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1 **Screening of pigs resistant to F4 enterotoxigenic *Escherichia coli***
2 **(ETEC) infection.**

3

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17

18 **Abstract**

19 The present study analysed quantitatively the mucin 4 polymorphism for determining the
20 F4ac/ab receptor status of a total of 63 pigs by comparing it with the *in vitro* villous adhesion
21 assay. The probability of a susceptible genotype for the mucin 4 increases significantly with
22 increasing F4ab or F4ac ETEC adhesion per 250 µm villi (P=0.029 for F4ab, P=0.030 for
23 F4ac), with the odds ratio for each unit increase of F4ab or F4ac equal to respectively 1.036
24 (95% CI [1.004-1.069]) and 1.018 (95% CI [1.002-1.034]). In the phenotypic *in vitro* villous
25 adhesion test, a cut-off value of 5 bacteria was chosen as a criteria for the distinction between

26 an F4R positive and F4R negative pig. The sensitivity and specificity for the *in vitro* villous
27 adhesion test, with the genotyping test for mucin 4 as golden standard, is 100% and 24%
28 respectively for F4ab as well as F4ac. Absence of adhesion of F4ac and F4ab ETEC to the
29 villous brush borders was not associated with genotypic resistance suggesting that there is at
30 least one other receptor for F4ab/ac *E. coli*. As a consequence, not only mucin 4 gene
31 polymorphism but also expression of these other receptor(s) has to be included in a screening
32 assay for F4ac/ab receptor negative pigs.

33

34 Keywords: mucin 4, F4ab/ac ETEC, *in vitro* adhesion assay.

35

36 **Introduction**

37 Infections with enterotoxigenic *Escherichia coli* (ETEC) that express the F4ab or F4ac
38 fimbriae, cause diarrhea and mortality in neonatal and newly weaned piglets. These pathogens
39 bind with their F4ab/ac fimbriae to F4 specific receptors (F4R) on the brush border of small
40 intestinal enterocytes, resulting in colonization and a secretory watery diarrhea (Gyles CL.,
41 1994). These receptors are not present in every pig and their absence causes resistance to F4
42 ETEC induced diarrhea (Sellwood et al., 1975). Their expression is genetically determined
43 and inherited in a dominant way (Bijlsma and Bouw, 1987; Gibbons et al., 1977). The gene
44 underlying resistance to F4ab/ac ETEC has been assigned by linkage analysis to porcine
45 chromosome 13 (Guérin et al., 1993), whereas the F4ad ETEC receptor is localized on
46 another chromosome (Peelman, 1999). For this reason, we did not focus on the F4ad ETEC in
47 our study. Moreover, a polymorphism in the mucin 4 gene has been linked to the F4ab/ac
48 ETEC adhesive phenotype and not for the F4ad ETEC adhesive phenotype. A DNA marker-
49 based test has been developed to allow genotyping for F4ab/ac ETEC resistance/susceptibility

50 (Jørgensen et al., 2004). Three different genotypes were observed and were called resistant
51 (RR), susceptible heterozygote (SR) and susceptible homozygote (SS).

52 Breeding programs with F4R negative pigs could result in prevention of F4ab/ac induced
53 ETEC diarrhea in the pig population. Therefore, an efficient screening assay for genetic
54 F4ab/ac resistance is needed. The current analysis for F4ab/ac susceptibility is based on an *in*
55 *vitro* adhesion assay using villi (Girardeau, 1980) or brush border epithelial cells (Sellwood et
56 al., 1975). The *in vitro* villous adhesion assay is performed by incubating small intestinal villi
57 with F4ab or F4ac ETEC. Then, villi are examined by phase-contrast microscopy and the
58 adhesion of bacteria is evaluated quantitatively. Since the adhesion assay either demands
59 major intestinal surgery or slaughter of the pig, it makes it difficult to incorporate it into
60 breeding programs. A DNA-based test is more preferable because it allows precise
61 genotyping of living animals.

