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Genotyping and molecular detection of polymorphism in FUT1 gene of swine

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Abstract: Alpha (1, 2)-fucosyltransferase (FUT1), as a candidate gene in controlling the expression of *Escherichia coli* F18 receptor has been identified to determine whether an animal (i.e., *Sus scrofa*, swine) is resistant or susceptible to enterotoxigenic *E. coli* (ETEC) infections. This study was conducted to determine the genotypes of 150 blood samples of three swine breeds. From these, 20 individuals were randomly selected for sequencing. Polymerase chain reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) results revealed that among the genotypes, seven of the Duroc x Pietrain samples carried the AG genotype which was previously reported to be susceptible to ETEC infection. Two AA genotypes were presumptively resistant and 50 that were presumptively susceptible, as these samples carried either AG or GG genotypes from the Landrace variety. Of the Large White samples, two samples carried the AA genotype and 89 had either AG or GG genotypes. Allelic frequency of allele A was 0.273, while, and allele G was 0.726. The DNA sequences registered 100% homologies to the *S. scrofa* FUT1 gene. Twelve of the sequenced samples exhibited a shift from G to A in the 117th nucleotide and one sample had a C to T shift in the 39th nucleotide. A change in the protein was observed from alanine to threonine at the 117th nucleotide position indicating a functional mutation. The FUT1 mutation was found in all the Duroc X Pietrain samples, as well as in samples with the AA genotype (Large White samples). Heterozygous forms of Landrace and Large White also exhibited this mutation. The constructed phylogenetic tree revealed 2 groupings based on the mutations at the 117th nucleotide.
Keywords: *ETEC* F18, FUT1 mutation, PCR-RFLP.

1. Introduction

Enteric pathogens can cause infection and disease depending on the type of immune responses they elicit and how vaccination may protect against infection and disease. The different bacterial enteropathogens can cause disease by: (a) colonizing the intestinal mucosa without invasion or morphological damage but eliciting diarrhea using powerful secreted enterotoxins by Enterotoxigenic *Escherichia coli* (ETEC); (b) adhering to the mucosa and inducing enterocyte effacement by "injecting" bacterial proteins into the epithelial cells (e.g., enteropathogenic *E. coli*); (c) inducing enterocyte killing by producing powerful exotoxins that block cellular protein synthesis (Holmgren and Lundgren, 2018).

ETEC infection-induced post-weaning diarrhea is one of the leading causes of morbidity and mortality in newly-weaned pigs and one of the significant drivers for antimicrobial use in swine production (Kim et al., 2022).In pigs, the alpha-(1,2) fucosyltransferase (*FUT1*) gene has been highlighted for its properties in controlling the intestinal expression of ETEC F18 receptors; a pathogen causing edema disease and post-weaning diarrhea. In this study, we hypothesized that pigs with different genotypes ETEC F18 resistant (AA) versus susceptible (AG/GG) differed in the following systemic and enteric responses: growth performance, plasma metabolic profiles, expression of candidate genes for intestinal mucosal homeostasis and immunity, number of selected bacteria and the concentration of short-chain fatty acids (SCFA) in feces and digesta in piglets pre- and post-weaning, and on the ETEC F18 adherence ex vivo (Poulsen et al., 2018).

Associations of the FUT1 gene with disease resistance can affect animal productivity and be breed-specific (Vashchenko et al., 2019). However, the problem of the influence of resistance-related genes on the productivity of pigs has not been sufficiently studied. Susceptibility to infectious diseases depends more or less on the genetic component of the animals. Infectious diseases are difficult to cure and cause significant economic losses in livestock species. Selective breeding is used to increase resistance against infectious disease and it may prove to be an economical and sustainable practice. Genetic methods, such as the selection of disease resistance in the pig, have not been widely used (Devi et al., 2018).

Hog raising is a lucrative business prevalent in the Philippines because everyone can start even in their backyards. Based on the data from the Philippine Statistics Authority (PSA) (2023), the country's total swine inventory was estimated at 10.18 million heads. Aside from the economic gains, pigs are also the main source of various food products in the Philippines. With the increasing population rate of 113 million (PSA, 2023), the country's demand for pork products also increases. With this, the production of good quality products must be taken seriously as they may cause the spread of food-borne diseases. The significance of detecting and analyzing polymorphisms in the genotypes of the involved genes is to predetermine the health of swine for livestock and for the breeder's industry. Moreover, these studies encourage the breeding of disease-resistant piglets. In this study, Polymerase Chain

Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) tests were undertaken in three pig breeds to determine the genotypes and to detect polymorphism by DNA sequencing.

