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Preliminary Trials for Sexing of *In Vitro* Produced Buffalo Embryos Using Multiplex PCR

[1]Samah F. Darwish, [2]Karima Gh. M. Mahmoud and [3]T.H. Scholkamy

[1]Biotechnology Research Unit, Animal Reproduction Research Institute, El-Haram, Giza, Egypt
[2]Department of Animal Reproduction and AI, National Research Center, Giza, Egypt
[3]Department of Artificial Insemination and Embryo Transfer,
Animal Reproduction Research Institute, El-Haram, Giza, Egypt

Abstract: The objective of this study was to identify the sex of *in vitro* produced buffalo embryos using an efficient polymerase chain reaction (PCR) assay. Buffalo oocytes collected from slaughtered animals were *in vitro* matured, fertilized and cultured *in vitro* for 7 days. On the day seven embryos were washed twice in PBS and once in 1X PCR buffer then each embryo was placed separately in 20 µl 1X PCR buffer and stored at -20°C for DNA extraction and PCR assay. A pair of satellite primers common to both male and female (Sat I and II) and a pair of male-specific primers (BuRYNI 1and 2) which targeted male-specific sequence in the buffalo DNA were used in PCR assay. The assay was carried out on both whole blood samples, collected from adult male and female buffaloes and 40 IVF-produced buffalo embryos. When DNA samples from blood were amplified, the sex determined by PCR always corresponded to the anatomical sex. The sex of 39 out of 40 embryos was successfully determined using the multiplex PCR assay. In conclusion, multiplex PCR assay could be used as a reliable and efficient tool for sex determination of IVF-produced buffalo embryos.

Key words: Sexing · Embryo · Buffalo · PCR

INTRODUCTION

Sex predetermination of offspring in agriculturally important species has an immense potential in the livestock industry. With the improvement of *in vitro* fertilization (IVF), *in vitro* culture (IVC) and embryo manipulation techniques, sexing of preimplantation embryos can assist the dairy producer in managing his resources more effectively [1]. Gender preselection has a clear application in several fields. Not only is it an important productive tool because it allows the adjustment of offspring gender to market demands, but it also contributes to minimizing gender-linked genetic diseases and might restore a balanced male-female ratio in endangered species.

Different methods were reported for determining the sex of preimplantation embryos [2]. These methods included cytogenetic analysis of blastomeres [3], using antibodies to detect male-specific antigens [4], detecting metabolic differences (x-linked enzyme activity) between male and female embryos [5] and using DNA hybridization by specific Y-linked probes [2]. All these methods were reported to be difficult, impractical and unreliable for daily routine use. With the development of molecular techniques, the need for quick, reliable, easy to perform method for sex determination can be accomplished using PCR. Different types of PCR including nested, multiplex and primer extension preamplification-PCR (PEP-PCR) have been used to determine the sex of preimplantation IVF-produced embryos in human, bovine, ovine and porcine [6-8]. More recently, loop-mediated isothermal amplification was applied in buffaloes [9], in which the amplification of a target sequence can be judged by measurement of turbidity in a reaction solution.

In Egypt, buffaloes represent one of the livestock hope to solve the problem of meat and milk shortage. However, the lower reproductive efficiency of domestic buffaloes is a major impediment to proper use of buffaloes for milk and meat production worldwide. The most efficient techniques to improve the buffalo production are artificial insemination (A.I), *in vitro* fertilization and embryo transfer (ET). These techniques were tried to raise the efficiency of artificial breeding of native buffaloes. Selection of the sex of offspring of such species might have a tremendous impact on buffalo dairy industry.

Corresponding Author: Dr. Samah F. Darwish, Biotechnology Research Unit, Animal Reproduction Research Institute, El-Haram, Giza, Egypt E-mail: samahtarek@hotmail.com

The aim of this preliminary study was to apply the PCR assay, as a fast, efficient and low cost method, to determine the sex of IVF-produced buffalo embryos.

