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Selective genotyping and logistic regression analyses to identify favorable SNP-genotypes for clinical mastitis and production traits in Holstein dairy cattle

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ABSTRACT

The objective of this study was to estimate effects of SNP-genotypes on clinical mastitis, somatic cell score (SCS) and on estimated breeding values (EBV) for production traits in Holstein dairy cattle based on selective genotyping methodology. For identification of SNPs, we focused on candidate genes for clinical mastitis. The data set was comprised of a total of 3823 Holstein cows from two Holstein contract herds located in two regions in Iran. Data included 305-d lactation records for the production traits milk yield, fat yield, protein yield, fat percentage, protein percentage, SCC, and the no. of cases of clinical mastitis per lactation. Selection of cows for selective genotyping was based on extreme values for clinical mastitis residuals (CMR) from mixed model analyses. Two extreme groups of 135 cows each were genotyped for two loci of both candidate genes for mastitis *TLR4* [=*TLR4*(*1*) and *TLR4*(*2*)] and *CACNA2D1* [=*CACNA2D*1(*1*) and *CACNA2D*1(*2*)] using PCR-SSCP or PCR-RFLP . Associations between SNP-genotypes and traits of interest were estimated by applying logistic regression analyses, i.e. estimating the probability of the heterogeneous genotype in dependency of the EBVs and of values for CMR. The heterozygous genotype was contrasted to both homozygous genotypes allowing the estimation of effects for dominance. Allele G of *TLR4*(*1*) was associated with fewer cases for CM, and showed desired effects in production traits, e.g. higher milk yield and protein yield, and lower values for CMR. Also allele G of *CACNA2D1*(*2*) was predominant in the resistant group, and favorably associated with milk yield. Those SNPs are interesting for breeding, because they simultaneously improve both antagonistic traits. Effects of dominance ranged from 0.05 SD to 0.13 SD for the production trait (milk yield), and from 0.04 to 0.19 SD the functional trait (CMR).

1. Introduction

Mastitis is the major health disorder in dairy cattle causing profound economic loss at various stages of production. The cost component includes in particular the treatment and application of antibiotics, reduced milk production and discarded milk, and an increased risk of subsequent diseases. The latter problem often results in

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too early dairy cow disposals. Besides improvements in dairy cattle husbandry and in the farm and feeding management, the implementation of sustainable breeding strategies is a pre-requisite to reduce incidences of mastitis in dairy cow populations and to improve the quality of milk production. However, the success of traditional breeding strategies based on quantitative genetics application is hampered due to a substantial lack of recording systems, and due to relatively low additive genetic variances and heritabilities for health disorders (e.g. Gernand et al., 2012). Only those projects or national genetic evaluations that are based on substantial datasets for clinical mastitis (CM), and basing selection decisions on CM over decades, reported phenotypic and genetic improvement (e.g. Heringstad et al., 2007). Population wide recording of health traits may be difficult to implement in most countries. As an alternative, breeding organizations in Germany intend to implement a system of so called "contract herds" (Schierenbeck et al., 2011) with the primary aim of high-quality health data recording. Also for countries like Iran, a focus on selected contract herds may generate phenotypes of higher quality compared to population wide recording systems. Data from new health disorder phenotypes can be combined with genomic data to establish innovative selection strategies.

One strategy using molecular high density single nucleotide polymorphism (SNP) markers more or less evenly distributed over the whole genome is referred to as genomic selection (Meuwissen et al., 2001). Such a dense marker set is the base for deriving genomic breeding values. As summarized by Wensch-Dorendorf et al. (2011), the concept of genomic selection is implemented in Holstein dairy cattle breeding programs in Europe and in North America. In these countries, genomic breeding values have moderate to high accuracies, because large calibration groups from sires across country borders were used. For new health traits like clinical mastitis, accurate conventional breeding values of sires do not exist in most countries, and even larger calibration groups of cow phenotypes have to be built up. Such a strategy is very difficult to realize in countries like Iran, and here, focusing on a few markers and on marker assisted selection (MAS), especially in bull dam contract herds, still might be a reasonable alternative. Also in a large Holstein population, Pimentel et al. (2011a) focused only on SNPs located within genes identified in gene expression profiling to explain genetic relationships between production and fertility. Using this approach, valuable SNP genotypes were identified that improved fertility without decreasing milk production, and vice versa. Those rare SNP genotypes that did not depict the general antagonistic relationship between production and fertility can be used for improving selection response simultaneously in both groups of traits. A similar strategy was introduced by Moe et al. (2009) in swine.

For CM, identified candidate genes have one or multiple roles in the host's immune system (Paape et al., 2000). As defined by Youngerman et al. (2004), of major importance are the histocompatability complex, cytokine and cytokine receptors, and the natural resistance associated macrophage protein-1 . Additionally, they referred to studies which found associations of alleles of the major histocompatability complex with incidences of CM (e.g. Park et al., 2004). These studies also focused on the physiological background, and they identified pathogen related differences for defense reactions of the immune system. One reason for differences might be due to the effect of the toll-like receptor (TLR). The bovine *TLR4* gene was identified in 2003 on chromosome 8 (McGuire et al., 2005; White et al., 2003). The *TLR4* gene encodes for an innate immune protein located on cell surfaces, which recognizes lipopolysaccharides (LPS) of gram-negative bacteria (Akira and Takeda, 2004; Shizuo et al., 2001). In addition, differences in expression profiles of the *TLR4* gene during mastitis episodes were reported by Goldammer et al. (2004). Another important gene involved in resistance to CM is the calcium channel, voltage dependent, alpha-2/delta subunit 1 (*CACNA2D1*), which is described in detail on the "genetics home reference" and by Buitkamp et al. (2003). Consequently, based on the impact of both candidate genes *TLR4* and *CACNA2D1* on udder health, linear or generalized linear mixed models for testing associations between SNPs located in the *CACNA2D1* gene and somatic cell score (Yuan et al., 2011; Zhang et al., 2009), and between SNPs located in the *TLR4* gene and clinical mastitis (Wang et al., 2007), were applied.

The objective of the present study was to estimate effects of single SNPs located within the *TLR4* and *CACNA2D1* genes for CM and production traits in Holstein dairy cattle from Iran based on the method of selective genotyping, and following the approach by Sharma et al. (2006a). Identification of favorable SNP-genotypes for both production and CM resistance would have the combined effects of improving production without neglecting udder health. Furthermore, we used our data including cow phenotypes and cow genotypes to estimate effects of dominance at selected marker loci.

2. Materials and methods

2.1. Data for selective genotyping

In total 3823 Holstein cows from two Holstein contract herds located in Tehran and Isfahan in Iran were recorded for CM over a period of 21 months from March 2008 to December 2010.

Cows from these two special contract herds are a source for bull dam selection, with a current focus on cows in higher parities. Cows from parities two to five were used for detailed recording of health disorders. In the case of repeated lactations per cow, the specific lactation no. including a complete lactation (305 d in milk) was used for further analyses. Clinical mastitis was diagnosed as described by Gernand et al. (2012), i.e. an obvious infection of the udder including dolor, rubor, change of color, and additionally flakes in the milk. When counting cases of CM, an interval of 5 d was required to consider occurrence of CM as a new case of CM (Gernand et al., 2012; Hinrichs et al., 2005), resulting in 1647 records for cases of CM.

According to Sharma et al. (2006a), the pool of cows used for selective genotyping, i.e. the most resistant and the most susceptible group for CM, was extracted based

on values for clinical mastitis residuals (CMR). For analysis of variance, the trait 'no. of CM cases per lactation' was analyzed by applying the procedure GLM in SAS (SAS Institute, 1999) using the following linear model:

$$Y_{ijk} = H_i + L_j + \beta X_k + e_{ijk}$$

where

Y_{ijk} = no. of CM cases per lactation for the kth cow
H_i = fixed effect of the ith herd-year
L_j = fixed effect of the jth lactation
X_k = 305-d lactation milk yield of cow k in previous lactation
β = linear regression of the no. of CM cases per lactation on 305 d milk yield
e_{ijk} = random residual effect of CM cases

305-d lactation milk yield was considered in the statistical model, because some authors, e.g. Fleischer et al. (2001) found pronounced associations between 305-d milk yield in previous lactation and occurrence of health disorders in the current lactation. Furthermore, a cow's production level may encourage breeders for application of preferential treatment improving both the cow's health and longevity. This is the main reason for developing the concept of 'functional longevity' by including production level as explanatory variable (e.g. Buenger et al., 2001) as done in the present study.

