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Associations between *CXCR1* polymorphisms and pathogen-specific incidence rate of clinical mastitis, test-day somatic cell count, and test-day milk yield

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ABSTRACT

The *CXCR1* gene plays an important role in the innate immunity of the bovine mammary gland. Associations between single nucleotide polymorphisms (SNP) *CXCR1*c.735C>G and c.980A>G and udder health have been identified before in small populations. A fluorescent multiprobe PCR assay was designed specifically and validated to genotype both SNP simultaneously in a reliable and cost-effective manner. In total, 3,106 cows from 50 commercial Flemish dairy herds were genotyped using this assay. Associations between genotype and detailed phenotypic data, including pathogen-specific incidence rate of clinical mastitis (IRCM), test-day somatic cell count, and test-day milk yield (MY) were analyzed. *Staphylococcus aureus* IRCM tended to associate with SNP c.735C>G. Cows with genotype c.735GG had lower *Staph. aureus* IRCM compared with cows with genotype c.735CC (rate ratio = 0.35, 95% confidence interval = 0.14–0.90). Additionally, a parity-specific association between *Staph. aureus* IRCM and SNP c.980A>G was detected. Heifers with genotype c.980GG had a lower *Staph. aureus* IRCM compared with heifers with genotype c.980AG (rate ratio = 0.15, 95% confidence interval = 0.04–0.56). Differences were less pronounced in multiparous cows. Associations between *CXCR1* genotype and somatic cell count were not detected. However, MY was associated with SNP c.735C>G. Cows with genotype c.735GG out-produced cows with genotype c.735CC by 0.8 kg of milk/d. Results provide a basis for further research on the relation between *CXCR1* polymorphism and pathogen-specific mastitis resistance and MY.

Key words: *CXCR1* single nucleotide polymorphism, dairy cow, udder health, milk yield

INTRODUCTION

Mastitis, an inflammation of the mammary gland mostly caused by bacterial IMI, impairs milk quality and production and increases the culling probability of dairy cows substantially (Beaudeau et al., 1995; Hortet et al., 1999; Ma et al., 2000; Santos et al., 2003; Seegers et al., 2003). The disease can be accompanied by local or systemic symptoms [clinical mastitis (**CM**)] or present itself without visible symptoms [subclinical mastitis (**SM**)]. In absence of clinical symptoms, mastitis can be detected by an increase in the concentration of somatic cells in milk (Schukken et al., 2003).

Resistance to mastitis is genetically determined (Detilleux, 2009), making a decrease in the incidence of mastitis by breeding a possibility (Windig et al., 2010). Identification of genetic polymorphisms linked with mastitis resistance would allow evaluation of the genetic make-up of male and female breeding animals for udder health, even before their offspring is born (Ogorevc et al., 2009). Polymorphisms in genes involved in the recognition of pathogens, recruitment of immune cells toward site of infection, elimination of the pathogens, and resolution of the inflammatory response are potential genetic markers for mastitis resistance (Pighetti and Elliott, 2011). In a recent genome-wide association study, polymorphisms located near *CXCR1* and *CXCR2* were highly associated with occurrence of CM (Sodeland et al., 2011). The latter genes encode the 2 receptors present on PMNL for IL-8 (CXCL-8), named chemokine (C-X-C motif) receptor 1 and 2 (CXCR1 and CXCR2). Binding of IL-8 causes chemotaxis, increases activity, and inhibits apoptosis of PMNL (Barber and Yang, 1998; Kettritz et al., 1998; Mitchell et al., 2003).

Polymorphisms in the coding region of *CXCR1* have been described (Pighetti et al., 2012; Verbeke et al., 2012). Eight of the detected SNP were nonsynonymous. The most frequently studied SNP, located at position 735 relative to the start codon [c.735C>G (rs208795699)], causes an AA change (p.His245Glu) in the third intracellular loop of the receptor and was in full linkage disequilibrium with nonsynonymous SNP

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c.37A>T (rs380621468), c.38T>A (rs110296731), and c.68G>A (rs133273369), causing changes in the N terminus (p.Ile13Tyr and p.Gly23Glu). Single nucleotide polymorphisms c.980A>G (rs43323012) and c.995A>G (rs43323013) cause changes in the C-terminus (p.Lys327Arg and p.His332Arg) and were in full linkage disequilibrium with SNP c.337G>A (rs207564206), causing a change in the first extracellular loop (p.Val113Ile). The SNP c.365T>C (rs211042414) encodes for p.Val122Ala in the third transmembrane domain (Verbeke et al., 2012). Amino acid changes potentially influence receptor activity through ligand binding, G-protein-related signaling, or receptor internalization (reviewed in Pighetti et al., 2012). Functional effects have yet to be studied. Additionally, (linked) polymorphisms could affect gene expression (Leyva-Baca et al., 2008a). Because of the results of the genome-wide association study and the important innate immune function of *CXCR1*, it is reasonable to believe that *CXCR1* polymorphisms alter mastitis resistance.

Youngerman et al. (2004) detected associations between SNP c.735C>G and the incidence of SM, SCS, and milk yield (**MY**). Holstein c.735CC cows showed a higher incidence of SM and higher MY compared with c.735GG cows. Additionally, c.735GG cows had lower SCS compared with c.735CG cows. The association with SCS was confirmed later in an Irish cow population (Beecher et al., 2010). Galvão et al. (2011) detected lower MY and higher incidence rate of clinical mastitis (**IRCM**) in c.735GG cows. Because innate immune responses heavily depend on the invading mastitis pathogen (Bannerman et al., 2004; Schukken et al., 2011), associations between polymorphisms in innate immune genes and udder health are ideally studied using pathogen-specific data. Recently, we revealed a pathogen group-specific association between SNP c.980A>G and the odds of IMI in early lactating heifers: c.980AG heifers were less likely to have IMI due to major mastitis pathogens (e.g., *Staphylococcus aureus* and *Streptococcus uberis*) compared with c.980GG heifers, but were not less likely to have IMI by CNS still considered as minor pathogens (Verbeke et al., 2012). In our study, associations between SNP c.735C>G and c.980A>G and pathogen-specific IRCM, test-day SCC, and test-day MY were studied using a fluorescent multiprobe PCR assay that was specifically designed and validated to genotype a large number of cows in a reliable, fast, and cost-effective manner.

