Comparison of Natural Resistance-associated Macrophage Protein (*NRAMP*)1 Expression between Cows with High and Low Milk Somatic Cells Counts

Y. S. Joo, J. S. Moon, L. K. Fox[1], G. H. Suh[2], N. H. Kwon[3], S. H Kim[3] and Y. H. Park*[,1]

National Veterinary Research and Quarantine Service, Anyang-si, 480, Gyeonggi-do, 430-824, Korea

ABSTRACT : Studies using natural resistance-associated macrophage protein (*NRAMP*) identification indicated that cattle could be selected for immunity. Several studies performed on intracellular organisms such as *Mycobacterium*, *Salmonella, Brucella* and *Leishmania* in human and mouse revealed that resistance against these bacteria was dependent on high activity of *NRAMP*1 in macrophages. However, hardly any researches have been done on *Staphylococcus aureus* in bovine mastitis, which is an intracellular organism and the main cause of bovine mastitis. The objectives of this study were to establish reverse transcriptase polymerase chain reaction (RT–PCR) methods, through which *NRAMP*1 mRNA expression could be compared and analyzed between mastitis-resistant and -susceptible cows. *NRAMP*1 gene and its expression were investigated using 20 cows (Holstein Friesian) in Korea. Cows were evenly split into two groups, with and without histories of clinical mastitis. Equivalent numbers of cows were randomly selected from each group. Monocytes were isolated from the bovine peripheral blood of each selected cows and activated with lipopolysaccharide (LPS). mRNA was separated from the monocytes and cDNA of *NRAMP*1 was synthesized and amplified using RT-PCR with amplification of β-actin as a control. The difference in *NRAMP*1 expressions of mastitis-resistant (n=10) and -susceptible (n=10) Holstein cows was analyzed. Results demonstrate that resistant cows produced more *NRAMP*1 mRNA than the susceptible ones, and ratios of *NRAMP*1:β-actin expression were higher in resistant cows with or without LPS activation. Therefore, this study could be applied to select bovine mastitis resistant cows before infection based on the expression of *NRAMP*1. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 12 : 1830-1836)***

Key Words : *NRAMP*1, RT-PCR, Bovine mastitis, *Staphylococcus aureus*

INTRODUCTION

Selection for disease resistance has been advocated as a mechanism to control mastitis. Studies using Bovine Leukocyte Antigen (BoLA) typing or natural resistance-associated macrophage protein (*NRAMP*) identification indicated that cattle could be selected for immunity (Lewin et al., 1999). The objective of both BoLA typing and *NRAMP* analysis was to reduce the pecuniary loss through the early detection of individuals with high resistance to the disease. Monocytes, macrophages, and neutrophils play important roles in the nonspecific immune responses of mammary mucosa during the early infection of mastitis and are essential for the eradication of pathogens and antigen processing. They are more bactericidal and/or bacteriostatic when the expression of *NRAMP* is induced by pathogens. However, they also offer refuge to intracellular pathogens such as *Staphylococcus aureus* thus causing difficulty in disease treatment (Sanchez et al., 1993). Therefore, studies on *NRAMP*1 of these cells, which participated in the phagolysosome activity against intracellular organisms was in need.

The *NRAMP* family, whose genetic sequences are highly conserved in humans, mice, sheep, porcine, and cattle, plays a key role on the homeostasis of iron and other metals as divalent metal transporters, among which *NRAMP*2 acts as an iron uptake protein in both the duodenum and peripheral tissues. *NRAMP*1, on the other hand, functions as a divalent-metal efflux pump in the phagosomal membrane of macrophages and neutrophils, and its mutation cause susceptibility to several intracellular pathogens. It is rapidly recruited into the membrane of maturing phagosomes, and is suggested to modulate microbial replication by altering the bacteriostatic and bactericidal properties of the fused phagosome. Mammalian and bacterial *NRAMP*s would compete for the same essential substrates within the microenvironment of the phagosome, in addition to the interface of host-pathogen interactions.