62 The aim of the present study was to perform a quantitative analysis for the mucin 4
63 polymorphism and the *in vitro* villous adhesion test for 63 pigs from 31 different litters to
64 investigate the relationship between the genotype test for mucin 4 on the one hand and the
65 villous adhesion test for F4ac and F4ab on the other hand.

66

67 **Materials and methods**

68 The *in vitro* villous adhesion assay for F4R characterization.

69 Sixty-three pigs (with a age difference of 2 months) from 31 different sows and 14 different
70 boars (Belgian Landrace x Piétrain, 20 pigs; Belgian Landrace x Dutch Landrace, 12 pigs;
71 Large White x Piétrain, 31 pigs), on three different pig farms in Flanders were tested. In order
72 to determine the presence or absence of the F4R on the brush border of small intestinal
73 villous enterocytes, an *in vitro* villous adhesion assay was performed as described by Van den
74 Broeck et al. (1999). Briefly, a 15 cm long intestinal segment was excised of the mid jejunum

75 at the moment of slaughter. The segment was washed twice with PBS and once with Krebs-
76 Henseleit buffer (0.12 M NaCl, 0.014 M KCl, 0.001 M KH₂PO₄ and 0.025 M NaHCO₃
77 adjusted to pH 7.4) containing 1 % vol/vol formaldehyde at 4°C. Subsequently, the villi were
78 scraped off with glass slides and washed 4 times in Krebs-Henseleit buffer without
79 formaldehyde whereafter they were resuspended in PBS supplemented with 1 % (wt/vol) D-
80 mannose (Fluka, Sigma-Aldrich, Bornem, Belgium) to prevent adhesion by type 1 pilli.
81 Subsequently 4 x 10⁸ F4ac bacteria (strain GIS 26, serotype O149:K91, F4ac⁺, LT⁺STa⁺STb⁺)
82 or F4ab bacteria (strain G7, serotype O8:K87:F4ab⁺, LT⁺) were added to an average of 50
83 villi in a final volume of 0.5 ml and were incubated at room temperature for 1 hour while
84 gently shaking. The adhesion of bacteria was evaluated by counting the number of bacteria
85 adhering along a 50 µm villous brush border length at 20 randomly selected places with a
86 phase-contrast microscope at a magnification of 600. Adhesion of more than 5 bacteria per
87 250 µm villous brush border length was noted as positive (Cox and Houvenaghel, 1993). Each
88 villous adhesion test included some negative controls. First, the villi were checked before
89 addition of F4ab/ac bacteria to exclude adhering bacteria from the intestinal flora. Second, F4
90 bacteria without the adhesin (FaeG) were added. Finally, strain E57 (serotype O138: K81,
91 STa⁺STb⁺), bacteria without fimbriae, was added to the villi.

92

93 The DNA marker-based test

94 The DNA marker-based test was performed as described by Jørgensen et al. (2004). It relies
95 on a *Xba*I polymorphism in intron 7 of the porcine mucin 4 gene. The PCR-RFLP assay was
96 performed on genomic DNA from pigs in a total volume of 20 µl using 1 x Supertaq buffer
97 (HT Biotechnology Ltd., Cambridge, England), 2 mM MgCl₂, 200 µM of each dNTP, 1.0 µM
98 of each primer: 5'-GTGCCTTGGGTGAGAGGTTA-3' / 5'-CACTCTGCCGTTCTCTTTCC-
99 3', and 0.25 units Supertaq (HT Biotechnology Ltd., Cambridge, England). Thermocycling

100 was performed using 15 min initial denaturation at 95 °C and subsequently 95 °C for 15 s in
101 the additional cycles. The annealing temperature was 65 °C and the extension was carried out
102 at 72 °C for 1 min for 35 cycles. The PCR product obtained from pig genomic DNA is 367 bp
103 and 5.5 µl of the PCR products is used for *Xba*I digest as recommended by the supplier (New
104 England Biolabs, MA, USA). The resistant allele (R) is indigestible by *Xba*I, whereas the
105 susceptible allele (S) is digested into 151 bp and 216 bp fragments. As a control for the *Xba*I
106 digestion, we used a plasmid with one restriction site for *Xba*I.

