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EFFECTS OF *FUT1* AND *MUC4* POLYMORPHISMS ON SPERM QUALITY TRAITS OF LANDRACE AND YORKSHIRE PIGS UNDER TROPICAL CONDITIONS IN NORTHERN VIETNAM

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ABSTRACT

This study was aimed to determine allelic and genotypic frequencies of two single nucleotide polymorphisms (SNPs) located in alpha (1) fucosyltransferase (FUTI) gene and mucin 4 (MUC4) gene, and effects of these SNPs on sperm quality traits of Landrace and Yorkshire pigs. A total of 4771 ejaculates from 63 Landrace and 34 Yorkshire boars have been collected from May 2015 to July 2018 at nucleus breeding pig farm in Vietnam. The sperm quality traits were ejaculate volume, spermatozoa motility, sperm concentration and total number of spermatozoa in ejaculate. Hardy-Weinberg equilibrium was assessed using Fisher exact test. A mixed model was used to test the effects of fixed and random factors on the sperm traits. The fixed factors were genotype, breed, age of boar, month of the year, while boar ID number was included in the model as a random effect, allowing to properly model repeated measurements. These resistant genotypes were absent in Landrace. While the frequency of resistant genotypes AA for FUT1 (0.03) and GG for MUC4 (0.16) was relatively low in Yorkshire boar population. The only detected significant effect of FUT1 and MUC4 on semen quality traits had a positive effect of the resistant genotype GG of MUC4 on sperm motility (P = 0.015). The two SNPs located in FUT1 and MUC4 did not affect sperm traits except sperm motility. Consequently, the selection based on these resistant genotypes does not seem to negatively affect the monitored sperm traits.

Keywords: SNP, diarrhea resistant, semen, swine

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INTRODUCTION

The increase in the prevalence of diarrhea in pigs as well as the societal requirement for sustainable pork production have resulted in a change in selective breeding approaches in which disease resistance is incorporated in the selection objectives (Guy et al., 2012). In Vietnam, diarrhea in pigs caused by Escherichia coli is common and occurs very frequently under intensive conditions, leading to extreme economic losses in many farms (Hong et al., 2006). Marker-assisted selection based on candidate genes has proven an accurate and effective method that has been widely applied in the pig industry around the world in recent years (Wang et al., 2012). The alpha (1) Fucosytransferase (FUT1) gene and Mucin 4 (MUC4) gene have been well documented as candidate genes for enterotoxigenic E. coli resistance in pigs due to their association to resistance and susceptibility patterns of small intestinal epithelium to E. coli F18 adhesion

(Meijerink et al., 1997). In a study of Meijerink et al. (2000) on 56 pigs (35 Large White × Landrace, 6 Large White and 15 Duroc), sequencing of FUT1 gene of pigs resistant to E. coli F18 infection related to the c.307G>A mutation. Similarly, Peng et al. (2007) reported that the g.243A>G mutation in intron 17 of MUC4 is significantly associated with susceptibility/resistance to E. coli F4 infection in the White Duroc × Erhualian pigs. Thus for the selection of *E.coli* adhesion resistant animals, alleles A and G were considered as good markers respectively for FUT1 (Edfors and Torremorell, 2010) and MUC4 (Liu et al., 2015). In Vietnam, several recent studies on the polymorphisms of the FUT1 gene in exotic pig breeds (Landrace and Yorkshire) reported that three genotypes (AA, AG and GG) were observed although with a low frequency of the A allele (Cuong et al., 2012; Luc et al., 2020).

However, breeding for disease resistance in pigs could have negative effect on other economic production traits. Prunier *et al.* (2010) stated that the identification of

possible consequences on other traits is needed when selecting for a related health trait of the animals. Most recent studies have confirmed that there is an association between polymorphisms of *FUT1* and production and reproduction performance of pigs (Bao *et al.*, 2011; Zhu *et al.*, 2014; Luise *et al.*, 2019; Luc *et al.*, 2020). Additionally, polymorphisms of *MUC4* associated with reproduction traits of the Iberian × Meishan pigs (Balcells *et al.*, 2011) while did not affect production traits of Italian Large White pigs (Geraci *et al.*, 2019).