Several studies have been performed on intracellular organisms such as *Mycobacterium*, *Salmonella, Brucella,* and *Leishmania* in human and mouse, which revealed the high activity of *NRAMP*1 (Belouch et al., 1995). However, hardly any have been done on *Stapylococcus aureus* in

* Corresponding Author: Yong Ho Park, Tel: +82-2-880-1257, Fax: +82-2-871-7524, E-mail: yhp@snu.ac.kr

[1] College of Veterinary Medicine, Washington State University, Field Disease Investigation Unit, Pullman, WA 99164-6610, USA.

[2] Veterinary Medicine Research Laboratory, Department of Livestock Improvement and Improvement Technology Research, National Livestock Institute, Rural Development Administration, Seongnam-myeon, Cheonan-si, Chungcheongnam-do, 330-891, Korea.

[3] Department of Microbiology, College of Veterinary Medicine and School of Agriculture Biotechnology, Seoul National University, Shillim-dong, Kwanak-gu, 151-742, Korea.

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Table 1. Rearing conditions of cows

Group	No.	Birth	Weight (kg)	Feeds (kg)			
				Commercial dry feed		Corn	Dry wheat
				Wet period	Dry period		
S[1]	1	1992	850	10	2	25	4
	2	1995	700	9	2	24	4
	3	1991	900	11	2	26	4
	4	1994	750	9.5	2	24.5	4
	5	1991	800	10	2	25	4
	6	1994	870	10	2	25	4
	7	1993	750	9.5	2	24.5	4
	8	1992	700	9	2	24	4
	9	1996	650	9	2	24	4
	10	1994	750	9.5	2	24.5	4
R[2]	1	1994	750	9.5	2	24.5	4
	2	1996	650	9	2	24	4
	3	1996	650	9	2	24	4
	4	1996	675	9	2	24	4
	5	1996	650	9	2	24	4
	6	1993	800	10	2	25	4
	7	1995	675	9	2	24	4
	8	1996	650	9	2	24	4
	9	1993	800	10	2	25	4
	10	1994	750	9.5	2	24.5	4

[1] Susceptible to mastitis. This group had more than three treatments for bovine mastitis. [2] Resistant to mastitis. This group had no history of medical treatment for mastitis. There were no restriction of feed and water. Energy level was calculated by means of body condition score (BCS), and BCSs of all experimental animals were ranged from 2.5-4.0 with no difference between mastitis-susceptible and -resistant cows.

Table 2. Somatic cell counts in raw milk during four-year period (×1 thousand cells/ml)

Group	No. of cows	1997	1998	1999	2000
Resistant to mastitis	10	172.6±88.2[1]	76.7±69.8	85.7±61.2	85.3±53.1
Susceptible to mastitis	10	314.5±435.6	527.2±580.6	525.2±605.7	440.2±533.6

[1] Mean±standard deviation (SD).

bovine mastitis, which is an intracellular organism and the main cause of bovine mastitis, although some studies on the genotyping of *S. aureus* associated with mastitis of cows had been presented (Joo et al., 2001). In addition, few studies have analyzed the expression of *NRAMP*1 in mammalian tissues. The objectives of this study were to establish reverse transcriptase polymerase chain reaction (RT–PCR) methods, through which *NRAMP*1 mRNA expression could be compared and analyzed between mastitis-resistant and -susceptible cows.

MATERIAL AND METHODS

Experimental animals

Holstein Friesian cows raised by the National Livestock Research Institute, the Rural Development Administration, Korea, were used (Table 1). Mastitis infection frequency, frequency of medical treatments, and treatment conditions recorded over a four-year period since 1997 were used to select animals. Animals were divided into two groups: resistant group, with no history of medical treatment for mastitis, and susceptible group, which had more than three treatments for bovine mastitis. Milk somatic cell counts (SCC) were determined using Foss 300 and Foss system 4000 (Foss Electric Co., Denmark). SCC of the resistant group averaged below 200 thousands/ml, which of the susceptible group was higher during the four-year period (Table 2). Furthermore, to verify the mastitic pathogen, bacteria were isolated when SCC was higher than 500 thousands/ml in whole milk during the lactating period. To establish the *NRAMP*1 mRNA detection method, we used five each bovine mastitis-resistant and -susceptible cows, which were randomly picked from each group. In addition, to analyze the expression capacity of *NRAMP*1 through the established method, 10 cows from each group were used.

