FINAL REPORT
DIPA BIOTROP 2021

IDENTIFICATION OF FERTILITY BIOMARKER PROTEINS IN SPERM OF SUPERIOR LOCAL BREED BULLS TO SUPPORT THE NATIONAL CATTLE BREEDING PROGRAM

Prof. Drh. Bambang Purwantara, M.Sc., Ph.D.
Dr. Ir. Dedy Duryadi Solihin, D.E.A.
Prof. Asep Gunawan, S.Pt. Ph.D.
Dr. Drh. Muhammad Agil, M.Sc.Agr.
Zulfi Nur Amrina Rosyada, S.Pt, M.Si.

MINISTRY OF EDUCATION, CULTURE, RESEARCH, AND TECHNOLOGY OF THE REPUBLIC OF INDONESIA
SECRETARIAT GENERAL
SEAMEO SEAMOLEC
SOUTHEAST ASIAN REGIONAL CENTRE FOR TROPICAL BIOLOGY (SEAMEO BIOTROP)
2021

APPROVAL SHEET

1.	Research Title	:	Identification of Fertility Biomarker Proteins in Sperm of Superior Local Breed Bulls to Support National Catttle Breeding Program
2.	Research Coordinator		
	a. Name	:	Prof. Drh. Bambang Purwantara, M.Sc., Ph.D.
	b. Gender	:	Male
	c. Occupation	:	SEAMEO BIOTROP Affiliate Scientist
3.	Institution		
	a. Name of Institution	:	Dept. of Reproduction and Pathology Clinic, Faculty of Veterinary Medicine, IPB University
	b. Address	:	Agatis Street, IPB University, Darmaga district, Bogor, 16680
	c. Telephone/Fax	:	+628129325449
	d. Email	:	purwantara@ipb.ac.id, purwantara1959@gmail.com
4.	Duration of Research	:	9 months
5.	Research Budget	:	

Bogor, 30 November 2021

Endorsed by,
Acting Manager of Research Hub
Innovation Department
SEAMEO BIOTROP

Ir. Sri Widayanti, M.Si.
NIP. 19670822 200701 2 001

Research Coordinator

Prof. Drh. Bambang Purwantara, M.Sc., Ph.D.
NIP. 19591006 198403 1 003

Approved by,
SEAMEO BIOTROP Director

Dr. Zulhamsyah Imran, S.Pi, M.Si.
Director of SEAMEO BIOTROP
NIP 19700731 199702 1 001

TABLE OF CONTENT

I. INTRODUCTION

1.1 Background

Currently, Indonesian beef cattle are showing a rapid development to meet the national demand for meat. The population of beef cattle in 2014 was 14.7 million, which then increased in 2018 to 17.1 million (Ministry of Agriculture 2018). This population increase does not entirely come from local beef cattle because as much as 55% of the demand for beef in Indonesia is still supplied from imports (Agus and Widi 2018). Therefore, efforts to increase Indonesian local beef cattle productivity continue to be made, one of which is under improvement.

Indonesia has many indigenous cattle breeds that already adapted to local conditions like Bali cattle, Madura cattle, Ongole crossbred cattle (PO), Aceh cattle, Pesisir cattle Sumba Ongole (SO), and, the less commonly found, Galekan cattle of Trenggalek. Also, there are many hybrids between zebu and Bali cattle, such as Jabres cattle of Brebes, Rambon cattle of Bondowoso, Banyuwangi, and the surrounding areas. Farmer's demand on local breed cattle are great because they have many benefits, such as having high reproduction efficiency, fast-breeding, potential in producing meat with high carcass percentage, and also have good adaptability to the environment (Nuraini *et al.* 2018).

Bali cattle breed, cattle with the highest population compared with other cattle such as Ongole cattle, Ongole crossbreed (PO) cattle, Aceh cattle, and Pasundan. Bali cattle are one of the original Indonesian beef cattle breeds with superior genetic quality in terms of productivity and reproducibility. A right level of reproducibility in Bali cattle is shown by the value of service per conception (S/C) of 1.66 (Siswanto *et al.* 2013) to 1.81 (Pratami *et al.* 2019) and the conception rate (CR), which varies from 55.37-57.93% (Pratami et al. 2019) to 80.00-83.38% (Wildan *et al.* 2020). Increasing the population of Bali cattle is carried out by maintaining the genetic purity of these breeds of cattle by concentrating breeding activities on the Island of Bali and controlling the expenditure and slaughter of Balinese cattle (Chamdi 2004).

The Ongole developed most ideally in Java, and the SO cattle were distributed to Java through a program called "Ongolization," in which male Javanese cattle were castrated, and the female was mated with male SO. The program led to the extinction of the remaining Javanese cattle and creation of a new species, the Ongole Crossbred (PO). Kebumen district is

feasible to be a source of PO cattle breeding because the reproduction of PO cattle in this area is good enough, and its population dynamics are expected to increase from 2015 to 2019. Local Ciamis cattle have carcass percentage, which is not different from Bali cattle, PO, and crosses cattle. Local Ciamis cattle have a closer genetic distance to PO cattle (Hilmia *et al.*, 2013). Each area has a specific cattle breed, such as Aceh, West Sumatra (Pesisir cattle), West Java (Pasundan cattle), and the breed's wider spread is a Bali cattle and Ongole cross breed cattle. Besides, local breed cattle's potential makes this livestock a meat source in Indonesia (Andreas *et al.* 2010).

