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Bovine lactoferrin gene polymorphism and expression in relation to *mastitis* resistance – a review

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Lactoferrin is an iron-binding protein present in many mammalian biological fluids, such as tears, saliva and milk, widely investigated due to its multiple activities. Human milk contains 1 to 5 mg of lactoferrin /ml, contrary to bovine milk, where lactoferrin concentration reaches maximum level of 0.1 mg/ml. Dramatic increase of lactoferrin content has been noticed in colostrum, mammary gland secretion during involution, and in milk yielded by females suffering from udder inflammation. Lactoferrin gene has developed during evolutional mutations in transferrin gene. It was mapped to bovine chromosome 22, contains 17 exons and spreads out on about 34.5 kilo base pairs (kbp) of genomic DNA. There are many polymorphisms in lactoferrin gene. Polymorphisms occurring in gene regulatory region seem to be particularly interesting, as they may affect a gene expression. It has been claimed, that lactoferrin gene expression in mouse and human uterus appeared under estrogen stimulation, but for bovine species the regulation of its expression has not been fully understood yet. Another possibility to find functional polymorphisms is to search for them in exons which code for lactoferrin antimicrobial peptides. Due to its relation to the innate immunity, lactoferrin gene is supposed to be a promising candidate gene for *mastitis* resistance trait.

KEYWORDS: cattle / gene polymorphism / lactoferrin gene / marker-assisted selection / *mastitis*

Lactoferrin, one of the common iron transporting proteins, is present in a wide variety of biological fluids – in saliva, bile, mucosal secretions and in mammalian milk. Protein molecule of lactoferrin contains two lobes, both built of two globular domains [Moore *et al*. 1997]. There is a galore of lactoferrin biological functions and among them a special attention is being paid to its antibacterial [Nonnecke and Smith

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1984, Bihmani 1999, Singh *et al*. 2002, Kutila *et al*. 2003, Orsi 2004, Al-Nabulsi and Holley 2005, Pan *et al*. 2007, Małaczewska and Rotkiewicz 2007, Gonzalez-Chavez *et al*. 2009], antiviral [Anderson et al. 2003, Pan *et al*. 2006, Mistry *et al*. 2007, Małaczewska and Rotkiewicz 2007, Gonzalez-Chavez *et al*. 2009], antitumor [Yoo *et al*. 1997, Tsuda *et al*. 2000, Małaczewska and Rotkiewicz 2007, Gonzalez-Chavez *et al*. 2009] and immunomodulatory properties [Wakabayashi *et al*. 2006, Małaczewska and Rotkiewicz 2007, Gonzalez-Chavez *et al*. 2009]. A direct antimicrobial activity of lactoferrin affecting the bacterial cell wall, occurs due to two antimicrobial peptides of an N-terminal part of amino acid chain of this protein, called lactoferricin and lactoferrampin. These peptides are released from native protein by pepsin-mediated digestion [Dionysius and Milne 1997, Kraan *et al.* 2004, Orsi 2004, Exposito and Recio 2006]. Lactoferrampin derived from bovine lactoferrin is bactericidal, whereas this human peptide is probably inactive under normal conditions [Haney *et al*. 2009].

In human milk, there is 1 to 5 mg of lactoferrin /ml [Teng 2002], contrary to bovine milk, where this protein concentration reaches maximum level of 0.1 mg/ml [Nonnecke and Smith 1984, Schanbacher *et al.* 1993, Molenaar *et al.* 1996]. A dramatic increase in lactoferrin has been noticed in colostrum, mammary gland secretion during involution [Smith *et al*. 1971, Welty *et al*. 1976, Schanbacher *et al.* 1993], and in milk obtained from females suffering from a mammary gland inflammation [Harmon *et al*. 1976, Smith *et al*. 1971, Welty *et al*. 1976, Schanbacher *et al.* 1993, Hagiwara *et al*. 2003, Malinowski *et al*. 2008]. Milk from quarters, in which *mastitis* pathogens are observed, contains more lactoferrin than that obtainable from uninfected ones, and protein concentration is to some extent pathogen-specific. According to some authors, quarters where *Streptococcus uberis* is present (a sub-clinical inflammation), contain significantly ($P<0.001$) more lactoferrin than quarters where no *mastitis* pathogens can be found, but there is no significant increase in protein concentration in the presence of the other *mastitis*-causing pathogens. On the other hand, there is no similar relation between a pathogen and a lactoferrin concentration in occurrence of clinical udder inflammation [Chaneton *et al*. 2008].

