Original Article

Rapid Single Cell Typing using SYBR® Green Real-Time PCR Together with Melt Curve Analysis for Sex Identification of Porcine Sperm

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Abstract

Identification of X or Y chromosome is a very useful technique to verify the sex of boar sperm, but common methods used in pig such as Fluorescence In situ Hybridisation (FISH) and whole semen Polymerase Chain Reaction (PCR) have some limitations. FISH is highly accurate, but time-consuming (>3 days). Whole semen PCR is faster than FISH (3-6 h), but not highly accurate (approximate methods). The objective of this study was to develop a fast and highly accurate protocol to identify sex of boar sperm. In the present study, our team developed an alternative sex identification protocol using single cell SYBR® green real-time PCR technique together with low resolution melt curve analysis. Primers specific for chromosome 1 and chromosome Y, a high performance KAPA SYBR® DNA polymerase and Rotor gene PCR platform were used. Male and female single white blood cells were used to calculate sensitivity and specificity. Single sperm was picked up under inverted microscope and transferred to 1 µl of lysis buffer, and real-time PCR was run according to the programmed protocol and analyzed with melt curve analysis. Results showed that our method was a fast (<50 min) accurate method with high sensitivity (95-99%) and specificity (100%) with low percentage of PCR failure (< 3%). Validation of this method using boar whole semen detected Y sperm at 52% and X sperm at 48%, which was comparable to the theory ratio of X and Y sperm (50:50) in semen. It may be concluded that the single cell SYBR® green real-time PCR technique together with melt curve analysis is fast and accurate that can be used to identify sex of boar sperm.

Keywords: pig, real-time PCR, sex identification, single cell

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Introduction

Sex identification in pig is very important for research and diagnosis such as sex identification of sperm (Parrilla et al., 2003) and embryo sexing (Torner et al., 2013) as well as for the control of boar taint in meat products (Langen et al., 2010). Separation of X and Y sperm (sexing technology) can be used to select and control sex of offspring in pig (Vazquez et al., 2009). Development of sexing technology is very useful and important for the pig industry because it provides flexible farm management and an increase in product value and productivity (Johnson et al., 2005, Vazquez et al., 2009). Sex identification technique is used to verify the accuracy and purity of sperm sexing technology (Parrilla et al., 2003).

Polymerase chain reaction (PCR) (Wang et al., 2011) and fluorescence in situ hybridisation (FISH) (Parrilla et al., 2003; Eisenberg et al., 2012) are effective techniques to validate sexing sperm. An easier, cheaper and faster technique is the whole semen sex ratio PCR (Parati et al., 2006; Choi et al., 2009), but this technique is less accurate than single cell PCR.

Single cell PCR and FISH are the most accurate techniques for sperm typing (Harton and Tempest, 2012) but FISH is the only method which has been used to identify sex of porcine single sperm (Parrilla et al., 2003; Mohammadi et al., 2011). However, the main disadvantages of FISH are time-consuming (3-5 days) and labor intensive (Parrilla et al., 2003; Mohammadi et al., 2011; Eisenberg et al., 2012).

Use of conventional single sperm PCR has been mainly reported in cattle and human (Colley et al., 2008; Cui 1997; Wang et al., 2011), but not in pigs. All of PCR sex identification in pigs has been done in samples which have a high number of cells such as whole semen (Choi et al., 2009), embryos (Pomp et al., 1995; Sembon et al., 2008) tissues (Sathasivam et al., 1995; Ballester et al., 2012) and meat.(Langen et al., 2010; Abdulmawjood et al., 2012).

Therefore, the use of rapid single cell PCR for sex identification in pigs will be useful to verify sperm sexing technology. The objective of this study was to develop a fast and highly accurate technique to identify sex of boar sperm. In the present study we developed an alternative sex identification technique by using single cell SYBR® green real-time PCR together with melt curve analysis. This technique is fast and accurate. Melt curve analysis is an assessment of the dissociation-characteristics of double-stranded DNA during slowly increasing temperature and can be used to validate PCR product (Mergny and Lacroix 2003; Vossen et al., 2009).

