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(54) **METHOD FOR THE DETECTION OF PRION DISEASES**

(75) Inventors: **Bram Edward Cornelis Schreuder**, Lelystad (NL); **Lucius Johannes Mattheus Van Keulen**, Bunnik (NL); **Maria Elisabeth Wilhelmina Vromans**, Lelystad (NL); **Johannes Pieter Maria Langeveld**, Harderwijk (NL); **Marinus Adrianus Smits**, Harderwijk (NL)

(73) Assignee: **Stichting Instituut Voor Dierhouderij en Diergezondheid**, Lelystad (NL)

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A61K 49/00 (2006.01)

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(58) **Field of Classification Search** 424/9.1, 424/9.2, 130.1, 139.1, 184.1; 435/7.1, 40.5; 530/387.1
See application file for complete search history.

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Primary Examiner—Rodney P Swartz
(74) *Attorney, Agent, or Firm*—TraskBritt

(57) **ABSTRACT**

The invention provides methods for the detection of prion diseases, such as scrapie of sheep, bovine spongiform encephalopathy of cattle, Creutzfeld-Jacob disease of man, whereby aberrant proteins or prion proteins are detected in tissues which can be sampled from live animals.

3 Claims, 2 Drawing Sheets

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Fig. 1A

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Multiple sequence alignment of prior proteins of various origin.

Perfectly conserved:  '*'          Disulfide-bond:    '$'
Well conserved:       '.'          N-Glycosylation:   '#'

            ---------sign-----------
SHPRP       MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGN   50
BTPRP       MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGN   50
MINKPRP     MVKSHIGSWLLVLFVATWSDIGFCKKRPKPGGGWNTGGSRYPGQGSPGGN   50
GORPRP      M--ANLGYWMLVLFVATWSDLGLCKKRPKPGG-WNTGGSRYPGQGSPGGN   47
HSPRP       M--ANLGCWMLVLFVATWSDLGLCKKRPKPGG-WNTGGSRYPGQGSPGGN   47
MAPRP       M--ANLSYWLLALFVAMWTDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGN   47
MMPRP       M--ANLGYWLLALFVTMWTDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGN   47
RRPRP       ------------------------------GGWNTGGSRYPGQGSPGGN   19+
                                          * ****************

SHPRP       RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG--------G   92
BTPRP       RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGG   100
MINKPRP     RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG--------G   92
GORPRP      RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGG---------G   88
HSPRP       RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGG---------G   88
MAPRP       RYPPQGGGTWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGG---------G   88
MMPRP       RYPPQGG-TWGQPHGGGWGQPHGGSWGQPHGGSWGQPHGG---------G   87
RRPRP       RYPPQSGGTWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGG---------G   60+
            *****.*  .***************.*******.*******         *

SHPRP       WGQGG-SHSQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPL   141
BTPRP       WGQGG-THGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPL   149
MINKPRP     WGQGGGSHGQWGKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPL   142
GORPRP      WGQGGGTHSQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPI   138
HSPRP       WGQGGGTHSQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPI   138
MAPRP       WGQGGGTHNQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPM   138
MMPRP       WGQGGGTHNQWNKPSKPKTNLKHVAGAAAAGAVVGGLGGYMLGSAMSRPM   137
RRPRP       WSQGGGTHNQWNKPSKPKTNLKHVAGAAAAGAVVGGLGGYMLGSAMSRPM   110+
            *.***  .*.**.********.**.*************************.

                                                       $ #
SHPRP       IHFGNDYEDRYYRENMYRYPNQVYYRPVDRYSNQNNFVHDCVNITVKQHT   191
BTPRP       IHFGSDYEDRYYRENMHRYPNQVYYRPVDQYSNQNNFVHDCVNITVKEHT   199
MINKPRP     IHFGNDYEDRYYRENMYRYPNQVYYKPVDQYSNQNNFVHDCVNITVKQHT   192
GORPRP      IHFGSDYEDRYYRENMHRYPNQVYYRPMDQYSNQNNFVHDCVNITIKQHT   188
HSPRP       IHFGSDYEDRYYRENMHRYPNQVYYRPMDEYSNQNNFVHDCVNITIKQHT   188
MAPRP       MHFGNDWEDRYYRENMNRYPNQVYYRPVDQYNNQNNFVHDCVNITIKQHT   188
MMPRP       IHFGNDWEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITIKQHT   187
RRPRP       LHFGNDWEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITIKQHT   160+
            .***.*.********* ********.*.*.*.*************.*.**

                  #              $
SHPRF       VTTTTKGENFTETDIKIMERVVEQMCITQYQRESQAYYQ--RGASVILFS   239
BTPRP       VTTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQ--RGASVILFS   247
MINKPRP     VTTTTKGENFTETDMKIMERVVEQMCVTQYQRESEAYYQ--RGASAILFS   240
GORPRP      VTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQ--RGSSMVLFS   236
HSPRP       VTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQ--RGSSMVLFS   236
MAPRP       VTTTTKGENFTETDIKIMERVVEQMCTTQYQKESQAYYDGRRSSA-VLFS   237
MMPRP       VTTTTKGENFTETDVKMMERVVEQMCVTQYQKESQAYYDGRRSSSTVLFS   237
RRPRP       VTTTTKGENFTETDVKMMERVVEQMCVTQYQKESQAYYDGRRSSA-VLFS   209+
            **************.*.*********.***..**.***.   *...  .***
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Fig. 1B

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---GPI-
SHPRP       SPPVILLISFLIFLIVG    256
BTPRP       SPPVILLISFLIFLIVG    264
MINKPRP     PPPVILLISLLILLIVG    257
GORPRP      SPPVILLISFLIFLIVG    253
HSPRP       SPPVILLISFLIFLIVG    253
MAPRP       SPPVILLISFLIFLMVG    254
MMPRP       SPPVILLISFLIFLIVG    254
RRPRP       SPPVILLISFLIFLIVG    226+
            .********.**.*.**
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Dictionary of the sequences which have been aligned
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[ 1]   SHPRP
DE     PROTEINSEQ OF SHPRP NT 72-839
       Size: 256 residues.
[ 2]   MINKPRP
DE     MINK TRANSL BY ALX 41-814
       Size: 257 residues.
[ 3]   GORPRP
DE     GORPRP TRANSL FROM 1-762 BY ALX
OS     GORILLA
       Size: 253 residues.
[ 4]   MAPRP
DE     MAPRP TRANSL FROM 11-733 BY ALX   AA MANLSYWLLALFVA ADDED
OS     SYRIAN GOLDEN HAMSTER
       Size: 254 residues.
[ 5]   BTPRP
DE     BOVINE PRP GENE FOR A PRION-PROTEIN.
OS     BOS TAURUS (CATTLE)
       Size: 264 residues.
[ 6]   HSPRP
DE     HOMO SAPIENS PRP GENE TRANSL FROM 50-811 BY ALX
OS     HOMO SAPIENS
       Size: 253 residues.
[ 7]   MMPRP
DE     MMPRP TRANSL FROM 107-871 BY ALX
OS     MURINE PRP
       Size: 254 residues.
[ 8]   RRPRP
DE     RAT PRION-RELATED PROTEIN (PRP) MRNA TRANSL <1? TO 678 FRAME 1
(ALX)
OS     RATTUS RATTUS (RAT)
       Size: 226 residues.
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METHOD FOR THE DETECTION OF PRION DISEASES

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation of application Ser. No. 09/155,794, filed May 20, 1999, U.S. Pat. No. 6,972,177, issued on Dec. 6, 2005, which is the national stage entry of PCT/NL97/00166, Apr. 2, 1997, published in English as WO 97/37227 on Oct. 9, 1997, the entire contents of the entirety of which is incorporated by this reference, which claims priority to EP 96200917.1, filed on Apr. 3, 1996.

