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Sex Determination in Ovine Embryos Using Amelogenin (*AMEL*) Gene by High Resolution Melting Curve Analysis

Yao Jing Yue, Jian Bin Liu, Ting Ting Guo, Rui Lin Feng, Jian Guo,
Xiao Ping Sun, Chun E. Niu and B.H. Yang
Chinese Academy of Agricultural Sciences,
Lanzhou Institute of Animal and Veterinary Pharmaceutics Sciences,
335# Jiangouyan Street, Qilihe District, Lan Zhou City,
730050 Gan Su Province, China

Abstract: In the study, researchers have established and tested the reliability of a method for sex determination of ovine embryos using the *AMEL* gene by melting curve analysis of PCR amplification. It can be carried out in a regular laboratory or under farm conditions within 1.5 h for 96 samples. The PCR amplicons of 99/99 and 99/54 base pairs produced from female and male sheep, respectively are easily distinguished by both melting curve analysis and gel electrophoresis. The specificity of the method was earlier demonstrated by testing 9 blood samples from small-tailed sheep (5 males and 4 females). No amplification failures and very high agreement between genotypic and phenotypic sex was found (9/9). The sensitivity of the AMEL sexing assay was established for values >10 pg ovine genomic DNA. Forty five biopsied embryos were transferred into 22 recipient sheep on the same day that the embryos were collected and sex of the kid was confirmed after parturition. About 17 kids of predicted sex were born. The sex, as determined by PCR corresponded to the anatomical sex in all cases. To the knowledge, this was the first time that sex determination using the amelogenin gene was performed in ovine embryos by melting curve analysis.

Key words: Ovine embryos, amelogenin gene, sexing, high-resolution melting, blood, China

INTRODUCTION

The embryo transfer technology represents a powerful tool for the acceleration of various breeding programs in sheep. Known sex of embryos produced for use in ET programs can more effectively help to manage producer resources because more heifer calves per ET can be produced. This approach can improve the genetic potential of sheep breeds in shorter time intervals.

Several protocols have been established for sexing embryos in farm animals such as karyotyping (King, 1984), H-Y antigen detection (Andersib, 1987), X-linked enzymatic determination (Monk and Handyside, 1988) and based on the identification of the Y chromosome such as *SRY*, *ZFY* and *TSPY* genes include *in situ* hybridization, Southern dot blotting, Polymerase Chain Reaction (PCR), Loop-Mediated Isothermal Amplification (LAMP) (Miller, 1991; Bredbacka and Peippo, 1992; Gutierrez-Adan *et al*., 1996; Ng *et al*., 1996; Sohn *et al*., 2002; Jinming *et al*., 2007). Among of these methods, PCR-based sexing assays are generally favored because of the advantages of being relatively simple, rapid and inexpensive. Some of the existing protocols are only based on the PCR-detection of Y chromosome specific sequences such as genes: SRY (Takahashi *et al*., 1998; Mara *et al*., 2004) and TSPY (Lemos *et al*., 2005) or repeated sequences (Schroder *et al*., 1990; Bredbacka and Peippo, 1995; Kageyama *et al*., 2004). The presence of no signal does not necessarily mean that the sample has a female origin because experimental errors can also lead to negative results. Then, sexing protocols also need a PCR product being out of the Y chromosome as a positive control of template (DNA) in the sample or an X chromosome specific fragment. The amelogenin (*AMEL*) gene which exists on both X (AMELX) and Y (AMELY) chromosomes has been used to determine the sex in humans (Sullivan *et al*., 1993), cattle (Chen *et al*., 1999; Nicolai Z. Ballin), sheep and deer (Pfeiffer and Brenig, 2005), goats (Chang *et al*., 2006; Weikard *et al*., 2006) as well as in the related species (Weikard *et al*., 2006). The complexity of these methods and the need for multiple steps to perform them greatly increase the risk of

Corresponding Author: B.H. Yang, Chinese Academy of Agricultural Sciences,
Lanzhou Institute of Animal and Veterinary Pharmaceutics Sciences, 335# Jiangouyan Street,
Qilihe District, Lan Zhou City, 730050 Gan Su Province, China

cross-contamination and thereby misdiagnosis. Recently, a novel technique, High-Resolution Melting (HRM) has been investigated for the detection of point mutations, Single-Nucleotide Polymorphism (SNP), internal tandem duplications, simultaneous mutation scanning and genotyping in bacteriology, cancer research and human platelet antigens (Wittwer *et al*., 2003). The sample preparation consists of a standard PCR reaction with a dsDNA intercalation fluorescent dye and does not require any post-PCR handling. Products can be analyzed directly after PCR amplification using specially designed instruments for High-Resolution Melting (HRM) analysis. HRM is a mutation detection and scanning technique that has high reliability. It has been reported to have near 100% sensitivity and specificity when the analyzed PCR products were up to 400 bp in length. In this research, researchers tried to design a protocol of sex determination in ovine embryos using Amelogenin gene (*AMEL*) by melting curve analysis of PCR amplifications.