Lactoferrin inhibits *in vitro* growth of *mastitis*-causing pathogens, and the most potent activity of the protein is noticed against *Escherichia coli* and *Pseudomonas aeruginosa* [Nonnecke and Smith 1984, Kutila 2004, Chaneton *et al.* 2008], while more varying results have been obtained for other pathogens – *Staphylococcus aureus* and *Clebsiella pneumoniae* [Kutila 2004]. A direct inhibitory activity of lactoferrin has been reported against *Staphyloccocus aureus* both *in vitro* and *in vivo* [Lacasse *et al.* 2008]. A new method for fighting *mastitis* has been proposed by Zhang *et al.* [2007]. They managed to insert a lactoferrampin gene to mammary gland secretory cells, by a plasmid-mediated transfection, which led to a peptide expression only under conditions of inflammation. A success of such experiments strongly suggests that there is a feasibility of lactoferricin application by a gene transfer.

A lactoferrin gene has developed during evolutional mutations in a transferrin gene. There is 60-65% identity of nucleotide sequences between these two genes

[Baker and Baker 2005]. A similar homology in a gene sequence exists among different mammalian species – human, bovine, murine and porcine genes are identical in 65 to almost 100% [Teng 2002]. The lactoferrin gene size varies between 23 and 35 kbp among different species [Teng 2002]. The number of amino acids, encoded by 15 out of 17 exons is the same in human, bovine and murine lactoferrin genes. An intron-exon pattern of the lactoferrin gene of these species is identical in 12 regions. Main differences between species lie in exons 2 and 12, where in human genes there are two or three codons more than in bovine, murine and porcine genes [Teng 2002].

The bovine lactoferrin gene was mapped to bovine chromosome 22 (BTA 22) [Schwerin *et al*. 1994], contains 17 exons and spreads on about 34.5 kbp of a genomic DNA. Exons from 2 to 4 and from 9 to 12 code for the first globular domain of each lobe and exons from 12 to 15 code for another one. The amino acids, forming a hinge region of protein are encoded at the beginning of exon 9 [Seyfert *et al.* 1994]. There are 32 splice junctions in a bovine lactoferrin gene, all but one of them belonging to the canonical border site of 5' – GT and 3' – AG. There is also an unusual connection in exon 2: TTTCA/GAGA (3' – intron 2 / 5' – intron 3). Adequate similarities among species, as mentioned for the lactoferrin gene, also exist for its promoter. The most significant similarity occurs between human and bovine promoter sequences [Seyfert *et al*. 1994]. Zheng *et al*. [2005] analysed 4355 bp of the bovine promoter sequence. They claimed that a 5'-flanking region was rich in GC pairs and discovered a noncanonical TATA box in this region along with multiple SP1/GC binding sites [Zheng *et al.* 2005, also Seyfert *et al*. 1994]. Such mentioned features are characteristic for housekeeping gene promoters. These promoter features are related to the wide spectrum of tissues, in which the lactoferrin gene is being expressed. The promoter also contains some adjacent binding sites for transcription factors: NF-κB, AP1, STAT3, STAT5, an estrogen receptor, a progesterone receptor and a glucocorticoid receptor.

It has been claimed, that a lactoferrin gene expression in a mouse and human uterus happens under estrogen stimulation. Similar to many other typical estrogen-responsive genes, promoters of these genes contain an estrogen response element. An estrogen response module of a human and murine lactoferrin contains a Chicken Ovalbumin Upstream-Promoter Transcription Factor (COUP-TF) – Teng [2006] – whereas there is no such factor in a bovine lactoferrin gene promoter [Seyfert *et al*. 1994, Zheng 2005]. This situation suggests a need of further research on the potential bovine lactoferrin gene responsiveness under estrogen stimulation [Teng 2006]. Apart for an estrogen, the lactoferrin gene expression is stimulated by many other factors: a retinoic acid, a tumor necrosis factor alpha (TNF-alpha) and a MPTP neurotoxin. It is not precisely known how this lactoferrin gene expression is regulated in cattle.