The effects of FUT1 and MUC4 on male fertility have been identified in some species. In mice, several authors have examined the significance of epididymal (1,2) fucosylation in fertility of animals. Millette et al. (1987) reported that fucosylated glycans were related to sperm maturation in the rodent. However, Domino et al. (2001) concluded that uterine epithelial (1,2) fucosylated glycans were dispensable for fertility, and thus there was no requirement for FUT1-dependent epididymal fucosylation events in the spermatozoa maturation process of mice. To date, limited information about the effects of FUT1 and MUC4 genotypes on boar sperm traits is available. Stoyanova et al. (2010) initially reported that boars with AA genotypes of FUT1 gene might have decreased sperm quality compared to other genotypes in Danube white pig herd. Recently, Zinnatova et al. (2014) also found a negative effect of genotype GG of FUT1 gene on sperm traits of Large White and Landrace boars. According to our knowledge, there have not been any publications about the effects of FUT1 and MUC4 polymorphisms on sperm quality of Landrace and Yorkshire breeds under the tropical climate conditions. Therefore, this study aims at determining the allelic and genotypic frequencies of two polymorphisms (SNPs) located in FUT1 and MUC4, and the effects of these polymorphisms on sperm traits in Landrace and Yorkshire boars under industrial conditions in Northern Vietnam. The outcomes of this study should enable us to avoid a possible deterioration of the semen quality when selecting preferred boars with the diarrhea-resistance genotypes.

MATERIALS AND METHODS

Experiment and Animals: The experiment was carried out on 97 boars (63 Landrace and 34 Yorkshire) between 7 months and 3 years of age at Dabaco Nucleus Breeding Pig Company, Bac Ninh province, Vietnam (40 km north from Hanoi) from May 2015 to July 2018. In the beginning of the performance testing period, the bodyweight of these animals was 38.21±5.65kg (±s.d.) at an age of 81.27±5.69 day. At the end of the testing period, the body weight reached 97.84±9,61kg at the age of 148.03±6.67 days. The average daily gain during the testing period (66.76±7.85 days) was 895.1 gram. These boars were born from 63 sires (38 Landrace and 25

Yorkshire) and 86 dames (55 Landrace 31, Yorkshire). Among 97 boars, there were 9 full-sib including 3 Landrace and 6 Yorkshire. Boars were kept individually in a single pen of 6.25 m² (2.5 m x 2.5 m) in a closed housing system with a cooling system (cooling pad and exhaust fan). The animals had free access to water by nipple drinkers. They were fed twice per day at 9.30 am and 4.00 pm. All industrial feeds were supplied by Dabaco Company. The feed rations were boar diets (16.5% protein, 3200 kcal ME). Vaccines against porcine reproductive and respiratory syndrome, foot and mouth disease, Aujeszky's disease, and pestivirus were used. Young boars were trained at 7 months of age before starting semen collection. The interval between consecutive collections was 4 to 7 days.

