Association of the porcine C3 gene with haemolytic complement activity in the pig

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Abstract – The complement component C3 plays an essential role in the activated complement system, which is involved in phagocytosis, inflammation and immunoregulation to destroy infectious microorganisms. The C3 molecule has more implications in the general defence mechanisms. In this study, the porcine C3 cDNA sequences including 5'- and 3'- flanking regions were determined and the polymorphisms in this gene were identified to carry out an association analysis between C3 and complement activity traits. Porcine C3 gene has high homology with human C3. Five single nucleotide polymorphisms (SNPs) and one microsatellite were detected in the porcine C3 gene. Haemolytic complement activity of alternative and classical pathways (ACH, CCP) was measured in 416 F2 animals of a crossbred of Duroc × Berlin Miniature Pig, which were immunized with Mycoplasma, Aujeszky and PRRS vaccines. C3 markers were found to be significantly associated (P < 0.05) with both ACP and CCP. Animals with the more frequent haplotype present in Duroc and other commercial breeds exhibit higher ACP and CCP levels than the animals with haplotype specific to some Berlin Miniature Pigs. The association of C3 with complement activity reinforces the importance of C3 as a candidate gene for natural resistance to microorganisms.

porcine C3 gene / natural resistance / haemolytic complement activity / association / pig

1. INTRODUCTION

The complement cascade defines an important link between the innate and the specific immune system [13]. The complement component C3 is the key molecule of the three pathways of complement activation (alternative, classical and lectin pathways), which are involved in phagocytosis, inflammation and immunoregulation processes to destroy infectious microorganism [19].

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C3 deficiency in hosts causes increasing susceptibility to bacterial or viral infections [1,4,13,22]. C3 has more implication in general defence mechanisms. We have previously found polymorphic sites within the porcine C3 which were used for genetic mapping of the locus [25]. The present study was carried out to investigate the porcine C3 gene as a candidate gene for complement activity traits. Therefore, the full length cDNA sequence of the porcine C3 as well as 5′- and 3′- flanking regions were obtained, screened for additional polymorphisms in this gene and, finally, association analysis was conducted between C3 markers and complement activity traits.

2. MATERIALS AND METHODS

2.1. Animals

Sequence information of the porcine C3 gene was identified in a DUMI F2-animal of a reciprocal cross of Duroc × Berlin Miniature Pig [9]. Screening for polymorphisms in the porcine C3 gene was performed in Duroc (n = 6), German Landrace (n = 28), Pietrain (n = 23), DUMI F2 (n = 902) and Thai native (n = 32) pigs. For association study, haemolytic complement activity was recorded in 416 animals of the DUMI-F2 resource population.

2.2. cDNA sequence and 5′ and 3′ flanking regions of the porcine C3 gene

Total RNA was extracted from pig liver using TRIzol™ Reagent (Life Technologies, Karlsruhe, Germany). Full length porcine C3 cDNA sequence was determined by the SMART™ RACE cDNA Amplification Kit (Clontech, Heidelberg, Germany). RACE primers and gene specific primers were designed based on partial porcine C3 sequence information (GenBank accession number AF110278, F14640) (Fig. 1):

RACE: 5′-ctaatacgactcactatagggcaacgtagtggataacgcagagt-3′
C3-A2: 5′-ccttctccagacatccagatctacg-3′
C3-B1: 5′-ccaccaagaccatgaatgtg-3′
C3-B2: 5′-tagagcttctggccaggttc-3′
C3-C1: 5′-ggtgggtacccgatgttaccaacg-3′.

The 5′ and 3′ flanking regions of porcine C3 gene were isolated from a genomic DNA library, which was constructed using Lambda DASH II® (Stratagene, Amsterdam, Netherlands). Two positive clones of 5′ and 3′ flanking region were amplified with primers specific to porcine C3 (5′-flanking region 5′-tgtgccccctcttctgttgg-3′, 3′-flanking region 5′-gtccgatgatgtagggtatgg-3′) and T3/T7
standard primers. All the PCR fragments were ligated in pGEM®-T vector (Promega, Mannheim, Germany) and sequenced using the SequiTherm Excel cycle sequencing Kit (Epicentre Technologies, Biozym, Hessisch Oldendorf, Germany) and a LI–COR 4200 automated sequencer (LI–COR Biosciences, Bad Homburg, Germany).

