

IDENTIFICATION OF POLYMORPHISM OF FSH BETA-SUBUNIT GENE AS SPERM QUALITY MARKER IN BALI CATTLE USING PCR-RFLP

A.B.L. Ishak^{1,4}, C. Sumantri², R. R. Noor² and I. Arifiantini³

¹Graduate School, Bogor Agricultural University,
Jl. Agatis, Darmaga Campus, Bogor - Indonesia

²Faculty of Animal Science, Bogor Agricultural University,
Jl Agatis, Darmaga Campus, Bogor - Indonesia.

³Faculty of Veterinary Medicine, Bogor Agricultural University,
Darmaga Campus 16680, Bogor - Indonesia

⁴Assesment Institute for Agricultural Technology, South Sulawesi,
Jl. Perintis Kemerdekaan Km 17 Makassar, South Sulawesi - Indonesia
Corresponding E-mail : lompengengishak_genetika@yahoo.co.id

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ABSTRAK

Penelitian dilakukan untuk mengidentifikasi keragaman gen FSH beta-subunit dengan metode PCR-RFLP sebagai penanda kualitas sperma pada sapi Bali. Pengambilan sampel darah untuk ekstraksi DNA dari berbagai populasi sebanyak 470 sampel dan 127 sampel yang berasal dari Balai Nasional Inseminasi Buatan dan Balai Inseminasi Buatan Regional beserta data sperma. Frekuensi alel pada sapi Bali adalah alel A (0,000) dan alel B (1,000). Tidak ditemukannya alel A menunjukkan gen FSH beta-subunit pada sapi Bali bersifat monomorfik. Hasil yang berbeda pada sapi Brahman, FH, Simmental dan Limousin yang bersifat polimorfik dengan adanya alel A dan B. Heterozigositas pengamatan tertinggi pada sapi Limousin (0,318) dan heterozigositas harapan tertinggi pada sapi Simmental (0,420). Nilai chi-square tidak berpengaruh nyata pada sapi Bali dan Simmental, namun pada sapi Brahman, FH dan Limousin berpengaruh nyata. Pada bangsa sapi yang dianalisis persentase abnormalitas sperma lebih tinggi ditemukan pada sapi Simmental, Limousin, Brahman dibandingkan pada sapi Bali dan FH. Persentase abnormalitas sperma tipe abaxial lebih tinggi ditemukan pada sapi Brahman, Simmental dibandingkan pada sapi FH. Dari semua tipe abnormalitas sperma, persentase tipe abaxial dan microcephalus paling tinggi.

Kata kunci : Gen FSH beta-subunit, polimorfisme, abnormalitas sperma

ABSTRACT

The aim of study was to identify the association of FSH beta-subunit gene polymorphisms with sperm quality traits. A total of 470 samples of normal mature bull from several breeds were used for population study and 127 bulls from National and Regional AI centre of Indonesia for association study. To amplify, a PCR-RFLP method was used and digested with *Pst*I restriction enzyme. The allele frequency of the A and B in Bali cattle were (0.000) and (1.000), respectively. The absence of other allele A suggested that the Bali cattle was monomorphic, while Brahman, FH, Simmental and Limousine were polymorphic. The highest observed heterozygosity were found in Limousine (0.318) and the highest expected heterozygosity were in Simmental (0.420). The higher incident of percentage of sperm abnormalities were found in Simmental, Limousin, Brahman compared to Bali and FH. Among all types of sperm abnormalities, the abaxial and microcephalus were found in highest number.

Key words : FSH beta-subunit gene, polymorphism, sperm abnormalities

INTRODUCTION

Follicle stimulating hormone (FSH) is a glycoprotein hormone expressed in the pituitary,

and it is responsible for regulating reproduction in mammals (McLahlan and Meachem, 2003; Otha *et al.* 2007). In males, FSH in combination with testosterone is the most important tropic hormone

regulating Sertoli cell function, requires for the initiation and maintenance of the quality and quantity sperm in spermatogenesis processes. Follicle stimulating hormone (FSH) acts on the germinal cells in the seminiferous tubules of the testis and is responsible for spermatogenesis up to the secondary spermatocyte stage, and androgens from the testis support the final stages of spermatogenesis.

