Indonesian Journal of Agricultural Science
Vol. 20 No. 1 June 2019: 29–34
DOI: http//dx.doi.org/10.21082/ijas.v.20.n1.2019.p.29–34

SCREENING OF THE ACID MEAT CONDITION IN THE RENDEMENT NAPOLE GENE USING POLYMERASE CHAIN REACTION - RESTRICTION FRAGMENT LENGTH POLYMORPHISM

Penapisan Kondisi Daging Asam dalam Gen Rendement Napole *Menggunakan* Polymerase Chain Reaction - Restriction Fragment Length Polymorphism

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Submitted 07 January 2019; Revised 29 May 2019; Accepted 11 June 2019

ABSTRACT

A mutation in the rendement napole (RN) gene causes the acid meat condition which results to poor meat quality due to its reduced water holding capacity, low pH, pale color, reduced processing and cooking yield due to increased drip, and strong metallic taste. This study was conducted to detect the mutation in the RN gene in 535 commercial breeder pigs from the Philippines. Blood collection was done then subjected to DNA extraction and genotyping using polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) using the enzyme *BsrBI,* then validated by DNA sequencing. Results revealed that 97.01% of the breeder pigs did not have the mutation in their RN gene, while 2.69% had at least one copy of the defective allele in their gene. The acid meat condition has only been previously detected in the Hampshire breed whereas this study found the mutations predominantly in Pietrain and Landrace breed they were classified as normal (rn/rn), heterozygous mutants (RN/rn), and homozygous mutants (RN/RN) which allowed breeding systems to be developed ensuring that all offspring are free of the defect. This genetic screening will help in detecting the presence of the defect in a given swine population and reduce the unwanted effects on meat quality thus increasing its market value.

[**Keywords**: Acid meat, gene screening, PCR-RFLP, RN gene]

ABSTRAK

Mutasi pada gen rendement napole (RN) *menyebabkan kondisi daging menjadi asam sehingga kualitas daging menurun karena menurunnya kapasitas penahanan air, pH rendah, warna pucat, berkurangnya rendemen pengolahan dan pemasakan karena peningkatan tetesan, dan rasa logam yang kuat. Penelitian ini dilakukan untuk mendeteksi mutasi pada gen RN pada 535 babi peternakan komersial di Filipina. Darah dikumpulkan lalu dilakukan ekstraksi dan genotiping DNA menggunakan* polymorphism chain reaction - restriction fragment length polymorphism *(PCR-RFLP) menggunakan enzim* BsrBI*, kemudian divalidasi dengan sekuensing DNA. Hasil penelitian menunjukkan bahwa 97,01% dari babi tersebut tidak menunjukkan mutasi pada gen* RN*, sementara 2,69% memiliki setidaknya satu salinan alel yang rusak dalam gen mereka. Kondisi daging asam sebelumnya hanya terdeteksi pada Hampshire, sedangkan penelitian ini menemukan mutasi dominan pada Pietrain dan Landrace. Mereka diklasifikasikan sebagai normal (rn/rn), mutan heterozigot (RN/rn), dan mutan homozigot (RN/RN) yang memungkinkan dikembangkannya sistem pemuliaan untuk memastikan bahwa semua keturunan bebas dari cacat. Penapisan genetik ini akan membantu dalam mendeteksi adanya cacat pada suatu populasi babi dan mengurangi pengaruh yang tidak diinginkan terhadap kualitas daging sehingga meningkatkan nilai pasarnya.*

[**Kata kunci**: Daging asam, penapisan gen, PCR-RFLP, gen *rendement napole*]

INTRODUCTION

One of the main concerns of the pork industry is to improve pork quality as to satisfy the consumers' preference and strengthen their acceptance. Another is to increase the production yield which results to a greater profit. To attain these, genetic factors that affect meat quality and meat production are needed to be taken into consideration prior to breeding. The increase in the production of swine products, if not monitored properly, might lead to the adverse decline of meat quality in the presence of an unwanted gene in the gene pool of a swine population. To address this situation, one of the concerns is to reduce, and possibly for the longer run is to remove, the unwanted genes in the gene pool of a particular swine population. The screening of genetic diseases such as the acid meat condition (AMC) will help the determination of their presence in a given swine population and decrease their unwanted effects. Gene screening can be utilized to detect the genes that may have a negative economic effect in increasing the meat

production and quality of our swine (Jiang and Gibson 1999).

