Sex Ratio Determination by Quantitative Real Time PCR using Amelogenin Gene in Porcine Sperm

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ABSTRACT

Sex-sorting of sperm is an assisted reproductive technology (ART) used by the livestock industry for the mass production of animals of a desired sex. The standard method for sorting sperm is the detection of DNA content differences between X and Y chromosome-bearing sperm by flow cytometry. However, this method has variable efficiency and therefore requires verification by a second method. We have developed a sex determination method based on quantitative real-time polymerase chain reaction (qPCR) of the porcine amelogenin (AMEL) gene. The AMEL gene is present on both the X and the Y chromosome, but the length and sequence of its noncoding regions differ between the X and Y chromosomes. By measuring the threshold cycle (Ct) of qPCR, we were able to calculate the relative frequency of X chromosome. Two sets of AMEL primers were used in these studies. One set (AME) targeted AMEL gene sequences present in both X and Y chromosome, but produced PCR products of different lengths for each chromosome. The other set (AXR) bound to AMEL gene sequences present on the X chromosome but absent esholthe Y-chromosome. Relative product levels were calculated by normalizing the AXR fluorescence to the AME fluorescence. The AMEL method accurately predicted the sex ratios of boar sperm, demonstrating that it has potential value as a sex determination method.

(Key words : amelogenin (AMEL) gene, sex determination, quantitative real time PCR, boar sperm)

INTRODUCTION

Assisted reproductive technology (ART) using sex-typed spermatozoa has made it possible to control the sex of domestic animal reproduction for livestock management purposes. It has lowered livestock production costs and enhanced the ability of the livestock industry to meet market demands. In addition, sex determination is an important tool for the reduction of sex-linked genetic defects, forensic science application, and genotype analysis of livestock species.

Several molecular methods are available for sperm sex determination including an immunologically based system which detects different proteins on X and Y sperm cell surfaces (Blecher *et al.*, 1999), karyotyping after fertilization (Brandriff *et al.*, 1986), fluorescence *in-situ* hybridization (FISH) using X- or Y-chromosome-specific probes (Griffin *et al.*, 1991; Di Berardino *et al.*, 2004) and Chromogenic *in-situ* hybridization (CISH) (Kim *et al.*, 2007). However, PCR of X or Y-chromosome specific sequences has become the standard method for animal sperm sexing (Greenlee *et al.*, 1998; McClive and Sinclair, 2001). Its potential has been represented in several species including swine (Johnson *et al.*, 2005), mice (Kunieda *et al.*, 1992), cattle, pig, horse (Poloumienko., 2004), and primates (Villesen and Fredsted, 2006).

The SRY gene is a common PCR target for sexing because it is conserved between and Y-specific in a wide range of mammals (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990; Payen and Cotinot, 1993; Choi *et al.*, 2009). While other male-specific DNA sequences in pigs breeds have been used for PCR-based sexing (Horng and Huang, 2003), using these genes requires additional time and materials because it is difficult to distinguish the failure of amplification from the female sperm. The AMEL gene has recently been developed for PCR-based sexing because it has the potential to overcome this limitation. In most

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mammals, copies of AMEL are present on both the X (AMELX) and Y (AMELY) chromosomes, but differences in the introns of these two alleles enable them to be distinguished by PCR product size (Ennis and Gallagher, 1994; Pfeiffer and Brenig, 2005; Sembon *et al.*, 2008; Fontanesi *et al.*, 2008). The ability to design one pair of primers to positively identify both X and Y chromosome-bearing sperm is time-saving and economical.

Real-time qPCR has enabled the development of rapid and reproducible high throughput sexing methods. There are two common ways of analyzing qPCR data. First, absolute quantification involves the construction of a standard curve based upon known copy number of the transcript of interest. Second, relative quantification describes the change in expression level relative to some reference group, typically the experimental control group. This qPCR approach has been used to determine bovine semen sex ratio and avian sexing (Parati *et al.*, 2006, Chang *et al.*, 2008). In this study, we applied AMEL gene qPCR in the determination of porcine semen sex ratios. We used the relative method to demonstrate the utility of AMEL gene qPCR data for estimating the frequencies of X- and Y-chromosome-bearing spermatozoa.

