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Association of NRAMP1 gene polymorphism with the productive traits of the Ukrainian Large White pig

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Summary

The study aimed to determine the influence of *NRAMP1* gene polymorphisms on the pig's productive traits. Genomic DNA was isolated from blood samples (n = 50) from pigs of the Ukrainian Large White breed. Genotyping was performed at *NRAMP1* loci at positions 72 and 364 (*AvaII*) SNP and 176 and 334 (*HinfI*) SNP, counting from the beginning of the amplified fragment 536 bp. Polymorphism was found at the last two these loci. For *NRAMP1HinfI* 334 SNP, the frequency of the allele T was 1.9 times as high as that of the allele C. The observed genotype distribution deviated significantly from the expected one for *NRAMP1HinfI* 334 SNP ($\chi^2 = 10.150$; $p < 0.01$). Pigs of the Ukrainian Large White breed showed a rather high *NRAMP1HinfI* 334 SNP polymorphism (PIC = 0.35). Relationships were found between the Exon2 of the *NRAMP1* gene (*HinfI* 334 SNP) polymorphism and the productive traits of pigs (average daily gains, $p < 0.05$; fat thickness, $p < 0.01$). The TT genotype positively affected the growth rate of the experimental pigs from day 28 to day 120, and their average daily gains were higher by 17.7 grams or 3.2% ($p < 0.05$). At day 180, the body weight of pigs with the TT *NRAMP1HinfI* 334 SNP genotype was higher by 5.04 kg ($p < 0.05$). At the same time, they had thicker backfat ($p < 0.01$).

Keywords: Ukrainian Large White pig breed, DNA markers, *NRAMP1* gene, productivity

A significant cause of the rapidly rising bacterial resistance to antibiotics is the massive use of antibiotics in industrial pig production (11). Regulation (EU) 2019/6 continues and strengthens the EU's fight against antimicrobial resistance by introducing a ban on the preventive use of antibiotics in groups of animals, a ban on the prophylactic use of antimicrobials in medicated feed, and restrictions on the use of antimicrobials as a control treatment to prevent a further spread of infection. The search for new ways to protect farm animals against diseases continues to arouse interest among scientists (7).

The current fast development of molecular genetics, molecular biology techniques, and the discovery of genes (genetic markers) associated with disease resistance could produce practical solutions for improving genetic disease resistance. Researchers' main task is to discover the primary effective or candidate genes controlling disease-resistant capability (23).

One of the most promising genes that could be a candidate for an animal resistance marker is *NRAMP1*. The pig's Natural Resistance-associated Macrophage Protein 1 Encoding Gene (*NRAMP1*), also known as *SLC11A1* (solute carrier family 11 member A1) (9), is located on chromosome 15 q23-26 in pigs, and it was initially cloned by Tuggle et al., 1997 (19). The *NRAMP1* gene belongs to the family encoding divalent cation transporters localized to late endosomes/

lysosomes (1, 17). The NRAMP1 protein influences the replication of microorganisms in the phagosome by modifying the intra-phagosomal environment (4, 25).

In mammals, the *NRAMP1* gene is associated with the transport of iron and other divalent cations. The transition metal ions are essential for maintaining divalent metal homeostasis, including the regulation of transcription through DNA-binding proteins and metal response elements (2, 5). The results obtained by Xiaoling et al. (25) indicate that the *NRAMP1* gene could be a molecular marker for genetic selection in terms of disease susceptibility in pig breeding. They found that the polymorphism and expression of Natural Resistance-associated Macrophage Protein 1 Encoding Gene were significantly correlated with immune function and production performance. It can be regarded as a candidate gene for disease resistance. The *NRAMP1* gene is highly expressed in the digestive and intestinal tissues of Meishan piglets after weaning, which indicates its role in immune regulation during weaning stress, and in particular, its role in intestinal immunity (8). This gene is an essential factor associated with intracellular pathogens responsible for susceptibility to infectious diseases. Differences in transmembrane domains and tertiary structures of the NRAMP1 protein of the Tibetan and Yorkshire pig breeds could explain differences in the disease resistance of these two breeds (22). According to Wu et al. (23), the death rate of Large White pigs with the genotype BB was lower than that of pigs with the genotype AB. On the other hand, the death rate of Songliao Black pigs with the genotype AB was lower. The desirable genotype of the *NRAMP1* gene may be different for other pig breeds (14). Thus, to use the *NRAMP1* gene as a breeding marker, it is necessary to conduct preliminary studies in a given population.

