

The effect of estrogen receptor genotypes on the number of stillborn and mummified piglets in Topigs 20 sows

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MEŇČIK, S., V. VUKOVIĆ, M. MODRIĆ, M. ŠPEHAR, M. OSTOVIĆ, V. SUŠIĆ, I. ŠTOKOVIĆ, K. SALAJPAL, H. KABALIN, A. EKERT KABALIN: The effect of estrogen receptor genotypes on the number of stillborn and mummified piglets in Topigs 20 sows. Vet. arhiv 86, 311-322, 2016.

ABSTRACT

Steroid hormones such as estrogen play a central role in the postnatal female physiology and their effects are exerted through its receptors. The estrogen receptor gene is one of the first and most extensively investigated candidate genes for reproductive traits in pigs, especially for litter size. The aim of the study was to investigate the estrogen receptor gene polymorphism using endonuclease *PvuII*, and its association with the number of stillborn (NSB) and the number of mummified (NMUM) piglets in the first, second, third and subsequent parities and in overall parities. Topigs 20 sows (n = 101) from 1st to 7th parities were analyzed. Estrogen receptor genotypes were detected by the polymerase chain reaction-restriction fragment length polymorphism method. Two alleles (A and B) were identified with three genotypes. Alleles (A and B) and genotype frequencies were determined. Comparison of the observed and expected genotype frequencies was performed using the χ^2 -test and considerable deviation from the Hardy-Weinberg principle was found. All data were analyzed using the General Linear Model. Statistical analysis showed a significant difference (P<0.05) in NMUM between the

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AB and BB genotypes in the third and subsequent parities. The statistical significance of differences between AA and BB genotypes tended to be lower ($P < 0.1$) in the third and subsequent parities in NMUM. The sows with A allele had less NSB, indicating a beneficial effect of this allele, unlike the B allele in NMUM. The results obtained will contribute to the understanding of the effect of ESR genotype on NSB and NMUM, and substantiate genetic evaluation of litter size traits in pigs.

Key words: estrogen, genotype, stillborn and mummified piglets, Topigs 20

Introduction

The efficiency of production is influenced by the factors that cause losses during the perinatal and postnatal period. These losses are manifested as the number of stillborn piglets (NSB) and the number of mummified piglets (NMUM), which should be studied separately from the total number born (TNB) and the number of born alive (NBA) piglets (VALLET et al., 2006).

Two main points of view are used to improve the efficiency of reproductive traits, such as identification of genetic markers (CHVOJKOVÁ and HRAŠKA, 2008), quantitative trait loci (QTL) and candidate gene approaches (ROTHSCHILD et al., 2007; TERMAN and KUMALSKA, 2012). Analysis of the genome and different gene polymorphisms has revealed significant effects on litter size traits and has been included in the selection procedure in animals, known as marked-assisted selection (MAS) (ALFONSO, 2005).

The estrogen receptor gene (ESR) is located on the pig chromosome 1 and a specific polymorphism was established using the *Proteus vulgaris* II (*Pvu*II) restriction enzyme in the third intron of ESR, expressing two alleles (A and B), possibly associated with litter size traits (ROTHSCHILD et al., 1991 and 1996). The estrogen receptor gene is one of the most extensively analyzed candidate genes for litter size traits in pigs. Many studies have been performed in different populations of purebred, crossbred and hybrid sows concerning litter size traits, especially TNB and NBA, however, with quite ambiguous results (LEGAULT et al., 1996; SHORT et al., 1997; ALFONSO, 2005; OMELKA et al., 2005; SUWANASOPEE and KOONAWOOTRITTRIRON, 2011). Analysis of a particular candidate gene locus is important in the evaluation of genetic parameters for quantitative properties, at the level of additive and dominance genotype variance and its allele frequency in a population (PIERCE, 2003; SPÖTTER et al., 2009). Additive genotype variance describes the cumulative effect of a particular gene, in contrast to dominance variance, where the allele effect can be calculated as the difference in heterozygote value relative to homozygote average in the study population (HILL et al., 2008; PRIPWAI, 2012). Regression models are used to calculate deviation in phenotypic values, which are dependent on the frequency of a particular allele and other paragenetic factors (WELLER, 2009).

The aim of this study was to evaluate the genotype and allele frequencies of ESR as a candidate gene for NSB and NMUM in a Topigs 20 line of sows, in commercial

production conditions, with additive and dominance effects, and to test the significance of the effects included in the model of calculation.

