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Veterinary Microbiology 126 (2008) 210–215

veterinary microbiology

www.elsevier.com/locate/vetmic

The possibility of positive selection for both F18$^+$ *Escherichia coli* and stress resistant pigs opens new perspectives for pig breeding

Annelies Coddens [a], Frank Verdonck [a], Martin Mulinge [a], Els Goyvaerts [d], Cora Miry [e], Bruno Goddeeris [b], Luc Duchateau [c], Eric Cox [a,*]

[a] *Laboratory of Veterinary Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium*

[b] *Department of Biosystems, Faculty of Bioscience Engineering, K.U. Leuven Kasteelpark Arenberg 30, B-3001 Heverlee, Belgium*

[c] *Department of Physiology and Biometrics, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium*

[d] *Dierengezondheidszorg Vlaanderen, Hagenbroeksesteenweg 167, 2500 Lier, Belgium*

[e] *Dierengezondheidszorg Vlaanderen, Industrielaan 29, 8820 Torhout, Belgium*

Received 23 April 2007; received in revised form 19 June 2007; accepted 25 June 2007

Abstract

F18$^+$ *Escherichia coli* infections causing post-weaning diarrhoea and/or oedema disease are a major cause of economic losses in pig industry. To date, no preventive strategy can protect pigs from F18$^+$ *E. coli* infections. One of the most attractive approaches to eliminate F18$^+$ *E. coli* infections is the selection for pigs that are resistant to F18$^+$ *E. coli* infections. However, this strategy was not believed to be favourable because of reports of genetic association with the stress-susceptibility gene in the Swiss Landrace. To investigate this potential association more thoroughly, 131 randomly selected Belgian hybrid pigs were genotyped for both the F18$^+$ *E. coli* resistance alleles ($FUT1^A$) and the stress-susceptibility alleles ($RYR1^T$) and their association was investigated by determining the linkage disequilibrium. This linkage disequilibrium (LD = − 0.0149) is close to zero and does not differ significantly from 0 (likelihood ratio test $\chi^2_1 = 1.123$, $P = 0.29$), demonstrating no association between the $FUT1^A$ and $RYR1^T$ alleles. Furthermore, only a small fraction (4.6%) of the Belgian pigs was found to be resistant to F18$^+$ *E. coli* infections. Our results suggest that selection for F18$^+$ *E. coli* resistant pigs might be an attractive approach to prevent pigs from F18$^+$ *E. coli* infections, unlike to what has previously been postulated.

Keywords: F18$^+$ *Escherichia coli* (F107/86); Pig; *RYR1*; *FUT1*; Porcine stress syndrome

1. Introduction

Many pig farms suffer from severe economical losses due to outbreaks of post-weaning diarrhoea or

* Corresponding author. Tel.: +32 92647396; fax: +32 92647496.
E-mail address: Eric.Cox@UGent.be (E. Cox).

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doi:10.1016/j.vetmic.2007.06.021

oedema disease in young pigs. F18$^+$ *Escherichia coli* infections are a major cause of both diseases. A prerequisite for these infections is the adherence of F18$^+$ *E. coli* to specific F18 receptors (F18R) on the porcine gut epithelium, followed by colonization of the gut and excretion of entero- and/or verotoxins leading to diarrhoea and/or oedema disease, respectively.

The prevalence of F18$^+$ *E. coli* infections is estimated to be very high in Belgium (up to 96% of Flemish pig farms were seropositive) (Verdonck et al., 2003). Nevertheless, some pigs are found to be resistant to colonization by F18$^+$ *E. coli* due to lack of F18R expression. Extensive mating studies revealed that the F18R status of pigs is genetically determined with susceptibility dominating resistance (Bertschinger et al., 1993). The F18R locus has been genetically mapped to the halothane linkage group on porcine chromosome 6 and it was found that *FUT1*, encoding an α(1,2)fucosyltransferase, is a candidate gene for this locus (Meijerink et al., 1997; Meijerink et al., 2000). Sequencing of the *FUT1* gene revealed a polymorphism (G or A) at nucleotide 307. The F18$^+$ *E. coli* resistant pigs showed presence of the A nucleotide on both alleles ($FUT1^{A/A}$ genotype), whereas pigs susceptible to F18$^+$ *E. coli* had either the heterozygous $FUT1^{G/A}$ or the homozygous $FUT1^{G/G}$ genotype.

