Detection and Characterization of PCR-SSCP Markers of the Bovine Lactoferrin Gene for Clinical Mastitis

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ABSTRACT : A total of 80 cows, including 40 top mastitis resistant and 40 top mastitis susceptible animals as Group I and Group II, were selected from a population of 520 cows based on clinical mastitis occurrence. PCR-SSCP analysis on four fragments within the 5'region and two fragments of Exons 4,15 of bovine lactoferrin (bLF) revealed that four fragments-P1,P4,E4,E15-had polymorphisms which totally included six base mutations, and only two of them had significant differences in allele frequencies between resistant and susceptible groups, P1 (53.7% vs. 70.0%, p<0.05) and P4 (55.0% vs. 68.8%, p<0.05). Further study on these two promising markers combined with the milk performance traits of cows demonstrated that their selection would result in higher fat percentage (p<0.05), lower Somatic Cell Score (SCS) (p<0.05) and Clinical Mastitis Residuals (CMR) (p<0.01) indicating higher mastitis resistance and lower milk yield (p<0.05). The putative transcription factor binding sites in the 5'region were also studied by using MatInspector 7.2.2 software, and two signal pathways regulating the expression of bLF including the NF-κB pathway and nuclear hormone receptor pathway were predicted. (**Key Words :** Cow, Lactoferrin, Clinical Mastitis, Polymorphism, PCR-SSCP)

INTRODUCTION

Mastitis, defined as inflammation of the mammary gland caused by microorganisms, is a most frequent, complex and costly disease in dairy cows (Kossaibati et al., 1998). It was predicted that mastitis would cost dairy farmers US$200 per cow annually, due to veterinary cost, loss of milk production and milk quality, and early culling (Schutz 1994). Although mastitis is influenced by many factors including both hereditary and environmental (D. Hanajima, 2004), considering the very different resistances of cows in the same environment it is still possible to select for high-resistance cows.

Lactoferrin, mainly found in milk, is a ferric ion (Fe^{3+})-binding glycoprotein and one member of the lactoferrin family. Bovine lactoferrin has broad-spectrum antibacterial action especially against the pathogenic bacteria responsible for mastitis (Seyfert, 1996). Gaunt (1980) found that the concentration of bLF in mastitic cows was much higher than in normal cows and even for the same cow the concentration of bLf varied during different physiological phases which indicated that the Lf gene not only had a certain relationship with mastitis, but had high flexibility in expression. Seyfert (1994) described the structure of the bovine lactoferrin encoding gene and its promoter; Li (2004) studied polymorphisms in the lactoferrin gene and their relationship with mastitis using the somatic cell count (SCC) as an indicator and found no direct relationship between SCC and mastitis. However, recent studies (Klungland, 2001; Schulman, 2004) found an absence of common quantitative trait loci (QTL) positions for both clinical mastitis and SCC, which indicated that clinical mastitis and SCC were probably two different traits.

Therefore, the objectives of this study were to use PCR-SSCP technique to analyze the polymorphisms in the partial sequence of bLf, to screen the marker closely linked to the mastitis resistance and to make an estimation of the marker effect on function and production traits based on clinical cases. Due to lack information on the molecular mechanism of regulation and expression of the bLf gene, the MatInspector 7.1.1 (http://www.genomatix.de) was used to predict the putative transcription factor binding sites in the 5'-flanking region.
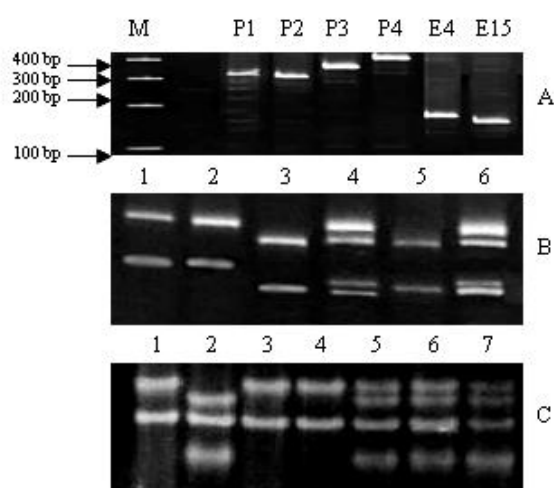
MATERIAL AND METHOD

