UNIVERSITY OF NOVA GORICA
GRADUATE SCHOOL

PRION DISEASES-EPIDEMIOLOGY AND PROPHYLAXIS

PRIONSKE BOLEZNI - EPIDEMIOLOGIJA IN PROFILAKSA

MASTER'S THESIS

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ABSTRACT

Prion diseases, as the name itself indicates, are caused by prions. Prion diseases are a group of severe neurodegenerative conditions of humans and animals . The most known disease among all prion diseases in animals is Bovine Spongiform Encephalopathy (BSE) and in humans Creutzfeldt-Jakob disease (CJD). The use of British Meat and Bone Meal (MBM) in the diets of cattle most probably initiated epidemics of BSE in Europe. Another possibility for infection of cattle was the use of the MBM after the prohibition in cattle ban used for poultry and pig fodder; cross contamination may have happened in the feedstuff factories or by lateral cross-contamination. The newest form of human prion disease called variant Creutzfeldt-Jakob disease (vCJD) is resulting from the consumation of BSE infected cattle products. Prions are responsible for the lenght of incubation times, neuropathology of the central nervous system and the transmissibility to other species; these specifications form the basis for the identification of the BSE and vCJD.

For my Master thesis 5 inqueries were performed: Questionnaire about dairycow fodder, Inquiry in the production of Meat-Bone Meal, Inquiry in manufactures of animal fodder, Inquiry in the slaughterhouse and Inquiry at the Veterinary administration board of Republic Slovenia (VARS). Slovenia is following criteria by the EU directive 999/2001. If Slovenia will continue to following the rules, of directive it can be put from the 4th category into 3rd of BSE status in near future (around the year 2014), if there will be no cases of BSE in next five years.

Key words : Bovine spongiform encephalopathy (BSE), variant Creutzfeldt-Jakob disease (vCJD), Meat and Bone Meal (MBM), slaughtery, animal fodder, dairy cow fodder

1 INTRODUCTION

Prion diseases are a group of severe neurodegenerative conditions (prion diseases are listed in Table 1) of humans (Creutzfeldt 1920; Gerstman 1928) and animals (Comber 1772; Wells 1997).

***Table 1** : Prion diseases*

Human	**Animal**
Creutzfeldt-Jakob disease (CJD)	Scrapie-goat, sheep
genetic (gCJD) sporadic (sCJD) iatrogenic (iCJD) variant (vCJD)	Bovine spongiform encephalopathy(BSE)-cattle
	Chronic wasting disease-mule deer**
	Feline encephalopathy-cat**
	Mink encephalopathy-mink **
Gerstmann-Sträussler Scheinker sindrom (GSS)	
Fatal familiar insomnia (FFI)	
Fatal sporadic insomnia (FSI)	
Kuru *	

** Kuru (CJD like disease) is disappearing by now (it was transmited by ritual kanibalism at Fore people-New Guinea)*

*** Rare cases*

All prion diseases are infectious. This was scientifically confirmed. They have in common a key role for the normal cellular prion protein, PrP^{C}, encoded by the prion gene, /PRNP/. The prion gene /PRNP/, encoding the prion protein (PrP) is located on the short arme of chromosome 20 (20p12-pter). The coding DNA (cDNA) consist of (subunits) nucleotides (purines Adenin-Guanin, AG) and pyrimidines (Thymin-Cytosine, TC) base pairs, phosphates and sugars (deoxyribose). The /PRNP/ consists

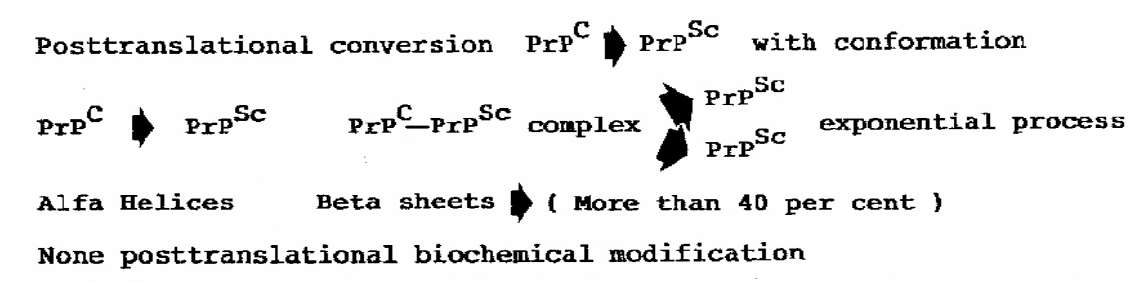
35,222 base pairs totally, the PrP coding exon contains 2,355 bp in lenght (Lee, 1998) or 2,301 (Pucket, 1991).

The DNA sequences of ORF (Open Reading Frame) generally exihibits circa 90 per cent similarity in mammals, but the similarity increases to 95 per cent when PrP of different primates are compared. The similarity is less than 70 per cent in non-placental mammals (opusum, kenguru). An even lower degree of similarity is found in chicken. Some authors are of the opinion that /PRNP/ was present before starting existence of mammal species. No evidence of disease association nor any association with the age of patient on onset, disease duration, or PrP strain type (Mead, 2000).

PrP^{Sc} produced in vitro lacks detectable infectivity (Hill, 1999). The mixture of PrP^{Sc} and recombinatly-derived PrP^{C} in vitro resulted in production of a protease-resistent prion protein, as typical for the pathogenic form. No infectivity was detected on bioassay. Some experiments performed later, could also not provoke a prion disease.

The PrP^{C} is converted in a post-translational process, possibly influenced also by the unknown protein X into its pathological isomer, PrP^{Sc} (Sc from scrapie) differing in conformation. Formation of PrP^{Sc} process is presented in Figure 1.

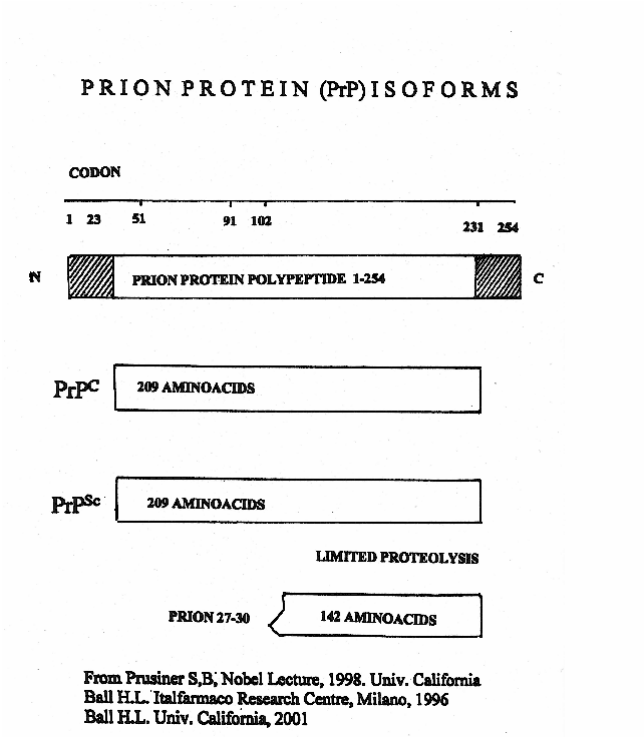
Figure 1*: Formation of* PrP^{Sc}



The most probable infectious agent, PrP^{Sc}, was called prion (proteinacens infectious) resistent to substances inactivating nucleic acids (Prusiner,1982). It has an unstructured N-terminal domain of around 100 aminoacids and a structural C-terminal part of similar

size including a disulphide bond and two glycosylation sites (one, two or without glycosides). It was presented in the Nobel Lecture (Prusiner, 1998) as shown in Figure 2.