Based on values for CMR, two extreme groups including 135 cows per group were extracted for selective genotyping. For cows assigned to the CM resistant group, CMR ranged from −1.34 to −0.93 with a mean value of −1.05. In the CM susceptible group, CMR of individual cows ranged from 1.28 to 5.60 with a mean value of 2.71 (Fig. 1). The CM resistant cows were never observed for occurrence of CM. The no. of CM cases in the selected susceptible group ranged from 3 to 6. The 270 selected cows were progeny of 159 sires. Hence, the majority of sires had only 1 or 2 daughter records; 80 sires were identified in the CM resistant group, and 99 sires were identified in the CM susceptible group with 41 sires being present in both extreme categories. Coefficients of genetic relationships within and across groups were on a similar and low level, i.e. $a = 0.050$ within the resistant group, $a = 0.046$ within the susceptible group, and $a = 0.039$ between both groups. In neither group there were any full-sibs .

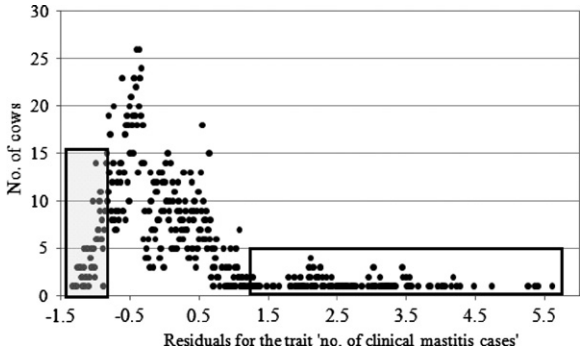

Fig. 1. Distribution of residuals for the trait 'no. of clinical mastitis (CM) cases per lactation'. Cows of extreme groups extracted for selective genotyping are within gray (=resistant) and transparent (=susceptible) boxes. (Style of figure according to Sharma et al. (2006a).

2.2. DNA extraction and genotyping

Genomic DNA was extracted using the improved salting out method (Miller et al., 1988). DNA concentration and DNA quality were assessed by micro-plate spectrophotometer light and 1% agarose gel electrophoresis. DNA was diluted to 50 ng/μL and stored at −20 °C. For both genes of interest (*TLR4* and *CACNA2D1*), primers were used according to Wang et al. (2007) and Yuan et al. (2011). In the following, the two loci are denoted as SNP 1 and SNP 2 of the *TLR4* gene [=*TLR4*(*1*) and *TLR4*(*2*), respectively], and as SNP 1 and SNP 2 of the *CACNA2D1* gene [=*CACNA2D1*(*1*), and *CACNA2D1*(*2*), respectively].

Polymerase chain reactions (PCR) were carried out in a total volume of 25 μL solution containing 50 ng templates DNA, 2.5 × buffer (Tris–HCl 100 mmol/L, pH 8.3; KCl 500 mmol/L), 1.0 μmol/L primers, 2.0 mmol/L $MgCl_2$, 1.0 mmol/L dNTPs, and 0.5 U Taq DNA polymerase. In chronological order, the reaction conditions of the PCR were: (1) an initial DNA denaturing using 95 °C for 5 min, (2) followed by 35 cycles of 94 °C for 30 s, with different annealing temperatures of 58 °C, 61 °C, 63 °C and 61.5 °C for *TLR4*(*1*), *TLR4*(*2*), *CACNA2D1*(*1*) and *CACNA2D1*(*2*), respectively, and (3) a final extension using 72 °C for 10 min or 72 °C for 8 min for *TLR4* and *CACNA2D1*, respectively. The PCR product was monitored by agarose gel electrophoresis using 2% agarose gel in 1 × TAE buffer at 100 V for 40 min. UV light of a trans-illuminator was used to visualize the amplified product.

The polymorphism at the *TLR4*(*1*) locus was detected by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). Molecular genetic analyses followed for most parts the procedures described by Wang et al. (2007): a total of 6.0 μL PCR product of *TLR4*(*1*) was mixed with 12 μL of the denaturation solution (50 mmol/L NaOH, 1 mmol/L EDTA), and 1 μL of the loading buffer containing 0.25% bromophenol blue and 0.25% xylene cyannol, denatured for 10 min at 95 °C, and subsequently, rapidly chilled for 10 min using an ice block. The samples were electrophoresed using a 12% sodium dodecyl sulfate-polyacrylamide gel kept at a constant temperature of 4 °C. The following conditions were used to run the gels: 250 V, 40 mA, 10 min (pre-electrophoresis) and 150 V, 24 mA, for 10 h. Subsequently, the gels were stained by silver stain. Sequences were analyzed by using 'BigDye® Terminator v3.1 Cycle Sequencing Kit' on an 'ABI PRISM® 3130 Genetic Analyzer'.

Polymorphisms at the other loci were detected by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP). The product of the PCR without purification was digested using 20 μL reaction containing 1.5 × buffer L, and 5 U Alu1, 6 U Rsa1 and 8 U Taq1 for *TLR4*(*2*), *CACNA2D1*(*1*) and *CACNA2D1*(*2*), respectively. Three hundred nanogram of PCR product was incubated with the restriction enzyme according to the manufacturer's instructions and subsequently electrophorized using a 3% agarose gel in 1 × TAE buffer at 90 V for

2.5 h. The pattern of the digested product was visualized under UV light on a trans-illuminator .

2.3. Estimation of marker effects

Effects of SNP-genotypes on estimated breeding values (EBV) for production traits (milk yield, fat yield, protein yield, fat%, and protein%), on SCC, and on CMR were studied for *TLR4*(*1*), *TLR4*(*2*), *CACNA2D1*(*1*), *CACNA2D1*(*2*). For genetic evaluation of production traits in Iran, a multiple-trait animal model was used including 305-d lactation yields from first lactation for milk yield, fat yield, and protein yield. EBVs for fat percentage and protein percentage were calculated by applying deterministic equations. Somatic cell score (SCS) is not included in the national genetic breeding goal in dairy cattle in Iran (Sadeghi-Sefidmazgi et al., 2011), but was recorded in the present study to verify effects of SNP-genotypes on CMR.

For estimation of SNP-effects, we followed the approach by Henshall and Goddard (1999) for selective genotyping data. At first, logistic regression analyses were carried out using a logistic model as implemented in the SAS Glimmix macro (Wolfinger and O'Connell, 1993). The statistical model for estimating the probability of e.g. a genotype AG vs. a genotype AA was defined as follows:

$$\text{logit}(\pi_r) = \log\left[\frac{\pi_\gamma}{1-\pi_\gamma}\right] = a + bY_r$$

π_r = probability of the genotype AG of a cow r
a = intercept
Y_r = EBV for production traits or CMR
b = linear regression of genotype AG on EBV or CMR

Test of significance of linear regression coefficients b was based on sum of square type I tests (Wald-type tests) as implemented in the Glimmix macro and as explained by König et al. (2005).

In a second step, the contrast α of the heterozygous genotype to the homozygous genotype, e.g. the effect of genotype AG in contrast to genotype AA, was estimated as described by Henshall and Goddard (1999) or by Sharma et al. (2006a) using the equation:

$$\alpha = \frac{-1+\sqrt{1+b^2\sigma_X^2}}{b}$$

with σ_X^2 denoting the variance of the EBV or CMR in the unselected base population.

All four loci analyzed were bi-allelic resulting in three different genotypes. Following the example given above, both homozygous genotypes (AA and GG) were contrasted to the heterozygous genotype AG in two consecutive runs, i.e. first contrasting AA to AG, and in a second run, contrasting AG to GG. Knowledge of both contrasts allows for the estimation of dominance effects (Falconer and Mackay, 1996). Dominance effects d were estimated for CMR and for the EBV for milk yield.

