Development of new primers for determination sex in bovine via Nested-PCR

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Introduction

The majority of protocols currently available for bovine embryo sexing involve amplification of repeated specific sequences of the Y chromosome using the PCR technique (Shea, 1999; Lu et al., 2007). However, the utilization of these sequences shows some disadvantages. One of them is the absence of an inner control for reactions, which causes a great difficulty in separating a female diagnosis, characterized by absence of amplification, from a miscarriage on reaction, which is either one characterized by absence of amplification. Thus, the error ratio of embryos diagnosed as female tends to be high (Garcia, 2001). An alternative that is been used to overcome this problem is the co-amplification of a specific Y chromosome sequence together with an autossomic sequence (Park et al., 2001). However, the utilization of an additional pair of primers could decrease the efficiency of reactions (Kageyama et al., 2004). The bovine amelogenin gene (bAMEL) has two alleles, one present on the X chromosome (AMELX) and the other present on the Y chromosome (AMELY), in which the allele presents a series of deletions in relation to the other allele present in the X chromosome (Chen et al., 1999). This difference in size between both alleles makes the amelogenin gene a genetic marker with a great potential for use in sexing procedures. Furthermore, the amelogenin gene also works as an inner control for reactions without using an additional pair of primers (Weikard et al., 2006; Fontanesi et al., 2008; Sembon et al., 2008). Since the amelogenin gene have only one copy in each sexual chromosome, the efficiency of reactions could be doubtful, especially in the case of embryos in which the DNA content is limited. One way to overcome this problem is the use of a technique called Nested-PCR (Almodin et al., 2005). The principal objective of this technique is to improve the sensitivity and specificity of reactions. This technique consists of two step-reactions, where the second step uses one pair of inner primers of the sequence amplified in the first reaction, which inhibits the non-specific sequences generated in the first step to be amplified again (Bredbacka, 2001). The present study aimed to develop a combination of reliable primers to determine bovine sex. For this reason, specific primers were projected for the amplification of the intron 5 of the bovine amelogenin gene (bAMEL) using the Nested-PCR technique.

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Material and methods

DNA extraction

Blood samples were obtained from 20 cross-bred cattle (10 males and 10 females). A commercial Kit was used for DNA extraction (Biotools - B & M Labs, S. A. - Spain).

Primers and amplifications

A total of seven primers (F1, F2, F3, R1, R2, R3 e R4) were developed for the intron 5 of the bovine amelogenin amplification. All primers were projected for the amplification of a fragment corresponding to the AMELX allele, and one fragment corresponding to the AMELY allele. The PCR program consisted of an initial step of denaturation of 5 minutes at 94ºC, followed by 40 cycles of denaturation at 94ºC for 60 seconds, annealing of primers for 60 seconds, and a new streamer synthesis at 72ºC for 90 seconds. After the last cycle, the reactions had an additional step of 7 minutes at 72ºC for the final stretch of streamers. The reaction mixture contained ultra pure water, PCR buffer [10 mM Tris-HCl (pH 8.3) and 50 mM KCl], 2mM $MgCl_2$, 200 µM of each dNTPs, 0.5 U *Taq* DNA polymerase, a pair of outer primers (20p/mol) and 0.5 µl DNA solution, to a final volume of 25 µl . Samples of 0.2 µl PCR products were used as a model for the Nested-PCR reactions. In this step, a pair of inner primers from the sequence amplified in the first reaction was used. The products of reactions were electrophoresed in 2.0% agarose gel containing ethidium bromide (0.5 µg/ml).

Results and discussion

Initially the assays were performed with DNA samples from two animals (one of each sex), so all primers projected were used in all possible combinations. Eight primers sets (F1R2/F2R4, F1R2/F3R4, F1R2/F3R3, F1R3/F2R4, F1R3/F3R4, F2R2/F3R3, F2R2/F3R4 e F2R3/F3R4) were specific for the amelogenin alleles amplification. However, only three sets (F2R2/F3R3, F2R2/F3R4 and F2R3/F3R4) showed consistent amplifications, so they were selected for the subsequent assays. To evaluate the efficiency and precision of these three primers sets, new reactions with DNA samples obtained from 10 animals (five of each sex) were made. In these reactions only the primers set F2R3/F3R4 maintained 100% efficiency, with very consistent streamers. With the objective to prove the reliability of primers set F2R3/F3R4 for sexing, a new assay with DNA samples from 20 animals (10 of each sex) was made with duplicate reactions and 100% efficiency was observed in the reactions. Females were identified by bands of 398 bp (AMELX), and males by bands of 398 bp (AMELX) and 281 bp (AMELY) (Figures 1 e 2). In all cases, the sex determined by Nested-PCR corresponded to phenotypic animal sex (100% precision). No amplification was detected in the negative control, which testified for the absence of contamination in the assays. The development of primers is a crucial step for the establishment of a protocol for sex determination. The studies on animals sexing have as a principal objective the improvement of reaction protocols for posterior application to embryos. For this reason, several studies have been carried out with many domesticated animals such as cattle

