Media Peternakan, December 2011, pp. 190-195
EISSN 2087-4634
Accredited by DGHE No: 66b/DIKTI/Kep/2011

Online version:
http://medpet.journal.ipb.ac.id/
DOI: 10.5398/medpet.2011.34.3.190

Genetic Polymorphisms of the Coding Region (Exon 6) of Calpastatin in Indonesian Sheep

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(Received 31-01-2011; accepted 09-05-2011)

ABSTRAK

Kalpastatin (CAST) berperan dalam menghambat fungsi enzim kalpain yang terlibat dalam mengatur *turn over* protein dan pertumbuhan. Tujuan penelitian ini adalah untuk mengidentifikasi keragaman genetik gen CAST khususnya pada seluruh daerah ekson 6 pada domba lokal. Metode PCR-SSCP digunakan untuk mengidentifikasi variasi pada gen CAST. Sebanyak 258 ekor domba lokal dari 8 subpopulasi digunakan dalam penelitian ini, tiga group berasal dari Domba Ekor Tipis (DET) dari Sukabumi, UP3J Jonggol dan Kissar, sedangkan sisanya antara lain domba Priangan dari Margawati dan Wanaraja serta Domba Ekor Gemuk (DEG) dari Donggala, Sumbawa, dan pulau Rote. Analisis SSCP menunjukkan tiga pola SSCP berbeda yang merujuk pada tiga alel berbeda pada lokus CAST yaitu alel CAST-1, 2 dan 3 dengan lima genotipe berbeda. Variasi genetik di antara populasi dihitung berdasarkan frekuensi alel dan genotipenya. Sebagian besar populasi yang diteliti menunjukkan polimorfisme gen CAST dengan frekuensi masing-masing genotipe CAST-11, CAST-12, CAST-22, CAST-32, dan CAST-33 adalah 0.286, 0.395, 0.263, 0.046, dan 0.007. Alel CAST-1 dan 2 adalah alel yang paling umum pada seluruh populasi dengan total frekuensi 0.970, sementara alel yang langka adalah CAST-3 (0.030) dan hanya ditemukan di populasi DET. Kesimpulan yang dihasilkan adalah bahwa gen CAST pada domba lokal bersifat polimorfik dan informasi keragaman tersebut dapat digunakan untuk mencari hubungan dengan sifat pertumbuhan, kualitas karkas dan daging.

Kata kunci: domba lokal, kalpastatin, PCR-SSCP, exon 6

ABSTRACT

Calpastatin (CAST) is an indigenous inhibitor of calpain that involved in regulation of protein turn over and growth. The objective of this research was to identify genetic polymorphisms in the entire exon 6 of calpastatin gene in Indonesian local sheep. A PCR-SSCP method was carried out to identify genetic variation of CAST gene. In total 258 heads of local sheep from 8 populations were investigated, three groups of samples were Thin Tail Sheep (TTS) from Sukabumi, Jonggol, and Kissar. The rest samples were Priangan sheep (PS) from Margawati (Garut meat type) and Wanaraja (Garut fighting type) and Fat Tail Sheep (FTS) from Donggala, Sumbawa, and Rote islands. SSCP analysis revealed that three different SSCP patterns corresponded to three different alleles in the CAST locus (CAST-1, 2, and 3 allele) with five different genotypes. Genetic variation between local sheep populations were calculated based on genotypic and allelic frequencies. Most populations studied were polymorphic, with genotype frequencies of CAST-11, CAST-12, CAST-22, CAST-32, and CAST-33 were 0.286, 0.395, 0.263, 0.046, and 0.007 respectively. CAST-1 and 2 alleles were most commonly found in all populations with total frequency was 0.970, while CAST-3 was a rare allele 0.030 and only found in TTS population. Variation in the CAST gene could be used for the next research as genetic diversity study or to find any association between CAST polymorphism with birth weight, growth trait and carcass quality in Indonesian local sheep.

