

Preliminary study of the *RBP4*, *EGF* and *PTGS2* genes polymorphism in pigs and its association with reproduction traits of sows*

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Genes of retinol-binding protein 4 (*RBP4*), epidermal growth factor (*EGF*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*) were examined as candidate genes for reproductive traits in sows. The material included a total of 300 sows of the Polish Landrace ($n=86$), Polish Large White ($n=64$) and commercial Line 990 ($n=150$). Included in the study was the total number of piglets born (TNB), number of piglets born alive (NBA), number of piglets on day 21 of age (NP21), number of piglets weaned (NPW), litter weight on day 21 of age (LW21), litter weight at weaning (LWW) and farrowing interval (FI). Calculations were based on records available for two farrowings (1st and 2nd) from each sow.

The *RBP4* genotype was found to have a significant effect on LW21 and LWW ($P \leq 0.05$ and $P \leq 0.01$, respectively). No significant effect of this genotype was identified for litter size traits (TNB, NBA). The effect of *EGF* genotype was found significant on TNB ($P \leq 0.01$) and NBA ($P \leq 0.05$). The *A/A* animals showed the highest reproductive efficiency compared to those of *B/B* and *A/B* genotypes. No significant effects of the *PTGS2* genotype were found due to a total absence of one of the homozygous genotypes at this *locus*.

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Molecular genetics technology is leading to the discovery of individual genes having a significant effect on traits of interest in pigs. The identification of such major genes and the underlying mutations can enhance rates of genetic improvement in the short term [Dekkers 2004] through the application of marker-assisted selection (MAS), which will increase as more associations between markers (genes) and traits are identified [Rothschild 1998]. Development of porcine genome maps offers also an opportunity to identify individual genes controlling reproduction. This technology seems to be especially promising for fertility traits such as litter size, as they are characterized by a low heritability [Drogemüller *et al.* 2001]. Over the past decade, a variety of genes named “candidate genes”, have been investigated for their possible association with reproductive traits in pigs [Isler *et al.* 2002].

In this study genes *RBP4*, *EGF* and *PTGS2* were selected as potential candidate genes for reproduction due to their integral role in several reproductive pathways.

In the organism the retinol acid (vitamin A) is derived from endogenous retinol, which is delivered to target cells by retinol-binding protein 4 (RBP4). The availability of retinol to *conceptus* is limited to that which can be transported from maternal capillaries across several boundaries of epithelium to the uterine lumen [Roberts *et al.* 1993]. Brief and Chew [1985] showed that supplementing the diet of pregnant sows with vitamin A can increase litter size. Assuming that *conceptuses* signal the uterine secretion of RBP4 and that they express high levels of RBP4 around day 12 of pregnancy, this indicates an important function of retinol and RBP4 at this stage [Trout *et al.* 1991]. The transport and buffering ability of RBP4 at this critical time of pregnancy makes *RBP4* a strong candidate gene for litter size in pigs [Rothschild *et al.* 2000].

Epidermal growth factor (EGF) is produced by *conceptus*. In foetus, EGF stimulates growth and proliferation of skin epithelium, whereas in neonate EGF stimulates the pulmonary maturation [Hadley 1996].

Prostaglandin-endoperoxide synthase 2 (PTGS2) also known as cyclooxygenase 2 (COX2), was chosen as it is the rate-limiting enzyme in the formation of prostaglandins [Lim *et al.* 1997] and is also involved in the synthesis of thromboxanes and prostacyclin. A null mutation was described [Wilson *et al.* 2002] producing multiple reproductive failures in mice - oocyte maturation was not complete and the first polar body was usually not extruded. An increased expression of cyclooxygenase 2 by the filamentous *conceptus* was associated with an increased content of prostaglandins (particularly prostaglandin E2) found in uterine luminal fluid.

The objective of the current study was to examine the associations between the polymorphism of genes *RBP4*, *EGF*, *PTGS2* and sow reproductive traits.

Material and methods

Animals

The animals examined were owned by the National Research Institute of Animal Production, Pig Hybridization Centre in Pawłowice and a single farm co-operating with the University of Technology and Agriculture in Bydgoszcz and comprised Polish Landrace (n=86), Polish Large White (n=64) and commercial Line 990 (n=150) sows. The sows from different breeds were kept in different herds. Two first litters from each sow were evaluated for the following reproduction traits: total number of piglets born (TNB), number of piglets born alive (NBA), number of piglets on day 21 of age (NP21), number of piglets weaned (NPW), litter weight on day 21 (LW21), litter weight at weaning (LWW) and farrowing interval (FI).