TECHNICAL FIELD

The invention relates generally to biotechnology and more particularly to the field of prion diseases, also called spongiform encephalopathies (SEs), such as scrapie of sheep, bovine spongiform encephalopathy (BSE, mad-cow disease) of cattle, Creutzfeld-Jacob disease (CJD) and kuru of man. Prion diseases are transmissible via among others ingestion of or inoculation with prion proteins, can occur iatrogenically, but can also happen occasionally or on a hereditary basis without evidence of transmission.

BACKGROUND

Prion diseases are a focal point of public interest, recently fuelled by the detection of unexpected cases of CJD in teenagers and in farmers, both in Great Britain, where transmission of prion proteins from cattle to humans via meat consumption is postulated, thus indicating the transmission of BSE to humans, thereby causing CJD.

Several factors enhance public concern:

a) the nature of the causative agent, the so called prion protein, of SEs is unknown or at least controversial,

b) whatever its nature, the agent is highly resistant to procedures that eliminate other infectious agents (e.g., heating);

c) therapeutical interventions are apparently not possible, once symptoms occur;

d) SEs have an extremely long incubation period;

e) practical, sensitive and specific diagnostic methods to be used during the preclinical phase are not available. This all adds to the general feeling of "living with a time-bomb". Not only the possible presence of prion proteins in meat and neat products poses a health threat, also the possible presence of prion proteins in blood and blood products used in transfusion, the presence in pharmaceutical products of animal origin, in cosmetics of animal origin, in sera used for cell culture, in short, in an extensive array of products of animal origin, pose possible threats to human and animal health.

Until now, confirmatory diagnosis of scrapie and also other transmissible spongiform encephalopathies depended on histological examination of the brain, collected during post-mortem examination from animals or humans with clinical signs of the disease. Deposits of an aberrant or altered protein (PrPSc, prion protein) can be detected in the brain of diseased animals. This protein is very insensitive to methods, such as proteinase K digestion, that otherwise denature, lyse or remove normal proteins. The aberrant protein is considered central in the pathogenesis of prion disease. Albeit not infectious in a classical microbiological way due to the absence of specific nucleic acid, the aberrant protein itself is seen as the causal agent, and when a

susceptible animal obtains such an aberrant protein in its body (i.e. by ingestion, inoculation or via mutation of the gene of the normal version of the PrP protein, PrP^{C}) a chain reaction may start that ultimately will lead to a clinical manifestation of prion disease. The chain reaction entails the formation of more aberrant proteins formed out of the normal protein present in the animal's body. Normal and aberrant forms will interact in such a way that more aberrant forms are produced. Since the aberrant form is very resistant to proteolysis, deposits of the converted prion protein will be formed, especially in the brain and other parts of the central nervous system (CNS), giving rise to the spongiform encephalopathy and thus clinical manifestations of brain disease.

As SE-infected or affected animals and man lack a disease-specific immune response, identifying individuals before they develop clinical signs (which can take years) has been practically impossible so far. No biochemical, haematological, or gross pathological abnormalities are consistently associated with SEs. The diagnosis of SEs, therefore, depends on the recognition of clinical signs, electro-encephalography or magnetic resonance imaging techniques (both used only in human patients), or the more invasive method of taking brain-biopsies. The final diagnosis is made during autopsy, by histological examination of the brain. The neuro-pathological lesions, consisting of vacuolation (spongiform change) of the grey matter associated with gliosis and neuronal loss, are generally sufficiently characteristic. Further confirmation is possible by demonstrating scrapie associated fibrils (SAFS) in brain extracts, or by demonstrating the presence of its constituent protein, PrP^{Sc}. PrP^{Sc} is associated with the disease and is an aberrant form of the host encoded prion protein (PrP), the aberrant form is induced by a conformational change. PrP^{Sc} can be detected by immunological techniques such as Western blotting or immunohistochemistry. The latter technique is gradually becoming more and more accepted as a reliable diagnostic tool for clinical cases, in both the human and veterinary SE field.

The search for a practical preclinical diagnostic test has been and continues to be a main topic of research. This generally focuses on the detection of infectivity using a bio-assay, or the detection of the disease associated PrP^{Sc}. The bio-assay, in spite of being the most sensitive detection method, is far too cumbersome and time-consuming to ever become a practical diagnostic method: test results might become available long after the patient has passed away.

Most researchers have therefore focused on techniques to detect PrP^{Sc}. Although not all researchers agree with the statement that PrP^{Sc} is the causative agent, most, if not all, agree that the association of the presence of PrP^{Sc} and disease has been firmly established. Detection of PrP^{Sc} in tissues outside the CNS would allow sampling through less invasive methods than brain biopsies, thereby brightening prospects for a practical preclinical diagnostic technique substantially. Various tissues have been used in an attempt to develop an early detection technique: blood, urine, tissue fibroblasts, and, particularly in the animal field, lymphoid tissue. A short summary of the most promising and striking ones is given here (for an extensive review see Schreuder, 1994a, 1994b).

Blood: In human SEs, there is the often disputed experimental transmission of CJD from buffy coat samples of human CJD-patients to rodents (Muaramoto et al., 1993), but there is little or no indication that blood and specifically, buffy coat contains any infectivity in animals affected naturally with scrapie, either in clinical or in preclinical stages

(Fraser and Dickinson, 1978; Hadlow et al., 1982). Interesting results have recently been reported by Meiner et al. (1992) who detected PrP^{Sc} in peripheral tissues, both in cultured fibroblasts and in monocytes, in a group of eight CJD patients carrying the codon 200 mutation and suffering from clinical disease. These authors used both Western blotting and immunocytochemistry techniques. Their publication, however, appears to have had no follow-up and even if these results could be confirmed, the chances for a reliable blood test seem remote, at least in the case of animal SEs and given the number of negative reports from literature (reviewed in Brown, 1995).

