Analysis of DNA sequence variants in candidate genes for bovine spongiform encephalopathy (BSE) susceptibility located in a QTL region on bovine chromosome 17q23-q24

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D7

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Table of contents

I List of Abbreviations

Akt	Protein family, protein kinases B
ARR	Alanine, Arginine, Arginine
B3GNT4	Beta 1,3-N-acetylglucosaminyltransferase 4
bp	Base pair
BSE	Bovine spongiform encephalopathy
BTA17	Bovine chromosome 17
°C	Degree celcius
Ca^{2+}	Calcium ion, two valent
cAMP	Cyclic adenosine monophosphate
CHST8	Carbohydrate (N-acetylgalactosamine 4-O) sulfotransferase 8
CJD	Creutzfeldt-Jakob disease
cM	Centimorgan
cm	Centimeter
COOH	Carboxyl group
^{Ctm}PrP	COOH-terminal transmembrane form of prion protein
Cu^{2+}	Copper ion, two valent
CWD	Chronic wasting disease
DNA	Deoxyribonucleic acid
g	Gravity x force or g - force
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulm associated degradation
Erk	Extracellular signal-regulated kinases
FFI	Fatal familial insomnia
Fyn	Membrane-associated tyrosine kinase
GPI	Glycosyl-phosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker syndrome
H	Histidine
HSA12	Human chromosome 12
Hsp60	Heat shock protein
Kb	Kilobase pair
LRP/LR	Laminin receptor precursor/laminin receptor

M	Methionine
min	Minutes
MMU5	Mouse chromosome 5
μL	Mikroliter
μM	Mikromolar
mL	Milliliter
NH_2	Amino group
^{Ntm}PrP	NH_2-terminl transmembrane form of prion protein
ng	Nanogram
pmol	Pikomol
PrP, PRNP	Prion protein
PrP^C	Cellular prion protein
PrP^d	Scrapie prion protein
Pint1	Prion interactor
PITPNM2	Phosphatidylinositol transfer membrane-associated 2
PSMD9	Proteasome 26S subunit, non-ATPase, 9
rpm	Rotations per minute
QTL	Quantitative trait locus
RNP24	Coated vesicle membrane protein
RP58	Repressor protein
s	Seconds
SNP	Single nucleotide polymorphism
SP1	SP1 transcription factor
Src	Sarcoma, proto-oncogene tyrosine kinases
SSCP	Single strand conformation polymorphism
TSE	Transmissible spongiform encephalopathy
UTR	Untranslated region
vCJD	Variant of Creutzfeldt-Jakob disease
VRQ	Valine, Arginine and Glutamine

II List of Figures

III List of Tables

1 List of Publications

Parts of PhD thesis have been published previously.

A) Peer reviewed journals:

R. Morina, C. Knorr, B. Haase, T. Leeb, T. Seuberlich, A. Zurbriggen, G. Brem, E. Schütz, & B. Brenig. (2009) Molecular analysis of carbohydrate N-acetylgalactosamine 4-0 sulfotransferase 8 (*CHST8*) as a candidate gene for bovine spongiform encephalopathy susceptibility. *Anim Genet, in press.* DOI:10.1111/j.1365-2052.2009.01951.x

B) Congress contribution:

R. Morina, C. Knorr, & B. Brenig. (2005) Positional and functional candidate genes for BSE susceptibility. *Vortragstagung der DGfZ und GfT am 21./22. September in Berlin.* Abstract No. A22;

R. Morina, C. Knorr, T. Leeb, E. Schütz, & B. Brenig. (2007) Analysis of DNA sequence variants in candidate genes in normal and BSE cattle. *13th International Conference, Production Diseases in Farm Animals 29. July - 04. August in Leipzig,* ISBN 978-3-934178-90-8, page 627;

R. Morina, C. Knorr, E. Schütz, & B. Brenig. (2007) Genetic analysis of candidate genes in normal and BSE cattle. *Vortragstagung der DGfZ und GfT am 26./27. September in Hohenheim*. Abstract No. A18;

R**.** Morina, C. Knorr, B. Haase, T. Leeb, T. Seuberlich, A. Zurbriggen, G. Brem, E. Schütz, & B. Brenig. (2008) Analysis of DNA sequence variants in candidate genes for bovine spongiform encephalopathy (BSE) susceptibility located in a QTL region on bovine chromosome 17. *XXXI Conference of the International Society for Animal*

Genetics. July 20.-24. in Amsterdam. Poster number 2053;

R. Morina, C. Knorr, B. Haase, T. Leeb, T. Seuberlich, A. Zurbriggen, G. Brem, E. Schütz, & B. Brenig. (2008) Analysis of DNA sequence variants in candidate genes for bovine spongiform encephalopathy (BSE) susceptibility located in a QTL region on bovine chromosome 17. *Vortragstagung der DGfZ und GfT am 17./18. September in Bonn*. Abstract No. D24.

2 Introduction

Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disease in cattle also known as Mad Cow Disease that causes a spongy degeneration of the brain and spinal cord and also causes red eyes. Chesebro (2004) proposed the following different theories on the initial occurrence of BSE in cattle:

- Diet induced cross-species infection, perhaps through contamination of meat and bone meal supplemented in the fodder.
- Existence as an endemic disease in cattle that went unnoticed because of its low level horizontal transmission.
- BSE might have originated from spontaneous misfolding of the normal cellular prion protein into the disease-associated abnormal isoform.

Although the incidence of BSE is now low (see chapter 2.4), it still brings trouble to governments and cattle farmers. BSE has strongly influenced medical, agricultural, economic and political issues in Europe. The control of the BSE in cattle and the attempts to avoid the infection of humans are conducted by three means: first, eliminating feeding of ruminants tissues to ruminants; second, remove high risk cattle tissues from human food-chain; and third, continue to test for BSE in cattle (Chesebro, 2004).

2.1 Transmissible spongiform encephalopathy

Prion diseases also termed transmissible spongiform encephalopathies (TSEs), are a group of neurodegenerative diseases that affect humans as well as domestic and wild animals (Prusiner, 1982; Prusiner, 1991a; Collinge, 2001). Traditionally, human spongiform encephalopathies have been classified into Creutzfeldt-Jakob disease (CJD), with more recent classification into variant CJD (vCJD), Gerstmann-Sträussler-Scheinker syndrome

(GSS), and Kuru (Collinge, 2001). Animal TSEs include transmissible mink encephalopathy, scrapie of sheep and goats (Collinge, 2001), chronic wasting disease of deer and elk (Williams and Young, 1980), feline spongiform encephalopathy (Wyatt et al., 1991), and BSE (Wells et al., 1987; Prusiner, 1998). The TSEs are unique in that their aetiology might be genetic, sporadic or infectious via ingestion of infected foodstuffs and via iatrogenic (e.g. blood transfusion) (Prusiner, 1998; Collinge, 2001). Central to the development of these diseases is the accumulation of PrP^{d} that arises by misfolding of the host-encoded cellular prion protein (PrP^{C}) in tissues of the central nervous system (Prusiner, 1982; Prusiner, 1998; Weissmann, 2004). The nature of the infectious agent, which modulates prion diseases has been a topic of heated debates among the scientific community for many years (Collinge, 2001). Failure of the scientific community to isolate a virus from infected materials as well as the inability of ultraviolet radiation or nucleases treatment to inactivate the agent (Collinge, 2001) has led researchers to ponder alternate hypotheses including the Nobel prize winning "prion-only hypothesis" (Prusiner, 1982; Prusiner, 1991b; Prusiner, 1998). Enriching homogenated brain material for infectivity, Bolton et al. (1982) were able to isolate a protease-resistant glycoprotein, which was subsequently termed the prion protein.

2.2 Prions and their role in neurodegenerative diseases

The central role in TSEs is the misfolding aggregation and brain accumulation of the prion protein (Soto, 2003). In studies with postnatal PrP knockout animals it was observed that depletion of PrP^{C} in mice with established prion infection reverses early spongiform degeneration and prevented neuronal loss and progression to clinical disease (Mallucci et al., 2003). The word "prion", proteinaceous infectious particle was coined by

Stanley Prusiner (1982) and used to distinguish the infectious agent identified from viruses (Collinge, 2001). To date, all evidence indicates that the infectious agent of prion diseases are abnormal protease-resistant isoforms of the host-encoded PrP^C (Prusiner, 1982; Oesch et al., 1985; Oesch et al., 1990). But a number of studies also showed that cytosolically accumulated PrP^C conducts toxic effects on the cells by a yet unknown mechanism (Ma and Lindquist, 2002; Ma et al., 2002). Once the misfolding has started, it has a self-perpetuating character, influencing more PrP^C proteins to adopt the PrP^d-like conformation (Hooper, 2003). The prion neurotoxicity on its molecular basis remains an important and controversial subject (Mallucci and Collinge, 2005).

Soto (2008) proposed three models to explain the implication of PrP^C:

a) The infectious and neurotoxic PrP species might not be the same, an undetectable misfolded intermediate in correlation with "protein X" is responsible for neurotoxicity;

b) PrP^C located in the cell surface may act as a receptor for PrP^d, triggering a signal transduction pathway leading to neurodegeneration;

c) Induction of endoplasmic reticulum (ER) stress by PrP^d may lead to translocation of nascent PrP^C molecules to the cytosol for proteasomal degradation. This mechanism turns negative under chronic ER stress condition, overwhelming the proteasome and leading to accumulation of potentially toxic PrP^d molecules.

Many reports try to identify the elusive "protein X" that is believed to be a necessary component of the pathogenic conformational conversion (Prusiner, 1998). A number of ligands were proposed to play the role of "protein X", some of them are; the prion interactor (Pint1), chaperons (Hsp60), the laminin receptor precursor/laminin receptor (LRP/LR), and glycosaminoglycans (Edenhofer et al., 1996; Gauczynski et al., 2001; Horonchik et al., 2005; Linden et al., 2008).

Interruption of neuronal PrP^C expression during an ongoing prion infection within the central nervous system prevents neuronal loss and reverses early spongiform changes (Mallucci et al., 2003). These findings jointly argue that PrP^C itself may play a critical role in the prion neuropathologic cascade. In support of this hypothesis, PrP^C-dependent signal transduction was identified following specific antibody-mediated cross-linking in a differentiated neuronal cell line (Mouillet-Richard et al., 2000; Solforosi et al., 2004).

Rane et al. (2008) postulated that TSE neurodegeneration might be dependent on the chronic ER stress produced by PrP^d accumulation, which in turn lead to persistent activation of a quality control system that aborts the ER translocation of PrP, allowing its proteasome mediated degradation in the cytosol.

2.2.1 Cellular prion protein

PrP^C is concentrated primarily in neurons, neural stem cells, hematopoietic stem cells, T cells, leukocytes, synaptic fields of the olfactory bulb, limbic structures and the strato-nigral complex (Borchelt et al., 1994). The mammalian *PRNP* encodes a protein of approximately 250 amino acids that contains several distinct domains, including an N-terminal signal peptide, a series of five proline-and glycine-rich octapeptide repeats, a central hydrophobic segment and a C-terminal hydrophobic region that is a signal for addition of a GPI anchor (Harris, 1999). The PrP^C is a glycoprotein that is usually monomeric in structure, sensitive to proteases and linked to cellular membranes through a GPI anchor (Figure 1). The C-terminal segment is folded into a largely alpha helical conformation. There are three alpha helices and short antiparallel beta-sheets in which helices are stabilized by a single disulfide bond (Collinge, 2001). PrP^C is found in non-, mono-, or diglycosylated forms (Pan et al., 2002).

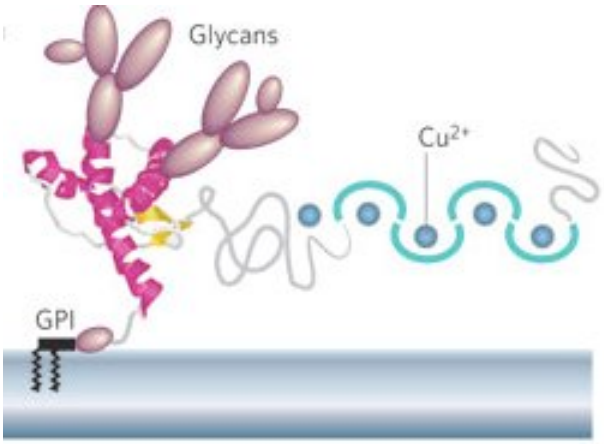

Figure 1 PrP^C as a GPI-anchored plasma-membrane glycoprotein (Caughey and Baron, 2006).

2.2.2 Subcellular trafficking of PrP^C

Little progress has been made in the understanding of initial events of PrP^C synthesis, raft insertion and localization. The PrP^C is synthesized in the rough ER and transits the Golgi apparatus (Figure 2) on its way to the cell surface (Harris, 1999). During normal expression, PrP^C is translocated into the ER lumen, where it undergoes several posttranslational modifications, including the addition of the GPI anchor, disulfide bond formation, and core glycosylation at two asparagines, before it passes to the Golgi apparatus for further sugar modification and sialation en route to the plasmalemma (Campana et al., 2005). The PrP^C can be synthesized with at least three topologies in the ER: a secreted form (PrP^C), COOH-terminal transmembrane form (^{Ctm}PrP) and NH_2-terminal transmembrane form (^{Ntm}PrP) (Hegde et al., 1998a).

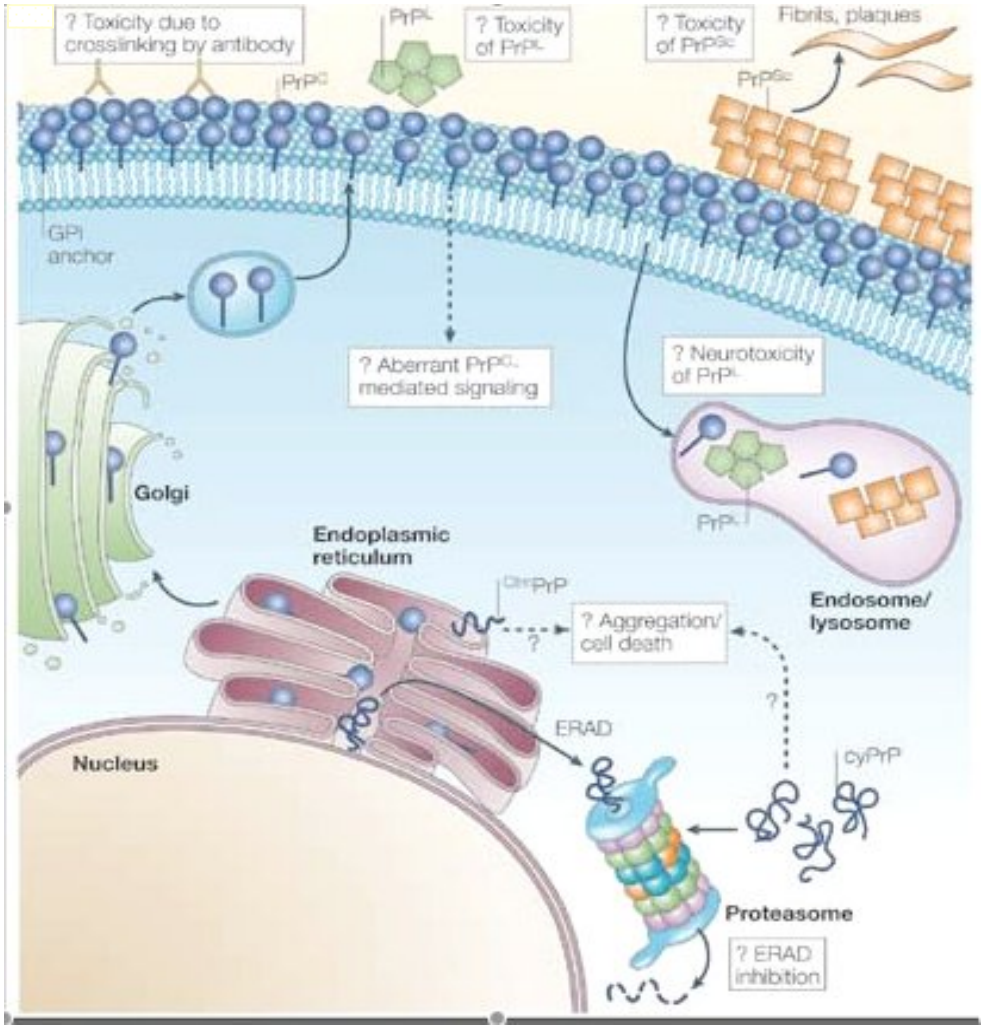

Figure 2 The way of PrP^C synthesis and potential neurotoxic mechanisms. PrP^C is synthesized, folded and glycosylated in the ER, where its GPI anchor is added prior to further modifications in the Golgi apparatus. Conversion of PrP^C to PrP^d could occur through a toxic intermediate PrP^L (green pentagons) with direct neurotoxic effects both on the cell surface and within late endosomes/lysosomes, where conversion is thought to occur. It has been suggested that misfolded PrP (cyPrP and ^{Ctm}PrP, dark blue coils) which would normally be degraded by the proteasomes through the ERAD (ER-associated degradation) pathway, aggregate in the cytoplasm and cause cell death (Mallucci and Collinge, 2005).

