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DNA fingerprinting of *Brucella abortus* isolated from bovine brucellosis outbreaks by repetitive element sequence (rep)-PCR

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Abstract : DNA fingerprint patterns of 8 *Brucella* reference strains and 15 *B. abortus* field isolates were characterized by repetitive element sequence-based PCR (rep-PCR) using BOX- and ERIC-primers in this study. AMOS PCR differentiated all *Brucella* field isolates from *B. abortus* RB51, a vaccine strain by producing a *B. abortus*-specific 498 bp band. Rep-PCR using BOX-primer produced 13 to 18 bands with sizes of between 230 and 3,300 bp, and discriminated *Brucella* strains to the species level except *B. canis* and *B. suis*. PCR products amplified with ERIC primers were, however, not appropriate for differentiating the *Brucella* isolates. DNA fingerprint patterns for all *B. abortus* field isolates were identical among them and were put on one cluster with *B. abortus* biovar 1 reference strain in the dendrogram, indicating they were highly clonal. These results suggested that rep-PCR using BOX primer might to be a useful tool for calculating genetic relatedness among the *Brucella* species and for the study of brucellosis epidemiology.

Key words : *Brucella abortus*, AMOS PCR, rep-PCR

Introduction

The bacterial genus *Brucella* is gram-negative, facultative intracellular bacteria that can infect many species of animals and human. There are six recognized species of *Brucella* based on biochemical characteristics and host specificity: *B. abortus* (cattle), *B. melitensis* (goats), *B. suis* (pigs), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotomae* (desert rats) [9]. Brucellosis is a typical zoonotic infection of domesticated and wild animals caused by these organisms. Human become infected by ingestion of animal food products and by direct contact with infected animals. According to World Health Organization (WHO) data, about 500,000 human cases have been reported throughout the world per year [28]. While all six species occur worldwide at least sporadically, the greatest economic impact results from bovine brucellosis caused by *B. abortus*. Infection decreases reproductive efficiency, mainly by abortion. During the period of 2000-2003, a total of 684 cases of bovine brucellosis including 3,936 positive cattle were reported in Korea [1].

Because the *Brucella* spp. share a high degree of DNA homology with more than 90% for all species, they have been proposed as a monospecific genus [23]. Several techniques have been employed to find DNA polymorphisms, which would enable the molecular typing of *Brucella* species and their biovars. One of the genetic targets frequently used for the identification and phylogeny of strain is the rRNA operon, particularly the 16S rRNA gene. These genes are highly conserved and diverge very slowly. The DNA sequences within a genus will differ by only a few percent [4]. Other DNA analysis methods included random amplified polymorphic DNA (RAPD) [20], outer-membrane proteins (OMPs) genes [25], insertion sequence (IS) 6501 and *rrs-rrl* ribosomal spacer DNA [19].

Short inter-spaced repetitive DNA sequences, such as repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences, or BOX element have been shown to be useful in the study of bacterial epidemiology [6, 9]. These sequences are small DNA repeats (approximately 35-125 bp) found dispersed throughout the chromosome of

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most bacteria [24]. In this study, we report the use of repetitive element sequence-based PCR (rep-PCR) with ERIC and BOX primers to characterize *B. abortus* field strains isolated from bovine brucellosis outbreaks in Korea between 2001 and 2003.

Materials and Methods

Bacterial strains

Strains used in this study are listed in Table 1. Samples were collected from bovine brucellosis outbreaks on the course of slaughtering due to positive serum tube test. The outbreaks occurred from two different regions and from different sources of dairy and Korean native cattle between 2001 and 2003. Bovine tissues were cultured on Brucella agar (Difco, USA) supplemented with antibiotics, and incubated at 37°C in an atmosphere of 10 percent carbon according to the methods and criteria described by Alton *et al*. [3] with minor modifications. Tissues included spleen, mammary gland, uterus as well as the regional lymph nodes of the reproductive and gastrointestinal tracts.

