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Review

Expression and polymorphism of defensins in farm animals

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Due to their activity against bacteria, viruses, and fungi, antimicrobial peptides are important factors in the innate resistance system of humans and animals. They are called "new generation antibiotics" for their potential use in preventive and therapeutic medicine. The most numerous group of antimicrobial peptides is a family of cationic peptides which include defensins and cathelicidins. Among them the most common are peptides with a beta-sheet structure containing three intra-molecular disulphide bonds, called defensins, comprising three classes: alpha, beta, and theta. The class of beta-defensins is the largest one. Their transcripts have been found in many tissues of humans and animals. The aim of this paper is to present the current knowledge about antimicrobial peptides from the defensin family in farm animals, their expression, polymorphism, as well as the potential of their use as genetic markers of health and production traits.

Keywords: farm animals, defensins, expression, polymorphism

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INTRODUCTION

Antimicrobial peptides (AMPs) and proteins are important players in immunological systems of humans and animals. They stimulate the innate immunity of an organism and demonstrate a direct activity against bacteria, enveloped viruses, and fungi (Kagan *et al*., 1990; Yang *et al*., 1999; Anbu *et al.,* 2003; Pawlik *et al.,* 2009). As summarized by Kamysz *et al.* (2003) and then by Lai and Gallo (2009) the numerous actions of the antimicrobial peptides such as cathelicidins, alpha- and beta-defensins include influence on: expression of adhesion molecules, production of adrenocorticoids, secretion of chloride ions, angiogenesis, wound repair, and DNA synthesis. They have also been shown to interact with cell membrane receptors, influencing diverse cellular processes such as releasing of cytokines, chemotaxis and antigen presentation (Lai & Gallo, 2009). The diversity of the biological activities of antimicrobial peptides was confirmed by a study carried out on human beta-defensin-2 (hBD-2) by Baroni *et al.* (2009). The hBD-2 stimulated *in vitro* endothelial cell migration, proliferation and formation of capillary-like tubes. Alpha- and beta-defensins have chemotactic activity also for immature dendritic cells (Lehrer & Ganz, 2002).

A majority of antimicrobial peptides demonstrate similar modes of action, based on an interaction with cell membranes of microorganisms. The interaction results in causing the membrane permeability by piercing and formation of channels by which the contents of a cell leaks (so called "aggregate channel model" or "barrel-stave model"), or total disruption of a microorganism's membrane (so called "carpet model") (Brogden, 2005; Shai, 1999; Helmerhorst *et al.,* 1999). However, it can not be excluded that other, still unknown mechanisms of their antimicrobial activity exist (Gordon & Romanowski, 2005; Lai & Gallo, 2009).

The average concentration of defensins in human tissues ranges from 10 to 100 μg/ml, while in granules of leucocytes where they are stored the concentration reaches as much as >10 mg/ml. The biological activity of defensins depends strongly on their concentration (Ganz, 2004). As an example, at low concentrations (<10 μg/ml) alpha-defensins stimulate expression of adhesion molecules in human endothelial cells and cytokine production in human monocytes. Also at low concentration they are chemo-attractants for immune cells (monocytes, T-lymphocytes, dendric cells) and/or inhibitors of protein kinase C activity. Human neutrophil peptides (HNPs; also called alpha-defensins), at concentrations between 10- and 100-fold below those required for antimicrobial activity, show chemotactic activity for $CD45RA^+$ and $CD8^+$ T lymphocytes. The hBD-2 effect on chemotaxis and wound healing depends on its concentration, with a maximum effect at 500 ng/ml and more. But the minimal hBD-2 concentration needed to kill bacteria appears much higher *in vitro* then *in vivo*. The reason for this may be that in a living organism it is co-expressed as a group with other AMPs that act synergistically (Lai & Gallo, 2009).

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Abbreviations: AMPs, antimicrobial peptides; AvBD, avian beta-defensins; BAC, bacterial artificial chromosome; BBD, bovine beta-defensins; BLAD, bovine leucocyte adhesion deficiency; BNBD, bovine neutrophil beta-defensins; BTA, *Bos taurus* autosome; CBD-103, canine beta-defensin 103; CDGs, combined defensin genotypes; DEFA, equine alpha-defensin; EBD, enteric beta-defensin; EBD-P and EBD-P2, enteric beta-defensin pseudogenes; ECA, *Equus caballus* autosome; GAL/CHP, gallinacins, chicken beta-defensins; GBD-1 and GBD-2, goat beta-defensin 1 and 2; H5N1, subtype of influenza A virus, also known as "bird flu"; hBD-2, human beta-defensin 2; HDP, host defense peptide; HSA, *Homo sapiens* autosome; LAP, lingual antimicrobial peptide; LD, lethal dose; LPS, bacterial lipolysaccharide; Mc1r, melanocortin 1 receptor; MDCK, cell line derived from canine kidney; Osp-1 and Osp-2, ostrich beta-defensins = ostricacins-1 and 2; pBD, porcine beta-defensins; RFLP, restriction fragment length polymorphism; RH, radiation hybrid; RTD, theta-defensins, mini-defensins or demidefensins; SBD-1 and SBD-2, sheep beta-defensin 1 and 2; SCC, somatic cell count; SMAP-29, sheep myeloid antimicrobial peptide-29 (sheep cathelicidin); SNP, single nucleotide polymorphism; STR, short tandem repeat; TAP, tracheal antimicrobial peptide; THP1, THP2, THP3 and GPV-1, turkey's beta-defensins; 5'UTR or 3'UTR, 5' or 3' untranslated region

As reviewed by Ganz (2004), defensins at concentrations ranging from 1 to 10 μg/ml exhibit a broad spectrum of antimicrobial activity that includes Gram-negative and Gram-positive bacteria and fungi. At 25 μg/ml they stimulate DNA synthesis. At very high concentrations (≥100 μg/ml) these peptides stimulate keratinocyte growth and cause the lysis of microbes and some tumor cells (Biragyn *et al*., 2002; Lehrer & Ganz, 2002; Kamysz *et al*., 2003; Ganz *et al.,* 2004; Wiechuła *et al*., 2006). As emphasized by Wiechuła *et al.* (2006) natural antimicrobial peptides may be especially effective due to their inter-leucocyte location; these cells are drawn to infection sites and inflammation stimulates synthesis of certain peptides in the epithelium.

Alpha- and beta-defensins, unlike theta-defensins, are sensitive to salt concentration. They show reduced antimicrobial activity even in the presence of physiological salt (Ganz & Lehrer, 1998; 1999; Tang *et al.,* 1999). Increasing concentrations of salts and plasma proteins inhibit the antimicrobial activity of defensins in a manner that is specific to both the particular defensin and its microbial target.

Based on their total net charge antimicrobial peptides can be differentiated into anionic and cationic ones (Hancock, 1997; Lehrer & Ganz, 1999). Cationic peptides constitute a very large group of antimicrobial factors consisting of several families, including defensins and cathelicidins. The most common family are peptides with beta-sheet structures stabilised by intra-molecular disulphide bonds between cysteine residues. They are called defensins and comprise three classes: alpha, beta and theta.

