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Veterinary Microbiology 123 (2007) 249–253

**veterinary
microbiology**

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Short communication

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

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Received 13 November 2006; received in revised form 13 February 2007; accepted 19 February 2007

Abstract

The present study analysed quantitatively the mucin 4 polymorphism for determining the F4ac/ab receptor status of a total of 63 pigs by comparing it with the *in vitro* villous adhesion assay. The probability of a susceptible genotype for the mucin 4 increases significantly with increasing F4ab or F4ac ETEC adhesion per 250 μm villi ($P = 0.029$ for F4ab, $P = 0.030$ for F4ac), with the odds ratio for each unit increase of F4ab or F4ac equal to, respectively, 1.036 (95% CI [1.004–1.069]) and 1.018 (95% CI [1.002–1.034]). In the phenotypic *in vitro* villous adhesion test, a cut-off value of 5 bacteria was chosen as a criteria for the distinction between an F4R positive and F4R negative pig. The sensitivity and specificity for the *in vitro* villous adhesion test, with the genotyping test for mucin 4 as golden standard, is 100% and 24%, respectively, for F4ab as well as F4ac. Absence of adhesion of F4ac and F4ab ETEC to the villous brush borders was not associated with genotypic resistance suggesting that there is at least one other receptor for F4ab/ac *Escherichia coli*. As a consequence, not only mucin 4 gene polymorphism but also expression of these other receptor(s) has to be included in a screening assay for F4ac/ab receptor negative pigs.

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Keywords: Mucin 4; F4ab/ac ETEC; *In vitro* adhesion assay

1. Introduction

Infections with enterotoxigenic *Escherichia coli* (ETEC) that express the F4ab or F4ac fimbriae, cause diarrhea and mortality in neonatal and newly weaned piglets. These pathogens bind with their F4ab/ac

fimbriae to F4 specific receptors (F4R) on the brush border of small intestinal enterocytes, resulting in colonization and a secretory watery diarrhea (Gyles, 1994). These receptors are not present in every pig and their absence causes resistance to F4 ETEC induced diarrhea (Sellwood et al., 1975). Their expression is genetically determined and inherited in a dominant way (Bijlsma and Bouw, 1987; Gibbons et al., 1977). The gene underlying resistance to F4ab/ac ETEC has been assigned by linkage analysis to porcine

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chromosome 13 (Guérin et al., 1993), whereas the F4ad ETEC receptor is localized on another chromosome (Peelman, 1999). For this reason, we did not focus on the F4ad ETEC in our study. Moreover, a polymorphism in the mucin 4 gene has been linked to the F4ab/ac ETEC adhesive phenotype and not for the F4ad ETEC adhesive phenotype. A DNA marker-based test has been developed to allow genotyping for F4ab/ac ETEC resistance/susceptibility (Jørgensen et al., 2004). Three different genotypes were observed and were called resistant (RR), susceptible heterozygote (SR) and susceptible homozygote (SS).

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

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

2. Materials and methods

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

Sixty-three pigs (with a age difference of 2 months) from 31 different sows and 14 different boars (Belgian Landrace × Piétrain, 20 pigs; Belgian Landrace × Dutch Landrace, 12 pigs; Large White × Piétrain, 31 pigs), on three different pig farms in Flanders were tested. In order to determine the presence or absence

