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Sex identification of Iraqi local cattle using PCR-RFLP technique

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ABSTRACT:

This study was conducted in the physiology lab. / department of animal production / college of Agriculture / Misan University and Genetic Engineering lab. / department of biology / college of Science / Misan University. The objective of the study was to identify the sex of bovine embryos using amniotic fluid samples by application of PCR-RFLP assay. Amniotic fluid samples were collected from pregnant uteri of cows brought from the slaughter house (25 samples). DNA was extracted and PCR-RFLP was done using SRY primers, the products of amplification furtherly restricted by (HINDIII) enzyme. The results showed three bands on Agarose gel electrophoresis diagnosed as male samples, while the presence of one band indicated female samples. Amelogenin X was used as internal control which appeared in both sexes but not restricted by the enzyme. These results furtherly confirmed by inspection of embryos anatomy, the results were accurate in 100%. This method will help producers to build a scientific strategy in management with better prediction of sex ratio in the coming season, also prevent slaughter cows pregnant with wanted embryos or vice versa.

Keyword: *sex identification, cattle, PCR-RFLP technique*

INTRODUCTION

Sex identification using non-invasive samples allows population studies based on sex ratio information without capturing and marking animals (Kim et al., 2008). Simple and precise methods for sex determination in animals are pre-requisite for a number of applications in animal production and forensics. However, some of the existing methods depend only on the detection of Y-chromosome specific sequences. Therefore, the absence of a signal does not necessarily mean that the sample is of female origin, because experimental errors can also lead to negative result (Pfeiffer and Brenig, 2005). Therefore, the detection of Y-chromosome specific sequence is not enough, and application of PCR-RFLP technique may be the best way to confirm the results (Ennis and Gallagher, 1994).

Amniotic fluid cells for sex determination

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 cytogenetically analyses of human anomalies (Stelle and Breg, 1966). The AML-X gene, found in chromosome X, has been used to determine sex in human (Sullivan et al., 1993).

SRY and AML-X genes

The male phenotype is determined by the expression of a testis – determining factor(TDF) found on the Y chromosome. Recently, a gene was discovered within the sex – determining region of the Y chromosomes (SRY) that is expressed at the onset of testicular differentiation (Coriat et al.,1993). 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). Amelogenin is a major protein constituent of the developing enamel matrix. The gene structure of this protein has been demonstrated. The X- specific target sequence was included as an internal control in discrimination of male and female with a higher accuracy. It also served as a PCR positive control ensuring that the absence of a Y- specific product was not due to PCR failure.

MATERIALS AND RESULTS

DNA extraction

Genomic DNA was extracted from amniotic fluid samples beside genomic DNA samples isolated from the blood of known phenotypic sex used as control for verification of accuracy and specificity of PCR. The DNA was extracted according to the technique modified from Sambrook et al. (2001). Atotal of 600 µL of amniotic fluid and blood 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, 1mM 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 15000 g during five minutes at 4°C. the next step was to transfer the supernatant another tube to which200 µl phenol-chloroform 1:1 was added; the sample was homogenized for one minute and centrifuged at 15000 g for five minutes. The supernatant was then transferred to another tube to which 300µl of chloroform merck was added; it was mixed for one minute and centrifuged at 15000 g during five minutes. The following substances were then added once again in other tube: 30µl 3m ammonium acetate. All of the supernatant from the previous tube (where the DNA is found) add 300µl isopropanol (merk). The mixture was homogenized during one minute and incubated in the freezer for 60minutes at -20°C; it was then centrifuged at 18,000g during 30 minutes. The supernatant was discarded and the sediment was washed with 500 µL 70% ethanol (Merck) by centrifugation at 18,000 g during 5 minutes at 4ºC. The ethanol was removed and the sediment was left at room temperature and then re- suspended 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.

PCR amplification

The PCR reactions were carried out in 50 µl of PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl and 0.1% TritonX-100),

MgCl2 (1.5 mM), four dNTPs (each 200 µM), Taq DNA polymerase (2 units) and oligonucleotide primers. The PCR amplification was carried out using (Eppendorf research thermal cycler) with the following amplification procedure: An initial denaturation for 5 min at 95°C was followed by 35 cycles of denaturation (30 secs at 94°C), primer annealing (30 secs at 56°C) and strand synthesis (30 secs at 72°C), and in the last cycle, the samples were held at 72°C for an additional 5 min. The amplification was confirmed by agarose gel electrophoresis (1.2%) stained with 0.5 µg/ml ethidium bromide. The PCR product were digested with HindIII Enzyme (for 1.5-2 hrs at 37 °C).

