Folia biologica (Kraków), vol. **55** (2007), No 3-4

Review

Effect of Bovine PRNP Gene Polymorphisms on BSE Susceptibility in Cattle

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Accepted April 16, 2007

GURGUL A., SŁOTA E. 2007. Effect of bovine PRNP gene polymorphisms on BSE susceptibility in cattle. Folia biol. (Kraków) **55**: 81-86.

Prion protein gene (PRNP) variants determine the susceptibility of humans, sheep and mice to prion diseases, whereas polymorphisms in the open reading frame (ORF) of bovine PRNP seem to be unrelated to the incidence of bovine spongiform encephalopathy (BSE). According to the latest reports, the genetic susceptibility of cattle to BSE is associated with polymorphisms of the regulatory region of the PRNP gene and the level of its expression. This review provides information on the bovine PRNP gene, its polymorphism, and recently identified genetic markers for BSE, and attempts to explain the mechanism behind the genetic resistance or susceptibility of cattle to this disease.

Key words: PRNP, BSE, susceptibility, cattle, polymorphism, promoter, prion protein gene.

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BSE is one of the transmissible spongiform encephalopathies (TSEs), a group of fatal prion neurodegenerative diseases, observed in different species of animals and in humans.

TSEs are characterized by the accumulation in the central nervous system (CNS) of a pathological, protease-resistant isoform of the prion protein (PrP^{sc}), generated as a result of post-translational modification and change in the stereochemical conformation of host-encoded, cellular prion proteins (PrP^{c}). As an infectious particle, PrP^{sc} is able to penetrate the CNS and trigger the chain reaction of conversion of PrP^{c} into PrP^{sc} (PRUSINER 1982; PRUSINER 1998). Feeding meat-and-bone meals contaminated with PrP^{sc} is considered to be the main cause of the BSE epidemic among cattle (WILESMITH *et al*. 1992). The disease, due to its infectious nature, poses a threat not only to animals but also to humans who eat meat products (SCOTT *et al*. 1999).

Because the cellular prion protein plays a strategic role in the development of the disease, it is essential to explore all factors affecting its synthesis and stability. Among possible factors are mutations within the cellular PRNP gene, which is responsible for PrP^{c} synthesis (BASLER *et al*. 1986).

Polymorphisms in the ORF of this gene, which cause amino acid substitution, have been associated with the susceptibility to TSE and the incubation period of the disease in humans (HILTON *et al*. 2004) and in sheep (BOSSERS *et al*. 1996). Sheep with valine at codon 136 of the ORF and glutamine at codon 171 (V136, Q171) show susceptibility to, and a short incubation period of scrapie, whereas sheep with alanine and arginine at the same codons (A135 and R171) are resistant to this disease (BOSSERS *et al*. 1996). It is believed that hereditary mutations in the PRNP gene are the main cause of familial prion diseases in humans. A very rare disease known as fatal familial insomnia (FFI) has been linked to the polymorphism of codons 178 (D178N) and 129 (M129) of the ORF (ZARRANZ *et al*. 2005). Studies of variant Creutzfeldt-Jakob disease (vCJD) have showed that it only develops in homozygotes for the predisposing allele (M129M) (HILTON *et al*. 2004). Although BSE is in the same group of diseases, the effect of genetic factors on its development in cattle is not completely clear and there are still only a limited number of studies showing a relationship between polymorphisms in the bovine PRNP promoter region and BSE susceptibility in cattle. It therefore seems appropriate to collect the available information on research aimed at exploring the genetic background of this disease and general

information on the bovine PRNP gene, providing the basis for further studies in this area.

Characteristics of the bovine PRNP gene and the relationship between its polymorphisms and susceptibility of cattle to BSE

The bovine PRNP gene was localized within syntenic group U11 (RYAN & WOMACK 1993) and mapped on BTA13 chromosome (13q17) (IANNUZZI *et al*. 1998; SCHLÄPFER *et al*. 1998). This gene extends over 20.2 kb, and full-length mRNA containing three exons is 4244 bp. Exon 1 spans 53 bp and exon 2 spans 98 bp (INOUE *et al*. 1997). The size of the second intron has been estimated to be approximately 14 kb (HORIUCHI *et al*. 1998). The whole ORF is located within exon 3 and has a size of 795 bp (YOSHIMOTO *et al*. 1992). The complete genomic sequence of 78 056 bp has also been determined and deposited in the EMBL/GenBank database under accession number AJ298878 (HILLS *et al*. 2001).