Table 1. Bacterial strains used in this study

Strains	Origin
Brucella abortus biovar 1	NVRQS[a]
Brucella abortus biovar 3	NVRQS
Brucella abortus biovar 5	NVRQS
Brucella abortus RB 51	NVRQS
Brucella melitensis biovar 3	NVRQS
Brucella suis biovar 4	NVRQS
Brucella canis RM 6/66	NVRQS
Brucella neotomae ATCC 23450	NVRQS
Brucella abortus KP 1	Field isolate (dairy cattle)
Brucella abortus KP 2	Field isolate (dairy cattle)
Brucella abortus KP 3	Field isolate (dairy cattle)
Brucella abortus KP 4	Field isolate (dairy cattle)
Brucella abortus KP 5	Field isolate (dairy cattle)
Brucella abortus KP 6	Field isolate (dairy cattle)
Brucella abortus KP 7	Field isolate (dairy cattle)
Brucella abortus KP 8	Field isolate (dairy cattle)
Brucella abortus KP 9	Field isolate (dairy cattle)
Brucella abortus KP 10	Field isolate (dairy cattle)
Brucella abortus KP 11	Field isolate (dairy cattle)
Brucella abortus KP 12	Field isolate (dairy cattle)
Brucella abortus KP 13	Field isolate (Korean native cattle)
Brucella abortus KP 14	Field isolate (Korean native cattle)
Brucella abortus KP 15	Field isolate (Korean native cattle)

[a]National Veterinary Research and Quarantine Service, Anyang, Korea

PCR

Isolates were tested by the *Brucella* AMOS (acronym for *B. abortus*, *melitensis*, *ovis* and *suis*) PCR to identify *B. abortus* isolates and differentiate them from *B. abortus* RB51, a vaccine strain [10]. PCR was performed as previously described [10]. When bacterial cells were used directly for PCR, template DNA was extracted from 2-3 colonies of each isolate from the agar plate in 500 μl of distilled water by boiling them for 5 min. The whole cell was used as the PCR template and stored at -20°C until use. The reaction mixture consisted of 60 mM Tris-HCl (pH 9.0), 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 250 μM of each deoxynucleoside triphopates (dNTPs), 1 U of *Taq* polymerase and 0.2 μM of each five-primer cocktail (*B. abortus*-, *B. abortus* RB 51-, *B. melitensis*-, *B. suis*- and IS*711*-specific primer). The mixture was cycled 35 times in a thermocycler (UNO II, Biometra, Germany) with following conditions: 1.2 min at 95°C, 2.0 min at 55.5°C and 2.0 min at 72°C. The amplified products were separated by electrophoresis through a 1.5% agarose gel and visualized by staining with ethidium bromide under UV light.

rep-PCR

PCR was performed as previously described [26]. Primers included ERIC 1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3'), ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') and BOX A1R (5'-CTA CGG CAA GGC GAC GCT GAC G-3'). PCR mixtures were prepared in a 25 μl volume containing 2 μl of whole cell suspensions of each isolate, 20 pmol of each primer, 1.25 mM dNTPs and 2U of *Taq* DNA polymerase (Bioneer, Korea). For the ERIC primers, PCR cycles used were as follows: 1 cycle at 95°C for 7 min, 30 cycles at 94°C for 1 min, 52°C for 1 min and 65°C for 8 min. For the BOX primer, 1 cycle at 95°C for 7 min was followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and at 65°C for 8 min. After the reactions, 10 μl of the PCR products were separated on 1.5% agarose gel. The gel was electrophoresed at 4°C for 10 h at 70 V and stained with ethidium bromide.

Pattern analysis

A dendrogram was constructed with Analysis software

(Biometra, Germany). The patterns were compared by means of the Dice coefficient of band-based similarity by unweighted pair group method using averages (UPGMA); a tolerance of 5% in the band position was applied.

Results

The results of AMOS PCR with DNA extracted from the isolates in Table 1 are shown in Fig. 1. PCR products were specifically amplified from three *Brucella* strains, *B. abortus* biovar 1 (498 bp), *B. melitensis* (731 bp) and *B. abortus* RB51 (498 and 364 bp). One nonspecific-band with size of about 650 bp was detected in *B. abortus* RB 51. All field isolates also produced a *B. abortus*-specific 498 bp band. However, *B. abortus* biovar 3, 5, *B. canis* and *B. suis* boivar 4 did not produce any band. We tested those field isolates with a PCR primer set specific for *B. abortus* RB51, a vaccine strain, to confirm that the outbreaks were not caused by the vaccine strain. The PCR did not produce a 364 bp band, confirming that all field isolates were not vaccine strain.