For verification of results from logistic regression analyses, three alternative conventional models were used. In verification models, the EBV, CMR, or SCS was defined as dependent trait in a linear model, and the SNP-genotype was the explanatory variable. The verification model (v1) for EBVs and CMR was used to analyze SNP-genotypes from different genes, considering one SNP at a time, whereas verification model (v2) for EBVs and CMR fitted all SNPs simultaneously. The statistical model (v1) was:

$$Y_{ij} = \mu + G_i + e_{ij} \quad [v1]$$

where Y_{ij} is the dependent trait of interest (EBV for production traits or CMR), μ is the overall mean effect, G_i is the fixed effect of the i-th SNP-genotype using different runs for *TLR4*(*1*), *TLR4*(*2*), *CACNA2D1*(*1*) and *CACNA2D1*(*2*), and e_{ij} is the random residual component. Model (v2) was:

$$Y_{ijklm} = \mu + TLR4(1)_i + TLR4(2)_j + CACNA2D1(1)_k + CACNA2D1(1)_l + e_{ijklm} \quad [v2]$$

where $TLR4(1)_i$ is the i-th SNP-genotype of SNP *TLR4*(*1*), $TLR4(2)_j$ is the j-th genotype of SNP *TLR4*(*2*), $CACNA2D1(1)_k$ is the k-th genotype of SNP *CACNA2D1*(*1*), and $CACNA2D1(2)_l$ is the l-th genotype of SNP *CACNA2D1*(*2*).

Effects of SNP-genotypes on CMR were verified by using the correlated trait SCS. Verification model (v3) estimating effects of SNP-genotypes on SCS was:

$$Y_{ijkl} = \mu + G_i + H_j + L_k + e_{ijkl} \quad [v3]$$

where Y_{ijkl} is the average value of SCS during lactation, μ is the overall mean effect, G_i is the fixed effect of the i-th SNP-genotype using different runs for *TLR4*(*1*), *TLR4*(*2*), *CACNA2D1*(*1*) and *CACNA2D1*(*2*), H_j is the fixed effect of the j-th herd, L_k is the fixed effect of the k-th lactation no., and e_{ijkl} is the random residual component.

3. Results

The 316 bp fragment (=SNP 1) of the *TLR4* gene [=*TLR4*(*1*)] was detected using PCR-SSCP . The PCR-SSCP results are shown in Fig. 2. Sequencing of PCR products showing different SSCP patterns revealed an A/G substitution within *TLR4*(*1*). The result of the blast of different genotypes of *TLR4*(*1*) is depicted in Fig. 3. Other SNP-loci were detected using the PCR-RFLP method. The differentiation of the 3 genotypes using PCR-RFLP for *TLR4*(*2*) is shown in Fig. 4. For *CACNA2D1*(*1*), the identified genotypes due to differences in base pair (bp) fragments were: TT (322 bp), CT (322, 236 and 86 bp), and CC (236 and 86 bp).

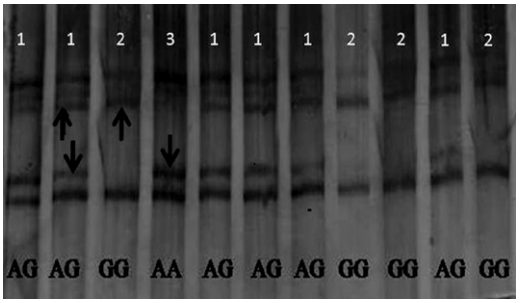

Fig. 2. Identification of the three genotypes AA, AG, and AG of SNP 1 in the gene *TLR4* by the PCR-SSCP technique. Arrows indicate the differentiation of the SSCP pattern by SSCP bands.

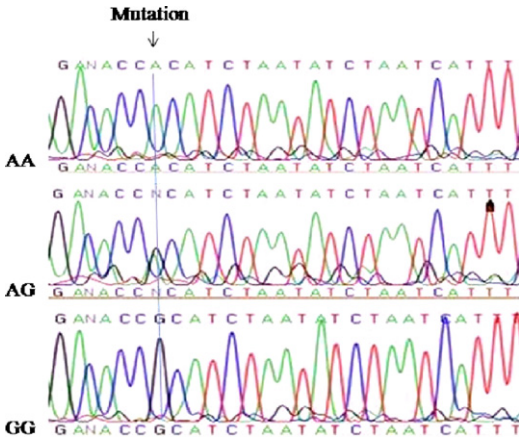

Fig. 3. Blast of the different genotypes of SNP 1 located in the gene *TLR4*.

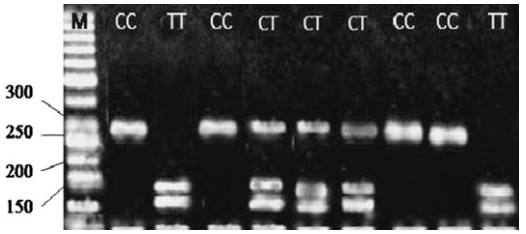

Fig. 4. Identification of the three genotypes CC, CT, and TT of SNP 2 in the gene *TLR4* by the PCR-RFLP technique.

For *CACNA2D1*(*2*), the following genotypes were differentiated: AA (386 bp), AG (386 bp, 229 bp and 157 bp), and GG (229 bp and 157 bp). Results for *CACNA2D1*(*1*) and *CACNA2D1*(*2*) are shown in Figs. 5 and 6, respectively.

Genotype and allele frequencies at the four loci in both groups CM resistant and CM susceptible cows are presented in Table 1. Chi-square statistics were applied to identify significant differences of genotype and allele frequencies in defined extreme groups. Both homozygous genotypes were found in both CM resistant and CM susceptible groups. However, apart from *CACNA2D1*(*1*), differences in genotype and allele frequencies were highly significant ($P<0.0001$) between the CM resistant and the CM susceptible group. Especially for *CACNA2D1*(*2*), only two resistant cows had genotype AA, whereas in the susceptible group, A was the predominant allele and 29 cows were genotyped for AA. Allele G was the predominant allele at the *TLR4*(*1*) locus, and characterizing the CM resistant cows. At the *TLR4*(*2*) locus, the frequency of allele T was generally higher compared to allele C, even though allele C was predominant in the CM resistant group. The homozygous genotype GG was predominant at loci *TLR4*(*1*) and *CACNA2D1*(*2*). For the remaining groups and loci, highest frequencies were observed for the heterozygous genotype.

Differences in EBVs for production traits and for CMR when comparing the heterozygous genotype to both homozygous genotypes of the SNPs *TLR4*(*1*), *TLR4*(*2*), *CACNA2D1*(*1*), and *CACNA2D1*(*2*) are given in Tables 2–5, respectively. Allele G was the predominant allele in the CM resistant group for *TLR4*(*1*), and accordingly, genotypes AG and GG had lower values for CMR compared to genotype AA (Table 2). Interestingly, allele G was associated with fewer cases for CM, and apart from protein percentage, allele G was also associated with desired effects on production traits, e.g. higher milk

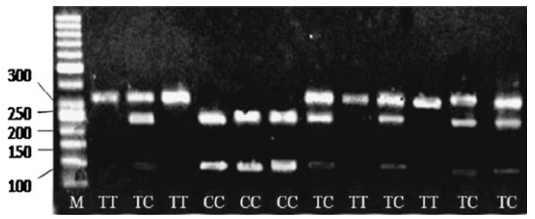

Fig. 5. Identification of the three genotypes CC, CT, and TT of SNP 1 in the gene *CACNA2D1* by the PCR-RFLP technique.

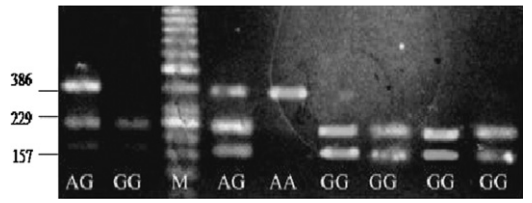

Fig. 6. Identification of the three genotypes of SNP 2 in the gene *CACNA2D1* by the PCR-RFLP technique.

Table 1
Genotypic and allele frequencies of the SNPs *TLR4*(*1*), *TLR4*(*2*), *CACNA2D1*(*1*), and *CACNA2D1*(*2*) in the clinical mastitis-resistant and in the clinical mastitis-susceptible cows. (Style of table according to Sharma et al. (2006a).