Urine: Only once has a claim been made that infectivity in urine was demonstrated in a case of CJD, by transmitting it to mice. The same author was, however, unable to repeat this experiment (Brown, 1995). A totally different approach was reported recently (Brugere et al., 1991). Urine from scrapie affected and control animals was tested in a voltametric method by repeated capillary micro-electrolysis, which allowed discrimination of these two groups. This approach appeared promising, but, its value in detecting preclinical stages of in particular BSE could not be confirmed.

Lymphoid tissue: Lymphoid tissue has apparently not been used in the field of diagnosing human SEs, it has, however, in the veterinary field. The already classical work by Hadlow has shown that in the lymphoid tissue of naturally infected scrapie sheep, infectivity was detectable by bio-assay as early as 10–14 months of age. This was before any infectivity in the CNS was found (Hadlow et al., 1980). Western blotting has revealed the presence of PrP^{Sc} in the spleen of scrapie-infected mice (Diringer et al., 1983; Doi et al., 1988), in some cases PrP^{Sc} was detected as early as 4 weeks after experimental infection. Pooled lymph nodes from these mice also contained PrP^{Sc}. Similarly, also using Western blotting, PrP^{Sc} was detected fairly consistently in a group of naturally injected sheep showing clinical signs of scrapie, in samples from the CNS, spleen, and lymph nodes (Ikegami et al., 1991). The value of this Western blotting technique was, at least for clinical cases, confirmed by other groups. The results, however, from a group of experimentally infected sheep that were killed at 16, 18 and 21 months after inoculation but before clinical signs developed, were inconsistent and difficult to evaluate: PrP^{Sc} was detected in spleen samples of only 3 out of 12 supposedly positive animals, with lymph node samples only weak or doubtful results, but no positive results were found, illustrating the insensitivity of this technique. Therefore, using Western blotting techniques in pre-clinical diagnoses of TSE give erratic and not reliable results.

The reason for these erratic results can be found in the method to prepare the PrP^{Sc} protein (present in the affected tissues) and dissociate or separate it from the normal cellular isoform PrP protein that is also immunoreactive with the same antisera used for the Western blotting.

Ikegami et al. (1991) and Muramatsu et al. (1993) need to prepare the samples for Western blot analysis by various steps. They first enrich the samples by preparing tissue extracts containing fractions relatively enriched for both PrP^{Sc} and PrP, after which the need to remove the PrP protein with a proteinase K treatment. This procedure entails at least 10 separate incubation and separation steps in which the absolute amount of the proteins to be detected in the sample is reduced at every step. Although this protocol works very well for the diagnosis of the clinical phase of SE's, where an abundance of PrP^{Sc} is present in relation to the normal cellular isoform PrP, in the preclinical phase of TSE, the absolute amount of PrP^{Sc} is so small that it usually gets lost during the preparation.

In BSE, the situation differs from that of scrapie: on the one hand, results from mice-transmission experiments using different tissues of BSE affected cattle, may indicate that distribution of the BSE agent tissues outside the CNS is not as extensive as in the case of scrapie in sheep, on the other hand it may be that the mice used in the bio-assays are far less sensitive for BSE than for scrapie. Experimental transmission of BSE to mice only succeeded when brain material was used (Fraser et al., 1988; Fraser et al., 1990); mice inoculated with other materials, including spleen, semen, buffy coat, muscle, bone marrow and placenta remained healthy.

However, all above techniques other than bio-assays have in common that diagnosis of SEs can only be established in the clinical phase of the disease, often at autopsy only. Considering the fact that bio-assays are very slow, due to the very slow progress of the disease in the experimental animal that is used for the bio-assay as such, no methods are currently available that offer immediate diagnoses of SEs in a pre-clinical phase of the disease. Thus, although the average expert in diagnostic test development has currently a wealth of diagnostic techniques available to detect all kinds of proteins in biological samples, using monoclonal or polyclonal antisera in enzyme- or label-linked immunoassays, using techniques with or without enriching methods for the protein under study, no gold-standard is available to give guidance to the development of those diagnostic techniques that would be applicable in the case of pre-clinical diagnosis of prion disease. In other words, methods to establish sophisticated diagnostic tests are currently well known to the general expert in the field; the expert lacks, however, methods to establish the sensitivity and specificity of those sophisticated diagnostic tests due to the lack of a "gold standard".

DISCLOSURE OF THE INVENTION

We have now found a reliable and fast diagnostic method for pre-clinical diagnosis of prion diseases or SE's. The invention offers a method for pre-clinical diagnosis in sheep scrapie but also for other SEs like BSE and CJD. We used scrapie in sheep as a model to study SEs. Knowledge of the group of SEs, which includes the human forms such as CJD and Kuru, has been largely obtained from studies with scrapie. Scrapie is a progressive and fatal neurological disease of sheep and goats and is considered the "archetype" of the group of SEs and the probable cause for the BSE epidemic in the United Kingdom. The control and sanitary measures taken during the outbreak of BSE in the UK were also largely based on what was known about scrapie. Taking into account the above mentioned data of Hadlow on the presence of infectivity in various peripheral tissues, we concluded that among others lymphoid tissue would be a candidate for the development of a preclinical test based on detection of PrP^{Sc}, but also other tissues, such as but not limited to retina, alveolar macrophages or monocytes, where PrP infectivity is found.

In our hands, immune histochemistry (IHC) using the immuno-peroxidase staining method, when used on histological sections of the brains for diagnosing clinical scrapie and BSE, proved a highly reliable and practical method for detecting PrP^{Sc} (Van Keulen et al., 1995) and less cumbersome than Western blotting. Using the same IHC-technique and the same antisera, we examined a number of lymphoid tissues in a group of naturally affected, clinically-positive scrapie sheep (n=55) (Van Keulen et al., in press, see also the experimental part). We demonstrated the presence of PrP^{Sc} in the spleen, the retropharyngeal lymph node, mesenteric lymph node and the palatine tonsils, in all but one of the animals (98%). Of all examined lymph nodes, tonsils were found having the highest PrP^{Sc} deposition rate that could be detected per number of follicles: in all positive cases, more than 60% of the tonsil follicles stained positive and in 95% of these cases this was even more than 80%. To assess the applicability of this method in the pre-clinical phase of scrapie, we embarked upon a study involving sequential biopsy taking of tonsils in sheep, tonsils were chosen while the experimental availability for sequential studies is guaranteed, however, using other tissues can as well be contemplated for pre-clinical diagnosis. We have detected the scrapie associated PrP^{Sc} in tonsils of 10 month old sheep, which is at less than half-way the incubation period as the sheep under study are expected to develop scrapie when approximately 25 months old. In sheep that are expected to develop scrapie at a much later stage or stay healthy during their whole life span, we did not detect this PrP^{Sc} protein.

With regard to scrapie, future control programs could profit from these findings. Control programs in several breeds could consist of a combination of breeding programs that make use of the established linkage between PrP genotype and increased scrapie-susceptibility or -resistance, and the above described method that detects the pathognomonic presence of PrP^{Sc} in tonsils of susceptible animals in the preclinical stage of the disease.

