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The Expression of Plasma Protein in Bali-polled Bulls Using 1D-SDS-PAGE

Athhar Manabi Diansyah[1], Muhammad Yusuf[2]*, Abdul Latief Toleng[2], Muhammad Ihsan Andi Dagong[2], and Tulus Maulana[3]

[1]*Post-Graduate School, Hasanuddin University, Jl. Perintis Kemerdekaan Km. 10 Tamalanrea Makassar, South Sulawesi, 90245 Indonesia*
[2]*Departement of Animal Production, Faculty of Animal Science, Hasanuddin University, Jl. Perintis Kemerdekaan Km. 10 Tamalanrea Makassar, South Sulawesi, 90245 Indonesia*
[3]*Research Center for Applied Zoology, National Research and Innovation Agency, Jl. Raya Bogor Km. 46, Cibinong, West Java, 16911 Indonesia*

*Corresponding author's Email: myusuf@unhas.ac.id

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ABSTRACT

The fertility rate of bulls in a breeding program is not only described by the quantity and quality of semen. Factors, such as the interstice factor of the sperm and the plasma component of semen, affect the fertility rate of bulls. The fertility rate can also be determined by identifying the protein content of semen plasma. Therefore, the current study aimed to identify the relationship between seminal plasma protein molecular weight and semen quality of Bali-polled bulls. The study was conducted at the Laboratory of Semen Processing, Faculty of Animal Science, Hasanuddin University, Makassar, Indonesia, the Research Center for Applied Zoology, National Research and Innovation Agency, Cibinong, Indonesia and the Laboratory of Animal Biotechnology Center, IPB University, Bogor, Indonesia from November 2021 to January 2022. The samples came from 5 Bali-polled and 5 Bali-horned bulls. Semen collection was conducted twice a week using an artificial vagina. The concentration of seminal plasma protein was determined by the Bradford method of 1D-SDS-PAGE. The study results showed that fresh semen of Bali-polled and Bali-horned bulls was considered a normal category. Seminal plasma proteins of Bali-polled and Bali-horned bulls were classified using 8 bands to categorize molecular weight; 150 kD (IGF-1), 110 kD (A-kinase anchoring protein 3), 93 kD (A-kinase anchoring protein 4), 54-87 kD (Arylsulfatase-a), 44-62 kD (N-Acetyl-ß-Guicosaminidase), 44kD (Phosphoglycerate kinase), 15-30 kD (BSP A1/A2, BSP-A3 and BSP-30 [BSP1, BSP3, and BSP5]) and 12-14 kD (Acidic seminal fluid proteins). The findings indicated that both Bali-polled and Bali-horned bulls could have a high reproductive rate. In conclusion, protein analysis based on molecular weight using 1D-SDS-PAGE can be used as a biomarker for semen quality in Bali-polled bulls. Therefore, evaluating the semen quality with a molecular basis as an additional indicator of superior bull in the selection process is an alternative method.

Keywords: Bali-polled bull, Seminal protein plasma, Sperm, 1D-SDS-PAGE

INTRODUCTION

Livestock reproductive biotechnology has now developed and opened up great opportunities to explore the potential for reproductive performance, as well as increasing population, and genetic quality of livestock. Artificial insemination (AI) is the first generation of reproductive technology that aims to efficiently utilize superior males, avoid the spread of reproductive diseases, and improve the genetic quality of livestock (Syahruddin, 2020). Artificial insemination technology has the potential to be used for the development of Bali-polled cattle in Indonesia. Thus, the selection of polled bulls becomes very important, especially in modern livestock management (Brockmann, 2000).

Conventionally, the breeding soundness evaluation (BSE) technique was used to test the reproductive ability of an animal. The BSE is repeatable, easy to perform, and correlates with male fertility (Thundathil et al., 2016). The assessment method of BSE is not limited to the bulls that can reach the BSE threshold or exceed it. The method provides an opportunity for males with low potential to be selected in case only one aspect of the BSE test is observed (Alexander, 2008).

According to Kaya and Memili (2016), the fertility rate of bulls in a breeding program is not only described by the quantity and quality of semen. In the same line, Viana et al. (2018) found that many factors contributed to determining the quality of sperm, such as the interstice factor of the sperm and the plasma component of semen. Moreover, the protein function affects the fertility rates in bulls depending on the protein content of semen plasma (Druart and de Graaf, 2018).