2.3. Polymorphism screening and marker genotyping

To identify polymorphisms in the porcine C3 gene, oligonucleotide primers were designed revealing 17 overlapping PCR fragments (Tab. I). They were amplified from individual liver cDNA and genomic DNA of six pig breeds. PCR was performed in a 25 µL reaction volume containing 50 ng of liver cDNA, 0.2 µM of each primers, 50 µM each dNTPs, 0.5 U of Pfu polymerase (Promega) in 1 × Pfu-PCR buffer. PCR cycling program was 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 52–60 °C (Tab. I) for 30 s, 72 °C for 1 min and final extension at 72 °C for 5 min. PCR products were gel purified, cloned and sequenced. The nucleotide sequences of each animal were compared to find out polymorphisms in the porcine C3 gene.

To genotype each polymorphic site in the porcine C3 gene, PCR-RFLP, allele specific PCR, SSCP and microsatellite analysis were performed. Oligonucleotide primers (Tab. II) were used to amplify PCR fragments from genomic DNA. PCR reactions were carried out in 20 µL volume using Taq polymerase (Promega). Amplified PCR fragments with primer C3-I (Tab. II) were mixed with 1:10 with loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol), denatured at 95 °C for 5 min, then cooled on ice immediately and separated on 12% polyacrylamide gel (49:1 acrylamide/bis-arylamide) at room temperature with a constant 12 W for 6 h at 0.5 × TBE. The SSCP-bands were stained by silver staining procedure. Amplicon C3-II was digested with restriction enzyme TaqI (Promega). The digested PCR fragments were analysed on 2% agarose gels. Genotyping the SNPs within amplicons C3-III and C3-IV was done as described previously [25]. Fragments with length polymorphism were amplified with primer C3-V (Tab. II) and were identified on 6% SequiGel®XR-denaturating polyacrylamide gels.
Table I. Primer sequences used for screening polymorphisms in the porcine C3 gene.

| Name  | Forward primer sequence \((5' \text{ to } 3')\) | Reverse primer sequence \((5' \text{ to } 3')\) | Annealing temp. \(\text{ (°C)}\) | cDNA \(\text{(bp)}\) | Genomic-DNA \(\text{(bp)}\) |
|-------|---------------------------------|---------------------------------|----------------|----------------|----------------|
| C3–5'| GCATCGACTTTGAATTCACAG          | CCATGATGGAGGGACGGTG            | 55             | –              | 477            |
| C3–01| TCCTTTCCCCCTGTCCTTTT          | GGGAGTCCCCGGTGTATGTC           | 58             | 577            | –              |
| C3–02| TGAGACACCTGAAGGCATTG          | GCTGTTCATACACACACTCTC          | 58             | 306            | ~ 700          |
| C3–03| TCAACATCATGCCCCAGGTTC         | GGGCTCTGGTGAAGTGGATC           | 58             | 324            | –              |
| C3–04| CATCTGACCTCCCTCTAATC          | GTGCGCAGGTGGAAGTTAAC           | 59             | 393            | ~ 1000         |
| C3–05| TCAAGCCAGGAGGAATCTC           | TCTCAGGTACATCGTGTGC           | 59             | 360            | ~ 1500         |
| C3–06| GAAGCAAGACAAGCAGCATC          | TGTCATCCTTTTCTCCATG           | 56             | 338            | ~ 900          |
| C3–07| GTGCACTGTATGGAGAAAAAG        | ATTCATGCTTTGCTTGGAG           | 56             | 360            | –              |
| C3–08| CTCAACAGACACATGAAAT            | TCTTCAAGGCAACAAATG            | 56             | 356            | –              |
| C3–09| CATTGTGACCTTGAAGGAAG         | GTGCTGTCCAGGTAGTGAC           | 55             | 396            | –              |
| C3–10| CAGTCTACGGTTGACATCA          | GTTCTTGAAGCACCACATC           | 55             | 343            | –              |
| C3–11| GATTTGGCTGGCTTCAAAGAC      | CTACCAGCAAGACCAAG             | 52             | 341            | –              |
| C3–12| CTGGGTCTCTGTGCTGTA          | CTGGGTACATGGTCAC              | 52             | 338            | ~ 1000         |
| C3–13| GTGACCATGTACACCG            | CCTCCAGGTTGTGAGAC             | 52             | 337            | ~ 1300         |
| C3–14| GATCTCACACACCCCGGAGG         | CACTTCACGGAGCCCTGATT          | 55             | 379            | –              |
| C3–15| AATCAGGCTCCGATGTAAGTG       | GGCAACCAAGACGACCAT            | 56             | 270            | –              |
| C3–3’| TGGCAACTTCTCTTGAGAGA         | TGCAGGGAGAATCAGGGT            | 60             | –              | 530            |
Table II. Nucleotide sequence primers for genotyping the polymorphisms in the porcine \(C3\) gene.

