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The use of amelogenin gene polymorphism in PCR embryo sexing in bovine IVF embryos

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Abstract. The present study describes a rapid, simple method of bovine IVF embryo sexing by use of PCR technique. A pair of primers corresponding to the bovine amelogenin sequence has been used. The Rapid Cycler (Idaho Technology, USA) used in the current experiment enabled the PCR programme consisting of 55 cycles to be completed in less than 40 minutes. Therefore the total sexing procedure could be performed in less than 90 minutes. The described method succeded in case of 85% analysed embryos.

Key words: amelogenin, bovine embryo, PCR, sex.

Introduction

Embryo sexing has recently become a procedure of economic importance in cattle breeding (HERR, REED 1991). PCR approach seems to be the best choice in this strategy (Van VLIET et al. 1989). Using this technique it is possible to sex over 80% of analysed embryos (BONDIOLI et al. 1989, AGRAWALA et al. 1992, KIRKPATRICK, MONSON 1993, MACHATY et al. 1993, BREDBACKA et al. 1995). Usually one pair of Y-chromosome-specific primers and one pair of bovine-specific primers are used at the same time. Multiplex PCR reaction may occasionaly fails to amplify at one or other locus which may lead to misdiagnosis. There is a gene – amelogenine (AMGL) – found in the human lacated on the sex chromosome pair with a deletion on the Y chromosome. This sequence is succesfuly used in the human for sex determination on DNA level (NAKAHORI et al. 1991, WITT 1994). It has been shown, that

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in cattle as well as in human, the amelogenin gene is located on both sex chromosomes (GIBSON et al. 1991). There is a 63bp deletion in this gene on Y chromosome that causes a polymorphism. Using a pair of primers specific for the amelogenin sequence, a 280 bp product for the X chromosome and a 217 bp product for the Y chromosome can be amplified. As a result of PCR reaction two bands (217 and 280 bp) are detectable from male DNA and one band (280 bp) detectable from female DNA (ENNIS, GALLAGHER 1994).

The aim of the current study was to establish a simple protocol for sexing bovine IVF embryos using the amelogenin primers.

Material and methods

Material – 34 bovine embryos produced in vitro at various developmental stages (from 2-blastomere until blastocyst stage) were submitted for analysis.

Methods

1) zona pellucida digestion – embryos were placed in Talp medium containing 0.5% protease and held until the zona appeared completely digested. As soon as possible after the zona was digested the cell mass was removed and rinsed in sterile water. The cells stayed together for about 2 min after the zona had gone. To verify the presence of the sample, the tubes were inspected under stereomicroscope after adding the blastomeres to the tube (0.2 ml thin walled plastic tubes, Biogene, U.K.). The whole embryo has been used each time.

2) DNA extraction – DNA was extracted from embryos in eppendorf tubes using a Hybaid thermocycler. Two protocols have been used:

a) 20 µl of lysate buffer (1 × PCR buffer + 0.005 g proteinase K/ml) was added to the tube containing blastomeres in 5 µl of water and sample was ovelaid by mineral oil. Afterwards samples were incubated at 55°C for 1 hour followed by 15 min at 94°C and cooled down on ice.

b) frozen-thawed (–20°C) samples containing blastomeres in 10 µl of water were covered with mineral oil and incubated according to the following program: 99°C for 3 min followed by 55°C for 3min repeated three times. No proteinase K was added. The whole lysate was used as a template.

3) PCR amplification – the remaining components of the PCR reaction (1 × PCR buffer, 1 µM of each primer, 200 µM of each dNTPs, 5 IU Taq polymerase) were added to the tube to give the final volume of 50 µl. The whole reaction mixture was transferred into a glass capillary (without oil) and sealed at both ends. We used the pair of primers according to the sequence published

by ENNIS and GALLAGHER (1994). The amplifications were carried out in a rapid cycler (Idaho Technology, USA). The pair of primers has been tested on 10 DNA samples derived from adult individuals of known sex. Each time positive (female and male genomic DNA) and negative controls (no DNA) were included. All samples were denatured at 94°C for 1 min followed by 55 cycles consisting of denaturation for 10 sec at 94°C, annealing for 10 sec at 62°C and extention at 72°C for 15 sec. After the last cycle the samples were incubated for further 1 min at 72°C.

4) analysis of PCR products – amplification products (10 µl) were electrophoresed on a 1.5% agarose gel stained with ethidium bromide.