Animals

A total of 523 cows were selected from the farm and were analyzed. The complete information on clinical cases,

A. The PCR result of six paired primers, P1, P2, P3, P4, E4, E15; M: marker; B. PCR-SSCP polymorphisms of P1, 1-6: AA,AA,BB,AB,BB, AB; C. PCR-SSCP polymorphisms of P3, 1-7: AA,BB,AA,AA,AB,AB,AB

Figure 1. The electrophoresis images of PCR results, SSCP polymorphisms.

year in risk, lactation number and production traits in four years was obtained. The factors affecting the number of CM cases were determined by SAS GLM (SAS 8.2) using the following model:

$$Y_{jkl} = L_j + \beta X_k + e_{jkl}$$

where Y_{jkl} = number of CM cases, L_j = fixed effect of jth lactation (k = 1, 2, 3+), X_k = number of years for which an individual remained in the herd, β = regression of years for which an individual remained in the herd, and e_{jkl} = random residual effects of clinical mastitis (CM) cases, referred to as clinical mastitis residual (CMR). Individuals were selected according to the distribution of the CMR. Two groups were formed by selecting 40 animals having the lowest CMR values as the resistant group, group I, and 40 animals having the highest CMR values as the susceptible group, group II. In group I, CMR was -0.96±0.12, ranged from -1.19 to -0.79 with no clinical cases. In group II, the CMR was 2.89±1.11, ranged from 1.67 to 6.96 with 2 to 6 clinical cases. Whole blood samples of 10mL per cow with ACD anticoagulant were collected for DNA isolation.

PCR-SSCP analysis

The primers of four fragments covering the 5' flanking region of 1149 bp including the P1 (forward 5'-ggc cac acc tca cag cat at-3' reverse 5'-ggc aat atg ag gtg gga gac a-3'), P2 (forward 5'-gga caa aat agg aca ttt at-3' reverse 5'- tct ttg cgg atc agc ttg ac-3'), P3 (forward 5'-tgg ttc cca agc act tta ga-3' reverse 5'-ccc gag gtc tgc tgg ttc ct-3'), P4 (forward 5'-cta ggc agc gct ggg gaa ct-3' reverse 5'-acc tgc act cac caa ggg ct -3') and two fragments for exons 4, 15

including E4 (forward 5'-gct ccc caa acc cac tat tag c-3' reverse 5'-gag ggg ctc gag tga ctc tgt-3'), E15 (forward 5'-gaa tcg tga gga ctt cag gt-3' reverse 5'-tta cct gct ggt gga gca gca -3') were designed according to the sequence published in the GenBank (AH000852) by using the Prime Primer 5.0 soft-assistant designer. All primers of these PCR amplifications were annealed at 58 to 61°C and then extended at 72°C for 30 s. The products were separated by native polyacrylamide gels and then detected by silver staining. Genotypic and allele frequencies of the polymorphisms were calculated for the two groups to find the most promising marker linked with mastitis resistance.

Sequence analysis

All PCR products of various genotypes found in the SSCP polymorphisms were cloned and sequenced. The PCR product sequences from wild and mutated homozygotes were initially analyzed by DNAStar3.0. The putative transcription factor binding sites within the 5' flanking region were analyzed by MatInspector professional 7.2.2 from the Genomatix Software Complex (http://www.genomatix.de) and the changes of binding sites induced by mutations were also predicted.

Estimation of marker associated effects

All milk performance records of 80 cows were analyzed to estimate marker effects on various milk performance and functional traits. A general trait-based analysis, which accounts for selective genotyping, was carried out by logistic regression. The following model (Henshall, 1999) was employed for predicting genotype of an individual as a response variable with production traits and CMR as explanatory variables:

$$\text{logit } [p(AB)] = \log[p(AB)/1-p(AB)] = a+bX$$

where, p = probability of AB genotype, a = intercept, b = regression coefficient, and X = production traits of interest. The effect of difference between various genotypes on various traits was estimated by principles described by Henshall (1999) as:

$$2\left[\left\{-1+\sqrt{(1+b^2\sigma^2)}\right\}\Big/b\right]$$

where σ^2 is an estimate of variance for a trait from all data from which individuals were selected. The significance of the effects was determined on the basis of significance level of b, given their direct relationship.

