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Molecular Confirmation Test of Sexing Method on Limousin Cattle Sperm with Swim Up Technique

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Abstract: The purpose of this study was to determine the quality of the sperm and confirm the results of the separation of the sperm carrying the X and Y chromosomes using the molecular swim up method. Determining the calves' sex is a strategic step in the development of artificial insemination technology. One of the efforts to separate an X chromosome spermatozoa from a Y gene was carried out using the swim-up method, in the isotonic medium. This method was a preparation procedure, which allowed motile spermatozoa to migrate or swim from the lower to the upper layer of semen. Furthermore, this research aimed to test for the molecular confirmation of sexing method, through the use of the swim up technique. The motility and the viability parameters of the separated spermatozoa, were also microscopically tested for the quality. The separated sperm DNA was extracted by using the spin-column method, which was amplified by Polymerase Chain Reaction (PCR) at Sex-determining Region Y (SRY) in the Y chromosome area, and the GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) in the HMGB (High Mobility Group Box) location. Also, the sperm separation results in the upper layer and control, showed that there were 2 bands, namely the SRY gene (318 bp) and GAPDH (415 bp). This result further showed that after the swim up method, more Y genes were detected in the lower layer of the technique process. Moreover, the results showed that there was only 1 band of GAPDH (415 bp), which indicated the presence of X genes in the lower layer. Also, these results showed that sexing with the swim-up method was verified molecularly, in order to separate the spermatozoa carrying X and Y chromosomes, which were then tested using the duplex PCR method

Keywords: XY chromosome, swim up, Polymerase Chain Reaction, Sex-Determining Region Y, glyceraldehyde 3-phosphate dehydrogenase.

游動技術對利木贊牛精子進行性別鑑定的分子確證試驗

摘要：這項研究的目的是確定精子的質量，並使用分子游動法確認帶有X和Y染色體的精子的分離結果。確定小牛的性別是人工授精技術發展的戰略性步驟。使用游動法在等滲培養基中進行了將X染色體精子與Y基因分離的工作之一。該方法是一種製備方法，其使運動精子從精液的下層遷移到上層或從上層游動。此外，該研究旨在通過使用游泳技術來測試性別鑑定方法的分子確認。還對分離的精子的運動性和生存力參數進行了顯微鏡測試以檢測其質量。通過旋轉柱法提取分離出的精子脫氧核糖核酸，並通過聚合酶鏈反應在Y染色體區域的性別決定區Y和甘油醛3-
磷酸脫氫酶中擴增。（高移動性分組框）位置。另外，在上層和對照中的精子分離結果表明，存在兩個條帶，即性別決定区基因（318 bp）和甘油醛3-磷酸脱氢酶（415 bp）。該結果進一步表明，在游動法之後，在工藝過程的下層中檢測到更多的Y基因。此外，結果顯示甘油醛3-磷酸脱氢酶只有1條帶（415 bp），表明在下層存在X基因。而且，這些結果表明，用游泳法進行了性別鑑定，以分離出帶有X和Y染色體的精子，然後使

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用雙鏈聚合酶链反应方法對其進行了测試.

关键词：XY染色体，游动，聚合酶链反应，性别决定区Y，3-磷酸甘油醛脱氢酶。

1. Introduction

Spermatozoa sexing is one of the reproductive technology results considered to be a promising alternative, in order to reproduce children with desired sex. With varying results, this was also applied in artificial insemination (AI) and embryo transfer. The percentage of pregnancy rates in horses through the AI technique using spermatozoa sexing method, reached 47.6%, while it achieved 52% in cattle [1-2]. The success of pregnancy through the embryo transfer technique was 35.4% [3].

Also, the spermatozoa sexing technique is carried out by separating the X and Y chromosomes, based on differences in morphological characteristics, DNA content, macromolecular proteins on the two genes, with weight and movement of the seminal fluid [4-5]. It is also estimated that the DNA content of the X chromosome is 3-5% more, compared to the Y gene [6]. Based on these criteria, various spermatozoa separation techniques have been developed, such as the flow cytometer method [7], the Gradient Percoll technique [8], and the BSA gradient model.