With regard to BSE, and SEs in general, changes and adjustments of the technique used can now be made to adjust to the specific circumstances and conditions of BSE, and SE, diagnosis. Those changes can be guided by specific knowledge about homologies and heterologies in the amino acid sequences of prion proteins from different species (for a selection of known sequences FIGS. **1**A and **1**B). Also, guidance may be found in selecting specific antisera by selecting for reactivity of selected continuous or discontinuous peptide sequences of those prion proteins. First of all, the IHC-technique may be further refined for use in BSE and in peripheral lymph nodes in particular. This could require adaptations of the protocol in use for immuno-staining of brain sections. PrP^{Sc} detection in lymphoid tissues has been tried only using immuno-blotting methods and in clinical cases (Mohri et al., 1992). These results were negative, indicating a detection problem with regard to sensitivity. No serious efforts have been made to detect PrP^{Sc} in preclinical stages of BSE. The technique of taking tonsillar biopsies in live cattle is feasible and even easier than in sheep, as cattle can do with a light sedation (Xylazine (Rompun). The possibility of an early diagnosis in case of BSE could alleviate the need for certain draconical measures proposed today with regard to the cattle population in the UK.

Far reaching implications of our invention lie in the field of human SEs. Also here the applicability of the IHC technique in the preclinical phase can now be established. In literature, we did not find any reference to the examination of lymphoid tissues in this context. With tonsils being more readily accessible and with almost always access to a pathologic-anatomical laboratory, the above described technique, applied in human SEs, could contribute to an early diagnosis of suspected cases of SEs. This allows the possibility of detecting individuals harboring the disease at a moment early in the incubation period; at least considerably long before clinical signs appear, which in turn would allow certain therapeutic measures to be applied for specific groups at risk (at least interventions that delay the progression of the disease, such as the use of amphotericin B).

The present invention thus provides methods f or the detection of prion disease whereby aberrant proteins are detected in various tissues, such as but not limited to lymphoid or tonsillar tissue, which can be sampled from live animals, in particular from farm animals or humans or other mammals. The invention also provides methods that distinguish between aberrant and normal protein, by removing the normal protein with methods that proteolyse, hydrolyse or denature the normal protein, or by immunologically detecting the aberrant protein. Immunological detection entails any method currently known by the expert in diagnostic test development, all methods employing immunological detection with enzyme- or label-linked or non-linked antibodies, even Western blotting techniques, may now be developed into sensitive and specific techniques, due to the fact that a gold-standard for pre-clinical diagnosis of prion disease has now become available. These methods may also be developed into diagnostic tests or test kits comprising the necessary elements of any of the above methods. The invention further provides use of any of the above methods, tests or test kits in the diagnosis of prion disease, in disease control programs, in the selection of meat fit for consumption and in the selection of blood or blood products.

BRIEF DESCRIPTION OF THE FIGURE

FIG. **1**A is a multiple sequence alignment of prion proteins of various origins, and FIG. **1**B is a continuation of FIG. **1**B. SHPRP corresponds to SEQ ID NO:1. BTPRP corresponds to SEQ ID NO:2. MINKPRP corresponds to SEQ ID NO:3. GORPRP corresponds to SEQ ID NO:4. HSPRP corresponds to SEQ ID NO:5. MAPRP corresponds to SEQ ID NO:6. MMPRP corresponds to SEQ ID NO:7. RRPRP corresponds to SEQ ID NO:8.

DETAILED DESCRIPTION OF THE INVENTION

Experimental

Immunohistochemical detection of prion protein in lymphoid tissues of sheep with clinical cases of natural scrapie

Materials and Methods

Sheep. Sixty seven sheep with nervous disorders resembling those of a scrapie infection were purchased. Fifty-five sheep were diagnosed with scrapie by histopathological and immunohistochemical examination of the brain. (van Keulen et al., 1995). One animal suffered from both a scrapie infection and a concurrent meningo-encephalitis probably caused by Listeria monocytogenes. Scrapie-positive sheep originated from 30 different flocks. The group consisted of 54 females and one male ranging in age from 2 to 5 years and comprised eight different breeds and crossbreeds. Twelve sheep did not show any histopathological signs of a scrapie infection nor did they display any PrP^{Sc} immunostaining in the brain. Five of these sheep were diagnosed with meningo-encephalitis, one had intramyelinic edema of unknown cause, and 6 sheep showed no histopathological abnormalities. Scrapie negative sheep were all females from 10 different flocks and two different breeds and crossbreeds, ranging in age from 1 to 5 years.

Necropsy. Necropsy was performed within 36 hours after natural death or immediately after killing the animal by intravenous injection of sodium pentobarbital and exsangination. The brain was removed from each sheep for scrapie diagnosis as described previously (van Keulen et al., 1995). Samples were taken from several lymphoid tissues including spleen, palatine tonsil, superficial cervical lymph node (prescapular lymph node), subiliac lymph node (prefemoral lymph node), medial retropharyngeal lymph node, tracheobronchial lymph node, mesenteric lymph node, and ileum.

Histological and immunohistochemical procedures. Tissue samples were immediately immersed for 24 hours in periodate-lysine-paraformaldehyde fixative (PLP) containing 2% paraformaldehyde (Merck, Darmstadt, Germany). Samples were then trimmed to a maximum thickness of 2 mm and fixed for another 24 hours in freshly prepared PLP. After fixation, tissue samples were washed in water, routinely dehydrated and embedded in paraffin. Three sections of 5 μm were cut, mounted on 3-aminoalkyltriethoxysilane-coated glass slides (Sigma, St. Louis Mo., USA), dried for at least 48 hours at 60° C. and deparaffinized. The first section was stained with hematoxylin-eosin (HE). Second and third sections were immunostained with anti-peptide serum directed against the ovine prion protein and pre-immune serum respectively according to the following procedure; after 30 minutes immersion in 98% formic acid (Merck), sections were washed and autoclaved immersed in water for another 30 minutes at 121° C. in a pressure cooker. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol (Merck). Incubation at room temperature for 1 hour with anti-peptide antiserum or pre-immune serum, diluted 1:1500 in phosphate-buffered saline (pH 7.2) containing 1% bovine serum albumin (Sigma), was followed by incubation, first with biotin-conjugated goat-anti-rabbit IgG and then with streptavidin-peroxidase for 10 and 5 minutes respectively (Dakopatts, Glostrup, Denmark). As substrate we used aminoethylcarbazole (Zymed Laboratories Inc., San Francisco Calif., USA) because its red color could easily be differentiated from the yellow-brownish ceroid/lipofuscin and hemosiderin pigment which was often present in the lymphoid tissues. Between the various steps, sections were thoroughly rinsed in phosphate-buffered saline containing 0.05% Tween-20 (Merck). Sections were counterstained with Mayers hematoxylin for 30 seconds and mounted in Glycergel (Dakopatts). With every immunohistochemical staining, a section of the medulla oblongata of a confirmed scrapie-affected sheep was simultaneously stained for PrP to check correct immunostaining procedures.