Isolation and identification of mastitis pathogens from milk

The method of Joo et al. (2001) was applied for the isolation of pathogens from mastitis-susceptible cows. In brief, each milk sample of quarters of mastitis-susceptible cows was cultured on 5% sheep blood agar (KOMED, Korea) and incubated at 37°C for 48 h. Bacterial colonies presumptively identified as staphylococci by colony characteristics, catalase reaction, and hemolytic patterns were speciated following the National Mastitis Council

protocols (Harmon et al., 1990). Those isolated were analyzed further using the API staph system (Biomerieux, France) and coagulase test.

Separation of monocytes from bovine peripheral blood and sensitization

Method used for leukocyte separation was based on that proposed by Barta (1993). Ten ml each blood samples were collected from both groups in heparinized vacutainer (Becton Dickinson, USA), into which 3 ml RPMI 1640 (Sigma, USA) medium was added. The collected samples were then transported to the laboratory. Final volumes of the samples were adjusted to 20 ml with RPMI 1640. Diluted blood samples were centrifuged at 330 g and 20°C for 30 min. Obtained buffy coat was diluted with Hank's Balance Salt Solution (Gibco BRL, USA) and centrifuged at 250 g for 10 min. Pellets were resuspended with RPMI 1640 and washed twice. A total 10 ml of RPMI 1640 with 10% fetal bovine serum (Gibco) was added and incubated in a 5% CO_2 incubator (Napco, USA) for 1 h. The cells attached to the bottom were collected. Monocytes were separated from the leukocyte based on their ability to attach to plastics. To activate *NRAMP*1 in the monocyte, 20 ng/ml *S. enterica* serovar Typhimurium lipopolysaccharide (LPS; Sigma) was added and incubated in a 5% CO_2 incubator for 6 h at 37°C. Controls were treated with RPMI 1640 only.

Pure separation of RNA

The method of Favaloro et al. (1980) was applied for the purification of RNA. Monocytes obtained were collected and centrifuged at 2,500 g for 10 min. Supernatant was aspirated, into which 100 μl of 3 M sodium acetate (pH 5.2) was added and mixed. Subsequently 500 μl cold ethanol was added and mixed well. After the mixture was centrifuged at 5,000 g for 10 min, the obtained pellet was dried at room temperature. The above procedure was repeated twice, the pellet was dissolved with 50 μl of 0.02 M EDTA (pH 8.0; Sigma) and an equal volume of 4-chloroform:1-1-butanol (4:1) (Gibco) was added. The mixture was then shaken and centrifuged. Collected supernatant was treated with 4 M sodium acetate (pH 7.0) and centrifuged. Obtained pellet was dissolved with 0.2% SDS (Bio-rad, USA) and 0.05 M EDTA (pH 8.0). RNA was isolated using cold ethanol. RNA purity and quality were determined using a spectrophotometer (Pharmacia, LKB Mod.80-2, USA) at 260/280 nm.

Synthesis of cDNA and RT-PCR

RT mixture for cDNA synthesis consisted of 1 μg RNA, avian myeloblastosis virus reverse transcriptase (15 U/μg; Gibco), 5 mM $MgCl_2$, 1 mM 2'-deoxynucleoside 5'-triphosphate (dNTP; Promega, USA), Oligo dT primer (0.5 μg; Bioneer, Korea), 10 mM Tris- HCl, 50 mM KCl, 0.1% Triton X-100 (Sigma), and 20 μl distilled water. Reaction was performed at 42°C for 30 min.

Primers for *NRAMP*1 were designed based on the complete bovine cDNA sequences in GenBank, with accession number U12862. The sense and antisense primers were 5'CGTGGTGACAGGCAAGGA3' (positioned at 406-423 bp) and 5'CGAGGAAGAGGAAGAAGAAAG3' (positioned at 626-606 bp), respectively. Control primers used for the production of β-actin were 5'ACGTGG CCCTGGACTTCGAGCAGG3' (positioned at 653-676 bp) and 5'GCTGGAAGGTGGACAGCGAGGCCAGGA3' (positioned at 1,060-1,034 bp), which were designed based on GenBank (accession number AY141970). The PCR mixture was composed of 10 μl RT product, 2 mM $MgCl_2$, 200 μM dNTP, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1.25 U *Taq* polymerase (Promega), and 0.5 mM primer pairs. Thermal cycler used was GeneAmp PCR System 2400 (Perkin Elemer, USA) under the following conditions: initial denaturation for 2 min at 95°C, followed by 35 cycles of 94°C for 1.5 min, 59°C for 1 min, and 72°C for 2 min.