MATERIALS AND METHODS

Herds and Animals

Sixty-seven Flemish dairy producers were invited to participate in a 1-yr cohort study to estimate the pathogen-specific IRCM in Flanders (Verbeke et al., 2014). All participating herds (n = 53) were visited at the beginning of the study in September or October 2012 by the first author. At that time, the study details were discussed with the producers and herd veterinarians, and blood samples of all lactating heifers and cows were taken. From then on, producers were asked to take a milk sample of each quarter showing signs of CM during the 12-mo study period (see further). To be able to include data of early lactating heifers, participating herds were revisited between February and March 2013 also by the first author. At that time, heifers that had calved between both visits were blood sampled. Data of 3 herds were omitted from the analysis along the study period because producers stopped sampling cases halfway through the study.

In total, 3,106 animals from 50 herds were blood sampled ranging between 18 and 250 per herd. Cow-level records [birth date, calving date(s), parity, culling date, and sire and dam (both when available)] were retrieved from DHI records for herds participating in the DHI program organized by the Cattle Breeding Organization (Oosterzele, Belgium; n = 32) or from the identification and registration system of the Animal Health Service Flanders (DGZ Vlaanderen, Drongen, Belgium) for other herds (n = 18).

Phenotypic Records

CM (Full Data Set, 50 Herds, 3,106 Cows). Producers were asked to take a milk sample from each quarter showing signs of CM during the 12-mo survey period. Signs of CM were defined as visible abnormalities of the udder or milk indicating udder inflammation. Sampling date and cow identification were recorded. Samples were frozen on farm and collected by the herd veterinarian. A courier of the Animal Health Service Flanders transported the samples from the veterinary practice to the Milk Control Center Flanders (Lier, Belgium) where bacteriological culture was performed according to National Mastitis Council standards (National Mastitis Council, 1999). From each thawed sample, 10 μL of milk was spread on blood-esculin and MacConkey's agar and incubated aerobically for 24 to 48 h at 37°C. Identification of bacteria was done by Gram staining, inspection of the colony morphology and biochemical testing. Catalase tests were performed to differentiate gram-positive cocci in catalase-positive or catalase-negative cocci. Staphylococci were identified as *Staph. aureus* or non-*aureus* staphylococci by colony morphology, hemolysis patterns, and DNase tests. Streptococci were differentiated in esculin-positive and esculin-negative streptococci (*Streptococcus agalactiae* and *Streptococcus dysgalactiae*). *Streptococcus uberis*

was distinguished from other esculin-positive cocci by incubation in NaCl 6.5% medium and bile esculin agar. Christie, Atkins, Munch-Petersen tests were used to differentiate *Strep. agalactiae* and *Strep. dysgalactiae*. Gram-negative bacteria were identified by colony morphology, lactose fermentation on MacConkey agar, incubation in sulfide-indole-motility medium, and oxidase, triple sugar iron, citrate, and urease testing. The API 20 E system (bioMérieux, Craponne, France) was used if the previously mentioned tests failed to identify the gram-negative bacterium. Samples yielding 2 different bacterial species were grouped as a mixed culture, whereas samples yielding 3 or more different bacterial species were considered to be contaminated.

The lactation-level IRCM was calculated by dividing the number of quarter cases by the days at risk during the lactation within the study and expressed as cases per 10,000 cow-days at risk. Samples taken from the same cow within 2 wk from a previous case were not considered new cases and therefore excluded from the analysis (Barkema et al., 1998). The number of days at risk for each lactation was equal to the time between the start of the study or calving and the end of the study, culling date, or the next calving minus 14 d per case. Overall IRCM (all cases), as well as pathogen-specific IRCM (*Staph. aureus*, *Strep. uberis*, *Strep. dysgalactiae*, and *Escherichia coli*, specifically) were calculated (Verbeke et al., 2014).

Test-Day SCC and MY (Subset of Data, 32 Herds, 1,961 Cows). Composite milk SCC (cells per milliliter) and MY (kilograms of milk) at test-day per cow were available from DHI records of all herds participating in the DHI program (n = 32 herds). Records between 14 and 285 DIM from cows with at least 2 measurements per parity were saved in a data set.

CXCR1 Genotyping

Genotype at SNP c.735C>G and c.980A>G was determined using a multiprobe PCR assay similar to the assay described by Van Poucke and colleagues (2005). The DNA was extracted from blood samples (100 μL) using a proteinase K digestion method (Van Poucke et al., 2005). Three PCR primers and 4 dual-labeled allele-specific oligonucleotide probes were designed and synthesized by Sigma-Aldrich (St. Louis, MO). Sequences, melting temperature (calculated using OligoAnalyzer 3.1; Integrated DNA Technologies, Coralville, IA), and binding positions are presented in Table 1. Regions forming potential secondary structures or including repeat sequences were identified with Mfold (Zuker, 2003) and RepeatMasker (Smit et al., 2010), respectively, and avoided. The forward primer was designed to bind *CXCR1* specifically. Because of SNP *6C>A (db SNP ss 974294528), *7A>C (db SNP ss 974294529), and *10C>T (db SNP ss 974294530), 2 reverse primers were used. Homology between *CXCR1* and *CXCR2*, *CXCR1* SNP and binding positions of primers and probes are visualized in Figure 1. To amplify a region including both SNP specifically, a relatively large amplicon was multiplied (523 bp).

The PCR was performed in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with a 10-μL PCR mix containing approximately 100 ng of genomic DNA, 1.0 μL of 10 × FastStart Taq DNA Polymerase Buffer (Roche Applied Science, Penzberg, Germany), dNTP Mix (0.2 m*M* each; 5 prime), 0.5 μ*M* forward primer (Integrated DNA Technologies), 0.5 μ*M* reverse primer 1 (Integrated DNA Technologies), 0.5 μ*M* reverse primer 2 (Integrated DNA Technologies), 0.6 μ*M* C735-probe, 0.3 μ*M* G735-probe, 0.4 μ*M* A980-probe, 0.2 μ*M* G980-probe, and 0.5 U Taq DNA Polymerase (Roche Applied Science). The real-time PCR program consisted of an initiation step of 3.5 min at 95°C, followed by 40 amplification cycles (denaturation for 30 s at 95°C, annealing-elongation for 40 s at 60°C, and detection of fluorescent signals generated by cleavage of the dual-labeled probes).

