATGAAGACGGCTATCGACTG
Bruce 43	NEDTCTCAAGCCCGATATGGAGAAT

*HEX, NED, and 6-FAM are fluorescent dyes covalently bound to the 5′ end of the synthetic primer. PCR = polymerase chain reaction.

the ABI PRISM BigDye Terminator Cycle Sequencing kit containing AmpliTaq DNA polymerase.

2.5. Cloning and sequencing analysis

The PCR product was cloned into a pCR 2.1-TOPO (Invitrogen, Carlsbad, CA, USA), and the plasmid was transformed into the competent *Escherichia coli* strain DH5a. Recombinant bacteria were selected and identified by blue/white screening.

2.6. Data analysis

The genetic diversity of VNTRs was calculated using online tools [16]. The diversity index (DI) is a measure of variations in the number of repeats at each locus. It ranges from 0.0 (no diversity) to 1.0 (complete diversity).

3. Results

3.1. Selection of MLVA primers appropriate for Korean Brucella subtyping

The MLVA standardization was conducted on the reference strain, *B. abortus* 2308, and on the 80 human isolates of biovar 1, which were identified in the following Korean regions between 2003 and 2007: Gyeonggi (GG), Gyeongbuk (GB), Gyeongnam (GN), CB, CN, DG, JB, GJ, JJ, and GW. In this study, MLVA primers that are appropriate for Korean *Brucella* subtyping were selected using the 78 MLVA primers, including MLVA-16. The 78 primers were applied to the reference strain and the human isolates, and the results were shown as the size of the PCR product. When genetic diversity was measured using an online analysis tool, the DI was shown between 0% and 75%. Among the primers, Bruce 01, Bruce 14, Bruce 22, Bruce 43, Bruce 71, and Bruce 72 had a high diversity (Figure 1). In addition, Bruce 22, Bruce 01, Bruce 14, Bruce 43, and Bruce 71 had a Simpson DI of more than 0.5. The nonspecific band size was shown in Bruce 06. In the case of Bruce 15, however, it was difficult to discriminate among the various Korean isolates because there were no data on PCR amplification. The highest diversity was seen in 14 MLVA primers, including Bruce 01, Bruce 04, Bruce 07, Bruce 09, Bruce 11, Bruce 14, Bruce 16, Bruce 22, Bruce 25, Bruce 30, Bruce 42, Bruce 43, Bruce 71, and Bruce 72. These primers seemed to be effective in Korean *Brucella* subtyping.

For further accurate analysis of 14 MLVA primers that were found to be appropriate for Korean *Brucella* subtyping, fluorescent-labeled genotyping was conducted using the 14 primers. Fluorescent labels, such as HEX, 6-FAM, and NED, were attached to the primers according to the size of PCR products (Table 1), and then the primer set was applied to the reference strain, *B. abortus* 2308, and the 80 human isolates. The genotyping data were analyzed using V-DICE software. The results of genotyping analysis were compared with those of electrophoresis analysis. More diverse discrimination was seen in genotyping using fluorescence labels. This may be attributed to the fact that a small difference that is difficult to be highly discriminated by electrophoresis analysis can be discriminated by fluorescent labeling. A diversity of $\geq$2 was observed in Bruce 2, Bruce 01, Bruce 14, Bruce 72, Bruce 71, Bruce 43, and Bruce 25 compared with that of electrophoresis analysis.

To examine the accuracy of genotyping analysis, 12 PCR products that had a size different from that of the reference strain, *B. abortus* 2308, were selected among MLVA PCR products of the isolates, and their nucleotide sequence was analyzed. As a result of nucleotide sequence analysis, 11 of 12 cases were consistent with those of fluorescent-labeling analysis, and two cases were consistent with those of electrophoresis analysis. This result suggested that fluorescent labeling could be used for accurate analysis.

3.2. Genomic stability of multiple passages

A previous study reported that the VNTR number of strains changes if multiple passages are performed [2]. Thus, the reference strain, *B. abortus* 2308, and human isolates 1/3 were examined after 30 cycles of multiple passages with intervals of 2−3 days. The DNA was then extracted from each strain, and mixed with the selected 14 primers to investigate consistency in PCR products. The change in PCR product size between the reference strain and human isolates 1/3 was observed in the ninth passage for Bruce 01, Bruce 04, Bruce 07, Bruce 14, Bruce 22, Bruce 25, Bruce 30, Bruce 71, and Bruce 72 among the 14 MLVA primers (Figure 2). Meanwhile, genomic stability without size change was observed for

