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SYBR® Green quantitative PCR for sex determination of bovine spermatozoa

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Abstract
Spermatozoa sexing technology in cattle breeding is being done by separation of X- and Y- chromosome bearing spermatozoa using flow-sorting technology. However, the sexing technique needed to be validated to ensure the accuracy of the technology. A technique to determine the sex of bovine spermatozoa using SYBR® Green real-time quantitative PCR (qPCR) was developed. Two sets of primers, ZFX and SRY were designed specifically to X- and Y- chromosome bovine genes respectively. Plasmid was inserted with ZFX and SRY gene fragment separately to create standard curves that ranged from 3.0 x 102 to 3.0 x 106 copies. The standards generated linear relationship with regression coefficient $r^2 = 0.984$ for ZFX and $r^2 = 0.996$ for SRY. Both standards of ZFX and SRY showed melting peak at temperature of 83 ºC and 85 ºC respectively. Real-time qPCR of bovine spermatozoa DNA samples and cloned plasmid ZFX and SRY genes for creating standard samples were performed simultaneously. The percentages of unsexed X- and Y- chromosome-bearing spermatozoa did not differ much from the 1:1 as reported in unsexed spermatozoa population. Therefore, the highly sensitive and fast method of real time PCR is a suitable technique for quantitating X- and Y- chromosome-bearing spermatozoa in semen samples.

Keywords: real-time PCR, quantitative PCR (qPCR), bovine spermatozoa

Introduction

Sex pre-selection procedure is economically important in cattle breeding. Sexed spermatozoa leads to the possibility of planned matings for a specific gender, which are used to increase productivity or efficiency of producing meat or milk. Spermatozoa sexing is made possible because of the differences in total DNA content, whereby spermatozoa carrying X- chromosome has approximately 4% more DNA than those carrying the Y- chromosome (Underwood et al. 2011).

Sex pre-selection of mammalian spermatozoa has been accomplished using different methods, which include albumin gradient separation, sex-specific antibody binding, multitube spermatozoa swim-up procedure, fractionation on discontinuous Percoll gradient, free-flow electrophoresis and flow cytometric separation (Joerg et al. 2004). Amongst the methods, flow

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cytometry remains the scientifically proven method of sex selection at the time of conception (Underwood et al. 2011).

The latest technology of flow cytometry provides an accurate and convenient verification analysis of sexed semen purity (Parati et al. 2006). However, for sex ratio evaluation of sorted semen, it should be done with a reliable method which does not rely on the same instrumentation to prevent carry-over of any errors that were resulted in improperly sorted spermatozoa (Colley et al. 2008).

The method to evaluate sex ration in ejaculated spermatozoa was developed in 1970 (Parati et al. 2006). Amongst the evaluation as well as validation of spermatozoa sex separation approaches available include sex determination by PCR (Reynolds and Varlaro 1996; Tan et al. 2006), Quinacrine mustard staining for Y- chromosome bearing spermatozoa (Ogawa et al. 1988), sexed spermatozoa analysis for DNA content (Welch and Johnson 1999), *in vitro* fertilisation and embryo transfer (Joerg et al. 2001), fluorescence *in situ* hybridisation (FISH) (Rens et al. 2001), and real-time quantitative PCR (qPCR) (Joerg et al. 2004).

The qPCR sex determination of spermatozoa by qPCR method was established subsequent to PCR and FISH (Parati et al. 2006). This technique combines the enormous sensitivity of PCR with the precise real-time monitoring of PCR products as they are generated (Leong et al. 2007). It offers the possibility of simultaneous amplification of the segment of interest and measurement of the amount of resulting DNA molecules through reaction cycles. Through the amplification of the signal with real time PCR, it is possible to quantitate the X- and Y- chromosome bearing spermatozoa.