Key words: Indonesian local sheep, calpastatin, PCR-SSCP, exon 6

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INTRODUCTION

Calpastatin (CAST) is a member of calpain calpastatin system involving three molecules, µ-calpain, m-calpain and calpastatin as specific inhibitor of the two calpain. This system implicated in various physiological and pathological processes (Kidd *et al*., 2000; Huang *et al*., 2001; Goll *et al*., 2003; Raynaud *et al*., 2004) and involved in regulation of protein turn over and growth (Goll *et al*., 1992), myoblast migration (Dedieu *et al*., 2003) and fusion (Temm-Grove *et al*., 1999). CAST is therefore believed to be an excellent candidate gene for growth and carcass trait in livestocks.

Many studies have demonstrated the association of CAST polymorphism with carcass and meat quality, especially tenderness in several livestocks (Schenkel *et al*., 2006; Casas *et al*., 2006; Curi *et al*., 2009). In the sheep, the polymorphism of CAST gene was reported to have significant association with birth weight (Byun *et al*., 2008), and body weight (Sumantri *et al*., 2008,) but did not influence lamb tenderness (Zhou *et al*., 2008a).

Previous study reported the polymorphisms of CAST in Indonesian local sheep but this study only concentrated in intron region (Sumantri *et al*., 2008), and no investigation yet reported in coding sequences of local sheep. Exon 6 was the largest exon in the ovine calpastatin, and known to be polymorphic in the sheep with five different alleles (Zhou *et al*., 2007; Byun *et al*., 2009a). Previous study has shown that sequences coded by exon 6, contained multiple phosporylation sites, and directly involved in determining the cell localization of calpastatin (Tullio *et al*., 2009). This study suggested that variation in these sequences may impact on the activity of Ca^{2+} channels and hence regulate or modulate calpain activity. The objectives of this research were to identify polymorphism of CAST gene in the coding region (Exon 6) in Indonesian local sheep population.

MATERIALS AND METHODS

Blood Samples and DNA Extraction

In total 258 sheep from 8 populations were investigated, i.e. (i) Thin Tail Sheep (TTS) from Sukabumi (50), Jonggol (26), and Kissar (32); (ii) Priangan sheep (PS) from Wanaraja (Garut fighting type) (35), Margawati (Garut meat type) (20); and (iii) Fat Tail Sheep (FTS) from Sumbawa (29), Donggala (54), and Rote (12). DNA extraction was carried out by using standar phenol chloroform method (Sambrook *et al*., 1989) with some modification by Andreas *et al.* (2010).

PCR Amplification

A pair of PCR primer, forward: 5′-GTTATGA ATTGCTTTCTACTC-3′ and reverse: 5′-ATACGATT GAGAGACTTCAC-3′ was designed to amplify part of intron 5 and whole exon 6 of CAST gene, as described by Zhou *et al*. (2007). PCR amplification was carried out in 25 µl reaction containing 50-100 ng genomic DNA, 0.25 µM of each primer, 200 µM dNTPs (Fermentas), 4.0 µM Mg^{2+}, 0.5 U of *Toptaq* DNA polymerase (Qiagen, Hilden, Germany), and 1x the reaction buffer. The condition of thermal cycling consisted of pradenaturation at 95 °C for 5 min, followed by 35 cycles of denaturation 95 °C for 30 s, annealing 56 °C for 45 s, and extension 72 °C for 45 s. The final extension step was at 72 °C for 5 min. Amplification was carried out in a thermal cycler (Mastercycler Personal 22331, Eppendorf, Germany). The PCR amplicon were checked on 1.5% agarose gels in 0.5 x TBE buffer containing 10% of ethidium bromide at 100 volt for 45 min and visualized by UV transiluminator.

Single Strand Conformational Polimorphism (SSCP) Analysis

A SSCP procedure was used to identify variation in the amplicon of CAST locus. A 5 µl aliquot of each amplimer was mixed with 5 µl of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol). After denaturation at 95 °C for 5 min, samples were rapidly cooled on ice bath and then loaded on 12% acrylamide : bisacrylamide (29 : 1) gels. Electrophoresis was performed by using Protean II xi cells (Bio-Rad), at 300 V for 18 h at refrigerator condition in 0.5 x TBE buffer. Gels were silver stained based on the method of Byun *et al.* (2009b) with modification in staining solution (0.1% $AgNO_3$, 0.04% NaOH 10 N, and 0.4% NH_3).

