J Bangladesh Agril Univ 20(4): 402-406, 2022 https://doi.org/10.5455/JBAU.109580

ISSN 1810-3030 (Print) 2408-8684 (Online)

Journal of Bangladesh Agricultural University

Journal home page: http://baures.bau.edu.bd/jbau

Research Article

Sex Determination of Unknown Ovine Samples Gathered from Slaughters

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ARTICLE INFO

Article history
Received: 26 Aug 2022
Accepted: 06 Dec 2022
Published: 31 Dec 2022

Keywords
Amelogenin gene,
PCR,
sex determination,
slaughterhouse,
sheep.

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ABSTRACT

Accurate and specific methods for sex determination in farm animals play a vital role in animal resources. Therefore, this study was conducted to differentiate between males and females for unknown sheep samples collected from slaughters based on the amelogenin (*AMELX/ AMELY*) gene by using the polymerase chain reaction (PCR) technique. A total of 200 blood samples of Awassi sheep, were collected from slaughterhouses, with an average age ranging between 1-3 years. Blood samples from the sheep were used to isolate the DNA. Specific PCR primers were used to amplify one fragment (262 bp) from the X- chromosome in ewes, and two fragments (262 and 202 bp) from the X and Y- chromosome in rams, respectively. The specificity of the primers was evaluated by using amelogenin amplicons for samples of known sex. The results revealed a single band for ewes (262 bp) and two bands for the rams (202 and 262 bp) after gel electrophoresis. In conclusion, this assay is proved to be a precise, inexpensive, and favorable method in sex determination, especially when unknown samples are collected from the slaughterhouse, and the samples are mixed so that it is difficult for the authors to determine the sex of the samples. This method can be employed in other vertebrates and sexing of offspring in animal breeding because the amelogenin gene is conserved among mammals.

Introduction

Sheep (*Ovis aries*) are important types of farm animals in their production and reproductive performance (Al-Thuwaini, 2021; Ajafar et al., 2022). Sex differences in the performance characteristics of sheep are reported in growth traits (Caro Petrović et al., 2015), reproduction performance (Adjibode et al., 2017), and meat quality traits (de Araújo et al., 2017). Females are homogametic, and possess two copies of sex X chromosomes, while males are heterogametic, and have a copy from each sex chromosome X and Y (Pajares et al., 2007). Due to these reasons, sexing methods for unknown samples tend to attract the most attention. Determining the sex of unknown samples is very useful especially when the samples are gathered from slaughters and to prevent economic fraud by misrepresenting cheaper meat as more expensive. The collection of samples from the slaughterhouse is often accompanied by mistakes in determining the sex for most authors due to the necessity to collect samples quickly without contamination. Various methods of determining animal sex have been developed mainly based on the detection of hormone-dependent methods that includes the determination of immunohistochemistry or DNA methods (Gokulakrishnan et al., 2012). While the molecular approaches for sex determination include semi-quantitative PCR, *in situ* hybridization, loop-mediated isothermal amplification (LAMP), and southern blotting (Singh et al., 2017). More recently, real-time PCR technology using SYBR Green and Taq Man has been utilized in sex determination in bovines (Khamlor et al., 2014). However, most of these methods have proven to be of high specificity and sensitivity in detection with limited application due to their high cost and large equipment requirements (Sachan et al., 2020).

The PCR-based techniques that are used for sex discrimination have received widespread attention because they are more accurate, fast, and inexpensive.

Cite This Article
Al-Awadi, J.H., Imran, F.S. and Al-Thuwaini, T.M. 2022. Sex Determination of Unknown Ovine Samples Gathered from Slaughters. *Journal of Bangladesh Agricultural University*, 20(4): 402-406. https://doi.org/10.5455/JBAU.109580

Previously, the sex is determined in sheep embryos based on specific restriction enzymes by using RFLP-PCR (Saravanan et al., 2003). However, this method is characterized by a lack of accurate diagnosis due to the limited amount of DNA in the embryos, the contamination that is possible to occur in the sample, and the long time required for analysis (Chen et al., 2007). Besides, error in the experiment can lead to false negative results so the lack of a specific band does not necessarily mean that the sample is female. Subsequently, PCR amplification methods are only intended to detect specialized Y-chromosome sequences such as the sex determining region Y (*SRY*) and testis specific protein Y-linked 1 (*TSPY*) gene or repeating sequences (Gokulakrishnan et al., 2012; Mohd Hafizal et al., 2016). Therefore, it is necessary to improve a reliable and inexpensive method for sexing in which there is positive control of a known male animal available in every experiment (Statham et al., 2007).

