Biotechnology in Animal Husbandry 24 (5-6), p 9-21 , 2008 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636.234

ACCOMPANIMENT OF THE POLYMORPHISM AT DEFENSIN GENE LOCI WITH MILK PRODUCTIVITY IN HOLSTEIN FRIESIAN AND EGYPTIAN COWS **

E. E. Hafez[1], S. M. Abdel-Rahman[2*], S. A. El-Sohaimy[1]

[1]Arid Lands Research Institute (ALRI), Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt.
[2]Genetic Engineering and Biotechnology Research Institute (GEBRI), Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt.
*Corresponding author: salahmaa@yahoo.com
**Original scientific paper - Originalni naučni rad

Abstract: Because of the antimicrobial role that defensins play in cows, genes encoding these peptides may be considered as molecular markers of a genetically determined susceptibility of the mammary gland to mastitis. In Friesian and Egyptian cows which were selected based on their milk productivity, 1638-bp beta-defensin genes were amplified. Two PCR amplicon sizes of the gene encoding beta-defensin (1638 bp and 429 bp) were observed in Friesian cows (high milk production), while in Egyptian cows (low milk production) one PCR amplicon size (429 bp) was observed. PCR–RFLP technique was used to discriminate between the common 429 bp band in both Friesian and Egyptian cows, but no difference between them had been observed. DNA sequencing for 1638 bp (B2) and 429 bp (B1) was carried out. Sequence analysis indicated that these two PCR amplicon sizes were two types of genes encoding beta-defensins and very tightly close to each other. Based on their sequence alignment (B1 and B2) with the presented defensin genes in the GenBank, phylogenic tree was constructed. A new gene (B1) belongs to the beta-defensin genes family was detected for the first time and associated to the low milk production in cattle population.

Keywords: Holstein Friesian, Egyptian cows, defensins, milk, PCR-RFLP, DNA sequencing.

Introduction

Defensins are a group of disulfide-linked cationic antimicrobial peptides that function in vertebrate innate immunity (*Aono et al. 2006*). Innate immunity is highly conserved from fruit flies to human and is the first line of defense against invading pathogens (*Yuan and Walker 2004*). Defensins act as direct antimicrobial effectors by disrupting membrane integrity and function, which ultimately leads to the lysis of the microorganisms (*Yang et al. 2002*). Two main defensin subfamilies, α- and β-defensins, differ in length and pairing of the six cysteines (*Selsted and Ouellette 2005*). Alpha- and beta-defensins are salt-sensitive and the direct antimicrobial effect occurs in vacuoles of phagocytes and on mucosal epithelia, where there is low ionic strength (*Goldman et al. 1997; Yang et al. 2002*). Beta-defensin was first isolated from bovine respiratory tract and was named tracheal antimicrobial peptide (TAP) (*Diamond et al. 1991*).

Because of the role played by defensins in defending humans and animals against bacterial, viral or fungal infections, the genes encoding defensins seem to be potential markers of the genetically determined susceptibility (or resistance) of the mammary gland. Defensins are found not only in the mammary gland, but also in milk (*Jia et al. 2001*), as well as in leukocyte granules (*Diamond et al. 2000; Frye et al. 2000*) and in macrophages (*Zhang et al. 1998*), which constitute a part of milk cell population. Both cell types are responsible for phagocytosis of microbes (*Fehlbaum et al. 2000*). Moreover, defensins are produced on all epithelial surfaces of the mammary gland (*Kaiser and Diamond 2000*). In particular, together with clearance mechanisms, barrier properties of epithelial surfaces and additional antimicrobial factors, beta-defensins are proposed to help maintain the respiratory and other mucosal surfaces free from infection (*Diamond et al. 2000*).

Mammary gland of the cow is highly susceptible to inflammation. Where, during the gland tissue infection with bacteria the number of polymorphonuclear leucocytes in milk increase dramatically (*Emanuelson et al. 1988; Hogan et al. 1992; Lund et al. 1999*). The effect of bacterial infection on the yield, composition and quality of cow milk is well documented (Kehrli and Schuster 1994). This prompted us to investigate whether the defensin genotype affects the milk yield and quality in dairy cows. This hypothesis is even more attractive, as our earlier investigation showed a significant relation between defensin genotypes and SCC of bovine milk (*Ryniewicz et al. 2002*).

