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Association of *CXCR1* polymorphisms with apoptosis, necrosis and concentration of milk neutrophils in early lactating dairy heifers

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ABSTRACT

Associations between polymorphisms in the candidate gene *CXCR1*, encoding the chemokine (C-X-C motif) receptor 1, and udder health have been identified before. In the present study, associations between the *CXCR1* genotype (whole coding region) and apoptosis, necrosis, and concentration of milk polymorphonuclear neutrophilic leukocyte (PMNL) of 292 quarters belonging to 73 early lactating dairy heifers were studied. In uninfected quarters, % milk PMNL apoptosis was higher in c.980GG heifers [least squares means (LSM) 27%] compared to c.980AG heifers (LSM 16%), whereas in infected quarters, % milk PMNL apoptosis was higher in c.642GG heifers (LSM 29%) compared to c.642AG heifers (LSM 18%). Differences in milk PMNL concentration between infected and uninfected quarters were smaller in c.980AG heifers than in c.980GG heifers. An association between the *CXCR1* genotype and necrosis of milk PMNL could not be demonstrated. Results indicate that *CXCR1* polymorphisms influence viability and concentration of milk PMNL and provide a foundation for future research.

1. Introduction

Innate immune responses are the predominant form of defense in the early stage of intramammary infection (IMI) (Sordillo et al., 1997), with migration of polymorphonuclear neutrophilic leukocytes (PMNL) from the blood to the site of infection being a key component (Paape et al., 2003). Both number and viability of PMNL that pathogens encounter are of importance (Mehrzad et al., 2004). Apoptosis and necrosis impair the phagocytic and respiratory burst capacity of PMNL (Mehrzad et al., 2004; Van Oostveldt et al., 2002). Percentage milk PMNL apoptosis and milk PMNL concentration differ among heifers (Piepers et al., 2009b) and are potentially genetically determined. In this context, genes involved in the innate immunity of the mammary gland form an interesting area of study. Recently, we reported 16 polymorphisms in the coding region of *CXCR1* and revealed an interesting association between polymorphism c.980A>G (dbSNP ID: rs43323012) and the odds of IMI in early lactating heifers: heifers with genotype c.980AG were less likely to have IMI due to major mastitis pathogens compared to heifers with genotype c.980GG but were not likely to have less IMI by coagulase-negative staphylococci (CNS), still considered as minor pathogens (Verbeke et al., 2012). The *CXCR1* gene actually encodes one of the two receptors present on PMNL for interleukin 8 (IL-8), named chemokine (C-X-C motif) receptor 1 (CXCR1). Interleukin 8 is known as the main chemoattractant for PMNL after intramammary invasion of a pathogen (Barber and Yang, 1998) and has also been shown to increase activity (Mitchell et al., 2003) and inhibits apoptosis of PMNL (Kettritz et al., 1998). There is strong *in vitro* evidence that polymorphism *CXCR1* c.735C>G (dbSNP ID: rs208795699) affects the survival and migration of bovine blood PMNL in response to IL-8 (reviewed in Pighetti et al., 2012). Polymorphisms in *CXCR1* potentially alter the functionality of the receptor and might hence be associated with PMNL viability and concentration in milk, and could explain our previous findings on susceptibility to IMI to some extent.

We aimed at analyzing whether or not variation in milk PMNL apoptosis, milk PMNL necrosis, and milk PMNL concentration in (quarters of) early lactating dairy heifers can be due to *CXCR1* polymorphisms.

2. Materials and methods

2.1. Phenotypic data

Phenotypic records from 73 heifers from 19 commercial Flemish dairy herds (Piepers et al., 2009b) were available. On average, four heifers per farm were included, ranging between 1 and 7. Forty-six different sires and 72 different dams were noted, indicating that the heifers were not closely related to each other. Apoptosis, necrosis, and concentration of milk PMNL were measured on quarter milk samples collected in early lactation (between 1 and 4 DIM).

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To determine the IMI status, additional quarter milk samples (5 ml) were aseptically collected between 1 and 4 DIM and between 5 and 8 DIM for bacteriological culturing. The interval between both samplings was at least 3 days. Samples were taken just before morning milking (Piepers et al., 2011; Verbeke et al., 2012).

The % milk PMNL apoptosis and necrosis were measured flow cytometrically using a double fluorescein isothiocyanate (FITC)-annexin-V and propidium iodide (PI) staining. Milk PMNL and other milk cells were differentiated by a two-step fluorescent immunolabeling as described before (Piepers et al., 2009a, 2009b). Approximate concentration of PMNL in milk, further referred as milk PMNL concentration, was quantified based on the absolute number of milk PMNL events as determined by flow cytometry, the sample acquisition time (t_{acq}, s), and the flow rate (FR, μL/s) (Piepers et al., 2009b). The IMI status of each quarter was determined using bacteriological culture of two consecutive milk samples as described before (Verbeke et al., 2012).

