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Genetic Screening of Halothane Gene on Selected Philippine Native Pig Herds

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Abstract

The establishment of nucleus herds (NHs) of Native Pigs (NPs) at various R&D stations in the Philippines is currently being undertaken for food security and genetic conservation advocacy. Marker-assisted selection (MAS) is being utilized to identify individuals carrying favorable alleles of genes associated with production traits and screen out genetic defects (GD) for breeding purposes. Porcine Stress Syndrome (PSS) caused by a mutation in Halothane (HAL) gene is a GD frequently found in commercial breeds that when expressed, causes pale, soft, exudative (PSE) meat. PSE is inferior quality meat undesirable in the market causing economic losses to the swine industry. Thus, this study was conducted to screen the HAL gene through mutagenically separated-polymerase chain reaction (MSPCR) in selected NP herds and assessed its repeatability in local breeds. Results showed that out of 577 screened individuals, 543 (94.11%) were normal (NN), 0 (0%) were homozygous mutant (nn) and 34 (5.89%) were heterozygous carriers (Nn). Therefore, the optimized PSS screening protocol using MSPCR is also applicable to local breeds. As such, the availability of genetic tests for PSS could be useful in improving the Philippine NPs breeding selection and inhibiting or eliminating PSS mutant incidence within its nucleus herd.

Keywords

Halothane Gene, Porcine Stress Syndrome, Native Pig

1. Introduction

Despite the setbacks and threats imposed by the African Swine Fever (ASF), efforts in establishing nucleus herds of Native Pigs (NPs) are being undertaken at various R&D stations in the Philippines. NPs are known for their valuable traits of being adaptable and resilient to sudden environmental changes. Also, unlike

commercial breeds, NPs only require inexpensive housing facilities, with minimal care, and can be fed with leftovers, vegetable scraps, and plant foliage [1]. Thus, the program encourages the repopulation of this species to promote their market, alleviate food security and strengthen their genetic conservation.

However, like any livestock industry, it faces challenges that can impact productivity and sustainability. One such challenge is the Porcine Stress Syndrome (PSS) also known as Malignant Hyperthermia (MH) or Halothane Stress Mutation (HSM). PSS is an autosomal recessive disorder that occurred in porcine animals [2]. PSS is caused by a mutation in a single gene called Ryanodine Receptor 1 (RYR1) or Halothane (HAL) Gene.

The porcine RYR1 locus was localized on chromosome 6p11-q21 with substitution occurrence at position 1843 (C/T) which corresponds to Arginine-Cysteine shifting at position 615 during translation [3]. The RYR1 gene is responsible for channeling the release of calcium ions (Ca^{2+}) stored and regulated by the sarcoplasmic reticulum (SR) of the skeletal muscle [4]. These Ca^{2+} were being utilized during muscle contraction, relaxation, and energy metabolism [3]. Mistranslated amino acids as a consequence of polymorphism cause RYR1 dysfunction which opens and inhibits the closing of the RYR1 channel resulting in the continuous outflow of Ca^{2+} from the SR altering the homeostasis of ions within cells and intracellular [3]. Therefore, exposure to stressors and volatile anesthetics like halothane triggered the onset of PSS.

The first incidence of PSS mutation was recorded in Belgium in Pietrain Breed through intensive selection and subsequent testing [5] [6] [7]. During the period of 1950, the demand for lean and muscled meat was very high despite the poor quality [5] [6]. A noticeable speed and prevalence of the spread worldwide were attributed to the pyramid structural scheme of the modern swine industry wherein the genetics from the small proportion of the population amplifies to a larger proportion due to the rapid national and international exchange of breeding stock [5]. Thus, without the proper screening and management, the disorder can spread through breeding lines continuously, leading to reduced productivity, compromised meat quality, and increased economic losses for pig farmers.

Hence, genetic screening serves as a powerful tool to identify carriers of the HAL gene and safeguard the breed's valuable traits. By employing modern genetic techniques, breeders can identify and eliminate carriers from their breeding programs, reducing the risk of passing on the defective gene to the next generation. Selective breeding based on genetic screening allows breeders to focus on preserving the positive attributes of the pig breed while mitigating the risk of PSS. Also, prioritizing the health and well-being of pigs through genetic screening demonstrates a commitment to responsible animal husbandry practices and sustainable development in the swine breeding sector. As such, guided the study of Manalaysay, *et al.*, (2014) [8] in optimizing a screening protocol of PSS in the Philippine context, and it successfully screened PSS incidence in several commercial breeds like Pietrain, Landrace, Large White, Duroc, and Chester White.

However, the study was limited to commercial breeds only leaving the Philippine Native pig still unscreened with this genetic condition. Meaning, the livelihood of countless farmers and backyard raisers who depend on this integral part of Philippine agriculture is still in jeopardy. So, the application of such technology to the local herd could minimize economic losses, ensure the breed's resilience, and secure livelihood for the raiser folks.