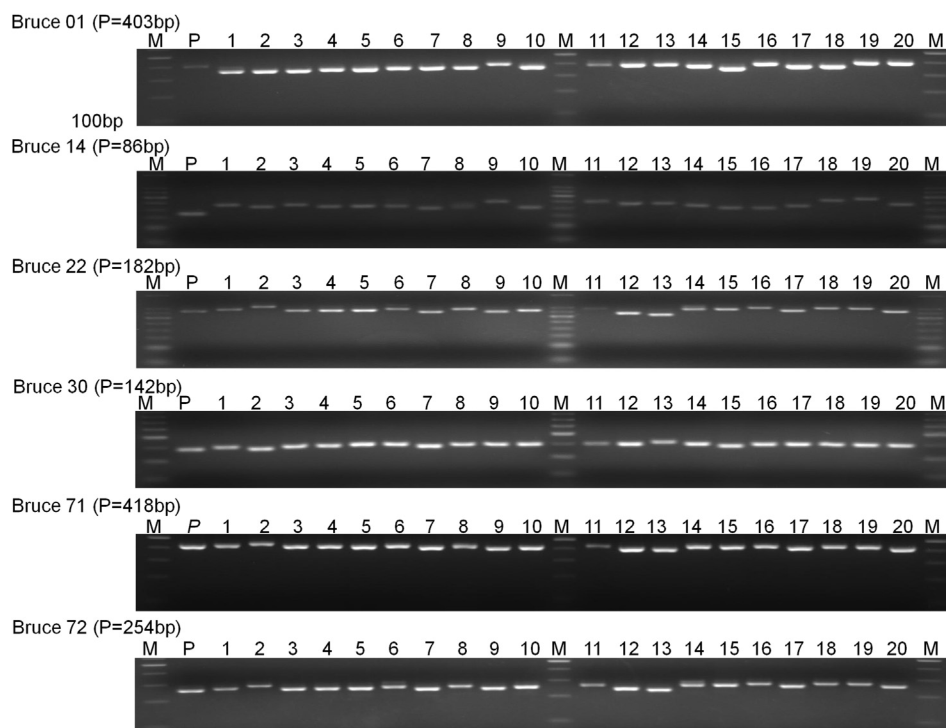

Figure 1. Amplification patterns of MLVA on *Brucella abortus* biovar 1. Lane M, 100-bp ladder; Lane P, *B. abortus* 2308; Lanes 1−20, *B. abortus* biovar 1 isolates. The indicated PCR products size of the reference strain was confirmed by DNA sequencing. The six VNTR loci (Bruce 01, Bruce 14, Bruce 22, Bruce 30, Bruce 71, and Bruce 72) show differentiation in *B. abortus* human isolates. MLVA = multilocus variable-number tandem-repeat (VNTR) analysis; PCR = polymerase chain reaction.

Bruce 09, Bruce 11, Bruce 16, Bruce 42, and Bruce 43 despite the 30 cycles of multiple passages.

3.3. Establishment of the condition of fluorescence-labeling multiplex MLVA

For the development of rapid and easy MLVA, primer sets that are appropriate for multiplex PCR were designed to be used in electrophoresis. Two primer sets were prepared using the finally selected five primers to allow discrimination in genotyping without the overlapping of fluorescent labeling, and then multiplex PCR was conducted. When electrophoresis was performed, the difference in the PCR product size was found with naked eyes. Meanwhile, accurate discrimination was

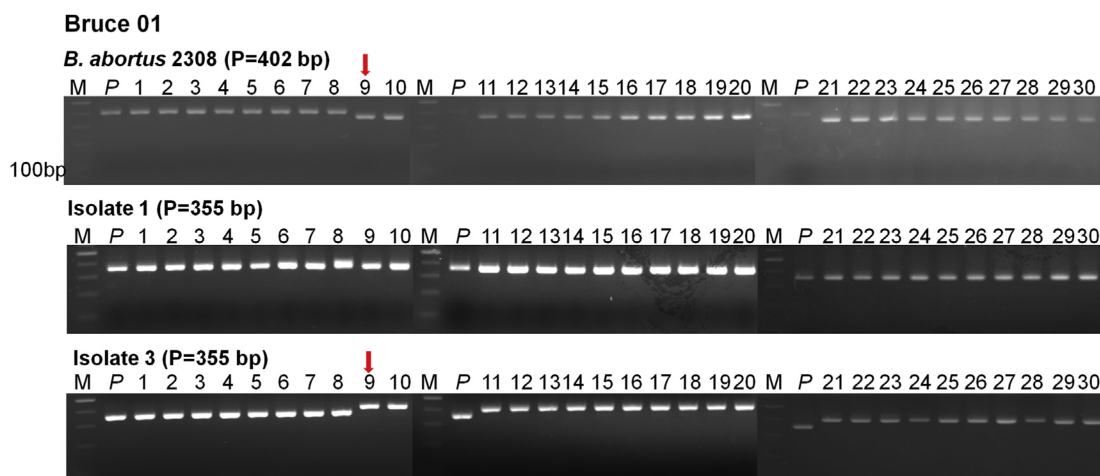

Figure 2. The stability of 15 VNTR loci on *Brucella*. Lane M, 100-bp ladder; Lane P, *Brucella abortus* 2308, isolate 1, isolate 3 positive control; Lanes 1−30, passages. The five VNTR loci (Bruce 09, Bruce 11, Bruce 16, Bruce 42, and Bruce 43) show stability during serial passages. VNTR = variable-number tandem-repeat.

