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Genetic Diagnosis of Band 3 Deficiency and Sexing in Bovine Preimplantation Embryos

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ABSTRACT. Band 3 deficiency with hereditary spherocytosis and hemolytic anemia in Japanese black cattle, band $3^{Bov.Yamagata}$, is caused by a total lack of band 3 protein with an autosomal dominant inheritance. Genotyping for band 3 deficiency and sexing were successfully achieved in biopsied embryo cells with efficiencies of 98.4% and 97.4%, respectively. Transfer of the embryo that was determined as homozygous for the mutant allele into a recipient cow resulted in the production of a fetus exhibiting the genotype and red cell phenotypes characteristic of band $3^{Bov.Yamagata}$. These results demonstrate that our procedure is reliable and applicable to produce animals free from or homozygous for the mutant allele by breeding carrier animals.

KEY WORDS: band 3 deficiency, cattle, embryo, genetic diagnosis, sexing.

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The molecular basis for band $3^{Bov.\ Yamagata}$ is a nonsense mutation at codon 664 for an Arg residue (R664X mutation), and a diagnostic procedure using polymerase chain reaction (PCR)-restriction fragment length polymorphism for this mutation has been established [7, 8]. Genetic diagnosis to detect carrier animals of band $3^{Bov.\ Yamagata}$ has achieved prevention of this disease [2]. Genotyping for the R664X mutation at an embryonic stage would facilitate the effective production of animals not carrying the mutant allele by embryonic selection, particularly when breeding between carrier animals is desired for improved productivity. This would also contribute to produce animals homozygous for the R664X mutation with a close genetic backbone to investigate the pathobiology of this disease, which has not yet been fully understood.

Although several different approaches have already been reported for analyses of genomic DNA in preimplantation embryos [1, 3, 4], these approaches can generate false-positive results because of contamination of exogenous DNA during complicated PCR amplification steps. However, we recently developed a procedure with a single step of PCR to determine the genotype for a genetic disease, claudin-16 deficiency, and sex, with high efficiency using as little as 5 *p*g of genomic DNA obtained from biopsied embryonic cells [5]. Our simple procedure appeared effective in improving breeding and the production of animals with selected phenotypes of various inherited diseases.

The purpose of this study was to develop a protocol to determine the genotype for band $3^{Bov.\ Yamagata}$ and the sex of embryos prior to embryo transfer and to demonstrate the reliability of this method.

MATERIALS AND METHODS

Genotyping for band 3 and sexing in embryos: PCR was performed using a HotStarTaq Master Mix kit (QIAGEN Inc., Valencia, CA, U.S.A.) according to the manufacturer's protocol with combinations of PCR primers, p17; 5'-AAACTCAGTGTACCTGAAGGC-3' and p14; 5'-GCAAACATCATCCAGATGGGA-3' [7] at a concentration of 0.75 μM. The reaction condition consisted of initial denaturing at 95°C for 15 min followed by 45 cycles of 94°C for 30 sec, 64°C for 30 sec and 72°C for 30 sec followed by final extension for 5 min at 72°C. Under these conditions, 3 *p*g of genomic DNA was enough to obtain a 107-bp PCR product that was clearly detectable on gel electrophoresis (data not shown).

The obtained PCR products were digested with *Dra*III (TOYOBO Inc., Tokyo, Japan) for 4 hr at 37°C. After digestion, products were separated on 12% polyacrylamide gels (TEFCO Inc., Tokyo, Japan) at 300 volts for 16 min, stained with ethidium bromide (0.5 μg/m*l*) and photographed under UV light. The PCR-amplified fragment derived from the mutant allele was divided into 63-bp and 44-bp fragments because the R664X mutation generates a *Dra*III recognition site, while that from the normal allele remains intact (107 bp).

Embryos were sexed as previously described [9]. In brief, PCR was performed as described above using a primer pair S4b1; 5'-CAAGTGCTGCAGAGGATGTGGAG-3' and S4b2; 5'-GAGTGAGATTTCTGGATCATATGGCTACT-3'. The reaction condition consisted of initial denaturing at 95°C for 15 min followed by 15 cycles of 97°C for 8 sec, 50°C for 25 sec and 72°C for 15 sec, and 30 cycles of shuttle PCR at 98°C for 8 sec and at 66°C for 20 sec followed by incubation for 5 min at 72°C. In addition to a 150-bp PCR product common to males and females, a male-specific fragment of 178 bp was amplified only when male DNA was included in the reaction.