The HAL-locus, which also belongs to the halothane linkage group, determines whether pigs are susceptible for malignant hyperthermia, an inherited neuromuscular disorder that is triggered by inhalational anaesthetics (halothane), skeletal muscle relaxants (succinylcholine), as well as stress (Reik et al., 1983). Therefore, this disease is often referred to as the porcine stress syndrome (PSS). The underlying cause of this disease is the dysfunction of the Ca^{2+} release channel (ryanodine receptor, RYR) of the skeletal muscle sarcoplasmatic reticulum (O'Brien, 1986; Murayama et al., 2007), due to a mutation (C → T) at nucleotide 1843, resulting in substitution of Cys^{615} for Arg in the ryanodine receptor (Fujii et al., 1991).

In the present study, the prevalence of both the F18$^+$ *E. coli* resistance allele ($FUT1^A$) and the malignant hyperthermia susceptibility allele ($RYR1^T$) in the Belgian pig population is estimated. Furthermore, the association of the $FUT1^A$ alleles and the $RYR1^T$ alleles is investigated in order to address the possibility to select for both stress resistance and F18$^+$ *E. coli* resistance. Association of both alleles would have the negative side effect that an increase of the stress-susceptibility allele frequency would occur when selecting for F18$^+$ *E. coli* resistant pigs. Our results will enable us to formulate advices on the usefulness or potential drawbacks towards positive selection for F18$^+$ *E. coli* resistant pigs in the Belgian pig population.

2. Materials and methods

2.1. Survey sampling

Blood samples from pigs out of five Belgian provinces were randomly collected by veterinary practitioners and were sent to our laboratory by the regional veterinary investigation centres. Twenty-one percent of the examined pigs were sows and 79% were fattening pigs. The pigs had different ages and were randomly selected from both open and closed farms. The number of collected samples per province was accorded to the number of pig-rearing farms present in each province (West-Flanders $n = 67$, East-Flanders $n = 33$, Antwerp $n = 18$, Limburg $n = 10$, Flemish Brabant $n = 3$). A total of 131 pigs from 36 different farms with a maximum of five pigs per farm were successfully genotyped for both the F18$^+$ *E. coli* and stress-susceptibility genes. The selected pigs were not purebred, but hybrid in nature.

2.2. DNA extraction

DNA was extracted from whole blood using the following procedure. One hundred microliters blood was washed three times by adding 1 ml TE buffer (10 mM Tris–HCl + 1 mM EDTA; pH 7.5) followed by centrifugation at 3800 × *g* for 1 min. Next, 200 μl K-buffer (50 mM KCl + 20 mM Tris–HCl + 2.5 mM $MgCl_2$ + 0.5% Tween 20; pH 8.3) was added to the cell pellet, followed by 2 μl proteinase K (Boehringer Mannheim) and this mixture was incubated at 56 °C during 1 h. Inactivation of proteinase K was established by a 10-min incubation at 95 °C. After centrifugation (3800 × *g*, 1 min), the supernatant containing the genomic DNA was collected.

2.3. Determination of the F18$^+$ *E. coli* genotype

PCR-RFLP based on the G → A mutation at nucleotide 307 of the *FUT1* gene was used for genotyping the pigs (Meijerink et al., 1997). Hereto, a fragment of the *FUT1* gene was amplified using specific primers (M307F: 5′-CTTCCTGAACGTC-TATCAAGACC-3′, M307R: 5′-CTTCAGCCAGGG-CTCCTTTAAG-3′), followed by digestion with the CfoI restriction enzyme (Promega) (Fig. 1A).

