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Research Article

Association of Single Nucleotide Polymorphism (SNP) in Exon 4 *IGF-1* Gene with Sperm Quality in Bali Bulls

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Sperm Quality is one of the primary traits of reproduction in bulls. Insulin-like growth factor-1 (IGF-1) gene is one of the markers candidates for sperm quality and fertility in bulls. This study was conducted to determine the association of the new single nucleotide polymorphism (SNP) in exon 4 *IGF-1* gene with sperm quality in Bali bulls. A total of 164 bulls were genotyped for *Rsa*I restriction site in the exon 4 of *IGF-1* by applying PCR-RFLP method. Semen samples were collected to analyze the characteristics of semen quality namely sperm volume, concentration, viability and motility. The results of this study showed the presence of SNP in exon 4 IGF-1 gene that caused by the *C*/*T* transition. Genotyping of Bali bulls on exon 4 IGF-1 genes produced two alleles, namely *C* and *T* alleles with the allele frequencies were 0.793 and 0.207, respectively and three individual genotypes namely *TT, CT* and *CC* with successive frequencies: 0.170; 0.074; and 0.756. Polymorphism of IGF-1/Rsa1 genotype has significantly effect the semen quality of Bali bulls.

Key word : gene, transition, restriction , polymorphism, markers, and selection

1. Introduction

Bali cattle (*Bos javanicus*) is the Indonesian indigenous cattle originating from domesticated wild banteng. These cattle has widespread in Indonesia (Siregar, 2008) and in several countries such as Papua (Samberi et al., 2010), Malaysia, Philippines and Australia (Sumantra & Sumitayati, 2005). Bali cattle is one of the tropical cattle breeds which have several advantages, among others, good adaptability to the new environment, high carcass production, high fertility rate , and low calf mortality, (Siregar, 2008; Purwantara et al., 2012).

Breeding program and genetic improvement in Indonesia and generally in developing countries are still being carried out through selection based on phenotype information for each generation. Selection of livestock based on observable physical traits directly is very ineffective and inefficient from both an economic and time perspective to produce the desired genetic quality improvement. However, DNA molecular markers (genetic markers) that have been identified to be associated with economically valuable *QTL* can be used to increase the accuracy, speed and intensity of selection so as to accelerate genetic quality improvement (Dekkers et al., 2004; Ranjbari et al., 2012).

Several reproductive traits in livestock such as calfing rate, sperm quality and others are economically valuable traits that are controlled by several genes (polygenic). One of the genes that control these traits in beef cattle is the insulin like growth factor-1 (IGF-I) gene. Some studies have indicated that IGF-1 play a pivotal role in reproductive physiology

regulation, since the onset of puberty until the end of the active reproductive period in the bulls. This implies that pre-pubertal IGF-1 serum concentration correlate with scrotal circumference and motility of sperm of bulls. Moreover, the pre-pubertal IGF-1 serum concentration in females (cows) also correlates with age at first calf and calving rate (Yilmaz et al., 2004). The IGF-1 has been recognized as a regulator of testicular growth that affects the development of seminiferous tubules and leydig cells in mammals (BAGU ET AL., 2010). A number of study have also shown the possible role of IGF-I in maintaining both the viability and motility of spermatozoa in mice (Baker et al., 1996), cattle (Henricks et al., 1998) and buffalo (Selvaraju et al., 2010) through energy metabolism (Escott et al., 2014; Rato et al., 2012) and its antioxidant effect (Cocuzza, et al., 2007).

The association of a single nucleotide polymorphism (SNP) in IGF-1 gene with growth traits, milk production and reproductive traits in several cattle breeds have been widely reported. The previous studies in order to identify any new mutations in exon 4 IGF-1 gene and its association with production traits on Bali cattle have been carried out. However, no studies have been reported on the influence of IGF-1 gene polymorphisms on quality of sperm, especially in Bali cattle.

This study was conducted to recognize (SNPs) in exon 4 IGF-1 gene using PCR-RFLP method and to determine their effects on Bali bull sperm quality. The results may confirm that IGF-1 could be a candidate gene for applications in assisted marker selection.

2. Material and methods

All procedures carried out with the use of animals had been approved by the board of ethics committee of Faculty of Medicine, Mataram University, Indonesia (approval No…..

2.1. DNA extraction, PCR and genotyping

Blood samples for DNA genotyping were collected from the jugular vein of each bull by an authorized veterinarian. Blood samples was collected on Venoject tube with K2EDTA_ and preserved at -25°C for several weeks. The extraction of Genomic DNA from blood sample was performed using Wizard Genomic kit following manufacturer instructions (Promega, Madison, WI, USA).