Genotype identification: Ear tissue samples from Landrace and Yorkshire boars were collected and stored at -20°C until genomic DNA was extracted. Genomic DNA was extracted following standard procedures (Sambrook et al., 1989). The polymorphisms c.307G>A (referred to as rs335979375 on position 6:54079650 in the last version Sscrofall.1 of the pig genome) located in FUT1 and g.243A>G located in (referred to as rs698037138 on position 13:134237729 in the last version Sscrofal1.1 of the pig genome) located in MUC4 were determined according to the methods of Meijerink et al. (1997) and Peng et al. (2007) respectively using the PCR-RFLP technique at Genetic laboratory of Vietnam National University of Agriculture. For SNP c.307G>A of FUT1, a 421 bp DNA fragment was amplified using primers: forward reverse F5'and CTTCAGCCAGGGCTCCTTTAAG-3' R5′-CTGCCTGAACGTCTATCAAGACC-3'. The fragments of 421 bp DNA were digested with the restriction enzyme Hin6I and identified genotyping by electrophoresis on 3% agarose gel. Two allele (A and G) and three genotypes (AA, AG and GG) were identified in two breeds. Allele A is characterized by fragments of 328 and 93bp, while allele G with a polymorphic restriction site is represented by fragments of 241, 93 and 87 bp. For SNP g.243A>G of MUC4, a 538 bp DNA fragment was amplified using forward and reverse primers: F5'-CAGGATGCCCAATGGCTCTAC-3' and CCCCGAAGTTGTGAAAGGAAG-3'. The fragments of 538 bp DNA were digested with the restriction enzyme HhaI and identified genotyping by electrophoresis on 3% agarose gel. Two allele (A and G) and three genotypes (AA, AG and GG) were identified in two breeds. Allele A is characterized by fragment of 538 bp, while allele G with a polymorphic restriction site is represented by fragments of 295 and 343 bp. A and G denote the resistance and susceptibility alleles respectively for FUT1 while, inversely, A and G stand for the susceptibility and resistance alleles respectively for MUC4.

Data collection: A total of 4771 ejaculates (3791 and 980 for Landrace and Yorkshire, respectively) were collected. The number of boars and ejaculates according to breeds (Landrace and Yorkshire) and genotypes (AA, AG and GG of FUT1 or MUC4) is detailed in Table 1. The sperm quality traits were ejaculate volume (ml), spermatozoa motility (%), sperm concentration (×10³/mm³), total number of motile spermatozoa in ejaculate (×10⁹ spz) and total number of spermatozoa in ejaculate (×10⁹ spz). All sperm traits were evaluated on the day of collection. Ejaculates were collected with the gloved hand method using dummy sow. The analysis of sperm

traits was carried out immediately after collection at the laboratory in the farm. Ejaculate volume after filtration of the gelatinous fraction was determined using a graduated measuring cylinder, sperm concentration and spermatozoa motility were estimated using Leja counting chambers with a computer-assisted sperm analyzer Ceros II Semen Analyzer (CASA, Hamilton Thorne CEROS Model, USA) according to guidelines of the supplier, total number of spermatozoa in ejaculate was calculated as the product of sperm concentration and ejaculate volume.

Table 1. Number of boars and ejaculates according to breeds (Landrace and Yorkshire) and genotypes (FUT1 and MUC4).

Itam		Landrace		`	Takal			
Item	AA	AG GG		AA	AG	GG	Total	
FUT1							•	
Number of boars	0	6	57	1	8	25	97	
Number of ejaculates	0	208	3583	18	358	604	4771	
MUC4								
Number of boars	59	0	0	5	22	5	91	
Number of ejaculates	3284	0	0	140	571	210	4205	

Statistical analyses: Hardy-Weinberg equilibrium was assessed using a Fisher exact test. For sperm traits, 12 statistical models were tested and the best fit model with the lowest Akaike information criterion (AIC) was selected as the final model. In all models, effect of FUT1 and MUC4 on sperm traits were treated separately. The final mixed model was used to test the effects on the sperm quality traits. The fixed factors were genotype (AA, AG and GG of FUT1 or MUC4), breed (Landrace and Yorkshire), age of boar $(1, 2 \text{ and } \ge 3 \text{ years})$, month of the year (from January to December), while boar ID number was included in the model as a random effect, allowing to properly model repeated measurements. Each ejaculate from the boar was considered as an observation. The between fixed interactions factors (breed*age, breed*month and month*age) were also included in the

model. The data were analyzed using SAS software version 9.3. The least-squares mean (LSM) and the standard error (SE) were estimated and LSM were compared using Tukey t-tests.