| Amplicon | Primer (5’ to 3’) | Size (bp) | Annealing temp. (°C) |
|----------|-------------------|-----------|----------------------|
| C3-I     | F: GCATCGACTTGAATTACAG  |
|          | R: CCATGATGGAGGGACGGTG  |
|          | 477                 | 55        |
| C3-II    | F: CACCCTGATTTGCTGCAATG |
|          | R: TACCTCAACTTACTGCAGTG |
|          | 383                 | 60        |
| C3-III   | F: TGAGAATGTGGATGGACCAG |
|          | R: GGACTTGAATGGCCCAAGATC |
|          | 384                 | 60        |
| C3-IV    | F1: AAGGATCTGAACCTGGATGTGA |
|          | F2: GGATCTGAACCTGGATGTG |
|          | R: ACCCCGCTAATCTGTGATGC |
|          | 454, 452            | 64        |
| C3-V     | F: TGGGCAACTTCTCTGAGAAC |
|          | R: TAGGATGAACCTGAGCTTG |
|          | 204, 207            | 60        |

(Biozym Diagnostik GmbH) by the LI-COR 4200 automated sequencer (LI-COR Biosciences). The fragment size of alleles was analysed by One-Dscan software (Scanalytics, MWG Biotech).

2.4. Haemolytic complement activity phenotypes

Total haemolytic complement activities were determined in 416 F2-animals of the DUMI-resource population produced from 11 F1 sows (2 full-sib groups) mated with 3 F1 boars. The animals were reared in the Frankenforst research farm of the University of Bonn. They were immunized with Mycoplasma, Aujeszky and PRSS vaccines at 6, 14 and 16 weeks of age, respectively. Blood samples were taken immediately before vaccination and on 4 and/or 10 days after vaccination (Fig. 2). Total haemolytic activity of the alternative (ACP) and classical complement pathway (CCP) was determined by a method modified from Liu and Young [15]. Non sensitised rabbit erythrocytes and sensitised sheep erythrocytes were used as target cells, for ACP and CCP respectively. The haemolytic complement activity was expressed as the titre that lysed 50% of erythrocytes (CH50 unit · mL\(^{-1}\)).

2.5. Association analysis between \(C3\) and complement activity traits

Association between \(C3\) markers and haemolytic complement activity traits was analysed using the REPEATED statement of the SAS® PROC MIXED procedure [14]. The statistical model included \(C3\) genotype, time of blood
sampling, sire, and interaction of $C3$ genotype and time of blood sampling as fixed effects, and dam nested within sire as random effect. A heterogeneous compound-symmetry (CSH) covariance structure was included in the statistical model to analyse fixed effects because it gave the best fit when compared to other covariance structures.