Beta-defensins have been isolated from neutrophils and other leucocytes, epithelial cells, blood plasma, urine and many tissues of vertebrates, including humans and domestic animals (Brogden *et al.,* 2003; Schneider *et al.,* 2005). Peptides of this subfamily are also found in invertebrates and plants (Lynn & Bradley, 2007). Alpha-defensins were first found in primates (humans, rhesus), lagomorphs and rodents, but then also in other mammals (Looft *et al.,* 2006; Bruhn *et al.,* 2007; Lynn & Bradley, 2007). They are widely distributed in phagocytes (neutrophils and macrophages), in Paneth's cells and on the mucosal surface of epithelia (Ganz & Lehrer, 1995, Schneider *et al.* 2005). Alpha-defensins comprise about 5–7% of all proteins present in mature neutrophils and 30–50% in azurophile granules (Rice *et al.,* 1987). The last class identified in animals (only in rhesus monkey) were cyclic mini-defensins, called theta-defensins, demidefensins or retrocyclins (RTD-1 and RTD-2), reported by Tang *et al.* (1999). Liang *et al.* (2010) tested the ability of retrocyclin 2, to protect chicken embryos from infection by a highly pathogenic strain of avian influenza virus H5N1. A fragment of the retrocyclin 2 gene cloned into a eukaryotic expression vector was shown to protect chicken cells and embryos from infection through inhibition of H5N1 replication and transcription.

Defensins contribute to the innate immunity, acting immediately after microbial invasion not only by direct killing of the pathogens but also as immune regulators (Lai & Gallo, 2009). Defensins are ancient components of immunity systems; they originated before the emergence of eukaryotes (Nava *et al*., 2009). The beta-defensin family is phylogenetically older than alpha-defensins, while theta-defensins are thought to arise in evolution from two mutated alpha-defensin genes (Patil *et al*., 2004). The Patil's paper provides evidence of true orthology among defensins and analyzes the molecular diversity of a mammalian-specific domain responsible for their antimicrobial activity. Specifically, this analysis demonstrates that eleven amino-acid residues of the antimicrobial domain have been subject to positive selection to confer specialization of different AMPs. These data support the notion that natural selection acts as an evolu-

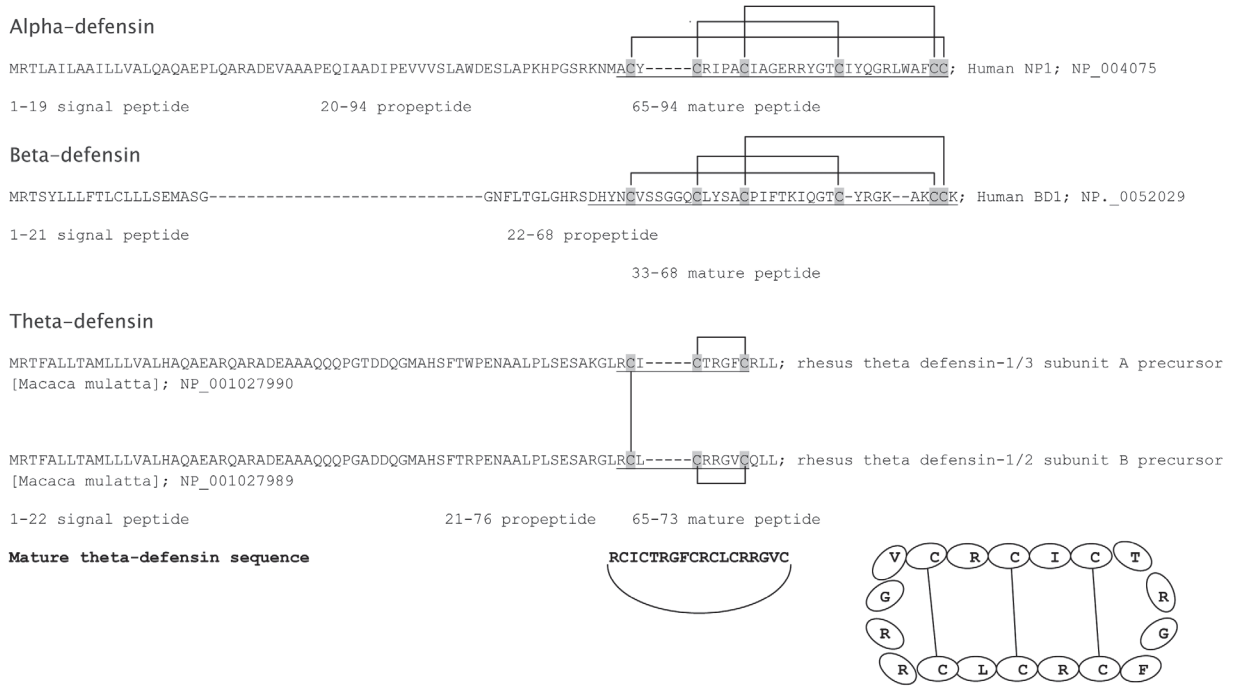

Figure 1. Amino-acid sequence of mammalian alpha, beta and theta defensins; braces and shading show disulfide bonds and cysteines involved

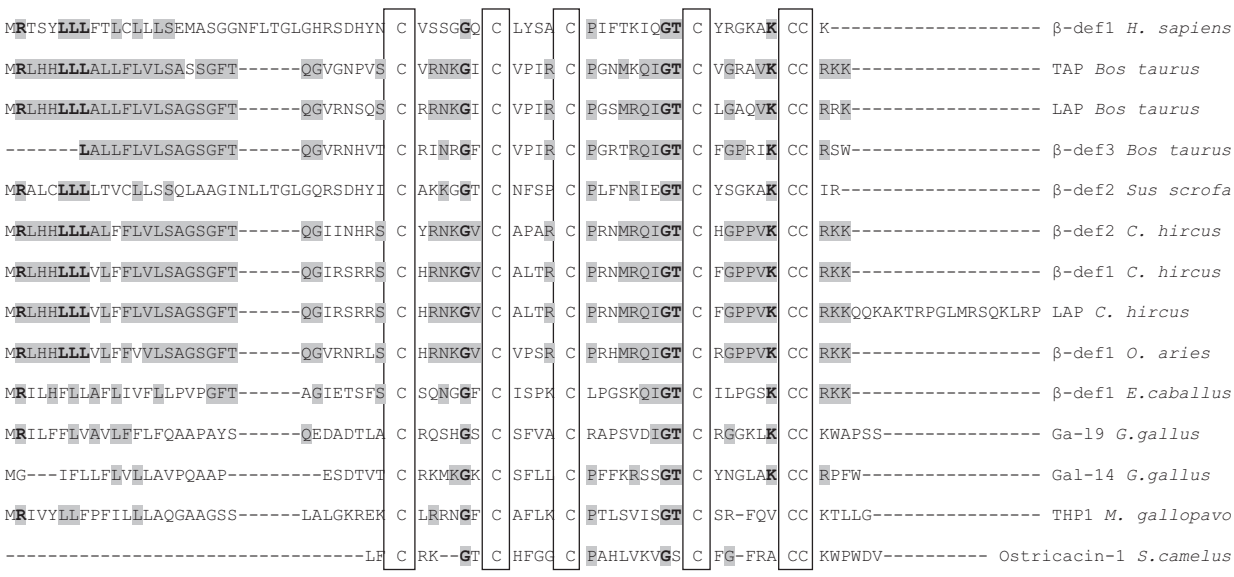

Figure 2. Alignment of amino-acid sequences of mammalian and avian defensis. Shaded — highly conserved amino acids; framed — cysteines participating in disulfide bonds.

tionary force driving the diversification of defensins, and may help to elaborate more effective antibiotics.