As one of Indonesia's local cattle and Indonesian germplasm, Madura bulls are being developed to meet the demand for meat in Indonesia. Madura bulls come from a crossbreed between *Bos indicus* (zebu) and *Bos javanicus* (banteng). Madura cattle are raised for three purposes: karapan, sonok, and beef cattle (Hartatik *et al.* 2010). Madura bulls are local beef cattle that can adapt to local conditions than other breeds. Madura cattle can grow well even with low diet quality and have a high carcass with good carcass quality (Sutarno and Setyawan 2016; Kutsiyah 2017). Madura bulls, which are mostly developed by small breeders on the island of Madura, are known to have not met the optimal value for efficient reproductive performance (Zuhri *et al.*, 2019). This condition is described by several parameters, including the percentage of service per conception (S/C) of 1.68%, the conception rate (CR) of 58%, a long calving interval (CI) of 14.56 months, and a calve mortality rate of 2, 23%. Besides, the gap between the natural increase (NI) (30.75%) and the output value (27.96%) indicates a depletion of the Madura cattle population (Kutsiyah 2017; Zuhri *et al.* 2019).

Efforts to maintain and increase the Madura cattle population are applying reproductive technology, namely artificial insemination (AI) using frozen semen from superior Madura bulls (Prihatin *et al.*, 2018). Artificial insemination is one of the reproductive technologies that can improve the genetic quality of livestock and reproductive efficiency (Diskin 2018). The success of AI is influenced by many factors, one of which is bulls fertility (Kaya and Memili 2016). Superior bulls are selected based on good quality frozen semen and proven by reproductive efficiency in the field (Somashekar *et al.* 2015).

Production of frozen semen at the Indonesian Artificial Insemination Center refers to the Indonesian National Standard for frozen semen number 4869-1: 2017. However, frozen semen from superior bulls passed the breeding soundness examination (BSE), and quality control processes still showed various reproductive performances (Fair and Lonergan 2018; Rosyada *et al.* 2020). Thus, the bull's fertility rate cannot be determined based on BSE. According to Chenoweth (2004), BSE is only a method for physical examination, libido, semen

quality, and heredity to determine which males have the potential to be used as superior bulls but cannot distinguish the level of potential fertility between males when used as a breed.

The fertility characteristics of sperm are quantitative and intricate processes controlled by multiple pairs of genes (polygene). It shows that bulls' fertility rate can be influenced by several factors, such as molecular factors, namely the complex and interacting protein content of spermatozoa, to modulate the fertilization process (Peddinti et al. 2008 and Moura and Memili 2016). Several pairs of candidate genes that play a role in controlling spermatozoa fertility are heat shock protein 70 (HSP70), and OPN (osteopontin). HSP 70 is considered a motility marker, concentration, and induces spermatozoa and egg cells' interaction in the capacitation process (Zhang *et al.* 2015, Agarwal 2020). OPN (osteopontin) is a gene found in the acrosomal cap, equatorial segment, and mid-piece of spermatozoa that protects spermatozoa cells and helps the fertilization process (Williams 2011, Souza *et al.* 2008). The analysis revealed decreased expression of HSPA2 that are crucial for sperm functions such as motility, acrosome reaction, and fertilization of oocyte (Agarwal *et al.* 2020). Zhang et al. (2015) also report that the HSP 70 (HSP A2) has a higher expression in semen with good motility than low motility.

Functional study of genomic, transcriptomic and proteomic of spermatozoa through RNA analysis and gene expression, DNA polymorphism, and spermatozoa protein profile can provide information about candidate genes for fertility markers bioinformatics systems as interpretations accurate on bull fertility. The efforts can increase breeding efficiency to meet the need of meat consumption in Indonesia. Moreover, the study is needed to keep the superior local breed of cattle as our local germplasm away from extinction.

1.2 Objectives

This study explores the potential marker fertility in the sperm of bulls of various local cattle breeds. This sperm molecular analysis to (1) describes the RNA fertility marker gene expression, (2) accurately can determine the potential and the single nucleotide polymorphism from the HSP 70-2 (HSP A2), and OPN as fertility marker, (3) Describe the profiles and interactions of sperm proteins that modulate fertilization.

1.3 Expected Outputs

The results of this study are expected to be able to provide several molecular marker information related to bulls fertility, which is essential as a basis for selection and reselection

for determining superior bulls that are accurate, have high fertility, and as a policy reference in the bulls refusal process used by the Indonesian Center for Artificial Insemination. Besides, it is also a measure to preserve Madura cattle as local Indonesian germplasm.

II. BENEFITS AND IMPORTANCE OF CONDUCTING THE RESEARCH

The benefits and importance of implementing this research is as an effort to increase the contribution of the livestock sector in the national food security program. One of the tangible manifestations in meeting the increasing demand for livestock products is the consumption of meat in Indonesia. So that it can support the achievement of self-sufficiency in meat. Another benefit in finding biomarkers of fertility for local breed in Indonesia is as a strategic concept for the preservation of local livestock and superior Indonesian germplasm.

III. METHODS

3.1 Time and Place

This research was conducted from March to November 2021. Sampling of frozen semen from Madura cattle was carried out at the Lembang National Insemination Center in Bandung, West Java, and the Singosari National Insemination Center in Malang, East Java, Indonesia. Then, the samples were stored at -196 C in a cryopreservation container. Speramtozoa analysis was carried out at LIPI, Cibinong. Molecular genetic analysis was carried out in the Lab. Molecular Biology-Center for Biological Resources and Biotechnology Research (PPSHB), IPB University, Dramaga, and Primate Research Centre (PSSP), IPB University, Lodaya, Bogor.

3.2 Grouping of Local Breed Bulls

The grouping of bull fertility rates was based on data processing results on reproductive efficiency in the form of the percentage of Sire Conception Rate (CR) using ISIKHNAS data.