MATERIALS AND METHODS

***In vitro* Embryo Production:** *In vitro* fertilized (IVF) embryos were produced according to the method described by Abdel Dayem *et al.* [10]. Briefly, buffalo ovaries were obtained from a slaughterhouse and transported to the laboratory in saline (0.9 % w/v, Nacl) at 38°C within one to two hours of slaughter. Cumulus-oocyte-complexes (COCs) were aspirated from antral follicles (2 to 6 mm in diameter) using sterile, disposable 18-G needle fixed to 10-ml disposable syringe. The COCs were washed twice in TCM 199 with Earl's salts and 25mM HEPES. The COCs were matured in a maturation medium (10/ 50µl drop) consisting of TCM 199 supplemented with 10 % heat-inactivated fetal calf serum (FCS) and 50 µg/ml gentamycin sulfate. All COCs were cultured in Falcon 4-well dishes covered with mineral oil and incubated at 38.5°C in 5 % Co_2, 95% humidity for 24 hours. Frozen semen was thawed in water bath at 37°C for 30 seconds and washed in BO medium [11] containing 0.02 mg/ml heparin and 3.884 mg/ml sodium caffeine benzoate and centrifuged at 1800 rpm for 10 min., then the supernatant was discarded and the sperm cells were adjusted to 12.5×10^6 cells/ ml [12]. Microdroplet of semen was prepared (100µl) and overlaid with mineral oil, incubated at 38.5 °C, 5% Co_2 and 95% humidity till preparing the oocytes for fertilization. Matured oocytes were washed three times in BO medium containing BSA and were introduced into the microdroplet of the semen under mineral oil, incubated under the same condition for 4 hours [13]. Thereafter, presumptive zygotes were washed in TCM-199 to remove loose unattached sperm. Groups of 10-20 oocytes were again cultured with previously prepared cocultures of 100 µl droplets consisting of TCM-199+10% fetal calf serum for further development. On the third day of development, uncleaved oocytes were discarded and embryos were transferred to fresh medium for further four days. On the day seven embryos were washed twice in PBS and once in 1X PCR buffer then each embryo was placed separately in 20 µl 1X PCR buffer and stored at -20°C for DNA extraction and PCR.

Extraction of Genomic DNA from Blood: Whole blood from five males and five females' adult buffalo were collected in heparinized vaccutainer tubes for optimization of the PCR conditions. Genomic DNA was extracted from the blood by phenol-chloroform method as described by Sambrook *et al.* [14]. It was based on removal of erythrocytes by successive washing in lysis buffer and subsequent lysis of the leucocytes using sodium duodecyle sulphate (SDS) and proteinase K followed by extraction with phenol chloroform. Finally, DNA concentration was adjusted by measuring the OD at 260 nm.

Extraction of DNA from Embryos: Total number of 40 IVF- produced buffalo embryos was used. Embryos were taken out of deep freeze and the volume was adjusted to 50 µl by adding 1X PCR buffer, then 0.13 µg proteinase K was added to each embryo and the reaction was covered with 30 µl mineral oil to avoid evaporation. All embryos were incubated at 50°C for 18 hours and a deactivation step for proteinase K was carried out at 95°C for 10 min. [15].

Primers: The primers used in the PCR reaction are shown in Table (1). Two pairs of primers were designed. The first pair is gender-neutral primers [16] which targeted bovine satellite sequences common to both male and female (Sat I and II). It amplified 216 bp DNA fragment in both male and female buffaloes. The other primer pair is male-specific primers (BuRYNI 1and 2) which targeted male-specific sequence [7] in the buffalo DNA. It amplified 164 bp DNA fragment in males only.