TLR4(*1*)	Genotype frequency			Allele frequency	
Group	AA	AG	GG	A	G
Resistant	7	49	63	63	175
Susceptible	40	80	14	160	108
Test-statistic		$\chi^2_2=61.13$		$\chi^2_1=56.47$	$P<0.0001$
P-value		$P<0.0001$			
TLR4(*2*)					
Group	CC	CT	TT	C	T
Resistant	32	65	22	129	109
Susceptible	10	73	51	93	175
Test-statistic		$\chi^2_2=22.70$		$\chi^2_1=19.46$	$P<0.0001$
P-value		$P<0.0001$			
CACNA2D1(*1*)					
Group	CC	CT	TT	C	T
Resistant	17	81	22	115	125
Susceptible	30	61	42	121	145
Test-statistic		$\chi^2_2=12.03$		$\chi^2_1=0.298$	$P<0.5848$
P-value		$P<0.0024$			
CACNA2D1(*2*)					
Group	AA	AG	GG	A	G
Resistant	2	56	61	60	178
Susceptible	29	94	11	152	116
Test-statistic		$\chi^2_2=67.21$		$\chi^2_1=51.40$	$P<0.0001$
P-value		$P<0.0001$			

Table 2
Difference in mean estimated breeding values (EBV) and mean CMR[a] between genotypes of the SNP *TLR4*(*1*) and regression coefficients (*b*-value) from the logistic model.

Trait	Difference AG vs. GG			Difference AG vs. AA		
	General units	SD units	*b*-value[b]	General units	SD units	*b*-value[b]
EBV Milk-kg	−14.37	0.02	−0.0004^{ns}	171.40	0.29	0.0005^{ns}
EBV Fat-kg	1.02	0.05	0.002^{ns}	13.42	0.65	0.04***
EBV Protein-kg	−2.68	0.10	−0.004^{ns}	3.31	0.12	0.005^{ns}
EBV Fat-%	−0.01	0.08	0.62^{ns}	0.07	0.49	3.89**
EBV Prot-%	−0.18	0.25	−0.36^{ns}	−0.52	0.73	−1.17^{ns}
CMR[a]	0.40	0.43	0.47***	−0.23	0.24	10.26**

[a] CMR=Residuals for the cases of clinical mastitis per lactation after accounting for effects of herd, parity, and production level in milk yield.
[b] Significance level: $^{***}=P\leq 0.001$, $^{**}=P\leq 0.01$, $^{*}=P\leq 0.05$, ns=$P>0.05$.

Table 3
Difference in mean estimated breeding values (EBV) and mean CMR[a] between genotypes of the SNP *TLR4*(*2*) and regression coefficients (*b*-value) from the logistic model.

Trait	Difference CT vs. TT			Difference CT vs. CC		
	General units	SD units	*b*-value[b]	General units	SD units	*b*-value[b]
EBV Milk-kg	44.13	0.07	0.0001^{ns}	3.31	0.01	0.0001^{ns}
EBV Fat-kg	−1.09	0.05	−0.003^{ns}	−3.71	0.18	−0.01^{ns}
EBV Protein-kg	−1.53	0.06	−0.002^{ns}	−1.51	0.06	−0.002^{ns}
EBV Fat-%	−0.04	0.29	−2.19^{ns}	10.03	0.23	−1.78^{ns}
EBV Prot-%	−0.36	0.50	−0.75^{ns}	−0.24	0.34	−0.48^{ns}
CMR[a]	−0.16	0.17	10.18*	0.24	0.25	0.27**

[a] CMR=Residuals for the cases of clinical mastitis per lactation after accounting for effects of herd, parity, and production level in milk yield.
[b] Significance level: $^{***}=P\leq 0.001$, $^{**}=P\leq 0.01$, $^{*}=P\leq 0.05$, ns=$P>0.05$.

Table 4
Difference in mean estimated breeding values (EBV) and mean CMR[a] between genotypes of the SNP *CACNA2D1*(*1*) and regression coefficients (*b*-value) from the logistic model.

Trait	Difference TC vs. TT			Difference TC vs. CC		
	General units	SD units	*b*-value[b]	General units	SD units	*b*-value[b]
EBV Milk-kg	−17.96	0.03	−0.0001^{ns}	−43.06	0.07	−0.0001^{ns}
EBV Fat-kg	−1.20	0.06	−0.003^{ns}	2.01	0.10	0.005^{ns}
EBV Protein-kg	−0.41	0.02	−0.0001^{ns}	1.39	0.05	0.002^{ns}
EBV Fat-%	0.01	0.09	0.70^{ns}	0.03	0.24	1.84^{ns}
EBV Prot-%	0.04	0.06	0.08^{ns}	0.08	0.10	0.15^{ns}
CMR[a]	−0.19	0.20	−0.22**	−0.17	0.18	−0.19*

[a] CMR=Residuals for the cases of clinical mastitis per lactation after accounting for effects of herd, parity, and production level in milk yield.
[b] Significance level: $^{***}=P\leq 0.001$, $^{**}=P\leq 0.01$, $^{*}=P\leq 0.05$, ns=$P>0.05$.

yield, fat yield, protein yield, and fat percentage. The difference in fat percentage when comparing genotype AG to genotype AA was almost 50% of one SD of the EBV for fat percentage. At locus *TLR4*(*2*), the CM resistant group was characterized by slightly higher no. of C-alleles than T-alleles, which is supported by lowest values for CMR for genotype CC (Table 3). Also here, mastitis resistance was in line with marginally higher milk yield, but lower EBVs for fat percentage and protein percentage. Significant lower values for CMR were found for the heterozygous genotype TC compared to both homozygous genotypes at locus *CACNA2D1*(*1*) (Table 4). The difference in CMR between genotype TC and genotype TT was −0.19 in units of the trait, and −0.17 between genotype TC and genotype CC. This result was expected, because the no. of T- and C-alleles in the CM resistant and in the CM susceptible group was not significantly different. Association studies for production traits only revealed minor allele substitution effects ranging between 0.02 and 0.24 SD. Pronounced differences in EBVs, and especially for CMR, were found when comparing genotypes GG, AG, and AA at locus *CACNA2D1*(*2*) (Table 5). The CM resistant group had substantially more G- than A-alleles, and accordingly, the value of genotype AG for CMR was 0.27 SD below the value of genotype AA. The lowest value for CMR was associated with genotype GG. Again, as found for genes *TLR4*(*1*) and *TLR4*(*2*), the favorable allele for CMR was also associated with

Table 5
Difference in mean estimated breeding values (EBV) and mean CMR[a] between genotypes of the SNP *CACNA2D1*(*2*) and regression coefficients (*b*-value) from the logistic model.

Trait	Difference AG vs. GG			Difference AG vs. AA		
	General units	SD units	*b*-value[b]	General units	SD units	*b*-value[b]
EBV Milk-kg	−215.56	0.36	−0.0006*	92.15	0.15	0.0003ns
EBV Fat-kg	−3.97	0.19	−0.01ns	4.61	0.22	0.01ns
EBV Protein-kg	−6.10	0.23	−0.009ns	2.21	0.08	0.003ns
EBV Fat-%	0.02	0.16	1.20ns	0.01	0.12	0.95ns
EBV Prot-%	−0.10	−0.15	−0.20ns	−0.23	0.33	−0.47ns
CMR[a]	0.51	0.54	0.62***	−0.26	0.27	−0.30**

[a] CMR=Residuals for the cases of clinical mastitis per lactation after accounting for effects of herd, parity, and production level in milk yield.
[b] Significance level: ***=$P \leq 0.001$, **=$P \leq 0.01$, *=$P \leq 0.05$, ns=$P > 0.05$.

Table 6
Probability values (*P*-values) for testing significance of mean differences in estimated breeding values (EBV) and CMR between heterozygous and homozygous genotypes: (a) logistic regression model=LRM, (b) linear model running one SNP at a time=ONE SNP, and (c) linear model considering all four SNPs simultaneously=MULTIPLE SNP. (Only *P*-values for traits with $P \leq 0.05$ from the logistic regression model are shown.)