Molecular test

Genomic DNA was isolated from blood leukocytes according to Kawasaki [1990] using the WIZARD Kit. PCR-RFLP technique was used to genotype sows at the *RBP4*, *EGF* and *PTGS2* loci. Primer sequences for amplifying the PCR products and reaction conditions followed those described by Rothschild *et al.* [2000], Mendez *et al.* [1999] and Gladney *et al.* [1999]. Some of the reaction conditions were modified according to authors' own experience.

The polymorphism of *RBP4* was identified using restriction enzyme *MspI* while that of *PTGS2* using the *MseI* restriction enzyme. In case of *EGF* only the PCR product was identified.

Statistical

The relationships between the genotypes of candidate genes and reproductive traits were evaluated with the LSM (SAS, GLM) procedure. The models included the fixed effects of *RBP4*, *EGF*, *PTGS2* genotypes of the given sow, the breed of the sow, parity, year and season of farrowing, number of boar as well as covariate of additive and dominance effect of *RYR1* genotypes (*HAL^A* and *HAL^D*). The *RYR1* genotype was introduced in the models due to its known strong effect on reproduction traits, demonstrated in various populations. Additionally, the farrowing interval was included in the models as covariate for traits TNB, NBA and NP21.

Results and discussion

Effects of the *RBP4* genotype

The designed primers were located in exon 2 and exon 4 what made it possible to receive a 550 bp-long fragment of the gene *RBP4*. In this study both alleles – 1 and 2 – were represented (Photo 1). For whole tested population the frequency of individual alleles at this locus amounted to 0.68 and 0.32 for allele 1 and 2, respectively.

The results of the association analyses are presented in Table 1. No significant effect of *RBP4* genotype was identified for the TNB, NBA and FI. A significant effect

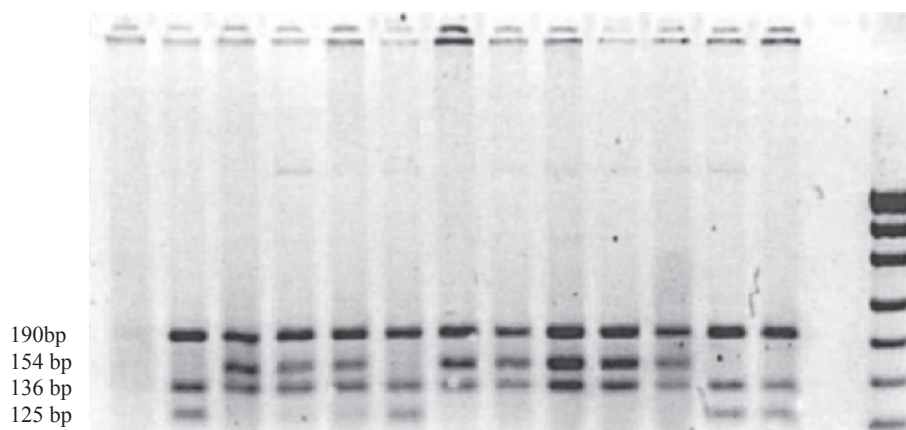


Photo 1. Digestion products of the fragment of porcine *RBP4* gene electrophoresed in a 3% agarose gel. The 190,154 and 136 bp-long bands are present in allele 1, while bands 190, 136 and 125 bp-long are characteristic for allele 2.

Table 1. Least square means (LSM) and their standard errors (SE) for the *RBP4/delo1* genotype effects on reproductive traits in cows (n=304*)

Trait ^a	Genotype 1/1 (n=228)		Genotype 2/1 (n=223)		Genotype 2/2 (n=53)	
	LSM	SE	LSM	SE	LSM	SE
INE	9.44	0.32	9.50	0.32	10.28	0.72
NBA	8.17	0.31	8.34	0.30	9.23	0.69
NP21	7.84	0.32	8.04	0.32	9.23	0.71
NPW	7.49	0.32	7.99	0.31	8.72	0.70
LW21	41.14 ^a	2.00	43.05	1.94	50.99 ^b	4.38
LWW	52.74 ^a	2.68	54.35	2.63	67.77 ^b	5.89
FI	158.10	2.27	154.90	2.23	153.67	4.94

* - Means in rows bearing different superscripts differ significantly at: small letters - $P \leq 0.05$; capital - $P \leq 0.01$.