Table 1: Oligonucleotide primers used in the PCR

Repeat sequence	Primer name	Primer sequence (5'-3')	PCR product (bp)
Satellite	Sat I	TGGAAGCAAAGAACCCCGCT	216 bp
	Sat II	TCGTGAGAAACCGCACACTG	
BuRYNI	BuRYNI 1	CGTGGTGGGTGACCCCACAGCCCC	164 bp
	BuRYNI 2	ACAGGTGCTTATGCTGCAGTGCTG	

Fig. 1: Agarose gel electrophoresis of multiplex PCR products amplified from buffalo genomic DNA isolated from blood using both the Sat I and II primers, amplifying a gender-neutral signal (216bp) and BuRYNI 1 and 2 primers, amplifying a male-specific signal (164 bp). M, 100 bp ladder DNA size marker, lanes 1, 2, 3 are males representing both 216 and 164 bands while lanes 4 and 5 are females representing only the 216 bp.

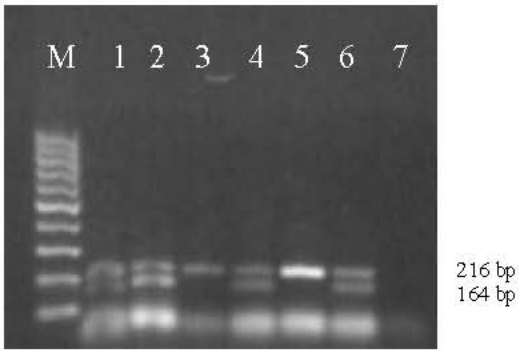

Fig. 2: Agarose gel electrophoresis of multiplex PCR products amplified from IVF- produced buffalo embryos using both the Sat I and II primers, amplifying a gender-neutral signal (216 bp) and BuRYNI 1 and 2 primers, amplifying a male-specific signal (164 bp). M, 100 bp ladder DNA size marker, lane 1 is male positive control, lanes 2, 4 and 6 are male embryos while lanes 3 and 5 are females, lane 7 is a negative control.

PCR Amplification: At first, multiplex PCR was carried out on the DNA extracted from blood of adult male and female buffaloes using the both primer pairs. For each IVF- produced buffalo embryo, three PCR amplifications were carried out. The first PCR was carried out using only the bovine satellite sequences common to both male and female DNA (Sat I and II) while the second PCR was carried out using the male-specific primers (BuRYNI 1 and 2). The third PCR was a multiplex PCR carried out using both the gender-neutral and the male-specific primers. The PCR amplification was carried out in a total of 25 µl reaction volume. The PCR components concentration were optimized to be 1X PCR buffer, 1.5 mM MgCl, 0.5 mM dNTPs and 12.5 pmol of each primer, 1.5 unit of Taq polymerase and either 100 ng of genomic DNA or 5 µl of embryonic lysate. The amplification cycles were carried out in a PTC-100 Thermocycler (MJ Research, USA). Reaction conditions were also optimized to be 95°C for 3 min. as initial denaturation followed, by 35 cycles of 94°C for 45 seconds, 58°C for 1 min. and 72°C for 1 min. A final extension step at 72°C for 10 min. was followed. Positive male DNA isolated from buffalo whole blood and negative control (no template) were included in each PCR run to ensure no cross contamination or amplification failure due to presence of inhibitors. All samples were repeated twice to ensure reproducibility of the PCR test.

Agarose Gel Electrophoresis: Amplification products were electrophoresed in 2% agarose gel containing 0.5 X TBE at 70 volts for 60 min. and visualized under ultraviolet light. To assure that the amplification products were of the expected size, a 100 bp DNA ladder was run simultaneously as a marker.

RESULTS

PCR Amplification: A multiplex PCR using both the gender-neutral and the male-specific primers was conducted on the genomic DNA to detect the sex. The results of PCR of all blood samples confirmed the anatomical sex of the adult animal. Male animals amplified both 216 and 164 bp DNA fragments while females amplified only the 216 bp DNA fragment as shown in figure 1. The agreement between the result of multiplex PCR and the anatomical sex of the examined animals confirmed the reliability and specificity of the PCR sexing method.