With the objective of checking spectrum of tissues in which the lactoferrin gene can be expressed, an RNA distribution has been specified for different tissues by Zheng *et al*. [2005]. A high expression level has been observed in a mammary gland and liver, an intermedial in intestine, and a very low, but still detectable – in lungs, kidneys

and spleen. A mammary gland expression occurs in epithelial cells and neutrophiles [Molenaar *et al*. 1996, Pfaffl *et al*. 2003] and its level level depends on the region (a gland site), and on the development stage of the alveoli. The expression is stronger in alveoli, which hold somatic cells within their lumen [Molenaar *et al*. 1996]. The analysis of the promoter function displayed that the expression happens under the stimulation of LPS (lipopolysaccharide) derived from an *E. coli* cell wall [Bruckmeier 2005]. It has also been shown that the lactoferrin gene promoter contains estrogen response elements [Zheng *et al*. 2005]. It is possible that the expression, especially in a periparturient period, can be stimulated by prolactin [Nakaima *et al*. 2008].

Due to the lactoferrin relation to the innate immunity, this protein gene is supposed to be a promising candidate gene for the *mastitis*-resistance trait [Seyfert *et al.* 1996, Sender *et al*. 2003]. The other interesting genes in the area of the *mastitis* resistance research are the major histocompatibility complex genes (BoLA) – Sender *et al*. [2005, 2006], Galal Abdel Hameed *et al*. [2008]. Not a strict relation has been found between known lactoferrin gene polymorphism and *mastitis* susceptibility yet, but the results of the already made research suggest an existence of such association [Rupp and Boichard 2003]. The lactoferrin gene polymorphism occurs in the coding and

Table 1. Polymorphisms found in the regulatory region of bovine lactoferrin gene, possibly significant for gene expression

Locus	SNP	Source	SNP location
-2151	G/A	O'Halloran *et al.* [2009]	GC rich region
-2009	C/T	O'Halloran *et al.* [2009]	GC rich region
-1155	G/A	O'Halloran *et al*. [2009]	GATA-1 and SP1 transcription factors putative binding site
-929	G/A	O'Halloran *et al.* [2009]	Adjacent to a GC rich region
-915	T/G	Li *et al.* [2004] Daly *et al.* [2006]	C/EPBα putative binding site
-838	C/- INDEL	Li *et al.* [2004]	NF-κB putative binding site
-765	C/T	Daly *et al.* [2006]	Adjacent to the putative glucocorticoid receptor binding site
-610	G/T	Daly *et al.* [2006]	PEBP2αA1 putative sequence
-599	G/A A/A	Daly *et al.* [2006]	PEBP2αA1 putative sequence
-585	C/T	Daly *et al.* [2006]	AP-2αA binding site
-478	-/G	Li *et al.* [2004], Daly *et al.* [2006]	MBF-I binding site
-457	C/T	Daly *et al.* [2006]	HNF-4α binding site
-190	G/A	O'Halloran *et al*. [2009] Daly *et al*. [2006]	Adjacent to SP1 binding site
-132	T/C	Daly *et al*. [2006]	Putative NF-ATc sequence recognizing region
-131	T/C T/- C/-	O'Halloran *et al*. [2009] Daly *et al.* [2006]	
-28	A/C	O'Halloran *et al*. [2009], Li *et al*. [2004], Daly *et al*. [2006]	Adjacent to a TATA-box, placed in putative sequence recognizing estrogen receptor

regulatory regions as well as in introns [Seyfert *et al*. 1994, Martin-Burriel *et al.* 1997, Li *et al*. 2004, Kamiński *et al*. 2006**]**. There is a strong suggestion that polymorphisms which are located in a regulatory region (Tab. 1) have an impact on a gene expression. Polymorphism in +32 position (G/C) plays an important role in the determination of milk protein yield and milk protein content, but is not related to the somatic cell count [Kamiński *et al*. 2006]. According to the author mentioned, a G allele reduces the lactoferrin gene expression, and promotes a lower somatic cell count in milk. On the contrary, C allele causes a lactoferrin concentration to increase, leads to a stronger immune response and in effect to a higher somatic cell count. Recently, Kaminski *et al*. [2008] reported that the higher milk protein yield was related to polymorphism at position +216. When it occurs along with another polymorphism (e.g. in the growth hormone receptor gene), it can increase the milk yield as well as elevate the milk protein and fat yield [Kamiński *et al*. 2008]. The lactoferrin polymorphisms have been characterized in various breeds of cattle. It has been proved that some of the polymorphic variants of this gene occur only in a few dairy cattle breeds. For example, polymorphisms in positions - 926 and - 915 have been found only in Holstein Friesian (HF), New Zealand HF and Montbeliarde cattle [Daly *et al*. 2006]. However, O'Halloran *et al.* [2009] found only one polymorphism in promoter region related to the breed – at position -1560. Referring to this polymorphism, all HF cows were homozygotes CC [O'Halloran *et al*. 2009]. Searching for polymorphisms in the gene promoter region, especially in transcription factors binding sites and adjacent transcription factors binding sites, seems to be promising. Polymorphisms which occur in such regions may affect the gene expression level under the conditions of immune system stimulation during infection [Daly *et al*. 2006, O'Halloran *et al.* 2009].

