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Real-time PCR Assay Based Glyceraldehydes 3-Phosphate Gene for Identification of *Brucella* sp.

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Abstract: Brucellosis is one of the most important zoonoses which affects both animals and humans and leads to serious economic and public health problems. The aim of this study was to design, optimize and evaluate real-time PCR assay for *Brucella* sp. detection by targeting gap gene and to compare to those of conventional PCR assays. A low variation in C_T values was observed for the gap gene target when the same quantity of DNA for 5 Brucella reference strains was used as template in the assays (C_T: 21-23 with 500 pg of Brucella DNA). No amplification products were observed in real-time PCR whatever the target with any of the 50 non-Brucella organisms tested. In the analytical sensitivity of real-time PCR assay based gap gene of *B. abortus* biovar 1 RB51, DNA concentration of 5 fg was successfully amplified and the sensitivity of the gap-based TaqMan real-time PCR assay was identical and 10-100 times higher than the sensitivity of the three conventional PCR. In the clinical trial, 9 (16.3%) and 11 (21.2%) among 52 blood samples from cows confirmed with *B. abortus* infection by Rose Bengal Spot agglutination test were positive in culture of *B. abortus* and gap real-time PCR, respectively. In conclusion, the use of the gap-based TaqMan real-time PCR assay appears promising due to it high sensitivity for the simple, faster and specific detection of the *Brucella* sp.

Key words: Real-time PCR, *Brucella* sp., glyceraldehydes 3-phosphate gene, conventional PCR assays, zoonoses

INTRODUCTION

Brucellosis is one of the most important zoonoses which affects both animals and humans and leads to serious economic and public health problems. The genus *Brucella* has classically been divided into six species based on host specificity including *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. neotomae* and *B. canis* (Corbel and Brinley-Morgan, 1984). About 2 new species, *B. cataceae* and *B. pinnipediae* have been discovered recently in marine mammals (Cloeckaert and Vizcaino, 2002).

Diagnosis of brucellosis in humans and animals is initially made by use of appropriate serological or other immunological tests and confirmed by bacteriological isolation and identification of the agent. Serological assays are rapid, sensitive and easy to perform but lack specificity due to cross-reaction with other bacteria, particularly with *Yersinia enterocolitica* O:9 that result from O chains antigenic similarity (Godfroid *et al*., 2002; Nielsen *et al*., 2004). Moreover, the identification of *Brucella* sp. relies on an array of approximately 25 phenotypic traits like biochemical characteristics, serological typing and phage typing and requires long times such as 10-14 days with well-equipped laboratories and highly skilled specialist (Lopez-Goni and Moriyon, 2004).

PCR-based methods are fast, simple, less hazardous and more sensitive (Bricker, 2002). Gene targets for detection and identification of pathogens using PCR have included *Brucella* sp. outer membrane proteins, 16S rRNA gene sequences, 16S-23S spacer regions, housekeeping genes, erythritol utilization genes and insertion sequences (IS711) (Al Dahouk *et al*., 2007; Debeaumont *et al*., 2005; Queipo-Ortuno *et al*., 2005). Glycealdehyde-3-phosphate dehydrogenase (gap) gene with various degrees of similarity is a kind of housekeeping genes and present *Brucella* sp. and present in a few other bacteria including *Yersinia enterocolitica* O:9, *Vibrio cholerae* O1 and *Escherichia coli* O:157 (Figge *et al*., 1999). Most recently, real-time PCR assays for the detection of *Brucella* sp. have been developed (Al Dahouk *et al*., 2007;

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Gopaul *et al*., 2008; Navarro *et al*., 2006). Real-time PCR is a variation of the standard PCR technique used to quantify DNA or messenger RNA in a specimen such as milk, blood, tissues and uses to compare relative copy numbers between tissues, organisms or different genes relative to a specific housekeeping gene that are generally always expressed and thought to be involved in routine cellular metabolism. The aim of this study was to design, optimize and evaluate real-time PCR assay for *Brucella* sp. detection by targeting the gap gene and to compare to those of conventional PCR assays already published.