The ^{Ctm}PrP is toxic and causes degeneration if it is overexpressed in the cell and passes the quality control machinery (Hegde et al., 1998b).

PrP^C is translocated to the ER due to the presence of an NH_2-terminal signal peptide, indicating that this subcellular location may be exclusively related to abortive translocation (Ma and Lindquist, 2002). The GPI anchor is added in the ER after removal of a COOH-terminal peptide signal (Stahl et al., 1987). PrP^C associates with lipid rafts early within the ER. Immature and mature PrP^C are differentially sensitive to cholesterol or sphingolipid depletions, suggesting that maturation of PrP^C in ER and Golgi plasma membrane is associated with distinct and changing membrane rafts (Sarnataro et al., 2004). PrP^C that is not associated with rafts in the ER undergoes conformational changes that modify sensitivity to proteases, indicating that immature protein may be misfolded and subjected to the ER quality control mechanism (Sarnataro et al., 2004).

2.2.3 Function of cellular prion protein

The function of PrP^C is very complex. Potential functions, have been proposed by several authors:

1) Activity of numerous signal transduction pathways, including cAMP/protein kinase A, mitogen-activated protein kinase, phosphatidylinositol 3-kinase/Akt pathways, as well as soluble non-receptor tyrosine kinases, Calcium (Ca^{2+}), extracellular signal-regulated kinases (Erk), membrane-associated tyrosine kinasees (Fyn), sarcoma, proto-oncogene tyrosine kinases (Src) (Chiarini et al., 2002; Martins et al., 2002);
2) Copper transport or metabolism (Brown, 2003);
3) The PrP^C has neuroprotective functions. Knockout mice ($Prnp^{-/-}$) suffered more extensive damage in brain as wild type mice (Sakurai-Yamashita et al., 2005);
4) Role during embryogenesis (Harris, 1999);

5) Role on behavior, and lack of PrP^C impair motor coordination (Katamine et al., 1998);

6) Role on sleep-wakefulness cycle. The fatal familial insomnia (FFI) is an infectious cerebral amyloidosis (Tateishi et al., 1995);

7) Role on long-term memory (Nishida et al., 1997).

2.2.4 Pathological form of prion protein

The misfolding from PrP^C to PrP^d has become one central issue in understanding the pathogenesis of prion diseases. Useful approaches to clarify which mechanisms are involved in the generation of PrP^d are: investigating the biosynthesis, the cellular localization, posttranslational processes and the trafficking of PrP^C and PrP^d. The site of conversion of PrP^C to the protease resistant PrP^d is unknown, but there is evidence that lipid rafts are necessary in this process (Taraboulos et al., 1995). PrP^C and PrP^d share the same amino acid sequence and posttranslational modifications, but they differ in their secondary and tertiary structure (Pan et al., 1993).

Several lines of evidence suggest that the presence of PrP in the cytoplasm is linked to prion disease, as initiating factor or toxic element (Norstrom et al., 2007). PrP^d accumulation is neither uniformly necessary nor sufficient for neuronal toxicity (Rane et al., 2004). It seems that other molecules are crucial for prion propagation, either as components or cofactors in the conformational conversion of PrP^C to PrP^d (Caughey and Baron, 2006). It is supposed that PrP^d can affect direct or indirect neurotoxic mechanisms;

One is that PrP^d is directly toxic to neurons, if produced within them in a GPI anchored state (Kristiansen et al., 2005).

A second possibility is that PrP^d itself is not the neurotoxic entity, but an intermediate or labile by-product, that must be generated in neuronal membranes to exert its effect (Caughey and Lansbury, 2003).

The third possibility is that the clinical disease is due to the corruption or subversion of the normal function (Harris and True, 2006).

2.3 Prion gene polymorphisms

The TSEs in small ruminants, based on the European Food Safety Authority, are divided into three categories: BSE in small ruminants, classical scrapie, and atypical scrapie (www.efsa.europa.eu/, accessed on 22 September, 2009). The atypical scrapie first was diagnosed in Norwegian sheep in 1998 (Benestad et al., 2003). Buschmann et al. (2004) found that atypical scrapie isolates are less resistant to proteinase K compared to classical scrapie. Atypical scrapie of sheep differs from classical scrapie in its neuropathological and biochemical features (Benestad et al., 2008).

To date, the only gene identified in mammals that has conclusively been linked to the expression of prion diseases such as CJD, GSS, scrapie, CWD, and BSE, is the prion gene (*PRNP*) (Goldmann et al., 1990; Belt et al., 1995; Collinge, 2001). In mouse, cattle and sheep the PRNP gene contains three exons, whereas in hamster and humans it contains two exons. In all species, only a single exon encodes the PrP^C protein (Westaway et al., 1994; Choi et al., 2006). Polymorphisms of the *PRNP* resulting in amino acid substitutions have been shown to influence the susceptibility/resistance to prion infections in humans and sheep (Bruce et al., 1997; Collinge, 2001). Sheep breeds exhibit variable degrees of susceptibility to scrapie, depending on polymorphisms at codons 136 (Alanine or Valine; A, V), 154 (Histidine or Arginine; H, R), and 171 (Glutamine, Arginine, or Histidine; Q,

R, H) (Goldmann et al., 1990). Sheep homozygous for the VRQ allele are highly susceptible to classical scrapie, whereas sheep homozygous for the ARR allele are resistant (Belt et al., 1995). Polymorphism at residue 129 (M or V) of the human PrP protein similarly influences susceptibility to human TSE (Collinge, 2001).

Polymorphisms encoded in exon 3 of the *PRNP* have been described. It has been demonstrated that the so called octapeptide region influences susceptibility to scrapie (Goldmann et al., 1990; Belt et al., 1995; Tranulis et al., 1999; Heaton et al., 2003). However, no amino acid polymorphisms associated with BSE have been identified in cattle. Several studies report a number of silent mutations, microsatellite variants, single nucleotide variants (SNPs) and in the noncoding regions of prion protein gene (Hills et al., 2001; Humeny et al., 2002; Sander et al., 2004; Geldermann et al., 2006). Among the polymorphisms in noncoding regions, two indels, a 23 bp long in the putative promoter region and a 12 bp long within the first intron have been described to be associated with susceptibility to prion infection in German cattle. Both deletions were more frequent in the affected animals (Sander et al., 2004; Juling et al., 2006). Both the 23 bp indel in the promoter region and the 12 bp indel in intron 1 affect binding sites for transcription factors, repressor protein (RP58) and transcription factor (SP1), respectively (Sander et al., 2005; Kashkevich et al., 2007). The polymorphism in the promoter region of *PRNP* seems to be more breed specific. Sander et al. (2004) found a correlation of polymorphisms in the *PRNP* promoter and BSE within breed Holstein Friesian and German Simmental, but not in Brown Swiss cattle.

As a result of the active surveillance of healthy slaughtered animals or fallen stock, two further BSE variants (atypical BSE) have been detected. They differ from that of classical BSE by having protease-resistant fragments of a higher (H) or a slightly lower (L) molecular mass, respectively, and different patterns of glycosylation (Biacabe et al., 2004;

Buschmann et al., 2004). Both types have been detected worldwide as rare cases in older animals, at a low prevalence consistent with the possibility of sporadic forms of prion diseases in cattle (Biacabe et al., 2008).

2.4 Bovine spongiform encephalopathy

The social concern about the transmissible spongiform encephalopathies reached the maximal visibility during the past decade with the BSE epidemic in the United Kingdom (UK) and in all European regions. BSE was first described 1987 in the UK (Wells et al., 1987). Exposure of cattle to the BSE agent had almost certainly begun in 1981 (Wilesmith et al., 1992). It reached its peak in 1992, when 36,680 cases were confirmed in UK, a steady decline is evident. More than 180,000 cases have been recorded in the UK, although the total number of infected animals has been estimated at around one million (Anderson et al., 1996). BSE has also subsequently been detected on a much smaller scale in 19 other European countries, in Israel, in Japan, and recently in Canada and the USA. The number of BSE cases detected in the European Union (EU), between 2001 to 2008 decreased from 2181 to 175 and the detection rate decreased from 2.55 to 0.18 positive BSE cases per 10,000 tests carried out (Figure 3) (http://www.defra.gov.uk, accessed on 22 September, 2009). In Germany from 2000 to 2009, 411 cases of BSE were detected (http://www.bmelv.de, accessed on 22 September, 2009). The primary origin of the BSE agent is still unclear. Some authors rebut the hypothesis that BSE originates from the scrapie agent. Especially, since the scrapie-agent cannot infect humans, no direct epidemiological links between scrapie and humans have become evident (Beringue et al., 2008). By contrast bovine PrP^d is the only known animal prion that has been transmitted to humans, leading to a variant form of CJD (Collinge, 2001; Beringue et al., 2008).

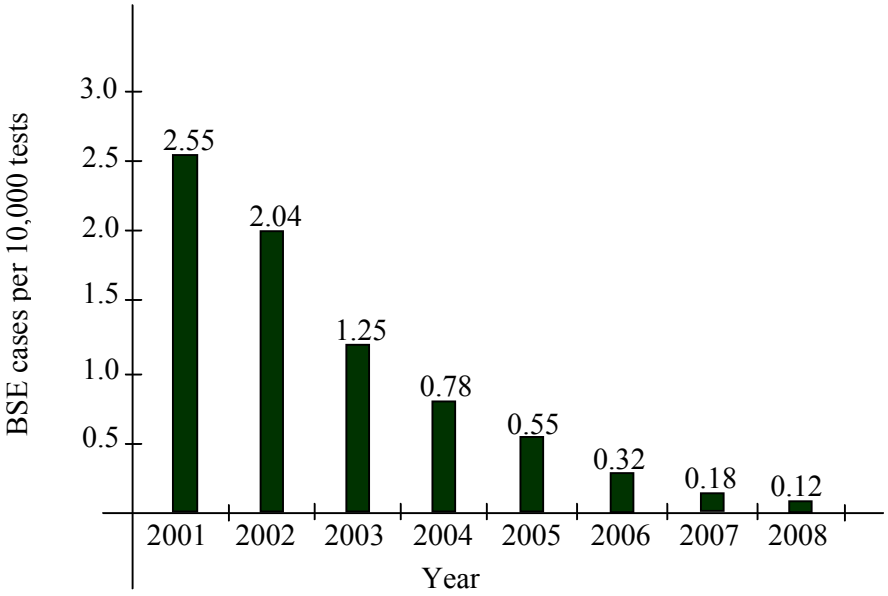

Figure 3 BSE cases detected per 10,000 cattle tested in the EU from 2001 to 2008.

Mathematical modeling indicates that the epidemic in humans might be small, but uncertainties, genetic effects on the incubation period and the results of screens for prion infection indicate the need for caution (Mallucci and Collinge, 2005). The epizooty is under control in most European countries. In North America even up to day there is no epidemic of BSE, however, there are major concerns with input to the high incidence of the cervid CWD in wild and captive populations of deer and elk (Chesebro, 2003). BSE occurs in adult animals in both sexes, typically in animals aged five years and more. Most researchers agree that the main route of propagation of the BSE epidemic was via the recycling of contaminated remains of BSE-infected cattle in the manufacture of cattle feed (Wilesmith et al., 1992; Smith and Bradley, 2003). It is a neurological disease in which affected animals show signs as: change in behaviour; apprehension or nervousness (flighty); repeated, exaggerated reactions to touch or sound; weakness or high stepping of the legs, particularly the hind legs; reluctance to cross concrete or drains/turn corners/enter yards/go through doorways/permit milking; occasionally, aggressive to cattle and humans;

manic kicking when milked; head shy, with head held low; difficulty in rising, progressing to recumbency (Wilesmith et al., 1988; Winter et al., 1989). The clinical disease usually lasts for several weeks and it is invariably progressive and fatal. The behavioral signs provide the first indication for livestock managers and veterinarians in the field to identify animals that are potentially infected with BSE (Braun et al., 1998).

2.4.1 QTL regions associated with BSE susceptibility/resistance

Experiments with prion inoculation in mice expressing homologous PrP^C molecules on different genetic backgrounds displayed different incubation times, indicating that the conversion reaction may be influenced by other gene products (Tamguney et al., 2008). Studies with inbred lines of mice revealed that large differences in incubation times occur even with the same amino acid sequence of the prion protein, suggesting that others genes may contribute to the observed variation (Stephenson et al., 2000; Baron, 2002). Microarray analyses indicated that 116 genes were up-regulated and 180 genes down-regulated in BSE-infected mouse brain tissues compared with normal controls (Sawiris et al., 2007). The different studies were done to prove possible associations of QTL regions in mice and cattle with BSE susceptibility or resistance. Zhang et al. (2004) estimated the multiple QTL analysis and found two genome-wide significant QTL on bovine chromosome 17 (BTA17) and the sex chromosomes X and Y_{ps} (BTA X/Y_{ps}). Their results were based on the linear regression method and gave un-coherent results compared to data analyzed with the TDT transmission equilibrium test (Hernandez-Sanchez et al., 2002). Marker *INRA025* located in the QTL region on BTA17 at position144 cM was found to be linked with susceptibility/resistance to BSE with the highest F-value (6.2) in small chromosomal region 91 to 164 cM (95% confidence intervals) (Zhang et al., 2004). Also

Moreno et al. (2003) found in mice two significant genome-wide QTL regions (MMU5 and MMU7), which are involved in the resistance to scrapie as well as to BSE. The QTL regions linked with susceptibility/resistance to TSE on BTA17 and MMU5 are syntenic. In synteny with both BTA17 and MMU5 are also parts of the human chromosome 12 (HSA12). HSA12 was used to identify candidate genes for further genome analysis. The positional candidate genes located in this QTL region of BTA 17q23-q24 are listed in Figure 4. The distance between positional candidate genes suspected to be associated with BSE and marker *INRA025* range from 8 to 10 cM.