Embryo production: Japanese black cattle were super-

ovulated by administering FSH (20 IU/cow, Antorin®R•10, Denka Pharmaceutical Co., Ltd., Kanagawa, Japan) twice daily in decreasing doses over 3 days. $PGF_{2\alpha}$ (cloprostenol 0.5 mg/cow, Resipron®-C, Teikoku Hormone Manufacturing Co., Ltd., Tokyo, Japan) was injected on the third day of superovulation. The cows were inseminated 24 hr after the onset of estrus, and were flushed at 6–7 days after artificial insemination.

Analysis of preimplantation embryos: *In vivo* derived embryos were recovered from normal cows inseminated with semen from a bull heterozygous for band 3 deficiency. Biopsied cells were obtained by cutting using a microbrade attached to a micromanipulator. Ten to twenty percent of the trophectoderm of blastocysts were removed and lysed for 10 min at 37°C in 10 μl of 0.1 mg/m*l* proteinase K (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.5% Tween 20 (Nacalai Tesque, Inc., Kyoto, Japan) (PK-TW method). Proteinase K was then inactivated by heating at 99°C for 10 min. Five μl of the DNA solution were used as a template for band 3 genotyping and sexing by the procedures described above.

Production of a fetus homozygous for band 3 deficiency: Embryos were recovered from cows heterozygous inseminated with semen from a bull heterozygous. Biopsied cells obtained by the same method described above were lysed in 10 μl of extraction solution by the PK-TW method. Five μl aliquots of the DNA solutions were used as templates for band 3 genotyping. Manipulated embryos were incubated for 5 hr in IVD-101 medium (Research Institute for the Functional Peptides, Yamagata, Japan) at 39°C under 5% CO_2 and stored in liquid nitrogen prior to use as described elsewhere [11]. Five frozen-thawed embryos that had been judged as homozygous for the R664X mutation were transferred into five recipient cows (one embryo/one recipient) at 6–7 days after estrus. Pregnancy was diagnosed by ultrasonography at 41 or 42 days after estrus. A 97-day-old fetus (0-day-old=estrus) was obtained from the recipient cow by Caesarian section, and the liver was collected from the fetus to analyze the genotype for band 3 deficiency.

Characterization of red cells from a fetus homozygous for R664X mutation: Microscopic examination of red cells was carried out on blood films stained with Giemsa. Immunofluorescence microscopy was performed to detect band 3 proteins for red cells fixed with methanol using a monoclonal antibody raised against the cytoplasmic domain of bovine band 3 (cdb3–64, manuscript in preparation) and Alexa Fluor 568-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, U.S.A.). Red cell membranes were prepared and analyzed for protein constituents by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie brilliant blue as described previously [7].

Statistical analysis: The statistical significance of differences among different band 3 genotypes was evaluated by the Chi-square test.

RESULTS

Determination of band 3 genotype and sex in preimplantation embryos: When genomic DNA from cells biopsied from embryos was used as a template, PCR amplification followed by *Dra*III digestion gave clear bands specific to genotypes of band 3 deficiency as shown in Fig. 1A. When embryos recovered from normal cows inseminated with semen from a bull heterozygous for band 3 deficiency, genotyping for the R664X mutation was successful in 61 of 62 embryos examined (98.4%), among which 36 embryos (59.0%) were determined to have wild/wild alleles, and 25 embryos (41.0%) were determined to have wild/mutant alleles. The remaining sample was not assessed because the PCR product content was less than that required for visualization. These data were consistent with the expected Mendelian ratio (p=0.16). Thirty-nine specimens out of 61 were also sexed by PCR and their sex was able to clearly distinguished (Fig. 1B) with a success rate of 97% (38/39).

*Production of a fetus homozygous for band 3 deficiency based on genotyping of preimplantation embryo*s: Five embryos homozygous for the R664X mutation were transferred into the uteri of recipients, one of which was diagnosed as pregnant by ultrasound at 41 days. The fetus obtained on day 97 by Caesarian section apparently had no jaundice. PCR analysis of DNA extracted from the liver demonstrated that the fetus was homozygous for the R664X mutation as expected by genotyping at the embryonic stage (Fig. 2).