Material and methods

Animals

In the present study, five Egyptian female wild type cows and five female breed cows (Friesian) were precisely selected from two different farms; Faculty of Veterinary Medicine farm and Faculty of Agriculture farm, Alexandria University, Abeas, Alexandria, Egypt, respectively. These ten animals were selected according to their high/low productivity of milk trait.

DNA isolation

In tubes containing K3EDTA, blood samples for DNA genotyping from theses animals were collected from jugular vein by an authorized veterinarian. Total genomic DNA was isolated from whole blood using DNA purification kit (Promega).

Amplification of defensin gene using specific PCR

Beta-defensin gene of the examined animals was amplified with the use of primer sequence (Ryniewicz et al. 2003): 5'-GCCAGCATGAGGCTCCAT-3' (forward) and 5'-AACAGGTGCCAATCTGT-3' (reverse). PCR was performed in a reaction volume of 25 µl using 25 ng genomic DNA of each sample, 25 pmol of each primer, 10X Taq DNA polymerase buffer including $MgCl_2$, 0.2 mM dNTPs and 5 unit/ µl Taq DNA polymerase (Promega Co.). Thermal cycling (Perkin Elmer 9700) was carried out by initial denaturation at 95°C for 5 min, followed by 34 cycles each at 94°C for 1 min, annealing temperature at 63 for 45s, polymerization temperature at 72°C for 1 min and final extension at 72°C for 10 min, then the samples were held at 4°C. The amplified DNA fragments were separated on 2% agarose gel (GibcoBRL), stained with ethidium bromide, visualized on a UV Transilluminator and photographed by Gel Documentation system (Alpha Imager M1220, Documentation and Analysis System, Canada).

RFLP for the enteric region of the defensin gene

To determine the polymorphism of the amplified enteric region of defensin genes (429 bp), restriction fragment length polymorphism (RFLP) technique was used. Where, six different restriction endonucleases (Fermentase): *Taq*I, *Hind*III, *Pst*I, *Pvu*II, *Nde*II, *Sma*I. However, one unit is defined as the amount of enzyme required to digest 1 µg of DNA in a total reaction volume of 50 µl for 2 hours at 65°C (TaqI), 37°C (HindIII, , PstI, PvuII, NdeII) and 25°C (SmaI). DNA fragments were separated on 2% agarose gel, stained with ethidium bromide, visualized on a UV Transilluminator and photographed.

Sequencing and analysis of the defensin genes

DNA sequence for the defensin genes were performed by Macrogene Company in south Korea. Where, sequencing was carried out for the enteric region (429 bp) of the wild Egyptian cows and the full gene of the Friesian breed cows (1638 bp). The sequences (429- and 1638- bp) were submitted to NCBI GenBank database to get the accession numbers. Sequence alignment was carried out using ClustaW (1.8). Phylogenetic tree was constructed using MEGA4.0 program and the sequence alignment was compared with five defensin genes that are available in the GenBank database (http://www.ncbi.nlm.nih.gov).

Results and discussion

PCR amplification using specific primers

In this study, the genomic DNA from five Egyptian cows (low milk production) and five Frisian cows (high milk production) was extracted to amplify beta-defensins gene. In Frisian cows, PCR amplification of the gene encoding beta-defensins yielded two major PCR products 1638 bp and 429 bp, approximately, in addition to several less abundant PCR products appeared (Figure 1A). While in Egyptian cows, only one band (429 bp-PCR product) was obtained (Figure 1B).

Band elution and purification

The bands were cut and eluted from the agarose gel using gel extraction kit (Promega) according to the manufacture procedure. Subsequently, these segments of PCR amplification (429- and 1638-bp) were subjected for more characterization using both RFLP and DNA sequence techniques.

Genotyping for the defensin gene using RFLP technique

Purified PCR bands (429 bp) from both Egyptian and Friesian cows were digested with six different restriction enzymes (*Taq*I, *Hind*III, *Nde*II, *Pst*I, *Pvu*II and *Sma*I). Four of them (*Taq*I, *Hind*III, *Nde*II and *Pvu*II) are able to digest the PCR products resulted in identical pattern fragments in the two types of the cows. Figure 2 represents three identical pattern fragments (200-, 150- and 79-bp) generated by *Taq*I in both Egyptian and Friesian cows.

