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Genetic Markers of Twinning Births of Local Beef Cattle and Its Crossbreds in Indonesia

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ABSTRACT: Genetic polymorphisms of Insuline Like Growth Factor 1 (IGF1) gene and Osteopontin (OPN or SPP1) gene were studied in Indonesian local beef cattle and its crossbreds. In regarding to twinning trait, the IGF1 gene is associated to ovulation rates, while the OPN gene is related to maintain fetal growth and pregnancy. Blood samples of local Ongole Grade (OG) cattle of historical twin (T) and non twin (NT) were collected from the Provinces of South Kalimantan (T=17 and NT=3) and East Java (T=28 and NT=12); and for its crossbreds from Central Java (T=28 and NT=9). Genotyping was done by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) Method, using restriction enzymes of SnaB1 (the IGF1 gene) and Bsr1 (the OPN gene). Genotyping at the intron1 IGF1 gene for all observed cattles was monomorphic, resulting entirely the BB genotype, with a DNA fragment of 249 bp. Genotyping at the intron4 OPN gene was polymorphic, resulting three genotypes, i.e. CC (200 and 90 bp), CT (290, 200, 90 bp) and TT (290 bp). The OG twin cattle was dominated by the TT genotype (97%) than the CT genotype (3%). By contrast, twin crossbreds had almost similar the TT and the CT genotypes. Instead of the monomorfic intron1 IGF1| *SnaB1* gene, the intron4 OPN|*Bsr1* gene could be considered as a genetic marker in exploring twinning traits in beef catlle observed.

Key Words: Beef Cattle, Twinning Births, IGF1 gene, OPN gene, and FCR-RFLP.

INTRODUCTION

Cattle is considered into a uniporous species, very commonly giving single calf per birth. Twin or multiple births in cattle therefore are very rare. Cows in giving twin (multiple) births factually were found in Indonesian local beef cattle. The ocurence was distributed widely, though its frequency was very low (about 1-4%). Cows calving twin or multiple births were also reported in a local Ongole Grade (OG) cattle and its crossbreds, so it was interesting to study genetic control of twinning trait in our local OG its crossbreds.

Twinning trait in cattle is known of following the pattern of a quantitative trait, controlled by many genes and interacting to environment. A number of genes have been identified possible in controlling twinning births, two of those are insuline Like Growth Factor 1 (IGF1) and Osteopontin (OPN or SPP1) genes. IGF-1 gene in cattle known is located at chromosome five (BTA-5) and known as regions where quantitative trait loci (QTL) controlling twinning or multiple births (Lien *et al.*, 2000). Study Echternkamp *et al.* (1990) proved that twinning (multiple) births in cattle is associated by the increasing IGF-1 concentrations in both blood serum and follicular fluid. The IGF-1 stimulates mitogenesis of granulosa cells and stereoidogenesis of ovarian cell cultures. This gene plays an important role in the regulation of folliculogenesis and may be involved in the process of multiple ovulation in cattle.

OPN gene in cattle is located at chromosome 6 (BTA6) close to quantitative trait gene (QTL) for milk production. OPN is generally in the form of a monomer with long ranges of 264-301 amino acids. Ongoing post-translational modification extensively involves phosphorylation, glycosylation and cleavage generating molecular variants ranging from 25-75 kDa. The OPN gene is hypothesized to affect uterine environment through histotropi components needed in the adhesion and signal transduction processes on the surface of the uterus-placenta. The resulting product is expressed as uterine stromal invasion caused its response to conceptus, products of sac placenta and uterine immune cells that are useful in regulating cytokine production. Regulation and functional implications of conditions involving instantaneous OPN probably result in a common activity to ensure developing and maintaining pregnancy (Johnson *et al.,* 2003).

This research was aimed to study genetic polymorphims of these two genes by PCR-RFLP method in the local OG and its crossbreds.

MATERIALS AND METHODS

Blood samples

Fresh bloods for DNA extraction were collected from a total number of 117 heads of historical twin (T = 83 hd) and non twin (NT = 24 hd) as control in Indonesian local beef cattle and its crossbreds. Blood samples of local Ongole Grade (OG) cattle were collected from Hulu Sungai Tengah District (T=17; NT=3) in South Kalimantan Province; Probolinggo (T=8; NT=1), Pasuruan (T=9; NT=1), and Tuban (T=21; NT=10) in East Java Province. For its crossbreds (mating by exotic males of Simmental, Brangus, Limousin dan Brahman), fresh bloods were collected from Kendal (T=15; NT=5) and Sragen (T=28; NT=9) in Central Java Province.

