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(54) Title: METHODS AND COMPOSITIONS TO IDENTIFY SWINE GENETICALLY RESISTANT TO F18 *E. COLI* ASSOCIATED DISEASES

(57) Abstract

The present invention provides non-invasive methods and compositions to differentiate, with a high level of sensitivity and specificity, swine that are genetically susceptible to diseases associated with F18 *E. coli* infection, from resistant swine. DNA polymorphisms in the swine alpha (1,2) fucosyltransferase 1 (*FUT1*) gene were used to differentiate resistant from susceptible swine. The invention includes a polypeptide with amino acid substitutions, encoded by the nucleotide polymorphisms, a molecular diagnostic assay, and a kit for the differentiation, of *E. coli* F18-adhesion resistant, heterozygous (carrier) and homozygous susceptible pigs. The molecular test identifies susceptibility to oedema disease and postweaning diarrhea with high sensitivity and specificity, therefore, is useful to swine breeders in their effort to enhance for resistance. Information on the polymorphisms of the present invention provides insight into causation and treatment of *E. coli* associated intestinal disorders.

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METHODS AND COMPOSITIONS TO IDENTIFY SWINE GENETICALLY RESISTANT TO F18 *E. COLI* ASSOCIATED DISEASES

Compositions and non-invasive methods are provided for the identification of swine genetically resistant to *E. coli* related diseases, in particular, intestinal diseases associated with a strain of *E. coli* bacteria supplied with fimbriae F18. DNA polymorphisms in the swine alpha (1,2) fucosyltransferase (*FUT1*) gene were identified that differentiate resistant from susceptible swine and provide a diagnostic test useful for swine breeders.

A major problem in breeding swine is to keep them disease-free. Intestinal disorders postweaning are a particular problem. A limited number of serotypes of toxigenic *Escherichia (E.) coli* strains are the causative agents of oedema disease and postweaning diarrhea in swine which induce serious economic losses, especially among piglets aged 4 to 12 weeks, in swine breeding farms all over the world. The typical clinical symptoms of oedema disease are neurological signs such as ataxia, convulsions and paralysis. At post mortem examination, oedema is typically present at characteristic sites such as eyelids and forehead, stomach wall and mesocolon. The diseases are caused by Shiga-like toxin-II variant and enterotoxins LT, STa, STb respectively, produced by *E. coli* that colonize the surface of the small intestine without effecting major morphological changes of the enterocytes (cells in the intestine). Certain types of bacterial *E. coli* strains, F18, F4 and K88 are major lethal villains in this regard. "Oedema disease of pigs is an enterotoxaemia characterized by generalized vascular damage. The latter is caused by a toxin, Shiga-like toxin II variant, produced by certain strains of *E. coli*" (Bertschinger *et al.*, 1993). The *E. coli* are distinguished by their pili types, a group of adhesive fimbriae that are related are designated *e.g.*, K88 or F18 (Vogeli *et al.*, 1997).

Not all swine succumb to *E. coli* infections. Colonization depends on adherence of the bacteria to the enterocytes which is mediated by the bacterial fimbriae designated *e.g.*, K88 or F18. Susceptibility to adhesion, *i.e.* expression of receptors in swine for binding the fimbriae, has been shown to be genetically controlled by the host and is inherited as a dominant trait with, in the case of F18, *B* being the susceptibility allele and *b* the resistance allele. (Vogeli *et al.*, 1996; Meijerink *et al.*, 1996). The genetic locus for this *E. coli* F18-receptor (*ECF18R*) has been mapped to porcine chromosome 6 (SSC6), based on its close genetic linkage to the *S* locus and other loci of the halothane (HAL) linkage group on chromosome 6. The receptor for K88 *E. coli* is on chromosome 13.

The mechanism for resistance appears to be that intestinal borders in resistant animals are not colonized by *E. coli*, *i.e.*, the bacteria do not adhere to intestinal walls of resistant swine. Glycoprotein receptors in the brush border membrane of the intestine were shown to be responsible for the differences between adhesive and non-adhesive phenotypes related to some *E. coli*, therefore, the genotype of the host swine determines resistance. The fimbriated bacteria also have been studied. (WO 9413811)

Current methods of identifying swine that are resistant to F18 *E. coli* associated diseases are either to 1) collect intestinal samples from swine at slaughter and perform the microscopic adhesion test, 2) challenge the animals with virulent *E. coli* ("colonization test"), or 3) perform blood typing of the A-O(S) blood group system. The first two methods are not practical for identifying resistant animals for use as breeding stock. Although the blood typing method does identify resistant animals, the test is unable to determine whether susceptible animals are homozygous or heterozygous for susceptibility. Knowledge of the genotype of animals with regard to these alleles (conditions of a gene) is essential to develop a successful breeding program. The purpose of the breeding program is to produce swine that are resistant to F18 *E. coli* associated diseases that decimate stock post-weaning.

In one publication the authors stated, in reference to oedema disease in swine, that "Searches are underway for appropriate genetic markers. . ." (Bertschinger *et al.*, 1993, page 87) and, citing Walters and Sellwood, 1982:

> Breeding resistant swine is an attractive method for prevention of diseases for which an effective prophylaxis is not available. The feasibility of this approach will depend on the prevalence of the gene(s) encoding resistance in the pig population, improved methods for the detection of resistant pigs, and absence of negative genetic traits co-selected with this resistance.

A genetic "marker" locus is a coding or non-coding locus that is close to a genetic locus of interest, but is not necessarily the locus itself. Detectable phenotypes include continuous or discontinuous traits, e.g. restriction length fragment polymorphisms, production traits, bacterial adhesion traits, colorimetric or enzymatic reactions, and antibiotic resistance. The *S* locus controls expression of the A and O blood group antigens. Swine homozygous recessive at the S locus do not express either A or O blood group antigens. A similar condition exists in humans and is due to mutations in the alpha (1,2) fucosyltransferase gene which encodes the human blood group H (Kelly *et al.*, 1994; *see also* WO 9628967). The porcine alpha (1,2) fucosyltransferase gene of swine has recently been sequenced (Cohney *et al.*, 1996). This gene is very likely the gene present at the *S* locus in swine.

The blood group *H* and *Se* loci have been mapped genetically and physically to human chromosome 19q13.3. This region is evolutionarily conserved, containing genes homologous to the HAL linkage group of genes in pigs. The blood group H encoding gene is the so called *FUT1* whereas the *Se* gene is equivalent to the *FUT2* gene. *FUT1* determines H antigen expression in the erythroid cell lineage, whereas *FUT2* regulates expression of the H antigen in the secretory epithelia and saliva. Conservation of the *FUT1* gene has been shown in lower mammals such as rat and rabbit, and mRNA expression has been shown in rabbit brain tissue and rat colon. In all these species two types of alpha (1,2) fucosyltransferase genes have been reported which are structurally very similar to the human *FUT1* and *FUT2* genes, but in particular the *FUT1* homologous genes show a species specific expression pattern. In humans the *FUT1* gene is responsible for synthesis of H antigens in the precursors of erythrocytes. However, in pigs erythrocytes passively adsorb H-like antigens from the serum, as is the case for the human *Lewis* antigens. In pigs all H-like antigens are related to exocrine secretory tissues, and expression of the *FUT2 (Secretor)* gene is seen in secretory tissue of other animal species. Therefore, expression of the porcine A-O blood group determinants which cross-react with anti-human blood group H and A antibodies might be influenced by the *FUT2* gene.

Further information about blood groups and *E. coli* swine diseases include that carbohydrate structures of blood group antigens mediate the adhesion of some pathogenic microorganisms to host tissues, *e.g. Helicobacter pylori* adhere to Lewis^b blood group antigens, and *E. coli* causing urinary tract infections adhere to blood group *P* substance. Genes encoding glycosyltransferases that are responsible for the formation of the blood group specific carbohydrate structures, therefore, represent candidate genes for the control of bacterial colonization by the host. The localization of these genes is in the same chromosomal region as the locus responsible for adhesion/non-adhesion of F18 positive *E. coli* in the swine small intestine.

Swine do not express blood group antigens A and O until after weaning, this is the same time that they become susceptible to disease caused by F18 *E. coli.*

New methods of diagnosis and treatment are needed for *E. coli* related intestinal diseases in swine. Detection of a genetic mutation was proposed as a diagnostic test for some swine disorders (malignant hypothermia) (Fujii *et al.*, 1991; U.S. Pat. No. 5,358,649), but polymorphic markers were not reported for diagnosis. Vaccines to develop resistance to *E. coli* colonization were described (U.S. Pat. No. 5,552,144; WO 8604604), but are unlikely to be a preferred method to prevent the *E. coli* disease because of difficulties in administering live vaccine orally to newborn swine, and because of regulatory restrictions. Antibiotics are available for treatment, but there is no successful prophylaxis.

SUMMARY OF THE INVENTION

The compositions and non-invasive methods of the present invention provide detection and elimination of swine that are susceptible to *E. coli* associated diseases. A non-invasive method for identifying a swine that is resistant to intestinal colonization by *E. coli* F18 includes the following steps: determining whether a genetic polymorphism associated with resistance to colonization is in a biological sample from the swine; and inferring that the swine is resistant if the swine is homozygous for the polymorphism.[1]

More particularly, the method is determining in a biological sample from the swine whether the nitrogen base at position 307 in the alpha (1,2) fucosyltransferase gene of the swine is only adenine or only guanine; and identifying the swine as resistant if the only nitrogen base at position 307 is adenine.

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[1] A polymorphism is a change in a nucleotide sequence that exists in a population due to mutation.

To determine whether a polymorphism is present in a biological sample, restriction fragment length polymorphisms are analyzed on a gel that separates them by molecular weight. Restriction endonucleases are enzymes that reproducibly cut nucleic acid molecules at specific sites, resulting in nucleic acid fragments of different molecular weights, depending on the location of the cuts.

The invention also relates to a method for breeding swine to be resistant to *E. coli* associated diseases by selecting for breeding swine that have a genetic polymorphism in the alpha (1,2) fucosyltransferase 1 gene that identifies them as swine that are resistant to *E. coli* related intestinal diseases; and breeding the selected swine.

An aspect of the invention is a DNA molecule which is polymorphic for the alpha (1,2) fucosyltransferase 1 gene in swine, in particular a sequence in accordance with FIG. 1. Other aspects of the invention are molecules with nucleotide sequences complementary to that of FIG. 1.

An aspect of the invention is an isolated DNA molecule with a substitution of adenine for guanine in position 307. This molecule may also bond a substitution of adenine for guanine in position 857. Other isolated DNA molecules of the present invention include those with a mutation at nucleotide position 229 of the sequence of FIG. 1, wherein the codon CTT is changed to TTT, encoding for the amino acid phenylalanine instead of leucine. A mutation at nucleotide position 714 is from GAT→GAC, but there is no accompanying amino acid substitution in the encoded product.

Polypeptides encoded by the DNA molecules of the present invention and having alpha (1,2) fucosyltransferase activity are also aspects of the invention.

A molecular assay for detecting *E. coli* F18 receptors in swine is to (a) isolate DNA from porcine nucleated cells; (b) amplify the DNA in a polymerase chain reaction (PCR) using oligonucleotides as primers which are complementary to a DNA sequence of the porcine alpha (1,2) fucosyltransferase gene 1; (c) perform a restriction enzyme digest with at least one restriction enzyme *e.g.*, CfoI; (d) separate the resulting fragments by gel electrophoresis; and (e) determine the respective numbers and lengths of fragments on the gel; and (f) determine from the numbers and length of fragments of F18, which receptors are present in the porcine cells. Use of the larger amplified fragments disclosed herein for restriction length polymorphism analysis (RFLP), rather than smaller fragments, is less expensive because the DNA bands can be run on agarose gels of relatively low concentration. Also, to produce some of the fragments, only one restriction enzyme is needed for a constant restriction site adjacent to the variable diagnostic site.

A kit for detecting polymorphisms associated with *E. coli* F18 receptors uses oligonucleotides in separate containers which are complementary to a DNA sequence of the porcine alpha (1,2) fucosyltransferase gene 1 that distinguishes resistant from sensitive swine. The test can be performed on swine of any age.

