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Determination of male-specific gene expression of White Lamphun cattle semen treated by monoclonal antibodies

SIRIKRON DUMRONGSRI[1], T KHAMLOR[2], P PONGPIACHAN[3] and K SRINGARM[4]

Chiang Mai University, Chiang Mai 50200 Thailand

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ABSTRACT

In the present study, male-specific monoclonal antibodies, P1C2B8 and P1C2C9, were used for sperm sexing via agglutination and cytotoxic reactions in White Lamphun cattle semen no. 862, no. 863 and no. KL. Live sperm percentage was used to find out an optimum dilution of antibody in agglutination and cytotoxic reactions and multiplex real time polymerase chain reaction was used to determine male-specific gene expression in each optimum dilution. In agglutination treatment, the results showed that all cattle had the same optimum antibody dilution while in cytotoxicity treatment, each cattle had different optimum antibody dilution. The results from multiplex real time polymerase chain reaction also showed that in both agglutination and cytotoxicity treatments, male-specific gene expression in semen samples was lower in cattle no. 862, no. 863 and no. KL when compared to control treatment. From these results, it was implied that antibodies P1C2B8 and P1C2C9 could alter the sperm sex ratio by reducing Y sperm in White Lamphun cattle semen.

Key words: Male-specific gene expression, Monoclonal antibodies, Multiplex real time polymerase chain reaction, Sperm sexing

White Lamphun cattle is one of the important native breeds of beef cattle in northern Thailand because they can be raised in malnutrition area and harsh environment. However, beef production is not enough for consumption because the numbers of beef cattle are low due to lack of parent stock (Department of Livestock Development, Thailand, 2015). Therefore, female calves are preferred for herd replacement and enlarging herd size. Several methods of sex pre-selection are based on sperm sexing which attempts to distort sperm sex ratio. Currently, the widely used method is flow cytometry, which is considered to be the most reliable procedure. However, the disadvantages of flow cytometry are the high cost of equipment, complicate procedure, low speed of sorting and small amount of sorted sperm/h (Johnson *et al*. 1989, Seidel Jr. 2003). Alternatively, agglutination reaction (Hoppe and Koo 1984) which uses male-specific antibody and cytotoxic reaction (Bennett and Boyse 1973) that uses male-specific antibody with complement are interesting procedures for sperm sexing. These methods are attractive due to low sperm damage and are cost-effective. The consequence of both methods is reduced Y sperm in the whole sperm population leading to increased female offspring. To investigate the result of sexed semen samples, real time polymerase chain reaction (real time PCR) was used. This technique can detect the amount of target DNA in each cycle with the increasing of fluorescence signal (Valasek and Repa 2005). Also, multiplex real time PCR performed by using fluorescent labeled-Taqman probes to investigate male-specific and female-specific genes in the same tube was reported by Khamlor *et al*. (2014). To carry out sex determination, the specific genes, i.e., sex-determining region Y (SRY) gene which is expressed on Y chromosome (Gubbay *et al*. 1990) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, which is expressed on autosomes (Robert *et al*. 2002) were used (Gokulakrishnan *et al*. 2012). SRY gene is widely used as it plays an important role in mammalian male development and is located on Y chromosome (Shende *et al*. 2014). Thus, the expression of SRY gene can be used to assess the amount of Y sperm in bovine semen (Li *et al*. 2011). Therefore, the objective of this study was to perform sperm sexing in White Lamphun cattle semen by using male-specific monoclonal antibodies. The optimum reaction of each cattle was determined by live sperm percentage and evaluated male-specific gene expression of sperm by multiplex real time PCR to determine the effect of antibodies on reducing Y sperm.

Present address: [1]Ph D Scholar (tang_oni@hotmail.com), [3]Associate Professor (ptpngpchn@gmail.com), [2,4]Lecturer (trisadee.kha@cmu.ac.th, korawan.s@cmu.ac.th), Department of Animal and Aquatic Science and Center of Excellence on Agricultural Biotechnology (AG-BIO/PERDO-CHE), Bangkok.

MATERIALS AND METHODS

White Lamphun cattle semen: White Lamphun cattle

semen no. 862 and no. 863 was obtained from Changrung farm, Doi Lo district, Chiang Mai and no. KL was acquired from Department of Livestock Development, Chang Phueak sub-district, Chiang Mai. Frozen-thawed semen with live sperm percentage of 35–40% were used in the study.

