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FULL PAPER

Genetic diagnosis of band 3 deficiency using a quenching probe (QProbe)-PCR assay in bovine embryos

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Abstract

The present study was conducted to develop a simple and rapid procedure to determine the genotype of band 3 deficiency in bovine embryos by a novel real-time PCR method using a fluorescent quenching-based probe (QProbe-PCR). QProbe-PCR successfully distinguished wild type and R664X mutant alleles by melting curve analysis. Minimal amounts of DNA template were required for the detection of wild type/wild type alleles, mutant/mutant alleles, and wild type/mutant alleles; their amounts were 10 pg, 25 pg, and 50 pg, respectively. When 10 blastomeres were used as a DNA sample, accuracies of genotyping by QProbe-PCR were 100% and 89% in embryos homozygous for the wild type allele and heterozygous for the wild type and mutant alleles, respectively. QProbe-PCR takes approximately 2 h for genotyping and requires lesser time than the conventional method using PCR–RFLP, which requires digestion with a restriction enzyme and electrophoresis. Our data showed that QProbe-PCR is a useful method for rapid analysis of the genetic deficiency in preimplantation embryos. Reduction in the time required for genotyping enabled the transfer of genetically selected embryos to recipient cows on the day of embryo collection. These results suggest that determination of the genotype for the genetic deficiency in embryos is useful to select animals free from the genetic disease, and it also makes it possible to produce an animal model homozygous for the mutation.

Key Words: Band 3, Cattle, Embryo, Preimplantation genetic diagnosis, quenching probe

Introduction

Genetic diversity in the Japanese Black cattle is small[20]. There are many hereditary diseases of Japanese Black cattle that are detrimental to breeding, health, and productive performance[17]. To avoid these problems, many analytical methods based on PCR for preimplantation

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genetic diagnosis (PGD) have already been developed[1,4,6]. Although these methods are highly sensitive and amplify a small amount of template DNA, contamination of exogenous DNA during complicated PCR amplification steps and electrophoresis may cause false-positive results.

We established methods for PGD with the single PCR procedure and restriction fragment length polymorphism (RFLP) analysis for claudin-16 deficiency or band 3 deficiency[12,15]. Using these methods, animals homozygous for the mutation causing claudin-16 deficiency or band 3 deficiency were successfully produced[12,15]. These are effective techniques to produce animal models of hereditary diseases. However, reduction in the amount of time required for genotyping and simplification of procedures are needed for using the techniques in the field. The immediate postbiopsy transfer of embryos without cryopreservation, significantly increases the pregnancy rate in recipient females as compared to the transfer of frozen/thawed embryos. Shea[21] obtained 49%–60% pregnancy rates when fresh transfers were made and 23%–41% for frozen/thawed transfers. PCR–RFLP is a complicated method and time consuming because it requires restriction enzyme digestion and electrophoresis. Therefore, a simple and rapid procedure to determine genotypes in preimplantation embryos is required.

Real-time PCR with its rapidity and high sensitivity has been widely used, and the risk of carryover contamination is low because specificity can be verified by melting temperature (*T*m) analysis[9,19,24]. Kurata *et al.*[18] and Crockett & Wittwer[5] developed novel real-time PCR methods using fluorescent quenching-based primers/probe (QProbe). The QProbe is an oligonucleotide with fluorescent dye-modified cytosine at the 3′ or 5′ end. It utilizes a reaction by which the fluorescence of the dye is quenched by transfer of an electron to a guanine base at a particular position. PCR amplification can be monitored by the quenching ratio of the hybridized probe to the fluorescence of the free probe. Because the method requires only a single dye that interacts with a nucleobase leading to decreases in fluorescence intensity, it is more cost-effective than other real-time PCR methods, such as TaqMan PCR, which requires two dyes (i.e., a reporter dye and a quencher dye) for intramolecular fluorescence resonance energy transfer.

We used band 3 deficiency (band 3 $^{\text{Bov. Yamagata}}$) as a genetic disorder model because it is one of the well known genetic diseases in cattle, and the experimental materials are relatively easily available. Band 3 deficiency is a hereditary disorder with spherocytosis and hemolytic anemia in Japanese Black cattle[2,13]. The molecular basis of band 3 deficiency is a nonsense mutation at codon 664 for an Arg residue (R664X mutation) in the *SLC4A1* gene. The purpose of this study was to develop a rapid procedure using real-time PCR and QProbe, QProbe-PCR, to determine the genotype of band 3 deficiency in bovine preimplantation embryos.

