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

Short Communication https://doi.org/10.47278/journal.ijvs/2023.123

Testing Motility Parameters of Post-Thawing Pesisir Bulls (*Bos indicus*) Semen with and without Sexing

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Article History: 23-321 Received: 15-Oct-23 Revised: 13-Dec-23 Accepted: 14-Dec-23

ABSTRACT

This study aimed to test the motility parameters of frozen semen from Pesisir bulls with and without sexing. The research material consisted of semen from two Pesisir bulls. The data obtained were statistically analyzed using ANOVA. The results of this study showed that the motility percentage of Pesisir bulls ranged from 53.66 to 58.87%, and progressive motility ranged from 41.20 to 48.87%. The values of distance curve line (DCL) ranged from 24.05 to 28.24µm, distance average path (DAP) ranged from 24.05 to 24.35µm, and distance straight-line (DSL) ranged from 18.49 to 22.78µm. The values of velocity curve line (VCL) ranged from 93.95 to 112.97µm/s, velocity average path (VAP) ranged from 53.90 to 62.57µm/s, and velocity straight-line (VSL) ranged from 41.97 to 50.51µm/s. The values of linearity (LIN) ranged from 0.43 to 0.45, straightness (STR) ranged from 0.75 to 0.80, and wobble (WOB) ranged from 0.54 to 0.59. Meanwhile, the amplitude of lateral head (ALH) value was between 4.22 and 5.14µm, and the beat cross frequency (BCF) value ranged from 21.34 to 26.99Hz. The motility parameters, progressive motility, DAP, DSL, VAP, VSL, and LIN of post-thawing Pesisir bulls semen did not show significant differences (P>0.05) both with and without sexing. However, there were significant differences (P<0.05) in the parameters of progressive motility, DCL, VCL, STR, WOB, ALH, and BCF. However, for future research, it is expected that studies could involve more significant and more diverse sample sizes, as well as variations in cryopreservation techniques that may be employed. Additionally, further research could consider factors such as the age and health condition of Pesisir bulls, which could influence semen quality after thawing.

Key words: CASA, Motility, Pesisir bulls, Sexing semen.

INTRODUCTION

Sexing or spermatozoa separation is one of the supporting technologies in reproduction, especially for livestock. This technology separates spermatozoa based on DNA content, namely spermatozoa carrying X and Y chromosomes. The aim of spermatozoa sexing is to determine livestock births according to the intended purpose of breeding. Thus, the availability of semen based on gender allows for the selection of male and female bulls births (Holden and Butler 2018). Despite the benefits of sexing spermatozoa, its usage percentage in artificial insemination is currently low (Seidel 2014).

The use of sexing technology can be performed through separation techniques such as flow cytometry, which is the only reliable method for determining the gender of offspring (Garner et al. 2013). However, this technique is still limited in Indonesia. Additionally, there are other separation techniques, such as albumin gradient. Separation using the albumin gradient technique can separate spermatozoa based on the differences in morphology and swimming speed of each spermatozoon in response to varying albumin concentrations. The use of albumin concentration as a sexing medium and the appropriate duration for sexing is predicted to yield optimal sexing results (Sunarti et al. 2016; Niu et al. 2023).

Separated spermatozoa must undergo a quality assessment before being applied in artificial insemination. This is because spermatozoa have minimum quality requirements that must be met for their use. Sperm carrying X and Y chromosomes exhibit various distinct characteristics, including variations in DNA content, shape, movement, mass, and the specific genes they have (Sharma and Sharma 2016). Spermatozoa testing is commonly performed through visual microscopic examination, which is subjective and dependent on the examiner's judgment (Sarastina et al. 2006). Subjectivity can be overcome by using Computer Assisted Semen Analysis (CASA).

Cite This Article as: Afriani T, Udin Z, Jaswandi, Wahyudi D and Asyraf M, 2024. Testing motility parameters of post-thawing Pesisir bulls (*Bos indicus*) semen with and without sexing. International Journal of Veterinary Science 13(4): 453-457. https://doi.org/10.47278/journal.ijvs/2023.123

CASA technology is now commercial and objectively evaluates sperm characteristics (Soler et al. 2018). The use of CASA is based on digital image technology, which yields rapid and accurate results. Additionally, it can assess relevant motility parameters related to fertility (Simmet 2004). Amann and Waberski (2014) stated that CASA (Computer-assisted semen analysis) technology enables the automated and precise assessment of the motile patterns of individual sperm within the ejaculate. CASA evaluation allows for the assessment of both the overall motility and progressive motility of sperm cells, as well as a comprehensive analysis of their motility characteristics (Syarifuddin et al. 2018; Suo et al. 2023).