All mammalian defensins are small (about 10 kDa), cationic and amphiphilic peptides. Most defensins have six cysteine residues and three disulphide bonds which are not essential for the antimicrobial activity but confer high resistance to bacterial proteolysis (Nava *et al.,* 2009). The three disulphide bonds are crucial for determining and maintaining the core configuration of their structure (Fig. 1 and 2). Alpha- and beta-defensins have similar three-dimensional structure and antimicrobial activity (Schneider *et al.,* 2005) but they differ markedly in the location of the intra-molecular disulphide bridges, structure of their precursors and sites of expression (Lehrer & Ganz, 1999). They derive from an ancestral gene which probably existed before reptiles and birds diverged (Zhao *et al.,* 2001).

The amphiphilic structure appears to be critical for permeabilization of pathogens' membrane. In mammals all known alpha- and beta-defensin genes have two exons (Mallow *et al.,* 1996; Huttner *et al.,* 1998b). The first exon encodes 5'UTR and leader domain of the pre-propetide. The second exon encodes the mature peptide. All known avian beta-defensin genes have four exons (Soman *al*., 2009) (Fig. 3).

Functions other than antimicrobial have been attributed to mammalian defensins. In domestic dog, apart from *Agouti* and melanocortin 1 receptor (*Mc1r*), a third gene (*K locus*) controls pigment type-switching. Candille *et al.* (2007) have identified the *K locus* as beta-defensin 103 (CBD-103) gene and showed that its protein product binds with high affinity to Mc1r having a direct strong effect on coat pigmentation of dogs.

Of all antimicrobial peptide families defensins are the most common. The presence of alpha- and beta-defensin transcripts has been documented in many tissues of different farm animal species. Therefore, the aim of the present review is to summarize the current knowledge about these antimicrobial peptides in farm animals (mammals and birds), their expression, polymorphism, as well as the potential of their application as genetic markers of health and production traits in animal breeding.

DEFENSINS IN CATTLE

The first defensin ever identified in a farm animal was a 38-amino-acid peptide isolated from bovine trachea, named tracheal antimicrobial peptide (TAP) (Diamond *et al*., 1991; Bals *et al*., 1998). This peptide demonstrated germicidal activity against Gram-positive and Gram-negative bacteria as well as against fungi — *Candidia albicans* (Diamond *et al*., 1991, 1993). The highest level of TAP mRNA was found in mucosal membrane of the bovine respiratory tract. The transcript level showed a strong increase after infection by bacteria or stimulation by bacterial lipolysaccharide (LPS; Diamond *et al*., 2000). Tarver *et al.* (1998) also demonstrated *TAP* gene transcript in the distal part of the small intestine.

In 1993 Selsted and co-workers presented data about thirteen structurally homologous cationic peptides isolated from the cytoplasmic fraction of bovine blood neutrophils rich in granules. These peptides, containing 38–42 amino acids with a highly conserved sequence, demonstrated *in vitro* antibacterial activity. Their consensus sequence was different from that of other defensins known then in other mammalian species. The anti-bacterial activity of some of those bovine neutrophil peptides equalled that of the most active rabbit neutrophil defensin NP-1 (one of the alpha-defensins). Due to the considerable similarity to the rabbit defensins as regards the structure and function, and to differentiate them from the alpha-defensin class, the authors suggested the name "beta-defensin" for the newly discovered family of antimicrobial peptides.

From the group of bovine neutrophil peptides reported by Selsted *et al.* (1993), the best known are beta-defensins 4 and 5 (BNBD-4 and BNBD-5). Ryan *et al.* (1998) showed their strong constitutive expression in macrophages located on the surface of bovine pulmonary alveoli. They were found irrespectively of the presence of pro-inflammatory factors, such as LPS, or air pollutants, residual oil fly ash (ROFA), SiO_2, or asbestos, resulting in an inflammation of the respiratory tract and stimulating release of cytokines. Goldammer *et al.* (2004) observed a high level of BNBD-5 mRNA in epithelium

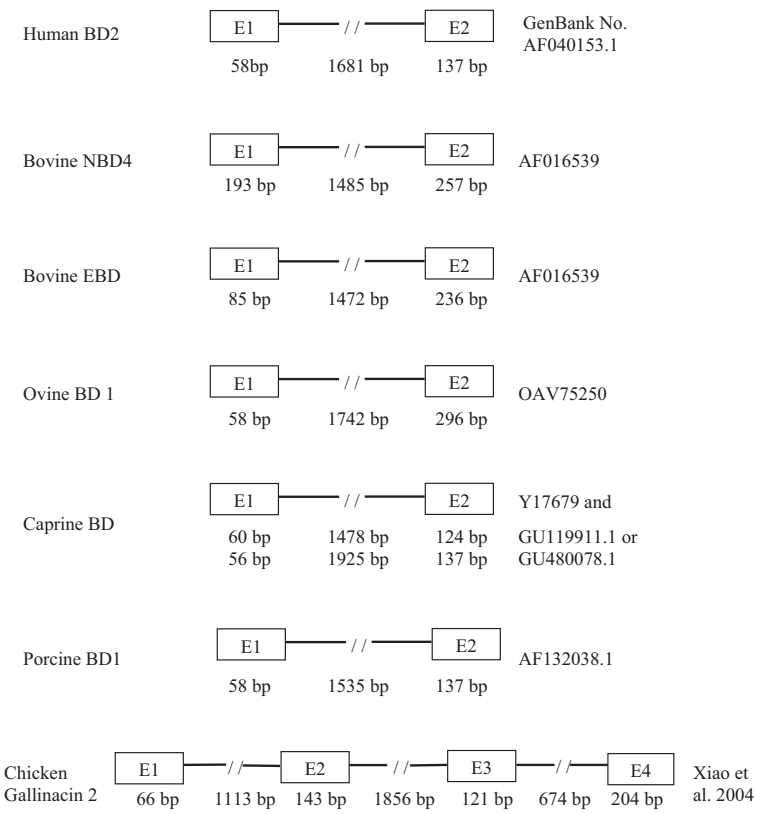

Figure 3. Structure of mammalian and avian genes encoding beta-defensins. E, exons; –//–, introns.