Materials and Methods

Animals and Samples:

One to three year old pigs from a commercial farm in Nakhon Pathom province were used in the experiment. Blood samples were collected from 5 male and 5 female domestic pigs (Landrace x Large White). Frozen semen samples from 3 boars (Duroc) were used. Three frozen-thawed semen straws of each boar were used. Semen collection and cryopreservation were performed according to previous studies (Wongtawan et al., 2006; Kaeoket et al., 2012). This experiment was performed under the permission of the Faculty of Veterinary Science Animal Care and Use Committee (FVS-ACUC) Mahidol University.

Whole blood, semen lysis and conventional PCR: DNA was extracted from whole blood and sperm using Azupure DNA purification kit (BIOTEC, Pathum Thani, Thailand). Conventional PCR was performed using 2xTaq Master Mix (Vivantis, Selangor Darul Ehsan, Malaysia) (Cat.no.PLMM01) with PTC-200 Thermal cycle (MJ Research, Quebec, Canada). The PCR reaction included 25 µl of Master Mix, 1 µl of 10 µM forward primer, 1 µl (10 µM) of reverse primer, 2 µl of sample and 25 µl of DEPC water. DNA and.The conventional PCR program was as follows: pre-denaturation at 94°C for 2 min, denaturation at 94°C for 2 sec, annealing at 60°C for 30 sec, extension at 72°C for 15 sec. The PCR was performed 30 cycles and final extension at 71°C for 7 min. The process was repeated three times.

PCR Primers: Primers were synthesized by Sigma-Aldrich (St. Louis, MO, USA), and the primer sequences were designed and used in previous publications (Rubes et al., 1999; Parrilla et al., 2003; Choi et al., 2009, Mohammadi et al., 2011).

Chromosome 1, product size is 244 bp.
Forward primer: 5′TTGCACTTTCACGGACGCAGC3′
Reverse primer: 5′CTAGCCCATTGCTCGCCATAGC3′

Chromosome Y, product size is 377 bp.
Forward primer: 5′AATCCACCATACCTCATGGACC 3′
Reverse primer: 5′ TTTCTCCTGTATCCTCCTGC 3′

Single cell collection and lysis: Single sperm and single white blood cells were collected. The cells were diluted in 0.1% Poly vinyl alcohol (PVA) (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS) (PAA, GE Health Care, Velizy-Villacoublay, France) approximately 1,500 cell/ml. Five milliliters of suspension were poured into a 10 cm Petridish (Sterilin, Newport, UK) and observed under an inverted microscope (Olympus, Tokyo, Japan) using 400x magnification, and a single cell was collected using mouth pipette. Approximately 1 µl buffer contained a single cell and was released into 200 µl PCR tube (Axygen®, Corning, CA, USA).

The single cell was lysed with 5 µl of lysis buffer (200 mM KOH (Sigma-Aldrich, St. Louis, MO, USA) and 50 mM DTT (Vivantis, Selangor Darul Ehsan, Malaysia)) and incubated at 65 °C for 10 min, then 5 µl of neutralising buffer (900mM Tris-Cl, pH 8.3 (Promega, Madison, WI, USA), 300 mM KCl (Sigma-Aldrich, St. Louis, MO, USA), and 200 mM HCl (Merck Millipore, Billerica, MA,USA) were added.

Single cell SYBR® green real-time PCR: KAPA SYBR® Fast qPCR Master Mix (2x) Universal (KAPA Biosystem, Woburn, MA, USA) (Cat.no.KK4600) was used. The PCR reaction included 10 µl of MasterMix,

0.4 µl (10 µM) of forward primer and 0.4 µl (10 µM) of reverse primer. Reagents were added into a 200 µl PCR tube that already contained the lysed single cell.

