Identification and Screening of Gene(s) Related to Susceptibility to
Enterotoxigenic Escherichia coli F4ablac in Piglets

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ABSTRACT: In 2004, Jorgensen and coworkers proposed the MUC4 gene as a candidate gene of enterotoxigenic Escherichia coli
(ETEC) F4ablac receptor in piglets and a mutation of G→C in intron 7 of MUC4 was identified to be associated with the ETEC F4ablac
adhesion phenotypes. In this study, we used 310 piglets of three breeds (Landrace, Large White and Chinese Songlino Black) to analyze
the relationship between this mutation and the F4ab/ac adhesion phenotype. The results show that the genotypes at this site and the
ETEC F4ab/ac adhesion phenotypes were not completely consistent, although they are very strongly associated. Among the individuals
with genotype CC, which was identified as a resistant genotype to F4ab/ac adhesion, only 72.1% (124/172) were non-adhesive to ETEC
F4ab and 77.9% (134/172) were non-adhesive to ETEC F4ac infections. This suggests that this mutation may not be the causative
mutation for ETEC F4ab/ac adhesion, rather, the actual causative mutation may be in another gene closely linked to MUC4, or at another
site within the MUC4 gene. Our results also suggest that the receptors of F4ab and F4ac may be determined by two different but closely
linked loci. In order to screen other genes related to F4ab/ac adhesion in piglets, the mRNA profiles from six full sib piglets, of which
three were adhesive to ETEC F4ab/ac and three non-adhesive, were analyzed by suppression subtractive hybridization (SSH). One
up-regulated gene, Ep-CAM, was selected for further analysis based on its role in the intestinal epithelial cells adhesion. Using real-time RT-
PCR, we found that the Ep-CAM gene was significantly up-regulated in the piglets adhesive to F4ab/ac. It was mapped to SSC3q11-q14
by radiation hybrid mapping. (Key Words: ETEC F4ab/ac, Susceptibility, Receptor, Muc4, Ep-CAM, Piglets)

INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) expressing fimbriae F4 (formerly K88) is the major pathogenic bacteria causing diarrhoea and death in neonatal and post-weaning piglets (Wilson et al., 1986; Cheng et al., 2006; Wang et al., 2006). Three F4 antigenic variants including F4ab, F4ac and F4ad have been identified (Mooi et al., 1978; Gunee et al., 1979). These fimbriae mediate the adhesion of ETEC F4 to F4 variant specific receptors (Bijlsma et al., 1982) and facilitate their colonization in the small intestine by preventing intestinal peristalsis from removing the ETEC (Isaacson et al., 1988). The colonized bacteria produce enterotoxins, a heat-stable and/or heat-labile toxin, which induce massive fluid and electrolytes into the gut lumen and result in diarrhea (Mooi et al., 1999).

The loci encoding for the porcine intestinal receptors for ETEC F4ab/ac were assigned to chromosome 13 by linkage analysis (Guérin et al., 1993; Edfors-Lilja et al., 1995) and refined to SSC13q41 by two research groups (Python et al., 2002; Jorgensen et al., 2003). Recently, the F4ac receptor locus was precisely located to a region flanked by SW207 and SW0283 on 13q41 (Joller et al., 2006) and our research group confirmed it and found that the locus was closely linked to SW0283 (Li, 2006). Within this region, Jorgensen et al. (2004) investigated a candidate gene MUC4 and found that a mutation of G→C in its intron 7 was strongly associated with ETEC F4ab/ac adhesion phenotypes. The CC genotype was supposed to be associated with non-adhesive phenotype and hence inferred as resistant genotype, the GG and GC genotypes were associated with adhesive phenotype and hence inferred as susceptible genotypes. However, whether the existence of F4abR (F4ab receptor) and F4acR (F4ac receptor) is under the control of one locus or two closely linked loci is still unclear. Evidences for two loci closely linked were reported by Bonneau et al. (1990) and Guérin et al. (1993). But the results of Python et al. (2002) were in favor of a single locus.
In this study, we analyzed the relationship between the mutation of G→C in intron 7 of the MUC4 gene and the adhesion phenotypes of ETEC F4ab/ac in a large experiment population in order to verify the association between them found by Jørgensen et al. (2004) and to clarify whether the existence of F4abR and F4acR is determined by a single locus or two closely linked loci. Furthermore, to further reveal the genetic mechanisms of the adhesion of ETEC F4ab/ac, we used the technique of suppression subtractive hybridization to screen the genes related to the adhesion of ETEC F4ab/ac.