SNP	Trait	Genotype	Statistical model		
			LRM	ONE SNP	MULTIPLE SNP
TLR4(*1*)	EBV Fat-kg	AG vs. AA	0.0004	0.0002	0.0002
	EBV Fat-%	AG vs. AA	0.0087	0.0082	0.0039
	CMR	AG vs. AA	0.0103	0.0059	0.0477
		AG vs. GG	0.0001	0.0001	0.0001
TLR4(*2*)	CMR	CT vs. CC	0.0098	0.0052	0.0187
		CT vs. TT	0.0172	0.0139	0.0254
CACNA2D1(*1*)	CMR	TC vs. CC	0.0247	0.0217	0.0541
		TC vs. TT	0.0059	0.0047	0.0863
CACNA2D1(*2*)	EBV Milk-kg	AG vs. GG	0.0142	0.0116	0.0149
	CMR	AG vs. AA	0.0099	0.0041	0.0084
		AG vs. GG	0.0001	0.0001	0.0001

CMR=Residuals for the cases of clinical mastitis per lactation after accounting for effects of herd, parity, and production level in milk yield.

Table 7
Difference in mean SCS between genotypes of the SNPs *TLR4*(*1*), *TLR4*(*2*), *CACNA2D1*(*1*), and *CACNA2D1*(*2*).

SNP	Genotype	General unit	Probability value
TLR4(*1*)	AG vs. AA	−0.30	0.0001
	AG vs. GG	0.19	0.0004
TLR4(*2*)	CT vs. CC	0.19	0.0057
	CT vs. TT	−0.11	0.0359
CACNA2D1(*1*)	TC vs. CC	−0.12	0.0790
	TC vs. TT	−0.16	0.0075
CACNA2D1(*2*)	AG vs. AA	−0.20	0.0055
	AG vs. GG	0.26	0.0001

Table 8
Estimates of dominance effects for the EBV in milk yield (in kg) and CMR for the genes *TLR4*(*1*), *TLR4*(*2*), *CACNA2D1*(*1*), and *CACNA2D1*(*2*). Values in brackets are dominance effects expressed in SD units.

Gene	Trait	
	EBV milk yield	CMR[a]
TLR4(*1*)	78.52 (0.13)	0.09 (0.10)
TLR4(*2*)	23.72 (0.06)	0.04 (0.04)
CACNA2D1(*1*)	−30.51 (0.05)	−0.18 (0.19)
CACNA2D1(*2*)	−61.71 (0.10)	0.13 (0.14)

[a] CMR=Residuals for the cases of clinical mastitis per lactation after accounting for effects of herd, parity, and production level in milk yield.

favorable EBVs in milk yield, fat yield, and protein yield. Only for protein percentage, highest EBVs were associated with genotype AA.

For the trait CMR, regressions coefficients from the logistic model were significant ($P \leq 0.05$) for all SNPs *TLR4*(*1*), *TLR4*(*2*), *CACNA2D1*(*1*), and *CACNA2D1*(*2*) when contrasting heterozygous genotypes to homozygous genotypes (Tables 2–5, respectively). Furthermore, significance of regression coefficients ($P \leq 0.05$) was observed for the following production traits and differences in EBVs: fat yield and fat% for genotype AG vs. genotype AA at SNP *TLR4*(*1*) (Table 2), and milk yield for genotype AG vs. genotype GG at SNP *CACNA2D1*(*2*) (Table 5). Differences in mean EBVs and CMR from the logistic model approach were verified by applying models [v1] and [v2]. Probability values for contrasts from all three models were in a narrow range and are presented in Table 6 for those contrasts which were significant in the logistic regression model. Apart from the contrasts within the *CACNA2D1*(*1*) locus from model [v2], where multiple SNPs were considered simultaneously, probability values $P \leq 0.05$ were confirmed. Results from the logistic model for CMR were verified by using the indicator trait SCS and model [v3]. Differences in solutions for SCS were significant except for the contrast of genotype TC vs. genotype

CC (Table 7), and same SNP-genotypes were favorable for both traits SCS and CMR.

Estimates for effects of dominance for the EBV for milk yield and for CMR for SNPs *TLR4*(*1*), *TLR4*(*2*), *CACNA2D1*(*1*), and *CACNA2D1*(*2*) are shown in Table 8. Effects of dominance were partly positive, and partly negative. In the case for milk yield, positive values for dominance are favorable, i.e. indicating a surplus in milk yield of the heterozygous genotype compared to the expected value (=average between both homozygous genotypes). This is valid for *TLR4*(*1*) and *TLR4*(*2*) showing dominance effects $d=78.52$ kg and $d=23.72$ kg, respectively. For *CACNA2D1*(*1*) and *CACNA2D1*(*2*), the EBV for milk yield of the heterozygous genotype was lower than the expected value (-30.51 kg and -61.71 kg, respectively). For CMR, lower values are favorable, and for effects of dominance, negative values indicate fewer than expected cases of CM for the heterozygous genotype. For example for *CACNA2D1*(*1*), the difference in CMR between genotype CC and genotype TT is of value -0.02, indicating an expected value of -0.01. In this specific case, the heterozygous genotype TC was decreased for CMR compared to both homozygous genotypes CC and TT. Values were $\alpha=-0.17$ and $\alpha=-0.19$, respectively (=over-dominance), resulting in an estimate for dominance of $d=-0.18$. For *TLR4*(*1*) and *CACNA2D1*(*2*), the value of the heterozygous genotype ranked between the two homozygous genotypes, but was higher than the expected value. Dominance of value $d=0.04$ was found for genotype CT of the SNP *TLR4*(*2*).

4. Discussion

4.1. The methodical concept

The concept of logistic regression for estimating the effects of quantitative trait loci (QTL) has been outlined by Henshall and Goddard (1999) for selective genotyping in a simulated half-sib design. Sharma et al. (2006a) put those ideas into practice by estimating allele substitution effects for markers associated with CM in dairy cattle. In their association study, only two genotypes (one homozygous and the heterozygous), were compared by neglecting the other homozygous genotype with extremely low allele frequencies. In the present study, we followed the approach by Sharma et al. (2006a), but additionally, we contrasted the heterozygous genotype to both homozygous variants. This approach not only allows estimating differences between SNP genotypes. In addition, knowledge of estimates for three genotypes provides estimates for effects of dominance.

The traditional approach for estimating effects of genotypes defines the trait of interest as dependent variable, and the genotype as explanatory variable (e.g. Pashmi et al., 2009). The idea developed by Henshall and Goddard (1999) is based on the probability of a heterozygous genotype as a linear function of the trait of interest. However, for verification of results from association analyses, we also applied the 'traditional approach' by modeling SNPs as explanatory variable to explain the variation of the trait of interest. Probability values for the contrasts of genotypes from the logistic regression model were comparable to those probability values from models [v1] and [v2].

4.2. Allele frequencies, genotype frequencies and differences between genotypes

The approach of selective genotyping in combination with logistic regression analyses identified useful marker genotypes in the regions of genes *TLR4* and *CACNA2D1*. Furthermore, estimates of allele and genotype frequencies in the CM resistant and in the CM susceptible group were in line with results from association analyses for CMR. Especially alleles A of the *TLR4*(*1*) and *CACNA2D1*(*2*) were relatively rare in the CM resistant group, and consequently, association analyses revealed favorable effects for CMR for allele G. Hence, allele G might be the beneficial allele for mastitis resistance in Iranian Holstein cows. Due to the multitude of physiological mechanisms attributed to *TLR4*, Ogorevc et al. (2009) proposed *TLR4* as a strong candidate gene to improve selection response for mastitis resistance in dairy cattle populations. Staudt (2001) highlighted the potential of gene expression profiling in gaining a deeper understanding of cellular physiology and the underlying disease processes. In the case of the gene *TLR4*, two gene expression experiments (Goldammer et al., 2004; Zheng et al., 2006) revealed differences in gene expression profiles depending on the status of infection. Goldammer et al. (2004) focused on expressions of pathogen recognition receptors, which have impact on the release of "effector molecules" of the innate immune system. In the process of udder infections, they found a strong increase of *TLR4* mRNA for infections with *Staphylococcus aureus*. Zheng et al. (2006) used a mouse model for proving the associations between the host and specific major pathogens via gene expression profiling. The increase of mRNA expression for genes of the immune system such as the toll-like receptor was reported by Swanson et al. (2009), but simultaneously, they observed a decrease of mRNA expressions for important milk protein genes. Hence, the general quantitative-genetic antagonism between udder health and production was confirmed on the level of gene expressions, underlining the necessity to identify single SNPs being favorable for both production and functionality. Individual markers can improve udder health without decreasing milk yield, as demonstrated e.g. for markers on chromosomes 6, 11, 15, and 26 (Lund et al., 2008). For SNPs located in the *TLR4* gene, Sharma et al. (2006b) identified favorable effects on both somatic cell score and lactation persistency for identical SNP polymorphisms. They referred to Dekkers et al. (1998), who found that cows with a persistent rather than precipitous milk yield were generally less susceptible for common health disorders. Based on the results from the present study as well as on previously published physiological background information (e.g. Sabroe et al., 2003) it can be concluded that the *TLR4* gene plays a central role in overall disease resistance in dairy cattle, and in particular in resistance to CM.

