



Erythropoietin receptor gene polymorphism in Indian pig lines

S PRAMOD¹, A P USHA² and R THIRUPATHY VENKATACHALAPATHY³

Kerala Veterinary and Animal Sciences University, Thrissur, Kerala 680 651 India

Received: 21 November 2014; Accepted: 28 September 2015

ABSTRACT

A restriction fragment length polymorphism (RFLP) based genotyping test was developed to assess a C/T polymorphism in the regulatory region of erythropoietin receptor (EPOR) gene of pigs. Homozygous CC animals were absent in all the genetic groups studied, whereas the locus appeared fixed with T alleles in indigenous animals. The association of allelic variants with traits like litter size at birth (LSB), litter size at weaning (LSW), litter weight at birth (LWB) and litter weight at weaning (LWW) were explored in Indian Large White Yorkshire (LWY), Duroc, Indigenous Ankamali and F₁ crosses between LWY boars and Ankamali sows (CB). All the populations under study had very high frequency of T alleles, with *desi* group being fixed with T allele. Genotypes of EPOR gene were not associated with any of the litter traits. The allele frequencies remained under HWE except in *desi* group.

Key words: Erythropoietin receptor (EPOR), Litter traits, Pig

The domestic pig is a litter bearing animal which is an excellent source of red meat. Even though pig is a litter bearing animal, prenatal mortality is very high among them. About 20–30% of foetuses die before day 30 and another 10–20% dies during the period from day 40 to parturition (Klemcke *et al.* 2001). Lack of space within uterus for all the developing foeti was identified as an important reason for such loss of piglets. Further, it was suggested that intrauterine crowding resulted in impaired fetal erythropoiesis and that breed differences existed in the rate of blood cell development (Vallet *et al.* 2003). The concentration of circulating RBC's is controlled by the hormone erythropoietin (EPO), which acts through the EPO receptor (EPOR). EPO enhances RBC count through inhibition of apoptosis or increased cell division. The coding sequence of EPOR gene was 1,843 bp, with a putative protein of 509 residues. A single nucleotide polymorphism (C/T) which created an extra GATA-1 site (T allele) in intron 4 of the swine EPOR gene was identified and a genotyping assay was described by them for the same, based on primer extension and mass spectrometry (Vallet *et al.* 2005). The foetal genotype was associated with a functional increase in the expression of the EPOR gene and improved foetal survival. The present article discusses the development of a PCR-RFLP based genotyping test for this polymorphism besides exploring its association with traits like litter size at birth (LSB), litter size at weaning (LSW), litter weight at birth (LWB), litter weight at weaning (LWW) pre-

weaning mortality (PWM) and weight gained till weaning (WGW).

MATERIALS AND METHODS

The 5,425 bp sequence of EPOR gene submitted by Xi and Di (2008) was retrieved from Genbank (accession no. EU407778.1) and the primers described by Vallet *et al.* (2005) for amplifying a 116 bp product were located using the tool, 'Primerblast' (Ye *et al.* 2012). The product was predicted to lie between 2,308 and 2,423 bp. The polymorphic site described by Vallet *et al.* (2005) was located inside the extracted sequence. A new reverse primer was designed 5'-GGATGAGAGGCGTGGTCAA-3' and used along with the forward primer, 5'-CTACCTGGTCCCGTTCTG-3' reported by Vallet *et al.* (2005), so as to amplify a 180 bp polymorphic fragment. Neb cutter (V2.0), a web based tool (Vincze *et al.* 2003) for restriction analysis was used to predict the expected restriction pattern. The set of fragments predicted were therefore 104 bp, 56 bp and 20 bp for C allele and 124 bp and 56 bp for T allele, upon digestion with the enzyme *Mni*I.

Data on LSB, LWB, LSW and LWW were recorded for the association study from 75 Large White Yorkshire (LWY) animals, 22 Ankamali sows, 45 crossbred (CB) and 18 Duroc sows. The CB animals were F₁ crosses between Ankamali sows and LWY boars. Blood samples were drawn from ear vein of these animals and DNA was isolated using Phenol-Chloroform method. Polymerase chain reactions (PCR) were performed in a volume of 10 µl, so as to contain about 75 to 100 ng of template DNA, 5 pM each of forward and reverse primers, 200 µM of each dNTP's, 2.5 mM of MgCl₂, 0.75 U Taq polymerase and polymerase buffer to a

Present address: ¹Veterinary Surgeon (dr.prs@hotmail.com), District Animal Husbandry Office, Kannur. ²Professor and Head (apusha@kvasu.ac.in), ³Associate Professor (thirupathy@kvasu.ac.in).