The PCR analysis for exon 4 IGF-1 gene was carried out in eppendorf mastercyclers nexus gradient (Eppendorf - Hamburg, Germany) using specific primer according to Reyna *et al*. (2010) as depicted in Tables 1. The reaction mixture was conditioned at 25 µl of total

volume and contained 100 ng of genomic DNA, 0.5 µM of each primers (forward and reverse), 1 x PCR buffer (10mM Tris-HCl pH 9.0), 50 mM KCl and 1.5 mM MgCl, 5% deionized Formamide, 200 µM dNTP, and 0.025 U of Taq polymerase (Pharmacia). The PCR programs was performed in the following cycles: initial cycle at 94ºC for 5 minutes followed by 33 cycles 94ºC for 30 seconds, 60ºC for 60 seconds, and 72º C for 90 seconds, then ended the next step at 72ºC for 7 minutes. Sequence and position of the primer is presented in Table 1.

A total 5 µl of amplified DNA (PCR products) were cut with 10 U of *Rsa*I (Sigma Aldrich) restriction enzyme at 37°C over night in 15 µl reaction mixture. The DNA fragments were separated on 2.5% agarose gels electrophoresis. with ethidium bromide staining. The gels were visualized and analyzed using an AlphaImager EP (Alpha Innotech Corporation, USA).

Table 1. PCR primer sequence information and annealing temperatures

Locus	Primer sequences	Tm (ºC)	Locations[a]
Exon 4	F : 5′-CCACTCTAAAGCTAGGCCTCTCTC-3′ R : 5′-GAAGTCTATGAGGGTATGAAT-3′	60	56127 bp – 56470 bp

2.2. Semen collection and evaluation

Samples of semen were obtained from Animal Breeding Center of NTB Province. Semen samples were collected using an artificial vagina with a frequency of 2 times/head/week at 8.00-10.00 WITA by a bull master. After collection, semen samples were assessed immediately for volume (ml), concentration (x 10^6/ml), pH, mass movement (0-3), individual motility (%) and sperm viability (%) according to Baracaldo *et al*. (2007).

Volume of sperm was measured directly from the scale indicated on the collection tube, while its concentration was calculated using a photometer HR 6. The degree of acidity (pH) of semen was determined using a special pH indicator paper. For mass movement assessment, 20µl of undiluted sperm samples was dropped to a pre-warmed glass slide (37°C) and verified under light microscope (100X). Assessment is based on the velocity and thickness of the mass waves of sperm using scales from 0 (thin and no velocity) to 3 (thick and excellent velocity). Individual motility is assessed by looking through a microscope and Computerized Assisted Sperm Analysis (CASA). Semen samples were diluted using physiological NaCl (1: 5), then transferred on pre-warmed slid (37°C), covered with cover slip and the sperms individual motility were assessed under phase-contrast microscope (10 x 40). The ratio of live and dead spermatozoa (viability) was assessed by making a mixture

of sperm and eosin nigrosin in a glass object with a ratio of 1: 4. the eosin-nigrosine stained slides were observed in 10 visual fields or until the minimum cell count was > 200 cells, where unstained spermatozoa were considered to be alive (evans and maxwell, 1987).

2.3. Statistical analysis

The allele frequencies were calculated directly from the observed genotype. PopGene software (Version 32) was used to determine expected and observed heterozygosities and diversity indexes of population for data as depicted in Tables 2 and 3. The associations analysis between IGF-1 gene with semen quality traits were performed using General Linear Model (GLM) procedure of SAS (2008). The following statistical model was used:

$$Yij=A+Gi+eij$$

where *Yij* is the semen quality trait of animal, *A* is the overall mean, *Gi* is the fixed effect of the *i* genotype, and *eij* is the random error effect for each observation.

3. Results and discussion

Allele and genotype frequencies at the *IGF-1*/*Rsa1* SNP in Bali cattle

The results of previous studies have found a new mutation in exon 4 IGF-1 gene in Balinese cattle, namely a transitional mutation which converts the base of Cytosin to Thymine (C/T) (Maskur et al., 2012). This mutation can be identified using the PCR-RFLP technique with the *Rsa*1 restriction enzyme (GT ↓ AC). The C/T transition in exon 4 IGF-1 gene is a point mutation that changes the nucleotide sequence in the 6th codon from exon 4, namely ACG (threonin) to AUG (methionine).

In this research, One hundred and sixty four Bali cattle bulls were genotyped for the snp of IGF-1/Rsa1 at the exon 4 of the IGF-1 gene. Genotyping of Balinese bulls using PCR-RFLP technique on exon 4 IGF-1 genes produced two alleles, namely C and T allele with very contrasting frequency distribution differences, respectively 0.793 and 0.207 and three individual genotypes in the population namely TT, CT and CC with successive frequencies: 0.170; 0.074; and 0.756.

