



A PCR-based method for fetal sex determination using ovine amniotic fluid⁽¹⁾

(Determinação do sexo fetal em líquido amniótico de ovinos através da técnica de PCR)

"Nota / Note"

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Abstract

Pre-natal sexing was undertaken using 19 samples of ovine amniotic fluid obtained through amniocentesis from pregnant uteruses of animals killed in a slaughterhouse. The aim was to evaluate the applicability and efficiency of the Polymerase Chain Reaction technique (PCR) for these purposes. Multiplex PCRs were carried out with SRY and Aml-X primers. The amplified samples were plotted in 2% electrophoresis agarose gel and viewed using an ultraviolet transilluminator. Ten samples (52.63 %) were identified as "male" (116bp and 300bp) and nine samples (47.37 %) as "female" (300bp); this information was confirmed by a morphological analysis of all fetuses. The results allow to conclude that PCR, when using SRY and Aml-X markers on ovine amniotic cells, is efficient and reliable to fetal sex identification.

Key-words: sexing, Aml-X, SRY, amniocentesis.

Resumo

Foi realizada a identificação pré-natal do sexo em 19 amostras de líquido amniótico ovino obtidas por amniocentese em úteros prenhes de animais abatidos em matadouro. Teve-se o objetivo de avaliar a aplicabilidade e a eficiência da técnica de Reação em Cadeia da Polimerase (PCR) para esta finalidade. Foram realizados PCR multiplex com os "primers" referentes aos genes SRY e Aml-X. As amostras amplificadas foram plotadas em gel de agarose 2% para eletroforese e posterior visualização em transluminador de luz ultravioleta. Foram identificadas 10 amostras (52,63 %) como macho (116pb e 300pb) e 9 (47,37 %) como fêmea (300pb), que foram confirmadas pela avaliação morfológica dos fetos. Os resultados permitem concluir que a técnica de PCR é eficiente e aplicável para sexar fetos através do fluido amniótico.

Palavras-chave: identificação do sexo, Aml-X, SRY, amniocentese.

Introduction

Among new technologies, the use DNA analysis has increased a lot during the last few years and the Polymerase Chain Reaction (PCR) method has been widely applied in the different fields of biology. Due to the advances in PCR it is presently possible to carry out several different types of

diagnoses, including paternity investigation, detection of genetic and infectious diseases, and embryo sexing through DNA analysis (GARCIA et al., 2003).

The possibility of selecting the sex of offspring presents practical and economic advantages for the animal production industry (ALMEIDA and ALVAREZ, 2003).

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The best known molecular methods for sexing involve viewing sexual chromosomes based on the construction of a karyotype (cytogenetics) or detecting a specific chromosome Y DNA sequence after PCR amplification (LUZ et al., 2000).

The targeted segments for amplification and choice of specific chromosomal fragments include SRY (located at the long portion of the Y chromosome) and Aml-X (found in chromosome X). The possibility of a simultaneous amplification of sequences matching both chromosomes allows their use as an efficient and duplicable method to determine sex through PCR in caprines (PHUA et al., 2003).

The AMEL gene, found in chromosome X, has been used to determine sex in bovines (ENNIS et al., 1994) and in humans (SULLIVAN et al., 1993).

Cells from the amniotic fluid were first used for fetal sexing in humans by Fuchs and Riis (1960). From then on, the amniocentesis procedure has improved considerably and a method to cultivate these cells *in vitro* has been introduced into cytogenetical analyses of human anomalies (STELLE and BREG, 1966).

The aim of this study was to evaluate the applicability and efficiency of multiplex PCR to sex ovine fetuses by using the amniotic fluid collected from pregnant uteruses coming from a slaughterhouse.

Material and Methods

Amniotic fluid samples

The ovine uteruses were obtained from undefined-race sheep of a private slaughterhouse from the town of Igarassu (State of Pernambuco, Brazil) and conserved at low temperature until removal to the Veterinary Medicine Department at UFRPE.

Nineteen samples of amniotic fluid (10 mL) were collected from 15 uteruses (eleven simple gestations and four multiple gestations) and stored in properly sterilized and labeled test tubes. The liquid was

conserved in a refrigerator for three hours in order to allow for cell sedimentation at the bottom of the tubes.

DNA extraction

The DNA was extracted according to a technique modified from Sambrook et al. (2001). A total of 600 µL of amniotic fluid was removed in each sample with cellular sedimentation and transferred to 1.5 mL tubes to which 300 µL of TE (Tris, 10 mM – Invitrogen Life Technologies and EDTA, 1 mM pH 8.0 – Sigma Chemical Co.) and 300 µL balanced (pH 8.0) phenol (Merck) was added. The samples were homogenized in a vortex during one minute and then centrifuged (SIGMA 2K15) at 18,000 g during five minutes at 4°C. The next step was to transfer the supernate to another tube to which 200 µL phenol-chloroform (1:1) was added; the sample was homogenized for one minute and centrifuged at 18,000 g for five minutes.