3. RESULTS

3.1. Sequence analysis of the porcine $C3$ gene

The complete porcine $C3$ cDNA sequence (accession No. AF154933) was determined. The 5127 bp cDNA contains an open reading frame coding for 1661 amino acids, including a 22-amino acids signal peptide, 643 amino acids $\beta$-chain, a 4 amino acids linker and 992 amino acids $\alpha$-chain. The ORF of porcine $C3$ shows 80, 78 and 77% homology with human, rat and mouse $C3$ nucleotide sequence, respectively [6,7,17]. The 63 bp 5′-UTR is a pyrimidine rich region (approximately 80% pyrimidines). A 56 bp of 3′-UTR was sequenced and a polyadenylation signal (AATAAA) was found at position 5074 to 5079 of cDNA sequence (AF154933). The sequence information of the 5′-flanking region (Fig. 3a) contained several features of promoter region. The putative TATA-box (GATAAA) sequence was located at −29 bp of the first nucleotide $C3$ sequence (AF154933) and various putative enhancer/transcription factor binding sequences were also found (Tab. III). Comparison of the porcine 5′-flanking sequence with the promoter region of human $C3$ sequence (X62904) [10] revealed 74% sequence identity. However, the 3′-flanking region (Fig. 3b) of porcine $C3$ had low homology with human $C3$ sequence (M63422) [21].

3.2. Polymorphism and allelic frequency of the porcine $C3$ gene

Six polymorphic sites were found in the porcine $C3$ gene including five SNPs and one microsatellite (Fig. 4a). Two SNPs were found in the 5′-flanking
Figure 3. Sequence of the 5′-flanking region upstream from ATG start codon (a) and of the 3′-flanking region downstream from TGA codon (b) of the porcine C3 gene (accession No. F154933). The 5′-UTR is indicated in italics and the putative transcription start site is indicated in italics, bold and underlined. A putative TATA box and polyadenylation signal are indicated in bold and underlined in the 5′- and 3′-flanking regions, respectively.

region at position −384 (G > A) and −44 (C > T) nucleotide (nt) upstream ATG start codon. The other three SNPs were located in the coding region at 1905 (C > A), 3882 (G > A) and the nt 204 (T > C) of the intron 13. A microsatellite with (T)_{14-21}-repeat units was found in the 3′-flanking region of C3 and was located at 71 bp downstream from the TGA stop codon.

The two SNPs of 5′-flanking region were genotyped by SSCP analysis (Fig. 4b). *Taq* I-RFLP was used to detect the variation of the nt 204 in the intron 13 of C3-cDNA sequence (amplicon C3-III) and showed either undigested PCR product (384 bp, allele T), and/or digested product (285 bp and 143 bp, allele C) (Fig. 4c). Similarly, restriction enzyme *Hsp*92 I was used to test polymorphism at position 1502 (amplicon C3-III) and revealed either
Table III. Position of transcription factor binding sites in the 5′-flanking region of the porcine C3 gene.

| Factor       | Consensus sequence | Position      | Homology (%) | Ref. |
|--------------|--------------------|---------------|--------------|------|
| Enhancer core| GGGGAAAA           | −110 to −103  | 88           | [8]  |
| C/EBP        | TTGAGAAAT          | −176 to −168  | 100          | [23] |
| IL-6-RE      | AGGGGGA            | −112 to −106  | 86           | [21] |
|              | TCTGGGG            | −168 to −162  | 100          | [21] |
|              | TGAGAAA            | −175 to −169  | 100          | [21] |
|              | TGAAAAA            | −197 to −191  | 100          | [21] |
|              | TGAGAGA            | −290 to −284  | 100          | [21] |
|              | TGGGGAA            | −371 to −365  | 100          | [21] |
| IL-6 RE rev  | TTCCCCCA           | −220 to −226  | 100          | [8]  |
|              | TCCTCCA            | −351 to −345  | 100          | [8]  |
| IFNγ RE      | GAAAAACTTTTGGA     | −196 to −192  | 87           | [8]  |
|              |                    |               |              |      |
| AP-2         | CCCTTAGG           | −132 to −139  | 88           | [18] |
| LF-A1        | TGGCCA             | −210 to −205  | 83           | [8]  |
| LF-B1/HNF-1  | GTTAAT             | −443 to −438  | 100          | [8]  |
|              | GTTATT             | −232 to −227  | 83           | [8]  |
| Estrogen RE  | TGTCCCTTGTCC       | −31 to −19    | 85           | [21] |

