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Single Cell PCR Using Y Chromosome Specific Repeat for Sexing

Elham Arbabi Aval[1], Frouzandeh Mahjoubi[1*] and Raheleh Torabi[1]

[1]Department of Medical Biotechnology, National Institute for Genetic Engineering and Biotechnology, Tehran, 14965/161, Iran.

Authors' contributions

This work was carried out in collaboration between all authors. Authors EAA and FM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors EAA and FM managed the analyses of the study. Author RT managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Embryo sexing is one of the important ways for sex selection of offspring. This is a potential method to considerably improve animal breeding and the efficiency of dairy and meat production. A novel repeated sequence specific to male cattle has been identified and named S4. S4 is a 1.5 Kb repeating unit contains various internal repeated sequence. S4 is localized on long arm of the Y chromosome in the region near to ZFY genes. The objective of this study was to establish a simple, sensitive, reliable, reproducible and cost effective PCR based technique for sexing.
Study Design: Case control study.
Place and Duration of Study: Sample: whole blood samples of male and female cattle. Medical Biotechnology Dept. National Institute for Genetic Engineering and Biotechnology, Tehran, 14965/161, Iran, between June 2010 and July 2011.
Methodology: Genomic DNA was extracted from the whole blood samples of male and female cattle. PCR and single cell PCR were performed using specific primers for this region.
Results: By this PCR based methods we could differentiate between female and male genomic DNA.
Conclusion: With this technique we can distinct male from female using as little as 0.1pg DNA.

**Corresponding author: E-mail: frouz@nigeb.ac.ir;*

Using this method we could determine the sex of embryo (4 blastomers). This method may optimize for the quantitative detection of Y chromosomes in semen.

Keywords: PCR; Y chromosome; sex determination; cattle.

1. INTRODUCTION

In bovine, as in other mammals, males have only one X and only one Y chromosome (XY) and females have two X chromosome (XX). In mammals sex determination depends on the Y chromosome [1,2].

Simple methods such as PCR are mainly used for sex determination and thus a novel repeated sequence specific to male cattle has a key role in management and a big potential for genetic improvement of bovine strains.

Many studies had represented various Y chromosome markers including expression of FBNY [3] and the amelogenin (AMEL) have been used for PCR sex typing test in humans, Bovines and Deers, because of the difference in length of PCR products from the X and Y chromosomes [4], DDX3Y, USP9Y, ZRSR2Y, EIF1AY, HSFY, SRY, TSPY and ZFY genes were detected in all male embryos examined and appeared to represent good candidates for RNA-based sexing [5], at least five dinucleotide (CA)n microsatellite repeat arrays Y-specific microsatellite polymorphisms in a range of bovid species [6] or repeated sequences [7-9].

In particular, PCR using highly repeated sequences on Y-chromosome made it possible to amplify the target sequence from a small number of blastomeres [10-12]; Kageyama et al. [14] In cattle, Herr et al. [13] reported the accurate sex prediction of calves by embryo sexing based on PCR. In comparison with earlier methods, PCR offered also the invaluable advantage of being so fast.

Y specific repetitive sequence called S4 with high copy number has been reported by [14] (Gen Bank accession number D16357). FISH analysis showed that S4 is localized on the whole long arm and the proximal region of the short arm of the Y chromosome [14]. The S4 sequence has been used for sex determination [15,16]. The aim of this study was to launch a sensitive and inexpensive technique with high precision for sex determination when only limited and minimal amount of DNA is available. The technique could have the potential for sex determination in multicellular embryos and single cell [15,16].

2. MATERIALS AND METHODS

2.1 DNA Isolation

Blood samples of female and male bovines were collected in the Dairy Husbandry Department of Zanjan University. Three embryo samples were provided by National Institute for Genetic Engineering and Biotechnology.

DNA from blood samples of female (n=4) and male (n=8) were extracted by Kit (Qiaamp DNA mini kit, Qiagen, USA) and salting out method. Ovaries of slaughtered bovines were collected and maintained in phosphate-buffered saline. The follicles were collected and incubated with oestrous serume bovine (20%) FSH (10µg/ml) calcium lactate (2.92mmol/l) sodium pyruvate (2mmol/l) sodium bicarbonate (33.9mmol/l) hepes (4.43mmol/l) and gentamycin (60mg/ml). Mutration was carried out at 39ºC in atemosphere (Ball et al.) [17]. The fertilization medium was with BSA (6mg/ml) adrenalin (1µmol/l) hypotaurine (10µmol/l) and heparin (10µg/ml) [18].