***Figure 2** : Prion protein (PrP) isoforms*



The PrP consist of 254 residues. The signal frequences are 1-23 (N terminal) and 231-254 (C terminal). The pathologic PrP appears, partially, protease resistant. The INDEL region contains 4 octapeptides and one nanopeptide (50-91) and is sensitive to the Proteinase K.

Because of the autologous identical proteins of the aetiological agent (prions) specific antibodies were difficult to be prepare. The proteins all appear extremelly resistant to physico-chemical methods of decontamination, particularly to preparations decontaminating nucleic acid substances.

Animal diseases are transmissible by lateral, connatal or exceptionally iatrogenic way. Some 15 percent of human prion diseases were transmitted hereditary, by autosomal dominant traits cosegregating with mutations in the /PRNP/ gene, that encodes the prion protein. The origin of sporadic CJD or GSS has not been known. Human iatrogenic infections have been well known and of great importance (particularly blood) but they are very rare (Pana, 2005).

Genetic factors have a great, easily measurable influence on prion diseases. In scrapie, they determine the sensitivity to infection very clearly. By a planed growing of scrapie resistant animals, the number of new scrapie cases may be significantly reduced. In New Zealand, there are numbers of scrapie genetically sensitive animals. In many millions of animals in herds there was not a single case of the scrapie disease.

In human prion diseases there are 10-15 percent of cases with a clearly evident point (missense) mutation in the C-terminal domain (102-) of prion protein or octapeptide insertions in the N-terminal domain (50-91). Octapeptide deletions have rarely, if ever, been involved in CJD like case. For details see Annex 5.

Human prion diseases of the CJD group were detected in first decades of this century. Scrapie is a clinically and pathologically similar disease in sheep and goats known over 200 years worldwide. The second animal prion disease, bovine spongiform encephalopathy (BSE) was detected in 1986. The disease was responsible for an epidemy of unexpected wideness in Europe and in the world. Characteristics of human and animal prion diseases are summarized in Table 2.

***Table 2**: Review of prion diseases*

Origin	Name	Types of disease
Human	Creutzfeldt-Jakob diseases (CJD)	Sporadic
		Inherrited-genetic
		Variant
		Iatrogenic
Animal	Scrapie	Epidemic
		Sporadic
		Known lateral, connatal
		Genotyped
Animal	Bovine spongiform encephalopathy (BSE)	Epidemic
		Sporadic-unknown origin
		Sporadic lateral Meat-Bone-Meal(MBM)
		Connatal
		Molecular-biology typed (BASE types, H&L strains,...)

1. 1 Prion stability in the environment

Prion infectivity may enter soil environment via shedding from infected animals or decomposition of infected carcasses. Burial of infected animals carcasses may result in introduction of prions into surface environment. The infectivity originated from sheep placenta or amniotic fluid, saliva or non-sterilized organic fertilizers. Natural transmission appears to occur via the alimentary tract in the majority of cases. Animals can be infected on pasture, thus confirming the contagion in the environment. Feeding of aqueous soil extract can also induce the disease. One BSE case at a goat (Brugère-Picoux, 2005), that was absolutely confirmed by European authorities. Animals could have been feeded with such MBM containing prion proteins. The experimental

transmission of BSE from cattle to sheep was easily accomplished. The scrapie has been also a true infectious disease (Hunter, 1997); genetic factors are, undoubtedly, responsible for the susceptibility to the scrapie infection. Scrapie, therefore, cannot be eradicated by genetic selection, but only through enormous financial investments.

A substantial PrP^{Sc} adsorbtion to clay minerals, quartz and soil samples was detected (Johnson, 2006). The bounded PrP^{Sc} remained infectious. The transmission studies of scrapie and chronic wasting disease (CWD) seem to include environmental spread in their epidemiology (Cooke, 2007). Burried carcasses may remain a potential reservoir of infectivity for many years. PrP^{Sc} binds strongly to one or more soil components. In the clay soil the PrP^{Sc} binding occurs via the N- terminal to a component, that is absent in the tested sandy soils. It has also been shown that the scrapie agent (263K) can trasmitt orally after persistence in soil over years (Siedel, 2007). Feeding of aqueous soil extracts was able to induce the disease. Publications confirm the experiment from 1989 with scrapie prions unbound with soil containing pots and burried in a garden for three years. Between two and three of five exposed log units to infectivity, survived the exposure. (Brown, 1991).

1.2 Bovine spongiform encephalopathy (BSE)

In 1986 a new, hitherto unknown, severe encephalopathy in cattle, was detected in widely separated geographic locations of England and Scotland. The clinical disease probably started in 1985.

Histopathology demonstrated degenerative changes and necrosis of neurons in different brain steam locations with ovoid or spherical vacuoles or microcavities causing balooning of the cells resembling a spongiosis phenomen. Gliosis also impaired degenerative changes (Wells, 1987). The disease was provisionally appellated Bovine Spongiform Encephalopathy (BSE). In Table 3 clinical criteria for diagnosis of BSE are presented.

Table 3: *Clinical criteria for the diagnosis of BSE*

At least one sign in each row must be present to meet the diagnostic criteria

Classical criteria	1,2,3
Veter. Labor Weybridge UK	4,5,2
Belgium	6,5,2,7,8,1,9,10
Germany	1,11,5,2
Switzerland	12,7,5,1,13
Recumbent cases (A)	14,15,2
Recumbent cases (B)	14,12,2

For details see Konold et al 2006

Legend to Table 3: Clinical signs

1) Temperament change to nervous or aggressive
2) At least two to three startled responses to hand clapping
3) Ataxia, tremor
4) Exaggerated menace response
5) Fear at head restraint
6) Kicking at milking
7) Increased fear toward humans
8) Hypermotile ears
9) Reduced milk production
10) Teeth grinding
11) Nose licking
12) Body condition is less compared to others in the group
13) Increased fear at entrances or doorways
14) One or both hind limbs are stretched backward
15) Heart rate below 60 beats per minute

Clinical and histopathological characteristics as well as the absence of inflammation strongly resemble on scrapie at sheep and goats, known as a typical prion disease. Scrapie also resembled on: (a) Kuru (Hadlow, 1959) transmitted by ritual canibalism (erradicated by now) and (b) human Creutzfeldt-Jakob disease (Hadlow, 1980). The BSE epidemic developed dramatically and almost simultaneously in different regions of England and Scotland. The characteristics of the epidemic were typical of a food borne event. In Great Britain over 184.00 BSE cases (see Table 4) were registered until recently but over one million of cattle, diseased or being at risk, were killed and incinerated in the field because of shortage of incineration capacities.