One of the efforts to separate spermatozoa with X and Y chromosomes is carried out by means of swim-up method, in the isotonic medium [9], This method is a preparation procedure, which allows motile spermatozoa to migrate or swim from the lower to the upper layer of semen [10]. Furthermore, the separation of spermatozoa by swim-up method is based on differences in the swimming speed of X and Y chromosome, towards the media surface [11-12]. The Y-chromosome spermatozoa also has a faster swimming speed than the X gene. When the method is carried out, the Y chromosome swims to the upper layer (surface), much more faster than the X gene. Also, the number is very significant in the upper layer, compared to the population of the X chromosome. However, the spermatozoa population with the Y chromosome obtained in the lower layer is significantly less, compared to the density of the X gene [13]. Therefore, the success rate of spermatozoa separation of X and Y chromosome carriers in the swim-up method, is strongly influenced by the length of time to swim up [11].

Furthermore, confirming the sex separation of sperm is carried out with the Polymerase Chain Reaction (PCR) method, in order to determine the purity of the DNA samples, using certain primers. The Sex-Determining Region Y (SRY) gene located in the Y chromosome layer determines the male sex in sperm. The SRY gene amplification was an important tool for determining sex [14]. This gene is in a non-recombinant region on the Y chromosome, and has a single exon that codes for 204 amino acids. Also, the SRY structure has a central region correlating with HMG (home box, housekeeping gene, or GADPH), which has a conservative area with abilities to differentiate between species. The characteristics of SRY-HMG are mostly used for sex-based DNA research [15-16].

Moreover, the advantage of amplification, through the use of the SRY gene and polymerase chain reaction (PCR) methods, is relatively easy, fast, accurate, and affordable [17], compared to sexing sperm by a flowcytometer. The duplex PCR amplifies DNA simultaneously, by using multiple primers to prevent refraction results, when there is no SRY gene in the sample [18-19]. However, duplex PCR only tends to detect DNA in certain mammals, and has not been widely reported in cattle [20-21]. Therefore, the purpose of this study is to confirm the spermatozoa carrying X and Y chromosomes in the semen of Limousin cattle, sexed with molecular swim-up method. The novelty of this research is for the application of making frozen semen that has been sexed and for artificial insemination in the field requiring male or female calves. Artificial insemination is an attempt by humans to insert spermatozoa into the female reproductive tract using special equipment. The development of AI technology is not only limited to increasing the birth rate, but can also be used to produce calves with the sex desired by the farmer. This can be done through the use of sexed semen. Various sexing methods have been tried to produce semen containing spermatozoa carrying similar chromosomes (X or Y) and one method that is considered quite simple to do is the swim up method. Proving the sex separation of sperm using the swim up method can be done by using the Polymerase Chain Reaction (PCR) method which can determine the purity of the sample DNA using certain primers. Genes for Sex Determination Region Y (SRY), which is in the Y chromosome region, is a gene that determines male sex in sperm and this study has never been conducted by other researchers.

2. Materials and Methods

This research was conducted at the Faculty of Veterinary Medicine, Airlangga University Surabaya, and Denpasar Bali Veterinary Centre. The main material in this this research was genomic DNA, which was obtained from Limousin cattle semen straws, from

the Institute for Artificial Insemination (BBIB) Singosari. The genomic DNA was extracted using the QIAamp Mini spin column DNA extraction Kit, in order to degrade cell walls, proteins, and fats. Also, the DNA sample was ready to proceed with the PCR reaction. The tools used in this research included centrifuge, cooler, Eppendorf tube, micropipette, tip, tube rack, PCR machine, electrophoresis device, autoclave, scale, gloves, test tube, artificial vagina for semen collection. Also, the main ingredient was a DNA sample obtained from limousine cattle semen. Other supporting materials included, Primers, DNA Extraction Materials (Proteinase K, Absolute Ethanol, Buffer Lysis, Wash buffer A&B), PCR components (dNTP Mix, Taq DNA polymerase enzymes), Electrophoresis variables (Triss Base, boric acid, agarose, Na_2 EDTA, Sybr Safe, DNA Marker, DNA Loading dye), tissue and plastic mica, with physiological NaCl. Cattle semen was obtained from the Singosari Centre for Artificial Insemination, through approximately 20 Limousin cattle straw samples

