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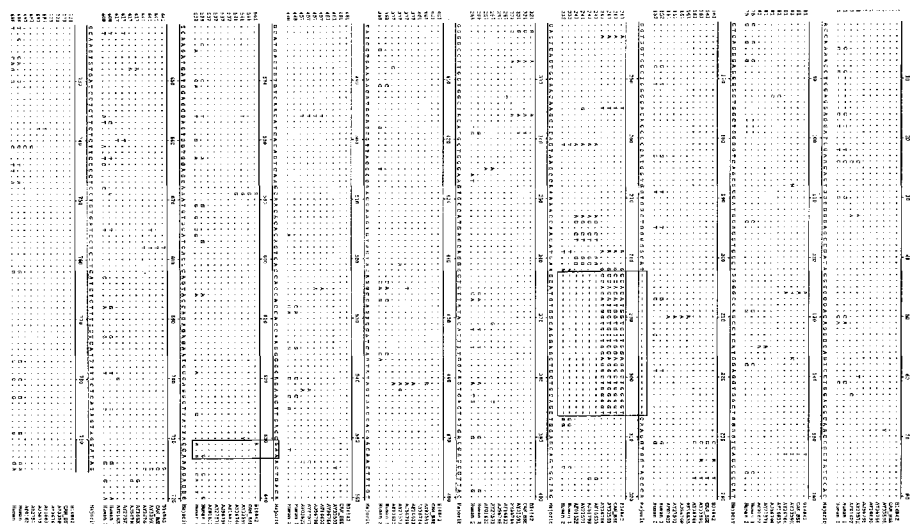
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(54) Title: NOVEL POLYMORPHISM IN BOVINE PRION PROTEIN GENE SEQUENCE



(57) Abstract: A specific, non-synonymous SNP in the Prnp gene encoding the bovine prion protein affects the susceptibility of bovine animals to bovine spongiform encephalopathy (BSE). Depending on the number of octapeptide repeat units present in the Prnp gene, the position of the SNP is either nucleotide 631 of exon 3 (codon 211) when the Prnp gene comprises six octapeptide repeat region sequences, nucleotide 607 of exon 3 (codon 203) when the Prnp gene comprises five octapeptide repeat region sequences, or nucleotide 655 of exon 3 (codon 219) when the Prnp gene comprises seven octapeptide repeat region sequences. Alleles of the bovine Prnp wherein the SNP at these positions is lysine (K) at the corresponding amino acids (i.e., 211, 203 or 219) in the bovine prion protein are all indicative of increased susceptibility to BSE in comparison to alleles which encode glutamic acid (E) at the same position. This SNP may be used as a marker for selecting bovines susceptible to BSE for disposal and/or removal from breeding, the human food and animal feed supplies.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

**NOVEL POLYMORPHISM IN BOVINE
PRION PROTEIN GENE SEQUENCE**

Field of the Invention

[0001] The invention relates to a method for detecting genetic variations in bovine which influence their susceptibility to bovine spongiform encephalopathy.

Background of the Invention

[0002] Transmissible spongiform encephalopathy (TSE) agents or prions induce fatal neurodegenerative diseases in humans and in other mammalian species. They are transmissible among their species of origin, but they can also cross the species barrier and induce infection and/or disease in other species. Human TSEs include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome, Kuru and fatal familial insomnia (36). In animals, 4 distinct TSE diseases are recognized: scrapie in sheep and goats, transmissible mink encephalopathy (TME) in mink, chronic wasting disease (CWD) in cervids, and bovine spongiform encephalopathy (BSE) in cattle. BSE was transmitted via BSE-contaminated feed to cats (feline spongiform encephalopathy, FSE) and exotic ungulates (exotic ungulate encephalopathy, EUE) and via contaminated food to humans (48, 49, Smith et al., 2004. CTMI 284: 161). BSE first emerged in the United Kingdom in 1986, and has subsequently spread to many countries, predominantly in Western Europe. These outbreaks, caused by the consumption of infected meat and bone meal containing a malformed protein, have resulted in the destruction of thousands of cattle and have caused significant economic losses.

Description of the Prior Art

[0003] Prions are proteinaceous infectious particles and are the causative agents of TSEs. They are host coded proteins that have undergone conformational changes and have biological and physicochemical characteristics that differ significantly from those of other infectious agents. For example, they are resistant to inactivation processes that are effective against conventional viruses including those that alter nucleic acid structure or function. These include ionizing and UV radiation (1) or inactivation by formalin (20). In addition, infectivity is highly susceptible to procedures that modify protein conformation. Protein denaturants are effective at reducing infectivity titers but complete inactivation requires extremely harsh conditions, such as up to 4 hours of autoclaving at 134°C or treatment with 2 N NaOH (39). In TSE disease, the normal cellular protein, PrP^C, is converted to abnormal prion protein, PrP^{Sc}. PrP^{Sc} exhibits increased beta sheet content, a change that may drive the additional changes in solubility and protease resistance (38). Unlike normal cellular protein, PrP^{Sc} is relatively insoluble in detergents, is relatively resistant to proteases (37) and is capable of causing a conformational change in additional molecules of PrP^C. The precise function of the normal PrP^C in healthy animals remains unknown. PrP^C might play a role in sleep physiology, in resistance to oxidative stress, in signal transduction and in self-renewal of hematopoietic stem cells (16, 29, 31, 53).

[0004] TSE disease involves the accumulation of PrP^{Sc} in the central nervous system (CNS) of the host, eventually leading to neurodegeneration and disease. In TSE-affected animals, PrP^C has a determinant role in the incubation time and species barrier (8). Transgenic mice lacking prion protein gene (*Prnp*) expression are not susceptible to TSE agents or prion infection,

demonstrating the key role of PrP in TSEs (8). Susceptibility to prions thus depends upon the presence of PrP^c on the cell membrane of the host; prions do not propagate in brains that lack PrP^c (6).

[0005] Widely referred to as "mad cow disease", BSE was first identified as a TSE of cattle in the mid 1980s in the U.K. and more than 180,000 positive cases have been diagnosed in the U.K. to date. BSE is a chronic degenerative disease affecting the central nervous system of cattle. Affected animals display changes in temperament, abnormal posture, incoordination and difficulty in rising, decreased milk production, and/or loss of body weight despite continued appetite (40). The average incubation period is about 4-6 years and all affected animals succumb to the disease (28). Following the onset of clinical signs, the animal's condition deteriorates until it either dies or is destroyed. This process usually takes from 2 weeks to 6 months. Most cases in Great Britain occurred in dairy cows between 3 and 6 years of age with the highest susceptibility to infection being in the first 6 months of life; adult cattle are at relatively low risk of infection (3).