In the present study, the objective was to develop a technique to determine the sex of bovine spermatozoa. This was done by quantifying X- and Y- chromosome bearing spermatozoa using Zinc Finger Protein X and Sex Determining Region Y genes via SYBR® Green technology. SYBR® Green is more economical as compared to Taqman probe and provides an equally accurate approach to qPCR, due to its high specificity and artifacts such as minimal primer dimers (Granfar et al. 2005). The developed technique will allow an accurate quantification of the frequencies of X- and Y- chromosome bearing spermatozoa in the bovine semen samples.

Materials and methods

A Kedah-Kelantan (KK) bull aged 8 years was sexually stimulated with a female in oestrus and semen was collected using an artificial vagina. Then semen was collected once in a week. Freshly ejaculated semen was immediately sent to the laboratory for quality evaluation based on parameter in *Table 1*. Semen samples with <30% mortality, <30% abnormal morphology and >50% motility were mixed with cryopreservation extender to a final concentration of 1.0 x 10^8 spermatozoa per ml and frozen in 0.25 ml straws using liquid nitrogen. The frozen semen straws were kept in liquid nitrogen at –196 °C and were used for spermatozoa sex determination.

DNA was extracted from the unsexed frozen semen in six replicates using Wizard® Genomic DNA purification kit (Promega, USA) according to the manufacturer's protocol. The purity and concentration of DNA was measured with Nanodrop ND-1000 spectrophotometer. The genomic DNA was stored at –20 °C until further use.

The forward and reverse primers were designed from the Zinc Finger Protein X (ZFX) (NM177490) and Sex Determining Region Y (SRY) (U15569) using the Primer Premier 5 software. The primer sequences and expected product sizes are showed in *Table 2*.

PCR amplification of genomic DNA was carried out using ZFX or SRY primers on Perkin Elmer GeneAmp® PCR System 9700 thermal cycler. The reaction mixture contained 70 ng template DNA, 0.4 mM

Table 1. Techniques used for each semen evaluation parameter

Parameter	Technique
Libido of ejaculation time	Time taken (second) starts from bull exposed to female to ejaculation
Semen volume	Total ml per ejaculation
Colour	Colour determined upon collection, usually whitish, creamy and yellowish for semen of *Bos indicus* (Brakmas) bulls
General motility	Collective movement of sperm observed under phase contrast microscope (10X) examination of undiluted semen, score 1 – 5; 0 = no swirl (nil or sporadic oscillation of individual sperm), 1 = no swirl (generalised oscillation of individual sperm only), 2 = very slow distinct swirl, 3 = slow distinct swirl, 4 = moderately fast distinct swirl, and 5 = fast distinct swirl (appearance of good quality bull semen).
Individual sperm motility	The percentage of motile (moving) sperm seen under phase contrast microscope (10X) examination after 10X dilution with normal saline (0.9% NaCl)
Mortality	A drop of semen sample is mixed with three drops of the eosin-negrosin stains on a pre-warmed slides using applicator stick and a thin smear is made using another slide. After air drying, the smear is observed under phase contrast microscope (40X) to get the percentage of stained or partial stained heads of spermatozoa
Abnormal morphology	The smear of eosin-negrosin stained sperm were observed under phase contrast microscope (40X) to get the percentage of abnormal sperm size, shape or appearance
Concentration	Sperm concentration (per ml) determined using hemacytometer chamber

Table 2. Primer sequences and expected PCR product size

Gene	Forward primer	Reverse primer	PCR product
ZFX	5'-caa gcg att tga aac gac a-3'	5'-cca act tct tta tga tgt cgc a-3'	475 bp
SRY	5'-aaa gag tag gtt gat ggg tt-3'	5'tgc ctc ctc aaa gaa tgg-3'	525 bp

Table 3. Optimised PCR program for ZFX and SRY primers on Perkin Elmer GeneAmp® PCR System 9700

Step	Temperature	Time	Cycle
Initial denaturation	95 °C	10 min	1
Denaturation	94 °C	30 sec	45
Anealling	55 °C	30 sec	
Elongation	72 °C	30 sec	
Final extension	72 °C	10 min	1

dNTP mix, 1X PCR buffer, 2 mM $MgCl_2$ and 2.5 unit Taq DNA polymerase in a final volume of 25 µl. The amplification reactions were performed using the conditions listed in *Table 3*. The PCR products were resolved by 2.0% agarose gel electrophoresis and visualized under UV illumination. The size of the products were determined by comparison with 100 bp molecular weight marker as a DNA ladder.

