Original Article

A Survey to Determine the Presence of the *N*-acetylglucosamine-6-sulfatase (G6S) Gene Mutation in Anglo-Nubian Goats in Southern Thailand

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Abstract

Mutation of the G6S gene in Anglo-Nubian (AN) goat causes non-function of the *N*-acetylglucosamide 6-sulfatase enzyme within lysosomes, which increases heparin sulfate accumulation and induces pathogenesis of the internal organs. Transversion of base C to T at nucleotide 322 changes decoding of an amino arginine to a stop codon (R322X) and produces a truncated protein. This mutation is detrimental to the health of a goat kid leading to delay in nervous development and death. It was found first time in goats of a Michigan herd in the US, but has not yet been reported in Thailand. This study aimed to determine the incidence of the G6S gene in AN and crossbred AN goats in southern Thailand. DNA samples of 39 purebred AN goats and 82 crossbred AN x Thai Native (TN) goats from 3 research farms in Yala and Songkhla province were tested by RFLP and 5 DNA samples were subsequently sequenced. No instances of the mutation at nucleotide 322 were found, however, polymorphism of base C at nucleotide 354 was detected, which was different from a previous report which noted this polymorphism at base T. This would indicate that the goat line in Thailand differs from the G6S mutant line in USA. Moreover, the pressure of high growth rate selection after many generations and the cell and death of those with poor growth might have removed this homologous recessive gene entirely from tested goat population.

Keywords: Anglo-Nubian goat, mucopolysaccharidosis type IIID, N-acetylglucosamine-6-sulfatase, restriction fragment length polymorphism, Thailand

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บทคัดย่อ

การสำรวจการผ่าเหล่าของยีน *N*-acetylglucosamine-6-sulfatase (G6S) ของแพะ Anglo-Nubian ในภาคใต้

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การกลายพันธุ์ของยีน G6S ในแพะพันธุ์ Anglo-Nubian (AN) ทำให้เอ็นไซม์ *N*-acetylglucosamide 6-sulfatase enzyme เสียสภาพและทำให้เกิดการสะสมของสาร heparin sulfate ใน lysosomes มีผลให้เกิดพยาธิสภาพของอวัยวะภายในหลายชนิด โดยการกลายพันธุ์แบบ transversion ของเบส C ไปเป็นเบส T ที่ตำแหน่งนิวคลีโอไทด์ 322 จะเปลี่ยนการถอดรหัสของกรดอะมิโน argenine ไปเป็นรหัสหยุด (R322X) ทำให้โปรตีนมีขนาดสั้นลง การกลายพันธุ์นี้มีผลต่อสุขภาพลูกแพะโดยทำให้ระบบประสาทมีพัฒนาการที่ช้าและแพะมักตาย พบการกลายพันธุ์นี้ครั้งแรกในแพะที่เลี้ยงในรัฐมิชิแกน สหรัฐอเมริกาแต่ยังไม่เคยมีรายงานพบในประเทศไทย งานวิจัยนี้มีจุดมุ่งหมายเพื่อศึกษาอุบัติการณ์ของการผ่าเหล่าของยีน G6S ในแพะ AN พันธุ์แท้และลูกผสมในภาคใต้ของประเทศไทยโดยเก็บตัวอย่างดีเอ็นเอจากแพะ AN พันธุ์แท้ 39 ตัวอย่าง และจากแพะลูกผสม AN x พื้นเมืองไทย 82 ตัวอย่าง จากฟาร์ม 3 ฟาร์มในเขตจังหวัดยะลาและสงขลา ตรวจด้วยวิธี RFLP และสุ่มตัวอย่างจำนวน 5 ตัวอย่างเพื่อหาลำดับเบสของดีเอ็นเอ จากการสำรวจไม่พบว่ามีการผ่าเหล่าที่ตำแหน่งนิวคลีโอไทด์ 322 แต่พบ polymorphism ของเบส C แทนที่เบส T ที่ตำแหน่งนิวคลีโอไทด์ 354 ซึ่งต่างจากที่เคยมีรายงานก่อนหน้านี้ อาจบ่งชี้ว่าสายพันธุ์แพะ AN ในประเทศไทยต่างจากของสหรัฐอเมริกา นอกจากนี้การคัดเลือกพันธุ์สัตว์ให้มีอัตราการเติบโตที่สูงอย่างต่อเนื่องหลายชั่วรุ่น รวมทั้งสัตว์ที่ผ่าเหล่าจะมีสุขภาพไม่ดีและตายไปเอง ทำให้เกิดการคัดทิ้งยีนที่ผ่าเหล่าออกจากฝูงแพะที่ทดสอบไปแล้ว

คำสำคัญ: แพะพันธุ์ Anglo-Nubian โรค Mucopolysaccharidosis type IIID เอ็นไซม์ *N*-acetylglucosamine-sulfatase Restriction fragment length polymorphism ประเทศไทย

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Introduction

Anglo-Nubian (AN) is the main goat breed in Thailand. The current herd is the result of over 30 years of cross between the Thai Native and male AN goats from many countries. This crossbred shows good performance under the moist heat environment of Thailand, and was promoted nationwide, especially in the southern region.