Peptide synthesis and anti-peptide antisera. Five peptides with sequences derived from the ovine prion protein (PrP 94–105, 100–111, 126–143, 145–177, 223–234) were synthesized and used to raise anti-peptide antisera in rabbits following previously published procedures (van Keulen et al., 1995). Antisera were confirmed to be specific for PrP (both undigested and after proteinase K treatment) on western blots of partially purified prion protein from scrapie-affected sheep brain according to established procedures (Hilmert and Diringer, 1984). Pre-immune sera were collected before immunization and served as negative control sera.

The sera used have advantages which are based on a mixture of empirical, theoretical and analytical values the combination of which makes them invaluable in the diagnostic application. The preparation of the sera has been described in a publication of van Keulen et al. 1995. The immunochemical properties of these sera are partly published. The specific sera used in this example have been designed for scrapie diagnosis, however, guidance can be found in the below given indications for the development of sera that are applicable in diagnosis of the other SEs, provided one selects the sequences as corresponding to the species specific sequence of the prion protein. When needed one may select other animals than rabbits to generate the specific sera.

1: the sera have been induced with synthetic peptides with sequences based on the sequence of PrP protein.

2: the sera have been induced in rabbits.

3: the peptides sequences have such differences with the rabbit PrP sequence that they induced not only antibodies which recognized these peptides but also the authentic PrP protein.

4: the peptides used for immunization are kept short (12 mers); this shortness is supposed to have a critical role in the high specificity for the scrapie forms of PrP and thus in the binding in the tissue sections even after harsh denaturing and degradative treatments.

5: the sequences used for immunization and yielding the specific scrapie PrP staining were selected from the protease K resistant domain of the PrP^{Sc}.

6: the sera of use in the diagnostic IHC are also well reactive in other immunochemical tests such as: Western blotting of both PrPc and PrP^{Sc}, ELISA with PrP protein, PEPSCAN with 12 mer peptides with overlapping sequences of sheep PrP.

7: the peptides selected have properties (hydrophilicity, flexibility, surface occurrence) which are—when used for immunization—advantageous for eliciting antibodies with binding to the antigen on which the sequences have been based.

8: the antisera elicited show the right specificity when analyzed in PEPSCAN with 12 mer peptides. The addition of a foreign dimeric glycine at either the N-terminus or the C-terminus of these peptides does not decrease the specificity of the peptides but more probably does make the immunization more effective, supposedly because it makes the peptides stand out farther away from the carrier protein and makes them more flexible on the carrier protein properties which are important determinants in antigenicity.

9: the sequences selected for peptide synthesis and immunization represent domains which have a low tendency to form secondary structure (a-helix or β-sheet) and are not part of the four regions described in the literature a being able to form β-sheet as synthetic peptides.

Results

Immunohistochemical Testing of Antipeptide Antisera.

An identical and distinct immunolabelling pattern was detected with all anti-peptide antisera in the lymphoid tissues of scrapie-affected sheep. Because the five antisera were directed against different epitopes of the PrP protein, cross-reactivity of the anti-peptide antisera with another protein can be excluded. We therefore classified the immunolabelled protein as PrP. We further defined this PrP as scrapie-associated PrP (PrP^{Sc}), because no PrP immunoreactivity was seen in any of the lymphoid tissues of scrapie-negative sheep. Replacing the anti-peptide antisera with pre-immune sera did not result in any immunolabelling.

Localization of PrP^{Sc} in the Lymphoid Tissues.

PrP^{Sc} was located within the primary and secondary lymphoid follicles of the spleen, palatine tonsil, lymph nodes, and solitary follicles or Peyer's patches of the ileum. The PrP^{Sc} immunolabelling pattern consisted of a reticular network in the center of the lymphoid follicle which varied in staining intensity. Apart from this network, fine to coarse granules of PrP^{Sc} were seen in the cytoplasm of nonlymphoid cells within the follicle. Several of these cells were identified as macrophages because of the simultaneous presence of ceroid/lipofuscin pigment in their cytoplasm. No immunolabelling of the B lymphocytes in the lymphoid follicle was seen.

Occasionally, additional immunolabelling was found in specific cells and regions of the lymphoid tissues. In the spleen, individual cells in the periarterial lymphatic sheath (PALS) and the marginal zone surrounding the splenic corpuscles contained granules of PrP^{Sc} sometimes combined with ceroid/lipofuscin pigment within the cytoplasm. No PrP^{Sc} was seen in the red pulp of the spleen. In the palatine tonsil and ileum, branches or granules of PrP^{Sc} were found interspersed between the lymphocytes of the dome area between the follicles and the crypt epithelium. In the lymph nodes, granules of PrP^{Sc} were seen between the lymphocytes of the paracortex.

Distribution of PrP^{Sc} in Lymphoid Tissues.

PrP^{Sc} was detected in 54 (98%) of the 55 scrapie-affected sheep in the spleen, tonsil, retropharyngeal lymph node and mesenteric lymph node. In the tracheobronchial, prefemoral and prescapular lymph node, PrP^{Sc} was seen in a slightly lower percentage of the sheep. PrP^{Sc} was found in solitary lymphoid follicles or Peyer's patches of the ileum in 24 (89%) of the 27 sheep in which lymphoid tissue was present in the sections of the ileum. In only 1 of the 55 scrapie-affected sheep, PrP^{Sc} could not be detected in any of the lymphoid tissues.

The percentage of lymphoid follicles that contained PrP^{Sc} was estimated for the sections of the spleen, tonsil and lymph nodes. In the palatine tonsil of 98% of the scrapie-affected sheep, over 60% of the lymphoid follicles contained PrP^{Sc}. In the tonsils of 93% of the sheep with scrapie the percentage of PrP^{Sc}-positive lymphoid follicles even exceeded 80%. In the spleen or lymph nodes, PrP^{Sc} accumulation in more than 60% of the lymphoid follicles was only present in less than 30% of the sheep.

Immunohistochemical detection of prion protein in lymphoid tissues of sheep with pre-clinical cases of natural scrapie.

Material and Methods

Sheep.