Table 2. Comparison between gel electrophoresis and genotyping assay of Simpson diversity index of each of the 15 VNTR loci with human *Brucella abortus* isolates

Capillary electrophoresis analysis			
Locus	Diversity	Confidence interval	*K*
Bruce 22	0.871	0.847−0.895	12
Bruce 01	0.864	0.840−0.888	12
Bruce 14	0.840	0.812−0.869	11
Bruce 72	0.829	0.793−0.866	11
Bruce 71	0.796	0.743−0.848	9
Bruce 43	0.590	0.513−0.643	7
Bruce 25	0.578	0.513−0.531	4
Bruce 30	0.408	0.285−0.531	4
Bruce 04	0.285	0.161−0.409	4
Bruce 07	0.140	0.039−0.241	4
Bruce 09	0.117	0.023−0.211	3
Bruce 42	0.116	0.024−0.208	2
Bruce 11	0.095	0.008−0.182	3
Bruce 16	0.094	0.009−0.179	2

VNTR = variable-number tandem-repeat.

observed in genotyping analysis. Thus, genotyping analysis was confirmed as an analysis method that can identify primers for discrimination among Korean Brucellosis cases.

4. Discussion

Methods for investigating pathogenesis have been significantly developed. Currently, MLVA has been mainly used for tracking the pathogenesis of bacterial genus with a high homology, such as the *Brucella* genus [11]. Because this method is applied to animals or humans to identify causative factors of infections where origin tracking of infection is difficult, that is, in cases involving infection of veterinarians or laboratory technicians and infection caused by food intake, appropriate molecular genetic analysis is required for tracking the route of transmission in Korea. Therefore, this study was conducted to investigate the origin tracking of endemic strains of biovar 1 human isolates collected from seven regions between 2003 and 2007. Based on a previous study indicating that discrimination among strains and origin tracking can be conducted using MLVA-16 that consist of three panels, the total MLVA, including the aforementioned MLVA-16, was applied to Korean human isolates. As a result, Bruce 01, Bruce 04, Bruce 07, Bruce 09, Bruce 11, Bruce 14, Bruce 16, Bruce 22, Bruce 25, Bruce 30, Bruce 42, Bruce 43, Bruce 71, and Bruce 72 were shown to be the MLVA primers that can discriminate between the reference strain, *B. abortus* 2308, and the isolates. These primers were also found to have a high diversity. The DI at 14 VNTR loci was shown to vary from 59% in Bruce 43 to 87% in Bruce 22 (Table 2). The 14 primers with a high diversity seem to be useful for discriminating between the reference strain and the Korean isolates and for the identification of the possible route of *Brucella* transmission.

It has been known that changes in repeat number by multiple passages induce DNA insertion, deletion, and DNA point mutation. To assess the usefulness of the selected 14 primers, the reference strain, *B. abortus* 2308, and the isolates 1/3 underwent 30 cycles of multiple passages, and then the DNA of each strain was extracted. The obtained DNA was mixed with the primers selected from subtyping, followed by PCR. As a result, despite the 30 cycles of multiple passages, Bruce 09, Bruce 11, Bruce 16, Bruce 42, and Bruce 43 showed genomic stability without changes in the size of PCR product (Figure 2). Fluorescent-labeling multiplex PCR was performed to develop a rapid and accurate MLVA using the selected five primers. As a result, a small difference that is hard to be discriminated by electrophoresis was identified by this method. Because molecular genetic analysis requires the accuracy and stability of genetic markers, this rapid and accurate analysis method could be useful for molecular genetic analysis.

In summary, of the 78 MLVA primers used in this study, Bruce 09, Bruce 11, Bruce 16, Bruce 42, and Bruce 43 allowed discrimination among the Korean isolates. Thus, a rapid and easy identification of strains was feasible by multiplex PCR using the five loci primers. In addition, these primers can be used for the identification of strains that cause an endemic outbreak and for tracking their origin, as well as for the identification of an outbreak of *Brucella abortus* isolates.

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