Real-time PCR reaction program protocol was as follows: step 1, pre-denaturation at 95°C for 1 min; step 2, cycling, 95°C for 5 sec (denaturation), 58°C for 20 sec (annealing), performed for 35 cycles; Step 3, Final extension at 72 °C for 3 min; step 4, post PCR melt setting, temperature was increased from 72°C to 99°C, 1°C step, waited for 15 sec on first step and 4 sec on each step afterwards.

PCR platform was Rotor gene 6000 (Qiagen, Hilden, Germany). PCR results and melt curve analysis were analyzed using Rotor gene software 6.1 (Qiagen, Hilden, Germany). The process was repeated three times of each sample.

Sensitivity and Specificity: Sensitivity is the ability of a test to identify positive results, while specificity is the ability of test to identify negative results. Sensitivity is calculated by dividing number of PCR positive PCR by total number of positive control samples. Specificity is calculated by dividing number of PCR negative PCR by total number of negative control samples. Fluorescence signal of PCR positive products of both chromosome 1 and chromosome Y should arise before 25 cycles. Positive melt peak of chromosome 1 should be between 88 to 90°C. Positive threshold (–dF/dT unit) of the melt peak of chromosome 1 should be higher than 0.4 unit. Positive melt peak of chromosome Y should be approximately 85 to 87°C and positive threshold (–dF/dT unit) of the melt peak of chromosome Y should be higher than 1 unit. Failure of PCR reaction was calculated by number of negative PCR / total number of positive control samples.

Results

Validation of primer by conventional PCR: DNA samples extracted from male and female white blood cells were used to validate PCR primers. The results revealed that each primer had a specific band for chromosome 1 (Fig 1, lane 1) or chromosome Y (Fig 1, lane 2). The product of chromosome 1 was found in both male and female blood samples while the product of chromosome Y was found only in male blood samples (Fig 1, lanes 5-6). Both primers were also validated with whole semen samples (both X and Y sperm) and the results showed specific bands for both chromosome 1 and chromosome Y (Fig 1, lanes 3 and 7). Conventional PCR was also tested for single cell level detection, but it could not detect it. We, then, tested several Taq polymerases in our lab and found that only KAPA SYBR® DNA polymerase could detect chromosome 1 and chromosome Y at single cell level.

Identification of PCR product and nonspecific products by real-time PCR and melt curve analysis DNA from male and female white blood cells was used to identify the fluorescence pattern of PCR products during real-time PCR and post-PCR melt. The results showed different fluorescence patterns for each PCR product and nonspecific products during PCR (Fig 2A) and post-PCR melt (Fig 2B). After calculating with melt curve analysis software, the melt peak was observed as the negative derivative (–dF/dT) of the melt curves (Fig 2C). The melt peak is the melting temperature of the DNA sample that causes the highest rate of fluorescence decrease, at this point the double stranded DNA is 50% dissociated (Ririe et al., 1997). These results showed that the melt peak of chromosome Y was approximately 85 to 87°C while the melt peak of chromosome 1 was approximately 88 to 90°C (Fig 2C). For nonspecific products which are usually a primer dimer, (Brownie et al., 1997), the melt peak was generally lower than 80°C (Fig 2C) and it could also be separated from the PCR product by the PCR fluorescence pattern (Fig 2A) and post-PCR melt curve (Fig 2B). Notably, the fluorescence signal of primer dimer arose after 30 PCR cycles and the exponential curve was not sharp (Fig 2A).

Duplex PCR: The advantage of duplex PCR is that it can detect chromosome 1 and chromosome Y at the same time for a single sample. To perform duplex PCR both chromosome 1 and chromosome Y primers were added. Male and female white blood cells were tested. For conventional PCR and electrophoresis, the duplex conventional PCR was successful to identify sex of samples. Male samples revealed double specific DNA bands which represented chromosome 1 and chromosome Y whereas female samples revealed a single specific DNA band which represented chromosome 1 (Fig 1, lanes 9-12).