RESULT

PCR results and SSCP polymorphisms

The predicted sizes of six fragments, P1, P2, P3, P4, E4,

Table 1. Genotype distributions and allele frequencies of PCR-SSCPs for the bLF gene in two groups

		P1		P4		E4		E15	
		I	II	I	II	I	II	I	II
Genotype	AA	0.15	0.48**	0.15	0.45**	0.30	0.33	0.43	0.40
frequencies	AB	0.78**	0.45	0.80**	0.48	0.40	0.48	0.47	0.43
	BB	0.07	0.07	0.07	0.07	0.30	0.19	0.10	0.17
Allele	A	0.54	0.70*	0.55	0.69*	0.50	0.56	0.66	0.61
frequencies	B	0.46*	0.30	0.45*	0.31	0.50	0.44	0.34	0.39

* Means significantly different (p<0.05); ** Means extremely different (p<0.01).

Table 2. Average difference between AB and AA genotypes for significant traits

Traits	Difference AB vs. AA		Corrected significance
	General unit	SD unit	
Average milk (kg)	-650	0.82	p<0.038
Average fat percent	0.15	0.47	p<0.041
Average SCS	-0.10	0.65	p<0.021
CMR	-0.24	0.28	p<0.001

E15 were 325 bp, 313 bp, 341 bp, 398 bp, 177 bp, 158 bp which were successfully amplified. PCR results are shown in Figure 1A. Nucleotide variances in P1, P4, E4, E15 were found through SSCP analysis. All of four polymorphism fragments had two allele genes A and B, and three genotypes, AA, AB and BB. In this paper, partial results are shown in Figure 1B and 1C.

Sequence analysis

After sequencing, there were two mutations, T to G at -945 and G to A at -928 in P1; two mutations, C to A at -28 and G to C at +33 in P4; one mutation, A to G at 118 in E4 which induced an amino acid mutation of bovine lactoferrin, Ile to Val at 126; one mutation, T to C at 166 in E15 which did not induce any change in amino acids of bLF. The putative transcription factor binding sites in the 5' flanking region were also analyzed by using the MatInspector professional 7.1.1. The results indicated that there were some putative transcription factor binding sites in the promoter of bLF including estrogen receptor, CRE receptor, RAR receptor, NF-κB, AP1.

Genotype and allele genes frequencies

The results of the genotype and gene frequencies of the four polymorphism fragments in two groups are shown in Table 2. By using a x^2 independent test, genotypic and allele frequencies of these four fragments were calculated and tested for both CMR groups. The results showed that only P1 and P4 fragments confirmed a significant difference in genotypic frequencies (p<0.01) and allele frequencies (p<0.05), whereas, E4 and E15 showed no difference in both genotypic and allele frequencies in the two groups. The results (see Table 1) indicated that the major genotypes of the resistant animals were heterozygous, AB genotypes in P1 and P4 fragments, and susceptible animals were more frequently homozygous, AA genotypes in P1 and P4

fragments. So the AB genotypes of P1 and P4 could be the markers of mastitis resistance. Meanwhile it was also found that the mutations of P1 and P4 fragments always occurred in the same individuals, and most of the heterozygous genotypes in P1 and P4 were also uniform in individuals, which indicated that the heredity of P1 and P4 were tightly linked.

