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Increased expression of a novel splice variant of the complement component 4 (*C4A*) gene in mastitis-infected dairy cattle

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ABSTRACT. The complement system helps in the direct lysis of invading pathogens and modulates phagocytic, humoral and cellular immune responses. Complement 4 is a critical component in complement activity and protection against many bacterial pathogens because it is essential to classical and lectin activation pathways. We used reverse transcription and PCR to investigate alternative splicing and expression of the complement component 4 (*C4A*) gene in Chinese Holstein cattle. The PCR products were cloned and sequenced. A novel splice variant involving intron 10 was identified, which we named C4A-AS. To examine how *C4A* gene activity is affected by bovine mastitis, six Chinese Holstein cattle were divided into healthy (non-mastitic) and *Staphylococcus aureus*-induced mastitic groups. Real-time quantitative PCR (qRT-PCR) revealed that the C4A-complete and C4A-AS transcripts are expressed at significantly different levels in healthy cows, while there were no significant differences in the mastitic

group (P = 0.257). Expression of C4A-AS increased significantly when mastitis developed. We also examined the expression of C4A-complete and C4A-AS in several tissues (liver, heart, spleen, lung, kidney, tongue, and muscle). The two transcripts were expressed in all of these tissues but there were no significant differences in expression between healthy and mastitic cows. We therefore conclude that the C4A-complete transcript is the main transcript under normal physiological conditions, while C4A-AS is augmented when mastitis develops.

Key words: *C4A* gene; Alternative splicing; Transcription pattern; Dairy cattle; Mastitis

INTRODUCTION

C4 is a component in the classical and lectin complement pathways, and it plays an important role in host defense. In humans, the *C4* gene exists in two copies *C4A* and *C4B* (Dodds and Law, 1990), whereas only the *C4A* gene is reported in bovine. The bovine complement component 4 (*C4A*) gene is closely linked to the major histocompatibility complex class II region on chromosome 23 (Andersson et al., 1988), which shows significant association with susceptibility to intramammary infections with major pathogens (Rupp and Boichard, 2003). Bovine mastitis is an inflammation of the mammary gland, and related economic losses are the costs of treatment, culling, death and decreased milk production (Bradley, 2002). In cows, the deposition of C4 on *Streptococcus agalactiae* was increased in the milk of infected glands compared to normal milk (Rainard and Poutrel, 1995). Mastitis could up-regulate the mRNA abundance of the *C4A* gene in *Escherichia coli*-infected udders (Günther et al., 2009). This implies a possible relationship between the *C4A* gene and mastitis resistance in dairy cattle.

In humans, C4 has been examined for association with various diseases, such as systemic lupus erythematosus (Petri et al., 1993), schizophrenia (Morera et al., 2007), 21-hydroxylase deficiency (Guerra-Junior et al., 2008), and diabetes (Vergani et al., 1983). C4 is very complex at both the genomic and protein levels. Different individuals can differ either in number or size of the *C4* gene (Yu et al., 1986). The *C4* gene demonstrates a high rate of polymorphism (Belt et al., 1985), and C4 protein was also found to be highly polymorphic by electrophoretic (Awdeh and Alper, 1980) and serological typing (Giles, 1984).

Alternative splicing in eukaryotes is a regulatory mechanism for generating potentially many transcript isoforms from a single gene, and it is highly relevant to disease and therapy (Garcia-Blanco et al., 2004; Kim et al., 2007; Ju et al., 2011). A large fraction of the protein-coding genes of multicellular organisms are alternatively spliced (Ast, 2004). Of the 21,755 bovine genes, 4567 (21%) are alternatively spliced (Chacko and Ranganathan, 2009). The types of AS include exon skipping, alternative 5'-and 3'-splice sites, alternative promoter usage, mutually exclusive exons, intron retention, and alternative polyadenylation (Keren et al., 2010). Thus, AS may be one of the mechanisms by which the diverse physiologic functions of the *C4A* gene are regulated as well. Taken together, we investigated the potential AS patterns of the *C4A* gene in Chinese Holsteins. In addition, the transcripts of the *C4A* gene in different tissues of healthy and mastitic cattle were analyzed to examine their effect on mastitis.

MATERIAL AND METHODS

Tissues sample collection

The milk, tissue of mammary gland in four areas, heart, liver, spleen, lung, kidney, muscle, and tongue were sampled aseptically from 10 culled Chinese Holstein cattle with clinical mastitis and 6 healthy control cows in the slaughterhouse. The milk was stored in an ice box, and tissue samples were obtained immediately after slaughter, snap-frozen in liquid nitrogen until RNA isolation and transported to the laboratory.