MATERIALS AND METHODS

Sheep blood samples and DNA extraction: In order to test the specificity of the technique, samples with known sex (male and female) from 9 Small Tailed Han (4 female, 5 male). Blood samples were obtained from. Genomic DNA was obtained from blood samples following the manufactured instruction of the Tiangen Biotech Commercial kit.

The DNA was quantified using NanoDrop ND-2000 Spectrophotometer (NanoDrop Technologies, Inc.). The sensitivity of the AMEL sexing assay was proven using dilution series of ovine genomic DNA, ranging from 1-10 pg (1 ng, 100 pg, 10 pg). DNA purity was evaluated by comparing the absorbance ratios A260/280 and A260/230.

Collection of sheep embryos: About 10 donors (Poll Dorset x Small Tailed Han intercross F1) were treated with CIDR (Fluorogestone acetate 300 mg) (New Zealand) sponges for 12 days and were super ovulated with oFSH (ovine Follicle Stimulating Hormone 20 mg mL^{-1}) (Canada) in 4 decreasing doses (2 days ×1.5 mL and 2 days ×1.0 mL) at 12 h intervals from 10-13th days. On the 12th day, the sponges were removed and PMSG (300 IU) (Canada) was injected intramuscular (i.m.) and oestrus was detected by rams at 12 h intervals after sponge withdrawal (Wang *et al*., 2006). Intrauterine insemination with fresh semen (Poll Dorset x Small Tailed Han intercross F1) was carried out twice at 12 h intervals 48 h after sponge withdrawal. Embryo recovery took place 7~8 days after sponge removal.

Embryo manipulation and DNA extraction: Four to ten cells from 67 compact morulae were aspirated through the zona pellucida using micro-manipulation systems equipment (TransferTip (ES), Eppendorf). After biopsy, embryos were incubated in1 X PBS (Phosphate Buffered Saline, PBS (Gibico) pH = 7.4) supplemented with 0.4% BSA (Bovine Serum Albumin, BSA) at 10°C during 10 h (Hong *et al*., 2005). In this period sexing was performed from the aspirated cells by high-rsesolution melting analysis. The following day, embryos were transferred to FGA+eCG (equine Chorionic Gonadotropin) treated recipients (2 embryos of the same predetermined sex/ewe) (Dervishi *et al*., 2008).

The DNA was isolated from the embryos by the single step method described by Saravanan *et al*. (2003) in 1×PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) containing proteinase K (150 mg mL^{-1}) and incubated for 30 min at 37°C. Then proteinase K was inactivated by incubating at 97°C for 10 min. The tubes were kept frozen at -20°C until sexing was carried out.

High-resolution melting analysis of *AMEL* gene: Primers were designed using Primer 5.0 (Rozen and Skaletsky, 2000). The primers AMEL-SF and AMEL-SR had the sequences 5'-ATCCAGCCRCAGCCTCACC-3' and 5'-GATGGGGTGCACGGGTGG -3', respectively.

All DNA was amplified in a 10 μL final volume containing 1×Tiangen Biotech taq PCR Master Mix, 0.1 μmol L^{-1} forward primer (AMEL-SF), 0.1 μmol L^{-1} reverse primer (AMEL-SR) and 1×LCGreenPlus+. The PCR program consists of an initial preheating at 95°C for 5 min to activate the Taq DNA polymerase, followed by 30 amplification cycles. Each cycle is comprised of an annealing step at 62.5°C for 15 sec, an elongation step at 72°C for 15 sec and denaturation at 94°C for 45 sec. The final melting program consists of three main steps beginning with a denaturation at 95°C for 1 min, renaturation at 25° for 1 min.

The PCR products were separated in 2% agarose gel for 1 h and then photographed. The Lightscanner (Idaho technology) is an instrument that measures high-resolution DNA melting curves from samples in a 96 well PCR plates. This is achieved by monitoring the fluorescence change of the fluorescent DNA intercalating dye, LCGreenPlus+ as the sample is melted. Turnaround time per sample is approximately 1-2 min, depending on how broad the temperature range is required to be. The Lightscanner was heated at 0.3°C sec^{-1}. *AMEL* gene was simultaneously analyzed between 40 and 98°C with a turnaround time of approximately 7 min/96 samples. The Light-Emitting Diode (LED) power was auto adjusted to 90% fluorescence.

