F4-related mutation and expression analysis of the aminopeptidase N gene in pigs[1]

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ABSTRACT: Intestinal infections with F4 enterotoxigenic *Escherichia coli* (ETEC) are worldwide an important cause of diarrhea in neonatal and recently weaned pigs. Adherence of F4 ETEC to the small intestine by binding to specific receptors is mediated by F4 fimbriae. Porcine aminopeptidase N (ANPEP) was recently identified as a new F4 receptor. In this study, 7 coding mutations and 1 mutation in the 3′ untranslated region (3' UTR)were identified in *ANPEP* by reverse transcriptase (RT–) PCR and sequencing using 3 F4 receptor-positive (F4R+) and 2 F4 receptor-negative (F4R–) pigs, which were F4 phenotyped based on the *MUC4* TaqMan, oral immunization, and the in vitro villous adhesion assay. Three potential differential mutations (g.2615C > T, g.8214A > G, and g.16875C > G) identified by comparative analysis between the 3 F4R+ and 2 F4R– pigs were genotyped in 41 additional F4 phenotyped pigs. However, none of these 3 mutations could be associated with F4 ETEC susceptibility. In addition, the RT-PCR experiments did not reveal any differential expression or alternative splicing in the small intestine of F4R+ and F4R– pigs. In conclusion, we hypothesize that the difference in F4 binding to ANPEP is due to modifications in its carbohydrate moieties.

Key words: aminopeptidase N, F4 enterotoxigenic *Escherichia coli*, F4 phenotyping, F4 receptor, mutation, pig

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INTRODUCTION

Enterotoxigenic *Escherichia coli* (**ETEC**) expressing F4 fimbriae are worldwide a major cause of diarrhea in neonatal and recently weaned pigs (Do et al., 2006; Zhang et al., 2007; Amezcua et al., 2008; Madoroba et al., 2009; de la Fe Rodriguez et al., 2011). The bacteria colonize the small intestine of the pig by binding with their F4 fimbriae to specific receptors and produce enterotoxins, inducing diarrhea (Jones and Rutter, 1972; Alexander, 1994). The F4 fimbriae, composed of major and minor subunit structures, exist in 3 serological variants, namely F4ab, F4ac, and F4ad (Orskov et al., 1964; Guinee and Jansen, 1979; Mooi and de Graaf, 1985). These variants differ in the amino acid composition of the major fimbrial subunit FaeG, which has adhesive properties and recognizes glycoconjugates on the surface of enterocytes (Kearns and Gibbons, 1979; Sellwood, 1980; Bakker et al., 1992; Van den Broeck et al., 1999a). Although different F4 receptor profiles were observed due to different F4 ETEC adhesion phenotypes, no causal mutation in previous proposed candidate genes has yet been identified (Ren et al., 2012; reviewed in Schroyen et al., 2012).

Recently, aminopeptidase N (**ANPEP**) has been found to act as an endocytotic F4 receptor by comparative proteomic analysis of the brush border proteins in F4ac receptor-positive and F4ac receptor-negative pigs (Melkebeek et al., 2012). Aminopeptidase N, belonging to the M1 family of zinc metallopeptidase, is a 936 amino acid membrane glycoprotein and is widely expressed on the surface of various cell types, includ-

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ing porcine enterocytes (Delmas et al., 1992; Rawlings and Barrett., 1993; Olsen et al., 1997). The gene encoding ANPEP (GenBank: NC_010449.4; Supplementary Fig. 1) is located on chromosome 7 and is composed of 20 exons (Poulsen et al., 1991).

The purpose of this study was to investigate whether a mutation in *ANPEP* could be associated with F4 ETEC susceptibility in pig.

MATERIAL AND METHODS

Animals, Sample Collection, and F4 Phenotyping

Forty-six mixed-breed pigs from 7 different litters were used in this study (Table 1). Blood samples were collected before euthanasia at 6 to 18 wk of age in EDTA blood tubes and stored at –20°C. After euthanasia, mid-jejunum samples for RNA analysis were collected and washed 3 times with Krebs–Henseleit buffer (0.12 *M* NaCl, 0.014 *M* KCl, 0.001 *M* KH_2PO_4, and 0.025 *M* $NaHCO_3$, pH 7.4). Next, they were frozen in liquid nitrogen and stored at –80°C. Mid-jejunum samples for the in vitro villous adhesion assay were washed twice with ice cold Krebs-Henseleit buffer followed by 1 washing step with Krebs–Henseleit buffer containing 1% (vol/vol) formaldehyde. Villi were then scraped from the mucosa and stored as mentioned in Van den Broeck et al. (1999b).

