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Comparative validation using quantitative real-time PCR (qPCR) and conventional PCR of bovine semen centrifuged in continuous density gradient

[Validação comparativa utilizando PCR quantitativo em tempo real (qPCR) e PCR convencional de sêmen bovino centrifugado em gradiente de densidade contínuo]

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ABSTRACT

The objective of the present study was to determine the sperm enrichment with X-bearing spermatozoa, after one centrifugation in a Percoll or OptiPrep continuous density gradient, using quantitative real-time polymerase chain reaction (qPCR) of sperm DNA and resultant *in vitro*-produced bovine embryos by PCR. Frozen/thawed sperm was layered on density gradients and the tubes were centrifuged. Supernatants were gently aspirated and the sperm recovered from the bottom of the tubes. Cleavage and blastocyst rates were determined through *in vitro* production of embryos and PCR was performed to identify the embryos' genetic sex. A difference in blastocyst rate was found in the Percoll treatment compared to OptiPrep ($P < 0.05$). The percentage of female embryos in the Percoll and OptiPrep groups was 62.0% and 47.1%, respectively. These results were confirmed by qPCR of spermatozoa DNA and underestimation was seen only in the Percoll group. It was possible to sexing sperm using simple approach.

Keywords: bovine, sperm sexing, centrifugation, embryo sexing, qPCR, PCR

RESUMO

O objetivo do presente estudo foi determinar o enriquecimento de espermatozoides portadores do cromossomo X após a centrifugação em gradiente de densidade contínuo de Percoll ou OptiPrep, utilizando reação em cadeia da polimerase quantitativa em tempo real (qPCR) do DNA do espermatozoide e dos embriões bovinos produzidos *in vitro* resultantes pela PCR convencional. Espermatozoides descongelados foram depositados em gradientes de densidade e os tubos foram centrifugados. Os sobrenadantes foram gentilmente aspirados e os espermatozoides recuperados do fundo dos tubos. As taxas de clivagem e de blastocisto foram determinadas pela produção *in vitro* de embriões e a PCR foi realizada para a identificação genética do sexo dos embriões. Verificou-se diferença na taxa de blastocistos entre os grupos Percoll e OptiPrep ($P < 0,05$). A porcentagem de embriões de fêmeas nos grupos Percoll e OptiPrep foi de 62,0% e 47,1%, respectivamente. Estes resultados foram confirmados pela qPCR do DNA de espermatozoides e uma subestimação foi observada no grupo do gradiente de densidade de Percoll. Foi possível a sexagem de espermatozoides utilizando uma metodologia simples.

Palavras-chave: bovino, sexagem espermatozoides, centrifugação, sexagem embriões, qPCR, PCR

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INTRODUCTION

Sperm sexing has aroused great interest due to the extensive application in animal production as well as medicine, and new separation techniques with both better accuracy and low cost are necessary. Density gradient centrifugation could be an approach to spermatozoa sexing because more DNA content of X-bearing spermatozoa can be related to more 0.06% density in those cells (Windsor *et al.*, 1993).

For the separation of bovine X- or Y-chromosome bearing spermatozoa (X or Y sperm) satisfactory results have been obtained through recentrifugation of spermatozoa on two Percoll discontinuous density gradients. In cattle, Schwiderski *et al.* (1991) used two centrifugations in Percoll discontinuous gradients, consisting of 10 layers of 0.6mL of Percoll solution with densities ranging from 1.034 to 1.068g/mL. This method has provided an enrichment of over 75% and 65% of X or Y sperm fractions above and below, respectively, as verified by in situ hybridization. Blottner *et al.* (1993) using the same methodology used the sperm of both fractions for in vitro production of bovine embryos that had the sex confirmed by PCR, which demonstrated that the use of sperm from the top and bottom fraction resulted in 75% and 92% of the sex of embryos male and female, respectively. Lange *et al.* (1995) repeated both experiments and obtained 70% accuracy in the selection of X sperm in the sediment and 65% of Y sperm in the supernatant, after evaluation by in situ hybridization.

Hossepian de Lima *et al.* (2000) and Hossepian de Lima (2007) reported an accuracy of 70% using discontinuous Percoll density gradient in one or two centrifugations, but suggested many inconveniences that difficult commercial application, such as the impossibility of gradient storage, immediate use after semen collection or thawing, in addition to complex preparation (too many layers) that can increase variability and be time-consuming.