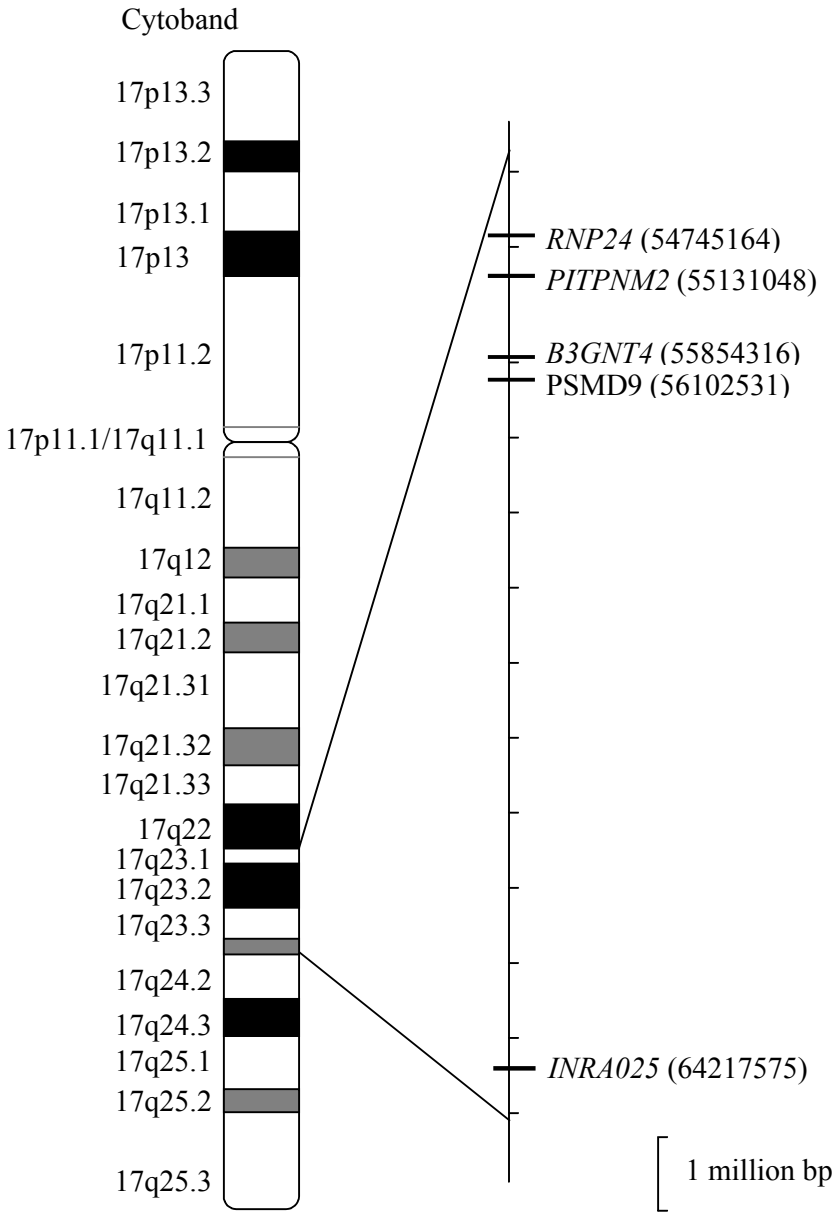

Figure 4 Physical map of genes and nucleotide distances from marker *INRA025*. The 5′end positions of genes are indicated, i.e *RNP24 5′*end position 54745164 (GenBank accession number NC_007315.3).

2.4.2 Positional and functional candidate genes

The search for potential candidate genes in QTL region of chromosome 17q23-q24 was limited to approximately 10 cM up- and downstream of marker *INRA025*. The search was

restricted to genes that are involved in transport of protein, protein modification, protein degradation, signal transduction, and calcium transportation. Using this specification four genes upstream of *INRA025* were identified, i.e. *RNP24*, *PITPNM2*, *PSMD9*, and *B3GNT4*.

The gene RNP24 or coated vesicle membrane protein is involved in the budding of coatomer-coated and other species of coated vesicles and binds cargo molecules to collect them into budding vesicles (Blum et al., 1996). During the synthesis of proteins, they are transported from the ER to Golgi structures by vesicles (Blum et al., 1999).

The PITPNM2 gene or phosphatidylinositol transfer protein, membrane-associated 2, has very complex functions. The protein is involved in calcium binding, lipid binding, can bind/exchange one molecule of phosphatidylinositol (PI) or phosphatidylcholine (PC) and thus aids their transfer between different membrane compartments, metabolic processes (Ocaka et al., 2005). Possible associations with the prion protein could be the affinity of PITPNM2 protein for ions of calcium, protein transport and trigger the signal transduction. Both, PITPNM2 and the prion protein were found in synaptic fields of olfactory bulbs, and both play a role in synaptic functions (Sales et al., 1998; Tian and Lev, 2002).

The PSMD9 gene or proteasome (prosome, macropain) 26S subunit, non-ATPase, 9, is part of the multicatalytic proteinase. This gene encodes a non-ATPase subunit of the 19S regulator, which is involved in multicatalytic processes and cleaves peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway (Coux et al., 1996). Mutated and misfolded proteins that arise as a result of mutations, immaturation, or posttranslational environmental stress are recognized specifically and removed efficiently by proteasomal degradation (Ciechanover et al., 1984; Ciechanover and Brundin, 2003). The proteasome as part of endoplasmic reticulum associated degradation, is involved in the routine quality control of prion (Yedidia et al., 2001). The inhibition of proteasomes results

in the accumulation of PrP in the cytosol, aggregates in the ER, Golgi apparatus and the nucleus (Zanusso et al., 1999; Yedidia et al., 2001).

The B3GNT4 gene or UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 4 gene encodes a member of the beta-1,3-N-acetylglucosaminyltransferase protein family (Shiraishi et al., 2001). This enzyme is involved in galactosyltransferase activity and transfer of glycosyl groups. It is also important for GPI anchoring to membranes (Stahl et al., 1987). Several investigators have described direct interactions between cellular or recombinant prions and glycosaminoglycans (Caughey and Race, 1994; Brimacombe et al., 1999). The production of PrP^{d} is increased in infected cell lines if the glycosilation is inhibited (Taraboulos et al., 1990).

The gene which is not part of QTL region on BTA17 is *CHST8* (carbohydrate (N-acetylgalactosamine 4-O) sulfotransferase 8). This gene was analyzed as candidate gene for BSE susceptibility, because it was shown that the expression was 17-fold down-regulated in cells infected with scrapie (Barret et al., 2005). The *CHST8* is involved in carbohydrate and sulfur metabolic processes, central nervous system development and proteoglycan biosynthesis.

3 Materials and Methods

3.1 Animals

DNA samples (n = 623) of three breeds (Holstein Friesian, German Simmental, Brown Swiss) were collected at different regions in Austria, Germany, and Switzerland. Samples of Holstein Friesian (BSE affected, n = 36, age 4 – 15 years; BSE non-affected, n = 125, age 1 – 9 years) were collected and DNA isolated at the Institute of Veterinary Medicine (Georg-August-University Göttingen, Germany). Samples from BSE positive tested German Simmental (n = 55, age 4 – 9 years) were collected and DNA isolated at the Friedrich-Loeffler Institute (Riems, Germany). Non-affected control samples of German Simmental (n = 191, age 1 – 8 years) were provided by Agrobiogen GmbH (Hilgertshausen, Germany). Brown Swiss samples (BSE affected, n = 48, age 3 – 5 years; BSE non-affected, n = 168, age 1 – 15 years) were collected and DNA isolated at the Neurocenter Berne (University of Berne, Switzerland). For effect of similarities the ratio of affected to non-affected animals was 1 : 3.5 for each breed. BSE-positive cattle have been detected during the routine European BSE surveillance programme using different approved BSE *post mortem* tests, i.e. the TeSeE test kit (Bio-Rad Laboratories, München, Germany), the HerdChek test kit (IDEXX, Ludwigsburg, Germany), and the Prionics-Check PrioSTRIP (Prionics, Zurich, Switzerland). Control samples were randomly collected from different farms to exclude possible interdependences.

3.2 Enzymes, Oligonucleotides, Kits, Chemicals and Glassware

3.2.1 Enzymes

Enzymes were purchased from the following companies:

- New England Biolabs GmbH, 65926 Frankfurt, Germany: Restriction enzymes; *Aci*I, *Ava*II, *Bce*AI, *Bsa*HI, *Bsp*1286I, *Eco*RV, *Hpy*8I, *Hpy*99I, *Fok*I, *Kas*I, *Mnl*I, *Msl*I, *Msp*I, *Not*I, *Taq*LI, *Tsp*45I;
- Fermentas GmbH, 68789 St. Leon-Rot, Germany: Restriction enzyme; *Sau*3AI

3.2.2 Oligonucleotides

Oligonucleotides (Table 1) were purchased from the following companies:

- MWG-Biotech GmbH, (Ebersberg, Germany);
- Sigma-Aldrich Biochemie GmbH, (Hamburg, Germany).

3.2.3 Kits

Kits were purchased from the following companies:

- Agilent DNA1000 Kit: Agilent Technologies, (Waldbronn, Germany);
- BigDye Terminator v3.1 cycle Sequencing kit: Applied Biosystems GmbH, (Darmstadt, Germany);
- BigDyeX Terminator Kit: Applied Biosystems GmbH, (Darmstadt, Germany);
- HerdChek test kit: IDEXX GmbH, (Ludwigsburg, Germany);
- pGEM-T and PGEM-T easy vector system: Promega Corporation, (Madison, USA);
- Prionics® Check PrioStrip test kit: Prioncs AG, (Schlieren-Zürich, Switzerland);
- QIAquick® PCR purification Kit: QIAGEN GmbH, (Hilden, Germany);
- QIAamp® DNA Mini kit: QIAGEN GmbH, (Hilden, Germany);
- Taq PCR core Kit: QIAGEN GmbH, (Hilden, Germany);
- TeSeE test kit: Bio-Rad Laboratories GmbH, (München, Germany);

- SURVEYOR™ Mutation Detection Kit for Standard Gel Electrophoresis: Transgenomic Ltd., (Elancourt, France).

3.2.4 Chemicals

Reagents were purchased from the following companies:

- Amersham Pharmacia Biotech Europe GmbH, (Freiburg, Germany);
- Bio-Rad Laboratories GmbH, (München, Germany);
- DIFCO Laboratories, (Detroit, USA);
- Fluka Biochemica AG, (Buchs, Switzerland);
- Merck GmbH, (Darmstadt, Germany);
- Carl Roth GmbH, (Karlsruhe, Germany);
- Sigma-Aldrich Biochemie GmbH, (Hamburg, Germany).

3.2.5 Glassware

Glassware were purchassed from the following companies:

- Schütt Labortechnik GmbH, (Göttingen, Germany);
- VITLAB GmbH, (Großostheim, Germany).

Table 1 Primer pairs for *RNP24*, *PITPNM2*, *PSMD9* and *B3GNT4* (GenBank accession number NC_007315.3), and methods of genotyping.

Primer name	Sequence (5´- 3`)	Product size (bp)	Genomic region	T_A(°C)	Method
proRNP24F1	ACAACCCAGATCTCCTCACG	235	54756336-54756102	55	Sequencing
proRNP24R1	TCCATGGGATTTTGTCCAT				
proRNP24F2	AGCAGCAGCCAAGGTAAAAT	718	54755476-54754759	55	Sequencing
proRNP24R2	CACCCTAGGGGGCTGATG				
RNP24ex2F	TAGTATATCTGCATCAAT	378	54752861-54752484	53	RFLP
RNP24ex2R	TAAATACTACATTCAGGTGG				
proPITPNM2F	CGCCATCAAATAGGCAGTTT	793	55123014-55123806	58	Sequencing
proPITPNM2R	AAGTGTCTGCCTGCAATGTG				
PITPNM2ex4F	TCTTGAGTACCCCAGAGCCAAGC	341	55135285-55135625	60	RFLP
PITPNM2ex4R	GCAGCACTGCCAAAAAGTAAA				
PITPNM2ex6F	TTCATAGCAGACAGAGAATGG	259	55136886-55137144	58	RFLP
PITPNM2ex6R	GACGCTGGTTCACTGGTTTG				
PITPNM2ex9F	CTGTCCTCTCTTTCTCTCTC	680	55178634-55179387	55	RFLP
PITPNM2ex9R	TTTGGGAGGTGTTTAGGTCA				
PITPNM2ex12F	ATTCGGGGATGGCAGGAACATG	422	55191486-55191907	53	RFLP
PITPNM2ex12R	CAGAGTAACAGAAACTTGGA				
PITPNM2ex14F	AGTGTCATCGCACCTGCCCGCTT	250	55195608-55195857	55	RFLP
PITPNM2ex14R	TCCCCACCGACACTTTCAGA				
PITPNM2ex15F	AGCCATAGAAGCATGGGGGCCC	579	55198356-55198934	55	RFLP
PITPNM2ex15R	GTGCCAAGTTCTGCCTCCATT				
PITPNM2ex16F	TCTTTCCCCAGCATGACAGAAGC	496	55201582-55202077	55	RFLP
PITPNM2ex16R	GACTAACACACTCTAACCTCA				
PITPNM2ex18F	TGAACCTGTGTGGGGCCATT	312	55203526-55203837	58	RFLP
PITPNM2ex18R	ACATTGGCCATCAGCACCAC				
PITPNM2ex20F	ACACACTTTTGGGTTTCTCTG	390	55206798-55207187	58	RFLP
PITPNM2ex20R	CCGGAACTTGGAGCACCCAA				
PSMD9ex2F	CTGTGCGAAGTGATCAGTACATG	286	56113792-56113407	54	RFLP
PSMD9ex2R	GTTCTCAGTCCCTCCATGGGGAC				

Table 1 continued

PSMD9ex3F	TGTAGATGAGTCCTCAGCGTCT	528	56110807-56110280	58	Sequencing
PSMD9ex3R	ATCGCCTTCCACCTTGTGG				
B3GNT4F1	CTCCTTGCAGGACCACCATGTTCC	104	55855547-55855444	60	
B3GNT4R1	CTGGGCCTCCTTCTTCAGTAAGAAC				
B3GNT4probe[1]	CCACCTTGCGGAACATGG	44	5585489-55855532	95	Melting[3]
B3GNT4anchor[2]	TGCAAGGCTGTACAGGACCAGCC				

1) Labeled 5´-Fluorescein and 3´-Phosphat

2) Labeled 3´-Rox

3) Melting curve determination

3.3 Laboratory equipments and consumables

3.3.1 Laboratory equipments

- Agilent 2100 Bioanalyzer: Agilent Technologies GmbH, (Waldbronn, Germany);
- Autoclave ´´Varioklav 75S´´: ThermoScientific GmbH, (Langenselbold, Germany);
- Basic pH Meter PB-11, Analytical Balances TE6101, Analytical Balances TE214S, Incubator BS-1: Sartorius GmbH, (Göttingen, Germany);
- Bench-Centrifuge 5415R and 5424, Cool-Centrifuge 5417R, Thermo-mixer compact: Eppendorf AG, (Hamburg, Germany);
- DNA Analyzer ABI PRISM 3100: Applied Biosystemss GmbH, (Darmstadt, Germany);
- Electrophoresis power supply, PowerPac basic: Bio-Rad Laboratories GmbH, (München, Germany);
- Incubator, Centrifuge (Megafuge 1.0R): Heraeus Instruments GmbH, (Bad Grund, Germany);
- Light Cycler 480 II: Roche Diagnostics GmbH, (Penzberg, Germany);
- Milli-Q Biocel A10: Millipore SAS, (Molsheim, France);
- Pipettes set ´´PIPETMAN´´ 10 µL, 50-200 µL and 100-1000 µL: Gilson, (Middelton, USA);
- Refrigerator 4 °C and -20 °C: Siemens GmbH, (Munich, Germany);
- Spectrophotometer Nanodrop ND-1000, Gel documentation for ethidiumbromide-stained agarose gel: PEQLAB Biotechnologie GmbH, (Erlangen Germany);
- Speed Vac Plus SC110A: Savant Instruments, (Farmingdale, USA);
- Thermocyler TGradient and Thermocyler T3000: Biometra GmbH, (Göttingen, Germany);

- Vortex Genie 2: Scientific Industries, (New York, USA);
- Water bath 5 liter: GFL-Labortechnik GmbH, (Burgwedel, Germany).

3.3.2 Consumables

Consumable materials were purchased from the following companies:

- BRAUN Melsungen AG, (Melsungen, Germany);
- Carl Roth GmbH, (Karlsruhe, Germany);
- Eppendorf AG, (Hamburg, Germany);
- Greiner Bio-One GmbH, (Frickenhausen, Germany);
- Kimberly-Clark Professional Europe Ltd., (Kent, United Kingdom);
- Microflex Corporation, (Vienna, Austria);
- Nerbe plus GmbH, (Winsen/Luhe, Germany);
- Sarstedt GmbH, (Nümbrecht, Germany);
- Terumo Europe N.V, (Leuven, Belgium).