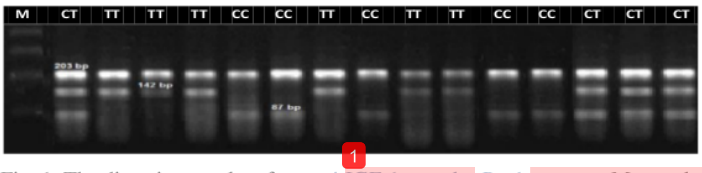

Fig. 1. The digestion results of exon 4 IGF-1 gene by *Rsa*1 enzyme. M= marker 100 bp. C allele (203, 87, and 55 bp) and T allele (203 and 142 bp).

The result of Chi-square (X2) test indicate a higly contrasting difference between the TT, CT and CC genotypes in the population. This means that the population is not in the balance of Hardy-Weinberg (Hardy-Weinberg Equilibrium/HWE) where there is an accumulation of homzygote CC genotypes in the population.

Table 2. The frequencies of genotype and allele of IGF-1 gene in Bali bulls (N = 164).

Locus	Allele	Allele frequency	Genotype	Genotype frequency	X^2(HWE)
Exon 4	C	0,793	CC	0,756	99,107**
	T	0,207	CT	0,074	
			TT	0,170	

**P<0,01

Many studies have also shown the presence of mutations in the coding and non-coding regions of IGF-1 genes in dairy and beef cattle. In Charolais and Beefmaster, the mutant allele frequencies for intron 4 were 0.48 and 0.30, while promotor region of IGF-1 were 0.26 and 0.03 respectively (Reyna et al., 2010). Mutant alleles in the promotor region of IGF-1 have also been reported in Angus cattle with a frequency of 0.44 (Liron et al., 2012) and in Holstein-Friesian cows 0.41 (Nicolini et al., 2013). The differences in allele frequencies between the current studies and the previous researches may be due to the different sample sizes, mutation locations and livestock breeds studied.

Table 3. The estimation of diversity indexes in Bali bulls population (N = 164).

Locus	Ob_He	Ex_He	Ne	I	Nei	PIC
Exon 4	0,073	0,329	1,490	0.510	0.329	0,275

Ob_He: Observed Heterozygosity, Ex_He: Expected Heterozygosity, Ne: Number of effective alleles, I = Shannon's index, Nei: Nei index, PIC: Polymorpic Information Content

In the present study, the value of Shannon information index was 0.510 for exon 4 of the IGF-1 gene, indicating that variation of IGF-1 gene in Bali cattle bull is categorized moderat. Also, the expected heterozygosity using Nei index for these positions was 0.329, indicating that the genetic diversity is on the moderat level as well (table 3). The variability and informativeness of the IGF-1 gene as a genetic marker for linkage analysis is shown by moderat level of the PIC value (0.25).

Association of genotypes at exon 4 of IGF-1 gene with Bali Cattle *(Bos Javanicus)* Fresh Semen Quality

The results of association analyses between the IGF-1/Rsa1 with the sperm quality in Bali cattle are shown in Table 5. The analysis of this study confirmed that the polymorphism of exon 4 IGF-1 gene showed significant effect on sperm quality traits.

Among the three genotype of animals, the CT genotype animal produced spermatozoa with the highest volume per ejaculated, individual motility and viability traits ($P < 0.05$) compared to TT genotypes, although no significant with CC genotype. also, animals with CT genotype produced spermatozoa with the highest concentration ($P < 0.1$) compared with CC and CT genotypes. This result was consistent with Bakhtiar et al., (2017) findings in Sanjabi breed rams who reported that the significant effect of exon 3 IGF-1 gene polymorphism on individual motility and concentration of spermatozoa.

Table 4. Least square means (±standard errors) for the association between exon 4 IGF-1 gene polymorphism with semen parameter in Bali Bull

Semen Characteristics	Genotypes (n)		
	CT (12)	CC (28)	TT (28)
Volume (ml)	4,80±0,92^{a}	4,23±0,78^{ab}	3,91±0,72^{b}
Concentration (x 10^{6}/ml)	1802,50±380,67^{A}	1553,30±318,22^{B}	1078,09±185,35^{C}
pH	7,0±0,0	7,0±0,0	6,8±0,1
Mass movement (0-3)	2,9±0,1	2,8±0,1	2,6±0,1
Individual Motility (%)	70,00±3,75^{a}	66,70±2,96^{ab}	62,39±2,89^{b}
Sperm Viability (%)	82,60±4,12^{a}	79,85±3,88^{ab}	77,70±3,65^{b}

^{a,b,c} Different superscripts within the same row represents a significant difference ($P<0.05$), and
^{A,B,C} Different superscripts within the same row represents a significant difference

The influence of exon 4 IGF_1 polymorphism on fresh semen quality of Bali cattle is apparent from the data of this study. The result of this study is supported by several previous studies which showed that the IGF_1 gene is involved in male reproductive regulation. IGF-1 and IGF-1 receptors have been founded on semen of cattle (Henricks *et al.*, 1998) and buffalo (Namagirilakshmi, 2013), it suggests that this factor has an important role in regulating functions of bovine spermatozoa. Also, Miah et al. (2008) showed that IGF-1 increased sperm progressive motility, induction of capacitation and acrosome reactions.