The application of spermatozoa sexing technology in local cattle breeds like Pesisir bulls (*Bos indicus*) has not been previously explored. This technology can be employed in artificial insemination to produce more male offspring, thereby enhancing the efficiency of artificial insemination programs (Afriani et al. 2022). Yurnalis et al. (2017) stated that the Pesisir bulls breed is an indigenous bull's breed in Indonesia known for its adaptation to challenging conditions, particularly in hot and humid climates and with limited access to nutritious feed, contributing to its ability to produce meat. Pesisir cattle exhibit notable qualities, such as remarkable physical robustness and the capacity to adapt to their surroundings, granting them unique advantages when compared to other cattle breeds (Zaituni et al. 2022). This study focuses on spermatozoa motility parameters' quality with or without sexing. The expected outcomes aim to provide more objective information regarding motility parameters in Pesisir bulls semen.

MATERIALS AND METHODS

This research utilized semen from two Pesisir bulls in West Sumatra, Indonesia that were collected once a week using an artificial vagina and met the criteria of good quality for further processing. The semen that met the requirements was divided into two stages: sexing spermatozoa using the albumin gradient method and without sexing (directly continuing to the dilution, equilibration, and freezing stages).

Semen Cryopreservation

Semen that had been evaluated and met the quality criteria was further processed using the albumin gradient separation method. Bovine Serum Albumin (BSA) solution with concentrations of 5 and 10% was used for this purpose. The prepared BSA solution was added to 15mL reaction tubes, with 10% BSA at the bottom and 5% BSA at the top, each with a volume of 2mL. Then, a 1mL suspension of diluted semen was added to the very top of the tube, and the tube was incubated in a water bath at 37°C for 45min. After incubation, the uppermost 1 mL was discarded, while the 5 and 10% solutions were each collected in 2mL tubes and transferred to centrifuge tubes containing semen diluent. They were then centrifuged at 1800rpm for 10min (Kaiin 2013). The supernatant was collected, and the pellet was retained, followed by further dilution. The semen was loaded into straws using a filling and sealing machine, equilibrated at 4°C for 4hours, and frozen in liquid nitrogen at -196°C.

Computer Assisted Semen Analysis (CASA)

The frozen semen was subsequently thawed for quality testing. CASA Sperm Vision 3.7 was used for testing to obtain more accurate results. CASA testing was performed on both sexed and non-sexed semen by capturing images of spermatozoa in four fields of view. Spermatozoa image capture was conducted across four different fields of view (Ratnawati et al. 2017). CASA testing covered motility parameters, including motility, progressive motility, Curve Linear Distance (DCL), Average Path Distance (DAP), Straight Linear Distance (DSL), velocity curve linear (VCL), velocity average pathway (VAP), velocity straight linear (VSL), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). Before the introduction of the CASA-Mot (Computer Assisted Semen Analysis for Motility) system, the evaluation of sperm motility relied on subjective methods. However, this technology has become a common tool in both clinical and research settings for objective assessment (Giaretta et al. 2017).

Data Analysis

The samples were measured using a Randomized Complete Block Design with 3 treatments consisting of spermatozoa without sexing, spermatozoa X and spermatozoa Y, with 6 replications for each treatment. The data obtained were analyzed using analysis of variance (ANOVA), and if the treatments showed a significant effect, a post-hoc Duncan's test was also conducted. The software used was SPSS 16.0.

RESULTS AND DISCUSSION

The percentage of motility and progressive motility are essential criteria for measuring the fertility level of male cattle (Abavisani et al. 2013). Based on the analysis of variance, it was found that the motility parameter did not significantly affect the results ($P>0.05$). However, as shown in Table 1, spermatozoa without sexing had a higher percentage than those after sexing. Meanwhile, progressive motility showed a significant difference ($P<0.05$) between spermatozoa without sexing and sexed spermatozoa. Spermatozoa without sexing (48.87%) was significantly higher compared to spermatozoa X (41.20%) and spermatozoa Y (41.52%). This indicates a decrease in quality during the sexing process due to the collision of the sexing medium and the washing effect on spermatozoa during centrifugation. Sunarti et al. (2016) stated that the decrease in the percentage of spermatozoa is believed to be due to the prolonged incubation process, which results in spermatozoa remaining active for a more extended period and requiring more energy to penetrate the egg albumin. Furthermore, the centrifugation process can increase free radicals or Reactive Oxygen Species (ROS) due to membrane damage (Zanella et al. 2016). Consequently, ROS are essential for the processes of sperm maturation, hyperactivation, capacitation, acrosome reaction, and fertilization (Dutta et al. 2019).