The study aimed to determine the effect of the polymorphism of the *NRAMP1* gene on the productive traits of pigs, which may be indirectly related to immunity.

Material and methods

This study used 50 pigs (non-related to each other, sex ratio 1 : 1) of the Ukrainian Large White breed from the breeding herd of the Plekhiv-Agro farm in the Poltava region of Ukraine. The pigs were housed in a system with slatted floors under conditions of optimal pig welfare. Piglets were weaned on day 28 of life. All procedures were carried out according to principles and ethical guidelines for animal research and approved by the Animal Care and Use Committee of the Pig Breeding and Agro-industrial Production Institute.

Blood samples (10 ml) were aseptically collected from the ear vein into a vacutainer tube (containing anticoagulant). Genomic DNA was isolated from 400 µl of blood by the sorbent Chelex 100 method (21). A 536 bp fragment of the sequence of the porcine *NRAMP1* gene was amplified using a pair of specific primers (18): forward (5'-GCGTCAGTCTTCCCTGCTCAG-3') and reverse (5'-ACGGCAGTTACCACTCTCCATCT-3'). PCR was performed in 25 µl reactions, including 1 × PCR buffer, 1.5 mM $MgC1_2$, 0.2 mMdNTPs, 10 pmol of each primer and 1.0 U of Taq polymerase. Two restriction enzymes, *AvaII* and *HinfI*, were used for PCR-RFLP analysis. SNP at positions (72, 364, 176 and 334) were counted from the amplified 536 bp fragment (14). Counting from the beginning of LOCUS NC_010457 (presented in GenBank NCBI: ACCESSION NC_010457 REGION: 120434100…120446396), SNP's position numbers are 337 and 626 for *AvaII*, and 441 and 599 for *HinfI*. Alleles were identified according to patterns proposed by Tuggle et al., 2005 (18). Our minor addition: allele D for *HinfI* was added after analyzing the information presented in the GenBank NCBI (Tab. 1, Tab. 2). When using *HinfI*, combinations of two alleles give unique sets of fragment lengths, as shown in Tab. 3.

The productivity and body development of pigs from birth to slaughter were determined on the basis of body weight (kg), daily gains (g/day), backfat thickness (mm) (measured with an ultrasonic device Renco Lean Meter), body length (cm) at day 180 (measured with a measuring tape from the occipital crest to the root of the tail), height at withers (cm) (measured with a measuring stick), and chest circumference (cm) (measured with a measuring tape).

The direct counting method was used to determine the genotypic and allelic frequencies of the *NRAMP1* gene

Tab. 1. Alleles of the *NRAMP1* gene detected with the enzyme *Ava II*

Allele	Position 72	Position 364	Fragments
1	G	A	411, 72, 53
2	G	G	291, 120, 72, 53
3	A	A	483, 53
4	A	G	363, 120, 53

Tab. 2. Alleles of the *NRAMP1* gene detected with the enzyme *HinfI*

Allele	Position 176	Position 334	Fragments
A	G	C	360, 100, 76
B	G	T	205, 155, 100, 76
C	A	T	231, 205, 100
D	A	C	436, 100

Tab. 3. Length fragments for different combinations of alleles with the enzyme *HinfI*

Genotype at position 76	Genotype at position 334	Allele 1	Allele2	Fragments
GG	CC	A (GC)	A (GC)	76, 100, 360
GG	CT	A (GC)	B (GT)	76, 100, 155, 205, 360
GG	TT	B (GT)	B (GT)	76, 100, 155, 205
AG	CC	D (AC)	A (GC)	76, 100, 360, 436
AG	CT	D (AC)	B (GT)	76, 100, 155, 205, 436
AG	CT	C (AT)	A (GC)	76, 100, 205, 231, 360
AG	TT	C (AT)	B (GT)	76, 100, 155, 205, 231
AA	CC	D (AC)	D (AC)	100, 436
AA	CT	D (AC)	C (AT)	100, 205, 231, 436
AA	TT	C (AT)	C (AT)	100, 205, 231

variant. The chi-square test (χ^2) was performed using GenAlEx6 to check whether the populations were in the Hardy-Weinberg equilibrium (15). The Fixation index (FST), the theoretically expected heterozygosity (H_e), and the PIC index (Polymorphism Information Content index) were also calculated using GenAlEx6 (15). The assumption of data normality was tested with the Shapiro-Wilk test, and it was found that the data were normally distributed ($p > 0.05$).