Quantification cycles (Cq values) for each probe were analyzed using CFX Manager Software v3.1 (Bio-

Table 1. Primers and probes used in the real-time PCR assay for genotyping SNP *CXCR1*c.735C>G and *CXCR1*c.980A>G

Primer or probe name	Sequence (5′ → 3′)[1]	Length (bp)	Tm[2,3] (°C)	avTm[2,4] (°C)	Binding position
Forward primer	TCCGACCTAGTCTGCTA	17	59.6	N/A	c.574→c.590
Reverse primer 1	CCGGTGTGGAGTCTC	15	59.2	N/A	c.1082←*13
Reverse primer 2	CGATGGTGAGTCTCAGA	17	58.7	58.7	c.1079←*12
C735-probe	*HEX*-CGGTGCTTGTGCCC-*BHQ1*	14	61.6	55.3	c.730←c.743
G735-probe	*FAM*-CGGTGCTTCTGCCCC-*BHQ1*	15	64.0	54.2	c.729←c.743
A980-probe	*Cy5*-CCATGATCTTGAGGAGTCC-*BHQ2*	19	60.3	56.5	c.970←c.988
G980-probe	*TexasRed*-CCATGATCCTGAGGAGTCC-*BHQ2*	19	62.2	55.0	c.970←c.988

[1]Fluorescent labels and quenchers are in italic; SNP are underlined.

[2]Melting temperatures were calculated using OligoAnalyzer 3.1, Integrated DNA Technologies (Coralville, IA).

[3]Tm = melting temperature when annealed to *CXCR1* for primers and to target variant for probes.

[4]avTm = melting temperature when annealed to *CXCR2* for primers and to allelic variant for probes.

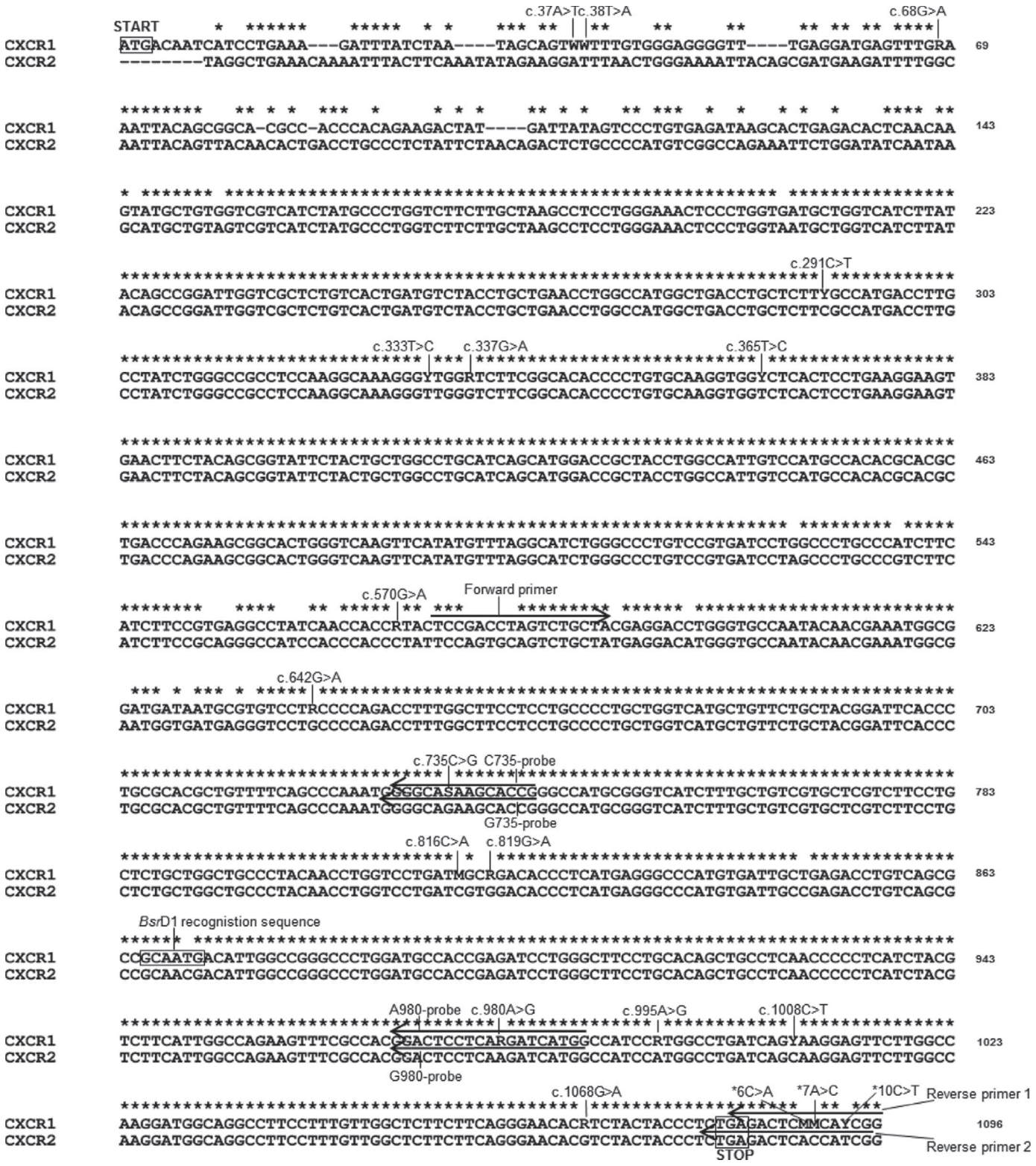

Figure 1. The coding sequence and 13 bp downstream from the stop codon of bovine *CXCR1*, as detected by DNA sequencing (Verbeke et al., 2012), was aligned with bovine *CXCR2* (AC_000159 region: 106913181… 106914285) using ClustalX2 (Larkin et al., 2007). The start codon, SNP in *CXCR1*, and stop codon are indicated. Binding positions of primers and probes used for *CXCR1* genotyping (see Table 2) are marked with arrows.

Rad). In each run, positive controls with genotype c.735CC980AA, c.735CG980AG, and c.735GG980GG and a no-template control were included to assign baseline cycles and threshold position, and check for PCR contamination. Potential unspecific amplification of *CXCR2* was analyzed by digesting a PCR amplicon with restriction enzyme *Bsr*D1 (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. In silico analysis showed that *Bsr*D1 cleaves the predicted *CXCR1* region, but not the homologous *CXCR2* region (NM_001101285.1; Figure 1). The sample was run on ethidium bromide-stained agarose (2%) gel (135 V, 40 min). None of the original fragments was visible, indicating that no *CXCR2* was multiplied. To validate the genotyping method, 26 DNA samples genotyped by direct sequencing (Verbeke et al., 2012) were tested in a blind manner. At least 3 samples of genotype c.735CC980AA, c.735CC980AG, c.735CC980GG, c.735CG980AG, c.735CG980GG, and c.735GG980GG were included. Results were concordant for all 26 samples. Real-time amplification plots for all detected genotypes are presented in Figure 2.