of bacteria-infected mammary glands of cows. Low levels of BNBD-3, BNBD-4, BNBD-9 mRNAs were found in the distal part of the small intestine, while a high abundance of BNBD-3 and BNBD-9 transcripts was observed in bone marrow (Tarver *et al*., 1998). Moreover, Roosen *et al*. (2004) identified six new defensin genes (*DEFB401-DEFB405* and *LAP-like*), highly homologous to the earlier known beta-defensin genes and two pseudogenes, named *EBD-P* and *EBD-P2*. Transcripts encoding several anti-microbial peptides (defensins): BNBD-3, BNBD-9, BNBD-12, DEFB-401, TAP, as well as the described below LAP and DEFB1 (enteric defensin), were detected in mammary gland of lactating cows. TAP mRNA was identified only in tissues from healthy mammary glands. Other mRNAs were found in both healthy and bacteria-infected udders (Roosen *et al*., 2004). Our studies demonstrated beta-defensin 4 (BNBD-4) transcripts in somatic cells derived from cow milk (Bagnicka *et al*., 2006).

The lingual antimicrobial peptide (LAP) is another bovine defensin; it was found in squamous tongue epithelium (Schonwetter *et al*., 1995) and in trachea (Russell *et al*., 1996), where its mRNA level was low but detectable. It was also found in distal part of the small intestine by Tarver *et al*. (1998). Schonwetter *et al*. (1995) showed that expression of LAP mRNA in the tongue is induced principally around wounds. A high abundance of LAP mRNA was reported in stomach, colon, rectum, nostrils, trachea, skin and some other tissues (Table 1). Schonwetter *et al*. (1995) have demonstrated a significant increase of LAP transcript in ileum tissues from cows infected by *Mycobacterium paratuberculosis* and in bronchial epithelium from calves infected by *Pasteurella haemolytica*. In cows suffering from bovine leucocyte adhesion deficiency (BLAD) syndrome an increased in level of LAP mRNA was found in bronchial epithelium and sub-mucosal gland after inoculation with *P. haemolytica* in comparison with healthy tissues (Stolzenberg *et al*., 1997). In the already mentioned studies by Roosen *et al*. (2004) a high content of LAP mRNA was found both in healthy and in infected mammary gland tissues. By contrast, Swanson *et al*. (2004) showed expression of this defensin only in infected mammary tissues and concluded that it was induced by mastitis. In somatic cells isolated from milk, LAP gene transcript was found only if the cell number was increased above the normal level (>100 000/ml of milk), indicating inflammation of the mammary gland (Bagnicka *et al*., 2006).

Enteric beta-defensin (EBD) was first isolated from bovine alveolar macrophages, colon, and small intestine (Tarver *et al*., 1998). A high level of EBD mRNA was demonstrated in the distal part of the small intestine. Moreover, infection of calves with *Cryptosporidium parvum* resulted in a 5–10-fold increase of the *EBD* mRNA in the intestine, compared with that observed in control animals. In our studies *EBD* gene transcript was found in somatic cells obtained from milk of healthy cows and those with clinical mastitis (Bagnicka *et al*., 2006).

Cormican *et al*. (2008) reported a novel group of bovine beta-defensins (BBD-119, BBD-120, BBD-122, BBD-122a, BBD-123, BBD-124 and BBD-142) and found their transcripts abundant in uterine tissues. They also found that BBD-123 inhibits growth of several bacteria species, especially that of *Escherichia coli* and *Listeria monocytogenes*. Another study showed the presence of transcripts of *LAP*, *TAP*, *BNBD-4*, *BNBD-5*, and *BNBD-123* in bovine uterus — in endometrial epithelial cells treated with *E. coli* LPS, as well as the *LAP* and *TAP* transcripts in uterine stromal cells (Davies *et al*., 2008).

Aono *et al*. (2006) compared the gene structure and amino-acid sequence of bovine beta-defensin 1 (BBD-1) with its human counterpart (hBD-1) and with other bovine beta-defensins. They showed that bovine *BD-1* gene has one long intron, as large as the intron of the human gene (8547 bp in *hBD-1 vs*. 6962 in *BBD-1*), about five-fold longer then introns of other known bovine beta-defensin genes. Bovine BD-1 shows a 57% similarity in amino acid sequence to hBD-1 and 34% to bovine LAP, while LAP shows 78, 81, 86 and 89% similarity to BNBD-4, DEF401, EBD and TAP, respectively.

By means of fluorescence *in situ* hybridization beta-defensin genes have been mapped to bovine chromosome 27 (Gallagher *et al*., 1995). The mapping data suggested clustering of the genes defining the location of DEFB@ to 27q13→q14. Recently, it was shown that coat color in cattle co-segregated with markers in a region of chromosome 27 (BTA27) that include the beta-defensin 103 gene (Dreger & Schmutz, 2010).

Das *et al*. (2005) cloned and characterized a beta-defensin mRNA from distal ileum of water buffalo (*Bubalus bubalis*). A sequence analysis indicated 29 nucleotide substitutions *vs*. cattle enteric beta-defensin (EBD) mRNA