[0006] Using epidemiological surveillance programs, many European and non-European countries have discovered BSE-positive animals within the last decade (17, 34). Validated diagnostic tests for BSE require brain tissue (33, 47). There are no validated *ante mortem* tests for BSE available at present. The original diagnostic test method was histopathology in which sections of brain were examined under a microscope, and the classical vacuoles and spongiform changes in specific areas of the brain would allow a diagnosis (33). In the mid-1990s, immunohistochemistry (IHC) and Western blotting were developed which allowed the detection of PrP^{Sc} in tissues (33). Both IHC and Western blot are considered confirmatory tests for BSE by

the World Organization for Animal Health-OIE (33). In the past decade, so-called "rapid tests" have been introduced commercially for BSE surveillance (33).

[0007] However despite these and other advances, the need remains for improved methods for diagnosing BSE infected animals, and particularly for detecting bovine animals having increased susceptibility to BSE.

Summary of the Invention

[0008] I have now discovered a specific, non-synonymous single nucleotide polymorphism (SNP) in the gene encoding bovine prion protein (Prnp) which might affect the susceptibility of bovine animals to bovine spongiform encephalitis (BSE). The precise location of the SNP varies with the number of octapeptide repeat sequences present in the Prnp gene. The octapeptide region polymorphisms result in encoded PrP proteins of different amino acid length. There are three known sequences of the bovine Prnp gene, those with five, six or seven of the octapeptide repeat units. Although six octapeptide repeat units are present in the Prnp gene of most bovines, a relatively small number of bovine possess Prnp genes having five octapeptide repeat units, and on rare occasions, bovine possessing Prnp genes having seven octapeptide repeat units have been observed. The SNP of this invention corresponds to position 322 nucleotides downstream from last nucleotide of the 3' end of the last octapeptide repeat region of the bovine Prnp sequence. This is equivalent to a net distance of 321 nucleotides between the last nucleotide of the last octapeptide repeat region and the SNP nucleotide (occurring in the 5' to 3' direction, i.e. in the bovine Prnp gene with 6 octapeptide repeat regions [e.g., GenBank Accession no. AJ298878, (SEQ. ID. No. 1)], the last

nucleotide at the 3' end of the 6th octapeptide repeat region is at nucleotide position 309 and the SNP mutation at nucleotide position 631) on exon 3 of the Prnp gene, wherein the nucleotide position of the SNP is measured relative to the Prnp sequence, GenBank Accession no. AJ298878, disclosed by Coulthart and coworkers (12) the contents of which are incorporated by reference herein. Thus, depending on the number of octapeptide repeat units present in the Prnp gene, the position of the SNP is either nucleotide 631 of exon 3 (codon 211) when the Prnp gene comprises six octapeptide repeat sequences, nucleotide 607 of exon 3 (codon 203) when the Prnp gene comprises five octapeptide repeat sequences, or nucleotide 655 of exon 3 (codon 219) when the Prnp gene comprises seven octapeptide repeat sequences.

[0009] Alleles of the bovine Prnp wherein the codons 203, 211 or 219, respectively, with the nucleotide Adenine (A) at the SNP position encode lysine (K) at the corresponding amino acids (*i.e.*, 211, 203 or 219) in the bovine prion protein (PrP), are all indicative of increased susceptibility to BSE in comparison to alleles of the bovine Prnp wherein the codons 203, 211 or 219, respectively, with the nucleotide Guanine (G) at the SNP position encode glutamic acid (E) at the same position. This SNP (nucleotide position 631 in Prnp gene with 6 octapeptide repeat region according to GenBank Accession no. AJ298878) may be used as a marker for selecting bovines susceptible to BSE for disposal and/or removal from breeding.

[0010] In accordance with this discovery, it is an object of this invention to determine an SNP in the Prnp gene in bovine which effects susceptibility to BSE.

[0011] It is also an object of this invention to provide methods for identifying this SNP in the Prnp gene in bovine which effects susceptibility to BSE.

[0012] In accordance with this discovery, it is an object of this invention to provide methods for identifying bovine exhibiting the trait of increased susceptibility to BSE.

[0013] Another object of this invention is to provide methods for screening bovine to select for those animals which possess alleles of the *Prnp* gene that are associated with increased susceptibility to BSE.

[0014] Other objectives and advantages of this invention will become readily apparent from the ensuing description.

Brief Description of the Drawings

[0015] Figure 1 A shows the alignment of bovine, ovine, cervid and human *Prnp* nucleotide sequences including BSE case B14842 as described in Example 1 (SEQ. ID. Nos. 2-12). All bovine alleles contained six octapeptide repeat units, while the ovine, cervid and human *PrnP* alleles contained five octapeptide repeat units. The box enclosing nucleotides 283-306 (codons 95-102) represents the additional octapeptide-repeat region in bovine (for a total of six octapeptide repeats) which is not present in ovine and cervids. The box surrounding nucleotides 631-633 represents the codon where the non-synonymous polymorphism ("G"AA/"A"AA) is located in the first position of bovine codon 211 of BSE case B14842. Standard single letter codes are used for nucleotides. Y=C or T; R=A or G; K=G or T; W=A or T.

[0016] Figure 1B shows the amino acid sequences for the corresponding prion protein encoded by the *Prnp* gene sequences depicted in Figure 1A (SEQ. ID. Nos. 13-23). Standard IUPAC single letter codes are used for amino acids. The box surrounding codon 211 represent the codon with the E211K polymorphism.

[0017] Codon numbering refers to the most common six-copy octapeptide repeat allele for *Bos Taurus* as described by Coulthart and coworkers (12). The following bovine, cervid, ovine and human GenBank entries were used for the alignments: BSE Case B14842, and GenBank accession numbers AY335912 (bovine: reports *Prnp* coding variation in a panel of 96 cattle chosen to represent most of the genetic diversity of the beef cattle breeds most commonly raised in North America), CAN_BSE = Canadian 2003 BSE case (see Coulthart et al., 2003; citation 12), AY335912 (bovine), AY367641 (bovine), AF166334 (ovine), AY275712 (white-tailed deer), and AF016227 (elk). The human sequences were derived from Figure 1 in Coulthart et al., 2003 (12). Human 1 = M13899; Human 2 = PRNPvar.

Definitions

[0018] Allele: the term coined by Bateson and Saunders (1902) for characters which are alternative to one another in Mendelian inheritance (Gk. Allelon, one another; morphe, form). Now the term allele is used for two or more alternative forms of a gene resulting in different gene products and thus different phenotypes. In a haploid set of chromosomes there is only one allele at its specific locus. Diploid organisms have 2 alleles at a given locus, and if they are homozygous for a defined gene, both alleles are identical. However, if heterozygous for a defined gene they have one normal and one mutant allele. A single allele for each gene locus is inherited separately from each parent (e.g., at a locus for eye color the allele might result in blue or brown eyes). An organism is homozygous for a gene if the alleles are identical, and heterozygous if they are different. (Birgid Schlindwein's Hypermedia Glossary of Genetic Terms).