final concentration of 1X, diluted with autoclaved ultra-filtered water. The preparations were amplified in a thermal cyclor. To avoid spurious amplification, 1.5% V/V formamide (Sarkar *et al.* 1990) was also included in the reaction mixture. The PCR products were co-incubated with one unit of *MniI* overnight in the same tube at 37°C, along with its buffer in a volume of 15 µl. The digested amplicons were resolved out in 6% non-denaturing polyacrylamide gel along with 50 bp DNA ladder and were silver stained according to the protocol developed by Qu *et al.* (2005). Allele frequencies were calculated using direct count method and were analysed using *Chi* square test for the identification of differences between breed. The gene frequencies were examined for deviations from Hardy-Weinberg equilibrium using *Chi* squared test for testing the equilibrium.

Association study was performed using the linear model $Y_{ijklm} = \mu + G_i + B_j + A_k + S_l + Si_m + e_{ijklmn}$ in SAS (Version 9.1) where μ is the general effect; G_i represented the genotypes of EPOR (CC or CT); B_j represented breed (j, 1–4); A_k represented groups based on age (k, 1–5); S_l represented season (l, 1–3); Si_m represented sires (m, 1–44) and e_{ijklmn} stands for random error. The sows were grouped on the basis of age as less than one year, 1 to 1_{1/2} years, 1_{1/2} to 2 years, 2 to 2_{1/2} years and 2_{1/2} to 3 years.

RESULTS AND DISCUSSION

The overall least square means and means observed in different genetic groups under study are presented in Table 1. Considering breed wise means, litter size at weaning ($P \leq 0.01$) and weight gained till weaning ($P \leq 0.05$) differed significantly among genetic groups (breeds). The mean LSW of LWY in the present study was marginally higher than that observed by Singh *et al.* (2002) and Palve *et al.* (2000) which were 7.84 and 7.48, respectively. The LSW of Large White pigs was lower than that reported by Wolf *et al.* (2008) in Czech population. The F₁ crosses between LWY and *desi* of Andhra Pradesh performed better than those from the present study (Prasanna *et al.* 2009).

The results point out that genotyping of polymorphic region of EPOR gene is also possible using PCR-RFLP, as against primer extension and mass spectrometry reported by Vallet *et al.* (2005). The predicted as well as the observed restriction pattern confirms the presence of a non polymorphic restriction site within the PCR product. The ‘C’ allele of the gene presented an additional restriction

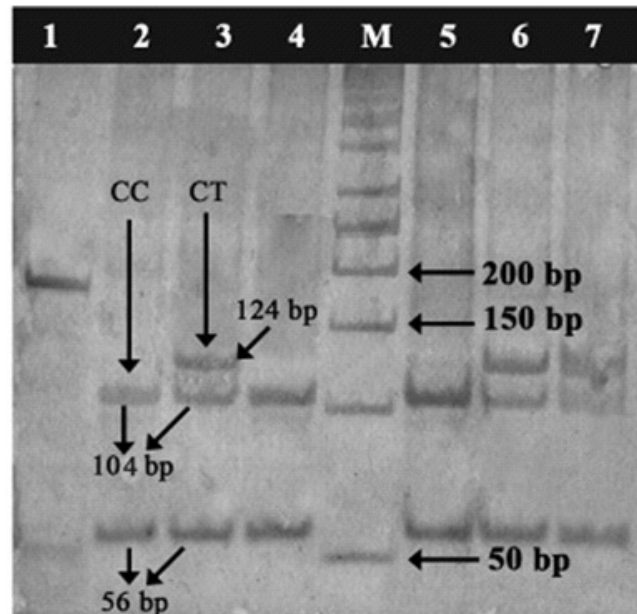


Fig. 1. Alleles from EPOR locus resolved on 6% polyacrylamide gel and visualized by silver stain. Lane 1, PCR product (180 bp); Lanes: 2,3,5, genotype CC–104 bp, 56 bp (20 bp fragment was not observed on gel); Lanes 3,6,7,8, genotype CT–124 bp, 104 bp, 56 bp.

site for the enzyme *MniI* (5' CCTC(N)₇) whereas this site would be absent in T allele in which the 5' C is replaced by the nucleotide T. The test provide a cheaper alternative to primer extension and mass spectrometry used by the original authors (Vallet *et al.* 2005) for the genotyping of this locus. The restriction pattern observed is presented in Fig. 1. The 20 bp fragment was not observed on gel, except for this the banding pattern observed was as predicted by Neb Cutter (V 2.0).