The parameters DCL, DAP, and DSL measure the displacement distance of spermatozoa per second. Based on the analysis of variance in Table 1, it is shown that the values of DAP and DSL are not significantly different ($P>0.05$), While the value of DCL shows a significant

Table 1: Parameter motility of Pesisir bull semen

Parameters	Average ±SD		
	Non Sexed	X-Sexed	Y-Sexed
Motility (%)	58.87±7.82	55.44±7.96	53.66±6.23
Progressive Motility (%)	48.87±6.45b	41.20±8.34a	41.52±3.50a
DCL (µm)	40.83±6.43a	42.11±6.02a	51.16±8.47b
DAP (µm)	24.35±4.04	24.05±3.14	28.24±4.54
DSL (µm)	18.49±3.88	18.72±2.62	22.78±3.77
VCL (µm/s)	95.04±13.5a	93.95±10.8a	112.97±17.0b
VAP (µm/s)	56.92±8.24	53.9±5.60	62.57±9.21
VSL (µm/s)	43.30±8.12	41.97±4.86	50.51±7.96
LIN	0.45±0.04	0.44±0.02	0.43±0.02
STR	0.75±0.03a	0.77±0.01a	0.80±0.02b
WOB	0.59±0.03b	0.56±0.02a	0.54±0.01a
ALH (µm)	5.14±0.40b	4.34±0.29a	4.22±0.21a
BCF (Hz)	21.34±1.41a	24.67±1.95b	26.99±1.21c

Values (Mean±SD) bearing different letter in a row differ significantly (P<0.05).

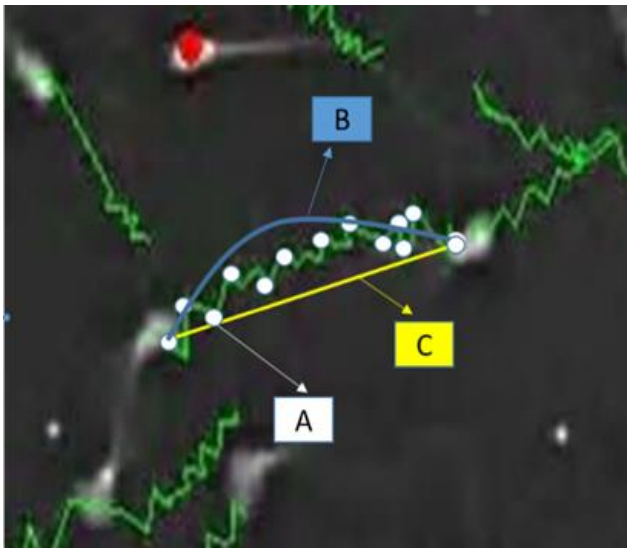

Fig. 1: Spermatozoa Velocity Parameters Using CASA. A) VCL = Spermatozoa velocity on a winding track per second, B) VAP = The average velocity of spermatozoa on its course, and C) VSL = The velocity of spermatozoa on a straight path per second.

difference (P<0.05). The DCL value for spermatozoa Y (51.16µm) is significantly higher than that of spermatozoa X (42.11 µm) and spermatozoa without sexing (40.83µm). Generally, the results indicate that spermatozoa Y has a higher swimming speed than spermatozoa X and spermatozoa without sexing. This result is attributed to the fact that spermatozoa Y exhibits higher swimming velocity compared to spermatozoa X (Wahyudi et al. 2023).

Velocity or spermatozoa speed in Table 1 shows that the values of VAP and VSL are not significantly different (P>0.05), while the value of VCL shows a significant difference (P<0.05). VCL for spermatozoa Y (112.97µm/s) is significantly higher than that of spermatozoa without sexing (95.04µm/s) and spermatozoa X (93.95µm/s). The findings of this study indicate a higher value compared to the report by Mustofa et al. (2020), who stated that the optimal VCL value for sexed semen of Ongole crossbreed was 55.2 µm/s. Similar to the values of DCL, DAP, and DSL, spermatozoa Y have higher velocity values compared to spermatozoa X. This is because velocity values correlate positively with distance values. Spermatozoa velocity is based on the trajectory followed by spermatozoa per second, as depicted in Fig. 1. Velocity is used to determine the value of spermatozoa's progressive motility. According to Aghazarian et al. (2021), spermatozoa are considered progressive when they have VAP>25µm/s, whereas spermatozoa with VAP<25µm/s are considered non-progressive. Spermatozoa velocity is influenced by various factors such as diluent viscosity, pH, energy source, and osmolarity (Pelumal et al. 2014). Semen diluents should possess high transparency and prolonged retention capability without compromising the semen's fertilization quality (Ondho et al. 2019). Amal et al. (2019) reported that the VCL parameter was found to be correlated with the permeability of spermatozoa to enter cervix mucus. Meanwhile, VAP and VSL were employed for the prediction of in vitro fertility (Singh et al. 2020).