2. Materials and Methods

2.1. Blood Sample Collection

The experimental samples comprised 150 swine blood collected from breeder farms in Luzon Island, the Philippines. Samples were stored in vacutainer tubes treated with Tris-EDTA buffer to prevent coagulation of the blood and refrigerated at 4ºC for further use. Genomic DNA was extracted using a standard DNA protocol following the manufacturer's instructions (Wizard Genomic DNA Extraction Kit Promega, Madison, WI, USA). Afterward, β-actin confirmatory test was done to ensure and confirm whether the extraction protocol was successful through the gene amplification after PCR.

2.2. PCR

Forward 5'-GTG CAT GGC AGG CTG GAT GA-3' and Reverse 5'-CCA ACG CCT CCG ATT CCT GT-3' primer sequences adapted from Bao et al. (2012) were used to initiate the amplification of genomic DNA. In a total reaction volume of 10 µl, the following volume of components was used: 4.4 µl of Sterile Double Distilled H_2O, 2.0 µl PCR Buffer, 1.0 µl Mg_2Cl, and 0.5 µl each of forward and reverse primers, 0.1 µl *Taq* DNA Polymerase, 0.5 µl dNTP and 1 µl DNA template. DNA and components were subjected to the following cycling conditions: Pre-denaturation at 94°C for 5 min followed by denaturation at 94°C for 30 sec. To allow annealing of primers, the temperature was adjusted at 60°C for 30 sec. An increase in temperature to 72°C for 30 sec was used to allow elongation, followed by a final elongation step at 72°C for 5 min. The amplified PCR products were separated based on their sizes using gel electrophoresis. Two percent agarose gel with TAE (Tris-Acetate EDTA) buffer was prepared as the medium with supplementary 0.8 µl of Gel Red Biotium™ to allow visualization under a UV transilluminator (FluorChem E, Santa Clara, CA, USA). Electrophoresis was initiated at a constant of 110 V for 30 min. Thereafter, the gel was viewed under a UV transilluminator to confirm the resulting products.

2.3. PCR-RFLP

PCR-RFLP was used to digest genomic DNA and separate the fragments based on their length. Specific restriction endonuclease *BstHHI* was used to digest the genomes. Samples were incubated in a 37°C water bath for 2 h before digestion. The following volume of reagents was used for PCR-RFLP: 2.85 µl sterile distilled water, 0.1 µl 10X Buffer, 0.05 µl restriction enzyme *BstHHI*, and 2 µl PCR product in a total reaction volume of 5 µl. Products were analyzed using 4% agarose gel at 110 V for 40 min. Gene fragments were stained with Gel Red Stain and visualized under a UV transilluminator.

2.4. DNA Sequencing and Screening of Polymorphisms in the FUT1 Gene

Twenty randomly selected PCR products were sent to the Philippine Genomic Center for the DNA sequencing at Quezon City, the Philippines. The results were analyzed using Mega6™ for the presence of polymorphisms in the FUT1 gene that causes susceptibility or resistance to *ETEC* F18.

2.5. Phylogenetic Analyses

The phylogenetic tree was constructed using the Tamura-3 parameter model and the Neighbor-joining method with 1000 bootstrap replicates (Tamura et al., 2013).

3. Results

3.1. PCR

Figure 1 shows 20 PCR samples. The PCR products were used for sequencing. A 100 bp ladder was used as a reference for the size of the amplified gene.

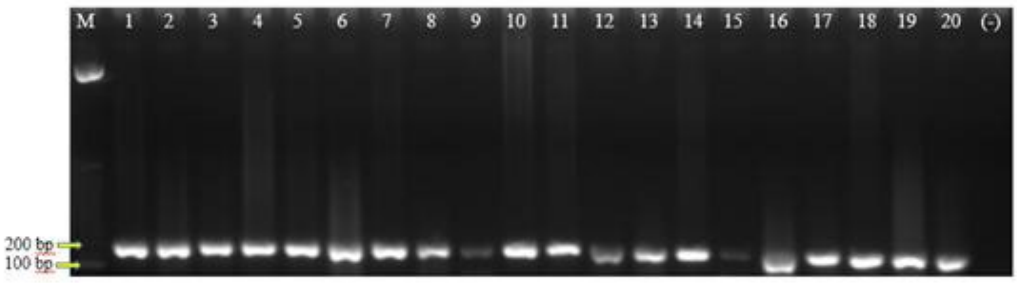

Figure 1 – Agarose gel electrophoresis of amplified FUT1 gene by PCR. Lane M: 100 bp; Lanes 1-20: Samples 1-20; (-): negative control.