3.3 Evaluation of Spermatozoa Viability and Abnormality

Frozen semen from eight Madura males was obtained from the semen processing and freezing laboratory at BIB Lembang and BBIB Singosari. Spermatozoa viability is the percentage of spermatozoa that live in spermatozoa samples, while abnormalities are abnormalities in the shape of spermatozoa cells. There are two types of spermatozoa

abnormalities: primary and secondary abnormalities. Viability and abnormality were determined using the eosin-nigrosine staining method (Björdahl et al., 2003). The steps taken were 5 l of frozen Madura semen from each level of fertility dropped into an object glass and mixed with 20 l of eosin-nigrosine solution. The prepared semen sample was smeared on another object glass and fixed with a heating table at 37°C for 10 minutes prior to observation. Live spermatozoa remain unstained, while dead cells are wholly or partially pink to red/brown. In addition, live spermatozoa were also classified as normal or morphologically abnormal.

3.4 Total RNA Extraction

Total RNA from spermatozoa was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. A modified protocol was performed to improve the yield and quality of the extracted RNA. The frozen semen was thawed in water for 30 seconds and then put into a microtube. Put 100 l of semen into a 2 ml tube. Then add 200 l and centrifuged at 10000 rpm for 10 minutes to clean the spermatozoa from the diluent. Next, the supernatant was discarded and added with 300 l of RLT buffer solution, then homogenized using a vortex for 1 minute and homogenized manually for 10 minutes. The mixture was then centrifuged at 10000 rpm for 1 minute. Ethanol 70% 400 L was added, and stirred gently with a micro-pipette for 5 to 10 minutes. The supernatant was then transferred to a spin column and centrifuged for 1 minute at 10000 rpm, RW1 700 l was added and centrifuged for 1 minute. Buffer E 700 L and centrifuged for 1 minute. The spin-column was transferred to a new 1.5 ml tube and added RNase-free water, allowed to stand for 10 minutes, and centrifuged at 13000 rpm for 1 minute.

The amount of RNA extracted from each sample was measured spectrophotometrically using Nanodrop ND-1000 (Thermo Scientific). RNA quality was measured using the Agilent 2100 Bioanalyzer and RNA Nano 6000 Labchip Kit (Agilent Technologies) with standard parameters RNA Integrity Number (RIN) more than 7 (RIN > 7) and the ratio of 28S rRNA: 18S rRNA was 2:1.

3.5 Synthesis of complementary DNA (cDNA)

Synthesis of cDNA was carried out through a PCR reverse transcriptase process using two µg RNA, and superscript II reverse transcriptase (Invitrogen) and oligo (dT) 12primers (Invitrogen).

Table 1. Primary designs of the HSP-70, OPN, PRM1 and GAPDH genes for qRT-PCR.

Gene	Accession no.	Primer (5'-3')	Product Sze
HSP-70.2	NM_174344.1	F: TTGGGGACAAGTCAGAGAATG R: ATCGTGGTGTTCCTTTTGATG	118 bp
OPN	AY878328	F: ATGCATGACGCACCTAAGAAG R: TCAATTGACCTCAGAAGAGGC	267bp
PPIA	XM_001252921.1	F: ATG CTG GCC CCA ACA CAA R: CCC TCT TTC ACC TTG CCA AA	100 bp

3.6 Quantification of the Expression of the HSP-70.2, PRM1, OPN Gene

cDNA was filled into the microtiter plate. qRT-PCR was run using the program design: 95°C for 3 minutes and 40 cycles at 95°C for 15 seconds or 60°C for 45 seconds on the StepOne Plus qPCR system (Applied Biosystems). A total of 10 g iTaqTM SYBR® Green Supermix with Rox PCR core reagent (Bio-Rad), 2 l cDNA (50 ng/µl) and primers from each target gene (Table 1) were mixed with ddH2O to a final volume of 20 µl/well. used for PCR reactions. All samples were analyzed in duplicate, and the geometric meaning of the threshold value (Ct) was used to profile the mRNA expression. Geometric analysis of two housekeeping genes, namely the PPIA gene, was used to normalize the target gene. The value of delta Ct (ΔCt) was calculated as the difference between the target gene and the reference gene (ΔCt = Ctgen target-reference Ctgen) (Nader et al., 2015).

3.7 Total DNA Extraction

Total genomic DNA from spermatozoa will be extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. A modified protocol will be carried out to improve the yield and quality of the extracted DNA. The frozen semen was thawed in water for 30 seconds and then put into a microtube. Put 100 L of cement into a 2 ml tube. Then add 200 l and centrifuged at 10000 rpm for 10 minutes to clean the diluent spermatozoa. Then the supernatant was discarded and 200 l of ATL buffer was added to the pellet, then ground with a micro pestle. Proteinase-K 30 l was added and the solution was incubated at 56°C for one hour, every 30 minutes the sample was inverted, then 200 l buffer AL was added and incubated at 56°C for 10 minutes. 200 l absolute ethanol was added and mixed manually, then stored in the freezer overnight. The supernatant was then transferred to a spin column and centrifuged for 1 minute at 8000 rpm, added with 600 l of AW1 and centrifuged for 1 minute. AW2 600 l was added and centrifuged at 8000 rpm for 2 minutes.

The spin-column was transferred to a new 1.5 ml tube and 100 l of buffer AE was added. Then the tube will be centrifuged for 1 minute at a speed of 10000 rpm.