Table 4: *Number of BSE cases reported in the United Kingdom data as of 30th September 2008*

Year	Number
and before 1987	446
1988	2514
1989	7228
1990	14407
1991	25359
1992	37280
1993	35090
1994	24438
1995	14562
1996	8149
1997	4393
1998	3235
1999	2301
2000	1443
2001	1202
2002	1144

Year	Number
2003	611
2004	343
2005	225
2006	114
2007	67
2008	25
Total	**184.576**

Source : http://www.oie.int/eng/info/en_esbru.htm (7.12.2008)

In other countries over 5 thousand cases were registered recently as presented in Table 5.

***Table 5**: Register of BSE cases in the world*

Country	Total number of cases (up to 7th of December 2008)	Year of first reported BSE case
Austria	6	2001
Belgium	133	1997
Canada	16	1993
Czech Republic	28	2001
Denmark	15	1992
Finland	1	2001
France	997	1991
Germany	416	1992
Greece	1	2001
Ireland	1635	1989
Israel	1	2002
Italy	142	1994
Japan	34	2001
Liechtenstein	2	1998
Luxemburg	3	1997

Country	Total number of cases (up to 7th of December 2008)	Year of first reported BSE case
Netherlands	85	1997
Poland	61	2002
Portugal	1055	1990
Slovakia	24	2001
Slovenia	8	2001
Spain	717	2000
Sweden	1	2006
Switzerland	464	1990
USA	2	2006
Total	**5.847**	

Source : http://www.oie.int/eng/info/en_esbmonde.htm (7.12.2008)

Still few states are beside above written states, that are performing control of BSE. These states are: Brazil, Chile, Chinese Taipei, Cyprus, Estonia, Hungary, Latvia, Lithuania, Malta and Mexico. There has not been positive BSE case yet.

It appears also of interest to learn the time of the development of BSE cases in Europe and otherwise. The first BSE case in Slovenia, as documented, was registered in 2001. In some countries single cases were declared to be imported; there is no data on cattle export (see Table 6).

***Table 6**: Countries/Territories having reported cases of BSE at imported animals only*

Country/Territory	Number of cases	Date
Falkland Islands	1	cases confirmed in 1989
Oman	2	cases confirmed in 1989

Early epidemiological studies etiologically excluded therapeutic or agricultural chemicals on affected farms exclusively as a common factor. Genetic analyses eliminated BSE from being exclusively determined by Mendelian inheritance. There was also no evidence that the disease was introduced by imported cattle or semen. The study supported the evidence of clinically and pathologically similarities with scrapie. These findings were consistent with exposure of cattle to a scrapie like agent via cattle feedstuffs containing ruminant derived protein (Wilesmith, 1988). Table 7 represents methods for detection of ruminant proteins. It appears safe to use the MBM without ruminant proteins; a prion infection could be avoided in this way. The methods are, except immunohistochemistry, on molecular basis as Enzyme ImmunoAssay (EIA) or Polymerase Chain Reaction (PCR) with corresponding primers. Controls of a significant number of MBM batches has not be possible at present.

***Table 7**: Methods to detect ruminant (sheep and cattle) proteins in human and animal foodstuffs*

Method	Author
PCR-meat derived from bovine	Tartaglia 1998
ELISA for GFAP 1999	Schmidt 1999
Immunohistochemistry	Wenisch 1999
ELISA for GFAP 2001	Schmidt 2001
PCR for bovine DNA	Krcmar 2001
165rDNA GENE	Bottero 2003
Reverse transcription PCR/GFAP	Seybold 2003
PCR 8 ATP synthesis	Kusama 2004
Bovine DNA in MBM PCR	Toyoda 2004
FLUORESCENT PCR	Sawyer 2004
PCR animals mtDNA	Myers 2004
DNA FORENSIC KIT	Myers 2004
Real time fluorescent PCR	Rensen 2005
PCR using DNA forensic fit	Yancy 2005

Method	Author
Lateral-flow test kit	Myers 2005
PCR DNA sequence	Ha 2006
EIA immunohistochemistry	Hossner 2006
(R) Ridascreen EIA for GFAP	Bozzetta 2006
PCR primers 134-154	Henk 2006
SANDWICH ELISA	Ofori 2007

For details about Methods to detect ruminant (sheep and cattle) proteins in foodstuffs see Annex 1

The infectivity was also indicated by simultaneous appearance of the disease in different regions, where the MBM was the only typical vehicle.

The MBM was introduced as cattle food at about 1970, because of price increase for fish and soya meal. MBM was prepared from slaughtered scrapes of animals that were to enter the human food chain. MBM was extracted with organic solvents for tallow and autoclaved at 120 ^{o}C following the hyperbaric steam to drive residual solvent. About 1980 the autoclave temperatures were decreased and the solvent extraction was omitted. The infectivity of the MBM was confirmed (Taylor, 1995). Temperature decrease and the omission of the solvent extraction also (probably in part) contributed to the persistence of infectivity in MBM. The use of British MBM most probably caused the disease in Europe (Taylor, 1997a).

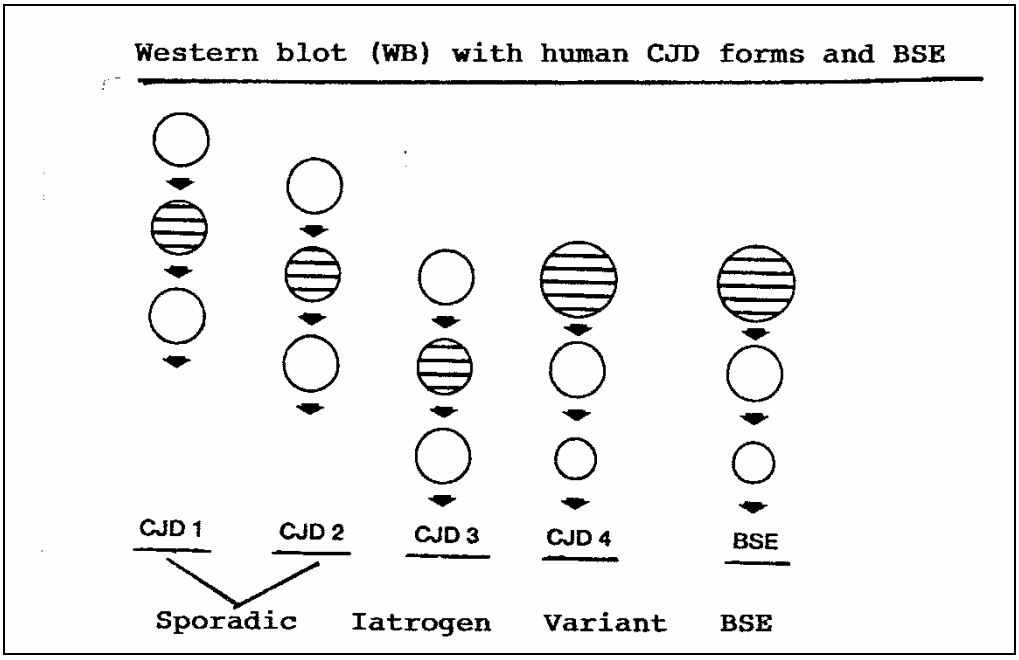
The use of MBM in Great Britain was immediately banned (MAFF 1988). Risk organs of cattle were also banned as brain, spinal cord, spleen, thymus and tonsils (Anon, 1989). The export of British MBM was not banned; however, the disease infected European animals. The proposal of British government not to use British MBM for cattle (Taylor, 2004) has not been everywhere known. Final MBM ban was generally introduced in 1996 and the ban for risk organs some years later. In the same year, the appearance of new variant Creutzfeldt-Jakob disease, vCJD, was published (Will, 1996).