Several researchers have targeted the amelogenin gene to identify sex in most vertebrates because of the conserved status among mammal species (Pfeiffer and Brenig, 2005; Grzybowski et al., 2006; Kashyap et al., 2006). The Amelogenin gene is positioned on the X chromosomes (*AMELX*) and Y-chromosomes (*AMELY*) consist of seven exons and encodes a protein of dental enamel (Grzybowski et al., 2006). The difference sequence between two sex chromosomes of the amelogenin gene enables it to be used in the sexing of unknown specimens (Grzybowski et al., 2006; Kashyap et al., 2006). In mammals, the difference in the length of the PCR amplicons of the amelogenin gene between two sex chromosomes has been utilized in sex determination in humans and cows (Salabi et al., 2014; Liu et al., 2015). Other studies have been performed using the amelogenin gene in livestock such as sheep and deer (Pfeiffer and Brenig, 2005), sheep and goats (Chang et al., 2006; Weikard et al., 2006). However, it is critical to note that these efforts are limited to embryos and blood samples gathered from live animals and do not include samples taken from slaughtered animals. Therefore, this study aims to evaluate the use of the amelogenin gene in sexing of unknown sheep samples that are collected from the slaughterhouse, to be the first study. Additionally, we used the primers established in sheep and red deer to evaluate their suitability for other sheep breeds, such as Awassi sheep.

Materials and Methods

Animals and blood collection

This study was approved by Al-Qassim Green University (Approval No. 12.10.15) in the Animal Production Department / College of Agriculture for the period from November 2018 to April 2019, according to Institutional Animal Care and Use Committee. A total of 200 blood specimens of Awassi sheep, were collected from slaughterhouses in the governorates of Karbala and Babylon, with an average age ranging between 1-3 years. Blood specimens were gathered via puncturing of the external jugular vein with 18-gauge sterile disposable needle and blood taking using vacutainer tubes with EDTA.

Genomic DNA extraction and PCR technique

The manual method was performed to isolate genomic DNA from the blood (Al-Shuhaib, 2017). Briefly, blood was mixed with distilled water to destroy red blood cells. Resuspend the pelleted leukocytes in WBC lysis buffer (Tris-Cl, NaCl, EDTA, SDS). The NaCl was used to denature the protein and then the absolute ethanol was used to precipitate the DNA. These precipitation DNA strands were washed with 70% ethanol and recovery via elution buffer (Tris-HCl, EDTA). The isolated DNA samples were evaluated uses a NanodropμLITE spectrophotometer (Biodrop, UK) and used as a template for polymerase chain reaction (PCR). One pair of specific primers were used to amplify the amelogenin gene based on the based on the sheep sequences previously developed by Saberivand and Ahsan (2016) with minor modifications. The sequences of primer used in this study were as follows: F:5'- CAGCCAAACC TCCCTCTGC -3', R:5'- CCCGCTTGGTCTTGTCTGTTGC -3'. The PCR reaction was conducted using PCR PreMix of Bioneer Company. The PCR amplification was initiated by denaturation at 94°C for 5 min, followed by 30 cycles of denaturation (94°C), gradient annealing (61.8°C), extension (72°C), for 30 s each, and a final extension step (72°C for 5 min). The amplicons were visualized by electrophoresis in 1.5 % agarose gel by staining with ethidium bromide. The photograph was performed by using the gel documentation unit (Chemidoc, Biorad, USA) (Al-Thuwaini, 2020).

Results

The primers used to amplify the amelogenin gene were based on sheep sequences, but the first time used in Awassi sheep. Therefore, a gradient PCR (53 - 64ºC) applied to the ovine amelogenin gene to identify the optimum primer annealing temperature. The annealing temperature with a clear band and highest intensity products ideally ranged between 59ºC to 61.8ºC, and for greater stringency a higher temperature at 61.8ºC was chosen. The results of the stepwise PCR in picking up the proper annealing temperature represent in (Figure 1). One pair of primers of the ovine amelogenin gene was used to study sex determination. PCR experiments were conducted for all 200 samples (100 known and 100 unknown samples). The results revealed a single band for ewes (262 bp) and two bands for the

rams (202 and 262 bp) after gel electrophoresis (Figure 2).