The protein in seminal plasma plays an important role in sperm protector regulations (Fu et al., 2019). Understanding the physiological way seminal plasma proteins' function can help breeder bulls become more fertile and have less infertility. Some specific proteins in semen have been linked to fertility although many seminal proteins still have unresolved functions and correlations with fertility indices (Kumar et al., 2012). Therefore, there is a need for

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further studies to investigate the relationship between semen plasma protein and bulls' fertility. The current study aimed to identify the relationship between seminal plasma protein molecular weight and semen quality of Bali-polled bull.

MATERIALS AND METHODS

Ethical approval

All procedures in the present study were approved by the University Animal Ethics Committee of Hasanuddin University, Makassar, Indonesia.

Study area and period

The study was conducted at the Laboratory of Animal Reproduction, Semen Processing Unit, Faculty of Animal Science, Hasanuddin University, Makassar, Indonesia. Determination of seminal plasma protein was analyzed at the Research Center for Applied Zoology, National Research and Innovation Agency, Cibinong, Indonesia, and the Laboratory of Animal Biotechnology Center, IPB University, Bogor, Indonesia, from November 2021 to January 2022. The seminal plasma samples were obtained from 5 Bali-polled bulls and 5 Bali-horned bulls aged 5-8 years. The sample size was based on the previous studies by Kasimanickam et al. (2019) and Westfalewicz et al. (2021).

Semen collection and evaluation

Bali-polled and Bali-horned bull semen samples were collected using an artificial vagina twice a week. Soon after collection, the semen samples were sent to the laboratory for evaluation. The evaluation of semen quality was performed both macroscopically and microscopically. Semen that passed the macroscopical evaluation was then evaluated for microscopic evaluation, including motility, concentration, abnormality, viability, and membrane integrity of the sperms.

Motility

The motility, progressive motility, and kinematics of sperms were determined by making 10 µl semen spot on the object glass. For the movement evaluation of sperms, the semen was then subjected to the CASA (Vision Version^{TM} 3.7.5 program Minitube, Germany) following Diansyah et al. (2022).

Concentration

The sperm concentration was evaluated using a photometer SDM 6 (Minitube, Germany). The cuvette containing 3 ml of physiological NaCl solution was inserted into the device with the line facing forward, and then the zero button was pressed. The cuvette was removed and then replaced with a cuvette containing a physiological NaCl solution in which 30 µl of fresh semen was added, and then the result button was pressed; the concentration of spermatozoa would be obtained in the amount per ml (Diansyah et al., 2022).

Abnormality and viability

The viability and abnormality of the sperms were evaluated by mixing 10 µl of semen and 10 µl of Eosin 2% in the object glass. After drying, the object glass was observed using a trinocular microscope (Primo Star, Zeiss, Germany) at 400x magnification with Indomicro View 3.7 software. Spermatozoa with red color were considered dead, and the colorless ones were considered alive. Spermatozoa with severed tails, broken tails, and abnormal head shapes were considered abnormal. For the accurate calculation, at least 200 sperms cells per observation were performed following Diansyah et al. (2022).

Membrane integrity

An evaluation of membrane integrity was performed microscopically in which 10 µl of semen was added into HOST solution (0.179g NaCl in 100 ml of aquabides). The solution was then incubated for 30 minutes at 37^{o}C in the oven. The evaluation was carried out using a 400x magnification of a trinocular microscope (Primo Star, Zeiss, Germany) by counting at least 200 spermatozoa cells. Sperms with membrane integrity were characterized by a circular tail, while sperms characterized by a straight tail were considered damaged (Diansyah et al., 2022).

Concentration of seminal plasma protein

Seminal plasma protein concentration was determined by centrifuging the semen at about 3-4 mL at 6500 rpm for 30 minutes. After centrifugation, the supernatant was put into the microtube and kept in a cryobox for storage at 20^{o}C. For characterization of seminal plasma protein, 1D-SDS-PAGE (SMOBIO, Hsinchu, Taiwan) based on molecular weight (MW) of protein was used. The gels were stained (Sigma-Aldrich®, United States) with Coomassie Brilliant Blue stain (Sigma-Aldrich®, United States), and molecular mass was determined by the MW marker (Karunakaran et al., 2019). Concentration of seminal plasma protein was determined by the Bradford method (Bradford, 1976). The

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Bradford protocol of analysis using Protein Assay Kit (Sigma-Aldrich®, United States) was based on the user guide of coomassie (Bradford, 1976) for sample preparation. The samples were then analyzed by Thermo Skanlt RE for Multiskan Go Software, 3.2 version (Thermo Scientific®, United States) to obtain seminal plasma concentration.