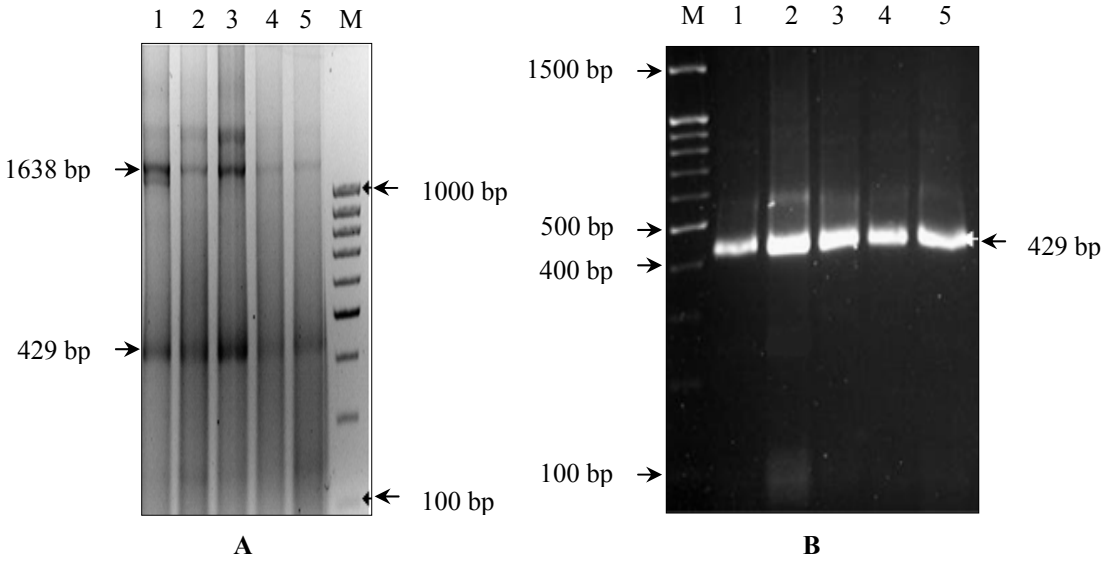

Figure 1. PCR amplification products generated by the beta-defensin gene primer. Where, A: Friesian cows (1-5), B: Egyptian cows (1-5) and M: DNA marker.

DNA sequence and accession numbers

The purified PCR products were subjected to DNA sequencing. About 429 bp for the small defensin gene in the Egyptian cows and 1638 bp of the long defensin gene in Friesian cows were sequenced and read. Consequently, DNA nucleotide sequences were submitted to the GenBank and the accession numbers were **EU599220** (B1) and **EU599221** (B2).

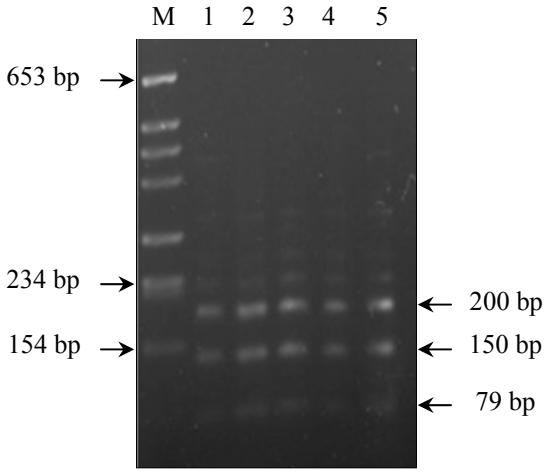

Figure 2. *Taq*I digestion of the PCR fragment (429-bp) as a representative DNA pattern from the examined animals. Where, M is DNA marker and 1-5 are random samples of the ten animals (Friesian and Egyptian cows).