The AMC has been discovered to negatively affect the quality and production yield of pork and processed meat. As a result, within the succeeding years, there has been an increased interest for the understanding of this genetic mutation because of its association with increased meat glycolytic content. This gene causes reduced water holding capacity, low pH, pale color, reduced processing and cooking yield due to increased drip, and strong metallic taste that often leads to poor meat quality (Du 2004; Granlund et al. 2011; Moeller et al. 2003; Stanton et al. 2010). Moreover, this gene causes the reduction in visual marbling and intramuscular fat which also decreases the meat quality (Hamilton et al. 2000).

The mutation in rendement napole (RN) gene causes the AMC. This defect is due to a non-conserved arginine to glutamine substitution (*R200Q).* Through the aid of various DNA technological advancements, it was recently found that the AMC is also associated with several missense mutations caused by amino acid substitutions at *T30N* (threonine to asparagine), *G52S* (glycine to serine) or *I199V* (isoleucine to valine) in the same *PRKAG3* polypeptide (Ciobanu et al. 2001; Meadus et al. 2002; Milan et al. 2000). The *Sus scrofa* AMPK gamma subunit (*PRKAG3*) gene sequence information is available in GenBank with an accession number of AF214521. The fact that the defective allele in the RN gene is a dominant gene shows a need for immediate action, such as genetic testing, for the possible elimination of these swine from the population. Hence, consumer satisfaction will be attained and will also reduce the drastic effects on production yields leading to a greater profit for the breeders.

Initial studies showed that the AMC has high prevalence in the Hampshire breeds and crossbreeds from Hampshire. However, recent studies showed that this condition is not limited to these breeds. Ciobanu et al. (3(2001) discovered other substitutions in the *PRKAG3* gene that are associated to the AMC in a three-generation intercross between Berkshire and Yorkshire pigs. Moreover, a study of Alarcón-Rojo et al. (2008) showed seven RN gene positive carriers out of 194 commercial animals they used in a local abattoir in Chihuahua, Mexico. Meadus et al. (2002) also cited that the prevalence of this gene has increased in retail pork chops that majorly supplied by the large composite breeds. Thus, these recent studies showed that the RN gene is not limited to the traditional Hampshire and crossbreeds from Hampshire but is now also present in the regular commercial pig population. There are still no known studies that have been conducted regarding this condition in the Philippines.

The polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) or cleaved amplified polymorphic sequence (CAPS) is a less expensive method capable of locating genes associated with certain genetic disorders. Mutations such as single nucleotide polymorphism, multiple nucleotide polymorphism, deletion, duplication and combination often cause the formation or elimination of a restriction enzyme recognition site. The method uses this circumstance as an advantage to analyze the DNA (Saraswathy and Ramalingam 2011).

In the Philippines, meat has been considered as principal food and by far, pork has been the dominant meat product. The increasing population, at a rate of 2% per annum is an indication that the demand for meat will steadily increase (Stanton et al. 2010). Thus, to meet the increasing demand and satisfy meat preference of most Filipinos, different possibilities to improve meat quality and meat production of breeders must be taken into consideration.

This study was conducted to screen mutation in the RN gene which causes the AMC, determine its frequency in a population of domesticated swine, and identify incidence in different breeds tested. The study also aimed to establish a genetic screening method for AMC in the Philippines that will prevent the transfer of these undesirable genes to future generations. This study would represent the sampled population of the selected breeders in the entirety of the swine population across Philippines.

MATERIALS AND METHODS

Animals

Five milliliters (5 ml) of blood samples were collected from 535 swine samples (Pietrain-98; Landrace-121; Large White-207; Duroc-28, and Chester White-81) from commercial breeders in the Philippines. The collection was done by licensed veterinarians to ensure proper and safe collection of blood.

DNA Extraction

Blood samples were placed in vacutainer tubes containing EDTA buffer. These tubes were then stored in a cooler maintaining a temperature of less than 4 ^{o}C prior to transportation to the Philippine Carabao Center (PCC) National Headquarters and Gene Pool in Science City of Munoz, Nueva Ecija for DNA extraction and analysis. The DNA from each collected blood sample was extracted using the Promega Blood Extraction Kit® following the manufacturer's instructions. The DNA quantity was measured through the use of Nanodrop.