DNA Sequencing and Analysis

Amplicon that produced SSCP patterns that could be confirmed as homozygous were used directly as templates for DNA sequencing. Prior to sequencing, one of the unique bands representing each allele was cut out of the polyacrylamide gel (PAGE) and purified by the method as described by Hu *et al.* (2010). This was then used as the DNA template for reamplification and sequencing. To ensure these templates were similar to original sequences and not the result of amplification error, the identity of the templates cut from PAGE was confirmed by matching the PCR SSCP patterns generated from the templates and the corresponding genomic DNA. Sequence alignments, translations and comparisons were carried out using MEGA software version 4.0 (Tamura *et al*., 2008). The BLAST (basic local alignment search tool) program was used to search the NCBI GenBank (http://www.ncbi.nlm.nih.gov/BLAST) databases for homologous sequences.

Statistical Analysis

The genotype and allele frequencies were calculated based on Nei & Kumar (2000) formulation.

$$X_{ii} = \frac{n_{ii}}{N} \times 100\%$$

$$X_i = \frac{\left(2n_{ii} + \sum_{j \neq i} n_{ij}\right)}{2N}$$

where Xi= iith genotype frequency, X_i= ith allele frequency, n_{ii}= number of sample of ii genotype, n_{ij}= number of sample of ij genotype, and N= total sample.

Test of Hardy-Weinberg equilibrium (HWE) with chi-square test (Kaps & Lamberson, 2004).

$$X^2 = \sum (\text{Obs} - \text{Exp})^2/\text{Exp}$$

where χ^2= chi-square , Obs= number of observation of iith genotype, and Exp= number of expected of iith genotype.

Observed (H_o) and Expected heterozygosity (H_e) based on Nei's heterozygocities (1973) and computed using PopGene32 software version 1.31 (Yeh *et al*., 1999).

$$H_o = \sum_{k}^{s} w_k \sum_{i \neq j}^{q} X_{kij}$$

$$H_e = 1 - \sum_{k}^{s} w_k \sum_{i}^{q} x_{ki}^2$$

where H_o= observed within-population heterozygocity, H_e= expected within-population heterozygocity, w_k= relative population size, X_{kij} ($i \neq j$)= the frequency of A_iA_j in the kth population.

RESULTS AND DISCUSSION

PCR-SSCP Analysis of CAST Gene

Part of intron 5 and entire exon 6 of CAST gene were amplified by PCR using forward and reverse primer, with predicted amplicons 254 bp in length (Figure 1). PCR-SSCP analysis showed polymorphism in this region with three unique SSCP banding patterns. Three unique banding patterns corresponding to three different alleles, CAST-1, CAST-2, and CAST-3 allele. Either one or two unique banding patterns were found in each individual sample that consistent with either homozygous or heterozygous with five different genotypes, CAST-11, CAST-12, CAST-22, CAST-32, and CAST-33. The CAST-31 genotype was not observed in this study. Figure 2 shows the electrophoresis of CAST genotypes after SSCP.

The level of polymorphisms that found in this study was lower than reported by Zhou *et al*. (2007) with five different allelic in Merino, Corriedale, Poll Dorset and NZ crossbreed sheep. However, it was higher than previously reported in Indonesian local sheep (using PCR-RFLP; Sumantri *et al*., 2008), Iranian Karakul sheep (Shahroudi *et al*., 2006), and Kurdi sheep (Nassiry *et al*., 2006).

Genetic Diversity of CAST Gene

The results of this study may indicate that the CAST gene in the local sheep is polymorphic in all populations. Genotype frequency and allele frequency of the CAST gene is presented in Table 1. The study observed only three alleles (CAST-1, 2 and 3) and five genotypes (CAST-11, 12, 22, 32, and 33) in Indonesian local sheep populations, but not genotype of CAST-31. The most frequent alleles were CAST-1 and CAST-2 that contribute 48.4% for each allele and both counted 97%, while CAST-3 was rare allele (3%). The most frequent genotype was CAST-12 (39.5%). Zhou *et al*. (2007) also found that CAST-1 and CAST-2 were most common alleles, and both counted for 82% of the allele population in Merino, Corriedale, Romney, Poll Dorset and NZ cross-breed, while the rare allele were CAST-3 (13%), CAST-4 (2%), and CAST-5 (3%).