DNA cloning and sequencing

The bands of expected sizes; 475 bp and 525 bp for ZFX and SRY respectively, were purified from the gel using Promega Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The purified PCR products were cloned into 2.1 TOPO®-TA vector and transformed into One Shot® TOP10 competent cells (Invitrogen,

USA) in accordance to the manufacturer's protocol. The plasmids were extracted using Wizard® Plus SV Minipreps DNA Purification System (Promega, USA) and were sent for sequencing (Macrogen, Korea). The specificity and the uniqueness of the sequence of ZFX and SRY genes was further confirmed by Basic Local Alignment and Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). *E. coli* containing ZFX and SRY plasmids were kept as stock for multiplication in Luria-Bertani (LB) media added with 15% of glycerol.

Assay optimisation and SYBR® Green melting curve analysis

Suitability of primers for their uniqueness to amplify a single PCR product in conventional thermal cycler were assured. qPCR was carried out on standards using a real time thermal cycler (Stratagene MxPro3005P System). Melting curve analysis was carried out after the final amplification. A confirmation step using 2% agarose gel electrophoresis in TAE buffer confirmed the appropriate fragment sizes of all real time assay products.

Standard curves

The concentration of plasmids carrying the ZFX and SRY genes was measured using Nanodrop ND-1000 spectrophotometer. Using the average molecular weight of the product and mathematical calculations in accordance to the method by Rose'Meyer et al. (2003) and Applied Biosystems (2003), the number of copies per unit volume was calculated. The volume of the purified linear double stranded ZFX and SRY plasmid DNA standards was adjusted to 3.0 x 10^6 copies. This stock solution was serially diluted by 10 folds to obtain a standard series from 3.0 x 10^2 to 3.0 x 10^6 copies. A corresponding standard was prepared under the same conditions using different copy numbers in triplicates. The optimum real-time program is shown in *Table 4*. The C_T values were plotted against the logarithm of their initial template copy numbers under the optimum threshold based on the highest regression coefficient, r^2. Each standard curve was generated by a linear regression of the plotted points.

Quantitative PCR

The qPCR was performed using 10 µl IQ™ SYBR® Green Supermix (Biorad), 200 nmol forward primer, 200 nmol reverse primer, and the mixture was topped up with sterile water to a final volume of 20 µl. The spermatozoa DNA with various concentrations of plasmid copy number were used as a DNA template. The qPCR step was carried out together with triplicate of standard dilution to create standard curve. PCR conditions were as described in the previous section. The absolute copy of X- and Y- chromosome bearing spermatozoa was obtained by comparing the C_T value in the standard curves. The percentages of X- and Y- chromosome bearing spermatozoa were obtained.

Data analysis

A standard curve of 3.0 x 10^2 to 3.0 x 10^6 serial dilutions of plasmid molecules was

Table 4. The real time PCR program for ZFX and SRY primers using Stratagene MxPro3005P System

Step	Temperature	Time	Cycle
Initial denaturation	95 °C	10 min	1
Denaturation	94 °C	30 sec	45
Anealling	55 °C	30 sec	
Elongation	72 °C	30 sec	
Melting curve	72 – 98°C	Every 0.2 °C hold 1 second	

obtained and compared to the samples in order to get absolute copy numbers of X- and Y- chromosome bearing bovine spermatozoa. The average of copy numbers of X- and Y- chromosome bearing bovine spermatozoa obtained among the replicates.

