Small Ruminant Research 136 (2016) 18–21

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Short communication

Association study between β-defensin gene polymorphisms and mastitis resistance in Valle del Belice dairy sheep breed

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ARTICLE INFO

Article history:
Received 28 September 2015
Received in revised form
23 December 2015
Accepted 29 December 2015
Available online 4 January 2016

Keywords:
β-Defensin genes
Mastitis resistance
Single nucleotide polymorphisms
Valle del Belice dairy sheep breed

ABSTRACT

Mastitis is generally caused by bacteria, and it is the most common disease in livestock species. Defensins are peptides with a broad spectrum of antimicrobial activity and β-defensin genes have been studied in several livestock species due to their important role in the innate immune response. The aim of this study was to establish an association between polymorphisms in the β-defensin 1 and 2 genes and mastitis resistance in the Valle del Belice dairy sheep. Data consisted of 1855 and 2804 observations for case and control group, respectively. Six single nucleotide polymorphisms and seven haplotypes were selected for association studies with mastitis. In particular, polymorphism G1747A on β-defensin 1 gene was associated with susceptibility to mastitis, while polymorphism G1659A on β-defensin 2 gene was associated with resistance to mastitis. Haplotypes ACAGGG and GCAGGG were associated with resistance to mastitis, whereas haplotype ACGGGG was associated with susceptibility to mastitis. The present study has firstly suggested the possible associations of β-defensin gene polymorphisms with mastitis resistance traits and showed the presence of interesting haplotypes in Valle del Belice dairy sheep breed. Results from association analysis provided preliminary evidence that β-defensins could be used as candidate genes or molecular markers for the improvement of ovine mastitis resistance traits in Valle del Belice dairy breed.

1. Introduction

Mastitis is the most common and costly infectious disease of mammary gland affecting dairy animals caused by bacteria. Mastitis alters the state of well-being and health of the animals, and it leads to economic loss mainly due to reduced milk yield and quality, veterinary treatments, milk disposal due to antibiotic treatments, and early culling. Moreover, milk and dairy products from animals affected by mastitis represent a significant risk for the consumers not only for the presence of the pathogens, in particular for dairy products made with raw milk, but also for the presence of bacterial toxins and the antibiotic residues (Mitchell et al., 1998). The contagious infection causes an increase in total somatic cell count (SCC) as a consequence of both leukocyte and epithelial cell numbers increasing, with or without clinical signs of mastitis. Mastitis remains a major challenge to the worldwide dairy industry despite the widespread implementation of mastitis control strategies (Bradley, 2002). Selection for genetic resistance to mastitis can be done directly or indirectly. Direct selection relates to the diagnosis of the infection, whereas indirect methods have been widely applied based on the evaluation of the degree of inflammation or of internal mammary lesions (Riggio and Portolano, 2015).

Antimicrobial peptides are important and effective components of innate immunity and are being evaluated as possible alternatives to conventional antibiotics due to the fact that bacteria have not developed resistance against antimicrobial peptides because they target components that are within to bacterial structures (Lai and Gallo, 2009). These endogenous host-defense molecules are encoded by distinct genes and translated from mRNA templates (Ramanathan et al., 2002). Based on their common features, two major families of antimicrobial peptides have been characterized in mammals, defensins and cathelicidins, which are the part of the antimicrobial arsenal of the leucocytes (Kościuczuk et al., 2014). Defensins are expressed in a variety of epithelial tissues, which serve as primary microbial interface sites (Kaiser and Diamond, 2000). They are cationic peptides, 18–45 amino acids residues in length, whose structure is stabilized by three intramolecular disulfide bonds formed by six strongly conserved cysteine residues

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http://dx.doi.org/10.1016/j.smallrumres.2015.12.037
0921-4488/

(Selsted et al., 1993; Nicholas and Mor, 1995). They are classified as α-, β- and θ-defensins based on structure, size, and disulfide bonds patterns (Kaiser and Diamond, 2000; Selsted and Ouellette, 2005). Defensins are peptides that exhibit a broad spectrum of antimicrobial activity, often including both gram-positive and gram-negative bacteria, fungi and enveloped viruses (Kaiser and Diamond, 2000). Moreover defensins have been suggested as effector molecules in host defense, interacting with many target cells and tissues (Yang et al., 2002). In recent years, β-defensin genes have been studied in several livestock species due to their important role in the innate immune response (Luenser et al., 2005). More than 20 β-defensins were found in cattle tissues, and several of them are expressed in the mammary gland (Bagnicka et al., 2010; Meade et al., 2014). In pigs, β-defensin 1 has been first characterized by Shi et al. (1999) and 11 more β-defensins were identified by Sang et al. (2006) in the same species. In goat, the precursors of β-defensin 1 and β-defensin 2 have been characterized by Zhao et al. (1999). In sheep, only two β-defensin genes have been described: β-defensin 1 (SBD1) and β-defensin 2 (SBD2). Both genes have been mapped on chromosome 26 and consist of two exons and one intron of approximately 1500 bp (Huttner et al., 1998). SBD1 and SBD2 expression was mainly described in the gastrointestinal tract and trachea (Huttner et al., 1998). Few studies have been conducted on β-defensin genes in sheep. However, Souza et al. (2015) performed a genotyping study on SBD2 gene in Amazon sheep. Monteleone et al. (2011), in a sequencing polymorphisms discovery study on Valle del Belice dairy sheep, reported a total of seven single nucleotide polymorphisms (SNPs), two in SBD1 and five in SBD2 genes. Only two of the five SNPs identified in SBD2 sheep gene (positions 1659 and 1667 referred to GenBank Acc. no. U75251), within coding region, were nonsynonymous mutations and leading to amino acid changes. Indeed, the SBD2 sequence carrying these two polymorphisms showed changes in mRNA secondary structure and suggested a possible role of these SNPs in modulation of the immune response (Monteleone et al., 2011). Considering that resistance to mastitis has a genetic background and genetic improvement is possible (Tolone et al., 2012; Sender et al., 2013), the identification of genetic markers that allow the inclusion of mastitis resistance in selection programs would help to reduce the economic loss linked with this intra-mammary infection disease. Genetic association studies, in which the allele or genotypic frequencies at markers are determined in infected individuals and compared with those of healthy ones (control case), may be an effective approach to identify the role of common variants with modest effects. Therefore, the aim of the present study was to investigate whether there is an association between SNPs or SNP haplotypes at SBD1 and SBD2 genes and mastitis resistance in Valle del Belice dairy sheep breed.