Sex Determination of Buffalo Embryos: Crude embryo lysates were prepared from all the examined embryos. PCR was conducted on crude DNA obtained from embryo samples. Three PCR experiments were carried out on each embryo. In the first PCR, carried out using only the gender-neutral primers, thirty nine embryos out of the

forty examined embryos amplified a 216 bp fragment. In the second PCR, carried out using only the male-specific primers, a total of 18 out of forty embryos amplified the male specific sequence (164 bp) and assigned as male embryos.

The results of the third PCR, which is a multiplex PCR carried out using both the male-specific and the gender-neutral primers, revealed that 18 out of the forty examined embryos amplified both the 216 and 164 bp DNA fragments and so assigned as male embryos while 21 embryos amplified only the gender-neutral fragment (216 bp) and so assigned as female embryos (Fig. 2). Only one embryo showed no amplification in all the conducted three PCR experiments.

DISCUSSION

In this study, male-specific primers (BuRYNI 1 and 2) derived from the repetitive Y-chromosome-specific sequence designated as BuRYNI were used for sex determination of IVF-produced buffalo embryos. This sequence was chosen because it was reported to be conserved in the buffalo genome [7]. Furthermore, this sequence was found to be repetitive in nature [17] and thus detection of this sequence by PCR proved to be efficient. This primer pair was designed to amplify a 164 bp DNA fragment in male buffalo DNA [7]. Because of the high sensitivity of the PCR, any contamination at the molecular level could give a positive signal in the absence of a template. Also, sub-optimal conditions might lead to a negative result even in presence of a template. Therefore, the use of internal control to monitor the PCR sexing assay was critical. Gender-neutral primers (Sat I and II) derived from the bovine satellite sequences, common to both male and female sexual DNA, were also used as internal control. This primer pair was designed to amplify 216 bp DNA fragment in both male and female buffalo DNA [16, 18]. This internal control facilitates the detection of absence of embryonic cells in the reaction mixture and / or amplification failure and thus, excludes the false negative female results. Both positive and negative (no template) control were included in each run to ensure absence of amplification failure and absence of cross contamination between samples.

At first, the PCR sexing method was carried out on genomic DNA extracted from whole blood samples collected form both adult male and female buffaloes as templates. Males amplified both the 216 and the 164 bp DNA fragments while females amplified only the 216 bp DNA fragment only. The results of PCR of blood samples were in complete agreement with the anatomical sex and thus confirming the reliability of the sexing method. Then, three PCR reactions were carried out on each IVF- produced buffalo embryos. The first PCR was carried out using only the gender-neutral primers (Sat I and II), common to both male and female. All the embryos except only one, amplified the 216 bp DNA fragment specific to this primer pair. The second PCR was carried out using only the male-specific primers (BuRYNI 1 and 2). Eighteen out of the forty IVF-produced buffalo embryos amplified the specific 164 bp DNA fragment and so assigned as male embryos. The third PCR was a multiplex PCR carried out using both primer pairs. Eighteen embryos amplified both the 216 and 164 bp DNA fragments and so assigned as male embryos while the twenty one embryos amplified only the 216 bp DNA fragment and so assigned as female embryos. Only one embryo failed to give any amplification in the PCR reaction. This could be attributed to be either amplification failure due to suboptimum PCR condition or due to absence of embryonic cells by mistake in the first step of DNA extraction.

In this study, out of the thirty nine embryos, 21 were identified as females and 18 were identified as males. The sex ratio was nearly 1:1. Also, it has been experimentally demonstrated in mammals [19] that during meiosis equal numbers of X and Y bearing spermatozoa are produced.

The Polymerase Chain Reaction is an efficient method for sexing embryos in both bovine [20] and buffaloes [21]. In this work, the forty embryos, except one gave positive amplifications. Previous studies in buffaloes reported that all the embryo stages in addition to single blastomere were sufficient for sex determination by PCR [7, 18]. So, further work is needed for amplification of single blastomere from embryos for the purpose of using such embryos after sexing.

In conclusion, multiplex PCR assay could be used as a reliable and efficient tool for sex determination of IVF-produced buffalo embryos.

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