of the F4R on the brush border of small intestinal villous enterocytes, an *in vitro* villous adhesion assay was performed as described by Van den Broeck et al. (1999). Briefly, a 15 cm long intestinal segment was excised of the mid jejunum at the moment of slaughter. The segment was washed twice with PBS and once with Krebs–Henseleit buffer (0.12 M NaCl, 0.014 M KCl, 0.001 M KH₂PO₄ and 0.025 M NaHCO₃ adjusted to pH 7.4) containing 1% (v/v) formaldehyde at 4 °C. Subsequently, the villi were scraped off with glass slides and washed four times in Krebs–Henseleit buffer without formaldehyde whereafter they were resuspended in PBS supplemented with 1% (w/v) D-mannose (Fluka, Sigma–Aldrich, Bornem, Belgium) to prevent adhesion by type 1 pili. Subsequently 4 × 10⁸ F4ac bacteria (strain GIS 26, serotype O149:K91, F4ac⁺, LT⁺STa⁺STb⁺) or F4ab bacteria (strain G7, serotype O8:K87:F4ab⁺, LT⁺) were added to an average of 50 villi in a final volume of 0.5 ml and were incubated at room temperature for 1 h while gently shaking. The adhesion of bacteria was evaluated by counting the number of bacteria adhering along a 50 µm villous brush border length at 20 randomly selected places with a phase-contrast microscope at a magnification of 600. Adhesion of more than 5 bacteria per 250 µm villous brush border length was noted as positive (Cox and Houvenaghel, 1993). Each villous adhesion test included some negative controls. First, the villi were checked before addition of F4ab/ac bacteria to exclude adhering bacteria from the intestinal flora. Second, F4 bacteria without the adhesin (FaeG) were added. Finally, strain E57 (serotype O138: K81, STa⁺STb⁺), bacteria without fimbriae, was added to the villi.

2.2. The DNA marker-based test

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

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

2.3. Statistical analysis

The relationship between the genotype test for mucin 4 on the one hand and the villous adhesion test for F4ac and F4ab on the other hand was investigated through a logistic regression model including sow as random effect. The receiver operating characteristic (ROC) curve was made to investigate the effect of

different cut-off values for F4ab and F4ac (distinction between F4R⁺ and F4R⁻) with respect to sensitivity and specificity. Repeatability of the *in vitro* villous adhesion test for F4ab and F4ac was studied through a mixed model with pig as random effect.