The objective of the present study was to determine the sperm enrichment with X sperm, after one centrifugation in a Percoll or OptiPrep continuous density gradient, using quantitative real-time polymerase chain reaction (qPCR) of

sperm DNA and resultant in vitro-produced bovine embryos by PCR.

MATERIAL AND METHODS

Chemicals and media were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. The semen samples were obtained from cryopreserved bovine semen of two Gir breed bulls and two Jersey breed bulls from a commercial company. The percentages of total motile sperm and sperm vigor were determined subjectively on a drop of semen placed in a pre-warmed glass-slide covered with a coverslip, and examined under a bright field microscope at 400x magnification. Sperm concentration was determined in a hemocytometer at a 1:200 dilution.

The preparation of continuous density gradient was as follows: Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradients were made by mixing a stock solution of Percoll with DMEM, pH 7.4, 280 to 290mOsm/kg/H₂O with 0.3% (wt/vol) of BSA (Calbiochem, Darmstadt, Germany) in order to obtain densities ranging from 1.110 to 1.123g/mL. OptiPrep was prepared by mixing different proportions in DMEM containing 0.3% (wt/vol) of BSA (Calbiochem, Darmstadt, Germany), pH 7.4, in order to obtain densities ranging from 1.110 to 1.123g/mL.

Discontinuous Percoll and OptiPrep density gradients were then consecutively layered in 15mL polystyrene centrifuge tubes. The tubes were maintained at 4°C for 24h to convert a discontinuous into a continuous density gradient, according to instructions in the manual for the preparation of density gradients (Density..., 2007). Continuous gradients are characterized by a smooth change in density from the top to the bottom of the tube. Instead of the obvious interfaces present in the discontinuous gradient, a continuous gradient can be thought of as having an infinite number of interfaces (Cell..., 2007).

Forty million thawed sperm were overlaid on Percoll and OptiPrep continuous density gradients and one centrifugation was performed. Motility was evaluated subjectively using light microscopy, and the concentration was determined by counting spermatozoa in a hemocytometer. They were then centrifuged 500 X g for 15min at 22°C. The supernatants were

carefully aspirated and sperm located at the bottom fraction were collected from the tubes. Sperm concentration, motility, and recovery rates were determined and the sperm was used for quality control by *in vitro* production (assessment of cleavage and blastocyst rates) and qPCR (assessment of X and Y sperm proportion) procedures.

For IVF, bovine ovaries were transported from the abattoir to the laboratory in a saline solution at 30 to 35°C. Antral follicles of 3 to 7mm were manually aspirated using a 19-gauge needle attached to a 20mL syringe. Oocytes with a compact cumulus and at least four layers of cells were selected for *in vitro* maturation (IVM). Groups of 15 to 20 oocytes were placed in 100µL droplets of IVM medium under mineral oil. IVM medium was TCM 199 (GIBCO BRL; Grand Island, USA) supplemented with 10% heat-inactivated (at 55°C for 30min) FCS, FSH (Folltropin™, Bioniche Animal Health, Belleville, Canada) (1.0µg/µL), hCG (Profasi HP™; Profasi™, Serono, São Paulo, Brazil) (10U/mL), estradiol (1.0µg/mL), sodium pyruvate (0.2mM) and 16.67µg/µL amikacin (Aminocina™, Instituto Biochimico, Rio de Janeiro, Brazil) and 5mM sodium bicarbonate. Oocytes were cultured for 22h at 38.5°C under an atmosphere of 5% CO₂ in the air.

The sperm centrifuged in Percoll and OptiPrep density gradients was counted, diluted IVF medium (100 x 10³ cells for a 90µL IVF droplet) and incubated for 60 to 90min for capacitation before IVF. The oocytes were washed three times in TCM-199³ medium supplemented with 25mM of HEPES, 100mM of sodium pyruvate, BSA (BSA fraction V, fatty acid free, Inlab, São Paulo, Brazil) (10mg/mL) and once in IVF medium. The oocytes and sperm were incubated for 20h in 5% CO₂ in humidified air at 38.5°C. Presumptive zygotes were denuded of cumulus cells by repeated pipetting, and washed three times in modified synthetic oviduct fluid (SOF) medium, and they were then transferred to 500µL SOF medium in four well dishes. Embryo culture was carried out under mineral oil in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 38.5°C and cleavage was assessed at 46 to 48 h and development to the blastocyst stage at day 7 to 8. Fetal calf serum (FCS) was not added during *in vitro* culture, as previously

described (Gutiérrez-Adán *et al.*, 2001). The blastocysts were frozen in liquid nitrogen individually in microtubes with 10µL of Milli-Q water and stored at -20°C until PCR analysis for sex determination. In order to obtain embryonic cell DNA for sex determination by PCR, 221 and 239 embryos from Percoll and OptiPrep groups, respectively.