107

108 Statistical analysis

109 The relationship between the genotype test for mucin 4 on the one hand and the villous
110 adhesion test for F4ac and F4ab on the other hand was investigated through a logistic
111 regression model including sow as random effect. The receiver operating characteristic (ROC)
112 curve was made to investigate the effect of different cut-off values for F4ab and F4ac
113 (distinction between F4R⁺ and F4R⁻) with respect to sensitivity and specificity. Repeatability
114 of the *in vitro* villous adhesion test for F4ab and F4ac was studied through a mixed model
115 with pig as random effect.

116

117 **Results and discussion**

118 Sixty-three pigs were tested in the *in vitro* villous adhesion assay and were genotyped
119 for the *Xba*I polymorphism in the mucin 4 gene. We tested the reliability of the genotyping by
120 checking all the resistant genotypes by DNA sequencing on the PCR products. Results are
121 presented for resistant (RR) or susceptible (SS, SR) animals using a box-and-whisker plot
122 (Figure 1). The box indicates the 25th and 75th percentiles and the central line is the median.
123 The points at the ends of the whiskers are the extreme values. Six of the 63 pigs had less than
124 5 F4ac or F4ab bacteria adhering per 250 µm villous brush border length in the *in vitro* villous

125 adhesion assay and had a resistant (RR) genotype. Fifty-seven pigs had more than 5 F4ac or
126 F4ab bacteria adhering per 250 μm villous brush border length. Nineteen of the 57 pigs had a
127 RR genotype and the number of F4ab adhering bacteria ranged between 8 and 95. The
128 number of F4ac bacteria in the 19 pigs with a RR genotype ranged between 5 and 125. Thirty-
129 eight pigs had at least one copy of the susceptible haplotype (SR or SS). The number of F4ab
130 adhering bacteria for these 38 pigs ranged between 6 and 105 with a median of 61. The
131 number of F4ac adhering bacteria ranged between 6 and 137 with a median of 61.

132 The probability of a SR/SS genotype for mucin 4 increases significantly with
133 increasing F4ab ETEC per 250 μm villi ($P=0.029$), with the odds ratio for each unit increase
134 of F4ab equal to 1.036 (95% CI [1.004-1.069]). For F4ac, the probability of a SR/SS genotype
135 for mucin 4 increases significantly with increasing F4ac ETEC per 250 μm villi
136 ($P=0.030$), with the odds ratio for each unit increase of F4ac equal to 1.018 (95% CI [1.002-
137 1.034]).

138 The sensitivity and specificity of the *in vitro* villous adhesion test for F4ab and F4ac
139 bacteria with the genotyping test for the mucin 4 as golden standard is represented in
140 Figure 2. The ROC curve is obtained by plotting the false positive rate (1-specificity) versus
141 the true positive rate (sensitivity) for various cut-off values. For a cut-off value of 95 for
142 instance, the estimated sensitivity and specificity for the adhesion test F4ac equal, is
143 respectively 23% and 84%. Based on the experiments of Cox and Houvenaghel (1993), a cut-
144 off value of 5 bacteria was chosen. With this cut-off value the estimated sensitivity and
145 specificity for the adhesion test for both F4ab and F4ac equal respectively 100% and 24%.

146 The estimated variance between measurements in the same pig equals 10.2 and 13.0
147 for F4ab and F4ac respectively. The estimated pig variance component (the additional
148 variability due to pig) equals 22.2 and 43.8, much larger than the within pig variance. This
149 variance is due to the fact that some pigs do not have any expression (due to the lack of the

150 gene) and that the expression of the F4 receptor genes varies when the gene is present
151 (Bijlsma and Bouw, 1987). Another factor which could explain the individual variation is the
152 age of the pigs. The villus length decreases with aging (Cera et al., 1988).