PCR-RFLP

Annealing temperature and cocktail mix optimization procedures were first done prior to the amplification of the extracted DNA samples. This method was used since it allows the determination of the optimum annealing temperatures on which oligonucleotide primers will work best (Prezioso and Jahns 2000). Annealing temperatures between 58 °C and 70 °C were tested for AMC. An annealing temperature of 69 °C was chosen as the ideal temperature because of the absence of non-specific amplicons and smearing.

The isolated DNA samples were subjected to thermal cycling using PCR for the amplification of the 249 bp region of the *PRKAG3* gene (Moeller et al. 2003). The primers used in this study were adapted from the study of Moeller et al. and to study the effect of the RN gene on performance, carcass, muscle quality, and sensory traits. Progeny (N = 118(2003). The sequence of the forward primer in 5' → 3' is AAATGTGCAGACAAGGATCTCG while the reverse primer is ACGAAGCTCTGCTTCTTGC amplifying a 249 bp segment of the *PRKAG3* gene. A PCR cocktail mixture was prepared with component volumes and concentrations as follows: 1.5 µl of 5x PCR buffer, 1 µl of 25 mM $MgCl_2$, 1.5 µl of dNTP mix, 0.3 µl of forward and reverse primers, 0.5 µl of Taq Polymerase, and 1 µl of genomic DNA to a final volume of 15 µl using dH_2O.

These samples were optimized under the following conditions: initial incubation for 4 minutes at 94 °C, followed by 35 repetitive cycles for denaturation at 94 °C for 45 seconds, annealing temperature of 62 °C for 1 minute, elongation of 45 seconds at 72 °C, then final elongation of 5 minutes at 72 °C and holding temperature of -4 °C.

An RFLP mixture containing 0.25 µl of the *BsrBI* (New England Biolabs Inc., USA) restriction enzyme, 1 µl RE buffer and 0.75 µl high power liquid chromatography (HPLC) water was used to detect the mutation on site 200 of the *PRKAG3* gene by cutting the restriction site 5'-GAGCGG-3' found in the normal allele (Figure 1). Eight µ of the PCR products were then mixed separately with RFLP mixtures and incubated for 2 hours at 37 °C. RFLP products were loaded into agarose gels using 3 µl for each sample and 5 µl of the DNA ladder. Gel electrophoresis subsequently followed where 3% agarose gel was utilized as the medium. Gel red was used for the visualization of the amplicons under the UV Gel Documentation System.

DNA Sequencing

Eight samples detected to have the mutation (4 homozygous mutants (RN/RN) and 4 normal samples) were sent to First Base Services in Malaysia for sequencing. Results from DNA sequencing were used to verify the results of the RFLP tests, whether the restriction enzyme *BsrBI* was able to discriminate the normal from mutated samples. The DNA sequences were assembled using Vector NTI® Software and aligned with the *PRKAG3* gene sequence downloaded from NCBI with accession numbers of AF214521.

RESULTS AND DISCUSSION

PCR-RFLP

This study screened 535 commercial breeder pigs for the presence of the mutation in the PRKAG3 gene. After the successful amplification of the 249 bp region of the PRKAG3 gene using PCR, the restriction enzyme BsrBI was used to cut the 5'-GAGCGG-3' restriction site of the amplified strand located from site 1849–1854 of the whole PRKAG3 gene. The inability of this enzyme to cut in the restriction site would be attributed to the point mutation in RN- positive (Moeller et al. 2003). Thus, samples wherein the amplified sequence was not cut are classified to have the acid meat condition (two alleles are mutants) as observed by a single band (249bp) in the gel electrophoresis image. On the other hand, normal samples resulted to two fragments with amplicon sizes of 215 bp and 34 bp. Heterozygous mutants have a

1701	TCATGTAGAG AGTACATCTC	GCAGGCCCGG CGTCCGGGCC	GAGGCGCCCG CTCCGCGGGC	GTGGAAGAAC CACCTTCTTG	CCTGGCTGGC GGACCGACCG	AGGGGACCTC TCCCCTGGAG
1801	GCGCTCAGAT CGCGAGTCTA	CAAGAAGGCC GTTCTTCCGG	TTCTTTGCCC AAGAAACGGG	TGGTGGCCAA ACCACCGGTT	CGGCGTCCGA GCCGCAGGCT	GCGCCACCTT CGCCGTGGAA
1901	GCTGGGGAGG CGACCCCTCC	CAGAGGTGGT GTCTCCACCA	GGGGAAGGGA CCCCTTCCCT	ATAGGGGGAC TATCCCCCTG	CTTGTGGGGT GAACACCCCA	GATTCTAGGG CTAAGATCCC

Figure 1.The restriction site of the restriction enzymes of the *PRKAG3* gene. The restriction enzyme *BsrBI* was used to cut the 5'-GAGCGG-3' restriction site of the amplified strand located from site 1849–1854 of the whole *PRKAG3* gene.

normal strand and a mutated strand, consisting of 249 bp, 215 bp and 34 bp (Figure 2).