DNA extraction

DNA Extraction Mini Kit (Geneaid) was used. Some procedures were by washing the blood from alcohol, 200 µl of blood sample was added 1000 µl of water (± 5 min), then centrifuged at 8000 rpm ((± 5 min) (2x). Sample was added by 10 µl ProtK 5 mg / ml, 200 µl buffer GT , and 200 µl GB buffer, then incubated at 60 º C (1 hour) and at 70 º C (10 minutes).

Polymerase Chain Reaction

Genomic DNAs of both twin and control were used as templates in DNA amplification reactions (PCR reactions), using primer pairs (mix) of nucleotide sequences of the IGF1 (Siadkowska *et al*., 2006) with F: ATT ACA AAG CTG CCT GCC CC and R: ACC TTA CCC GTA TGA AAG GAA; while those of the OPN gene (Leonard *et al*., 2005) with F: GCA AAT CAG AAG TGT GAT AGA C, and R: CCA AGC CAA ACG TAT GAG TT.

Genotyping fragments of the IGF1 and OPN genes

Amplification of the fragments of the IGF1 and the OPN genes was genotypied at the thermocycler engine by setting an initial denaturation temperature of 95ºC (5 minutes), while for 35 cycles at 95ºC (45 seconds). Amplicons of the IGF gene were restricted by the SnaBI enzyme while those of the OPN gene were by BsrI enzyme, then they were incubated at a temperature of 65ºC.

Frequencies of allele and genotype

Genotype frequency was calculated by dividing the number of a particular genotype to overall samples, whereas allele frequency was the ratio between a particular allele to all alleles at the observed locus (Nei, 1987):

$$x_{ii} = \frac{nii}{N} \qquad x_i = \frac{2nii + \sum nij}{2N}$$

Description :
x_{ii} = the iith genotype frequency; x_i = the ith allele frequency; n_{ii} = individual number of the iith genotype; n*ij* = individual number of the ij*th* genotype; and *N* = total number of individual samples.

RESULTS AND DISCUSSION

Genotyping IGF|*SnaBI* Gene**

DNA amplicons as the PCR products in Indonesian local OG beef cattle and its crossbreds that were genotyped through RFLP method using the SnaB1 enzyme did not showed any point mutations at the intron1 IGF1 gene. Genotyping results at the intron1 IGF1|SnaB1 in all of beef cattle observed produced only one DNA fragment with the length of 249 bp as presented in Figure 1. Table 1 showed that the result of the cutting SnaBI enzymes to PCR products at the intron1 IGF1 gene by RFLP method in all of the observed OG cattle and its crossbreds of twin or historical twin cows as well as single calving cows from three provinces of East Kalimantan, East Java and Central Java produced only one fragment with the dna size of 249 bp. This meaned that each individual of both historical twin or non twin of the local OG and its crossbreds had only one type of genotype, namely a BB genotype.

Table 1. Frequencies of genotype and allele of the intron4 OPN|Bsr1 gene in twin and non twin of Local Ongole Grade and its crossbreds

Province/District	Twin					Non twin				
	Genotype Freq (%)			Allele Freq (%)		Genotype Freq (%)			Allele Freq (%)	
	CC	CT	TT	C	T	CC	CT	TT	C	T
					Ongole Grade					
SK:H.S.Tengah	0 (0)	12 (2)	88 (15)	6	94	0 (0)	0 (0)	100 (3)	0	100
EJ: Probolinggo	0 (0)	0 (0)	100 (8)	0	100	0 (0)	0 (0)	100 (1)	0	100
Pasuruan	0 (0)	0 (0)	100 (9)	0	100	0 (0)	0 (0)	100 (1)	0	100
Tuban	0 (0)	0 (0)	100 (21)	0	100	0 (0)	0 (0)	100 (10)	0	100
Sub Total	0 (0)	0 (0)	100 (38)	0	100	0 (0)	0 (0)	100 (12)	0	100
Total (OG)	0 (0)	4 (2)	96 (53)	2	98	0 (0)	0 (0)	100 (15)	0	100
					Crossbred					
CJ : Kendal	0 (0)	60 (9)	40 (6)	30	70	0 (0)	0 (0)	100 (5)	0	100
Sragen	0 (0)	62 (8)	38 (5)	31	69	0 (0)	0 (0)	100 (4)	0	100
Sub Total	0 (0)	61 (17)	39 (11)	30	70	0 (0)	0 (0)	100 (9)	0	100
Total (CrossB.)	0 (0)	61 (17)	39 (11)	30	70	0 (0)	0 (0)	100 (9)	0	100
Overall	0 (0)	23 (19)	77 (64)	11	89	0 (0)	0 (0)	100 (24)	0	100

Description : (…) number in parentheses was number of cattle (head).
SK : South Kalimantan; EJ : East Java; and CJ : Central Java.
OG : Ongole Grade; CrossB : Crossbreds.