3.2. Genotyping using RFLP

From the total blood samples 59.3, 34.7, and 6.0% were from Large-White, Landrace, and the Duroc x Pietrain cross, respectively. The restriction enzyme *BstHHI*, an isoschizomer of *Hin6* was used to cut DNA at or near the restriction site. Different fragments after the digestion discriminates the genotype of the samples. Results showed that seven Duroc x Pietrain were found carrying either AG or GG genotypes and two AA genotypes. On the other hand, 50 samples from the Landrace breed have either AG or GG genotype, the Large White breed had two AA genotypes, and 89 with either AG or GG genotype (Table 1). As shown in Figure 2, lanes 67, 68, 71, 72, 74, 75, and 76 had an AG genotype showing three bands after gel electrophoresis. Lanes 70, 79, and 80 had a genotype GG with two fragments. Moreover, lanes 69 and 73 had an AA genotype showing a single band on the gel. Allele frequencies of three breed types were computed using the Hardy-Weinberg model (Table 1).

	Genotype Frequency			Allele frequency		
Breed	Resistant	Susceptible		Resistant	Susceptible	Total
	AA	AG	GG	A	G	
Large White	2	34	55	0.208	0.79	91
Landrace	2	35	15	0.375	0.625	52
Duroc x Pietrain	0	5	2	0.357	0.642	7
Total	4	74	72	0.273	0.726	150

Table 1 – Genotype and allele frequencies of 150 samples of the three swine breeds using the restriction enzyme BstHHI.

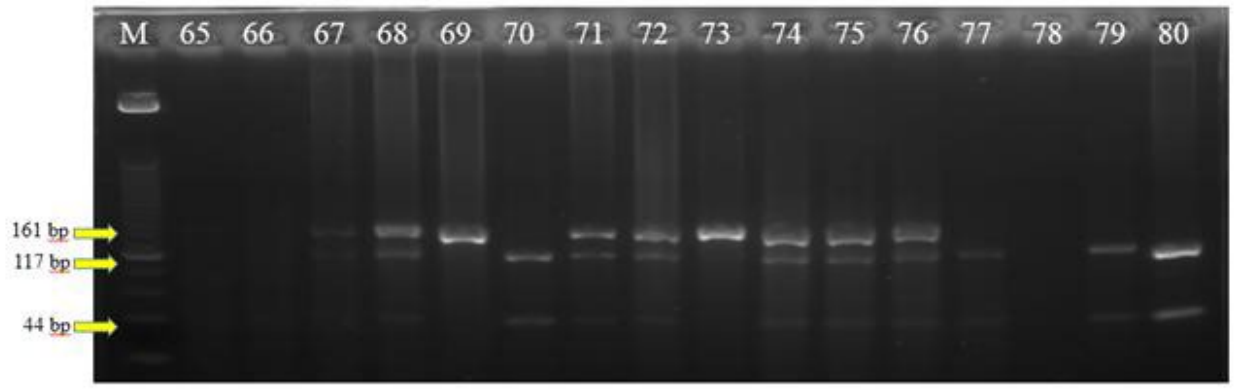

Figure 2 – Gel electrophoresis image of the PCR-RFLP samples 65-80, using a 25 bp ladder as a marker. Lanes 69 and 73 exhibit AAgenotypes; Lanes 70, 77, 79, and 80 exhibits GG genotypes, and Lanes 67-68, 71-72, and 74-76 exhibits AG genotypes.

3.3. Confirmation of DNA samples via Basic Local Alignment Search Tool (BLAST)

The online bioinformatics tool BLAST was used to search for regions of similarity between the sequences as well as to ensure that the sequences were really from a swine DNA. The data presented 100% similarity with the *S. scrofa* FUT1 gene available in the National Center for Biotechnology Information (NCBI, USA).

3.4. Detection of Polymorphism using DNA Sequencing

Mega6^{TM} software was used to assemble and align sequences. Based on the alignment, samples 5 and 10 to 20 showed polymorphism where a shift of G to A appeared at nucleotide 117. This particular polymorphism was not observed in sample 4, however, nucleotide C shifted to T located in the 39th nucleotide of the gene. These polymorphisms were noticed in heterozygous Landrace and Large-White breed, both heterozygous and homozygous GG Duroc x Pietrain, homozygous AA Landrace and Large, and homozygous GG Landrace respectively (Figure 3). A change in the protein was observed from alanine to threonine at the 117th nucleotide position which indicates a functional mutation (Figure 4).