Extensive molecular-biological studies on BSE prions were performed in 1996 and 1997 in connection with the BSE epidemics and the appearance of a new variant of human CJD. Collinge (1996) studied the PrP^{Sc} strain variations in different forms of human CJD and compared this with the results of PrP^{Sc} in BSE (see Figure 3). Studying the molecular mass of PrP^{Sc} and the glycoform patterns, he found the identity with the type 4 of human CJD (variant CJD). Bruce (1997) repeated this study with strains of scrapie, BSE and human vCJD considering to study molecular mass, incubation time, lesion profile in the brain and transmissibility to laboratory animals. This study also significantly confirmed the identity of the BSE and human vCJD. Somewhat later (Bruce, 2002) he could also clearly confirm the strain variations in natural scrapie strains, which may have changed over the past 20 years.

Variant Creutzfeldt-Jakob disease in humans has resulted from consuming of BSE infected meat (Wells, 1996). This was scientifically confirmed by transmission parallel experiments human-mice (Bruce, 1997), by Western Blot typing of sporadic, iatrogen, variant prion strains (see Figure 3) with strains of mice infected with BSE prions (Collinge, 1996), and by evidence for transmission of BSE prions to transgenic manipulated mice carrying human prion protein, to avoid the species barrier (Scott, 1999).

WB studies on PrP^{Sc} prions were first performed simultaneously with all types of the human CJD (sporadic, iatrogen, variant CJD and BSE; sporadic CJD was of the types 1 and 2, iatrogen CJD of type 3 and variant CJD and BSE of type 4 (see Figure 3).

***Figure 3**: Western blot (WB) with human CJD forms and BSE*



Source : Jung (1997) Summarized from Collinge 1996

Case study in 2006 showed evidence of an increased risk for vCJD associated with the frequency of consuming beef products which contain mechanically recovered meat and head meat such as burgers, meat pies and sausages (Ward, 2006). Bioassay and molecular-biology tests have enabled identification of what is classified as "highly infectivity" and "lower infectivity" tissues of cattle with BSE. The highly infectivity tissues include the brain, spinal cord, retina, optic nerve, dorsal root and trigeminal ganglia. The lower infectivity tissues include peripheral nerves, tonsils, nictitating membrane, distal ileum, bone narrow and possibly thigh muscle (http://www.who.int.blood product/TSE reports 7.12.2008).

There are over 162 vCJD cases in Great Britain, some 20 in France, 4 in Ireland, and 2 in United States and 1 each in Canada, Italy, The Netherlands, Portugal, Saudi Arabia and Spain. Seven of the non UK cases patients were most likely exposed to the BSE agent in the UK because of their having resided there during a key exposure period of

the UK population (http://www.cdc.gov 7.12.2008) to the BSE agent as shown on Figure 4.

***Figure 4**: BSE and vCJD potential exposure through international trade*

Source: http:// *www.who.int/zoonoses/diseases/impact/en* (07.12.2008)

The homozygote codon 129 m/m, probably for the sensitivity, was detected in 98% of infected persons. The vCJD was also transmitted by transfusion of blood products (Liewelyn, 2004; Peden, 2004). The disease appeared as a severe neurological disease and is invariably fatal. There is a single patient over 50 but all others are considerable younger. The reason for this age distribution is not well understood but it suggests that older adults are much less susceptible to vCJD through the oral route of exposure than are children and young adults.

Criteria for diagnosis of variant CJD

- Progressive neuropsychiatric disorder
- Early symptoms (depression, anxiety)
- Persistent painful sensory symptoms

- Ataxia
- Myoclonus, chorea or dystonia
- Dementia
- Atypical EEG
- Duration of disease + 6 months
- Age mostly +/- 30 years

BSE has caused a harrowing fatal disease for humans. The link between BSE and vCJD is well stablished, although the mode of transmission remains unclear.The BSE epidemic developed because of the recycling of animal protein in ruminant feed.A cohort study to examine maternally associated risk factors were performed between 1988 and 1997 (Wilesmith, 1997). The incidence of offspring of cows, which developed BSE, was compared with that in offspring of cows, born in the same calving season and herd cows, which have reached at least 6 years of age without developing BSE. The results indicated a statistically significant difference between the two cohorts. These results were later confirmed by experiments in transgenic animals. The rate of connatal transfer appears close to 10 percent. These, connatal infected animals will be responsible for the maintenance of new BSE cases in the future, because in the dairy cows, without clinical symptoms, the laboratory diagnosis appears extremely difficult. Successful prion diagnostic of BSE in asymptomatic cattle with Immunohistochemistry (IHC) appears very safe for the general prion diagnostic, but it is very time-consuming and requires excellent microscopic possibilities and experience. It does not enable a significant lesion profile diagnostics. It has not been used for the strain differentiation. Molecular biology and transmission experiments in transgenic mice appear significantly more successful for this purpose. (Schulz-Schaeffer, 2001)

The prophylaxis in prion diseases appears to be also of importance. Scrapie is a prion infection known for long time. Fortunately there are no symptoms to indicate human infection. Prophylactic measures are very important to avoid lateral infections by contact, secrets of the organism, the pasture on known areas with clinically diseased

animals in the past two years (Siedel, 2007), introducing an unknown animal into a known healthy herd, particularly animal with mastitis.

Prophylaxis is most effective in BSE. Excepting connatal infections, which cannot be excluded, the infection is caused by cattle food called Meat and Bone Meal if containing ruminant proteins contaminated with BSE prions.

For human protection it appears ideal to test for BSE prions in all animals at slaughter house before entering in the human food chain. Great efforts have been done, scientifically and politically in this respect. Modern automatisation has enabled the use of molecular biologic methods (Western blot (WB) and immunological methods (EIA)) of sufficient capacities for multiple, highly sensitive testing at reasonable prices. A large study, financed by EU, was performed (Raeber, 2006). A serious problem was the false negativity, because the diagnosis at BSE infected, but clinically asymptomatic animals, appeared practically impossible. The number of such tests, however, decreased continuously and in some countries (Switzerland) practically disappeared.

1.2.1 Early studies and regulations

If BSE is a form of scrapie in cattle, the question was whether the species barrier may be broken to produce a similar disease in humans. Epidemiological evidence was studied (slaughter house workers, veterinarians) and suggested, that the transmission from cattle to human was unlikely (Taylor, 1989).
MBM ban in 1988 in UK resulted in continuous decline in number of BSE cattles, but at the same time the exportation increased in amounts and prices in 1985-1990, particularly in France and in Switzerland (6.600 tonnes). The use of risk organs and tissues was also banned, but not rigorously. The cattle brains and tongues (!) were free available. In 2001 the MBM was banned for all animals because of lateral infections from swine and other animals. The MBM should be incinerated and used in the industry; this has not been always followed. Germany exported 2004-2005 about 80.000 tonnes MBM to Thailand,

Vietnam and Bangladesh (Spiegel, 2007). In Croatia 2006, 40 tonnes of MBM disappeared during transport from the rendering company to the industry for incineration (Jutranji list, 2006).