Diagnostic tests for identification of *Staphylococcus aureus* in milk

The milk isolates of bovine mastitis mammary areas were tested for hemolysis after overnight incubation at 37°C on sheep blood agar. Hemolysis was recorded as α-hemolysis, β-hemolysis, double hemolysis (α + β), and negative (no hemolysis). DNase activity was tested on DNase test agar following manufacturer recommendations (Tianhe Microorganism, Hangzhou, China). Only strong DNase activities (clear zone around growth similar to that of the *S. aureus* control strain ATCC 29213) were recorded as positive. Weak DNase activities with clear zones noticeably smaller than that of the positive control were recorded as negative.

Based on the result of diagnostic tests for identification of *S. aureus* in milk, a total of six Chinese Holstein cattle were selected and divided into two groups: normal (N = 6) and *S. aureus*-induced mastitis (N = 6). Total RNA of tissues was extracted with the E.Z.N.A®. Total RNA kit II (Omega Bio-tech).

Primer design

Based on mRNA sequence of bovine *C4A* and *β-actin* gene (GenBank accession Nos. NM_001166485.1 and NM_173979.3), the primers (Table 1) were designed with the Primer Premier 5.0 software and synthesized by Sangon Biological Engineering Technology (Shanghai, China).

Preparation and amplification of cDNA

The isolated RNA was transcribed into cDNA with PrimeScript® RT Master Mix (TaKaRa, Dalian, China) according to the manufacturer instruction. PCR was performed in a volume of 50 µL consisting of 1.0 µL cDNA, 1.0 µL 10 µM of each primer (C4A1 to C4A4), 5.0 µL 10X PCR buffer, 1.8 µL 50 mM Mg^{2+}, 1.0 µL 10 µM dNTP, 0.5 µL 5 U/µL Taq DNA polymerase, and ddH_2O added up to 50 µL. An initial denaturation for 5 min at 94°C, was followed by 35 cycles of denaturation at 94°C for 1 min, annealing at Ta for 1 min, and extension at 72°C for 90 s, and a final elongation at 72°C for 7 min (Table 1). The amplification was confirmed by electrophoresis on a 1% agarose gel with ethidium bromide.

Cloning and sequencing

The PCR product was purified with the E.Z.N.A.™ Gel Extraction kit (Omega Bio-tech)

and ligated to *pEASY*-T3 vector (TransGen, Beijing, China), and their mixture was subsequently transformed into DH 5α strain of *E. coli*. The positive clones were selected randomly and sequenced using an ABIPRISM3730 DNA Sequencer (BGI Biotech Co., Ltd., Qingdao, China).

Real-time quantitative PCR (qRT-PCR)

PCR was performed using the SYBR® Premix Ex Taq™ II (TaKaRa) and the Applied Biosystems 7300 Real-Time PCR System in reaction volumes of 20 µL. The reaction system contained 10.0 µL SYBR® Premix Ex Taq™ II, 0.8 µL 10 µM forward and reverse primers, 0.4 µL ROX Reference Dye II, 1.0 µL cDNA or plasmid DNA, and 7.0 µL ddH_2O. Each sample was amplified in triplicate.

The thermal cycling parameters were as follows: a pre-run at 95°C for 30 s, 40 cycles with a 5 s denaturation step at 95°C followed by a 60°C for 31 s. In addition, bovine *β-actin* was used as an internal control. A no-template control (negative control) was included in each assay.

Data mining and statistical analysis

Alternative splicing detection and sequence analysis were conducted using the BLAST search online resource (http://blast.ncbi.nlm.nih.gov) and the DNAMAN software package (version 6.0, Lynnon Biosoft, Quebec, Canada). The relative quantification of *C4A* gene expression was calculated using the standard curve-based method for real time PCR (Larionov et al., 2005). The analyses were performed using the SPSS 17.0 statistics software. The data were subjected to Student independent-sample *t*-test to determine differences between the means of different groups. The values of the expression level were presented as the mean ± SEM. Significance was concluded at $P < 0.05$.

RESULTS

Identification of *C4A* gene transcript variants

To investigate the possible splicing variants of the bovine *C4A* gene, RT-PCR was performed in the liver and mammary gland tissues. A total 67 clones were selected to sequence (RT-PCR with primer C4A1; other fragments had no detectable AS in the present study; data not shown) and one splice variant was obtained in the liver and mammary gland. The sequence alignment results showed that the splice variant retained intron 10 (Figure 1). Although the fragment length of C4A-AS was longer than the complete sequence transcript, the C4A-AS transcript encoded 492 amino acids, only a part of the *β*-chain of bovine C4. In the current study, the detected frequency of C4A-AS was 4.5%. The sequence of C4A-AS was submitted to the National Center of Biotechnology Information (GenBank accession No. HQ722913).

