P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2023.074

Molecular Verification of Spermatozoa Sexing Method in Pesisir Cattle Bull Using Bovine Serum Albumin Column

Mangku Mundana[1*], Tinda Afriani[1], Yurnalis[1], Adisti Rastosari[1], Fani Oktavianti[1], Muhammad Cadilac Al Razzak[1], Mylaufa Asyraf[2] and I Made Merdana[2]

[1]Department of Livestock Production, Faculty of Animal Science, Andalas University, Padang, West Sumatra, Indonesia
[2]Department of Veterinary Clinic, Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia
***Corresponding author:** mangkumundana@ansci.unand.ac.id

Article History: 23-227 Received: 10-Jun-23 Revised: 16-Jul-23 Accepted: 18-Jul-23

ABSTRACT

An accurate sexing process is crucial for preserving the purity of Pesisir cattle bulls and enhancing their productivity to meet the increasing beef demand in Indonesia. Therefore, this study aims to verify the separation of spermatozoa carrying X and Y chromosomes molecularly, using the Bovine Serum Albumin (BSA) column. The spin-column method was employed to isolate the separated X and Y spermatozoa. Amplification through PCR was performed using two sets of primers targeting the Sex-determining Region Y (SRY) gene, located on the Y chromosome, and the Ausal Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene on the X chromosome. The assessment of spermatozoa quality in coastal cattle bulls showed an average fresh semen volume, and sperm concentration to be 3±0.7mL, and 1.58±1.05x10^6/mL, respectively. While the other parameters of assessment of spermatozoa quality including average motility, spermatozoa survival, sperm abnormalities, and mean progressive motility (MPU) were to be 80±10, 85.83±7.59, 6.36±3.50, and 79.81±5.97%, respectively. The quality evaluation after sexing included the average motility of spermatozoa with X at 56.66±11.54 and Y at 66.66±11.54%, as well as their average intact plasma membrane (IPM) being 55.58±1.01 and 59.35±7.78%, respectively. The quality of spermatozoa decreased by 20-30% after sexing. The results of spermatozoa separation with the 5% BSA column confirmed one GAPDH band (415bp), indicating the content to be X. In contrast, the 10% BSA column and the non-sexed spermatozoa exhibited two bands, SRY (318bp) and GAPDH (415bp), indicating a higher proportion of Y in the 10% BSA column. These demonstrated molecular verification of sexed spermatozoa using the 5 and 10% BSA column, enabling the separation of those carrying X and Y chromosomes in Pesisir bulls through the duplex PCR method.

Key words: Spermatozoa Sexing, Molecular Verification, BSA Column, Pesisir Cattle, Spermatozoa Separation.

INTRODUCTION

Pesisir cattle are primarily raised by many farmers for beef production in West Sumatra, specifically in the Pesisir Selatan Regency. These local breeds are considered among five germplasms of native Indonesian cattle, alongside Bali, Aceh, Sumbawa, and Madura cattle. They possess several advantageous traits, including resilience to extreme environmental stresses such as heat and the ability to thrive on low-quality feed ingredients. Additionally, their exceptional body resistance and environmental adaptability require no special maintenance compared to other breeds (Udin et al. 2022).

Preserving the purity of Pesisir cattle and enhancing their productivity is crucial for meeting the increasing beef demand in Indonesia. These can be achieved through the application of reproductive biotechnology, known as spermatozoa sexing (Rodiah et al. 2015). Spermatozoa sexing represents a promising reproductive technology that enables the production of livestock with desired sexes, catering to the specific needs of breeders, such as obtaining bulls for beef or female cattle for dairy purposes. This increases the livestock population according to business requirements. The recommended breeding age for Pesisir bulls ranges from 36 to 48 months (Budhiyadnya et al. 2021).