2.2 Quantitative PCR

Two set of primers were designed (S4c and S4B). S4c is male specific and S4B has sequence for both sexes (Table 1) for the common male and female sequence. PCR reactions for both segments were prepared in a 50µl total volume contain 0.5µmol/l of each primer, 0.2µmol/l of each dNTPs, 0.625U Taq polymerase (Sinagen, Iran), 5mM MgCl2 and 1x PCR buffer (Sinagen, Iran), 200ng of genomic DNA, separately. PCR program was started by the initial denaturation step at bromide. The sensitivity of the PCR was investigated using different concentrations of male DNA (1pg to 100ng) with S4C primer sets (male specific) (Fig 1 and 2).

Table 1. Primers sequences

	Sequence	Tm	Amplicon
S4C	F5′CCATGATAGTTCAGAGGTTAGGAC3′	67.8	524bp
	R 5′GTCCATGGGGTCGCAAAGAGTCGG3′	61	
S4B	F 5 ′CAAGTGCTGCAGAGGATGTGGAC 3′	63.9	178bp
	R5′GAGTGAGATTTCTGGATCATCTGGCTACT3′	64.7	145bp

2.3 Single Cell PCR

In this experiment 3 embryos producted from IVF cycle were analyzed with both primers for embryo sexing (S4c and S4B). One was transferred to the PCR master mix and PCR was run for 45 cycles (Fig. 4).

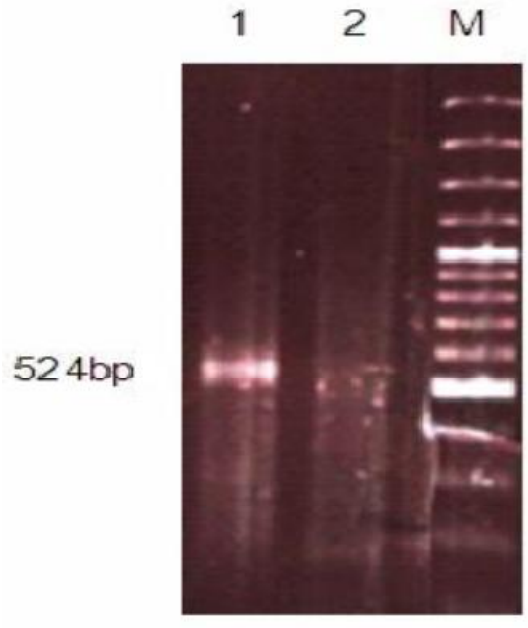

Fig. 1. Sex determination by S4C primer. Lane 1: male DNA, Lane. 2: female DNA, M: size marker (100bp)

3. RESULTS AND DISCUSSION

In this research S4 repetitive sequence were used for differentiation between male and female sex. The serial dilution of male DNA was prepared and PCR was employed for the amplification. As little as 0.1pg male DNA could give male specific band on the agarose gel (Fig. 2). However, when this sensitive PCR assay was used for sex determination in a single cell and multi cell embryo (four cell blastomere) could not distinguish between male and female sexes. Therefore another set of primer S4B was employed (Fig. 3).

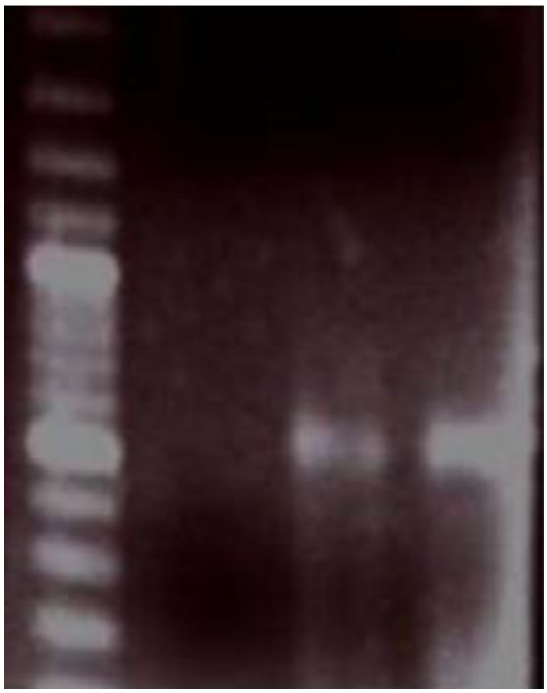

Fig. 2. Sensitivity of PCR by S4C primer. M: size marker (100bp), Lane 1: negative control, Lane 2: 0.1 pg DNA, Lane 3: 0.5pg DNA

The S4B primer sets gave 145bp segments in addition to 178bp segment in the male DNA and gave 145bp segments only in the female DNA (Fig. 3)

This S4B primer sets can be used for positive control in sex determination. When S4B primer was used for a single cell PCR a 145bp band indicating female sex and both 145bp sequence and 178bp band indicating male sex could be detected. So cultured oocyte was used for positive control in test. This sample showed only 145bp segment that matches to expected results of this test (Fig. 4).