2.1. Spermatozoa Separation using the Swim-Up Technique

The swim-up method is to separate the X and Y sperm chromosomes. The medium used to separate spermatozoa by the swim-up method was physiological NaCl solution. A total of 0.5 ml of fresh semen suspension was placed in a sterile test tube for each treatment group. Afterwards, 3 ml of physiological NaCl solution was added in a test tube containing 0.5 ml of fresh semen suspension, through the tube wall. Furthermore, the filled tube was placed on the rack in an upright position according to the swim-up time treatment and left for 10 minutes, at room temperature (27.0-28.5°C). Then the upper layer is taken to determine the XY chromosome and the lower layer for the XX chromosome. This research needs to be done because the molecular confirmation test of Sperm X chromosome or Y chromosome that has been sexed with Swim UP has many advantages, namely it is relatively easy, fast, accurate and affordable in terms of cost compared to sexing sperm using a flow cytometers. The duplex PCR is able to amplify DNA simultaneously using multiple primers to prevent refraction results if there is no Y fragment (SRY gene) in the sample [18]. However, duplex PCR can only detect DNA in certain mammals and has not been widely reported in cattle [14-21], in order to observe its quality parameters (motility, live and abnormal spermatozoa), by using a binocular microscope.

2.2. Spermatozoa Quality Examination Results in Separation with Swim-Up

The spermatozoa motility was calculated, using a glass object dripped with 10-15 µl of semen. Furthermore, the examination was carried out under a binocular microscope at 400x magnification, as the motile spermatozoa was observed to be moving forward. Also, the minimum observed spermatozoa were 100-200 cells in 5 fields of view, using a binocular microscope, as the number of motile seminal fluid was divided by the total number of visible sperm, and expressed in percent (%).

2.3. Viability and Motility

Calculation of the spermatozoa's live percentage was carried out through a staining technique, by mixing semen with eosin nigrosin solution on a glass object, making a preperate, and drying it. The dead spermatozoa showed a pink colour, while the living fluids were transparent. Moreover, the minimum number of spermatozoa observed was 100-200 cells in 5 fields of view, using a binocular microscope. Also, the living spermatozoa were counted and divided by the total number of the visible fluid (live spermatozoa + dead spermatozoa), and expressed in percent (%).

2.4. Spermatozoa Abnormalities

The morphology of spermatozoa abnormalities was also evaluated using eosin nigrosin stain method. It was observed with a binocular microscope at 400x magnification, as the minimum number of spermatozoa observed was 100-200 cells in 5 fields of view. Moreover, the abnormal spermatozoa obtained was divided by the total number of this normal fluid (normal spermatozoa + abnormal spermatozoa), and expressed in percent (%).

2.5. DNA Extraction

According to the work instructions contained in the tool, a total of 10 million cells/mL of sperm were extracted by the spin column method, through the Qiamp DNA Mini Kit. Also, the total sperm DNA obtained were stored at a temperature of -20°C, until the next stage was carried out.