The arginine to glutamine substitution (R200Q) which causes the acid meat condition was detected in this study. Specifically, the amino acid substitution was due to the guanine to adenine (G-A) mutation 1849 bases from the ATG start codon of the *PRKAG3* gene (Meadus et al. 2002). This gene is responsible for coding the regulatory gamma subunit of protein kinase AMP activated (AMPK). The AMPK functions for the regulation of glucose and lipid metabolism in skeletal and an energy level sensor (Alarcón-Rojo et al. 2008). Milan et al. (2000) found out that AMPK kinase activity is three fold lower in the RN positive and carriers than the normal swine. Thus the carriers have significantly lower AMP/ATP ratio (increased number of AMP over ATP). They have high ATP and low AMP, thus the *R200Q* substitution causes the decrease in AMPK kinase activity which in turn leads into glycogen synthesis resulting to more glycogen content. This results to the 70% increase in glycogen of muscles that is converted to lactic acid 24 hours after slaughter. When more lactic acid is produced in RN carriers, this results to lower ultimate pH, thus the name "AMC" as termed by Enfält et al.(1997).

Frequency of RN-Allele

The result of the RFLP assay showed the presence of the RN-allele in the non-Hampshire tested sample population. Out of the 535 samples with successful 249 bp fragment amplification, 16 samples carried at least one RN-allele, in which two were heterozygous mutants (RN/rn) and the remaining 14 were homozygous mutants (RN/RN). The remaining 519 samples were found to be normal.

Initial studies showed that the AMC has high prevalence in the Hampshire breeds and crossbreeds from Hampshire (Lundström et al. 1996). However, recent studies showed that this condition is not limited to these breeds but is now also present in the regular commercial pig population. Meadus et al. (2002) cited that the prevalence of this gene has increased in retail pork chops that are majorly supplied by the large composite breeds. This claim is supported by the results of this study as there were non-Hampshire samples found to be positive for the AMC. The non-Hampshire breeds found to be positive for the AMC were the Pietrain and Landrace. Pietrain breed had the highest incidence as out of 98 samples tested, 11 (11.22%) were found to be positive. While the Landrace breed had three positive samples two carriers out of 121 samples tested (Tables 1 and 2).

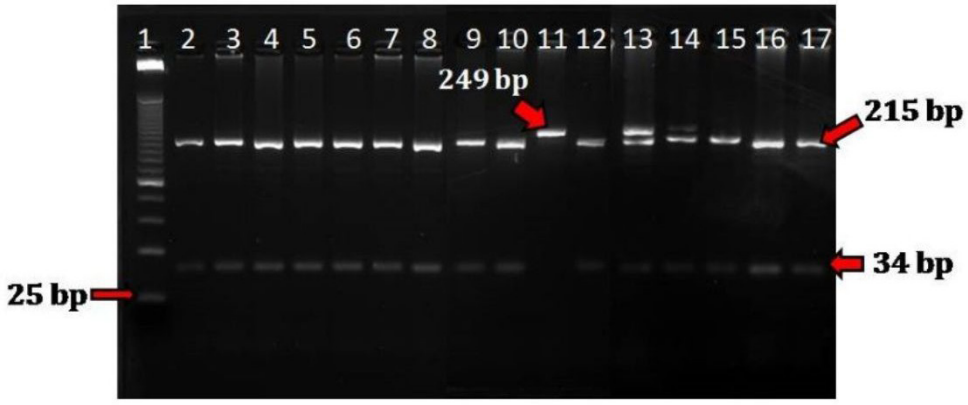

Figure 2. Agarose gel electrophoresis of PCR-RFLP of the *PRKAG3* gene; lane 1= 25 bp ladder; Lanes 2–10, 12, 15, 16, and 17= normal with 215bp and 34 bp; lane 11= positive with uncut 249 bp; lanes 13 and 14= Carriers with 249 bp, 215 bp, and 34 bp.