3.4 DNA extraction with QIAamp® DNA mini kit

DNA was extracted from EDTA stabilized blood using the QIAamp® DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

3.5 Polymerase chain reaction

The polymerase chain reaction (PCR) can generate a large number of copies from even the smallest amounts of DNA (Saiki et al., 1988). PCR primers (Table 1) were developed using the online software tool Primer3 (http://frodo.wi.mit.edu/, accessed on 22 September, 2009) (Rozen and Skaletsky, 2000) using the DNA sequence of BTA17 deposited with GenBank accession number NC_007315.3. PCR was carried out in a 25 µL reaction volume containing 20 ng DNA, 0.4 µM of each primer, 200 µM of each dNTP, and 1 unit *Taq* DNA polymerase, using the buffer supplied by the manufacturer. Amplifications were performed in a Biometra T3000 thermocycler (Biometra, Göttingen, Germany) with a initial denaturation at 94 °C for 3 min, followed by 35 cycles consisting of 30 s at 94 °C, 30 s at 53 - 60 °C (annealing, depending of optimum for each pair of primers, see Table 1), elongation for 30-60 s at 72 °C, and a final extension step of 10 min at 72 °C. PCR products were analysed on 1.5% agarose gels, purified using QIAquick® PCR purification kit (QIAGEN, Hilden, Germany) spin columns and quantified photometrically using Nanodrop ND-1000 instrument (PEQLAB, Erlangen, Germany).

3.6 DNA sequencing

After purification, amplicons were subjected to bi-directional sequencing on an ABI PRISM 3100 DNA Analyzer (Applied Biosystems, Darmstadt, Germany). Cycle sequencing was conducted using 10 ng purified PCR fragments, 10 µM forward or reverse

primer, and 4 μL BigDye Terminator (v.3.1) reaction mix in a total reaction volume of 10 μL. Amplifications were performed in a Biometra T3000 thermocyler (Biometra, Göttingen, Germany) with initial denaturation 96 °C for 1 min, following for 25 cycles 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. After purification using the BigDyeX Terminator Kit (Applied Biosystems, Darmstadt, Germany), reactions were analyzed using POP-6 polymer and 50 cm capillaries.

3.7 Alignment with SeqMan

The raw data were analysed with ABI Sequencing Analysis (v.3.7) software and the program package SeqMan, DNASTAR LASERGENE^{TM}6 (DNASTAR, Madison, USA). The SeqMan is an assembly program, the primary purpose of the program is to assemble and align number of shorter (up to 10 kb) overlapping sequences into one or more contiguous segments (contigs).

3.8 BLAST alignments

The Basic Local Alignments Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 22 September, 2009) was used to find sequences similar to a query DNA sequence, e.g. in order to compare amplicon sequences with the bovine genome database. BLAST uses a heuristic algorithm. BLAST finds homologous sequences, not by comparing sequence in its entirety, but rather by locating short matches between the two sequences (Altschul et al., 1990).

3.9 RFLP analysis

RFLP (restriction fragment length polymorphism) involves cutting a particular region of DNA with known variability, with restriction enzymes, then separating DNA fragments by 2% agarose gel electrophoresis. RFLP was applied after PCR product amplification using the appropriate enzyme for genotyping.

3.10 Melting curve determination

Melting curve analysis was done on a LightCycler 480 II (Roche, Penzberg, Germany). After PCR, amplicons were transferred (18 μL) to the LightCycler after adding a pre-made probe mixture containing of 0.4 μM of each labeled anchor oligonucleotide and the probe (Table 1). The melting curve was recorded after 45 s of denaturation at 95 °C followed by re-annealing at 37 °C for 1 s and detection during heating continuously to 70 °C. SNPs were detected by the lower melting temperature obtained for the sequence specific probe.

3.11 Detection of polymorphisms and genotyping

Detection for unknown mutations and polymorphisms on double stranded DNA fragments was done by mismatch specific cleavage of heteroduplex DNA using endonuclease *CEL I* (Oleykowski et al., 1998). Equal amounts of case and control DNA were mixed, to form hetero- and homo-duplexes. The mixture was heated to 95 °C for 2 min and was allowed to cool (- 0.1°C per 1 s) to 25 °C. The annealed heteroduplex/homoduplex mixture was treated with endonuclease *CEL I*. *CEL I* cleaves specifically on sites of mismatches in a heteroduplex DNA strands. The homozygous control DNA alone, treated similarly, served as a background control. The DNA fragments were analyzed by 2% agarose gel

electrophoresis. The formation of cleavage products indicates the presence of a mutation, while their size indicates the location of the mismatch.

However, all polymorphisms detected on PCR fragments by *CEL* I were identified for exact position by comparative DNA sequencing (see chapter 3.6 and 3.7).

3.12 Statistical methods and haplotype analysis

Statistical analysis were performed for each breed and each SNP separately using Fisher exact test (Fisher, 1922). The P values were corrected for multiple testing by using the Bonferroni method. We considered $p < 0.1$ as moderately significant, $p < 0.05$ as significant, $p < 0.01$ as highly significant. Only the most informative alleles were used to construct haplotypes inferred by the Partition-Ligation-Expectation-Maximization Algorithm (Qin et al., 2002) within the software HAPANALYZER system (NGRI, Seoul, Korea; http://hap.ngri.go.kr, accessed on 22 September, 2009) (Ho-Youl Jung, 2004). Several haplotypes and their frequencies were identified using iHaplor program (Ho-Youl Jung, 2004). An association test was performed for all haplotypes, which had frequencies of > 0.05. The HAPANALYZER includes a Yates´ continuity correction to improve the approximation.

4 Results

4.1 Identification of DNA sequence variants

As described in Materials and Methods DNA sequence variants were identified by comparative DNA sequencing and genotyping (see chaper 3.6 – 3.11) of four positional and functional candidate genes in BSE affected and BSE non-affected cattle of three different breeds (Holstein Friesian, German Simmental, and Brown Swiss). Figure 4 shows the locations, and distances of the four analysed genes with regard to microsatellite marker *INRA025*. The genes are located approximately 8 to 10 cM upstream of *INRA025*.

With the intention to focus on DNA sequence variants that could have an influence on protein function and transcription, we primarily analysed the coding regions, exon-intron boundaries, and 1-2 kb of the 5´-flanking regions. Table 2 shows the exact positions according to Btau_4.0 (08 August, 2008).

In *RNP24* we detected four sequence variants, i.e. three in the 5´-flanking region (g.54,756,194T>C, g.54,755,280G>A, and g.54,754,830G>A,) and one in exon 2 (g.54,752,610G>A, c.333G>A). In *PITPNM2,* 15 sequence variants were identified, five in the 5´-flanking region (g.55,137,106A>G, g.55,135,434G>A, g.55,123,651G>A, g.55,123,355T>C, g.55,123,304T>C) six in exons 5, 7, 8, 10, 13 and 15 (g.55,191,700C>T, c.531C>T, g.55,195,765T>C, c.1,029T>C g.55,198,570C>T, c.1,125C>T, g.55,201,759C>T, c.1,314C>T, g.55,203,705G>A, c.1,938G>A and g.55,206,987T>C, c.2226T>C) and four in introns 1, 2 and 11 (g.55,123,252G>A, g.55,123,115A>G, g.55,178,941C>T and g.55,203,767C>T). During our analyses the annotation of *PITPNM2* was updated. In Btau_2.1 *PITPNM2* harboured 31 exons, similar to the *Drosophila melanogaster* orthologue rdgB. In Btau_3.1 and Btau_4.0 the number of exons was corrected to 25 according to the human orthologue. With this adaptation, exon 9

in Btau_2.1 was updated to a start codon in Btau_4.0 and consequently most of the sequence variants that have been detected were now located in intron 1 and 2 of *PITPNM2*. In *PSMD9* we observed four sequence variants, i.e. two in exon 3 (g.56,110,475G>A, c.426G>A, g.56,110,484G>A, c.435G>A) and one in intron 2 (g.5,6113,507C>T), and intron 3 (g.56,110,399T>G), respectively. In *B3GNT4* a sequence variant was detected in the coding region (g.55,206,987A>G, c.8A>G). This sequence variant was located at the second position of the codon resulting in an amino acid substitution from histidin to arginin (p.His3Arg).

In summary, a total of twenty-four sequence variants were identified in *RNP24* (GenBank accession number FJ851394), *PITPNM2* (GenBank accession number FJ861208), *PSMD9* (GenBank accession number FJ851395) and *B3GNT4* (GenBank accession number GQ214815). Only one sequence variant in *B3GNT4* was non-synonymous, all others were synonymous DNA sequence variants.

Following the identification of the different sequence varaints, we performed statistical analyses using Fisher´s exact test, to determine significant associations between each sequence variant and the occurrence of BSE. Results were corrected for multiple testing (Bonferroni correction). Results of each sequence variant in the four candidate genes, breeds, and individuals are summarized in Table 2.

In summary, nine of twenty-four sequence variants, i.e. two in *RNP24* and seven in *PITPNM2*, showed highly significant associations with BSE in Brown Swiss. In German Simmental four sequence variants showed significant (*RNP24*, *B3GNT4* and *PSMD9*) and one moderately significant (*PITPNM2*) association. In Holstein Friesian only three sequence variants showed significant association (*PITPNM2* and *PSMD9)*.

As indicated in Table 2 nine of the 24 sequence variants did not show any association with the occurrence of BSE, 12 sequence variants showed a significant association at least in

one breed, three sequence variants showed a significant association in two breeds, but none of the sequence variants was significantly associated with BSE in all breeds.

Table 2 Genotype frequencies of genes and their P value.

Breed		**Holstein Friesian**								**German Simmental**							
		Genotype[2]								Genotype							
		BSE affected			Healthy					BSE affected			Healthy				
Gene	Position[1]	11	12	22	11	12	22	*P*	α[3)]	11	12	22	11	12	22	*P*	α
RNP24																	
5´- flanking[4]	g.54756194T>C	0.22	0.61	0.17	0.34	0.58	0.80	0.17	0.99	0.15	0.76	0.09	0.30	0.54	0.16	0.01	0.21
		8	*22*	*6*	*42*	*73*	*10*			*8*	*42*	*5*	*57*	*103*	*31*		
5´- flanking	g.54755280G>A	0.72	0.22	0.06	0.8	0.18	0.02	0.29	1.00	0.44	0.40	0.16	0.60	0.67	0.03	**0.001**	**0.02**
		26	*8*	*2*	*100*	*23*	*2*			*24*	*22*	*9*	*115*	*70*	*6*		
5´- flanking	g.54754830G>A	0.94	0.06	0.00	0.92	0.08	0.00	1.00	1.00	0.89	0.09	0.02	0.83	0.16	0.01	0.29	1.00
		34	*2*	*0*	*115*	*10*	*0*			*49*	*5*	*1*	*158*	*31*	*2*		
Exon 2	g.54752610G>A	0.44	0.36	0.2	0.33	0.57	0.1	0.05	0.71	0.24	0.60	0.16	0.37	0.47	0.16	0.16	0.98
		16	*13*	*7*	*42*	*71*	*12*			*13*	*33*	*9*	*71*	*90*	*30*		
PITPNM2																	
5´- flanking	g.55123115G>A	0.36	0.50	0.14	0.48	0.42	0.10	0.35	1.00	0.69	0.31	0.00	0.67	0.31	0.02	0.84	1.00
		13	*18*	*5*	*61*	*52*	*12*			*38*	*17*	*0*	*127*	*60*	*4*		
5´- flanking	g.55123252G>A	0.94	0.06	0.00	0.98	0.02	0.00	0.31	1.00	0.98	0.02	0.00	0.98	0.02	0.00	1.00	1.00
		34	*2*	*0*	*122*	*3*	*0*			*54*	*1*	*0*	*188*	*3*	*0*		
5´- flanking	g.55123304T>C	0.11	0.47	0.41	0.03	0.54	0.43	0.17	0.99	0.73	0.23	0.04	0.75	0.21	0.04	0.91	1.00
		4	*17*	*15*	*4*	*67*	*54*			*40*	*13*	*2*	*143*	*40*	*8*		
5´- flanking	g.55123355T>C	0.97	0.03	0.00	0.95	0.05	0.00	1.00	1.00	0.78	0.18	0.04	0.72	0.25	0.03	0.53	1.00
		35	*1*	*0*	*119*	*6*	*0*			*43*	*10*	*2*	*138*	*48*	*5*		
5´- flanking	g.55123651G>A	0.25	0.58	0.17	0.3	0.52	0.18	0.84	1.00	0.56	0.40	0.04	0.49	0.41	0.10	0.28	1.00
		9	*21*	*6*	*37*	*65*	*23*			*31*	*22*	*2*	*93*	*78*	*20*		
Intron 1	g.55135434G>A	0.53	0.39	0.08	0.25	0.3	0.45	0.09	0.90	0.38	0.38	0.24	0.38	0.42	0.20	0.82	1.00
		19	*14*	*3*	*31*	*38*	*56*			*21*	*21*	*13*	*72*	*80*	*39*		
Intron 1	g.55137106A>G	0.50	0.42	0.08	0.36	0.54	0.10	0.35	1.00	0.35	0.56	0.09	0.42	0.53	0.05	0.42	1.00
		18	*15*	*3*	*45*	*67*	*13*			*19*	*31*	*5*	*80*	*101*	*10*		

Table 2 continued

Intron 2	g.55178941C>T	0.28 *10*	0.69 *25*	0.03 *1*	0.29 *37*	0.54 *67*	0.17 *21*	0.05	0.71	0.44 *24*	0.51 *28*	0.05 *3*	0.38 *73*	0.46 *88*	0.16 *30*	0.14	0.97
Exon 5	g.55191700C>T	0.75 *27*	0.14 *5*	0.11 *4*	0.48 *60*	0.4 *50*	0.12 *15*	**0.006**	0.13	0.80 *44*	0.15 *8*	0.05 *3*	0.75 *143*	0.20 *38*	0.05 *10*	0.68	1.00
Exon 7	g.55195765T>C	0.86 *31*	0.06 *2*	0.08 *3*	0.77 *96*	0.16 *20*	0.07 *9*	0.29	1.00	0.98 *54*	0.02 *1*	0.00 *0*	0.94 *180*	0.05 *9*	0.01 *2*	0.68	1.00
Exon 8	g.55198570C>T	0.50 *18*	0.33 *12*	0.17 *6*	0.47 *59*	0.46 *58*	0.07 *8*	0.11	0.94	0.73 *40*	0.27 *15*	0.00 *0*	0.62 *119*	0.32 *61*	0.06 *11*	0.12	0.95
Exon 10	g.55201759C>T	0.58 *21*	0.42 *15*	0.00 *0*	0.33 *41*	0.54 *67*	0.14 *17*	**0.003**	0.07	0.15 *8*	0.78 *43*	0.07 *4*	0.16 *28*	0.07 *133*	0.15 *30*	0.27	1.00
Exon 13	g.55203705G>A	0.95 *34*	0.05 *2*	0.00 *0*	0.78 *97*	0.21 *26*	0.01 *2*	0.09	0.90	0.67 *37*	0.33 *18*	0.00 *0*	0.73 *139*	0.23 *44*	0.04 *8*	0.14	0.97
Intron 13	g.55203767C>T	0.92 *33*	0.08 *3*	0.00 *0*	0.85 *106*	0.14 *17*	0.01 *2*	0.74	1.00	0.93 *51*	0.05 *3*	0.02 *1*	0.81 *154*	0.18 *35*	0.01 *2*	0.04	0.62
19. Exon 15	g.55206987A>G	0.97 35	0.03 1	0.00 0	0.88 110	0.12 15	0.00 0	0.12	0.95	0.96 54	0.04 1	0.00 0	0.91 173	0.09 18	0.00 0	0.08	0.86
B3GNT4																	
20. Exon 1	g.55855523T>C	0.30 *11*	0.56 *20*	0.14 *5*	0.24 *30*	0.45 *56*	0.31 *39*	0.95	1.00	0.23 *13*	0.44 *24*	0.32 *18*	0.33 *63*	0.51 *97*	0.16 *31*	**0.02**	0.38
PSMD9																	
Intron 2	g.56113507C>T	0.33 *12*	0.58 *21*	0.08 *3*	0.46 *58*	0.44 *55*	0.10 *12*	0.32	1.00	0.31 *17*	0.51 *28*	0.18 *10*	0.23 *44*	0.45 *85*	0.32 *62*	0.10	0.92
Exon 3	g.56110475G>A	0.72 *26*	0.28 *10*	0.00 *0*	0.66 *83*	0.3 *37*	0.04 *5*	0.71	1.00	0.96 *53*	0.04 *2*	0.00 *0*	0.99 *189*	0.01 *2*	0.00 *0*	0.22	1.00
Exon 3	g.56110484G>A	0.50 *18*	0.42 *15*	0.08 *3*	0.22 *27*	0.54 *68*	0.24 *30*	**0.002**	**0.05**	0.84 *46*	0.16 *9*	0.00 *0*	0.78 *149*	0.19 *37*	0.03 *5*	0.64	1.00
Intron 3	g.56110399T>G	0.36 *13*	0.58 *21*	0.06 *2*	0.48 *60*	0.42 *53*	0.10 *12*	0.26	1.00	0.16 *9*	0.38 *21*	0.46 *25*	0.34 *65*	0.45 *86*	0.21 *40*	**0.0009**	**0.02**