Optimizing PCR condition for repetitive sequences has both advantages and disadvantages. It has advantage since it could work with very little amount of DNA. But it has disadvantage of giving sometimes non specific

and often shorter PCR products. Using S4C sequence we could amplified as little as 0.1pg DNA (Fig. 2). Weikard et al. [3] reported primer sets of FBNY gene that amplifies male specific sequence, they required at least 10pg DNA for precise embryo sexing. Lemos et al. [19,20] reported that 1pg DNA was required for TSPY sequence. Kageyama et al. [14] used S4 male specific sequence for embryo sexing using 0.5pg DNA, Therefore, the sensitivity of our technique is about 5 times more than their technique, hence, it will reduce the amount of required DNA (Fig. 2). This highly sensitive was useful for sex determination in single cell and multicellular samples. In previous studies, [19,20] they do determining the sex of beef in eight-cell blastomer by TSPY gene with 96.3% accuracy but we do this by novel S4 repetitive sequence in bastomers at the four-cell stage of embryo with 100% accuracy. Park et al. [21] raise the PCR sensitivity by rate 94 of DNA 5pg in each reaction and 95 then determining the sex in eight-cell embryos and Hartshorn et al. [22] do determining the sex in eight-cell blastomer by Oct4 and Xist RNA in mouse embryos.

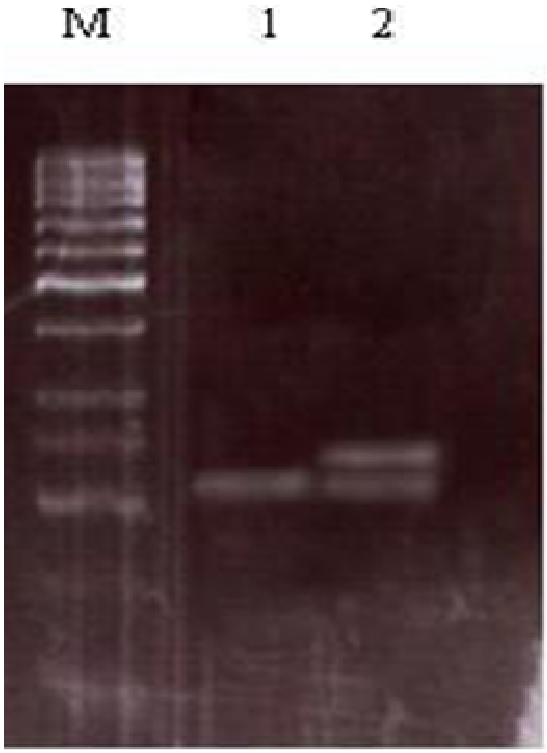

Fig. 3. Sex determination by S4B primer M: size marker (100bp), lane 1: female DNA, Lane 2: male DNA

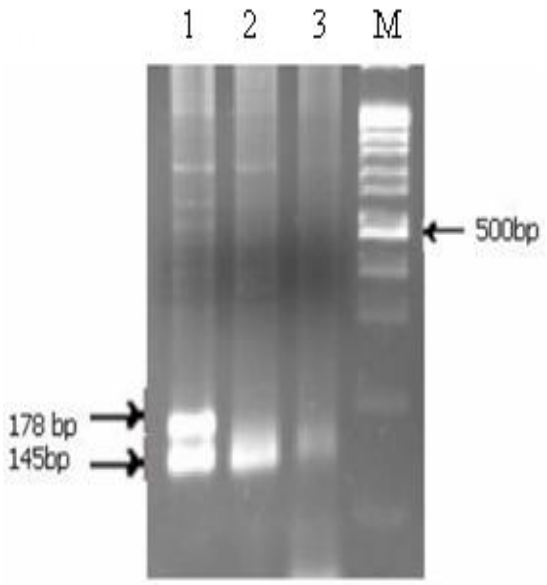

Fig. 4. Embryo sexing by S4B primer. Lane1: male embryo, Lane 2: female embryo, Lane3: cultured oocyte (positive control), M: marker size (100bp)

Single cell PCR is method for using a low amount of starting material that cells lyses into PCR directly without DNA extraction. This strategy offers valuable advantages; first, it requires low genomic DNA.

Second, it is possible to decrease the amount of needed biopsy sample for embryo sexing and thus lower risk to embryo tissues.

Therefore, we believe that our technique is useful for sex determination of blastomere in fewer amounts of cells in IVF implantation. Furthermore accuracy in sex determination not only leads to economic benefit proportional with needs of animal husbandry unit but also controls sex dependent diseases through this technique. Sex ratio predicted by our method was almost 100%. Our novel method is therefore more suitable than previous work in determining the sex of embryos.

4. CONCLUSION

In conclusion, we improved the sex determination using a sensitive, accurate, inexpensive and fast technique by simple PCR method and a pair of primers with novel S4 repetitive sequence. We also improved sensitivity power in sex determination with less amount of DNA.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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