This current study proves that IGF1-*Rsa*1 influences in sperm volume per ejaculated and sperm concentration of Bali bull. It is related to spermatogenic actions of IGF-1 in the somatotropic axis of male reproduction. IGF-1 is conventionally associated with growth and gonadotropin secretion, development of seminiferous tubules and Leydig cells. In addition, IGF-1 also plays an important role in several male reproductive processes such as pubertal transition, spermatogenesis, gonadal steroidogenesis, metabolism, and sexual behavior (Bagu et al., 2010; Choubey, 2020). A study conducted by Rodrigues et al., (2019) showed that paracrine/autocrine production of IGF-1 stimulated spermatozo maturation, and increased in IGF-1 level correlated linearly and significantly with total amount of sperms (Rodríguez et al., 2019).

The significant influence of IGF-1 gene on bovine sperm motility and viability was also demonstrated in this study. The results of this research appear to be in line with those of Selvaraju *et al*., (2009) who found that in vitro addition of IGF-1 at physiological concentrations increased the percentage of motile sperms of bovine semen. Similar findings were confirmed in several previous study on bovine (Henricks *et al*., 1998), human (Miao *et al*.,1998), equine (Chanpion *et al*., 2002) and swine (Miah *et al*., 2008). Also, Lee et al., (1995) shown that seminal IGF-1 had a significant effect on the total of motile and rapid linear progressive spermatozoa.

The possible mechanism of IGF-1 in maintaining sperm viability and motility needs to be explained. One possible way that IGF-1 is assumed to be through energy metabolism. Energy is required for testicular function and spermatogenic cell development, and the main metabolite energy comes from lactate (Choubey et al., 2019). Igfs plays a role in converting glucose to lactate by activating glucose transporter 8 (glut8) of Sertoli mature cells (Escott et al., 2014; Rato et al., 2012). IGFs is also reported to increase glucose uptake, lactate production, pyruvate dehydrogenase activity, and conversion of glucose to glucose 6 phosphate (Stewart et al., 1996).

Another possible mechanism to maintains sperm viability and motility is the antioxidant effect of IGF-I. There is much evidence that oxidative stress reduces sperm cell activity and men fertility. This occurs due to the lack of antioxidants that protect sperm cells and the increased production of reactive oxygen species (ROS) (Cocuzza, et al., 2007). Mitochondrial function will be harmed by oxidative stress through the impairment mechanism of oxidative phosphorylation directly on protein and lipid membranes. The subsequent destruction of the mitochondria will result in mitochondrial dysfunction, decreased MMP and ATP synthesis and increased production of ROS. NOTABLY, IGF-I PLAYS AN IMPORTANT ROLE IN PROVIDING MITOCHONDRIAL PROTECTION (García, et al., 2008). Some researchers have also confirmed that IGF-1 reduces the production of mitochondrial superoxide (Csiszar, et al., 2008) AND INCREASE IN OXIDATIVE STRESS DAMAGE ASSOCIATED WITH LOW LEVELS OF IGF-1 (García, et al., 2008; Pérez et al., 2008). Moreover, Shin et al., (2014) confirmed that IGF-1 might be able to maintain progressive motility of canine spermatozoa stored hypothermically by stimulated mitochondrial membrane potential (MMP).

The results of this study showed a significant influences of exon 4 IGF-1 polymorphism on Bali bull fresh sperm quality. This study confirmed that IGF-1 could be a

candidate gene for application in marker assisted selection. However, the physiological effects and level of IGF-1 gene expression due to mutation at these positions isn't well known, more detailed studies will clarify infertility and/or subfertility cases that will improve the accuracy of prediction of bulls reproductive performance.

4. Conclusion

This current study proved the existence of genotype polymorphism in IGF-1/Rsa1 due to the C/T transition and produced three individual genotypes, namely TT, CT and CC genotypes. The CT genotype animals generating semen with highest volume per ejaculation, concentration, individual motility and viability ($P < 0.05$) compared to TT and CC genotype. There are a significant influence of genetic polymorphism of IGF-1 gene on fresh semen quality in bali bull. For these reasons, IGF-1/Rsa1 has been considered a strong marker candidate for genetic improvement of fertility in bali cattle.

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