Therefore, this study was conducted to screen the prevalence of the PSS in Philippine native pig herds by determining and calculating the allelic and genotypic frequency of PSS variants each and within samples. And, to test the repeatability and application of the optimized PSS-screening protocol of Manalaysay, *et al.*, (2014) [8] to the local breed.

2. Materials and Methods

2.1. Ethical Statement

The study performed blood and tissue collection of pig samples for HAL gene screening. The restraining procedure and materials used were decorous. Protocols implemented were based on PCC ethical guidelines of the Animal Care and Use Program adapted from Department of Agriculture Administrative Order No. 40, Series of 1999, "Rules and Regulations on the Conduct of Scientific Procedures using Animals". Ethics approval was under the code BG-190001-ROG. Also, the PCC-National Water Buffalo Gene Pool is a registered facility recognized by DA-RFO III and the Bureau of Animal Industry pursuant to the provisions of the Republic Act 8485 otherwise known as the Animal Welfare Act of 1998, as amended by Republic Act 10631.

2.2. Sample Collection

Samples were collected from the different native pig herds inter-island of the Philippines. Stratified random sampling was applied per herd and each were proportionally represented by subsamples. The housing facility for the animals was mixed type consisting of concrete, metal, and bamboo house pen and some farms practice the free range type of raising. The herds are well-ventilated with the free-flowing air within the area. The feeding programs of the animals were mixed with commercial feeds and organic feeding materials like cut and carry grasses and vegetable scraps. The water system in each herd was a combination of deep-well type, barn-fed watering system, and rainwater-fed system.

Samples were collected in the form of blood and hair follicle. A portable and adjustable animal crate was used to isolate the animal from the herd and to hold them for collection. Using forceps, hair follicles were plucked and put in a labeled skirted 5 ml vials. Blood samples, on the other hand, were collected by lifting the head of the animals and performing venipuncture (the needle was punctured at the jugular vein of the animal). The collection kit was composed of a 21 G × 1 1/2" disposable venous blood collection needle, a holder, and a blood collection tube. The tube contains EDTA which acts as an anticoagulant. All

samples collected were kept in a cooler with 5 - 10 pieces of frozen ice packs and then immediately transferred in refrigerated condition with 2˚C - 8˚C upon arrival at the laboratory or field station.

2.3. DNA Extraction

The genomic extraction of tissue and blood samples was performed using the respective optimized extraction protocol in the Molecular Genetics laboratory. Extraction procedures used were indicated in the kits with minor modifications. For the extraction of tissue samples, QIAGEN, DNeasy Blood and Tissue Kit was utilized. A 5 - 10 pcs hair follicles were cut into pieces and placed in 1.5 microcentrifuge tubes (MCT). Buffer ATL and proteinase K were added and mixed via vortexing. It was incubated at 56˚C until it was completely lyzed. Occasional vortexing was performed. Buffer AL was also added and mixed by vortexing. Afterwards, it was supplemented with ethanol and subjected to another mixing. To remove the debris from the tissue and to isolate the genomic DNA, a DNeasy mini spin column was used. Buffer AW1 and AW2 were utilized to wash the sample thoroughly. For the final elution, the spin columns were transferred to a 1.5 ml MCT. Buffer AE was used to elute the DNA and it was incubated for 1 minute at room temperature and then stored at 2˚C - 8˚C.

Conversely, the extraction of blood samples was done using the Promega Wizard Genomic DNA Purification Kit with minor modifications. In a 2 ml MCT, 500 µl of homogenized blood samples were transferred. Ammonium chloride (N^4Cl) was used to wash and pool white blood cells (WBC) from the samples. Cell lysis, nuclei lysis, and protein precipitation were used to isolate the genomic DNA from the WBC. After centrifugation, the supernatant from the mix was transferred to another MCT. Isopropanol was added and mixed by inversion. It was stored overnight and subjected to another centrifugation. For the final washing, 70% ethanol was used and then air-dried inside the laminar flow hood. The DNA samples were eluted using 50 µl of DNA rehydration solution. Tapped the tube to mix the solution then stored at 2˚C - 8˚C.

2.4. Mutagenically Separated Polymerase Chain Reaction and Gel Documentation

Amplification of the HAL gene was done using mutagenically separated-polymerase chain reaction (MS-PCR). The PCR mix and conditions were based on the optimized protocol of Manalaysay, *et al.* (2014). The PCR products were verified using 3.5% agarose gel (in 100 ml of TAE add 3.5 g Pronadisa Agarose D1 Low EEO and 3.5 µl Gel Red Biotium Solution) and electrophoresed using Mupid-exU submarine type electrophoresis system. Gel results were viewed using Enduro™ GDS Gel Documentation System. Genotype scoring was performed using standard and manual computation.