Materials and methods

The study included 101 Topigs 20 line sows bred and raised at the Krmiva pig breeding farm (Croatia). Raising and feeding conditions were the same for all animals throughout the study period. Blood sampling was performed in the service unit during implementation of the Decree on the Measures of Animal Protection from Infectious and Parasitic Diseases, issued by the Ministry of Agriculture, Fisheries and Rural Development (Official Gazette 135/2006).

Genomic DNA was extracted from the blood using a sterile tube. DNA was isolated using the Dneasy Blood® & Tissue Kit (Qiagen, GmbH, Hilden, Germany). The ESR gene genotype was determined using the polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP). Primer sequences were designated according to SHORT et al. (1997). The amplification conditions, the composition of the reaction mixture for PCR reaction, restriction using endonucleases *PvuII*, and the conditions of electrophoresis with visual determination of the fragments have been described previously (MENČIK et al., 2012).

Reproductive parameters were collected from the documentation of the breeding farm. Farrowings were supervised periodically, and the first check-up was about 12 hours post-partum. At that time, NSB and NMUM were recorded for each litter. Piglets that were found lying behind the sow covered by placenta with a wet umbilical cord present were classified as stillborns. Mummified piglets, with skin discoloration and pressure sores associated with small vesicles, were defined as mummies (LE COZLER et al., 2002; BORGES et al., 2005). The phenotype data for NSB and NMUM were analyzed separately for the first, second, third with subsequent parities, and all parities. Effects included in the model were analyzed by the least square method using the General Linear Model (GLM) procedure in the SAS statistical package (SAS, 2010). The model ^[1] that fitted the NSB and NMUM best was represented in the following scalar equation:

$$y_{ijklmn} = \mu + ESR_i + P_j + YS_k + AGEF_l + SP_m + B_n + e_{ijklmn} \quad [1]$$

where: y_{ijklmn} - observed trait (NSB or NMUM); μ - population mean; ESR_i - genotype ($i = AA, AB, BB$); P_j - effect of parity ($j = 1, 2, 3 \leq$), which was not included in the analyses of all parities; YS_k - year-season interaction ($k = 1, 2, 3, \dots, 12$). The year-season effect was formed as the interaction between a period of three years and three consecutive months of farrowing. $AGEF_l$ - age at first farrowing ($l = 1, 2, 3$) included in the model as

a fixed class effect with three levels. The first level included sows aged ≤ 352 days, the second level those aged 353-383 days, and the third level those aged ≥ 384 days at first farrowing; SP_m - service period ($m = 1, 2, 3$) was included in the analyses for the second parity, the third with subsequent parities, and all parities. The service period was divided into three levels according to successful insemination: first (within 30 days), the second (between 31 and 50 days), and the third (after 51 days and more), B_n - effect of the sire ($n = 1, 2, 3$) and e_{ijklmn} - residual. For each level of the effect, the least square mean with standard errors ($LSM \pm SE$) was computed. The allele and genotype frequencies were calculated and the Hardy Weinberg equilibrium was tested to compare the observed and expected genotype frequencies using the χ^2 -test, according to the method described by RODRIGUEZ et al. (2009). According to LIU (1998), both additive and dominance effects were estimated using the REG procedure of SAS (SAS, 2010), where the additive effects were estimated as -1, 0, and 1 for AA, AB and BB genotypes, while dominance effects were represented as 1, -1, and 1 for AA, AB, and BB genotypes.

The effects with a probability ranging from 0.051 to 0.10 tended to be significantly different, while values lower than 0.05 were considered as a significant difference (VINCENT et al., 1998; VAN RENS et al., 2003). The F-test was used to assess the significance of the effects included in the model of calculation.

Results

In the investigated population of sows, two alleles (A and B) with three genotypes (AA, AB and BB) were determined, with the size of fragments presented in Table 1. The frequencies of alleles and genotypes for the analyzed traits and results of the χ^2 -test are shown in Table 2. A lower frequency was detected for the B allele, especially the BB genotype. The heterozygous genotype was more abundant in comparison to sows with the AA genotype. The Hardy-Weinberg principle did not show genetic equilibrium with regard to alleles and genotype frequencies for ESR ($\chi^2 = 5.73$, $P < 0.05$), as shown in Table 2. The traits analyzed in relation to ESR genotypes are illustrated in Table 3.