Amplified cDNA fragments were fractionated through 1.5% agarose gel (Sigma) electrophoresis in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA), at 50 V/cm for 30 min. Gels were stained with ethidium bromide (Sigma) and imaged with Advanced American Biotechnology Software Restriction Fragment Length Polymorphism (AAB software, USA). Based on the image analysis, band concentration was calculated in terms of optical density (OD) and normalized with β-actin.

Twenty μl of the *NRAMP*1-PCR product was cleaved with 20 U restriction enzyme *Msp* I (Promega) according to the manufacturer's instruction, and was subsequently precipitated in 2.0 M ammonium acetate and 2.5 volumes of ethanol. The pellet was dissolved in 12 μl of TE buffer, mixed with gel loading buffer, and separated on a 3% agarose gel in 0.5X TBE at 50 V/cm for 30 min. The gel was then stained and photographed.

Statistical analysis

*NRAMP*1 mRNA expression were subjected to SAS (ver 6.12 SAS institute, NC) for *t*-test to compare mastitis-susceptible and -resistant cows, and paired *t*-test was used to analyze the difference between before and after activation with LPS ($p<0.05$).

RESULTS

Identification of mastitis pathogens from mastitis-susceptible cows

S. aureus and *S. epidermidis* were the major pathogens isolated from milk sample of mastitis-susceptible cows. Especially, *S. aureus* were identified from more than one

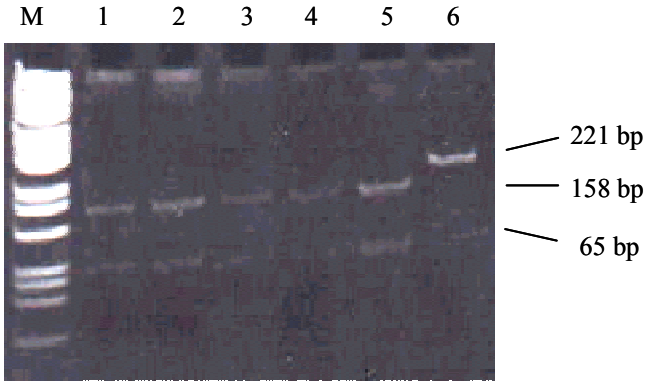

Figure 1. Electrophoretic analysis of RT-PCR products of NRAMP1 gene after digestion with *Msp* I (lane M: moleculer maker (pGEM) lanes 1-5: RT-PCR products of NRAMP1 gene after digestion with *Msp* I, lane 6: No digestion).

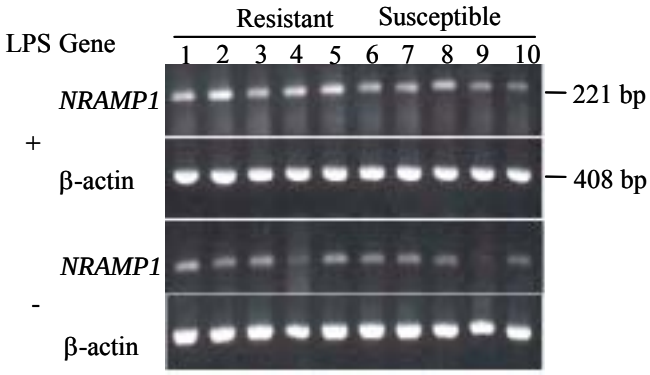

Figure 2. Expression of *NRAMP*1 mRNA in monocytes before and after activation with lipopolysaccharide (LPS) from bovine (lanes 1-5, mastitis-resistant cows; lanes 6-10, mastitis-susceptible cows).