CAST-11 genotype frequencies in FTS group from Sumbawa, Rote, and Donggala population ranged from 0.08 to 0.166. Those values were lower than the PS group from Margawati (0.850) or TTS group from Jonggol (0.423) population. CAST-12 genotype frequencies in FTS group with the range of 0.103-0.833 was higher than PS group from Wanaraja (0.571) and TTS group from Kissar (0.531) population. While the CAST-22 frequencies in FTS with the range of 0.083-0.351 were higher than MTS and TTS group. CAST-32 genotype only found in TTS group (Sukabumi, Kissar, and Jonggol), with the highest frequencies in Kissar population (0.156). Frequency of CAST-33 genotype was 0.040 and only found in Sukabumi population.

Genotype and allele frequency differences in populations studies demonstrated the high diversity of local sheep. Local sheep population in Indonesia has CAST-1 and 2 alleles in the same frequency (48.4%) and spread throughout population, while the CAST-3 was rare

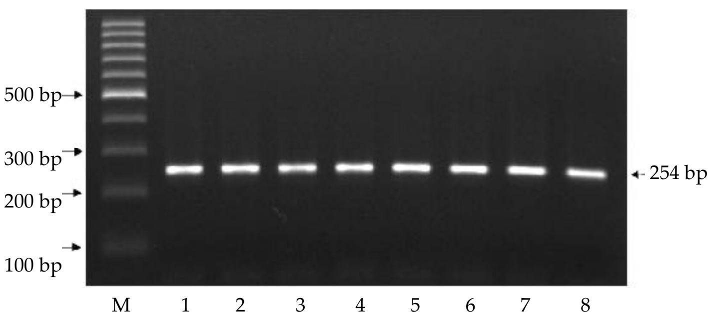

Figure 1. PCR product of CAST gene, M (marker)= 100 bp ladder, 1-8= individual samples

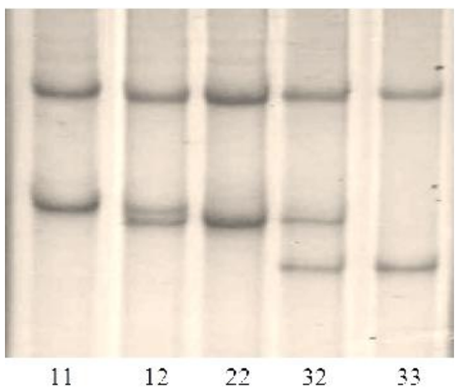

Figure 2. PCR-SSCP (single strand conformational polymorphism) of the ovine CAST gene

allele and only found in the thin tail sheep population (Sukabumi, Jonggol, and Kissar).

Another study using PCR-RFLP reported the polymorphisms of calpastatin (CAST-M*sp*1 locus) in Indonesian local sheep. With two types of alleles (M and N), but only found two types of genotypes (MN and NN) with the frequency of 25% and 75% for MN and NN genotypes, and 13% and 87% for M and N alleles (Sumantri *et al*., 2008).

The result of chi-square (X^2) test showed the distribution of six genotypes in the population were not in Hardy-Weinberg Equilibrium (Tabel 2). CAST-31 genotype was not found, probably due to a non-random mating system or because of direct selection (Bourdon, 2000). According to Nei & Kumar (2000), genetic diversity can be measured by using heterozygosity value. Observed heterozygosity (44.2%), expected heterozygosity (53.0%), Nei's expected heterozygosity (52.9%),