The BSE has been registered by now in 24 countries out of United Kingdom (Brown, 2006) in Europe, North America and Japan. Most BSE cases, except in the UK, were detected in France, Portugal, Spain, Germany and Switzerland. Prions of infected cattle appeared extremely infectious (10^9) if the brain tissue was tested. One infected cattle brain can infect ten thousands of cattle. Its inclusion in the MBM might be critical for animal health.

Bovine import from the UK may have been an important source of human exposure to BSE as well as the disease has contributed to the global risk for disease. Another possibility to infect cattle was the use of the MBM (after the cattle ban) for poultry and pig. It may happen in the feedstuff factories or by lateral, cross-contamination (Abrial, 2005). Only the feeding of pigs was statistically significant.

1.2.2 Sample preparation

Correct taking of sample is very important. For the preparation of material for diagnostic examination, cattle suspected of having the disease should be killed with an intravenous injection of a concentrated barbiturate solution following sedation, if necessary. The technical procedures concerned with collection, fixation, and histological processing and are revised and summarized below.

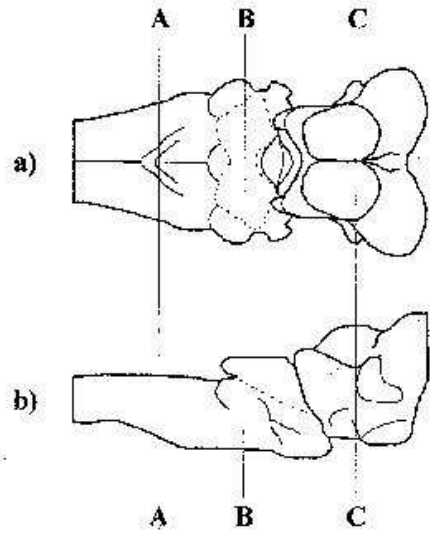
In all circumstances of surveillance of neurological disease in adult cattle where the occurrence of BSE within a country or state has not been established or is of low incidence, it is important that a standard neuropathological approach is followed in which representative areas of the whole brain are examined. A decline from this is dependent on the local national circumstances, including whether or not a differential

diagnosis is required. Additionally, where rapid immunoassays are selected as the primary method of choice, care will be needed in ensuring that sampling and tissue preparation for one test do not compromise the ability to confirm pathological phenotype by histopathological means.

Brain tissue should be removed as soon as possible after death. Fresh material for potential use in tests to detect disease-specific PrP should be taken ideally as a complete coronal section (2-4 g) from the medulla, caudal to the obex, specifically avoiding damage to the obex region. The cervical spinal cord and the lateral hemisphere of cerebellum also offer optimal sampling areas that will not encroach on histopathological requirements. This tissue is stored frozen prior to testing; precautions must be taken to insure that the tissues for histological or IHC examination are not frozen as this will provide artefactual lesions that may compromise the identification of vacuolation, and/or target site location. It is possible (but not desirable) to undertake immunohistochemistry for PrP on material that has been frozen prior to fixation (Debeer, 2002). However, it is still important to be sure that target sites have been identified and checked before negative results can be recorded. If the remaining whole brain is sampled for the histopathological examination, it should be placed in approximately 4-6 litres of 10% formal saline fixative, which should be changed twice weekly. After fixation for 2 weeks, the brain is cut into coronal slices. The fixation time may be shortened by cutting the fresh brainstem into smaller coronal pieces, leaving intact the diagnostically important areas at the obex, the cerebellar peduncles and the rostral colliculi. Depending on some other factors (temperature, agitation, and use of microwave) the fixation time for these small pieces of brainstem may be reduced to 2-5 days. The other formal-fixed parts of the brain may be used for differential diagnosis after completing the standard 2 weeks' fixation. Initially, a single block cut at the obex of the medulla oblongata (Fig. 5) should be selected for histological processing by conventional paraffin wax embedding methods for neural tissue. Sections, cut at 5 µm thickness and stained with haematoxylin and eosin, are examined for characteristic spongiform change and neuronal vacuolation. If results are inconclusive because of minimal lesions, or the material is histologically uninterpretable due to autolysis or damage, or if there are no histological lesions present,

it is necessary to carry out additional tests, including Immunohistochemistry (IHC) or immunoblotting.

Figure 5: *Brainstem after the removal of the cerebellum, from a) dorsal and b) lateral aspects.*



Recommended levels at which sections should be taken:
A-A = medulla, at the obex;
B (B = medulla through caudal cerebellar peduncles;
C (C = midbrain through rostral colliculi.

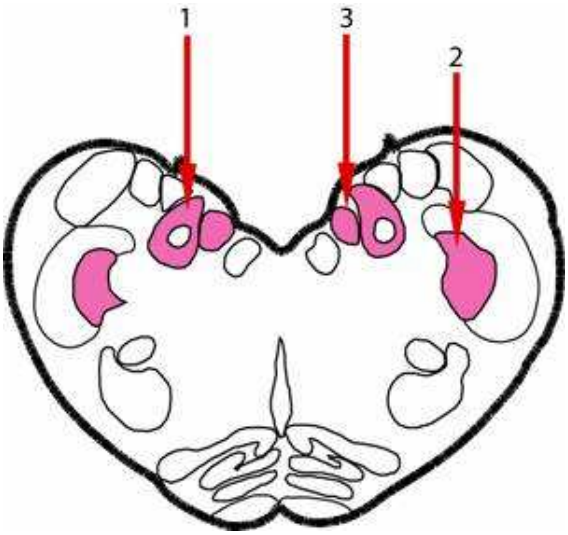
When the occurrence of BSE in a particular country has been established in the indigenous cattle population, and there is evidence that the distribution of lesions is consistent with what was seen in the brains of cattle from the UK epizootic, it is adequate for monitoring purposes to remove the hind brain alone (Fig.5).This can be achieved via the *foramen magnum* without removal of the calvarium. This will reduce the amount of fixative required, thereby lowering costs and improving safety, while maintaining representation of the major target areas for histological examination. The diagnosis may be confirmed if completely typical changes are present in the medulla at the level of the obex. When lesions are not obvious in the medulla (obex), immunohistochemistry should be performed. However, given the constant lesion pattern,

this is unlikely to contribute additional confirmation in more than 0.5% of cases of BSE where lesions are absent in the medulla (obex) section (Wells, 1989a). Clearly this abridged protocol does not allow a full neuropathological examination for differential diagnoses to be established, nor does it represent a comprehensive phenotypic characterization of any TSE.