Table 2 continued

Breed		**Brown Swiss**								**Pooled sample**							
		Genotype[2]								Genotype							
		BSE affected			Healthy					BSE affected			Healthy				
Gene	Position[1)]	11	12	22	11	12	22	*P*	α[3)]	11	12	22	11	12	22	*P*	α
RNP24																	
5′- flanking[4]	g.54756194T>C	0.21 *10*	0.62 *30*	0.17 *8*	0.39 *65*	0.46 *78*	0.15 *25*	0.05	0.71	0.17 *24*	0.68 *94*	0.15 *21*	0.27 *132*	0.52 *254*	0.20 *98*	**0.006**	0.13
5′- flanking	g.54755280G>A	0.27 *13*	0.73 *35*	0.00 *0*	0.47 *79*	0.43 *72*	0.10 *17*	**0.0003**	**0.007**	0.45 *63*	0.47 *65*	0.08 *11*	0.61 *294*	0.34 *165*	0.05 *25*	**0.005**	0.11
5′- flanking	g.54754830G>A	0.85 *41*	0.15 *7*	0.00 *0*	0.64 *108*	0.23 *39*	0.13 *21*	**0.003**	0.07	0.89 *124*	0.10 *14*	0.01 *1*	0.79 *381*	0.16 *80*	0 *23*	**0.009**	0.20
Exon 2	g.54752610G>A	0.17 *8*	0.60 *29*	0.23 *11*	0.31 *52*	0.45 *75*	0.24 *41*	0.09	0.90	0.27 *37*	0.54 *75*	0.19 *27*	0.34 *165*	0.49 *236*	0.17 *83*	0.244	1.00
PITPNM2																	
5′- flanking	g.55123115A>G	0.27 *13*	0.60 *29*	0.13 *6*	0.52 *88*	0.41 *68*	0.07 *12*	**0.005**	0.11	0.46 *64*	0.46 *64*	0.08 *11*	0.57 *276*	0.37 *180*	0.06 *28*	0.06	0.77
5′- flanking	g.55123252G>A	0.52 *25*	0.46 *22*	0.02 *1*	0.82 *138*	0.17 *29*	0.01 *1*	**0.0001**	**0.002**	0.81 *113*	0.18 *25*	0.01 *1*	0.92 *448*	0.07 *35*	0.01 *1*	**0.0004**	**0.01**
5′- flanking	g.55123304T>C	0.73 *35*	0.21 *10*	0.06 *3*	0.55 *92*	0.38 *64*	0.07 *12*	0.06	0.77	0.65 *90*	0.29 *40*	0.06 *9*	0.60 *289*	0.35 *171*	0.05 *24*	0.31	1.00
5′- flanking	g.55123355T>C	1.00 *48*	0.00 *0*	0.00 *0*	0.99 *166*	0.01 *2*	0.00 *0*	1.00	1.00	0.91 *126*	0.08 *11*	0.01 *2*	0.88 *423*	0.11 *56*	0.01 *5*	0.44	1.00
5′- flanking	g.55123651G>A	0.35 *17*	0.46 *22*	0.19 *9*	0.26 *44*	0.50 *84*	0.24 *40*	0.43	1.00	0.41 *57*	0.47 *65*	0.12 *17*	0.36 *174*	0.47 *227*	0.17 *83*	0.31	1.00
Intron 1	g.55135434G>A	0.52 *25*	0.38 *18*	0.10 *5*	0.59 *99*	0.34 *57*	0.07 *12*	0.58	1.00	0.47 *65*	0.38 *53*	0.15 *21*	0.47 *227*	0.36 *175*	0.17 *82*	0.87	1.00
Intron 1	g.55137106A>G	0.29 *14*	0.67 *32*	0.04 *2*	0.47 *79*	0.46 *78*	0.07 *11*	0.05	0.71	0.37 *51*	0.56 *78*	0.07 *10*	0.42 *204*	0.51 *246*	0.07 *34*	0.51	1.00

Table 2 continued

Intron 2	g.55178941C>T	0.23 *11*	0.71 *34*	0.06 *3*	0.46 *78*	0.44 *74*	0.10 *16*	**0.004**	0.09	0.32 *45*	0.63 *87*	0.05 *7*	0.39 *188*	0.47 *229*	0.14 *67*	**0.001**	0.02
Exon 5	g.55191700C>T	0.29 *14*	0.50 *24*	0.21 *10*	0.57 *96*	0.36 *61*	0.07 *11*	**0.0004**	**0.01**	0.61 *85*	0.27 *37*	0.12 *17*	0.62 *299*	0.31 *149*	0.07 *36*	0.16	0.98
Exon 7	g.55195765T>C	0.81 *39*	0.15 *7*	0.04 *2*	0.73 *123*	0.27 *45*	0.00 *0*	**0.01**	0.21	0.89 *124*	0.07 *10*	0.04 *5*	0.83 *399*	0.15 *74*	0.02 *11*	**0.02**	0.38
Exon 8	g.55198570C>T	0.56 *27*	0.40 *19*	0.04 *2*	0.79 *133*	0.14 *24*	0.07 *11*	**0.001**	**0.02**	0.61 *85*	0.33 *46*	0.06 *8*	0.64 *311*	0.30 *143*	0.06 *30*	0.72	1.00
Exon 10	g.55201759C>T	0.69 *33*	0.31 *15*	0.00 *0*	0.19 *32*	0.73 *123*	0.08 *13*	**0.00***	**0.00****	0.45 *62*	0.52 *73*	0.03 *4*	0.21 *101*	0.67 *323*	0.12 *60*	**0.00***	**0.00****
Exon 13	g.55203705G>A	1.00 *48*	0.00 *0*	0.00 *0*	0.99 *166*	0.01 *2*	0.00 *0*	1.00	1.00	0.86 *119*	0.14 *20*	0.00 *0*	0.83 *402*	0.15 *72*	0.02 *10*	0.25	1.00
Intron 13	g.55203767C>T	1.00 *48*	0.00 *0*	0.00 *0*	0.99 *166*	0.01 *2*	0.00 *0*	1.00	1.00	0.95 *132*	0.05 *6*	0.00 *1*	0.88 *426*	0.11 *54*	0.01 *4*	**0.03**	0.52
Exon 15	g.55206987A>G	0.96 46	0.04 2	0.00 0	0.98 164	0.02 4	0.00 0	0.61	1.00	0.97 135	0.03 4	0.00 0	0.92 447	0.08 37	0.00 0	0.05	0.71
B3GNT4																	
Exon 1	g.55855523T>C	0.19 *9*	0.62 *30*	0.19 *9*	0.33 *55*	0.54 *91*	0.13 *22*	0.14	0.97	0.24 *33*	0.53 *74*	0.23 *32*	0.31 *148*	0.50 *244*	0.19 *92*	0.06	0.23
PSMD9																	
Intron 2	g.56113507C>T	0.33 *16*	0.52 *25*	0.15 *7*	0.29 *48*	0.47 *80*	0.24 *40*	0.40	1.00	0.26 *36*	0.53 *74*	0.21 *29*	0.22 *104*	0.45 *220*	0.33 *160*	**0.02**	0.38
Exon 3	g.56110475G>A	0.98 *47*	0.02 *1*	0.00 *0*	1.00 *166*	0.00 *0*	0.00 *0*	0.22	1.00	0.91 *126*	0.09 *13*	0.00 *0*	0.91 *440*	0.08 *39*	0.01 *5*	0.60	1.00
Exon 3	g.56110484G>A	0.85 *41*	0.15 *7*	0.00 *0*	0.90 *152*	0.09 *14*	0.01 *2*	0.41	1.00	0.76 *105*	0.22 *31*	0.02 *3*	0.68 *328*	0.24 *119*	0.08 *37*	**0.03**	0.52
Intron 3	g.56110399T>G	0.25 *12*	0.58 *28*	0.17 *8*	0.24 *40*	0.48 *80*	0.28 *48*	0.24	1.00	0.25 *34*	0.50 *70*	0.25 *35*	0.35 *165*	0.45 *219*	0.20 *100*	0.08	0.86

Values are given as frequency and *number of observations.*

1) Genotype nomenclature: 11 = wild type homozygous, 12 = heterozygous, 22 = mutant homozygous.
2) Position and definition of wild type and mutant nucleotide according to GenBank accession number NC_007315.3. Wild type nucleotide first, mutant nucleotide second.
3) Bonferroni correction for multiple testing.
4) 5´- flanking region of gene

* P value is less than < 0.00000000001

** P value is less than < 0.0000000001

4.2 Haplotype analysis

Additional to the calculation of associations of sequence variants and BSE occurrence using Fisher´s exact test, we performed a haplotype analysis with HAPANALYZER. A total of 675 haplotypes was identified when all 24 allele variants were included. The frequencies were between 0.0008 and 0.014. No specific haplotype with a high frequency was identified that correlated with BSE affected cattle. However, if the statistically significant alleles were pooled and non-significant alleles were excluded, we were able to identify haplotypes showing significant association with BSE susceptibility/resistance. Haplotype CCGC (Table 3) in Holstein Friesian was more frequent in case group (46%) suggesting a susceptible effect ($P < 0.0001$) when compared with control group (18%). In German Simmental haplotype GCCT was more frequent in the control group (27%) suggesting a protective effect against BSE ($P < 0.0001$) when compared with case group (7%). In Brown Swiss the number of statistically significant alleles (Table 3) was highest and consequently haplotypes with a combination of 6 allele variants could be determined. Haplotype GGGTCT was observed more frequently in the case group (25%) suggesting a higher susceptibility to BSE ($P < 0.037$) than controls (15%).

Table 3 Statistically significant allele and haplotype frequencies

Breed	**Holstein Friesian**									
		Allele[1]								
		BSE affected		Healthy						
Gene	Position[2]	1	2	1	2	*P*	Haplotype[3]	BSE affected	Healthy	*P*
PITPNM2										
Exon 5	g.55191700C>T	0.82	0.18	0.68	0.38	**0.03**	CCGC	0.46	0.18	**0.0001**
		59	*13*	*170*	*80*			*33*	*46*	
Exon 10	g.55201759C>T	0.79	0.21	0.60	0.40	**0.003**	CTGC	0.11	0.22	0.07
		57	15	149	101			*8*	*54*	
Exon 13	g.55203767G>A	0.97	0.03	0.88	0.12	**0.04**	CCAC	0.13	0.12	0.99
		70	*2*	*220*	*30*			*9*	*29*	
PSMD9							CCGT	0.13	0.09	0.55
Exon 3	g.56110484C>T	0.71	0.29	0.49	0.51	**0.001**		*9*	*23*	
		51	*21*	*122*	*128*		CTAT	0.0	0.07	**0.01**
								0	*18*	
							Residual	0.18	0.32	N/A[4]
								13	*80*	
							Total	1.00	1.00	N/A
								72	*250*	

Table 3 continued

Breed	**German Simmental**									
		Allele[1]								
		BSE affected		Healthy						
Gene	Position[2]	1	2	1	2	*P*	Haplotype[3]	BSE affected	Healthy	*P*
RNP24										
5´-flanking region	g.54755280G>A	0.64	0.36	0.79	0.21	**0.002**	GCCT	0.07	0.27	**0.0001**
		70	*40*	*301*	*81*			*8*	*101*	
B3GNT4							GTTG	0.22	0.21	0.99
Exon 1	g.55855523T>C	0.45	0.55	0.59	0.41	**0.02**		*24*	*81*	
		50	*60*	*224*	*158*		GCTG	0.21	0.15	0.96
PSMD9								*17*	*56*	
Intron 2	g.56113507C>T	0.44	0.56	0.55	0.45	**0.05**	ACCT	0.21	0.13	0.68
		48	*62*	*209*	*173*			*17*	*51*	
Intron 3	g.56110399T>G	0.45	0.55	0.57	0.43	**0.0001**	GTCT	0.05	0.13	**0.03**
		49	*61*	*216*	*166*			*6*	*51*	
							Residual	0.35	0.11	N/A[4]
								38	*42*	
							Total	1.00	1.00	N/A
								110	*382*	

Table 3 continued

Breed	**Brown Swiss**									
		Allele[1]								
		BSE affected		Healthy						
Gene	Position[2]	1	2	1	2	*P*	Haplotype[3]	BSE affected	Healthy	*P*
RNP24										
5′-flanking region	g.54754830G>A	0.93	0.07	0.76	0.24	**0.0005**	GGGTCT	0.25	0.15	**0.037**
		89	*7*	*255*	*81*			*24*	*51*	
PITPNM2										
5′-flanking region	g.55213115A>G	0.84	0.16	0.56	0.44	**0.0001**	GAGCCT	0.04	0.18	**0.001**
		81	*15*	*187*	*149*			*4*	*59*	
5′-flanking region	g.55123252G>A	0.76	0.24	0.86	0.14	**0.02**	GGGCCT	0.13	0.12	0.95
		73	*23*	*290*	*46*			*12*	*39*	
Exon 5	g.55191700C>T	0.54	0.46	0.75	0.25	**0.0001**	GGGCCC	0.06	0.09	0.47
		52	*44*	*253*	*83*			*6*	*31*	
Exon 8	g.55198570C>T	0.75	0.25	0.91	0.09	**0.0001**	AAGCCT	0.00	0.10	**0.003**
		72	*23*	*305*	*31*			*0*	*33*	
Exon 10	g.55201759C>T	0.57	0.43	0.73	0.27	**0.006**	GGGTCC	0.10	0.04	**0.002**
		55	*41*	*244*	*92*			*10*	*13*	
							Residual	0.42	0.33	N/A[4]
								40	*110*	
							Total	1.00	1.00	N/A
								96	*336*	

Table 3 continued

Breed	**Pooled Samples**									
		Allele[1]								
		BSE affected		Healthy						
Gene	Position[2]	1	2	1	2	*P*	Haplotype[3]	BSE affected	Healthy	*P*
RNP24										
5′-flanking region	g.54755280G>A	0.69	0.31	0.78	0.22	**0.003**	GGACAC	0.17	0.22	**0.06**
		192	*86*	*755*	*213*			*46*	*213*	
PITPNM2							GGGCGC	0.22	0.15	**0.01**
5′-flanking region	g.55123115A>G	0.69	0.31	0.76	0.24	**0.04**		*61*	*150*	
		192	*86*	*732*	*236*		GGGCAC	0.11	0.08	0.15
5′-flanking region	g.55123252G>A	0.90	0.10	0.96	0.04	**0.0002**		*30*	*76*	
		251	*27*	*930*	*38*		GGACGC	0.04	0.09	**0.003**
Exon 10	g.55201759C>T	0.71	0.29	0.54	0.46	**0.0001**		*10*	*90*	
		197	*81*	*526*			AGGCGC	0.06	0.08	**0.49**
PSMD9								*18*	*77*	
Intron 2	g.56113507C>T	0.47	0.53	0.56	0.44	**0.01**	GGACGT	0.03	0.08	**0.01**
		132	*146*	*540*	*428*			*9*	*74*	
Exon 3	g.56110484G>A	0.87	0.13	0.80	0.20	**0.01**	AGGCAC	0.13	0.06	**0.0002**
		241	*37*	*775*	*193*			*35*	*55*	
							Residual	0.25	0.24	N/A[4]
								69	*233*	
							Total	1.00	1.00	N/A
								278	*968*	

Values are given as frequency and *number of observations*.