Mucopolysaccharidoses (MPS) are a group of lysosomal storage diseases characterized by malfunctioning of lysosomal enzymes to degrade glycosaminoglycans. Caprine mucopolysaccharidosis type IIID (MPS IIID) or *N*-acetylglucosamine-6-sulfatase (G6S) enzyme deficiency disease was first identified in Anglo-Nubian goat in 1992 (Thompson et al., 1992). It is an autosomal recessive trait, in which the heterozygous goat is normal and thus retains the genetic defect within the herd as a carrier, but a homologous recessive animal will show the disease symptoms. The goat with this disease will go through delayed motor development, growth retardation and premature death, although some can reach sexual maturity before dying suddenly of congestive heart failure (Thompson et al., 1992; Jones et al., 1998).

A nonsense mutation at nucleotide 322 (C to T) results in the change of the arginine codon to a stop codon (Cavanagh et al., 1995), which truncates the G6S protein and stops the enzyme function. However, this also creates a cut site for the *Alu*I restriction enzyme for a RFLP test. A survey of the frequency of this mutation in pure-bred Anglo-Nubian goats in Michigan, USA, found 25.2% heterozygous and 1.3% homozygous recessive goats (Hoard et al., 1998) and

other studies in Taiwan found 8.2-15.0% heterozygous goats (Lin et al., 2002; Lin et al., 2004).

Although Anglo-Nubian goats have been raised in Thailand for a long time, to date there has been no study attempting to determine the presence or incidence of this mutation. This study aimed to survey Anglo-Nubian goats from three main herds in southern Thailand, using RFLP to determine the incidence of the G6S mutation in our ongoing work to improve goat genetics.

Materials and Methods

Animal Samples: Anglo-Nubian goats from 3 major research farms in southern Thailand were used in the study: Yala Livestock Research and Breeding Center Farm in Yala province (39 samples); Department of Animal Sciences Farm, Faculty of Natural Resources, Prince of Songkla University (PSU), Hat-Yai district, Songkhla province (42 samples); and Small Ruminant Research and Development Center Farm of the Faculty of Natural Resources, PSU, Klonghoikhong district, Songkhla province (40 samples). Blood collection, DNA extraction, PCR and RFLP methods were done following protocol of Hoard et al (1998).

Blood Collection and DNA Extraction: Three milliliter of blood samples from all of the goats were collected in EDTA collection tubes. Whole blood (100 µl) was combined with 100 µl of red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4) and incubated on ice for 10 min. White blood cells were isolated by centrifuging at 12,000 x g at 4^0C for 30 sec. The supernatant was removed and the pellet was suspended in 50 mM NaOH 200 µl. The white blood cells were lysed by boiling for 10 min and then neutralized with 10 µl of 2 M Tris-HCl, pH 8.0 and frozen at -20^0C.

PCR Protocol: The PCR primers, 5/-CTTATGTGC CAAGTGCTCTC-3/ and 5/-CCTCCAGAGTGTTGT TAACC-3/, which are specific to sequences around the mutation site, were used to amplify a 96 bp PCR product. The PCR reaction was carried out using 1 µl of genomic leukocyte DNA in 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.1 µM of each primer and 1.25 units of *Taq* DNA polymerase to make final volume of 50 µl. Amplification was performed for 35 thermal cycles of 30 sec at 94^0C, 30 sec at 55^0C and 30 sec at 72^0C. The PCR product was precipitated by absolute ethanol and suspended in distilled water.

Restriction Fragment Length Polymorphism (RFLP) and DNA Sequencing: A 17 µl aliquot of the amplified DNA was digested with 1 µl (5 units) of *Alu*I and 2 µl of 10x buffer at 37^0C for 12 hours. Digestion of the *Alu*I produced 66 and 30 bp amplicons. The restriction fragments were resolved by electrophoresis in 4% agarose gel in 0.5x TBE buffer at 80 V for 45 min. After staining the gel with ethidium bromide, the fragments were visualized by UV transilluminator and photographed. Five DNA samples were chosen at random, sequenced and aligned with DNA data of normal G6S goat genes from GenBank (accession number U17694).