The most frequently observed polymorphism within the ORF of the bovine PRNP gene is an octapeptide-repeat polymorphism (PREMZL *et al*. 2000; VRTKOVA *et al*. 2001; LEONE *et al*. 2002; WALAWSKI & CZARNIK 2003; JEONG *et al*. 2005; NAKAMITSU *et al*. 2006).

The ORF of the bovine gene contains five or six copies (R1-R6) of tandem sequences encoding octapeptide repeats in the N-terminal part of the prion. These sequences are C-G-rich and consist of 24 or 27 bp. Depending on the variant (24 or 27 bp), they encode the peptide Pro-His-Gly-Gly-Gly-Trp-Gly-Gln (PHGGGWGQ) or Pro-Gln/His-Gly-Gly-Gly-Trp-Gly-Gln. The 27-bp variant encodes the R1 and R6 octapeptide repeat. The polymorphism detected in this region results from the insertion/deletion (indel) of one octapeptide repeat (24-bp indel) and usually it is the R3 repeat (GOLDMANN *et al*. 1991).

The most frequent genotype in cattle is PRNP 6/6 and the least frequent is PRNP 5/5. In 1163 Polish Black-and-White cattle analyzed, the frequency of genotypes was found to be 0.808 for 6/6, 0.181 for 5/6 and 0.011 for 5/5 (WALAWSKI & CZARNIK 2003). Although this breed of cattle has been considerably improved using Holstein-Friesian cattle, compared to purebred Holstein cattle it is characterized by a relatively high frequency of the 5/5 genotype. By way of example, no PRNP 5/5 genotype has been found in Holstein-Friesian cattle in Croatia or Italy (PREMZL *et al*. 2000; LEONE *et al*. 2002). Despite small differences in the frequency of the PRNP 5/5 genotype, the frequency of separate alleles in modern cattle breeds is similar. In Japan, in 869 Holstein cattle examined, the frequency of alleles was 0.94 (PRNP 6) and 0.06 (PRNP 5) and in 186 Japanese Balck cattle 0.97 and 0.03, respectively (NAKAMITSU *et al*. 2006). In Korea, it was 0.915 and 0.085 in 53 Holstein cattle (JEONG *et al*. 2005) and in Poland, it was 0.894 and 0.106 in 1163 Black-and-White cattle analyzed (WALAWSKI & CZARNIK 2003). In native or rare cattle breeds the frequency of the PRNP 5 allele seems to be higher than in intensively selected modern breeds (VRTKOVA *et al*. 2001).

Interesting results were obtained when studying the familial segregation of octapeptide-repeat alleles in Polish Black-and-White cattle. Among 58 progenies from parents with PRNP 5/6 genotypes, the distribution of genotypes (48% 6/6, 32.8% 5/6 and 22.4% 5/5) differed significantly ($\chi^2 = 12{,}72$; 2 df.) from the predicted distribution of 25% : 50% : 25%. The authors suggest that this may be associated with a possible lethal cis-trans linkage effect (WALAWSKI *et al*. 2003).

Polymorphism in the region of octapeptide repeats seems to be unrelated to the susceptibility of cattle to BSE, although HUNTER *et al*. (1994) found that in the material studied, none of the infected cows was homozygous for the PRNP 5 allele. However GELDERMANN *et al*. (2006) observed the PRNP 5/5 genotype in both healthy and BSE-affected animals. This discrepancy may result from a very low frequency of this allele in the population. It is intriguing that the AA genotype, determined using single-strand conformation polymorphism (SSCP) analysis in the region of octapeptide repeats, was significantly more frequent ($P<0.001$) in 56 diseased than in 38 healthy cattle of British Friesian breed (NEIBERGS *et al*. 1994). It was also found that the disease incubation period after BSE inoculation in transgenic mice carrying a modified bovine gene containing 7 octapeptide repeats is much shorter than in mice with a bovine gene containing 6 repeats (CASTILLA *et al*. 2004) and what is more, in mice with a gene construct containing 10 octapeptide repeats, a disease with symptoms typical of TSE develops spontaneously but is not transmissible (CASTILLA *et al*. 2005). Rare cases of an allele with 7 repeats in cattle and a single case of a gene variant with 4 repeats were observed in the Brown Swiss breed (SEABURY *et al*. 2004).