For the SYBR® green real-time duplex PCR, the florescence pattern during PCR was different between chromosome 1 and chromosome Y (Fig 3A). However, we found that both male and female samples gave a similar fluorescence pattern of post PCR melt (Fig 3B). The melt peaks were also found at the same temperature approximately 88 to 89°C whereabouts of chromosome 1 peak (Fig 3C). Therefore, SYBR® green real-time duplex PCR and its melt curve analysis could not distinguish between male and female samples.

Method sensitivity and specificity of single cell PCR: In this experiment, we investigated the sensitivity and specificity of this technique to detect chromosome 1 and chromosome Y in a single cell sample. The fluorescence pattern and melt curve analysis are shown in Fig 4 for chromosome 1, and Fig 5 for chromosome Y. Two hundred single female white blood cells were used as positive samples. Results showed that 198 cells were positive while 2 cells were negative. In the negative samples, no cell was added to 200 PCR tubes, and PCR reaction showed that all tubes were negative for chromosome 1. Therefore, the sensitivity of this technique for chromosome 1 was 99% while specificity was 100%.

DNA from male single white blood cells was used as positive samples and 200 of PCR reaction were tested. Results showed that 196 cells were positive for chromosome Y whereas 4 cells were negative. DNA from female white blood cells was used as a negative control.The results revealed that 200 female blood cells were negative for chromosome Y. Hence, the sensitivity of this method for chromosome Y was 97.5% and specificity was 100%. Single sperm real-time PCR with chromosome Y primer for whole semen sample.

In theory, the ratio of X and Y sperm in semen is approximately 50:50. To test the accuracy of this

technique, 400 of the single sperm were randomly picked and tested by real-time PCR. Two hundred sperm were used to identify chromosome 1 and another 200 sperm were used to identify the Y chromosome.

Results showed that 190 sperm were positive for chromosome 1, this means that the sensitivity of this technique for sperm is about 95% (Fig 6). For the sex ratio, 52% of the sperm (n=104) were positive for chromosome Y and 48% (n=96) were negative (chromosome X). The results are comparable to theory's ratio of X and Y sperm in pig semen. The fluorescence pattern and melt curve analysis were shown in Fig 6.

Discussion

The present study showed that using SYBR® green real-time PCR together with melt curve analysis was fast and accurate to identify the sex of a single cell such as sperm and white blood cells. High sensitivity and specificity were obtained with very low failure percentage using this method. The approximately 3% failure is considered lower than previous conventional single bovine sperm PCR (approximately 10%) (Wang et al., 2011), and comparable to FISH (approximately 2%) (Parilla et al., 2003).

One problem of the single sperm PCR technique is a low yield of PCR causing a failure of the test. As a result , several studies in human sperm (Li et al., 1990, Lien et al., 2002) applied two or more rounds of PCR to increase the sensitivity of detection, but multiple rounds of PCR were not necessary for our method. A single cell sample can be collected by manual or automated technique such as flow cytometer. Some previous studies collected single sperm from agarose gel (Lien et al., 2002, Wang et al., 2011), but we found that extraction of DNA directly from fresh samples produced better DNA yield than from agarose gel (unpublished observation). Picking up a single cell directly from the dilution buffer with a mouth pipette was not difficult and in our hand we could collect approximately 10 to 20 cells per minute.
The disadvantage of the SYBR® green PCR is that it can detect only one product at a time. In the identification of the sex of a single cell using SYBR® green dye, only chromosome X or Y can be detected for one sample. In this experiment, in which chromosome Y was used, if the sample was negative for chromosome Y, the sample was expected to contain chromosome X, with about a 97% possibility due to the failure of PCR (3%). Therefore, further development of this technique is to develop a way to identify chromosome 1, X and Y atthe same time. Using two to three different fluorescent primer probes might be possible. Duplex real-time PCR with two colour fluorescent primer probes has been used in determination of pig sex in meat and meat products (Abdulmawjood et al., 2012). However, the sensitivity of the probe for a single cell sample is still an unanswered question and the cost is more expensive than SYBR® green.