1) Position of sequence variant and definiton of wild type resp. mutant nucleotide according to GenBank accession number NC_007316.3. Wild type nucleotide first, mutant nucleotide second. Number of alleles is double the number of observations.

2) Allele nomenclature: 1 = wild type nucleotide, 2 = mutant nucleotide. Number of alleles equals the double number of observations.

3) Number of haplotypes is double the number of observations.

4) Not calculated.

5 Discussion

Since the beginning of the BSE crisis in Europe in the mid 1980-ies it was scrutinized whether the propagation of BSE is influenced or modified by additional genetic factors besides the exclusive presence of the cellular (PrP^C) or infectious prion (PrP^d). These questions were raised because in other species, e.g. sheep, goat, deer, man, mouse, it was shown that variations in the prion gene (*PRNP*) greatly influence resistance, susceptibility, incubation period, and pathogenesis of TSE infections. Especially in the closely related ruminants, i.e. the sheep, and deer, variants at different codons of *PRNP* influence resistance or susceptibility to a TSE infection (Goldmann, 2008). Hence, the question was addressed whether variants of the bovine prion protein gene exist and if so, whether these would have any influence on the pathogenesis of BSE. Early studies by Neibergs et al. (1994) identified a polymorphism of the octapeptide repeat region using single strand conformation polymorphism (SSCP) (Neibergs et al., 1994). The number of repeats varied between 5 and 6 copies of the motif. Although the data were not significant, it was suggested that BSE-affected animals and their relatives more likely would have the AA SSCP genotype corresponding to homozygous 6 repeats. However, studies in other populations resulted in other conclusions regarding the impact of the octapeptide repeat polymorphism on the occurence of BSE (Walawski and Czarnik, 2003; Walawski et al., 2003; Nakamitsu et al., 2006). After the complete bovine prion protein gene sequence became available (Horiuchi et al., 1998; Comincini et al., 2001), several projects were initiated to identify DNA sequence variants and their associations with BSE (Humeny et al., 2002; Geldermann et al., 2003; Heaton et al., 2003; Naharro et al., 2003; Takasuga et al., 2003). Currently, at least 388 DNA sequence variants at the bovine prion protein gene locus have been identified (Clawson et al., 2006). However, none of these variants have

been shown to have any influence on the pathogenesis of BSE. The first relevant polymorphisms that were shown to be significantly associated with BSE pathogenesis were identified in a study by Sander et al. (2004). They identified a 23 bp indel polymorphism in the putative promoter region and a 12 bp indel polymorphism in the first intron of the bovine prion protein gene (Sander et al., 2004). In a subsequent study, it was shown that there is an interaction between both sites harbouring binding sites for transcription factor RP58 and SP1, respectively. The haplotype 23ins:12ins showed a significant lower expression rate of the prion protein gene than the other possible haplotypes and was more often present in healthy cattle (Sander et al., 2005). These findings induced a plethora of studies in other cattle breeds to repeat and confirm the results (see e.g. Jeong et al., 2006; Juling et al., 2006; Nakamitsu et al., 2006; Czarnik et al., 2007; Haase et al., 2007; Kerber et al., 2008; Muramatsu et al., 2008; Un et al., 2008; Xue et al., 2008; Czarnik et al., 2009). Another study in Japanese Black cattle showed that an additional promoter polymorphism in a putative SP1 binding site down regulated expression of the prion gene (Nakamura et al., 2007). Hence, the only potential DNA sequence variants that seem to have a significant, although, moderate influence on the pathogenesis of BSE by down or up regulation of prion protein gene expression are altered transcription factor binding sites in the regulatory region of the bovine prion protein gene. It is comprehensible that this situation was rather unsatisfactory compared to the results in other species. Therefore, genome wide linkage studies were conducted to identify potential ”modifier genes“. This approach was supported by findings in mice and recently also in humans that other chromosomal regions influence the pathogenesis of TSE infections. In mice, six QTL were detected that influence incubation time, two of them at a genome-wide significant and four at a genome-wide suggestive level (Moreno et al., 2003; O'shea et al., 2008). In humans additional genetic risk factors besides the *PRNP* polymorphic codon 129 influencing vCJD

were described in a recently published genome-wide association study (Mead et al., 2009). And also the age of onset and death seems to be inherited in humans (Webb et al., 2009). In cattle two linkage studies were performed so far. In both studies, using the same population of cattle, genome-wide significant QTL regions have been identified with differences in the location of the QTLs depending on the statistical models applied (Hernandez-Sanchez et al., 2002; Zhang et al., 2004). One gene, i.e. *HEXA* on BTA10, which was suggested as potential candidate, was analysed in detail by Juling et al. (2008).

In the study presented here, positional and functional candidate genes on BTA17q23-q24, which is syntenic to mouse chromosome 5 (MMU5F) harbouring one of the murine TSE incubation time QTLs, were analysed. The region on BTA17q23-q24 was selected because it showed the genome-wide significant QTL with the highest *F*-value (6.2) in a relatively small chromosomal region (91-164 cM, 95 % C.I.) around microsatellite marker *INRA025* (144 cM) (Zhang et al., 2004). The exact position of *INRA025* is currently at 64,217,575 - 64,217,705 bp (Btau_4.0). The 5 % genome-wide significance level was approx. flanked by marker ILSTS58/BOVILS58 (60,983,439-60,983,566 bp) and BM1233 (98.6 cM) (Zhang et al., 2004). The *in silico* search for potential candidate genes in this chromosomal region was therefore limited to approximately 10 cM up- and downstream of marker *INRA025* (interval from 54 Mbp – 74 Mbp). According to the latest release of the bovine genome (Btau_4.0), this region harbours 273 genes. In addition, the search was restricted to genes that are involved in protein trafficking, protein modification, and protein degradation, because it has been shown in a number of studies that these steps greatly influence prion protein folding, deposition, and degradation and consequently the pathogenesis of the disease (see e.g. Aguzzi et al., 2007; Tatzelt and Schatzl, 2007; Linden et al., 2008; Caughey et al., 2009). Using this specifications four genes upstream of *INRA025* were identified, i.e. *RNP24*, *PITPNM2*, *B3GNT4*, and *PSMD9*, and

comparatively sequenced. To identify functional DNA variants, we mainly focused on the analysis of the regulatory (5´-flanking region, 5´-UTR and promoter) as well as coding regions (exons including 5´- and 3´-splice sites).

So far, eight DNA sequence variants have been reported in *PITPNM2* and three in *PSMD9* (see NCBI SNP database, http://www.ncbi.nlm.nih.gov/SNP/, accessed on 22 September, 2009). From these eleven DNA sequence variants only one was within the regions that were analysed here. However, this DNA sequence variant, i.e. rs41847263 in exon 25 of *PITPNM2,* was not polymorphic in our samples.

A total of 24 DNA variants were identified in our samples and analysed. Unfortunately, only one of these resulted in an amino acid exchange (B3GNT4), whereas all of the others were silent and therefore have to be considered as markers for other associated candidate genes. Nevertheless, the statistical analysis comparing the BSE affected and healthy control cattle showed highly significant associations for 15 of the SNPs in at least one or two of the breeds. However, none of the SNPs showed significant association over all three breeds. In a first analysis we compared the genotype data for each breed separately to avoid Simpson´s paradoxon and then the samples were pooled independently (Wagner, 1982). The analysis clearly showed that there are breed specific differences. Holstein Friesian, for instance, showed the lowest number of highly significant associated sequence variants (3 of 24), whereas Brown Swiss had a high number of associated sequence variants with high significance (9 of 24). Differences in the significance of associations have been described previously for other DNA variants (23 bp indel, 12 bp indel in the bovine *PRNP*) between UK Holstein, German Holstein, German Simmental, German Brown, and Brown Swiss (Sander et al., 2005; Juling et al., 2006; Haase et al., 2007). Juling et al. (2006) did not find an association of the 23/12 bp indel polymorphisms in the prion protein gene in German Simmental. Whereas in the other studies in German

Simmental both polymorphisms showed a significant association (Sander et al., 2005; Haase et al., 2007).

In summary, the data presented here support the findings that BTA17q23-q24 seems to play an important role in the susceptibility/resistance of cattle towards BSE. However, as the DNA variants identified are not ”functional mutations“ that would change any protein structure and function or are positioned in regulatory important elements, the search for other potential candidates has to continue. In addition, one has to keep in mind that BSE is primarily an infectious disease and mainly resulted from feeding cattle with infectious prions through contaminated milk replacer or concentrate. The use of any polymorphisms, either in the prion protein gene itself or any other modifier genes, for selection in breeding programmes, will therefore at most result in cattle that might be less susceptible, but most likely never completely resistant to a BSE infection. Therefore, other measures, e.g. routine BSE testing, correct inactivation of meat and bone meal, offal feed ban for ruminants etc., have to be implemented continuously to prevent the recurrence of a BSE crisis.

6 Abstract

Bovine spongiform encephalopathy (BSE) belongs to a group of diseases called transmissible spongiform encephalopathies (TSE). With the occurrence of the BSE crisis, several studies have been started to evaluate sequence variabilities in the prion protein gene (*PRNP*) to assess their possible associations with BSE. In contrast to sheep, goat and humans no polymorphisms in the coding sequence of *PRNP* leading to a susceptibility/resistance towards TSE have been reported in cattle. However, promising associations between polymorphisms in the regulatory region of *PRNP* and the disease have been reported. In parallel to these investigations, several genome-wide DNA marker scans have been reported and QTL regions that are significantly associated with BSE susceptibility/resistance (BTA 17 and X/Y_{ps}) were detected.

The project focused on the molecular analysis of functional and positional candidate genes located in the bovine QTL region on BTA 17q23-q24. The genes *RNP24* (coated vesicle membrane protein), *PITPNM2* (phosphatidylinositol transfer protein, membrane-associated 2), *PSMD9* (proteasome, 26S subunit, non-ATPase, 9), and *B3GNT4* (UDP-Gal beta-1,3-N-acetylglucosaminyltransferase 4) were analyzed. In addition *CHST8* (carbohydrate N-acetylgalactosamine 4-0, sulfotransferase 8) was analyzed as candidate gene for BSE susceptibility, which is not part of QTL region, but was found to display a 17-fold down-regulation of expression in cells infected with scrapie. DNAs from case and control cattle (Holstein Friesian, German Simmental, and Brown Swiss) were genotyped for mutations in *RNP24*, *PITPNM2*, *PSMD9*, *B3GNT4,* and *CHST8*. The screen for SNPs included all exons, exon-intron splice junctions and 1 to 2 kb of the 5`-flanking region for each gene. Only one polymorphism was non synonymous (SNP in B3GNT4: p.His3Arg), all others were synonymous. The association analysis results indicated that the sequence variants

(g.55191700C>T; g.55201759C>T; g.56110484G>A) in Holstein Friesian, sequence variants (g.54756194T>C; g.54755280G>A; g.55855523T>C; g.56110399T>G) in German Simmental, and sequence variants (g.54755280G>A; g.54754830G>A; g.55123115A>G; g.55123252G>A; g.55178941C>T; g.55191700C>T; g.55195765T>C; g.55198570C>T; g.55201759C>T; g.37254017G>T) in Brown Swiss showed significant associations with BSE susceptibility/resistance.

7 Zusammenfassung

Die bovine spongiforme Enzephalopatie (Bovine Spongiform Encephalopathy - BSE) gehört zu der Gruppe der übertragbaren spongiösen Enzephalopathien (Transmissible Spongiform Encephalopathies – TSE). Mit dem Aufkommen der BSE-Krise in Europa wurden verschiedene Studien durchgeführt, um mögliche Zusammenhänge zwischen Polymorphismen des Prion Protein Gens (prion protein gene - *PRNP*) und BSE aufzuzeigen. Im Gegensatz zu Schafen, Ziegen und Menschen wurde bei Rindern bisher kein Polymorphismus in der kodierenden Region des *PRNPs* identifiziert, der zu einer TSE-Prädisposition oder -Resistenz führt. Allerdings konnten vielversprechende Zusammenhänge zwischen Varianten in regulatorischen Bereichen des *PRNP* Gens (Promotor, Intron 1) und der Krankheit aufgezeigt werden. Weiterhin wurden parallel zu diesen Analysen verschiedene, das gesamte Genom umfassende, Marker-Scans durchgeführt, bei denen signifikante Assoziationen zwischen chromosomalen Regionen auf BTA17 und BTAX/Y_{ps} und der Prädisposition oder Resistenz gegenüber BSE ermittelt wurden.

Die vorliegende Arbeit konzentriert sich auf die molekulare Analyse von funktionellen und positionellen Kandidaten-Genen in der QTL Region BTA17q23-q24. Die Gene *RNP24* (coated vesicle membrane protein), *PITPNM2* (phosphatidylinositol transfer protein, membrane-associated 2), *PSMD9* (proteasome, 26S subunit, non-ATPase, 9) und *B3GNT4* (UDP-Gal beta-1,3-N-acetylglucosaminyltransferase 4) wurden analysiert. Zusätzlich wurde *CHST8* (carbohydrate N-acetylgalactosamine 4-0, sulfotransferase 8) als Kandidaten-Gen einer Prädisposition für BSE analysiert. *CHST8* befindet sich zwar nicht in einer der QTL Regionen, seine Expression ist jedoch in Scrapie-infizierten Zellen ca. 17-fach herunterreguliert. DNA von erkrankten Tieren und Kontrolltieren (Holstein

Friesian, Deutsches Fleckvieh und Brown Swiss) wurde auf Mutationen in den Genen *RNP24*, *PITPNM2*, *PSMD9*, *B3GNT4* und *CHST8* überprüft. Die Untersuchung umfaßte alle Exons, Exon-Intron Spleißübergänge und ca. 1-2 kb des 5`-Bereiches jedes Gens. Nur ein Polymorphismus war nicht synonym (SNP in *B3GNT4*: p.His3Arg). Bei allen anderen Polymorphismen handelte es sich um stille Mutationen. Die Ergebnisse der Assoziationsanalyse zeigen, dass die Sequenzvarianten (g.55191700C>T; g.55201759C>T; g.56110484G>A) bei Holstein Friesian, die Sequenzvarianten (g.54756194T>C; g.54755280G>A; g.55855523T>C; g.56110399T>G) beim Deutschem Fleckvieh und die Sequenzvarianten (g.54755280G>A; g.54754830G>A; g.55123115A>G; g.55123252G>A; g.55178941C>T; g.55191700C>T; g.55195765T>C; g.55198570C>T; g.55201759C>T; g.37254017G>T) bei Brown Swiss signifikante Assoziationen zu einer Prädisposition/Resistenz gegenüber BSE aufweisen.

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Molecular analysis of *carbohydrate N-acetylgalactosamine 4-O sulfotransferase 8* (*CHST8*) as a candidate gene for bovine spongiform encephalopathy susceptibility

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Summary

Endogenous prion proteins (PrP) play the central role in the pathogenesis of transmissible spongiform encephalopathies. The carbohydrate *N*-acetylgalactosamine 4-O sulfotransferase 8 (CHST8) promotes the conversion of the cellular PrP^C into the pathogenic PrP^d. Six sequence variants within the *CHST8* gene were identified by comparative sequencing and genotyped for a sample of 623 animals comprising bovine spongiform encephalopathy (BSE)-affected and healthy control cows representing German Fleckvieh (German Simmental), German Holstein (Holstein-Friesian) and Brown Swiss. Significant differences in the allele, genotype and haplotype frequencies between BSE-affected and healthy cows indicate an association of sequence variant g.37254017G>T with the development of the disease in Brown Swiss cattle.

Keywords bovine spongiform encephalopathy, BSE, *carbohydrate N-acetylgalactosamine 4-O sulfotransferase 8* (*CHST8*), prion, transmissible spongiform encephalopathy, TSE.