Estimation of milk performance traits on predicting genotypes and marker associated effects

Due to the tight linkage between P1 and P4, they had the same associated effects on production traits. So, only the result of P1 fragments is shown here. Eighty cows which consisted of 25 AA, 49 AB, 6 BB were accessed. Given that the logistic regression model only permits two response categories (Henshall, 1999) and the small number of homozygous BB cows, the most frequent genotype classes (AA and AB) were used in the analysis. After the principle component analysis of the correlation matrix of 8 traits, the first three principal components including the average milk yield, average fat percent and average SCS which were obtained accounted for over 61 percent of the total variation. To control false positives when testing the marker for association with multiple-traits, a modified three-level Bonferroni correction was applied (the critical P-value 0.05/3 = 0.0167). The maximum likelihood estimates of regression coefficients (b) for the various traits and their significance, where AB genotype was considered as success (i.e. 1) and AA genotype as failure (i.e. 0), were obtained. The difference between AB and AA (Table 2) indicated that the selection would result in significantly higher fat percent (p<0.05), lower SCS (p<0.05) and CMR (p<0.01) indicating higher mastitis resistance, and lower milk yield (p<0.05).

DISCUSSION

A new mutation in the bLF promoter (T to G at -945) was found, but a G insertion at -478 reported by Li (2004) was not detected. PCR-SSCP is a method to detect DNA sequence changes based on the shift in electrophoretic mobility. Not only was the electrophoretic mobility sensitive to the size and shape of the particle, but it is also strongly dependent on environmental conditions within the

gel such as temperature and ion concentration (Hayashi, 1991). So it could be one reason that most of fragments we amplified were more than 300 bp instead of the best length for SSCP, 100 to 300 bp, due to the requirements of primer design.

The effective selection of mastitis resistance has been taken more seriously due to the detrimental impact of mastitis (Rachel, 2003). So far, somatic cell counts (SCCs) in milk have been integrated in breeding programs as indicators for assessing mastitis resistance (Jong, 1996; Shem, 2002), because many studies indicated a positive and moderate to high genetic correlation between clinical mastitis (CM) and SCC with estimates averaging 0.7 (Heringstad, 2000; Shem, 2001). However, recent studies (Klungland, 2001; Schulman, 2004) declared the absence of common quantitative trait loci (QTL) positions for both CM and SCC. Meanwhile, some authors (Kelm, 1997; Schukken, 1997) argued that continuously decreasing SCC by selection could impair the whole cow's immunity, because the SCC represents the capacity for leukocyte recruitment. Elbers (1998) found that the quarters with lower initial SCC had higher risk of being infected by mastitis pathogens, and the SCC is very varied according to age, lactation phase and even different times in a day (Othmane, 2002). Therefore, in this study, the statistical model based on the clinical cases instead of the SCC was used because the clinical mastitis is easier to diagnose than SCC, and has a tighter relationship with mastitis resistance than SCC. Li et al. studied the polymorphisms in the lactoferrin gene and its relationship with mastitis on the basis of the somatic cell count (SCC) and concluded that there was no direct correlation between SCC and mastitis, whereas in our study, based on the clinical cases, two markers closely linked with clinical mastitis were obtained. The inconsistent results may be induced by the different screening standards. Selection using markers would result in higher resistance and lower production performance after analyzing the associated marker effect combined with the production traits, which is a very common problem in mastitis resistance selection (Rachel, 2003). So it would be dependent upon the economic analysis of including these markers in the breeding objectives to verify whether these markers could be practical for mastitis resistance selection.

The putative transcription factor binding sites in the 5' flanking region were studied due to lack of study on the molecular mechanisms of bLf gene expression and regulation. It was definitely known that the concentration of bLf in mastitic milk was much higher than in normal cows. At the same time, even for the same cow, the concentrations of bLf were variable during the different lactation phases which were regulated by hormones (Tsui, 1990; Seyfert, 1994). Therefore, probably two pathways including the

infection responsive pathway and hormone regulation pathway were involved in regulating the expression of bLf. Correspondingly, in the 5' regulation region, two kinds of transcription factor binding sites were obtained, including steroid hormone receptors (estrogen receptor, CRE receptor, and RAR receptor) and infection responsive transcription factors (NF-κB, AP1). Estrogen receptor and RAR receptor are the key proteins in the nuclear hormone pathway (Caelles, 1997) and NF-κB plays a central role in immune and inflammatory responses through its ability to enhance the expression of proinflammatory genes (Caamano, 2002). So the expression of bLf gene was probably regulated by these two signal pathways including the NF-κB pathway and nuclear hormone receptor pathway which were predicted.

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