digested PCR product (237 bp and 146 bp, allele C) and/or 383 bp (allele A) of undigested product (Fig. 4d). The SNP located at 3882 was genotyped by allele specific PCR technique with two different primer sets (Fig. 4e). Microsatellite analysis in the 3′-flanking region of porcine C3 revealed three alleles of 211, 207 and 204 bp (amplicon C3-V), and these alleles corresponded to (T)$_{21}$-, (T)$_{17}$-, and (T)$_{14}$-repeat units, respectively (Fig. 4f, allele 204 not shown).

Five of these polymorphic sites (at positions −44, 204 of intron 13, 1509, 3882 and +71) were segregating in the DUMI F2-resource population, and these alleles were coming from some Berlin Miniature Pig of the grandparent generation. All six polymorphic sites were segregating only in Thai native pigs, but no polymorphism was found among the 86 Duroc, German Landrace, Large White and Pietrain pigs. Allelic frequencies of the polymorphic sites in porcine C3 gene among pig breeds are given in Table IV.

### 3.3. Association analysis

Means and standard deviation of the haemolytic complement activity of alternative and classical pathways were estimated (Tab. V). Only 3 SNPs
Figure 4. Polymorphism in the porcine C3 gene including the 5'- and 3'-flanking regions (a); SSCP analysis at positions −384 and −44 bp in the 5'-flanking region (b); TaqI-PCR-RFLP test at position 204 in intron 13 (c); Hsp92 I-PCR-RFLP test at position 1905 (d); allele specific PCR test at position 3882 (e); and SSLP analysis at position +71 bp in the 3'-flanking region (f).

and one microsatellite (nt 204 of intron 13, 1905, 3882, +71) were genotyped. These four alleles were segregating with two haplotype patterns that can be described [2] as follows: allele C3*I, [IVS13+204T; 1905C; 3882G; c.5038+71(T)₂₁] and allele C3*I, [IVS13+204C; 1905A; 3882A; c.5038+71(T)₁₇]. The numbers of pigs for each genotype class were 290, 109 and 17 for C3*I/C3*I, C3*I/C3*I, and C3*I/C3*I genotypes respectively. Subsequently, the genotype C3*I/C3*I was excluded from the analysis because of its low frequency and unequal distribution over parities and litters.
Table IV. Allele frequencies at the porcine C3 gene in six genetic groups of pigs.

| Position | Allele | F2-DUMI Frequency | Duroc Frequency | German Landrace Frequency | Large White Frequency | Pietrain Frequency | Thai native pig Frequency |
|----------|--------|------------------|----------------|--------------------------|----------------------|-------------------|-------------------------|
| −384     | G      | 1.00             | 1.00           | 1.00                     | 1.00                 | 1.00              | 0.99                    |
| −44      | G      | 0.99             | 1.00           | 1.00                     | 1.00                 | 1.00              | 0.97                    |
| 204 (intron 13) | T   | 0.75             | 1.00           | 1.00                     | 1.00                 | 1.00              | 0.82                    |
| 1905     | C      | 0.74             | 1.00           | 1.00                     | 1.00                 | 1.00              | 0.70                    |
| 3882     | G      | 0.74             | 1.00           | 1.00                     | 1.00                 | 1.00              | 0.82                    |
| +71(T)_{21} | T_{21} | 0.76             | 1.00           | 1.00                     | 1.00                 | 1.00              | 0.00                    |
| +71(T)_{17} | T_{17} | 0.24             | 0.00           | 0.00                     | 0.00                 | 0.00              | 0.12                    |
| +71(T)_{14} | T_{14} | 0.00             | 0.00           | 0.00                     | 0.00                 | 0.00              | 0.17                    |