MATERIALS AND METHODS

Bacterial strains: The Brucella strains used in this study included the reference strains of *B. abortus* biovar 12308, *B. abortus* biovar 1 RB51, *B. melitensis* biovar 3 ether, *B. suis* biovar 1 1330, *B. ovis* 63/290 and *B. canis* RM6/66 received from National Veterinary Research and Quarantine Service in Korea and the field isolates of *B. abortus* GB1, *B. abortus* GB2, *B. abortus* GB3, *B. canis* GB1 and *B. canis* GB2. Non-*Brucella* sp. tested were 4 *Shigella sonnei*, 15 *Escherichia coli*, 15 *Salmonella* sp. 4 *Staphylococcus aureus*, 1 *Bacillus anthrax*, 4 *Yersinia enterocolitica*, 4 *Bacillus cereus*, 1 *Vibrio cholera* and 2 *Listeria monocytogenes*.

DNA extraction and quantification: Genomic DAN of all micro-organisms was extracted using QIAamp DNA Mini kit (Qiagen, France) according to manufacturer's directions. The DNA concentration was measured spectrophotometrically at A260 and a volume of 2 μL of aliquot was used as the template DNA in the conventional and real-time PCR.

Primers and probes: The primers using as targets the *Brucella* cell surface 31 kDa protein (bcsp31), 16s rRNA and IS711 for conventional PCR have been previously described (Baily *et al*., 1992; Mackay *et al*., 2002; Romero *et al*., 1995). By contrast, primers used for real-time PCR based gap gene were newly designed on the basis of gap sequences available in the GenBank by using multiple sequence alignment (Fig. 1). The gap probe was a TaqMan probe (Applied Biosystems, France) incorporating a 5' FAM reporter and a minor groove binder group with a non-fluorescent quencher at the 3' end.

Conventional PCR assay: Amplification was performed in 50 μL volume with a PCR buffer containing 10 mM Tri-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 250 mM concentration of each deoxynucleoside triphosphate (Promega,USA), 5 U of Taq polymerase (Qiagen, France), a 50 pmol concentration of each primer and 4 μL of DNA. The DNA quantities used for the analytical sensitivity corresponded to 10 fold dilution from 5 μg (1.5×10^7 genome equivalents) to 5 fg (1.5×10 genome equivalents).

Real-time TaqMan PCR: Real-time PCR was set up in a final volume of 25 μL with 7 μL of RNase free water, 8 μL of PowerChek™ *Brucella* sp. real-time PCR Kit (KOGEN Biotech Co., Korea) each primer and TaqMan probe at concentration of 10 and 1 pmol, respectively and 2 μL of DNA template. The reaction mixture was incubated for 5 min at 95°C. Amplication was performed for 40 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. The PCR reaction was performed on an ABI 7500 Real-time PCR systems (ABI, USA). Detection limit was assessed using pGEM®-T-Easy Vector (Promega Co. Madison, USA)-based plasmid, containing the target sequences of *B. abortus* RB51 that were cloned according to the manufacturer's protocol and determined three times by analyzing a dilution series of the prepared plasmid DNA in PCR-grade water ranging from 500-5 fg. Negative controls were also included and contained all the elements of the reaction mixture except template DNA.

Clinical samples: About 52 blood samples from cows in this study confirmed with *B. abortus* antibody positive by Rose Bengal Spot agglutination Test (RBT) were tested in this study. All cows were in the farms where brucellosis