The statistical model used to calculate the effect of polymorphism of the analyzed gene was

$$y_{ij} = \mu + NRAMP_{1i} + a_j + e_{ij}$$

where y_{ij} – trait phenotypic value, μ – population average, $NRAMP_{1i}$ – constant genotype effect at the examined locus *NRAMP1* (i = 1, 2), a_j – fixed effect of sex (j = 1, 2), e_{ij} – random error. Because the CC genotype was found in only 1 individual, it was not included in the statistical analysis.

The model did not consider the effects of the herd, season, or year, because the study was carried out simultaneously within one herd. Duncan's multiple comparison test was used to determine group differences for all traits. The calculations were made with the SAS statistical package.

Results and discussion

There were no polymorphisms at positions 72 and 364 of the *NRAMP1* gene (identified with *AvaII*). All animals analyzed in the experiment had genotype s*NRAMP1* (*AvaII*) 72 GG and *NRAMP1* (*AvaII*) 364 AA. Polymorphism was found at positions 176 and 334 (identified with *HinfI*). The results of our genetic-population analysis of the gene fragment examined in this study for the Ukrainian Large White pig breed are presented in Tables 4 and 5.

The frequency of the allele G in the *NRAMP1HinfI* 176 SNP was 8.1 times as high as the frequency of allele A. For SNP *NRAMP1HinfI* 334, the frequency of the allele T was 1.9 times as high as that of the allele C. At position 176 (*NRAMP1HinfI* SNP), genotype AA was not found. There were no significant deviations in genotype frequencies from the Hardy-Weinberg equilibrium. The frequency of the GG genotype was 3.6 times as high as that of the GA genotype. Significant deviations of the observed genotype distribution from the expected distribution were found in *NRAMP1HinfI* 334 SNP ($\chi^2 = 10.150$; $p < 0.01$). The significant number of heterozygotes in *NRAMP1HinfI* 334 SNP is consistent with a negative fixation index (Fst = –0.451) in the micropopulation analyzed here.

We found a significant deviation in the observed heterozygosity for *NRAMP1HinfI* 334 SNP, (Tab. 5). It may indicate some selection pressure at this locus in the micropopulation of the Ukrainian Large White pig breed.

The level of polymorphism required for associative studies is determined by PIC. (Polymorphism Information Content). The *NRAMP1 HinfI* 334 SNP locus was within optimal values, which range from 0.25 to 0.75, according to Botstein et al., 1980 (3). Therefore, this locus was chosen to search for the effect of its polymorphism on productive traits that are indirectly associated with greater disease resistance (Tab. 6).

Tab. 4. Allele and genotype frequencies at different positions

Locus	Allele frequencies	Genotype frequencies			χ^2	Fixation index (Fst)
NRAMP1 HinfI Position 176	A = 0.11 G = 0.89	AA = 0.00 (0.01)*	GA = 0.22 (0.20)	GG = 0.78 (0.79)	0.382	–0.124
NRAMP1 HinfI Position 334	C = 0.35 T = 0.65	CC = 0.02 (0.12)	CT = 0.66 (0.46)	TT = 0.32 (0.42)	10.150**	–0.451

Explanations: * – in parentheses are the expected genotype frequencies calculated using the Hardy-Weinberg equilibrium; ** – the χ^2 values were calculated to assess the statistical significance of the deviation of the observed genotype distribution from the expected one

Tab. 5. Observed heterozygosity (H_o), expected heterozygosity (H_e), and the PIC index at different loci

Locus	H_o	H_e	PIC
NRAMP1 HinfI 176	0.220	0.196	0.18
NRAMP1 HinfI 334	0.660	0.455	0.35

Tab. 6. Dependence of productive qualities on the *NRAMP1* genotype at position 334

