

## Multiple-locus variable number of tandem repeat analysis assay as a tool for the epidemiological study of *Brucella canis* in Korea

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Received: 31.12.2014 • Accepted/Published Online: 19.02.2015 • Printed: 28.08.2015

**Abstract:** Multiple-locus variable number of tandem repeats analysis (MLVA) is useful for epidemiological investigations. In this study, MLVA-18 was used to assess the genetic and epidemiological relationships of *Brucella canis* isolates among a domestic dog and dogs from two breeding farms. MLVA-18 assay showed variation in copy numbers in 6 of the 18 loci. Interestingly, the YJ02-1 strain isolated from the pet dog from Yangju-si showed only one tandem repeat change in Bruce 09 locus as compared with that of NYJ-1 strain isolated from a dog from the Namyangju-si farm. The two strains were shown to have originated from the same source or a closely related strain.

**Key words:** *Brucella canis*, epidemiological, MLVA

*Brucella canis* is an etiological agent of canine brucellosis, a zoonotic disease that can be transmitted to humans. *B. canis* is associated with dogs, which are its natural host. In some cases, dogs can also be infected with other *Brucella* species such as *B. abortus*, *B. melitensis*, and *B. suis*; however, *B. canis* is the primary etiological agent responsible for canine brucellosis (1,2). The clinical symptoms of canine brucellosis are abortion in late pregnancy, epididymitis, and prostatitis, all of which can cause reproductive failure (2,3). Thus, canine brucellosis can result in economic losses with respect to kennels. Transmission among dogs occurs while mating or through direct contact with vaginal discharge after abortion, infected placenta, or fetus. Infections can also be transmitted through urine, since *B. canis* can be shed in the urine for several years (2–4). Therefore, brucellosis can be easily transmitted among dogs living in close proximity.

Although *B. canis* infection in humans is rare, it can occur through exposure to contaminated canine tissues or fluids such as products exuded during conception and abortion or vaginal discharge. *B. canis* infection in humans can be difficult to diagnose because of the nonspecific disease symptoms such as undulant fever, headache, and weakness (5,6). Therefore, identification of the source of infection, treatment, and eradication of canine brucellosis may be an effective treatment strategy.

Many methods have been developed for molecular subtyping in the last decade and multiple locus variable number of tandem repeat analysis (MLVA) has proven to be highly appropriate for analyzing phylogenetic relationships among isolates and epidemiological trace-back investigations (7,8). MLVA has been used in epidemiological studies of *Brucella* species (9,10), *Streptococcus agalactiae* (11), *Pseudomonas aeruginosa* (12), *Bacillus anthracis* (13,14), and *Salmonella typhimurium* (15,16). The discriminatory power of the assay has been increased by the inclusion of more markers. However, MLVA has not been commonly used in epidemiological studies with *B. canis*. In this study, MLVA-18 was employed to analyze *B. canis* isolates from different areas and trace the infection source.

Blood of a 1-month-old domestic dog was sent to the diagnostic laboratory for bacteriological testing, asking especially for *B. canis*. The serological test was positive (data not shown) and *B. canis* was isolated from blood culture. Following this, an epidemiological survey was conducted, which traced the source of infection to a Yangju-si breeding farm, which was also the location of the domestic dog with brucellosis. The epidemiological survey also identified Namyangju-si breeding farm as the birthplace of the infected domestic dog, after which the dog was moved to the Yangju-si breeding farm, which was the location of its adoption. All the dogs from the two breeding farms were

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serologically tested for canine brucellosis. Six dogs from the Yangju-si farm and two dogs from the Namyangju-si farm tested positive serologically and blood samples were collected from these dogs for further analysis. Blood samples from the dogs were collected by veterinarians at the local animal hospitals with the dog owners' consent and approved by the animal experiment ethical committee of the Animal and Plant Quarantine Agency (QIA).

A total of nine *B. canis* isolates were obtained from the canine blood samples: one strain from the domesticated dog, six strains from the Yangju-si farm, and two strains from the Namyangju-si farm. All the isolates were obtained by blood culture and identified using multiplex PCR (17), real-time PCR (18), and classical biotyping methods based on CO<sub>2</sub> requirement, H<sub>2</sub>S production, urease activity, oxidase production, dye sensitivity, and lysis by phage (Table). The genomic DNA of *B. canis* isolates was extracted using a DNeasy blood and tissue kit (QIAGEN Korea Ltd., Korea) following the manufacturer's instructions and was stored at -20 °C until further use.