Spermatozoa sexing is employed to separate spermatozoa carrying the X and Y chromosomes based on differences in morphological characteristics, DNA content, movement, weight, and macromolecular proteins (Aini et al. 2016). The method used for this purpose involves separating spermatozoa through exposure to 5 and 10% BSA column, incubation for 45 min, and subsequent

Cite This Article as: Mundana M, Afriani T, Yurnalis, Rastosari A, Oktavianti F, Razzak MCA, Asyraf M and Merdana IM, 2024. Molecular verification of spermatozoa sexing method in Pesisir cattle bull using bovine serum albumin column. International Journal of Veterinary Science 13(1): 7-12. https://doi.org/10.47278/journal.ijvs/2023.074

centrifugation at 1800 rpm for 10 min. The Y spermatozoa tend to swim towards the higher concentration (10%), while the X remains in the lower concentration (5%). Spermatozoa sexing is advantageous for male Pesisir cattle (Pesisir bulls) because it permits the provision of frozen semen, which can be used for controlling the sex of the desired calf according to required maintenance during breeding. However, the process may lead to changes in motility and viability, acrosome reaction acceleration, elevated spermatozoa capacitation, membrane damage, and fragmentation (Yekti et al. 2022; Sallam et al. 2023).

To ensure the validity and reliability of inbreeding (IB) results obtained with sexing through the 5-10% BSA column, molecular verification needs to be conducted using the duplex PCR method. The applications of IB combined with spermatozoa sexing in several regions of Indonesia have achieved a sex match rate of 76-89% in field calves (Gunawan et al. 2015; Sawitri et al. 2021). Therefore, this study aims to molecularly verify sexing results of spermatozoa carrying X and Y chromosomes using the PCR method.

MATERIALS AND METHODS

Semen Collection

In this study, semen was collected from three Pesisir bulls using an artificial vaginal device, which has been assembled and lubricated on the outer rubber surface covering up to one-third of the length and filled with 40-52ºC warm water. Successfully collected semen was transported immediately to the laboratory for further analysis.

Fresh Semen Evaluation

The macroscopic evaluation conducted included examining volume, color, odor, consistency, and pH. Meanwhile, microscopic evaluation involved motility tests and the determination of live spermatozoa percentage and IPM. Motility was assessed by placing one drop of semen on a glass slide and observing it under a microscope at magnifications of 200x to 400x. A mortality rating of 70-90% was considered satisfactory. IPM was evaluated by adding semen into a hypoosmotic solution which was incubated at 37°C for 30min, then placed on a glass slide, and covered with a slip to be observed under a light microscope at 400x magnification.

Separation of Spermatozoa using the BSA Column Method

The collected fresh semen was diluted according to the respective concentrations and introduced into 5 and 10% BSA columns. The 10% BSA column was positioned at the bottom, while the 5% BSA column was placed on top, and the topmost layer was filled with diluted semen. The setup was incubated at 37°C for 45min in a water bath. Subsequently, the top layer (approximately 1mL) was discarded, while the middle and bottom layers were suspected to contain X and Y spermatozoa, respectively.

Sexing Semen Evaluation

The microscopic evaluation included the assessment of motility, survival percentages, and IPM. Motility was measured by placing one drop of semen on a glass slide and observing it under a microscope at magnifications of 200x to 400x. A mortality rating of 70-90% was considered satisfactory, then IPM was evaluated using the same method described earlier.

DNA Extraction

Sperm DNA was extracted using the Geneaid Presto™ Sperm DNA extraction kit according to the instructions of the manufacturer, then stored in the refrigerator at 20°C until further analysis.

