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Bacteriological detection of *Brucella abortus* and its characterization by PCR in the sporadic outbreak of bovine brucellosis in Gyeonggi province

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Abstract

Bovine brucellosis has occurred for years in Gyeonggi province under the national test and slaughter scheme. The serum agglutination test (SAT) is a diagnostic tool to confirm the disease despite the argument on its specificity. We selected 8 farms where only one or two individuals were diagnosed as brucellosis through SAT at the primary regular herd check and isolated the causative organism and characterized the species by species-specific PCR. The pathogen isolation was successful in 6 farms out of 8 farms by microbiological culture, showing the successful rate of 75%. The isolation rate of the causative organism represents 70% from supra-mammary lymph node and 60% from uterine tissues. They were characterized as *Brucella abortus* biovar 1 after biotyping by PCR, showing the fragment of 498 bp. Five of 8 farms were diagnosed as brucellosis two to four times more over the intervals of two or three months. Here in this study we briefly showed the correlation of the sporadic outbreak of brucellosis tested by SAT and the isolation of the causative organism. Moreover one or two reactors against brucellosis among considerable size of herd may indicate that SAT failed to detect potentially infected individuals in the incubation stage or chronic phase of the disease.

Key words : Isolation of *Brucella* spp, *Brucella* specific-PCR, *B abortus* biovar 1._

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Introduction

Brucellosis is recognised as an important zoonosis worldwide resulting in massive economic losses to the livestock industry and posing a serious human health hazard since human infection of *Brucella* spp manifests highly variable clinical signs such as intermittent fever and arthritis. *Brucella* spp are classically classified into 6 species: *Brucella abortus, B melitensis, B suis, B canis, B ovis, B neotomae*. Recently, *B pinnipediae* and *B cetaceae* from marine mammals were reported[1,2].

Bovine brucellosis is mainly caused by *Brucella abortus*, leading to economic losses, abortion, reproductive organ disorder, decreased milk production and miscarriage[3]. Brucella infection in a cattle population is not expected to be treated effectively since *Brucella abortus* is an intercellular parasite, resulting in resistance to an antibiotic treatment. Therefore, test and slaughter policy has been applied to eradicate the disease in Korea. Currently, diagnosis of bovine brucellosis is entirely based on serological tests. Briefly, rose bengal test (RBT) is primarily carried out and the results are confirmed by serum agglutination test (SAT) and complement-fixation test (CFT). However SAT is the most common diagnostic method to confirm bovine brucellosis in veterinary service labora- tories in Korea.

Bovine brucellosis is not easily diagnosed due to a highly variable latency of the causative agent and no remarkable clinical symptoms except for the abortion.

Bacteriological isolation of the causative organism and its identification is regarded as the "gold-standard" for definitive diagnosis of the disease.[4] However this method is time-consuming and requires a biosecurity lab containment since *Brucella* spp affect both animals and man. Currently, many laboratories in other countries have been using PCR with the bacterial isolation, ELISA[5,6], fluorescence polarization assay (FPA)[4].

Therefore there has been increased interest in developing rapid and accurate methods for detecting *Brucella* spp Serological tests have been carried out for a herd-check of brucellosis since the methods are simple and relatively inexpensive. Recently, conventional diagnosing tools such as rose bengal test (RBT), serum agglutination test (SAT) and complement fixation test (CFT) are substituted by the indirect ELISA and competitive ELISA, which are considered as usual tests[4,7]. In the serological tests for diagnosis of brucellosis, *Pasteurella* spp, *Salmonella* spp, *Yersinia* spp, *Escherichia* spp have the same antigenicity, especially *Yersinia enterocolitica* O:9 strain with O-chain of *Brucella* spp, causes false positivity in the test[7-9]. There were some difference in experimental results among the various diagnostic tools. It was published that *Brucella* spp were isolated from the cows which were negative via serological tests[10]. Moreover it was reported that ELISA represented higher specificity than CFT and SAT[7].