2.6. Amplification of Polymerase Chain Reaction (PCR)

The total sperm DNA that had been extracted and amplified by the Duplex PCR method with a total volume of 25 µl, contained 12.5 µl of GoTag Green Master Mix (Promega), 1 µl of both F (10 pmol), and R primers (10 pmol), 8.5 µl of nuclease-free water (Promega), and 2 µl of DNA sample. Also, the two pairs of primers used were SRY F 5 'AAGGGAGAACAGTTAGGGAGAG 3' and SRY R 5 'ATCGGGTTGCATAGTATTGAAG 3', with GADPH F 5 'GTGGCGCCAAGA GGGTC ATCATC 3' and GADPH R 5 'GGTTTCTCCAGGCGGCAGGT 3' (Bioneer). Furthermore, amplification was carried out by a PCR (Applied Biosystem) machine. The PCR program consisted of predenaturation of 94°C for 5 mins, denaturation of 94°C for 30 secs, annealing at 60°C for 30 secs, and extension of 72°C for 30 secs,

with repeated cycles of 45 times. Afterwards, the cycle ended with a final extension of 72°C for 10 mins, accompanied by a hold time at 4°C [15].

2.7. Electrophoresis

The PCR product was then analyzed by electrophoresis on 1.5% agarose gel, using SYBr dye (Invitrogen S7563, North America). Molecular markers (Ladder, Invitrogen) for measuring 100 bp were also electrophoresed, in order to determine the size of the PCR product. Furthermore, the gel electrophoresis results were observed, using the gel documentation system, with the amplicon size determined through the use of the software provided in the gel doc.

2.8. Data Analysis

The X gene band only has 1 band at 415 bp, while the Y and unsexing sperm produced 2 bands at 415 bp and 318 bp, respectively.

3. Results

3.1. Sperm Sexing with the Swim Up Method

Limousin cattle semen with a total of 10 samples were sexed between male (XY) and female (XX) sperm cells, using the swim-up method. The result showed that 0.5 ml of sperm swimming up (upper layer) were collected and placed in a tube marked XY, as the seminal fluid in the lower layer were obtained and inserted in an identified apparatus, XX. However, the 10 samples of Limousin cattle semen with non-sexing did not use swim up method (Fig. 1).

Fig. 1 Limousin cattle spermatozoa separation using the swim up method (upper layer of male sperm (XY) cells; lower layer of female sperm (XX) cells)

3.2. Motility and Viability of Limousin Cattle Spermatozoa

The motility and viability of limousine cattle spermatozoa are shown in the Table 1.

Table 1 The motility and viability percentage of limousine cattle spermatozoa

No	Spermatozoa Non Sexing		Spermatozoa XX(lower part)		Spermatozoa XY (upper part)	
	Motility	Viability	Motility	Viability	Motility	Viability
1	45	51	46	50	45	51
2	44	50	44	53	46	52
3	46	50	43	55	43	55
4	46	52	43	51	47	51
5	43	48	44	50	45	54
6	44	50	42	52	46	52
7	45	51	46	53	44	54
8	46	49	44	50	43	51
9	44	50	45	51	46	52
10	45	51	44	54	45	54
Average	44.8	50.2	44.1	51.9	45	52.6

Based on Table 1 above, it was observed that the average percentage of motility and viability of limousin cattle spermatozoa in non-sexing were 44.8 and 50.2, XX were 44.1 and 51.9, while XY were 45 and 52.6.

3.3. Abnormalitas Spermatozoa Sapi Limousin

Abnormalities of limousine cattle spermatozoa are also shown in the Table 2 below.

Table 2 Percentage of abnormalities in limousine cattle spermatozoa

	Spermatozoa Non Sexing	Spermatozoa XX (lower	Spermatozoa XY (upper

		part)	part)
1	5	4	5
2	4	4	4
3	5	5	4
4	6	6	5
5	3	4	6
6	4	3	4
7	5	5	5
8	5	4	4
9	4	5	6
10	6	5	5
Average	4.7	4.5	4.8

Based on Table 2 above, it was observed that the average abnormalities percentage of limousin cattle spermatozoa on non-sexing was 4.7, XX was 4.5, and XY was 4.8.