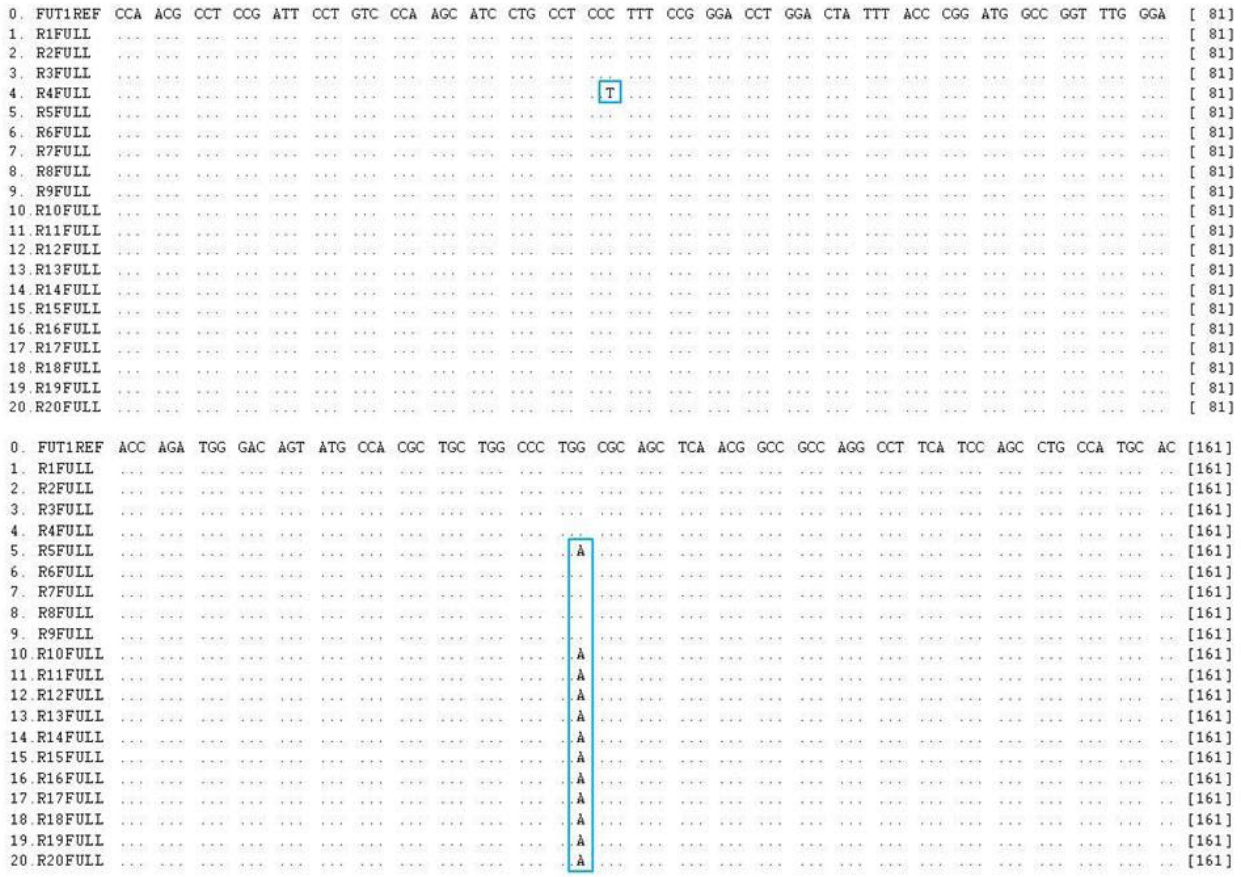

Figure 3 – Twenty sequenced samples using Mega6 show C to T shift in the 39th nucleotide of sample 4, and a G to A shift in the 117th nucleotide (samples 5, 10-20). The 0-sample labeled FUT1REF is the sequence of the FUT1 gene obtained from the NCBI to serve as the basis for polymorphisms. Polymorphisms are enclosed in a blue box.