Table 1. Expression of defensins in different farm animals species

Species	Peptide or gene name	Localization in tissues (peptide or mRNA)	Sources
Beta-defensins			
Cattle	TAP	mucosal membrane of respiratory tract, distal part of small intestine, bovine uterus — endometrial cells, stromal cells	Tarver *et al*., 1998; Diamond *et al*., 2000; Roosen *et al*., 2004; Davies *et al*., 2008
	Bovine neutrophil beta-defensins BNBD1–12,	mammary gland epithelium, milk somatic cells, distal part of small intestine, bone marrow, uterus, macrophages	Ryan *et al*., 1998; Tarver *et al*., 1998; Goldammer *et al*., 2004; Bagnicka *et al*., 2006
	LAP	squamous tongue epithelium, trachea, distal part of small intestine, palate, oesophagus, stomach, colon, rectum, nostrils, trachea, conjunctiva, skin, columnar epithelium of intestinal follicles, cerebral choroid plexus, cerebral cortex, cerebral Purkinje cells, sub-mucosal gland, milk somatic cells, uterus, placenta	Schonwetter *et al*., 1995; Russell *et al*., 1996; Tarver *et al*., 1998; Stolzenberg *et al*., 1997; Roosen *et al*., 2004; Bagnicka *et al*., 2006; Davies *et al*., 2008
	Enteric beta-defensin (EBD)	alveolar macrophages, colon, small intestine, milk somatic cells	Tarver *et al*., 1998; Bagnicka *et al*., 2006
	BBD119, BBD120, BBD122, BBD122a, BBD123, BBD124, BBD142	uterus	Cormican *et al.,* 2008
	DEFB401, DEFB405,	mammary gland	Roosen *et al*., 2004
Sheep	SBD-1,	epithelial cells, rumen, reticulum, omastum, small and large intestine, tongue, trachea	Huttner *et al*., 1998a
	SBD-2	epithelial cells, rumen, reticulum, omastum, tongue, trachea, ileum, large intestine	Huttner *et al*., 1998a
Goat	GBD-1	milk somatic cells, tongue, trachea, bronchi, lungs	Zhao *et al*., 1999; Bagnicka *et al*., 2005
	GBD-2	milk somatic cells, stomach, jejunum, ileum, large intestine, rectum	Zhao *et al*., 1999; Bagnicka *et al*., 2005
Pig	pBD-1	tongue, trachea, intestine, epithelial tissue	Shi *et al*., 1999; Zhang *et al*., 1999
	pBD2	liver, intestine (duodenum, jejunum, ileum), lungs, bone marrow	Sang *et al*., 2006
	pBD3	bone marrow, liver, lungs, lymphatic system	
	pBD4	lungs, epididymis	
	pBD104	pancreas, liver teste	
	pBD108	liver and epididymis	
	pBD114	ileum, pancreas, liver, lungs, tissues of the male reproductive system	
	pBD123	ileum, pancreas, lungs, tissues of the male reproductive system	
	pBD125	lungs, thymus, epididymis	
	pBD129	epididymis, duodenum, jejunum, pancreas, skin	
Horse	horse beta-defensin-1	many tissues and organs, including heart, pancreas, lymphoglandula, liver, lungs and digestive tract (small and large intestine)	Davis *et al*., 2004
Chicken	GAL 1 and GAL2	heterophils	van Dijk *et al*., 2007
	GAL3	tongue, bursa Fabricii, trachea, skin, oesophagus, air sacs, large intestine, kidneys	Zhao *et al*., 2001
	Gal6	digestive tract	
	Gal11	small intestine, liver, gallbladder, spleen	van Dijk *et al*., 2007
	Gal13	colon	
Duck	AvBD-2	bone marrow, spleen, kidney, lung, brain, bursa Fabricii, ovary	Ma *et al*., 2009a, 2009b
	AvBD-9	liver, kidney, crop, trachea	
	AvBD-10	liver, kidney	
Alpha-defensins			
Horse	DEFA1	intestine	Bruhn *et al*., 2007, 2009a, b

(sequence identity 86.2%). The sequence identity was 92.1%, 81.6%, and 84.6% with bovine LAP, bovine TAP, and goat BD-2, respectively. The deduced amino acid sequence encoded a 64-amino acid precursor peptide.

DEFENSINS IN SHEEP

In sheep, two beta-defensin genes have been identified and named *SBD-1* and *SBD-2* (Huttner *et al*, 1998a, 1998b). Iannuzzi *et al.* (1996) reported that sheep beta-defensin genes are located on chromosome 24q, while Huttner *et al.* (1998b) claimed their localization on chromosome 26. SBD-1 and SBD-2 pre-propeptides contain 64 amino acids. Their nucleotide sequence is identical in 87%, while the degree of identity of the amino acid sequence is 78%, which indicates a positive selection pressure. The transcripts of both ovine defensins were found in epithelial cells, but their levels differed between organs and between individual animals. The highest transcript abundance was found in the rumen as well as in small and large intestine (Table 1). In rumen the highest *SBD-1* and *SBD-2* mRNA levels were recorded during the first 6–8 weeks of life. Studies conducted on pregnant sheep demonstrated significant differences in expression levels of both defensins between tissues and between animals in the same period of pregnancy. The highest expression tended to appear during the third trimester. The highest level of *SBD-1* gene transcripts in adult sheep was observed in the tongue, large intestine epithelium and in trachea. A lower mRNA level was recorded in rumen epithelium. In ileum the *SBD-1* transcript was not found. In turn, the *SBD-2* gene transcript was shown only in the ileum and large intestine. No expression of beta-defensins 1 and 2 was recorded in sheep pancreas, heart or liver (Huttner *et al.*, 1998a).

Luenser *et al.* (2005) identified thirteen beta-defensin encoding sequences in six animal species belonging to the tribe *Caprini*, including sheep (*Ovis aries*) and goat (*Capra hircus*). In this number, two had already been known as sheep and goat beta-defensins 1 and 2. All the remaining beta-defensin sequences were identified as variants of SBD-1 and SBD-2 (sheep) or GBD-1 and GBD-2 (goat). A high conservation of beta-defensin exons was demonstrated; therefore discrimination of the different beta-defensin genes was possible only due to intron-specific differences.

DEFENSINS IN GOAT

Two beta-defensins have been identified in goats — GBD-1 and GBD-2. Genes of their precursors — pre-proGBD-1 and pre-proGBD-2 — were 96.8% identical in nucleotide and 88.2% in amino-acid sequence (difference of eight amino acids), again indicating a positive selection pressure. GBD-1 transcript was found in the tongue, trachea, bronchi and lungs. That of *GBD-2* was found in the stomach, large intestine, and rectum (Zhao *et al.*, 1999; Table 1). Anbu *et al.* (2003) studying cationic peptides isolated from goat tongue, demonstrated their germicidal activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*E. coli*) bacteria.

In our studies we found transcripts of *GBD-1* and *GBD-2* in kidneys, trachea, tongue epithelium, spinal cord, and in mammary gland of non-lactating (dry) goats (Bagnicka *et al.*, 2005). The highest mRNA levels of both beta-defensins were recorded in the trachea, slightly lower in tongue, mammary gland and kidneys, and the lowest in the spinal cord. Moreover, a varying level of defensin transcripts was found in the goat milk cells, depending on the somatic cell count (SCC); the higher the SCC the higher was the level of the transcripts.

The mRNA sequence of a gene encoding caprine lingual antimicrobial peptide — *LAP* was cloned and characterized (Sharma *et al.*, 2006; GenBank DQ836129). The information on the goat *LAP* includes the whole cds and translation product. Our comparison of the nucleotide sequence of *LAP* and *GBD-1* genes and the amino-acid sequences of goat defensins has revealed that GBD-1 is 18 amino acids shorter than that of LAP. This difference may be due to the presence of the stop codon UAG at position 209–211 in *GBD-1* mRNA. In the *LAP* mRNA counterpart, this triplet (CAG) encodes glutamine. Therefore, the sequence of the first 64 amino acids is identical in both LAP and beta-defensin-1 (GBD-1). The nucleotide sequence of *GBD-1* and *LAP* mRNAs differs only at two nucleotide positions (209 and 238, according to GenBank DQ836129). Thus, our analysis of the mRNA and peptide sequences suggests that in goat, GBD-1 and LAP may be products of two recently duplicated genes of which one acquired the nonsense mutation to create the TAG stop codon (Bagnicka *et al.*, submitted for publication).