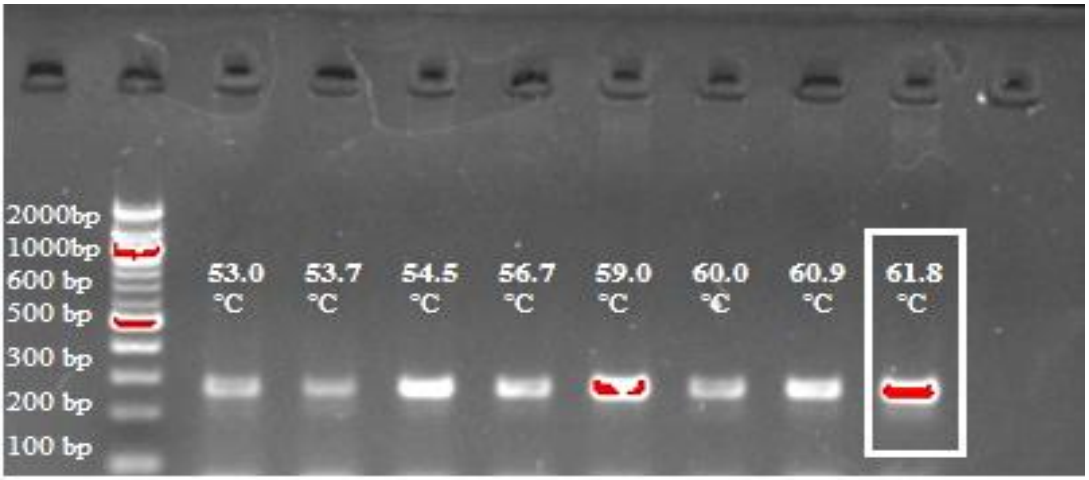

Figure 1. Gradient PCR (53 - 64°C) was applied to the ovine amelogenin gene to identify the optimum primer annealing temperature. M; Refers to DNA 100-bp ladder (Bioneer – Cat. No.D-1030). The square-bound refers to the optimum annealing temperature of the PCR fragments. Electrophoresis conditions: 1.5% agarose concentration, power applied: 100V (7V / cm), time to run: 30 min. Staining method; precast ethidium bromide