Quantification of C4A-complete and C4A-AS

Selective amplification of C4A-complete and C4A-AS was achieved by qRT-PCR using specific primers (Table 1). The relative expression quantities of healthy and *S. aureus*-

induced mastitic groups are shown in Figure 2A. Data showed that the expression of the two transcripts was significantly different ($P < 0.001$) in non-mastitic cows, while there was none in the mastitic group ($P = 0.257$). It is worth mentioning that C4A-AS increased conspicuously when mastitis occurred ($P = 0.030$). To evaluate whether C4A-complete and C4A-AS were expressed in other tissues, RT-PCR was performed in tissues of healthy and *S. aureus*-induced mastitic Chinese Holsteins (Figure 2B). The results confirmed that C4A-AS expression was lower compared to C4A-complete in the healthy cows but insignificant ($P > 0.05$). In addition, to make certain that the AS was authentic, RT-PCR products were sequenced.

Table 1. Sequence of primers used in the experiment.

Primer	Primer sequence (5'-3')	Fragment size (bp)	Ta (°C)	Function
C4A1	S:CATCTTGCTTCTTCGCCTTGT A:GACGATACGCCTTGGAACTGT	1713	59.8	RT-PCR 65→1777
C4A2	S:CCATTGAGTGGCAGAATCCGC A:CATCATCCCGTTGCTGCGACA	1923	65.5	RT-PCR 1451→3373
C4A3	S:CAAGACAGAGCAGTGGAGCAT A:TGCCCCATAAAACACAGAACA	1627	59.6	RT-PCR 3117→4743
C4A4	S:GTGAGAGGAAACAGCAAAGGA A:AGAGCCTTAGATGATGCCAAC	1343	57.0	RT-PCR 4136→5482
β-actin	S:GCACAATGAAGATCAAGATCATC A:CTAACAGTCCGCCTAGAAGCA	173	60.0	Internal control
C4A-complete	S:TAGCACTGATGACCTCCC A:AAAACAACTTGGCAGACAC	248	60.0	qRT-PCR
C4A-AS	S:GAAGGGGGAGGATGAGCA A:CGCTCCCATCTGTGTTCTG	221	60.0	qRT-PCR

Ta = annealing temperature.

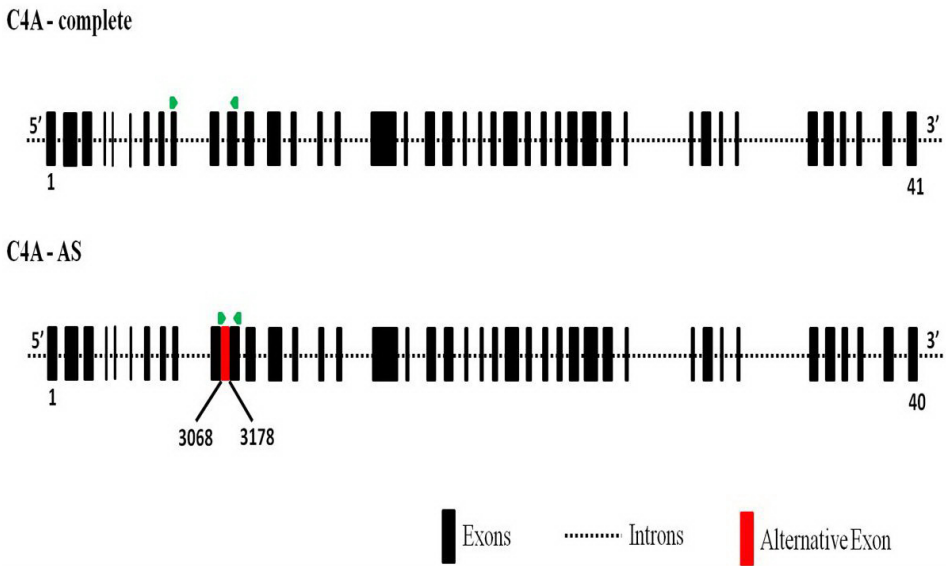

Figure 1. Gene structure and alternative splicing pattern of the *C4A* gene in Chinese Holstein cattle. Green arrows indicate forward and reverse primers for qRT-PCR, and numbers of the first exon and last exon are shown below as corresponding black boxes. C4A-AS is intron 10 retention (111 bp was retained), so the total exon number is 40.