Materials and Methods

Preparation of genomic DNA: Bovine genomic DNA was extracted using the QIAamp DNA minikit (Qiagen Inc., Valencia, CA, USA) from the peripheral blood leukocytes obtained from cows with *SLC4A1* genotypes determined by a previously reported method[14].

Embryo production: Japanese Black cattle were superovulated by the administration of follicle stimulating hormone (20 IU/cow; Antorin®R•10, Kyoritsu Seiyaku Corporation, Tokyo, Japan) twice daily in decreasing doses over 3 days. Prostaglandin F2α (cloprostenol 0.5 mg/cow, Resipron®-C, ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) was injected on the third day of superovulation. Cows were inseminated 24 h after the onset of estrus and were flushed 6–7 days after artificial insemination. Cows homozygous for the wild type allele and those heterozygous for the wild type and R664X mutant allele of the

SLC4A1 gene were inseminated with frozen thawed semen derived from a heterozygous carrier of the R664X mutant allele.

Genotyping of the SLC4A1 gene in embryos: Zonae pellucidae of morulae were removed using a holding pipette and glass needle attached to a micromanipulator. Blastomeres were separated by pipetting in Ca^{2+}- and Mg^{2+}- free Dulbecco's phosphate buffered saline supplemented with 1 mg/ml polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, MO, USA). Blastomeres (3, 5, or 10) were collected and treated for 10 min at 37°C in 10 μl of 0.1 mg/ml proteinase K (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.5% Tween 20 (Nacalai Tesque, Inc., Kyoto, Japan). Proteinase K was then deactivated for 10 min at 99°C. DNA samples were then added to 10 μl of water before use. The *SLC4A1* genotype of these DNA samples were analyzed with QProbe-PCR or PCR–RFLP. The rest of the cells in corresponding embryos were lysed using proteinase K and Tween 20 and the *SLC4A1*genotype was analyzed with PCR–RFLP.

QProbe-PCR: QProbe-PCR was performed using a primer set (forward: AAACTCAGTGTACCT GAAGGC and reverse: GCAAACATCATCCAG ATGGGA), which was previously reported[4]. QProbe (CTAACCCCACGGAGCGAGAC) was designed using QProbe design software (J-Bio21, Tokyo, Japan) supported by Fujitsu (Tokyo, Japan) and labeled at the 3′ end with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY FL D-6140, Molecular Probes, Eugene, OR, USA). Monitoring of PCR amplification was facilitated by an increase in the quenching ratio of the QProbe hybridized to the target with respect to free QProbe. A LightCycler (Roche Diagnostics, Mannheim, Germany) was used for real-time PCR. Uracil-DNA glycosylase (UNG; Roche Diagnostics), added to the reaction mixture, was used to prevent carryover contamination of PCR products. For the QProbe-PCR assay, the reaction solution (20 μl) contained 2 μl of sample DNA, 200 μM each of dATP, dCTP, dGTP, and dTTP, 1× PCR Buffer (10× TitaniumTaq Buffer; BD Biosciences Clontech, Palo Alto, USA), 0.2 U UNG and 1× Titanium Taq Polymerase (BD Biosciences, Clontech), 0.3 μM of forward primer, which elongated the strand hybridized to the probe, and 1 μM of reverse primer. The QProbe was added to the reaction solution at a final concentration of 0.1 μM. The glycosylase reaction was performed at room temperature for 20 min. PCR conditions were as follows: an initial denaturation step at 95°C for 5 min, 50 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

Melting curve analysis for the *T*m measurement was performed using the LightCycler program. The reaction mixture was heated to 95°C for 30 s, cooled to 40°C for 60 s, and then slowly heated (0.1°C/s) back to 95°C under continuous fluorescence monitoring. Identification of genotypes was performed on the basis of differences in the fluorescence-quenching rate that was calculated according to a method described in a previous report[23].

PCR–RFLP: PCR–RFLP to determine the *SLC4A1* genotype was conducted in accordance with a previous report[16]. Briefly, PCR products were digested with *Dra*III (TOYOBO Inc., Tokyo, Japan) for 4 hr at 37°C. After digestion, PCR products were separated on 12% polyacrylamide gels (TEFCO Inc., TOKYO, Japan) at 300 volts for 16 min, stained with ethidium bromide (0.5 μg/ml) and photographed under UV light. PCR products derived from the R664X mutant allele were divided into 63-bp fragments and 44-bp fragments because the R664X mutation generates a *Dra*III recognition site, while those from the normal allele remained intact (107 bp).