Red cells from the fetus revealed anisocytosis in which

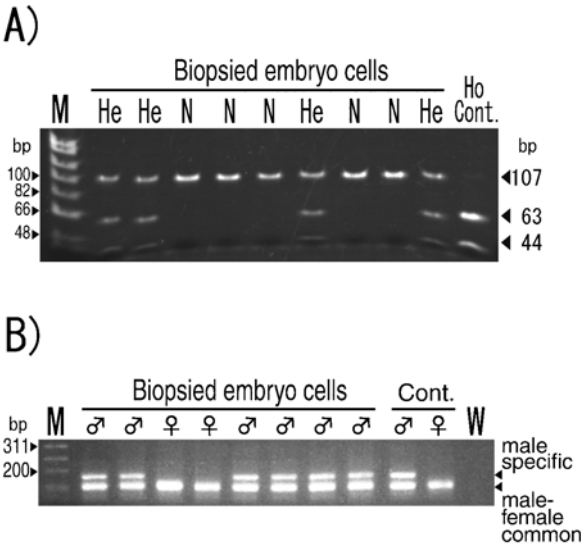

Fig. 1. Band 3 genotyping and sexing of embryos. Typical electrophoretic patterns for genotyping of band 3 deficiency (A) and sexing (B) for embryonic cells are presented. Indications are as follows: M, DNA size marker (øX174/Hinf I digest); N, homozygous for the wild-type allele; He, heterozygous for R664X mutation; Ho, homozygous for R664X mutation. Lane W indicates the reaction with water instead of DNA as the control.

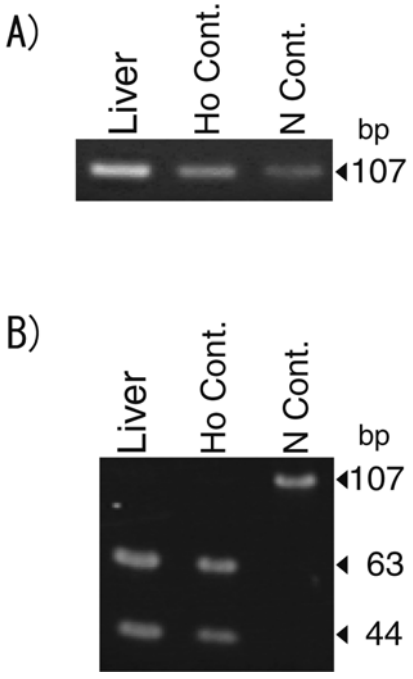

Fig. 2. Band 3 genotyping of a fetus produced from a genetically diagnosed embryo. A, PCR products of genomic DNA extracted from the liver of the 97-day-old fetus. B, genotyping for band 3 deficiency by digestion of PCR products with *Dra*III. Ho, homozygous for R664X mutation; N, homozygous for wild-type allele.

40%–50% of the red cells were larger in size than the cells from an age-matched normal fetus with polychromatophilic staining, and the remaining population had microcytic and spherocytic features (Figs. 3A and 3B). Immunofluorescence microscopy showed that fetal red cells homozygous for the mutation had no signals for band 3 polypeptides, whereas normal bovine red cells exhibited obvious and membranous distribution of the band 3 (Figs. 3C and 3D). Red cell membranes from the fetus totally lacked the band 3 polypeptide with nearly missing of protein 4.2, glyceraldehyde 3-phosphate dehydrogenase, and protein 4.1a (Fig. 3E), being consistent with earlier findings on band 3-deficient red cells [7].

These results demonstrate that the fetus obtained by embryo transfer in this study actually had the genotype and red cell phenotypes characteristic of band 3 deficiency, band $3^{Bov.Yamagata}$.

DISCUSSION

The procedure using PCR and restriction fragment length polymorphism analysis in this study showed high sensitivity and accuracy for the determination of band 3 genotypes and sex in preimplantation embryos. Genotyping at an embryonic stage was achieved using DNA from a small number of cells. Furthermore, we were able to obtain a fetus that had been diagnosed as homozygous for the R664X mutation by band 3 genotyping of the embryo before transfer. These results indicate that the fetus produced by embryo transfer in this study had phenotypes characteristic of band 3 deficiency.