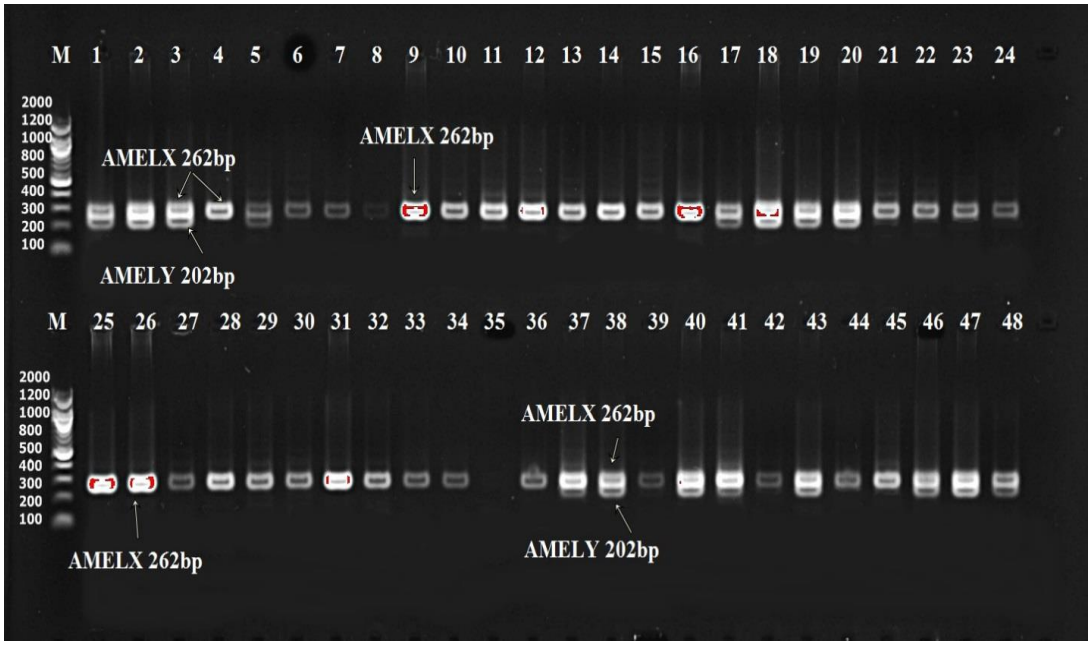

Figure 2. Electrophoresis assessment of the sex determination of sheep samples using the amelogenin gene. The symbol M refers to the ladder marker. Lanes (1-24 unknown and 25-48 known samples). Lanes 1-3,17-20,37-38,40-41,43,46-48 refer to rams DNA samples with two bands 202 and 262 bp. Lanes 4,6-16,21-24,25-36,39,42,44-45 refer to ewes DNA samples with one band 262 bp. Electrophoresis conditions: 1.5% agarose concentration, power applied: 100V (7V / cm), time to run: 30 min

Discussion

Growth and reproductive performance in animal production require knowledge of sex identification (Marufa et al., 2017; Mahala et al., 2019; Al-Thuwaini et al., 2020). Many of the existing approaches for sexing are performed based on PCR methods (Statham et al., 2007; Sachan et al., 2020). Sex determination based on PCR methods using the amelogenin locus has become a rapid and accurate method (Chen et al., 2007). The differences in length of PCR amplicons of the amelogenin gene have been performed to identify the sex in several mammals such as humans (Gibbon et al., 2009), cattle (Weikard et al., 2006), horses (Hasegawa et al., 2000), and sheep or goats (Pfeiffer and Brenig, 2005). Amelogenin genes (*AMELX* and *AMELY*) of sheep are present on both the X- and Y-chromosomes with a

significant sequence variation. This result is comparable to those reported in goats and sheep by other authors (Pfeiffer and Brenig, 2005; Chen et al., 2007; Saberivand and Ahsan, 2016). Furthermore, this method does not need another primer present on the somatic chromosomes as positive control and does not need restriction enzymes (Pfeiffer and Brenig, 2005; Chen et al., 2007). Several authors employed similar primer sequences (Pfeiffer and Brenig, 2005; Chen et al., 2007; Khaledi et al., 2009; Farahvash et al., 2016; Saberivand and Ahsan, 2016). However, these methods are performed for determining sex in the embryo and blood samples from live animals without focusing on samples collected from slaughter. Often, it is not easy to collect samples from slaughterhouses, and errors could have occurred during sex determination, especially when large samples are collected or as samples are transported to the laboratory, which may result in the author forgetting the label or fading it, and the animal never being alive to reconfirm the accuracy of the data collected.

This method has shown its efficiency in all samples used with higher accuracy. An evaluation of the method for known sexing confirms the accuracy of sexing. Differences in sequences of the amelogenin gene in mammals may be due to the deletion of 63 base pairs in exon 5 leading to the formation of two different fragments in the male and one fragment in females (Mohd Hafizal et al., 2016). In cattle, the deletion of 63 bp in exon 6 could be responsible for the sequence differences between the coding regions of the two chromosomes X and Y (Grzybowski et al., 2006). Moreover, the ins/del of 55 bp within the coding regions of the Y- chromosome of sheep and red deer causes sequence differences that could be dependent on sex determination (Pfeiffer and Brenig, 2005). The current study has unraveled the accuracy of the amelogenin gene in sex identification and refers to the possibility of using this method for other sheep breeds.

Conclusion

This assay is proved to be a precise, inexpensive, and favorable method in sex determination, especially when unknown samples are collected from the slaughterhouse, and the samples are mixed so that it is difficult for the authors to determine the sex of the samples. An accurate determination of the sex is crucial, especially for studies of known carcass traits but unknown which blood samples belong and in food authenticity.

Conflict of Interest

The author declares that no conflict of interests exists.

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