The sequence analysis and alignment with the other beta-defenisn genes

The sequence alignment showed that B2 defensin gene (1638-bp) is closed to the other beta-defensin genes. Because of the similarity of sequence does not exceed more than 70 percent, we are motivated to expect or to say that B2 is a new gene added to the other beta-defensin genes (Figure 3). To demonstrate the similarity between B1 defensin gene (429-bp) and B2 defensin gene, sequence alignment was carried out using ClustalW program (version 1.8). The result showed that, high similarity was observed between the two examined defensin genes B1 and B2 (Figure 4). Moreover, B2 and B1 were found in one group with the isolated defensin genes in Bos Taurus which had the same ancestor, but both **gi|34420978** and **gi|4959237** (adenovirus and kappa-casein genes of Bovine and Bos Taurus, respectively) were considered as out of this group (see Figure 3). ***The sequence analysis and alignment with the other beta-defenisn genes***

The sequence alignment showed that B2 defensin gene (1638-bp) is closed to the other beta-defensin genes. Because of the similarity of sequence does not exceed more than 70 percent, we are motivated to expect or to say that B2 is a new gene added to the other beta-defensin genes (Figure 3). To demonstrate the similarity between B1 defensin gene (429-bp) and B2 defensin gene, sequence alignment was carried out using ClustalW program (version 1.8). The result showed that, high similarity was observed between the two

examined defensin genes B1 and B2 (Figure 4). Moreover, B2 and B1 were found in one group with the isolated defensin genes in Bos Taurus which had the same ancestor, but both **gi|34420978** and **gi|4959237** (adenovirus and kappa-casein genes of Bovine and Bos Taurus, respectively) were considered as out of this group (see Figure 3).

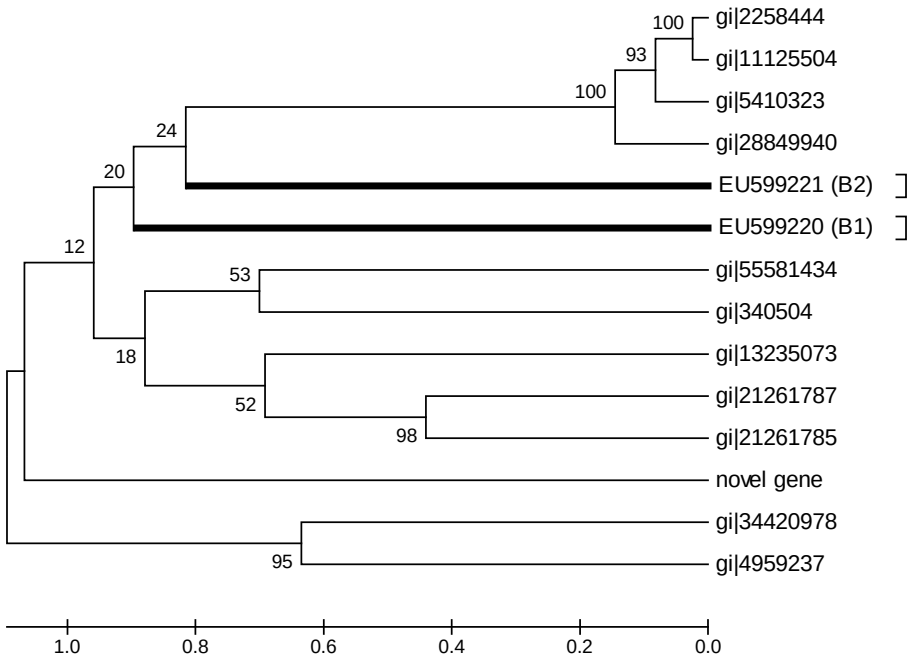

Figure 3. Phylogentic tree of the beta-defensin genes (B1 and B2) with the other beta-defensin genes family and their accession numbers based on the sequence alignment (GenBank). Where, **gi|2258444**: Bos taurus neutrophil beta-defensin 4 (BNBD4) gene, **gi|11125504**: Bos taurus bnbd5 gene for beta-defensin (exons 1-2), **gi|5410323**: Bos taurus neutrophil beta-defensin 12 (NBD12) gene, **gi|28849940**: Bos taurus neutrophil beta-defensin 4 (BNBD-4), mRNA, **EU599221**: B2, **EU599220**: B1, **gi|55581434**: Bos taurus SCD gene promoter region, **gi|340504**: Bovine tropoelastin gene, **gi|13235073**: Bos taurus partial mRNA for 5-lipoxygenase (ORF1), **gi|21261787**: Bos taurus partial mRNA for alpha-2C adrenergic receptor (adra2c gene), **gi|21261785**: Bos taurus partial mRNA for alpha-1B adrenergic receptor (adra1b gene), **gi|34420978**: Bovine adenovirus 2 BovD hexon gene and **gi|4959237**: Bos taurus kappa-casein gene (promoter and exon 1).

