Research article

PCR-based sex determination in Cattle using gene specific markers

Aftab Ahmad [1], Murad Ali Rahat [1], Adnan Wahab [1], Subhanuddin [3], Muzafar Shah [1], Akhtar Rasool [1,4], Muhammad Israr [5*]

[1]Centre for Animal Sciences and Fisheries, University of Swat.

[2] Department of Biotechnology and Genetic Engineering, Hazara University Mansehra.

[3]Department of Zoology, Islamia College Peshawar.

[4] Centre for Biotechnology and Microbiology, University of Swat.

[5] Department of Forensic Sciences, University of Swat.

Corresponding author: israr@uswat.edu.pk

Abstract: Forensics regarding animals must be considered important for the tracing of mixed cattle specimens to its gender. In our current experiment, amelogenin (AMELX/Y) gene is used for the investigation of cattle gender through polymerase chain reaction (PCR) of DNA isolated from blood remnants. The samples were subjected from a local slaughterhouse. A pair of primers was employed for the amplification of amelogenin gene. Whom homologs are found over both sex-linked chromosomes. The primer efficiency was checked on DNA samples from known gender prior to applying to unknown DNA samples. In terms of PCR band patterns, the X-linked gene partially produced 241 bp (base pair) fragment and Y-linked gene produced 178 bp fragment. In the results of blind testing, all male samples were observed with two bands (241 bp and 178 bp) and female samples were observed with only one band (241 bp). These findings marked that employing AMELX/Y gene in PCR-based testing is a reliable technique for gender determination in cattle.

Keywords: Blood remnants, DNA extraction, PCR, *Amelogenin*, Gender identification.

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1. Introduction

Sex determination of cattle meat is always prioritized by the public for utilization [1]. This is because of the economic policies, which designate female cattle beef of low price and male cattle beef of high price [2]. It is important to label the meat according to its original gender at butcher shop and also the meat products in the market. And in case of illegal marketing the analyst should require to trace back its remnants in the concerned slaughter house. The analysts should provide an easy and authentic approach for the authentic determination of cattle sex from mixed samples for forensic investigation. Different approaches based over polymerase chain reaction are established. Some of these are reported over Zinc finger gene [3], SRY gene [4], BRY-1 gene [5], BOV97M [6], and the amelogenin gene [7, 8]. Some studies also reported innovative tests for gender identification like TaqMan [9] and CYBR Green [10]. However, the PCR (polymerase chain reaction) based technique

and employing the *amelogenin* gene is a reliable and accurate test for gender identification and that's why have attracted practitioner's attention for the sex determination.

The gene, *amelogenin* is found in almost all mammalian species which is responsible for the coding of enameline proteins. These proteins appear as the outer white covering of teeth and act as protectors [11]. It is a member of protein family termed as "extra cellular matrix proteins" [12]. This gene is located over both Y and X chromosomes in the form of homologs [13]. Unlike others, this technique is dependent on a simple PCR test comprising only one primer's set targeting the rare mutation in the gene [14]. Different size bands for AMELX and AMELY represent female and male individual samples respectively [15, 16]. In our experiment, the work on *amelogenin* gene has been expand to the identification of cattle gender identification from mixed blood remnant samples collected from a local slaughterhouse.

2. Materials and Methods

Specimen's collection

A total of eighteen specimens were collected in the current experiment. Two samples of known genders (1 male and 1 female) were taken as referenced blood samples and collected intravenously from ear marginal vein with the help of a veterinary practitioner. The remaining specimens were randomly subjected from a slaughterhouse. All specimens were stored under 4°C temperature in 3ml EDTA (Ethylinediaminetetraacetate) tubes until further analysis.