Statistical Analysis

Deviations from Hardy-Weinberg equilibrium were analyzed using chi-squared goodness of fit tests. Chi-squared values were calculated to analyze linkage disequilibrium between loci. The relative value of disequilibrium, d, was calculated as introduced by Kaplan and Weir (1992).

In all models, statistical significance was assessed at $P \leq 0.05$, whereas a P-value between 0.05 and 0.10 was considered as a tendency toward significance. Nonsignificant fixed effects, except genotype as a variable of main interest, were removed from the models. Genotypes c.735CC and c.980AA encoding the conserved AA p.245Glu and p.327Lys (Pighetti et al., 2012) were set as reference groups in the models with c.735C>G and c.980A>G, respectively. Other genotypes were compared with the reference (2 independent post-hoc tests).

Clinical Mastitis. Associations between SNP c.735C>G and c.980A>G and 5 different outcome variables—(1) overall IRCM, (2) *Staph. aureus* IRCM, (3) *Strep. uberis* IRCM, (4) *Strep. dysgalactiae* IRCM, and (5) *E. coli* IRCM—were determined in several models using multilevel Poisson regression (MlwiN 2.16, Centre for Multilevel Modeling, Bristol, UK). Herd and cow were added as a random effect to correct for clustering of cows within herds and lactations within cows. The models included the cow genotype (c.735C>G and c.980A>G) at the position of the polymorphism, parity (1, 2, and ≥3), season of calving (January–March, April–June, July–September, and October–December), and the interaction between genotype and parity as categorical fixed effects and the natural logarithm of the number of days at risk as an offset variable (Barkema et al., 1999). To avoid nonconvergence of the statistical models, records on rare genotypes (c.980AA) were omitted in the pathogen-specific models. In latter models, c.980AG was set as reference. Results are presented as rate ratio (**RR**) with 95% CI.

Test-Day SCC and MY. Associations between SNP c.735C>G and c.980A>G (predictor variables of main interest) and test-day SCC and MY (separate outcome variables) were determined using 2 mixed regression models per outcome variable with herd, cow, and lactation as random effects to correct for clustering of cows within herds, lactations within cows, and multiple observations per lactation, respectively (MlwiN 2.16). A natural logarithmic transformation of SCC (**LnSCC**) was performed to obtain a normalized distribution. The models included DIM and the quadratic term for DIM as continuous fixed effects and the cow genotype (c.735C>G and c.980A>G) at the position of the polymorphism, parity (1, 2, and ≥3), season of calving (January–March, April–June, July–September, and October–December), season of the test day (January–March, April–June, July–September, and October–December), and the interaction between genotype and parity as categorical fixed effects.

RESULTS

Phenotypic Data

The average herd size was 60 lactating cows (range 16–240). All producers milked Holstein-Friesian cows. During the survey, participating herds had an average bulk milk SCC of 236,000 cells/mL (range 85,000–453,000 cells/mL).

In total, 685 CM cases from 534 cows were sampled by the producers. During the survey, 421, 94, 18, 3, and 2 cows suffered from 1, 2, 3, 4, and 5 cases, respectively. Seventy-nine percent (n = 538) of the samples were culture-positive, including 10% of contaminated samples (n = 69). *Streptococcus uberis* was the most frequently isolated pathogen (n = 121, 18% of all cases), followed by *E. coli* (n = 98, 14%), *Staph. aureus* (n = 55, 8%), and *Strep. dysgalactiae* (n = 51, 7%). Two different pathogens were isolated in 35 CM samples (5%). Non-*aureus* staphylococci (n = 32, 5%), *Corynebacterium bovis* (n = 17, 2%), yeast (n = 14, 2%), *Prototheca* spp. (n = 12, 2%), other esculine-positive cocci besides *Strep. uberis* (n = 9, 1%), *Klebsiella* spp. (n = 7, 1%), *Trueperella pyogenes* (n = 5, 1%), *Bacillus* spp. (n = 3, <1%), *Pasteurella* spp. (n = 3, <1%), *Streptococcus*