[0019] Amplicon: a term to define the amplification product generated by the polymerase chain reaction. The physical boundaries of an amplicon extend to the base sequence at the 5' ends of each of a pair of primers (short, 18-20 oligonucleotides) in the reaction.

[0020] Centimorgan (cM): a unit to measure the recombination frequency. One centimorgan is equal to a 1% chance that a marker at one genetic locus will be separated from a marker at a second locus due to crossing over in a single generation. In human beings, 1 centimorgan is equivalent, on average, to 1 million base pairs. (Birgid Schlindwein's Hypermedia Glossary of Genetic Terms).

[0021] Crossing over: the term coined by Morgan and Cattell (1912) for the occurrence of new combinations of linked characters. With the acceptance of the chromosome theory, the term is applied to the breaking during meiosis of one maternal and one paternal chromosome, the exchange of corresponding sections of DNA, and the rejoining of the chromosomes. This process can result in an exchange of alleles between chromosomes and gives rise to new character combinations. (Birgid Schlindwein's Hypermedia Glossary of Genetic Terms).

[0022] DNA or RNA sequence: a linear series of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

[0023] Genotype: the term proposed by Johannsen (1909) for the hereditary constitution of an individual, or of particular nuclei within its cells. (Birgid Schlindwein's Hypermedia Glossary of Genetic Terms).

[0024] Identity by descent: two alleles at a single locus are *identical by descent* if there are identical copies of the same allele in some earlier generation, *i.e.*, both are copies that arose by DNA replication from the same ancestral sequence

without any intervening mutation. The animal is homozygous for this defined locus.

[0025] Identity by type: two alleles at a single locus are identical by type, (i.e. "the same") if they have the same phenotypic effects.

[0026] Locus: the position of a gene on a chromosome or other chromosome markers; also, the DNA at that position. The use of the term locus is sometimes restricted to main regions of DNA that are expressed. (Birgid Schlindwein's Hypermedia Glossary of Genetic Terms).

[0027] Marker: an identifiable physical location on a chromosome (e.g., restriction enzyme cutting site, gene, minisatellite, microsatellite) whose inheritance can be monitored. Markers can be expressed regions of DNA (genes) or some segment of DNA with no known coding function but whose pattern of inheritance can be determined. (Birgid Schlindwein's Hypermedia Glossary of Genetic Terms).

[0028] Nucleic acid: a deoxyribonucleotide or ribonucleotide polymer in either single-or double-stranded form, including known analogs of natural nucleotides unless otherwise indicated.

[0029] Nucleotide: a monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is a nucleoside. The base characterizes the nucleotide. The four DNA bases are adenine ("A"), guanine ("G"), cytosine ("C") and thymine ("T"). The four RNA bases are A, G, C and uracil ("U").

[0030] Oligonucleotide: a single-stranded nucleic acid ranging in length from 2 to about 500 bases, usually 2-100 bases.

[0031] Phenotype: the term coined by Johannsen (1909) for the appearance (Gk. *phainein*, to appear) of an organism with respect to a particular character or group of characters (physical, biochemical, and physiologic), as a result of the interaction of its genotype and its environment. Often used to define the consequences of a particular mutation. (Birgid Schlindwein's Hypermedia Glossary of Genetic Terms).

[0032] Polymorphic marker or site: the locus at which divergence occurs. Preferred markers have at least two alleles, each occurring at frequency of greater than 1%, and more preferably greater than 10% or 20% of a selected population. A polymorphic locus may be as small as one base pair. Polymorphic markers include restriction fragment length polymorphisms, variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and insertion elements such as Alu. The first identified allelic form is arbitrarily designated as the reference form and other allelic forms are designated as alternative or variant alleles. The allelic form occurring most frequently in a selected population is sometimes referred to as the wildtype form. Diploid organisms may be homozygous or heterozygous for allelic forms. A diallelic polymorphism has two forms. A triallelic polymorphism has three forms (U.S. Patent No. 6,368,799).

[0033] Probe: a DNA fragment or an oligonucleotide capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, by hybridization or complementary base pairing, usually through hydrogen bond formation. Oligonucleotides probes are often 10-50 or 15-30 bases long. An oligonucleotide probe may include natural (*i.e.* A, G, C, or T) or modified bases (7-deazaguanosine, inosine, etc.).

[0034] Recombination: the process by which progeny derive a combination of linked genes different from that of either parent. In higher organisms, this can occur by crossing over between their loci during meiosis. Recombination may come about through random orientation of non-homologous chromosome pairs on the meiotic spindles, from crossing-over between homologous chromosomes, from gene conversion, or by other means. (Birgid Schlindwein's Hypermedia Glossary of Genetic Terms).

[0035] Single nucleotide polymorphism (SNP): occurrence of a polymorphic site occupied by a single nucleotide, constituting the site of variation between allelic sequences. The site is usually preceded by and followed by highly conserved sequences of the allele (e.g., sequences that vary in less than 1/100 or 1/1000 members of the populations). A single nucleotide polymorphism usually arises due to substitution of one nucleotide for another at the polymorphic site.

[0036] Specific hybridization: binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions such that the probe will hybridize to its target subsequence, but not to other sequences. Stringent conditions are sequence-dependent and are different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5EC lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. Typically, stringent conditions include a salt concentration of at least about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the

temperature is at least about 30EC. for short probes (e.g., 10 to 50 nucleotides). Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. For example, conditions of 5xSSPE (750 mM NaCl, 50 mM NaPhosphate, 5 mM EDTA, pH 7.4) and a temperature of 25-30EC are suitable for allele-specific probe hybridizations. A perfectly matched probe has a sequence perfectly complementary to a particular target sequence (U.S. Patent No. 6,368,799).

[0037] Transition: the term proposed by Freese (1959) for a mutation caused by the substitution in DNA or RNA of one purine by the other, and similarly with the pyrimidines. (Birgid Schlindwein's Hypermedia Glossary of Genetic Terms).

[0038] Transversion: the term proposed by Freese (1959) for a mutation caused by the substitution of a purine for a pyrimidine, and vice versa, in DNA or RNA. (Birgid Schlindwein's Hypermedia Glossary of Genetic Terms).

Detailed Description of the Invention

[0039] The entire exon 3 (coding region for the bovine prion protein) of the bovine PrnP gene was amplified by PCR from animal B14842 and has been sequenced. The nucleotide sequence and the corresponding amino acid sequence are shown in SEQ. ID. Nos. 3 and 14.