We selected a group of 10 purposely bred lambs, six of them homozygous for the PrP allele with valine (V) at position 136 and glutamine (Q) at position 171. In several breeds, this PrP^{VQ} allele is significantly associated with an increased susceptibility for scrapie (Belt et al., 1995). The remaining four lambs were heterozygous and possessed one PrP^{VQ} allele and one PrP^{AR} allele (alanine at position 136 and arginine at position 171). The PrP^{AR} allele is significantly associated with increased resistance of sheep for scrapie. In a flock with natural scrapie we observed that sheep with the genotype $PrP^{VQ/VQ}$ died from scrapie at approximately 25 months of age and that the majority of the sheep with the genotype $PrP^{VQ/AR}$ were still healthy at 70 months of age. Since we expected that the $PrP^{VQ/VQ}$ sheep would almost certainly develop clinical signs of scrapie within approximately 25 months after birth and that the $PrP^{VQ/AR}$ sheep would stay healthy, we regarded these two groups of sheep as a suitable model to study changes at known stages of the incubation period. All 10 sheep-were born and raised on the same farm, in an environment where scrapie has been occurring for several years. They were kept here until six months old, when they were transferred to our Institute, to a paddock where various scrapie positive animals had spent their last days.

Sampling and Testing of Tonsils of the Live Animal

Tonsil biopsies were collected under general anaesthesia, which was achieved by intravenous application of a combination of Ketalar (Ketamine-HC1) 4 mg/kg, Xylazine (Rompun) 0.05 mg/kg and Atropine 0.1 mg/kg. We used a mouth gag, a laryngoscope, and a biopsy forceps with a head of approximately 4 mm in diameter. Tonsils in sheep are not as readily accessible as in some other species, such as man, where they often protrude into the pharyngeal lumen. In sheep, they are hidden, surrounding a small cavity. It proved, however, sufficient to take a biopsy of the edge of the entrance to this cavity, the fossa tonsillaris, thereby collecting in general sufficient material to allow examination. Some experience in the technique was obtained by collecting, just before the animals were euthanized, tonsillar biopsies from 11 sheep, among them clinically affected scrapie sheep. Histological procedures included immunostaining with specific (anti-PrP^{Sc}) anti-peptide-sera, as described above and in Van Keulen et al., 1995. From the 11 sheep, eight proved to be scrapie positive while three turned out negative, as was confirmed histologically and by IHC of brain tissue during post mortem examination. The tonsillar biopsies of all eight positive animals showed a positive immune-staining in the IHC, whereas no immuno-staining could be detected in the three negative cases.

In the actual experiment, we planned to take tonsillar biopsies sequentially, at regular intervals and starting at an age of six months. For logistic reasons this was delayed. We collected biopsies from both groups for the first time at approximately 10 months after birth, when none of the sheep showed clinical signs of scrapie. The youngest sheep were nine-and-half months, the oldest sheep was 10 months and one week.

Results

After IHC-staining we found clear, already extensive, PrP^{Sc} staining in the tonsillar biopsies of all six susceptible $PrP^{VQ/VQ}$ sheep, whereas no immune-staining was detectable in the tonsillar biopsies of any of the resistant $PrP^{VQ/AR}$ sheep. We have thus detected the scrapie associated PrP^{Sc} in tonsils of 10 months old sheep, which is at less than half—way the incubation period as they are expected to develop scrapie when approximately 25 months old. In sheep that are expected to develop scrapie at a much later stage or stay healthy during their whole life span, we did not detect this PrP^{Sc} protein. We conclude that IHC-staining and related methods provide the possibility for pre-clinical diagnosis in sheep scrapie as well as for other SEs like BSE -and CJD.

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        35                  40                  45

Gly Asn Arg Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly Gln Pro His
    50                  55                  60

Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His
65                  70                  75                  80

Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Gly Trp Gly Gln Gly
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Gly Ser His Ser Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn Met
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Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Arg Tyr Ser Asn Gln Asn
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Asn Phe Val His Asp Cys Val Asn Ile Thr Val Lys Gln His Thr Val
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Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Ile Lys Ile
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Met Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr Gln Arg Glu
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Ser Gln Ala Tyr Tyr Gln Arg Gly Ala Ser Val Ile Leu Phe Ser Ser
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Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly Ser Pro Gly
        35                  40                  45

Gly Asn Arg Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly Gln Pro His
     50                  55                  60

Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His
 65                  70                  75                  80

Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His
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            100                 105                 110

Pro Ser Lys Pro Lys Thr Asn Met Lys His Val Ala Gly Ala Ala Ala
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Tyr Arg Glu Asn Met Tyr Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro
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Val Asp Gln Tyr Ser Asn Gln Asn Asn Phe Val His Asp Cys Val Asn
            180                 185                 190

Ile Thr Val Lys Glu His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn
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Phe Thr Glu Thr Asp Ile Lys Met Met Glu Arg Val Val Glu Gln Met
    210                 215                 220

Cys Ile Thr Gln Tyr Gln Arg Glu Ser Gln Ala Tyr Tyr Gln Arg Gly
225                 230                 235                 240

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Gly Asn Arg Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly Gln Pro His
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Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His
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Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Gly Trp Gly Gln Gly
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Gly Gly Ser His Ser Gln Trp Gly Lys Pro Ser Lys Pro Lys Thr Asn
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Met Lys His Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly
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Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Leu Ile His
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Phe Gly Asn Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr Arg
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Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn Gln
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Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Val Lys Gln His Thr
            180                 185                 190

Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Met Lys
        195                 200                 205

Ile Met Glu Arg Val Val Glu Gln Met Cys Val Thr Gln Tyr Gln Arg
    210                 215                 220

Glu Ser Glu Ala Tyr Tyr Gln Arg Gly Ala Ser Ala Ile Leu Phe Ser
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Gly
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Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Gly Gly Gly Thr His
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Ser Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn Met Lys His Met
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145                 150                 155                 160

Val Tyr Tyr Arg Pro Met Asp Gln Tyr Ser Asn Gln Asn Asn Phe Val
                165                 170                 175

His Asp Cys Val Asn Ile Thr Ile Lys Gln His Thr Val Thr Thr Thr
            180                 185                 190

Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Val Lys Met Met Glu Arg
        195                 200                 205

Val Val Glu Gln Met Cys Ile Thr Gln Tyr Glu Arg Glu Ser Gln Ala
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Tyr Tyr Gln Arg Gly Ser Ser Met Val Leu Phe Ser Ser Pro Pro Val
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Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly
     50                  55                  60

Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly
 65                  70                  75                  80

Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Gly Gly Gly Thr His
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Ser Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn Met Lys His Met
            100                 105                 110

Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly Leu Gly Gly Tyr
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Met Leu Gly Ser Ala Met Ser Arg Pro Ile Ile His Phe Gly Ser Asp
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Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met His Arg Tyr Pro Asn Gln
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Val Tyr Tyr Arg Pro Met Asp Glu Tyr Ser Asn Gln Asn Asn Phe Val
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His Asp Cys Val Asn Ile Thr Ile Lys Gln His Thr Val Thr Thr Thr
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Tyr Tyr Gln Arg Gly Ser Ser Met Val Leu Phe Ser Ser Pro Pro Val
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&lt;213&gt; ORGANISM: Syrian Golden Hamster

&lt;400&gt; SEQUENCE: 6

Met Ala Asn Leu Ser Tyr Trp Leu Leu Ala Leu Phe Val Ala Met Trp
  1               5                  10                  15