The values of LIN, STR, and WOB are parameters that indicate the movement patterns of spermatozoa. Table 1 shows that the LIN value is not significantly affected (P>0.05). The values of linearity (LIN) ranged from 0.43 to 0.45. According to Maulana et al. (2022), the linearity (LIN) value for sexed semen of Ongole Crossbreed Bulls was approximately 0.36-0.40 in the upper layer and around 0.64-0.67 in the lower layer. However, the values of STR and WOB are significantly affected (P<0.05). The STR value for spermatozoa Y (0.80) is significantly higher than that of spermatozoa X (0.77) and spermatozoa without sexing (0.75), while the WOB value for spermatozoa without sexing (0.59) is higher than that of spermatozoa X (0.56) and Y (0.54). The movement pattern of spermatozoa X and Y in this study tends to be linear because they have LIN>0.35 and STR>0.5, whereas the head wobble of spermatozoa without sexing is greater compared to after sexing.

Based on Table 1, it is evident that the values of ALH and BCF are significantly different (P<0.05). ALH for spermatozoa without sexing (5.14µm) is significantly higher compared to spermatozoa X (4.34µm) and Y (4.22µm). The ALH values for spermatozoa X and Y in this study are relatively stable as they are still below 5 µm, while spermatozoa without sexing are less stable, possibly due to cold storage, which increases the ALH value, indicated by the presence of a star-shaped pattern in head movement (Tardiff et al. 1997). Increased ALH values indicate lower quality, which can disrupt the progressive movement of spermatozoa (Amal et al. 2019). On the other hand, BCF for spermatozoa Y (26.99Hz) is higher than spermatozoa X (24.67Hz) and without sexing (21.34Hz), while spermatozoa X is higher compared to spermatozoa without sexing. The BCF value is used to measure the frequency of spermatozoa movement. BCF is also a valuable parameter for identifying changes in the flagellar rhythm pattern (Kathiravan et al. 2011). Meanwhile, the BCF value is influenced by the frame rate used in CASA (Perreault 2002).

It is essential to acknowledge several limitations encountered during this research. First, the sample size used in the study was relatively small, which may limit the generalizability of the findings to a larger population of Pesisir cattle. Additionally, the duration of semen post-thaw evaluation was relatively short-term, and a longer-term assessment could provide more comprehensive insights into the semen quality. Another limitation is related to the separation process, as it may introduce additional variability in the results due to the handling and techniques employed. Furthermore, the study did not

account for potential variations in environmental factors that could influence semen quality. Despite these limitations, the research contributes valuable insights into the motility parameters of post-thawed Pesisir cattle semen, highlighting the need for further investigations to address these constraints and improve our understanding of the subject.

Conclusion

The parameters of motility, progressive motility, DAP, DSL, VAP, VSL, and LIN for Pesisir cattle semen post-thawing did not show significant differences in averages, both with and without sexing. However, there were significant differences in the average values of the parameters for progressive motility, DCL, VCL, STR, WOB, ALH, and BCF. These findings provide valuable initial insights into the characteristics of Pesisir cattle semen post-thawing and its potential differences based on sex sorting. However, for future research, it is expected that studies could involve more significant and diverse sample sizes, as well as variations in cryopreservation techniques that may be employed. Additionally, further research could consider factors such as the age and health condition of Pesisir cattle, which could influence semen quality after thawing. This would aid in developing more effective cryopreservation methods and selecting semen based on desired characteristics.

Acknowledgement

The authors wish to thank the rector of Andalas University Padang, Indonesia and LPPM Andalas University who funded this research with scheme contract T/15/UN16.19/Pangan-PDU-KRP2GB-Unand/2023.

Author's Contribution

TA and ZU designed the experimental framework. Data collection was carried out by J, DW and MA. Statistical analysis and interpretation of results were performed by J and DW. The initial draft of the manuscript was prepared by TA, with critical revisions provided by ZU and J. All authors reviewed and approved the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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