MATERIALS AND METHODS

Pigs

The experimental herd for this study was bred at the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences. A two-generation population was used, comprised of 310 piglets and 77 parents from three different breeds. Landrace, Large White and Chinese Songliaoshan. All piglets were weaned at 28 days of age, and slaughtered a week after weaning and their jejunal segments free of contents were collected for adhesion test and RNA isolation.

Adhesion test

The phenotypes (adhesion, weak-adhesion and non-adhesion) were established by means of a microscopic enterocyte adhesion test. Three E. coli strains included 195 (O8: K87: F4(ab), 200) (O149: K91. F4ac) and 238 (O38: K99: F4-negative) from China Institute of Veterinary Drug Control in Beijing were used. Preparation of enterocytes and adhesion test were carried out using the procedure described by Baker et al. (1997) with slight modifications described by Li et al. (2007). For each piglet, 20 or more epithelial cells from its jejunal cell specimen were checked. A single epithelial cell was considered adhesive when more than five bacteria adhering to its brush border membrane. A pig was classified as adhesive if at least 10% of the checked epithelial cells were judged as adhesive (Vögel et al., 1996), as weak-adhesive if less than 10% of the epithelial cells were judged as adhesive, yet more than 10% bind one to four bacteria, or as non-adhesive if no epithelial cells bound bacteria.

MUC4 gene test

The MUC4 gene test was performed based on the Xbal-polymorphism in intron 7 of the porcine MUC4 gene just as described by Jørgensen et al. (2004) and Jensen et al. (2006). The PCR-RFLP assay was performed and the 367 bp product was obtained from pig genomic DNA. The resistant allele (C) was indigestible by XbaI, whereas the susceptible allele (G) was digested into 151 and 216 bp fragments.

Suppression subtractive hybridization (SSH)

The total RNAs were isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions from the jejunal segment of the piglets. Teste cDNA and driver cDNA were synthesized using the pool of total RNAs from three piglets adhesive to ETEC F4ab/ac and three piglets non-adhesive to ETEC F4ab/ac, respectively. All of these six piglets were from the same litter of Large White. PolyA+ RNA was purified by Oligotex mRNA mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s guidelines. SSH was performed as described in the PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Palo Alto, CA, USA). The differentially expressed PCR products were inserted into pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into Escherichia coli DH10B. White clones were picked and 1 μl of each bacterial LB culture was amplified by using Nested PCR Primers 1 and 2R (Clontech Laboratories, Palo Alto, CA, USA). All positive clones were directly sequenced using an ABI 3730 DNA Analysers at BGI LifeTech Company Limited in Beijing.

Sequence analysis and database search

Searches for sequence similarity were performed with the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST) against the non-redundant sequence and the pig-specific sequences (http://www.ncbi.nlm.nih.gov/genome/guide/pig/).

Real-time RT-PCR

The total RNAs of the six piglets of Large White were prepared as described above for SSH and further treated with DNase I (Promega, Madison, WI, USA) for 30 min at 37°C. RT-PCR transcripts from the six piglets were served as templates for amplification using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) and the QuantiTect SYBR Green RT-PCR kit (Qiagen). The primers used for Ep-CAM were as follows: forward, 5'-TCAATGCAGGGTCTACAG-3', reverse, 5'-CCTAAA TTACGGTTTATG-3'. And the PCR conditions were 50°C for 2 min, 95°C for 2 min and 40 cycles of 94°C for 30 s, 61 °C for 30 s and 72°C for 20 s, followed by one cycle of 95°C for 15 s, 60°C for 20 s and 95°C for 15 s for disassociation curve analysis. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous assay control (primer sequence: forward, 5'-TGAGCACCAGTGTGTCG-3' and reverse, 5'-CACACCCCGTGTGTCG-3'). Each sample was run in triplicate to ensure quantitative accuracy, and the threshold cycle numbers (Ct) were averaged. The results were calculated using 2^-ΔΔCt method (Livak et al., 2001).