2. Materials and methods

2.1. Animal and sampling

The data consisted of 4659 observations from 299 Valle del Belice dairy ewes. The genotyping data of SBD1 and SBD2 genes used in this study belonged to the previous SNP discovery study of Monteleone et al. (2011). Information about sampling and genotyping protocols were reported in Monteleone et al. (2011). Milk samples were collected aseptically for each animal from the two udder halves in sterile containers for bacteriological analyses following an A4 recording procedure (ICAR, 2014). Standard procedures were used for isolation and identification of bacteriological colonies: 5% sheep blood agar plates, incubated at 37 °C, and examined after 10–24 h and 36–48 h incubation. The bacteriological colonies observed were mainly: *Staphylococcus aureus*, *coagulase negative staphylococci*, *Staphylococcus intermedius* and other *staphylococci*; *Streptococcus canis*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Streptococcus agalactiae* and other *streptococci*; *Corynebacterium spp.*, *Pasteurella spp.*, and *Pseudomonas spp.*

Milk samples were considered infected if more than five colony forming units (CFU) per 10 μl of milk of one species of bacteria were isolated and they were included in case group (1855 observations) otherwise they were considered healthy and included in control group (2804 observations).

Table 1
Positions and regions of single nucleotide polymorphisms (SNPs) identified in SBD1 and SBD2 genes in Valle del Belice dairy sheep breed.

Gene	SNP position	Region
SBD1	1747 A → G	3′-UTR
	1757 T → C	3′-UTR
SBD2	89 C → T	Coding
	1659 G → A	Coding
	1667 G → A	Coding
	1750 G → A	3′-UTR
	1761 G → A	3′-UTR

2.2. Statistical analyses

The online software platform SHEsis (www.analysis.bio-x.cn/myAnalysis.php) was used to calculate allelic and genotypic frequencies and to infer haplotypes for case and control groups separately, and to conduct genetic association study. Only haplotypes with a frequency greater or equal to 0.03 were considered for the analysis. Haplotypes were inferred using the Partition–Ligation Combination-Subdivision EM as implemented in the SHEsis software (Shi and He, 2005; Li et al., 2009). The use of haplotypes allows multiple potentially causal variants to be tested simultaneously for association; moreover, haplotypes can be tested for association because they may be a proxy for untyped causal markers.

Association analysis was performed using the Case Control procedure in SAS 9.2, with the overall association with genotype based on the Armitage trend test and odd ratios (OR) based on allele counts, reflecting additive effects. Allelic association tests assume that the two alleles per marker within each individual are independent i.e., that they are in Hardy–Weinberg Equilibrium (HWE). Armitage's trend test and other tests that assume additivity of allele effects are alternatives that do not impose this assumption (Sasieni, 1997) and are, therefore, preferred. Under HWE, the allele-based test and the trend test are asymptotically equivalent. Association test is a χ^2 test of independence computed on a cross-classification table of outcome versus alleles or genotypes.

3. Results

In Table 1, SNP positions and regions of SBD1 and SBD2 sheep genes were reported by Monteleone et al., 2011. As reported by these authors, the functional analysis of the novel identified missense mutations in SBD2 gene was obtained using SIFT program (Ng and Henikoff, 2003) while the evolutionary analysis of coding SNPs was obtained with PANTHER tool (Thomas et al., 2003). According to software results, we concluded that these mutations may not have effects on protein function. SNPs in the 3′-UTRs (i.e., at position 1747 and 1757 in SBD1, and at position 1750 and 1761 in SBD2) were analyzed with RNAstructure software (Mathews et al., 2004) to check if they could influence mRNA structure. According to software predictions, SBD1 SNPs did not affect the mRNA shape whereas both SBD2 SNPs (at position 1750 and 1761) changed the mRNA secondary structure when they are present alone. When these mutations are both present, mRNA folds as when only the SNP at position 1750 is present demonstrating that it has a greater effect on RNA folding. Therefore, the structural difference in SBD2 messenger RNA may be related to a possible role in translation efficiency with a modulation in the protein produced amount.

After editing, the data consisted of 1855 and 2804 observations for case and control groups, respectively. Considering the linkage disequilibrium between the two SNPs on SBD1 gene, only the ones in position 1747 was considered for the analysis. Allelic and genotypic frequencies for case and control groups, for all SNPs identified in the SBD1 and SBD2 genes are reported in Table 2. Minor allele frequencies for all SNPs in the two genes ranged from 0.014 to 0.315 in case and from 0.012 to 0.343 in control group (Table 2). Haplotypes and their frequencies for case and control group are shown in Table 3. In both case and control groups, the results showed that ACGGGG haplotype has the highest frequencies (0.484) whereas the lowest one was found for GCAGGG (0.020) (Table 3).