Sequencing of *AMEL* gene PCR products: The samples were directly sequenced from the Lightscanner PCR amplification. The PCR products were mixed with Nucleic Acid Purification kit (Tiangen Biotech) to remove the remaining primers and bidirectional sequenced with forward and reverse primers using ABI PRISM terminator cycle sequencing kit Version 1.1 (Takra Biotechnology (DaLian) Co., Ltd.) on the ABI PRISM 3730 genetic analyzer (Takra Biotechnology (DaLian) Co., Ltd.).

RESULTS AND DISCUSSION

Sexing using sheep blood sample: As expected from the sheep sequence, a 99 bp product, representing amplification from the X chromosome amelogenin was detected for the ewe. For the ram, researchers detected the 99 bp, representing X chromosome and a new 54 bp band, representing specific Y chromosome amelogenin amplification. As shown in Fig. 1, in total, 9 animals (4 females and 5 males) were analyzed, showing no amplification failures and a high agreement between genotypic and phenotypic sex (10/10) indicating that the sexing method based PCR amplified amelogenin gene was 100% reproducible and reliable. Both amplified fragments were sequenced and analyzed showing that the 54 bp (AMELY) and 99 bp (AMELX) fragments had a 100% of identity with variant 2 of AMELY (GenBank acc DQ469593) and *AMELX* genes (GenBank acc DQ469591), respectively.

Amplicons from PCR analysis of ram (AMELX and AMELY) DNA with the primer pair AMEL-SF/R showed two distinct peaks in the high-resolution melting curve analysis (Fig. 2).

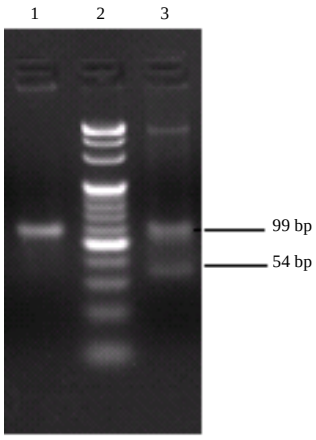

Fig. 1: Total 2% gel electrophoresis of PCR amplicons; Lane 1, a 20 bp ladder; Lane 2 and 3, PCR amplicons from beef male and female DNA, respectively

Gel electrophoresis of the PCR amplicons confirmed the melting curve results by showing two distinct fragments (Fig. 1) around the expected 54 and 99 base pairs (Fig. 1). Ewe (2 X AMELX) DNA subject to PCR analysis with the primer pair AMEL-SF/R showed as expected a single peak in the melting curve analysis (Fig. 2) and a single band on the gel electrophoresis (Fig. 1). The sensitivity of the AMEL sexing assay was established for values >10 pg ovine genomic DNA (Fig. 3).

Sexing of sheep embryos: A total of 51 embryos were used to test the efficiency and accuracy of the method. After biopsy, all embryos were incubated during 24 h and their developmental ability was evaluated. Five embryos

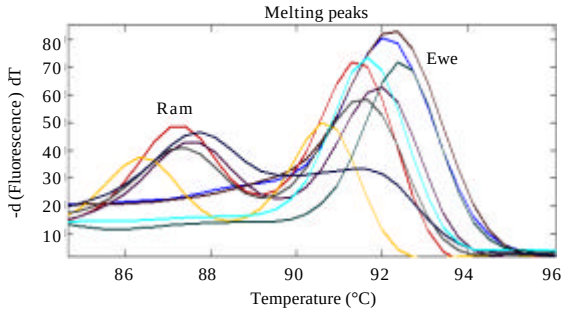

Fig. 2: Melting curve analysis of PCR amplicons. The different male and female curves represent DNA products from 9 Small Tailed Han