Productive traits	Genotype		ANOVA	
	CT (n = 33) (mean ± SE)	TT (n = 16) (mean ± SE)	F	p
Birth weight (kg)	1.48 ± 0.044	1.51 ± 0.060	1.45	0.245
Weight at day 28 (kg)	7.88 ± 0.163	8.05 ± 0.202	0.57	0.567
Weight at day 120 (kg)	50.38 ± 0.488^a	52.14 ± 0.620^b	4.44	0.017
Weight at day 180 (kg)	93.60 ± 1.099^a	98.64 ± 2.315^b	3.66	0.033
Average gain from birth to weaning at day 28 (g/day)	228.84 ± 5.271	235.85 ± 6.906	1.42	0.252
Average gain from day 28 to day 120 (g/day)	548.09 ± 5.365^a	565.80 ± 4.623^b	3.89	0.027
Average gain from day 120 to day 180 (g/day)	716.72 ± 18.260	770.00 ± 33.860	1.43	0.250
Body length at day 180 (cm)	117.73 ± 1.182	117.31 ± 2.410	0.36	0.697
Height at withers at day 180 (cm)	70.61 ± 1.153	72.13 ± 1.791	0.52	0.596
Chest circumference at day 180 (cm)	101.73 ± 1.408	97.06 ± 1.843	1.96	0.153
Backfat at 100 kg live weight	19.26 ± 0.562^A	22.82 ± 0.808^B	7.22	0.002
Average gain from birth to day 180 (g/day)	510.82 ± 6.029^a	537.98 ± 12.333^b	3.71	0.032
Age reaching 100 kg (days)	190.23 ± 1.816	183.79 ± 3.199	2.85	0.068

Explanations: $^{a, b}$ – $p < 0.05$, $^{A, B}$ – $p < 0.01$

We found that pigs with the TT genotype were characterized by higher gains from weaning to day 120. This led to significant differences in live weight at the age of 4 and 6 months. At the same time, those pigs had thicker backfat (Tab. 6). However, there was no significant effect of *NRAMP1* gene (*HinfI* 334 SNP) polymorphism on the other traits analyzed here. We found that the age of reaching a body weight of 100 kg was most favorable in pigs with the TT genotype, but differences in body weight were not statistically significant.

The high level of polymorphism at position 334, which we found in the Ukrainian Large White pig breed, is consistent with other researchers' conclusions that the level of polymorphism in indigenous pig populations is higher than it is in Western pig breeds (13, 20, 24, 26).

Typically, the effect of porcine *NRAMP1* gene polymorphism on immunity has been studied by hematological analysis. For example, in four pig populations, including Large White and three Chinese indigenous breeds (Wannan Black, Huai pig, and Wei pig), the *NRAMP1* SNP genotype (ENSSSCG00000025058: g.130 C > T) was significantly associated with the level of white blood cells ($p = 0.031$) and monocytes ($p = 0.024$) and the rate of cytotoxin in monocytes ($p = 0.013$). The other SNP (ENSSSCG00000025058: g.657 A > G) were significantly associated with the levels of MO% ($p = 0.012$), MC% ($p = 0.019$), and CD4−CD8+ T lymphocytes ($p = 0.037$) (21). At the same time, indirect signs, such as weight gain, feed efficiency, and others, may indicate a strong immune system (18). In our experiment, animals with the TT genotype showed higher gains, which can probably explain their better resistance.

The significant deviation of the observed genotypic distribution from the expected one towards a larger number of heterozygous at locus 334 (Tab. 4) can probably be explained by the fact that heterozygous pigs have a smaller backfat thickness compared to animals with the TT genotype. On the other hand, they show a better average daily gain compared to carriers of the CC genotype. The selection of animals for breeding in this herd is based on a selection index that includes these indicators (i.e. average daily gain and backfat).

Our results indicate an association between thicker backfat and greater disease resistance. Our results are consistent with those from other researchers, who observed that indigenous breeds often had better resistance (12, 16, 24) as well as greater fat thickness (6, 10, 14).

Pigs of the Ukrainian Large White breed are characterized by a relatively high level of polymorphism at the *NRAMP1Hinf1* 334 SNP locus, as evidenced by PIC = 0.35. The relationships that we found between the polymorphism in the Exon2 of the *NRAMP1* gene (*HinfI* 334 SNP) and the productive traits of pigs (average daily gains, $p < 0.05$; fat thickness, $p < 0.01$) can be used in breeding work with the Ukrainian Large White pig breed to obtain animals with good productive qualities and increased resistance to disease.

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