Results

ZFX and SRY primers successfully amplified fragments of 474 bp and 525 bp from the genome of Kedah-Kelantan cattle (*Bos indicus*) respectively. The amplified fragments of ZFX and SRY were successfully cloned into vectors and transformed into *E. coli* competent cells. The sequencing and BLAST bioinformatic analysis results of ZFX and SRY cloned plasmids showed that the sequences were 99% and 98% homologous to ZFX gene with accession number NM177490 and SRY gene with accession number U15569 in GenBank sequences respectively (*Figure 1*).

The amplification specificity of plasmid DNA was verified by SYBR® Green melting curve analysis (*Figure 2*). Both ZFX and SRY primers showed a single melting peak at the melting temperature of 83.0 ºC and 85.0 ºC respectively. The results showed specific binding in the amplification of products. The electrophoresis bands from the end point of qPCR were done to reveal any formation of multiple non-specific amplification products (*Figure 3*). Agarose gel electrophoresis generated prominent bands with expected sizes from unclear band to obvious intake band due to higher copy of template plasmid. These results indicated that non-specific PCR product with the primer pairs was not detected in the analysed temperature range. Negative control of the SRY primers did not generate any visible peak, indicating that no contamination occurred in the reactions. The results of combined melting curve and

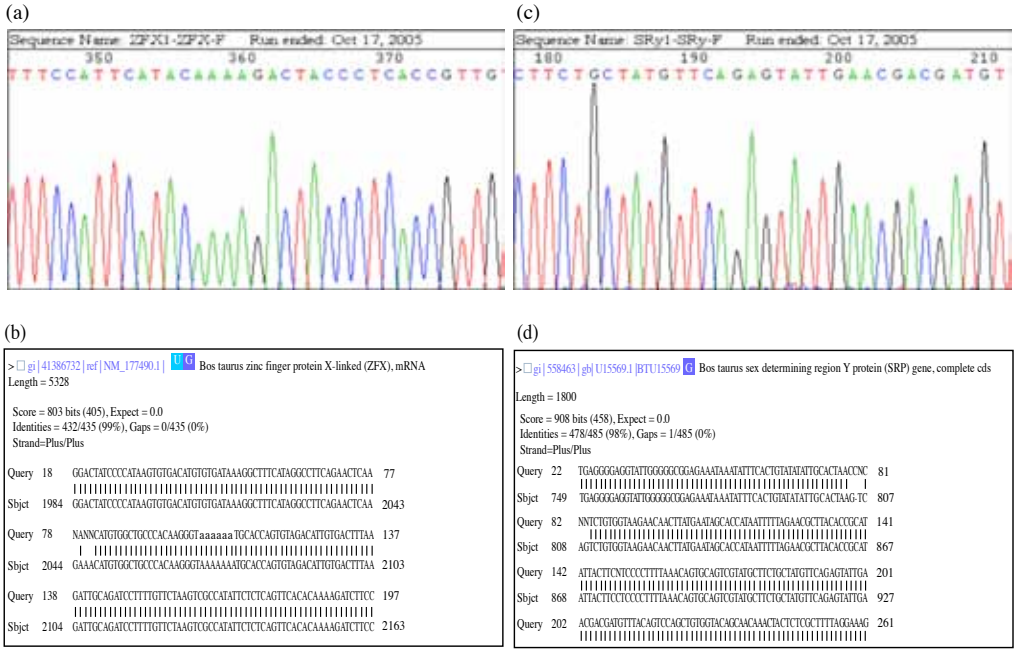

Figure 1. The (a) Sequencing result of ZFX; (b) BLAST analysis of ZFX (c) Sequencing result of ZFX; and (d) BLAST analysis of SRY plasmid. The results showed that sequences were 99% homologous to ZFX gene with accession number NM177490; and SRY gene with accession number U15569 in GenBank sequences, respectively