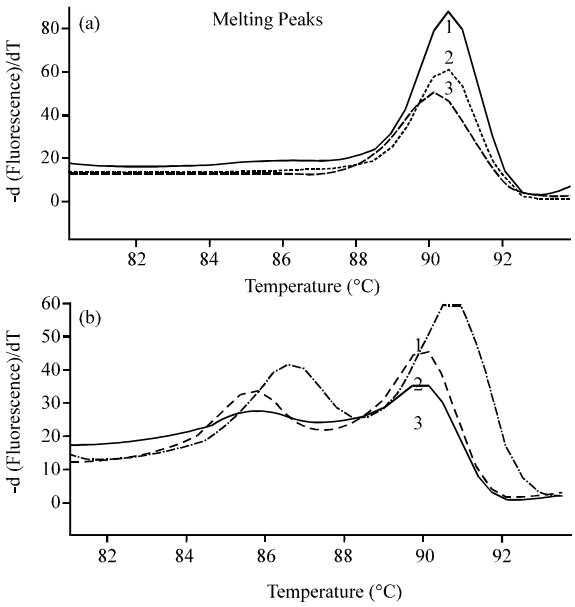

Fig. 3: Dilution series of the amelogenin assay; a) Lines 1-3, male DNA dilution series, 1) 1 ng; 2) 100 pg and 3) 10 pg; b) Lines 1-3, male DNA dilution series; 1) 1 ng ; 2) 100 pg and 3) 10 pg

were discarded because they showed abnormal development for their age. Forty five were transferred to twenty two recipient ewes. About 5 weeks after the transfer of the sexed blastocysts, pregnancy rate of the recipient of does was confirmed. About 17 lambs were born, 6 of which were males and 11 were females. The sex as determined by PCR corresponded to the anatomical sex in all cases. The sex determination took <3 h including DNA extraction and PCR amplification.

The amelogenin gene encodes an important protein in the developing mammalian tooth enamel matrix that has been conserved during the evolution of vertebrates. The amelogenin (*AMEL*) gene which exists on both X (AMELX) and Y (AMELY) chromosomes has been used to determine the sex in humans (Sullivan *et al*., 1993), cattle (Chen *et al*., 1999), sheep and deer (Pfeiffer and Brenig, 2005), goats (Chang *et al*., 2006; Weikard *et al*., 2006) as well as in the related species (Weikard *et al*., 2006). Pfeiffer and Brenig described a 45 bp deletion in the amelogenin gene at Y chromosome. In this way females amplify only a 263 bp band while males produce two bands of 263 and 218 bp. In this study, researchers also fund that females amplify only a 99 bp band while males produce two bands of 99 and 54 bp. The major advantage of this method is the co-amplification, in a single tube, of two specific fragments, one from Y-chromosome and one for the X chromosome using a single primer pair and making the use of a PCR control unnecessary (Gerardo *et al*., 2007). But all analyses of amplifications must be done by gel electrophoresis and therefore a potential future alternative. In the study, researchers have established and tested the reliability of a method for sex determination of ovine embryos using the *AMEL* gene by melting curve analysis of PCR amplification. This assay provides a rapid and sensitive method for sexing. It can be carried out in a regular laboratory or under farm conditions within 2-3 h for 96 samples. This is especially important for the future application of the protocol to sheep embryos sexing.The protocol was showing that the efficiencies in sex determination were 100% by evaluating genomic DNA from 4 females and 5 males. This result was comparable to those reported by Nicolai Z. Ballin with the same primers. Assuming a DNA content of about 6 pg per cell, this is in agreement with the result in the dilution series assay displaying a detection limit of 10 pg genomic DNA (Fig. 3). The results showed that the threshold of the amelogenin assay using DNA samples extracted from the embryos seems to be 2 cells.

The method of biopsy and sex determination of goat embryos that we used can accurately and efficiently predict the sex of embryos before transfer. The microblade used to biopsy the embryo is easy to use and has proven to be effective with bovine and sheep embryos (Herr and Reed, 1991; Kochhar *et al*., 2000). Quality of the embryo and the size of the biopsy can influence the outcome of the procedure (Ju *et al*., 2001). Researchers used morphologically normal blastocysts and obtained a 37.3% kidding rate after transfer. These results are comparable with *in vitro* produced ovine embryos, biopsied and sexed by similar technique (Mara *et al*., 2004).

CONCLUSION

In conclusion, researchers have established and tested the reliability of a method for sex determination of ovine embryos using the *AMEL* gene by melting curve analysis of PCR amplification. The advantage of this assay is that neither additional control amplicons with a second locus specific autosomal primer pair nor the gel electrophoresis is necessary for sex determination and control of the PCR reaction. This assay provides a rapid and sensitive method for sexing. It can be carried out in a regular laboratory or under farm conditions within 2-3 h for 96 samples. The rapid sex determination using amelogenin gene allows transferring sexed fresh embryos in MOET and IVF (*In Vitro* Fertilization) programmers to make them more efficient (Dervishi *et al*., 2008).

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