^aINE - total number of piglets born; NBA - number of piglets born alive; NP21 - number of piglets at the age of 21 days; NPW - number of piglets weaned; LW21 - litter weight on day 21 (kg); LWW - litter weight at weaning (kg); FI - farrowing interval (days).

*Total number of observations (litters):

was observed for LW21 ($P \leq 0.05$) and LWW ($P \leq 0.01$) – in both cases the values for 2/2 genotype were higher than those for genotype 1/1. As regards NP21 and NPW the animals of genotype 2/2 showed slight and not significant tendency for the higher reproductive efficiency.

The gene *RBP4* was studied as a possible candidate gene affecting litter size due to its relationship with embryo development. Yelich *et al* [1997] stated that most embryonic

losses in pigs occur between day 10 and 18 of gestation, concurrent with trophoblast elongation. RBP4 as a major protein produced by the *conceptus* may play a role in trophoblast elongation [Harney *et al.* 1990]. Harney *et al.* [1993] showed that from day 10 to 12 there is an increasing *RBP4* expression in gravid porcine endometrium. Their results confirm the important role of this vitamin A transport-protein in the uterine and *conceptus* physiology during the establishment of pregnancy. Therefore, owing to the integral role of RBP4 at the time of high embryonic mortality rate [Drogemüller *et al.* 2001] the gene *RBP4* was investigated as a candidate gene for litter size. The fact that in the present study its polymorphic variants had no significant effects on TNB and NBA is, compared to results of other studies, somewhat unexpected. Messer *et al.* [1996] and Ollivier *et al.* [1997] reported effects associated with gene *RBP4* to be 0.52 ± 0.45 for litter size in French Large White sows. Rothschild *et al.* [2000] using records obtained for 2500 litters, also reported an additive effect associated with *RBP4*. Allele 1, mentioned by Rothschild as favourable, had a higher frequency while the differences between genotype 1/1 and 2/2 reached approximately 0.55 piglets/litter for TNB and 0.26 piglets/litter for NBA in some commercial lines of pigs. In turn, Drogemüller *et al.* [2001] reported that the frequency of allele 1 also was the highest, but the *RBP4* locus showed no significant effect on litter size in the synthetic line examined. Linville *et al.* [2001] presented similar results.

Effects of the *EGF* genotype

In the present study two fragments of gene *EGF* were received with the PCR-RFLP method: for allele *A* a 1527 bp-long fragment was amplified, while for allele *B* – a 652 bp-long fragment (Photo 2). For whole tested population the frequency of individual alleles at this locus amounted to 0.30 and 0.70 for allele *A* and *B*, respectively.

The *EGF* genotype had a significant effect on TNB ($P \leq 0.01$) and NBA ($P \leq 0.05$). The *A/A* animals showed the highest reproductive efficiency compared to those of *B/B* and *A/B* genotypes. Simultaneously, *B/B* sows had more numerous litters (TNB) as compared to animals with genotype *A/B*. A similar situation was observed as regards NPW. However, the difference proved to be significant only between sows of genotype *A/A* and *A/B* ($P \leq 0.01$) or *B/B* and *A/B* ($P \leq 0.05$). No significant difference was observed between homozygotes (Fig. 1).

The results presented justify the choice of the gene *EGF* as a candidate gene. It was identified as a candidate gene on positional (chromosome 8) as well as physiological basis. Earlier studies have provided evidence that on chromosome 8 are located QTLs affecting several reproductive traits in pigs [Wilkie *et al.* 1999, Rathje *et al.* 1997, Rohrer *et al.* 1999, King *et al.* 2003]. According to Wollenhaupt *et al.* [2002] the *EGF* receptor system is involved in regulating the proliferation and differentiation in oviductal and endometrial tissues. However, in the study by Linville *et al.* [2001] the association between polymorphism in gene *EGF* and reproductive traits could not be estimated because only two genotypes occurred in the tested population – the effect of *A/A* homozygote which proved most favourable in the present study could not be

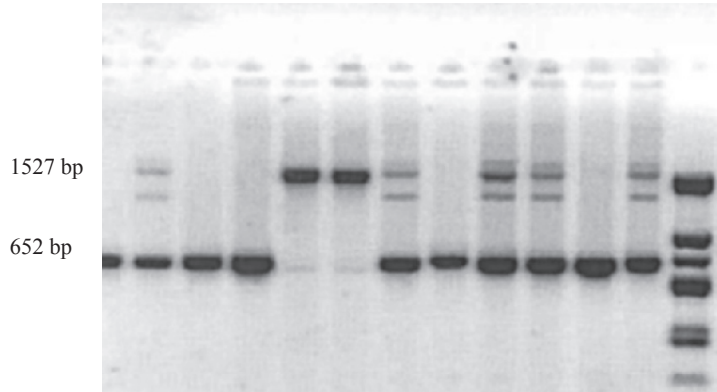


Photo 2. PCR product of the fragment of the porcine *EGF* gene. The 1527 bp bands are present in allele *A* while bands 652 bp-long are characteristic for allele *B*.