Also strong evidence was provided by association analyses for detected differences in allele frequencies in

CM resistant and CM susceptible cows for *CACNA2D1*(*2*). Following both types of analyses, allele G was predominant in the susceptible group and associated with lower values for CMR. According to e.g. Yuan et al. (2011), the *CACNA2D*1 gene is considered to be one of the most potential candidate genes influencing both mastitis and the indicator trait somatic cell score. The mapping position of the *CACNA2D*1 gene is in close distance to the chromosomal region where QTL for SCS were found (e.g. Rupp and Boichard, 2003). *CACNA2D1* is involved in a multitude of processes regulating health in farm animals as well as in humans. For example in Swine, Fujii et al. (1991) found mutations of the *CACNA2D1* gene and of the ryanodine receptor gene which are associated with the porcine stress syndrome. Associations between polymorphisms of the *CACNA2D1* gene and meat quality traits recently also have been reported in cattle (Hou et al., 2010). A study focusing on relationships between mutations of the *CACNA2D1* gene and udder health in the three breeds Holstein, Simmental and Sanhe cattle was conducted by Yuan et al. (2011). They used the indicator trait SCS, and they found that genotype GG of SNP *CACNA2D1*(*2*) was related to improved udder health, i.e. lower values for somatic cell score. Templin et al. (2011) recently studied in detail the physiological background of the *CACNA2D1* gene for disorders in humans, and disorders were caused by "loss of function mutations". As a major conclusion of their study, the authors suggested further analyses to get a deeper insight into the underlying molecular mechanisms caused by *CACNA2D1*. From a more applied perspective, and in addition to the major histocompatibility complex (e.g. Pashmi et al., 2009) and to the *TLR4* gene complex, also mutations of the *CACNA2D1* gene should be taken into account when developing MAS-strategies for the improvement of udder health in dairy cattle populations.

4.3. Dominance effects

To our knowledge, this is a first study estimating effects of dominance for CMR and milk yield based on results from a selective genotyping approach. Estimation of dominance requires availability of cow genotypes as well as cow phenotypes. This pre-requisite is met for CMR, but from a strictly theoretical background, an EBV representing only additive genetic values cannot be used for studying dominance. However, we also used EBVs for milk yield. Compared to AI-sires, EBVs of cows have low reliabilities. Hence, such values should be interpreted in a sense of a corrected phenotype, and also for milk yield, we found effects of dominance. In large genomic selection projects, up to now, estimation of dominance has not been possible yet because calibration sets are usually a group of sires with highly reliable conventional EBV. For new health traits, calibration groups of cow phenotypes will play a major role in the near future allowing the estimation of dominance for all SNP-loci . As a first step, Wellmann and Bennewitz (2011) conducted a comprehensive, theoretical study for understanding dominance, e.g. investigating the impact of additive genetic effects and of allele frequencies.

Following the theoretical background of genetics (Falconer and Mackay, 1996), higher values of dominance are assumed for low heritability traits. Results from our study revealed estimates for the moderate heritability trait (milk yield) and the low heritability trait (CM) in a narrow interval ranging from 0.04 to 0.19 SD units. It should be kept in mind that cows used in the present study are kept in two selected contract herds characterized by superior management conditions. Higher impact of dominance for health traits compared to production is assumed for cows from harsh environments or production systems. Lopez-Villalobos and Spelman (2010) estimated effects of heterosis of 10.7% for CM in Holstein × Jersey crosses in grazing production system in New Zealand, but only 5% for milk yield. Information on the genomic level, i.e. estimates for the traits of interest for both homozygous and the heterozygous genotype, will contribute to a deeper understanding of differences within breeds, but may also be helpful to explain effects of heterosis for across-breed comparisons.

4.4. Selection strategy

Selection on specific alleles of the gene complexes *TLR4* and *CACNA2D1* could improve the status of udder health in dairy cattle populations, but correlated response in other traits of economic importance has to be verified in advance. In most Holstein dairy cattle breeding programs worldwide, there is still a high emphasis on production traits covering almost 50% of the weighting factors in a combined breeding goal (Miglior et al., 2005). Based on quantitative genetics, genetic correlations between protein yield or milk yield and CM were generally antagonistic (e.g. Gernand et al., 2012). In a selection experiment conducted in Scandinavia (Heringstad et al., 2007), selection of sires on high EBVs for production traits was associated with an increase of CM on the phenotypic scale. Simultaneous response in selection in two antagonistic traits requires the inclusion of both traits with appropriate economic weights in the overall breeding goal. However, the lack of both requirements, the availability of phenotypic data and of economic weights for new health traits, hamper the process of putting such ideas into practice. Direct selection on genes or genetic markers carrying alleles which improve both traits simultaneously in the favorable direction could be applied in small but influential selection groups of animals. This group includes bull dams and young sires selected for AI, which have the strongest impact on genetic gain in dairy cattle breeding programs. Such an approach based on genotyped animals has been introduced by Moe et al. (2009) for the successful selection against boar taint without neglecting fertility. Valuable variants of SNPs located within candidate genes for female fertility and improving both fertility and production in dairy cattle have been identified by Pimentel et al. (2011a). Those SNP variants within, and therefore in close linkage with causal mutations are extremely valuable, following the same inheritance over decades. Also human geneticists focus on detection of so called 'functional' SNPs, e.g. for effective therapy against prostate cancer (Xu and Taylor, 2009). Results from our study revealed two valuable alleles within candidate genes improving both lactation milk

yield and resistance to CM, i.e. allele G of *TLR4*(*1*), and allele G of *CACNA2D1*(*2*). However, with these two genes it is not possible to improve simultaneously all three targeted traits milk yield, CM, and protein percentage. Favorable alleles for milk yield and CM had negative effects on protein percentage. Despite undesirable effects on protein percentage, we still recommend to select on alleles G of the genes *TLR4*(*1*) and *CACNA2D1*(*2*), because such a selection strategy is associated with an increase in protein yield and fat yield. Protein yield is the ultimate breeding goal for production traits in Holstein dairy cattle breeding programs (Miglior et al., 2005), because it considers protein percentage as well as the absolute amount of milk production. The pronounced negative genetic correlation between milk yield and percentage traits is well known in dairy cattle breeding, and also on the genomic scale, individual markers or genes are associated with antagonistic phenotypes (e.g. Pimentel et al., 2011a). Even for *DGAT1*, the most well known gene with a large effect on fat percentage (Grisart et al., 2002; Thaller et al., 2003), antagonistic associations between allele substitution effects on milk yield and fat percentage, and on milk yield and protein percentage, were reported. Interestingly, we found variants of genes improving both CM and milk yield or protein yield, but not improving simultaneously CM and percentage traits. Also in a study using data from German contract herds, genetic correlations between test-day protein percentage and CM, and between test-day fat percentage and CM, were close to zero (Gernand et al., 2012).

Based on the complex physiological background of genes *TLR4* and *CACNA2D1*, also their effect on other health traits should be clarified before implementing MAS. In addition to udder health, a main focus is on claw disorders and on metabolic diseases. König et al. (2005) found that a cow being susceptible for udder infections is also more susceptible for other types of health disorders, meaning that breeding for mastitis resistance is associated with general improvements in a cows' health status. In contrast, in a quantitative-genetic approach, Gernand et al. (2012) found genetic correlations between CM and other categories of health disorders close to zero. Hence, most of the genes with positive impact on CM might be neutrally associated with claw disorders or metabolic diseases, but also antagonistic relationships may occur.