The genetic factors influence on production and sperm quality have been reported by several studies (Kim *et al.*, 1988; Fries, 1989). Dai *et al.* (2009) and Dai *et al.* (2011) examined the gene polymorphism FSH beta-sub unit of production and sperm quality in Simmental, Charolais and Limousin. Some researchers also associate the diversity of FSH beta-sub unit genes with litter size in sow (Yaofeng *et al.*, 1998; Li *et al.*, 2000; Li *et al.*, 2008; Liu *et al.*, 2009), sperm quality in boar (Wimmer *et al.*, 2005; Lin *et al.*, 2006), litter size in ewes (Xiaopeng *et al.*, 2010; Zhang *et al.*, 2011) and equine sperm quality (Samper, 2010). FSH beta-sub unit gene specific unit is located on chromosome 15 that comprises two introns and three exons with a length of 6601 bp (Kim *et al.*, 1988; Fries, 1989; Hediger *et al.*, 1991). Variants of this gene was three alleles, namely A, B, and C. Dai *et al.* (2009) found 9 SNPs mutations, *i.e.* 4 mutations in promoter section ('5 URR), 3 in intron 2, and 2 in exon 3. Polymorphism of FSH beta-sub unit gene in exon 3 on the study significantly influenced the fresh and frozen semen quality. The AA and AB genotypes showed better quality of fresh as well as in frozen semen when compared to the BC. Based on the previous study in *Bos taurus* and *Bos indicus*, this research aimed to investigate the polymorphisms of FSH beta-sub unit gene as marker of sperm quality in Bali cattle (*Bos javanicus*).

MATERIALS AND METHODS

Sources of Animal and Data Collection

A total of 470 heads of normal mature bulls (225 Bali, 69 Brahman, 92 FH, 44 Limousin and 40 Simmental) for population studied. While, for association studied were used normal mature bulls

from National and Regional AI centre of Indonesia (24 Bali, 6 Brahman, 13 FH, 44 Limousin and 40 Simmental).

Isolation of Genomic DNA

Blood samples were collected from each bull in 10-ml non anticoagulant polypropylene tubes. Blood samples then were mixed with 96% ethanol. Genomic DNA was isolated by the phenol-chloroform extraction method (Sambrook *et al.*, 1998; Andreas *et al.*, 2010) and then were dissolved in TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA). Genomic DNA was stored at -20 °C until amplification with polymerase chain reaction (PCR).

PCR Amplification and RFLP Analysis

The amplification of PCR was carried out using specific primer (Dai *et al.*, 2009) for part intron 2 and complete coding of exon 3 (313 bp) (Table 1). The PCR was performed in a final volume of 25 µl for each reaction containing 2 µl DNA templates, 0.2 µl primer, 0.2 µl dNTPs, 0.1 µl Taq polymerase, 1 µl MgCl₂, 2.5 µl 1 X reaction buffer and 18.1 µl dilution of sterile water. The reaction mixture was subjected to an initial 5 min of denaturation 95 °C, followed 35 cycles of denaturation at 95 °C for 30 sec, annealing 30 sec at 63 °C, extension 1 min at 72 °C and final extension 5 min at 72 °C. Amplicons were visualized by electrophoresis in 1.5 % agarose gels containing 500 ng/ml of ethidium bromide in 1× TAE buffer with a 100-bp ladder as the molecular weight marker for confirmation of the length of the PCR products. For digestion by using enzyme and determination of RFLP, 7 µl of PCR product was added to 1 µl Pst1 enzyme and 2 µl buffer 10X with final volume 10 µl. The PCR products of FSH beta-sub unit gene digested at 37°C overnight by Pst1 enzyme. The digestion products were separated by horizontal electrophoresis (85 volts, 50 min) in 2% agarose gels in 1 × TBE and 10% ethidium bromide.

Semen Preparation

Methods of fresh semen collections and smears preparations was according to Arifiantini *et al.* (2010) and Bhakat *et al.* (2011), and were