3.8 Amplification, DNA Sequencing and Evaluation of Genotyping

Polymerase Chain Reaction (PCR) will be used to amplify the target gene. Each PCR amplification will be carried out at a total volume consisting of 25µL of PCR mix 1X PCR buffer (Promega), 1X GC enhancer, 0.2mM dNTP (Qiagen), 10pg DNA template, 0.02U/µl Taq polymerase (BioLabs; UK), 0 .02 M of each primer (Table 1). The amplification conditions consisted of initial pre-termination for 5 min at 94 ℃ followed by 35 cycles: 45 s at 94 ℃, 45 s at annealing temperature (based on optimization) and 6 min at 72 ℃, subsequent final incubation at 72 ℃ for 10 minutes.

The PCR product was detected with 1.2% agarose using 1xTBE buffer (89 mM Tris, 89 mM boric acid, and two mM EDTA, pH 8.0) in the electrophoresis apparatus (Hoefer USA). Observations will be made under UV light with the Quantity One program GelDoc (Biorad) after the gel is stained with ethidium bromide (0.5 g/ml). The PCR product size marker to be used is 100 bp. PCR products resulting from electrophoresis analysis will be sorted using ABI Prism version 3.4.1 (USA) on the First Base Singapore sequencing service through the sequencing service in Indonesia, PT. Genetika Science, Jakarta.

3.9 Analysis of Spermatozoa Protein Profiles

Spermatozoa protein has a contribution to male fertility. Differences in protein profiles of various fertility categories can be used as a predictor of fertility markers. In addition, protein profile analysis was intended to determine the relationship between gene profiles and gene expression from spermatozoa RNA.

Two samples of frozen semen straw were thawed, each was put into a 1.5 ml microtube for washing three times. Spermatozoa protein extraction was carried out according to Selvam et al. (2019) with modifications. The first step was centrifugation at 3000 rpm 4 ℃ for 10 minutes to dissolve the spermatozoa pellet with the supernatant. Then washing is done to remove the cryopreservation media (diluent) attached to the spermatozoa cells. Washing was carried out by adding 1200 l pH 7.4 PBS to the sample and centrifuged at 3000 rpm at 4 ℃ for 10 minutes to obtain spermatozoa pellets. Spermatozoa pellets were suspended in a buffer consisting of a radio-immunoprecipitation assay (RIPA, Sigma Aldrich, USA) supplemented with a protease inhibitor mixture. 100 L of RIPA buffer was added to 106 spermatozoa cells

and left overnight at 4 °C to lyse the cells. Then the suspension was vortexed for 10 minutes and continued with 20 seconds of sonication which was stopped three times. Then the samples were centrifuged at 10,000 rpm for 30 min at 4 °C. The collected supernatant was transferred into a microtube, and the precipitated cell debris was discarded. Spermatozoa protein extract was then quantified by the Bradford test and continued with SDS Page analysis based on the Laemmli method (1970).

The next step for liquid chromatography (LC-MS/MS) tandem mass spectrometry analysis was an aliquot sample of the digested spermatozoa protein extract using trypsin to produce peptides from all the proteins present in the sample. The peptides obtained were then separated by liquid chromatography (LC). The separation is based on charge and relative hydrophobicity. The amino acid sequence of the peptide was then determined by tandem mass spectrometry (MS/MS). The data obtained from LC-MS/MS were analyzed using software to identify proteins based on the database and analyze the molecular and biological functions of these proteins (Brewis and Gadella 2009).

3.10 Data Analysis

Between the high and low fertility groups, differences in gene expression levels of HSP-70.2 and OPN were analyzed using t-test. The relationship between HSP-70.2 gene expression, and OPN and male fertility was analyzed using Pearson correlation (Oliveira et al. 2013).

IV. RESULT AND DISCUSSION

4.1 Grouping of Madura Bulls

Local bulls that have been grouped into fertility levels in this study are Madura cattle. Madura cattle in this study were classified into three groups of fertility levels, namely High Fertility (HF), Medium Fertility (MF) and Low Fertility (LF) as shown in Table 2. Classification was carried out based on the calculation of the percentage of first service conception rate (% FSCR) using data secondary iSIKHNAS 2018-2020. The range of the number of IB for each Madura bull is 100 to 1500 times IB. The %FSCR value of Madura cattle has a mean-1SD value of 65.20, and a mean+1SD value of 79.81; males with %FSCR value < 65.20 were classified as Low Fertility (LF) (n=2), males with %FSCR value >80.26% were classified as High Fertility (HF) (n=2) males. Meanwhile, the rest are classified as Medium Fertility (MF).

Table 2. Classification of Madura bulls based on the results of the calculation of the percentage of first service conception rate (%FSCR)

Bulls ID	Bulls Name	%FSCR	Fertility Rate
160830	Pajudan	80.66	*High Fertility*
161001	Montehai	81.06	*High Fertility*
161035	Pasean	77.26	*Medium Fertility*
161033	Lombang	72.73	*Medium Fertility*
161204	Mangar	74.13	*Medium Fertility*
161002	Manding	68.35	*Medium Fertility*
161034	Siring	64.86	*Low Fertility*
160831	Jengka	60.97	*Low Fertility*

(Source: ISIKHNAS year 2018-2020)
Information: mean-1SD: 65.20; mean+1SD: 79.81

Accurate fertility prediction for Madura cattle bulls in the field is very important, especially for economic purposes. Semen with low fertilizing ability and/or failure to fertilize causes significant losses to farmers and the breeding industry and insemination centers. Breeders and the breeding industry have to pay for repeating AI, while the insemination center has to pay for the treatment of males who may be subfertile. Several times, markers, methods, or tests have been identified that can help predict male fertility, but the results are inconsistent. Therefore, further research is needed (Rodríguez-Martínez, 2013).