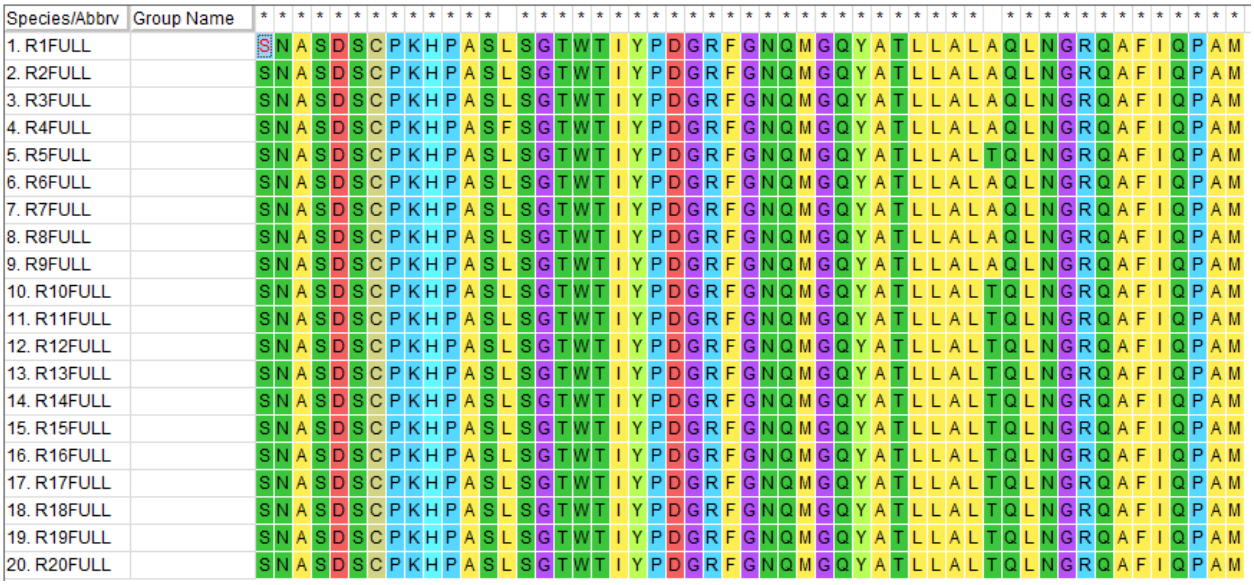

Figure 4 – A protein change coding for Alanine (A) to Threonine (T) in the 117th nucleotide position for samples 5, 10-20.

3.5. Phylogenetic Tree

The phylogenetic tree shows the 2 groups generated with the first group at 64% bootstrap value (Figure 5). This group comprised of samples 5, and 10 to 20 is consistent with the mutation observed at the 117th nucleotide shift from G to A. Moreover, the second group yielded a 69.0% bootstrap value. This group comprised samples 1 to 4, and 6 to 9 which had no mutation or polymorphisms. The outgroup with the accession number NM 177499.3 of *Bos taurus* species was obtained from NCBI.