The objective of this study is to

evaluate the brucella isolation rate from SAT reactors among the herds with sporadic occurrence, and promptly confirm the species and biotype of isolates through detecting the brucella specific gene.

Materials and Methods

Select herds and collect materials

From 2005 until March, 2006 8 farms were selected. Each farm had at least 10 heads and 1~2 reactors were found in the primary test (one reactor was in 5 farms and two were in each of 3 farms. The directory of the farm was presented in Table 1. For the isolation of the *Brucella* spp, samples (supr-amammary lymph node and uterine tissue) were collected from slaughtered cows. The samples were stored at 4℃ until analysis.

Table 1. Sporadic cases of bovine brucellosis in Gyeonggi province

Farms	Species	No of heads		M/D/Y	Reoccurrence	
		Examined	Positive		Frequency	Total reactors
GMHan05_1	Hanwoo	73	1	04/13/2005	4	32
HSHan05_2	Hanwoo	14	1	04/18/2005	1	5
HSHan05_3	Hanwoo	33	2	06/17/2005	3	9
HSHol05_4	Holstein	67	1	07/21/2005	4	19
ICHol05_5	Holstein	69	2	10/20/2005	1	2
HSHol05_6	Holstein	11	1	11/26/2005	0	0
HSHol06_7	Holstein	65	1	02/10/2006	0	0
HSHan06_8	Hanwoo	49	2	01/24/2006	0	0

Isolation and identification of *Brucella* spp

To prepare the specimens 3-5g tissue were homoginised into 1.5 ㎖ PBS with seasand (Junsei Chemical Co, Ltd). These specimens were inoculated into brucella broth (Difco Lab) with antibiotics and incubated at 37℃ for 5 days in incubator of 5% CO_2. This emulsified mixture was cultivated for 5-10 days in brucella selective agar (Difco Lab) with 30 ㎎/㎖ cyclohexmide, 7.5U/㎖ bacitracin, 1.8U/㎖ polymixin B sulfate antibiotics at the same condition. Isolated bacteria was gram negative cocobacillus, urease (+) and catalase (+)[3].

Preparation of Genomic DNA

After suspect colony was dissolved into 200 ㎖ sterile PBS, DNA was extracted using genomic DNA extraction kit (Bioneer). The specimen was stored at -20℃ and freezing-thawing was not

repeated 3 times more.

PCR assay

The primers used in PCR are described DYADTM)[11-13] in 20㎕ reaction mixture of PCR Premix(bioneer), in Table 2[11-13]. PCR was performed with a thermal cycler (MJ Reserch, DNA Engine 50 pmol of each primer and 5㎕ of template DNA. Following the reaction, the 8 ㎕ mixture was electrophoresed in the 1% agarose gel added ethidium bromide for 20 minutes at 100V. Then it was observed using image analyser (Kodak 2000R) equipped with UV.

Table 2. Sequences of oligonucleotide primers for PCR

A) Primers used to detect *Brucella* spp

Primers	Sequence(5'-3')	Species	Length	Ref
F4	TCG AGC GCC CGC AAG GGG	*B abortus* biovar 1-9 *B melitensis* biovar 1-3,	905 bp	(5)
R2	AAC CAT AGT GTC TCC ACT AA	*B suis* biovar 1-5, *B ovis, B neotomae,* *B canis*		

B) Primers used to differentiate the species of *Brucella* spp

Specific primers for	Sequence (5'-3')	Length	Ref
B abortus	GAC GAA CGG AAT TTT TCC AAT CCC	498 bp	(7)
B melitensis	AAA TCG CGT CCT TGC TGG TCT GA	731 bp	
B ovis	CGG GTT CTG GCA CCA TCG TCG	976 bp	
B suis	GCG CGG TTT TCT GAA GGT TCA GG	285 bp	
IS711	TGC CGA TCA CTT AAG GGC CTT CAT		