Figure 4. DNA sequence alignment between the two defensin genes (B1 and B2). Where, the asterisks represent the similarity between these two genes.

In particular, milk contains large number of somatic cells (leukocytes and epithelial mammary cells) from mammary glands. These somatic cells play a protective role against infectious disease in the dairy animals mammary gland (*Abdel-Rahman and Ahmed 2007*). Mammary gland of a cow is highly susceptible to inflammation. Where, during the gland tissue infection with bacteria the number of polymorphonuclear leucocytes in milk increases dramatically (*Emanuelson et al. 1988; Hogan et al. 1992; Lund et al. 1999*). Furthermore, the effect of bacterial infection on the yield, composition and quality of cow milk is well documented (*Kehrli and Schuster 1994*). However, defensins that are recognized as one of the innate defensive response systems against the pathogenic microorganisms can affect the quality of cow milk (*Ryniewicz et al. 2003*). As well as the milk production in correlation with the defense system especially the innate immune system against the bacteria which infect the mammary glands. That's encouraged us to study the influencing of defense system rather than the conditional genes on milk productivity between Egyptian cows (low milk production) and Friesian (high milk production) cows which living under the same conditions in Egypt.

Since many years ago, using specific primers, PCR amplification of the B-defensin gene in Holstein-Friesian was carried out by *Ryniewicz et al*. (2003). In our study, using the same primers, beta-defensin gene was amplified in both Friesian (encompassing parts of exons 1 and 2 and the interweaving intron) and Egyptian cows. Two PCR amplification sizes (1638 bp and 429 bp) were obtained in Friesian cows, while in Egyptian cows only the PCR amplification size (429 bp) was observed. The PCR amplifications of the gene encoding beta-defensin in Holstein-Friesian cows were in agreement with the results which obtained by *Ryniewicz et al.* (2003) who worked on Black–and-White cows to examine the association of the polymorphism at defensin gene loci with dairy production traits and milk somatic cell count. The different band patterns which appeared in the Frisian cows refer to many defensin genes resist the infection of Friesian cow's mammary gland against pathogenic bacteria. Because of the antimicrobial role that defensins play in animals, genes encoding these peptides may be considered as molecular markers of a genetically determined susceptibility (or resistance) of the mammary gland (*Tunzi et al. 2000*).

We turn now to the PCR amplification size (429 bp) in both Friesian and Egyptian cows. To differentiate between these two bands, PCR-RFLP technique was carried out using six different restriction endonucleases (*Taq*I, *Hind*III, *Pst*I, *Pvu*II, *Nde*II, *Sma*I). The same band patterns were generated in the two types of the animals (Friesian and Egyptian cows). On the other hand, the sequence analysis of this fragment (429 bp) revealed high similarity and tightly close to the enteric beta-defensin gene (1638 bp), whereas less similarity with the other beta-defensin genes family was observed (Figure 3). Consequently, it could be say that this fragment of DNA (429 bp) is the same in both Friesian and Egyptian cows and represents one gene (B1) of the beta-defensin genes.

Because of the low productivity of milk in Egyptian cows, the beta-defensin gene (B1) is not strong enough to resist the bacterial infection of the memory gland in the Egyptian cows, compared to the high productivity of milk in Friesian cows which have the enteric beta-defensin gene (B2). This result is in agreement with the result which obtained by both *Exner et al.* (2000) and *Zhang et al.* (2000), who postulated that segregating QTL's affecting dairy form and milk yield could exist near BM203 on chromosome 27. Many other previous studies were carried out to map or identify QTL's affecting milk production and health of dairy cattle. However, several QTL's have been found and located to the bovine chromosome 27, as well as, the group of genes which coding for defensins has been localized. This makes beta-defensin genes attractive candidates for genetic markers of the udder health traits and perhaps its susceptibility to inflammations (*Ashwell et al. 1998 and 1999*).