Amplification of X and Y Spermatozoa

The extracted sperm DNA was amplified through PCR with a total volume of 25µL consisting of 2µL DNA sample, 7.5µL master mix, 3µL F and R primer mixture, and 12.5µL nuclease-free water. Two pairs of primers were used, namely SRY F 5’AAGGGGAGAACAGTTAGGGAGAG3’, SRY R 5'ATCGGGTTGCATAGTATTGAAG3', GAPDH F 5'GTGGCGCCAAGAGGGTCATCATC3', and GAPDH R 5'GGTTTCTCCAGGCGGCAGGT3' (Bioneer). The in vitro amplification was conducted using a PCR machine, starting with pre-denaturation at 94°C for 5min, denaturation at 94°C for 30s, annealing at 60°C for 30s, and initial extension at 72°C for 30s, followed by 45 repeated cycles and a final extension at 72°C for 10min. The amplification results were visualized by electrophoresis using 2% agarose gel stained with the ethidium bromide dye for observation under a UV transilluminator. Successful amplification was indicated by the presence of bands on the agarose gel at the expected size corresponding to the target DNA product. The band position was compared with the DNA ladder marker to determine the target length of bp, then the electrophoresis results were documented using a camera.

RESULTS AND DISCUSSION

Fresh Semen Quality Evaluation

The assessment of semen quality involved both macroscopic and microscopic evaluation, and the results are presented in Table 1.

The macroscopic evaluation results showed an average volume of 3mL for the semen collected from three Pesisir bulls, which was classified as normal. However, it was observed that the 3mL average volume of Pesisir semen was lower than the 4.93mL obtained from 3-year-old Brahman bulls (Islam et al. 2018) and 5.21mL from 5-year-old Bali bulls (Nugraha et al. 2019).

The fresh semen of Pesisir bulls in this study appeared milky white, which was within the range of normal semen color. According to Susilawati (2013), normal male cow (bull) semen primarily has a milky white or yellowish-white color because of riboflavin content, while reddish yellow indicated abnormalities i.e., the presence of water, pus, and blood (Zulyazaini et al. 2016) also described the color of fresh Aceh cattle semen as creamy-white with a medium to thick consistency.

The fresh semen of the Pesisir bulls had a characteristic odor and a moderate consistency. According to Susilawati (2013), fresh semen with medium consistency majorly contains $1000x10^6$-$1500x10^6$ spermatozoa/mL

Table 1: Fresh semen evaluations of Pesisir cattle bull

Semen evaluation		Score
		Mean±SD
Macroscopic		
a. Volume (mL)		3±0.7
b. Color		Milky white
c. Odor		Characteristic
d. Consistency		2.3±0.57
e. PH		7±0
Microscopic		
a. Mass movement		2.33±1.15
b. Motility (%)		80±10
c. Alive percentage (%)		85.83±7.59
d. Abnormality		6.36±3.50
e. Concentration (million/mL)		1.58±1.05
f. IPM		79.81±5.97
Description	(+) : 1	Watery: 1
	(++) : 2	Medium: 2
	(+++) : 3	Thick: 3

semen. The average pH value of the fresh semen in this study was around 7±0 and considered to be good, aligning with the range of 6.4-7.8 reported for normal bull semen by Garner and Hafez (2000). In addition, Bearden et al. (2004) stated that the normal pH range for dairy and beef bulls is between 6.5-7.0. The acidity level of pH influences spermatozoa survival in semen greatly and deviations from the normal range may lead to reduced sperm viability.

The microscopic evaluation results showed an average mass movement value of 2.33±1.15, indicating that the semen of Pesisir cattle bull was in quite good condition. According to Zulyazaini et al. (2016), the more active, numerous, and forward-moving spermatozoa, the better the mass movement will be. The mass movement in this study had a very good score, ranging between +2 and +3.

In the present study, the motility obtained from the three individual bulls was an average of 80% and it was considered good. According to Samardzija et al. (2015), fresh ejaculates from bulls typically exhibit over 80% progressively motile spermatozoa, with 85% displaying morphologically normal shapes. Further, Susilawati (2013) stated that the percentage of fresh semen motility below 40% indicates poor quality and is associated with infertility, while motile spermatozoa under normal circumstances range from 70 to 90% of the total population.