2.4. Determination of the malignant hyperthermia genotype

In order to detect the C → T mutation on base pair 1843 of the *RYR1* gene, a PCR-RFLP-test was executed as described by Otsu et al. (1992). A fragment of the *RYR1* gene was amplified by PCR using specific primers (RYR1F: 5′-TCCAGTTTGC-CACAGGTCCTACCA-3′, RYR1R: 5′-ATTCACCG-GAGTGGAGTCTCTGAG-3′). This resulted in a PCR product of 659 bp, which was subsequently digested using Alw21I (HgiAI) (Fermentas) (Fig. 1B).

2.5. Data analysis

Confidence intervals for the prevalence of the alleles were based on the binomial distribution assumption. Heterogeneity of the prevalence of the alleles between farms was studied by a generalized mixed model with a binomially distributed error term and with farm as normally distributed random effect. The intraclass correlation coefficient (ICC), a measure for the clustering of the allele in a farm, was obtained from this model and it was tested whether it differed significantly from zero.

To investigate the association between the $FUT1^A$ alleles and the $RYR1^T$ alleles, the linkage disequilibrium (LD) between the alleles was estimated and it was further tested whether the LD differs from zero according to the methodology discussed by Hill (1979), which is based on the likelihood ratio test. In addition, the correlation coefficient between the two alleles was derived.

3. Results

3.1. Prevalence of the F18$^+$ *E. coli* and stress-susceptibility alleles

The prevalence of the F18$^+$ *E. coli* susceptibility genotypes $FUT1^{G/G}$ and $FUT1^{G/A}$ in Northern Belgium was estimated to be 62.6% (confidence interval (CI) 95% 53.7–70.9) and 32.8% (CI 95% 24.9–40.9), respectively. The $FUT1^{A/A}$ genotype referring to resistance to F18$^+$ *E. coli* infections was present in 4.6% (CI 95% 1.7–9.7) of the cases (Fig. 1). The frequency of the $FUT1^A$ and $FUT1^G$ allele was 0.21 and 0.79, respectively.

The prevalence of the stress resistance genotypes $RYR1^{C/C}$ and $RYR1^{C/T}$ in Northern Belgium was found to be 67.2% (CI 95% 58.4–75.1) and 30.5% (CI 95% 22.8–39.2), respectively, whereas the stress-susceptibility genotype $RYR1^{T/T}$ accounted for 2.3% (CI 95% 0.5–6.5). Allele frequencies for the $RYR1^C$ and $RYR1^T$ alleles were 0.81 and 0.19, respectively.

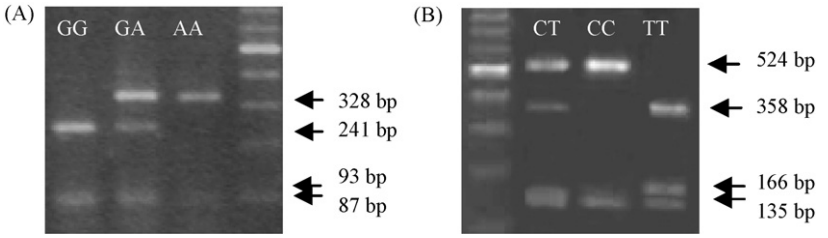

Fig. 1. Detection of (A) the FUT1 G to A and (B) the RYR1 C to T mutation by RFLP. (A) Digestion of amplified *FUT1* fragments with CfoI results in fragments of 241, 93 and 87 bp for the $FUT1^{G/G}$ genotype, whereas the $FUT1^{G/A}$ genotype generates fragments of 328, 241, 93 and 87 bp and the $FUT1^{A/A}$ genotype creates fragments of 328 and 93 bp. (B) Digestion of *RYR1* PCR-product with *Alw21*I generates fragments of 524, 358, 166 and 135 bp for the $RYR1^{C/T}$ genotype, while the $RYR1^{C/C}$ genotype results in fragments of 524 and 135 bp and the $RYR1^{T/T}$ genotype generates fragments of 358, 166 and 135 bp.