The *MUC4* TaqMan assay was performed as described by Nguyen et al. (2013) and is based on the g.8227G > C mutation of *MUC4* (Genbank: DQ848681) associated with F4ab/ac ETEC susceptibility (Jorgensen et al., 2004).

For the oral immunization, pigs were orally given 1 mg of F4ac fimbriae (strain Gis26) in 10 mL PBS on 3 consecutive days and once again at 15 d after primary immunization. Blood was collected before immunization and at 15 and 21 d after immunization from the jugular vein to determinate seropositive in F4-specific ELISA.

The presence of the F4 receptor was determined by performing the in vitro villous adhesion assay for the 3 F4 variants (F4ab/ac/ad; Van den Broeck et al., 1999b; see Table 1).

Experimental and animal management procedures were approved by the animal care and ethics committee of the Faculty of Veterinary Medicine, University of Ghent (EC2010/042), Heidestraat, Belgium.

Deoxyribonucleic Acid Isolation, RNA Isolation, and cDNA Synthesis

Deoxyribonucleic acid isolation of porcine blood for *MUC4* mutation detection and *ANPEP* mutation screening was performed as described by Van Poucke et al. (2005).

Total RNA isolation of mid-jejunum samples of 5 pigs (pig 5, 8, 15, 23, and 26) was performed by using the Aurum Total RNA Fatty and Fibrous Tissue kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the instructions of the manufacturer. Residual DNA was removed with an on-column deoxyribonuclease (**DNase**) treatment. The concentration of total RNA (between 400 and 1,600 ng/µL) and the optical density $(\mathbf{OD})_{260/280}$ ratio for purity (between 2.09 and 2.14) were measured by the Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, De Meern, The Netherlands). Ribonucleic acid quality was assessed by comparison of the 28S and the 18S ribosomal bands from 1 µg of total RNA on a 0.8% agarose gel stained with ethidium bromide. All RNA samples were confirmed to be DNA free by a minus reverse transcriptase (**RT–**) PCR with *Sus scrofa* (**Sscr**) ANPEP ± 5 primers on 20 ng RNA (Supplementary Table 1). A 10-µL PCR mix containing 0.5 U FastStart Taq DNA polymerase (Roche Diagnostics, Brussels, Belgium), 10x FastStart Buffer with 20 m*M* $MgCl_2$ (Roche Diagnostics, Brussels, Belgium), 200 µ*M* deoxynucleotide triphosphates (Bioline, London, UK), 0.5 µ*M* primers, and the RNA template was used. Positive and negative controls were included. Polymerase chain reaction conditions were 3.5 min at 95°C, 30 cycles with denaturation at 95°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 1 min, and 1 cycle at 72°C for 4 min. The PCR product was examined by gel electrophoresis.

Then, cDNA was synthesized from 1 µg high quality RNA with the Improm-II Reverse Transcriptase kit (Promega, Merelbeke, Belgium) using Random and Oligo dT primers (each 0.5 µg per reaction), verified by PCR (similar to the minus RT-PCR but with 10x diluted cDNA as a template and 40 PCR cycles), and examined by gel electrophoresis.

Structural Mutation Detection of ANPEP *by reverse transcriptase-Polymerase Chain Reaction and Sequencing*

Complementary DNA was used for amplifying the whole coding sequence of ANPEP in 4 overlapping amplicons with SscrANPEP ± 1, 2, 3, and 4 primers designed with Primer3Plus (Untergasser et al., 2007; Supplementary Table 1) in 3 F4 receptor-positive (**F4R+**) piglets (pig 5, 8, and 15) and 2 F4 receptor-negative (**F4R–**) piglets (pig 23 and 26). The composition of the PCR mix and the PCR conditions with 40 PCR cycles were similar as described above (see Supplementary Table 1 for annealing temperatures). Polymerase chain reaction products were examined by gel electrophoresis for differential gene expression and alternative splicing. After purification of the PCR products with Exonuclease I (4 U) and Antarctic Phosphatase (2 U; New England Biolabs, Ipswich, UK) at 37°C for 30 min and 80°C for 15 min, 1 µL PCR product was directly sequenced with 2 pmol of the corresponding *ANPEP* primers and an additional sequence primer (See