Table 1. ESR gene with the size of PCR products, size of allele and genotype DNA fragments (according to SHORT et al., 1997)

Candidate gene	PCR products size (bp*)	Endonuclease	Allele size (bp)	Genotype (size in bp)
ESR	120	<i>PvuII</i>	A - 120 B - 65, 55	AA - 120 AB - 120, 65, 55 BB - 65, 55

*bp - base pair

Table 2. Frequencies of ESR-*PvuII* genotypes and alleles in 101 sows of Topigs 20 line and assessment of genotype balance

ESR- <i>PvuII</i> genotype (n/f)*			ESR allele frequencies		χ^2	P-value
AA (n/f)	AB (n/f)	BB (n/f)	A	B		
33/0.3267	59/0.5842	9/0.0891	0.6188	0.3812	5.73	P<0.05

*n - number of sows; f - genotype frequency

Table 3. Number of stillborn (NSB) and mummified piglets (NMUM) in sows with different ESR genotypes and parity number, and additive and dominance effects

Parity number	Analyzed traits	Genotype (LSM \pm SE)			Additive effects (LSM \pm SE)	Dominance effects (LSM \pm SE)
		AA	AB	BB		
First parity n = 101	n	33	59	9		
	NSB	1.00 \pm 0.5	1.15 \pm 0.37	1.63 \pm 0.56	0.31 \pm 0.25	0.05 \pm 0.14
	NMUM	0.20 \pm 0.13	0.11 \pm 0.10	0.15 \pm 0.15	0.06 \pm 0.07	0.03 \pm 0.05
Second parity n = 97	n	32	56	9		
	NSB	0.54 \pm 0.44	0.58 \pm 0.34	0.55 \pm 0.58	0.28 \pm 0.2	0.18 \pm 0.14
	NMUM	0.01 \pm 0.13	0.01 \pm 0.1	0.06 \pm 0.17	0.02 \pm 0.06	0.008 \pm 0.004
Third with subsequent parities n = 228	n	84	126	18		
	NSB	0.74 \pm 0.29	0.82 \pm 0.28	0.88 \pm 0.34	0.38 \pm 0.37	0.09 \pm 0.17
	NMUM	0.22 ^b \pm 0.06	0.24 ^a \pm 0.06	0.04 ^{a,b} \pm 0.08	0.02 \pm 0.02	0.01 \pm 0.04
All parities n = 426	n	149	241	36		
	NSB	0.83 \pm 0.21	0.96 \pm 0.19	1.23 \pm 0.25	0.12 \pm 0.1	0.04 \pm 0.07
	NMUM	0.12 \pm 0.05	0.11 \pm 0.05	0.11 \pm 0.06	0.008 \pm 0.03	0.01 \pm 0.02

LSM \pm SE with superscripts (a) differ significantly (P<0.05); LSM \pm SE with superscripts (b) differ with tendency (P<0.1); NSB-number of stillborn piglets; NMUM-number of mummified piglets; n - number of analyzed litter

Primiparous sows with the BB genotype showed higher NSB compared to heterozygotes and the AA genotype. The highest NMUM was found in primiparous sows with AA genotype. In the second parity, similar results for NSB and NMUM were recorded in the three genotypes, with the lowest NMUM and NSB in comparison with other parities analyzed. In the third and subsequent parities, the sows with AA and AB genotypes farrowed less NSB in comparison to the BB genotype, while lower NMUM was recorded in the BB genotype than in AA and AB genotypes.

Table 4. Differences in analyzed traits (LSM \pm SE) according to ESR-*PvuII* genotypes and the analysis of variance for the effects included in the model of calculation (ANOVA-EF)

Parity number	Analyzed traits	Differences between genotypes (LSM \pm SE)			ANOVA-EF
		BB - AB	BB - AA	AB - AA	
First parity n = 101	NSB	0.48 \pm 0.19	0.63 \pm 0.06	0.15 \pm 0.09	*
	NMUM	0.04 \pm 0.05	-0.05 \pm 0.05	-0.09 \pm 0.02	*
Second parity n = 97	NSB	0.13 \pm 0.1	0.22 \pm 0.1	0.09 \pm 0.04	P = 0.06 (G)
	NMUM	-0.07 \pm 0.02	-0.05 \pm 0.02	0.02 \pm 0.02	*
Third with subsequent parities n = 228	NSB	0.06 \pm 0.08	0.14 \pm 0.08	0.08 \pm 0.04	P = 0.02 (YS)
	NMUM	-0.2 ^a \pm 0.01	-0.18 ^b \pm 0.02	0.02 \pm 0.008	P = 0.10 (G) P = 0.004 (S)
All parities n = 426	NSB	0.27 \pm 0.04	0.4 \pm 0.04	0.13 \pm 0.02	*
	NMUM	0 \pm 0.1	-0.01 \pm 0.01	-0.01 \pm 0.005	*