In addition, the heterogeneity over farms for the $FUT1^A$ and $FUT1^G$ alleles and the $RYR1^C$ and $RYR1^T$ alleles was investigated. For the $FUT1^G$ and $FUT1^A$ alleles, no differences could be found between the farms. However, when evaluating the heterogeneity of the $RYR1^C$ and $RYR1^T$ alleles on Belgian farms, an intraclass correlation coefficient of 0.76 ($P < 0.0001$) was found, revealing clustering of $RYR1^C$ or $RYR1^T$ alleles on the Belgian farms.

3.2. Association between the F18$^+$ *E. coli* resistance alleles and stress-susceptibility alleles

Linkage disequilibrium refers to the non-random association of alleles at distinct loci and is a reflection of the differences between the observed and expected haplotype frequencies, supposed that the two loci are not independent (Pritchard and Przeworski, 2001). In the present study, the linkage disequilibrium LD equals −0.0149, and does not differ significantly from 0 ($\chi^2_1 = 1.123$, $P = 0.29$). Accordingly, the correlation coefficient ($r = -0.096$) showed no significant correlation between both alleles. In Table 1, it can be seen that 67% of the $FUT1^{A/A}$ pigs ($n = 6$) showed the $RYR1^{C/C}$ genotype, while 33% the $RYR1^{C/T}$ genotype and none the stress-susceptible $RYR1^{T/T}$ genotype. For pigs with the heterozygous F18$^+$ *E. coli* susceptible genotype ($FUT1^{G/A}$ genotype, $n = 43$), it was found that 60% were homozygous stress resistant ($RYR1^{C/C}$), 35% were heterozygous stress resistant ($RYR1^{C/T}$) and 5% stress-susceptible ($RYR1^{T/T}$), whereas for pigs with the homozygous F18$^+$ *E. coli* susceptible genotype ($FUT1^{G/G}$ genotype, $n = 82$), it was shown that 71% had the $RYR1^{C/C}$ genotype, 28% had the $RYR1^{C/T}$ genotype and 1% the $RYR1^{T/T}$ genotype.

Table 1
Correlation between F18$^+$ *E. coli* and stress-susceptibility genotypes

FUT1	RYR1		
	CC ($n = 88$)	CT ($n = 40$)	TT ($n = 3$)
GG ($n = 82$)	58	23	1
GA ($n = 43$)	26	15	2
AA ($n = 6$)	4	2	0

4. Discussion

The discovery of the halothane challenge test for phenotyping PSS pigs (Webb, 1980), even as the identification of the causal mutation in the porcine ryanodine receptor (Fujii et al., 1991; Rempel et al., 1993) was highly important to decrease the high prevalence of stress susceptibility in the Piétrain and Landrace pigs in the late seventies (Van Zeveren et al., 1988). From that moment, the frequency of the stress-susceptible genotype in the Belgian landrace was monitored accurately and decreased strongly. However, the majority of the Piétrain boars used for production of fattening pigs is still stress susceptible because of the relation of this gene with excellent carcass meat quality (De Smet et al., 1996). The combination of these boars with a homozygous stress resistant sow leads to a mainly heterozygous stress resistant offspring. Belgian pig farmers are encouraged to select for stress negative pigs by the ban on tranquilizers during transportation and by the demand for stress-negativity to achieve the Belgian CERTUS-quality label for porcine meat products.