Proteinase K (Invitrogen, Cleveland, USA) was added at a final concentration of 5µg per embryo. The micro tubes were incubated at 37°C for 60min (for enzyme activity) and at 98°C for 10min (for enzyme inactivation). The sample of each micro tube was separated in two samples. Two pairs of primers of Y-specific sequences were split into two distinct samples. The first pair was 5'-CCT CCC CTT GTT CAA ACG CCC GGA ATC ATT-3' and 5'-TGC TTG ACT GCA GGG ACC GAG AGG TTT GGG-3' (Bondioli *et al.*, 1989), and the second pair was 5'-ATC AGT GCA GGG ACC GAG ATG- 3' and 5'-AAG CAG CCG ATA AAC ACT CCT T-3' (Schwerin *et al.*, 1991; Luz *et al.*, 2000). The first pair detected a sequence of 210 bp specific to the bovine Y-chromosome and the second one detected a sequence of 196bp specific to Y-chromosome. A third pair of primers detected an autosomal sequence of 280bp, indicating the presence of bovine genomic DNA. The third pair was 5'-AGG TCG CGA GAT TGG TCG CTA GGT CAT GCA-3' and 5'-AAG ACC TCG AGA GAC CCT CTT CAA CAC GT-3' (Ellis *et al.*, 1988). Multiplex PCR was carried out in the same tube with first (210bp) and third (280bp) primers and the PCR of the second pair of primers (196bp) was carried out in a second tube. Amplifications were performed in a thermocycler (MJ Research PTC 100 Thermal Cycler, GMI Inc., Ramsey, MN) as follows: a) for first and third primers: an initial step at 94°C for 10sec, 40 denaturation cycles at 94°C for 1 min, annealing at 58°C for 1min and synthesis at 72°C for 1min. A time extension of 7min at 72°C was added at the end of the final cycle; b) for the second primer: an initial step at 94°C for 10sec, 38 denaturation cycles at 94°C for 1min, annealing at 58°C for 1min and synthesis at 72°C for 1min. A time extension of 7min at 72°C was added at the end of the final cycle as well.

Comparative validation...

Before DNA extraction from spermatozoa, there were two washes with PBS by centrifugation at 900x g for 5min to remove as much residues of the density gradient as possible. DNA extraction from sperm samples was performed using the DNeasy Blood and Tissue Kit (Qiagen, GmbH, Germany). DNA concentration and purity were determined using a biophotometer (Eppendorf, Hamburg, Germany). The concentration of samples was around 100 ng. DNA samples were subjected to electrophoresis in 2% agarose gel for visualization of genomic DNA. The qPCR procedure (Parati *et al.*, 2006) was used to determine the enrichment of samples with X sperm, after centrifugation in density gradients, without the need to perform validation by means of in vitro production of embryos and subsequent sexing by conventional PCR. For internal control a negative control with no DNA added was used, a control with DNA extracted from the blood of

a male bovine and with DNA extracted from spermatozoa not centrifuged in density gradients.

The results of amplification (Ct values – cycle threshold) were analyzed in 7500 Sequence Detection System Software (Applied Biosystems, Foster City, USA) using a relative quantification ($\Delta\Delta C_t$) study assay. Figure 1 shows an example of amplification curves. The quality control of the centrifuged sperm evaluation was made by the proportion of X- and Y-sperm by qPCR. The calculation for predicting the proportion of X and Y sperm was performed based on the $2^{-\Delta\Delta C_t}$ method described by (Livak and Schmittgen, 2001). The calculation of the proportion of relative quantification of X and Y sperm in the samples also took into account the efficiency of each amplification, where 2.0 is 100%, 1.9 is 90%, etc.