4.2 Evaluation of Spermatozoa Viability and Abnormality

The mean percentage of dead spermatozoa in the semen samples of Madura cattle was statistically significantly different between the HF group of 16.46 ± 1.05%% and LF of 34.02% ± 2.37. However, the percentage of dead MF spermatozoa was around 19.59%. ± 2.51. HF Madura males had a lower percentage of dead spermatozoa ($P<0.05$) compared to LF but not significantly different from MF. The percentages of viable normal spermatozoa in HF, MF and LF were 80.84±2.18, 77.29±2.04 and 60.96± 1.20 while the percentages of viable abnormal spermatozoa in HF, MF and LF were 2.7±1.89, 3.12±1.42 and 5.02±1.67% (Fig. 1). Collectively, the data presented here indicate that Madura males with HF have viable normal spermatozoa compared to Madura males with LF.

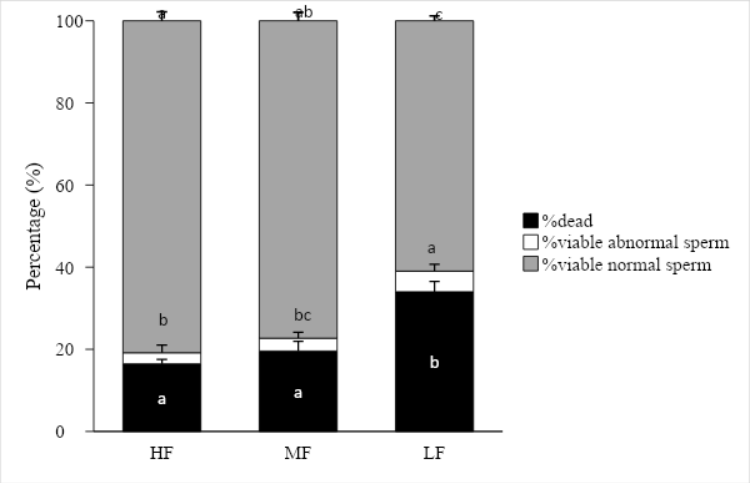

Figure 1. Viability and abnormalities of spermatozoa of Madura bulls with different fertility rates. Means with different letters differ significantly from each other (P<0.05), and the error bar is the standard deviation of the mean. HF: high fertility; MF: medium fertility; LF: low fertility

Pearson correlation coefficients for dead spermatozoa, abnormal live spermatozoa (AVS), normal live spermatozoa (NVS) and frozen semen fertility in Madura bulls are given in Table 3. Dead spermatozoa were reported to have a weak and negative correlation with fertility rate (r = -0.257). Similarly, AVS was negatively correlated with fertility (r = -0.606). On the other hand, the correlation of NVS with fertility was positive and strong (r = 0.867). The data presented here indicate that fertility is affected by AVS.

Table 3. Pearson correlation coefficient of dead spermatozoa, abnormal viable spermatozoa, normal viable spermatozoa, and fertility rate of frozen semen in eight Madura bulls

Parameter	**Dead**	**AVS**	**NVS**	**Fertility rate**
Dead	1			
AVS	0.332	1		
NVS	-0.515	-0.764**	1	
Fertility rate	-0.257	-0.606*	0.867**	1

Spermatozoa quality is a contributing factor to the male fertility profile (Franken 2014). The high percentage of live and morphologically normal spermatozoa (NVS) in Madura cattle HF group indicated that these cows had good fertility ability. Spermatozoa are alive and physiologically normal will be able to carry out their functions normally so that they can fertilize oocytes.

4.3 Optimization of Primary Fertility Marker Genes

Madura cattle are part of the germplasm of beef cattle that live in dry and infertile environments such as Madura Island. Madura Island is a hot and dry area with a temperature of 27-34°C, rainfall of 1600 mm/year, 80% humidity and dry soil conditions (Kutsiyah, 2017). This cow has good adaptability to heat stress, poor feed and small body with good reproductive ability. Thus, Madura cattle become local beef cattle with very high adaptability to the environment (Maylinda et al. 2019). HSP70 can also be a good indicator of thermotolerance and thermo-resistance (Subekti et al. 2019) as well as protection against thermal stress in the sperm of Madura bulls. Therefore, it is possible to be a potential biomarker for animal fertility and heat stress. Thus, HSP70-2 is also known as a dual-function gene.

It can be said that the spermatozoa DNA extraction method was successfully used to detect traces of HSP 70-2 in spermatozoa. DNA was extracted from post-thawing frozen semen samples using the Qiagen Blood and Tissue kit (Qiagen, USA) as described in the manufacturer's protocol with some modifications. The concentration and purity of DNA were read using Nano-drop (Nano Drop Thermo Scientific 200, USA). The best purity of extracted DNA with A260/280 ratio should be 1.8-2.0 (Table 4).

Table 4. Results of Reading Concentration of Madura Bulls Spermatozoa DNA with NanoDrop

Sample Name	Concentration	A260/280
Pajudan	17,851	1,871
Mangar	7,700	1,994
Jengka	5,650	1,844

Purification of PCR products using the main design of Zhang *et al.* (2015) with an anneling temperature of 53 °C and then sorted at First BASE Laboratories/Malaysia. Alignment and comparison of nucleotide sequences was carried out using the Mega 7 software. BLAST analysis was carried out on the website http://www.ncbi.nlm.nih.gov. Multiple Sequence Alignment was performed on the website http://www.ebi.ac.uk/Tools/msa/clustalo/ and compared with GenBank HSP70-2 with accession number NM_174344.1. The PCR product was detected on 1.2% ethidium bromide agarose gel using 1xTBE buffer (89 mM Tris, 89 mM boric acid, and two mM EDTA, pH 8.0) in an electrophoresis device (Hoefer, USA). Observations will be made under UV light using the GelDoc Quantity One (Biorad) program after the gel is stained with ethidium bromide (0.6 g/ml). The size of the electrophoretic DNA sample was estimated by visual comparison of the sample DNA band with the band on the DNA ladder and verified by sequencing results. The size of the HSP 70-2 PCR product was

118 bp. The DNA sequencing results of Madura bull spermatozoa were aligned with forward and reverse Hsp70-2 genes of Bos Taurus bulls in the gene bank (Access No. NM_174344.1) and OPN of Bos Taurus bulls in the gene bank (Access No. AY878328) (Figure 2.)