Where the index case is identified through active surveillance, the necessary brain areas for full phenotypic characterization may not be available. In most countries, hind brain alone is collected, even before the first confirmation of BSE. Ideally, provision should be made for heads that have been sampled in the course of active surveillance to be retained until the outcome of initial testing is available. This would enable much more comprehensive sampling of the brain of positive animals and enable this recommended approach to the characterization of cases. This is particularly important if un-validated tests are used, and where in the absence of direct comparison with the methods described here results in claims that new strains have been identified.

The processing of the brain tissue for use in the rapid test should be carried out precisely as specified by the supplier or manufacturer of the test method or kit. Details of this procedure vary from method to method and should not be changed without supportive validation data for the variant methodology. The preferred sample for immunoassay should be at, or within 1.5 cm anterior to, the obex. The choice of target site should take into account the preferred method of confirmation, where the inability to examine brainstem histologically at the obex may prevent the detection of bilateral vacuolation. Sampling the rostral medulla for rapid test does not compromise examination by histological or immunohistochemical means. Hemisection of the brainstem at the level of the obex will result in loss of the ability to assess the symmetry of lesions, but the need for such assessment is less if immunohistochemistry is used. If this approach is adopted however, it becomes critical to ensure that the target site is not compromised. Both the dorsal nucleus of the vagus nerve (the target area for scrapie) and the nucleus of the solitary tract (the target area in cattle) are small, and lie close to midline (Fig. 6).

Figure 6: *Cross section of the brain-stem at the level of the obex identifying the key target sites for diagnosis by histopathology and immunohistochemistry in BSE (nucleus of the solitary tract [1] and the nucleus of the trigeminal tract V [2]) and scrapie (dorsal nucleus of the vagus [3]).*



Inaccurate hemisection could easily result in the complete loss of target area for confirmatory testing, and significantly reduce the effectiveness of the surveillance programme. Such an approach needs to be implemented with a very clear policy and monitoring programme for training and QA of sampling procedures. Because of the unknown distribution of PrP^{Sc}, sample size should be specified in the diagnostic kit or if not specified, should be at least 0.5 g. Performance characteristics of all of the tests may be compromised by autolytic changes. In order to reduce hazard to the operators, collecting large numbers of samples for an active surveillance programme, bovine brains should be sampled without opening the cranium. This is readily achieved, even at abattoirs, following training of operators in the use of a specially designed spoon, which can be inserted through the foramen magnum of the severed head. The following is a protocol that has been drafted by the OIE Reference Laboratory, Bern, Switzerland. Some manufacturers that make rapid test also sell disposable spoons for brain removal.

The interpretation of observed vacuolar changes in the bovine brain must be approached with caution. Vacuoles within the perikarya, indistinguishable from those of BSE, have

been reported in neurons of the red and oculomotor nuclei of the midbrain and other brainstem nuclei as an incidental finding in cattle (Fankhauser, 1972; Gavier-Widen, 2001; McGill, 1993; Wells, 1987). Thus, like the diagnosis of scrapie, which may be confounded by the occurrence of such neuronal vacuolation scattered in the medullae of healthy sheep (Vicari, 1997; Wells, 1989a), histopathological diagnosis of BSE must not rely on the presence of occasional solitary vacuolated neurons. Even relatively numerous vacuolated neurons in the red nucleus and in the habenular nuclei must be disregarded. The presence of spongiform change in the neuropil in specific neuroanatomical locations in BSE provides the most confidence of minimizing false-positive diagnoses.

As with scrapie of sheep, the possibility of BSE cases occurring in which brain lesions are minimal or undetec by light microscopy is a potential problem that can be resolved only by diagnostic criteria independent of histopathology (Scientific Veterinary Committee, 1994; Wells 1994).

1.2.3 BSE field diagnostics

European Union (EU) has approved 12 rapid tests for BSE monitoring the cattle in slaughterhouses; the approval for some new tests is pending. The tests were used in active surveillance of BSE for the detection of infected cattle before they entry in the human food chain. One of the approved tests is based on the Western blot (WB) technique (Prionics) and other on EIA / Elisa techniques with various modifications. It must be stressed, that brain sample does not necessarily reflect pathologic prion protein PrP^{Sc}, characteristic for the infection, in a preclinical stage, although the animal has already been infected.

Most diagnostic test approved by the EU exploit the selective protease resistence of PrP^{Sc} in combination with immunological detection of the protease resistant part of PrP^{Sc}. Sample preparation seems to be very important; tissue solubilisation requires detergent extraction. Proteinase K digestion should remove the N-terminal part of PrP^{Sc}

leaving the protease resistant core (PrP 27-30). In the WB the visible size shift constitutes an indepednent criteria for the positive results thus greatly increasing the specificity. The digestion conditions in EIA (ELISA) tests because of a slightly incomplete digestion of PrP^{C} can lead to false positive results.

1.2.3.1 The 12 EU approved tests include

1. Prionics-Check Western (Prionics).

The diagnostic criteria include a decreased molecular weight (due to digestion of N-terminus of PrP^{Sc}) and a three band patterns (due to different glycosylation forms).

2. EIA Biorad USA.

It includes centrifugation and precipitation for the enrichment of the analyte.

3. Enfer TSE kit.

One step sample preparation. For the detection polyclonal antibodies are used and an enzyme-coupled secondary antibody (chemiluminescence).

4. Prionics-Check LIA (ELISA).

Sandwich ELISA using 2 monoclonal antibodies. Detection by chemiluminescence.

5. Conformation dependent immunoassay CDI-5 (ELISA), Inpro USA.

Differential binding of antibodies to native or denatured PrP^{Sc}. The antibody recognizes a conformation-dependent epitope. A signal difference (denatured PrP minus native PrP = PrP^{Sc}) is used as diagnostic criterion.

6. CedeTect BSE Test, The Netherlands.

A sample is treated with a chaotropic agent (T) and another used as a control (N). The ratio of the T and N value based on the ratio of the T and N value using an enzyme conjugated monoclonal antibody.

7. IDEXX HerdChek BSE USA.

Selective capture of the PrP^{Sc} without proteinase K digestion.

8. Institut Pourquier, France.

The detection uses a classical immunoassay with two monoclonal antibodies. Capture and detection of PrP^{Sc} in a single step in microplate.

9. PrioSTRIP, PrionicsMicroplate.

This is chromatographic immunoassay using two monoclonal antibodies to detect the core of PrP^{Sc}. The resulting immuno »sandwich« appears as a coloured line and the reading is done visually or automated. The cartridge holder is for 12 cartridges (96 strips) with software PrioSCAN.

10. Roboscreen Beta Prion EIA, Germany.

After treatment with Proteinase K the homogenate is precipitated and delipidated. The test is classical immunoassay using two monoclonal antibodies.

11. Roche Applied Science Prion Screen, Germany.

A classical 96-wells plate is used for a two sided immunoassay with two monoclonal antibodies. The detection is based on a colourimetric enzyme reaction.

12. Enfer TSE Kit, Ireland.

The homogenisation process is automated and the samples are transferred to a liquid handling robot for subsequent ELISA.

At present, no diagnostic test exists for the detection of prions in live animals or humans. New surrogate markers are under development.