MATERIAL & METHOD

1. DNA Isolation from Boar Semen

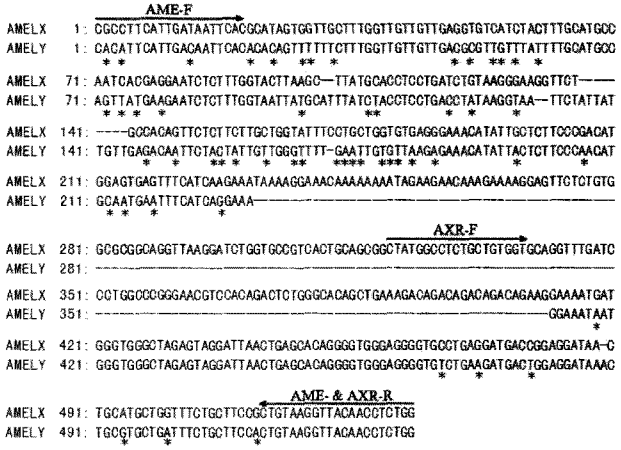
Genomic DNA was extracted from domestic pigs (*Sus scrofa domesticus*) tissue and boar spermatozoa. All reagents were from Sigma unless otherwise noted. A total of 1×10^9 sperm were diluted in 250 μl phosphate buffer saline (PBS) and combined with an equal volume of lysis buffer #1 (20 mM Tris-HCl pH 8.0, 20 mM ethylenediaminetetraacetic acid, 200 mM NaCl, 4% sodium dodecyl sulfate, 80 mM dithiothreitol and 20 μg proteinase K (Invitrogen). Then 500 μl of the tissue homogenates was added to an equal volume of lysis buffer #2 (10mM Tris- HCl pH 8.0, 0.5% sodium dodecyl sulfate, 0.1M ethylenediaminetetraacetic acid pH 8.0 and 20 μg proteinase K. Samples were incubated overnight at 55℃ and then exreacted with 25: 24:1 phenol:chloroform:isoamyl alcohol. An equal volume of isopropanol was added to the aqueous phase. The DNA pellet was recovered, washed in 70% ethanol, and air-dried. The pellet was resuspended in 10 mM Tris plus 1 mM ethylenediaminetetraacetic acid pH 8.0 and incubated overnight at 4℃.

2. Primers Design

A set of primers were designed to amplify the porcine AMEL genes (EMBL/GenBank accession numbers, AB091791 [AMELX] and AB091792 [AMELY]) and yield PCR products of different sizes (Sembon *et al.*, 2008). The primer sequence were: AME : forward primer (5'-CGCCTTCATTGATAATTCAC-3') and the reverse primer (5'-CCAGAGGTTGTAACCTTACAG -3'). Oligonucleotide X-specific primers were designed to amplify an AMELX specific sequence. The sequences of the forward and reverse primers were : AXR: forward : 5'-CGCCTTCATTGA TAATTCAC-3' and reverse 3'-ACCACAGCAGAGGCCATA G-5') (Fig. 1).

3. Quantitative Real-time PCR And Melting Curve Analysis (MCA)

Real-time qPCR was performed using an iQ^{TM}5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories) which monitored the PCR reactions, for two set of primers simultaoriously, and produced separate fluorescence amplification plots for each primer set product. The quantification was performed by raperimental determ fotion of the thressetd etcle (Ct), defined as the PCR etcle number. The quantities relative to control sample were automatically estimated using the iQ^{TM5} Optical System Software. To determine the normalized expression of the target gene, the relative quantity of target sequence was divided by relative quantity of the reference sequence. After completion of the PCR reaction, a melting curve was recorded by holding at 95℃ for 1min, cooling to 55℃ for 1min, and then heating slowly at 0.5℃/s up to 95℃ with a maximal ramp rate by default for the iQ^{TM5} instrument (80 repeats of



*, single-base substitution;-, deleted base.

Fig. 1. DNA sequence comparison between the X- and Y-specific amelogenin gene fragments of pigs. AME : primer set targeting both X- and Y-chromosome. AXR : primer set of X-specific sequence

counts). The melting peaks were plotted as the −dF/dT versus T (F is fluorescence; T is temperature).