Table 1. Detailed list of the pigs used in this study

Information pig				*ANPEP* mutations[1]			F4 binding profiling				
									F4 adhesion[4]		
Pig	Litter	Sow	Boar	M1	M2	M3	*MUC4*[2]	IR[3]	ab	ac	ad
1	1	Sow A	Boar A	C/C	A/G	C/C	SS	+	+++	+++	+
2	1	Sow A	Boar A	C/C	A/G	C/C	SS	+	+++	+++	+
3	1	Sow A	Boar A	C/T	A/G	C/G	SS	+	+++	+++	+++
4	1	Sow A	Boar A	C/C	A/G	C/C	SR	+	+++	+++	+
5	1	Sow A	Boar A	C/C	A/A	C/C	SS	+	+++	+++	+
6	2	Sow B	Boar B	C/C	A/A	C/C	SS	+	+++	+++	+
7	2	Sow B	Boar B	C/C	A/G	C/C	SR	+	+++	+++	+++
8	2	Sow B	Boar B	C/C	G/G	C/C	SS	+	+++	+++	+++
9	3	Sow C	Boar A	C/C	A/A	C/C	SS	+	+	+++	+++
10	3	Sow C	Boar A	C/C	A/G	C/C	SR	+	+++	+++	+++
11	5	Sow E	Boar D	C/T	G/G	C/G	SR	+	+++	+++	+++
12	5	Sow E	Boar D	C/C	G/G	C/C	SR	+	+	+++	+++
13	5	Sow E	Boar D	C/T	G/G	C/G	SR	+	+	+++	+++
14	5	Sow E	Boar D	C/C	G/G	C/C	SR	+	+	+++	+++
15	5	Sow E	Boar D	C/C	G/G	C/C	SR	+	+++	+++	+++
16	6	Sow F	Boar E	C/C	A/G	C/C	SR	+	+	+++	+
17	6	Sow F	Boar E	C/C	A/G	C/C	SR	+	+++	+++	+
18	6	Sow F	Boar E	C/T	A/G	C/C	SR	+	+	+++	+
19	7	Sow G	Boar F	C/C	A/A	C/C	SR	+	+++	+++	+++
20	7	Sow G	Boar F	C/C	A/G	C/C	SR	+	+	+++	+
21	7	Sow G	Boar F	C/C	A/G	C/C	SR	+	+	+++	+
22	4	Sow D	Boar C	C/C	A/G	C/C	RR	–	–	–	–
23	4	Sow D	Boar C	C/C	G/G	C/C	RR	–	–	–	–
24	4	Sow D	Boar C	C/C	A/G	C/C	RR	–	–	–	–
25	4	Sow D	Boar C	C/C	G/G	C/C	RR	–	–	–	–
26	5	Sow E	Boar D	C/T	G/G	C/G	RR	–	–	–	–
27	5	Sow E	Boar D	C/C	A/G	C/C	RR	–	–	–	–
28	1	Sow A	Boar A	C/C	A/G	C/C	RR	+	+++	+++	+++
29	2	Sow B	Boar B	C/C	A/G	C/C	RR	+	+++	+	+++
30	2	Sow B	Boar B	C/C	G/G	C/C	RR	+	+++	+++	+++
31	2	Sow B	Boar B	C/C	A/G	C/C	RR	+	+	+	+++
32	4	Sow D	Boar C	C/C	G/G	C/C	RR	+	+	+++	+
33	4	Sow D	Boar C	C/C	A/A	C/C	RR	+	+	+++	+++
34	4	Sow D	Boar C	C/T	A/G	C/G	RR	+	+	+++	+
35	4	Sow D	Boar C	C/C	G/G	C/C	RR	+	+++	+	+++
36	5	Sow E	Boar D	C/C	G/G	C/C	RR	+	+	+	+
37	7	Sow G	Boar F	C/C	A/G	C/C	RR	+	+	+	+
38	4	Sow D	Boar C	C/C	G/G	C/C	RR	+	–	–	–
39	4	Sow D	Boar C	C/C	A/G	C/C	RR	+	–	–	–
40	6	Sow F	Boar E	C/C	A/G	C/C	RR	+	–	–	+
41	6	Sow F	Boar E	C/C	A/G	C/C	RR	+	–	–	–
42	7	Sow G	Boar F	C/C	A/G	C/C	RR	+	–	–	+
43	4	Sow D	Boar C	C/C	G/G	C/C	RR	–	+	+++	+
44	4	Sow D	Boar C	C/T	G/G	C/G	RR	–	+	+++	+++
45	5	Sow E	Boar D	C/C	G/G	C/C	RR	–	+	+++	+++
46	3	Sow C	Boar A	C/C	A/G	C/C	SR	+	–	–	+