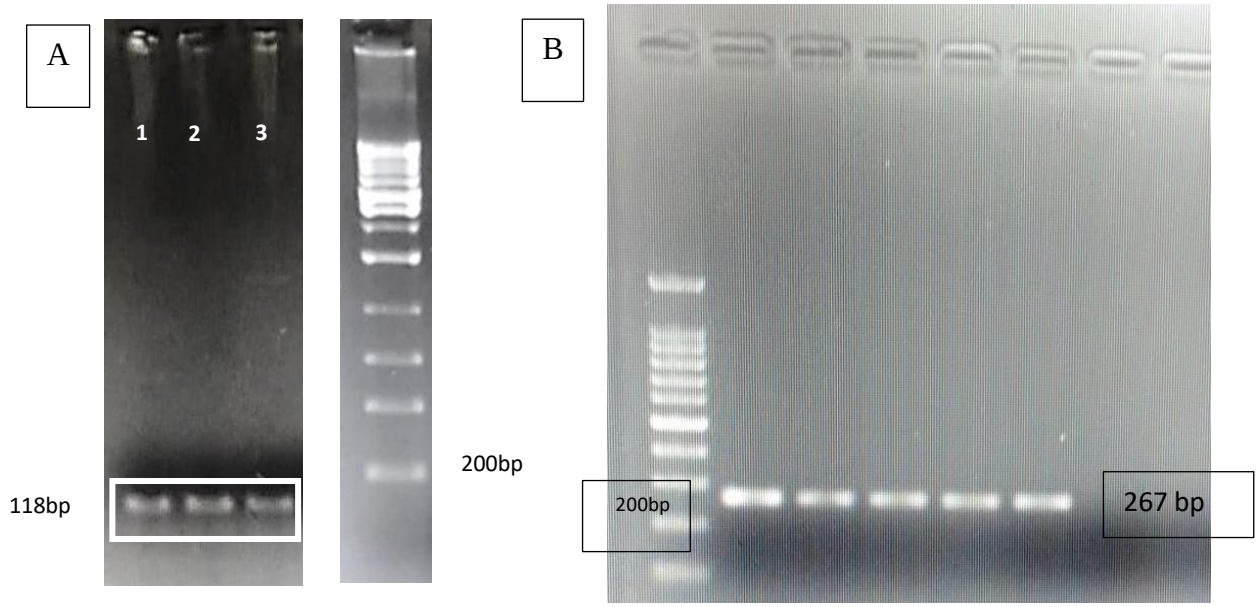

Figure 2. PCR product gel electrophoresis. A) the Hsp70-2 gene and B) the OPN gene.

4.4 Madura Bulls Spermatozoa RNA Extraction and Expression

Based on the process of extracting spermatozoa RNA using the RNeasy Mini Kit, then testing the quality of the RNA extraction results and the concentration of RNA obtained using NanoDrop. The data on the quality of RNA extraction results are presented in Table 5. These results indicate good RNA concentration and quality where the A260/230 value is in the range of 1.5-2.0. The RNA extraction results obtained were then diluted to equalize the RNA concentrations due to the difference in RNA concentrations obtained from 23.5 ng/µl to 184.5 ng/µl. The concentration of RNA that has been generalized is then processed for further transcriptomic analysis by converting it into cDNA, according to the research conducted by Pang et al. (2020).

Table 5. RNA concentration and quality

No	Sample Name	Fertility Group	RNA Concentration (ng/µL)	A260/230
1	Pajudan	High Fertility	184,5	1,85
2	Montehai	High Fertility	63,8	1,75
3	Pasean	Medium fertility	95,9	1,67
4	Lombang	Medium fertility	65,6	1,76
5	Manding	Medium fertility	23,5	1,57
6	Mangar	Medium fertility	165,2	1,56
7	Siring	Low fertility	85,6	1,65
8	Jengka	Low fertility	153,3	1,75

4.5 Extraction and Analysis of Post-Thawing Spermatozoa Protein with SDS-PAGE

Based on the optimization results of HSP70-2 and OPN primers on DNA and RNA of Madura cattle spermatozoa, it can be confirmed that HSP70-2 and OPN genes are present/expressed in Madura cattle spermatozoa. Several studies have shown that protein is correlated with RNA, but several studies have refuted this (Nagaraj et al. 2011; Vogel and Marcotte 2012; Payne 2015). Therefore, in this study, after successfully confirming the expression of HSP70-2 and OPN genes in Madura cattle spermatozoa, protein analysis still needs to be done to strengthen the description of these fertility marker candidates.

Spermatozoa protein expression of Madura cattle HF, MF and LF groups showed protein bands with various protein molecular weights (Figure 3). On SDS PAGE agar it can be seen that the protein HSP70-2 with a molecular weight range of 75.37 kDa and OPN at 60.69 kDa. The bands at specific molecular weights were then analyzed using ImageJ to estimate the levels of each of these proteins in the three fertility groups.