Fig. 1 shows the percentage of live spermatozoa in the present study, i.e., 85.83±7.59%, which was smaller than 88.0% reported by Ratnawati et al. (2017) in Bali cattle bull and 94.08% by Sukmawati et al. (2014). The percentages of live spermatozoa are often higher than the motile ones because motile spermatozoa are alive, while live spermatozoa may not necessarily exhibit the same motility.

Abnormalities of spermatozoa found in the present study have been presented in Fig. 2. We found 6.36±3.50% sperm abnormalities, which was less than the 6.56% obtained from Bali cattle bull semen in Indonesia (Ratnawati et al. 2017). Spermatozoa abnormalities are categorized into three types, namely primary, secondary, and tertiary. These abnormalities arise from physical deformities occurring during spermatozoa formation (spermatogenesis) within the seminiferous tubules (primary) and while traveling through the male reproductive tract (secondary).

The concentration of fresh Pesisir semen obtained in this study averaged 1.58±1.05x10^9 cells/mL, which aligned

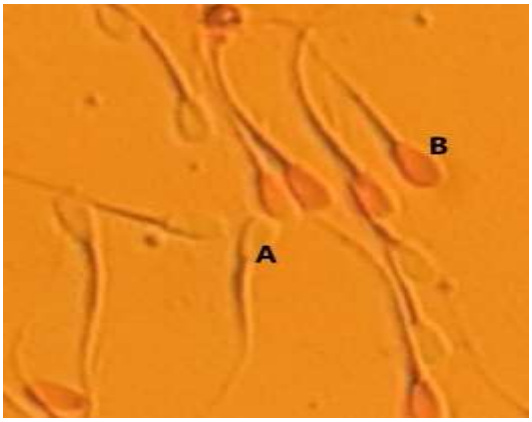

Fig. 1: Microphotograph of live spermatozoa**:** (A) Live spermatozoa, (B) Dead spermatozoa

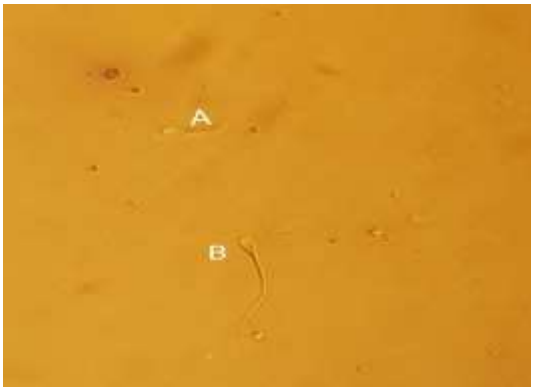

Fig. 2: Abnormality of Pesisir cattle spermatozoa: (A) Abnormal spermatozoa, (B) Normal spermatozoa

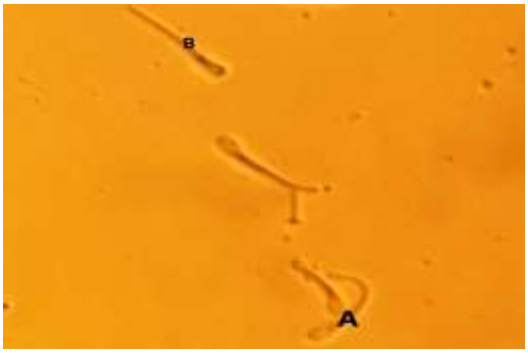

Fig. 3: Spermatozoa with IPM: (A) Spermatozoa with IPM, (B) Spermatozoa without IPM

with the 800 to 2000x10^9 cells/mL of semen reported by Garner and Hafez (2000). According to Ismaya (2014), spermatozoa concentration refers to the number of spermatozoa cells per unit volume or milliliter of semen. The concentration of fresh semen is one of the important indicators for quality assessment and the determination of appropriate diluent ratios to apply for further procedures. A hemocytometer with a 10:990 l formol saline ratio is commonly used for concentration calculations.