The polymorphisms are also useful to develop drugs to treat swine that have *E. coli*-associated disease. A mutated form of porcine alpha 1,2 fucosyltransferase could interfere with the normal enzyme, preventing it from producing the intestinal receptor for F18.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the nucleotide sequence (*FUT1*) (below) and the predicted amino acid sequence (above) of the swine $\alpha 1 \rightarrow 2$fucosyltransferase polymorphism of the present invention using the *one-letter* amino acid code. The *solid double line* below the amino acid sequences (=) is the putative transmembrane region; the *dotted* line below the amino acid sequence shows three potential N-linked glycosylation sites (....).

□ is where an adenine (A) is substituted for guanine (G) in resistant swine.

*Indicates the termination codon

Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Molecular analysis of DNA polymorphisms associated with resistance of swine to *E. coli* associated diseases facilitated diagnostic assays to select resistant pigs for breeding. Resistant pigs differ from sensitive pigs at the *E. coli* F18 receptor locus as identified by the polymorphic markers of the present invention.

The present invention provides non-invasive methods and compositions to distinguish with a high level of sensitivity and specificity, swine that are genetically susceptible to diseases associated with F18 *E. coli* infection from resistant swine. A DNA polymorphism in the swine alpha (1,2) fucosyltransferase (*FUT1*) gene was identified that differentiates resistant from susceptible swine. The polymorphism arose by a mutation (change) in a nucleotide sequence leading to a new allele. An allele is a condition of a gene. In a population there may be many alleles of a gene differing by nitrogen base substitutions, presumably caused by mutations in an ancestral DNA molecule. The coexistence in a population of more than one allele (sometimes referred to as a "variant") is called a genetic polymorphism. Loci at which more than one allele may exist as apparently stable components of a population, is a polymorphic locus. Usually, one of the polymorphic loci is at a low frequency in the population.

As determined from a biological sample, preferably blood, the resistant swine have a polymorphism in their genomes in which the only base detected at position 307 (see FIG. 1) in the nucleotide sequence is adenine, whereas the base in the same position in homozygous susceptible swine is guanine. Heterozygous swine will show both types of DNA and will be susceptible. The polymorphism is a variation of a porcine gene sequence (Cohney *et al.* 1996).

Genetic linkage analysis was performed on families of swine and genetic associations between polymorphisms in FUT1 and disease resistance in outbreed swine were determined. According to the present invention, polymorphisms have been found in the alpha (1,2) fucosyltransferase 1 gene (*FUT1*). A polymorphism that has a single nucleotide base substitution at position 307 was used to establish a close linkage between the fucosyltransferase gene and the *S*-system, the *ECF18R* locus and other loci of the *HAL* linkage group.

The detection of the close linkage of the mutation at *FUT1* and *ECF18R* allowed a molecular test to be developed for the identification of *E. coli* F18 adhesion resistant, heterozygous (carrier) and homozygous susceptible pigs. This diagnostic test identifies, with high sensitivity and specificity, pigs that are susceptible to oedema disease and postweaning diarrhea. The incidence of the polymorphisms of the present invention differs among swine breeds. Vogeli, *et al.* (1997) presented frequencies of the M307 allele in 5 pig breeds from herds that were not related to one another. The availability of the diagnostic test for the polymorphism of the present invention provides breeders with the opportunity to effectively eliminate the *ECF18R* susceptible allele from their swine herds, thereby eliminating a prerequisite for

E. coli F18 bacterial adhesion causing oedema disease and postweaning diarrhea.

The present invention further includes nucleotide sequences that are variants of a sequence of the alpha (1,2) fucosyltransferase gene 1 representing the various polymorphisms at bp 307, and diagnostic molecular based kits to identify polymorphisms in the alpha (1,2) fucosyltransferase gene.

In order to obtain candidate genes for the *E. coli* F18 receptor locus (*ECF18R)* 5 cosmids and one genomic clone containing the gene were isolated containing the alpha (1,2) fucosyltransferase genes, *FUT1* and *FUT2* (Meijerink *et al.*, 1997), from a porcine genomic library. Mapping by fluorescence *in situ* hybridization placed all these clones in band q11 of porcine chromosome 6 (SSC6q11). Sequence analysis of the cosmids resulted in the characterization of (a) an open reading frame (ORF), 1098 base pairs in length, that is 82.3% identical to the human *FUT1* sequence, and (b) a second ORF, 1023 base pairs in length which is 85% identical to human *FUT2* sequence. The *FUT1* and *FUT2* loci therefore seem to be porcine equivalents of the human blood group *H* and the *Secretor* locus. Direct sequencing of the two ORFs in swine either susceptible or resistant to adhesion and colonization by F18 fimbriated *E. coli* (*ECF18R*) revealed two polymorphisms at base pair 307 (M307) and base pair 857 (M857) of the *FUT1* ORF. The nucleotide positions are numbered from ATG (encoding methionine). Analysis of these mutations in 34 matings of Landrace families with 221 progeny showed close linkage with the locus controlling resistance and susceptibility to *E. coli* F18 adhesion and colonization in the small intestine (*ECF18R*) and with the locus of the blood group inhibitor *S*. Therefore, the M307 mutation is a good marker for marker-assisted selection of *E. coli* F18 adhesion resistant animals. Another mutation at nucleotide position 229 was found leading to a polymorphism in which the codon encoding leucine (CTT) was changed to

TTT (encoding phenylalanine). A mutation at position 714(GAT→GAC) (encoding aspartic acid) did not produce an amino acid substitution. No polymorphisms were identified in FUT2 that differentiated susceptible and resistant pigs.

Examples

The following examples provide embodiments of the invention.

Example 1: An Assay For Resistant Swine

The polymorphisms of the present invention are easily identified using PCR-RFLP tests. One embodiment of the tests used a 160bp fragment of porcine alpha (1,2) fucosyltransferase 1 amplified using PCR with the following primers; 5'CCAACGCCTCCGATTCCTGT3' and 5'GTGCATGGCAGGCTGGATGA3'. Preferred PCR conditions for this embodiment are 25 cycles at the following times and temperatures: 94°C, 30 sec; 60°C, 45 sec; 72°C, 90 sec. The amplified DNA from resistant swine was digested by the restriction enzyme HgaI, but was not digested by the restriction enzyme HinPI. The amplified DNA from homozygous susceptible swine was digested by the restriction enzyme HinPI. The amplified DNA from heterozygous susceptible swine was partially digested by both enzymes.

Alternatively, DNA was isolated from porcine nucleated cells according to standard procedures. Direct sequencing of porcine *FUT1* and *FUT2* sequences and their flanking regions in animals of different *ECF18R* genotype (Bb, bb) resulted in the identification of two G --> A transitions at positions 307 and 857 (termed *M307* and *M857*, respectively) of the *FUT1* ORF. The *M307* transition eliminates a restriction site for CfoI. Amplification of DNA isolated from porcine nucleated cells was performed according to standard procedures with primers P6 and P11 (3 min at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 56°C and 30 sec at 72°C, followed by a 7 min final extension at 72°C) followed by CfoI digestion and separation on a 3% agarose gel resulted in a restriction fragment length polymorphism (RFLP). Homozygous $M307^{AA}$ animals showed 2 bands. Homozygous $M307^{GG}$ animals showed 93-, 241- and 87bp fragments. Heterozygous animals showed all four fragments.

Example 2: Sensitivity And Specificity Of An Assay Using Alpha (1,2) Fucosyltransferase In Detecting Swine Resistant To *F18 E. Coli*

A study was conducted to determine the association between disease resistance and the polymorphism at position 307 of the FUT1 gene. 183 weaned swine (ranging in ages 2-6 months) were obtained from six different breeding herds. Only one of these herds was known to contain resistant animals before the start of the study, and this herd is known to have a high incidence of porcine stress syndrome. The other 5 herds had no evidence of porcine stress syndrome, and the incidence of disease resistance was unknown. Swine from each herd were randomly selected, humanely euthanized and spleens and samples of small intestine were removed. DNA was extracted from splenic tissue and used in a PCR-RFLP assay described in Example 1. Intestinal cells were purified by scraping the mucosal surface off the intestine, lysing the cells in a hypotonic EDTA solution and washing by centrifugation. The purified intestinal cell brush borders were incubated with F18 *E. coli*. This mixture was examined by phase contrast microscopy. This assay determined if swine were susceptible (intestinal samples had adhering bacteria) or resistant (intestinal samples had no adhering bacteria). The PCR-RFLP assay for the polymorphism correlated with the bacteria-intestinal cell binding assay in 53 of 53 resistant swine and 128 of 130 susceptible swine. Two swine that were determined susceptible using the bacteria-intestinal cell binding assay were incorrectly predicted to be resistant using the PCR-RFLP assay. Two of the six herds examined contained resistant pigs, while only one herd had porcine stress syndrome, demonstrating that the PCR-RFLP assay can identify disease resistant animals in animals that do not have porcine stress syndrome.

Example 3: Localization Of *FUT1* On Chromosome 6 (SSC6)

Cosmids ETHsl, -s2, -s3, -s4 and -s6 were identified after screening of the cosmid library with a *FUT1* nucleotide probe obtained from porcine genomic DNA with primers P7 and P10 and were mapped by FISH and DISC-PCR to chromosome 6 in band q11.

Example 4: Identification of the Porcine *FUT1 ORF*

Hybridizing KspI, EcoRI and KspI/EcoRI cosmid digests with radiolabelled porcine *FUT1* fragments P6-P11 and P7-P10 for Southern blot analysis revealed identical autoradiography signals for ETHs2, -s4 and -s6, whereas different signals were obtained from cosmids ETHs1 and -s3. From cosmid ETHs2 KspI, subclones 940 bp and 6.2 kb in length were isolated, corresponding to the estimated length of hybridizing KspI fragments on the Southern blot. The sequence results of both subclones were combined to yield a 1501 bp sequence, which was in agreement with results of direct sequencing of genomic PCR products. The 1501 bp sequence contains an open reading frame (ORF) of 1098 bp corresponding to the human *FUT1* ORF, with 82.3% nucleotide and 80.8% amino acid identity. The ORF encodes a polypeptide.

Example 5: Identification of a Porcine *FUT2* and a Pseudogene *FUTP*

ETHs1 has one DNA fragment (2.7 kb) that hybridizes to *FUT1* sequences, whereas ETHs3 has two (2.7 kb and 8.2 kb). Subcloning and partial sequencing of the 2.7 kb EcoRI fragment of ETHs1 and -s3 confirmed that these two fragments are identical. The sequence is highly similar to the human *FUT2* but shows several changes in the NH_2- and -COOH terminal regions. These changes lead to frame shifts that are not compatible with a conserved ORF, therefore an assumption is that the sequence obtained from the 2.7 kb fragment represents a psuedogene (*FUT2P*). After subcloning of ETHs3 BamHI digests, the hybridizing sequences contained in the 8.2 kb EcoRI fragment were identified. The sequence of the subclones obtained represents a 1023 bp ORF and is 85% identical at the nucleotide- and 83% identical at the amino acid level to the human *FUT2* sequence. Many differences in the NH_2- and -COOH terminal regions were observed between the porcine *FUT2* sequence and the *FUT2P* sequence derived from the 2.7 kb fragment. The predicted amino acid sequence corresponds to the partially determined amino acid sequence of the porcine *Secretor* enzyme (Thurin and Blaszczyk-Thurin, 1995). The porcine *FUT1, FUT2* and *FUTP* sequences obtained were submitted to GenBank and have accession numbers U70883, U70881 and U70882, respectively. The *FUT1* and *FUT2* genes have highly homologous sequences. This has to be considered in, for example, primer development. Furthermore, *FUT1* and *FUT2* enzyme activity need to be differentiated in further studies.

Example 6: Identification of M307 and
<u>M857 Mutations and Characterization of M307</u>

DNA was isolated from porcine nucleated cells according to standard procedures. Direct sequencing of porcine *FUT1* and *FUT2* sequences and their flanking regions in animals of different *ECF18R* genotypes (*Bb*, *bb*) resulted in the identification of two G → A transitions at positions 307 and 857 (termed *M307* and *M857*, respectively) of the *FUT1* ORF. The *M307* transition eliminates a restriction site for the enzyme CfoI. Amplification of DNA isolated from porcine nucleated cells was performed according to standard procedures with primers P6 and P11 (3 min at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 56°C and 30 sec at 72°C, followed by a 7 min final extension at 72°C) followed by CfoI digestion and separation on a 3% agarose gel resulted in a restriction fragment length polymorphism (RFLP). Homozygous *M307*^{AA} animals showed 2 bands (93- and 328-bp fragments). Homozygous *M307*^{GG} animals showed 87-, 93-, and 241-bp fragments. Heterozygous animals showed all four fragments.