Thr Asp Val Gly Leu Cys Lys Lys Arg Pro Lys Pro Gly Gly Trp Asn
             20                  25                  30

Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly Ser Pro Gly Gly Asn Arg
         35                  40                  45

Tyr Pro Pro Gln Gly Gly Gly Thr Trp Gly Gln Pro His Gly Gly Gly
     50                  55                  60

Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly
 65                  70                  75                  80

Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Gly Gly Gly Thr His
                 85                  90                  95

Asn Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn Met Lys His Met
            100                 105                 110

Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly Leu Gly Gly Tyr
        115                 120                 125

Met Leu Gly Ser Ala Met Ser Arg Pro Met Met His Phe Gly Asn Asp
    130                 135                 140

Trp Glu Asp Arg Tyr Tyr Arg Glu Asn Met Asn Arg Tyr Pro Asn Gln
145                 150                 155                 160

Val Tyr Tyr Arg Pro Val Asp Gln Tyr Asn Asn Gln Asn Asn Phe Val
                165                 170                 175

His Asp Cys Val Asn Ile Thr Ile Lys Gln His Thr Val Thr Thr Thr
            180                 185                 190

Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Ile Lys Ile Met Glu Arg
        195                 200                 205

Val Val Glu Gln Met Cys Thr Thr Gln Tyr Gln Lys Glu Ser Gln Ala
    210                 215                 220

Tyr Tyr Asp Gly Arg Arg Ser Ser Ala Val Leu Phe Ser Ser Pro Pro
225                 230                 235                 240

Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu Met Val Gly
                245                 250


&lt;210&gt; SEQ ID NO 7
&lt;211&gt; LENGTH: 254
&lt;212&gt; TYPE: PRT
&lt;213&gt; ORGANISM: Murinae gen. sp.

&lt;400&gt; SEQUENCE: 7

Met Ala Asn Leu Gly Tyr Trp Leu Leu Ala Leu Phe Val Thr Met Trp
  1               5                  10                  15
```

-continued

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Thr Asp Val Gly Leu Cys Lys Lys Arg Pro Lys Pro Gly Gly Trp Asn
            20                  25                  30

Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly Ser Pro Gly Gly Asn Arg
        35                  40                  45

Tyr Pro Pro Gln Gly Gly Thr Trp Gly Gln Pro His Gly Gly Gly Trp
    50                  55                  60

Gly Gln Pro His Gly Gly Ser Trp Gly Gln Pro His Gly Gly Ser Trp
65                  70                  75                  80

Gly Gln Pro His Gly Gly Gly Trp Gly Gln Gly Gly Gly Thr His Asn
                85                  90                  95

Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn Leu Lys His Val Ala
            100                 105                 110

Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly Leu Gly Gly Tyr Met
        115                 120                 125

Leu Gly Ser Ala Met Ser Arg Pro Met Ile His Phe Gly Asn Asp Trp
    130                 135                 140

Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr Arg Tyr Pro Asn Gln Val
145                 150                 155                 160

Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn Gln Asn Asn Phe Val His
                165                 170                 175

Asp Cys Val Asn Ile Thr Ile Lys Gln His Thr Val Thr Thr Thr Thr
            180                 185                 190

Lys Gly Glu Asn Phe Thr Glu Thr Asp Val Lys Met Met Glu Arg Val
        195                 200                 205

Val Glu Gln Met Cys Val Thr Gln Tyr Gln Lys Glu Ser Gln Ala Tyr
    210                 215                 220

Tyr Asp Gly Arg Arg Ser Ser Ser Thr Val Leu Phe Ser Ser Pro Pro
225                 230                 235                 240

Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu Ile Val Gly
                245                 250


<210> SEQ ID NO 8
<211> LENGTH: 226
<212> TYPE: PRT
<213> ORGANISM: Rattus rattus

<400> SEQUENCE: 8

Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly Ser Pro
  1               5                  10                  15

Gly Gly Asn Arg Tyr Pro Pro Gln Ser Gly Gly Thr Trp Gly Gln Pro
            20                  25                  30

His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro
        35                  40                  45

His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Ser Gln Gly
    50                  55                  60

Gly Gly Thr His Asn Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn
65                  70                  75                  80

Leu Lys His Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly
                85                  90                  95

Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Met Leu His
            100                 105                 110

Phe Gly Asn Asp Trp Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr Arg
        115                 120                 125

Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn Gln
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-continued

```
    130                 135                 140

Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln His Thr
145                 150                 155                 160

Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Val Lys
                165                 170                 175

Met Met Glu Arg Val Val Glu Gln Met Cys Val Thr Gln Tyr Gln Lys
            180                 185                 190

Glu Ser Gln Ala Tyr Tyr Asp Gly Arg Arg Ser Ser Ala Val Leu Phe
        195                 200                 205

Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu Ile
    210                 215                 220