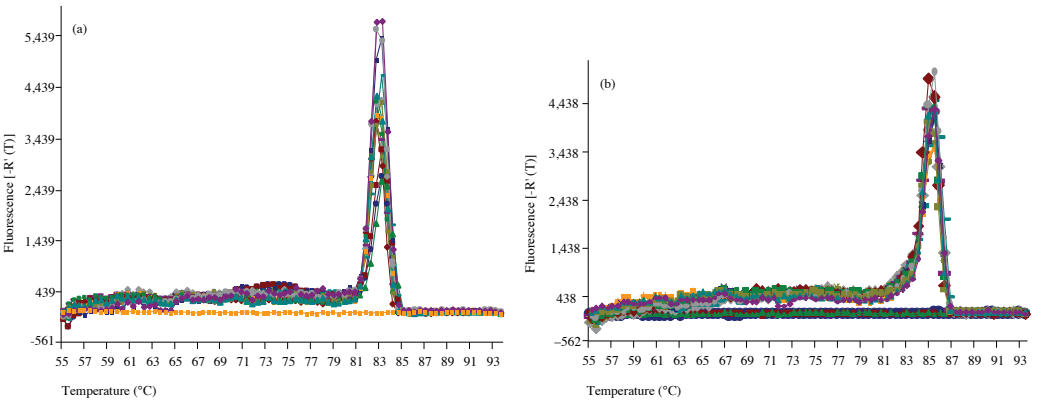

Figure 2. Melting curve of (a) ZFX and (b) SRY genes performed using Stratagene MxPro3005P System

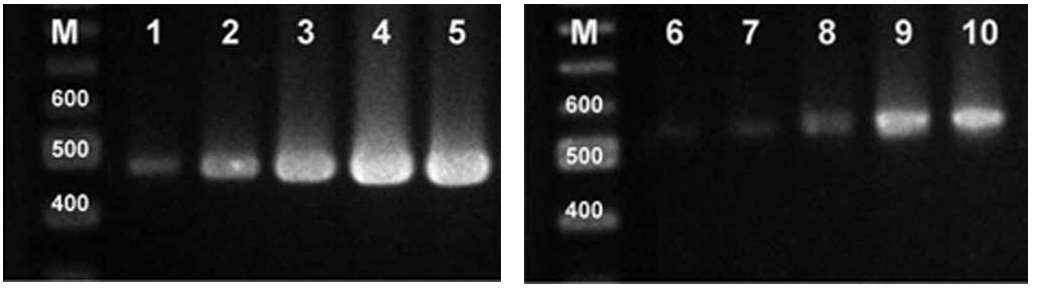

Figure 3. Gel electrophoresis on 2% agarose gel confirmed the specificities of real time PCR amplification for ZFX and SRY primers. Wells 1 – 5 indicated the standard of ZFX plasmid range from 3.0 × 10^2 to 3.0 × 10^6; 6 – 10 indicated the SRY plasmid range from 3.0 × 10^2 to 3.0 × 10^6; M is the 100 bp marker

gel electrophoretic analysis showed specific binding and amplification of both ZFX and SRY gene fragments.

The construction of standards curves of ZFX and SRY generated the linear relationship between the input plasmid DNA and the C_T values. The C_T values of assays ranged from 13.45 ± 0.40 (3.0 x 10^6) to 27.60 ± 0.50 (3.0 x 10^2) for ZFX, and 11.69 ± 0.10 (3.0 x 10^6) to 24.75 ± 0.06 (3.0 x 10^2) for SRY. *Table 5* shows the mean C_T values of replicate assays ranged from 3.0 x 10^2 to 3.0 x 10^6 for recombinant plasmid ZFX and SRY on the threshold of 409.0 generated from the qPCR software. From the standard curves, the mean coefficients of variation (CV) resulted were 2.43% and 1.39% for ZFX and SRY recombinant plasmid respectively

Table 5. The C_T value of standard curves for ZFX and SRY marker fragments recombination plasmid using quantitative real time PCR