Example 7: Characterization Of Mutation M857

The M857 mutation is a transition that eliminates an AciI site. Primer PBEST was designed to mismatch two additional AciI sites at positions 866 and 872. PCR with primers P7 and PBEST (3 min at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 56°C and 30 sec at 72°C, followed by a 7 min final extension at 72°C) followed by AciI digestion enables PCR-RFLP analysis on a 3% agarose gel. Homozygous *M857*^{AA} animals show a 174 bp fragment while amplification products of *M857*^{GG} animals show 136- and 38- bp fragments.

Example 8: Genetic Mapping Of The *FUT1* Gene

In Landrace swine families, recombination events between *M307* and the loci of the HAL linkage group (*S, ECF18R, RYR1, GPI, PGD*) revealed recombination fractions $\theta<0.04$ (Table 2). The lodscores *Z* for the overall recombination fractions were between 24.5 and 50.6, showing strong evidence for linkage between these loci. These data allow genetic mapping of the *FUT1* gene to the HAL linkage group in close proximity of S and *ECF18R* which are both influenced by *FUT1*. In the experimental Landrace families, allelic association was found between *ECF18R* and *RYR1*. An excess of genotypes *RYR1*^{TT} at position 1843 in *RYR1* (halothane susceptible genotype) was observed among pigs resistant to oedema disease and postweaning diarrhea (genotype *ECF18R*$^{b/b}$) (Table 3). This allelic association is a result of linkage disequilibrium, that is, deviation of observed haplotype frequencies from expected haplotype frequencies under independent assortment of alleles. Therefore, linkage disequilibrium designates a non random association of alleles belonging to linked loci. Owing to low recombination rates however, no locus order could be determined as being significantly better than others.

Example 9: Association Of M307^{A} With ECF18^{b} And M307^{G} With *ECF18R*^{B}

In Landrace (SL) and Large White (LW) parental pigs, *ECF18R*^{b} (the oedema and postweaning diarrhea **resistance** allele) is 100% associated with *M307*^{A}, and *ECF18R*^{B} (the oedema and postweaning diarrhea **susceptibility** allele) is 100% associated with *M307*^{G} (wherein A=adenine; G=guanine). In SL pigs 88% (30/34) of S^{s} accounted for all *ECF18R*^{b} and *M307*^{A} haplotypes, respectively. The corresponding values for both the S^{s}-*ECF18R*^{b} and S^{s}-*M307*^{A} haplotypes were 82% (9/11) in Large White pigs. In the experimental SL families, the occurrence of the *M857*^{A} allele at the *FUT1* locus was low, and even absent, in LW pigs. Therefore, a significant gametic association was not observed between the alleles of *M857* and the alleles of the flanking genes. The G->A transitions at positions *FUT1 307* and *FUT1 857* were found with variable frequencies also in Duroc, Hampshire and Pietrain pigs, making it likely that those transitions also occur in other pig breeds.

Example 10: Distribution Of *FUT1* Genotypes

Table 4 shows that the distribution of *FUT1* genotypes at nucleotide position 307 among *ECF18R* types was significantly different from the expected ratio under a hypothesis that the two are independent. Of the 119 oedema disease and postweaning diarrhea resistant *ECF18R*$^{b/b}$ animals, 118 were determined to have the genotype *M307*^{AA} in the DNA-based test. One resistant animal had the genotype *M307*$^{A/G}$. Of the 131 susceptible pigs, 130 were *M307*$^{A/G}$ or *M307*$^{G/G}$. One animal, susceptible to *E. coli* adhesion, was shown to be homozygous *M307*$^{A/A}$ by the DNA-test. The data from this example and example 2, together with past studies suggested that the *FUT1* gene is the gene present at the *S* locus in swine and the *ECF18* locus. While 4 animals in this example and example 2 contradict this hypothesis, it is probable these animals were incorrectly phenotyped in regards to disease resistance/susceptibility.

Example 11: Amino Acid Exchanges in Alpha (1,2) Fucosyltransferase

The G → changes at bp +307 and bp +857 of the alpha (1,2) fucosyltransferase gene 1 results in a predicted amino acid substitution of threonine (neutral-polar) instead of alanine (neutral-nonpolar) and glutamine (neutral-polar) instead of arginine (basic), respectively which may have functional consequences in the encoded product. A C → T change at bp 229 results in an amino acid substitution of leucine (neutral-nonpolar) instead of phenylalanine (neutral-nonpolar).

Table 1: Sequences Of Forward-(F) And Reverse-(R) Primers And Their Relative Position To The Porcine *FUT1* And *FUT2* Start Codons.[2]

Primer name	Primer sequence	Position
FUT1 P6 (R)	5'-CTTCAGCCAGGGCTCCTTTAAG-3'	+489
FUT1 P7 (F)	5'-TTACCTCCAGCAGGCTATGGAC-3'	+720
FUT1 P10 (R)	5'-TCCAGAGTGGAGACAAGTCTGC-3'	+1082
FUT1 P11 (F)	5'-CTGCCTGAACGTCTATCAAGATC-3'	+69
FUT1 P16 (F)	5'-AGAGTTTCCTCATGCCCACAGG-3'	-90
FUT1 P18 (R)	5'-CTGCTACAGGACCACCAGCATC-3'	+1203
FUT1 PBEST (R)	5'-ACCAGCAGCGCAAAGTCCCTGAC GGGCACGGCCTC-3'	+893
FUT2 P16 (R)	5'-CTCCCTGTGCCTTGGAAGTGAT-3'	+1094
FUT2 P17 (F)	5'-AACTGCACTGCCAGCTTCATGC-3'	-83

Table 2: Overall Recombination Fractions (θ), Lodscores (Z) And Number Of Informative Animals (N) For *M307* And Loci Of The *HAL* Linkage Group In The Landrace Experimental Population

Locus pair	N	θ	Z
S-ECF18R	183	0.01	50.6
M307-S	183	0.01	50.6
M307-ECF18R	216	0.01	57.1
M307-RYR1	198	0.02	47.2
M307-GPI	147	0.03	34.2
M307-PGD	147	0.04	24.5

[2] Primers *FUT1* P10 and *FUT1* P11 are derived from the human *FUT1* gene.

Table 3: Haplotype Frequencies At The Four Loci (*S-FUT-1* (*M307, M857*)-*ECF18R-RYR1*) In The Landrace (SL) Experimental Population And Randomly Selected Large White (LW) Pigs.

Breed	Haplotype[3] at *S*, *FUT1* (*M307*, *M857*), *ECF18R*, *RYR1*	Frequency[4] (number)
SL	*sAGbT*	70 (28)
	sAGbC	5 (2)
	SGGBC	15 (6)
	sGABC	10 (4)
LW	*sAGbC*	56 (9)
	SGGBC	31 (5)
	sGGBC	13 (2)

[3] *S*: Suppressor locus for A and O blood types (*S* and *s*).
FUT1 (*M307*): alteration of adenine (A) to guanine (G) at nucleotide 307 of the alpha (1,2) fucosyltransferase (*FUT1*) gene. *FUT1* (*M857*): alteration of adenine (A) to guanine (G) at nucleotide 857 of the *FUT1* gene. *ECF18R*: *E. coli* F18 receptor. The dominant susceptible allele is indicated by *B* and the resistant allele by *b*. *RYR1*: skeletal muscle ryanodine receptor. C (cytosine) is the dominant resistant and T (thymine) the susceptible allele for malignant hyperthermia.

[4] Haplotype frequencies in % and absolute number of haplotypes between brackets.

Table 4: Distribution Of The Genotypes, Tetrachoric Correlation (R) And Significance Of The Association (χ^2 And w x χ^2) Of The Associated Polymorphic *FUT1* (*M307*) And *ECF18R* Loci In Landrace (SL) Experimental Population And Randomly Selected Large White (LW) Swine.

		FUT1/M307					
Breed	Locus	Genotype	*A/G*	*A/A*	r	χ^2	χ^2 x w[5]
SL	*ECF18R*[6]	*b/b*	1	113	0.98	213.1	42.6***
		B/b	106	1			
			A/G				
		Genotype *(A/G)*	*G/G*	*A/A*			
LW	*ECF18R*[6]	*b/b*	0	5	1.00	29.0	11.6***
		B/b,B/B	24	0			

METHODS

1. Primers

Primers derived from the human *FUT1* gene were used for the amplification of its porcine counterpart from genomic DNA. From the resulting porcine sequences specific primers were designed which were used in further amplification and sequencing reactions (Table 1).

2. Screening of a Porcine Genomic Library

Porcine genomic libraries were screened with either a porcine FUT1 probe obtained with primers P7 and p10 or a porcine FUT1 cDNA. A porcine

[5] A weight factor of w = 0.2 (SL) and 0.4 (LW) was applied to correct for the lack of precision resulting from inclusion of related animals in the data, according to Cotterman (1947). *** p<0,001.

[6] Animals of genotype *b/b* at the *ECF18R* locus are resistant and those of genotype *B/b* and *B/B* are susceptible to adhesion of F18ab *E. coli* bacteria.

genomic library, constructed in SuperCos 1 (Stratagene, La Jolla, Ca, USA), was screened with an $\alpha^{32}P$ dATP labeled (Prime It II, Stratagene) *FUT1* probe otained from porcine genomic DNA with primers P7 and P10. After hybridization of replica filters at 42°C for 15h (50% formamide, 6 x SSC, 5 x Denhardt's, 0.5% SDS, 0.1 mg/ml Salmon Sperm) and washing twice at 65°C for 30 min. (1 x SSC, 0.1% SDS), positive colonies were identified after exposure (15h, -80°C) to X-ray film.

3. *In situ* Hybridization of Porcine Metaphase Chromosomes

Cosmid clones ETHs 1, ETHs2, ETHs3, ETHs4 and ETHs6 were subjected to fluorescence *in situ* hybridization (FISH) (Solinas Toldo, *et al.* 1993) or direct *in situ* chromosomal PCT (DISC PCR) on porcine metaphases. Metaphase chromosomes were Q-banded and photographed before hybridization. The probes were labeled by random priming using biotin-16-dUTP. Signal detection and amplification was performed using avidin-FITC and biotinylated anti-avidin. The chromosomes were counterstained with 4,6-diamidino-2-phenylindole, and the relative positions of the cosmids were determined as described by Solinas Toldo, 1993.

4. Subcloning

Enzymatic digests of probe positive genomic colonies were separated on agarose gel, transferred to a nylon membrane, and probe positive bands were subcloned into plasmids for FUT1 sequencing. The sequence of FUT1 derived from this method is shown in FIG. 1.

KspI-, EcoRI- and KspI/EcoRI digests of all cosmids were separated on a 0.8% agarose gel and transferred to a Hybond N nylon membrane. (Meijerink *et al.*, 1997). This blot was hybridized with $\alpha\,^{32}P$ dATP labeled porcine *FUT1* PCR products (primers P6-P11 and P7-P10). Based on the autoradiographic signals, ETHs1, -s2 and -s3 were subjected to further subcloning into pBluescript SK- (Stratagene), and *FUT* sequences were determined from subclones. The sequences of two *FUT*-like open reading frames (ORFs) (*FUT1* and *FUT2*) obtained from cosmids ETHs2 and -s3 were compared in *ECF18R* positive (*BB/Bb*) and negative (*bb*) animals by direct sequencing of PCR products.

5. Polymerase Chain Reaction and Direct Sequencing

Using the Perkin Elmer Ready Reaction Dye Terminator kit (Perkin Elmer Cetus, Norwalk, CT, USA) and 10 pmol of primer, cycle sequencing was performed with a thermal program consisting of an initial denaturation of 5 min at 95°C, followed by 25 cycles of 30 sec 95°C, 15 sec 50°C and 4 min 60°C. Primers used for amplification and sequencing of the porcine alpha (1,2) fucosyltransferase genes are listed in Table 1. Additional primers were designed taking the possibility of cross-annealing of primers due to the high similarity of *FUT1*, *FUT2* and the *FUT2* pseudogene into account. Samples were analyzed on a 373A ABI sequencer (Applied Biosystems Inc.) and sequence analysis was performed with the GCG package (Devereux, 1984).