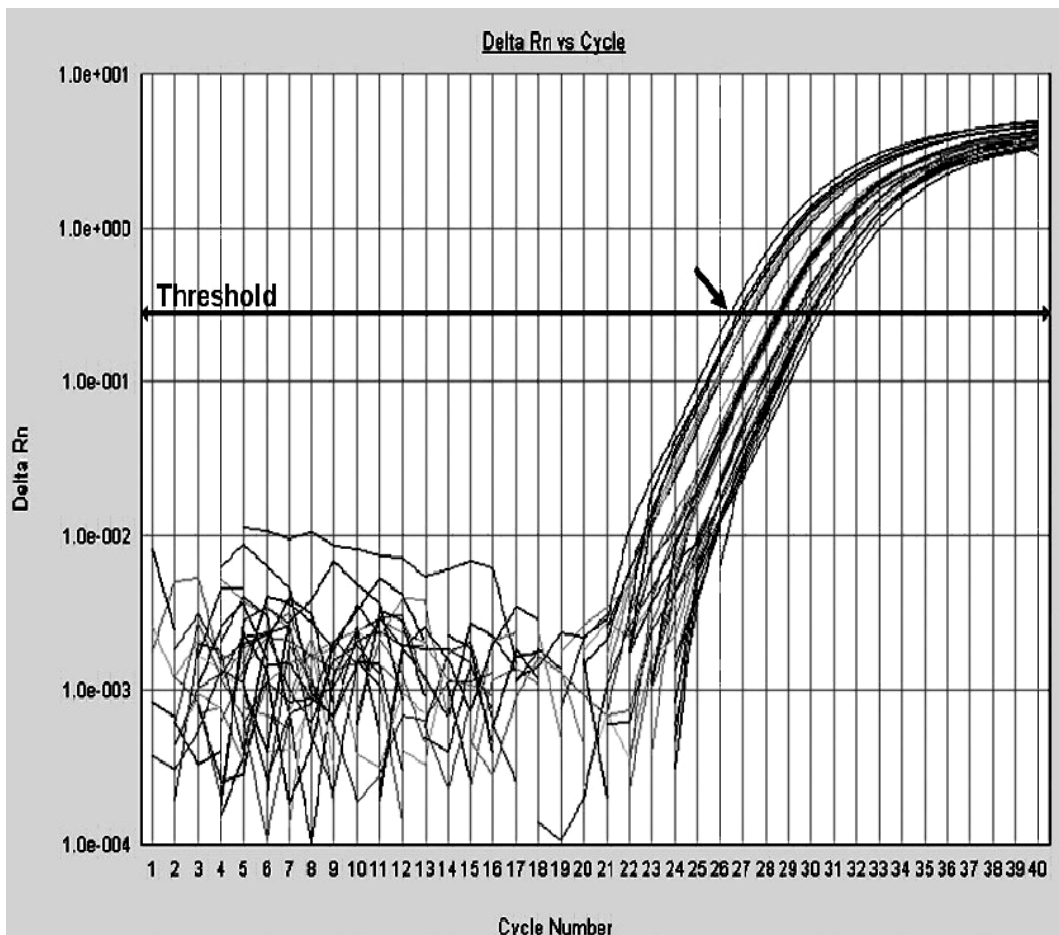


Figure 1. Sample of amplification curves (in logarithm of the fluorescence) of target genes X- (PLP) and Y- (SRY) chromosomes using real-time PCR and the threshold line. The cycle threshold (Ct) for each sample is the point where the amplification curve crosses the line (indicated by an arrow).

Five trials were performed for each bull in the Percoll and OptiPrep groups, totaling forty centrifugation procedure trials. Cleavage and blastocyst rates were analyzed through a chi-square test using an SAS program (SAS Inst. Inc., Cary, USA). The chi-square test was used to analyze the embryo PCR results, comparing the percentages of male and female embryos of sexing groups (Percoll and OptiPrep) with the expected 1:1 ratio. Means of female embryos obtained in the Percoll and OptiPrep groups were compared with means for DNA amplification of X sperm by qPCR, and analyzed by analysis of variance (PROC GLM of SAS program - SAS Inst. Inc., Cary, USA) followed by the Tukey's test. A 5% level of significance was used in all statistical analysis.

RESULTS

The mean motility before density gradient centrifugation was 64% and after centrifugation

it was 78% and 30% for the Percoll and OptiPrep groups, respectively. The sperm recovery rate after centrifugation was 5.7% and 19.4% for the Percoll and OptiPrep groups, respectively.

The findings of in vitro embryo production (cleavage and blastocyst rates) are shown in Table 1. A difference in blastocyst rate between the Percoll group and OptiPrep group was observed ($P < 0.05$), and no differences ($P > 0.05$) in cleavage and blastocyst rates after grouping the results of Gir and Jersey breeds were found between groups.