153 It can not be excluded that there could be some difference in results of the *in vitro*
154 villous adhesion assay compared to the adhesion assay used in the study of Jørgensen (2003).
155 They performed the adhesion assay by incubating single enterocytes with bacteria as
156 described by Edfors-Lilja (1986). Ten to 20 epithelial cells were examined for adhesion by
157 interference contrast microscopy. Their results were scored from 1 - 4, where 1 = no bacteria
158 and 4 = bacteria adhering to the whole brush border of all cells. We examined 250 µm villous
159 brush border, which contains a lot more than 20 enterocytes and our criteria for a receptor
160 negative animal was less than 5 adhering bacteria per 250 µm brush border.

161 In a study of Erickson (1994), a receptor from porcine enterocyte membranes was
162 identified and characterized as a mucin-type sialoglycoprotein which binds F4ac. However,
163 there was no correlation found between the expression of the mucin-type sialoglycoprotein
164 receptor and adherence of bacteria to the brush border (Francis et al., 1998). Our results do
165 not exclude mucin 4 as a candidate receptor, but the absence of the gene in 30,2 % pigs
166 positive in the *in vitro* villous adhesion assay for F4ac and F4ab, indicates that there is at least
167 one other receptor for F4ac/ab *E. coli*. Grange (1996) already demonstrated that F4ab can
168 bind to a receptor of the transferrin family but there should also exist an additional receptor(s)
169 for F4ac. As a consequence, not only mucin 4 gene polymorphism but also expression of
170 these other receptor(s) has (have) to be included in the screening assay for F4ac/ab receptor
171 negative pigs.

172

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176

177 **References**

178 Bijlsma I.G., Bouw J., 1987. Inheritance of K88-mediated adhesion of *Escherichia coli* to
179 jejunal brush borders in pigs: a genetic analysis. *Vet Res. Commun.* 11: 509-518.

180

181 Cera K., Mahan D., Cross R., Reinhart G., Whitmover R., 1988. Effect of age, weaning and
182 postweaning diet on small intestinal growth and jejunal morphology in young swine.
183 *J.Anim.Sci.* 66: 574-84.

184

185 Cox E., Houvenaghel A., 1993. Comparison of the in vitro adhesion of K88, K99, F41 and
186 P987 positive *Escherichia coli* to intestinal villi of 4- to 5- week-old pigs. *Vet. Microbiol.* 34:
187 7-18.

188

189 Edfors-Lilja I., Petersson H., Gahne B., 1986. Performance of pigs with or without the
190 intestinal receptor for *Escherichia coli* K88. *Anim Prod.* 42: 381-387.

191

192 Erickson A.K., Baker D.R., Bosworth B.T., Casey T.A., Benfield D.A., Francis D.H., 1994.
193 Characterization of porcine intestinal receptors for the K88ac fimbrial adhesin of *Escherichia*
194 *coli* as mucin-type sialoglycoproteins. *Infect. Immun.* 62(12): 5404-5410.

195

196 Francis D.H., Grange P.A., Zeman D.H., Baker D.R., Sun R., Erickson A.K., 1998.
197 Expression of mucin-type glycoprotein K88 receptors strongly correlates with piglet
198 susceptibility to K88⁺ enterotoxigenic *Escherichia coli*, but adhesion of this bacterium to
199 brush borders does not. *Infect. Immun.* 66(9): 4050-4055.