Table 1. Primer Sequence Used in PCR

Primer	Sequence	PCR Product	Annealing	Reference
Forward	F: 5`CTTCCAGACTACTGTAACATC`3	313 bp	63	Dai <i>et al.</i> (2009)
Riversed	R: 5`GTAGGCAGTCAAAGCATCCG`3			

performed from bull in AI centers. The samples were then delivered to the research Animal Genetic and Molecular Laboratory in Bogor Agricultural University. Sample collections were performed based on the standard protocol: a drop of semen was placed on the a glass slide, mixed with four drops of physiologic saline, homogenized by using a stick, and smeared on different glass slides. Smeared semen samples were air dried coded according to the bull ID, and packed in a glass slides box. Smeared samples were stained with carbofuchsin-eosin according to the method described by Williams in 1920 and modified by Lagerlof in 1934 (Kavak *et al.*, 2004). Steps in Williams staining protocol were as follow: The air-dried, thin smears of fresh semen on glass slides from AI centers were fixed in flame, washed with absolute alcohol for 4 minutes, and air dried. Then, the smears were treated with 0.5% chloramines solution for 2 minutes until the mucous disappeared and the smears looked fairly clear. The smears were washed in distilled water, rinsed in 95% alcohol, and stained with Williams for 8-10 minutes. Finally, the smears were washed in running water and allowed to dry.

Data analysis.

PCR-RFLP data were analyzed by calculating allele and genotype frequencies (Nei and Kumar, 2000). The allele and genotype frequencies were calculated by the following formulas :

$$X = \frac{(2n_{ii} + \sum n_{ij})}{2n}$$

x = allele frequencies

n_{ij} = Number of genotype A_iA_j

n_{ii} = Total of genotype A_iA_i

n = Total sample

$$\hat{h} = 2n - \frac{(1 - \sum x_i^2)}{2n}$$

x_i = allele frequencies

n = Total sample

\hat{h} = heterozygosity

Hardy-Weinberg equilibrium

Hardy-Weinberg equilibrium was tested by the χ^2 (Chi-square) (Noor, 2008).

$$\chi^2 = \frac{\sum (O-E)^2}{E}$$

χ^2 = Hardy-Weinberg equilibrium test

O = observed number of genotype A11

E = expected number of genotype A11

Degree of freedom (df) was according to Allendorf and Luikart (2007) :

$df = (\text{Number of genotype} - i) - (\text{Number of allele} - j)$

Sperm Abnormalities

The sperm were counted on each smeared using a light microscope at 400 X magnification (Olympus CH 20). Sperm morphology was examined and all types of abnormalities were recorded and classified. The classification sperm abnormalities was based on Bart and Oko (1989), Chenoweth (2005) and Arifiantini *et al.* (2010) such as : abaxial, macrocephalus, microcephalus, round head and knobbed acrosome defect. Sperm abnormalities were calculated from the type of abnormality in each field of view or 500 cells.

Sperm abnormalities =

$$\frac{\text{number of type sperm abnormalities}}{500 \text{ cell}} \times 100$$

RESULTS AND DISCUSSIONS

Amplification of FSH beta-sub unit Gene

The amplicon of FSH beta-subunit was 313 bp in length, in which this result was consistent with Dai *et al.* (2009). FSH beta-sub unit gene was located in chromosomes 15th with 3 exons and 2 introns (Kim *et al.*, 1988; Xiao-peng *et al.*, 2010). The nucleotide sequences of FSH sub-beta unit gene were 6610 base pair in length (GenBank No : M83753).

In this research, it has been amplified part of intron 2 and the complete coding region of exon 3 (313 bp). The amplification fragment of FSH beta-sub unit gene carried on GeneAmp® PCR System 9700 (Applied Biosystem) is shown in Figure 1.

Genotype and Allele Frequencies

The PCR product that has been digested by *Pst*I enzyme incubated for 14 hours at 37 °C resulted in 3 fragments. Genotype data of FSH beta-sub unit gene showed 313 bp for genotype BB, 202 bp and 99 bp for genotype AA, 313 bp, 202 bp and 99 bp for AB genotype. Visualization of RFLP result is shown in Figure 2.

Results of FSH beta-sub unit gene analysis showed that only B allele and BB genotype was found in Bali cattle. The absence of other alleles

suggested that Bali was monomorphic. This result was different from Brahman (*Bos indicus*), FH, Simmental and Limousine (*Bos taurus*) showing polymorphic. There was variation of alleles A and B as well as genotype variation, those were AA, AB and BB. The highest frequency of allele A was found in FH (0.891) and the lowest one in Bali cattle (0.000). The highest allele B was found in Bali (1.000) and the lowest one in Brahman cattle (0.174). These findings were similar to those previous reported in Germany FH cattle and Gelbvieh cattle (Schlee *et al.*, 1994), frequency of allele A was 0.000 and B was 1.000. Schlee *et al.* (1994) found the frequencies of allele A and B in Simmental were 0.831 and 0.147, respectively; in Limousin were 0.910 and 0.431, respectively. The allele and genotype frequency of each breed presented in Table 2.