A total of 460 embryos were sexed by conventional PCR analysis (genetic sex identification). The results are summarized in Table 2. Data from sexing groups were compared to that of the expected 1:1 ratio. Female sex ratio deviation was found in the Percoll group (62.0%) ($P < 0.05$) but not in the OptiPrep group (47.3%) ($P > 0.05$).

Table 1. Results of in vitro-produced bovine embryos (mean \pm standard deviation) using sperm centrifuged in continuous Percoll and OptiPrep density gradient

Group	Oocytes (n)	Cleavage rate n (% \pm SD)	Blastocyst rate n (% \pm SD)
Percoll	1,182	817 (69.12 \pm 9.65)	244 (20.64 \pm 8.92)
OptiPrep	1,150	802 (69.74 \pm 9.62)	247 (25.6 \pm 10.21)
Total	3,451	-	-

$P < 0.05$. SD: standard deviation.

Table 2. Sex ratio obtained after PCR analysis of in vitro-produced bovine embryos with sperm sexed by centrifugation in continuous Percoll and OptiPrep density gradient

Group	Total embryos (n)	Male embryos (% and SD)	Female embryos (% and SD)
Percoll	221	84(38.0 \pm 3.02)a	137(62.0 \pm 3.02)
OptiPrep	239	126(52.7 \pm 6.28)b	113(47.3 \pm 6.28)
Total	460	-	-

$P < 0.05$. SD: standard deviation.

Data from qPCR was grouped in Table 3. The relative quantification of X- and Y-chromosome from blood DNA samples (internal control) was 50.3% for each chromosome. In the samples containing sperm DNA (not centrifuged - internal control), it was 50.1% for each chromosome, showing that qPCR was able to correctly identify X and Y sperm, as the expected proportion in males is 1:1.

In the samples centrifuged in Percoll or OptiPrep density gradient, the percentage of X-chromosome was 52.2 and 47.2%, respectively. In the Percoll gradient, the percentage of X-chromosome was statistically lower than in PCR of embryos (62% of females) ($P < 0.05$). However, in the OptiPrep gradient, the percentage obtained in qPCR was not different ($P > 0.05$) from that found in conventional PCR of embryos (47.3% of female embryos) (Table 4).

Comparative validation...

Table 3. Average efficiency of *SRY* and *PLP* gene amplification and percentage of X- and Y-chromosome subjected to qPCR containing blood DNA and sperm DNA (centrifuged and not centrifuged)

Group	Efficiency of X-chromosome reaction (<i>PLP</i> gene)	Efficiency of Y-chromosome reaction (<i>SRY</i> gene)	X-chromosome %±SD	Y-chromosome %±SD
Blood DNA (internal control)	1.99	1.99	50.3±0.6	49.7±0.6
Sperm DNA from non-centrifuged sample (internal control)	2.00	2.00	50.1±1.1	49.9±1.1
Sperm DNA from Percoll gradient	2.00	2.00	52.2±2.3	47.8±2.3
Sperm DNA from OptiPrep gradient	2.00	2.00	47.2±2.4	52.8±2.4

SD: standard deviation

Table 4. Percentage of female embryos obtained by conventional PCR of in vitro-produced bovine embryos and percentage of X-chromosome from sperm DNA by qPCR in Percoll and OptiPrep groups

Group	Female embryos in conventional PCR, %±SD	X-chromosome in qPCR, %±DP
Percoll	62.0±3.02a	52.2±2.3b
OptiPrep	47.3±6.28a	47.2±2.4a

SD: standard deviation. Means within rows with different letters are statistically different (P<0.05).

The analysis of male bovine blood DNA amplification (internal control) and samples of sperm DNA from Percoll, OptiPrep and sperm DNA from non-centrifuged sample groups showed that the average efficiency was close to 100% for the two genes used (*PLP* and *SRY*), with a correlation (r^2) average of 0.996.

Table 4 shows a data comparison between conventional PCR of embryos and qPCR from sperm DNA subjected to centrifugation.

DISCUSSION

The objective in the present study was to determine the sperm enrichment with X-bearing spermatozoa, after one centrifugation in a Percoll or OptiPrep continuous density gradient, using quantitative real-time polymerase chain reaction (qPCR) of sperm DNA and resultant *in vitro*-produced bovine embryos by PCR. Also, results of qPCR of spermatozoa DNA centrifuged and sex ratio of *in vitro*-produced embryos by conventional PCR were compared, and how they affected the *in vitro* production of bovine embryos was studied.

