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EFFECT OF FSH β-SUB UNIT AND FSHR GENES POLYMORPHISMS ON SUPEROVULATORY RESPONSE TRAITS

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ABSTRAK

Follicle Stimulating Hormone (FSH) merupakan hormon glikoprotein yang dihasilkan oleh kelenjar pituitary, yang berfungsi mengatur reproduksi pada mamalia. Hormon tersebut terdiri dari α dan β-sub unit. β-sub unit berperan dalam menentukan spesifisitas ikatan dengan reseptor (FSHR). Penelitian ini bertujuan untuk mengidentifikasi keragaman gen FSH β-sub unit dan FSHR, dan pengaruhnya terhadap respon superovulasi pada sapi yang tersuperovulasi. Penelitian dilakukan pada 32 sapi Angus, Friesian Holstein (FH), Limousin, Simmental dan Brahman di Balai Embrio Ternak (BET) Cipelang. Sapi betina yang digunakan telah disuperovulasi dan dikawinkan secara inseminasi buatan. Respon superovulasi (SR), tingkat ovulasi (OR), persentase pembuahan (FP) dan persentase embrio yang layak transfer (VP) dianalisis untuk dipeajari pengaruh gen FSH β-sub unit dan FSHR. Frekuensi alel FSH β - sub unit|PstI dan FSH|AluI berlawanan pada spesies yang diamati. Alel B dan alel C untuk gen FSH β-sub unit dan FSHR memiliki jumlah yang besar pada spesies *Bos taurus* sedangkan sebaliknya pada spesies *Bos indicus*. Heterozigositas tertinggi ditemukan pada sapi FH (0,250) untuk FSH β-sub unit dan Brahman (0,333) untuk FSHR. Pengaruh yang nyata ditemukan antara keragaman gen FSHR dengan tingkat ovulasi, dimana genotipe CC lebih tinggi (P<0,05) daripada genotipe CG dan GG.

Kata kunci: FSH, reproduksi, keragaman gen, superovulasi, kualitas embrio

ABSTRACT

Follicle stimulating hormone (FSH) is a pituitary expressed glycoprotein hormone that regulates reproduction in mammals which composed of α and β-sub unit. The β-sub unit dictates its binding specificity with their receptor (FSHR). This study aimed to identify polymorphism of FSH β-sub unit and FSHR genes, and its effect to superovulatory response traits on superovulated cows. Study was done on 32 cows including Angus, Friesian Holstein (FH), Limousin, Simmental and Brahman in Cipelang Livestock Embryo Center. Cows used have been treated superovulation and mated by artificial insemination. Superovulation response (SR), ovulation rate (OR), fertilization percentage (FP) and viable transfer embryo percentage (VP) were analyzed to investigate the effect of FSH β-sub unit and FSHR polymorphism. Allele frequency of FSH β-sub unit|*Pst*I and FSH|*Alu*I were opposite within species. Mostly B allele and C allele for FSH β-sub unit and FSHR respectively have a high number in *Bos taurus* species while those were in contrast in *Bos indicus* species. The highest heterozygosity was found in FH cattle (0.250) for FSH β-sub unit and Brahman (0.333) for FSHR. Significant effect was

found between FSHR gene polymorphism with ovulation rate where CC genotype was higher (P<0.05) than CG and GG genotypes.

Keywords: *FSH, reproduction, gene polymorphism, superovulation, embryo quality*

INTRODUCTION

Follicle stimulating hormone (FSH) has an important role in reproduction in mammals either for male of female. It is expressed in pituitary gland (Ulloa-Aguirre *et al*., 1995). In females, FSH is responsible for proliferation and survival of follicular somatic cells and plays important role in development of follicle till ovulation (McGee and Hsueh, 2000). Whereas in males, combination between FSH and testosterone is the most important tropic hormone regulating sertoli cell function, required for the initiation and maintenance of the quality and quantity in spermatogenesis (Ohta *et al*., 2007).

Interaction between FSH and its receptor (FSHR) have a major role in follicles development and steroidogenesis regulation in the ovary. FSH is composed of a common α subunit and a hormone-specific β-sub unit, and although both of subunits contribute to bind the FSH receptor (FSHR), the β-sub unit dictates its binding specificity (Fan and Hendrickson, 2005). Bulls with mutation in exon 3 gene FSH β-sub unit identified have a lower fresh semen concentration, lower percentage of acrosome integrity in both fresh and frozen semen, lower sperm motility in frozen semen, poor quality and resistance on freeze treatment and lower fertility (Dai *et al*., 2009). Huang *et al*. (2002), Wimmers *et al*. (2005) and Lin *et al*. (2006) suggested FSH β-subunit as a candidate marker for semen quality and fertility in boars.