RESULTS

Genotype and allele frequencies of *FUT1* and *MUC4*: *FUT1* genotypes of 63 Landrace and 34 Yorkshire boars were identified, while *MUC4* genotypes could be obtained for 59 Landrace and 32 Yorkshire only. For *MUC4*, the genotypes of 6 boars could not be identified in the laboratory. The genotype and allele frequencies of *FUT1* and *MUC4* in Landrace and Yorkshire boars are presented in Table 2.

Table 2. Genotype and allele frequencies of FUT1 and MUC4 in Landrace and Yorkshire boars.

				Lan	drace	Yorkshire								
Genes	n	Genotype frequency			Allele frequency		P	n	Genotype frequency			Allele frequency		Р
		AA	AG	GG	A	G	•		AA	AG	GG	A	G	=
FUT1		0	0.09	0.91	0.05	0.95	1.00		0.03	0.24	0.73	0.15	0.85	1.00
	63	(0)	(6)	(57)				34	(1)	(8)	(25)			
MUC4		1.00	0	0	1.00	0	1.00		0.16	0.68	0.16	0.50	0.50	0.114
	59	(59)	(0)	(0)				32	(5)	(22)	(5)			

Numbers in parentheses indicate the number of boars; the column P provides the p-value when assuming Hardy-Weinberg equilibrium For FUT1, three genotypes (AA, AG, and GG) were observed in Yorkshire boars, while the AA genotype was absent from Landrace. The frequency of the susceptible allele (G) was much higher than that of disease resistant allele (A) in both breeds (0.95 and 0.85 for Landrace) and Yorkshire respectively).

For MUC4, the genotype distribution was also different between the two breeds. In Landrace, only AA genotype was found, while the AG and GG genotypes were absent from the sample. In Yorkshire, the three genotypes (AA, AG and GG) were present, and the AG was the most prevalent genotype (with a frequency of 0.68). The resistance allele (G) was relatively frequent in Yorkshire (50%). This is potentially helpful to select favorable boars carrying the disease resistance allele for the breeding purpose.

The genotype frequencies of FUT1 and MUC4

were in Hardy-Weinberg equilibrium for both breeds (all P>0.114).

Effects of FUT1 and MUC4 genotypes on sperm quality traits: A wide range of factors may influence the sperm quality traits of boars. Among these factors, the genotype, breed, age of animals, and months of the year were the most significant factors and were used and tested in the model. Tables 3 and 4 present the effects of FUT1 and MUC4 and other factors on sperm quality traits of Landrace and Yorkshire boars.

Table 3. Sperm quality traits according to effects of *FUT1* genotype, breed (B), age (A), month (M) and interaction (B*A, B*M and M*A).

Variable	AA (n=18) AG (n=566)			GG (n:	=4187)		Level of significance						
	LSM	SE	LSM	SE	LSM	SE	FUT1	Breed	Age	Month	B*A	B*M	M*A
VOL	239	62.2	247	16.4	270	7.43	0.419	0.023	<.001	<.001	0.012	0.478	0.002
MOT	90.7	2.59	88.7	0.65	88.3	0.32	0.539	0.014	0.022	<.001	0.863	0.013	<.001
CON	427	90.7	409	23.6	388	11.0	0.692	0.001	<.001	<.001	<.001	<.001	<.001
NT	102	22.0	94.9	5.71	99.5	2.66	0.762	0.365	<.001	<.001	<.001	0.014	0.001

Least squares means (LSM) and standard error (SE) of ejaculate volume (VOL, ml), spermatozoa (spz) motility (MOT, %), sperm concentration (CON, ×10³ spz/mm³) and total number of spz (NT, ×109)

Table 4. Sperm quality traits according to effects of *MUC4* genotype, breed (B), age (A), month (M) and interaction (B*A, B*M and M*A).