Table 3. Comparison of *NRAMP*1 m RNA expressions of monocytes activated with lipopolysaccharide in bovine mastitis-resistant and -susceptible cows

No.	1	2	3	4	5	6	7	8	9	10	Mean±SD
R^2 OD[1]	1.475	1.370	0.500	1.100	0.700	1.275	1.730	0.520	1.850	0.275	1.080±0.551
S^3	0.075	0.275	1.575	1.220	0.125	0.050	0.350	0.175	0.275	0.200	0.432±0.524

[1] Optical density: $OD_{after\ activation}$-$OD_{before\ activation}$. [2] Resistant to mastitis. [3] Susceptible to mastitis. $p<0.05$ ($p=0.01491$).

quarter of each dairy cow with high SCC (>500 thousands cellls/ml).

Detection of *NRAMP*1 mRNA by RT-PCR

*NRAMP*1-specific primers designed for RT-PCR revealed the existence of *NRAMP*1 specific 221 bp through electrophoretic analysis. Detected gene products digested with Msp I also showed *NRAMP*1-specific band pattern (Figure 1). Based on this result, a method which could measure the capacity of *NRAMP*1 mRNA expression in bovine monocytes was established.

Production of *NRAMP*1 mRNA derived from activated monocytes with LPS

To detect the individual bovine capacity for *NRAMP*1 expression, monocytes from peripheral blood were sensitized with LPS for 6 h. The mean OD values of pre- and post-activations of *NRAMP*1 expression were 0.3 and 0.98, respectively. Results showed that the degree of *NRAMP*1 gene expression after sensitization with LPS was significantly elevated than that of pre-sensitization ($p<0.05$), although the increased amounts were different among individuals within resistant or susceptible groups.

Comparison of *NRAMP*1 mRNA expression between bovine mastitis-resistant and -susceptible cows

*NRAMP*1 mRNA expression data (Table 3) showed that OD values of mastitis-resistant cows varied from 0.275 to 1.850 (1.080 average). On the other hand, OD values of susceptible cows varied from 0.05 to 1.575 with a mean value of 0.432, which were more than twofold lower than that of the resistant ones. The *t*-test indicated that the high *NRAMP*1 gene activity was more resistant to mastitis ($p<0.05$).

The *NRAMP*1 mRNA expression was compared between bovine mastitis-resistant and -susceptible cows and also between post- and pre-activations of both cow types (Figure 2). The average ratios of *NRAMP*1:β-actin expression for each group are as follows: those of resistant and susceptible groups after activation were 1.046±0.254 and 0.503±0.122, and before sensitization were 0.454±0.185 and 0.152±0.053, respectively. The ratios of *NRAMP*1:β-actin expression were different between resistant and susceptible cows with or without LPS activation ($p<0.05$), whereas no difference was observed in β-actin expression between the two groups regardless of activation.

DISCUSSION

Bovine mastitis, one of the most significant diseases in livestock industries, has been a subject of many researchers' studies as milk has been because it has caused much pecuniary loss to farmers (Kalorey et al., 2001; Shem et al., 2001; Dhaka et al., 2002; Mukhopadhyaya and Mehta, 2002; Shem et al., 2002; Mukherjee and Dash, 2003). Endeavors to select individuals genetically resistant to mastitis were taken as a line in the chain of these studies,

and we established the RT–PCR methods for the detection of *NRAMP*1 as a marker of mastitis-resistant characteristics. The primers used in this study amplified the exact size of *NRAMP*1, and digested product was also revealed expected products. Digested sizes of the two fragments were 158 and 65 bp, which were similar to those of humans (Belouchi et al., 1995; Kishi et al., 1996). According to our results, mastitis-resistant cows showed higher level of *NRAMP*1 expression than susceptible ones. It suggests that individuals with high *NRAMP*1 gene activity are not predisposed to mastitis, and cope with the infection of mastitis-causing pathogens effectively, because elevated *NRAMP*1 expression was confirmed especially after LPS treatment. Therefore, detection of *NRAMP*1 gene activity with RT-PCR could be a good indicator for the early detection of mastitis-resistant cows.

In porcine alveolar macrophages, peripheral blood mononuclear cells, and peripheral blood polymorphonuclear neutrophils, *NRAMP*1s were expressed in response to LPS (Zhang et al., 2000). Up-regulation of *NRAMP*1 occurred after a 6 h stimulation, and the induction was highly sensitive to LPS; as little as 10 ng/ml of LPS induced a level of expression close to the maximum induced by 1 μg/ml. This was similar with our study, in which mononuclear cells were sensitized with 20 ng/ml of LPS for a 6 h incubation period.

