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Dual priming oligonucleotide system for the multiplex detection of tuberculosis in Hanwoo

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Abstract

In present study, we described the reliability of the dual priming oligonucleotide (DPO) multiplex polymerase chain reaction (PCR) for the detection of *Mycobacterium tuberculosis* complex (MTC) and non-*Mycobacterium tuberculosis* (NMT) in blood samples of the Korea native cattle, Hanwoo. Among 340 samples 22 (6.5%) were positive in using DPO multiplex PCR, 21 (6.2%) were positive in PCR. The relative agreement between 2 tests was 99.7%, and the agreement quotient (kappa), was 0.95 (excellent). In these results, we demonstrated the successful application of DPO multiplex PCR for the diagnosis of bovine tuberculosis in Hanwoo. Multiplex PCR, using DPO primers, can be useful for the simple diagnosis of bovine tuberculosis in bovine blood samples.

Key words: Bovine tuberculosis, Dual priming oligonucleotide, Multiple PCR.

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Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis that has not known geographical boundaries. *M bovis* infection occurs in diverse groups of animals, which include farm animals, wildlife, and humans[1-3]. *M bovis* and *M tuberculosis* pose a potential health hazard to both animals and humans[3,4]. Detection of *M bovis* in bovine samples is necessary for the control and eradication of bovine tuberculosis, as infected animals are potentially capable of infecting humans (zoonotic tuberculosis)[5].

Besides *M bovis*, *M tuberculosis* is communicable from humans to animals and the reverse had been reported (reverse zoonosis)[4,6]. The identification of the species close-

ly related to MTC has remained a challenging task in diagnostic laboratories[7]. MTC includes a variety of sorts closely related to mycobacteria, namely, *M tuberculosis*, *M bovis*, *M canetti*, *M africanum*, and *M microti*. A panel of classical tests based on microbiological features such as growth rate, phenotypic and biochemical characteristics has conventionally been utilized to distinguish specific members among MTC[7]. However, these tests may be slow, cumbersome, unreliable, and time-consuming. The high degree of variability among these tests leaded to the development of molecular biological tools for identification of MTC members. In this regard, multiple gene targets have been used to detect and differentiate genetically identical species such as *M tuberculosis* and *M bovis*. The gene targets include *pncA*[8], *gyrB*[9], *oxyR*[10], and *katG*[11]. However, no single accepted protocol that can unambiguously differentiate each species among MTC is available. Detection of MTC is more important in terms of public health.

Successful PCR starts with proper priming between an oligonucleotide primer and the template DNA. However, the inevitable risk of mismatched priming cannot be avoided in the currently used primer system, even though considerable time and efforts are devoted to primer design and optimization of reaction conditions. Recently, DPO has been developed, which contains two separate primer segments joined by a polydeoxyinosine linker[12]. This structure made one primer anneal on the two sites with its two segments which have distinct properties. The primer applying this technology generated high PCR specificity under suboptimal conditions and improved the specificity of the multiplex PCR. The present study describes the reliability of the DPO multiplex PCR for the detection of MTC and NMT in blood samples of Hanwoo.

Materials and Methods

Three hundred and forty blood samples were obtained from 340 Hanwoo (298 male and 42 female) during 2006-2007. Ten ㎖ of blood samples for buffy coat preparations were mixed with 5㎖ of ACD solution (0.24 M sodium citrate, 0.24 M dextrose, pH 7.2). Buffy coats were aspirated following a 20 min centrifugation (1,500 rpm) and washed twice in 2.5㎖ of cold buffered water (0.0132M sodium phosphate, pH 7.2). Isotonicity was restored after each wash with 1.25㎖ of cold buffered 2.7% sodium chloride solution. The BC pellets, free of erythrocyte contamination, were digested overnight at 50℃ in 1㎖ of 0.01 M Tris (pH 9.0), 0.5% Tween 20, and 250 ㎍/㎖ of proteinase K.b Digests were then boiled 10 min to inactivate the proteinase K.

The DNA was extracted from buffy coat and reference bacteria using the DNAzol extraction kit (MRC, Cincinnati, Ohio) according to the manufacturer''s instructions. The isolated DNA was used as template in the in PCR and DPO multiplex PCR.