DEFENSINS IN PIG

Until recently, only one beta-defensin *pBD-1* transcript was detected in pig tongue epithelium by Northern blot technique (Shi *et al.*, 1999). This defensin is active against *E. coli*, *Salmonella typhimurium*, *L. monocytogenes* and *C. albicans*. Thus, it is likely that it co-creates the antimicrobial barrier of the tongue and oral cavity in pigs. The gene encoding pBD-1 consists of two short exons (82 bp and 234 bp) separated by a 1535 bp-long intron and is located on chromosome 15q14-q15.1. An expression of *pBD-1* mRNA was demonstrated during inflammation and bacterial infections of the tongue, trachea, and intestine epithelium (Zhang *et al.*, 1999). Only recently did Sang *et al.* (2006) record transcripts of eleven other beta-defensins in different porcine tissues. Abundant *pBD-2* transcript was found in the liver, intestine, lungs and bone marrow; *pBD-3* — in bone marrow, liver, lungs, and lymphatic system; *pBD-4* — in lungs and epididymis; *pBD-104* — in pancreas, liver and testes; *pBD-108* — in liver and epididymis; *pBD-114* — in ileum, pancreas, liver, lungs and tissues of the boar reproductive tract (Table 1). The presence of *pBD-123* transcript was recorded in the same tissues (with the exception of liver) in which *pBD-114* mRNA occurred. The transcript of the *pBD-125* gene was detected in lungs, thymus and epididymis, while that of *pBD-129* — in epididymis, duodenum, jejunum, pancreas, and skin (Table 1). Transcription of the *pEP2C* pseudogene was detected in the thymus, skin, testes, and certain secretions of the epididymis, but not in other tissues.

Recently, about 30 host defense peptides (HDPs) have been identified in the pig and their structure and functions partially characterized (Sang & Blecha, 2009). The antimicrobial activity of the porcine peptides has been evaluated extensively against a broad spectrum of microorganisms *in vitro* and for their protective role *in vivo*. Veldhuizen *et al.* (2008a) studied the antimicrobial activity of pBD-2, a porcine beta-defensin produced in the intestine. The peptide showed high antimicrobial activity

against a broad range of pathogenic bacteria. The highest activity was observed against *S. typhimurium*, *L. monocytogenes* and *Erysipelothrix rhusiopathiae*. Salmonellosis is a serious problem in pig breeding, therefore more detailed studies were conducted on the antimicrobial activity of pBD-1 and pBD-2 against *S. typhimurium* (Veldhuizen *et al.*, 2008a; 2008b). A 4–8 μM concentration of pBD-2 was sufficient to lyse these bacteria in 3 h. At higher ionic strengths the antimicrobial activity of pBD-2 decreased, and disappeared completely at the physiological concentration of NaCl (150 mM).

Inter-breed differences have been found in beta-defensin gene expression levels in various pig tissues (Chen *et al.*, 2010). In most tissues the abundance of *pBD-1, 2* and *3* mRNAs was higher in Meishan pigs than in the Duroc x Yorkshire x Landrace crossbreds. As the authors concluded, the higher expression of pBDs might be the reason why Meishan pigs have a higher resistance to disease than other pig breeds.

Galindo *et al.* (2009) have analyzed global gene expression profiles in the spleen of European wild boar naturally infected with *Mycobacterium bovis* using real-time RT-PCR analysis and an expression microarray (Pigoligoarray) representing 20 400 genes. Immune response genes were the most differentially expressed. Beta-defensin 129, T-cell surface glycoprotein CD8 and B-cell receptor-associated protein 29 were overexpressed in naturally infected animals compared with non-infected. That study has identified new mechanisms by which naturally infected wild boar respond to *M. bovis* infection and how the pathogen circumvents the host immune responses to establish infection.

DEFENSINS IN HORSE

Milenkovic *et al.* (2002) analyzed a horse bacterial artificial chromosome (BAC) DNA library and found a gene having 93% nucleotide sequence identity with those encoding goat and human beta-defensins. In the horse genome it was localised on the long arm of chromosome 9, band 14 (GTG staining). This was the first report on a defensin gene in horse.

In 2004 Davis and co-workers reported a full sequence of horse beta-defensin-1 cDNA. This defensin is about 60% identical with defensins of other animal species and humans. Horse *BD-1* cDNA shows the highest level of identity with pig *BD-1* (69.5%). The same authors demonstrated expression of beta-defensin-1 mRNA in many tissues and organs of the horse, including heart, pancreas, liver, lungs and the digestive tract.

The equine defensin gene cluster has been localized on ECA 27q17 using fluorescence *in situ* hybridization and confirmed by RH-mapping of a marker from the *DEFB1* (AY170395) gene (Looft *et al.*, 2006). This is in agreement with the results of Swinburne *et al.* (2006) indicating homology of the defensin-harboring human autosome 8 (HSA 8) with equine autosomes — ECA 9 and ECA 27.

Looft *et al.* (2006) using a horse BAC clones identified nine genes, of which six showed a high sequence similarity with the gene of human beta-defensin 4. For most of those genes no transcripts were detected in horse tissues. The authors identified also ten pseudogenes of high homology with human beta-defensin 4 (*DEFB-4*). Moreover, they identified a sequence showing high similarity with human alpha-defensin genes. That novel gene was named *DEFA-5L*; its pseudogene (*DEFA-5LP*) was also identified, without experimentally proven expression. These could be the first alpha-defensins found outside the species belonging to primates, lagomorphs (rabbit and hare) and rodents. Further studies found another thirty-eight transcripts of alpha-defensins in equine intestinal. At least 20 of them may code for functional peptides. For 14 genes present in the horse genome, however, no transcripts were found and 10 transcripts have no matching genomic sequence. These findings indicate that the assembly of the horse alpha-defensin gene cluster is not yet complete. To date, in addition to the species listed above, alpha-defensin genes have also been identified in opossum, elephant and hedgehog tenrec (Bruhn *et al.,* 2009b).

Studies by Bruhn *et al.* (2007; 2009a, 2009b) confirmed the presence of alpha-defensins in equine intestine. The first indentified equine alpha-defensin – DEFA1 – has a broad spectrum of antimicrobial activity, with the highest sensitivity at peptide concentrations <1 μM (LD90) shown by Gram-positive bacteria.

DEFENSINS IN POULTRY

Until recently, only peptides from the beta-defensin family have been identified in birds (Harwig *et al.*, 1994). But now several avian cathelicidins family members are also known (Meade *et al.*, 2009). The first beta-defensins identified in the chicken were gallinacins: GAL1/CHP1, GAL1 alpha/CHP2, GAL2 and GAL3, while in turkeys — THP1, THP2, THP3 and GPV-1. These peptides contain 36–39 amino acids and have a strong cationic nature due to the presence of numerous lysine and arginine residues (Brogden *et al.*, 2003). GAL3 was isolated from chicken (*Gallus gallus*) epithelium. In healthy birds this peptide was found in many organs and tissues (Table 1). The expression of GAL3 in the trachea increased significantly after infection with *Haemophilus paragallinarun*, while in tongue, bursa Fabricii and oesophagus it remained constant (Zhao *et al.*, 2001). Some of the peptides found in chicken (CHP1 and 2) and turkey (THP1) are active against *S. aureus* and *E. coli*, while THP2 and THP3 — only against *S. aureus*; they also show a fungicidal activity. However, no activity of those peptides was observed against the virus causing contagious bronchitis in birds (Evans *et al.*, 1995).