Results and discussion

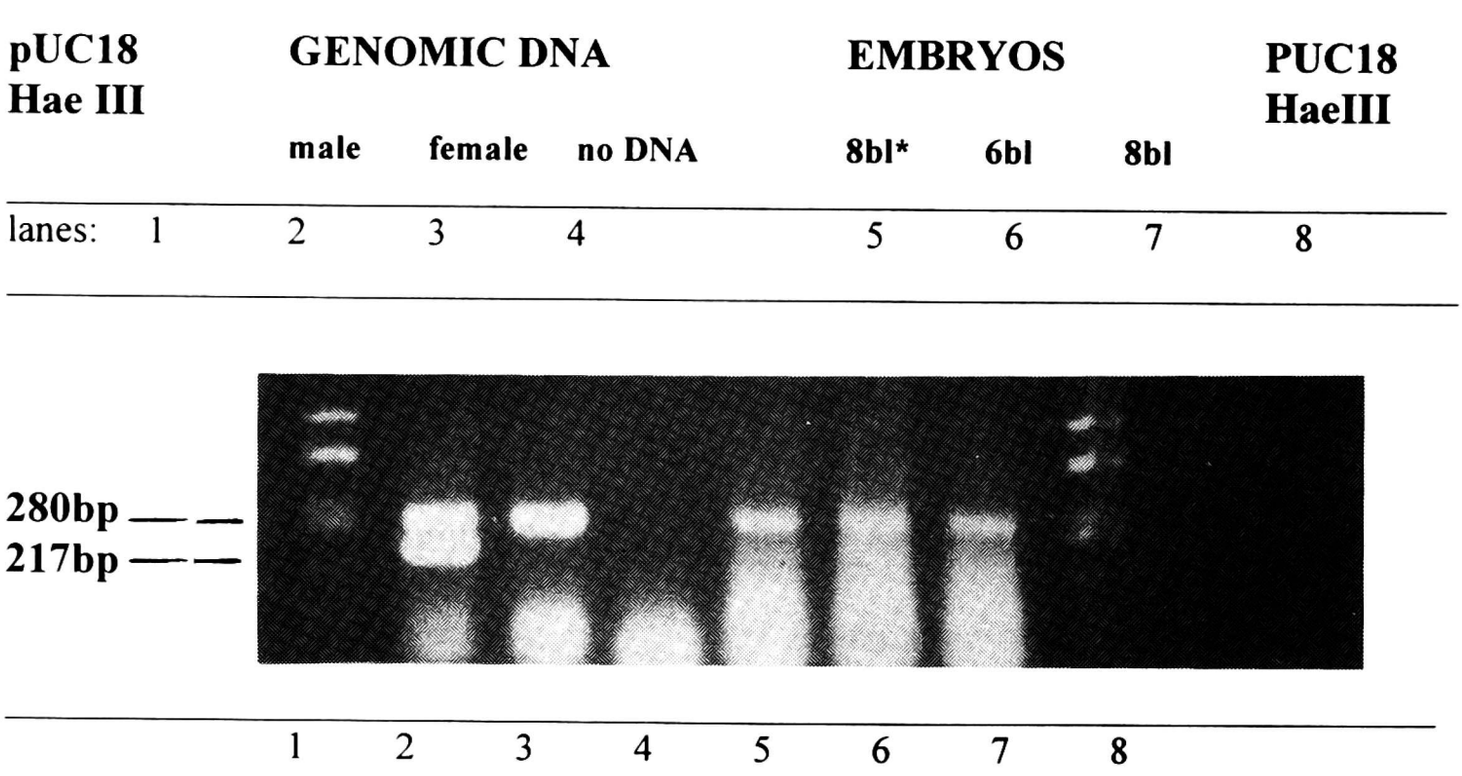
The sex determination assay presented here yielded a results for 29 of 34 embryos (85%, Table 1). The efficiency of the presented protocol falls into range of results shown by other authors when PCR was used (85%, BONDIOLI et al. 1989; 98%, AGRAWALA et al. 1992; 95%, MACHATY et al. 1993). Figure 1 shows the results of amplification with amelogenin primers: the 280 bp product is visible in both sexes whereas the 217 bp product exists only in males. The presence of any nonspecific amplification was not evident. The number of PCR cycles (35) that is often reported when handling with DNA derived from a single blastomere (MACHATY et al. 1993) appeared to be insufficient in the reported protocol for reliable identification of embryonic sex. In a simple

Table 1. The results of bovine embryo sexing assay using the amelogenin primers

The number of embryos submitted for analysis	No. of embryos succesfily sexes		
	total	male (%)	female (%)
34	29	20 (69%)	9 (31%)

experiment where PCR was conducted over 30, 40 and 50 cycles, respectively, faint bands started to appear after 40 cycles and after 50 cycles the amount of product was sufficient to detect.

In DNA extraction protocols proteinase K has often been used, which makes the whole procedure more complicated (PEURA et al. 1991, FULLER et al.



Note: * blastomeres

Fig. 1. Agarose gel electrophoresis of amelogenin specific fragments amplified from bovine embryo samples (the amount of DNA template ranged between 0.02 ng – two blastomere stage and about 1.4 ng – blastocyst stage in 50 µl reaction; lanes 5-7) and genomic DNA (170 ng template in 50 µl reaction; lanes 2-3).
Lanes 1, 8: DNA molecular weight marker (pUC 18 HaeIII), lane 4: negative control – no DNA. Note two bands (280 and 217 pb) amplified from male DNA (lanes: 2, 6) and one band (280 pb) produced from female DNA (lanes: 3, 5, 7).

1993). The protocol used here appeared to be simpler to work as no proteinase K was used.

The frequencies of male and female embryos detected in our study differs from the expectation of 50% each. Usually some sperms remain attached to the surface of zona pellucida which might be a source of male DNA in a female embryo. The risk of such errors has been minimalized by enzymatic removal of the zona pellucida before DNA extraction. It is considered that the shift in the sex ratio is due to the small number of embryos sampled.

A sex determination assay should be capable of functioning with a minimum amount of DNA, such as that obtained in a biopsy of 2-4 cells to ensure the maximal embryo viability. The usefulness of the current method has been proved by producing good amplification signals from some 2-blastomere embryos. It is very important when handling such a small amount of template to simplify the procedure in order to avoid losing DNA during manipulations. It is advaisable to keep the embryonic DNA in the same tube as long as possible and preferably through the whole procedure. In this experiment it was necessary

to transfer samples from tubes into capillaries after DNA extraction and the addition of all the PCR reaction components. However, the risk of losing DNA at this point was considered minimal.

In this study, the use of the Rapid Cycler enabled the PCR programme to be completed in less than 40 minutes. The total sexing procedure can be therefore performed in less than 90 minutes without sacrificing accuracy in any way.

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