In contract herds in Iran, high quality phenotypes for health traits are available, which is a crucial prerequisite for innovative selection strategies combining aspects of molecular and quantitative genetics. One successful breeding strategy on dairy cattle health in such a situation may be the following: (1) generating high quality health data in contract herds that allow a pronounced genetic differentiation (Schierenbeck et al., 2011), (2) focusing on and identifying polymorphisms within candidate genes and study their associations with traits of interest (Pimentel et al., 2011b), and (3) use of these genes or markers in combination with 'traditional' EBVs in an overall breeding goal (König and Swalve, 2009). Knowledge of effects of individual markers could also be used for optimizing in genomic evaluations. VanRaden (2008) compared three methods for computing the genomic relationship matrix **G**, where one method weighted markers on the diagonal according to their reciprocal variance. Also Pimentel et al. (2011b) focused on this idea, and they estimated the variance of single chromosomes. Based on their results, they suggested weighting factors when computing the genomic relationship matrix for single step procedures. However, genomic selection for improving low heritability health traits requires large calibration groups of 20,000 or more cow phenotypes to achieve sufficiently reliable genomic breeding values (e.g. Goddard and Hayes, 2009). Such a design is not feasible due to costs and no. of animals required in countries such as Iran. For the situation in Iran, with two contract herds for elite cows, and an own laboratory for SSCP- and RFLP-analyses, MAS still might be an alternative in the era of genomic selection. With this infrastructure, the costs per data point for genotyping is below 50 cent depending on the no. of probes as shown earlier by Ragot and Hoisington (1993). However, when implementing MAS, not only opportunities but also risks may exist, as highlighted by Boichard et al. (2006). Major risks address the small proportion of variance explained by a few markers whereas most traits follow an infinitesimal model, and effects of recombination. Hence, linkage phase between markers and genes is of crucial importance, and consequently, in most MAS breeding designs, within family selection strategies in combination with reproduction biotechnologies (larger families due to embryo transfer) have been suggested (e.g. Spelman and Garrick, 1998). To our knowledge, and presumably due to these risks, classical MAS on a grand scale only has been implemented in dairy cattle breeding programs in France (Boichard et al., 2006).

5. Conclusions

Genomic selection is successful in those countries or collaborative projects were large calibration groups of sires with highly reliable conventional EBVs exist. In developing countries, selective genotyping might be an alternative, especially when focusing on health traits. In the present study, recording of clinical mastitis from cows in contract herds was combined with PCR-SSCP or PCR-RFLP for the successful identification of two SNPs located in the gene *TLR4*, and two SNPs located in the gene *CACNA2D1*. Selective genotyping in combination with logistic regression analyses revealed polymorphisms of SNP *TLR4*(*1*) and of SNP *CACNA2D1*(*2*) being associated with desirable effects on both traits of interest, CM and protein yield. For CM, all contrasts between heterozygous and homozygous genotypes were significant, and also effects of dominance were found. Those can be used for the calculation of 'genomic production values', i.e. a combination of additive genetic effects and of dominance. Furthermore, we suggest to use information from identified markers within candidate genes for optimizing genomic evaluations. Classical MAS implementations in Iranian Holstein dairy cattle might be feasible for the selection of bull dams in contract herds for elite matings, but such a strategy might be associated with risks when effects are not validated in the entire population.

Conflicts of interest

We confirm that we have no conflicts of interest.

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References

Akira, S., Takeda, K.T., 2004. Toll-like receptor signaling. Nat. Rev. Immunol. 4, 499–511.

Boichard, D., Fritz, S., Rossignol, M.N., Guillaume, F., Colleau, J.J., Druet, T., 2006. Implementation of marker-assisted selection: practical lessons from dairy cattle. In: Proceedings of the 8th World Congress on Genetics Applied to Livestock Production, 13–18 August, Belo Horizonte, Brazil.

Buenger, A., Ducrocq, V., Swalve, H.H., 2001. Analysis of survival in dairy cows with supplementary data on type scores and housing systems from a region of Northwest Germany. J. Dairy Sci. 84, 1531–1541.

Buitkamp, J., Ewald, D., Masbanda, J., Bishop, M.D., Fries, R., 2003. FISH and RH mapping of the bovine alpha (2)/delta calcium channel subunit gene (CACNA2D1). Anim. Genet. 34 (4), 309–310.

Dekkers, J.C.M., ten Haag, J.H., Weersink, A., 1998. Economic aspects of persistency of lactation in dairy cattle. Livest. Prod. Sci. 53, 237–252.

Falconer, D.S., Mackay, T.F.C., 1996. Introduction to Quantitative Genetics, 4th ed. Longmans Green, Harlow, Essex, UK.

Fleischer, P., Metzner, M., Beyerbach, M., Hoedemaker, M., Klee, W., 2001. The relationship between milk yield and the incidence of some diseases in dairy cows. J. Dairy Sci. 84, 2025–2035.

Fujii, J., Otsu, K., Zorzato, F., Leon, S.D., Khanna, V.K., Weiler, J.E., O'Brien, S.D., Maclennan, D.H., 1991. Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. Science 253, 448–451.

Gernand, E., Rehbein, P., von Borstel, U.U., König, S., 2012. Incidences of and genetic parameters for mastitis, claw disorders, and common health traits recorded in dairy cattle contract herds. J. Dairy Sci. 95, 2144–2156.

Goddard, M.E., Hayes, B.J., 2009. Mapping genes for complex traits in domestic animals and their use in breeding programmes. Nat. Rev. Genet. 10, 381–391.

Goldammer, T., Zerbe, H., Molenaar, A., Schuberth, H.J., Brunner, R.M., Kata, S.R., Seyfert, H.M., 2004. Mastitis increases mammary mRNA abundance of betadefensin 5, toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle. Clin. Diagn. Lab. Immunol. 11, 174–185.

Grisart, B., Coppieters, W., Farnir, F., Karim, L., Ford, C., Berzi, P., Cambisano, N., Mni, M., Reid, S., Simon, P., Spelman, R., Georges, M., Snell, R., 2002. Positional candidate cloning of a QTL in dairy cattle: identification of a missense mutation in the bovine DGAT1 gene with major effects on milk yield and composition. Genet. Res. 12, 222–231.

Henshall, J.M., Goddard, M.E., 1999. Multiple-trait mapping of quantitative trait loci after selective genotyping using logistic regression. Genetics 151, 885–894.

Heringstad, B., Klemetsdal, G., Steine, T., 2007. Selection responses for disease resistance in two selection experiments with Norwegian Red cows. J. Dairy Sci. 90, 2419–2426.

Hinrichs, D., Stamer, E., Junge, W., Kalm, E., 2005. Genetic analyses of mastitis data using animal threshold models and genetic correlations with production traits. J. Dairy Sci. 88, 2260–2268.

Hou, G.-H., Youan, Z.-R., Gao, X., Li, J.-R., Gao, H.-J., Chen, J.-B., Xu, S.-Z., 2010. Genetic polymorphisms of the CACNA2D1 gene and their association with carcass and meat quality traits in cattle. Biochem. Genet. 48, 751–759.

König, S., Sharifi, A.R., Wentrot, H., Landmann, D., Eise, M., Simianer, H., 2005. Genetic parameters of claw and foot disorders estimated with logistic models. J. Dairy Sci. 88, 3316–3325.

König, S., Swalve, H.H., 2009. Application of selection index calculations to determine selection strategies in genomic breeding programs. J. Dairy Sci. 92, 5292–5303.

Lopez-Villalobos, N., Spelman, R.J., 2010. Estimation of genetic and crossbreeding parameters for clinical mastitis, somatic cell score and daily yields of milk, fat and protein in New Zealand dairy cattle. In: Proceedings of the 9th World Congress on Genetics Applied to Livestock Production, 1–6 August, Leipzig, Germany.

Lund, M.S., Guldbrandtsen, B., Buitenhuis, A.J., Thomsen, B., Bendixen, C., 2008. Detection of clinical mastitis, somatic cell score, udder conformation traits, and assessment of associated effects on milk yield. J. Dairy Sci. 91, 4028–4036.