Bovine spongiform encephalopathy (BSE) belongs to the group of transmissible spongiform encephalopathies triggered by the pathogenic form PrP^d of the cellular prion protein PrP^C (Prusiner *et al.* 1998; Weissmann 2004). PrP^C is a glycolipid-anchored plasma membrane protein that is widely expressed on neurons and glia in the central nervous system (Oesch *et al.* 1985; Stahl *et al.* 1987; Moser *et al.* 1995). The post-translationally modified isoform PrP^d is β-sheet enriched, insoluble in detergents and partially resistant to proteinase K (Pan *et al.* 1993; Huang *et al.* 1996; Cohen & Prusiner 1998). There is evidence that the conversion of PrP^C into PrP^d is promoted by hyposulfation of certain glycoconjugates such as chondroitin (Winklhofer *et al.* 2003; Neuendorf *et al.* 2004; Barret *et al.* 2005). The sulfation of chondroitin is mediated by carbohydrate *N*-acetylgalactosamine 4-O sulfotransferase (CHST8). It has been reported that transcription of *CHST8* is 17.13 times down-regulated in prion-infected ScGT1 cells compared with uninfected cells (Barret *et al.* 2005).

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The *CHST8* gene is located on bovine chromosome 18 (GenBank accession number NC_007316.1) and is organized in three exons encoding 424 amino acids (GenBank accession number XP_591309.2). A total of 139 cattle with BSE and 484 unaffected control cattle, both confirmed by post-mortem laboratory investigations of brain tissue, were investigated to detect sequence variants in *CHST8*. All animals (unaffected control cattle and cattle with BSE) were proven unrelated by the pedigree data and represented the breeds German Holstein, German Fleckvieh and Brown Swiss (Table 1). The animals of different ages (Table 1) came from different farms and regions scattered all over Germany (German Holstein and German Fleckvieh) and Switzerland (Brown Swiss). The samples were collected at different time points during the BSE epidemic in mainland

Table 1 Number of samples per breed, age distribution and BSE status.

Breed	Healthy	Age in years	BSE-affected	Age in years	Total
Holstein	125	1–9	36	4–15	161
Fleckvieh	191	1–8	55	4–9	246
Brown Swiss	168	1–15	48	3–5	216
Total	484		139		623

BSE, bovine spongiform encephalopathy.

Table 2a *CHST8* allele and genotype frequencies.

Breed	Position[1]	Allele[2]					Genotype						
		BSE-affected		Healthy			BSE-affected			Healthy			
		1	2	1	2	*P*	11	12	22	11	12	22	*P*
Holstein													
5′-UTR	g.37183315A>G	0.76	0.24	0.76	0.24	1.00	0.58	0.36	0.06	0.58	0.36	0.06	1.00
		55	17	190	60		21	13	2	73	44	8	
5′-UTR	g.37183319C>T	0.75	0.25	0.68	0.32	0.29	0.61	0.28	0.11	0.48	0.39	0.13	0.41
		54	18	169	81		22	10	4	60	49	16	
5′-UTR	g.37183634T>C	0.78	0.22	0.73	0.27	0.53	0.67	0.22	0.11	0.66	0.15	0.19	0.40
		56	16	183	67		24	8	4	82	19	24	
Intron 2	g.37253224C>T	0.93	0.07	0.88	0.12	0.32	0.86	0.14	0.00	0.80	0.16	0.04	0.65
		67	5	220	30		31	5	0	100	20	5	
Exon 3	g.37254017G>T	0.86	0.14	0.89	0.11	0.61	0.78	0.17	0.05	0.82	0.14	0.04	0.67
		62	10	223	27		28	6	2	103	17	5	
Exon 3	g.37254398G>A	0.90	0.10	0.87	0.13	0.56	0.83	0.14	0.03	0.76	0.22	0.02	0.53
		65	7	217	33		30	5	1	95	27	3	
Fleckvieh													
5′-UTR	g.37183315A>G	1.00	0.00	0.98	0.02	0.36	1.00	0.00	0.00	0.98	0.01	0.01	1.00
		110	0	375	7		55	0	0	187	1	3	
5′-UTR	g.37183319C>T	0.96	0.04	0.93	0.07	0.24	0.93	0.07	0.00	0.89	0.08	0.03	0.55
		106	4	354	28		51	4	0	169	16	6	
5′-UTR	g.37183634T>C	0.87	0.13	0.86	0.14	0.88	0.76	0.19	0.05	0.79	0.15	0.06	0.26
		96	14	329	53		42	12	1	150	29	12	
Intron 2	g.37253224C>T	0.85	0.15	0.81	0.19	0.34	0.73	0.25	0.02	0.67	0.28	0.05	0.30
		94	16	309	73		40	14	1	128	53	10	
Exon 3	g.37254017G>T	0.97	0.03	0.94	0.06	0.23	0.95	0.05	0.00	0.89	0.11	0.00	0.39
		107	3	358	24		52	3	0	168	22	1	
Exon 3	g.37254398G>A	1.00	0.00	0.99	0.01	1.00	1.00	0.00	0.00	0.98	0.02	0.00	1.00
		110	0	379	3		55	0	0	188	3	0	
Brown Swiss													
5′-UTR	g.37183315A>G	0.92	0.08	0.95	0.05	0.41	0.83	0.17	0.00	0.91	0.07	0.02	0.13
		88	8	318	18		40	8	0	153	12	3	
5′-UTR	g.37183319C>T	0.93	0.07	0.94	0.06	0.81	0.85	0.15	0.00	0.91	0.06	0.03	0.09[4]
		89	7	316	20		41	7	0	153	10	5	
5′-UTR	g.37183634T>C	0.98	0.02	0.96	0.04	0.74	0.96	0.04	0.00	0.96	0.02	0.02	0.58
		94	2	324	12		46	2	0	160	4	4	
Intron 2	g.37253224C>T	0.86	0.14	0.80	0.20	0.18	0.77	0.19	0.04	0.68	0.23	0.09	0.46
		83	13	268	68		37	9	2	115	38	15	
Exon 3	g.37254017G>T	0.94	0.06	0.84	0.16	0.03[3]	0.88	0.12	0.00	0.75	0.19	0.06	0.10[4]
		90	6	283	53		42	6	0	126	31	11	
Exon 3	g.37254398G>A	0.98	0.02	0.97	0.03	0.74	0.96	0.04	0.00	0.93	0.07	0.00	0.73
		94	2	325	11		46	2	0	156	12	0	
Pooled samples													
5′-UTR	g.37183315A>G	0.91	0.09	0.91	0.09	1.00	0.84	0.15	0.01	0.85	0.12	0.03	0.39
		253	25	883	85		116	21	2	413	57	14	
5′-UTR	g.37183319C>T	0.90	0.10	0.87	0.13	0.24	0.82	0.15	0.03	0.79	0.15	0.06	0.42
		249	29	839	129		114	21	4	382	75	27	
5′-UTR	g.37183634T>C	0.88	0.12	0.86	0.14	0.41	0.80	0.16	0.04	0.81	0.11	0.08	0.07[4]
		246	32	836	132		112	22	5	393	52	39	
Intron 2	g.37253224C>T	0.88	0.12	0.82	0.18	0.04[3]	0.78	0.20	0.02	0.71	0.23	0.06	0.13
		244	34	797	171		108	28	3	344	111	29	
Exon 3	g.37254017G>T	0.93	0.07	0.89	0.11	0.07[4]	0.88	0.11	0.01	0.82	0.14	0.04	0.22
		259	19	864	104		122	15	2	397	70	17	
Exon 3	g.37254398G>A	0.97	0.03	0.95	0.05	0.33	0.94	0.05	0.01	0.91	0.08	0.01	0.41
		269	9	921	47		131	7	1	440	41	3	

Values are given as frequency and number of observations.

[1]Position of sequence variant and definiton of wild-type resp. mutant nucleotide according to GenBank accession number NC_007316.2. Wild-type nucleotide first, mutant nucleotide second.

[2]Allele nomenclature: 1, wild-type nucleotide; 2, mutant nucleotide. Number of alleles is double the number of observations.

[3]Significance level of 5%.

[4]Significance level of 10%.

Europe between 1993 and 2006. Control samples were randomly collected from further farms located in Austria, Germany and Switzerland to exclude possible interdependences. Finally, the ratio between case and control per breed amounted to 1:3 in the sampling. Both the *CHST8* cDNA (GenBank accession number XM_591309.2) and the genomic DNA sequence (accession number NC_007316.1) were used to develop primers for six PCR products using PRIMER3 (http://frodo.wi.mit.edu/) (Rozen & Skaletsky 2000) to re-sequence a total of 2896 bp of the bovine *CHST8* promoter region and the coding regions including the intron–exon boundaries (Table S1). PCR products were purified with the ExoSAP-IT kit (USB) and then bi-directionally sequenced on an ABI Prism 3100 capillary analyzer (ABI) using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (ABI). The raw data were analysed with the appropriate ABI software and the program package DNASTAR LASERGENE™6 (DNASTAR Inc). Six sequence variants (GenBank accession number FJ744152 and Table 2a) were detected (three in the 5′-UTR, one in intron 2, and two in exon 3) and investigated for all animals; the two exonic sequence variants proved to be synonymous. Except for sequence variant g.37253224C>T (*Aci*I-RFLP; New England Biolabs), the other sequence variants were investigated by diagnostic sequencing. Statistical tests were performed for each breed and on the whole DNA repository (pooled data). Fisher's exact probability test and chi-squared test (pooled data) were applied to identify possible associations in the allele and/or genotype frequencies between BSE-affected and healthy cows (Table 2a). Such differences existed only in Brown Swiss cows and in the pooled data set: allele frequencies for g.37253224C>T and g.37254017G>T were distinctly different on a 5% significance level in the pooled data and in Brown Swiss, respectively. Allele frequencies for g.37254017G>T (pooled data) and genotype frequencies for sequence variants g.37183319C>T and g.37254017G>T (both Brown Swiss) as well as g.37183634T>C (pooled data) differed significantly on a 10% significance level. Only the most informative sequence variants (g.37253224C>T, g.37254017G>T and g.37254398G>A) located in intron 2 and exon 3 were used to construct haplotypes [inferred by the Partition-Ligation-Expectation-Maximization Algorithm within the software HAPANALYZER (http://hap.ngri.go.kr/) (Qin *et al.* 2002)] to estimate possible associations. HAPANALYZER includes a Yates' continuity correction to improve the approximation (Table 2b). In total, six haplotypes were deduced, but only haplotypes with a frequency of at least 0.05 in the DNA repository (data not shown in Table 2b) were taken for statistical analyses, i.e. CGG (0.77), TGG (0.09), and TTG (0.07). Significant associations ($P = 0.05$) between BSE-affected and healthy cows are reported for TGG in Brown Swiss and also highly significant distributions exist for haplotype TTG both in the Brown Swiss sampling and the pooled data. The results indicate a putative breed-specific association between *CHST8* gene sequence variants and BSE resistance/susceptibility in Brown Swiss. Allele, genotype and haplotype estimates as well as the absolute numbers of observations strongly support an association of sequence variant g.37254017G>T with the development of the disease. Allele T seems hereby to be the protective sequence variant. BSE is, however, an infectious disease, which is triggered after exposure to the pathogen (Wilesmith *et al.* 1988). A possible lack of exposure or individual-specific incubation times must therefore be considered and the data have to be interpreted accordingly. Nevertheless, here and by others – several candidate genes for BSE resistance/susceptibility have been investigated and associations

Table 2b *CHST8* haplotype frequencies.

	Breed											
	Holstein			Fleckvieh			Brown Swiss			Pooled samples		
Haplotype	BSE-affected	Healthy	*P*	BSE-affected	Healthy	*P*	BSE-affected	Healthy	*P*	BSE-affected	Healthy	*P*
CGG	0.72	0.73	0.98	0.85	0.79	0.16	0.78	0.75	0.58	0.80	0.76	0.25
	52	183		94	301		75	251		221	735	
TGG	0.06	0.03	0.27	0.12	0.14	0.64	0.14	0.06	0.03[2]	0.11	0.08	0.28
	4	7		13	54		13	21		30	82	
TTG	0.01	0.09	0.05	0.03	0.05	0.43	0.00	0.14	0.0002[3]	0.01	0.09	0.0001[3]
	1	23		3	19		0	447		4	89	
Residual	0.21	0.15	N/A	0.00	0.02	N/A	0.08	0.05	N/A	0.08	0.07	N/A
	15	37		0	8		8	17		23	62	
Total[1]	1.00	1.00	N/A	1.00	1.00	N/A	1.00	1.00	N/A	1.00	1.00	N/A
	72	250		110	382		96	336		278	968	

Values are given as frequency and number of observations.

N/A, not calculated.

[1]Number of haplotypes is double the number of observations.

[2]Significance level of 5%.

[3]Significance level of 1%.

between sequence variations and BSE status have been reported in several breeds including Brown Swiss (Sander *et al.* 2004; Juling *et al.* 2006; Haase *et al.* 2007). In addition, studies by others propose an increased susceptibility of Brown Swiss cattle towards BSE in terms of an increased prevalence and a lower incubation time (Geldermann *et al.* 2006; Sauter-Louis *et al.* 2006). Our data support this hypothesis, as the mean age of outbreak of the disease was the lowest in Brown Swiss, at 51.2 months, compared with the mean age of German Fleckvieh (71.5 months) and German Holstein (77.5 months).

Acknowledgements

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Primer pairs for *CHST8,* product size, nucleotide positions, annealing temperature (T_A).

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Vortragstagung der DGfZ und GfT am 21./22. September in Berlin.

Positional and functional candidate genes for BSE susceptibility

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Introduction

Since the advent of the BSE crisis potential genetical determinants for susceptibility towards the disease have controversially been discussed. Contrary to the well established association between polymorphisms in the ovine or caprine prion protein gene and TSE susceptibility (Baylis and Goldmann 2004) no such associations have been found in cattle although several polymorphisms in the orthologous gene have been described (e.g. Heaton et al. 2003). Only a weak - but, however, not significant association or correlation has been reported between variations of the so-called octapeptid-repeat in the prion protein gene and BSE susceptibility (Neibergs et al. 1994). Recently, first hints have been found that there are significant associations between ins/del polymorphisms in the bovine prion protein promoter and the occurrence of BSE (Sander et al. 2004; Seabury et al. 2004). Parallel to investigations of the bovine prion protein gene, there are several genome-wide DNA marker scans under way and significant QTL regions for susceptibility towards BSE are already postulated (Sanchez-Hernandez et al. 2002; Zhang et al. 2004). In addition to the bovine QTL studies, several studies in mice propose associations between chromosomal regions and different parameters of TSE susceptibility (e.g. Lloyd et al. 2001; Manolakou et al. 2001).

Intensive in-silico analysis of the QTL studies reveal several positional and functional candidate genes. The purpose of our project is the in-depth molecular analysis of such genes located in the respective QTL intervals.

Materials and Methods

Animals: DNA samples of 98 phenotypically BSE unaffected individuals belonging to divergent bovine breeds (Limousin, Schwarzbunte, Rotbunte, Blonde D'Aquitaine,

Fleckvieh, Scottish Highlands, Galloway, Charolais, Aubrac, Shorthorn, and Braunvieh) as well as DNA samples of 27 BSE affected individuals of different genetically origin are included in the analysis.

Amplification, sequencing and detection of polymorphisms: cDNA or genomic DNA information are used to develop specific primers for the amplification of promoter and coding regions of the candidate genes. Prior to sequencing, PCR products were purified with the QIAquick PCR purification kit and subcloned into the pGEM-T Easy TA-cloning vector (Promega, Mannheim). Alternatively, PCR products were directly sequenced using sequence specific oligonucleotides. All sequencing reactions were done on an ABI Prism 3100 capillary analyzer (ABI, Weiterstadt). Editing of sequences was performed with the SEQUENCHER software (Gene Codes, Ann Arbor) and subjected to BLAST analysis.

Results and Discussion

The coated-vesicle membrane protein 24 (RNP24) encoding for the coated vesicle protein involed in vesicular transport processes was comparatively sequenced using the total of 125 individuals (27 BSE affected animals and 98 unaffected animals). Based on the GenBank entries (gi/53490052 and gi/53463004) four primer combinations were designed that amplified the exonic regions plus additional 5' and 3' regions (estimated fragment lengths of 486 bp for exon 1, 340 bp for exon 2, 260 bp for exon 3, and 464 bp for exon 4). One SNP (single nucleotide polymorphism) was detected in exon 2 at position 153 (C>T). The frequencies of the genotype variants was 35% for CC and 16 % for TT, and for heterozygote's CT was 49 %. SNP C153T is a silent mutation and present in both BSE affected animals and in individuals of the unaffected control group. We managed to establish an EcoRV-PCR-RFLP enabling us to quickly genotype a large amount of individuals. Allele C is defined by the uncut 360 bp long PCR amplicon, whereas in case of allele T two fragments of 234 bp and 106 bp were detected.