[0040] Prion protein gene alleles containing a variety of polymorphisms have been associated with increased susceptibility to TSE in sheep and humans (28A, 32A). However, to date, polymorphisms effecting susceptibility to BSE have not been identified in exon 3, the coding region of bovine Prnp. So far, only certain polymorphisms described in the promoter region of the bovine Prnp gene have been associated with increased or reduced susceptibility to BSE (12, 23, 41). Upon Prnp gene

sequence analysis of a recently diagnosed BSE case, designated animal B14842, I have discovered a previously unknown non-synonymous polymorphism within the Prnp gene coding sequence from this animal. One Prnp gene allele of the animal contained an SNP designated E211K (GAA/AAA) at nucleotides 631-633 (codon 211) in the 6 octapeptide repeat region containing bovine Prnp gene as described by Coulthart and coworkers (12). As previously described in the art, normal bovine possess coding sequences for the Prnp gene which include six octapeptide repeat regions and guanine at position 631. The resultant 211 codon is GAA, encoding glutamic acid (E) at amino acid 211 of the prion protein. However, the BSE positive B14842 animal possessed an SNP at position 631 substituting adenine for guanine, and the Prnp gene codon 211 is AAA and consequently encodes the basic amino acid lysine (K) rather than the acidic amino acid glutamic acid (E).

[0041] It is believed that the presence of this SNP is associated with a case of genetic BSE in bovine B14842 and older animals carrying this SNP and such a SNP may increase the susceptibility of younger animals to BSE. Moreover, while a polymorphism for codon 211 has not been previously described for the bovine Prnp gene, a non-synonymous polymorphism at the corresponding codon 200 in the human Prnp gene (E200K; GAG/AAG) is well known. This E200K mutation has been shown to lead to genetic TSE's in humans (> 50% of individuals carrying the E200K polymorphism develop a human genetic TSE when advanced in age (see review by Kovacs et al., 2005; 28A), and is the most common mutation in human patients with genetic Creutzfeldt-Jakob disease (gCJD), fatal familial insomnia (FFI), and Gerstmann-Straussler-Scheinker (GSS) disease (28A). Considering that the human Prnp gene possesses only five octapeptide repeat units vs.

the typical six in the bovine (and also in the B14842 animal) Prnp gene, and that the human Prnp gene has three codon deletions in its N-terminus as compared to the bovine Prnp, the E211K SNP of the invention is homologous to the E200K SNP described for the human Prnp gene. This finding indicates that genetic BSE or gBSE could exist in cattle similar to the genetic prion diseases or gTSE described in humans (28A).

[0042] While the SNP of this invention was discovered in a bovine possessing a Prnp gene having the typical six octapeptide metal-binding repeat units, there are two other known bovine Prnp gene sequences with five and seven of the octapeptide repeats. Thus, depending on the number of octapeptide repeat units present in the Prnp gene, the position of the SNP is either nucleotide 631 of exon 3 (codon 211) when the Prnp gene comprises six octapeptide repeat sequences, nucleotide 607 of exon 3 (codon 203) when the Prnp gene comprises five octapeptide repeat sequences, or nucleotide 655 of exon 3 (codon 219) when the Prnp gene comprises seven octapeptide repeat sequences. Because the SNP exists at homologous codon positions in the bovine Prnp gene, the SNP may be described relative to the position of the last octapeptide repeat nucleotide (measured from the 5' to 3' direction) on exon 3 of the Prnp gene. In the bovine Prnp gene with 6 octapeptide repeat regions (GenBank Accession no. AJ298878), the last nucleotide at the 3' end of the 6th octapeptide repeat region is at nucleotide position 309 and the SNP mutation at nucleotide position 631 on exon 3 of the Prnp gene. The nucleotide position of the SNP is measured relative to the Prnp sequence, GenBank Accession no. AJ298878, disclosed by Coulthart and coworkers (12) the contents of which are incorporated by reference herein. In bovine possessing a Prnp gene having five, six or seven octapeptide repeat units on exon 3, the last (*i.e.*, on the 3' end) nucleotide of the last

(5th, 6th or 7th) octapeptide repeat region sequence is at nucleotide position 285, 309, and 333, respectively. Consequently, the SNP of this invention corresponds to a position 322 nucleotides downstream from this nucleotide at the 3' end of the last octapeptide repeat sequence. For the purposes of this invention, it is understood that the nucleotide position of the final 3' nucleotide of the last octapeptide repeat region (5th, 6th or 7th), and thus the nucleotide position of the SNP, is measured relative to the Prnp sequence, GenBank Accession no. AJ298878, disclosed by Coulthart and coworkers (12). It is also understood that animals which are either heterozygous or homozygous for the SNP of this invention (the E211K mutation) may exhibit increased susceptibility to BSE.

[0043] This invention is also drawn to a method for determining alleles of the bovine Prnp gene encoding prion protein (the amino acid sequence of which is shown in Figure 1B) which affects the susceptibility of bovines to BSE. In accordance with this method, a sample of nucleic acids from a bovine is assayed to determine the nucleotides present at the SNP in the Prnp gene which is disclosed herein. As noted above, the position of the SNP is either nucleotide 631 of exon 3 (codon 211) when the Prnp gene comprises six octapeptide repeat region sequences, nucleotide 607 of exon 3 (codon 203) when the Prnp gene comprises five octapeptide repeat region sequences, or nucleotide 655 of exon 3 (codon 219) when the Prnp gene comprises seven octapeptide repeat region sequences.

[0044] Because the polymorphism occurs at homologous codons on the Prnp gene, the codon containing the SNP may encode either glutamic acid (E) or lysine (A) at the corresponding amino acids 203, 211 or 219 of the bovine prion protein (for prion protein encoded by Prnp genes having five, six or seven octapeptide repeat units, respectively). Specifically, the above-mentioned

SNP at positions 607, 631 or 655 may be guanine, yielding a "G"AA codon encoding glutamic acid (E), or the SNP may be adenine, yielding an "A"AA codon encoding lysine (K). It is believed that alleles of the bovine Prnp wherein the SNP at these positions encode lysine at the corresponding amino acids (i.e., 211, 203 or 219) in the bovine prion protein, are all associated with increased susceptibility to BSE in comparison to alleles which encode glutamic acid (E) at the same position (as the latter is typically present in normal, healthy bovine). This SNP may be used as a marker for selecting bovines with a higher susceptibility to BSE for disposal and/or removal from breeding.