Male-specific antibodies: Monoclonal male-specific antibodies from clone no. P1C2B8 and P1C2C9 were prepared (Dumrongsri *et al*. 2014). The concentration of each antibody was quantified spectrophotometrically at 260 nm for 2.2 mg/ml.

Complement: To prepare complement, blood samples from healthy female New Zealand White rabbits were collected. Blood samples (10 ml) were collected and centrifuged at 100 × g for 5 min. Subsequently, the serum was collected and stored at –20°C until use. Diluted complement was optimized prior to use by selecting the dilution that caused lower toxicity to sperm (10% sperm death).

Sperm preparation: White Lamphun cattle semen was thawed at 37°C for 40 sec. Then, the semen was added into 10 ml of Beltsville-TS (BTS) extender (Johnson *et al*. 2000). Sperm was centrifuged at 100 × g for 5 min and the supernatant was discarded to collect the sperm pellet. A final concentration of 5×10^6 sperm/100 µl was obtained by adding BTS extender.

Primers and probes: Primer and probe sequences for amplification and detection of house-keeping gene and Y-chromosome specific gene used are shown in Table 1 (Li *et al*. 2011). Taqman probes in the multiplex real time PCR were labeled at the 5´ end with different fluorophores, i.e., HEX for Y-chromosome specific probe and FAM for house-keeping probe. BHQ was incorporated at the 3´ end of all probes to permit fluorescent detection of specific product. All primers and probes were synthesized by Bioline, USA.

Table 1. Primers and probes in real time PCR

Name and nucleotide sequence
GAPDH Primer Forward: 5´-GGCGCCAAGAGGGTCAT-3´
GAPDH Primer Reverse: 5´-GGTGGTGCAGGAGGCATT-3´
GAPDH Probe: 5´ TACTTCTCGTGGTTCACGCCCATCACA -3´
SRY primer Forward: 5´-GAAAATAAGCACAAGAAAGTCCAG G-3´
SRY Primer Reverse: 5´-CAAAAGGAGCATCACAGCAGC-3´
SRY Probe: 5´-TCCGCCGAAATCCGTGTAGCCA-3´

Evaluation of the specificity of male-specific monoclonal antibodies on sperm: To evaluate specificity of the antibodies, the experiment was designed as shown in Table 2. Each treatment was tested in triplicate. Briefly, the sperm concentration at 5×10^6 sperm/100µl was used in each treatment. The diluted antibodies from clone no. P1C2B8 or no. P1C2C9 were added (100 µl) into agglutination and cytotoxicity treatments. The samples were then incubated at 37°C for 15 min. Subsequently, the complement was added (100 µl) into both complement and cytotoxicity treatments, and the samples were continuously incubated at 37°C for 15 min. After reaction, each sample

Table 2. Evaluation of antibody specificity on sperm

Treatment	Antibody P1C2B8	Antibody P1C2C9
Control	1. Control	1. Control
Complement	2. Complement	2. Complement
Agglutination	3. Antibody 1:250	3. Antibody 1:10
	4. Antibody 1:1000	4. Antibody 1:100
	5. Antibody 1:5000	5. Antibody 1:1000
Cytotoxicity	6. Antibody 1:250 + Complement	6. Antibody 1:10 + Complement
	7. Antibody 1:1000 + Complement	7. Antibody 1:100 + Complement
	8. Antibody 1:5000 + Complement	8. Antibody 1:1000 + Complement

was centrifuged at 150 × g for 5 min and incubated at 37°C for 10 min. The swim-up sperm (100 µl) of each treatment was collected.

To investigate live sperm percentage, the sperm samples were stained with fluorescence color, i.e., SYBR 14 (green color stained live sperm) and propidium iodide (red color stained dead sperm) according to manufacturer's protocol. After staining, the samples were washed with 1 ml of phosphate-buffered saline (PBS), then centrifuged at 250 × g for 30 min. The sperm pellet was determined for live sperm percentage under the fluorescence microscopy. The reactions that caused highest sperm death were expected to maximally reduce Y sperm, so those reactions were continuously determined by multiplex real time PCR.