6. Production of Informative Offspring

Single nucleotide polymorphisms were analyzed in 221 Landrace swine produced from 4 boars and 16 sows, and in 29 Large White swine produced from 9 matings between unrelated swine. In order to produce a large number of informative offspring for the examination of linkage between porcine genes encoding ECF18 receptors and selected polymorphic loci, only informative Landrace matings of the type *B/b* x *b/b* were produced.

7. Colonization Test

In a study of Bertschinger *et al.*, 1993, the above mentioned Landrace swine were also tested for ECF18 susceptibility in a colonization test. For this, swine were inoculated shortly after weaning with bacteria of *E. coli* strain 124/76 of serotype O139:K12(B):H1:F18. (Rippinger, *et al.*, 1995). Faecal shedding of the bacteria was monitored daily. The extent of colonization was calculated as the mean of the two highest faecal scores. Swine with a mean faecal score of 3.5, corresponding to 6.7 log colony forming units (CFU)/g or more, were considered susceptible to colonization. This limit was based on a lack of mortality below this value, and on scores obtained from completely resistant litters.

8. Linkage Analysis of Nucleotide Polymorphisms

The results of the single nucleotide polymorphisms were compared with typing data for ECF18R, which were identified in an *in vitro* adhesion assay described by Vögeli *et al.*, (1996), and with typing data for the *GPI*-, *PGD*-, α-1-B-glycoprotein-(A1BG), ryanodine receptor (*RYR1*), *EAH*-and *S*-loci as published by Vögeli *et al.* (1996). Pairwise linkage analysis and calculation of recombination fractions was performed using the CRI-MAP version 2.4 programme (Green *et al.*, 1990). Multipoint linkage analysis was performed by sequential insertion of the above loci into the map. Haplotype frequencies were calculated from the parental animals, in the Landrace families and from the 8 parental Large White animals which were haplotyped for *ECF18R* from progeny information. Tetrachoric correlations of *ECF18R* and mutations in *FUT1* (*FUT1/M307*)(polymorphisms) were calculated on all Landrace and Large White progeny.

9. Southern Blot Analysis

Southern blot analysis was performed of cosmids ETHs1 (1-3), ETHs2 (4-6) and ETHs3 (7-9) after digestion with enzymes KspI (1, 4, 7), EcoRI (2, 5, 8) and KspI/EcoRI (3, 6, 9) and separation on 0.8% agarose. Hybridization with an α^{32} PdATP labeled 5' *FUT1* fragment (primers P6-P11) results in the same hybridizing 940 bp band in both the KspI digest (lane 4) and the KpsI/EcoRI digest (lane 6). However, hybridization with a 3' *FUT1* fragment (primers P7-P10) (Table 1) shows a 6.2 kb KspI band in lane 4 and a 1.1 kb KspI/EcoRI band in lane 6. Both the 5' and 3' *FUT1* fragments hybridize to the same 4.6 kb EcoRI fragment in lane 5. This indicates the presence of a KspI site in the *FUT1* gene contained in cosmid ETHs2. Cross hybridization of the 3' *FUT1* fragment detects 2.7 kb (lanes 2, 3, 8 and 9) and 8.2 kb (lanes 8 and 9) bands, resulting in the identification of the *FUT2* psuedogene (incomplete ORF) and the *FUT2* gene sequences, respectively.

10. <u>Restriction Fragment Polymorphisms</u>

Detection of (A) the M307 G to A and (B) the M857 G to A mutation in the porcine *FUT1* gene was achieved by restriction length polymorphism analysis, rising various restriction enzymes. Digestion of amplified *FUT1* fragments with CfoI (A) and AciI (B) results in a restriction fragment polymorphism. In the first lane is a 100 bp marker. Fragment lengths are indicated in base pairs. (A) The $M307^{A/A}$ genotype (lane 2) generates 328 and 93 bp restriction fragments while the $M307^{G/G}$ genotype (lane 4) generates 93, 241 and 87 bp fragments and heterozygous $M307^{A/G}$ genotypes (lane 3) shows all four fragments.

(B) Digestion of the $M857^{A/A}$ genotype (lane 2) generates 174 bp fragments, while it generates 136 and 38 bp fragments in the $M857^{G/G}$ genotypes (lane 4), and in $M857^{A/G}$ genotypes (lane 3) all three fragments are generated.

11. <u>Source of Swine</u>

Data of the Swiss Landrace experimental population came from two pedigrees, which were built up at the Institute of Veterinary Bacteriology, University of Zurich. All other pigs of the Large White, Swiss Landrace, Duroc, Hampshire and Pietrain breeds came from different breeding herds of Switzerland. Other swine were randomly obtained from farms in the U.S. Midwest.

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WE CLAIM

1. A method for identifying a swine that is resistant to intestinal colonization by *E. coli*, said method comprising:

a. determining whether a genetic polymorphism associated with resistance to colonization is in a biological sample from the swine; and

b. inferring that the swine is resistant if the swine is homozygous for the polymorphism.

2. A method for identifying a swine that is resistant to *E. coli* associated intestinal disorders, said method comprising:

a. determining in a biological sample from the swine whether the only nitrogen base at position 307 in the alpha (1,2) fucosyltransferase gene 1 of the swine is adenine ;and

b. identifying the swine as resistant if the only nitrogen base at position 307 is adenine.

3. A method for breeding swine that are resistant to *E. coli* associated diseases, said method comprising:

a. selecting for breeding swine that have a genetic polymorphism in the alpha (1,2) fucosyltransferase 1 gene that identifies them as swine that are resistant to *E. coli* related intestinal diseases; and

b. breeding the selected swine.

4. The method of claim 1, 2 or 3, wherein *E. coli* is strain F18.

5. An isolated DNA molecule which is polymorphic for the alpha (1,2) fucosyltransferase gene 1 in swine.

6. An isolated DNA molecule having a nucleotide sequence in accordance with FIG. 1.

7. The isolated DNA molecule of claim 6 wherein adenine is in nucleotide position 307 instead of guanine.

8. An isolated DNA molecule which is complementary to the nucleotide sequence of claim 6.

9. The isolated DNA molecule of claim 6 with a substitution of adenine for guanine in nucleotide position 857.

10. An isolated DNA molecule according to claim 6 with a threonine in nucleotide position 229.

11. A polypeptide encoded by the DNA molecule of claim 5 or 6, said molecule having alpha (1,2) fucosyltransferase activity.

12. A portion of the isolated DNA molecule of claim 5 or 6, said portion including the nucleotide sequence that distinguishes *E. coli* colonization resistant from sensitive swine.

13. A molecular assay for detecting *E. coli* F18 receptors, said assay comprising isolating DNA from porcine nucleated cells, amplifying said DNA in a polymerase chain reaction using oligonucleotides as primers which are complementary to a DNA sequence of the porcine alpha (1,2) fucosyltransferase 1 gene, performing a restriction enzyme digest with at least one restriction enzyme, separating the resulting fragments by gel electrophoresis, determining the respective numbers and lengths of fragments,

and determining from the numbers and lengths of fragments, which receptors are present.

14. The molecular assay of claim 14 wherein the restriction enzyme is CfoI.

15. A kit for detecting *E. coli* F18 receptors, said kit comprising in separate containers oligonucleotides which are complementary to a DNA sequence of polymorphisms of the porcine alpha (1,2) fucosyltransferase gene 1.

16. A porcine polypeptide having alpha (1,2) fucosyltransferase activity and an amino acid substitution in position 103.

17. The polypeptide of claim 17, further characterized as having an amino acid substitution in position 286.

18. Use of polymorphisms of porcine alpha (1,2) fucosyltransferase to develop drugs to treat swine that have *E. coli*-associated disease.