3.4. Amplification of Limousin Cattle Sperm DNA

DNA had been extracted accurately from 8 samples of each male and female sexing sperm cells (XY & XX), with 10 from non-sexing spermatozoa. Also, the results of electrophoresis showed that the sperm collected from the swim up method (X sperm) only had 1 band at 415 bp, while Y and non-sexing spermatozoa produced 2 bands at 415 and 318 bp (Figure 2, 3, 4), respectively, in all samples collected from Singosari Artificial Insemination Centre. Furthermore, the band at both 415 and 318 bp showed the GADPH and SRY gene, respectively, as these results indicated that the duplex PCR method used was able to verify the spermatozoa separation outcomes of the X and Y chromosomes, through the swim up technique. Therefore, this method was sensitive in determining the SRY gene in the collected sperm sample.

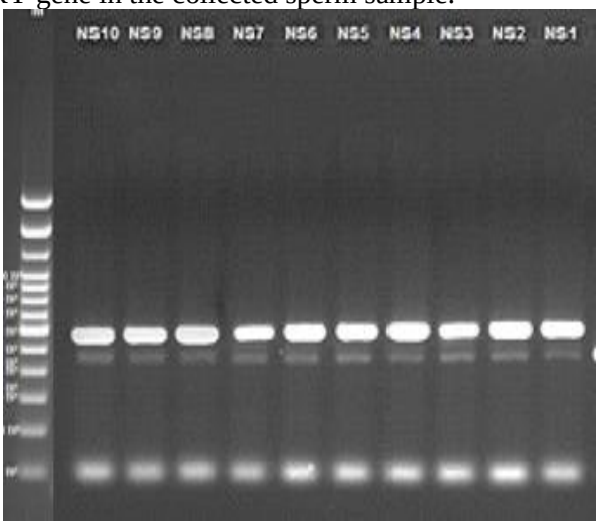

Fig. 2 The PCR results of Limousin cattle spermatozoa with non sexing (upper layer 415 bp; lower layer: 318 bp)

Note:
M = Marker
NS1 = Sperm of Non Sexing Limousin Cattle 1
NS2 = Sperm of Non Sexing Limousin Cattle 2
NS3 = Sperm of Non Sexing Limousin Cattle 3
NS4 = Sperm of Non Sexing Limousin Cattle 4
NS5 = Sperm of Non Sexing Limousin Cattle 5
NS7 = Sperm of Non Sexing Limousin Cattle 7
NS 8 = Sperm of Non Sexing Limousin Cattle 8
NS 9 = Sperm of Non Sexing Limousin Cattle 9
NS 10 = Sperm of Non Sexing Limousin Cattle 10

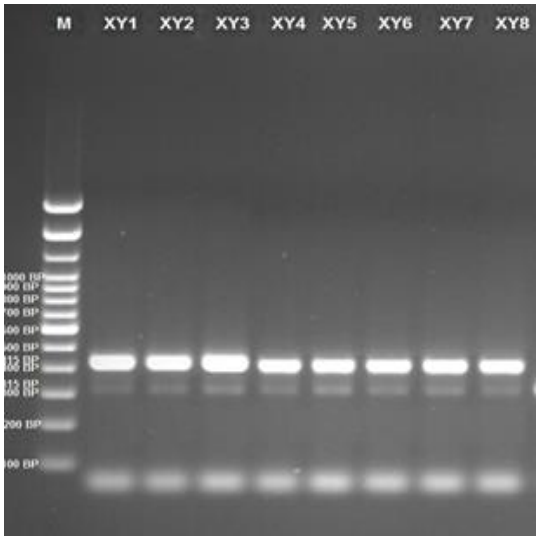

Fig. 3 The PCR results of male sexing Limousine cattle spermatozoa (XY) (upper layer 415 bp; lower layer: 318 bp)

Note:
M = Marker
XY1 = Sperm of Male Sexing Limousin Cattle 1
XY2 = Sperm of Male Sexing Limousin Cattle 2
XY3 = Sperm of Male Sexing Limousin Cattle 3
XY4 = Sperm of Male Sexing Limousin Cattle 4
XY5 = Sperm of Male Sexing Limousin Cattle 5
XY6 = Sperm of Male Sexing Limousin Cattle 6
XY7 = Sperm of Male Sexing Limousin Cattle 7
XY8 = Sperm of Male Sexing Limousin Cattle 8