Evaluation of male-specific gene expression using multiplex real time PCR: The optimum dilution of each treatment used is shown in Table 3 and the sexing method was done as described in previous experiment. Each treatment was tested in triplicate. The swim-up samples of all treatments were collected and washed by using 1 ml of PBS and centrifuged at 250 × g for 30 min. The DNA pellet of each sample was collected and extracted by adding 100 µl of 5% (w/v) chelex, 7 µl of proteinaseK (10 mg/ml) and 7 µl of 1 M dithiothreitol (DTT). The samples were then incubated at 56°C overnight. Then, the samples were boiled at 90°C for 10 min. The DNA samples were collected. The DNA sample of 4 µl was used as a template for amplification.

In brief, the real time PCR chemicals per reaction and process are described in Table 4 and Table 5.

Determination of male-specific gene expression: Gene expression in each sample was determined by using multiplex real time PCR-based technique. GAPDH primers were used to amplify a reference gene and SRY primers were used to amplify male-specific gene. Cycle threshold (C_t) value via delta-delta Ct ($\Delta\Delta C_t$) method was used to quantify target gene in specimen as previously described by Livak and Schmittgen (2001), Pfaffl (2006).

$\Delta\Delta C_t$= Delta C_t (ΔC_t) of treatment- Delta C_t (ΔC_t) of control

Whereas ΔC_t= C_t of target gene- C_t of reference gene

Table 3. The optimum dilution in agglutination and cytotoxicity treatments of antibodies P1C2B8 and P1C2C9

Treatment	Cattle no. 862	Cattle no. 863	Cattle no. KL
Control	1. Control	1. Control	1. Control
Agglutination	2. Antibody P1C2B8 1:250	2. Antibody P1C2B8 1:250	2. Antibody P1C2B8 1:250
	3. Antibody P1C2C9 1:10	3. Antibody P1C2C9 1:10	3. Antibody P1C2C9 1:10
Cytotoxicity	4. Antibody P1C2B8 1:5000 + Complement	4. Antibody P1C2B8 1:1000 + Complement	4. Antibody P1C2B8 1:250 + Complement
	5. Antibody P1C2C9 1:100 + Complement	5. Antibody P1C2C9 1:10 + Complement	5. Antibody P1C2C9 1:10 + Complement

Table 4. Real time PCR chemicals per reaction

Chemical	Amount
SensiFAST^{TM} probe No-Rox kit	10 µl
SRY forward primer (10 µM)	0.8 µl
SRY reverse primer (10 µM)	0.8 µl
SRY probe (10 µM)	0.2 µl
GAPDH forward primer (10 µM)	0.8 µl
GAPDH reverse primer (10 µM)	0.8 µl
GAPDH probe (10 µM)	0.2 µl
Deionized water	2.4 µl

Table 5. Real time PCR process

Step	Condition	Cycle (s)
Denaturation	5 min at 95°C	1
Denaturation and annealing	15 sec at 95°C and 45 sec at 56.5°C	40
Final extension	10 min at 72°C	1

Statistical analysis: The live sperm percentage data were compared by Chi-square test. The male-specific gene expression was shown in mean±SE, then compared by one-way ANOVA and followed by Least Significant Difference test for multiple comparison. All statistical analyses were considered significant at P<0.05 by using the Statistical Package for the Social Sciences (SPSS), version 17.0 software.

RESULTS AND DISCUSSION

Evaluation of antibody specificity: The specificity of the antibodies on sperm in both agglutination and cytotoxic treatments were determined by live sperm percentage. The lowest live sperm percentage was calculated to define the optimum reaction in each bull. In agglutination treatment, the results from monoclonal antibody P1C2B8 showed that optimum dilution for cattle no. 862, no. 863 and no. KL was 1:250 with sperm death at 14.96, 23.57 and 15.12% (P<0.05), respectively, when compared to the control treatment. Moreover, monoclonal antibody P1C2C9 showed that optimum dilution for cattle no. 862, no. 863 and no. KL was 1:10 with sperm death at 32.41, 43.71 and 13.49% (P<0.05), respectively, when compared to the control treatment. The difference of antibody dilution between clone no. P1C2B8 and no. P1C2C9 used in the experiment showed that each clone produced antibody differently. From antibody preparation process, many clones occurred in the same well before limiting to achieve a single cell. The same antigen can activate a variety of antibody (Roitt *et al*. 1996), so clone no. P1C2B8 and no. P1C2C9 had produced antibody differently. To sex sperm, antibody could immobilize the sperm and cause agglutination (data not shown) which was similar to the study of Yakirevich and Naot (1999). Therefore, the live sperm percentage was used to evaluate the effect of antibody on sperm. The results showed that sperm had a high death rate at the high dilution of antibodies, which was similar to the study of Saling and O'rand (1982). The study showed that percentage of immobilized sperm was high according to the concentration of antibody. In cytotoxicity treatment, the optimum monoclonal antibody dilution of P1C2B8 in cattle no. 862, no. 863 and no. KL was 1:5000, 1:1000 and 1:250, respectively. These dilutions caused sperm death at 12.99, 23.14 and 13.15% (P <0.05), respectively, when compared to the control treatment. Moreover, the optimum antibody