FIGURE 1

```
           M   W   V   P   S   R   R   H   L   C   L   T   F   L   L   V   C     17
CT  CGA GCC ATG TGG GTC CCC AGC CGC CGC CAC CTC TGT CTG ACC TTC CTG CTA GTC TGT    59
 V   L   A   A   I   F   F   L   N   V   Y   Q   D   L   F   Y   S   G   L   D     37
GTT TTA GCA GCA ATT TTC TTC CTG AAC GTC TAT CAA GAC CTC TTT TAC AGT GGC TTA GAC   119
 L   L   A   L   C   P   D   H   N   V   V   S   S   P   V   A   I   F   C   L     57
CTG CTG GCC CTG TGT CCA GAC CAT AAC GTG GTA TCA TCT CCC GTG GCC ATA TTC TGC CTG   179
 A   G   T   P   V   H   P   N   A   S   D   S   C   P   K   H   P   A   S   F     77
GCG GGC ACG CCG GTA CAC CCC AAC GCC TCC GAT TCC TGT CCC AAG CAT CCT GCC TCC TTT   239
 S   G   T   W   T   I   Y   P   D   G   R   F   G   N   Q   M   G   Q   Y   A     97
TCC GGG ACC TGG ACT ATT TAC CCG GAT GGC CGG TTT GGG AAC CAG ATG GGA CAG TAT GCC   299
 T   L   L   A   L   A   Q   L   N   G   R   Q   A   F   I   Q   P   A   M   H    117
ACG CTG CTG GCC CTG GCG CAG CTC AAC GGC CGC CAG GCC TTC ATC CAG CCT GCC ATG CAC   359
 A   V   L   A   P   V   F   R   I   T   L   P   V   L   A   P   E   V   D   R    137
GCC GTC CTG GCC CCC GTG TTC CGC ATC ACG CTG CCT GTC CTG GCG CCC GAG GTA GAC AGG   419
 H   A   P   W   R   E   L   E   L   H   D   W   M   S   E   D   Y   A   H   L    157
CAC GCT CCT TGG CGG GAG CTG GAG CTT CAC GAC TGG ATG TCC GAG GAT TAT GCC CAC TTA   479
 K   E   P   W   L   K   L   T   G   F   P   C   S   W   T   F   F   H   H   L    177
AAG GAG CCC TGG CTG AAG CTC ACC GGC TTC CCC TGC TCC TGG ACC TTC TTC CAC CAC CTC   539
 R   E   Q   I   R   S   E   F   T   L   H   D   H   L   R   Q   E   A   Q   G    197
CGG GAG CAG ATC CGC AGC GAG TTC ACC CTG CAC GAC CAC CTT CGG CAA GAG GCC CAG GGG   599
 V   L   S   Q   F   R   L   P   R   T   G   D   R   P   S   T   F   V   G   V    217
GTA CTG AGT CAG TTC CGT CTA CCC CGC ACA GGG GAC CGC CCC AGC ACC TTC GTG GGG GTC   659
 H   V   R   R   G   D   Y   L   R   V   M   P   K   R   W   K   G   V   V   G    237
CAC GTG CGC CGC GGG GAC TAT CTG CGT GTG ATG CCC AAG CGC TGG AAG GGG GTG GTG GGT   719
 D   G   A   Y   L   Q   Q   A   M   D   W   F   R   A   R   Y   E   A   P   V    257
GAC GGC CGT TAC CTC CAG CAG GCT ATG GAC TGG TTC CGG GCC CGA TAC GAA GCC CCC GTC   779
 F   V   V   T   S   N   G   M   E   W   C   R   K   N   I   D   T   S   R   G    277
TTT GTG GTC ACC AGC AAC GGC ATG GAG TGG TGC CGG AAG AAC ATC GAC ACC TCC CGG GGG   839
 D   V   I   F   A   G   D   G   R   E   A   A   P   A   R   D   F   A   L   L    297
GAC GTG ATC TTT GCT GGC GAT GGG CGG GAG GCC GCG CCC GCC AGG GAC TTT GCG CTG CTG   899
 V   Q   C   N   H   T   I   M   T   I   G   T   F   G   F   W   A   A   Y   L    317
GTG CAG TGC AAC CAC ACC ATC ATG ACC ATT GGC ACC TTC GGC TTC TGG GCC GCC TAC CTG   959
 A   G   G   D   T   I   Y   L   A   N   F   T   L   P   T   S   S   F   L   K    337
GCT GGT GGA GAT ACC ATC TAC TTG GCT AAC TTC ACC CTG CCC ACT TCC AGC TTC CTG AAG  1019
 I   F   K   P   E   A   A   F   L   P   E   W   V   G   I   N   A   D   L   S    357
ATC TTT AAA CCC GAG GCT GCC TTC CTG CCC GAG TGG GTG GGC ATT AAT GCA GAC TTG TCT  1079
 P   L   Q   M   L   A   G   P   *                                                365
CCA CTC CAG ATG TTG GCT GGG CCT TGA ACC AGC CAG GAG CCT TTC TGG AAT AGC CTC GGT  1139
CAA CCC AGG GCC AGC GTT ATG GGT CTC CGG AAG CCC GAG TAA CTT CCG GAG ATG CTG GTG  1199
GTC CTG TAG CAG GCT GGA CAC TTA TTT CAA GAG TGA TTC TAA TTG GCT GGA CTC AGA GGA  1259
AAC CCT GCA G                                                                    1269
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[54]发明名称 鉴定F18大肠杆菌相关疾病遗传抗性猪的方法和组合物

[57]摘要

本发明提供了以高水平的敏感性和特异性将在基因上对F18大肠杆菌感染的相关疾病敏感的猪与抗性猪区分开来的非侵害性方法和组合物。使用α(1,2)岩藻糖基转移酶(FUT1)基因的DNA多态性区分抗性猪和易感猪。本发明包括由核苷酸多态性编码的具有氨基酸取代的多肽，区分大肠杆菌F18－粘附抗性，杂合(载体)和纯合易感猪的分子诊断试验和试剂盒。分子试验以高敏感性和特异性鉴定猪对水肿病和断奶后腹泻的易感性，因此猪饲养者可利用该试验提高猪的抗性。本发明多态性的有关资料可以让人们深入了解大肠杆菌相关肠紊乱的病因和治疗方法。

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权 利 要 求 书

1. 鉴定大肠杆菌肠定居的抗性猪的方法，所述方法包括:

a. 测定猪生物样品中是否存在与定居抗性相关的遗传多态性；和

b. 如果猪的多态性是纯合的，则可推断猪是抗性的。

2. 鉴定大肠杆菌相关肠紊乱的抗性猪的方法，所述方法包括:

a. 测定猪生物样品中猪α(1, 2)岩藻糖基转移酶基因1第307位的含氮碱基是否仅是腺嘌呤；和

b. 如果第307位的含氮碱基仅是腺嘌呤，即可将猪鉴定为抗性。

3. 繁殖对大肠杆菌相关疾病具有抗性的猪的方法，所述方法包括:

a. 选择α(1, 2)岩藻糖基转移酶1基因中具有遗传多态性因而将其鉴定为对大肠杆菌相关肠疾病具有抗性的猪的繁殖猪；和

b. 繁殖所选择的猪。

4. 权利要求1，2或3的方法，其中大肠杆菌是菌株F18.

5. 分离的DNA分子，其中猪α(1, 2)岩藻糖基转移酶1基因具有多态性。

6. 分离的DNA分子，其具有根据图1的核苷酸序列。

7. 权利要求6的分离的DNA分子，其中第307位的鸟嘌呤被腺嘌呤取代。

8. 分离的DNA分子，其与权利要求6的核苷酸序列互补。

9. 权利要求6的分离的DNA分子，其中第857位的鸟嘌呤被腺嘌呤取代。

10. 权利要求6的分离的DNA分子，其第229位的核苷酸为苏氨酸。

11. 由权利要求5或6的DNA分子编码的多肽，所述分子具有α(1, 2)岩藻糖基转移酶活性。

12. 权利要求5或6的分离的DNA分子的部分，所述部分包括将大肠杆菌定居抗性猪和敏感猪区分开的核苷酸序列。

13. 检测大肠杆菌F18受体的分子测定法，所述测定法包括从猪成核细胞中分离DNA；使用与猪α(1, 2)岩藻糖基转移酶基因1的DNA序列互补的寡核苷酸作为引物，在聚合酶链反应中扩增所述DNA；用至少一种限制性酶进行限制性酶消化；通过凝胶电泳分离所得片断；测定各片断的数目和长度；和由片断的数目和长度确定存在哪一种受体。

14. 权利要求 14 的分子测定法，其中限制性酶是 CfoI.

15. 检测大肠杆菌 F18 受体的试剂盒，所述试剂盒在独立的容器中含有与猪α(1, 2)岩藻糖基转移酶基因 1 的多态性 DNA 序列互补的寡核苷酸.

16. 猪多肽，其具有α(1, 2)岩藻糖基转移酶活性和第 103 位的氨基酸取代.

17. 权利要求 17 的多肽，其进一步的特征在于第 286 位具有氨基酸取代.

18. 猪α(1, 2)岩藻糖基转移酶的多态性在开发治疗患有大肠杆菌相关疾病的猪的药物中的用途.

说　明　书

鉴定F18大肠杆菌相关疾病遗传抗性猪的方法和组合物

本文提供了鉴定对大肠杆菌相关疾病，尤其是与具菌毛F18的大肠杆菌菌株相关的肠道疾病具有遗传抗性的组合物和非侵害性方法。已鉴定出猪α(1，2)岩藻糖基转移酶(FUT1)基因中的DNA多态性，它可将抗性猪和易感猪区分开，从而H为猪养殖人员提供有用的诊断试验。

养猪过程中的主要问题是使猪免患疾病，断奶后的肠道紊乱是尤其严重的问题。有限数目的毒性大肠杆菌菌株血清型是猪水肿病和断奶后腹泻的致病原，它可导致全世界养猪业遭受严重的经济损失，尤其是4至12周龄幼猪的损失。水肿病典型的临床症状是神经学病征，如运动失调，惊厥和麻痹。在随后的尸检中发现，水肿一般存在于特征性位点，如眼睑和前额，胃壁和结肠系膜。此疾病分别由定居于小肠表面但不影响肠细胞(肠中的细胞)主要形态变化的大肠杆菌产生的志贺样毒素-II变体和肠毒素LT，Sta，STb导致。在此方面，某些类型的大肠杆菌菌株F18，F4和K88是主要的致死性的villain。“猪水肿病是肠毒素血症，其特征在于延及全身的血管损伤，后者是由某些大肠杆菌菌株产生的毒素，志贺样毒素-II变体导致的”(Bertschinger等，1993)。可以菌毛类型区分大肠杆菌，相关的一组粘附菌毛被称为例如K88或F18(Vogeli等，1997)。

不是所有的猪都易感大肠杆菌，定居依赖于细菌对肠细胞的粘附，这种粘附是由被称为K88或F18的细菌菌毛介导的。已证实对粘附的易感性，即猪中结合菌毛的受体的表达受到宿主的遗传控制，对F18而言，上述易感性作为显性性状被遗传下来，B表示易感的等位基因，b表示抗性等位基因(Vogeli等，1996; Meijerink等，1997)。根据其与染色体6上卤烷(HAL)连锁群中的S基因座和其它基因座的紧密遗传连锁，将大肠杆菌F18-受体(ECF18R)的基因座作图至猪染色体6 (SSC6)，K88大肠杆菌受体位于染色体13。

抗性机理可能是大肠杆菌未定居于抗性动物的肠表面，即细菌未粘附于抗性猪的肠壁上。某些大肠杆菌粘附和非粘附表型之间的区别取决于肠刷状缘膜的糖蛋白受体，因此，宿主猪的基因型决定抗性。另外还

研究了带菌毛的细菌(WO 9413811)。

鉴定 F18 大肠杆菌相关疾病的抗性猪的最新方法是 1)收集刚被屠宰的猪的肠样品，在显微镜下进行粘附试验，2)用强毒性的大肠杆菌攻击动物(“定居试验”)，或 3)进行 AO(S)血型系统血液定型。前两种方法对鉴定抗性动物以用作繁殖原种来说并不实用。尽管血液定型法能鉴定抗性动物，但此试验不能确定易感动物的易感性是纯合的还是杂合的。关于这些等位基因的动物基因型知识(基因状况)是发展成功育种计划所必需的。育种计划的目的是繁殖对大批杀死断奶后牲畜的 F18 大肠杆菌相关疾病具有抗性的猪。