[0045] The SNP's may be detected by assaying for the presence of the above-mentioned nucleotides in a sample of nucleic acids from a subject bovine animal at the loci of the SNP, wherein the loci correspond to nucleotide 631 of exon 3 (codon 211) when the Prnp gene comprises six octapeptide repeat sequences, nucleotide 607 of exon 3 (codon 203) when the Prnp gene comprises five octapeptide repeat sequences, or nucleotide 655 of exon 3 (codon 219) when the Prnp gene comprises seven octapeptide repeat sequences. Suitable nucleic acids for use in the assay include genomic DNA, cDNA, or RNA, as well as nucleic acids that encompass, or are encompassed by the bovine Prnp gene sequences of Figure 1 or the complement thereof, as well as bovine Prnp gene sequences which are the same as in Figure 1 except for the presence of five or seven octapeptide repeat sequences or their complement. As will be described in greater detail herein below, use of genomic DNA is preferred.

[0046] It is also envisioned that the SNP may also be detected by analysis of the encoded gene product, i.e., the amino acid sequence of prion protein in a sample obtained from the subject animal. Sample materials which may be collected

from the animal for the assay include, but are not limited to, milk, blood, tissue, cells, urine, or other biological samples from the subject such as described by Novakofski et al (32A, the contents of which are incorporated by reference herein).

[0047] The presence of the allelic forms of the above-described SNP can be determined by any of a number of diagnostic assays. These assays may use otherwise known techniques, including direct sequencing of the nucleic acids in the sample, or using probes which overlap the position of the SNP's on those nucleic acids. For example, Arnold et al. (U.S. Patent No. 6,410,231, herein incorporated by reference) is drawn to SNP detection by means of an array-based sandwich assay. Arnold et al. also makes mention of a variety of other techniques that had been previously developed for SNP detection and analysis; specifically: Sapolsky et al. (1999) U.S. Patent No. 5,858,659; Shuber (1997) U.S. Patent No. 5,633,134; Dahlberg (1998) U.S. Patent No. 5,719,028; Murigneux (1998) WO 98/30717; Shuber (1997) WO 97/10366; Murphy et al. (1998) WO 98/44157; Lander et al. (1998) WO 98/20165; Goelet et al. (1995) WO 95/12607 and Cronin et al. (1998) WO 98/30883. In addition, ligase based methods are described by Barany et al. (1997) WO 97/31256 and Chen et al. Genome Res. 1998; 8(5):549-556; mass-spectroscopy-based methods by Monforte (1998) WO 98/12355, Turano et al. (1998) WO 98/14616 and Ross et al. (1997) Anal. Chem. 15:4197-4202; PCR-based methods by Hauser, et al. (1998) Plant J. 16:117-125; exonuclease-based methods by Mundy U.S. Patent No. 4,656,127; dideoxynucleotide-based methods by Cohen et al. WO 91/02087; Genetic Bit Analysis or GBA.TM. by Goelet et al. WO 92/15712; Oligonucleotide Ligation Assays or OLAs by Landegren et al. (1988) Science 241:1077-1080 and Nickerson et al. (1990) Proc. Natl. Acad. Sci. (USA) 87:8923-8927; and primer-guided nucleotide incorporation procedures by Prezant et al. (1992) Hum.

Mutat. 1:159-164; Ugozzoli et al. (1992) GATA 9:107-112; Nyreen et al. (1993) Anal. Biochem. 208:171-175, all of which are incorporated herein by reference. Other potential assay techniques are described below. McCutchen-Maloney (U.S. Patent No. 6,340,566, herein incorporated by reference) teaches detection and quantification of SNP's, DNA sequence variations, DNA mutations, DNA damage and DNA mismatches using mutation binding proteins alone or as chimeric proteins with nucleases on solid supports. Also, Poponin (U.S. Patent No. 6,376,177, herein incorporated by reference) teaches a method and apparatus for SNP detection by means of spectroscopic analysis of hybridized nucleic acid using high density nucleic acid chips. Numerous conventional assay techniques for detecting SNP's which are also suitable for use herein are described by Aguirre et al. (U.S. Patent 6,428,958) and Rothenberg (U.S. Patent 6,355,425). The contents of each of the above-mentioned publications and patents are incorporated by reference herein.

[0048] In accordance with one preferred embodiment, the presence of the SNP is detected by PCR amplification as described in Example 1. It is envisioned that a variety of primers and PCR assays may be suitable for use in the amplification, including bovine Prnp specific primers disclosed by Heaton et al. (23), Sander et al. (41) or Coulthart et al (12), the contents of which are incorporated by reference herein. However, in a preferred embodiment, at least one of the primers is designed to hybridize to a region of the gene outside of exon 3, such as an intron (here intron 2). Although not reported in bovine to date, the recent disclosure of pseudogenes in cervidae (32A) raises the specter of their possible presence in genomes of other animals as well, including bovine. Because pseudogenes are non-functional, but heritable, genes believed to have been generated by reverse transcription of mRNA from

previous generations of the species, they may have different nucleotide sequences than the corresponding functional gene in the subject animal of interest. Consequently, if pseudogenes are present, the possibility exists that primers generated strictly from the exons of a gene of interest may in fact be selective for the pseudogene rather than the active gene. To guard against the possible presence of any such pseudogenes, at least one of the primers used herein is preferably generated to bind to a region of the gene outside of the coding sequence of the genomic DNA, such as an intron. In accordance with this embodiment, preferred primers for use herein include, but are not limited to 5'-CAT ATG ATG CTG ACA CCC TC-3' (SEQ. ID. No. 24) and 5'-AGA AGA TAA TGA AAA CAG GAA G-3' (SEQ. ID. No. 25), wherein the first primer is the intron 2-specific forward primer, and the second primer is the exon 3-specific reverse primer.

[0049] The presence of the SNP on exon 3 of the Prnp gene may also be detected by assaying for the same nucleotides described above at the loci corresponding to nucleotide 631 of exon 3 (codon 211) when the Prnp gene comprises six octapeptide repeat sequences, nucleotide 607 of exon 3 (codon 203) when the Prnp gene comprises five octapeptide repeat sequences, or nucleotide 655 of exon 3 (codon 219) when the Prnp gene comprises seven octapeptide repeat sequences, in an RNA molecule which is a transcript of a sequence encompassed by, or encompassing, the complementary strand to the bovine Prnp gene such as shown in Figure 1. Alternatively, any of the SNP's may be detected in the DNA strand complementary to the sequence shown in Figure 1 by assaying for the complementary nucleotides at the loci corresponding to position nucleotide 631 of exon 3 (codon 211) when the Prnp gene comprises six octapeptide repeat sequences, nucleotide 607 of exon 3 (codon 203) when the Prnp gene

comprises five octapeptide repeat sequences, or nucleotide 655 of exon 3 (codon 219) when the Prnp gene comprises seven octapeptide repeat sequences.