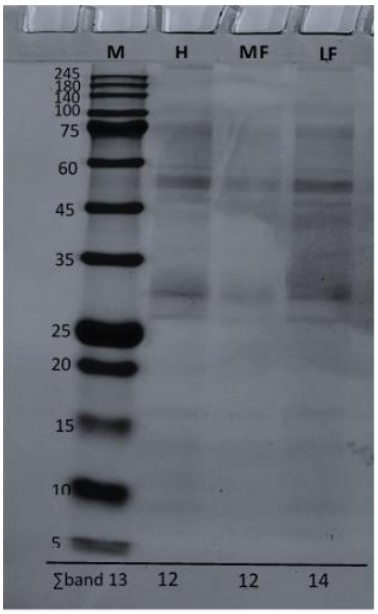

Figure 3. Protein band profile of Madura bulls spermatozoa with different fertility rates. M: markers; HF: high fertile; MF: medium fertile; LF: low fertile

The results of visualization of protein bands on SDS PAGE gel by measuring the gray density spectra on ImageJ software by adapting the working principle of Tsai et al. (2007). The agar image is converted to grayscale color and then its density is measured to predict the levels of each protein. From the processing results, the gray color density spectra produced from the HSP70-2 protein band with a weight of 75.35 kDa in HF were higher (thick) than MF and LF, while the OPN protein band with a weight of 60.69 kDa did not show a related pattern. Thus, this study shows that the HSP70-2 gene and protein can be used as fertility markers for Madura bulls, while the OPN gene and protein cannot be used as fertility markers for Madura bulls.

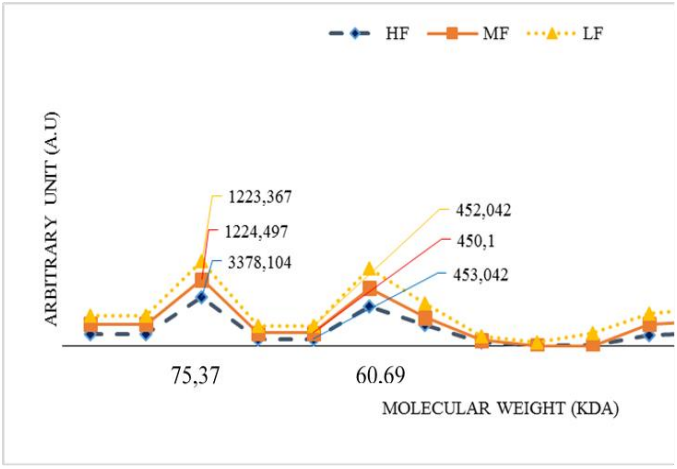

Figure 4. Grayscale spectral density from each protein bands from each fertility groups

V. CONCLUSION

The Madura bulls could be grouped into three fertility rates based on the field conception rate data (iSIKHNAS data): highly fertile, medium fertile, and low fertile. The sperm protein with an MWs 75.35 kDa and 5.36 kDa is predicted as HSP70 and PRM1 sperm protein biomarker that abundance in the high fertile of Madura bulls. They were expected as a specific protein biomarker that influences the Madura bull's fertility. Thus, the presence of specific sperm proteins and the viability of normal sperm are determiners of sperm fertilization abilities.

VI. REFERENCES

Agarwal A, Selvam MKP, Baskaran S. 2020. Proteomic Analyses of Human Sperm Cells: Understanding the Role of Proteins and Molecular Pathways Affecting Male Reproductive Health a review. *Int. J of Mol Sci.* 21 (1621): 1-21.

Agus A, Widi TSM. 2018. Current situation and future prospects for beef cattle production in Indonesia - A review. *Asian-Australasian J Anim. Sci*. 31(7): 976-983.

Andreas E, Sumantri C, Nuraini H, Farajallah A, Anggraini A. 2010. Identification of GH|ALUI dan GHR|ALUI genes polymorphisms in Indonesian buffalo. *JITA.* 35(4):215-221.

Artika IM, Susilo H, Setyo AVD, Hasan AEZ. 2011. Antibacterial activity of propolis chewing candy supplemented against *Streptococcus mutans*. *Microbiol.* 5: 99 – 102.

Brewis IA, Gadella BM. 2009. Sperm surface proteomics: from protein lists to biological function. *Mol.Hum Reprod.* 16(2):68–79. DOI: 10.1093/ molehr/ gap077.

Chamdi AN. Karakteristik sumberdaya genetic ternak sapi bali (*Bos-bibos banteng*) dan alternative pola konservasinya. *Biodiversitas.* 6 (1): 70-75.

Chenoweth PJ.2004. Bull breeding soundness exams and beyond. In Proceed. The Applied Repro. Strategies in Beef Cattle Workshop. Sept 5-6 2002. Manhattan (US): North Nebraska.

Diskin MG.2018. Review: Semen handling, time of insemination and insemination technique in cattle. *Animal*. 12(S1): s75–s84.

Dogan SP, Vargovic P, Oliveira R, Belser LE, Kaya A, Moura A, Sutovsky P, Parrish J, Topper E, Memili E. 2015. Sperm protamine-status correlates to the fertility of breeding bulls. *Bio Reprod*. 92 (4): 1-9.

Fair S, Lonergan P. 2018. Review: understanding the causes of variation in reproductive wastage among bulls. *Animal*. 12(s1):s53–62. DOI: 10.1017/ S1751731118000964

Fortes MRS, Satake N, Corbet DH, Corbet NJ, Burns BM, Moore SS, Boe-Hansen GB. 2014. Sperm protamine deficiency correlates with sperm DNA damage in Bos indicus bulls. *Andrology*: 1-9.

Hartatik, T, Widi TSM, Widayati DT, Baliarti E. 2010. The exploration of genetic characteristics of Madura cattle. The 5th International Seminar on tropical Animal Production. 578–584.