The genotypes of the nine isolates were analyzed by MLVA-18. The primer sets of 18 loci, including previously known loci Bruce 04, Bruce 06, Bruce 07, Bruce 08, Bruce 09, Bruce 11, Bruce 12, Bruce 16, Bruce 18, Bruce 19, Bruce 21, Bruce 30, Bruce 42, Bruce 43, Bruce 45, Bruce 55, and Hoof 3 (4,8) and the new VNTR16 marker, were used for MLVA assay. The forward primer of each primer set was labeled with one of three fluorescent dyes: HEX (green), 6-FAM (blue), or NED (yellow). Amplification

was carried out as described in a previous study (4). The amplicon analysis was performed using a 3730xl DNA analyzer and GeneMapper software ver. 4.0 (Applied Biosystems, USA). The tandem repeat copy numbers of the 18 loci for the isolates were analyzed using Bionumerics ver. 6.1 (Applied Maths, St-Martens-Latem, Belgium) and clustering analysis was performed using the UPGMA algorithm. The genetic diversity of the isolates was evaluated using Simpson's diversity index (DI). Variation of tandem repeats for 18 loci was observed at 6 loci. Bruce 04, Bruce 07, Bruce 09, Bruce 16, Bruce 19, and Hoof 3 were shown to have three, three, four, four, two, and five allelic patterns, respectively. Hoof 3 appeared to have the highest variability, with a DI value of 0.716. The DI values were 0.642, 0.617, 0.593, and 0.346 for Bruce 07, Bruce 09, Bruce 04, and Bruce 19, respectively.

Two isolates, NYJ-1 and NYJ-2, from the Namyangju-si farm were found to have different genotypes, whereas the six isolates, YJ03-1 through YJ03-6, from the Yangju-si farm were divided into 5 genotypes by cluster analysis. Interestingly, the NYJ-1 strain from the Namyangju-si farm showed only one tandem repeat change in Bruce 09 as compared with the YJ02-1 strain from the domesticated dog in Yangju-si. Therefore, it was concluded that the YJ02-1 strain originated from the Namyangju-si farm (Figure).

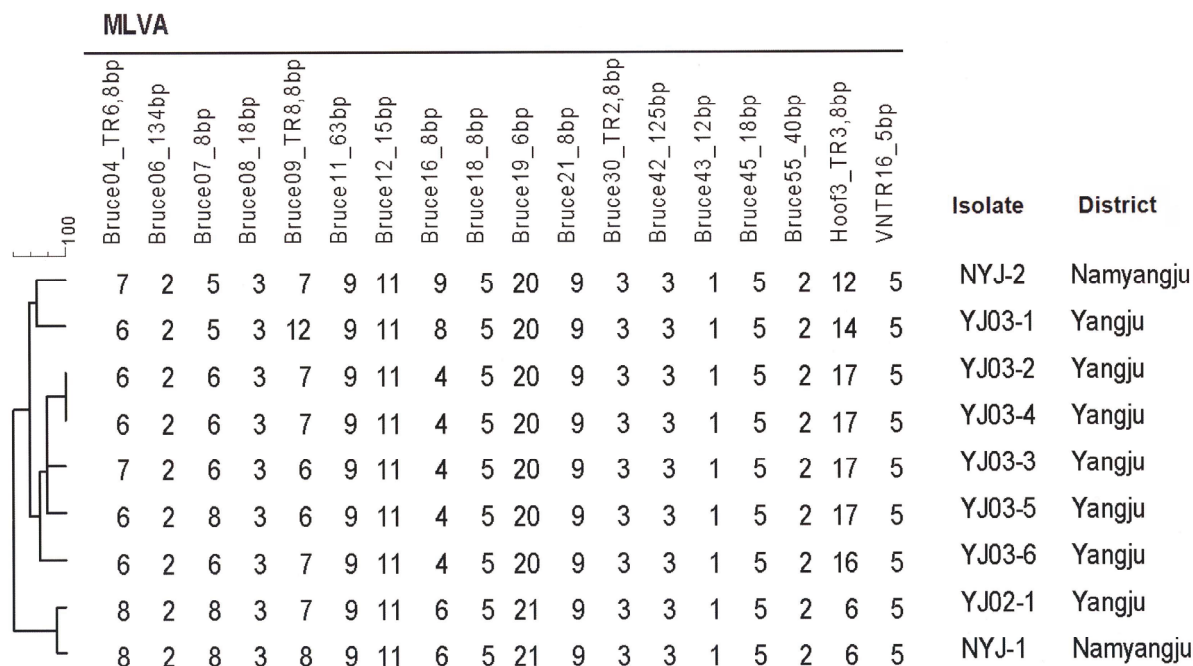
In this study, *B. canis* was isolated from the blood of a domesticated dog diagnosed with brucellosis and, by conducting an epidemiological survey, the possible sources of infection were identified as two breeding farms, which