Tabel 1. Genotype and allele frequency of CAST gene in Indonesian local sheep

Population	N	CAST Genotype						CAST Allele		
		11	12	22	31	32	33	1	2	3
Donggala (FTS)	54	0.166	0.481	0.351	0.00	0.00	0.00	0.407	0.592	0.00
Sumbawa (FTS)	29	0.103	0.103	0.793	0.00	0.00	0.00	0.155	0.844	0.00
Rote (FTS)	12	0.083	0.833	0.083	0.00	0.00	0.00	0.500	0.500	0.00
Sukabumi (TTS)	50	0.320	0.300	0.220	0.00	0.120	0.040	0.470	0.430	0.100
Kissar (TTS)	32	0.281	0.531	0.031	0.00	0.156	0.00	0.546	0.375	0.078
Jonggol (TTS)	26	0.423	0.346	0.192	0.00	0.038	0.00	0.596	0.384	0.019
Margawati (PS)	20	0.850	0.100	0.050	0.00	0.00	0.00	0.900	0.100	0.00
Wanaraja (PS)	35	0.228	0.571	0.200	0.00	0.00	0.00	0.514	0.485	0.00
Total	258	0.286	0.395	0.263	0.00	0.046	0.007	0.484	0.484	0.030

Note: n= individual number, FTS= Fat tail sheep, TTS= Thin tail sheep, and PS= Priangan sheep.

Tabel 2. Observed and expected genotype frequency of CAST gene in local sheep

Genotype	Observed freq. (O)	Expected freq. (E)	X2 (chi-square)	X2 tabel (0.01; 3)
CAST-11	74	60.43	30.55	11.345
CAST-12	102	121.35		
CAST-22	68	60.43		
CAST-31	0	7.76		
CAST-32	12	7.76		
CAST-33	2	0.23		
Total	258	258		

and average heterozygosity (44.5) value of CAST locus in Indonesian sheep were medium. Observed heterozygosity, expected heterozygosity, and Nei's expected heterozygosity value of CAST locus in each population are presented in Tabel 3.

Sequences Analysis

Sequence analysis revealed that amplicons varied from 253 to 254 bp in length. These were the expected size based on previously reported by Zhou *et al*. (2007). All of the sequences identified were shared high similarity or identical to the published ovine and bovine CAST gene sequences (Figure 3). Based on homology of bovine CAST gene sequences with GenBank Accession Nos. EF443057 and AY834770 (Zhou *et al*., 2008c & Raynaud *et al*., 2005), exon 6 was the largest exon of ovine CAST gene, with 114 bp in length and coding around 38 amino acid residues.

Based on sequence analysis, it identified that three SSCP patterns represented three allelic sequences of ovine CAST, and identified four single nucleotide polymorphisms (SNPs). All of the nucleotide variation identified in this study was similar to that reported previously by Zhou *et al*. (2007). SNP position of CAST-1 allele were at 62 bp (G>A) position (GenBank acc. no. DQ414513) relative to the CAST-2 allele sequences (GenBank acc. no. DQ414514), and SNPs position of CAST-3 allele were at 65 bp (G>T), 69 bp (indelT) and 96 bp (A>T) position (GenBank acc. no. DQ414517) relative to the CAST-1 and CAST-2 sequences.

Mutation in CAST-3 allele in the exon 6 region at 96 bp (A>T) position was a non synonymous mutation which would induced Gln/Leu substitution (Figure 3), Tabel 3. Observed and expected heterozygosity value of CAST gene in Indonesian sheep

Population	N	Heterozygosity			
		Ho	He	Nei*	Average
Donggala (FTS)	54	0.481	0.487	0.482	
Sumbawa (FTS)	29	0.103	0.266	0.262	
Rote (FTS)	12	0.833	0.521	0.500	
Sukabumi (TTS)	50	0.420	0.590	0.584	
Kissar (TTS)	32	0.687	0.563	0.554	
Jonggol (TTS)	26	0.384	0.506	0.496	
Margawati (PS)	20	0.100	0.184	0.180	
Wanaraja (PS)	35	0.571	0.506	0.499	
Total	258	0.442	0.530	0.529	0.445

Note: n= individual number, Ho= Observed heterozygosity, He= Expected heterozygosity according to Levene (1949) and Nei's (1973).