[0050] As noted above, the SNP of the bovine Prnp gene may also be detected by analysis of the prion protein product. In bovine possessing a prion protein having five, six or seven octapeptide repeat units, the last (*i.e.*, C-terminal) amino acid of the last (C-terminal) octapeptide region is at position 95, 103, and 111, respectively. Consequently, the SNP of this invention corresponds to a position 108 amino acids downstream from this amino acid at the C-terminal end of the last octapeptide repeat sequence. Again, for the purposes of this invention, it is understood that the amino acid position of the final C-terminal amino acid of the final C-terminal octapeptide region, and thus the amino acid position of the SNP, is measured relative to the prion protein sequence encoded by the Prnp sequence, GenBank Accession no. AJ298878, disclosed by Coulthart, 12. For example, the glutamic acid/lysine (E/K) amino acid substitution caused by the SNP at the above-identified positions may be identified by contacting the biological samples with immunolabelling agents, such as monoclonal or polyclonal antibodies, raised against the variant protein (*i.e.*, the protein resulting from the Prnp gene with the aforementioned glutamic acid/lysine substitutions). Such antibodies may be obtained using standard techniques and may be polyclonal or monoclonal. Polyclonal antibodies can be obtained, for example, by the methods described in Ghose *et al.* (Methods in Enzymology. Vol. 93:326-327, 1983). A prion protein polypeptide, or an antigenic fragment thereof, is used as an immunogen to stimulate the production of prion protein reactive polyclonal antibodies in the antisera of animals such as rabbits, goats, sheep, rodents and the like. Anti-prion protein

antibodies specific for Prnp gene products are raised by immunizing animals with a polypeptide spanning site of the variation (i.e., amino acids 203, 211 and/or 219). Monoclonal antibodies may be obtained by the process described by Milstein and Kohler (1975. Nature. 256:495-497) or as modified by Gerhard (Monoclonal Antibodies. Plenum Press. 1980. pages 370-371). Hybridomas are screened to identify those producing antibodies that are highly specific for the selected prion protein immunogen, which is characteristic of increased or decreased susceptibility to BSE, i.e. specific for the E or K allele of the bovine Prnp gene.

[0051] Antibody binding may also be detected using known methods. For example, an ELISA assay utilizing a substrate (e.g., a plastic dish) coated with antigen comprising a bovine-derived biological sample containing the Prnp gene product. An antibody preparation specific for a known Prnp gene product is added to the well, whereupon the antibody will bind or fail to bind to the sample in the well. Non-binding material is washed away and a marker enzyme (e.g., horse radish peroxidase or alkaline phosphatase, coupled to a second antibody directed against the antigen-specific primary antibody) is added in excess and the nonadherent material is washed away. An enzyme substrate is added to the well and the enzyme catalyzed conversion is monitored as indicative of presence of the variant.

[0052] The SNP in the bovine Prnp gene of this invention may be used as a marker for identifying bovine animals having increased or reduced susceptibility to BSE. In a preferred embodiment, the SNP is used as a marker to select for cattle having the alleles associated with increased susceptibility to BSE (encoding lysine), and such animals would not be selected for breeding, may be prevented from use in the preparation of

animal feed or human food products, and/or may be destroyed. In contrast, bovine possessing the SNP encoding glutamic acid may be selected for use in breeding programs to produce progeny which will also exhibit reduced susceptibility to BSE. While it is envisioned that the invention may be practiced with any species of *Bovidae*, and particularly any species of the genus *Bos*, it is preferably practiced with *Bos taurus* and *Bos indicus*, and particularly bulls, cows or calves.

[0053] The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention which is defined by the claims.

Example 1

[0054] This example describes the identification and characterization of a recently diagnosed BSE case, bovine animal no. B14842. Western Blot analyses by the USDA-Agricultural Research Service-National Animal Disease Center confirmed that this animal was BSE positive.

Material and Methods

[0055] Animals and tissues. Brainstem tissue samples were taken from a bovine, animal no. B14842, suspected to be infected with BSE according to the USDA BSE surveillance plan.

[0056] Frozen samples from the medulla oblongata (obex) were available for analysis.

[0057] Western Blot Analyses. Brain homogenates from this BSE case were prepared and analyzed using the OIE-recommended Scrapie Associated Fibril (SAF)-Immunoblot method

³³(http://www.oie.int/eng/normes/mmanual/A_summry.htm) with minor modifications. This method enriches brain samples for PrP^{Sc} by ultracentrifugation prior to loading them onto a SDS-PAGE gel.

The SDS-PAGE electrophoresis conditions, subsequent transfer and immunodetection of PrP^{res} were carried out as described elsewhere (Hamir et al, 2006. Vet Pathol. 43: 118-126). Therefore, only the enrichment method will be described in more detail. Material for analysis was taken from the brainstem area and cut into small pieces with a new razor blade after removal of dura mater. A 10% (w/v) tissue homogenate in 10 mM Tris, pH 7.5, containing 5 mM MgCl₂ was prepared using a homogenizer with a disposable probe (5 times, 30 sec). The homogenate was mixed well and then again sonicated for 30 seconds on ice bath (5 -10 times).

Benzonase® was added to the mixture for a final concentration of 100 Units/ml and incubated for 1 h at 37 °C while shaking. An equal volume of 20% (w/v) N-Lauroylsarcosine^m in 10 mM Tris, pH 7.5 and 1 mM DTT was added to each homogenate, vortexed for 1 min every 10 min for a total of 30 min at room temperature. Homogenates were transferred to polyallomer tubes and centrifuged at 20,000 x g for 25 min at 10 °C. Supernatant was centrifuged again using polyallomer tubesⁿ at 200,000 x g for 55 min at 10 °C. The resultant supernatant was discarded, the pellet was resuspend in sterile, distilled H₂O (1 µl per mg tissue equivalent) and sonicated until suspended. Sample was split into two aliquots into microcentrifuge tubes and one sample was treated with PK (concentration 0.4 Units/ml) by incubation at 37 °C for 60 min with agitation while the control sample was not treated with PK. Phenylmethanesulphonyl fluoride (PMSF) was added to a final concentration of 5 mM, incubated on ice for 15 min and transferred to a new 1.5 ml ultracentrifuge tube. Volume was brought up to 500 µl with H₂O and centrifuged at 200,000 x g for 1 hr at 10 °C. Pellet was resuspended in SDS-PAGE sample buffer to at least 10 mg tissue equivalent per µl. Samples were sonicated on wet ice before loading on SDS-PAGE

gel. For both Western Blot techniques, detection was performed either on Biomax films or scanned images were obtained with a Typhoon imaging system.