**Table.** Identification of *B. canis* isolated from the dogs by classical biotyping and PCR methods.

Farms	Districts	Isolates	Growth characteristics				Growth on dyes <sup>a</sup>		Lysis by phages <sup>b</sup>		PCR		
			CO <sub>2</sub>	Oxidase	H <sub>2</sub> S	Urease	TH	BF	Tb	Wb	R/C	Multiplex	Real time
Domesticated dog	Yangju-si	YJ02-1	-	+	-	+	-	+	-	-	+	+	+
		YJ03-1	-	+	-	+	-	+	-	-	+	+	+
		YJ03-2	-	+	-	+	-	+	-	-	+	+	+
		YJ03-3	-	+	-	+	-	+	-	-	+	+	+
		YJ03-4	-	+	-	+	-	+	-	-	+	+	+
		YJ03-5	-	+	-	+	-	+	-	-	+	+	+
Yangju-si farm	Yangju-si	YJ03-6	-	+	-	+	-	+	-	-	+	+	+
		NYJ-1	-	+	-	+	-	+	-	-	+	+	+
		NYJ-2	-	+	-	+	-	+	-	-	+	+	+

<sup>a</sup>Thionin (TH), basic fuchsin (BF)

<sup>b</sup>Phages: Tbilisi (Tb), Weybridge (Wb), and R/C (routine test dilution).



**Figure.** Genetic profiles of *B. canis* isolates based on the dataset of 18 loci. NYJ-1 and NYJ-2 are from the Namyangju-si farm, YJ02-1 from the domesticated dog, and YJ03-1 through YJ03-6 from the Yangju-si farm. The YJ02-1 strain from the domesticated dog had the same copy number of tandem repeat units as the NYJ-1 strain from the Namyangju-si farm, except Bruce 09.

were the domesticated dog's previous residences. Eight *B. canis* isolates were identified from eight dogs from the two breeding farms, which had tested serologically positive for canine brucellosis. To assess the genetic similarity or difference, the isolates were analyzed by MLVA assay. The results of cluster analysis indicated that it was likely that the two isolates from the Namyangju-si farm originated from different sources and that the six isolates from the Yangju-si farm originated from two or three infection sources, since the difference of one repeat number for each locus can be considered as the same allelic type (19). Based on the MLVA-18 data, two strains, YJ02-1 from the domesticated dog and NYJ-1 from the Namyangju-si farm, showed only one tandem repeat change in Bruce 09. In a previous study, Bruce 09 showed some variation after in vitro passage and showed pattern variation in strains from the same farm (4). Therefore, the extent of similarity between the two strains, although originating from different areas, helped us identify that they might be from the same source.

Canine brucellosis is a zoonotic disease transmitted to humans. The risk of infection is high in dog breeders, immunosuppressed individuals, children, and pregnant women (2,3,5). In an effort to protect public health, it is important to survey the prevalence, identify the origin of the infection, and develop countermeasures for the

eradication of brucellosis. To prevent the introduction of brucellosis into breeding farms, the introduction of untested dogs should be avoided. Therefore, brucellosis testing is indispensable, and dogs should be kept quarantined until the test results are determined. It is therefore necessary to establish diagnostic and epidemiological tools for brucellosis. A regular test for dogs of breeding farms should be made mandatory. MLVA enables the comparison of genotypes among strains isolated from different areas, analysis of epidemiological relationships, and identification of the source of infection (20). Therefore, it can provide clues to prevent the spread of the disease, thereby contributing to the eradication of canine brucellosis.

Through the application of MLVA-18, our study demonstrated a genetic relatedness between the two *B. canis* isolates obtained from different areas. MLVA is a very promising tool considering its usefulness to investigate the epidemiological relationship between isolates and to trace the infection sources.

**Acknowledgment**

This work was supported by a fund of the Veterinary Science Technical Development Research Project from the Animal and Plant Quarantine Agency, Republic of Korea.

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