3. Results and discussion

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

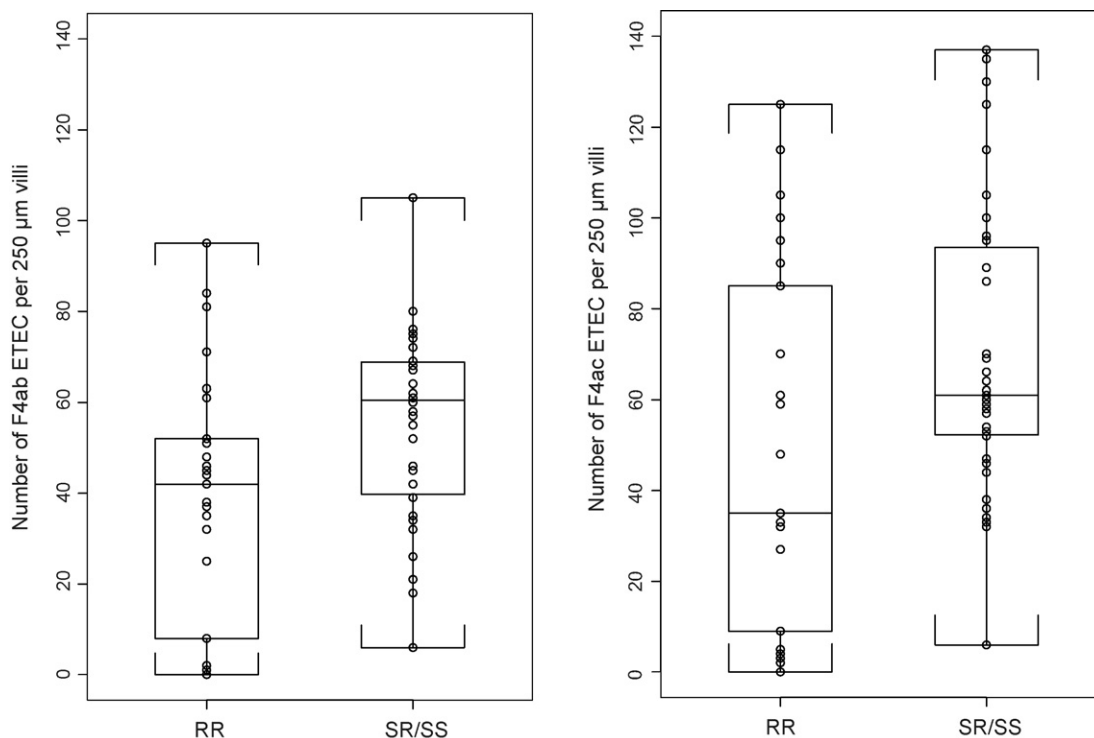


Fig. 1. Box-and-whisker plots of the *in vitro* villous adhesion test for F4ab and F4ac fimbriae for the resistant genotype (RR) and the susceptible (SR/SS) genotype of 63 pigs. The box indicates the 25th and 75th percentiles and the central line is the median. The points at the ends of the whiskers are the extreme values. One point represents one pig.

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

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

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

The estimated variance between measurements in the same pig equals 10.2 and 13.0 for F4ab and F4ac, respectively. The estimated pig variance component (the additional variability due to pig) equals 22.2 and 43.8, much larger than the within pig variance. This variance is due to the fact that some pigs do not have any expression (due to the lack of the gene) and that the expression of the F4 receptor genes varies when the gene is present (Bijlsma and Bouw, 1987). Another factor which could explain the individual variation is the age of the pigs. The villus length decreases with aging (Cera et al., 1988).

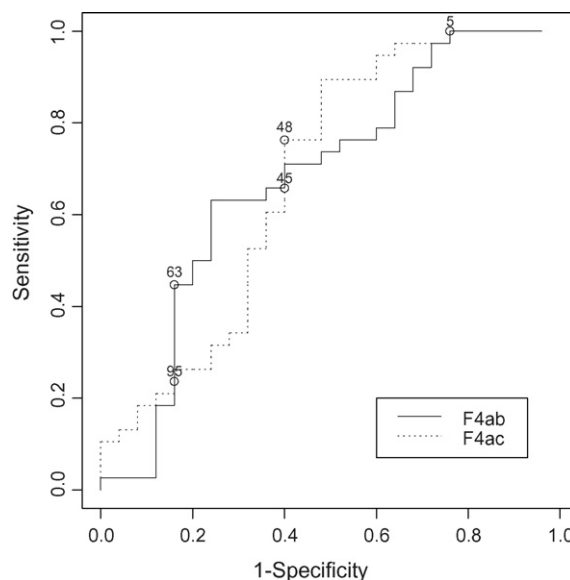


Fig. 2. The receiver operating characteristic (ROC) curve, obtained by plotting the false positive rate (1-specificity) versus the true positive rate (sensitivity) for various cut-off values of F4ab (full line) or F4ac (dotted line). The numbers in the plot refer to specific cut-off values. For instance,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

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

In a study of Erickson et al. (1994), a receptor from porcine enterocyte membranes was identified and characterized as a mucin-type sialoglycoprotein which binds F4ac. However, there was no correlation found between the expression of the mucin-type sialoglycoprotein receptor and adherence of bacteria to the brush border (Francis et al., 1998). Our results do not exclude mucin 4 as a candidate receptor, but the

absence of the gene in 30.2% pigs positive in the *in vitro* villous adhesion assay for F4ac and F4ab, indicates that there is at least one other receptor for F4ac/ab *E. coli*. Grange and Mouricourt (1996) already demonstrated that F4ab can bind to a receptor of the transferrin family but there should also exist an additional receptor(s) for F4ac. As a consequence, not only mucin 4 gene polymorphism but also expression of these other receptor(s) has (have) to be included in the screening assay for F4ac/ab receptor negative pigs.

Acknowledgements

This work was supported by the Research Fund of the Ghent University and the “FWO-Flanders”.

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