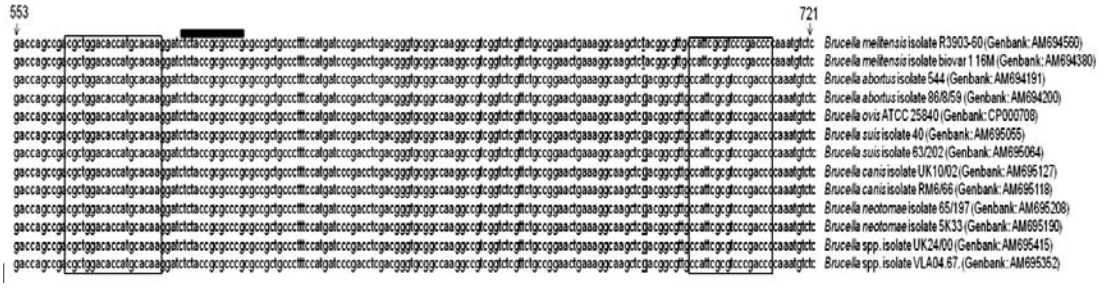

Fig. 1: Aligments of various gap gene sequences around of the $gap_{586/600}$ probes. The alignment contains 13 sequences taken directly from Genbank. In this figure, the variable region of *Brucella* sp. are bold and underlined and the black bar above figures represents the location of probe hybridization

was occurred before. For cultivation of blood samples, 1 mL of whole blood mixed with 4 mL of leukocyte lysis solution (0.32 M sucrose, 10 mM tris base, 1% triton X-100, 5 mM magnesium chloride in distilled water, pH 7.5) and incubated for 5 min at room temperature. The mixture was centrifuged at 3,000 rpm for 30 min and the supernatant was removed. The cell sediment was spread evenly on 5% sheep blood agar medium and the plates were incubated at 37°C for 48-72 h (Gaviria-Ruiz and Cardona-Castro, 1995). Suspicious colonies sized 1-2 mm in diameter, appearing nonhemolytic and light gray were confirmed by conventional PCR method tested in this study. DNA from blood sample was extracted from 200 μL of EDTA anticoagulated whole blood and used QIAamp DNA Mini kit (Qiagen, France) according to manufacturer's direction. Sera were tested for antibodies against *Brucella* sp. using competitive ELISA (SVANOVIR®Brucella-Ab c-ELISA, Svanova Biotech AG Uppsala, Sweden) and RBT as recommended by the OIE Manual of Standards for Diagnostic Tests and Vaccines, 2008 (Office International des Epizooties OIE, 2008). The cut-off value for the c-ELISA was determined according to the manufacturer's guidelines.

RESULTS AND DISCUSSION

A low variation in C_T values was observed for gap gene target when the same quantity of DNA for reference strains of *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis* and *B. canis* was used as a template in the assays (C_T: 21-23 with 500 pg of Brucella DNA) (Fig. 2). No amplification products were observed in real-time PCR whatever the target with any of the 50 non-Brucella organisms tested. The analytical sensitivity of real-time PCR assay based gap gene of *B. abortus* biovar 1 RB51 was tested at least three times for each serial dilution from 500-5 fg (Fig. 3). In fact, DNA concentration of 5 fg was successfully amplified. In every instance, the amplification was clearly distinguishable from the no-template controls. The respective lower limit of detections of conventional and real-time PCR assay observed with 10 fold dilutions of *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis* and *B. canis* DNA are shown in Table 1. In all cases, the real-time PCR was more sensitive than conventional PCR assay. In conventional PCR assays, the lower limit of detection was identical to IS711 target. But the sensitivity of the gap-based TaqMan real-time PCR assay was identical and 10-100 times higher than the sensitivity of the three conventional PCR assays.

The result of *B. canis* isolation and gap real-time PCR from 52 blood samples with *B. abortus* seropositve are shown in Table 2. Only 9 (16.3%) and 11 (21.2%) samples were positive in isolation of *B. abortus* and gap real-time PCR, respectively. Otherwise, 49 (94.2%) samples were positive in c-ELISA.

The aim of this study was to develop a rapid and sensitive detection method for multiple Brucella serotypes based on real time RCR and to compare it to traditional PCR methods. Real-time PCR methods have proven themselves valuable in general laboratories as a rapid diagnostic method and substitute to traditional PCR assays. The basis of rapid specific region detection of real-time PCR assay is principally due to reduced cycle times and omitted post-PCR detection procedures as electrophoresis of agarose gel in the ethidium bromide for analysis of the resulting specific band after irradiation by ultraviolet light (Mackay *et al*., 2002).