LSM \pm SE with superscripts (a) differ significantly ($P < 0.05$); LSM \pm SE with superscripts (b) differ with tendency ($P < 0.1$); NSB - number of stillborn piglets; NMUM - number of mummified piglets; G - genotype; P - parity; S - sire; YS-year-season; n - number of analyzed litters; * - variance of effects included in the model was not significant

There were no significant differences between genotypes for NSB and NMUM, except for the third and subsequent parities for NMUM between AB and BB genotypes ($P < 0.05$). Also, in the third and subsequent parities, a lower level of difference ($P < 0.1$) was recorded between AA and BB genotypes. Differences between genotypes in all parities were not significant for the study traits of NSB and NMUM. The highest NSB was recorded in sows with the BB genotype as compared to sows with AB and AA genotypes.

Additive and dominance effects are shown in Table 3. The highest additive effects were found on NSB for the B allele in the first, second and third with subsequent parities at the level of 0.28 to 0.38. The dominance effect on the observed traits was extremely low, with the lowest dominance effect on NMUM in second parities. Differences between genotypes in analyzed traits are shown in Table 4. The statistical model used to analyze the significant effects of the genotype, parity, age at first farrowing, farrowing year-season interaction and service period. The number of stillborn piglets and NMUM as analyzed traits showed only the sire had any significant effect on NMUM ($P < 0.05$) and year-season interaction on NSB ($P < 0.05$) in third- and higher parities, with a lower level of statistical significance ($P < 0.1$) and a tendency for genotype effect on NSB in second parities and on NMUM in third and subsequent parities.

Discussion

Many studies have been conducted to assess litter size traits, especially TNB and NBA. However, only a few studies have demonstrated the influence of the ESR genotype on NSB and NMUM, especially in a high fertility line of sows under commercial conditions. The relative frequency of the A allele was in agreement with the results reported for the population of Large White breed, and crossbred with Yorkshire pigs (ISLER et al., 2002; MATOUŠEK et al., 2003; OMELKA et al., 2005; SANTANA et al., 2006; CHVOJKOVA and HRAŠKA, 2008), and crossbred American Yorkshire with Landrace pigs (HERNANDEZ-LOPEZ et al., 2006), ranging from 0.63 to 0.6828. The frequency of the heterozygous genotype was in accordance with the study on a population of Large White breed (LEGAULT et al., 1996; GOLIAŠOVA and WOLF, 2004). A significant influence of parity and year-season ($P < 0.05$) interaction was established for NSB in Yorkshire and Landrace breeds of sows (ISLER et al., 2002). ISLER et al. (2002) found the highest NSB and lowest NMUM in the sows with the AA genotype of Large White, Yorkshire and their crossbreds, in comparison to AB and BB genotypes, while the lowest NSB and highest NMUM were detected in the BB genotype as compared with other observed genotypes; however, the differences were not statistically significant. The results of the present study are in contrast with the results of ISLER et al. (2002) for all parities analyzed. SUWANASOPEE and KOONAWOOTRITRIRON (2011) recorded the highest NSB and NMUM in the Yorkshire and Landrace breeds of sows with AA genotype and the lowest number of NSB and NMUM in BB genotype, in comparison with heterozygotes. In the study by VAN RENS et al. (2002), the population of Large White and F₂ Meishain crossbred showed the highest NSB and NMUM in gilts with AA genotype.

The results of the present study pointed to the unfavorable effect of the presence of the B allele on NSB, while the opposite was established for the A allele and NMUM. The negative impact of the presence of the B allele in Large White lines of sows for litter size traits was reported by SHORT et al. (1997). In the present study, the B allele indicated an additive effect on NSB in the parities analyzed, but the value was not significant. These results indicate that in the analyzed population, any significant impact on reducing the rate of NSB and NMUM may depend on parity number. Large litters with more than 12 piglets born alive have higher NSB and NMUM in comparison to small litters ($P < 0.05$) (RUIZ-FLORES and JOHNSON, 2001; LUCIA et al., 2002; PETRY et al., 2004).

VANROOSE et al. (2000) report that 70 % of the losses, in the form of stillbirths and mummies immediately before and after farrowing, are related to various factors. This is in agreement with the fact that the highest NSB in first parities may be caused by many physiological factors. The most common factors are specified placental traits, a narrow birth canal in younger females, asphyxiation during delivery, and hypoxia at birth (PEJSAK, 1984; VAN DER LENDE and VAN RENS, 2003). The lower reproductive efficiency

of sows during first and second parity is a possible consequence of the insufficient maturity of young sows, and the effect of the year-season interaction on farrowing (SEGURA-CORREA et al., 2014).