Recombinant plasmid	C_T values	Copies number
ZFX	27.60 ± 0.50	3.0 × 10^2
ZFX	23.56 ± 0.03	3.0 × 10^3
ZFX	21.40 ± 0.47	3.0 × 10^4
ZFX	16.68 ± 0.87	3.0 × 10^5
ZFX	13.45 ± 0.40	3.0 × 10^6
SRY	24.75 ± 0.06	3.0 × 10^2
SRY	21.96 ± 0.28	3.0 × 10^3
SRY	18.48 ± 0.35	3.0 × 10^4
SRY	14.75 ± 0.40	3.0 × 10^5
SRY	11.69 ± 0.10	3.0 × 10^6

(*Figure 4*). A linear relationship between the input plasmid DNA and the C_T values with regression coefficient (r^2) more than 0.98 was obtained for both markers. The coefficient of determinations of standard curves for ZFX and SRY are $r^2 = 0.984$; $y = 3.517x + 36.28$ and $r^2 = 0.996$; $y = 3.333x + 33.25$, respectively (*Figure 4*). The results show that C_T values are the parameter by which quantified values are assigned in qPCR assay.

Absolute quantification was successfully performed using the standard curves constructed. The result from the absolute quantification of the DNA sample from spermatozoa is shown in *Table 6*. The percentages of X- and Y- chromosome bearing spermatozoa in the semen sample were 50.38 ± 5.38% and 49.62 ± 5.38% in six replicates, respectively.

Discussion

The ZFX and SRY genes were identified on the X- and Y- chromosomes and were used as gender markers in unsexed spermatozoa in this study. The ZFX fragment was specific for female that located on the X- chromosome and was not amplified in Y- chromosome bearing spermatozoa and vice-versa for the SRY amplification. Both ZFX and SRY genes exist as single copy on X- and Y- chromosome respectively in the bovine genome. Therefore, every single copy of ZFX and SRY sequence detected in qPCR indicated the presence of respective quantity of X- and Y- chromosome bearing spermatozoa. ZFX and SRY genes are commonly used as DNA markers in gender determination. Previous studies had demonstrated that ZFX gene had been used in sex determination of bovine embryo