2.2. Genotypic data

Genotyping of the whole single-exon coding region (1083 bp) of bovine CXCR1 was performed by sequencing as described by Verbeke et al. (2012). In brief, DNA was extracted from blood samples (100 μl) using a proteinase K digestion method (Van Poucke et al., 2005) and its concentration was estimated with a ND spectrophotometer (NanoDrop). A PCR mix containing approximately 100 ng genomic DNA, 1.0 μl 10× FastStart Taq DNA Polymerase Buffer (Roche Applied Science), dNTP Mix (0.2 mM each; BIOLINE), 0.25 μM forward primer (Integrated DNA Technologies, 5′-TCCTTGATGAGAGT GATTTGGA-3′), 0.25 μM reverse primer (Integrated DNA Technologies, 5′-TTGACATGGGACTGTGAACG-3′) and 0.5 U Taq DNA Polymerase (Roche Applied Science) was made.

The PCR program consisted of an initiation step of 5 min at 95 °C followed by 30 amplification cycles (denaturation for 30 s at 95 °C, annealing for 30 s at 62 °C and extension for 75 s at 72 °C) and a final 4 min elongation step at 72 °C. All amplicons were sequenced by direct sequencing with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a 16-capillary 3130xl DNA Analyzer (Applied Biosystems) according to the manufacturer's protocol. Sequencing reactions per sample were performed in duplicate using the forward and reverse primers. Deviations from Hardy–Weinberg equilibrium of each studied polymorphism were analyzed using chi-square goodness of fit tests.

2.3. Statistical analysis

Associations between phenotypic traits and genotype were analyzed for polymorphisms c.642G>A, c.735C>G, c.816C>A and c.980A>G (dbSNP ID: rs109587131, rs208795699, rs210501501, and rs43323012) only. All other polymorphisms were in full linkage disequilibrium (correlation 100%) with one of these polymorphisms, and results would be the same for that reason. In all models, statistical significance was assessed at $P \leq 0.05$ while a P-value between 0.05 and 0.10 was considered as a tendency toward significance. Transformation were performed to obtain normalized distributions. Percentage milk PMNL apoptosis and necrosis were square-root transformed whereas milk PMNL concentration was log transformed.

The association between the different polymorphisms (predictor variables of main interest) and the % milk PMNL apoptosis and necrosis, and milk PMNL concentrations (outcome variables) were determined using similar linear mixed regression models. Herd and heifer were included as random effects to correct for clustering of heifers within herds and quarters within heifers, respectively (RANDOM statement) (PROC MIXED, SAS 9.2, SAS Institute Inc., NC, USA). The different models included IMI status (0 = uninfected quarter versus 1 = infected quarter, all pathogens combined) and polymorphism c.642G>A, c.735C>G, c.816C>A or c.980A>G, respectively, as categorical fixed effects. The interaction between both variables was also tested. If the interaction term was significant, post-hoc pairwise comparisons were performed to test differences between quarters with different IMI statuses or genotypes. Post-hoc comparisons were adjusted according to Bonferroni.

3. Results

3.1. Descriptive statistics

Participating herds consisted on average of 48 lactating cows with an average 365-d milk production of 8368 kg of milk. The average bulk milk SCC was 278,736 cells/ml between 2006 and 2007 (Piepers et al., 2009b).

The IMI status in early lactation could be determined for 245 out of 292 quarters (84%). Two quarters showed signs of clinical mastitis between 1 and 4 DIM. One-hundred-and-two quarters (42%) were uninfected. The large majority (91%) of the IMI were caused by CNS ($n = 130$). Of the remaining 13 infected quarters, *Corynebacterium bovis* ($n = 2$), *Staphylococcus aureus* ($n = 5$), *Streptococcus dysgalactiae* ($n = 1$) and esculine-positive cocci ($n = 5$), respectively, were identified as the cause. The average milk PMNL necrosis was 29% (IQR 14–46%) for uninfected quarters, and 28% (IQR 17–43%) for infected quarters. The average milk PMNL apoptosis was 23% (IQR 15–34%) for uninfected quarters, and 22% (IQR 15–34%) for infected quarters. The average milk PMNL concentration was higher in infected quarters (log10 = 3.7; IQR 2.9–4.3) compared to uninfected quarters (log10 = 3.3; IQR 2.9–3.8).

3.2. CXCR1 genotypes

All 16 polymorphisms (c.37A>T, c.38T>A, c.68G>A, c.291C>T, c.333T>C, c.337G>A, c.365T>C, c.570G>A, c.642G>A, c.735C>G, c.816C>A, c.819G>A, c.980A>G, c.995A>G, c.1008C>T, and c.1068G>A) described by Verbeke et al. (2012) were observed in this subset of heifers. The studied population was in Hardy–Weinberg equilibrium ($P > 0.05$) for all polymorphisms. Four (H1–H4, [GenBank: HM13954-7]) of the five haplotypes reported and labeled (H1–H5) by Verbeke et al. (2012) were present (Table 1).

Table 1
CXCR1 haplotype sequences detected in 73 Belgian dairy heifers.

Label	37_38[a]	68	291	333	337	365	570	642	735	816	819	980	995	1008	1068	Freq.	Accession[b]
H1	AT	G	C	T	G	T	G	G	C	C	G	A	A	C	G	0.16	HM013954
H2	AT	G	T	C	A	T	G	G	C	A	G	G	G	T	G	0.29	HM013955
H3	TA	A	C	C	A	C	A	A	G	C	A	G	G	C	A	0.36	HM013956
H4	TA	A	C	C	A	C	A	G	G	C	A	G	G	C	A	0.19	HM013957

[a] Numbers indicate positions of the polymorphisms relative to the start codon.
[b] GenBank accession number.