[1]*ANPEP* mutations are defined as follows: M1= g.2615C > T; M2 = g.8214A > G; M3 = g.16875C > G.

[2]*MUC4* genotypes are defined as follows: SS denotes homozygous susceptible, SR denotes heterozygous susceptible, and RR denotes homozygous resistant.

[3]Immune response (IR) is defined as follows: + denotes positive immune response on oral immunization with F4 fimbriae and – denotes negative immune response.

[4]F4 enterotoxigenic *Escherichia coli* adhesion is defined as follows: +++ denotes strong adhesion, + denotes weak adhesion, and – denotes no adhesion.

Table 2. Result of *ANPEP* mutation detection in 3 F4 receptor-positive and 2 F4 receptor-negative pigs[1]

Item[2]	EX1 g.2380 C > T (Y33Y)	EX1 g.2419 G > A (Q46Q)	EX1 g.2602 C > A (F107L)	EX1 g.2603 A > C (I108L)	EX1 g.2615 C > T (P112S)	EX4 g.4328 C > T (P330S)	EX 12 g.8214 A > G (I621V)	EX 20 g.16875 C > G (–)
PIG 5 (R+)	TAC/TAC (Y33Y)	CAG/CAG (Q46Q)	TTA/TTA (L107L)	CTT/CTT (L108L)	CCC/CCC (P112P)	CCC/TCC (P330S)	ATC/ATC (I621I)	C/C
PIG 8 (R+)	TAC/TAC (Y33Y)	CAG/CAG (Q46Q)	TTA/TTA (L107L)	CTT/CTT (L108L)	CCC/CCC (P112P)	CCC/CCC (P330P)	GTC/GTC (V621V)	C/C
PIG 15 (R+)	TAC/TAT (Y33Y)	CAG/CAA (Q46Q)	TTA/TTA (L107L)	CTT/CTT (L108L)	CCC/CCC (P112P)	CCC/CCC (P330P)	GTC/GTC (V621V)	C/C
PIG 23 (R–)	TAC/TAC (Y33Y)	CAG/CAG (Q46Q)	TTA/TTA (L107L)	CTT/CTT (L108L)	CCC/CCC (P112P)	CCC/CCC (P330P)	GTC/GTC (V621V)	C/C
PIG 26 (R–)	TAC/TAT (Y33Y)	CAG/CAA (Q46Q)	TTA/TTA (L107L)	CTT/CTT (L108L)	CCC/TCC (P112S)	TCC/TCC (S330S)	GTC/GTC (V621V)	C/G

[1]Mutation in codon and substituted amino acid are underlined.

[2]R+ denotes receptor-positive for F4 ETEC; R- denotes receptor-negative for F4 ETEC.

Supplementary Table 1) using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). The sequence reactions were analyzed on a 16-capillary 3130xl DNA Analyzer (Applied Biosystems) according to the manufacturer's protocol. Sequences were processed using BioEdit software (Hall, 1999).

Mutation Screening by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

Potential differential mutations (g.2615C > T, g.8214A > G, and g.16875C > G) found in the structural mutation detection were genotyped in 41 additional F4-phenotyped pigs. Polymerase chain reaction-RFLP assays were developed for screening the mutations on genomic DNA. The primers, restriction enzymes (New England Biolabs), and cutting positions are listed in Supplementary Table 2. Digested PCR products were analyzed on 3% agarose gels.