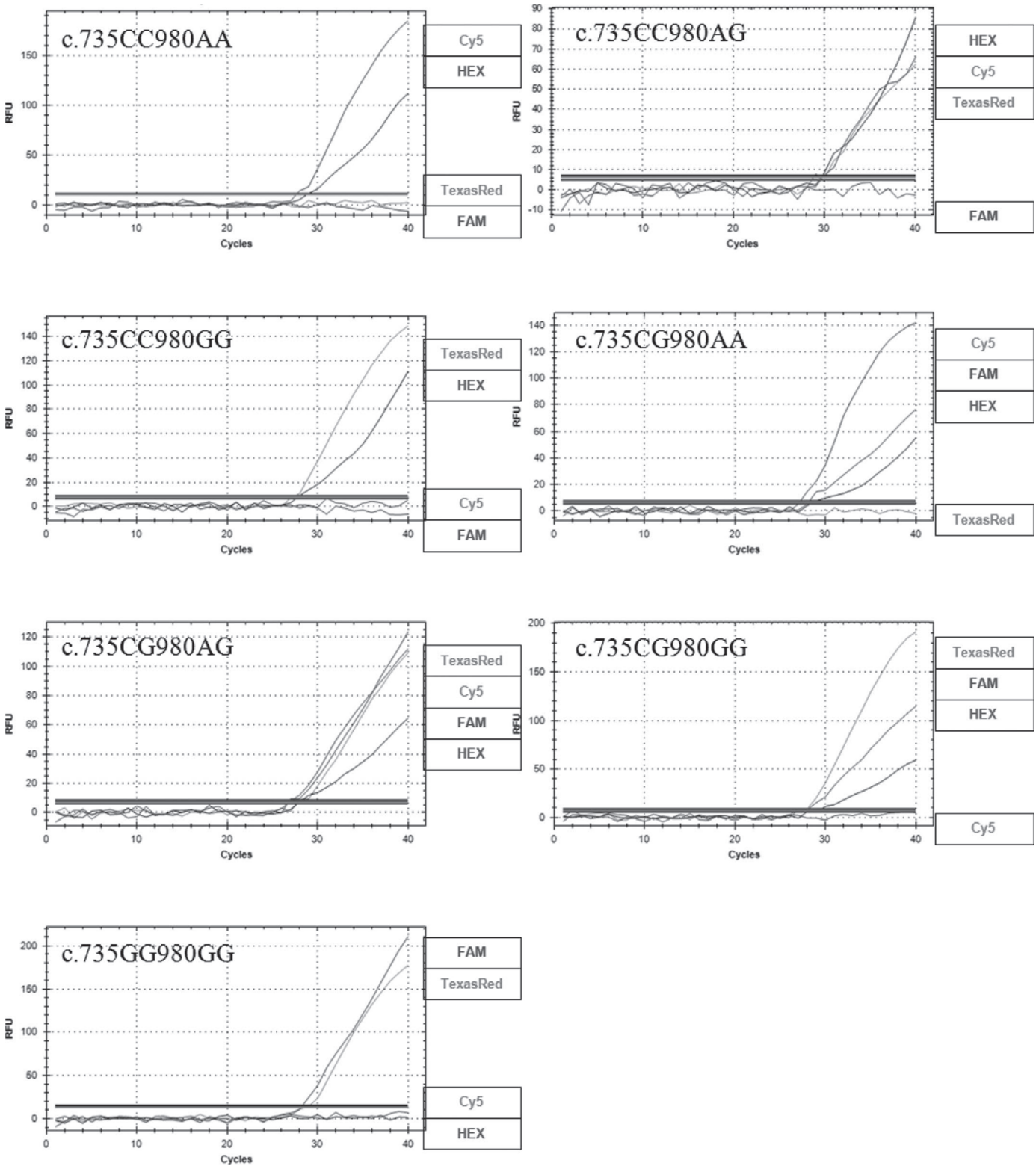

Figure 2. Amplification plots obtained with the fluorescent multiprobe PCR assay for bovine *CXCR1* genotyping of 7 cows with different genotype at SNP *CXCR1*c.735C>G or *CXCR1*c.980A>G. Alleles c.735C, c.735G, c.980A, and c.980G were detected using probes labeled with fluorescent labels HEX, FAM, Cy5, and TexasRed, respectively. Genotype c.735GG980AA and c.735GG980AG were not detected in the study population. RFU = relative fluorescence unit.

canis (n = 3, <1%), *Pseudomonas aeruginosa* (n = 3, <1%), *Strep. agalactiae* (n = 2, <1%), *Serratia* spp. (n = 2, <1%) ,and molds (n = 1, <1%) were isolated in the remaining samples. Overall, *Staph. aureus*, *Strep. uberis*, *Strep. dysgalactiae*, and *E. coli* IRCM was calculated at 7.1, 0.5, 1.3, 0.5, and 1.1 quarter cases per 10,000 cow-days at risk, respectively. In herds participating in the DHI program, genotyped cows had a geometric mean SCC of 79,000 cells/mL (range: 2,000–9,590,000) and an average MY of 27.8 kg (range: 1.9–63.5) at test day.

Genotypic Data

The sire and dam of 957 (31%) and 1,964 (63%) cows, respectively, were available from DHI records. Among these cows, 460 different sires and 1,951 different dams were noted, indicating that they were not closely related to each other.

All cows were genotyped using the multiprobe PCR assay. Frequencies of genotype c.735CC, c.735CG, and c.735GG were 25, 48, and 27%, respectively, in the total population. Frequencies of genotype c.980AA, c.980AG, and c.980GG were 5, 34, and 61%, respectively, in the total population. Frequencies of c.735C>G and c.980A>G were not significantly different in the subpopulation of cows with DHI records compared with the total population $[P\left(\chi^2_{2df} > 0.45\right) = 0.80$ and $P\left(\chi^2_{2df} > 0.95\right) = 0.62$, respectively]. Both SNP were in Hardy-Weinberg equilibrium ($P > 0.05$). In total, 7 combined genotypes were detected: c.735CC980AA (5%), c.735CC980AG (12%), c.735CC980GG (8%), c.735CG980AA (<1%), c.735CG980AG (22%), c.735CG980GG (26%), and c.735GG980GG (27%). Combined genotypes c.73GG980AA and c.735GG980AG were not observed in the study population. The SNP were in strong linkage disequilibrium ($P < 0.05$, d = 0.65).

Associations with Udder Health and MY

Descriptive statistics on pathogen-specific IRCM, LnSCC, and MY by SNP c.735C>G and c.980A>G are given in Table 2.

Incidence Rate of CM. Season of calving was not associated with (pathogen-specific) IRCM and was therefore removed from all IRCM models. Overall, *Strep. uberis*, *Strep. dysgalactiae*, and *E. coli* IRCM were significantly associated with parity ($P < 0.05$), but not with SNP c.735C>G, c.980A>G or the interaction between genotype and parity (Table 3). Results were different for *Staph. aureus* IRCM. In the model with c.735C>G, the interaction between c.735C>G and parity was not significant. Parity was significant ($P < 0.01$) and genotype tended to be significant ($P = 0.08$). Cows with genotype c.735GG had, on average, a lower *Staph. aureus* IRCM compared with cows with genotype c.735CC (RR = 0.35, 95% CI = 0.14–0.90; Table 3). In the model with c.980A>G, the interaction between genotype and parity was significant ($P < 0.05$). Heifers with genotype c.980GG had on average a lower *Staph. aureus* IRCM compared with heifers with genotype c.980AG (RR = 0.15, 95% CI = 0.04–0.56). The association between c.980A>G and *Staph. aureus* IRCM was less pronounced in cows with parity 2 and ≥3 (Table 3).

SCC. Season of calving, season of test day, DIM^2, and the interaction between genotype and parity were not associated with LnSCC and were therefore removed from both LnSCC models. Test-day LnSCC was significantly associated with parity ($P < 0.01$) and DIM ($P < 0.01$), but not with genotype (Table 4).

MY. The interaction between genotype and parity was not associated with MY and was therefore removed from both MY models. Test-day MY was significantly associated with parity, season of calving, season of test-day, DIM, and DIM^2 ($P < 0.01$). Milk yield was associated with SNP c.735C>G ($P < 0.05$), but not with SNP c.980A>G ($P = 0.13$). Cows with genotype c.735CG and genotype c.735GG produced, on average, 0.30 (SE 0.27) and 0.81 (SE 0.31) more kilograms of milk per day compared with c.735CC cows (Table 4).