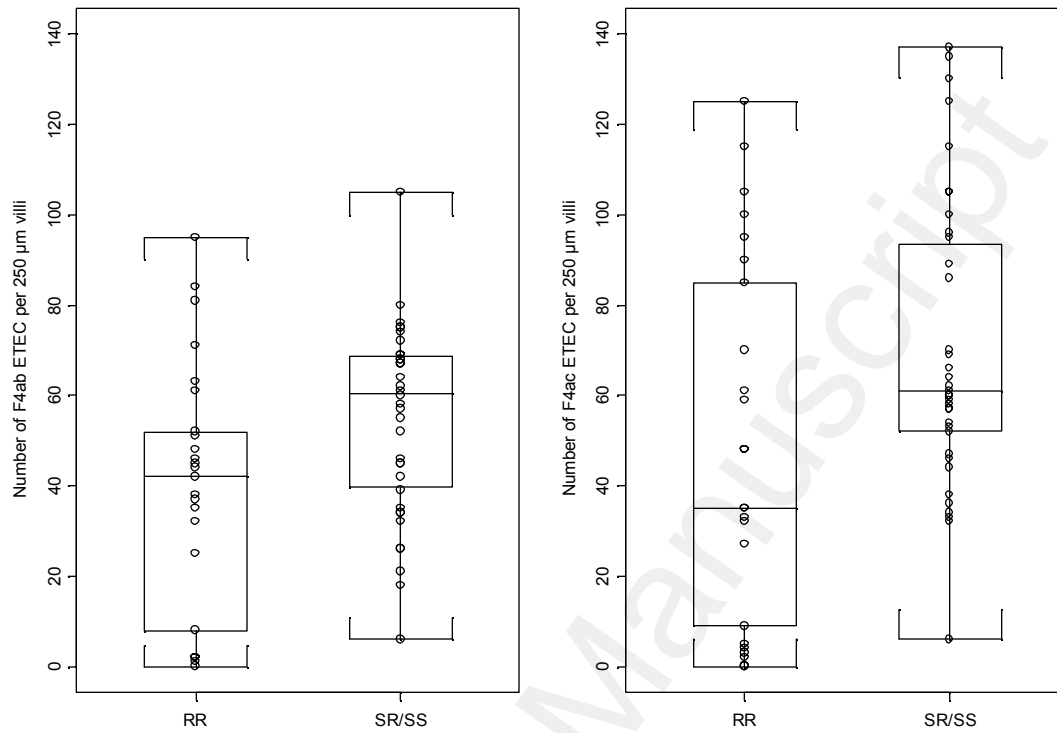
200

201 Gibbons R.A., Sellwood R., Burrows M., Hunter P.A., 1977. Inheritance of resistance to
202 neonatal *E. coli* diarrhea in the pig: examination of the genetic system. *Theor. Appl. Genet.*
203 51: 65-70.

204

- 205 Girardeau J.P., 1980. A new *in vitro* technique for attachment to intestinal villi using
206 enteropathogenic *Escherichia coli*. *Ann. Microbiol.* 131 (B):31-37.
207
- 208 Grange P.A., Mouricourt M.A., 1996. Transferrin associated with the porcine intestinal
209 mucosa is a receptor specific for K88ab fimbriae of *Escherichia coli*. *Infec. Immun.* 64: 606-
210 610.
211
- 212 Guérin G., Duval-Iflah Y., Bonneau M., Bertaud M., Guillaume P., Ollivier L., 1993.
213 Evidence for linkage between K88ab, K88ac intestinal receptors to *Escherichia coli* and
214 transferrin loci in pigs. *Animal Genetics.* 24: 393-396.
215
- 216 Gyles CL. 1994. *Escherichia coli* enterotoxins. In: CL Gyles (Editor), *Escherichia coli* in
217 domestic animals and humans. CAB International, Wallingford, Oxon, UK, pp. 337-64.
218
- 219 Jørgensen C.B., Cirera S., Anderson S.I., Archibald A.L., Raudsepp T., Chowdhary B.,
220 Edfors-Lilja I., Anderson L., Fredholm M. 2003. Linkage and comparative mapping of the
221 locus controlling susceptibility towards *E. coli* F4ab/ac diarrhea in pigs. *Cytogenet. Genome*
222 *Res.* 102: 157-163.
223
- 224 Jørgensen C.B., Cirera S., Archibald A.L., Anderson L., Fredholm M., Edfors-Lilja I. 2004.
225 Porcine polymorphisms and methods for detecting them. International application published
226 under the patent cooperation treaty (PCT). PCT/DK2003/000807 or WO2004/048606-A2.
227
- 228 Peelman L.J., 1999. Genetic investigation of the resistance mechanisms of the pig against
229 diarrhea caused by *E. coli*. *Verh. K. Acad. Geneesk. Belg.* 61: 489-515.
230
- 231 Sellwood R., Gibbons R.A., Jones G.W., Rutter J.M., 1975. Adhesion of enteropathogenic
232 *Escherichia coli* to pig intestinal brush borders: the existence of two pig phenotypes. *J. Med.*
233 *Microbiol.* 8: 405-411.
234
- 235 Van den Broeck W., Cox E., Goddeeris B.M., 1999. Receptor-specific binding of purified F4
236 to isolated villi. *Vet. Microbiol.* 68: 255-263.
237
238