Moreover, the effectiveness of PCR reaction may increase by optimising PCR conditions such as MgCl2 and primer concentration. Most of the present study performed real-time PCR for 35 cycles, but we

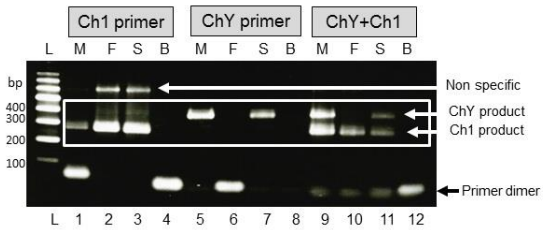

Figure 1 Conventional PCR used to detect chromosome 1 (Ch1) and chromosome Y (ChY). L=DNA Ladder, M=male white blood cell samples, F=female white blood cell samples, S=semen samples and B=blank. Lanes 1-4, chromosome 1 detection. Lanes 5-8, chromosome Y detection. Lanes 9-12, both chromosome 1 and Y detection (duplex PCR)

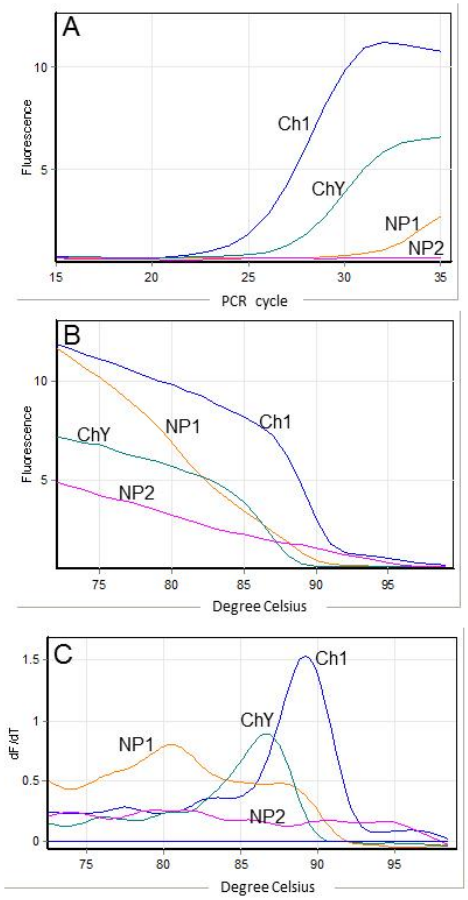

Figure 2 Identification of chromosome 1 (Ch1) and chromosome Y (ChY) using SYBR® green simplex real-time PCR and melt curve analysis. Samples are male white blood cells (Male), female white blood cells (Female) and no template control (NTC). (A) Fluorescence pattern of PCR product during PCR. (B) Fluorescence pattern of PCR product during PCR product melt. (C) Graph of melt curve analysis revealing specific peak for each PCR product. NS1 and NS2 are non-specific PCR products or primer dimer from NTC. NP1 represents samples with high amount of non-specific products while NP2 represents samples with small amount of nonspecific products