Fig. 2 shows the band patterns of BOX-PCR amplified products from 16 *Brucella* species strains including 8 representative field isolates. Thirteen to 18 bands with sizes of between 230 and 3,300 bp were detected. A pattern of 5 sharp bands with about 340 bp, 420 bp, 1,100 bp, 1,500 bp and 2,100 bp was common for all of the *Brucella* species tested. Two regions of polymorphism with sizes of between 950 bp and 1,300 bp, and with sizes of between 2,500 and 3,300 bp were observed. In *B. abortus* RB 51 (lane 1), the band sizes of more than 2,100 bp were not observed. These bands, however, were present in the other *Brucella* species. Also, in *B. abortus* biovar 5 (lane 4), a band of 960 bp present in other *Brucella* species was not detected. *B. canis* and *B. suis* showed an identical band pattern between them (lane 7, 8). DNA fingerprint patterns for *B. abortus* field isolates generated by using BOX-PCR showed that the patterns of all isolates were identical, indicating the isolates were closely related. Each isolate approximately contained about 13 bands with the sizes ranging from about 350 bp to 3,300 bp, and showed a very sharp band of 2,500 bp band. *B. abortus* RB51, however, showed a different fingerprint pattern with *B. abortus* field isolates. It could be distinguished from other isolates by the absence of two bands of approximately 3,000 and 3,300 bp. ERIC PCR-amplified products with the same strains generated about 3 to 4 bands. Those band patterns were, however, very smear or light that they were not appropriate for differentiating the *Brucella* isolates (data not shown).

Fig. 3 shows the dendrogram of *Brucella* strains

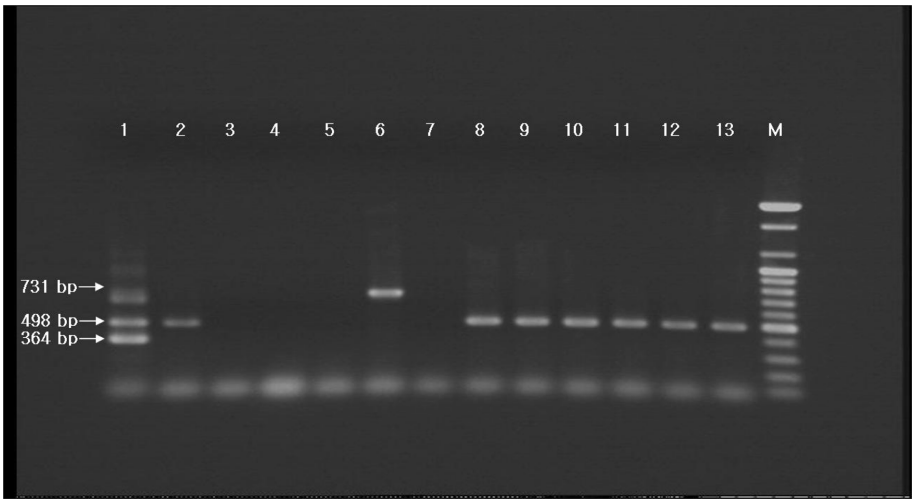

Fig. 1. PCR amplification products from 13 *Brucella* strains including 6 representative *Brucella abortus* field isolates by AMOS PCR. Lane 1, *B. abortus* RB 51; Lane 2, *B. abortus* biovar 1; Lane 3, *B. abortus* biovar 3; Lane 4, *B. abortus* biovar 5; Lane 5, *B. canis* RM 6/66; Lane 6, *B. melitensis* biovar 3; Lane 7, *B. suis* biovar 4; Lane 8 to 13, 6 representative *B. abortus* field isolates; Lane M, 100 bp ladder.