No genetic variation or monomorphic nature in the base fragment of the intron1 IGF1|SnaB1 was likely due to the nucleotides as a cutting site of the SnaBI restriction enzyme without the C/T base transition. Another possibility was that the observed OG cattles had unique nucleotide sequences, especially at the cutting sides of the SnaBI restriction enzyme (no transition C/T), so this enzyme could not cut specific fragments at intron1 of the IGF1 gene.

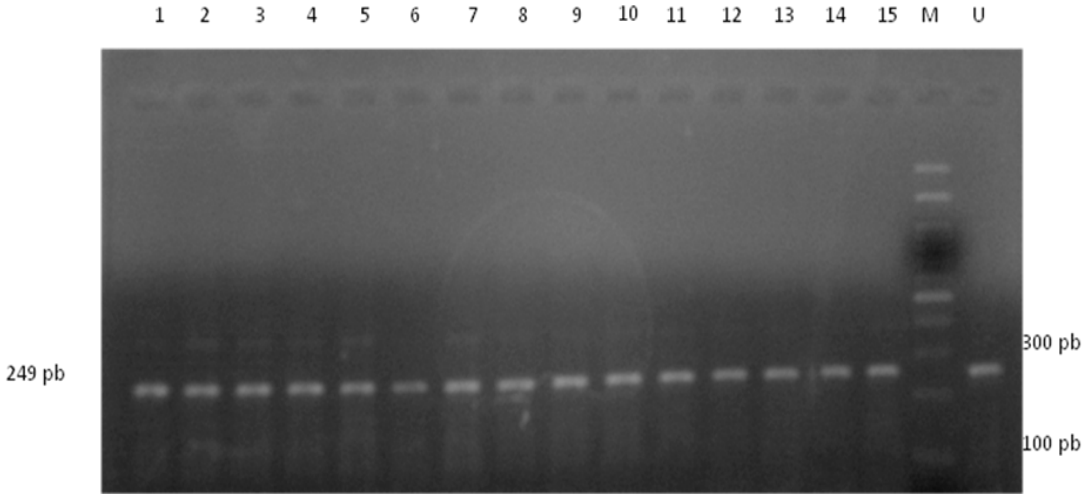

Figure 1. RFLP results of the intron1 IGF1 gene. All columns with DNA fragment of 249 bp for BB genotype. M = Marka (300pb)

Results from this study however different to the previous study by Siadkowska *et al.* (2006). By genotyping the intron1 IGF1|SnaBI gene in Holstein Friesian (HF) Norway (662 head) from that study identified three type of genotypes. In the case of genotyping PCR products resulted two DNA bands (223 and 26 bp) then be identified as a homozypous AA (TT) genotype, three DNA bands (249, 223 and 26 bp) as a heterozygous AB (CT) genotype, and only one DNA band (249 bp) as a homozygous BB (CC) genotype. Frequencies of the AA, AB and BB genotypes were reported succesively 29%, 47% and 24%; while the fequencies for A and B alleles were 52% and 48% respectively.

Monomorphic of the intron1 IGF1|*SnaB1* gene was also identified in both twin (27 heads) and non twin (15 heads) of Holstein Friesian (HF) cattle by Anggraeni *et al*., (2012) by resulting only the BB genotype, without the AB and the AA genotypes. It was stated that as no genetic polymorphims identifying at this fragment gene made unable for the intron1 IGF1|*SnaB1* gene to be functioned as a genetic marker in exploring twinning trait in the observed HF cattle.

Genetic polymorphisms of the IGFI gene in Angus cattle previously was identified by Ge *et al.* (1997) as single strand conformation polymorphism (SSCP), which was known as C/T transition at the position -472 relative to the early transcription (at position 512 bp from the ATG codon, corresponding to the gene bank sequences at AF210383) (Ge *et al.*, 2001).