DISCUSSION

Several research groups studied associations between SNP in *CXCR1* and udder health (Youngerman et al., 2004; Leyva-Baca et al., 2008b; Goertz et al., 2009; Beecher et al., 2010; Galvão et al., 2011; Verbeke et al., 2012). However, we were the first to study pathogen-specific data in a relative large population from multiple commercial dairy herds. The 50 study herds were randomly selected. No inclusion criteria were applied in our study, underlining the external validity of our results. A downside of this selection procedure was the lack of DHI records and pedigree information in a large number of herds. Consequently, we could not study test-day MY and LnSCC of all animals, nor account for additive polygenic effects in the statistic models.

Different methods were used to genotype animals in previous studies. Sequencing allows detection of new SNP (Youngerman et al., 2004; Verbeke et al., 2012), but is relatively expensive and time consuming. The PCR-RFLP assay used by Goertz et al. (2009), Beecher et al. (2010), and Galvão et al. (2011) is cheaper and contains only 4 steps (DNA extraction, PCR, restriction enzyme digestion, and gel electrophoresis). To further reduce the time required per sample, we designed a

Table 2. Descriptive statistics of pathogen-specific incidence rate of clinical mastitis (IRCM; cases/10.000 cow-days at risk) and test-day LnSCC[1] and milk yield between 14 and 285 DIM by *CXCR1* genotype and parity

		c.735C>G genotype			c.980A>G genotype			
Outcome variable	Parity	CC	CG	GG	AA	AG	GG	Total
Herds with CM data		50	50	50	41	50	50	50
Cows with CM data		772	1,502	832	149	1,053	1,904	3,106
Lactations with CM data	1	363	701	390	62	511	881	1,454
	2	348	659	348	64	453	838	1,355
	≥3	465	931	508	103	622	1,179	1,904
Total IRCM (cases)	1	4.0 (36)	4.1 (69)	3.5 (34)	3.8 (6)	4.3 (54)	3.7 (79)	3.9 (139)
	2	6.4 (43)	6.2 (80)	7.8 (52)	5.0 (6)	5.6 (48)	7.3 (121)	6.7 (175)
	≥3	10.9 (93)	11.0 (192)	9.0 (86)	7.7 (15)	12.0 (129)	10.0 (227)	10.6 (371)
Staphylococcus aureus IRCM (cases)	1	0.4 (4)	0.6 (10)	0.1 (1)	0.0 (0)	1.0 (12)	0.1 (3)	0.4 (15)
	2	0.7 (5)	0.4 (5)	0.2 (1)	0.8 (1)	0.5 (4)	0.4 (6)	0.4 (11)
	≥3	1.1 (9)	0.9 (15)	0.5 (5)	0.0 (0)	0.7 (8)	1.0 (21)	0.8 (29)
Streptococcus uberis IRCM (cases)	1	0.7 (6)	0.7 (11)	0.9 (9)	0.0 (0)	0.6 (8)	0.9 (18)	0.7 (26)
	2	0.7 (5)	0.7 (9)	1.1 (7)	0.8 (1)	0.5 (4)	1.0 (16)	0.8 (21)
	≥3	1.8 (15)	2.3 (39)	2.1 (20)	2.6 (5)	2.1 (24)	2.1 (45)	2.1 (74)
Streptococcus dysgalactiae IRCM (cases)	1	0.2 (2)	0.2 (4)	0.4 (4)	0.0 (0)	0.2 (3)	0.3 (7)	0.3 (10)
	2	0.9 (6)	0.6 (8)	0.2 (1)	1.7 (2)	0.6 (5)	0.5 (8)	0.6 (15)
	≥3	0.6 (5)	1.1 (19)	0.2 (2)	0.0 (0)	0.9 (10)	0.7 (16)	0.7 (26)
Escherichia coli IRCM (cases)	1	0.9 (8)	0.4 (7)	0.6 (6)	0.0 (0)	0.6 (8)	0.6 (13)	0.6 (21)
	2	0.9 (6)	1.2 (16)	1.4 (9)	0.0 (0)	1.4 (12)	1.2 (19)	1.2 (31)
	≥3	2.0 (17)	1.7 (28)	1.2 (11)	2.0 (4)	2.0 (22)	1.4 (30)	1.6 (56)
Herds with DHI records[2]		32	32	32	28	32	32	32
Cows with DHI records		474	966	521	93	691	1,177	1,961
Lactations with DHI records	1	181	371	198	31	291	428	750
	2	163	326	168	35	215	407	657
	≥3	184	417	236	43	294	521	858
Test-days with DHI records	1	976	1,902	1,017	163	1,506	2,226	3,895
	2	718	1,444	751	145	941	1,827	2,913
	≥3	896	1,815	1,007	200	1,296	2,222	3,718
LnSCC (± SEM)	1	4.13 (0.04)	4.08 (0.03)	4.12 (0.04)	4.14 (0.09)	4.14 (0.03)	4.08 (0.02)	4.10 (0.02)
	2	4.47 (0.05)	4.41 (0.03)	4.32 (0.04)	4.21 (0.10)	4.43 (0.04)	4.40 (0.03)	4.40 (0.02)
	≥3	4.80 (0.05)	4.75 (0.03)	4.65 (0.04)	4.74 (0.10)	4.80 (0.04)	4.70 (0.03)	4.74 (0.02)
Milk yield[3] (± SEM)	1	24.4 (0.2)	24.7 (0.1)	25.5 (0.2)	23.8 (0.4)	24.8 (0.2)	25.0 (0.1)	24.8 (0.1)
	2	27.0 (0.3)	28.6 (0.2)	29.1 (0.3)	27.7 (0.7)	27.9 (0.3)	28.6 (0.2)	28.4 (0.2)
	≥3	29.6 (0.3)	30.9 (0.2)	31.2 (0.3)	30.4 (0.7)	30.6 (0.3)	30.8 (0.2)	30.7 (0.2)

[1]Natural logarithmic transformed test-day composite SCC (LnSCC).

[2]Thirty-two out of 50 study herds participated in a DHI program.

[3]In kilograms of milk per day.