[0058] DNA isolation and PCR amplification. Genomic DNA was extracted from 200 μ l of a 10% brain homogenate using the DNeasyTM tissue kit (Qiagen) according to the manufacturer's instructions. PCR was performed in a 100 μ l final reaction volume containing 0.2 pmole of forward primer (5'-CAT ATG ATG CTG ACA CCC TC -3'), 0.2 pmol of reverse primer (5'-AGA AGA TAA TGA AAA CAG GAA G -3') 1X Easy-A PCR buffer, 2.5 mM MgCl₂, 0.8 mM each deoxyribonucleotide triphosphate (dNTP Master Mix, 2.5 U of Easy-ATM high-fidelity cloning Taq DNA polymerase, and 0.4 μ g of total DNA. Amplification was performed with the following conditions: 94 °C for 5 min, followed by 30 Cycles of 94 °C for 30 sec (denaturation), 59 °C for 30 sec (annealing), and 72°C for 1 min. A final extension step at 72°C was performed for 10 minutes. Amplified DNA product was purified using a GENECLAN[®] spin kit^u and sequencing was performed using the ABI 3700 DNA sequencer with a cycle sequencing kit. The fragment was sequenced in duplicate using the original two primers and two internal primers 4142 and 9612 for a total of 8 reactions. Databases were searched using standard nucleotide-nucleotide BLAST at the National Center for Biotechnology Information Web Site) (<http://www.ncbi.nlm.nih.gov>). The database is a collection of sequences from several sources, including GenBank and Reference Sequence. The nucleotide sequence of the BSE case was aligned using both CLUSTAL V ^{24,25} and CLUSTAL W ⁴⁶ with the following GENBANK accession numbers: AY335912 (bovine), AY367641 (bovine), AF016227 (elk), AY275712 (white-tailed deer), AF166334 (ovine), and the Canadian BSE case using Lasergene version 5.07 software (DNASTAR-Madison, WI).

Results

Western Blot Analysis.

[0059] Western blot analysis of brainstem homogenate of the BSE case revealed a definite positive reaction. All three isoforms of PrP^{Sc} were definitely present at the milligram brain tissue equivalent tested.

Analysis of the *Prnp*.

[0060] In order to confirm the material from the BSE case was derived from cattle and to determine whether the BSE case of the affected animals might be associated with a spontaneous germline mutation, the full coding sequence from exon 3 of the *Prnp* was amplified and aligned with known PrP sequences from cattle, sheep and cervids. DNA was isolated from fresh brainstem material. The prion protein (PrP) alleles of animal B14842 were found to have two polymorphisms, a synonymous polymorphism Q78Q (CAA/CAG) at codon 78 (as described in Genbank submission AY335912), and a non-synonymous polymorphism E211K (GAA/AAA) at codon 211 and both alleles contained the six-copy octapeptide repeat region. A polymorphism for position 211 has not been described for the cattle prion protein gene so far, but a non-synonymous polymorphism at the same codon, designated codon 200 in the human prion protein gene (*Prnp*) has been described previously (E200K; GAG/AAG). The E200K mutation is the most common mutation in human patients with genetic Creutzfeldt-Jakob Disease (gCJD), fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker (GSS) disease (Kovacs et al., 2005, *Hum. Genet*, 118, 166-174).

[0061] It is understood that the foregoing detailed description is given merely by way of illustration and that modifications and variations may be made therein without departing from the spirit and scope of the invention.

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I claim:

1. A method for determining an allele of the gene encoding the prion protein in bovines comprising assaying a sample of nucleic acid molecules from a bovine for the presence of a single nucleotide polymorphism in the bovine Prnp gene encoding prion protein, wherein said single nucleotide polymorphism corresponds to a position 322 nucleotides downstream from the 3' end of the last nucleotide of octapeptide repeat region sequence, occurring in the 5' to 3' direction on exon 3 of said Prnp gene.
2. The method of claim 1 wherein said position is selected from the group consisting nucleotide 631 of exon 3 (codon 211) when said Prnp gene comprises six octapeptide repeat region sequences, nucleotide 607 of exon 3 (codon 203) when said Prnp gene comprises five octapeptide repeat region sequences, and nucleotide 655 of exon 3 (codon 219) when said Prnp gene comprises seven octapeptide repeat region sequences.
3. The method of claim 2 said position is nucleotide 631 of exon 3 (codon 211) and said bovine Prnp gene comprises six octapeptide repeat region sequences.
4. The method of claim 2 said position is nucleotide 607 of exon 3 (codon 203) and said bovine Prnp gene comprises five octapeptide repeat region sequences.
5. The method of claim 2 said position is nucleotide 655 of exon 3 (codon 219) and said bovine Prnp gene comprises seven octapeptide repeat region sequences.

6. The method of claim 1 wherein said nucleic acid molecules are selected from the group consisting of genomic DNA, cDNA, RNA, and combinations thereof.
7. The method of claim 1 wherein said nucleic acid molecules comprise genomic DNA.
8. The method of claim 1 wherein said nucleotide at said position is selected from adenine and guanine.
9. The method of claim 1 wherein said nucleic acid molecules comprise a DNA molecule that encompasses, or is encompassed by, the bovine Prnp sequence shown in Figure 1 or a complement thereof, and further wherein said position is nucleotide 631 (codon 211) and said bovine Prnp gene comprises six octapeptide repeat region sequences.
10. The method of claim 9 wherein said nucleotide at said position is selected from adenine and guanine.
11. The method of claim 9 wherein said nucleic acid molecules comprise genomic DNA molecules.
12. The method of claim 1 wherein said nucleic acid molecules comprise an RNA molecule that is a transcript of a sequence that encompass, or are encompassed by, the bovine Prnp sequence shown in Figure 1 or a complement thereof, and further wherein said position is nucleotide 631 (codon 211) and said bovine Prnp gene comprises six octapeptide repeat region sequences.
13. The method of claim 12 wherein said nucleotide at said position is selected from adenine and guanine.

14. The method of claim 1 wherein said bovine is *Bos Taurus* or *Bos indicus*.

15. The method of claim 14 wherein said bovine is a bull, cow or calf.

16. A method for identifying the genotypic status of a single nucleotide polymorphism in bovine comprising:

- a) obtaining a nucleic acid sample from a bovine; and
- b) assaying said sample of nucleic acids for the presence of a single nucleotide polymorphisms in the bovine Prnp gene encoding the prion protein, wherein said single nucleotide polymorphism corresponds to a position 322 nucleotides downstream from the 3' end of the last nucleotide within the last octapeptide repeat region sequence, occurring in the 5' to 3' direction on exon 3 of said Prnp gene;

further wherein said polymorphism encodes either lysine (K) or glutamic acid (E) at amino acid 211 of said bovine prion protein when said protein comprises six octapeptide repeat region sequences, at amino acid 203 of said prion protein when said protein comprises five octapeptide repeat region sequences, and amino acid 219 of said prion protein when said protein comprises seven octapeptide repeat region sequences.