Several hypotheses have been made on the sites where *NRAMP* functions, including the phagosome, lysosome, cell membrane, and nuclear membrane. One report described that large quantity of *NRAMP*1 existed in phagolysosomes of macrophage and neutrophil, which reinforced the bactericidal activities about intracellular pathogens (Cellier et al., 1994; Hu et al., 1995; Govoni et al., 1995). Antibodies raised against *NRAMP*1 demonstrated that the protein was localized to late endosomal and lysosomal compartments, but not to early endosomes and outer macrophage membranes (Gruenheid et al., 1997; Blackwell et al., 2000). These results have important implications for any direct role that *NRAMP*1 might have in determining intracellular pathogenic survival.

Although it is certain that *NRAMP*1 plays a key role during intracellular pathogenic infection, some controversial results have also been reported. Vidal et al. (1995) analyzed *NRAMP*1 sequences of 27 mice and discovered that *Salmonella*-susceptible mouse had mutation at the second domain, in which Asp replaced Gly; that is, mutation of *NRAMP*1 had an effect on the susceptibility. Whereas the bactericidal activity of macrophage against *Mycoplasma* and *Salmonella* decreased in the *NRAMP*1 mutant, no effects were observed on *Listeria monocytogenes* and *Legionella pneumophila* (Swanson et al., 1983; Sanchez et al., 1988; Roach et al., 1991; Malo et al., 1994). Furthermore, the bactericidal activity of bovine macrophages regulated by *NRAMP*1 was superior against *S. dublin, B. abortus,* and *M. bovis,* but not against *S.* Typhimurium (Qureshi et al., 1996). In addition, recent studies failed to show evidence of association between *NRAMP*1 and tuberculosis resistance or susceptibility in cattle (Barthel et al., 2000) and higher *NRAMP*1 expression in healthy cattle than in *M. bovis*-infected bovines (Estrada-Chavez et al., 2001). These conflicting results might be due to little available information on *NRAMP*1 and its varying interactions with different intracellular pathogens. From this point of view, the present study is able to present a correlation of *NRAMP*1 and resistance against intracellular pathogens such as *S. aureus* in which few studies have been undertaken.

According to Brown et al. (1997), who studied the mRNA expressions of *NRAMP*1, TNF-α, and iNOS against *Mycobacterium*, much higher expression of *NRAMP*1 mRNA was observed in bacille Calmette-Guerin (BCG)-resistant (Bcg^R) mouse than in BCG-susceptible (Bcg^S) mouse after sensitization with LPS, IFN-γ, and corticosterone. The continuation of expression was also observed upto 50 h in Bcg^R mouse, but dipped below 20% in Bcg^S mouse after 20 h. This result corresponded to our study that revealed the high expression of *NRAMP*1 gene in mastitis-resistant cows.

Some evidences were presented that *NRAMP*1 played a role in autoimmune diseases as well as infectious disease susceptibility (Blackwell et al., 2000). In human *NRAMP*1, Z-DNA-forming polymorphic repeats in the promoter region, which influence gene expression as functional polymorphism, were discovered (Searle et al., 1999). Four alleles and their different frequencies have been observed. *NRAMP*1 expression was enhanced or reduced by these alleles, whose response was highly dependent on the presence of external stimuli including LPS. Understanding the function of *NRAMP*1 in macrophages is an important prerequisite to understanding its complementary role in the infectious disease of cows. Therefore, further study should be carried out on bovine *NRAMP*1 function to control diseases, particularly major mastitis-causing intracellular pathogenic infection caused by *S. aureus*.

In conclusion, we established RT–PCR methods through which the aspect of *NRAMP*1 mRNA expression could be detected and the difference between mastitis-resistant and -susceptible cows analyzed. Results revealed that monocytes of resistant cows produced more *NRAMP*1 mRNA than susceptible ones in response to LPS challenge *in vitro*, and the average ratio of *NRAMP*1:β-actin expression was also higher in resistant group than the other group regardless of LPS stimulation. We estimate that the resistant group will show far better bactericidal and resistant capacities due to the enhanced phagolysosome activity when intracellular mastitic pathogens such as *S. aureus* invade the udder or alveolar cells.

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