Hilmia N, Noor RR, Sumantri C, Gurnadi RE, Priyanto R. 2013. Productivity and genetic diversity of local cattle in ciamis-West Java. *JITA*. 38(1):10-19.

Kaya A, Memili E. 2016. Sperm macromolecules associated with bull fertility. *Anim Reprod* Sci. 169:88–94. DOI: 10.1016/j.anireprosci.2016.02.015.

Kutsiyah F. 2017. Dynamic of population and productivity of Madura Bull in conservation area of Sapudi Islands (*In Indonesian*). *Sains Peternakan*. 15(2):70-77.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227(5259): 680-685. DOI: 10.1038/227680a0.

Ministry of Agriculture. 2018. Ministry of Agriculture statistics. Ranch. Jakarta (ID): Ministry of Agriculture of the Republic of Indonesia.

Moura AA, Memili E. 2016. Functional aspects of seminal plasma and sperm proteins and their potential as molecular markers of fertility. *Anim Reprod*. 13(3):191–199. DOI: 10.21451/1984-3143-AR884.

Nader LM, Mohamed AS, Ismail MM, Fatah DSA, Ibrahim TM. 2015. Molecular biomarkers: the development of mRNA multiplex RT-PCR for the definitive identification of semen. *MOJ Toxicology*. 1 (5): 169-177.

Nuraini H, Aditia EL, Brahmantiyo B. 2018. Meat quality of Indonesian local cattle and buffalo. Bovine Science - A Key to Sustainable Development, Sadashiv S. O. and Sharangouda J. Patil, IntechOpen, DOI: 10.5772/intechopen.79904.

Peddinti D, Nanduri B, Kaya A, Feugang JM, Burgess SC, Memili E. 2008. Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkas associated with fertility. *BMC Syst Biol*. 13:1–13. DOI: 10.1186/1752-0509-2-19.

Pratami R, Kurnia D, Anwar P. 2019. Evaluasi tingkat keberhasilan pelaksanaan program inseminasi buatan pada sapi bali *Bos javanicus* di Kecamatan Logas Tanah Darat dan Kecamatan Singingi Hilir. *JAC*. 1(2): 91-104.

Prihatin KW, Maylinda S, Hakim L. 2018. The SRY gene variations amongst selected Madura cattle populations. *J Ked Hewan*. 12(4):101–3. DOI: 10.21157/j.ked.hewan.v12i4.9674.

Rodríguez-Martínez, H., 2013. Semen evaluation techniques and their relationship with fertility. Anim. Reprod. 46, 148–159.

Rosyada ZNA, Ulum MF, Tumbelaka LITA, Purwantara B. 2020. Sperm protein markers for Holstein bull fertility at national artificial insemination centers in Indonesia. *Vetworld*. 13(5):947-955. DOI: 10.1402/vetworld.2020.947-955.

Selvam MKP, Agarwal A, Pushparaj PN, Baskaran S, Bendou H. 2019. Sperm proteome analysis and identification of fertility-associated biomarkers in unexplained male infertility. *Genes*. 10 (522): 114

Siswanto M, Patmawati NW, Trinayani NN, Wandia IN, Puja IK. 2013. Penampilan reproduksi sapi bali pada peternakan intensif di instalasi pembibitan Pulukan. *JIKH*. 1(1): 11-15.

Somashekar L, Selvaraju S, Parthipan S, Ravindra JP. 2015. Profiling of sperm proteins and association of sperm PDC-109 with bull fertility. *Syst Biol Reprod Med*. 61(6):376–387. DOI:10.3109/ 19396368.2015.1094837.

Souza CEA, Moura AA, Monaco E and Killian GJ. 2008. Binding patterns of bovine seminal plasma proteins A1/A2, 30 kDa and osteopontin on ejaculated sperm before and after incubation with isthmic and ampullary oviductal fluid. *Anim Reprod Sci*. 105. 72-89. doi:10.1016/j.anireprosci.2007.11.027

Subekti K, Solihin DD, Afnan R, Gunawan A, Sumanri C**.** 2019. Pengaruh Polimorfisme Gen Heat Shock Protein 70 (HSP70) SacII terhadap Toleransi Panas Itik Lokal Sumatera Barat. *Jurnal Agripet* 19:122–128. doi:10.17969/agripet.v19i2.14876

Sutarno S, Setyawan AD. 2016. The diversity of local cattle in Indonesia and the efforts to develop superior indigenous cattle breeds. *Biodiv J Biol Div*. 17(1): 275-295.

Tsai H, Low TY, Freeby S, Paulus A, Ramnarayanan K, Cheng CP, Leung HE. 2007. Increase in local protein concentration by field-inversion gel electrophoresis. *Prot. Sci.* 5(18):1-14. doi:10.1186/1477-5956-5-18

Wildan H, Anwar P, Jiyanto. 2020. Evaluation of success level of implementation of made insemination in bali cows in Kuantan Singingi District. *Green Swarnadwipa*. 9 (1): 71-77.

Williams, Chance L., "Effects of Osteopontin Single Nucleotide Polymohisms on Bull Semen Quality" (2011).Theses and Dissertations. 109. http://scholarworks.uark.edu/etd/109

Zhang XG, Hong JY, Yan GJ, Wang YF, Li QW, Hu H. 2015. Association of heat shock protein 70 with motility of frozen-thawed sperm in bulls. *Czech J. Anim Sci.* 60(6): 256 – 262.

Zuhri MS, Ihsan MN, Isnaini N. 2019. Evaluation of the reproductive performance of Madura cattle raised by small-scale farmers in Madura, Indonesia. Livestock *Research Rural Dev*. 31(5): 1-7.