	1810				1860
PRKAG3 Gene (AF214521)	CAAGAAGGCC	TTCTTTGCCC	TGGTGGCCAA	CGGCGTCCGA	GCGGCACCTT
Sample 1 (Assembled)	CAAGAAGGCC	TTCTTTGCCC	TGGTGGCCAA	CGGCGTCCGA	GCGGCACCTT
Sample 2 (Assembled)	CAAGAAGGCC	TTCTTTGCTT	TGGTGGCCAA	CGGCATCCGA	GCGGCACCTT
Sample 3 (Assembled)	CAAGAAGGCC	TTCTTTGCCC	TGGTGGCCAA	CGGCGTCCGA	GCGGCACCTT
Sample 4 (Assembled)	CAAGAAGGCC	TTCTTTGCTT	TGGTGGCCAA	CGGCATCCGA	GCGGCACCTT
Sample 5 (Assembled)	CAAGAAGGCC	TTCTTTGCCC	TGGTGGCCAA	CGGCGTCCAA	GCGGCACCTT
Sample 6 (Assembled)	CAAGAAGGCC	TTCTTTGCCC	TGGTGGCCAA	CGGCGTCCAA	GCGGCACCTT
Sample 7 (Assembled)	CAAGAAGGCC	TTCTTTGCTT	TGGTGGCCAA	CGGCATCCAA	GCGGCACCTT
Sample 8 (Assembled)	CAAGAAGGCC	TTCTTTGCTT	TGGTGGCCAA	CGGCGTCCAA	GCGGCACCTT

Figure 3. DNA alignment of the eight assembled nucleotide sequences. Samples 5, 6, 7, and 8 which were positive samples (RN/RN) showed the G->A substitution in the 1849th base of the *PRKAG3* gene.

Table 1. Incidence of positive to acid meat condition among different breeds.

Breed	No. of samples	Positive	Incidence (%)
Pietrain	98	11	11.22
Landrace	121	3	2.48
Large White	207	0	0
Duroc	28	0	0
Chester White	81	0	0

Table 2. Incidence of carrier of acid meat condition among different breeds.

Breed	No. of samples	Carrier	Incidence (%)
Pietrain	98	0	0
Landrace	121	2	1.65
Large White	207	0	0
Duroc	28	0	0
Chester White	81	0	0

As stated in the study conducted by Meadus et al. (2002), the AMC is due to the G-A mutation in the 1849th base from the ATG start codon of the *PRKAG3* gene. All of the sequenced samples coincided with the RFLP results for the AMC. The DNA sequence confirmed the mutated genotypes of four samples among the total eight samples sent for sequencing. This can be seen by the G -> A point mutation on the 1849th base of the *PRKAG3* gene (Figure 3).

Although the preliminary studies observed the AMC in the Hampshire breeds and crossbred from Hampshire swine (Ellis et al. 1999), it is not only limited to this breed. Single nucleotide polymorphism or point mutations might have also occurred in different breeds as their way of adapting to the changing environment. The incidence of the AMC, though of low frequency, indicates the presence of the condition in the non-Hampshire swine population tested in the Philippines, showing the importance of conducting genetic screening in other populations. The establishment of a genetic screening for acid meat should be utilized by all the private swine breeders in the Philippines to fully eliminate the negative gene in the population.

CONCLUSION

The study was able to establish a genetic screening method for acid meat condition in the Philippines which will prevent the transfer of the undesirable genes to future generations. Out of 535 commercial breeder pigs, 519 (97.01%) did not have the mutation in their RN gene, while 16 (2.69%) had at least one copy of the defective allele in their gene. As the acid meat condition has only been previously detected in the Hampshire breed, it is also important to note that in this study, mutations were predominantly found in the Pietrain and Landrace breeds. The genetic screening applied in the study should be used to remove the presence of the mutated gene in the swine breeding population.

ACKNOWLEDGEMENT

We thank Dr. Arnel N. Del Barrio, PCC Executive Director, for the support to finish the study. Thanks to the Department of Science and Technology-Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development (DOST-PCAARRD) for the research grant under the Swine Genomics Project 2 and for monitoring the progress of this research. Also, special thanks to all the staff of the Biosafety and Environment Section of PCC for their technical support.

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