在一篇文献中，作者在谈及猪水肿病时说到：“正在寻找适当的遗传标记”(Bertschinger 等，1993, p.87)，并提到 Walters 和 Sellwood, 1982:

> 繁殖抗性猪是预防无法有效被预防的疾病的好方法，此方法的可行性取决于猪群体中编码抗性的基因的大量存在，检测抗性猪的改良方法，和用此抗性同时选择的阴性遗传性状的缺乏。

遗传“标记”基因座是靠近所需基因座的编码或非编码基因座，但基因座本身并不是必需的。可测的表型包括连续的或不连续的性状，如限制性长度片断多态性，生产性状，细菌粘附性状，生色或酶促反应和抗生素抗性。S 基因座控制着 A 和 O 血型抗原的表达。S 基因座隐性纯合的猪不表达 A 或 O 血型抗原。由于编码人血型 H 的α(1, 2)岩藻糖基转移酶基因中具有突变，类似的状况也存在于人中(Kelly 等，1994, 也见于 WO9628967 中)。最近测定了猪α(1, 2)岩藻糖基转移酶基因的序列(Cohney 等，1996)，此基因与猪 S 基因座上存在的基因非常相似。

在遗传和物理图谱上，血型 H 和 Se 基因座位于人染色体 19q13.3，此区域在进化中是保守的，含有与猪基因 HAL 连锁群同源的基因。编码血型 H 的基因是所谓的 FUT1，而 Se 基因等同于 FUT2 基因。FUT1 决定红细胞谱系中的 H 抗原表达，而 FUT2 调节分泌上皮和唾液中 H 抗原的表达。在如大鼠和兔的低等哺乳动物中，FUT1 基因是保守的，兔脑组织和大鼠结肠中显示出 mRNA 的表达。在所有这些物种中，已报道了两种

类型的α(1, 2)岩藻糖基转移酶基因，其结构非常类似于人FUT1和FUT2基因，但FUT1同源基因特别地显示出物种特异性的表达模式。人FUT1基因负责红细胞前体中H抗原的合成，然而，猪红细胞被动地吸附血清中的H样抗原，此时为人Lewis抗原。猪中所有的H样抗原都与外分泌组织相关，在其它动物的分泌组织中也观察到FUT2(secretor)基因的表达。因此，FUT2基因可能会影响到与抗人血型H和A抗体交叉反应的猪A-0血型决定簇的表达。

有关血型和猪大肠杆菌疾病的其它信息包括血型抗原的碳水化合物结构介导一些病原体微生物与宿主组织的粘附，如幽门螺杆菌与Lewis[b]血型抗原的粘附，和导致尿道感染的大肠杆菌与血型P物质的粘附。因此，负责血型特异性碳水化合物结构形成的糖基转移酶的编码基因代表着宿主控制细菌定居的候选基因。这些基因与负责猪小肠中F18阳性大肠杆菌的粘附/非粘附的基因座定位于相同的染色体区域。猪直至断奶后才表达血型抗原A和0，此时猪容易感染F18大肠杆菌所致的疾病。

迫切需要诊断和治疗猪大肠杆菌相关肠疾病的新方法，有人建议将基因突变的检测作为一些猪疾病(恶性过热)的诊断试验(Fujii等，1991；美国专利5,358,649)，但无人报道过用于诊断的多态性标记。对大肠杆菌定居产生抗性的疫苗已被描述(美国专利5,552,144；WO 8604604)，但由于给新生猪口服活疫苗的困难和调节的限制性，使其不可能成为预防大肠杆菌疾病的优选方法。抗生素可以用于治疗，但无法成功地预防疾病。

发明简述

本发明的组合物和非侵害性的方法可以检测和排除易感大肠杆菌相关疾病的猪。鉴定大肠杆菌F18肠定居的抗性猪的非侵害性方法包括下列步骤：测定猪生物样品中是否存在与定居抗性相关的遗传多态性；如果猪的多态性[1]([1]多态性是种群中存在的核苷酸序列因突变所致的变化)是纯合的，则可推断猪是抗性的。

更具体地说，此方法是测定猪生物样品中猪α(1, 2)岩藻糖基转移酶基因第307位的含氮碱基仅是腺嘌呤还是仅为鸟嘌呤；如果第307位的含氮碱基仅是腺嘌呤，即可将猪鉴定为抗性。

为了测定生物样品中是否存在多态性，在通过分子量进行分离的凝胶上分析限制性片断长度的多态性。限制性内切核酸酶是能在特异性位

点再现地切割核酸分子的酶，根据切割的位置产生不同分子量的核酸片断。

本发明还涉及繁殖对大肠杆菌相关疾病具有抗性的猪的方法，所述方法包括选择α(1，2)岩藻糖基转移酶 1 基因中具有遗传多态性因而将其鉴定为对大肠杆菌相关肠疾病具有抗性的猪的繁殖猪；并繁殖所选择的猪。

本发明的一个方面是猪α(1，2)岩藻糖基转移酶 1 基因具有多态性的 DNA 分子，尤其是根据图 1 的序列。本发明的另一方面是具有与图 1 序列互补的核苷酸序列的分子。

本发明的一个方面是第 307 位的鸟嘌呤被腺嘌呤取代的分离的 DNA 分子，此分子第 857 位的鸟嘌呤也被腺嘌呤取代。本发明其它分离的 DNA 分子包括图 1 序列第 229 位的核苷酸具有突变的分子，其中编码亮氨酸的密码子 CTT 被变为编码苯丙氨酸的密码子 TTT。第 714 位核苷酸的突变是由 GAT 变为 GAC，但编码的产物中没有相伴随的氨基酸取代。

由本发明的 DNA 分子编码并具有α(1，2)岩藻糖基转移酶活性的多肽也是本发明的内容。

检测猪中大肠杆菌 F18 受体的分子测定法是(a)从猪成核细胞(nucleated cell)中分离 DNA；(b)使用与猪α(1，2)岩藻糖基转移酶基因 1 的 DNA 序列互补的寡核苷酸作为引物，在聚合酶链反应(PCR)中扩增 DNA；(c)用至少一种限制性酶，如 CfoI 进行限制性酶消化；(d)通过凝胶电泳分离所得片断；和(e)测定凝胶上各片断的数目和长度；和(f)由 F18 片断的数目和长度确定猪细胞中存在哪一种受体。使用本文公开的较大的扩增片断而不是较小的片断进行限制性长度多态性分析(RFLP)较为便宜，因为 DNA 带可在相对低浓度的琼脂糖凝胶上进行电泳。另外，为了产生一些片断，与可变诊断位点相邻的恒定限制性位点仅需要一种限制性酶。

检测与大肠杆菌 F18 受体相关的多态性的试剂盒在各个独立的容器中使用了与猪α(1，2)岩藻糖基转移酶基因 1 的 DNA 序列互补的寡核苷酸，它能将抗性猪和敏感猪区分开来。可对任何年龄的猪进行此试验。

多态性也可用于开发治疗患大肠杆菌相关疾病的猪的药物。突变形式的猪α(1，2)岩藻糖基转移酶可以干扰正常的酶，防止其产生 F18 的肠受体。

附图简述

图 1 显示了本发明的猪α(1, 2)岩藻糖基转移酶多态性的核苷酸序列(FUT1)(下)，并使用单字母氨基酸密码显示了推定的氨基酸序列(上)。氨基酸序列下方的双实线(=)是推定的跨膜区；氨基酸序列下方的点线表示3个潜在的N联糖基化位点(...)。

□是抗性猪中鸟嘌呤(G)被腺嘌呤(A)取代的位置。

*表示终止密码子。

氨基酸残基的缩写如下：A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp;和 Y, Tyr.

优选实施方案的描述

与猪对大肠杆菌相关疾病的抗性有关的 DNA 多态性分子分析便于进行选择繁殖所用抗性猪的诊断试验。通过本发明的多态性标记鉴定出：抗性猪与敏感猪的大肠杆菌 F18 受体基因座不同。

本发明提供了以高水平的敏感性和特异性将在遗传上对 F18 大肠杆菌感染的相关疾病敏感的猪与抗性猪区分开来的非侵害性方法和组合物。鉴定出猪α(1, 2)岩藻糖基转移酶(FUT1)基因的 DNA 多态性，籍此区分抗性猪和易感猪。多态性由核苷酸序列中导致新等位基因的突变(变化)引起。等位基因是基因的一种状况，在一个种群中，一个基因有很多等位基因，其通过含氮碱基的取代而有所不同，所述取代可能是由亲代 DNA 分子中的突变引起的。一个种群中共存有一个以上的等位基因(有时称之为“变体”)被称为基因的多态性。在种群中，一个以上的等位基因作为明显稳定的成分存在的基因座是多态性基因座。通常，其中一个多态性基因座在种群中是低频的。

由生物样品，优选为血液中测得：抗性猪的基因组具有多态性，其中在核苷酸序列第 307 位(见图 1)测定的唯一碱基是腺嘌呤，而纯合易感猪的相同位置的碱基是鸟嘌呤。杂合猪会显示出两种类型的 DNA，并且会是易感的。多态性是 Cohney 等，1996 所报道的猪基因序列的变异。

对猪家族进行遗传连锁分析，测定 FUT1 的多态性和远系繁殖猪的疾病抗性之间的遗传联系。根据本发明，已在α(1, 2)岩藻糖基转移酶 1 基因(FUT1)中发现多态性。第 307 位具有单个核苷酸碱基取代的多态性被用于确定岩藻糖基转移酶基因和 S-系统，ECF18R 基因座和 HAL 连

锁群的其它基因座之间的紧密连锁.

检测 FUT1 和 ECF18R 的突变的紧密连锁使得分子试验发展成为可鉴定大肠杆菌 F18 粘附抗性，杂合(载体)和纯合的易感猪. 此诊断试验以高敏感性和特异性鉴定出易感水肿病和断奶后腹泻的猪. 在猪群中，本发明多态性的发生率有差异. 据 Vogeli 等(1997)报道，猪群中 5 只猪的 M307 等位基因频率互不相关. 本发明多态性诊断试验的可用性为养猪者提供了从猪群中有效消除 ECF18R 易感等位基因，从而消除导致水肿病和断奶后腹泻的必要条件:大肠杆菌 F18 细菌粘附.

本发明还包括核苷酸序列，其为α(1, 2)岩藻糖基转移酶基因 1 的序列的变体，代表 bp307 处的多种多态性，本发明还包括基于诊断分子试验的试剂盒，可用于鉴定α(1, 2)岩藻糖基转移酶基因中的多态性.

为了得到大肠杆菌 F18 受体基因座(ECF18R)的候选基因，从猪基因组文库中分离出含基因的 5 个粘粒和 1 个基因组克隆，其含有α(1, 2)岩藻糖基转移酶基因 FUT1 和 FUT2(Meijerink 等, 1997). 通过荧光原位杂交作图将所有这些克隆定位于猪染色体 6 的 q11 带上(SSC6q11). 对粘粒的序列分析鉴定出(a)开放阅读框(ORF)，其长度为 1098 个碱基对，与人 FUT1 序列 82.3%相同，和(b)第二个 ORF，其长度为 1023 个碱基对，与人 FUT2 序列 85%相同. 因此，FUT1 和 FUT2 基因座似乎是人血型 H 和 Secretor 基因座的猪等同物. 对易感或抗具有 F18 菌毛的大肠杆菌(ECF18R)的粘附和定居的猪中的两个 ORF 进行直接测序，揭示出 FUT1 ORF 的碱基对 307(M307)和碱基对 857(M857)处的两个多态性. 核苷酸位置是从 ATG(编码甲硫氨酸)开始计数的. 在具有 221 个后代的 Landrace 家族 34 次交配中分析这些突变，结果表明与控制对大肠杆菌 F18 粘附和定居于小肠的抗性和易感性的基因座(ECF18R)和血型抑制剂 S 的基因座紧密连锁. 因此，M307 突变是标记-辅助选择大肠杆菌 F18 粘附抗性动物的良好标记. 发现第 229 位核苷酸的另一突变也导致多态性，其中编码亮氨酸的密码子(CTT)变为 TTT(编码苯丙氨酸). 第 714 位(编码天冬氨酸)的突变(GAT 变为 GAC)不产生氨基酸取代. 在 FUT2 中未鉴定出可以区分易感猪和抗性猪的多态性.

实施例

下列实施例提供了本发明的实施方案.

实施例 1: 抗性猪试验

使用 PCR-RFLP 试验可轻易地鉴定本发明的多态性，试验的一个实施方案使用了下列引物：5'CCAACGCCTCCGATTCCTGT3' 和 5'GTGCATGGCAGGCTGGATGA3'。此实施方案的优选 PCR 条件是在下列时间和温度下进行 25 轮循环：94℃，30 秒；60℃，45 秒；72℃，90 秒。通过限制性酶 HgaI 消化抗性猪的扩增 DNA，但不用限制性酶 HinPI 消化此 DNA。用限制性酶 HinPI 消化纯合易感猪的扩增 DNA，用上述两种酶部分消化杂合易感猪的扩增 DNA。

或者，根据标准方法从猪成核细胞中分离 DNA，对不同 ECF18R 基因型(Bb，bb)动物的猪 FUT1 和 FUT2 序列及其侧翼区直接测序，结果鉴定出 FUT1 ORF 的第 307 和 857 位(分别称之为 M307 和 M857)有两个 G→A 的转换。M307 转换消除了 CfoI 限制性位点，根据标准方法，用引物 P6 和 P11 扩增分离自猪成核细胞的 DNA(95℃ 3 分钟，然后是 95℃，30 秒；56℃，30 秒；72℃，30 秒共 30 轮循环，接着是 72℃最后延伸 7 分钟)，接着进行 CfoI 消化，在 3%琼脂糖凝胶上分离，产生限制性片断长度多态性(RFLP)。纯合 $M307^{AA}$ 动物显示出 2 条带，纯合 $M307^{GG}$ 动物显示出 93-，241-和 87bp 的片断，杂合动物显示出所有 4 个片断。

实施例 2：使用α(1，2)岩藻糖基转移酶检测 F18 大肠杆菌抗性猪的试验的敏感性和特异性

研究测定疾病抗性和 FUT1 基因第 307 位多态性之间的联系。183 头断奶的猪(年龄为 2 至 6 个月)得自 6 个不同的猪群。研究开始前只知道其中一个猪群含有抗性动物，已知该猪群压力综合征的发病率高，其它 5 个猪群没有猪压力综合征的迹象，疾病抗性的发生率是未知的。从各猪群中随机选择猪，人为地使其安乐死，取出脾脏和小肠样品。从脾组织中提取 DNA，并用于实施例 1 所述的 PCR-RFLP 试验。通过从小肠上刮下粘膜表层，在低渗 EDTA 溶液中裂解细胞并通过离心洗涤以纯化肠细胞。将纯化的肠细胞刷状缘与 F18 大肠杆菌一起保温，通过相差显微镜检查此混合物，此试验可测定猪是易感的(肠样品粘附有细菌)还是抗性的(肠样品未粘附细菌)。在所有 53 个抗性猪和 130 头易感猪的 128 头中，多态性的 PCR-RFLP 试验与细菌-肠细胞结合试验相关。使用细菌-肠细胞结合试验测定为易感猪的两头猪被 PCR-RFLP 试验不正确地测定为抗性猪。被检查的 6 个猪群中有 2 个含有抗性猪，而只有 1 个猪群具有猪压力综合征，这表明 PCR-RFLP 试验可鉴定不具有猪压力综合征的

动物中的疾病抗性动物。

实施例 3: 将 FUT1 定位于染色体 6(SSC6)

用得自猪基因组 DNA 的 FUT1 核苷酸探针筛选粘粒文库之后，用引物 P7 和 P11 鉴定粘粒 ETHs1，-s2，-s3，-s4 和-s6，并通过 FISH 和 DISH-PCR 将其作图至染色体 6 的 q11 带上。