Variable	AA (n=3424)		AG (n=571)		GG (n=210)		Level of significance							
	LSM	SE	LSM	SE	LSM	SE	MUC4	Breed	Age	Month	B*A	B*M	M*A	
VOL	250	14.5	300	19.7	263	30.9	0.185	0.016	<.001	<.001	<.001	0.497	0.005	
MOT	88.9^{ab}	0.59	86.7^{b}	0.82	90.0^{a}	1.20	0.015	0.023	0.027	<.001	0.980	0.018	<.001	
CON	407	20.9	340	28.6	431	43.7	0.062	0.012	<.001	<.001	<.001	0.001	<.001	
NT	96.5	5.18	98.2	7.08	108	10.8	0.644	0.972	<.001	<.001	<.001	0.009	<.001	

Least squares means (LSM) and standard error (SE) of ejaculate volume (VOL, ml), spermatozoa (spz) motility (MOT, %), sperm concentration (CON, ×10³ spz/mm³) and total number of spz (NT, ×10⁵) Means with differing letters in each row differ (P<0.05)

No effect of FUT1 on all tested sperm quality parameters was found in Landrace and Yorkshire boars, while MUC4 gene had an effect on spermatozoa motility (P = 0.015). Note that this effect is not strong and might even vanish if we consider a correction for multiple testing. For FUT1, the number of animals carrying the AA genotype (that is resistant to ETEC E18) was very low in the studied populations (only one Yorkshire boar with 18 ejaculates), much less than those of AG and GG genotypes. With this low frequency of AA, the statistical power for the comparison of the genotypes is low. Since no genotype effect could be found on sperm quality, it means either that the breeding selection of the boars according to FUT1 does not have any (negative) impact on the sperm quality or that the power of the statistical test did not allow to unravel such an effect.

For MUC4, the GG (i.e. resistant) animals showed a higher spermatozoa motility than the two other genotypes (AA and AG), although only the difference

between the GG and AG animals was significant (P = 0.020). There were no significant differences for other sperm quality traits (volume, concentration, total number of spermatozoa, number of motile spermatozoa in ejaculate) among three genotypes (all P>0.05). This is of significance in breeding selection program since selection of the boars carrying the GG genotype could improve spermatozoa motility without negatively affecting other traits.

The effect of breed, age of boars and month of the year on sperm quality parameters of Landrace and Yorkshire are also provided in Tables 3 and 4. Breed significantly affects the ejaculate volume, spermatozoa motility, and sperm concentration (all P<0.05). Yorkshire boars had a lower ejaculate volume (Fig. 1A), but a higher spermatozoa motility (Fig. 1B) and sperm concentration (Fig. 1C) than Landrace.

When taking the age of the animal into account, we observed that age had a significant effect on all tested

sperm quality parameters (all P<0.05). In the first year, motility (Fig. 1B) and number of spermatozoa (Fig. 1D) of the boars were significantly lower (all P<0.05) than those in the second and third years while ejaculate volume was largest in the second year (Fig. 1A). Additionally, sperm concentration was highest in the third year (Fig. 1C). Furthermore, the sperm quality traits

were significantly different between months (P<0.001). Ejaculate volume (Fig. 2) and number of spermatozoa (Fig. 3) in the summer and autumn seasons (i.e. June to November) tended to be lower than in winter and spring seasons (December to May). The trend was similar for the sperm concentration (Fig. 2).

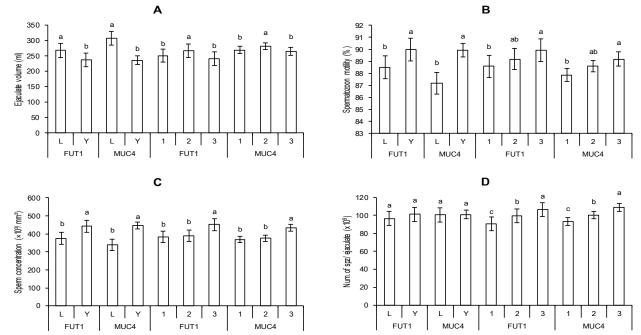


Fig. 1. Within a gene (FUT1 or MUC4), effect of breed (L: Landrace and Y: Yorkshire) or age (1, 2 and 3 years) on Ejaculate volume (A), Spermatozoon motility (B), Sperm concentration (C) and Number of spz in ejaculate (D)