Thirteen beta-defensin genes have been described in the chicken — *GAL1-13*, and showed to comprise a gene cluster. Polymorphisms of those genes and especially of three of them — *GAL11, GAL12* and *GAL13* were correlated with the resistance of the animals to *Salmonella enteritidis* infection (Hasenstein & Lamont, 2007; Derache *et al.*, 2009a). Transcripts of *Gal1* and *2* were found in heterophils, *Gal11* — in small intestine, liver, gallbladder and spleen, *Gal13* — in colon, while *Gal6* — in the digestive tract (Table 1). The sequence of the *GAL6* gene showed the most similarity with human *BD-1* (43% identity), with bovine neutrophil beta-defensin genes (43 to 45%), and with sheep beta-defensin genes (41%) (van Dijk *et al.,* 2007). According to the current knowledge, the avian beta-defensin family (previously referred to as gallinacins) consists of 14 peptides (*AvBD1-14*) encoded by genes located on chromosome 3 in a single 85-kb region (Meade *et al.*, 2009).

Gong *et al.* (2010) have found in the chicken genome, three copies of a gene encoding gallins, antimicrobial peptides closely related to avian beta-defensins, of a new avian antimicrobial peptide family.

Gallins contain six cysteine residues found in all defensins, although their spacing differs. They were expressed in tubular cells of the magnum region of oviduct and their mRNA levels were 10 000 times greater in magnum than in shell gland. These peptides have potent antimicrobial activity against *E. coli* and form antimicrobial barrier in the avian innate immune system, particularly in the egg white.

Three biologically active beta-defensins have been purified by chromatography from chicken bone marrow: avian beta-defensins AvBD-1, AvBD-2 and AvBD-7 (Derache *et al.*, 2009b). Their antibacterial activities were assessed against a large panel of Gram-positive and Gram-negative bacteria. All three defensins displayed similar activity against Gram-positive strains, but AvBD-1 and AvBD-7 exhibited stronger activity against Gram-negative bacteria then AvBD-2. Those authors also studied expression of two beta-defensins, AvBD-1 and AvBD-2C, in embryonic intestinal cells isolated from chickens of two inbred lines of different susceptibility to *S. enteritidis*. Primary intestinal cell cultures were found to differentially express the two beta-defensin genes, depending on the line. Furthermore, *S. enteritidis* interfered with AvBD-2 expression only in the cells from the susceptible line 15I, suggesting that these antimicrobial peptides may play a crucial role in immunoprotection against bacteria.

Ebers *et al.* (2009) determined mRNA expression profiles of 14 avian beta-defensins (AvBDs) in primary chicken oviduct epithelial cells before and after infection with *Salmonella enterica*. The infection temporarily inhibited expression of certain AvBDs but induced expression of other minimally expressed defensins. Distinct expression patterns of innate immune genes, including Toll-like receptors, AvBDs, and both pro- and anti-inflammatory cytokines during early chicken embryonic development, were shown by Meade *et al.* (2009). Expression of AvBD-9 was significantly increased on day 9; and AvBD-10 was increased on day 12 in embryo abdomen, relative to day 3 expression levels.

The presence of two beta-defensins in ostrich — ostricacins-1 and 2 (Osp-1 and Osp-2) has been described (Sugiarto & Yu, 2007). The ability to disrupt bacterial membrane integrity by those defensins was shown to be weaker than that of sheep cathelicidin SMAP-29, but stronger than that of human neutrophil peptide-1 HNP-1 (alpha-defensin).

Two beta-defensins homologous to other avian beta-defensins were isolated from duck liver by Ma *et al.* (2009a, 2009b); they were named duck AvBD-9 and AvBD-10. The *AvBD-9* mRNA was differentially expressed in many tissues of ducks, with especially high levels of expression in the liver, kidney, crop, and trachea. Duck AvBD-10 was only expressed in the liver and kidney (Table 1). Both defensins exhibited antimicrobial activity against several bacterial strains: *Bacillus cereus*, *Pasteurella multocida*, *E. coli*, *Salmonella choleraesuis*, and *S. aureus*. Soman *et al.* (2009) identified and characterized a duck beta-defensin 2 homologue gene with a 195-base pair open reading frame, which was 83% identical with chicken and 85% with turkey beta-defensin 2. The peptide encoded by this gene had the classical beta-defensin core motif formed by a beta-sheet-rich structure. Apart from moderate expression in the kidney, lung, brain, bursa Fabricii and ovary; duck *AvBD-2* mRNA showed a very high constitutive expression in bone marrow and spleen, indicating that it is a myeloid defensin.

DEFENSINS AS GENETIC MARKERS

The nucleotide sequence polymorphism of human defensin genes has been studied intensively. Single nucleotide polymorphisms (SNPs) of beta-defensins have been correlated with increased susceptibility to certain diseases. Associations have been reported between beta-defensin gene polymorphisms and susceptibility to diabetes, melanoma, oral squamous carcinoma, prostate cancer, Crohn's disease, psoriasis, dermatitis, leprosy, and infectious diseases — *Helicobacter pylori*-induced gastritis, HIV infection, and many others. Numerous studies have reported altered expression of beta-defensins in cancers suggesting their involvement in carcinogenesis. At least in one case, the functionality of a mutation in a defensin encoding gene has been shown in that it modifies the gene expression level (Sun *et al.*, 2006). In the human beta-defensin 1 (*hBD-1*) gene, a candidate tumor suppressor, the C/G polymorphism at position –688 upstream of the ATG translation start codon affected *hBD-1* gene promoter activity in a reporter gene (luciferase) transcription test in DU145 and TSU-Pr1 cells. The transcription rate was 40 to 50% lower when the promoter variant with the G nucleotide was used compared with the wild-type promoter variant with nucleotide C. In addition, the C/G polymorphism at position –44 modified the transcriptional activity of the *hBD-1* promoter; the C→G transversion enhanced transcription level up to 2.3-fold. The effect of the –44C/G transversion on the human beta-defensin 1 (*hBD-1*) gene expression was then confirmed in many different types of normal and cancerous cells.

Very little study has been carried out on the polymorphism of defensin genes in farm animals and its effect on disease susceptibility and production traits. Hasenstein and Lamont (2007) analyzed two lines of chickens for association of gallinacin genotypic variation with resistance to *S. enteritidis* infection. In the search for nucleotide sequence polymorphism, thirteen chicken gallinacin genes, which are the functional equivalents of mammalian beta-defensins, were sequenced from individuals of different hen breeds and production lines. On average, seventeen SNPs per kilobase were found in the chicken gallinacin gene cluster. The SNP genotypes of the *GAL11*, *GAL12*, and *GAL13* genes showed an association with bacterial load in the cecal content suggesting a role of the gallinacins in the defense of poultry against enteric pathogens. The authors concluded that the polymorphisms of the chicken gallinacin (beta-defensin) genes *GAL11, GAL12* and *GAL13* could be used as a marker assisting in selection of poultry for resistance to *S. enteritidis* infection.