Single nucleotide polymorphisms (SNPs) found in the PRNP gene that cause amino acid substitution affect the susceptibility to prion diseases in sheep and humans. The search for point mutations in the ORF of the bovine PRNP gene revealed 13 SNPs in indigenous Indonesian cattle, including two nonsynonymous substitutions (TAKASUGA *et al*. 2003), 10 SNPs in the native Brazilian Caracu breed (KUES *et al*. 2006) and a total of 12 SNPs in various breeds of American cattle, including two that were predicted to alter the amino acid se-

quence of PrP. In most of these SNPs, the frequency of the less common allele was low and ranged from 0.01 to 0.14 (HEATON *et al*. 2003). In the Holstein-Friesian breed in which the highest incidence of BSE was found, the only detected polymorphism that altered the amino acid sequence is the octapeptide-repeat polymorphism, and the loci of silent SNPs are not as numerous.

The most frequently observed silent SNPs are substitutions of nucleotides in codons 78 and 129 of the ORF (HUMENY *et al*. 2002; HEATON *et al*. 2003; HILLS *et al*. 2003; TAKASUGA *et al*. 2003; SANDER *et al*. 2004; JEONG *et al*. 2005; NAKAMITSU *et al*. 2005). The first of these polymorphisms is a guanine to adenine substitution (CAG→CAA) in the third position of codon 78 (A234G). This position corresponds to the last nucleotide in the sequence of the third octapeptide repeat (R3) (HUMENY *et al*. 2002). The same mutation was earlier described as an isoform of the R3 repeat (RN1) and was linked to the presence of an allele containing 7 octapeptide repeats (SCHLÄPFER *et al*. 1999). In subsequent studies, HUMENY *et al*. (2002) found that the RN1 motif occurs regardless of the presence of the PRNP 7 allele and seems characteristic of some breeds. The polymorphism of codon 78 does not result in amino acid substitution and the allelic variants occur in different breeds and in different populations with similar frequency. The frequency of the allele containing adenine (A234) was 0.26 in the U.S. MARC (Meat Animal Research Center) Beef Cattle Diversity Panel version 2.1 (HEATON *et al*. 2001; HEATON *et al*. 2003), 0.19 in Japanese Holstein cattle (NAKAMITSU *et al*. 2005) and 0.283 in Korean Holstein cattle (JEONG *et al*. 2005).

The polymorphism at codon 192 involves the substitution of thymine instead of cytosine (AAC→AAT) at the third position of this codon (C576T). The frequency of the allele containing thymine (T576) in Holstein cattle is low with a value of 0.032 in Korean Holstein cattle (JEONG *et al*. 2005) and null in Japanese cattle. By way of comparison, in Japanese Black cattle the frequency of this allel was 0.4 (NAKAMITSU *et al*. 2005). This polymorphism does not cause amino acid substitution and has been found to be unrelated to the susceptibility of cattle to BSE (HUNTER *et al*. 1994).

Because there is no evidence that polymorphisms in the ORF of bovine PRNP affects BSE susceptibility in cattle, studies were begun to explore the remaining part of the gene. Non-coding parts of the gene and flanking sequences contain regulatory elements that may affect its function. In order to determine polymorphism of the PRNP gene, HILLS *et al*. (2003) sequenced samples from 13 Holstein-Friesian cows and from two of each of the following cattle breeds: Aberdeen Angus, Ayrshire, Hereford, Longhorn, South Devon and Shorthorn. Within the 22 kb spanning the entire bovine gene and small fragments of 5' and 3' flanking regions, they localized 51 polymorphic sites, including two (described earlier) within the ORF. Forty-two of the fifty-one polymorphisms observed were SNPs, and the other nine were indels. Of the SNPs identified, 79% were transitions and 21% were transversions. The localization of these variations in the reference sequence, and their characteristics, are included in the GenBank database under accession number AJ298878.

Analysis of the complete genomic sequence of the bovine PRNP gene showed that over 37% of its DNA is composed of repetitive DNA motifs. In this sequence, 24 microsatellite *loci* with three or more repeats and over 90% motif homology were localized. Eight of these are polymorphic, of which four have two alleles and one each has three, four, six (R18 in intron 2) and ten (R16 in intron 1) alleles. The sequences of these alleles are accessible at GenBank under accession numbers from AF532794 to AF532816 (GELDERMANN *et al*. 2003).