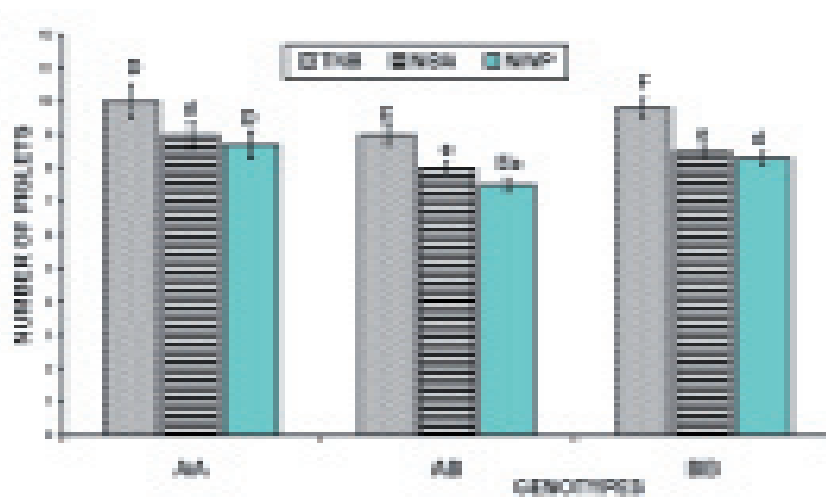


Fig. 1. Effect of *EGF* genotype on litter size. Total no. of observations = 550 (AA = 71, AB = 173, BB = 306).

^{D,e..}Within traits means represented by bars bearing different letters differ significantly at: small letters

analysed by the authors mentioned.

Effects of the *PTGS2* genotype

The 1550 bp-long fragment (spanning exon 3 and exon 4) was amplified. At this locus both alleles were present (Photo 3), but no homozygous *A/A* animal was detected.

The frequency of individual alleles at this *locus* amounted to 0.2 and 0.8 for *A* and *B* allele, respectively. Preliminary data concerning gene *PTGS2* as a candidate gene reported by Gladney *et al.* [1998], showed opposite results - allele *A* occurred with a higher frequency (0.68). In turn, Linville *et al* [2001] showed allele *A* to be favourable at the *PTGS locus*. Moreover, in their study the homozygote *B/B* was not found.

No significant effect of genotypes *PTGS2* was observed on litter size and piglet weight (Tab. 2), but animals of the *B/B* genotype showed no significant tendency to a lower efficiency of reproduction. TNB, NBA and NP21 were significantly affected by both the sow's breed and successive farrowing (parity), while NPW, PW21 and PWW only by successive farrowing. The FI was significantly affected by the sow's breed.

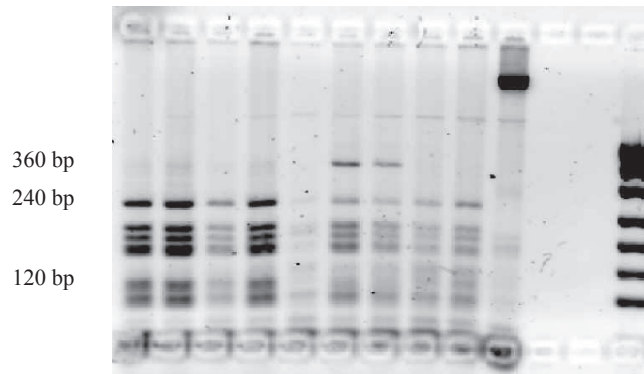


Photo 3. The PCR product of the fragment of porcine *PTGS2* gene electrophoresed in a 3% agarose gel. A 360 bp band is present in allele *A* while bands 240 and 120 bp-long are characteristic for allele *B*.