Isolation of DNA

DNA of 200 µl blood from all the collected specimens were extracted through *WizPrep™ gDNA Mini Kit (blood)*) Cat# W71050-100 (Wizbiosolutions, South Korea). Manufacturer instructions were considered as mentioned for good quality DNA extraction. A negative reaction was also considered in the process. The DNA was stored at -20°C in Tris-EDTA after resolving the bands under UV-light in the trans-illuminator (OmniDoc software Version 1.1.3.9). The electrophoresis of 0.8 % agarose gel was carried out via TBE buffer in gel tank (JY-SPDT, JUNYI- China) connected with a power supply (JY300, JUNYI-China) supplying voltage 100V, time 35min and current was adjusted automatically by the machine.

Genetic Marker Amplification

A forward and reverse set of cattle amelogenin primers were employed in our experiment as shown in table 1.1. Purpose of the single pair of primer was to successfully generate or amplify the gene present over both sex chromosomes.

Table 1.1: Showing primer's sequences for cattle amelogenin gene.

Program	Step #	Step name	Procedure	
Basic PCR	Step 1	Initial denaturation	04 min. @ 94 °C	
	Step 2c	Denaturation	30 sec. @ 94 °C	35 Cycles
	Step 3c	Annealing	30 sec. @ 59 °C	
	Step 4c	Extension	30 sec. @ 72 °C	
	Step 5	Final extension	10 min. @ 72 °C	
	Step 6	Final hold	Infinity @ 4 °C	

The polymerase chain reaction for gender identification was carried out in a PCR machine (PERKIN ELMER, USA). The reaction was set in a volume of 10 µl reaction mixtures containing 10 picoMol/µl each primer, 5.0 µl 2x Taq Master Mix (Taq DNA Polymerase, PCR Buffer with 4 mM $MgCl_2$ and dNTPs 400 µM each), 3.5 µl PCR water and 1.0 µl eluted DNA. The PCR program was initiated in six steps as shown in table 1.2.

Table 1.2: Showing thermal cycler Paremeters for the amelogenin gene.

Gene	Primer's sequences	Reference
Cattle *Amelog-enine*	forward 5′- GGCCAACACTCCATGACTCCA-3′ reverse 5′-TGGGGAATATYGGAGGCAGAG -3′	[1]

The resultant PCR products or bands were resolved on 2.5 % agarose gel through electrophoresis and visualized in the trans-illuminator machine.

3. Results

The DNA was isolated successfully from both, the reference samples and the random samples. Following the instructions of the manufacturer, good quality of DNA bands was visualized in the trans-illuminator system and documented as shown in figure 1.1. After performing polymerase chain reaction, two different size bands were observed. Upon electrophoresing of a 100bp (GENEONE Cat# 300009) DNA ladder simultaneously with PCR products, these bands were successfully identified as 178 bp and 241 bp. Male reference specimen's DNA generated 178 bp and 241 bp bands while female reference specimen's DNA generated only 241 bp band. The rest of specimen's DNA that showed two bands were classified as male cattle and that showed single band were classified as

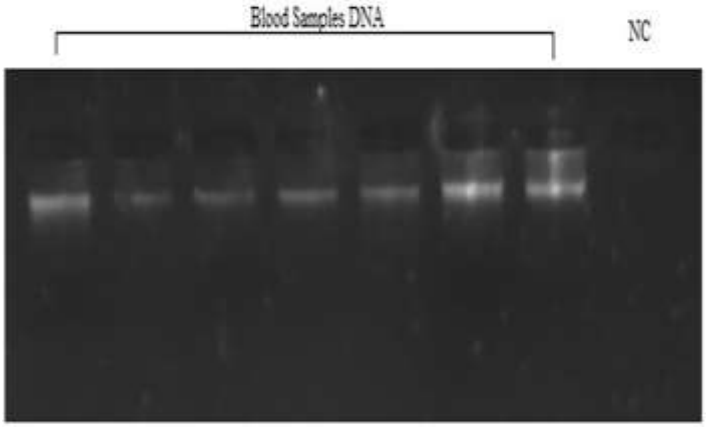

Figure 1.1: Genomic DNA on 0.8% agarose gel, extracted from cattle blood, whereas NC stands for negative control.

female cattle as shown in figure 1.2. Of the total 18 samples (excluding reference samples), five samples were identified as male and eleven samples were identified as female samples. These bands were observed on a 2.5 % agarose gel following 1X TBE buffer and 5V/cm/hr.

formula for agarose gel (8cm) electrophoresis. The gel was visualized in gel documentation system and file was documented (OmniDoc Software_Version 1.1.3.9).