The DNA of each sample was primed in separate wells for the two sequences of interest, using a defined volumetric quantity of the same DNA sample (250 ng). The PCR mixture (20 μl total volume) contained for each target sequence, 200 μM (AMX) or 1 μM (AXR) primers, 10 μl 2×Universal PCR Master Mix (Bio-rad). Cycling parameters were followed: 94℃ (4 min); 50 cycles of 94℃ (10s), 48℃ (1 min), 72℃ (30s); and 72℃ (5 min). To confirm the presence of amplicons and compare with melting curve analysis data, PCR products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and photographed under UV light.

4. Calculation of X-chromosome Bearing Sperm Frequency

The $2^{-\Delta\Delta CT}$ method was used to calculate the relative ratio of X-bearing sperm in semen samples using the real-time qPCR data. Normalized expression was calculated as the relative quantity of X-specific sequence normalized to the relative quantities of the X/Y common sequence (AMELX and AMELY). We estimated the sex ratio of unsorted sperm sample from this value. We derived a mathematical model which was used to correct for the error introduced into the sex ratio value by a discrepancy between the PCR efficiencies of each primer set. The mathematical model was based on the efficiency of primer sets for amplification of each individual AMEX and AMEY gene (Table 1~3).

Table 1. Definitions of primer set's efficiency to amelogenin X and amelogenin Y gene.

	Primer	
	AME	AXR
Amelogenin X	A	C
Amelogenin Y	B	0*

These value are used to correct for the error. *There is no sequence in amelogenin Y for AXR primer set.

Table 2. Definition and theoretical value of normalized expression.

Condition	Definition	Theoretical value
Sow tissue	α	$\frac{2C}{2A}=\frac{C}{A}$
Boar tissue	β	$\frac{C}{A+B}$
Unsorted sperm	γ	$\frac{xC}{xA+yB}$

α, β, and γ is measured by Detection System iQ^{TM}5 Multicolor Real-Time PCR and A, B, and C value is settled by these values. x and y are proportion of X- and Y- chromosome to total sex chromosome, respectively.

Table 3. Theoretical value of sex ratio in unknown sample.

Condition	Theoretical value
X gene ratio (x)	$\frac{1}{1+\frac{(\alpha-\gamma)\times\beta}{(\alpha-\beta)\times\gamma}}$
Y gene ratio (y)	$\frac{1}{1+\frac{(\alpha-\beta)\times\gamma}{(\alpha-\gamma)\times\beta}}$

x and y are equal to those in Table 2. A, B, and C are substituted by α, β, and γ to determine sex ratio (x, y).

RESULTS

To assess sperm sex ratio in semen samples, we performed qPCR of the AMELX and AMELY specific gene sequences. The reference gene quantities could be estimated amplifying AMEL gene sequences on both the X and Y chromosome with one primer set (AME). X chromosome quantities were evaluated with the X-specific AMEL gene primer set (AXR). The melting curve analysis showed that the AXR end products had the same melting temperature (83.5℃) and the AME primer set produced 3 peaks (80.0℃, 83.0℃, 88.0℃) (Fig. 2). Two peaks represented the end products amplified from AMELX and AMELY genes (melting temperature 80.0℃, 88.0℃), but the third peak represents unknown product (melting temperature 83.0℃).

All peaks were coincident with products resolved by agarose gel electrophoresis (Fig. 3). Two PCR products of 522 bp and 350 bp were amplified by the AME primer set (Fig. 3, lane 2, 3, 4). The 522-bp product was amplified from the X-chromosome and its melting temperature was 88.0℃. The 350-bp product was amplified from the Y chromosome and its melting temperature was 80.0℃. The agarose gel electrophoresis appeared as a band between the two sex chromosome PCR products, so its melting temperature was 83.0℃. An additional 328-bp band was amplified by the AXR primer set and has a melting temperature of 83.5℃ (Fig. 3, lanes 5, 6, 7).

