SHORT COMMUNICATION

Diagnosis of Freemartinism in Korean Native Cattle by Amplification of Two Different Male-Specific DNA Sequences

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ABSTRACT

The freemartinism is the most frequent form of intersexuality found in cattle, and females of heterosexual twins become sterile. With increase of twinning rates due to transfer of multiple embryos derived from *in vitro* fertilization, it is of great economic value to establish early diagnosis of freemartins to remove infertile individuals from breeding stock. In the present study polymerase chain reaction (PCR) of two different Y-chromosome specific segments (BRY.1 and AMX/Y) was performed to identify freemartins from twins and less common single born freemartins in Korean Native Cattle (KNC). Two male-specific sequences were amplified in all heterosexual twins tested (n=5). In addition, Y-specific PCR products were detectable in one of the single born females (n=4) with visible genital abnormalities. These results suggest that the sensitivity of PCR-based assay may be sufficient to detect freemartinism in single born females as well as female partners of heterosexual twins in KNC.

(Key words : Korean Native Cattle, Freemartin, PCR, Y chromosome)

A syndrome representing sterilization in female partner of heterosexual twin has referred to as the freemartinism. This condition arises when vascular connections form between the placentae of developing male and female fetuses. Presumably due to Mullerian inhibiting substance (MIS) from the male inhibiting Mullerian duct development in the female when the male external genitalia are masculinized, the female tubular reproductive tract is also masculinized to varying degrees. The freemartin, animal with such syndrome, is frequently found in cattle, and less commonly in other species, including sheep (Marcum, 1974; Parkinson *et al.*, 2001) and deer (Stewart-Scott *et al.*, 1990).

In cattle early identification and removal of animals with freemartinism is often beneficial because such animals would be of no potential for breeding. Freematinism is currently diagnosed by clinical examination, karyotyping, blood grouping, detection of Y-chromosome DNA, fluorescent *in situ* hybridization (FISH) and hormonal methods including measurement of steroid hormones, gonadotro-phins, histocompatibility-Y (H-Y) antigen and MIS. Among these methods the detection of Y-chromosome DNA segment by polymerase chain reaction (PCR) may have advantages in rapidity, sensitivity and efficiency over other methods (Padula, 2005). In the present study freemartinism in Korean Native Cattle (KNC) was diagnosed using the procedure involving PCR of two different Y-chromosome specific DNA segments.

Five female heifers co-twinned to males were clinically diagnosed as freemartins. In addition, four single born female heifers from transfer of two embryos were diagnosed as anatomical anormalies in external genitalia and hair color. Besides normal male and female KNC controls, blood samples from these animals were collected in 10-mL EDTA containers. Genomic DNA samples were prepared using QIAamp blood mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. Using primers which amplify the two DNA segments on Y-chromosome (Table 1), PCR was performed as previously reported by Ennis *et al.* (1999). Briefly, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10 pmol each primer, 200 mM dNTPs and 0.9IU Taq polymerase (Promega, Madison, WI, USA) were mixed. The mixtures with DNA samples were denatured at 97 °C for 3 min followed by 35 cycles of 94°C for 1 min, 65°C (BRY.1)/57°C (AMX/Y) for 1 min and 72°C for 1 min,

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Table 1. The sequences of PCR primers to amplify Y-chromosome specific locus

Locus	Primer sequence (5'-3')	Fragment size
BRY.1	Forward GGATCCGAGACACAGAACAGGCTGC Reverse TTGATCAAGCTAATCCATCCATCCTAT	307 bp
AMX/Y	Forward CAGCCAAACCTCCCTCTGC Reverse CCCGCTTGGTCTTGTCTGTTGC	280 bp 217 bp

and then extended for 10 min at 72°C. The amplified PCR products were analyzed by gel electrophoresis on 3% agarose, stained with ethidium bromide and visua- lized under ultraviolet light.

Fig. 1 and 2 show PCR analyses of heterosexual twins using Y-chromosome specific primers BRY.1 and AMX/Y, respectively. The male specific DNA segments were amplified in female as well as male co-twin samples, indicating chimerism. The fact that male specific sequences were present in all five female co-twin samples tested comfirms that these females are freemartins in molecular basis in addition to clinical diagnosis.