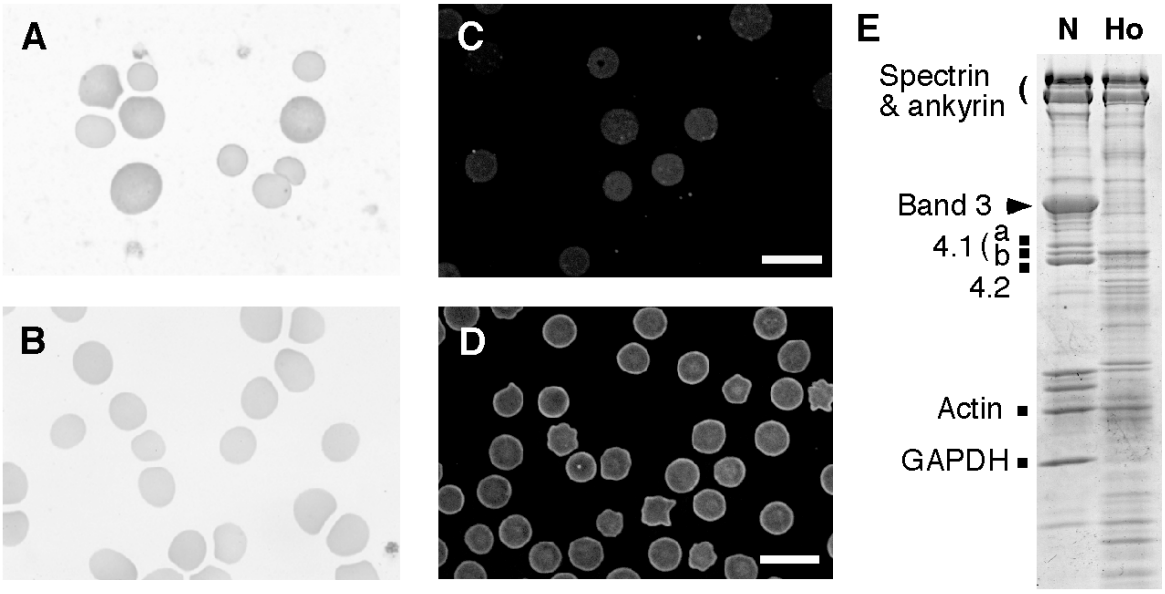

Fig. 3. Characterization of red cells from the fetus homozygous for R664X mutation. Red cells from the fetus homozygous for R664X mutation (A and C) and a fetus free from band 3 deficiency (B and D) on blood films were stained with Giemsa (A and B) or detected for band 3 proteins with immunofluorescence microscopy (C and D). Bars in C and D indicate 5 μm. Red cell membrane proteins from a normal cattle (N) and the fetus homozygous for R664X mutation (Ho) were separated on an 8% SDS-gel and stained with Coomassie brilliant blue (E). Major polypeptides including band 3 are indicated.

Primer extension preamplification PCR [3, 4] and nested PCR [1] have been used to determine genotypes in preimplantation embryos. However, these techniques involve many steps and have the risk of generating a false positive result because of DNA contamination during the handling of PCR products in duplicate PCR procedures. Although our method requires restriction enzyme digestion and electrophoresis, the risk of contamination is low because it consists of a single PCR step. We established the single PCR procedure to determine the *CL-16* genotype and sex in bovine preimplantation embryos [5]. It appears possible enough to diagnose 2 or 3 traits during preimplantation stage by reliable operation of cell sampling and DNA extraction. There are various ways to extract DNA from the blastomeres, for example, boiling the biopsies for 1 min, or snap-freezing the biopsies in liquid nitrogen followed by heating. Among them PK-TW is most effective procedure to extract DNA from small amount of cells (data not shown). In addition, HotStarTaq DNA Polymerase is provided in an inactive state with no polymerase activity at ambient temperatures, so the amplification reaction is only started after the initial denaturation step. This effectively prevents extension of nonspecifically annealed primers. In addition, it would be effective to change the diagnostic procedure from the single PCR described here to a real-time PCR protocol, which reduces the time for enabling fresh embryo transfer. The real-time PCR assays eliminate post-PCR processing and the need for electrophoresis of amplification products. Although only one out of five recipients conceived in the present study, a higher conception rate would be obtained if the embryos were transferred as immediately as possible after recovery.

Diagnosing a genetic disease in preimplantation embryos is useful for the effective practical use of a genetic resource. Several inherited disorders including progressive degenerative myeloencephalopathy (weaver) [6] in cattle reportedly linked to carcass traits. Although association of band 3 deficiency and the quality of beef remains unknown, the current procedure proved effective in production of an animal with an expected genotype. This procedure would facilitate the generation of offspring with desired traits and free from specific genetic diseases.

In addition, this method may contribute to the production of animal models of hereditary diseases. In humans, congenital hemolytic anemia and distal renal tubular acidosis are due to band 3 anomaly [15]. While previous investigators had suggested that a total lack of band 3 was lethal [12–14], band $3^{Bov.Yamagata}$ [7, 8] was reported as the first case in nature with complete deficiency of band 3, although the precise pathogenesis and mechanism by which affected animals compensated for the aberrant defects of band 3 are not known. Therefore, pathobiological studies on fetuses obtained as described in this study may provide relevant information about the physiological functions and pathological significance of band 3 defects generating hemolytic anemia and acidosis.

In conclusion, we established a procedure to determine the band 3 genotype and sex in bovine preimplantation embryos. This procedure can be used to produce animals free from inherited diseases involving band 3 deficiency and to promote animal breeding.

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