The normalized fold expression of products amplified by AXR primers was estimated by iQ^{TM}5 Multicolor Real-Time

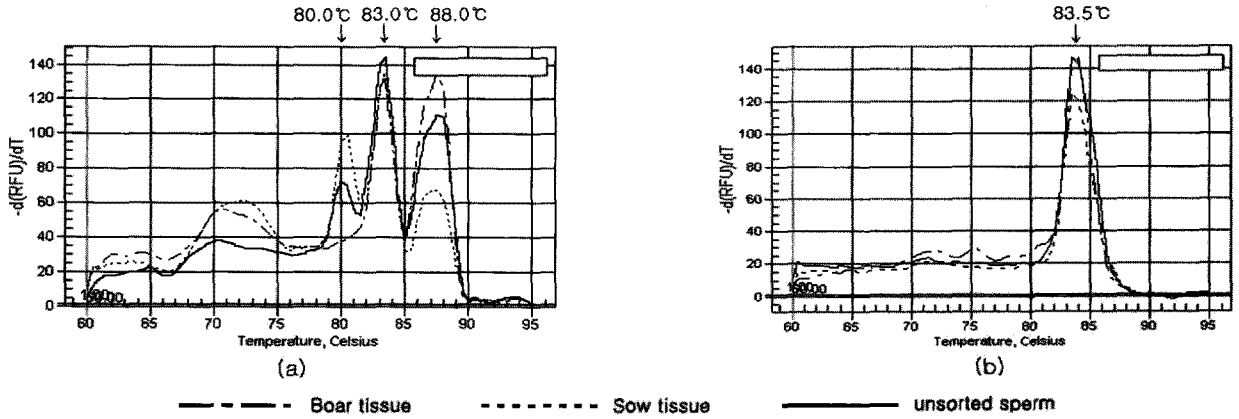


Fig. 2. Real-time melting curve analysis data using primer sets AME (a) or AXR (b). DNAs were chosen from sow, boar and unsorted sperm. The Tm of PCR products amplified by Afied by Afied are 80.0℃, 83.0℃, 88.0℃. The Tm of PCR products amplified by AXR primer set is 83.5℃. RFU, Relative fluorescence unit.

PCR Detection System (Bio-Rad Laboratories) (Fig. 4). This value was based on five experimental replicates for sow tissue, boar tissue, and unsorted sperm. The mean value of sow tissue, boar tissue, and unsorted sperm were 1.88, 0.91 and 0.94 respectively. The standard error value was 0.64 for sow tissue, 0.40 for boar tissue, and 0.36 for unsorted sperm. To determine the accuracy of this test, we could mix sow and boar DNA sample in 1:5 or 3:3 ratio and, then applied to AMEL qPCR method. The mean value of '1F:5M' (sow DNA:boar DNA=1:5) was 1.28 and that of '3F:3M(sow DNA:boar DNA= 3:3) was 1.31. The amplification efficiencies of AME primer were similar for AMELX and AMELY, but differed from the efficiency of AXR primers. (AME primer efficiency:AXR primer efficiency = 1: 1.88) (Table 4). The amplification efficiency was calculated relative to the efficiency of amplification of the AMEX by the AME primers (value = 1). The higher amplification efficiency of the AXR primer was confirmed by gel electrophoresis. The product bands for the AXR primer set were thicker than that of AME primer set for all tested samples.

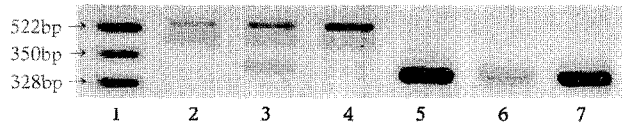


Fig. 3. Gel view showing PCR products of amelogenin gene fragments from sow, boar and unsorted sperm. 1 : DNA ladder, 2: Sow tissue with AME primer, 3: Boar tissue with AME primer, 4: unsorted sperm with AME primer, 5: Sow tissue with AXR primer, 6: Boar tissue with AXR primer, 7: unsorted sperm with AXR primer.