For the development of the real-time PCR assay, primers were designed specifically for Brucella. To the knowledge, no previously published real-time PCR presented primers based gap gene while this sequence is specific and its specificity was confirmed with at least 50

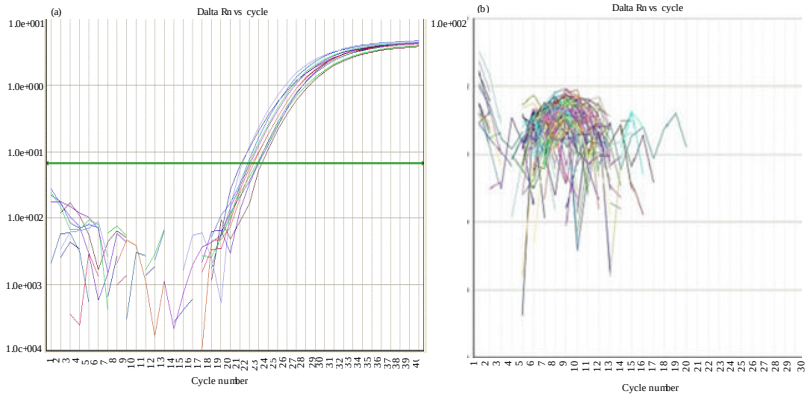

Fig. 2: Real-time PCR amplification curves of *B abortus*, *B melitensis*, *B suis*, *B ovis* and *B canis* reference strains (a) and 50 non-*Brucella* sp. genomic DNA (b) tested in this study. The concentration of genomic DNA was adjusted to 5 ng

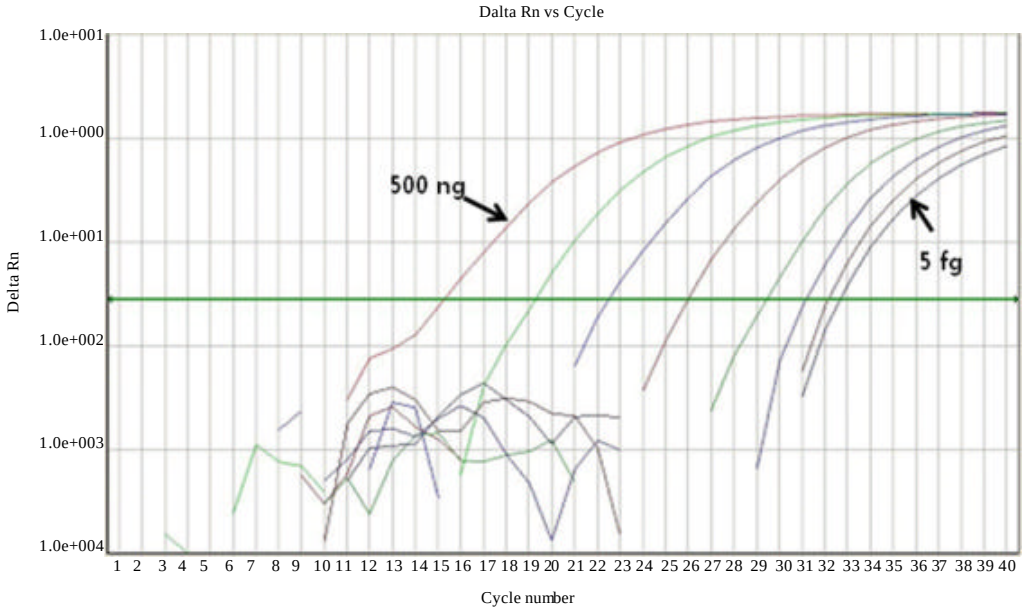

Fig. 3: Real-time PCR amplification curves of *B. abortus* biovar 1 RB51 genomic DNA. A 10 fold dilution series from 500 ng-5 fg was used as a template

Table 1: Comparison of convention and real-time PCR assays lower limit of detection (fg)