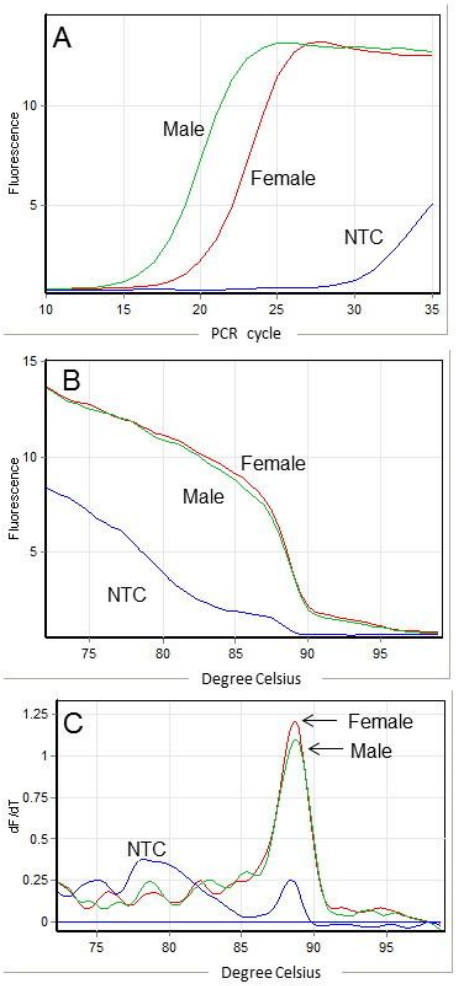

Figure 3 Fluorescence pattern of PCR product produced by SYBR® green duplex real-time PCR and melt curve analysis. Samples are male white blood cells (Male), female white blood cells (Female) and no template control (NTC). (A) Fluorescence pattern of PCR product during PCR. (B) Fluorescence pattern of PCR product during PCR product melt. (C) Melt curve analysis of real-time duplex PCR revealing similar melt peak pattern of male and female samples.

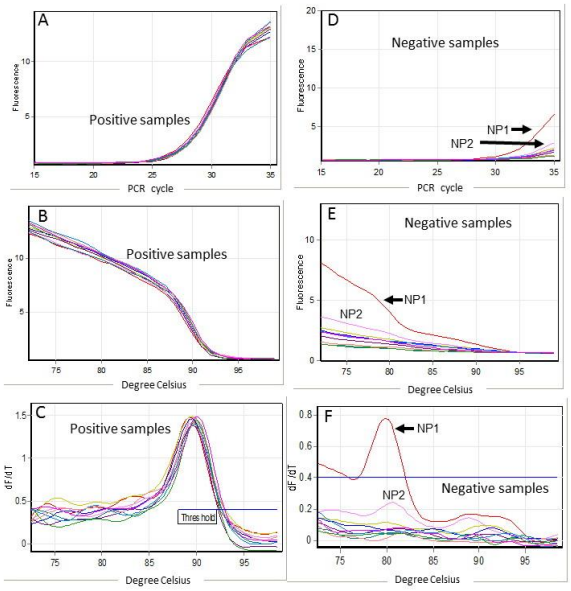

Figure 4 Sensitivity and specificity of SYBR® green real-time PCR and melt curve analysis for chromosome 1detection. (A-C) Positive samples. (D-F) Negative samples. (A and D) PCR fluorescence pattern during PCR reaction, PCR products were raised before 25 cycles whereas non-specific product (NP) were raised after 30 cycles. (B and E) Post PCR melt fluorescence pattern. (C and F) Melt curve analysis, the melt peak of PCR products ranged from 88 to 90°C and the threshold for df/dt unit of melt curve was 0.4. The melt peak of primer dimer is 80°C. NP1 represents samples with high amount of nonspecific products while NP2 represents samples with small amount of nonspecific products