The association of microsatellite polymorphism and other DNA variants in the PRNP region with the susceptibility of cattle to BSE was also investigated by GELDERMANN *et al*. (2006). They studied material obtained from 252 BSE-affected and 376 healthy cattle of four breeds (Simmental, Brown Swiss, Holstein Black and Holstein Red). The frequency analysis of different alleles of R16 and R18 microsatellites and *locus* REG2 revealed significant differences between diseased and healthy animals. In the group of cattle with BSE, alleles R16 164 bp, R18 173 bp and REG2 128 bp occurred with higher frequency ($P<0.05$; $P<0.01$; $P<0.001$ respectively), while alleles R16 167 bp, R18 174 and 175 bp as well as REG2 140 bp occurred with lower frequency ($P<0.05$; $P<0.01$; $P<0.001$) than in the control group. Significant differences were found in the frequency of some genotypes between the group of cattle with BSE and the control cattle. REG2 128/128 and R18 173/174 genotypes were more frequent ($P<0.01$), and REG2 140/140 and R18 174/175 genotypes were less frequent ($P<0.01$) in cattle with BSE than in healthy cattle. Likewise, the REG2 128 bp – R18 173 bp haplotype occurred more frequently ($P<0.001$) in diseased than in healthy animals, while the REG 140 bp – R18 175 bp haplotype was overrepresented ($P<0.05$) in healthy animals. It was also found that animals with the REG2 128/128 genotype developed BSE at a younger age ($P<0.05$).

REG2 is the *locus* of a polymorphism created by the insertion/deletion of 12 bp in intron 1. The REG2 140-bp allele corresponds to a 12-bp insertion (12-bp ins), while the REG2 128-bp allele corresponds to a 12-bp deletion (12-bp del). The association of the 12-bp indel polymorphism with BSE was reported earlier by SANDER *et al*. (2004),

who suggested that due to its localization within the promoter, this polymorphism can alter the expression level of the PRNP gene and thus affect the BSE incubation period.

Polymorphism of the promoter region and its effect on PRNP gene expression

The region showing major promoter activity in the bovine PRNP gene was localized between nucleotides -88 to -30 in relation to the transcription start site. The region from nucleotide -88 to -1 has a high CG content (C+G 78%) and contains three potential binding sites for SP1 (specific protein 1, CCGCCC), but has no potential CCAAT or TATA - box. Promoter activity is also shown by the segment between nucleotides +123 and +891, which is necessary for normal function of the appropriate promoter (INOUE *et al*. 1997). The size of the CG-rich promoter region, which contains many regulatory elements, has been estimated to be around 2.6 kb (GELDERMANN *et al*. 2002).

In transgenic mice, the length of the disease incubation time is inversely proportional to the amount of PrP^c generated in the brain (PRUSINER *et al*. 1990; MANSON *et al*. 1994). The amount of protein synthesized in a cell largely depends on the level of expression of the gene encoding this protein. Research has suggested that polymorphisms of the promoter region of the PRNP gene may affect its expression (INOUE *et al*. 1997; MEAD *et al*. 2001; MAHAL *et al*. 2001; SANDER *et al*. 2005; BRATOSIEWICZ-WASIK *et al*. 2007) and alter the level of PrPc synthesis.

The function of the bovine PRNP promoter is affected by insertion/deletion-type polymorphisms localized in intron 1 (12-bp indel, REG2) and in the region upstream of exon 1 (23-bp indel). The 12-bp indel polymorphism contains a binding site for transcription factor Sp1 (position +300), while the 23-bp indel contains the binding site for RP58 (repressor protein 58, position –1594). The EMSA (electrophoretic mobility shift assay) experiments have shown that an oligonucleotide of approximately 50 bp (obtained on the basis of the bovine PRNP gene) containing a 12-bp insertion binds Sp1, whereas an oligonucleotide containing a 12-bp deletion does not bind this transcription factor. The oligonucleotide with the 23-bp insertion binds strongly to RP-58, while the 23-bp deletion weakens this bond (SANDER *et al*. 2005). Promoter – reporter gene assays in a PT cell line (kidney cells of calf) demonstrated that the modified promoter fragment that contains no 23-bp polymorphism and has a 12-bp insertion intensifies ($P<0.0001$) the expression of the luciferase gene compared to a similar construct that contains a 12-bp deletion (SANDER *et al*. 2005). This finding is consistent with the fact that Sp1 is known to be a strong activator of gene transcription (ANDERSON & FREYTAG 1991) and by binding to the 12-bp insertion allele it enhances mRNA synthesis. A promoter with insertions at both polymorphic sites (23-bp ins – 12-bp ins) surprisingly reduced ($P<0.001$) the expression of the reporter gene in relation to the promoter with both deletions (23-bp del – 12-bp del). The lower expression of the gene controlled by the promoter with the 23-bp insertion – 12-bp insertion is most likely the result of strong binding of RP58 by the 23-bp insertion (SANDER *et al*. 2005). A similar regulatory mechanism in which RP58 inhibits the expression of a gene has previously been observed for other promoters (AOKI *et al*. 1998).