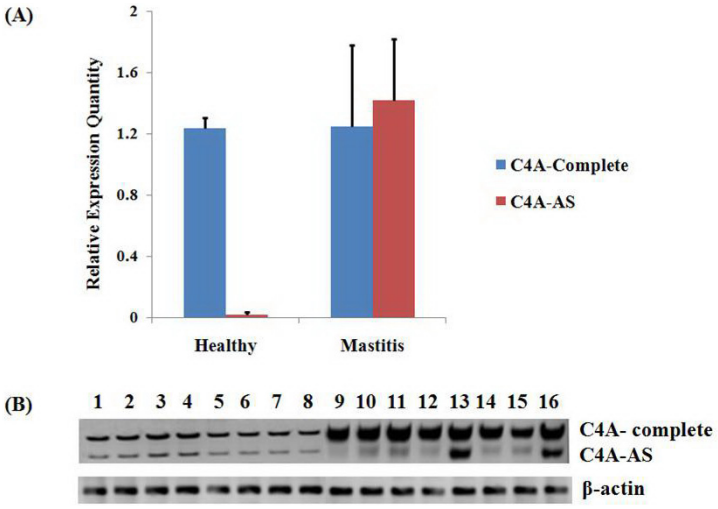

Figure 2. Results of relative C4A-complete and C4A-AS transcript quantification. **A.** qRT-PCR relative quantification of C4A-complete and C4A-AS in mammary glands of healthy and *Staphylococcus aureus*-induced mastitic cows. **B.** Comparison of C4A-complete and C4A-AS transcript levels in several tissues (*Lanes 1* and *9* = heart; *lanes 2* and *10* = tongue; *lanes 3* and *11* = lung; *lanes 4* and *12* = spleen; *lanes 5* and *13* = liver; *lanes 6* and *14* = kidney; *lanes 7* and *15* = muscle; *lanes 8* and *16* = mammary glands) in non-mastitic (*lanes 1-8*) and *S. aureus*-induced mastitic cattle (*lanes 9-16*).

DISCUSSION

To date, little is known about *C4A* mRNA expression in cattle, and splice variants of bovine *C4A* have not yet been characterized. In the present study, we reported for the first time the detection of a transcript variant of the bovine *C4A* gene, which shows intron 10 retention. Although this kind of variant may be derived from unspliced or partially spliced pre-mRNAs (Galante et al., 2004), some cases of intron retention with known biologic effect have been proven (Rio, 1991; Le Hir et al., 2002). Therefore, we analyzed the expression quantity of C4A-complete and C4A-AS in healthy and *S. aureus*-induced mastitic Chinese Holstein cows. Our findings suggest that the C4A-AS may have some actions against *S. aureus*-induced mastitis, owing to the significant increase in diseased individuals.

In humans, people with a short *C4B* gene have higher C4 serum protein levels and higher C4 hemolytic activity than those with a long *C4A* gene, which contains an endogenous retrovirus HERV-K (C4) in intron 9 (Yang et al., 2003). In H-2^k mice, the insertion of the B2 sequence into intron 13 results in low C4 expression in serum (Pattanakitsakul et al., 1992). These findings imply that splicing of the *β*-chain could regulate C4 concentration, consistent with our results. Other components in the complement system also have AS, such as MASP-3, which is generated through alternative splicing of the MASP-1/3 gene (Dahl et al., 2001).

The presence of C4A-AS may be associated with a single nucleotide polymorphism (SNP), which we have identified in exon 10 of Chinese Holstein cattle, *C4A* gene (A→G, ss262957630). This coincides with the results of Liu et al. (2001), who considered that a coding-region SNP may affect the patterns or efficiency of AS. Our conclusion is also consistent with the results of Hull et al., who believe that there is a highly significant association between splice phenotype and neighboring SNPs (Hull et al., 2007).

Liver is the major source of C4. In mice, C4 is expressed in peritoneal macrophages, liver, mammary gland, lung, spleen and kidney (Cox and Robins, 1988). In humans, C4 is enriched in liver, kidney, thyroid, brain, and breast (Witte et al., 1991). Our RT-PCR results showed that *C4A* is also expressed in heart, liver, spleen, lung, kidney, muscle, tongue, and mammary gland of dairy cattle. Moreover, there were expression differences between the two transcripts of the *C4A* gene in mammary gland tissues from healthy and mastitic groups. Wang et al. (2008) found that the expression of C4 mRNA was first downregulated by lipopolysaccharide challenge and then fluctuated in the zebrafish *Danio rerio*. And LPS is the main component of *E. coli*, which is a mastitis-causing bacterium. Herein, we proposed a hypothesis that the C4A-complete transcript was a dominating transcript under normal physiologic conditions, while the expression of C4A-AS was enhanced when the mastitis-causing bacteria such as *S. aureus* invaded or other diseases occurred.

In conclusion, this study showed that one splice variant was identified in Chinese Holstein cattle, which we named C4A-AS. We detected the expression level of two transcripts in several tissues and found that the C4A-AS may have a relationship with mastitis resistance. Since our present study included small samples and lacked randomization, the biologic significance of the C4A-AS transcript requires further study.

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