Table 6. Male-specific gene (SRY gene) expression in agglutination and cytotoxicity treatments as determined by real time PCR

Treatment	Cattle no. 862 mean±SE (n=3)	Cattle no. 863 mean±SE (n=3)	Cattle no. KL mean±SE (n=3)
Control	1±0.03^{a}	1±0.03^{a}	1±0.02^{a}
Antibody P1C2B8	0.47±0.04^{bc}	0.61±0.04^{b}	0.84±0.19^{a}
Antibody P1C2C9	0.44±0.01^{c}	0.55±0.03^{b}	0.63±0.07^{a}
Antibody P1C2B8 with complement	0.40±0.04^{c}	0.56±0.03^{b}	0.81±0.19^{a}
Antibody P1C2C9 with complement	0.57±0.04^{b}	0.63±0.03^{b}	0.61±0.07^{a}

^{a,b,c}, column means with different lower case superscripts are significantly different (P<0.05).

dilution of P1C2C9 for cattle no. 862, no. 863 and no. KL was 1:100, 1:10 and 1:10, respectively, with sperm death at 24.73, 35.12 and 12.47% ($P < 0.05$), respectively, when compared to the control treatment. The result from this study was similar to the study of Piedrahita and Anderson (1985) who showed that the highest death of sperm depend on an optimum dilution in cytotoxic reaction. Rooney *et al*. (1992) showed that the cytotoxic reaction activates the formation of membrane attack complex (MAC) and deposition of MAC on the sperm surface. This phenomenon could lyse the sperm and lead to sperm death (Goldberg *et al*. 1971, Rooney *et al*. 1992). From these results, the difference of optimum antibody dilution which was used in each bull implied that each bull had different amount of H-Y antigen on the sperm surface according to Manca *et al*. (1991). The study showed that the optimum antibody/antigen ratio could activate the proper reaction. Therefore, the determination of optimum antibody prior to use in the experiment should be carried out.

Evaluation of male-specific gene expression in sperm using multiplex real time PCR: In our previous experiment, the reactions which caused highest sperm death in each bull were selected to quantify gene expression by using multiplex real time PCR method. The SRY primers were used to determine SRY gene expression in the samples to identify male-specific gene which is located on Y chromosome. In cattle no. 862 and cattle no. 863, the results showed that antibodies P1C2B8 and P1C2C9 could lower SRY gene expression with statistical significance ($P<0.05$) while cattle no. KL showed that antibodies P1C2B8 and P1C2C9 could lower SRY gene expression with no statistical significance ($P>0.05$) in both agglutination and cytotoxicity treatments (Table 6). The SRY gene expression between agglutination and cytotoxic reaction had no difference in cattle no. 863 and no. KL. The result confirmed that using of male-specific monoclonal antibody in agglutination and cytotoxicity treatments could reduce Y sperm in 3 bulls because the expression of SRY gene was lower which showed that Y sperm was reduced. To evaluate gene expression on target DNA, the delta-delta C_t method was used to determine gene expression of interested gene via real time PCR process (Lawniczak and Begun 2007, Makarova *et al*. 2011), which was found unnecessary to use because it relies on the relative quantification of a target gene versus normalization genes (Makarova *et al*. 2011). The advantage of this method are reduction of the cost of chemicals, less time-consuming and easier procedure. Therefore, the lower SRY gene expression could imply that the Y sperm was reduced by agglutination reaction, leading to an increase of female offspring which was similar to the previous study of Hoppe and Koo (1984). Bennett and Boyse (1973) and Shelton and Goldberg (1984) showed that cytotoxic reaction could reduce Y sperm and lead to an increase of female offspring.

In conclusion, we assumed that the agglutination and cytotoxic treatments in this study could reduce Y sperm which should make a great impact on the economics of beef production, so this method can be used as an alternative method for sperm sexing.

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