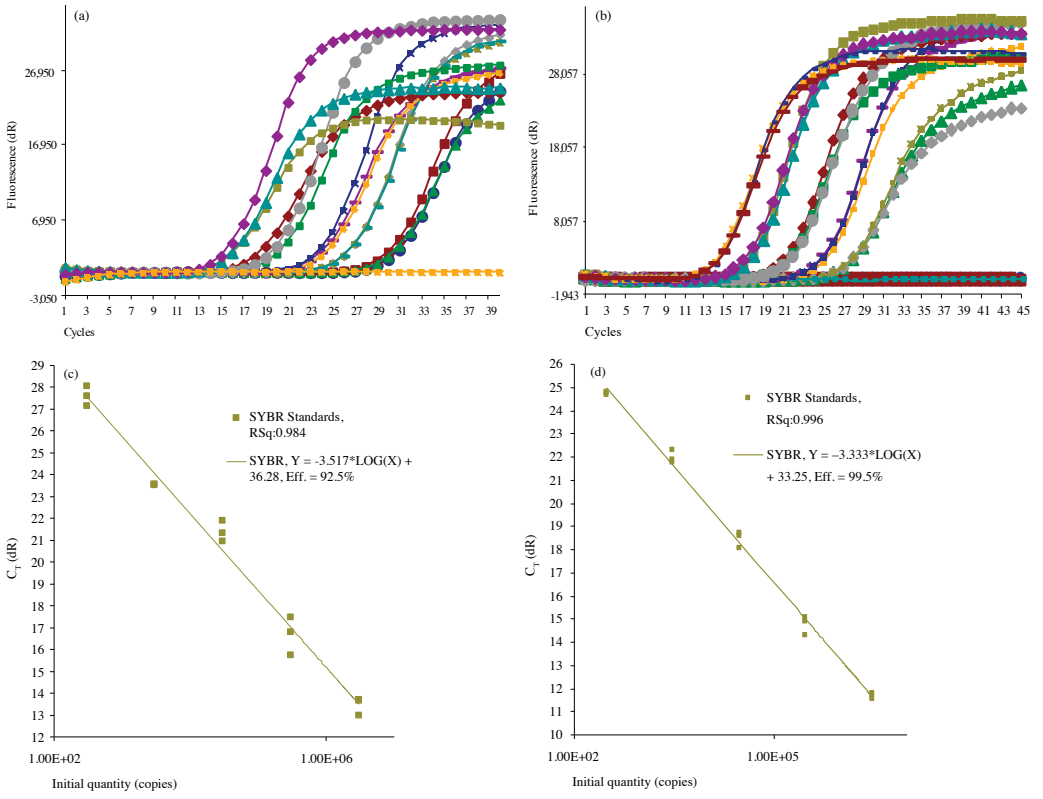

Figure 4. Amplification plot of (a) ZFX and (b) SRY; and standard graph of (c) ZFX ($r^2 = 0.984$; $y = 3.517x + 36.28$) and (d) SRY ($r^2 = 0.996$; $y = 3.333x + 33.25$) performed using Stratagene MxPro3005P System

Table 6. The percentage of X- and Y- chromosome bearing spermatozoa determined using quantitative real-time PCR

Sex	Time of reading						Mean
	1	2	3	4	5	6	
% X- spermatozoa	46.6	51.9	53.8	45.6	45.6	58.8	50.38 ± 5.38
% Y- spermatozoa	53.4	48.1	46.2	54.4	54.4	41.2	49.62 ± 5.38

blastomeres (Virta et al. 2002) and SRY gene has been used as a DNA marker for embryo sexing by PCR amplification (Bai et al. 2010).

In this study, the recombinant plasmid for ZFX and SRY were used to generate the standard curves where the cloned plasmids acting as carrier of the marker fragments. Plasmid containing cloned of a target sequence is commonly used as standard in qPCR (Whelan et al. 2003) and *E. coli* is the most popular host cell for bacterial recombination (Lee et al. 2006). In qPCR analysis, SYBR® Green was chosen as a DNA interchelating fluorescent dye due to several advantages over sequence-specific probes when singleplex PCR are performed. The dye is the simplest and least expensive compared to the presently known dyes (Leong et al. 2007). Based on the survey by Colborn et al. (2008), the SYBR® Green was frequently used in qPCR assays (85%) as compared to the TaqMan method that often cited as the reasons for favored usage of the SYBR® Green method (Hatt and Loffler 2012).

SYBR® Green dye tends to bind to all double-stranded nucleic acid molecules (Whittwer et al. 1997), hence the accumulation of primer dimers and the amplification of non-specific PCR products can be detected in SYBR® Green (Deprez et al. 2002). It had been reported that SYBR® Green detection was used to determine DNA contamination in plasmid preparations and provides a reliable alternative to both the traditional blotting methods and expensive TaqMan protocols (Lee et al. 2006). However, a comparative analysis by Hatt and Loffler (2012) of the melting curve and agarose gel electrophoresis results demonstrated that a single peak in post amplification does not always signify specific target gene amplification. Therefore, in this study the electrophoresis from the amplification fragments of the end point of qPCR were done to reveal any formation of multiple non-specific amplification products as a step for further validation and confirmation.

Preliminary qPCR analysis showed that the copy numbers below 3.0×10^2 gave inconsistent C_T values within the triplicates (Workenhe et al. 2008). Therefore, the copy numbers of the plasmid standards in this study were prepared from the ranged starting from 3.0×10^2. However, to maximise the accuracy in preparing standards, the 10-fold serial standard curve dilutions of ZFX and SRY plasmids were prepared over the range of copy numbers that include the amount of target DNA expected, of which in this study, reached up to 3.0×10^6. Besides, the C_T values were taken during log-linear reaction phase. Therefore, this procedure has the advantage of greater precision and more objectivity. These markers exist as single copies in X- and Y- chromosome bearing spermatozoa, where it is possible to quantify accurately the copy number of X- and Y- chromosome bearing spermatozoa in the semen sample, as the number of ZFX and SRY detected using the current method will illustrate the copy number of X- and Y- chromosome bearing spermatozoa in the samples.