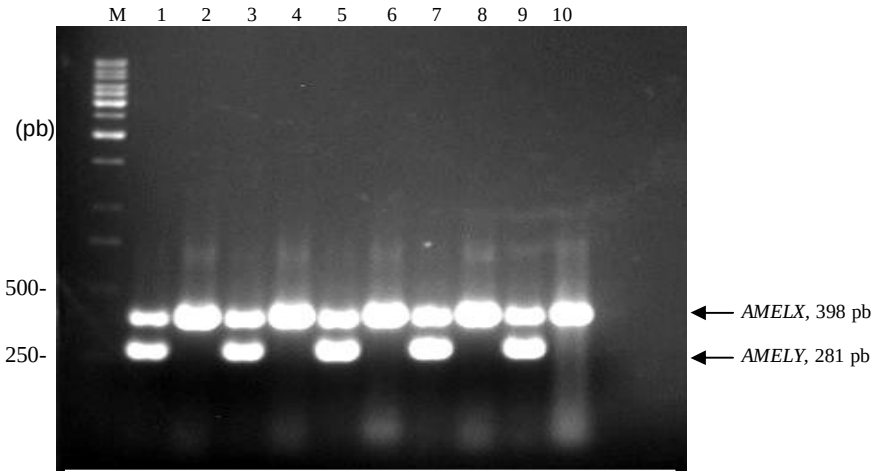

Figure 1: Agarose gel electrophoresis of the bovine amelogenin gene fragments amplified by Nested-PCR (1º assay). M: molecular weight marker (1Kb). Lanes 1-10: DNA samples from 10 animals. The length of each fragment in base pairs is given.

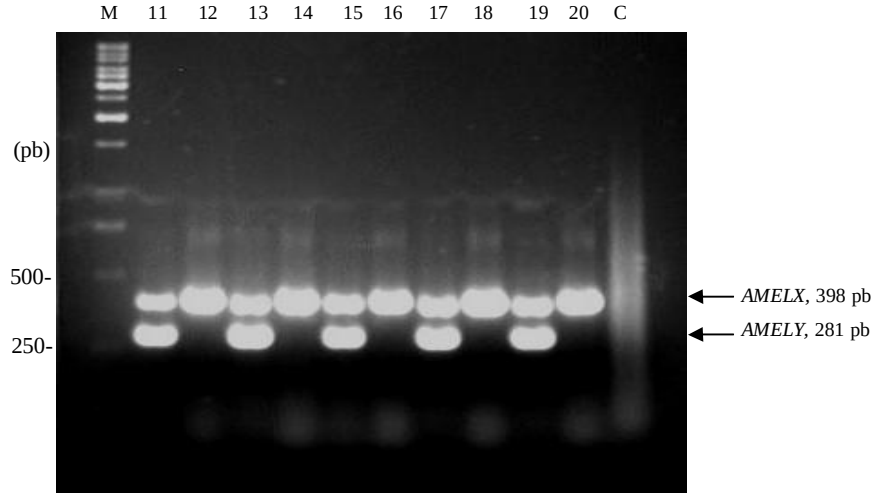

Figure 2: Agarose gel electrophoresis of the bovine amelogenin gene fragments amplified by Nested-PCR (1º assay). M: molecular weight marker (1Kb). C: negative control. Lanes 11-20: DNA samples from 10 animals. The length of each fragment in base pairs is given.

(Horng et al., 2004), pigs (Sathasivam et al., 1995), buffalos (Horng et al., 2004), goats (Phua et al., 2003) and sheep (Pfeiffer e Brenig, 2005). Even with a greater availability of DNA for assay, the utilization of Nested-PCR technique was very justified, since the reactions were being improved for embryo sexing.

Conclusion

The results obtained prove the specificity and precision of the protocols utilized, and the next step will be to test the efficiency and sensitivity in assay with embryo biopsy.

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