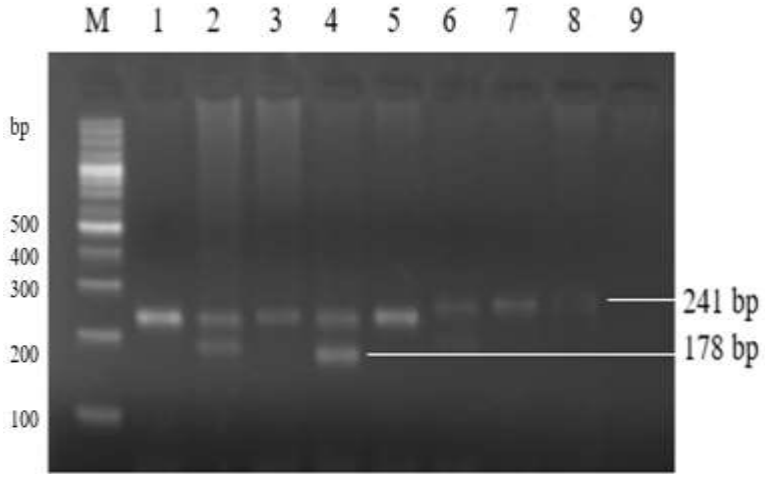

Figure 1.2: PCR amplicons on 2% agarose gel, M stands for 100bp DNA marker, well 1 and 2 represent female and male reference samples, well 3-8 represent random sampling and well 9 represent

4. Discussion

The *amelogenin* gene is present on both sex-linked X/Y chromosomes. Whom PCR amplicons are different for male and female cattle in terms of band pattern. Because of the presence or absence of 63 bp sequence over X-linked or Y-linked chromosomes respectively. Due to these insertions/deletions AMELX homologs in female shows single bands and AMELX/Y homologs in male shows two bands [17]. This difference in the amelogenin gene homologs have been targeted using single primer set in PCR. Of which Y-linked homolog always generate smaller size fragment than the X-linked homolog. Because of this differentiation in the *AMELX/Y* gene, it is used to identify the gender of almost all mammals

The cattle *amelogenin* gene primers are employed from the experiment of [1] to generate amplicons of both homologs in single PCR test. Through electrophoresis of the PCR products both male and female individuals were observed with different band size on 2.5 % agarose gel. The accurateness of the primers was checked by isolating genomic DNA of a male, female cattle reference specimens and sixteen random samples. The test subjects of this experiment were observed with178 bp fragment for Y-linked chromosome and 241 bp band for X-linked chromosome that determine the female and male cattle specimens accurately. These findings showed similar results regardless of the source of DNA with that reported in the experiment of [1]. As they have used or designed primers in the same region

of the selected gene. Some experiments have showed results different than ours regarding the amelogenin gene, because of primers used in their studies are different. They amplified 280 bp for AMEL-X homolog and 217 bp for AMEL-Y homolog [15]. Another study in the same line of work generated 417 bp for AMEL-X homolog and 340 bp for AMEL-Y homolog in cattle [16]. After all these results have something in common and that is the difference between homologs of the same gene at both sex-linked chromosomes. The different base pair sizes are because of the selection of the position for primer's designing in the desired gene. Studies have been carried out in other mammals for gender identification such as human [18] cattle [17], horse [19], sheep and goat [7].

Conclusions

In our experiment, it is concluded that employing the *amelogenin* gene specific primers for PCR based sex determination of cattle is a reliable approach. Due to the 63 bp difference between the X and Y homologs of the same gene, determine the male and female individuals prominently and showing no ambiguity. No additional markers unlike the SRY gene and post PCR tests such as RFLP (restriction fragment length polymorphism) are required for the study validation.

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