Polymorphisms within binding sites of the other transcription factors that can be related to the function of the PRNP gene (POZ -1980T→C, neurogen-1 -85G→T, AP2 +571A→G and ERG4 +709A→G) did not affect promoter activity (SANDER *et al*. 2005).

An earlier report from German researchers indicated a relationship between 12-bp and 23-bp indel polymorphisms and BSE susceptibility in cattle. When studying the frequency of alleles, they found that 23-bp deletion and 12-bp deletion alleles are more frequent ($P<0.05$) in BSE-affected ($n = 43$) than in healthy animals ($n = 48$) of six different popular German cattle breeds (SANDER *et al*. 2004). Similar findings were reported by JULING *et al*. (2006) who studied material obtained from a greater number of animals (641 BSE and 1130 healthy cattle of UK Holstein, German Holstein, German Brown and German Fleckvieh breeds). In their study, the frequency of alleles with deletions (23-bp deletion and 12-bp deletion) was significantly higher in infected than in control animals ($P = 2.01 \cdot 10^{-3}$ and $P = 8.66 \cdot 10^{-5}$ respectively). They also found that an increased risk of BSE incidence is related to the 23-bp deletion - 12-bp deletion haplotype, and the most vulnerable animals are those carrying 23-bp deletion - 12-bp deletionl/23-bp deletion - 12-bp deletion diplotype. According to the same authors, the risk of BSE incidence in a population increases with an increase in the number of 12-bp deletion alelles, and of the two polymorphisms analysed, 12-bp indel polymorphism is the main genetic factor modulating the susceptibility of cattle to this disease.

No significant differences in the frequency of alleles of 12- and 23-bp indel polymorphisms in healthy or infected Japanese Holstein cattle were found by NAKAMITSU *et al*. (2006), although in their experiment the number of BSE-infected animals was too low ($n = 6$) to perform a reliable statistical analysis.

The activity of the promoter region can be affected by other factors which together control gene expression. The mechanism controlling the expression of the PRNP gene was investigated in PC12 cells (pheochromocytoma cell line) using a luciferase reporter system. The study revealed activation of the cloned fragment of the human promoter in cells stimulated with nerve growth factor (NGF) and showed that NGF acts through mitogen-activated protein kinase (MEK1) (ZAWLIK *et al*. 2006). The PRNP promoter was also activated by copper ions, but not through the metal responsive transcription factor-1 (MTF-1) as expected (VARELA-NALLAR *et al*. 2006).

Recently identified genetic markers allow for the diagnosis of animals particularly threatened with BSE. However this does not mean that animals classified as resistant (i.e. animals with genotypes less frequently observed among infected cattle) will not develop the disease and cannot carry the infectious form of prions. The available evidence suggests that the "genetic resistance" of cattle to BSE results from the longer incubation time after infection, resulting from the decreased expression of the PRNP gene and not from the increased stability of the prion protein. Susceptibility or resistance to disease may be a result of the combined action of a greater number of genes (HERNANDEZ-SANCHEZ *et al*. 2002; ZHANG *et al.* 2004), and determination of the *loci* of those genes that in addition to PRNP affect the development of BSE will provide more markers and show the cause-and-effect relationships. In some populations, the PRNP polymorphism is much greater than previously described. CLAWSON *et al.* (2006) identified 287 hitherto unobserved polymorphic sites (in a non-coding region of PRNP) in different breeds of American beef and dairy cattle. The presence of these polymorphisms and their association with BSE susceptibility in other populations should be investigated. Special attention should be given to the SNPs in the putative promoter region. These mutations may alter consensus binding sites for regulatory elements and in consequence change the level of PRNP expression.

The PRNP *locus* is conserved among mammalian species (LEE *et al*. 1998; CHOI *et al*. 2006). PREMZL and GUMULIN (2007) identified conserved regions, which may be potential silencers, enhancers (in upstream region of PRNP) or cytoplasmatic polyadenylation element sites (in 3'-untranslated region). Their effect on PRNP function needs to be experimentally confirmed. Previous studies of the bovine PRNP gene do not fully explain the effect of its polymorphism on the susceptibility of cattle to BSE. Therefore, further studies are needed to identify all the genetic factors that may affect the development of the disease, which poses a threat also to humans.

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