These results are similar to those recorded by Linville *et al* [2001], who had not found any significant association between the polymorphism within the gene *PTGS2* and reproduction traits. Also consistent with the present results are those obtained by Casady [1999] who performed a complete genome scan for QTLs affecting reproduction traits in an F2 population of Nebraska lines and found evidence ($P < 0.05$) of the presence of a QTL affecting ovulation rate on chromosome 9. The *PTGS2 locus* is located on chromosome 9. However, the QTL for ovulation rate found by Cassady [1999] was approximately at 1 cM position, whereas *PTGS2* has been mapped between markers S0295 and S0114, which are located at the positions 100 and 123 cM, respectively, to the USMARC linkage map [Gladney *et al* 1999]. It may be possible that the position of this gene is the reason for which candidate gene studied here does not have large effect on reproduction traits in the population analysed.

To conclude, significantly associated with genotypes at *locus RBP4* appeared only traits connected with the weight of piglets. The allele frequency and significant relationships between traits and genotypes were different from those reported by other authors. The results obtained in the present study demonstrate the difficulties in confirming the effect of candidate gene when testing various genetic groups of pigs.

Table 2. Least squares means (LSM) and their standard errors (SE) for the *PTGS2*696c1 genotype effects on reproductive traits in sows (n=309*)

Trait ¹	Genotype A/B (n=103)		Genotype B/B (n=404)	
	LSM	SE	LSM	SE
INE	10.92	0.84	10.48	0.48
NEA	9.74	0.77	9.23	0.43
NP21	9.74	0.83	8.29	0.44
NPW	9.39	0.81	8.14	0.43
LW21	51.34	5.53	43.91	3.07
LWFW	47.50	7.40	37.74	4.21
FL	182.45	7.49	179.12	3.90

¹Trait abbreviations are explained at the bottom of Table 1.

*Total number of observations (litters).

No significant inter-genotype differences were found.

The study presented here of candidate gene *PTGS2* has equivocally validated the results of genome-wide QTLs searches. There has been a relatively small correspondence between candidate genes and QTL locations, identified for litter size or its component traits. This lack of correspondence between them could be due to the fact that the effect of some candidate genes is too small to be detected for the sample size employed in this research, to the lack of segregation of the candidate genes alleles in some populations, or to the fact that the originally observed candidate gene effects may, in some cases, have been detected due to chance [Kirpatrick 2002]. However, sometimes the results of association between gene and traits of interest confirm the results of the QTL analysis. The *EGF* gene was identified as a candidate gene both on positional (chromosome 8) and physiological basis. The present study provides evidence supporting the assumption that on porcine chromosome 8 is located a QTL for prolificacy.

In the current study all the nucleotide polymorphisms analysed in the porcine genes *RBP4*, *EGF* and *PTGS2* were found in introns. Although the SNPs in introns do not alter directly any functionally important amino acid residue, they may prove useful as markers for functional SNP *via* linkage disequilibrium mapping. In addition, some introns play a role in regulating gene expression and thus their constituent SNPs may be directly related to a functional variation [Jiang *et al.* 2002].

The results presented in this paper are only part of a major research project, but they show that the genes analysed should be further examined as possible candidate genes. A more conclusive proof of the effect of those genes on litter size would necessitate the examination of a large sample population. Further investigations will comprise data obtained from genotyping about 800 sows and at least four parities for each sow. It gives possibility of breed-specific statistical analysis on different genetic background.

This should render it possible to conclude about genes which have a major impact on the improvement of reproductive efficiency.

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Wstępna charakterystyka polimorfizmów w obrębie genów *RBP4*, *EGF* i *PTGS2* i ich związku z cechami reprodukcyjnymi świń

Streszczenie

Geny *RBP4* (retinol-binding protein 4), *EGF* (epidermal growth factor) i *PTGS2* (prostaglandin-endoperoxide synthase 2) badano jako geny kandydujące cech reprodukcyjnych macior. Materiał stanowiło

RBP4, EGF and PTGS2 genes polymorphism in pigs

300 macior ras pbz , wbp i linii syntetycznej 990 (odpowiednio n = 86, 64 i 150) Na podstawie danych pochodzących z dwóch pierwszych miotów, analizowano zależność między genotypami badanych białek a poziomem wybranych cech związanych z liczebnością i masą miotu, a także okresem międzymiotu. Istotne zależności stwierdzono między genotypami *RBP4* a masą miotu i między genotypami *EGF* a liczebnością miotu. W obrębie genu *PTGS2* nie zaobserwowano żadnych istotnych zależności. Jednak w przypadku *PTGS2*, w testowanym materiale stwierdzono występowanie homozygot tylko jednego typu.