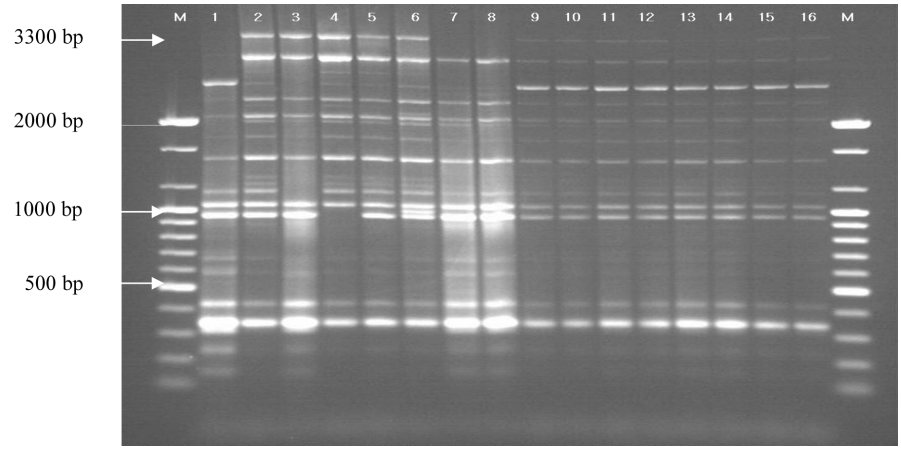

Fig. 2. DNA fingerprint patterns of *Brucella* species by rep-PCR with BOX primer. Lane M, 100 bp ladder; Lane 1, *B. abortus* RB 51; Lane 2, *B. abortus* biovar 1; Lane 3, *B. abortus* biovar 3; Lane 4, *B. abortus* biovar 5; Lane 5, *B. melitensis* biovar 3; Lane 6, *B. neotomae* ATCC 23450; Lane 7, *B. canis* RM 6/66; Lane 8, *B. suis* biovar 4; Lane 9 to 16, 8 representative *B. abortus* field isolates.

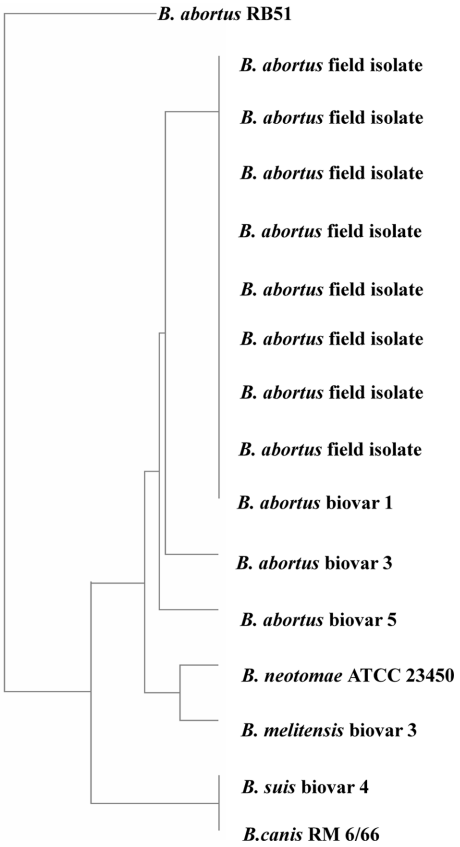

Fig. 3. Similarity dendrogram of *Brucella* strains produced by DNA fingerpringing using BOX PCR based on the DNA band patterns shown in Fig. 2.

produced by the DNA fingerprinting based on BOX-PCR band patterns in Fig. 2. A total of 7 clusters were found. All *B. abortus* field strains were put on one cluster. Strains belonging to the same *B. abortus* group were 75-100% similar. Other *Brucella* species were clustered separately except *B. canis* and *B. suis* biovar 4, which were clustered as identical. *B. abortus* RB 51 strain was the most remotely clustered among *Brucella* species. In general, it was observed that BOX PCR resulted in a clear delineation between the genetic distances calculated for members of different *Brucella* species corresponding to the host preferences of *Brucella*.

Discussion

The AMOS PCR was performed to confirm the identification of *B. abortus* in this study. It was based on the fact that the genetic element IS*711* occurs at several species-specific (or biovar-specific) chromosomal loci. The assay was designed to amplify species-specific-sized products by using five primers, one of which hybridizes to one of four species-specific regions adjacent to the element. Thus, the products were composed of a portion of the IS*711* element and a predetermined number of nucleotides flanking the 3′ end of the element at a species-specific locus [5]. The PCR result was in 100% agreement with the conventional biochemical identification procedures in identifying the *B. abortus* field isolates tested. Previous study [5],

however, reported that *B. abortus* biovar 3, 5 and *B. suis* boivar 4 did not produce any band by the AMOS PCR, coinciding with our result.