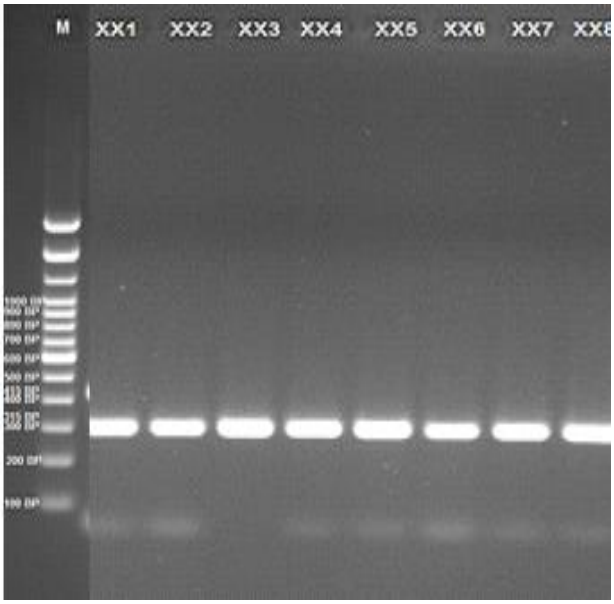

Fig. 4 The PCR results of female sexing Limousine cattle spermatozoa (XX) (415 bp)

Note:
M = Marker
XX1 = Sperm of Female Sexing Limousin Cattle 1
XX2 = Sperm of Female Sexing Limousin Cattle 2

XX3 = Sperm of Female Sexing Limousin Cattle 3
XX4 = Sperm of Female Sexing Limousin Cattle 4
XX5 = Sperm of Female Sexing Limousin Cattle 5
XX6 = Sperm of Female Sexing Limousin Cattle 6
XX7 = Sperm of Female Sexing Limousin Cattle 7
XX8 = Sperm of Female Sexing Limousin Cattle 8

4. Discussion

The mean percentage of motility and viability of limousine cattle semen in non-sexing, XX, and XY spermatozoa were 44.8 & 50.2, 44.1 & 51.9, and 45 & 52.6, respectively. Also, the average percentage of the semen abnormalities in non-sexing, XX, and XY spermatozoa was 4.7, 4.5, and 4.8, respectively. Furthermore, the research by Malik [22], separated the sex of sperm with both vaginal mucus and Percoll gradient (45-90%), resulting in a X and Y ratio of 58.33% and 56.67%, respectively [22].

Moreover, the research that used blood samples of male and female cattle, and obtained similar results, as regards the formation of 2 bands of GAPDH (218 bp) and SRY (122 bp), in the masculine materials [14]. The results of this research indicated that SRY-GADPH multiplication was able to differentiate the sex of sperm DNA samples, with GADPH amplification used as a positive control for all materials. Furthermore, it was observed that GADPH primers do not affect the Y chromosome amplification. Therefore, GAPDH as a positive control, determines PCR amplification works accurately.

Also, it is an advantage to use 2 short primary targets, which are distinguished by about 100 bp [14], therefore clearly differentiating the outcomes of the electrophoresis obtained. The PCR method should be used to determine the sex ratio in sperm and embryos. A study to evaluate the sex variations of sperm during ejaculation, and discovered a significant difference in the percentage of Y spermatozoa in each ejaculate [15]. This was also observed in the SRY band thickness on each sample. Therefore, further research needs to be carried out to determine the Y chromosome quantity. Also, the swim up method was molecularly verified, in order to separate the spermatozoa carrying X and Y chromosomes. The duplex PCR method was also able to differentiate sex from Limousine cattle spermatozoa, using the swim up technique.

5. Conclusion

Conclusively, molecular methods were used to confirm X and Y chromosome from the Limousin cattle semen, through the use of the swim up method. The swim up method should be used in the separation of X and Y chromosomes, which are present in Limousin cattle spermatozoa.

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