Table 3. Multilevel Poisson regression models describing the associations between the pathogen-specific incidence rate of clinical mastitis (IRCM) and SNP *CXCR1*c.735C>G and *CXCR1*c.980A>G, respectively

IRCM	Independent variable	Subset	β[1]	SE	RR[2]	95% CI RR[3]	*P*-value[4]	Independent variable	Subset	β[1]	SE	RR[2]	95% CI RR[3]	*P*-value[4]
Overall	Intercept		−7.87	0.16				Intercept		−8.17	0.26			
	c.735C>G[5]	CC		Reference			0.73	c.980A>G[6]	AA		Reference			0.29
		CG	0.04	0.11	1.04	0.84–1.28			AG	0.36	0.23	1.43	0.91–2.27	
		GG	−0.05	0.13	0.95	0.75–1.22			GG	0.30	0.23	1.35	0.86–2.10	
	Parity	1		Reference			<0.01	Parity	1		Reference			<0.01
		2	0.60	0.12	1.83	1.44–2.32			2	0.61	0.12	1.83	1.45–2.33	
		≥3	1.05	0.11	2.86	2.30–3.56			≥3	1.06	0.11	2.88	2.32–3.58	
	Interaction						NS	Interaction						NS
Staphylococcus aureus	Intercept		−9.83	0.37				Intercept		−9.30	0.34			
	c.735C>G[5]	CC		Reference			0.08	c.980A>G[6]	AA					<0.01
		CG	−0.16	0.34	0.85	0.44–1.65			AG		Reference			
		GG	−1.05	0.48	0.35	0.14–0.90			GG	−1.91	0.68	0.15	0.04–0.56	
	Parity	1		Reference			<0.05	Parity	1		Reference			<0.05
		2	0.01	0.42	1.00	0.44–1.65			2	−0.67	0.61	0.51	0.16–1.69	
		≥3	0.73	0.35	2.08	1.05–4.12			≥3	−0.26	0.51	0.77	0.29–2.07	
	Interaction						NS	Interaction	GG*2	1.63	0.95	5.08	0.79–32.82	<0.05
									GG* ≥ 3	2.23	0.82	9.29	1.87–46.26	
Streptococcus uberis	Intercept		−9.55	0.37				Intercept		−9.44	0.28			
	c.735C>G[5]	CC		Reference			0.59	c.980A>G[6]	AA					0.49
		CG	0.20	0.23	1.22	0.78–1.89			AG		Reference			
		GG	0.25	0.25	1.28	0.78–2.09			GG	0.13	0.19	1.14	0.78–1.66	
	Parity	1					<0.01	Parity	1		Reference			<0.01
		2	0.07	0.28	1.07	0.62–1.85			2	0.03	0.28	1.03	0.59–1.79	
		≥3	1.03	0.22	2.80	1.83–4.29			≥3	1.01	0.22	2.75	1.78–4.23	
	Interaction						NS	Interaction						NS
Streptococcus dysgalactiae	Intercept		−10.45	0.42				Intercept		−10.39	0.39			
	c.735C>G[5]	CC		Reference			0.13	c.980A>G[6]	AA					0.77
		CG	0.20	0.36	1.22	0.61–2.44			AG		Reference			
		GG	−0.68	0.49	0.51	0.19–1.33			GG	−0.09	0.32	0.91	0.49–1.71	
	Parity	1		Reference			<0.05	Parity	1		Reference			<0.05
		2	0.69	0.42	1.99	0.87–4.57			2	0.57	0.44	1.78	0.75–4.19	
		≥3	0.96	0.39	2.62	1.22–5.63			≥3	1.00	0.39	2.72	1.26–5.88	
	Interaction						NS	Interaction						NS
Escherichia coli	Intercept		−9.63	0.34				Intercept		−9.56	0.32			
	c.735C>G[5]	CC		Reference			0.73	c.980A>G[6]	AA					0.24
		CG	−0.20	0.27	0.82	0.48–1.39			AG		Reference			
		GG	−0.20	0.31	0.82	0.45–1.51			GG	−0.27	0.23	0.76	0.48–1.21	
	Parity	1		Reference				Parity	1		Reference			<0.01
		2	0.79	0.31	2.21	1.22–4.02			2	0.79	0.30	2.20	1.21–3.99	
		≥3	1.03	0.29	2.81	1.60–4.96			≥3	0.97	0.29	2.64	1.49–4.65	
	Interaction						NS	Interaction						NS

[1]Regression coefficient.

[2]RR = rate ratio.

[3]95% confidence interval of RR.

[4]Overall *P*-value of the fixed effect.

[5]Genotype at SNP *CXCR1*c.735C>G.

[6]Genotype at SNP *CXCR1*c.980A>G. Cows with genotype c.980AA were excluded from the analysis to allow for convergence.

Table 4. Mixed regression models describing the associations between test-day LnSCC[1] and milk yield between 14 and 285 DIM and SNP *CXCR1*c.735C>G and *CXCR1*c.980A>G, respectively

		LnSCC			Milk yield		
Polymorphism	Independent variable	β[2]	SE	*P*-value[3]	β[2]	SE	*P*-value[3]
c.735C>G	Intercept	3.61	0.08		30.90	0.92	
	Genotype			0.29			<0.05
	CC	Reference			Reference		
	CG	−0.06	0.05		0.30	0.27	
	GG	−0.10	0.06		0.81	0.31	
	Parity			<0.01			<0.01
	1	Reference			Reference		
	2	0.32	0.05		3.35	0.26	
	≥3	0.72	0.05		5.65	0.26	
	Interaction of genotype × parity			NS[4]			NS
	Season of calving			NS			<0.01
	January–March				Reference		
	April–June				−0.47	0.29	
	July–September				−1.40	0.29	
	October–December				−0.14	0.32	
	Season of test-day						<0.01
	January–March				Reference		
	April–June				0.82	0.11	
	July–September				−0.23	0.14	
	October–December				−0.92	0.11	
	DIM	0.0036	0.0001	<0.01	−0.035	0.002	<0.01
	DIM[2]			NS	-5×10^{-5}	1×10^{-5}	<0.01
c.980A>G	Intercept	3.53	0.12		30.80	1.03	
	Genotype			0.44			0.13
	AA	Reference			Reference		
	AG	0.06	0.11		0.33	0.52	
	GG	0.01	0.10		0.71	0.51	
	Parity			<0.01			<0.01
	1	Reference			Reference		
	2	0.33	0.05		3.33	0.26	
	≥3	0.73	0.05		5.65	0.26	
	Interaction of genotype × parity			NS			NS
	Season of calving			NS			<0.01
	January–March				Reference		
	April–June				−0.47	0.29	
	July–September				−1.41	0.29	
	October–December				−0.15	0.32	
	Season of test-day			NS			<0.01
	January–March				Reference		
	April–June				0.82	0.11	
	July–September				−0.24	0.14	
	October–December				−0.92	0.11	
	DIM	0.0036	0.0001	<0.01	−0.035	0.002	<0.01
	DIM[2]			NS	-5×10^{-5}	1×10^{-5}	<0.01

[1]Natural logarithmic transformed test-day composite SCC (LnSCC).