In our earlier study (Ryniewicz *et al.*, 2003) we revealed twenty different combined genotypes (CDGs) in bovine defensin genes. We showed that several CDGs significantly associated with dairy performance traits of Holstein-Friesian cows, as well as with the milk somatic cell count (SCC), a well recognized indicator of clinical or sub-clinical mastitis in cow udders. The results speak for using defensin genes as markers of disease susceptibility and productivity of cows. This could help in early selection of high yielding animals with high resistance to mastitis. Our results were confirmed by Wojdak-Maksymiec *et al.* (2006) who investigated associations between the same combined defensin genotypes and somatic cell count (SCC) in Jersey cows. The highest SCC was found in the milk of cows with the A1-B1-C1C2 genotype, whereas the lowest in cows with the genotype

A2-B1B2C1C2. Also studied were associations between the defensin genotypes and milk production traits. Combined genotypes were found to associate with daily milk yield and with fat and protein content in milk.

Later on we found ten SNPs in the bovine *BNBD-4* gene intron by sequencing of the gene from ten Holstein-Friesian (HF) cows (Bagnicka *et al*., 2007; 2008). Two SNPs generated new endonuclease digestion sites and therefore could easily be genotyped using the restriction fragment length polymorphism (RFLP) technique. This enabled genotyping of a numerous cohort of dairy HF cows and studying associations of the *BNBD-4* gene polymorphism with milk production traits and resistance/susceptibility to mastitis. The A→C transversion at position 1674 (according to GenBank No. AF008307; recognized with RFLP-*Bsr*I) was associated with daily milk, fat and protein yield, average daily fat, protein, lactose, and dry matter contents. On the other hand the C→T transition at position 2239 (RFLP-*Nla*III) affected milk fat, protein and lactose contents and also the number of somatic cells in the milk. Therefore, they were shown to be promising bio-markers for early selection of dairy cows less susceptible to mastitis and producing milk of good quality (Bagnicka *et al*., 2007; 2008). In general, an important message from our studies is that the defensins may be used as genetic markers in breeding programs aimed at selecting highly productive dairy cattle with increased resistance to udder infections.

Recently, two microsatellites (short tandem repeats — STRs) and five SNPs newly found in the bovine beta defensin 103 (*DEFB103*) gene were used as genetic markers for linkage mapping with red coat phenotype in Holstein cattle (Dreger & Schmutz, 2010). The variant red (VR) phenotype, caused by a mutation in melanocortin 1 receptor, was shown to co-segregate with markers in a region of chromosome 27 that included *DEFB103*. One haplotype was inherited in VR cattle in a 6-generation pedigree.

PERSPECTIVES

One of the largest and most studied group of antimicrobial peptides are defensins, especially the beta-defensin subfamily. In addition to the beta-defensins many other types of anti-microbial peptides are known, including alpha- and theta-defensins, cathelicidins and others. Defensins are common in plants and animals. The high variety of the antimicrobial peptides is obviously a response of organisms to the diversity of pathogens. The antimicrobial peptides are thought to have diverged during evolution under the pressure of natural selection to maintain a host-pathogen balance (Nava *et al*., 2009). On the other hand, the universal character of the antimicrobial peptides contributed to their conservation throughout evolution.

The antimicrobial peptides that participate in the innate immunity of most metazoa justly hold claim to the name of "new generation antibiotics". They could be used both in preventive and therapeutic treatments (Kamysz *et al*., 2003; Koczulla & Bals, 2003). The benefits of the use of antimicrobial peptides in therapy include the wide spectrum of their activity, direct antimicrobial action, stimulation of phagocytosis, and anti-inflammatory activity (Kamysz *et al*., 2003). Despite having an ancient origin the defensins remain the most efficient component of antimicrobial defense because they have not induced resistance in most microorganisms. This is possibly due to their high diversity. The high diversity of antimicrobial peptides and their several modes of action against bacteria might have impeded the evolution of resistance in natural bacterial populations. On the other hand, an increasingly wide future therapeutic use of only some of AMPs may lead to the evolution of bacterial resistance (Perron *et al.,* 2006; Nava *et al.,* 2009).

Intensive studies are being conducted in medical research centres and pharmaceutical companies aimed at production of synthetic or recombinant peptides in order to use them in human treatment. Some antimicrobial peptides are in the pre-clinical and clinical phases of study. Most, but not all such studies, have shown positive results. Their results seem very promising and researchers are beginning to see the reality of using such peptides in human medicine (Gordon & Romanowski, 2005; Jenssen *et al.,* 2006; Wiechuła *et al.,* 2006). Studies have been conducted on the synergistic effects of defensins and classical antibiotics (Leszczyńska, 1998) and the use of defensins as immunostimulators to induce the natural resistance of the human organism (Gordon & Romanowski, 2005). Understanding all aspects of the biological activity of antimicrobial peptides, then the synthesis or production of recombinant peptides is very important, but is only the first step that must be taken. Using exogenous peptides in human treatment is a challenge as it is still necessary to develop appropriate methods for their administration. It is also still necessary to develop ways of avoiding their possible toxicity to the host organism. Moreover, production of such compounds is very expensive. So far, their use has been limited to local treatments (creams, ointments, mouth rinsing preparations). Antimicrobial peptides administered, for example, intravenously would have to permeate healthy tissues to reach infection sites, thus exerting undesirable side effects. Moreover, the peptides are rather large molecules. They have a strong positive charge and enter tissues slowly. Some promising signals have emerged indicating the possibility of solving these problems.

Antimicrobial peptides, especially defensins, constitute an important innate, non-specific system in the natural defense in animals and human. No doubt, their use in human therapy is only a question of time. Studies on antimicrobial peptides carried out in farm animals are still a step behind those in humans or laboratory animals. So far, there are no plans to use such peptides to treat diseases in farm animals. Nevertheless, the results of recent studies, including ours, indicate that the polymorphism of beta-defensin genes may be a marker in selection for milk production performance and/or susceptibility to mastitis of farm ruminants (Ryniewicz *et al*., 2003; Bagnicka *et al*., 2007; 2008) and those of chicken AMPs — in selection for resistance to *Salmonella* infections (Hasenstein & Lamont, 2007). To reach these goals, further association studies are needed to be carried out on numerous groups of farm animals of different species, supported by basic research of biological activities of defensin variants. The most challenging seem to be efforts to elucidate the causative relationships between the nucleotide sequence polymorphisms in defensin genes and the anti-microbial functions of encoded peptides. For such studies advanced molecular methods of functional genomics, such as transcriptomics, proteomics and next-generation sequencing, would be very helpful.

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