From the X- and Y- chromosome frequencies in the samples, the quantity of spermatozoa did not differ much in the 1:1 ratio of X- to Y- chromosome bearing spermatozoa as reported in a normal unsexed mammals' spermatozoa population

(Rosenfeld and Roberts 2004; Luna-Estrada et al. 2006; Whyte et al. 2007). Unsorted semen samples were expected to be in the ratio of 1:1, according to the standard meiotic model, in which equivalent number of X- and Y- chromosome bearing spermatozoa are produced. The results of unsorted spermatozoa population is also supported by previous study using different method of spermatozoa determination (Parati et al. 2006; Colley et al. 2008). One of the advantages of using unsorted semen in this study is that it is easier to optimise qPCR reactions by using the samples with a known fraction of X- and Y- chromosome bearing spermatozoa ratio.

The copy number of ZFX and SRY plasmids was obtained in the standard curve using C_T values. Although qPCR using SYBR® Green assay is suitable for automation, this technique has to be optimised for other samples due to the efficiency and sensitivity of qPCR that may differ between samples (Joerg et al. 2004). In this study, ZFX and SRY gave different C_T value with the same gene copy number due to different length of ZFX and SRY fragments. The SYBR® Green dye will bind to the double stranded DNA and resulted in the variation of C_T value of both fragment with the same gene number copy. Therefore, different standard curves were obtained for ZFX and SRY genes in quantifying the X- and Y- chromosome bearing spermatozoa.

Conclusion

A quantitative analysis of sex determination bovine spermatozoa by real-time PCR was successfully performed using double-strand DNA-binding dye SYBR® Green. The qPCR technique in the present study is a rapid and reliable technique in quantifying the sex ratio of X- and Y- chromosome bearing spermatozoa in semen sample. This method is a suitable tool for routine verification of bovine X- and Y- chromosome bearing spermatozoa in sorted semen samples or for validation and calibration of other related techniques, not only in chromosome studies, but in any analysis of quantitative detection of various genes for other applications.

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Abstrak

Pengasingan jantina spermatozoa dalam pembiakan lembu dijalankan melalui teknologi pengisihan aliran bagi pengasingan spermatozoa pembawa kromosom -X dan -Y. Bagaimanapun, teknik pengasingan jantina tersebut perlu disahkan bagi menjamin kejituan teknologi. Kaedah penentuan jantina spermatozoa menggunakan teknologi kuantitatif PCR (qPCR) SYBR® Green telah dibangunkan. Dua set primer, ZFX dan SRY, masing-masing khusus untuk kromosom -X dan -Y lembu telah direka bentuk. Plasmid telah diselitkan dengan fragmen gen ZFX dan SRY secara berasingan dalam menjana graf piawai bagi julat daripada 3.0×10^2 hingga 3.0×10^6 salinan. Graf piawai hubungan linear menghasilkan pekali regresi $r^2 = 0.984$ untuk ZFX dan $r^2 = 0.996$ untuk SRY. Suhu lebur qPCR untuk kedua-dua plasmid ZFX dan SRY masing-masing diperoleh pada bacaan 83.0 ºC dan 85.0 ºC. qPCR masa nyata untuk DNA spermatozoa lembu dan plasmid yang diklon telah dijalankan. Peratusan spermatozoa pembawa kromosom -X dan -Y menunjukkan perbezaan yang tidak ketara daripada nisbah 1:1 seperti yang dilaporkan dalam populasi spermatozoa tidak terasing jantina. Oleh itu, sensitiviti yang tinggi dan kepantasan kaedah PCR masa nyata telah menjadikannya kaedah yang sesuai untuk mengkuantitatif spermatozoa pembawa kromosom -X dan -Y.