实施例 4: 鉴定猪 FUT1 ORF

将 KspI,EcoRI 和 KspI/EcoRI 粘粒消化物与经放射性标记的猪 FUT1 片断 P6-P11 和 P7-P10 进行杂交以进行 Southern 印迹分析，结果表明 ETHs2，-s4 和-s6 的放射自显影信号相同，而得自粘粒 ETHs1 和-s3 的信号不同。从粘粒 ETHs2 KspI 中分离出长度为 940bp 和 6.2kb 的亚克隆，相当于 Southern 印迹上杂交 KspI 片断的估计长度。结合两个亚克隆的序列结果，产生 1501 bp 序列，这与基因组 PCR 产物直接测序结果一致。1501bp 序列含有 1098bp 的开放阅读框(ORF)，相当于人 FUT1 ORF，与其具有 82.3%的核苷酸和 80.8%的氨基酸同一性。ORF 编码多肽。

实施例 5: 鉴定猪 FUT2 和假基因 FUTP

ETHs1 具有 1 个与 FUT1 序列杂交的 DNA 片断(2.7kb)，而 ETHs3 具有两个上述片断(2.7kb 和 8.2kb)。对 ETHs1 和-s3 的 2.7kb EcoRI 片断进行亚克隆和部分测序，证实了这两个片断是相同的。此序列与人 FUT2 非常相似，但在 NH_2 和-COOH 末端区域显示出几个变化。这些变化导致与保守 ORF 不相容的移码，因此，假定得自 2.7kb 片断的序列表示假基因(FUT2P)。亚克隆 ETHs3 BamHI 消化物之后，鉴定 8.2kb EcoRI 片断中所含的杂交序列。所得亚克隆序列表示 1023bp ORF，它与人 FUT2 序列在核苷酸水平上 85%相同，氨基酸水平上 83%相同。在猪 FUT2 序列和衍生自 2.7kb 片断的 FUT2P 序列之间观察到 NH_2 和-COOH 末端区域的很多差异。推测的氨基酸序列相当于猪 Secretor 酶部分测定的氨基酸序列(Thurin 和 Blaszcyk-Thurin，1995)。将所得猪 FUT1，FUT2 和 FUTP 序列登记于 GenBank，登记号分别为 U70883，U70881 和 U70882。FUT1 和 FUT2 基因具有高度同源性序列。在例如引物开发中已考虑到这一点。另外，在进一步的研究中需要区分 FUT1 和 FUT2 酶活性。

实施例 6: 鉴定 M307 和 M857 突变和表征 M307

根据标准方法从猪成核细胞中分离 DNA，对不同 ECF18R 基因型(Bb，bb)的动物的猪 FUT1 和 FUT2 序列及其侧翼区直接测序，结果鉴定出 FUT1

ORF 的第 307 和 857 位(分别称之为 M307 和 M857)有两个 G→A 的转换。M307 转换消除了 CfoI 限制性位点，根据标准方法，用引物 P6 和 P11 扩增分离自猪成核细胞的 DNA(95℃ 3 分钟，然后是 95℃，30 秒；56℃，30 秒；72℃，30 秒共 30 轮循环，接着是 72℃最后延伸 7 分钟)，接着进行 CfoI 消化，在 3%琼脂糖凝胶上分离，产生限制性片断长度多态性(RFLP)。纯合 M307^{AA}动物显示出 2 条带(93-和 328-bp 片断)，纯合 M307^{GG}动物显示出 93-，241-和 87bp 的片断，杂合动物显示出所有 4 个片断。

实施例 7：鉴定突变 M857

M857 突变是消除 AciI 位点的转换。设计引物 PBEST 以错配第 866 和 872 位两个多余的 AciI 位点。用引物 P7 和 PBEST 进行 PCR(95℃ 3 分钟，然后是 95℃，30 秒；56℃，30 秒；72℃，30 秒共 30 轮循环接着是 72℃最后延伸 7 分钟)，接着进行 AciI 消化，在 3%琼脂糖凝胶上进行 PCR-RFLP 分析。纯合 M857^{AA}动物显示出 174bp 的片断，而 M857^{GG}动物的扩增产物显示出 136-和 38-bp 片断。

实施例 8：绘制 FUT1 基因的遗传图谱

在 Landrace 猪家族中，M307 和 HAL 连锁群(S，ECF18R，RYR1，GPI，PGD)基因座之间的重组事件揭示出重组分数θ<0.04(表 2)。全部重组分数的优势对数评分 Z 为 24.5 至 50.6 之间，这可以充分证明这些基因座之间的连锁。这些数据可将 FUT1 基因作图于 HAL 连锁群中与皆受 FUT1 影响的 S 和 ECR18R 紧邻的位置。在实验性 Landrace 猪家族中，发现了 ECF18R 和 RYR1 之间的等位基因联合。在水肿病和断奶后腹泻的抗性猪(基因型 ECF18R$^{b/b}$(表 3))中观察到 RYR1 第 1843 位有过量的基因型 RYR1^{TT}(卤烷易感基因型)。这种等位基因联合是连锁不平衡的结果，即在独立分类等位基因时，所观察到的单元型频率与预期的单元型频率有偏差的结果。因此，连锁不平衡设计了属于相连基因座的等位基因的非随机联合。然而由于低重组率的缘故，无法确定某种基因座顺序显著优于另一种基因座顺序。

实施例 9：M307^{A}与 ECF18^{b}和 M307^{G}与 ECF18R^{B}的联合

在 Landrace(SL)猪和亲代 Large White(LW)猪中，ECF18R^{b}(水肿病和断奶后腹泻抗性等位基因)与 M307^{A} 100%联合，而 ECF18R^{B}(水肿病和断奶后腹泻易感等位基因)与 M307^{G} 100%联合(其中 A=腺嘌呤；G=鸟嘌呤)。在 SL 猪中，88%(30/34)的 S^{s}分别是 ECF18R^{b}和 M307^{A}单元型。在

Large White 猪中，S^s-ECF18R^b和 S^s-M307^{A}单元型的相应值为 82%(9/11)。在实验性 SL 家族中，FUT1 基因座的 M857^{A}等位基因的发生率低，在 LW 猪中甚至为零。因此，未观察到 M857 等位基因和侧翼基因的等位基因之间的显著配子联合。在 Duroc，Hampshire 和 Pietrain 猪中发现 FUT1307 和 FUT1857 位的 G→A 转换具有可变的频率，使其它猪群中也出现这种转换成为可能。

实施例 10：FUT1 基因型的分布

表 4 显示出 ECF18R 型中的 FUT1 基因型分布于核苷酸第 307 位，这与这两者为独立的假说中预期的比例显著不同。在 119 头水肿病和断奶后腹泻的抗性 ECF18R^{bb}动物中，在基于 DNA 的试验中测定其中的 118 头具有基因型 M307^{AA}，1 头抗性动物的基因型为 M307$^{A/G}$。在 131 头易感猪中，130 头为 M307$^{A/G}$或 M307$^{G/G}$。通过 DNA 试验显示出 1 头对大肠杆菌粘附易感的动物为纯合的 M307$^{A/A}$。本实施例和实施例 2 的数据与过去的研究结果一起表明 FUT1 基因是存在于猪 S 基因座和 ECF18 基因座上的基因。尽管本实施例和实施例 2 中有 4 头动物同此假说相矛盾，但这可能是因为这些动物在疾病抗性/易感性方面被错误地划定表型的缘故。

实施例 11：α(1，2)岩藻糖基转移酶中的氨基酸交换

α(1，2)岩藻糖基转移酶 1 中 bp+307 和 bp+857 处的 G→ 变化导致推测的氨基酸取代，即苏氨酸(中性-极性)取代丙氨酸(中性-非极性)和谷氨酰胺(中性-极性)取代精氨酸(碱性)，其分别对编码产物产生功能上的影响。bp229 处的 C→T 变化导致的氨基酸取代为亮氨酸(中性-非极性)取代苯并氨酸(中性-非极性)。

表 1: 正向(F)和反向(R)引物的序列及其相对于猪 FUT1 和 FUT2 起始密码子[2]的位置

引物名称	引物序列	位置
FUT1 P6 (R)	5'-CTTCAGCCAGGGCTCCTTTAAG-3'	+489
FUT1 P7 (F)	5'-TTACCTCCAGCAGGCTATGGAC-3'	+720
FUT1 P10 (R)	5'-TCCAGAGTGGAGACAAGTCTGC-3'	+1082
FUT1 P11 (F)	5'-CTGCCTGAACGTCTATCAAGATC-3'	+69
FUT1 P16 (F)	5'-AGAGTTTCCTCATGCCCACAGG-3'	-90
FUT1 P18 (R)	5'-CTGCTACAGGACCACCAGCATC-3'	+1203
FUT1 PBEST (R)	5'-ACCAGCAGCGCAAAGTCCCTGAC GGGCACGGCCTC-3'	+893
FUT2 P16 (R)	5'-CTCCCTGTGCCTTGGAAGTGAT-3'	+1094
FUT2 P17 (F)	5'-AACTGCACTGCCAGCTTCATGC-3'	-83

[2]引物 FUT1 P10 和 FUT1 P11 衍生自人 FUT1 基因

表 2: 在 Landrace 实验群体中，M307 和 HAL 连锁群基因座的全部重组分数(θ)，优势对数评分(Z)和提供数据的动物数(N)

基因座对	N	θ	Z
S-ECF18R	183	0.01	50.6
M307-S	183	0.01	50.6
M307-ECF18R	216	0.01	57.1
M307-RYR1	198	0.02	47.2
M307-GPI	147	0.03	34.2
M307-PGD	147	0.04	24.5

表 3: 在 Landrace(SL)实验群体和随机选择的 Large White(LW)猪中，4 个基因座[S-FUT1(M307, M857)-ECF18R-RYR1]处的单元型频率

品系	*S, FUT1 (M307, M857), ECF18R, RYR1* 处的单元型[3]	频率[4] (数目)
SL	*sAGbT*	70 (28)
	sAGbC	5 (2)
	sGGBC	15 (6)
	sGABC	10 (4)
LW	*sAGbC*	56 (9)
	sGGBC	31 (5)
	sGGBC	13 (2)

[3]S: A 和 0 血型抑制基因(S 和 s)的基因座.

FUT1(M307): α(1, 2)岩藻糖基转移酶(FUT1)基因中核苷酸 307 处的腺嘌呤(A)改变为鸟嘌呤(G). FUT1(M857): FUT1 基因中核苷酸 857 处的腺嘌呤(A)改变为鸟嘌呤(G). ECF18R: 大肠杆菌 F18 受体. B 表示显性易感等位基因, b 表示抗性等位基因. RYR1: 骨骼肌 ryanodine 受体. C(胞嘧啶)是恶性过热的显性抗性等位基因而 T(胸腺嘧啶)是易感等位基因.

[4]单元型频率以%表示, 括号中为单元型的绝对数目.

表 4: Landrace(SL)实验群体和随机选择的 Large White(LW)猪中相关多态性的 FUT1(M307)和 ECF18R 基因座的基因型分布，tetrachoric 相关性(R)和联合的显著性(χ^2和 w × χ^2)[5]

		FUT1/M307					
品系	基因座	基因型	A/G	A/A	*r*	χ^2	χ^2 x w[5]
SL	*ECF18R*[6]	*b/b*	1	113	0.98	213.1	42.6***
		B/b	106	1			
		基因型	A/G, G/G	A/A			
LW	*ECF18R*[6]	*b/b*	0	5	1.00	29.0	11.6***
		B/b,B/B	24	0			

[5]根据 Cotterman(1947)，将重量因子 w=0.2(SL)和 0.4(LW)用于校正因数据中包括相关动物而失去的精确性，***p<0.001.

[6]ECF18R 基因座处的基因型为 b/b 的动物对 F18ab 大肠杆菌细菌的粘附具有抗性，而基因型为 B/b 和 B/B 的动物对上述粘附是易感的.

方法

1. 引物

使用衍生自人 FUT1 基因的引物从基因组 DNA 中扩增其猪对应物。由所得猪序列设计特异性引物，将该引物用于进一步的扩增和测序反应中(表 1).

2. 筛选猪基因组文库

用引物 P7 和 P10 得到猪 FUT1 探针，用该探针或猪 FUT1 cDNA 筛选猪基因组文库. 用引物 P7 和 P10 从猪基因组 DNA 中得到 FUT1 探针，用经α^{32}P dATP 标记的(Prime It II, Stratagene)该探针筛选构建于 SuperCos 1(Stragene, La Jolla, Ca, USA)中的猪基因组文库。于 42℃将复本滤膜杂交 15 小时(50%甲酰胺，6 × SSC，5 × Denhardt's，0.5% SDS，0.1mg/ml 鲑精 DNA)，65℃洗涤两次达 30 分钟(1 × SSC，0.1%SDS)之后，暴露于(15h，-80℃)X 射线胶片后鉴定阳性菌落。

3. 猪中期染色体原位杂交

对粘粒克隆 ETHs1，ETHs2，ETHs3，ETHs4 和 ETHs6 进行荧光原位杂交(FISH)(Solinas Toldo 等，1993)或对猪中期染色体进行直接原位染色体 PCT(DISC PCR)．杂交前对中期染色体进行 Q-显带和照相．使用生物素-16-dUTP 通过随机引物法标记探针．使用亲和素-FITC 和生物素化的抗亲和素进行信号检测和扩增．用 4,6-diamidino-2-phenylindole 负染染色体，按 Solinas Toldo，1993 所述测定粘粒的相对位置．

4. 亚克隆

在琼脂糖凝胶上分离探针阳性基因组菌落的酶消化物，转移至尼龙膜上，将探针阳性带亚克隆至质粒中以进行 FUT1 测序．由此方法得到的 FUT1 序列示于图 1.

在 0.8%琼脂糖凝胶上分离所有粘粒的 KspI-，EcoRI-和 KspI/EcoRI 消化物，转移至 Hybond N 尼龙膜上(Meijerink 等，1997)．将此印迹与经 $\alpha^{32}P$ dATP 标记的猪 FUT1 PCR 产物(引物 P6-P11 和 P7-P10)杂交．根据放射自显影信号，将 ETHs1，-s2 和-s3 进一步亚克隆至 pBluescript SK-(Stratagene)，由亚克隆测定 FUT 序列．通过直接测序 PCR 产物，比较 ECF18R 阳性(BB/Bb)和阴性(bb)动物得自粘粒 ETHs2 和-s3 的两个 FUT-样开放阅读框(ORF)(FUT1 和 FUT2)的序列．