Molecular techniques are a very useful tool for bacterial typing. Restriction endonuclease analysis of chromosomal DNA using conventional agarose electrophoresis requires microbial cultivation and often generates complex fingerprints that are difficult to interpret [17]. Ribotyping also requires restriction endonuclease digestion of the bacterial DNA, southern blotting, hybridization with ribosomal DNA directed probes and subsequent autoradiography [18]. These procedures require several days. Microbial cultivation, the use of rare cutting restriction enzymes, tedious cell preparations in agarose-embedding plugs and lengthy electrophoretic separations are necessary for pulsed field gel electrophoresis (PFGE) [22]. Arbitrary primed PCR (AP-PCR) uses a short randomly chosen oligonucleotide primer to create species- or strain-specific DNA fingerprints, but may require the initial testing of many individual primers [11, 27]. Rep-PCR amplifies the repeated sequences that are very similar but not identical and dispersed throughout the chromosome of most bacteria. There have been reports of using rep-PCR fingerprinting technique as a useful epidemiological tool for several bacterial pathogens including *Brucella* species [14, 15, 21]. Mercier *et al*. [15] and Tcherneva *et al*. [21] investigated ERIC- and REP-PCR for differential typing of *Brucella* species. They both used identical primer pairs for amplification and found that most of *Brucella* strains could be discriminated at least to the species level. Interestingly, they had different results. Mercier *et al*. [15] found ERIC-PCR to have greater discriminating power among isolates while Tcherneva's group found more diversity with REP-PCR.

We used ERIC- and BOX primers to distinguish *Brucella* strains that belong to different species and those isolated from bovine brucellosis outbreaks. The results in this study showed that BOX-PCR provided the distinct patterns for *Brucella* species. All *Brucella* reference strains tested could be discriminated at least to the species level except *B. canis* and *B. suis* biovar 4, suggesting that BOX-PCR is also a promising fingerprinting method for the study of brucellosis epidemiology. Furthermore, it is suggested that BOX-PCR is more powerful than either REP- or ERIC-PCR from the viewpoint of number of bands produced. Those two PCR produced about between 10 and 15 bands [15, 21], however, BOX PCR in this study amplified about 13 to 18 bands. The results of ERIC-PCR showed that it was rarely discriminating than BOX-PCR, a result different from that obtained by Mercier *et al*. [15]. This might be explained by the fact that we used different primer cycling parameters, annealing temperatures and template concentration for amplification. Another fact to be considered is that the degenerative and mismatched primers of these rep-PCR assays are hypersensitive in PCR conditions with consequential effects on the PCR performance. We could not distinguish *B. suis* and *B. canis* in this study. A close relationship of *B. suis* and *B. canis* has been reported based on phenotypic characteristics [2], and these organisms were not distinguishable by their physical maps [16]. Our dendrogram obtained by rep-PCR using BOX primer was in good agreement with the one analyzed on the basis of the sequence of *omp*2 gene [12]. The relationship between *B. abortus* RB51 and other *Brucella* species was compared because this strain had been used as a vaccine strain in cattle. It was differentiated from other *Brucella* species by the absence of two bands of more than 3,000 bp, and was most remotely clustered among them. Jesen *et al*. [13] also reported the same results by PFGE with our finding

DNA fingerprint patterns for *B. abortus* field isolates generated by using BOX-PCR found to be identical among them, indicating all isolates were highly clonal. Originally, 15 *B. abortus* field strains were considered to be unrelated each other because they were isolated from the outbreaks of two different regions, at least 150 km distance apart between them, and from two different sources, dairy cattle and Korean cattle, respectively. The results in this study suggested that the bovine brucellosis outbreaks were caused by one clone and cross infection might have occurred between two species. Characterization of much more isolates from broad regions and diverse sources will be required to determine this hypothesis. AMOS PCR could detect *B. abortus* biovar 1, but not detect biovar 3 and 5 [5]. All field isolates in this study produced a *B. abortus*-specific band, and those strains were put on one cluster with *B. abortus* biovar 1 reference strain in the dendrogram. These results also raised a possibility that all *B. abortus* field might belong to biotype 1. Chung *et al*. [7] found the most bovine brucellosis in this country to be caused by *B. abortus* biovar 1. Also, the fact that all field

isolates showed a different fingerprint pattern with *B. abortus* RB51, a vaccine strain, demonstrated that the outbreaks were not caused by a vaccine strain.

In conclusion, BOX-PCR is a useful and promising fingerprinting method for the study of brucellosis epidemiology. Furthermore, Korea is not free from brucellosis and implementing a thorough test and slaughter strategy program for eradication of this infectious disease. Execution of a far strict and thorough test program collaborating with the proper application of epidemiological tool such as BOX-PCR could eradicate the brucellosis in the near future.

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