RESULTS

F4 Phenotyping in Pigs

To obtain a reliable F4 phenotype, 3 tests were performed in 46 pigs, namely the *MUC4* TaqMan assay, the oral immunization with F4 fimbriae and the in vitro villous adhesion assay (Table 1). In this study, all homozygous susceptible (SS) pigs (n = 7) for the *MUC4* TaqMan assay were F4 seropositive and showed adhesion towards F4ab/ac/ad ETEC. Also, all heterozygous susceptible (SR) pigs (n = 15) were F4 seropositive: 14 pigs showed F4ab/ac/ad ETEC adhesion and 1 pig showed only F4ad ETEC adhesion. Twenty-four pigs were genotyped homozygous resistant (RR): 6 seronegative and 3 seropositive pigs showed no F4 ETEC adhesion, 10 seropositive and 3 seronegative pigs showed F4ab/ac/ad ETEC adhesion, and 2 seropositive pigs showed only F4ad ETEC adhesion. Only pigs (n = 21) with 3 positive tests (F4R+) and pigs (n = 6) with 3 negative tests (F4R–) were used in the structural mutation detection and screening.

Structural Mutation Detection of ANPEP

A total of 8 mutations were found in 3 F4R+ and 2 F4R– pigs (Table 2): 2 silent mutations (exon 1), 5 missense mutations (exon 1, 4, and 12), and 1 mutation in the beginning of the partially sequenced 3′ untranslated region (**UTR**; exon 20). The 5 missense mutations resulting in amino acid substitutions had no effect on the glycosylation sites predicted by NetNglyc 1.0 and NetOGlyc 4.0 (R. Gupta, E. Jung, S. Brunak, 2004, personal commmunication; Steentoft et al., 2013).

The mutation in the 3′ UTR was found in a region where no microRNA binding sites were detected by miRbase (Griffiths-Jones, 2004). Comparative analysis between F4R+ and F4R– identified 3 possible differential mutations (Table 2).

During the structural mutation detection, the expression of *ANPEP* in the small intestine was semiquantitative analyzed via gel electrophoresis of the RT-PCR products. No obvious expression difference or alternative splice variants were observed between F4R+ and F4R– pigs as illustrated in Fig. 1.

Mutation Screening in 41 Additional F4-Phenotyped Piglets

Three mutations (g.2615C > T, g.8214A > G, and g.16875C > G) were screened by PCR-RFLP in 41 additional F4 phenotyped pigs (Table 1). No TT homozygotes (g.2615C > T) and no GG homozygotes (g.16875C > G) were observed in this study. When comparing F4R+ (n =

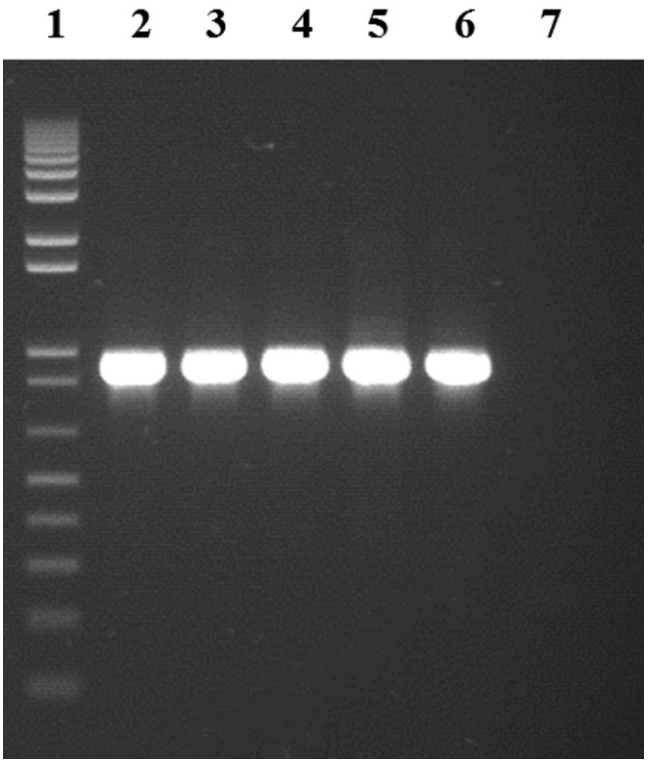

Figure 1. Agarose gel of *Sus scrofa* aminopeptidase N +/-3 (primer) ± 3 PCR products using cDNA as a template representing the absence of differential gene expression and alternative splice variants in the small intestine of F4 receptor-positive (F4R+) and F4 receptor-negative (F4R–) pigs. Lane 1: 1Kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA); lane 2: F4R+ pig 5; lane 3: F4R+ pig 8; lane 4: F4R+ pig 15; lane 5: F4R– pig 23; lane 6: F4R– pig 26; lane 7: water.