		Conventional PCR			
Organisms	References	Bcsp31	16S-rRNA	IS711	Real-time PCR
B. abortus biovar 1 2308[a]	-	500	500	50	5
B. abortus biovar 1 RB51[a]	-	500	500	50	5
B. melitensis biovar 3 ether[a]	ATCC 23458	500	500	500	50
B. suis biovar 1 1330[a]	ATCC 23444	500	500	50	5
B. ovis 63/290[a]	ATCC 25840	500	500	50	5
B. canis RM6/66[a]	ATCC 23365	500	500	50	50
B. abortus GB1[b]	Field strain	500	500	50	5
B. abortus GB2[b]	Field strain	500	500	50	5
B. abortus GB3[b]	Field strain	500	500	50	5
B. canis GB1[b]	Field strain	500	500	NT[a]	50
B. canis GB2[b]	Field strain	500	500	NT	50
Non-*Brucella* sp.[b, c]	-				

[a]Strains were obtained from National Veterinary Research and Quarantine Service (Korea). [b]Strains were kept in Kyungsangbuk-do Veterinary Service Laboratory (Korea). [c]Non-*Brucella* sp. tested in this study were 4 *Shigella sonnei*, 15 *Escherichia coli*, 15 *Salmonella* sp. 4 *Staphylococcus aureus*, 1 *Bacillus anthrax*, 4 *Yersinia enterocolitica*, 4 *Bacillus cereus*, 1 *Vibrio cholera* and 2 *Listeria monocytogenes*

Table 2: Comparison of isolation of *B. abortus* and real-time PCR from 52 blood samples in cows confirmed with *B. abortus* antibody positive by Rose Bengal Spot agglutination test

	Positive samples detected	
Methods	Number	Percentage
c-ELISA	49	94.2
Culture	9	16.3
gap real-time PCR	11	21.2

non-Brucella organisms of closely related or clinically relevant bacteria. A greater sensitivity was observed for the gap gene detection by real-time PCR and could reliably detect DNA down to 5 fg. The one set of real-time PCR gap primer presented in this study was selected among many pairs after intensive testing. This careful design of the gap primers allowed for a significant improvement of the specificity of the assay since no cross-reaction was observed. Gopaul *et al.* (2008) described a detection limit of 50 fg for *B. melitensis* with real-time PCR based single nucleotide polymorphism analysis. In IS711 real-time PCR assay described by Newby *et al.* (2003) was able to detect 7.5 fg of *B. abortus* DNA. Bogdanovich *et al.* (2004) described a detection limit of 2,000 fg for *B. abortus*, *B. melitensis* and *B. suis* DNA and 200 fg for *B. neotomae* and *B. ovis* DNA with a per gene-based real-time PCR.

Bounaadja *et al.* (2009) reported that the specificity of conventional PCR assays presented similar results to in this study and 10 fold maximum variation was observed

according to the species tested with a 50-500 higher sensitivity for the real-time PCR. In this study, the sensitivity of the conventional PCR with IS711 target was also identical or 10 times higher than two other targets because of copy numbers among *Brucella* species and biovars but presented lower sensitivity than real-time PCR based gap gene.

But, the results of the study suggest that whole blood is not a good template for the detection of *B. abortus* DNA. Culture is considered the gold standard in the laboratory diagnosis of brucellosis in human, although many cases can be missed by culture. Yagupsky (1999) reported the sensitivity of blood culture has ranged from 40-90%. Blood is also the first sample for diagnosis of bovine brucellosis without slaughter of suspicious cows.

But bacterial detection by blood samples may be influenced by the number of circulating bacteria, the host's immune response, the intracellular character, etc (Born *et al*., 1976; Herlich *et al*., 1982). Leal-Klevezas *et al*. (1995) reported that the PCR performance with the Brucella DNA extracted from blood samples is very often compromised by the presence of PCR inhibitors and further complicated because Brucella is an intracellular pathogen. O'Leary *et al*. (2006) also reported that the presence of large amount of bovine genomic DNA may have inhibitory affects on the PCR assay. Therefore, the real-time PCR assay based gap gene should be more carefully validated again on clinical samples.

CONCLUSION

In conclusion, the use of the gap-based TaqMan real-time PCR assay appears promising due to its high sensitivity for the simple, faster and specific detection of Brucella and the optimal clinical specimen for real-time PCR diagnosis of bovine brucellosis remains to be determined.

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