Regarding the separation of seminal plasma protein, two polyacrylamide gels 12% (containing sodium dodecyl sulphate [SDS]) were used. A 20 µg of protein mass was analyzed with SDS-PAGE using Thermo Prestained Protein Ladder marker (5 µL, Thermo Scientific®, United States). Subsequently, the protein separation was performed using 120 v electricity in 70 minutes. The photo of gel color was dipped in an acidic liquid and then shaken until protein bands appeared to ease for identifying protein bands. Each protein band was determined using ImageJ software (Schneider et al., 2012).

Statistical analyzes

The data of fresh semen quality both in Bali-polled and Bali-horned bulls were reported as mean and standard deviation. T-Test was used to compare each parameter regarding the quality of fresh semen in the two bulls. The parameter was considered significant when the p-value was lower than 0.05. All statistical analyzes were calculated using SPSS for Windows software (version 25). The specific protein in each seminal plasma of the bulls was analyzed descriptively.

RESULTS AND DISCUSSION

The quality of fresh semen and concentration of seminal plasma protein in Bali-polled and Bali-horned bulls

This current study intended to explore the quality of Bali-polled bull semen for predicting the fertility level using plasma protein expression. In order to achieve this intention, Bali-horned bulls that have been known as having high fertility level was used for comparison. Table 1 shows the quality of Bali-polled and Bali-horned bulls' fresh semen. Statistical analysis showed that sperm motility in the Bali-polled bulls did not differ significantly ($p > 0.05$) from the Bali-horned bulls (82.91% vs. 83.18%). Likewise, sperm concentration (1578.72 x 10^6 mL^{-1} vs. 1475.80 x 10^6 $mL^{-1)}$, sperm viability (91.97% vs. 92.03%), sperm intact membrane (80.90% vs. 81.52%), and seminal plasma concentration (567 mg mL^{-1} vs. 547 mg mL^{-1}) did not differ significantly between the two groups ($p > 0.05$). However, sperm abnormality in Bali-polled bulls was significantly ($p < 0.05$) lower than in Bali-horned bulls (4.17% vs. 5.19%). The results of the current study revealed that the quality of fresh semen in both groups of bulls was likely higher than the other local bulls reported previously (Romadhoni et al., 2014; Zulyazaini et al., 2016).

According to Indonesian Minister of Agricultural Regulation Number:10/Permentan/PK.210/3/2016 and Indonesian National Standardization 4868.1:2007 for frozen bull semen (Baharun et al., 2021), fresh semen quality can be processed as frozen semen with sperm motility value >70% and sperm abnormalities value <20%. Based on these regulations, the quality of Bali-polled and Bali-horned bulls' fresh semen in the present study was considered a normal category. Therefore, the semen of the two bulls used in this study can be processed further as frozen semen.

As can be seen in Table 1, the seminal plasma protein concentration of Bali-polled and Bali-horned bulls was 567 mg mL^{-1} and 547 mg mL^{-1}. The plasma protein concentration was used only as the basis for further electrophoresis or mass spectrometry analysis by Bradford method. The amount of plasma protein concentration cannot be relied upon to analyze the quality of semen (Westfalewicz et al., 2016). However, several studies have demonstrated that plasma protein components in semen are considered effective in improving sperm quality (Codognoto et al., 2018; Viana et al., 2018; Panda et al., 2020). Seminal plasma is mostly composed of testicular, epididymal, and accessory sex gland secretions. Proteins involved in sperm metabolism and motility, membrane restructuring and function, protection against reactive oxygen species and immunological responses, capacitation, and the acrosome reaction are expressed in the fluid surrounding sperm cells in semen (Moura et al., 2018). Many proteins in the seminal plasma bind the sperm, affecting membrane structure and sperm function. The study of Purdy (2006) has shown that the role of seminal plasma proteins in regulating sperm function is highly complex. Several studies have provided solid evidence that seminal plasma proteins were adsorbed to the sperm surface and affected its function and properties (Purdy, 2006; Moura et al., 2018).

Table 1. The quality of fresh semen and seminal plasma protein concentration of Bali-polled and Bali-horned Bulls

Parameters / Breed	Sperm motility (%)	Sperm concentration (10^6 mL^{-1})	Sperm viability (%)	Sperm abnormality (%)	Sperm intact membrane (%)	Seminal plasma protein concentration (mg mL^{-1})
Bali-Polled	82.91 ± 1.34	1578.72 ± 65.83	91.97 ± 10.6	4.17 ± 2.39[a]	80.90 ± 1.04	567
Bali-Horned	83.18 ± 1.44	1475.80 ± 59.93	92.03 ± 6.96	5.19 ± 1.45[b]	81.52 ± 0.71	547
p value	0.944	0.219	0.943	0.014	0.313	

Means in a column with different superscripts differ significantly at $p < 0.05$.