1 Figure 1



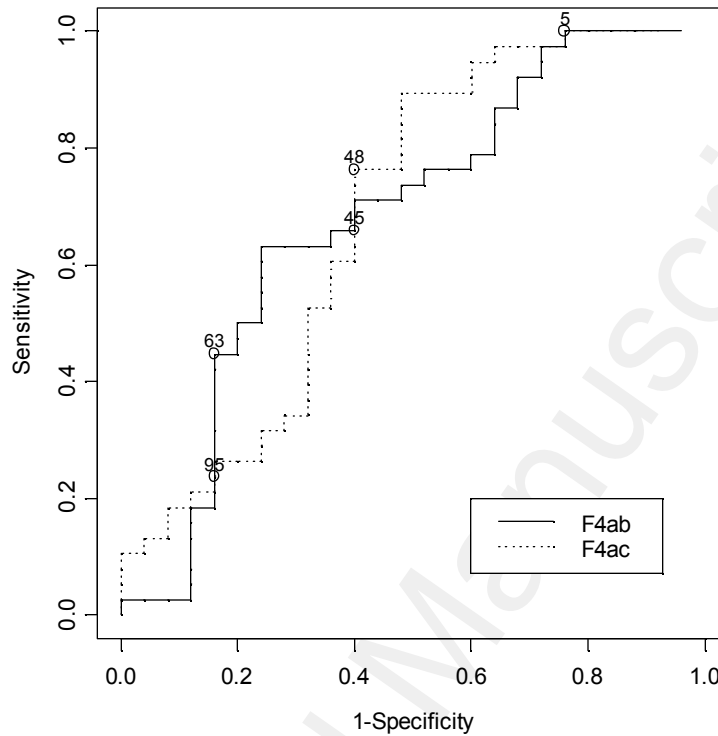
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3 Fig.1. Box-and-whisker plots of the *in vitro* villous adhesion test for F4ab and F4ac fimbriae
 4 for the resistant genotype (RR) and the susceptible (SR/SS) genotype of 63 pigs.

5 The box indicates the 25th and 75th percentiles and the central line is the median. The points at
 6 the ends of the whiskers are the extreme values. One point represents one pig.

7

1 Figure 2



2

3 Fig. 2. The receiver operating characteristic (ROC) curve, obtained by plotting the false
 4 positive rate (1-specificity) versus the true positive rate (sensitivity) for various cut-off values
 5 of F4ab (full line) or F4ac (dotted line). The numbers in the plot refer to specific cut-off
 6 values. For instance, for a cut-off value of 48 for the F4ac test, the sensitivity and specificity
 7 are 66% and 40% respectively, whereas for a cut-off value of 45 for the F4ab test, the
 8 sensitivity and specificity are 76% and 40% respectively.

9