Study of β-defensin polymorphisms in Valle del Belice dairy sheep

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ABSTRACT - The aim of this work was to sequence the exons of β-defensin 1 and 2 genes (SBD1 and SBD2) in Valle del Belice dairy sheep in order to identify polymorphisms. The study was conducted on 60 samples from three flocks. Six SNPs were identified: two in SBD1 and four in SBD2. Both genes consist of two exons and one intron. In SBD1 gene, SNPs were found only in the exon 2, whereas in SBD2, SNPs were detected in both exons. In both genes, SNPs were located in the coding regions and in the 3'-UTR. The SNP in SBD2 located at position 1659 determined a change in the protein sequence. Further studies will be necessary to investigate if the amino acid change modifies the biological function of the protein and the association with SCC, in order to use this information in a breeding program for mastitis resistance in Valle del Belice sheep.

Key words: β-defensin, SNP, Sheep.

Introduction - Defensins are a class of small peptides belonging to the antimicrobial peptides family. They are involved in the innate immunity mechanisms and act directly against bacteria, viruses, and fungi, due to their bactericidal and cytotoxic activity (Brodgen *et al.*, 2003). These proteins are classified into α-, β-, and θ-defensins on the basis of structure, size, and disulfide bonds pattern (Kaiser *et al.*, 2000; Selsted and Ouellette, 2005). Defensin genes are arranged in clusters (Maxwell *et al.*, 2003; Patil *et al*., 2005) and are expressed in epithelial cells lining various organs such as kidneys, pancreas, trachea and mammary gland, oral mucosa, respiratory, gastrointestinal and urogenital tract, and leukocytes. Their expression can be constitutive and/or inducible by inflammatory mediators or bacterial origin molecules (Kaiser *et al.*, 2000). Due to their important role in the immune response, β-defensin genes have been characterized in different domestic animals like cattle (Yount *et al.*, 1999), pig (Zhang and Wu, 1998), and goat (Zhao *et al.*, 1999). In sheep, only two β-defensin genes have been described so far: β-defensin 1 (SBD1) and β-defensin 2 (SBD2) (GenBank Acc. no. U75250 and U75251, respectively). Both genes have been mapped on chromosome 26, and consist of two exons and one intron of approximately 1500 bp (Huttner *et al.*, 1998). Exon 1 encodes the signal sequence; exon 2 encondes the pro-peptide and the mature peptide (Luenser *et al.*, 2005). The aim of this study was to sequence the exons of SBD1 and SBD2 in Valle del Belice dairy sheep in order to identify polymorphisms.

Material and methods - A total of 60 samples of Valle del Belice sheep from three flocks were analyzed. Genomic DNA was extracted from whole blood using buffy coat DNA isolation method. PCR reactions were performed in final volume of 20 µl containing approximately 50 ng of genomic DNA, 10 µM of each primer and 1X PCR Master Mix (Fermentas). A set of eight primers were designed to amplify each specific exon (Table 1). Amplifying conditions were: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 62-68°C for 30 sec, and 72°C for 1 min, a final extension of 72°C for 5 min. PCR products were checked by electrophoresis on 2% agarose gel stained with ethidium bromide. Amplified fragments

were purified using 10 U of Exonuclease I and 1 U of Shirmp Alkaline Phosphatase. DNA sequencing reaction was performed using BigDye Terminator v3.1 Cycle Sequencing Kit in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Sequences were analyzed with SeqScape v2.5 software and aligned with Clustal W software (Thompson *et al.*, 1994). Hardy-Weinberg equilibrium was calculated.

Table 1. Primer sequences, amplified fragments, and annealing temperature for SBD1 and SBD2.

Gene region		Primer sequence 5'-3'	Amplified fragment	Annealing temperature
SBD1	Exon 1	Fw-CAGCCTCTTCTCCAGCATCA Rev-GAATTTTGCAGGACGGTTCT	282 bp	66°C
	Exon 2	Fw-ATTGTCATGAAGCCGTGTCCG Rev-ATTCACCTGGGATCAGACACCACA	423 bp	68°C
SBD2	Exon 1	Fw-CAGCCTCTTCTCCAGCATCA Rev-AAATTTTGCAGGACAGTTCT	279 bp	62°C
	Exon 2	Fw-GTTGTCATGAAGCCGTGTCCA Rev-ACCTCAATGACCAGTGGGCAAGAT	392 bp	66°C

Results and conclusions – In total, four fragments were analyzed and sequenced. The obtained sequences were aligned with SBD1 and SBD2 available in the GenBank database (Acc. no. U75250 and U75251, respectively). Overall, six SNPs were identified: two SNPs in the SBD1 gene and four in the SBD2 gene. Table 2 shows SNP positions and genotypic frequencies in the analyzed samples. No SNPs were found in the exon 1 of SBD1 gene. However, two SNPs were found in the exon 2: A→G at position 1747 in the coding region, T→C at position 1757 in the 3'-UTR. It is likely that these mutations are tightly linked. Our results, indeed, showed that individuals that present the transition A→G at position 1747 present the transition 1757 T→C as well. In SBD2 gene, SNPs were found in both exons. The only nucleotide substitution found in the exon 1, a transition C→T, is located at position 89, in the coding region. This mutation was found in 8 individuals: two with T/T homozygous genotype and six with C/T heterozygous genotype. In the exon 2, three SNPs were detected. The first one, G→A at position 1659, determines an amino acid change in the protein ($Arg^{42} \rightarrow Lys^{42}$) as described by Luenser *et al.* (2005) in *Ovis ammon*. This SNP was detected in 29 individuals with A/G heterozygous genotype, and in four individuals with A/A homozygous genotype. The SNPs G→A at position 1750 and G→A

Table 2. SNP positions in SBD1 and SBD2 and genotypic frequencies.

SNP position		Genotypic frequencies		
		Wild type	Heterozygote	Mutated homozygote
SBD1	1747 A→G	AA (0.82)	GA (0.15)	GG (0.03)
	1757 T→C	TT (0.82)	TC (0.15)	CC (0.03)
SBD2	89 C→T	CC (0.87)	CT (0.10)	TT (0.03)
	1659 G→A	GG (0.45)	GA (0.48)	AA (0.07)
	1750 G→A	GG (0.47)	GA (0.53)	AA (0)
	1761 G→A	GG (0.90)	GA (0.10)	AA (0)

at position 1761 are located in the 3'-UTR and were not found in A/A homozygous condition. Polymorphisms detected in the exon 2 of SBD2 gene are probably correlated, as the substitution at position 1761 seems to exclude the presence of the other two. Although the genotypic frequencies seem to be not balanced, the analyzed population was in equilibrium according to Hardy-Weinberg rule.

In cows, the polymorphisms in β-defensin genes have been associated with milk production traits, such as milk composition and somatic cell count (SCC) (Wojdak-Maksymiec *et al.*, 2006; Bagnicka *et al*., 2007). In particular, SCC reflects the health status of the udder and is considered as an indirect indicator of mastitis. Therefore, the association of β-defensin polymorphisms with SCC suggests that these genes could be used as candidate genes for mastitis resistance. Further studies will be necessary to check if the amino acid change identified in the present work modifies the biological function of the protein. Moreover, the association of these SNPs with SCC will be investigated, in order to use this information in a breeding program for mastitis resistance in Valle del Belice sheep.

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