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', and 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

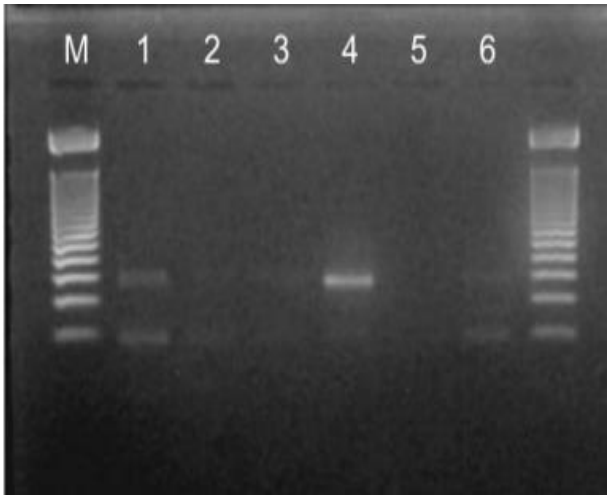

Figure 1: PCR amplification, band1 ladder100 bp, band 2,3,4 and7 are from male samples (two bands) of amelogenin and SRY genes, band 5 is from female sample (one band) of amelogenin gene. band 6 control.

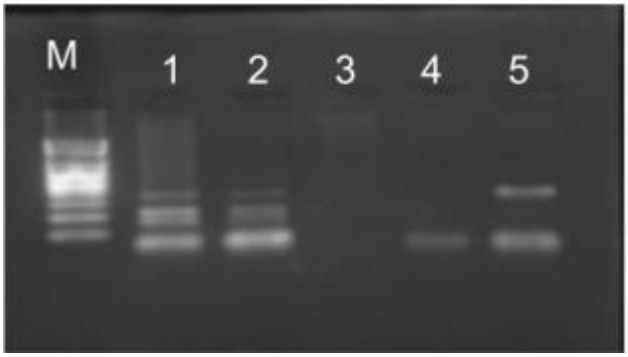

Figure 2: PCR products digestion with HindIII enzyme, band1 is 100bp ladder, band 2,3,6 are from male samples (digestion of SRY in to three bands), band 4 is control. band 5 is from female samples (Amelogenin gene did not digested with HindIII enzyme).

PCR amplification of SRY gene and Amelogenin gene resulted in 300 bp and 100bp fragments respectively. As shown in (figure 1), male samples presented both bands while female samples had only 100 bp band. PCR products were digested with HindIII enzyme to be sure of our results and exclude all the possibilities of errors, the digested PCR products were electrophorized on agarose gel 0.8 % our results revealed that the male samples showed three fragments while female samples showed only one fragment figure (2), for confirming the accuracy of the PCR, 10 blood samples of adult animals (5 females and 5males) were detected. All sex determination by the PCR was in agreement with the actual sexes of the animals from which blood samples were obtained, indicating that the sexing method based PCR-RFLP amplified SRY and Amelogenin and digested with HindIII enzyme was 100% accurate, reproducible and reliable.

Discussion

PCR is a rapid, easy procedure for large scale sexing, and primers derived from many Y-specific sequences have been used to screen blood, meat, and blastomere samples (Zeleny et al., 2002; Alves et al., 2003). Amplification of the Amelogenin and SRY genes was sufficient to determine the gender and it has been done successfully in bovine embryos and ovine embryos (Pfeiffer and Brening, 2005) sexing studies. Using X-amelogenin gene as an internal control did not interfere with the amplification of the Y-specific sequence (Phua et al., 2003). The objective of the study was to develop a bovine sexing assay that was accurate, sensitive, and relatively fast. The most common approach in sexing involves the co amplification of the Y-chromosome specific sequence containing the Y-linked genes (SRY) and an autosomal sequence that acts as a control for the presence of DNA (Mara et al.,2004). In the present study, we employed primers derived from a sequence for X-and Y-specific amelogenin, and verified the accuracy of the assay by evaluating genomic DNA from well-defined adult male and female cattle. After digestion of the PCR products there were more differentiating results indicating the possibility of sex identification in all animal tissues or traces with a very high accuracy reach to 100%. This result was comparable to those reported by other authors (Ennis et al.,1994; Pfeiffer et al., 2005; Weikard et al.,2006) with the same primers. This assay provides a rapid and sensitive method for sexing, because of the presence of the X-chromosome band. Moreover, it can be carried out in a regular laboratory or under farm conditions within 4 hours. This is especially important for the application of the protocol to bovine sexing. Also our study showed there was no contamination from human DNA during the laboratory analysis. These were minimized the risk of contamination, and insured the accuracy of sexing agree in this thought with (Chen et al. 2007). This method can be used in cattle breeding programs to manipulate sex ratios of offspring since it depends on amniotic fluid samples.

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