The results of cleavage rate did not differ between the centrifuged groups that ranged from 68 to 70%, and 70 to 73%, respectively, indicating that this parameter was not affected by centrifugation. These results were similar to those obtained using two centrifugations in Percoll discontinuous density gradient (Schwidorski *et al.*, 1991; Blotner *et al.*, 1993; Lange *et al.*, 1995; Hossepian de Lima, 2007). The only difference showing lower rate of blastocysts was observed in the Percoll gradient in Jersey breed. This was due to one of the bulls used in the study, which had a blastocyst rate of 11% compared to an average of 23% in the other three bulls (data not shown).

Despite the sex ratio deviation in the Percoll gradient (62% of females, P<0.05), the sexing results demonstrated that there was sex ratio towards females after PCR analysis. In bovine, one centrifugation on 12-layer discontinuous Percoll gradient produced a female sex ratio deviation that ranged from 55.7 to 74.3% (Hossepian de Lima *et al.*, 2000; Kobayashi *et al.*, 2004). In the present study, the discontinuous density gradient with only 3 layers was converted into continuous density gradient and

could be stored for 24 h at 4°C, which may simplify routine procedures of *in vitro* production in specialized companies.

OptiPrep has been used in medicine to separate motile and viable sperm as a substitute of Percoll, but since 1996 its clinical human use has been withdrawn due to potential endotoxin contamination (McCann and Chantler, 2000). Mousset-Simeon *et al.* (2004) reported that OptiPrep was less effective than Percoll to separate sperm with regular motility and morphology. In contrast, the present study obtained similar results in the comparison of motility between Percoll and OptiPrep, which was consistent with other reports in humans (Andersen and Byskov, 1997; Harrison, 1997; Claassens *et al.*, 1998). However, OptiPrep was not effective to separate X sperm. The qPCR has recently been used as a reliable technique for the quantification of X and Y sperm cells in semen samples, especially to validate techniques for sexing sperm (Joerg *et al.*, 2004; Parati *et al.*, 2006; Puglisi *et al.*, 2006).

For the first time the use of qPCR to validate sexing bovine sperm submitted to density gradient centrifugation was demonstrated. Furthermore, it was possible to quantify the proportion of X and Y sperm in semen samples before centrifugation without any adjustments by using linear regression, as described in other studies (Parati *et al.*, 2006; Puglisi *et al.*, 2006).

The results of predicting the proportion of X and Y sperm through qPCR confirmed the findings of genetic identification of sex in *in vitro*-produced bovine embryos with the use of sexed sperm in OptiPrep groups. In Percoll density gradient, the percentages of X-chromosomes found in the samples by qPCR (52%) were underestimated compared to the sex ratio of embryos obtained in conventional PCR (62%). This can be explained by the effect of some components found in Percoll, such as silica and PVP, which may have interfered with the DNA extraction process or amplification by qPCR, despite successive washes. There have been reports that Percoll at 80% concentration produced great variability during sexing sperm by flow cytometry (Stap *et al.*, 1998; Puglisi *et al.*, 2006). In the present study, centrifuged sperm were recovered in a fraction containing 90% Percoll, which theoretically could have

affected qPCR results, as seen in sexing by flow cytometry. However, the interference of Percoll in the DNA extraction process and qPCR has not been described in literature.

In a complementary study samples enriched with X sperm separated by flow cytometry were used. The percentage of X sperm using qPCR was 89%, which was within the expected 85% to 95% obtained by flow cytometry (Cran, 2007; Hamano, 2007; Seidel Jr., 2009).

These results in estimating the percentage of X and Y sperm in semen samples with sexed sperm through flow cytometry were higher than those reported by Joerg *et al.* (2004). In this study, the percentages of both X and Y sperm were underestimated, and a maximum of 80% and 70% were obtained, respectively, when the expected percentage was 85% to 95% (Cran, 2007; Seidel Jr., 2009).

CONCLUSIONS

The centrifugation procedure did not interfere with cleavage rates during *in vitro* embryo production, but a lower rate of blastocysts was seen in the Percoll gradient. There was a sex ratio deviation in the Percoll gradient, confirmed by conventional PCR of *in vitro*-produced bovine embryos. These results were confirmed by the qPCR of spermatozoa DNA, and an underestimation was seen only in the Percoll group. It was possible to sexing sperm using a simple approach.

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