The increased number of stillborn piglets in a high fertility line of sows could be ascribed to the uterine environment, especially the stretched uterine horns at the end of pregnancy (VAN RENS et al., 2002). Also, the extended length of parturition in primiparous sows, especially those with very large litters, may result in a higher number of stillborn piglets (HERPIN et al., 2001; LUCIA et al., 2002; CHU, 2005). Further analyses, including larger samples, may hopefully identify the effects of different ESR genotypes and their alleles on NSB and NMUM.

As suggested by DAMARIO et al. (2001), the ESR gene could be associated with physiological processes, in particular with the rate of survival during the first third of gestation. Analyzing different genetic types of pigs using a molecular approach, especially in sows selected for high fertility rate, will help clarify whether the ESR gene is a marker or major gene for NSB and NMUM. All these findings will contribute to solving the issue of how to reduce pig losses and maintain a high level of reproductive efficiency (DISTL, 2007). Also, through the represented scalar equation, the impact of other factors must be considered on statistical data analysis, including genotype, year-season interaction and the effects of the sire on the analyzed traits for the candidate gene under study (WANG et al., 2006).

Conclusion

The study traits of NSB and NMUM indicated that the B allele is associated with higher NSB, in contrast to the A allele, which has a significant impact on NMUM in third- and higher parities. However, additional studies on a larger data set are needed for functional validation of polymorphism and a better understanding of the ESR-*PvuII* genetic effects on NSB and NMUM.

Acknowledgements

The authors are thankful to Topigs Danubia and Krmiva Ltd., Zagreb (Croatia) for enabling the conduct of the study. The study was supported by grants from the Ministry of Science, Education and Sport of Croatia (053-0532265-2238).

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Received: 29 January 2015

Accepted: 10 December 2015

MEŇČIK, S., V. VUKOVIĆ, M. MODRIĆ, M. ŠPEHAR, M. OSTOVIĆ, V. SUŠIĆ, I. ŠTOKOVIĆ, K. SALAJPAL, H. KABALIN, A. EKERT KABALIN: Učinak genotipa estrogenog receptora na broj mrtvooprasenih i mumificiranih odojaka u krmača linije Topigs 20. *Vet. arhiv* 86, 311-322, 2016.

SAŽETAK

Steroidni hormoni imaju ključnu ulogu u postnatalnom razvitku i fiziologiji rasplodivanja ženskih jedinki. Djelovanje ovih hormona očituje se kroz podražaj njihovog receptora. Odsječak gena za estrogeni receptor među prvim je i najistraživanijim kandidatnim genima za reprodukcijску učinkovitost svinja, naročito za pokazatelje veličine legla. Cilj ovoga istraživanja bio je utvrditi polimorfizam gena estrogenog receptora uporabom

restriksijske endonukleaze i njegovu povezanost s brojem mrtvooprasenih (BMO) i mumificiranih (BMUM) odojaka u prvo-, drugo-, treće- i višepraskinja te skupnom analizom prasenja. Istraživanjem je bila obuhvaćena 101 krmača hibrida Topigs 20 od prvog to sedmog prasenja. Polimorfizam gena estrogenog receptora utvrđen je cijepanjem umnoženih odsječaka gena restriksijskim enzimom *PvuII* nakon postupka lančane reakcije polimerazom. Utvrđena je prisutnost dvaju alela, A i B, te triju genotipova. Učestalost genotipova i alela provjerena je χ^2 -testom. Usporedbom je utvrđeno značajno odstupanje od Hardy-Weinbergova zakona utvrđenih i očekivanih frekvencija genotipova. Prikupljeni podatci analizirani su pomoću općeg linearnog modela izračuna (GLM). Statističkom obradom podataka utvrđena je značajna razlika ($P < 0,05$) između genotipa AB i BB te između genotipa AA i BB ($P < 0,1$) u treće- i višepraskinja s obzirom na BMUM. Krmače s prisutnim alelom A, genotipa AA imale su manji BMO, što ukazuje na poželjan učinak ovoga alela. Dobiveni rezultati omogućit će bolje razumijevanje učinka gena, odnosno genotipa estrogenog receptora na broj BMO i BMUM te njegovu moguću ulogu u genetskoj procjeni za obilježja veličine legla u svinja.

Ključne riječi: estrogen, genotip, mrtvoopraseni i mumificirani odojci, Topigs 20