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              Forward primer
              ------------------->
DQ414513  gttatgaattgctttctactcctcagagaaaagtagcaatatgtgacatattgttaaccgtgtgggggtg  70
Cast 3    ----------------------------------------------------------------t---.-  69
Cast 2    -------------------------------------------------------------a--------  70
Cast 1    ----------------------------------------------------------------------  70
EF443057  ---------------------------------------------t------a------cca-----.--  69

                                                            Exon 6
                          ↓ P  K  S  Q  P  K  H  P  S  D  T  R  S  K  H  A  P  K
DQ414513  tgtgtgtgttttcag CCAAAAAGCCAACCCAAGCACCCCTCAGATACAAGAAGCAAGCATGCTCCTAAGG  140
                                    L
Cast 3    --------------- ----------T--------------------------------------------  139
Cast 2    --------------- -------------------------------------------------------  140
Cast 1    --------------- -------------------------------------------------------  140
EF443057  -a--c---------- ----------T----------T-A---------G---------------------  139

           E  K  A  V  S  K  S  S  E  Q  P  P  S  E  K  S  T  K  P  K  ↓
DQ414513  AAAAAGCTGTTTCCAAGTCAAGCGAGCAGCCACCATCAGAGAAATCAACAAAACCAAAG gtaaataaagc  210
Cast 3    ----------------------------------------------------------- -----------  209
Cast 2    ----------------------------------------------------------- -----------  210
Cast 1    ----------------------------------------------------------- -----------  210
EF443057  -------C--------A-----T------------------------------------ -----------  209

                                         Reverse primer
                              <--------------------
DQ414513  agacagat...agat.gaaagaaagaaagtgaagtctctcaatcgtat 254
Cast 3    --------...----.-------------------------------- 253
Cast 2    --------...----.-------------------------------- 254
Cast 1    --------...----.-------------------------------- 254
EF443057  --------gaa---aa-------------------------------- 257
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Figure 3. Nucleotide sequences of the ovine CAST-1, 2, and 3 allele and predicted amino acid sequences. DQ414513 and EF443057 were GenBank accession nos. of published ovine and bovine CAST sequences (Zhou *et al.*, 2007 & 2008c).

and it functional significance was unknown (Zhou *et al.*, 2007). However, it has been suggested that calpastatin has a Ca2+ channel regulating function located in the L domain (Hao *et al.*, 2000), and reported by Tullio *et al.* (2009) that sequences were coded by exon 6, containing multiple phosporylation sites, and directly involved in determining the cell localization of calpastatin. This suggests that variation in this sequences may impact on the activity of Ca2+ channels and hence regulate or modulate calpain activity. All variation in the intron region may affect RNA processing and consequently the function and level of expression of calpastatin (Zhou *et al.*, 2007).

Byun *et al.* (2008) reported that allele A (CAST-1) and C (CAST-3) had a significant effect on birth weight, but did not significantly affect the growth rate to weaning, while allele B (CAST-2) did not significantly affect the birth weight and growth rate. All of these three alleles (CAST-1, 2, and 3) or genotypes variation in the CAST locus did not significantly affect lamb tenderness (Zhou *et al.*, 2008a).

The polymorphism of CAST exon 6 in goat also reported by Zhou *et al.* (2008b), who identified a non-synonymous amino acid variation in the caprine CAST which would result in a Ser/Arg amino acid change in the domain L of the protein. A synonymous SNP (T>C) mutation was also indentified in bovine CAST exon 6 sequences (Zhou *et al*., 2008c).

CONCLUSION

Calpastatin (CAST) in the intron 5-exon 6 regions show polymorphism in Indonesian local sheep. Five genotypes were observed in this study, i.e CAST-11, CAST-12, CAST-22, CAST-32, and CAST-33 with the genotype frequencies were 0.286, 0.395, 0.263, 0.046, and 0.007 respectively. CAST-1 and CAST-2 were the most common alleles with total frequency in population 0.970, while the rarest allele was CAST-3 (0.030). Variation in the CAST gene could be used for the next research as genetic diversity study or to find any association between CAST polymorphism with birth weight, growth trait and carcass quality in Indonesian local sheep.

ACKNOWLEDGEMENT

The work was supported by Directorate General of Higher Education (DGHE), Ministry of National Education Republic of Indonesia through the *Hibah Kompetensi* 2010 project with contract no. 224/SP2H/PP/DP2M/III/2010. We thank H. Bunyamin (Tawakkal Farm) and JASTRU (Jonggol Animal Science Teaching and Research Unit) of Animal Science Faculty, Bogor Agricultural University for blood samples and E. Andreas for technical assistance.

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