Detailed information on animals including sexes, ab-normalities, body weights and results of PCR-based diagnosis was given in Table 2. Notably one of the Y-chromosome specific DNA sequences was detected by

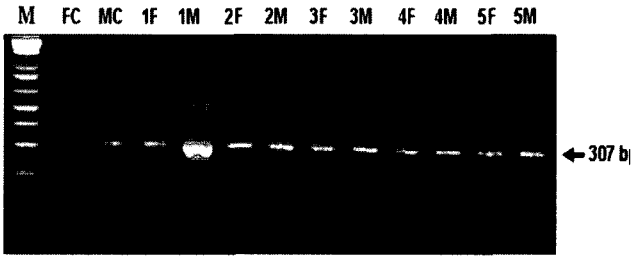

Fig. 1. Detection of freemartins by PCR amplifying BRY.1 locus. M: 100-bp ladder size marker, FC: normal female control, MC: normal male control, 1F-1M, 2F-2M, 3F-3M, 4F-4M, 5F-5M: freemartin twins (F and M represent female and male co-twins, respectively).

Table 2. Description of animals and PCR-based diagnosis of freemartinism

Animal ID	Description	Body weight (kg)	Site of abnormality	Detection of male-specific locus	
				BRY.1	AMX/Y
FC	♀ normal	70	none	-	-
MC	♂ normal	70	none	+	+
1F	♀ co-twin	24	genitalia	+	+
1M	♂ co-twin	23	genitalia	+	+
2F	♀ co-twin	26	genitalia	+	+
2M	♂ co-twin	18	genitalia	+	+
3F	♀ co-twin	23	genitalia	+	+
3M	♂ co-twin	20	genitalia	+	+
4F	♀ co-twin	21	genitalia	+	+
4M	♂ co-twin	21	genitalia	+	+
5F	♀ co-twin	50	genitalia	+	+
5M	♂ co-twin	22	genitalia	+	+
1SBF	♀ single born	28	genitalia	-	-
2SBF	♀ single born	26	genitalia	-	NA*
3SBF	♀ single born	27	genitalia	-	+
4SBF	♀ single born	18	hair color	-	-

* Not available.

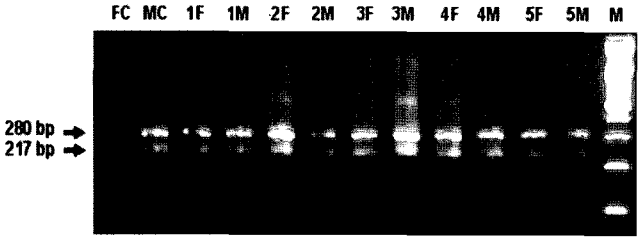

Fig. 2. Detection of freemartins by PCR amplifying AMX/Y locus. FC: normal female control, MC: normal male control, 1F-1M, 2F-2M, 3F-3M, 4F-4M, 5F-5M: freemartin twins (F and M represent female and male co-twins, respectively), M: 100-bp ladder size marker.

PCR in a single born female offspring from a transfer of multiple embryos. The heifer was visibly abnormal in genitalia presumably due to freemartinism. This suggests the method of amplifying male-specific DNA sequences used in this study may be sensitive enough to detect rather uncommon freematinism in single born females.

Due to a variation in the degree of abnormality in freemartins, physical examination of the reproductive tract may not sufficient to confirm freemartinism. Such variation is often observed in entire reproductive organs including vagina, vulval hair, clitoris, cervix, uterus and ovaries (Reick, 1963; Laster *et al.*, 1971). Although results from karyotyping and subsequent FISH may be definitive for diagnosis of freemartinism, such methods are often expensive and time-consuming. The PCR-based method as used in this study is simple, rapid and relatively inexpensive. Sensitivity of the method has been known to be sufficient to detect 0.05% of male cell chimerism (Ennis *et al.*, 1999). Identification of AMX/Y locus has been originally used for sexing bovine embryos (Ennis and Gallagher, 1994), and later BRY.1 locus in addition to AMX/Y been used to determine freemartinism in Holstein heifers (Ennis *et al.*, 1999). The same primers to detect BRY.1 and AMX/Y locus were successfully applied to detect freemartinism in KNC in the present study.

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