2.5. Statistical Analysis

For the statistical Analysis of this study, a Completely Randomize Design was

used and descriptive analysis was done. Hardy-Weinberg Equilibrium Equation was used to determine the Allelic frequency among samples. Genotypic frequency was also computed.

Hardy-Weinberg Equilibrium

$$p+q=1$$

$$1=p^2+2pq+q^2$$

p is the frequency of the dominant allele,

q is the frequency of recessive allele,

p^2 is the frequency of individuals with the homozygous dominant genotype,

$2pq$ is the frequency of individuals with the heterozygous genotype,

q^2 is the frequency of individuals with the homozygous recessive genotype.

$$\text{Genotypic Frequency} = \frac{\text{Number of genotype}}{\text{Total Population}} \times 100$$

3. Results and Discussion

This HAL gene has two possible alleles, the dominant N allele, and the recessive n allele. These variants are located in a single locus and could transcribe into three possible genotypes which are homozygous normal (NN), heterozygous carrier (Nn), and homozygous mutant/positive (nn) [4]. A total of ten (10) sampling sites were coordinated for the sample collection of this study. There were five hundred seventy-seven (577) samples collected and it consists of 285 sows, 158 boars, and 134 unknown sexes. Genomic DNA was extracted and genotyped using MSPCR. There were three genotypes determined using the HAL gene, the NN (normal), Nn (carrier), and the nn (mutant) with sizes 114 bp, 114 and 134 bp, and 134 bp respectively (**Figure 1**). While, genotypic and allelic frequency were illustrated in **Figure 2**.

Incidence genotypes and genotypic frequency among herds were calculated and tabulated in **Table 1**. Out of 577 samples, there were 34 carriers (Nn), zero occurrences of mutant (nn), and the rest were normal (NN) with 543 heads. It was shown that Farms 2, 3, 4, 9, and 10 were no incidence of PSS due to zero occurrence of the n allele among the screened samples. While Farm 1, 5, 6, 7, and 8 screened to have a single copy of the n allele among their samples. Noticeably, Farm 7 has the highest incidence of the PSS variants followed by Farm 5, 6, 1, and 8 (**Table 1**). Moreover, the incidence of genotypes with regards to sex in each farms were also determined (**Table 2**). The total incidence among sows and boars within samples where 17 and 16 heads, respectively.

These findings suggest that certain pig farms may have a higher likelihood of producing PSS mutant variant due to higher frequency of the Nn genotype across sexes which imply that both are potential carrier and contributor to the transmission of the mutant allele.

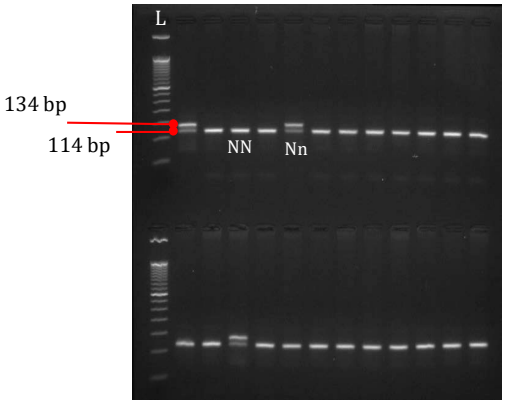
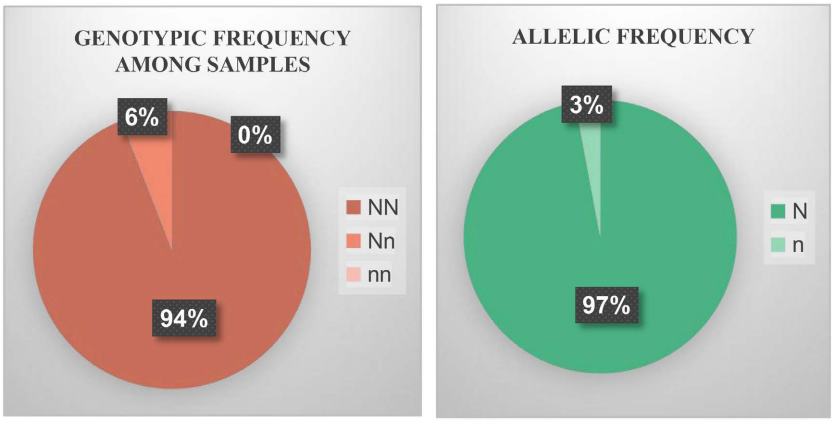


Figure 1. MSPCR gel products of samples having normal (NN) and carrier (Nn) genotypes with 114 bp, 114 and 134 bp sizes respectively.