Results

When the QProbe hybridized with the

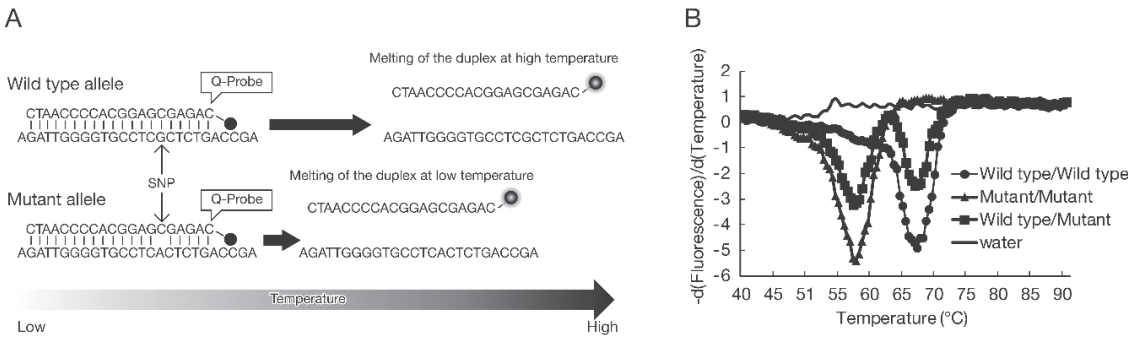

Fig. 1. Schematic representation of melting curve analysis in QProbe-PCR. (A) The fluorescent 3′-tailed QProbe is designed to possess a cytosine base at the 3′ end in the target-specific part containing a single nucleotide polymorphism (SNP). A fluorescent dye is quenched via electron transfer between the dye and a guanine base, which is complementary to a cytosine base at the 3′ end in the QProbe. The QProbe, which is complementary to the wild type allele, anneals to PCR products at low temperatures and the dye is quenched. In melting curve analysis, the QProbe dissociates from PCR products with increasing temperature and becomes fluorescent. Melting temperature in the mutant allele that contains a base mismatch to the probe is lower than that in the wild type allele. (B) Homozygotes for the wild type allele and the R664X mutant allele show distinct melting temperatures, and heterozygote dissociates between the two melting temperatures.

amplified target at the annealing temperature, the fluorescence of the dye at the 3′ end in the QProbe was quenched by the guanine base in the target (Fig. 1A). In melting curve analysis of the amplified products of QProbe-PCR, melting peaks were homozygous for the R664X mutation; this mutation had a mismatched nucleotide to the probe, homozygous for the wild type, which was complementary to the probe, as well as heterozygous (Fig. 1A). The position of the melting peak provided *T*m. Wild type (67.4°C) and R664X mutant (57.9°C) were successfully distinguished by differences in *T*m, and heterozygotes showed both *T*m values (Fig. 1B).

We examined detection sensitivity for the *SLC4A1* genotyping by QProbe-PCR using genomic DNA, which was extracted from blood cells. Homozygous wild type and mutant alleles were determined correctly from 10 pg and 25 pg of genomic DNA, respectively (Table 1). Heterozygotes required 50 pg of genomic DNA for accurate determination, and the accuracies of analysis using 25 pg and 10 pg of heterozygous genomic DNA were 88% and 94%, respectively.

When 10 blastomeres were used as a DNA sample, the accuracies of genotyping by QProbe-PCR were 100% and 89% in embryos homozygous for the wild type allele and heterozygous for wild type and mutant alleles (Table 2), respectively. PCR–RFLP showed 100% accuracy when 5 blastomeres were used as a DNA sample (Table 2).

Discussion

We developed a specific and simple real-time PCR method using QProbe to determine the genotypes of the *SLC4A1* gene in bovine embryos. To the best of our knowledge, this study is the first to report a method with QProbe-PCR for PGD in early embryos. QProbe-PCR takes approximately 2 h for genotyping and requires less time than that of PCR–RFLP following digestion with a restriction enzyme and electrophoresis. Embryo biopsy is essential for PGD and decreasing the number of cells in biopsied embryos reduces viability after cryopreservation[8,21]. Therefore, the rapidity of PGD is important in enabling embryo transfer without cryopreservation on the same day as embryo collection.