17. The method of claim 16 wherein said bovine prion protein comprises six octapeptide repeat region sequences, and further comprising selecting for breeding, or for the human food and animal feed supplies those bovine wherein said polymorphism encodes glutamic acid (E) at amino acid 211 of said bovine prion protein.

18. The method of claim 16 wherein said bovine prion protein comprises six octapeptide repeat region sequences, and further comprising selecting those bovine for removal from breeding or from the human food and animal feed supplies those bovine wherein said polymorphism encodes lysine (E) at amino acid 211 of said bovine prion protein.

19. The method of claim 16 wherein said bovine prion protein comprises five octapeptide repeat region sequences, and further comprising selecting for breeding or for the human food and animal feed supplies those bovine wherein said polymorphism encodes glutamic acid (E) at amino acid 203 of said bovine prion protein.

20. The method of claim 16 wherein said bovine prion protein comprises five octapeptide repeat region sequences, and further comprising selecting those bovine for removal from breeding or from the human food and animal feed supplies those bovine wherein said polymorphism encodes lysine (K) at amino acid 203 of said bovine prion protein.

21. The method of claim 16 wherein said bovine prion protein comprises seven octapeptide repeat region sequences, and further comprising selecting for breeding or for the human food and animal feed supplies those bovine wherein said polymorphism encodes glutamic acid (E) at amino acid 219 of said bovine prion protein.

22. The method of claim 16 wherein said bovine prion protein comprises seven octapeptide repeat region sequences, and further comprising selecting those bovine for removal from breeding or

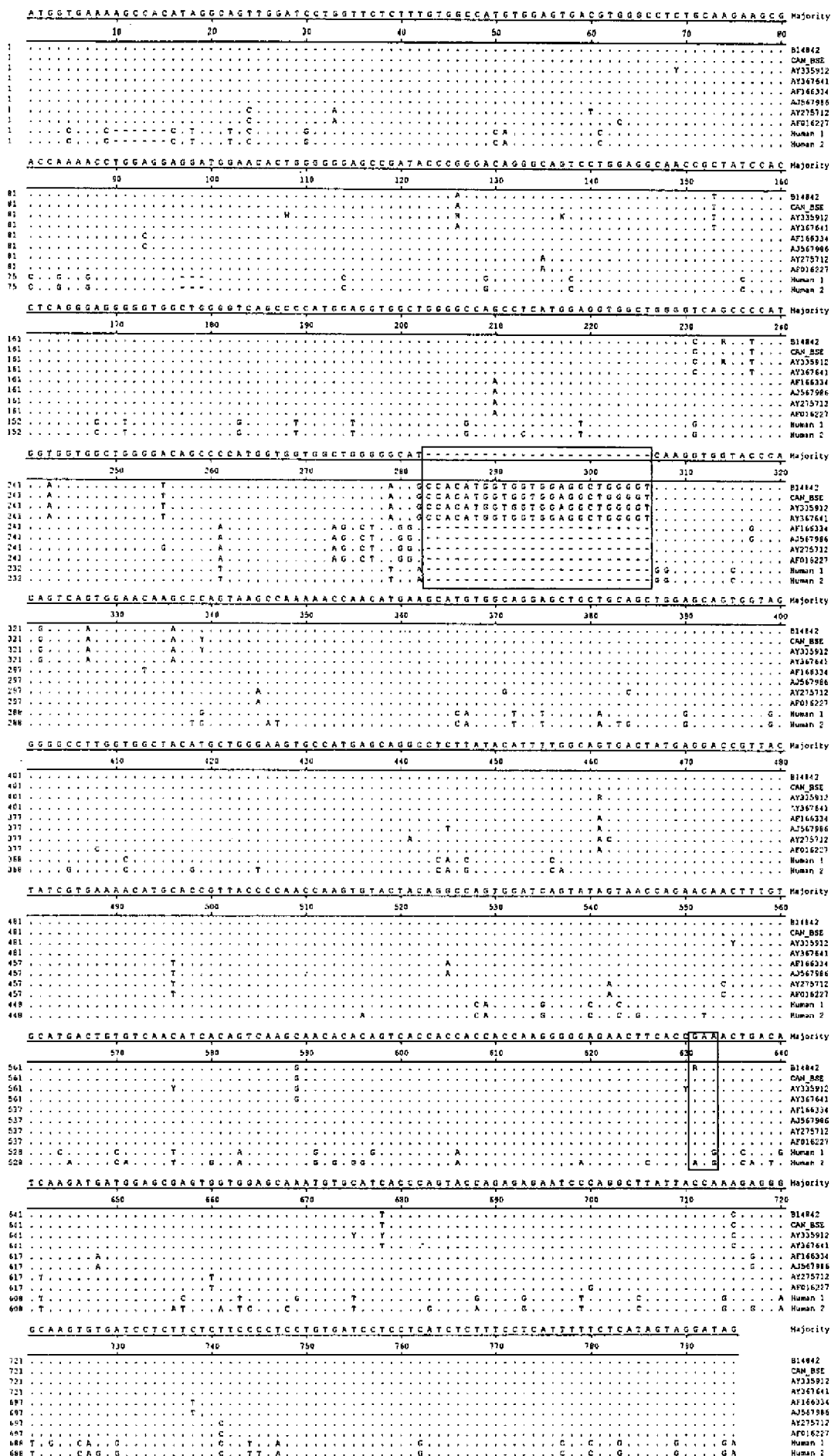
from the human food and animal feed supplies those bovine wherein said polymorphism encodes lysine (K) at amino acid 219 of said bovine prion protein.

23. A method for identifying a mutation in the prion protein of bovine, which said mutations affect susceptibility to bovine spongiform encephalopathy, comprising determining if the prion protein of a bovine comprises five, six, or seven octapeptide repeat region sequences, and further determining the presence either lysine (K) or glutamic acid (E) at amino acid 211 of said bovine prion protein when said protein comprises six octapeptide repeat region sequences, at amino acid 203 of said prion protein when said protein comprises five octapeptide repeat region sequences, and at amino acid 219 of said prion protein when said protein comprises seven octapeptide repeat region sequences.

24. The method of claim 23 wherein the presence of lysine (K) at said amino acid is indicative of increased susceptibility to bovine spongiform encephalopathy.

25. A method for identifying a mutation in the prion protein of bovine, which said mutations affect susceptibility to bovine spongiform encephalitis, comprising determining the presence either lysine (K) or glutamic acid (E) at amino acid 211 of said bovine prion protein.

26. The method of claim 25 wherein the presence of lysine (K) at amino acid 211 of said bovine prion protein is indicative of increased susceptibility to bovine spongiform encephalopathy.



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