Mutation on FSH β-subunit gene in women was reported affecting primary amenorrhoea and infertility phenotype (Matthews *et al*.,1993; Layman *et al*., 1998, 2002) with lower basal estradiol, progesterone and inhibin, having a high level of luteinizing hormone (LH) while FSH level is undetectable. In addition, Aittomäki *et al*. (1995) reported phenotypic similarity in patient with FSHR inactivation. Polymorphism study of FSH β-subunit and FSHR gene and its effect in animal livestock, especially cattle, is still limited. The objectives of this study were to identify polymorphism of these genes and effect of genotype on superovulatory response traits.

MATERIALS AND METHODS

Animal and Data Collection

A total of 32 animals consisted of Angus (3 heads), FH (8 heads), Limousin (10 heads), Simmental (8 heads) and Brahman 3 (heads) cattle in Cipelang Livestock Embryo Center were used in this study. Cows have been treated superovulation with foltropin hormone, then were mated by artificial insemination. Cows were maintained and fed in the same condition to minimize the effect of environment. Parameters observed were superovulation response (SR), ovulation rate (OR), fertilization percentage (FP) and viable transfer embryo percentage (VP). All of the experimental animals and data collection were handled according to standard operating procedure of Cipelang Livestock Embryo Transfer (BET Cipelang), Indonesian Ministry of Agriculture.

Blood and DNA Samples

Two ml of blood samples was obtained from the jugular vein by using multiple needle which collected in vaccutainer tubes containing K3EDTA anti co-agulan (VACUETTE®, Greiner Bio-One). Blood was homogenized and kept in refrigerator to prevent DNA molecules damage. The DNA was extracted from 200 µl of blood which lysed then added by proteinase K, phenol and choloroform and isoamyl alcohol solution to separate the DNA from other organic materials. Washing and purification of DNA molecules was carried out by using alcohol precipitation method (Andreas *et al*., 2011). Quality and quantity of DNA were measured by using Gene Quant type 100 spectrophotometer (GE Health) before used in subsequent analysis.

Amplification and Genotyping

Specific fragment amplification of FSH β-sub unit and FSHR were done by using polymerase chain reaction (PCR) methods. Primers were used in this study described in Table 1. A total of 25 µL reaction PCR component consisted 0.5 pmol of each primers, 0.2 mM of dNTPs, 2 mM of $MgCl_2$, 0.5 unit of taq polymerase (*Go Taq PCR Core System II*,

Table 1. Primer Information for Analysis

Gene	Primer sequences (5′ - 3′)	Position of Mutation	PCR product	Annealing	Restriction Enzyme	Reference
FSH β-sub unit	F: CTT CCA GAC TAC TGT AAC TCA TC R: GTA GGC AGC TCA AAG CAT CCG	Exon 3	313 bp	60°C	*Pst*I	Dai *et al*. (2009)
FSHR	F: CTG CCT CCC TCA AGG TGC CCC TC R: CCC CCT AAG ACA TTT AGC CAA GAA CT	Exon 10	306 bp	60°C	*Alu*I	Marson *et al*.(2008)

F: Forward; R: Reverse

Promega) and its buffer. Amplification process was run on GeneAmp® PCR System 9700 (*Applied Biosystems*™) with 35 cycles consisted of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and elongation at 72°C for 30 sec.

Genotyping was done by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods. Restriction enzyme used were *Pst*I and *Alu*I for FSH β-sub unit (FSHβ|*Pst*I locus) and FSHR (FSHR|*Alu*I locus) respectively. Allele and genotype identification was done through electrophoresis analysis on agarose gel 2% (v/w) which stained by EtBr above transiluminator and photographed by Alpha Imager® EP.

Data Analysis

Genotype data was analyzed for allele polymorphism information such as allele frequencies, χ^2 and heterozygosity by using Population Genetic Analysis (POPGENE Version 1.32). Effect of single gene (α_i) on superovulation response and embryo quality (γ_{ijk}) were analyzed using general linear model (GLM) method which were grouped based on breed of cattle (β_k) with mathematics model for GLM described as:

$$\log (\gamma_{ijk}) = (\alpha_i) + (\beta_k) + \varepsilon_{ijk}$$

Where

γ_{ijk} = Response of overovulation and embryo quality

α_i = Random effect of single gene

β_k = Random effect of breed of cattle

ε_{ijk} = Random error effect

RESULTS AND DISCUSSION

Amplification and Genotyping

Targeted fragment both of FSH β-sub unit and FSHR genes were successfully amplified by using PCR methods. Allele and genotype were identified by using PCR-RFLP methods generating two alleles and three genotypes for both two genes observed. Allele A in FSH β-sub unit was indicated by 313 bp band (unrestricted) in electrophoresis gel, while restricted fragment with 202 bp and 99 bp were named allele B (Figure 1). Moreover, for FSHR locus, C allele was indicated by 243 and 63 bp bands, while G allele has a three bands at 193, 63 and 50 bp (Figure 2).