Primers specific to mycobacteria were designed based on DPO technology[12]. The *CESA3* gene of *Arabidopsis thaliana* was used as an internal positive control target to confirm the fidelity of the PCR. The primer sequences and their predicted PCR product sizes in which they were included are shown in Table 1. Single PCR reactions were performed on reference strains

(*M bovis* [ATCC 19210], *M tuberculosis* [ATCC 27294], *M avium* subsp *avium* [ATCC 25291] *M intracellulare* [ATCC 13950], *Escherichia coli* with each primer pair to evaluate the specificity of the primer[13]. Each reaction mixture contained 1㎕ of prepared template DNA, 0.5 μM of each primer, 5㎕ of 10 x buffer (Takara, Kyoto, Japan), 2 mM $MgCl_2$ (Takara, Kyo-to, Japan), 200 μM each of dGTP, dATP, dTTP, and dCTP (Takara, Kyoto, Japan), and 0.5 U of EX Taq DNA polymerase (Takara, Kyoto, Japan). The final volume was adjusted to 50㎕ with distilled water. The PCR conditions were as follows: initial denaturation at 94°C for 10 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 10 min in a thermal cycler (Palm-cycler, Corbett Research, Australia). The amplified products were resolved by 1.5% agarose gel electrophoresis and visualized by ethidium-bromide staining. The PCR products were purified using a Gene-Clean II kit (Bio 101 Inc., La Jolla, CA, USA) according to the manufacturer's instructions. DNA sequencing was performed using an ABI-3700 automated sequencer (Applied Biosystems, Foster City, CA, USA) and the data were analyzed using the DNA-Man software (Lynnon Biosoft, Quebec, Canada).

Multiplex PCR reactions were performed with 1㎕l of the prepared template DNA, 10 ㎕ of 5x primer mixture (0.5 μM of each primer), and 25 ㎕ of 2x Multiplex Master Mix (Seegene, Seoul, Korea) and the final volume was adjusted to 50 ㎕ with distilled water.

The PCR conditions were as follows: initial denaturation at 94°C for 10 min, followed by 30 cycles of 94°C for 30 sec, 60 °C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 10 min in a thermal cycler (Palm-cycler, Corbett Research, Australia). The amplified PCR products were resolved by electrophoresis in a 1.5% agarose gel at 100 V for 30 min. The multiplex PCR was performed with serial dilutions of template DNA (24 steps from 45 ng/㎕ to 5 fg/㎕) from reference strains to determine the sensitivity. The sensitivity and specificity between the two assays in this study were compared. The agreement between DPO mPCR and conventional PCR was examined using kappa statistics[14].

Results and Discussion

The primer sets successfully amplified the target genes in the DPO multiplex PCR without nonspecific or additional bands on the reference bacteria. The sequencing results confirmed that all of the sequences of the PCR products showed 100% homology with the sequences in GenBank (data not shown).

The sensitivity of the DPO multiplex PCR was estimated by the minimal concentrations that could produce all of the expected bands. The reaction that had the most minimal concentration of template DNA was about 200 fg/㎕ (data not shown).

Using DPO multiplex PCR, 22 of 340 blood samples (6.5%) were positive, whereas 21 of 340 blood samples (6.2%) were positive by PCR (Table 1). The relative agreement between 2 tests was 99.7%, and the agreement quotient (kappa), which measures agreement beyond chance, was 0.95 (excellent) (Table 2).

This study was applied to a new diagnostic method, the DPO system. The DPO system

Table 1. Primer sequences and predicted product size

Primer name	Target gene	Direction	Primer sequence	Accession no.	Product size (bp)
MTC	IS6110	Foward	5′-TGCATCTGGC CACCTCGAIIIIITCACGGTTCA-3′	AM408590	366
		Reverse	5′-GACAACGCCGAATTGCGIIIIICGAACGCGAT-3′		
MBT	16S rRNA	Foward	5′-GGCGTAAAGAGCTCGTAGGTIIIIIGTCGCGTTGT-3′	X52918	496
		Reverse	5′-ACAGCCATGCACCACCTGIIIIIAGGCCACAAG-3′		
IC	CESA3	Foward	5′-ATGGAATCCGAAGGAGAAACIIIIIGAAAGCCGAT-3′	NM120599	800
		Reverse	5′-ACAAGCCGCAGCATAATAACIIIIITGTAAGGATT-3′		