Using the mathematical model described in the Materials and Methods, we calculated the sex ratio for unsorted sperm, with the X-bearing sperm comprising 52.1% and Y-bearing sperm comprising 47.9% of unsorted semen samples. We also calcu-

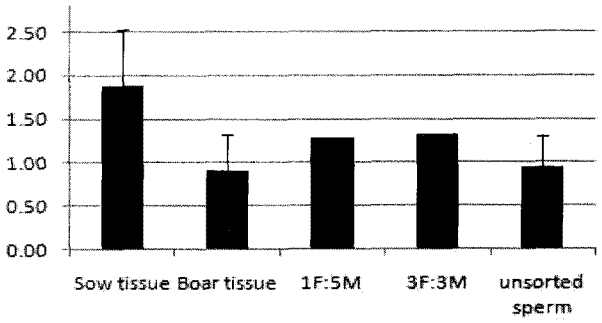


Fig. 4. Normalized gene expression of real-time PCR data for determination of the fold induction of PCR product of AXR primer set relative to AME primer set. The mean value of sow tissue, boar tissue, and unsorted sperm, 1F:5M (sow DNA:boar DNA=1:5), and 3F:3M (sow DNA:boar DNA=3:3) were 1.88, 0.91, 1.28, and 1.34 respectively.

Table 4. Efficiency of primer sets AME/AXR to amelogenin X and Y gene.

	Primer	
	AME	AXR
Amelogenin X	1 (Standard value)	1.88
Amelogenin Y	1.07	0 (none)

Those are represented as relative values according efficiency of AME primer set to amelogenin X gene.

Table 5. Sex ratio in unsorted sperm.

Condition	Ratio		
Condition	Unsorted sperm	1F:5M	3F:3M
X bearing	52.1 %	69.9 %	71.2 %
Y bearing	47.9 %	30.1 %	28.8 %

Using mathematical model, the sex ratio for unsorted sperm, 1F : 5M (sow DNA : boar DNA = 1 : 5), and 3F : 3M (sow DNA: boar DNA = 3 : 3) are calculated.

lated the sex ratio for '1F:5M' (sow DNA:boar DNA=1:5) with the X-chromosome comprising and Y-chromosome comprising 69.9% and 71.2% ,respectively. The ratio of X-chromoses to Y-chromosomes for '3F:3M' (sow DNA:boar DNA= 3:3) was 71.2:28.8. (Table 5).

DISCCUSION

In this study, qPCR of the AMELX and AMELY genes was used to determine the sperm sex ratios in boar ejaculates. Results were affirmed and corrected for primer efficiency by using positive control ratios determined for sow and boar tissue. The AME primer set, amplified a portion of intron 2 which differed in length between the two AMEL alleles (Sembon *et al.*, 2008). In addition to the two expected PCR products, a third nonspecific PCR product was detected and was likely to have resulted from a low annealing temperature. In previous studies, primer annealing was conducted at 60℃ for 30s but the annealing temperature used in this study was 48℃ for 60s. The amplification of this nonspecific sequence may account for the difference between the efficiencies of the AME and AXR primer sets. To minimize the effect of this discrepancy, a mathematical model was developed to correct for the different amplification efficiencies when calculating sex ratios for samples.

The calculated sex ratio for unsorted sperm was 52:48. This result corresponded well with the PCR products from sow tissue. The expected sex ratio of offspring is 1:1, but the actual ratio can vary. The average secondary sex ratio for swine has been reported to be 48.8 to 52.8% (Nalbandov *et al.*, 1964). The sex ratio in this study was within this range. To confirm the accuracy of AMEL qPCR method, we could mix sow and boar DNA sample in 1:5 or 3:3 ratio and, then applied to this sexing method. We expected the sex ratio for '1F:5M' (sow DNA:boar DNA=1:5) and '3F:3M' (sow DNA:boar DNA=3:3) with the X-chromosome comprising 58.8% and 75.0% but a calculated value was 69.9% and 71.2%. The difference between the expected ratio and the calculated ratio was about 6%, but the calculated ratio depended to the concentration of sow tissue DNA. To acquire more accurate results, we suggest this experiment execute repeatedly. Sex ratios of offspring are known to differ among breeds, breed crosses, strains, lines, line crosses, families, and individuals. Thus, pre-selection methods such as the qPCR method described in this study could enhance the ability to define sex ratios for livestock management. Pre-selection of X-bearing spermatozoa would be particularly useful for economical improvements of conventional AI, intrauterine insemination, intra-tubal insemination, and IVF. This technique could be also applied to confirmation of sexed-sperm sorted by other sex determination systems such as flow cytometry.

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