Homozygous CC genotype was not observed in any of the genetic groups under study. Ankamali pigs were apparently fixed with T allele in this locus. The frequency of C allele was highest in LWY sows. Between group allelic and genotypic frequencies were analyzed using *Chi* square test. Results indicated that the frequency of alleles and genotypes in case of Ankamali differed significantly from that of other genetic groups under study. The allele frequencies and genotype frequencies observed in the study are presented in Table 2. The figures in parenthesis represent the number of observations.

Table 1. Least squares means±SE of litter traits

Breed	LSB	LSW	LWB	LWW	PWM	WGL
LWY (75)	10.53±1.03	8.05±0.85 ^{a*}	10.71±1.28	68.58±7.41	2.56±0.80 ^a	46.71±5.54
Ankamali (22)	6.97±0.55	4.87±0.54 ^{b*}	4.57±0.78	35.40±4.00	3.46±0.94 ^b	41.26±6.52
CB (45)	10.45±0.99	7.40±0.82 ^{c*}	10.46±1.22	53.6±17.11	1.85±0.82 ^c	59.51±5.67
Duroc (18)	10.49±1.13	6.13±0.94 ^{d*}	11.64±1.40	48.98±8.15	5.03±1.42 ^d	31.78±9.87
Overall (160)	9.41±1.12	6.46±1.05	8.66±1.43	50.38±9.21	3.27±0.18	42.07±1.36

Means with different superscript within a column differ significantly $P \leq 0.01$; $*P \leq 0.05$; Figures in parenthesis represent number of observations.

Table 2. Frequency of genotypes and alleles at EPOR locus

Breed	Genotype frequency		Allele frequency	
	CT	TT	C	T
LWY (75)	0.27 (20)	0.73 (55)	0.13	0.87
Desi (22)	0.00** (0)	1.0** (22)	0.00*	1.0*
CB (45)	0.11 (5)	0.89 (40)	0.06	0.94
Duroc (18)	0.22 (4)	0.78 (14)	0.11	0.89
Overall (160)	0.18 (31)	0.82 (162)	0.09	0.91

** $P \leq 0.01$; * $P \leq 0.05$.

The allele frequencies were in equilibrium among Large White Yorkshire, Duroc and crossbred genetic groups. *Chi* square values were not calculated for Ankamali group as the observed frequency was zero for two of the genotypes.

Results from statistical analysis using general linear model revealed that genotype had no significant effect on any of the litter traits studied. Sire apparently had a significant effect on LSB ($P \leq 0.01$), LWB and PWM ($P \leq 0.05$). Wolf and Wolfova (2012) had observed that sires contribute to variance in Czech Large White and Landrace breeds, which supports the results of the present study. In contrast, studies on LWY, Crossbred and Duroc pigs by Raghavendran *et al.* (2013) ruled out significant effect of sire on litter traits.

Age of sows appeared to be a significant ($P \leq 0.05$) factor affecting pre, weaning mortality in piglets. Parity or age of sows consistently appeared to have a significant effect on litter traits as observed by Raghavendran *et al.* (2013) and Akdag *et al.* (2009). Season of farrowing was not associated with any of the litter traits covered in the present study. The report by Sai *et al.* (2009) that higher litter size was observed on rainy season was also in contrast with the findings of the present study. Wolf *et al.* (2008) also observed significant effect of herd-year-season on litter traits.

The Ankamali pigs used for the present study were obtained from the state of Kerala. The state is a narrow stretch of land, separated from Deccan plateau by the Western Ghats, which acts as a continuous natural boundary in the east, except for a few gaps. This should have allowed the animals to live in relative isolation resulting in the loss of C allele due to random drift or founder effect resulting in the fixation of T allele. Fixation of loci was reported earlier in indigenous pig breeds of China. The missing alleles, however, were found among European pig population of China, as reported by Bao *et al.* (2008) while working on Fucosyl transferase 1 gene. Therefore, the loss of allelic diversity in *desi* pigs of Kerala could not be viewed as an unusual finding.

The frequency of T allele reported in unselected pig populations of USA was as low as 0.028 (Vallet *et al.* 2005), whereas the values were much higher among all genetic groups covered in the present study (Table 2). The selective pressure exerted by environment in human populations of

different habitats are well documented (Hancock *et al.* 2010). Adaptation to a particular environment could actually depend on the alleles of genes possessed by the individual. Considering the results of present study, the T allele apparently has some sort of selective advantage under the geographical and climatic conditions prevailing in Kerala, whereas C allele could be the favored one in USA.

The conditions for HWE was reported to hold good for most human populations and deviations usually resulted from changes in population structure, sampling and genotyping errors (Wigginton *et al.* 2005). Since, HWE is maintained in all genetic groups except *desi*, it could be assumed that pig populations by and large remain in HWE, possibly due to high fecundity. Since, the *desi* animals were monomorphic at this locus, HWE could not be tested.