The diagnostic methods for the prion diagnostic have been the same as described earlier, but they were considerably improved (and complicated) requesting a prolonged working

time of days or weeks, to obtain a safe reproducible results. Only a Western blot with phosphotungstenacid precipitation appears satisfactory for quick diagnostic (in slaughter houses). Pathohistology or immuno-cytochemical tests require days and are very usable in research. The EIA method was recently improved by commercial availability of primers specific for most frequently encountered genetic cases (codon 102; 205). For details about diagnostic methods see Annex 2.

1.2.3.2 BSE testing of cattle in Slovenia

In Slovenia 309.780 rapid tests (mostly from Prionics Zürich) were performed in a) slaughter houses, b) in cattle aged over 24 months, succombed from unknown reasons and c) in five cattle succombed with typical heavy clinical BSE symptoms in owners stamble (one of these animals was imported from Germany) and in three cases at the slaughtery (two in Graz, Austria and one in Celje, Slovenia).The results are summarized in the Table 8.

***Table 8** : 8 confirmed cattle with BSE in Slovenia*

N°	Location	Year of birth and country	Data about disease	Quick test	Confirmation test	Age of cattle when died in years
1	Dravsko polje (Gornji grad)	1996 in Slovenia	BSE 2001	Prionics check	Histopathology, Immunohistopathology	5
2	Savinjska dolina (Ormož)	1995 in Slovenia	BSE 2002	Prionics check-Western	Histopathology, Immunohistopathology	7
3	Šaleška dolina	1999 in Slovenia	BSE 2003	Prionics check-Western	Platelia test (ELISA), Histopathology, Immunohistopathology	4
4	Gorenjska	2000 in Slovenia	BSE 2004	Enfer, Prionics check-Western	Histopathology, Immunohistopathology	4
5	Prekmurje	1998 in Germany	BSE 2004	Enfer, Prionics check-Western	Histopathology, Immunohistopathology	6
6	Prlekija	2000 in Slovenia	BSE 2005 Slaughter house Graz	Prionics check-Western, Prionics check LIA, PrioSTRIP	Immunohistopathology, Western blot	5
7	Dravsko polje	2000 in Slovenia	BSE 2006 Slaughter house Graz	Prionics check LIA	Histopathology Immunohistopathology	6
8	Celje	2000 in Slovenia	BSE 2007 Slaughter house Celje			7

It is evident and very important to see that positive tests were obtained only once in the Slovenian slaughtery house (2007) and twice in Austrian slaughtery (Graz). All the other cases in Slovenia were typical clinical BSE cases. It is also evident that the organized testing of over 300.000 cattle in Slovenia (2001-2007), which were clinical inapparent, gave totally negative results. This again confirms the previosly reviewed text that BSE diagnosis in apparently healthy cattle appears extremely diffucult. On the other hand this fact caused, particularly in Switzerland, the further testing of questionable importance increasing only the price for the meat to be sold.

Slovenian infections may have started between 2001-2005 or, in the last cases later. Lateral infections, as with the other prion diseases Scrapie (direct contact or by body fluids as saliva, urine), pasture on infected areas (where diseased or clinically asymptomatic animals pastured the last two or three years) because of the prions infectivity persistence in soil, contaminated by animal excretions. Connatal BSE infections in Slovenia might be excluded. The use of MBM in Slovenian BSE cases was denied by owners, but this could not be confirmed. The facts presented here indicate the MBM as the only reasonable infection source. This theory cannot be absolutely accepted as the solely responsible.

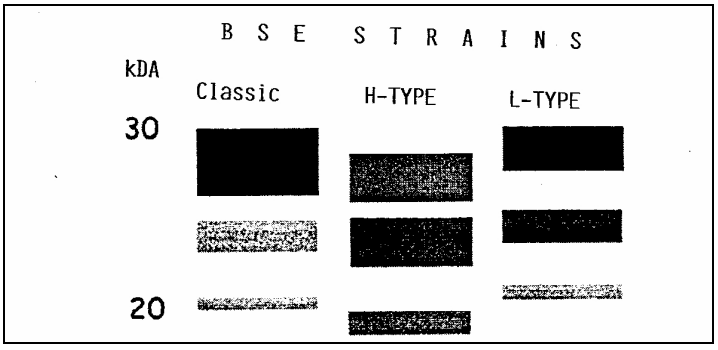
From large epidemiological studies on free living animals, in time of BSE epidemics in Europe, by isolation attempts of BSE prions (Western blot and EIA), supported by the EU Commission, we have learned that animals can be infectious, but clinically inapparent; the BSE diagnostic in such cases is extremely difficult or even impossible. Such sporadic, prion-positive but clinically inapparent animals were also detected in Slovenia, but very rarely. This also, may had happened years ago and animal rests after slaughtering, may have entered the home-produced MBM for decades until recently, because the BSE tests in slaughtery houses have been introduced a few years ago. We do not have any data about testing of MBM in Slovenia for the presence of prions.

1.2.4 Prion strain gene typing in animals

In France (Baron, 1999) the glycoform patterns of BSE were studied in cattle and in natural scrapie isolated; in some of these the glycoforms were comparable to those found in BSE linked disease.

The PrP^{Sc} types can be classified as classical BSE type C, H and L (as presented in Figure 7) according to the higher or lower molecular mass and positions of the nonglycosylated PrP^{Sc} bands. Atypical strains were found in several European countries (Jacobs, 2007). Béringue (2006) tested French cattle isolated (H-type) in transgenic mice expresing bovine or ovine PrP. All mice developed neurological symptoms and succumbed thus exhibiting specific features clearly distinct from that of BSE. Brunelle (2007) studied the polymorphisms in the prion gene promoter region, that influences classical BSE susceptability but they are not associated with atypical BSE cases. The results are consistent with the fact that atypical BSE originates spontaneously in cattle. Another publication (Brunelle, 2008) communicated about allele, genotype and haplotype data for BSE resistence polymorphism from health US Holstein cattle. Molecular – biology and genetic studies of the bovine prion gene will certainly be continued.

***Figure 7**: Western blots of PrP^{Sc} patterns of typical (classic) and atypical bovine spongiform encephalopathy strains*



Since 1990 the European community has adopted a series of measures to protect human and animal health from the risk of BSE. These measures have been based on the safeguard provisions of Directives on animal-health measures. It is appropriate, in view of the magnitude of the risk posed to human and animal health by certain TSEs, to adopt specific rules for their prevention, control and eradication.

Rules should be applied to the production and placing on the market of live animals and products of animal origin. However, it is not necessary to apply them to cosmetic or medical products, medical devices or their starting materials or intermediate products, for which other specific rules, in particular on the non-use of specified risk material, apply. Nor should they apply to products of animal origin which do not pose a risk to animal or human health since they are intended for purposes other than the production of food, feed or fertiliser.

Member States of the European Union should institute education programmes for those involved in the prevention and control of TSEs, as well as for veterinarians, farmers and workers involved in the transportation, marketing and slaughter of farm animals.

Certain ruminant tissues should be designated as specified risk material on the basis of the pathogenesis of TSEs and the epidemiological status of the country or region of origin or residence of the animal concerned. The specified risk material should be removed and disposed of in a manner which avoids any risk to human or animal health. In particular, it should not be placed on the market to be used in the production of food, feed or fertiliser. However, provision should be made for an equivalent level of health protection by means of a screening test for TSEs carried out on individual animals as soon as it has been fully validated. Slaughter techniques presenting a risk of causing brain material to contaminate other tissues should not be permitted in countries or regions other than those presenting the lowest risk of BSE.