Statistical Analyses: General data from each farm were used to evaluate the effect of breed and management. Body weight at birth, weaning, one year and two year were analyzed and compared by analysis of variance (ANOVA) followed by Duncan's multiple range tests (Steel and Torrie, 1980).

Results

General information of goat farm

The relevant backgrounds of each farm, as of the time of the study, were summarized as follows:

1. Yala Livestock Research and Breeding Center, Department of Livestock Development, Ministry of Agriculture and Co-operation, has for over 25 years been using pure-bred Anglo-Nubian (AN) goats from many countries to improve the goat breeds in southern Thailand, for example 4 goats from the USA (1988), 18 goats from the USA (1993), 5 goats from Australia (2006) and 15 goats from China (2006). The AN line was crossed with the Thai Native line to produce 50% AN x 50% TN. Good performance offspring were selected to cross with 100% ANs to continually increase the level of AN blood in the herd, until currently (2012) the level of AN blood in the Yala herd is 93.75% or higher.
2. Research Farm of the Department of Animal Science, Faculty of Natural Resources, Prince of Songkhla University (PSU), Hat-Yai, Songkhla province, was established in 1977 for goat and sheep research. In 1982, the Faculty of Natural Resources collaborated with the University of Queensland, Australia, in the Thai-Australia Prince of Songkhla Project to improve goat production in the villages of southern Thailand. The project studied the optimum level of AN suitable for the local environment and management. Semen of 6 pure-bred AN bucks from one farm in Queensland, Australia, were crossed with 100% TN goats to produce an F1 50% cross (Sripongpun, 1987). Then, selected 50% cross-bred males were crossed with various blood levels of AN females to produce 75, 62.5, 50 and 25% AN cross-bred.

In 1991, most 50% cross-bred goats were moved to the new Small Ruminant Research and Development Center, another research farm established by the Faculty of Natural Resources, PSU, at Klonghoikhong, Songkhla province, and only a small number of goats were left at the original farm. Presently, the variation of AN cross-bred in the research herd at Hat-Yai is very high, ranging from 23-59% purity.
3. Research Farm of the Small Ruminant Research and Development Center, of the Faculty of Natural Resources, PSU, Klonghoikhong, Songkhla, was established in 1991 as noted above, has maintained an AN level at 50% constantly by selecting 50% AN x 50% TN offspring to be sire and dam for in-breed mating. Some new 50% AN cross-bred males from the Yala center were introduced into the herd to reduce inbreeding problem in 1999.

Data of birth weights, weaning weights, and one and two year weights of a sample of goats from each

farm were analyzed in Table 1. No reports of deaths from MPS IIID in goat kids was recorded, but this is not entirely conclusive, because MPS IIID has similar signs with many diseases, and the low mortality rate from any of these diseases obscured observation.

RFLP and DNA sequence result

Every sample analyzed showed a negative result for the 322 (C to T) mutation of G6S on both heterozygous and homozygous recessive forms when tested by RFLP (Fig 1). DNA sequencing of random samples from each farm also found no mutations at nucleotide 322. However, a DNA polymorphism was found at nucleotide 354 (T to C) when compared with sequence reported by Friderici et al. (1995) (GenBank record number U17694) (Fig 2). This is a silent mutation (CAT to CAC) because it still decodes the amino acid histidine.

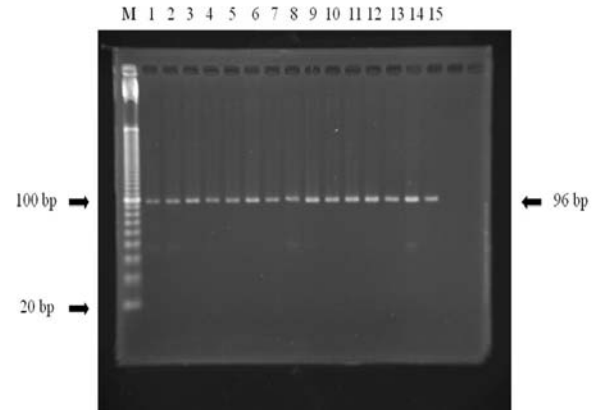

Figure 1 RFLP results from 15 samples. The PCR products were digested by *Alu*I for 12 hours and resolved by electrophoresis in 4% agarose gel in 0.5x TBE buffer at 80 volt, DNA marker (M) is 10 bp ladders.