FSH β Sub unit and FSHR Genes Polymorphism

The PCR-RFLP analysis showed that primers and restriction enzyme could be used to identify the point mutation in FSHβ|*Pst*I and FSHR|*Alu*I loci as described by Dai *et al*. (2009) and Marson *et al*. (2008). In Angus breed, genotype of both two genes observed were monomorphic, whereas frequency of B allele of FSHβ|*Pst*I locus and C allele C of FSHR|*Alu*I locus were 1 (one). Heterozygosity was which found in FSHR|AluI locus gene for almost in all population was higher than those in FSHβ|PstI locus, except in FH breed population. The χ2 analysis showed that Simmental in FSHβ|*Pst*I and FH in FSHR|*Alu*I loci were in unequilibrium with Hardy-Weinberg´s equation. These conditions indicated the tendency of higher selection intensity on both populations. Details of polymorphism of FSHβ|*Pst*I and FH in FSHR| *Alu*I loci are described in Table 2.

Effect of Gene Polymorphism on Superovulation and Embryo produced

Effect of gene polymorphism on superovulation and embryo quality were analyzed by using GLM methods. Both of single and

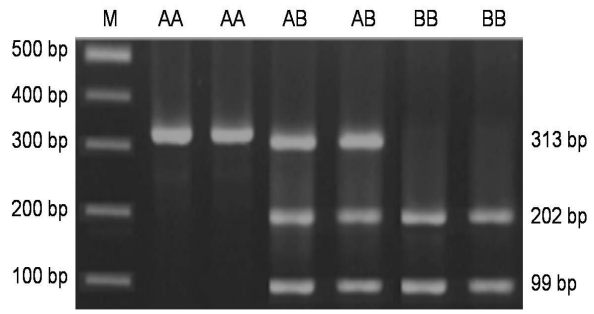

Figure 1. The Visualization Genotyping FSHβ using Agarose 2% (M : Ladder 100 bp)

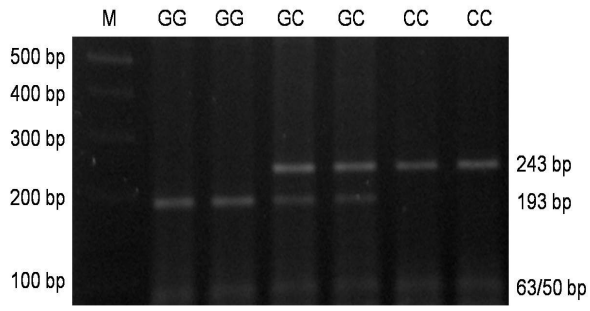

Figure 2. The Visualization of Genotyping FSHR using Agarose gel 2% (M: Ladder 100 bp)

Table 2. Polymorphism of FSHβ|*Pst*I and FSHR|*Alu*I loci.

Population (n)	FSHβ|*Pst*I					FSHR|*Alu*I				
	Alelle Freq.		χ^2	H_0	H_e	Alelle Freq.		χ^2	H_0	H_e
	A	B				C	G			
Angus (3)	0.00	1.00	na	0.000	0.000	1.00	0.00	na	0.000	0.000
FH (8)	0.13	0.88	0.077	0.250	0.233	0.69	0.31	5.091*	0.125	0.458
Limousin (10)	0.05	0.95	0.000	0.100	0.100	0.90	0.10	0.059	0.200	0.190
Simmental (8)	0.13	0.88	15.077*	0.000	0.233	0.75	0.25	1.432	0.250	0.400
Brahman (3)	1.00	0.00	na	0.000	0.000	0.17	0.83	0.000	0.333	0.333
Overall population (32)	0.17	0.83	15.874*	0.094	0.289	0.75	0.25	8.708*	0.188	0.381

n = number of sample, na = not analyzed, * = significantly different at $\chi^2_{0.05}$

interaction genes were analyzed. There is no significant effect of interaction genes in observed parameter (data are not shown). Significant effect was found only at ovulation rate in FSHR gene of all breed without being grouped (Table 3). Individual animal with CC genotype of FSHR|*Alu*I has higher ovulation rate (P<0.05) than CG and GG genotypes.