5. 聚合酶链反应和直接测序

使用 Perkin Elmer Ready Reaction Dye Terminator 试剂盒(Perkin Elmer Cetus，Norwalk，CT，USA)和 10pmol 引物，用由 95℃起始变性 5 分钟，接着 95℃ 30 秒，50℃ 15 秒和 60℃ 4 分钟进行 25 轮循环组成的热程序进行循环测序．用于猪α(1，2)岩藻糖基转移酶基因扩增和测序的引物示于表 1．考虑到因 FUT1，FUT2 和 FUT2 假基因的高度相似性所致的引物交叉退火的可能性，设计了其它的引物．在 373A ABI 测序仪(Applied Biosystems 公司)上分析样品，用 GCG 程序包(Devereux，1984)进行序列分析．

6. 产生可提供资料的后代

在由 4 头公猪和 16 头母猪产生的 221 头 Landrace 猪和由不相关的猪之间 9 次交配产生的 29 头 Large White 猪中分析单个核苷酸的多态性。为了产生大量可提供资料的后代以检查编码 ECF18 受体的猪基因和选定多态性基因座之间的连锁，仅进行了 B/b × b/b 型可提供资料的

Landrace 交配。

7. 定居试验

在 Bertschinger 等，1993 的研究中，也在定居试验中检测了上述 Landrace 猪的 ECF18 易感性. 为此，断奶后立即用血清型为 0139:K12(B):H1:F18 的大肠杆菌菌株 124/76 接种猪(Rippinger 等，1995). 每天监测猪粪便中排出的细菌. 将定居的程度计算为两个最高粪便评分的平均值. 平均粪便评分为 3.5，相当于菌落形成单位(CFU)/g 的对数为 6.7 或更高被认为是对定居敏感. 此界限的根据是低于此值即无死亡率和得自完全抗性小猪的评分.

8. 核苷酸多态性的连锁分析

将单个核苷酸多态性的结果与 Vögeli 等(1996)所述的体外粘附试验中鉴定的 ECF18R 定型数据和 Vogeli 等(1996)所述的 GPI-，PGD-，α-1-B-糖蛋白-(AIBG)，ryanodine 受体(RYR1)，EAH-和 S-基因座的定型数据比较. 使用 CRI-MAP 2.4 版本的程序(Green 等，1990)进行成对连锁分析并计算重组分数. 通过将上述基因座依次插入图谱中以进行多点连锁分析. 计算 Landrace 家族的亲代动物和 8 头亲代 Large White 猪的单元型频率，所述动物的 ECF18R 单元型是由得自后代的资料确定的. 计算所有 Landrace 和 Large White 猪后代的 ECF18R 和 FUT1(FUT1/M307)中的突变(多态性)的四氯相关性.

9. Southern 印迹分析

用 KspI(1，4，7)，EcoRI(2，5，8)和 KspI/EcoRI(3，6，9)消化并在 0.8%的琼脂糖凝胶上分离之后，对粘粒 ETHs1(1-3)，ETHs2(4-6)和 ETHs3(7-9)进行 Southern 印迹分析. 与经$\alpha^{32}P$ dATP 标记的 5' FUT1 片断(引物 P6-P11)的杂交导致 KspI 消化物(泳道 4)和 KspI/EcoRI 消化物(泳道 6)中有相同的 940bp 杂交带. 然而，与 3' FUT1 片断(引物 P7-P10)(表 1)的杂交在泳道 4 显示出 6.2kb KspI 片断和在泳道 6 显示出 1.1kb KspI/EcoRI 带. 5'和 3' FUT1 片断都与泳道 5 中相同的 4.6kb EcoRI 片断杂交. 这表明粘粒 ETHs2 中含有的 FUT1 基因中存在 KspI 位点. 3' FUT1 片断的交叉杂交检测到 2.7kb(泳道 2，3，8 和 9)和 8.2kb(泳道 8 和 9)的带，分别鉴定出 FUT2 假基因(不完全的 ORF)和 FUT2 基因序列.

10. 限制性片断的多态性

使用多种限制性酶通过限制性长度多态性分析检测到猪 FUT1 基因

中的(A)M307 G至A和(B)M857 G至A的突变．用CfoI(A)和AciI(B)消化扩增的FUT1片断导致限制性片断的多态性．第一泳道是100bp的标记物，片断长度以碱基对表示．(A)$M307^{A/A}$基因型(泳道2)产生了328和93bp的限制性片断，而$M307^{G/G}$基因型(泳道4)产生了93，241和87bp的片断，杂合的$M307^{A/G}$基因型(泳道3)显示出所有4个片断．

(B)$M857^{A/A}$基因型(泳道2)的消化产生了174bp的片断，而$M857^{G/G}$基因型(泳道4)产生了136和38bp的片断，$M857^{A/G}$基因型(泳道3)产生了所有3个片断．

11.猪的来源

Swiss Landrace 实验群体的数据得自两个种，这两个种是苏黎世大学兽医细菌学研究所建立的．Large White，Swiss Landrace，Duroc，Hampshire 和 Pietrain 猪群的其它猪得自瑞士的不同猪群．其它猪随机得自美国中西部的农场．

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说　明　书　附　图

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               M   W   V   P   S   R   R   H   L   C   L   T   F   L   L   V   C        17
CT  CGA GCC ATG TGG GTC CCC AGC CGC CGC CAC CTC TGT CTG ACC TTC CTG CTA GTC TGT        59
 V   L   A   A   I   F   F   L   N   V   Y   Q   D   L   F   Y   S   G   L   D        37
GTT TTA GCA GCA ATT TTC TTC CTG AAC GTC TAT CAA GAC CTC TTT TAC AGT GGC TTA GAC       119
 L   L   A   L   C   P   D   H   N   V   V   S   S   P   V   A   I   F   C   L        57
CTG CTG GCC CTG TGT CCA GAC CAT AAC GTG GTA TCA TCT CCC GTG GCC ATA TTC TGC CTG       179
 A   G   T   P   V   H   P   N   A   S   D   S   C   P   K   H   P   A   S   F        77
GCG GGC ACG CCG GTA CAC CCC AAC GCC TCC GAT TCC TGT CCC AAG CAT CCT GCC TCC TTT       239
 S   G   T   W   T   I   Y   P   D   G   R   F   G   N   Q   M   G   Q   Y   A        97
TCC GGG ACC TGG ACT ATT TAC CCG GAT GGC CGG TTT GGG AAC CAG ATG GGA CAG TAT GCC       299
 T   L   L   A   L   A   Q   L   N   G   R   Q   A   F   I   Q   P   A   M   H       117
ACG CTG CTG GCC CTG GCG CAG CTC AAC GGC CGC CAG GCC TTC ATC CAG CCT GCC ATG CAC       359
 A   V   L   A   P   V   F   R   I   T   L   P   V   L   A   P   E   V   D   R       137
GCC GTC CTG GCC CCC GTG TTC CGC ATC ACG CTG CCT GTC CTG GCG CCC GAG GTA GAC AGG       419
 H   A   P   W   R   E   L   E   L   H   D   W   M   S   E   D   Y   A   H   L       157
CAC GCT CCT TGG CGG GAG CTG GAG CTT CAC GAC TGG ATG TCC GAG GAT TAT GCC CAC TTA       479
 K   E   P   W   L   K   L   T   G   F   P   C   S   W   T   F   F   H   H   L       177
AAG GAG CCC TGG CTG AAG CTC ACC GGC TTC CCC TGC TCC TGG ACC TTC TTC CAC CAC CTC       539
 R   E   Q   I   R   S   E   F   T   L   H   D   H   L   R   Q   E   A   Q   G       197
CGG GAG CAG ATC CGC AGC GAG TTC ACC CTG CAC GAC CAC CTT CGG CAA GAG GCC CAG GGG       599
 V   L   S   Q   F   R   L   P   R   T   G   D   R   P   S   T   F   V   G   V       217
GTA CTG AGT CAG TTC CGT CTA CCC CGC ACA GGG GAC CGC CCC AGC ACC TTC GTG GGG GTC       659
 H   V   R   R   G   D   Y   L   R   V   M   P   K   R   W   K   G   V   V   G       237
CAC GTG CGC CGC GGG GAC TAT CTG CGT GTG ATG CCC AAG CGC TGG AAG GGG GTG GTG GGT       719
 D   G   A   Y   L   Q   Q   A   M   D   W   F   R   A   R   Y   E   A   P   V       257
GAC GGC CGT TAC CTC CAG CAG GCT ATG GAC TGG TTC CGG GCC CGA TAC GAA GCC CCC GTC       779
 F   V   V   T   S   N   G   M   E   W   C   R   K   N   I   D   T   S   R   G       277
TTT GTG GTC ACC AGC AAC GGC ATG GAG TGG TGC CGG AAG AAC ATC GAC ACC TCC CGG GGG       839
 D   V   I   F   A   G   D   G   R   E   A   A   P   A   R   D   F   A   L   L       297
GAC GTG ATC TTT GCT GGC GAT GGG CGG GAG GCC GCG CCC GCC AGG GAC TTT GCG CTG CTG       899
 V   Q   C   N   H   T   I   M   T   I   G   T   F   G   F   W   A   A   Y   L       317
GTG CAG TGC AAC CAC ACC ATC ATG ACC ATT GGC ACC TTC GGC TTC TGG GCC GCC TAC CTG       959
 A   G   G   D   T   I   Y   L   A   N   F   T   L   P   T   S   S   F   L   K       337
GCT GGT GGA GAT ACC ATC TAC TTG GCT AAC TTC ACC CTG CCC ACT TCC AGC TTC CTG AAG      1019
 I   F   K   P   E   A   A   F   L   P   E   W   V   G   I   N   A   D   L   S       357
ATC TTT AAA CCC GAG GCT GCC TTC CTG CCC GAG TGG GTG GGC ATT AAT GCA GAC TTG TCT      1079
 P   L   Q   M   L   A   G   P   *                                                    365
CCA CTC CAG ATG TTG GCT GGG CCT TGA ACC AGC CAG GAG CCT TTC TGG AAT AGC CTC GGT      1139
CAA CCC AGG GCC AGC GTT ATG GGT CTC CGG AAG CCC GAG TAA CTT CCG GAG ATG CTG GTG      1199
GTC CTG TAG CAG GCT GGA CAC TTA TTT CAA GAG TGA TTC TAA TTG GCT GGA CTC AGA GGA      1259
AAC CCT GCA G                                                                        1269
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图　1