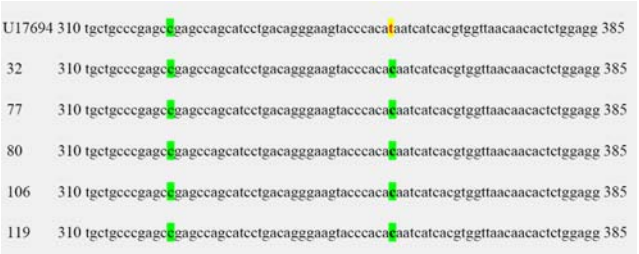

Figure 2 Multiple DNA alignment sequencing of five samples (sample numbers 32, 77, 80, 106, and 119) from the three study farms, compared with a DNA sequence from GenBank (accession number U17694).

Discussion

DNA screening for G6S mutation in Michigan State, USA, detected a high incidence of carriers and affected goats, which prompted our concern and led to the current study. Neither selection, random mating, low mutation, migration, nor large amount of animals in herd can maintain constant frequency of mutation through many generations following Hardy-Weinberg law (Hoard et al., 1998). However, animal selection by humans to improve production is a strong influence that can interfere gene frequency.

Our failure to find any mutations at nucleotide 322 of the G6S gene in our study might be due to many reasons:

1. There were no G6S mutations in the AN bucks or semen introduced to the Thailand stock over the years. There are two ways this could occur:
   1.1. The AN bucks or semen brought into Thailand from the various foreign countries over the years were G6S mutation free. This would mean that the herds in Thailand and in Michigan probably have different lineages. This is also indicated by the differences noted in nucleotide 354 between the DNA samples from the USA (U17694) (base T) and from the Thai samples (base C) (Fig 2).
   1.2. Or it may be that some or all of the AN bucks or semen introduced to Thailand had the G6S mutation, but through good fortunate none of those carrying the mutation were selected as breeders here.

If either or both of these conditions are true, it could be implied that there are no animals in the Thai-Native goat population with the G6S mutation at nucleotide 322.

Table 1 AN blood levels, gender and body weights of goats selected for G6S screening test from the three study farms

Farm	Gender (heads)	Body Weight ($\bar{X}$ ± SD, kg)			
		At birth	At weaning	At one year	At two years
Farm of PSU at Hat-Yai, Songkhla	Male (6)	2.26 ± 0.39^{ab}	9.50 ± 3.03^{ab}	23.17 ± 3.53^{b}	31.63 ± 2.29^{c}
	Female (36)	2.02 ± 0.45^{a}	8.67 ± 2.00^{a}	15.14 ± 4.10^{a}	22.15 ± 4.41^{a}
Farm of PSU at Klonghoikhong, Songkhla	Male (8)	2.47 ± 0.54^{bc}	10.96 ± 3.35^{b}	17.33 ± 5.44^{a}	25.94 ± 6.14^{bc}
	Female (32)	2.13 ± 0.47^{ab}	8.85 ± 2.63^{ab}	16.49 ± 4.60^{a}	23.58 ± 5.53^{ab}
Yala Livestock Research and Breeding Center	Male (8)	3.13 ± 0.88^{c}	17.92 ± 4.84^{c}	No record	No record
	Female (31)	2.92 ± 0.61^{c}	16.76 ± 2.13^{c}	No record	No record

a, b, c means within column not followed by the same superscript differ ($p < 0.05$)

2. It is known that strong in-line selection for high production of animals can preserve or eliminate some alleles of a gene from a herd. It has been observed that the G6S mutation is very prevalent in lines which are selected for high milk production, meaning that breeders who select goats for milk might inadvertently be selecting for the G6S defect also (Vinidish, 2002). This type of selected breeding has not occurred in Thailand, where the main goal for genetic improvement has been, and continues to be, a high growth rate and high weight for meat purposes.
3. When the G6S mutation does get introduced into a herd, the homologous recessive goats tend to die young, which reduces the occurrence of recessive alleles in the herd. When this tendency to die young is further combined with selection for meat type goats for several generations, the occurrence of the recessive G6S gene might diminish rapidly.

Although no G6S mutation was found in this study, local crossbred herds may still have this gene mutation from earlier introductions of Anglo-Nubians to the area, which have not been removed through the kind of selection talked about, for meat, but simply for no attributes other than survival to breeding age. However, the mortality rate of the homologous G6S mutation is high and the gene can be eliminated easily from a herd through simply using bucks confirmed to be G6S free. Farm owners, goat breeders and government centers should carefully test all bucks before introducing them to local goat populations, so that Anglo-Nubian goat line in Thailand remains G6S-free.

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