Besides the prevalence, clustering of $FUT1^A$ or $FUT1^G$ alleles and $RYR1^C$ or $RYR1^T$ alleles within farms was studied. For the *RYR1* alleles, clustering of the C as well as T alleles within farms could be found. This illustrates that testing of the stress gene is nowadays common in pig industry. Some pig breeders prefer to include the $RYR1^T$ allele in their herds in a controlled manner in order to obtain optimal muscularity, whereas other pig farmers decide to exclude the stress-susceptibility allele to avoid economic losses caused by pale, soft and exudative meat.

The prevalence of the F18$^+$ *E. coli* resistant genotype in the Belgian pig population was estimated to be 4.6% and the allele frequency of the $FUT1^A$ allele was 0.21. This is in accordance with data obtained by Meijerink et al. (1997) and Vögeli et al. (1997), showing an $FUT1^A$ allele frequency of 0.21 after examination of 455 animals. In addition, no clustering of the $FUT1^G$ and $FUT1^A$ allele was found, indicating a random distribution of these alleles in the population. This reflects absence of selection for F18$^+$ *E. coli* resistant pigs in the studied pig population. Furthermore, it is important to note that the prevalence of the F18$^+$ *E. coli* resistant genotype is strongly

dependent on the breed. The Polish Zlotnicka Spotted pigs, for instance, demonstrated a high occurrence (37.5%) of the $FUT1^{A/A}$ genotype (Klukowska et al., 1999), whereas almost all native Chinese breeds show complete absence of the $FUT1^{A}$ allele (Yan et al., 2003). Unfortunately, the potential association with the stress-susceptibility allele was not assessed in these studies.

Analysis of the association between the stress susceptibility and F18$^+$ *E. coli* resistance alleles in the Belgian pig population by determining the linkage disequilibrium demonstrated that both alleles are not associated. This is in contrast with earlier findings of Meijerink et al. (1997), who revealed a 93% association between $FUT1^{A}$ alleles and $RYR1^{T}$ alleles in the Swiss Landrace population and probably therefore, the selection for F18$^+$ *E. coli* resistant pigs has not been taken into practice until now. This difference between our data and the findings of Meijerink et al. (1997) could be explained by the different population history of Swiss Landrace pigs compared to Belgian pigs. It is possible that a founder effect in the studied pedigree of Swiss Landrace pigs was causative for this high association. Founder effects in pigs can arise because of the economically driven extensive selection programmes, which reduce the effective population sizes strongly. In Belgian commercial pigs for example, the effective population size is diminished from thousands to less than 200 animals (Harmegnies et al., 2006).

Selection for $FUT1^{A/A}$ pigs implies that a positive selection for pigs deficient in the *FUT1* gene is carried out. These $FUT1^{A/A}$ pigs show decreased levels of FUT1 activity (Meijerink et al., 2000), even as an almost complete absence of expression of histo-blood group antigens (HBGAs) on their intestinal epithelial cells (Coddens et al., 2007). The necessity of expression of these HBGAs on the intestinal epithelium for normal development and reproduction of pigs is not studied yet, although *FUT1* null mice and *FUT2* null mice are proved to be viable, healthy and fertile, indicating that the *FUT* loci and their fucosylated glycans are not essential for normal development in mice (Domino et al., 2001).

Since no association between the F18$^+$ *E. coli* resistance allele and the stress-susceptibility allele was found in the Belgian pig population, the authors believe that selection for F18$^+$ *E. coli* negative pigs can be taken into consideration. Hereto, we recommend to establish small scale breeding programmes focussing on selection for F18$^+$ *E. coli* negative pigs in order to extend the knowledge about economical important traits such as reproduction rates and meat- or carcass quality of F18$^+$ *E. coli* resistant pigs. If no negative effects on these qualities are found, these studies could pave the way for a general positive advice on selection for F18$^+$ *E. coli* resistant pigs. Since no vaccine is available against F18$^+$ *E. coli*, this strategy to provide protection against F18$^+$ *E. coli* infections could be of great economical importance for the pig industry.

Acknowledgements

This research was funded by a PhD grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen).

F. Verdonck has a postdoctoral grant from the FWO Vlaanderen.

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