Sequences Alignment of the FSH Beta-sub Unit Alleles

The result of PCR product sequences showed mutation. Sequences based on GenBank access number M83753, the genotype AA at position 237 and 238 nucleotides were CA, but the BB genotype was TG (Figure 3).

Association polymorphism FSH beta-sub unit gene with sperm abnormalities

Sperm abnormalities have long been associated with male infertility and sterility in most species studied. These abnormalities vary from morphological defects that are evident upon clinical examination, to those, which are more subtly defective. In general, sperm structure can play a substantial role in both fertilization and pregnancy outcome (Chenoweth, 2005). Eventhough the heritability of bull fertility is generally considered to be low, certain aspects of bull fertility, including sperm morphological abnormalities are under genetic control. Sperm abnormalities according to Chenoweth (2005) and

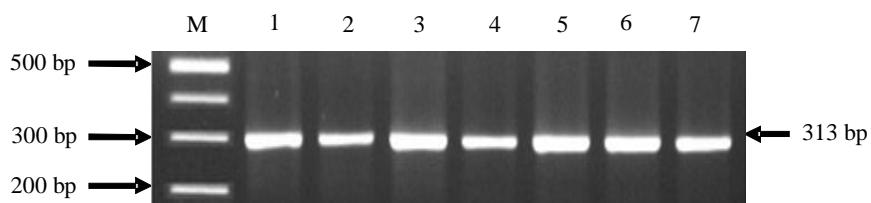


Figure 1. Amplification of FSH Beta-subunit Gene

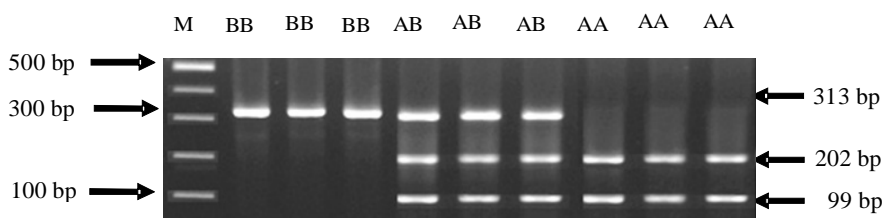


Figure 2. Genotyping of FSH Beta-subunit Gene with *PstI* Enzyme

Table 2. Allele and genotype Frequencies of FSH Beta-subunit Gene

Breed	Allele A	Genotype			X^2	PIC	
		B	AA	AB			BB
Bali	0.000	1.000	(0) 0.000	(0) 0.000	(225) 1.000	-	-
Brahman	0.174	0.826	(6) 0.087	(12) 0.174	(51) 0.739	10.75 *	0.246
FH	0.891	0.109	(81) 0.880	(2) 0.022	(9) 0.098	72.51*	0.175
Simmental	0.700	0.300	(24) 0.600	(8) 0.200	(8) 0.200	10.97*	0.332
Limousin	0.818	0.182	(29) 0.659	(14) 0.318	(1) 0.023	0.212	0.253

Note : () = number of sample, * = significant ($P < 0.05$), PIC = Polymorphism Information Content

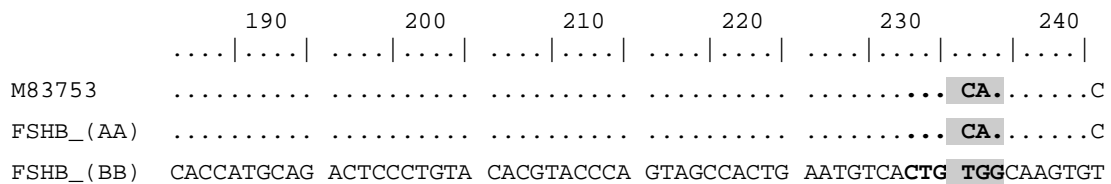


Figure 3. The Position of Polymorphic FSH Beta-sub Unit Gene

Table 3. Percentage of Sperm Abnormalities in Different Breed

Breed	Genotype	Sperm Abnormality (%)				
		Abaxial	Macro chepalus	Micro chepalus	Round head	Knobbed acrosome defect
Bali	BB (24)	0.34	0.17	0.17	0	0.37
	AA (3)	0.09	0	0.02	0	0.06
Brahman	AB (2)	0.01	0	0	0	0
	BB (3)	0.01	0.01	0.01	0	0
Holstein Frisian	AA (9)	0.03	0.03	0.08	0.05	0.09
	BB (4)	0.05	0	0.03	0	0.10
Simmental	AA (18)	0.72	0.50	0.50	0.21	0.22
	AB (9)	0.14	0.07	0.22	0.07	0
	BB (9)	0	0.22	0.29	0.65	0
Limosin	AA (10)	0	0.1	0.21	0.21	0.20
	AB (9)	0.31	0.05	0.26	0.10	0
	BB (7)	0.05	0.10	0.26	0.10	0.10