Table V. Haemolytic complement activities (mean ± SD) of alternative and classical pathways in DUMI resource population.

| Blood sampling | Haemolytic complement activity (Unit · mL$^{-1}$) |
|----------------|-----------------------------------------------|
|                | ACH50                                         | CCH50                                         |
| Time-1         | 53.64 ± 28.31                                 | 40.70 ± 23.13                                 |
| Time-2         | 53.22 ± 28.43                                 | 48.23 ± 28.81                                 |
| Time-3         | 55.05 ± 24.86                                 | 47.97 ± 29.19                                 |
| Time-4         | 56.86 ± 30.13                                 | 59.08 ± 36.46                                 |
| Time-5         | 62.31 ± 32.08                                 | 64.91 ± 35.71                                 |
| Time-6         | 68.46 ± 47.63                                 | 59.79 ± 39.10                                 |
| Time-7         | 69.75 ± 41.70                                 | 62.53 ± 35.58                                 |
| Time-8         | 69.71 ± 37.42                                 | 60.99 ± 40.89                                 |

Table VI. Significance in analysis of variance of alternative (ACH50) and classical (CCH50) complement activity traits.

| Traits      | Test of fixed effects (P value) |
|-------------|---------------------------------|
|             | C3 marker | Time | C3 × time | Sire    |
| ACH50       | 0.0255    | < 0.0001 | < 0.0001 | < 0.0001 |
| CCH50       | 0.0286    | < 0.0001 | 0.0014   | 0.0151   |

Analysis of variance revealed that hemolytic complement activity of both alternative and classical pathways was significantly affected ($P < 0.05$) by C3 marker, time of blood sampling and their interaction, and by sire effect (Tab. VI). The profiles of the haemolytic complement activities between the different C3
Figure 5. Haemolytic complement activity profiles of alternative (a) and classical (b) pathways for pigs with $C3^*1/C3^*1$ and $C3^*1/C3^*2$ genotypes were not parallel over time (Fig. 5). Complement activities in the alternative and classical pathway were 5.8 and 6.9 units · mL$^{-1}$ higher for animals homozygous for the $C3^*1$ allele than for $C3^*1/C3^*2$ animals.

### 4. DISCUSSION

Activated complement system plays an important role in killing and neutralization of microorganisms, and $C3$ is a key molecule of this system. Therefore, $C3$ can be regarded as a candidate gene for complement activities. The 5.1 kb sequence of full-length porcine $C3$ gene has a 4983-bp ORF which coded for $\alpha$- and $\beta$-chain of $C3$ protein. The porcine $C3$ is a conserved gene and has high homology with the other mammalian species $C3$ gene. Indeed, the putative promoter region contains one TATA-box and several cis-regulating
elements similar to that of the human C3 promoter [8,21]. The variation of haemolytic complement activities before and after vaccination within the F2-DUMI resource population was found to be associated with C3 markers. Although these C3 markers were silent at the amino acids level, another nucleotide exchange in the 5′-flanking promoter region of the C3 gene and in linkage disequilibrium with these C3 variants might be responsible for its function. Animals with the more frequent C3*1/C3*1 haplotype, present in Duroc and other commercial breeds, exhibited higher complement reactivity than those carrying the allele C3*2 coming from Berlin Miniature Pigs.

Genetic variation in C3 affecting the complement activity has been observed in human [3], rabbit [11], and mouse [5]. These reports indicated that the genetic defect of C3 gene caused low (or lack of) haemolytic complement activity and increased susceptibility to microorganisms. Phenotypic variation of haemolytic complement activity in pigs has been associated with effects due to vaccination, genomic variation at the swine leukocytes antigen locus (SLA) [16,20], breed [12], age as well as sire or/dam [16,24].

Although the complement is a complex system and is controlled by many genes, the present work showed there was an association of C3 and haemolytic complement activity – a defence mechanism active against many pathogenic agents – which reinforces the importance of C3 as a candidate gene for natural resistance to microorganisms.

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