Cattle are economically valuable livestock and the gestation period is over 280 days. Therefore, PGD to predict the phenotype of offspring is of great value as well as high

Table 1. Sensitivity of genotyping of the *SLC4A1* gene by QProbe-PCR

Amounts of genomic DNA (pg)	Successful detection/Examination (%)					
	Wild type/Wild type		Mutant/Mutant		Wild type/Mutant	
50	5/5	(100)	5/5	(100)	16/16	(100)
25	5/5	(100)	5/5	(100)	14/16	(88)
10	5/5	(100)	4/5	(80)	15/16	(94)
5	4/5	(80)	2/5	(40)	6/16	(38)
0	0/5	(0)	0/5	(0)	0/ 5	(0)

Table 2. Sensitivities of genotyping of the *SLC4A1* gene in bovine embryos by QProbe-PCR and PCR–RFLP

Method	Number of biopsied cells	Successful detection/Examination (%)	
		Wild type/Wild type	Wild type/Mutant
QProbe-PCR	3	4/8 (50)	1/5 (20)
	5	6/7 (86)	4/6 (67)
	10	5/5 (100)	8/9 (89)
PCR–RFLP	3	6/7 (86)	5/6 (83)
	5	6/6 (100)	6/6 (100)
	10	6/6 (100)	7/7 (100)

reliability is essential. All steps of QProbe-PCR can be performed in a single reaction tube and genotypes are then discriminated by melting curve analysis. Because of the elimination of handling amplified DNA products, QProbe-PCR lowers the possibility of obtaining false-positives because of DNA contamination.

Embryo sexing in cattle, which may have come the furthest in PGD; blastocyst-stage embryos are most frequently used for biopsy. Several researchers reported that biopsy of approximately 10 blastomeres satisfied both the accuracy of PGD and the pregnancy rate in fresh embryo transfer[8,21]. Although nested-PCR and preliminary amplification with whole genome amplification enable sensitive PGD with fewer blastomeres[4,26], handling of PCR products may cause false-positive results. PGD based on amplification of multi-copy sequences also shows high sensitivity[16]. However, all known causative genes of genetic disease in Japanese Black cattle are single-copy genes. In this study, QProbe-PCR correctly determined the genotypes of the *SLC4A1* gene using 10 blastomeres as the template, except in one of the samples from a heterozygous embryo in which no amplification was observed. A possible reason for this result was the loss of some DNA during DNA extraction. Although the detection sensitivity of QProbe-PCR was lower than that of PCR–RFLP using blastomeres, 100% detection was achieved using genomic DNA. Because the amount of DNA per single cell is from 6.3 pg to 7.86 pg in cattle[7], more than 8 cells are sufficient to obtain 50 pg. The average total cell number of bovine blastocyst on day 7 after estrus is 164.1[25]. In general, between 10% and 20% of the embryonic mass in bovine blastocysts is removed for PGD[21], thus biopsied samples contain above 10 cells on average. These findings suggest that QProbe-PCR appeared to have enough sensitivity for PGD in bovine blastocysts. This method can also be used for other single nucleotide polymorphism typing in bovine embryos such as ionotropic glutamate receptor AMPA-1 (GRIA1), which is associated with ovulation rates in cattle[22].

Various genetic diseases, such as claudin-16 deficiency[10,11], molybdopterin cofactor sulfurase deficiency[27], and Chediak-Higashi syndrome[28] were reported in Japanese Black cattle, which

were improved with a particular emphasis on beef marbling and rapid weight gain in small populations. These genetic diseases were carried by sires with an excellent inherent capacity for beef production and widely diffused by using their frozen semen. Therefore, the elimination of the carrier animal represents a loss of important genetic material. Since 2004, the Wagyu Registry Association has disallowed the registration of young bulls carrying causative genes of genetic disease. Therefore, these genetic diseases are unlikely to develop by breeding between carrier animals in the future. However, there are dams still carrying the causative genes because the registration of female calves in Wagyu is not restricted for the carriage of the genes. Because of high levels of inbreeding, the possibility of new genetic diseases occurring in the future is high. However, it is important to maintain carrier animals as a genetic resource for the improvement of cattle. PGD is an important technique to produce young bulls from embryos, which were collected from carrier donors.

Moreover, there are causative genes of genetic diseases, which are common in cattle and humans, including band 3 deficiency. Preclinical studies of gene therapy have benefited from the observation that several human genetic disorders have counterparts in large animals, likely resulting from inbreeding and the propagation of recessive alleles[3]. Large animals provide the translational bridge from in vitro and mouse experiments to human patients. The production of animals homozygous for the mutant allele as an animal model may provide clues for the underlying cause of diseases.

In conclusion, QProbe-PCR is a useful method for rapid analysis of the genetic deficiency in preimplantation embryos to produce animals free from the genetic disease, and it also makes it possible to produce an animal model homozygous for the mutation.

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