McGuire, K., Jones, M., Werling, D., Williams, J.L., Glass, E.J., Jann, O., 2005. Radiation hybrid mapping of all 10 characterized bovine Toll-like receptors. Anim. Genet. 37, 47–50.

Meuwissen, T.H.E., Hayes, B., Goddard, M.P., 2001. Prediction of total genetic value using genome-wide dense marker maps. Genetics 157, 1819–1829.

Miglior, F., Muir, B.L., Van Dormaal, B.J., 2005. Selection indices in Holstein cattle of various countries. J. Dairy Sci. 88, 1255–1263.

Miller, S.A., Dykes, D.D., Polesky, H.F., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16, 1215.

Moe, M., Lien, S., Aasmundstad, T., Meuwissen, T.H.E., Hansen., M.H.S., Bendixen, C., Grindflek, E., 2009. Association between SNPs within candidate genes and compounds related to boar taint and reproduction. BMC Genet. 10, 32.

Ogorevc, J., Kunej, T., Razpet, A., Dovc, P., 2009. Database of cattle candidate genes and genetic markers for milk production and mastitis. Anim. Genet. 40, 832–851.

Paape, M.J., Shafer-Weaver, K., Capuco, A.V., Van Oostveldt, K., Burvenich, C., 2000. Immune surveillance of mammary tissue by phagocytic cells. Adv. Exp. Med. Biol. 480, 259–277.

Park, Y.H., Joo, Y.S., Park, J.Y., Moon, J.S., Kim, S.H., Kwon, N.H., Ahn, J.S., Davis, W.C., Davies, C.J., 2004. Characterization of lymphocyte subpopulations and major histocompatibility complex haplotypes of mastitis-resistant and susceptible cows. J. Vet. Sci. 5, 29–39.

Pashmi, M., Qanbari, S., Ghorashi, S.A., Sharifi, A.R., Simianer, H., 2009. Analysis of relationships between bovine lymphocyte antigen DRB3.2 alleles, somatic cell count and milk traits in Iranian Holstein population. J. Anim. Breed. Genet. 126, 296–303.

Pimentel, E.C.G., Bauersachs, S., Tietze, M., Simianer, H., Tetens, J., Thaller., G., Reinhardt., F., Wolf, E., König, S., 2011a. Exploration of relationships between production and fertility traits in dairy cattle via association studies of SNPs within candidate genes derived by expression profiling. Anim. Genet. 42 (4), 346–356.

Pimentel, E.C.G., Erbe, M., König, S., Simianer, H., 2011b. Genome partitioning of genetic variation for milk production and composition traits in Holstein cattle. Front. Genet. 2, 9.

Ragot, M., Hoisington, D.A., 1993. Molecular markers for plant breeding: comparisons of RFLP and RAPD genotyping costs. Theor. Appl. Genet. 86, 974–984.

Rupp, R., Boichard, D., 2003. Genetics of resistance to mastitis in dairy cattle. Vet. Res. 34, 671–688.

Sabroe, I., Read, R.C., Whyte, M.K., Dockrell, D.H., Vogel, S.N., Dower, S.K., 2003. Toll-like receptors in health and disease: Complex questions remain. J. Immunol. 171, 1630–1635.

Sadeghi-Sefidmazgi, A., Moradi-Sharbabak, M., Nejati-Javaremi, A., Miraei-Ashtiani, S.R., Amer, P.R., 2011. Estimation of economic values and financial losses associated with clinical mastitis and somatic cell score in Holstein dairy cattle. Animal 5 (1), 33–42.

SAS Institute, 1999. SAS User's Guide, Statistics, Version 8 Developer's Edition. SAS Inst. Inc., Cary, NC.

Schierenbeck, S., Reinhardt, F., Reents, R., Simianer, H., König, S., 2011. Identification of informative cooperator herds for progeny testing based on yield deviations. J. Dairy Sci. 94, 2071–2082.

Sharma, B.S., Jansen, G.B., Karrow, N.A., Kelton, D., Jlang, Z., 2006a. Detection and characterization of amplified length polymorphism markers for clinical mastitis in Canadian Holsteins. J. Dairy Sci. 89, 3653–3663.

Sharma, B.S., Leyva, I., Schenkel, F., Karrow, N.A., 2006b. Association of toll-like receptor 4 polymorphisms with somatic cell score and lactation persistency in Holstein bulls. J. Dairy Sci. 89, 3626–3635.

Shizuo, A., Kiyoshi, T., Tsuneyasu, K., 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immun. 2, 675–680.

Spelman, R.J., Garrick, D.J., 1998. Genetic and economic responsed for within-family marker-assisted selection in dairy cattle breeding schemes. J. Dairy Sci. 81, 2942–2950.

Staudt, M., 2001. Gene expression physiology and pathophysiology of the immune system. Trends Immunol. 22 (1), 35–40.

Swanson, K.M., Stelwagen, K., Dobson, J., Henderson, H.V., Davis, S.R., Farr, V.C., Singh, K., 2009. Transcriptome profiling of *Streptococcus* uberis-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model. J. Dairy Sci. 92, 117–129.

Templin, C., Ghadri, J.-R., Rougier, J.-H., Baumer, A., Kaplan, V., Albesa, M., Sticht, H., Rauch, A., Puleo, C., Hu, D., Barajas-Martinez, H., Antzelvitch, C., Lüscher, T.F., Abriel, H., Duru., F., 2011. Identification of a novel loss-of-function calcium channel mutation in short QT-syndrome. Eur. Heart J. 32, 1077–1088.

Thaller, G., Krämer, W., Winter., A., Kaupe, B., Erhardt, G., Fries, R., 2003. Effects of DGAT1 variants on milk production traits in German cattle breeds. J. Anim. Sci. 81, 1911–1918.

VanRaden, P.M., 2008. Efficient methods to compute genomic predictions. J. Dairy Sci. 91, 4414–4423.

Wang, X., Shangzhong, X., Hongyan, R., Jinbao, C., 2007. Genetic polymorphism of TLR4 gene and correlation with mastitis in cattle. J. Genet. Genom. 34, 406–412.

Wellmann, R., Bennewitz, J., 2011. The contribution of dominance to the understanding of quantitative genetic variation. Genet. Res. 93, 139–154.

Wensch-Dorendorf, M., Yin, T., Swalve, H.H., König, S., 2011. Optimal strategies for the use of genomic selection in dairy cattle breeding programs. J. Dairy Sci. 94, 4140–4151.

White, S.N., Taylor, K.H., Abbey, C.A., Gill, C.A., Womack, J.E.S., 2003. Haplotype variation in bovine Toll-like receptor 4 and computational prediction of a positively selected ligand-binding domain. Proc. Natl. Acad. Sci. USA 100, 10364–10369.

Wolfinger, R., O'Connell, M., 1993. Generalized linear mixed models: a pseudo-likelihood approach. J. Stat. Comput. Simul. 48, 233–243.

Youngerman, S.M., Saxton, A.M., Oliver, S.P., Pighetti, G.M., 2004. Association of CXCR2 polymorphisms with subclinical and clinical mastitis in dairy cattle. J. Dairy Sci. 87, 2442–2448.

Yuan, Z.-R., Li, J., Zhang, L.-P., Zhang, L.-M., Chen, C., Chen, X.-J., Gao, X., Li, J., Chen, J.-B., Gao, H.-J., Xu, S.-Z., 2011. Novel SNPs polymorphism of bovine CACNA2D1 gene and their association with somatic cell score. Afr. J. Biotechnol. 10, 1789–1793.

Xu, Z., Taylor, J.A., 2009. SNPinfo: integrating GWAS and candidate gene information into functional SNP selection for genetic association studies. Nucleic Acids Res. http://dx.doi.org/10.1093/nar/gkp290.

Zhang, L.P., Gan, Q.F., Ma, T.H., Li, H.D., Wang, X.P., Li, J.Y., Gao, X., Chen, J.B., Ren, H.Y., Xu, S.Z., 2009. Toll-Like Receptor 2 Gene Polymorphism and its Relationship with SCS in Dairy Cattle. Anim. Biotechnol. 20, 87–95.

Zheng, J.A.D., Watson, A.D., Kerr, D.E., 2006. Genome-wide expression analysis of lipopolysaccharide-induced mastitis in a mouse model. Infect. Immun. 74, 1907–1915.