In this study, IPM was evaluated using a hypoosmotic solution by observing the reaction of spermatozoa with curved tails. The average IPM obtained was 79.81%, which was higher than the study by Diansyah et al. (2021) with IPM values of 43, 44, 38, 48, 51 and 56%, respectively reported in six different cattle bull types, namely Bali, Limousine,

Friesian Holstein, Brahman, Angus and Simental cattle, with an overall average of 46.67%. The integrity of the plasma membrane is vital for protecting and maintaining spermatozoa motility within the female reproductive tract, facilitating fertilization through interactions and direct or indirect attachment to cumulus cells.

Evaluation of Semen Quality after Sexing in Pesisir Bulls

The results of semen quality evaluation after sexing presented in Table 2, showed that the percentage of motile spermatozoa in sexed semen from three individual Pesisir bulls was 56.66±11.54% for X and 66.66±11.54% for Y. These were smaller than the motility rates of 75% for X and 67% for Y observed by Agasi et al. (2020) in Sumba Ongole cattle using albumin column. They were also slightly different from the motility rates of 58.33±10.32% for X and 60.83±13.57% for Y reported by Afriani et al. (2022). This current study indicated a 20-30% decrease in the motility percentage of sexed and non-sexed spermatozoa. This aligned with the findings of Susilawati (2000) and Patriani et al. (2019) who stated that sexing treatments could decrease spermatozoa quality.

Sexing is time-consuming; therefore, the quality of spermatozoa needs to be initially examined to determine their suitability for the process. The sex separation process for X and Y spermatozoa using a 5-10% BSA column requires 45min (Kaiin et al. 2017), which is longer than normal semen freezing. This method is more flexible and easier to carry out in the field. Further, it is based on the difference in motility between X and Y spermatozoa because of disparities in mass and size.

The average quality of IPM in sexed Pesisir cattle bull spermatozoa was determined to be 55.58±1.01 for X and 59.35±7.78 for Y, as presented in Table 2. These results differed slightly from the 53.63±16.54% X and 52.76±8.86% Y reported by Afriani et al. (2022). Furthermore, they were smaller than IPM percentages of 83.49% in the upper layer (X) and 83.62% in the lower layer (Y) obtained by Purwoistri et al. (2013) from the Limousine bull spermatozoa sexed using the albumin gradient method. Differences in the average quality of IPM among several studies could be attributed to variations in the individual cattle used, their age, and bull quality. The average IPM percentage of Pesisir bulls' spermatozoa reduced after sexing conducted with the BSA column. This was in accordance with the observations of Susilawati et al. (2001) that sexing treatments could lead to a decrease in spermatozoa quality.

DNA Isolation

The DNA electrophoresis results of the isolated Pesisir cattle bull sperm presented in Fig. 4, showed thick and clear bands, indicating the amount of DNA detected. This suggested a successful purification process to produce good DNA. However, some isolation results showed thin bands, indicating a small amount of DNA. Successful DNA isolation is achieved when there are no contaminants such as protein and RNA (Hidayati et al. 2016). Contaminants can be identified by the smearing patterns produced in electrophoresis results. Setiati et al. (2020) stated that the presence of smears observed below the DNA band in electrophoresis suggests that the isolated DNA is not intact, indicating that DNA fragments have formed due to physical treatment during the isolation process, such as storing samples in freezing and repeated thawing. Additionally, the presence of sediment at the bottom of each row indicates contamination in the form of RNA. It is widely recognized that the appearance of a smear on the electrophoresis gel may indicate that the extracted DNA has undergone degradation, thereby significantly impacting the quality of the subsequent molecular applications and their outcomes (Devi et al. 2013).

Table 2: The examination results of semen sexing in Pesisir cattle

Evaluation of semen sexing	Mean±SD	
	X	Y
Motility	56.66±11.54%	66.66±11.54%
IPM	55.58±1.01%	59.35±7.78%

Fig. 4: The result of DNA Isolation electrophoresis of X and Y spermatozoa in Pesisir cattle.