Note: Within a gene (FUT1 or MUC4), bars with different letters differ significantly at P<0.05

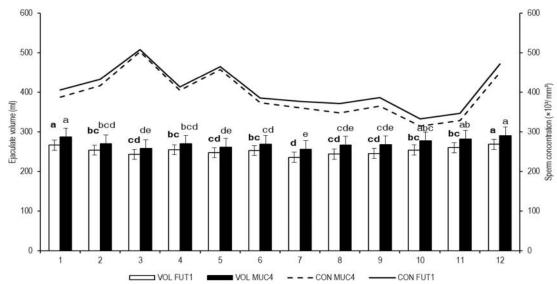


Fig. 2. Within a gene (FUT1 or MUC4), effect of month of the year on Ejaculate volume (VOL) and Sperm concentration (CON)

Note: Within a gene (FUT1 or MUC4), bars with different letters (**bold** and normal for FUT1 and MUC4 respectively) differ significantly at P<0.05

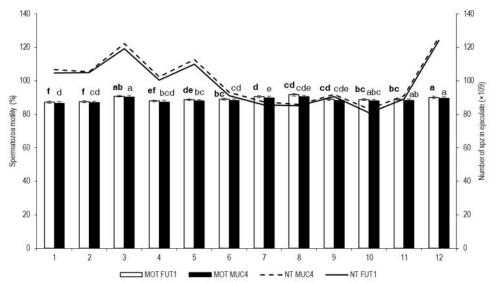


Fig. 3. Within a gene (*FUT1* or *MUC4*), effect of month of the year on Spermatozoa motility (MOT) and Number of spz in ejaculate (NT)

Note: Within a gene (FUT1 or MUC4), bars with different letters (bold and normal for FUT1 and MUC4 respectively) differ significantly at P<0.05

DISCUSSION

Genotype and allele frequencies of FUT1 and MUC4: The genotype frequency of FUT1 and MUC4 in Landrace and Yorkshire breeds have been repeatedly reported in recent studies (Luo et al., 2010; Cuong et al., 2012; Luc et al., 2020). For FUT1, most studies found a very low frequency of AA genotype in these two breeds. Meijerink et al. (1997) found a low frequency of AA genotype in Large White and Landrace. Luo et al. (2010) found a very low frequency of AA genotype in Landrace breed (0.02%). Cuong et al. (2012) and Luc et al. (2020) reported that the Yorkshire pig herds in Vietnam had a rather low frequency of AA genotype (13% and 0.98%, respectively). This low frequency in our population triggered a research question: why is the frequency of a favorable allele low in the population? It might be that the allele has been introduced only recently in our population. Therefore, it did not have sufficient time to reach higher frequency. This situation is favorable for future selection. Alternatively, previous selection has selected against this allele because it has an adverse effect on another trait.

In this study, frequency of the G allele of *MUC4* which indicates a resistance to Enterotoxigenic *E. coli*, was 0.5 in Yorkshire boars, but absent in Landrace. Fontanesi *et al.* (2012) reported that the frequency of susceptible allele was low in local Italian pigs (0.05 to 0.28), while close to 0.5 in Italian Large White and Italian Landrace. Joller (2009) indicated that a large fraction of Landrace and Large White boars carried the susceptibility allele (more than 70% out of 193 studied boars) in their study.