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The specific protein profile of seminal plasma in Bali-polled and Bali-horned bulls

The specific profile of the targeted protein in this study affected the quality of spermatozoa, as indicated by the molecular weight of the protein using 1D-SDS-PAGE (Figure 1). The seminal plasma protein-specific profile of Bali-polled and Bali-horned bulls, as determined by 1D-SDS-PAGE, shared the same particular protein from 8 protein candidates. The specific profile of the targeted protein is shown in Table 2. Selvaraju et al. (2016) indicated protein with MW 150 kD represented IGF-1. This protein improves sperm motility by reducing oxidative stress, maintaining structural membrane integrity and mitochondrial membrane potential, and protecting calmodulin, dermcidin, and the sperm acrosome membrane (Selvaraju et al., 2016). IGF-1 is found in plasma and tissue fluid, influencing steroidogenesis, metabolism, cell proliferation, and differentiation (Colombo and Naz, 1999). IGF-1 also affects various reproductive processes and plays an essential role in the onset, progress, and regulation of spermatogenesis (Dyck et al., 1999).

Table 2. Seminal plasma protein-specific profile of Bali-polled and Bali-horned bulls assessed by 1D-SDS-PAGE

Protein	MW (kD)	Bali-Polled	Bali-Horned
IGF-1	150	+	+
A-kinase anchoring protein 3	110	+	+
A-kinase anchoring protein 4	93	+	+
Arylsulfatase-a	54-87	+	+
N-Acetyl-ß-Guicosaminidase	44-62	+	+
Phosphoglycerate kinase	44	+	+
BSP A1/A2, BSP-A3 and BSP-30 (BSP1, BSP3 and BSP5)	15-30	+	+
Acidic seminal fluid proteins	12-14	+	+
Total Protein		8/8	8/8

MW: Molecular weight, +: Protein expressed, -: Protein non-expressed, BSP: Binder sperm protein

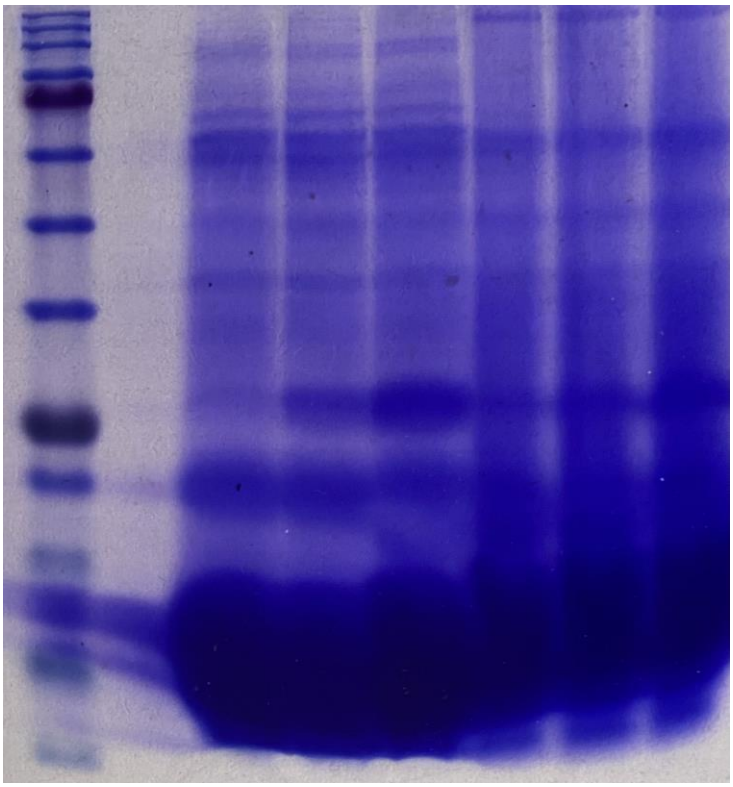

Figure 1. 1D-SDS-PAGE of Bali-polled and Bali-horned bulls

The protein with MW 110 kD and 93 kD contains AKAP 3 and AKAP 4 (Pujianto et al., 2018). These proteins contain the fibrous coating of the sperm's outer dense fiber, which forms the axoneme (Carr and Newell, 2007). During capacitation, AKAP3 and AKAP4 are phosphorylated, which is crucial for maintaining sperm motility (Pujianto et al., 2018). Molecular function mediates sperm motility through A-Kinase Anchoring Protein 4 (AKAP4) setting. It correlates with Adenosine triphosphate (ATP) for sperm motility and ATP's dephosphorylation (Freitas et al., 2017). AKAP3 is degraded in bovine sperm incubated under capacitation conditions (Hillman et al., 2013). The AKAP3 and AKAP4 isoforms are uniquely expressed by spermatids and spermatozoa, localize in the flagellum, and are involved in sperm motility. AKAP3 is mainly localized at the principal piece of the tail (Lea et al., 2004).