21) and F4R– pigs (n = 6), AA homozygotes of g.8214A > G mutation were absent in the F4R– pigs (Table 3). The AA genotype was also absent in the 3 seropositive pigs with RR genotype and with no F4 ETEC adhesion (Table 1).

The other genotypes (g.2615C > T: CC and CT; g.8214A > G: AG and GG; and g.16875C > G: CC and CG) were present in both F4R+ and F4R– pigs.

DISCUSSION

The F4 binding profile of 46 pigs was determined based on the *MUC4* TaqMan assay, the oral immunization with purified F4ac fimbriae and the in vitro villous adhesion assay for the 3 F4 variants. The results of the *MUC4* TaqMan assay confirm that the g.8227G > C mutation of *MUC4* is associated with F4ab/ac ETEC susceptibility (Jorgensen et al., 2004). Nevertheless, the genotypes, especially the RR genotype, were not completely consistent with the results of the oral immunization and the in vitro villous adhesion assay. Ten of the RR pigs (41.67%) became seropositive and showed adhesion towards F4ab/ac ETEC. This result confirms previous findings that the g.8227G > C mutation is a marker but not the actual causative mutation (Rasschaert et al., 2007; Li et al., 2008). Recently, a new strongly associated marker (g.28784 T > C) has been identified in *MUC13* and has shown a very high accuracy for distinguishing the F4ac susceptible and resistant pigs (Ren et al., 2012).

Three RR pigs (12.5%) showed in vitro no F4 ETEC adhesion and became nevertheless seropositive. Blotting of brush border membrane proteins of these pigs with F4 fimbriae displayed similar F4 binding patterns as the F4R– group described in Nguyen et al. (2013). It has been described that oral administration of F4ac antigens to F4R– pigs (based on the in vitro villous adhesion test) can prime the immune system resulting in a secondary antibody response following a subsequent intramuscular immunization (Van den Broeck et al., 2002). Looking at the F4-specific serum IgA response of these piglets 7 d after boosting a weaker response occurred (from a titer of 7 to 30), in comparison with F4R+ piglets (titer increased until 43). Consequently, we hypothesize that, at least during the second oral immunization, F4ac antigens boosted the immune system via small wounds in the mouth or mucosa of these pigs.

The positive immune response with F4ac fimbriae in F4adR+ pigs (2 RR pigs and 1 SR pig) can either be explained as for 3 seropositive F4 adhesion-negative RR pigs (described above) or might be due the high homology in the protein sequences of the F4 fimbrial subunit protein in the 3 F4 variants (Gaastra et al., 1981, 1983; Josephsen et al., 1984).

One seropositive SR pig (6.67%) and 2 seronegative RR pigs (8.3%) showed no F4ab/ac ETEC adhesion. Three seronegative RR pigs (12.5%) were F4ab/ac/ad ETEC adhesion positive. Although the in vitro villous adhesion test has been proven to be reliable (Van den Broeck et al., 1999b), it has been described that the correlation between in vitro F4 ETEC adhesion to isolated brush border vesicles and F4 ETEC susceptibility is not absolute (Francis et al., 1998).

Having well-characterized case-control groups is necessary to find a strong genotype–phenotype correlation in genetic association studies. We propose to use the *MUC4* TaqMan assay, the oral immunization, and the in vitro villous adhesion assay for F4 phenotyping the case-control groups in linkage studies for F4 ETEC.

Because ANPEP was recently identified as an endocytotic receptor for F4ac ETEC (Melkebeek et al., 2012), the cDNA sequence of *ANPEP* was investigated. Although binding of F4ab/ad ETEC to ANPEP is still unknown, pigs susceptible or resistant towards the 3 F4 ETEC variants were used in this study.