Conclusion

One of the most important findings in this study was that the sequence alignment of both B1 (**EU599220**) and B2 (**EU599221**) defensin genes (http://www.ncbi.nlm.nih.gov/sites/entrez) was compared with available defensin genes presented in the GenBank database. Where, the phylogenetic tree was constructed and demonstrate that B1 and B2 were found in one group with the other defensin genes in Bos Taurus which had the same ancestor, but both **gi|34420978** and **gi|4959237** were considered as out group of these genes as shown in Figure 3. The significant effect of the polymorphism of defensin genes on milk productivity performance trait found in this study in both Friesian (high milk production) and Egyptian (low milk production) cows may lead to the use of defensin genes as genetic marker (s) in the breeding programmes aiming at selecting highly productive dairy cattle with increasing resistance to udder infections.

Acknowledgement

The authors would like to thank Miss Shimaa H. El.Hofey, Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt, for her kind help.

Veza između polimorfizma na defensin gen lokusu i mlečnosti holštajn-frizijskih i egipatskih krava

E. E. Hafez, S. M. Abdel-Rahman, S. A. El-Sohaimy

Rezime

Zbog antimikrobiološke uloge koju defensin ima kod krava, geni koji kodiraju ove peptide se mogu smatrati molekularnim markerima za genetski određenu osetljivost mlečne žlezde na mastitis. Kod frizijskih i egipatskih krava kod kojih se radi selekcija na osnovu njihove mlečnosti, 1638-bp beta-defensin geni su pojačani. Kod frizijskih krava su registrovani geni koji

kodiraju beta-defensin i to dve PCR veličine amplikona (1638 bp i 429 bp) (visoka proizvodnja mleka), dok kod egipatskih krava (niska proizvodnja mleka) registrovana je jedna PCR veličina amplikona (429 bp). PCR–RFLP tehnika je korišćena za razlikovanje običnog 429 bp banda kod frizijskih i egipatskih krava, ali nije utvrđena razlika između njih. DNK sekvenciranje za 1638 bp (B2) i 429 bp (B1) je izvršeno. Analiza sekvence je ukazala da ove dve PCR veličine amplikona su dva tipa gena koji kodiraju beta-defensine i veoma se blizu jedna drugom. Na osnovu različitosti sekvenci (B1 i B2) sa defensin genima predstavljenim u GenBank, konstruisano je filogenetičko stablo. Novi gen (B1) pripada porodici beta-defensin gena i otkriven je po prvi put I povezan sa niskom proizvodnjom mleka u populaciji goveda.

Ključne reči: holštajn-frizijske krave, egipatske krave, defensini, mleko, PCR-RFLP, DNK sekvenciranje.

References

ABDEL-RAHMAN S.M., AHMED M.M. (2007): Rapid and Sensitive Identification of buffalo's, cattle's and sheep's milk using species-specific PCR and PCR-RFLP techniques. Food Control 18, 1246-1249.

AONO S., LI C., ZHANG G., KEMPPAINEN R., GARDD J., LU W., HU X., SCHWARTZ D., MORRISON E., DYKSTRA C., SHI J. (2006): Molecular and functional characterization of bovine b-defensin-1. Veterinary Immunology and Immunopathology 113, 181–190.

ASHWELL M.S., DA Y., VANRADEN P.M., REXROAD C.E., MILLER JR R.H. (1998): Detection of putative loci affecting conformational type traits in an elite population of United States Holsteins using microsatellite markers. Journal of Dairy Science 81, 1120-1125.

ASHWELL M.S., VAN TASSELL C.P. (1999): Detection of putative loci affecting milk, health and type traits in a US Holstein population using 70 microsatellite markers in genome. Journal of Dairy Science 82, 2497-2502.

DIAMOND G., LEGARDA D., RYAN L.K. (2000): The innate immune response of the respiratory epithelium. Immunol Rev. 173, 27-38.

DIAMOND G., ZASLOFF M., ECK H., BRASSEUR M., MALOY W.L., BEVINS C.L. (1991): Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. Proc. Natl. Acad. Sci. U.S.A. 88, 3952–3956.