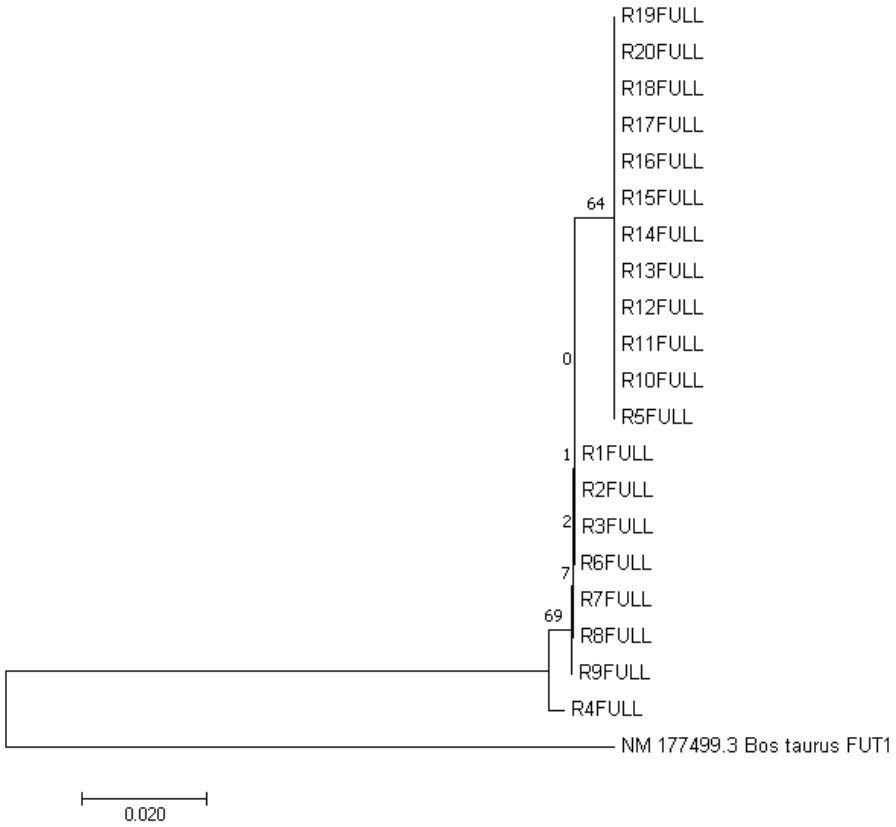

Figure 5 – Phylogenetic tree of 20 sequenced samples using Neighbor-joining method with 1000 bootstrap replicates.

4. Discussion

From the150 blood samples, 59.3 (n=91), 34.7 (n=52), and 6.0% (n=7) were from Large-White, Landrace, and Duroc x Pietrain, respectively. The very low genotype frequency for the resistant genotype AA indicates that commercial pig breeds in the Philippines are susceptible to ETEC-caused diarrhea. These results concur with previous studies were done by Petit et al. (2020), that the resistant A allele could have been lost during demographic bottlenecks. These bottlenecks are suspected to result from the associated genetic drift, in changes in the genetic composition including the loss of alleles. Another scenario can be attributed to the hypothesis that the A allele would have appeared and/or been selected in domestic pig populations following domestication. (Petit et al., 2020).

The FUT1 gene plays an important role in regulating the resistance of pigs to ETEC, expressing FUT1 associated with postweaning diarrhea (PWD) in piglets The variation of the FUT1 gene on the M307 locus is correlated with resistance to ETEC (Le et al., 2021). Also, it was found that the piglet survival rate of individuals with genotype AA was almost two times greater than that of GG individuals.

Also, the positive effect of the FUT1 AA genotype was observed in studies on Yorkshire pigs. The age of reaching 100 kg of AA genotype pigs was shorter by 4.23 days ($P< 0.05$) than that of animals with AG genotype, whereas back fat thickness and depth of the *longissimus dorsi* were similar. On the contrary, in another experiment on Yorkshire pigs (Luc et al., 2020), no significant effect of gene polymorphism on different types of productivity was found. In research, the body weights at birth, weaning, initial fattening period, and final fattening period were collected from 611, 516, 479, and 18 animals, respectively, whereas back fat thickness, depth of the *longissimus dorsi*, and lean meat percentage were recorded from 328 animals. The effect of the FUT1 genotype was not observed for all production traits ($P > 0.05$), whereas final body weight and depth of the *longissimus dorsi* were significantly different between females and males ($P <0.05$) (Sukhno et al., 2022).

FUT1 was used in marker-assisted selection (MAS) in a new pig population of Italian Large White pigs (Geraci et al., 2019). Therefore, can have a potential marker to improve post-weaning piglet survival (Le et al., 2021).

5. Conclusion

This study was able to obtain the genotype of 150 swine samples obtained from different breeding farms in Luzon using PCR-RFLP as well as detect polymorphisms in 20 samples in the FUT1 gene by nucleotide sequencing through PCR-RFLP, 146 of the samples were found to have either AG or GG genotype that are presumptively susceptible to ETEC infection, of which, 7, 50 and 89 were Duroc X Pietrain, Landrace and Large White, respectively. The four samples that carried AA genotypes that are presumptively resistant were from the Landrace and Large White. However, no AA genotype was found in Duroc X Pietrain. Twenty samples were randomly selected for sequencing. Afterward, the samples were subjected to BLAST to ensure that the sequences and genes used were really from swine (*S. scrofa*). Through molecular detection by nucleotide sequencing, the study was able to observe a shift from C to T and G to A in the 39th and 117th nucleotide, respectively. The mutation is present in heterozygous Landrace and Large White, both heterozygous and homozygous GG Duroc X Pietrain, and in the resistant Landrace and Large White. A very low frequency of A allele of 0.273 was found across 150 samples. Also, the phylogenetic tree yielded two groupings based on the observed mutation in the 117th nucleotide position. The detection of genotype and polymorphism in the FUT1 gene by RFLP and sequencing may be a useful tool in detecting PWD-resistant piglets that could be used as breeders, reducing disease incidence.

Compliance with Ethical Standards

The authors have no conflicts of interest to declare. All procedures and protocols used in this study were approved and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, Publication no. 85-23, revised 1996).

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