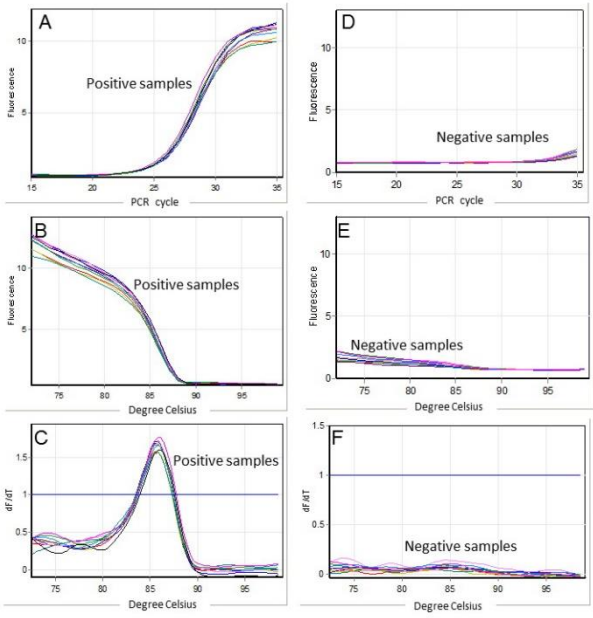

Figure 5 Sensitivity and specificity of SYBR® green real time PCR for chromosome Y detection. (A-C) Positive samples. (D-F) Negative samples. (A and D) PCR fluorescence pattern during PCR reaction, PCR product was raised before 25 cycles whereas nonspecific product was raised after 30 cycles. B and E are post PCR melt fluorescence pattern. C and F are melt curve analysis, the melt peak of PCR products ranged from 85-87°C. The threshold for df/dt unit of melt curve was 1.

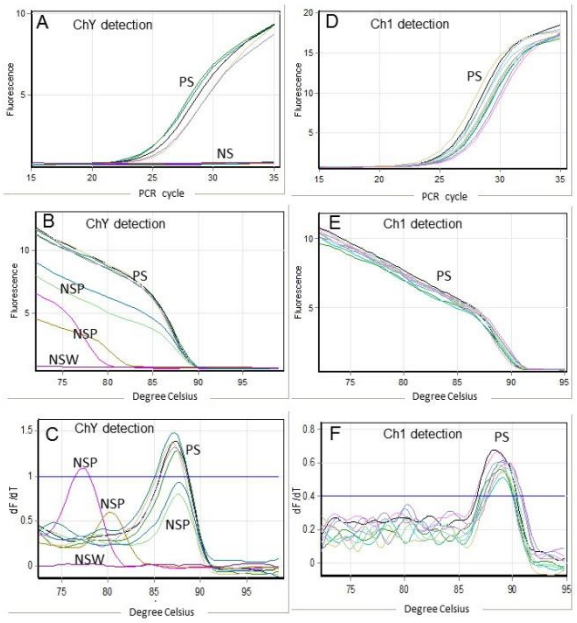

Figure 6 Sex identification of semen samples using single sperm SYBR® green real-time PCR. (A-C) Chromosome Y detection. (D-F) Chromosome 1 detection. PS represents positive sample (Sperm Y). NS represents a negative samples (Sperm X). NSP represents a negative sample with nonspecific products (Sperm X). NSW represents a negative sample without nonspecific products (sperm X).

found that it was possible to reduce the cycle to 30 cycles.

In conclusion, this is the first report using single sperm SYBR® green real-time PCR technique together with melt curve to identify the sex of sperm. This technique is fast, accurate and effective. It can be used to identify the sex of a single sperm and other cells. Further development of this method might be applicable to apply in single sperm typing in other genes or chromosomes and other species for research and diagnosis.

Acknowledgements

We thank the staff from the Semen laboratory and Molecular unit for their collaboration and expertise on this project. Dr. Neel Aziz and Dr. Nlin Arya for their English proof reading. This project was funded by the Thailand Research Fund (TRF; MRG5580026) and the Faculty of Veterinary Science, Mahidol University to Dr. Tuempong Wongtawan.