Li *et al*. [2004] found polymorphisms in exons 4, 8, 9, 11, 15, and in intron 4. A mutation occurring in exon 4 led to the amino acid substitution (isoleucine to valine), while other mutations were silent. The most important point the above mentioned authors arrived at, was that the mutation in exon 15 occurs always along with another mutation in regulatory regions of lactoferrin gene. This may play an important role in the gene translation and regulation.

An association between the lactoferrin gene polymorphism occurring in intron 6, and susceptibility to *mastitis* has been investigated by Wojdak-Maksymiec *et al*. [2006] who claimed that homozygous AA animals have a lower somatic cell count than heterozygotes AB. Investigations of the same polymorphism, reported by Sender *et al*. [2006] provided contrary results as the genotype BB animals showed the lowest somatic cell count and heterozygotes AB – the highest. Because of the low frequency of the BB genotype, investigations on this polymorphism need to be continued.

In this area, the lactoferrin gene can be of interest also for another reason. As inflammation of the mammary gland induces the lactoferrin expression in epithelial cells, the regulatory region of this gene can be useful in an expression of the other antimicrobial proteins, more potent in an anti-inflammatory action than lactoferrin itself [Kerr and Wellnitz 2003]. The investigations into a functional polymorphism

of the lactoferrin gene have been done on the human protein gene by Liu *et al*. 2002 and Velliyagounder *et al*. 2003]. The latter proved that a mutation leading to an amino acid substitution in the N-terminal region of the protein (arginine to lysine) has a positive impact on lactoferrin bactericidal activity against gram-positive pathogens. An arginine-containing variant of lactoferrin is also more potent in a beta-defensin TAP (tracheal antymicrobial peptide) transcription activation [Velliyagounder *et al*. 2003]. There is a suggestion that a functional polymorphism which may cause a change of protein activity features, is to be found in exons coding lactoferricin and lactoferrampin, facing the fact that the interruptions in these peptides' structures can alter their functions [Liu *et al*. 2002].

Due to lactoferrin relation to the innate immunity, its gene is supposed to be a promising candidate gene for *mastitis* resistance trait. The investigations on a functional polymorphism of the bovine lactoferrin gene have to be continued.

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Związek polimorfizmu i ekspresji genu laktoferyny bydła z występowaniem *mastitis*

Streszczenie

Laktoferyna jest białkiem wiążącym żelazo, obecnym w wielu płynach biologicznych ssaków – łzach, ślinie i mleku – szeroko opisanym ze względu na swoje właściwości. W mleku ludzkim znajduje się 1-5 mg laktoferyny, w przeciwieństwie do mleka krowiego, gdzie stężenie tego białka osiąga poziom nie wyższy niż 0,1mg/ml. Gwałtowne zwiększenie ilości laktoferyny następuje w siarze, w wydzielinie wymienia podczas inwolucji oraz w mleku zwierząt cierpiących na zapalenie wymienia. Gen laktoferyny powstał w wyniku ewolucyjnych przemian genu transferyny. Znajduje się w 22 chromosomie bydła, składa z 17 eksonów i zawiera około 34,5 tysiąca par zasad genomowego DNA. Dotychczas wykryto wiele polimorfizmów tego genu. Ciekawe wydają się zwłaszcza jego polimorfizmy występujące w rejonach regulatorowych, z racji ich potencjalnego wpływu na poziom ekspresji. Stwierdzono, że ekspresja genu laktoferyny w macicy ludzkiej i mysiej stymulowana jest przez estrogen, podczas gdy w przypadku bydła, regulacji ekspresji tego genu dotąd nie rozpoznano. Potencjalną możliwością odnalezienia funkcjonalnego polimorfizmu laktoferyny jest poszukiwanie go w eksonach, kodujących przeciwdrobnoustrojowe peptydy tego białka. Z racji związku laktoferyny z odpornością wrodzoną, gen jej uważa się za obiecujący gen-kandydat odporności krów na *mastitis*.

Powołano się na 56 pozycji piśmiennictwa.