EMANUELSON U., DANELL B., PHILIPSON J. (1988): Genetic parameters for clinical mastitis somatic cell counts and milk production estimated by

multiple-trait restricted maximum likelihood. Journal of Dairy Science 71, 467-476.
EXNER K., THOMSEN P.D., PAUL S., ROOSEN S., KALM E., LOOFT C.H.R. (2000): Charac-terization of β-defensins – a family of peptide antibiotics also expressed in the epithelium of the bovine mammary gland. ISAG Conference, Minnesota, July 22-26, Book of Abstracts, Abstract C006, p. 60.
FEHLBAUM P., RAO M., ZASLOFF M., ANDERSON M. (2000): An essential amino acid induces epithelial β-defensin expression. Proceedings of the National Academy of Sciences, USA 97, 12723-12728.
FRYE M., BARGON J., LEMBCKE B., WAGNER T.O.F., GROPP R. (2000): Differential expres-sion of human α and β-defensins mRNA in gastrointestinal epithelia. European Journal of Clinical Investigations 30, 695-701.
GOLDMAN M.J., ANDERSON G.M., STOLZENBERG E.D., KARI U.P., ZASLOFF M., WILSON J.M. (1997): Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. Cell 88, 553–560.
HOGAN J.S., WEISS W.P., TODHUNTER D.A., SMITH K.L., SCHOENBERGER P.S. (1992): Efficacy of an Escherichia coli 15 mastitis vaccine in an experimental challenge trial. Journal of Dairy Science 75, 415-422.
JIA H.P., STARNER T., ACKERMANN M., KIRBY P., TACK B., MCCRAY B. (2001): Abundant human β-defensin-1 expression in milk and mammary gland epithelium. Journal of Pediatrics 138, 109-112.
KAISER V., DIAMOND G. (2000): Expression of mammalian defensin genes. Journal of Leukocyte Biology 68, 779-784.
KEHRLI M.E., SHUSTER D.E. (1994): Factors affecting milk somatic cells and their role in health of the bovine mammary gland. Journal of Dairy Science 77, 619-627.
LUND M.S., JENSEN J., PETERSEN P.H. (1999): Estimation of genetic and phenotypic parameters for clinical mastitis, somatic cell production deviance, and protein yield in dairy cattle using Gibbs sampling. Journal of Dairy Science 82, 1045-1051.

RYNIEWICZ Z., ZWIERZCHOWSKI L., BAGNICKA E., FLISIKOWSKI K., MAJ A., KRZYŻEWSKI J., STRZAŁKOWSKA N. (2003): Association of the polymorphism at defensin gene loci with dairy production traits and milk somatic cell count in Black-and-White cows. Animal Science Papers and Reports 21, 209-222.
RYNIEWICZ Z., ZWIERZCHOWSKI L., BAGNICKA E., KRZYŻEWSKI J., STRZAŁKOWSKA N. (2002): Preliminary investigations on the polymorphism of defensin genes in cattle – relation with milk somatic cell count. Animal Science Papers and Reports 20, 125-131.

SELSTED M.E., OUELLETTE A.J. (2005): Mammalian defensins in the antimicrobial immune response. Nat. Immunol. 6, 551-557.
TUNZI C.R., HARPER P.A., BAR-OZ B., VALORE E.V., SEMPLE J.L., WATSON-MACDONELL J.L., GANZ T., ITO S. (2000): β-defensin expression in human mammary gland epithelia. Pediatric Research 48, 30-35.
YANG H.W., LIN Y.W., YEN C.D., MIN M.Y. (2002): Change in bi-directional plasticity at CA1 synapses in hippocampal slices taken from 6-hydroxydopaminetreated rats: the role of endogenous norepinephrine. Eur J Neurosci 16, 1117–1128.
YUAN Q., WALKER W.A., PEDIATR J., NUTR G. (2004): Innate immunity of the gut: mucosal defense in health and disease. May 38, 463-73.
ZHANG G.L., ROSS C.R., BLECHA F. (2000): Porcine antimicrobial peptides: New prospects for ancient molecules of host defense. Veterinary Research 31, 277-296.
ZHANG Q., BIOCHARD D., HOESCHELE I., ERNEST C., EGGEN A., MURKVE B., PFISTER-GENSKOW M., WITTE L.A., GRIGNOLA F.E., UIMARI P., THALLER G., BISHOP M.D. (1998): Mapping quantitative trait loci for milk production and health of dairy cattle in a large outbred pedigree. Genetics 149, 1959-1973.