Radiation hybridization mapping

Here, we report the mapping of Ep-CAM gene that may
Table 1. Numbers of piglets and percentages (in the parentheses) with respect to different MUC4 genotypes and ETEC F4ab/ac adhesion phenotypes in three pig breeds

<table>
<thead>
<tr>
<th>Breed</th>
<th>Genotype</th>
<th>N</th>
<th>Adhesive</th>
<th>Weak-adhesive</th>
<th>Non-adhesive</th>
<th>Adhesive</th>
<th>Weak-adhesive</th>
<th>Non-adhesive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Landrace</td>
<td>GG</td>
<td>30 (35.7)</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>37 (44.0)</td>
<td>35</td>
<td>1</td>
<td>1</td>
<td>33</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>17 (20.2)</td>
<td>6</td>
<td>1</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>84</td>
<td>71 (84.5)</td>
<td>2 (2.4)</td>
<td>11 (13.1)</td>
<td>66 (78.6)</td>
<td>1 (1.2)</td>
<td>17 (20.2)</td>
</tr>
<tr>
<td>Large White</td>
<td>GG</td>
<td>11 (7.4)</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>59 (40.3)</td>
<td>59</td>
<td>0</td>
<td>1</td>
<td>54</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>78 (52.3)</td>
<td>14</td>
<td>1</td>
<td>63</td>
<td>6</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>149</td>
<td>84 (56.4)</td>
<td>1 (0.7)</td>
<td>64 (43.0)</td>
<td>71 (47.7)</td>
<td>12 (8.1)</td>
<td>66 (44.3)</td>
</tr>
<tr>
<td>Songliao Black</td>
<td>GG</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>CC</td>
<td>77 (100)</td>
<td>20</td>
<td>6</td>
<td>51</td>
<td>6</td>
<td>14</td>
<td>57</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>77</td>
<td>20 (26.0)</td>
<td>6 (7.8)</td>
<td>51 (66.2)</td>
<td>6 (7.8)</td>
<td>14 (18.2)</td>
<td>57 (74.0)</td>
</tr>
</tbody>
</table>

Table 2. Association of MUC4 genotypes and ETEC F4ac adhesion phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>41</td>
<td>41</td>
<td>0</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>GC</td>
<td>97</td>
<td>95</td>
<td>2</td>
<td>91</td>
<td>6</td>
</tr>
<tr>
<td>CC</td>
<td>172</td>
<td>48</td>
<td>124</td>
<td>38</td>
<td>134</td>
</tr>
<tr>
<td>Total</td>
<td>310</td>
<td>184</td>
<td>126</td>
<td>170</td>
<td>140</td>
</tr>
</tbody>
</table>

be related to the process of adhesion of E. coli F4ab/ac. The primers (forward, 5'-TGTCAGTCCAGTATCGTACCT-3' and reverse, 5'-TCAGAGTCCTTATCGGTCCT-3') only amplified the Ep-CAM gene in pig, and not the hamster controls. The PCR was performed in a total volume of 20 μl using 1-xPCR buffer, 200 μM of each dNTP, 0.2 μM of each primer, 1 U Taq DNA polymerase, and 25 ng of genomic DNA of the full INRA-University of Minnesota porcine radiation hybrid (IMpRH) panel. PCR was carried out at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 59.2°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 7 min. The PCR products were analysed on 2.5% agarose gels containing ethidium bromide. Data analysis of all genes was performed through the IMpRH website (http://www.toulouse.inra.fr/lgc/pig/RH/IMpRH.htm). The gene was typed in duplicate with the IMpRH panel.

RESULTS

Adhesion test and MUC4 gene test

The results of ETEC F4ab/ac adhesion test and MUC4 gene test are summarized in Table 1. There are significant differences in distributions of MUC4 gene genotypes and F4ab/ac adhesion phenotypes in the three pig breeds. In Landrace, most of the pigs possessed the susceptible genotypes (GG or GC) and were adhesive to F4ab/ac as well. On the other hand, in Songliao Black, all of the pigs possessed the resistant genotype (CC) and most of them were non-adhesive to F4ab/ac. In Large White, about half of the pigs possessed the susceptible genotypes and were adhesive to F4ab/ac.

Associations between the MUC4 genotypes and the F4ab/ac adhesion phenotypes

For testing the associations between the MUC4 genotypes and the F4ab/ac adhesion phenotypes, the adhesive and weak-adhesive phenotypes were merged into one phenotype which was inferred as F4ab/ac positive. In correspondence with this, the non-adhesive phenotype was inferred as F4ab/ac negative. The associations were tested using Chi-Square test and the results were given in Table 2. It can be seen that the MUC4 genotypes were very strongly associated with the F4ab and F4ac phenotypes (p = 3.94E-35 and 3.78E-37, respectively). All individuals with genotype GG were both F4ab and F4ac positive, 98% and 94% individuals with genotypes GC were F4ab and F4ac positive, respectively. 72% and 78% individuals with genotype CC were F4ab and F4ac negative, respectively.