First, a structural mutation detection and screening were performed for finding a mutation that could alter the binding site or change the protein structure and therefore sterically blocking the binding site. Previously, it has been shown that ANPEP is a functional cell surface receptor for group I coronaviruses and that this interaction is dependent on species-specific amino acid differences in the receptor

Table 3. Genotype frequency of g.2615C > T (M1), g.8214A > G (M2), and g.16875C > G (M3) mutations in the different F4 enterotoxigenic *Escherichia coli* (ETEC) adhesion groups of 21 F4 receptor-positive and 6 F4 receptor-negative pigs

F4 ETEC adhesion[1]			F4ab +++	F4ab +	F4ab –	F4ac +++	F4ac +	F4ac –	F4ad +++	F4ad +	F4ad –
M1	CC	22	11/22 (50%)	6/22 (27%)	5/22 (23%)	17/22 (77%)	/	5/22 (23%)	8/22 (36%)	9/22 (41%)	5/22 (23%)
	CT	5	2/5 (40%)	2/5 (40%)	1/5 (20%)	4/5 (80%)	/	1/5 (20%)	3/5 (60%)	1/5 (20%)	1/5 (20%)
	TT	0	/	/	/	/	/	/	/	/	/
M2	AA	4	3/4 (75%)	1/4 (25%)	0/4 (0%)	4/4 (100%)	/	0/4 (0%)	2/4 (50%)	2/4 (50%)	0/4 (0%)
	AG	14	7/14 (50%)	4/14 (29%)	3/14 (21%)	11/14 (79%)	/	3/14 (21%)	3/14 (21%)	8/14 (57%)	3/14 (21%)
	GG	9	3/9 (33%)	3/9 (33%)	3/9 (33%)	6/9 (66%)	/	3/9 (33%)	6/9 (66%)	0/9 (0%)	3/9 (33%)
M3	CC	23	11/23 (48%)	7/23 (30%)	5/23 (22%)	18/23 (78%)	/	5/23 (22%)	8/23 (35%)	10/23 (43%)	5/23 (22%)
	CG	4	2/4 (50%)	1/4 (25%)	1/4 (25%)	3/4 (75%)	/	1/4 (25%)	3/4 (75%)	0/4 (0%)	1/4 (25%)
	GG	0	/	/	/	/	/	/	/	/	/

[1]F4 ETEC adhesion is defined as follows: +++ denotes strong adhesion, + denotes weak adhesion, and – denotes no adhesion.

protein (Delmas et al., 1992, 1994; Yeager et al., 1992; Tresnan et al., 1996; Benbacer et al., 1997; Li et al., 2007; Tusell et al., 2007). For instance, a T742V or a T742R substitution in feline ANPEP, a common receptor for group I coronaviruses, abrogated the receptor activity for feline enteric coronavirus, canine coronavirus, and porcine transmissible gastroenteritis coronavirus (Tresnan and Holmes, 1998; Tusell et al., 2007).

Comparative analysis of F4R+ (n = 21) and F4R– pigs (n = 6) in the *ANPEP* mutation screening showed that no structural mutation in ANPEP is associated with F4 ETEC susceptibility.

Second, alternative splicing could play a role in F4 ETEC susceptibility by removing or adding specific domains of the F4 fimbriae binding site or by altering the steric structure of ANPEP. Although human ANPEP is subjected to alternative splicing (Dybkaer et al., 2001), we observed no porcine *ANPEP* alternative splice variants in the small intestine during structural mutation detection.

Third, a regulatory mutation could explain susceptibility or resistance to F4 ETEC by altering the ANPEP expression in the small intestine. According to Nam and Lee (2010), the density of ANPEP could play an important role in contributing to an efficient porcine epidemic diarrhea virus infection. Because an obvious difference in expression between F4R+ and F4R– pigs was expected, the expression of *ANPEP* was only semiquantitatively analyzed during structural mutation detection. We could conclude that no apparent expression difference was observed, hence the F4ac susceptibility is not governed by a regulatory mutation.

Previously, other candidate receptors for the 3 F4 variants were suggested, all glycoconjugates (reviewed in Van den Broeck et al., 2000). The carbohydrate moiety of these glycoconjugates appeared to be necessary for establishing adhesion with the F4 adhesin (Erickson et al., 1992; Grange and Mouricout, 1995; Grange et al., 1999; Coddens et al., 2011).

For binding to ANPEP, it was shown that F4ac ETEC, like porcine transmissible gastroenteritis CV, is binding on the carbohydrate moieties of ANPEP in a sialic acid-dependent manner (Schultze et al., 1996; Melkebeek et al., 2012). Given the results described above, we hypothesize that the susceptibility towards F4 ETEC mediated by aminopeptidase N is not due to modifications in the protein itself but due to modifications in the carbohydrate structures attached to it.

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