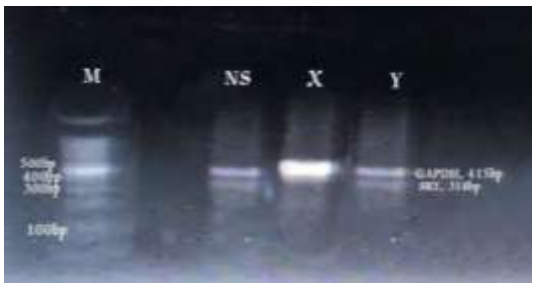

Fig. 5: Electrophoresis results of samples from sexing in Pesisir cattle using 5-10% BSA column. Description: M=Marker, NS=non-sexing, X=X Spermatozoa, Y=Y Spermatozoa.

Amplification of X and Y Chromosomes

Fig. 5 shows that the X spermatozoa collected from the 5% BSA column only had a single band at 415bp. Meanwhile, Y from the 10% BSA column and unsexed spermatozoa displayed two bands at 415bp and 318bp. The band at 415bp corresponded to the GAPDH gene, and the one at 318bp indicated the SRY gene. Based on the observations, the duplex PCR method employed in this study effectively confirmed the separation of X and Y chromosome spermatozoa using 5 and 10% BSA columns. Therefore, this method was sensitive in determining the SRY gene in samples collected from each BSA column. The results suggested that the 5 and 10% BSA columns contained higher X and Y spermatozoa, respectively. Similarly, Kaiin et al. (2017) molecularly verified Simmental cattle spermatozoa sexing using BSA, where the 5% BSA column displayed one band at 415bp and the 10% BSA column exhibited two bands at 415bp and 318bp, while unsexed spermatozoa had two bands at 415bp and 318bp.

The currently obtained results were consistent with the study conducted on Limousine bulls using the swim-up method, which showed two bands for Y and unsexed spermatozoa at 415 bp and 318bp, and one band for X at 318bp (Utomo et al. 2021). The differences between both spermatozoa included speed, hence, Y reached the 10% BSA column first because of being faster than X. These were consistent with the electrophoresis results where the 10% BSA expressed the presence of SRY gene in the samples collected from column.

Similar results were observed in the investigation conducted by Prashant et al. (2008) using blood samples of bulls and female cattle, which showed two bands for GAPDH (218 bp) and SRY (122bp) in the blood sample of bulls. The results of this study indicate that SRY-GAPDH multiplication differentiated the sex of spermatozoa DNA examined, and GAPDH amplification could serve as a positive control for all samples. GAPDH, as a housekeeping gene, ensures a proper PCR amplification process. Moreover, it is advantageous to use two short primary target sequences that can be distinguished by approximately 100bp (Prashant et al. 2008), enabling a clear distinction of the electrophoresis results. According to Esfahani et al. (2016), PCR methods can be employed for determining the sex ratio in embryos and spermatozoa. In this study, the duplex PCR method successfully distinguished the sex of Pesisir bull spermatozoa using the BSA column.

Conclusion

The quality of fresh semen spermatozoa decreased by 20-30% after sexing with the 5-10% BSA column method. Molecular verification using the duplex PCR demonstrated the ability to differentiate between X and Y sperm in Pesisir bulls. The 5% BSA column predominantly contained X sperm, while the 10% BSA column contained a higher proportion of Y sperm. The presence of SRY (318bp) and GAPDH (415bp) genes allowed for accurate identification of the sex of spermatozoa DNA.

Acknowledgment

The authors are grateful to the rector and LPPM of Andalas University Padang, Indonesia for funding this study under grant number 001.13/UN.16.06.D/PT.01/ SPP.RD/FATERNA/2022.

Conflict of interest

There is no conflict of interest about the publication of this study.

Authors Contribution

MM and TA conducted investigations, while AR, FO, MCAR, and MA collected the data. MM drafted the manuscript, IMM, and Y provided critical feedback and revisions, then MM finalized the manuscript.

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