Freneau *et al.* (2010) was divided into major (15 type) and minor abnormality (16 type), where abnormality was caused by genetic factors ie. abaxial (abnormalitas on the tail), round head, microchepalus, macrochepalus, knobbed acrosome defect (abnormalities in the head). Abaxial is a type of sperm abnormality whereas the implantation fossa of the tail is off-center. Variable size is a term to describe sperm possessing head abnormalities which are bigger (macrocephalus) or smaller (microcephalus) than the normal size. Knobbed acrosome defect occurs in the acrosome region of sperm; the apex of the acrosome is flatte or inden (Table 3).

Among all types of sperm abnormalities, the microchepalus and abaxial were found in highest number, especially in Brahman bull, in kontras the round head was found in low number in Bali bull cattle. While in FH semen, knobbed acrosome defect and microchepalus were found in highest number. This research demonstrated that the Simmental and Limousin have a highest number of microchepalus and round head. Arifiantini *et al.* (2010) reported the sperm abnormalities from

14 AI centre in Indonesia, it was found that the Simmental, Limousine and Brahman cattle have a high number of primary sperm abnormalities which were 4.8%, 3.6%, 2.6% respectively, compare to Bali only was 1.8%, even so, its classified as low primary sperm abnormality. Classification as high primary sperm abnormality if the percentage is larger than 10.1-15 and very high if larger than >15%. Purwantara *et al.* (2010) found 1.00 to 8.40% primary sperm abnormality mostly pearshaped of FH bull from 20 samples collected from three different AI centers.

In this study the primary sperm abnormality such as the abaxial and microchepalus were found mostly in AA and BB genotypes and only abaxial was found the highest number in AB genotype (Figure 4).

CONCLUSION

The FSH beta-subunit gene in Bali cattle was monomorphic, in contrast to Brahman, FH, Limmousin and Simmental, in which were

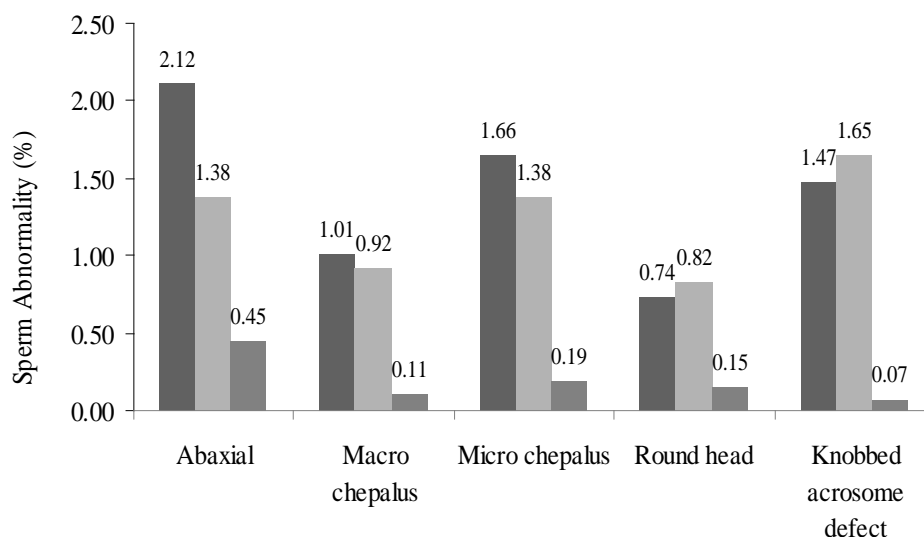


Figure 4. Type of Sperm Abnormalities Type of Bull Semen in Different Genotype

■ : AA, □ : BB, ▒ : AB

polymorphic with PCR-RFLP. In association to polymorphisms of FSH beta-sub unit gene in different genotypes with the percentage of the highest sperm abnormality, it was found in BB and AA genotypes.

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