Effects of FUT1 and MUC4 genotypes on sperm quality traits: A recent survey on Danube white boar population detected that animals carrying AA genotype of FUT1 had a lower sperm quality, which could lead to the low frequency of A allele in that population (Stoyanova et al., 2010). Inversely, Zinnatova et al. (2014) reported that the ejaculate volume of AA boars was higher than that of AG and GG. The association between the FUT1 genotypes and the sperm quality has been identified not only in pigs, but also in other species of mammals (Millette et al., 1987; Domino et al., 2001). In mice, several authors have examined the significance of epididymal (1,2) fucosylation in fertility of animals. Millette et al. (1987) reported that fucosylated glycans were related to the sperm maturation in the rodent. However, Domino et al. (2001) concluded that uterine epithelial (1,2) fucosylated glycans were dispensable for fertility, and thus there was no requirement for FUT1dependent epididymal fucosylation events in the spermatozoa maturation process of mice. In our case, no significant effect of FUT1 on sperm quality parameters has been seen in the examined population, maybe due to the low frequency of AA genotype in Landrace and Yorkshire boars. Therefore, the selection based on the FUT1 gene either does not affect the sperm quality of the boars, or the low frequency of the allele in our population does not allow to highlight potentially harmful effects.

There was no effect of MUC4 genotypes on sperm quality of Landrace and Yorkshire boars, except the motility. We found the highest spermatozoa motility in the boars carrying the GG genotype. For other parameters, we did not find any difference among three

genotypes. In our literature review, there was no reference reporting on the effects of *MUC4* genotypes on pig sperm quality. Therefore, the selection of Landrace and Yorkshire boars based on *MUC4* genotypes does not seem to influence the sperm quality and could even help improve the spermatozoa motility.

A number of studies confirmed that the breed had an influence on sperm quality. Knecht *et al.* (2014) showed that Polish Landrace had a higher sperm concentration, total number of spermatozoids and total number of live spermatozoids than those of Polish Large White. Jaishankar *et al.* (2018) reported that Large White boars had a significant higher semen volume and sperm concentration than Landrace, while the live sperm count was significantly higher in Landrace compared to Large White. Kondracki (2003) also found significant differences of semen quality among breeds and concluded that Landrace and Large White had a relatively high volume and a concentration that are satisfactory for insemination.

Effects of age on sperm quality of boars have also been well documented by many authors. Tsakmakidis *et al.* (2012) demonstrated that the young (7-10 months) and old (51-61 months) boars were more susceptible to sperm chromatin instability than the mature (18-33 months) boars. The result of this study was also consistent with research of Savić *et al.* (2013), in which the lowest volume of ejaculate was observed in the young Large White boars (10-13 months of age) and the highest volume was determined in boars at the age of 26 to 29 months.

Season was also an important factor influencing the sperm quality traits and well-documented by many authors. Savić et al. (2013) indicated that the lowest semen volume and sperm motility of Large White boars were observed in winter due to the low temperature. However, in our study, under tropical conditions, the sperm quality of boars seems to be better in winter and spring. Tretipskul et al. (2012) also reported that there were significant differences in semen volume, concentration, and total number of sperm of boars in tropical condition, in which the highest semen volume was observed in winter (November and December) and lowest semen concentration was seen during middle rainy to early winter (August to December). These concordant observations seem to indicate a negative effect of high temperature on semen quality of boars during hot summer.

Conclusion: The frequency of resistant genotypes AA for FUT1 (0.03) and GG for MUC4 (0.16) was relatively low in Yorkshire boar population examined. These resistant genotypes were absent in Landrace. The only detected significant effect of two SNPs located in FUT1 and MUC4 on semen quality traits was a positive effect of the resistant genotype GG of MUC4 on sperm motility.

Consequently, a selection based on these resistant genotypes is not expected negatively affect sperm quality of Landrace and Yorkshire.

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Ethical Approval: Not applicable for this type of study in Vietnam.

Informed Consent: These data are published with consent of Dabaco Nucleus Breeding Pig Company, Bac Ninh province, Vietnam.

Conflict of interest: The authors declare that they have no conflict of interest.

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