Genotyping of the OPN|*Bsr1* gene

Genetic polymorphism of the inton4 OPN gene was identified by examining a base mutation of C/T transition at a non-code area 5‘ of the OPN gene through the PCR-RFLP method following the study by Leonard *et al.* (2005) in *Bos taurus* dairy cattle. According to the previous study, as presented in Figure 2, a homozygous CC genotype was identified if the cutting by SbrI enzyme to the PCR product (290 bp) resulting two DNA bands (200 bp and 90 bp); a heterozygous CT genotype was for resulting three DNA bands (290 bp, 200 bp and 90 bp); and a homozigous TT genotype was for the obtaining only one DNA band (290 bp) in which the SbrI enzymes failured in cutting the PCR product.

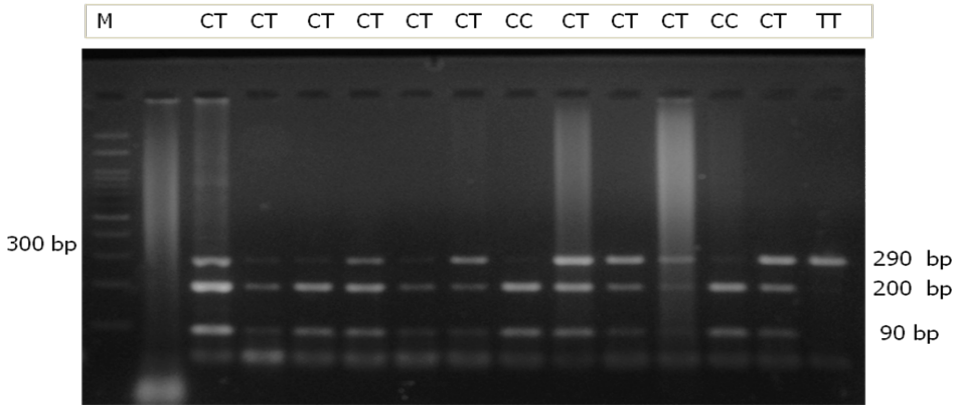

Figure 2. RFLP results of the intron 4 OPN gene. Columns with DNA fragments of 200 and 90 bp for CC; 290, 200 and 90 bp for CT; and 290 bp for TT genotypes. M = Marker (300bp).

Frequencies of genotype and allele of the intron4 OPN|*Bsr1* gene of twin and non twin in both local OG and its crossbreds were listed in Table 1. The results explained that the genotyping results at the intron4 OPN|*Bsr1* gene in twin (multiple) OG cattle from H.S. Tengah in South Kalimantan Province was polymorphic, resulting two genotypes, namely TT and CT genotypes. Genotyping of this fragment gene hence resulted two type of alleles, namely T and C alleles. For this cattle group, the frequency of the TT genotype was predominantly to that of the CT genotype (88% vs. 12%). Contrastly, monomorphic nature was identified in the twin OG from East Java Province, of which all of observed cattle had the only TT genotype (100%).

While for the twin crossbreds from Central Java Province, the intron4 OPN|*Bsr1* gene was polymorphic, resulting also the TT and the CT genotypes. However, the frequency of the CT genotype was higher than that of the TT genotype (61% vs. 39%). Further, the overall of non twin

(as control) cattles neither in OG nor its crossbred were consistently monomorphic. These results informed that by the existing genetic polymorphism at the intron4 OPN|*Bsr1* gene identifying in historical twinning OG cattle and its crossbreds could be considered as a genetic marker with regarding to explore twinning or multiple births in beef cattle observed.

In a previous study in HF dairy cattle in West Java Province by Anggraeni *et al.* (2012) also reported that genotyping at the intron4 OPN|*Bsr1* gene either in historical twin cattle (27 head) and non twin cattle (15 heads) were polymorphic. Both groups resulted three genotypes, i.e. CC, CT and TT genotypes. However, the first group had the frequencies of the CC and the CT genotypes were higher than the second one (30% and 56% vs. 20% and 40%).

By considering the abone results, studies for the association of twinning or multiple births to genetic marker in cattle seemingly have shown a fairly complex process. It is considerably required to exploit marker genes closely associated with various fertility traits, that will give the success of follicle development, fertilization, embryo development and by the ending twinning (multiple) births in cattle.

CONCLUSION

Genotyping the intron1 IGF1|*Snab1* gene in local OG and its crossbeds was monomorfic resulting solely the BB genotype (249 bp), whilst genotyping the intron4 OPN|*Bsr1* gene in in twin OG and its crossbeds was polimorfic resulting the CT and the TT genotypes, so this gene could be considered as a genetic marker in exploring twinning births in observed cattle.

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