The supernate was then transferred to another tube to which 300 µL of chloroform (Merck) was added; it was mixed for one minute and centrifuged at 18,000 g during five minutes. The following substances were then added, once again in another tube: 30 µL 3M ammonium acetate (Cromato Produtos Químicos LTDA), all of the supernate from the previous tube (where the DNA is found), and 300 µL isopropanol (Merck).

The mixture was homogenized during one minute and incubated in the freezer for 60 minutes at -20°C; it was then centrifuged at 18,000g during 30 minutes. The supernate was discarded and the sediment was washed with 500 µL 70% ethanol (Merck) by centrifugation at 18,000 g during 5 minutes at 4°C. The ethanol was removed and the sediment was left at room temperature and then resuspended in 30 µL TE. The DNA extracted was analyzed in 1% agarose gel (Invitrogen life Technologies) with a phage lambda marker, dyed with ethidium bromide for analysis using ultraviolet light, and then photographed (Olympus Digital Camera – C-7070 Wide) to verify its quality.

DNA amplification

Both the SRY gene sequences, as well as the Aml-X sequences as a control, were amplified during the PCR process. For each sample, multiplex PCR was carried out in a final volume of 25 μ L containing 2.5 mM of $MgCl_2$, 10 pmoles of each primer, 200 μ M of each dNTP, 1U of Taq-polimerase, 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 2 mM $MgCl_2$), and 7 μ L of DNA; ultra-pure water was added to reach the final volume.

The amplification protocol consisted in an initial denaturation at 94°C for five minutes, followed by 34 cycles of 94°C for 45 seconds, 58°C for 45 seconds, and 72°C for one minute, with a final extension of 72°C for seven minutes at the end of the cycle. The amplification products were separated by electrophoresis in 2% agarose gel, dyed with ethidium bromide, viewed in an UV transilluminator, and compared with a 50 bp DNA ladder marker (Invitrogen, USA) (PHUA et al., 2003).

Oligonucleotide sequences

The primers used had the sequences described below (PHUA et al., 2003):

Aml-X.5: 5' CAGTAGCTCCAGCTCCAGCT 3'

Aml-X.3: 5' GTGCATCCCTTCATTGGC 3'

SRY.5: 5' ATGAATAGAACGGTGCAATCG 3'

SRY.3: 5' GAAGAGGTTTTCCCAAAGGC 3'

Animals were considered male when two bands were formed: one with 116bp (from the SRY gene amplification sequence) and another with 300bp (from the Aml-X gene amplification sequence found in both sexes). Animals were considered female when only the 300bp band was formed (PHUA et al., 2003).

Results and Discussion

The DNA extraction from amniotic cells, in 1% agarose gel, show less than 50ng/ μ L DNA when compared in the phago lambda quantification, which is considered a small amount (PHUA et al. 2003).

From the 19 samples analyzed, ten (52.63%) showed the formation of two bands, one with 116bp and another with 300bp,

which correspond to the SRY and Aml-X gene sequence amplifications, respectively, and characterize males; nine (47.37%) samples only amplified the 300bp section, which corresponds to the Aml-X gene – this characterized females (Figure 1). In all cases the sex was confirmed through a morphological analysis of the fetuses.

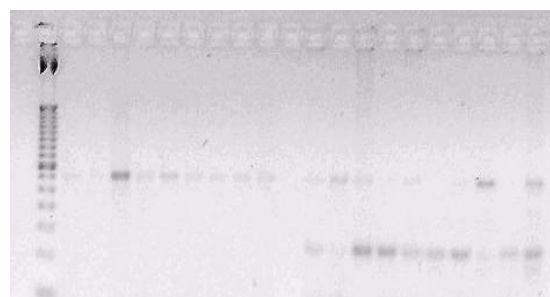


Figure 1 – Band formation using 300bp (Aml-X) in all samples and bands with 116bp (SRY) present only in male animals, from 11 to 20.

The Aml-X gene served as a parameter, since its amplified sequence showed up in all samples, as suggested by Pfeiffer and Brenig (2005); thus, it is a quantity control for the amplification reaction. It was possible to undertake sexing using a SRY gene sequence in all samples, which confirms the proposal of Luz et al. (2000).

Despite the small amount of DNA found in the amniotic fluid, PCR is an efficient tool to copy desired target sequences, which corroborates with Champe and Harvey (1997).

The sex determination in ovine amniotic liquid was not previously related using this method. Otherwise, it was showed that is possible utilize in ovine, the same primers that amplified the caprine SRY and Aml-X.

The results allow to conclude that the PCR technique, when using SRY and Aml-X markers on ovine amniotic cells, allows for quick and reliable fetal sex determination.

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