Reports indicate that foetal, rather than maternal genotype of EPOR gene had significant effect on litter size at birth (Vallet *et al.* 2003; Vallet *et al.* 2005). The present study tested the hypothesis that EPOR genotype of dam had significant effect on litter traits. The study focused on maternal genotype rather than foetal genotype and had failed to identify any such association. Other litter traits like LSW, LWB and LWY were also tested for significant associations but nothing of that sort could be identified.

ACKNOWLEDGEMENT

The authors acknowledge INSPIRE Fellowship awarded by Ministry of Science and Technology (India), Funds provided by KVASU, ICAR and Laboratory facility provided by CASAGB, Mannuthy.

REFERENCES

- Akdag F, Arslan S and Demir H. 2009. The effect of parity and litter size on birth weight and the effect of birth weight variations on weaning weight and pre-weaning survival in piglets. *Journal of Animal and Veterinary Advances* **8** (11): 2133–38.
- Bao W B, Wu S L, Musa H H, Zhu G Q and Chen G H. 2008. Genetic variation at the alpha (1) fucosyltransferase (FUT1) gene in Asian wild boar and Chinese and Western commercial pig breeds. *Journal of Animal Breeding and Genetics* **125**: 427–30.
- Hancock A M, Alkorta-Aranburu G, Witonsky D B and Rienzo A D. 2010. Adaptations to new environments in humans: the role of subtle allele frequency shifts. *Philosophical Transactions of Royal Society of London B Biological Science* **365** (1552): 2459–68.
- Klemcke H G, Vallet J L, Christenson R K and Pearson P L. 2001. Erythropoietin mRNA expression in pig embryos. *Animal Reproduction Science* **66**: 93–108.
- Palve S, Maitra D N, Pyne A K, Roy S K and Majumder S C. 2000. Effect of season on litter traits of large white yorkshire pigs in West Bengal. *Indian Veterinary Journal* **77** (7): 640–42.
- Prasanna S J, Prakash M G, Gupta B R and Mahender M. 2009. Genetic study on reproductive traits in crossbred pigs. *Livestock Research for Rural Development*. [online] **21** (9). Available: <http://www.lrrd.org/lrrd21/9/pras21142.htm> [23 Nov. 2012].

- Qu L, Li X, Wu G and Yang N. 2005. Efficient and sensitive method of DNA silver staining in polyacrylamide gels. *Electrophoresis* **26**: 99–101.
- Raghavendran V B, Thirupathi Venkatachalapathy R, Usha A P and Pramod S. 2013. Genetic polymorphism of retinol binding protein 4 locus and its association with litter traits in pigs. *Indian Journal of Animal Sciences* **83** (11): 1179–82.
- Sai P J, Prakash M G, Gupta B R and Mahender M. 2009. Genetic study on reproductive traits in crossbred pigs. *Livestock Research for Rural Development*. [online] 21 (9). Available: <http://www.lrrd.org/lrrd21/9/pras21142.html> [23 Nov. 2012].
- Sarkar G, Kapelner S and Sommer S S. 1990. Formamide can dramatically improve the specificity of PCR. *Nucleic Acids Research* **18** (24): 7465.
- Vallet J L, Klemcke H G and Christenson R K. 2005. Allelic variation in the erythropoietin receptor gene is associated with uterine capacity and litter size in swine. *Animal Genetics* **36**: 97–103.
- Vallet J L, Klemcke H G, Christenson R K and Pearson P L. 2003. The effect of breed and intrauterine crowding on fetal erythropoiesis on day 35 of gestation in swine. *Journal of Animal Science* **81**: 2352–56.
- Vincze T, Posfai J and Roberts R J. 2003. NEB cutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Research* **31**: 3688–91.
- Wigginton J E, Cutler D J and Abecasis G R. 2005. A note on exact tests of Hardy-Weinberg Equilibrium. *American Journal of Human Genetics* **76**: 887–83.
- Wolf J, •áková E and Groeneveld E. 2008. Within-litter variation of birth weight in hyperprolific Czech Large White sows and its relation to litter size traits, stillborn piglets and losses until weaning. *Livestock Science* **115**: 195–205.
- Xi G and Di L. 2008. Clone polymorphism analysis on pig GnRH receptor gene and EpoR gene and the construction of tissue expression spectrums of relating genes. Submitted (JAN–2008) to the EMBL/GenBank/DDBJ databases.
- Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S and Madden T. 2012. Primer-BLAST: A tool to design target specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**:134.