Polymorphism in FSH β-sub unit has been reported previously by Dai *et al*. (2009) that found 9 single nucleotide polymorphisms (SNPs) in whole FSH β-sub unit sequence. Two SNPs were found in 5′-upstream regulation region (URR) and seven SNPs in exon 3. Mutation in position 4453A>C in exon 3 predicted replaced Ser103Arg in protein sequence while the other mutation were synonymous. Mutation in this region was suggested having an important role in regulation of normal male fertility through affecting alteration of FSH function. Since same homozygous in human affecting azoospermia in male (Lindstedt *et al*., 1998; Phillip *et al*., 1998; Layman *et al*., 2002), did not detect CC genotype in bulls, it might have been caused by elimination in selection process (Dai *et al*., 2009). In the present study, frequency of A allele, which was similar to C allele in Dai *et al*. (2009), was lower than G allele in *Bos taurus* breed. On the other hand, in *Bos indicus* cattle, such as Brahman *breed,* the frequency of A allele was higher than those of G allele.

Inactivating of FSHR gene in women has affected quite similar phenotype of primary amenorrhoea and infertility (Matthews *et al*., 1993; Aittomäki *et al*., 1995; Layman *et al*., 1998, 2002). Several non-synonimous mutation on c.337C>G, c.871A>G and c.1973C>G in FH cow FSHR gene have been described by Cory *et al*.

Table 3. Effect of Single Gene of FSHβ|*Pst*I and FSHR|*Alu*I on Response of Superovulation and Embryo Quality

FSHβ|*Pst*I

Parameters	Angus	FH		Limousin		Simmental		Brahman	Overall		
	BB (3)	AB (2)	BB (6)	AB (1)	BB (9)	AA (1)	BB (7)	AA (3)	AA (4)	AB (3)	BB (25)
SR	50.0±50.0	37.5±53.0	61.5±40.6	100	73.3±35.7	100	74.8±25.4	75.0±25.0	81.3±23.9	58.3±52.0	68.1±34.8
OR	7.8± 6.9	1.0± 1.4	4.9± 4.8	12.5	5.9± 3.0	19.25	6.2± 3.1	5.0± 2.0	8.6± 7.3	4.8± 6.7	6.0± 3.9
FP	55.2±47.8	41.7±58.9	59.7±39.6	72	71.0±36.5	51.95	67.4±22.5	100.0± 0.0	88.0±24.0	51.8±45.2	65.4±33.5
VP	64.7±56.1	50.0±70.7	63.3±49.3	61.1	77.9±30.5	67.5	69.9±34.9	79.7± 9.4	76.7± 9.8	53.7±50.4	70.6±37.7

FSHR|*Alu*I

Parameters	Angus	FH		Limousin			Simmental			Brahman		Overall		
	CC (3)	CC (5)	CG (1)	GG (2)	CC (8)	CG (2)	CC (5)	CG (2)	GG (1)	CG (1)	GG (2)	CC (21)	CG (6)	GG (5)
SR	50.0±50.0	33.8±36.2	75	100.0± 0.0	76.2±37.0	75.0±35.4	86.7±18.3	70.0±42.4	50	75	75.0±35.4	64.9±38.8	73.3±24.8	80.0±27.4
OR	7.8± 6.9	4.9± 5.6	2	2.5± 0.7	6.7± 4.0	5.9± 0.2	10.2± 5.5	4.3± 3.2	3	3	6.0± 1.4	7.3± 5.1^{A}	4.2± 2.1^{B}	4.0± 2.0^{B}
FP	55.2±47.8	41.6±44.8	83.3	75.0±35.4	75.7±33.6	52.5±43.6	70.6±16.4	74.0± 1.4	22.2	100	100.0± 0.0	63.5±35.8	72.7±26.7	74.4±36.3
VP	64.7±56.1	56.0±51.3	100	50.0±70.7	72.8±31.7	90.0±14.1	80.9±12.3	76.3±33.5	0	77.8	80.7±13.1	63.5±35.8	85.1±19.0	52.3±48.8

Different superscript indicate significantly different at P<0.05, SR: super ovulation rate, OR: ovulation rate, FR: fertilized percentage and VP: viable transfer embryo percentage

(2013). These mutations have a significant effect on percentage of viable embryos and unfertilised oocytes, embryo yield after superovulatory treatments. SNP c.1973C>G corresponds to a threonine-to-serine (p.The658Ser) modification in the intracellular carboxyl-terminal domain of the FSHR protein, and homozygous GG Holstein cows were associated with a lower embryo yield and a higher percentage of unfertilised oocytes. Our result showed that the frequency of C allele within *Bos taurus* cattle was higher than those of G allele, and having the same respect within *Bos indicus* cattle. In addition, statistical analysis on overall breed showed that genotype of CC affects higher ovulation rate ($P<0.05$) than CG and GG genotypes.

CONCLUSION

The allele of B and C for FSH β-sub unit and FSHR respectively have a high number in *Bos taurus* species while in contrast in *Bos* i*ndicus* species. There is no significant effect of individual gene either FSHβ|*Pst*I or FSHR|*Alu*I on the observed parameters within each breed except for ovulation rate.

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