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บทคัดย่อ

การตรวจเพศตัวอสุจิในระดับเซลล์เดี่ยวแบบรวดเร็วโดยใช้เทคนิคปฏิกริยาลูกโซ่พอลิเมอเรสแบบเรียลทามโดยใช้สีไซบ้ากรีนร่วมกับการวิเคราะห์กราฟของการสลายดีเอ็นเอ

วราภรณ์ กอชื่นจิตร[1] กัมพล แก้วเกษ[2,3] ยินดี กิติยานันท์[4,5] เติมพงศ์ วงศ์ตะวัน[1,2,6]*

การตรวจแยกเพศอสุจิ เป็นเทคนิคที่สำคัญที่จะใช้ในการหาปริมาณและอัตราส่วนของเพศตัวอสุจิสุกร แต่เทคนิคที่ใช้กันทั่วไปในการตรวจเพศอสุจิของสุกร เช่น การติดฉลากดีเอ็นเอด้วยสารเรืองแสง (FISH) และ การใช้เทคนิคปฏิกริยาลูกโซ่พอลิเมอเรสตรวจตัวอสุจิเป็นกลุ่มในน้ำเชื้อ (whole semen PCR) ยังมีข้อจำกัดอยู่หลายอย่าง การติดฉลากดีเอ็นเอด้วยสารเรืองแสงเป็นเทคนิคที่แม่นยำ แต่ใช้เวลาในการตรวจนาน (มากกว่า 3 วัน) ในขณะที่การใช้เทคนิคปฏิกริยาลูกโซ่พอลิเมอเรสตรวจตัวอสุจิเป็นกลุ่มในน้ำเชื้อเป็นเทคนิคที่ทำได้เร็วกว่า (ใช้เวลา 3-6 ชม.) แต่มีความแม่นยำน้อยกว่าเพราะเป็นเทคนิคที่ใช้การประมาณการ จุดประสงค์ของการทดลองนี้คือ เพื่อพัฒนาเทคนิคใหม่ที่เร็วกว่าการติดฉลากดีเอ็นเอด้วยสารเรืองแสง แต่แม่นยำกว่าการใช้เทคนิคปฏิกริยาลูกโซ่พอลิเมอเรสตรวจตัวอสุจิเป็นกลุ่มในน้ำเชื้อ ในการทดลองนี้คณะวิจัยได้พัฒนาเทคนิคที่รวดเร็วและแม่นยำในการตรวจเพศอสุจิ โดยใช้เทคนิคปฏิกริยาลูกโซ่พอลิเมอเรสแบบเรียลไทม์กับเซลล์เดี่ยวโดยใช้สีไซบ้ากรีน (single cell SYBR® green real-time PCR) ร่วมกับการวิเคราะห์กราฟการสลายดีเอ็นเอ (Melt curve analysis) โดยใช้ไพรเมอร์ที่จำเพาะต่อโครโมโซม 1 และโครโมโซมวาย เอ็นไซม์ KAPA SYBR®DNA polymerase และ เครื่อง Rotor gene PCR platform ในการทำการทำปฏิกริยาลูกโซ่พอลิเมอเรส และใช้เซลล์เม็ดเลือดขาวเดี่ยวจากสุกรทั้งเพศผู้และเพศเมียในการวิเคราะห์ความไวและความจำเพาะของเทคนิค จากผลการทดลองพบว่า เทคนิคนี้จะใช้เวลารวดเร็ว (<50 นาที) และแม่นยำ โดยความไวของเทคนิคประมาณร้อยละ 95-99 และร้อยละความจำเพาะ 100 มีโอกาสในการวิเคราะห์ตัวอย่างผิดพลาดประมาณ ร้อยละ 3 เมื่อทำการทดสอบกับน้ำเชื้อสุกร พบว่าในน้ำเชื้อมีตัวอสุจิวายร้อยละ 52 และตัวอสุจิเอ็กร้อยละ 48 ซึ่งตรงกับทฤษฎีว่าอัตราส่วนของตัวอสุจิเอ็กและตัวอสุจิวายในน้ำเชื้อสุกรจะมีประมาณ 50:50 ผลการทดลองอาจสรุปได้ว่าการตรวจเพศเซลล์อสุจิด้วยเทคนิคปฏิกริยาลูกโซ่พอลิเมอเรสแบบเรียลทามกับเซลล์เดี่ยวโดยใช้สีไซบ้ากรีนนั้นเป็นเทคนิคที่รวดเร็วและแม่นยำ

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