(a) (b)

Figure 2. (a) Genotypic frequency among samples; (b) Allelic frequency.

Table 1. Porcine Stress Syndrome incidence among selected Native Pigs Herd.

FARM NO.	N	NORMAL	INCIDENCE %	CARRIER	INCIDENCE %	POSITIVE	INCIDENCE %
1	105	98	93.33	7	6.67	0	0
2	4	4	100.00	0	0.00	0	0
3	12	12	100.00	0	0.00	0	0
4	51	51	100.00	0	0.00	0	0
5	28	25	89.29	3	10.71	0	0
6	105	96	91.43	9	8.57	0	0
7	96	82	85.42	14	14.58	0	0
8	115	114	99.13	1	0.87	0	0
9	28	28	100.00	0	0.00	0	0
10	33	33	100.00	0	0.00	0	0
TOTAL	**577**	**543**	**94.11**	**34**	**5.89**	**0**	**0**

Table 2. Porcine Stress Syndrome Incidence among selected Native Pigs Herd with regards to sex.

FARM NO.	SEX	n	NORMAL	INCIDENCE %	CARRIER	INCIDENCE %	POSITIVE	INCIDENCE %
	F	63	57	90.48	6	10.53	0	0
1	M	32	31	96.88	1	3.23	0	0
	UNKNOWN	10	10	100.00	0	0.00	0	0
2	UNKNOWN	4	4	100.00	0	0	0	0
3	UNKNOWN	12	12	100.00	0	0	0	0
4	UNKNOWN	51	51	100.00	0	0	0	0
	F	17	17	100.00	0	0.00	0	0
5	M	10	7	70.00	3	30.00	0	0
	UNKNOWN	1	1	100.00	0	0	0	0
6	F	60	57	95.00	3	5.00	0	0
	M	45	39	86.67	6	13.33	0	0
	F	33	26	78.79	7	21.21	0	0
7	M	23	17	73.91	6	26.09	0	0
	UNKNOWN	40	39	97.50	1	0	0	0
	F	80	79	98.75	1	1.25	0	0
8	M	33	33	100.00	0	0.00	0	0
	UNKNOWN	2	2	100.00	0	0	0	0
	F	5	5	100.00	0	0.00	0	0
9	M	9	9	100.00	0	0.00	0	0
	UNKNOWN	14	14	100.00	0	0	0	0
10	F	27	27	100.00	0	0.00	0	0
	M	6	6	100.00	0	0.00	0	0
N = 577			**FEMALE = 285**		**MALE = 158**		**UNKNOWN = 134**	

In general, prompting genetic defects carrier into the population is sometimes subjected to culling because it has a perceivable threat to the entirety of the population. However, in the case of HAL gene mutation, it has a binary outturn that gives options to raisers on whether to utilize or eliminate the PSS variant. It is given that the presence of the single copy of the n allele is associated with meat quality such as lean, heavy muscling, and increased growth efficiency [5] [9] which are advantageous traits to raisers. While PSS-mutant (nn) could lead to deleterious effects such as stress-susceptibility producing poor quality meat [5] [6] [10] that may have caused significant losses. Providentially, this negative outturn is highly variable and could be modified by environmental and management factors [5] [6].

Indeed, the identification of HAL gene mutation causing PSS in local breeds has several potential impacts and implications for breeding practices and genetic conservation efforts. Understanding alleles and genotypes within the population

allows us to realize the genetic diversity of the animals which is crucial for apprehending the genetic health and potential risks associated with excessive inbreeding.

Additionally, this information serves as primary guidelines for implementing selective breeding strategies which could be achieved through careful selection and systematic mating plans for homozygous normal individuals (NN) and carriers (Nn). Somehow, throughput permit breeders to reduce, manage and prevent PSS-related issues. Likewise, farms with a higher incidence of carriers might implement specific management practices to mitigate the risks associated with the dysfunction.

Also, this provides an opportunity to inform and educate swine breeders about the importance of responsible breeding practices, the consequences of genomic variabilities, and the potential risk associated with PSS. Acquired knowledge through this study may lead to a more informative and concrete basis for decision-making among breeders.

Overall, the identification of PSS variants in the local breed and its incorporation into breeding and conservation practices can contribute to the long-term sustainability and health of the population.

4. Conclusion

Therefore, findings imply the presence of a single copy of the n allele in Philippine native pigs. This may suggest the prevalence of mutant genotype when neglected. Also, the results emphasize the significance of the study as the first recorded incidence of PSS variants within the population. Indeed, the optimized PSS screening protocol of Manalaysay, *et al.* (2014) [8] has been tested and repeated for the result's accuracy and reliability to local breed. This signifies effective screening protocol for HAL gene across breeds which could be a basis of animal retention and culling within breeding programs.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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