[2]Regression coefficient.

[3]Overall *P*-value of the fixed effect.

[4]Nonsignificant fixed effects except genotype were removed from the models.

fluorescent multiprobe PCR assay allowing us to genotype SNP c.735C>G and c.980A>G simultaneously in only 2 steps (DNA extraction and PCR). Using this assay, we could process the relative large number of samples cost and time effectively. Although the use of small amplicons (75–150 bp) is recommended (Livak, 1999), we used a relatively large amplicon to be able to genotype the SNP simultaneously. Purification of DNA and usage of special qPCR mixes generally result in lower Cq-values and higher RFU (relative fluorescence units) values (own observations) but was not required in our assay, which reduced the cost per sample. Comparison with sequence data demonstrated that results were reliable and repeatable. The assay can be used in further research or in breeding programs.

When studying associations between gene polymorphisms and mastitis resistance, different udder health parameters can be measured (Pighetti and Elliott, 2011). By monitoring clinical signs, CM incidence can be estimated directly. In the Nordic countries Denmark,

Finland, Norway, and Sweden, disease-recording systems provide CM data on the majority of dairy herds (Wolff et al., 2012). In Belgium and other countries, CM data are not available on a larger scale. Yet, conducting our study on the same herds as a study estimating the IRCM in Flanders (Verbeke et al., 2014) enabled us to analyze associations between SNP in *CXCR1* and IRCM in a relatively large population. However, we could not confirm the overall higher IRCM in c.735GG cows compared with c.735CG and CC cows observed by Galvão et al. (2011). Because different mastitis pathogens elicit different immune responses (Bannerman et al., 2004; Schukken et al., 2011), we also analyzed associations with pathogen-specific IRCM. This allowed us to detect a lower *Staph. aureus* IRCM in c.735GG compared with c.735CC cows. Additionally, genotype c.980GG was associated with lower *Staph. aureus* IRCM in heifers but not in multiparous cows. The interaction between SNP c.980A>G and parity could be explained by differences in innate immunity between heifers and multiparous cows. Phagocytosis and killing of *Staph. aureus* by milk PMNL was shown to be higher in heifers compared with multiparous cows (Mehrzad et al., 2009). Although *Staph. aureus* IMI elicits little to no IL-8 response (Bannerman, 2009), SNP in the gene encoding its receptor were associated with *Staph. aureus* IRCM. However, we should mention that the number of *Staph. aureus* cases was limited in our study and that we could not account for additive polygenic effects in the statistic models. The association does not prove causality and needs further substantiation (e.g., through conducting an experimental challenge study in which several cows per genotype are infected with *Staph. aureus*). Polymorphisms linked with SNP c.735C>G and c.980A>G might cause differential immune responses following *Staph. aureus* IMI and explain the differences in *Staph. aureus* IRCM, and could be pictured in such an experiment. Additionally, it could elucidate why genotype c.980AG was associated with a lower *Staph. aureus* IRCM in our study yet with a higher likelihood of IMI by major pathogens in a previous study (Verbeke et al., 2012). Hypothetically, heifers with genotype c.980AG are more likely to develop clinical signs in cases of *Staph. aureus* IMI without being more susceptible to the pathogen. Considering *Staph. aureus* is a major cause of CM (Olde Riekerink et al., 2008; Keane et al., 2013; Oliveira et al., 2013) associated with considerable milk losses worldwide (Hertl et al., 2014), more research on this topic is warranted and justified.

Besides (pathogen-specific) IRCM, we also studied SCC at test day. Similar to Goertz et al. (2009), Galvão et al. (2011), and Verbeke et al. (2012), no associations between SCC and SNP in *CXCR1* were detected. This does not necessarily indicate that the SNP are not associated with incidence of SM, as SCC is merely an indirect test for subclinical mastitis that does not give information on the pathogens associated with the SCC elevations. Yet, using culture data, c.735C>G was found to be associated with overall incidence of SM (Youngerman et al., 2004), whereas c.980A>G was found to be associated with the prevalence of SM caused by major pathogens in early lactating heifers (Verbeke et al., 2012). Culture data on a large number of SM cases were not collected in our study, but would have allowed to test pathogen-specific associations between SNP in *CXCR1* and SM incidence as well.

Marker-assisted selection against mastitis is a noble goal, but would receive little attention if it would decrease the genetic merit for production (Detilleux, 2009). Unfavorable genetic correlations between CM and MY traits have been described (Koivula et al., 2004). Therefore, we also tested associations between SNP c.735C>G and c.980A>G and MY. Besides a lower *Staph. aureus* IRCM, c.735GG had higher MY compared with c.735CC cows, contradicting findings by Galvão et al. (2011). Whether the lower *Staph. aureus* IRCM partly explains the higher MY is hard to test because the traits were recorded in somewhat different populations.

As mentioned in the introduction, functional effects of *CXCR1* SNP have yet to be analyzed. As suggested by Pighetti et al. (2012), AA change p.His245Glu caused by c.735C>G could affect G-protein binding and signal transduction, whereas AA change p.Lys327Arg caused by c.980A>G could interfere with adaptin-2 binding and receptor internalization. In silico analysis showed an important role of the N terminus of CXCR1 in the first steps of binding IL-8 (Liou et al., 2014). Hence, AA changes p.Ile13Tyr and p.Gly23Glu in this region caused by SNP c.37A>T, c.38T>A, and c.68G>A being in strong linkage disequilibrium with c.735C>G could alter ligand binding. Latter and other processes could affect receptor functionality and therefore mastitis resistance or susceptibility.

CONCLUSIONS

A fluorescent multiprobe PCR assay was designed, validated, and used to genotype *CXCR1* SNP c.735C>G and c.980A>G in 3,106 dairy cows. Because of the important function of *CXCR1* in innate immune responses, mutations in the encoding gene might affect mastitis resistance and milk yield. In our study, SNP c.735C>G tended to associate with *Staph. aureus* IRCM and was associated with MY. Cows with genotype c.735GG had a lower *Staph. aureus* IRCM and a higher MY compared with cows with genotype c.735CC. Additionally,

SNP c.980A>G was associated with *Staph. aureus* IRCM in heifers. Genotype c.980GG resulted in lower *Staph. aureus* IRCM compared with genotype c.980AG. The results provide a basis for further research on the relation between *CXCR1* polymorphism and pathogen-specific mastitis resistance and MY.

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