C) Primers used to detect *B abortus* biovar 1

Primers	Sequence(5'-3')	Length	Ref
DSF	GCG CTC AGG CTG CCG ACG	689 bp	(14)
DSR	CAC CCC AGA CAG CCC AA	804 bp	

Results

Isolation and identification

For the isolation and PCR of the *Brucella* spp, samples were collected from reactors at the slaughtering procedure getting the material 10 supramammarian lymph node (LN), 5 uterine tissue (Table 3). *Brucella* spp were isolated at 7 samples (70%) out of 10 supramammary LN, and 3 samples (60%) out of 5 uterine tissue, respectively. Isolated colonies were bio-

chemically confirmed as cocobacillus urease(+), catalase(+). Gene fragment universal to six species of *Brucella* spp was amplified for each sample and finally identified as *Brucella* spp(Fig 1)5). *Brucella* spp was isolated from six out of eight brucella positive farms (75%). Moreover there was no correlation between SAT titers and the rate of bacterial isolation.

Table 3. Isolation of *Brucella* spp from Brucella positive farms

Farms	Positive reactors ID No	SAT	Bacterial Culture		PCR [a]
			Supramammary lymph node	Uteral tissue	
GMHan05_1	2184	1:400	+	n/a	+
HSHan05_2	6140	1:200	+	+	+
HSHan05_3	30	1:200	−[b]	n/a	n/a
	1908	1:100	n/a	n/a	
HSHol05_4	44	1:200	+	−	+
ICHol05_5	7908	1:400	−	−	n/a
	709	1:400	+	+	+
HSHol05_6	3	1:100	−	n/a	n/a
HSHol06_7	82	1:400	+	+	+
HSHan06_8	3	1:100	+	n/a	+
	33	1:200	+	n/a	+
Positive rate(%)	7/10 (70%)		7/10 (70%)	3/5 (60%)	7/7 (100%)

[a] PCR was used to confirm *Brucella* spp from the suspicious bacterial colonies[11]. Bacterial culture negative samples showed no colony on the agar plate.

[b] A single supramammary lymph node was collected from one positive reactor only, resulting in the bacterial culture negative.

Identification of *B abortus* biovar 1

To investigate the effect of potential bacterial contaminants three bacterial genera having similar antigenicity were subjected for *B abortus*-specific PCR assay. Three bacterial genera such as *Y enterocolitica*, *E coli*, *Salmonella* spp were amplified using *B abortus*-specific primers at the same condition as farm isolates were tested. As shown in Fig 2, there was no reaction with the three bacterial genera and only *B abortus* specific gene was amplified by *B abortus*-specific primers.

According to the previous report[13] *Brucella* spp have two copies of Omp2 gene, 804bp. whereas *B abortus* biovar 1 has two different sizes of Omp2 gene, 689bp and 804bp. In the PCR assay

using *B abortus* biovar 1 specific primers, DNA fragments of all isolates were amplified at the size of 689bp and 804bp as shown in Fig 3. Therefore all isolates were identified as *B abortus* biovar 1.

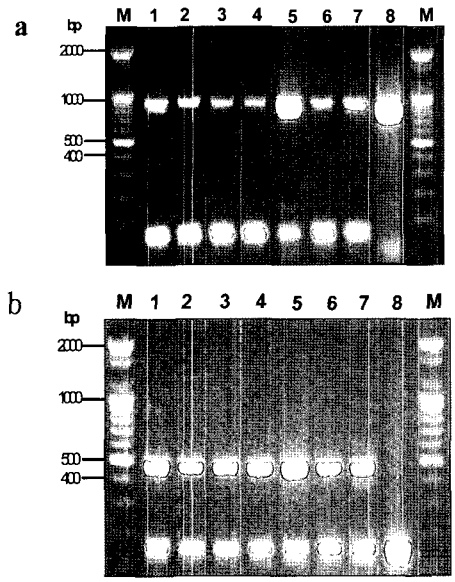

Fig 1. Electrophoresis of amplified PCR products (a) PCR using the primers which detect the common 16S rRNA portion of *Brucella* species, yielded the fragment size around 905 bp. (b) Species specific PCR to discriminate *B abortus*, yielded the fragment size around 498 bp.