Generation of SSH cDNA libraries

In order to isolate differential genes between the piglets adhesive to ETEC F4ab/ac and those non-adhesive, forward and reverse subtractive cDNA libraries were constructed. In this study, we drew our attention to the preferentially up-regulated genes in the forward subtractive cDNA library, and a total of 246 cDNA clones were obtained. After DNA
The relative expression of Ep-CAM to GAPDH in the intestinal epithelial cells of pigs with different phenotype. Group1: piglets adhesive to ETEC F4ab/ac; Group2: piglets non-adhesive to ETEC F4ab/ac. The difference in the expression level between the two groups is significant (p<0.05).

Real-time RT-PCR

The expression of Ep-CAM was quantified based on the Ct values obtained for the total RNA transcripts of the intestines of the six piglets. The expression profiles of Ep-CAM were significantly different (p<0.05) between the piglets adhesive to ETEC F4ab/ac and those non-adhesive using Student's t-test (Figure 1).

Radiation hybridization mapping

Based on a combination of the IMpRH results and the cytogenetic position of the flanking markers, Ep-CAM was located to SSC3q11-q14 closely linked to microsatellite SW236, with a LOD score of 5.89 and a distance of 66cR. This result was consistent with the known homology between HSA2p and SSC3q.

DISCUSSION

The results of this study confirm the finding of Jørgensen et al. (2004) that the mutation of G→C in intron 7 of the MUC4 gene was strongly associated with ETEC F4ab/ac adhesion phenotypes. However, in spite of the very strong associations, the genotypes and the phenotypes were not completely consistent, particularly for genotype CC, which is considered as resistant genotype to F4ab/ac. Of the 172 CC individuals, 48 (27.9%) were adhesive to F4ab, and 38 (22.1%) were adhesive to F4ac. This result suggests that the MUC4 gene may not be the causative gene or the mutation of G→C in intron 7 of the MUC4 gene may not be the causative mutation responsible for the existence of F4abR/F4acR. Rather, it might be just a marker closely linked to the actual causative mutation, either within the MUC4 gene or in another gene.

Several previous studies on the adhesion patterns of F4ab and F4ac revealed the existence of the patterns of F4ab+/F4ac- and F4ab-/F4ac+ (+ means positive and - means negative) in different pig populations (Bonneau et al., 1990; Baker et al., 1997, Li et al., 2007). Therefore, they suggested that F4ab and F4ac should have different receptors. Erickson et al. (1992, 1994) identified two intestinal mucin-type glycoproteins (IMTGP-1, IMTGP-2) as receptors for F4ac. Grange et al. (1996) found that a 74-kDa glycoprotein (GP74) purified from pig intestinal membranes was specifically detected in vitro by F4ab fimbriae, while F4ac and F4ad fimbriae did not bind to GP74. These results provided strong evidence for the two receptors hypothesis. In this study, we found that although the mutation of G→C in intron 7 of the MUC4 gene was very strongly associated to both F4ab and F4ac adhesion phenotype, the proportions of F4ab and F4ac positive individuals among the individuals with genotype CC were different (Table 3). When the weak-adhesive phenotype classified as positive, the two proportions were 27.9% and 22.1%, respectively, which were not significantly different (p = 0.1336). However, in consideration of the possible errors in judging the weak-adhesive phenotype, it might be more reasonable to remove the weak-adhesive individuals when comparing the proportions. In doing so, the two proportions were 21.4% and 10.3%, respectively, which were highly significantly different (p = 0.001). These results prompt that the F4abR and F4acR should not be controlled by the same gene (or mutation).

By suppression subtractive hybridization, we found the Ep-CAM gene was significantly up-regulated in the piglets adhesive to ETEC F4ab/ac. Ep-CAM was regarded as a physical homophilic interaction molecule between intestinal epithelial cells and intrapithelial lymphocytes at the mucosal epithelium for providing immunological barrier as a first line of defense against mucosal infection (Nochi et al., 2004). Although Ep-CAM was located to SSC3q11-q14, not in the mapping region of ETEC F4ab/ac receptor gene (SSC13q41), it may play an important role in the adhesion of ETEC F4ab/ac to the intestinal epithelial cells. Further investigation is needed to reveal their relationship.

ACKNOWLEDGMENTS

We are grateful to Dr Martine Yerle (INRA, Castanet-
REFERENCES