Several other candidate genes are currently comparatively screened for SNPs and convenient test assays are developed. On the short run, the primary goal is to include the SNPs into the ongoing QTL studies to test their associations with the BSE phenotype.

Later in vivo and in vitro studies in appropriate test systems will follow to estimate the effects of the variants on BSE susceptibility.

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18.2 ANALYSIS OF DNA SEQUENCE VARIANTS IN CANDIDATE GENES IN NORMAL AND BOVINE SPONGIFORM ENCEPHALOPATHY CATTLE

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Problem: With the occurrence of the BSE crisis, several studies have been started to evaluate sequence variabilities in the prion protein gene (PRNP) to assess their possible associations with BSE. In contrast to sheep, goat and humans no functional polymorphisms in PRNP leading to a susceptibility/resistance towards transmissible spongiform encephalopathies (TSE) have been reported in cattle. Recently, however, a promising association between polymorphisms in the regulatory region of PRNP and the disease have been reported. In parallel to these investigations, several genome-wide DNA marker scans have been performed and QTL regions that are significantly associated with BSE susceptibility/resistance (BTA 5 and 17) were detected. Aim Our project was focused on the molecular analysis of functional and positional candidate genes located in the bovine QTL region on BTA 17q23-q24. This region harbors marker INRA025, which is significantly associated with BS E susceptibility/resistance.

Materials and methods: DNA from 84 BSE cattle and 284 non-diseased control cattle (Holstein, Fleckvieh, and Braunvieh) were genotyped for mutations in the candidate genes coated vesicle membrane protein (RNP24), Proteasome 26S subunit, non-ATPase, 9 (PSMD9), and phosphatidylinositol transfer protein, membrane-associated 2 (PITPNM2). Prior to that, discovery of polymorphisms was done by heteroduplex cleaving (Tilling).Results: SNPs were detected in all three genes, and allele frequencies were calculated for each SNP stratified according to disease. A single silent SNP was detected in exon 2 (C153T) of RNP24. No significant association between this SNP and BSE susceptibility/resistance was found (Chi2, p is equal to 0.5). Four SNPs were detected in PSMD9 (one SNP in intron 2, two SNPs in exon 3, and one in intron 3). The exonic SNPs (C184T and C193T) are silent, whereas SNP C184T is significantly associated with BSE susceptibility/resistance (Chi2, p is less than or equal to 0.01). The significance we found also in SNP in intron 2(Chi2,p is less or equal to 0.01). A total of 12 SNPs was identified in PITNM2 (7 exonic, 3 intronic and 2 SNPs in the promoter region). The SNP in exon 4 (G32A) causes an amino acid exchange (R to Q). The SNP C113T in intron 9 revealed a significant association with BSE (Chi2, p is equal to 0.01) and the SNP in exon 17 (C90T) also reval signifcance (Chi2, p is equal to 0.000).

Conculsions: A total of 17 SNPs have been characterized in three candidate genes and their association with BSE susceptibility/resistance was estimated. Chi-square calculations revealed significant associations between four SNPs and BSE. After analysis of further genes located in the targeted QTL region, haplotypes will be constructed to assess their possible effects.

Vortragstagung der DGfZ und GfT am 26./27. September 2007 in Hohenheim

Genetic analysis of candidate genes in normal and BSE cattle

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Introduction

With the occurrence of the BSE crisis, several studies have been started to evaluate sequence variabilities in the prion protein gene (*PRNP*) to assess their possible associations with BSE. In contrast to sheep, goat and humans no functional polymorphisms in *PRNP* leading to a susceptibility/resistance towards transmissible spongiform encephalopathies (TSE) have been reported in cattle (Baylis and Goldman 2004, Tranulis 2002), although several polymorphisms in the orthologous gene have been descreibed (Heaton et al 2003, Sander et al. 2004). Recently, however, a promising association between polymorphisms in the regulatory region of PRNP and the disease have been reported (Sander et al. 2005, Juling et al. 2006, Kashkevich et al. 2007). In parallel to these investigations, several genome-wide DNA marker scans have been performed and QTL regions that are significantly associated with BSE susceptibility/resistance (BTA 5 and 17) were detected (Sanchez-Hernandez et al. 2002, Zhang et al. 2004).
Our project was focused on the molecular analysis of functional and positional candidate genes located in the bovine QTL region on BTA 17q23-q24. This region harbors marker INRA025, which is significantly associated with BSE susceptibility/resistance.

Materials and Methods

Animals: DNA from 139 BSE cattle and 294 non-diseased control cattle (Holstein, Fleckvieh, and Braunvieh) were genotyped for mutations in the candidate genes *coated vesicle membrane protein* (*RNP24), Proteasome 26S subunit, non-ATPase, 9 (PSMD9)*, and *phosphatidylinositol*

transfer protein, membrane-associated 2 (PITPNM2). Prior to that discovery of polymorphisms was done by heteroduplex cleaving (Tilling).

Table 1 Number of animals.

Breeds	Non-BSE	BSE	Total
Holstein	125	36	161
Fleckvieh	121	55	176
Braunvieh	48	48	96
Total	294	139	433

Results and Discussion

SNPs were detected in all three genes, and allele frequencies were calculated for each SNP stratified according to disease. A single silent SNP was detected in exon 2 (C153T) of *RNP24*. No significant association between this SNP and BSE susceptibility/resistance was found (Chi2, p is less than or equal to 0.5). Four SNPs were detected in *PSMD9* (one SNP in intron 2, two SNPs in exon 3, and one in intron 3). The exonic SNPs (C184T and C193T) are silent, whereas SNP C184T is significantly associated with BSE susceptibility/resistance (Chi2, p is less than or equal to 0.01). A total of 15 SNPs was identified in *PITNM2* (5 in 5´upstream region, 7 exonic, and 3 intronic). The SNP in exon 4 (G32A) causes an amino acid exchange (R to Q). SNP C113T in intron 9 and SNP in exon 17 C101T revealed a significant association with BSE (χ^2, p is less than or equal to 0.002) .

Table 2 Number oand locations of SNPs

	Number of SNPs		
Gene	5´upstream	Exonic	Intronic
PITPNM2	5	7	3
PSMD9	-	2	2
RNP24	-	1	-
Total	5	10	5

A total of 20 SNPs have been characterized in three candidate genes and their association with BSE susceptibility/resistance was estimated. Chi-square calculations revealed significant associations between two SNPs and BSE. After analysis of further genes located in the targeted QTL region, haplotypes will be constructed to assess their possible effects.

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Title: Analysis of DNA sequence variants in candidate genes for bovine spongiform encephalopathy (BSE) susceptibility located in a QTL region on bovine chromosome 17

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Abstract:
With the occurrence of the BSE crisis, several studies have evaluated sequence variations in the prion protein gene (*PRNP*) to assess their associations with BSE. In contrast to other mammals no functional polymorphisms in *PRNP* leading to a susceptibility/resistance towards BSE have been reported. Recently, associations between polymorphisms in the regulatory region of *PRNP* and BSE have been shown. In addition, several genome-wide DNA marker scans have been performed and quantitative trait loci (QTL) regions significantly linked to BSE susceptibility (BTA 5 and 17) were detected. We have analyzed functional and positional candidate genes located in one of these QTL regions on chromosome 17q23-q24. The region flanks several cM upstream and downstream marker *INRA025*, which showed a genome-wide significant linkage disequilibrium in BSE positive cattle. DNA from 139 BSE cattle and 484 unaffected control cattle (Holstein, Fleckvieh, and Braunvieh) were genotyped for mutations in *RNP24, PSMD9*, *PITPNM2*, and *B3GNT4*. SNPs were detected in all four genes, and allele frequencies were calculated for each SNP stratified according to disease. Different statistical analysis showed that several of the SNPs are significantly associated with BSE susceptibility/resistance. **Acknowledgments:** The study was supported by the German Research Foundation DFG (BR992/14-1,2) and German TSE platform.

Vortragstagung der DGfZ und GfT am 17./18. September 2008 in Bonn

Analysis of DNA sequence variants in candidate genes for bovine spongiform encephalopathy (BSE) susceptibility located in a QTL region on BTA17

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1 Introduction

BSE is a chronic, neurodegenerative disorder affecting the central nervous system of cattle. It was first described in 1987 (Wells et al. 1987) and confirmed to be a transmissible spongiform encephalopathy (TSE) in 1988. According to the aberrant protein agent hypothesis, a disease-specific isoform of the prion protein PrP^{Sc} interacts with normal host PrP^{C}, resulting in its conversion to PrP^{Sc} (Prusiner 1991). PrP^{Sc} is a posttranslationally modified isoform of PrP^{C}, a glycolipid-anchored, plasma membrane protein that is widely expressed on neurons and glia in central nervous system (Oesch et al. 1985; Stahl et al. 1987). Several studies have evaluated sequence variations in the prion protein gene (*PRNP*) to assess their possible associations with bovine spongiform encephalopathy (BSE). In contrast to sheep, goat and humans no functional polymorphisms in *PRNP* leading to a susceptibility/resistance towards transmissible spongiform encephalopathies have been reported in cattle (Baylis and Goldman 2004; Tranulis 2002). Recently, however, promising associations between polymorphisms in the regulatory region of *PRNP* and BSE have been reported (Sander et al. 2005; Juling et al. 2006; Kashkevich et al. 2007). Genome-wide DNA marker scans have been performed and a quantitative trait locus (QTL) region linked to BSE susceptibility has been detected around INRA025 on BTA 17q23-q24 (Zhang et al. 2004). Here, we have analyzed functional and positional candidate genes located in this QTL region of chromosome 17q23-q24.

2 Materials and Methods

DNA from 139 BSE cattle and 484 unaffected control cattle (Holstein, Fleckvieh, Brown Swiss, see Table 1) were genotyped for mutations in the coated vesicle membrane protein (*RNP24*), the phosphatidylinositol transfer protein membrane-associated 2 (*PITPNM2*), the proteasome (prosome, macropain) 26S subunit, non-ATPase 9 (*PSMD9*) and the UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 4 (*B3GNT4*). Genotyping was done with direct sequencing, RFLP or melting curve analysis. PCR products were bi-directionally sequenced using the Big Dye Terminator (v 3.1) cycle sequencing kit. All sequencing reactions were performed on an ABI PRISM® 3100 DNA analyzer. The Fisher´s exact probability test with contingency table 2x3 and odds ratios were calculated for the identification of association between SNP and phenotypes.

Table 1: Number of samples per breed and status of affection

Breeds	Non-BSE	BSE	Total
Holstein (HO)	125	36	161
Fleckvieh (FV)	191	55	246
Brown Swiss (BS)	168	48	216
Total	484	139	623

3 Results and Discussion

We have analyzed functional and positional candidate genes located in the BSE QTL region of chromosome 17q23-q24 (Figure 1). DNAs from case and control cattle (HO, FV, BS) were genotyped for mutations in *RNP24, PITPNM2*, *PSMD9*, and *B3GNT4*. The screen for SNPs included all exons, exon-intron splice junctions, and 1 to 2 kb of the 5´-UTR for each gene. Twenty-four polymorphisms were identified in *RNP24*, *PITPNM2*, *PSMD9* and *B3GNT4* (Table 2 and Table 3). Only one polymorphism was non synonymous (SNP in *B3GNT4*: Arg to His), all others were synonymous. The largest differences of SNPs in BSE animals and the control group animals were observed in BS. Nine of the twenty-four SNPs showed high or moderate significance (Table 2). Two significant SNPs were found in *RNP24*, seven in *PITPNM2,* but none were detected in *PSMD9* and *B3GNT4*. FV showed four SNPs significantly associated with BSE and one moderately significant SNP in four genes. The lowest number of SNPs was detected in HO, with three SNPs in *PITPNM2* and *PSMD9.* Our study shed light on positional and functional candidate genes on bovine chromosome 17q23-

24. Most of the SNPs found in the analysed genes and their correlation with resistance/susceptibility to BSE were breed specific.

Table 2: Odds ratio (OR) and p values (Fisher's exact test) for each SNP in the respective breed

Breed	HO		FV		BS	
Genes	OR	p value	OR	p value	OR	p value
RNP24						
1. 5′-UTR	**2.07**	0.17	**2.43**	**0.01**	**2.39**	0.05
2. 5′-UTR	0.27	0.29	0.16	**0.001**	0.41	**0.0003**
3. 5′-UTR	1.47	1.00	**1.71**	0.29	**3.25**	**0.003**
4. Exon 2	**1.58**	0.05	0.95	0.16	1.08	0.09
PITPNM2						
5. Exon 13	**4.77**	0.12	**5.62**	0.08	0.56	0.61
6. Intron 11	**1.97**	0.74	**3.06**	**0.04**	0.50	1.00
7. Exon 11	**4.90**	0.09	0.78	0.14	0.50	1.00
8. Exon 8	**3.32**	**0.003**	**2.28**	0.27	**14.6**	**0.0000**
9. Exon 7	1.15	0.11	**1.61**	0.12	**1.60**	**0.001**
10. Exon 6	**1.78**	0.29	**3.30**	0.68	**1.53**	**0.01**
11. Exon 4	**3.67**	**0.006**	1.34	0.68	0.26	**0.0004**
12. Intron 1	**7.06**	0.05	**3.11**	0.14	**1.58**	**0.004**
Intergenic part						
13. 5′-UTR	**1.84**	0.35	0.55	0.42	1.45	0.05
14. 5′-UTR	**3.47**	0.09	0.92	0.82	0.67	0.58
15. 5′-UTR	1.40	0.84	**3.10**	0.28	**1.74**	0.43
16. 5′-UTR	1.45	1.00	1.30	0.53	0.50	1.00
17. 5′-UTR	0.94	0.17	1.16	0.91	**2.28**	0.06
18. 5′-UTR	0.36	0.31	0.86	1.00	0.28	**0.0001**
19. 5′-UTR	0.66	0.35	1.20	0.84	0.54	**0.005**
PSMD9						
20. Intron 2	1.17	0.32	0.46	0.10	0.88	0.40
21. Exon 3	**3.48**	**0.002**	1.44	0.64	0.50	0.41
22. Exon 3	1.32	0.71	0.28	0.22	0.00	0.22
23. Intron 3	**1.97**	0.26	0.33	**0.0009**	**2.00**	0.24
B3GNT4						
24. Exon 1	1.00	0.95	0.61	**0.02**	0.47	0.14

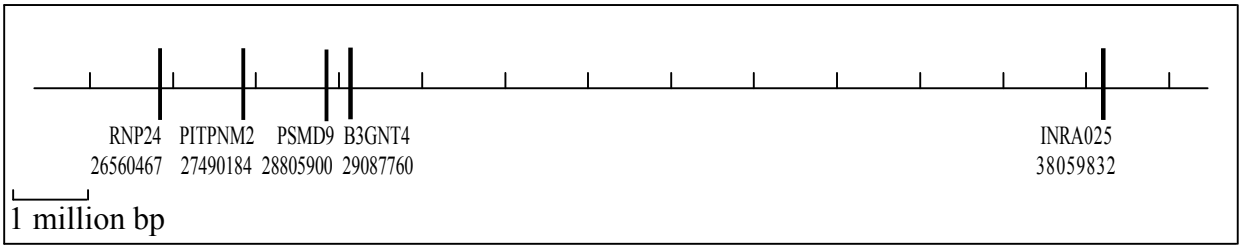

Figure 1: Physical map of genes on BTA17 and their distance from marker INRA025. The 3′-end position of genes is shown (GenBank entry NC_007315)

Table 3: Association between SNPs and breeds. '+', means that SNP is significant at the respective position for the specific breed. '-', means that SNP is not significant at the respective position for the specific breed (Numbering of SNPs is according to Table 2)

Gene	*RNP24*				*PITPNM2*															*PSMD9*				*B3GNT4*	
Breed/SNPs	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
HO	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-
FV	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
BS	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-

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