* MTC : *Mycobacterium tuberculosis* complex; MBT : *Mycobacteria*

Table 2. Sensitivity and specificity of DPO multiplex PCR compared with conventional PCR analysis for the detection of *Mycobacteria* in the bovine blood samples

DPO multiplex PCR		PCR		
		+	-	Total
	+	21	1	22
	-	0	318	318
	Total	21	319	340

Percent observed agreement = (21+318)/340 = 99.7%. Relative sensitivity = 21/21 = 100%. Relative specificity = 318/319 = 99.7%. Kappa = 0.95 (excellent).

differs structurally and functionally from the conventional primer system by including a poly(I) linker which is one of the most commonly used universal bases between two segments of primer sequences[12]. In general, primers >25 bases are rarely used since the primers longer than 25 mers can be annealed over 70°C but that is too high for proper annealing[12]. This is a fundamental limitation in current conventional primer design. However, the long DPO primer (34-42 mer) is divided into two distinct target-specific priming segments by the presence of the poly(I) linker, leading to no limitations of a high Tm. In addition, the two priming segments of differing lengths have distinct priming functions. For example, at the general annealing temperatures of 55-65 °C, stable annealing is initiated only by the longer 5′-segment since it has a high enough Tm (over 50°C) to bind to the template. Target-specific extension is then determined by the shorter 3′-segment, resulting in unparalleled high specificity. In order to demonstrate the usefulness of DPO, we compared the performance of the DPO system to that of the conventional primer system in PCR.

Multiplex PCR is a rapid and economical tool[13], but when a large bank of genes is amplified with multi-primer sets, conventional primers often produce false positives due to primer competition, to primer dimers or to the different melting temperatures of the different primers. Therefore, current multiplex PCR-based assays require further validation such as nested PCR or a probe hybridization assay[12,15]. However, DPO allows specific detection of a large number of pathogens without any false result because the bubble-like structure of the poly (I) linker in DPO efficiently prevents primer-dimer and hairpin structure formation. The example presented here (Fig 1) demonstrates the successful use of the DPO-based multiplex PCR for simultaneous detection of

Mycobacterium spp with one PCR step. The high specificity without production of any non-specific bands or false-positive products clearly demonstrates the great potential of DPO-based multiplex PCR approaches to be a reliable, rapid, practical and cost-effective detection method. Overall our results demonstrated that the DPO multiplex PCR had dramatically improved performance.

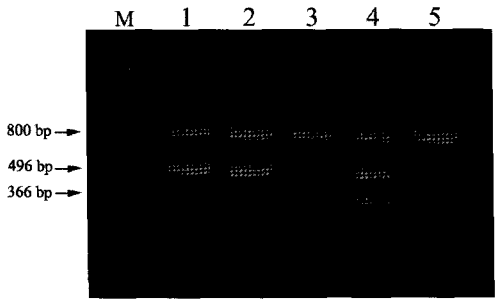

Fig 1. Amplification of *Mycobacterium* spp by DPO multiplex PCR. Lane 1, *Mycobacterium tuberculosis complex* (MTC) positive sample; Lane 2, *Mycobacterium spp.* positive sample; Lane 3, *Mycobacteria* negative sample; Lane 4, Positive control; Lane 5, Negative control; Lane 6, 100 bp marker

In Korea, bovine tuberculosis in cattle has been reported since 1913. From then, control measure for tuberculosis has been focused mainly on dairy cattle. Recently, a case of tuberculosis in Hanwoo was reported[16]. Eight Hanwoo affected with bovine tuberculosis were found by inspectors at slaughterhouses. Adequate surveillance program and control scheme should be performed on bovine tuberculosis for Hanwoo including daily cattle. However, it is not easy to confine Hanwoo for tuberculin test because of free raising compared with dairy cattle. For the test of bovine tuberculosis, especially Habwoo, the method described in this study may be more useful.

We have demonstrated the successful application of DPO multiplex PCR for the diagnosis of bovine tuberculosis in Hanwoo. Multiplex PCR, using DPO primers, can be useful for the simple diagnosis of bovine tuberculosis in bovine blood samples. To achieve reliable results with multiplex PCR, feasible guidelines and standardization are of major importance. Further studies will be required to define the usefulness of molecular tests for bovine tuberculosis.

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