Lanes are: M: DNA marker; track 1-6: field isolates of *Brucella* spp; track 7: *B abortus* 1119-3 strain; track 8: *B canis* (field isolate). Products of less than 100 bp were presumed to be primer-dimer complexes.

Discussion

This study was aimed to confirm the diagnosis of brucellosis in sporadic cases, which only one or two serological reactors were found. As described in Table 3 *Brucella* abortus was isolated from six out of eight herds (75%) that brucellosis has occurred sporadically, and the rate of isolation in supra-mammary LN was 70% (7/10 samples), uterine tissue 60% (3/5 samples). It could say that the number of samples were too small to estimate the effectiveness of isolation and infection in this study.

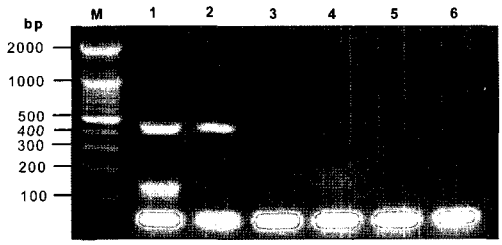

Fig 2. Specificity of the *Brucella* species specific PCR assay. Bacterial cells from three genera (*Y enterocolitica, E coli, Salmonella* spp) were tested by PCR. *B abortus* field strains were included as positive controls. Lanes are M: DNA marker; track 1-2: *B abortus* field strains; track 3: *Y enterocolitica*; track 4: *E coli*; track 5: *Salmonella* spp; track 6: water as negative control. Products of less than 100 bp were presumed to be primer-dimer complexes.

In HSHan05_3 farm isolation of *Brucella* spp was not successful however nine reactors were further detected through 3 times of regular SAT every two or three months under the brucellosis eradication scheme. This could view that bacterial culture can have its experimental limitation even though it was a positive case. However, there was no additional SAT reactor in HSHo105_6 farm, another bacterial isolation negative

one (Table 1 and Table 3). *B abortus* was identified from a cow with low SAT titer (1:100), but it was not isolated from a cow with high titer (1:400) (Table 3). In this study the bacterial isolation rate was not consistent with the SAT results by which the reactors were identified. This was supported by the other report[14].

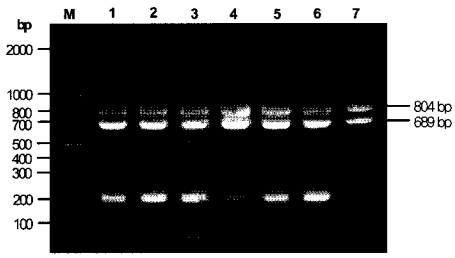

Fig 3. PCR assay to discriminate *B abortus* biovar 1. Six strains of *B abortus* and *B abortus* 1119-3 strain were subjected to PCR, resulting in the fractionated PCR products, 804 bp and 689 bp. This clearly indicated that the pathogen associated with the infection was *B abortus* biovar 1[13].

There was reoccurrence in five herds among 8 herds where the reactors were found by SAT whereas no reactors have been detected in others 3 farms since the primary detection of brucellosis was carried out (Table 1). The frequency of reoccurrence was one to four times, and average was 2.6 times. Especially the number of reactors were increased as time goes, ie, the number of that was 1~2 in the first time of detection , but it was 2~8 later.

SAT may not confer ideal sensitivity or specificity to detect the antibodies presenting in early stage of infection. It should be taken account that many potentially infected cows still remained in the herd when only small number of reactors were confirmed by SAT. This could be a critical information to conduct a successful control of bovine brucellosis.

Therefore, it is recommended that the alternative methods such as standard ELISA should be officially used with SAT for the effective detection of bovine brucellosis.

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