Val Gly
225
```

What is claimed is:

1. A method for the immunological detection of prion disease, said method comprising:

extracting tissue from a live animal; and

screening for prion disease, said screening comprising detecting aberrant prion proteins in the tissue extracted from the live animal with an antibody induced against an epitope of said aberrant prion protein, but not an epitope of a normal prion protein,

wherein said antibody binds to an epitope of aberrant prion protein, but not to an epitope of normal prion protein.

2. A method for the immunological detection of prion disease, said method comprising:

collecting tissue from a live animal;

inducing production of an antibody with specificity against an epitope of an aberrant prion protein, but not an epitope of a normal prion protein, wherein said antibody binds to an epitope of aberrant prion protein, but not to an epitope of normal prion protein; and

screening the tissue for the presence of an aberrant prion protein with said antibody;

wherein said antibody is also reactive in an immunochemical test when analyzed with 12 mer peptides with overlapping sequences of sheep PrP.

3. A diagnostic test for performing a method of immunologically detecting prion disease in a live animal, said method comprising extracting tissue from a live animal and screening the tissue for prion disease, wherein the screening comprises detecting aberrant prion proteins in the tissue with an antibody induced against and that binds to an epitope of aberrant prion protein, but not induced against and not binding to an epitope of a normal prion protein, said diagnostic test comprising:

the antibody induced against the epitope of the aberrant prion protein, wherein said antibody binds to an epitope of aberrant prion protein, but not to an epitope of normal prion protein, and

said tissue extracted from the live animal.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,211,401 B2
APPLICATION NO. : 10/949880
DATED : May 1, 2007
INVENTOR(S) : Bram Edward Cornelis Schreuder et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the title page:
In ITEM (30),
Foreign Application Priority Data, change “96200917” --96200917.1--

In Item (56) **References Cited**,
OTHER PUBLICATIONS
1st page, 2nd column, line 1, change “#Brown, Can Creutzfeldt-Jakob disease can be transmitted by” to --#Brown, Can Creutzfeldt-Jakob disease be transmitted by--

1st page, 2nd column, line 4, change “protection, Nature,” to --protein, Nature,--

In the specification:

COLUMN 8,	LINES 13-14,	change “(12 mers);” to --(12mers);--
COLUMN 8,	LINE 23,	change “PrPc” to --PrP^C--
COLUMN 8,	LINE 24,	change “12 mer peptides” to --12mer peptides--
COLUMN 8,	LINE 32,	change “12 mer peptides.” to --12mer peptides.--

In the claims:
CLAIM 2, COLUMN 24, LINE 24, change “12 mer peptides” to --12mer peptides--

Signed and Sealed this

Thirtieth Day of September, 2008

JON W. DUDAS
Director of the United States Patent and Trademark Office

patsnap

专利名称(译)	检测朊病毒病的方法		
公开(公告)号	US7211401	公开(公告)日	2007-05-01
申请号	US10/949880	申请日	2004-09-24
[标]申请(专利权)人(译)	CORNELIS SCHREUDER BRAM EDWARD VAN KEULEN LUCIUS JOHANNES MATTHEUS WILHELMINA VROMANS MARIA ELISABETH MARIA LANGEVELD JOHANNES PIETER SMITS MARINUS ADRIANUS		
申请(专利权)人(译)	CORNELIS SCHREUDER BRAM EDWARD VAN KEULEN LUCIUS JOHANNES MATTHEUS WILHELMINA VROMANS MARIA ELISABETH MARIA LANGEVELD JOHANNES PIETER SMITS MARINUS ADRIANUS		
当前申请(专利权)人(译)	STICHTING INSTITUUT VOOR DIERHOUDERIJ EN DIERGEZONDHEID		
[标]发明人	SCHREUDER BRAM EDWARD CORNELIS VAN KEULEN LUCIUS JOHANNES MATTHEUS VROMANS MARIA ELISABETH WILHELMINA LANGEVELD JOHANNES PIETER MARIA SMITS MARINUS ADRIANUS		
发明人	SCHREUDER, BRAM EDWARD CORNELIS VAN KEULEN, LUCIUS JOHANNES MATTHEUS VROMANS, MARIA ELISABETH WILHELMINA LANGEVELD, JOHANNES PIETER MARIA SMITS, MARINUS ADRIANUS		
IPC分类号	G01N33/53 A61K49/00 G01N1/30 G01N33/569 G01N33/68		
CPC分类号	G01N33/6896 G01N2800/2828 G01N2333/47		
优先权	1996200917 1996-04-03 EP PCT/NL1997/000166 1997-04-02 WO		
其他公开文献	US20050048582A1		
外部链接	Espacenet USPTO		

摘要(译)

本发明提供了检测朊病毒疾病的方法，例如绵羊的瘙痒病，牛的牛海绵状脑病，人的Creutzfeld-Jacob病，其中在可从活体动物取样的组织中检测到异常蛋白质或朊病毒蛋白质。

Fig. 1A

Multiple sequence alignment of prior proteins of various origin.

```
Perfectly conserved:  '*'          Disulfide-bond:    '$'
Well conserved:       '.'          N-Glycosylation:   '#'

               ---------sign-----------
SHPRP          MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGN   50
BTPRP          MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGN   50
MINKPRP        MVKSHIGSWLLVLFVATWSDIGFCKKRPKPGGGWNTGGSRYPGQGSPGGN   50
GORPRP         M--ANLGYWMLVLFVATWSDLGLCKKRPKPGG-WNTGGSRYPGQGSPGGN   47
HSPRP          M--ANLGCWMLVLFVATWSDLGLCKKRPKPGG-WNTGGSRYPGQGSPGGN   47
MAPRP          M--ANLSYWLLALFVAMWTDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGN   47
MMPRP          M--ANLGYWLLALFVTMWTDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGN   47
RRPRP          ------------------------------GGWNTGGSRYPGQGSPGGN   19+
                                              * *****************

SHPRP          RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG--------G   92
BTPRP          RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGG   100
MINKPRP        RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG--------G   92
GORPRP         RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGG---------G   88
HSPRP          RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGG---------G   88
MAPRP          RYPPQGGGTWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGG---------G   88
MMPRP          RYPPQGG-TWGQPHGGGWGQPHGGSWGQPHGGSWGQPHGG---------G   87
RRPRP          RYPPQSGGTWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGG---------G   60+
               *****.* .**************.*******.*******          *

SHPRP          WGQGG-SHSQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPL   141
BTPRP          WGQGG-THGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPL   149
MINKPRP        WGQGGGSHGQWGKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPL   142
GORPRP         WGQGGGTHSQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPI   138
HSPRP          WGQGGGTHSQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPI   138
MAPRP          WGQGGGTHNQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPM   138
MMPRP          WGQGGGTHNQWNKPSKPKTNLKHVAGAAAAGAVVGGLGGYMLGSAMSRPM   137
RRPRP          WSQGGGTHNQWNKPSKPKTNLKHVAGAAAAGAVVGGLGGYMLGSAMSRPM   110+
               *.***  .*.**.********.**.**************************.

                                                    $ #
SHPRP          IHFGNDYEDRYYRENMYRYPNQVYYRPVDRYSNQNNFVHDCVNITVKQHT   191
BTPRP          IHFGSDYEDRYYRENMHRYPNQVYYRPVDQYSNQNNFVHDCVNITVKEHT   199
MINKPRP        IHFGNDYEDRYYRENMYRYPNQVYYKPVDQYSNQNNFVHDCVNITVKQHT   192
GORPRP         IHFGSDYEDRYYRENMHRYPNQVYYRPMDQYSNQNNFVHDCVNITIKQHT   188
HSPRP          IHFGSDYEDRYYRENMHRYPNQVYYRPMDEYSNQNNFVHDCVNITIKQHT   188
MAPRP          MHFGNDWEDRYYRENMNRYPNQVYYRPVDQYNNQNNFVHDCVNITIKQHT   188
MMPRP          IHFGNDWEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITIKQHT   187
RRPRP          LHFGNDWEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITIKQHT   160+
               .***.*.********* ********.*.* *.*************.*.**

                       #              $
SHPRF          VTTTTKGENFTETDIKIMERVVEQMCITQYQRESQAYYQ--RGASVILFS   239
BTPRP          VTTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQ--RGASVILFS   247
MINKPRP        VTTTTKGENFTETDMKIMERVVEQMCVTQYQRESEAYYQ--RGASAILFS   240
GORPRP         VTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQ--RGSSMVLFS   236
HSPRP          VTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQ--RGSSMVLFS   236
MAPRP          VTTTTKGENFTETDIKIMERVVEQMCTTQYQKESQAYYDGRRSSA-VLFS   237
MMPRP          VTTTTKGENFTETDVKMMERVVEQMCVTQYQKESQAYYDGRRSSSTVLFS   237
RRPRP          VTTTTKGENFTETDVKMMERVVEQMCVTQYQKESQAYYDGRRSSA-VLFS   209+
               **************.*.*********.***..**.***.   *...  .***
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