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The MW 54-87 kD protein may represent arylsulfatase-a (Moura et al., 2010). This protein can be attached to the superficial sperm head (Weerachatyanukul et al., 2003). During the binding/penetration process, arylsulfatase-a functions as either lectins or hydrolases (Nickolajczyk and O'Rand, 1992). Arylsulfatase-a is involved in the stability and permeability of the spermatozoa plasma membrane (Gadella et al., 1991). Arylsulfatase-a quantitative detection and distribution during in vitro sperm capacitation could be used to get a better insight into molecular changes during the fertilization process and improve artificial reproductive technologies (Gómez-Torres et al., 2021). The protein N-acetyl-β-guicosaminidase (62-44 kD) is glucose hydrolyze enzyme for glycoprotein membrane during sperm maturation in the epididymis (Moura et al., 2010). These proteins are potential mediated sperm-oocyte interactions (Abascal et al., 1998).

The protein with MW 15-30 kD (Druart et al., 2013) is indicated as binder sperm protein (BSP) A1/A2, BSP-A3, and BSP-30 (BSP1, BSP3, and BSP5). After ejaculation, BSP proteins bind to the sperm membrane at the acrosome, post-acrosome, and midpiece area, which is crucial for beginning motility (Manjunath et al., 1994). All biochemical studies and binding properties have provided good insight into the putative functions of BSP proteins in fertility. The BSP proteins are multifunctional proteins used for various purposes, including sperm motility, formation of the sperm reservoir, but most importantly, sperm capacitation (Plante and Munjunath, 2014). The function of BSP-30 kD as exhibits a significantly broader binding specificity to choline phospholipids, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, and cardiolipin, whereas BSP-A1, BSP-A2, and BSP-A3 bind specifically to the phosphorylcholine groups (Desnoyers and Manjunath, 1992).

According to Moura et al. (2010), a protein with MW 12-14 is Acidic Seminal Fluid Proteins (aSFP). Jobim et al. (2004) suggested the aSFP as a marker of good semen freezability. Furthermore, the aSFP is a marker for bovine semen freezability, possibly due to its antioxidant activity and effect on sperm mitochondrial function (Moura et al., 2010). In bulls, aSFP represents a major sex-specific seminal protein secreted mainly from the ampulla and seminal vesicle in variable but high concentrations (Einspanier et al., 1994). This protein seems to protect spermatozoa from free oxygen radicals in such an in vitro system (Schoneck et al., 1995).

Regarding the profile of seminal protein expression, Bali-polled and Bali-horned bulls contain similar total protein candidates. This suggests that the two bulls are linked and have a similar reproductive rate.

CONCLUSION

Protein analysis using 1D-SDS-PAGE based on molecular weight can be used as a biomarker for semen quality in Bali-polled bulls. The semen of both bulls contains IGF-1, AKAP 3, AKAP 4, arylsulfatase-a, N-acetyl-β-guicosaminidase, BSP A1/A2, BSP-A3, and BSP-30 (BSP1, BSP3, and BSP5) and aSFP candidates that are linked to a high reproductive rate. Therefore, the evaluation of the semen quality with molecular basis as an additional indicator of superior bull in the selection process is one of the alternatives.

DECLARATIONS

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Authors' contribution

Athhar Manabi Diansyah contributed to collecting data, drafting method, analysis, writing-original draft, writing-review, and editing the manuscript. Muhammad Yusuf contributed to performing data analysis, writing-original draft, reading-original draft, writing-review, and reading-review draft and approved the final manuscript. Abdul Latief Toleng contributed to performing data analysis, writing-original draft, reading-original draft, writing-review, and reading-review draft and approved the final manuscript. Muhammad Ihsan Andi Dagong performed data analysis, writing-original draft, reading-original draft, writing-review, and reading-review draft, and approved the final manuscript. Tulus Maulana performed data analysis, writing-original draft, reading-original draft, writing-review, reading-review draft, and approved the final manuscript.

Competing interests

The authors declared no competing interests.

Ethical consideration

The authors have confirmed ethical issues, such as plagiarism, misconduct, information fabrication and/or falsification, consent to publish, duplicate publishing and/or submission, and redundancy.

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