Measures should be taken to prevent the transmission of TSEs to humans or animals by prohibiting the feeding of certain categories of animal protein to certain categories of

animal, and by prohibiting the use of certain ruminant materials in food. These prohibitions should be proportional to the risks involved.

The suspected presence of any TSE in any animal should be notified to the competent authority, which should immediately take all appropriate measures, including placing the suspect animal under movement restrictions while awaiting the results of the investigation or having it slaughtered under official supervision. If the competent authority cannot exclude the possibility of a TSE, it should have the appropriate investigations carried out and should keep the carcase under official supervision until a diagnosis has been made.

In the event of official confirmation of the presence of a TSE, the competent authority should take all the necessary measures, including having the carcase destroyed, carrying out an investigation in order to identify all animals at risk and placing movement restrictions on the animals and the products of animal origin identified as such. Owners should be compensated, as soon as possible, for the loss of animals and products of animal origin destroyed pursuant to EU Regulation 999/2001.

Provisions should be laid down covering the placing on the market of certain live animals and products of animal origin. Existing Community rules on the identification and registration of bovine animals provide for a system enabling the animals to be traced back to the dam and herd of origin in accordance with international standards. Equivalent guarantees should be provided for bovine animals imported from third countries. The animals and products of animal origin covered by Community rules, moving in intra-Community trade or imported from third countries, should be accompanied by the certificates required by the said rules, supplemented as appropriate in accordance with Regulation for prevention, control and eradication of TSEs.

The placing on the market of certain products of animal origin derived from bovine animals in high risk regions should be prohibited.

It is necessary, in order to ensure that the rules concerning the prevention, control and eradication of TSEs are observed, for samples to be taken for laboratory testing on the basis of an established protocol which would give a full epidemiological picture of the situation regarding TSE. In order to guarantee uniform testing procedures and results, national and Community Reference Laboratories and reliable scientific methods, including rapid tests specifically for TSEs, should be established. Rapid tests should be used as far as possible.

Animal suspected of being infected by a TSE: live, slaughtered or dead animals, which show or have shown neurological or behavioural disorders or a progressive deterioration of the general condition linked to impairment of the central nervous system and for which the information gathered on the basis of a clinical examination, response to treatment, a post-mortem examination or an ante or post-mortem laboratory analysis do not allow an alternative diagnosis to be established. Bovine spongiform encephalopathies (BSE) shall be suspected in bovine animals which have produced a positive result from a rapid test specifically for BSE.

1.2.5 Determination of BSE status

The BSE status of a Member State of European Union or a third country or of one of their regions, here and after referred to as ‘country or region’, shall be determined on the basis of the following **criteria:**

(a) The outcome of a risk analysis identifying all the potential factors for the appearance of BSE referred to factors written below and their development over time;

(b) An education programme for veterinarians, breeders and those who transport, trade in and slaughter bovine animals, which seeks to encourage them to report all cases of neurological manifestations in adult bovine animals;

(c) The compulsory reporting and examination of all bovine animals showing clinical signs of BSE;

(d) A system of continuous surveillance and monitoring of BSE with particular reference to the risks, taking account of the guidelines or in accordance with the appropriate international standards; reports on the number of examinations carried out and the results thereof must be kept for at least seven years;

(e) The examination in an approved laboratory of samples of encephala or other tissues collected under the surveillance system mentioned in point (d).

The risk analysis referred to factors written above shall be based on the following factors:

- The consumption by bovine animals of meat and bone meal or greaves derived from ruminants;
- The importation of meat and bone meal or greaves potentially contaminated by a TSE or animal feed containing meat and bone meal or greaves;
- The importation of animals or ova/embryos potentially infected by a TSE;
- The epidemiological status of the country or region in regard to animal TSEs;
- The extent of knowledge about the structure of the bovine, ovine and caprine population in the country or region;
- The source of animal waste, the parameters of the processes for treating such waste and the methods of producing animal feed.

1.2.6 Definition of categories

The BSE status of Member States or third countries or one of the regions shall be determined by classification into the following categories:

1.2.6.1 CATEGORY 1: Country or region free of BSE

A country or region where a risk analysis has been conducted which demonstrated that appropriate measures have been taken for the relevant period of time, to manage any risk identified and

1. EITHER no BSE case has been recorded and:
(i) The criteria in points (b) to (e) have been complied with for at least seven years, or
(ii) The criteria in point (c) have been complied with for at least seven years and it has been demonstrated that for at least eight years no meat and bone meal or greaves derived from ruminants or mammals has been fed to ruminants;

2. OR where all cases of BSE have been clearly demonstrated to originate directly from the importation of live bovine animals or bovine embryos/ova, and all the affected bovine animals as well as, if these are females, their last progeny born within two years prior to, or after, the first clinical signs of onset of the disease, if alive in the country or region, have been killed and completely destroyed and, either
(i) The criteria in points (b) to (e) have been complied with for at least seven years, or
(ii) The criteria in point (c) have been complied with for at least seven years and it has been demonstrated that for at least eight years no meat and bone meal or greaves have been fed to ruminants;

3. OR where the last indigenous case of BSE was reported more than seven years ago, the criteria in points (b) to (e) have been complied with for at least seven years and the feeding of ruminants with meat and bone meal and greaves derived from ruminants has been banned and the ban has been effectively enforced for at least eight years.

1.2.6.2 CATEGORY 2: BSE provisionally free country or region where no indigenous case has been reported

Country or region where a risk analysis has been conducted which demonstrates that appropriate measures have been taken for the relevant period of time to manage any risk identified, and

1. EITHER where there has been no case of BSE and:
(i) The criteria in points (b) to (e) are complied with, but have not been complied with for seven years, or
(ii) It has been demonstrated that for at least eight years no meat and bone meal or greaves has been fed to ruminants, but the criteria in point(c) have not been complied with for seven years;

2. OR where all cases of BSE have been clearly demonstrated to originate directly from the importation of live bovine animals or bovine embryos/ova, and all the affected bovine animals as well as, if these are females, their last progeny born within two years prior to, or after, the first clinical signs of onset of the disease, if alive in the country or region, have been killed and completely destroyed, and either:
(i) The criteria in points (b) to (e) are complied with, but have not been complied with for seven years, or
(ii) It has been demonstrated that for at least eight years no meat and bone meal or greaves has been fed to ruminants, but the criteria in point (c) have not been complied with for seven years.

1.2.6.3 CATEGORY 3: BSE provisionally free country or region where at least one indigenous case has been reported

Any country or region where a risk analysis has been conducted which demonstrates that appropriate measures have been taken for the relevant period of time to manage any risk Identified and:

1. EITHER the last indigenous case of BSE was reported more than seven years ago, the criteria in points (b) to (e) are complied with and the ban on feeding ruminants with meat and bone meal and greaves derived from ruminants is effectively enforced, but:

(i) The criteria in points (b) to (e) have not been complied with for seven years, or
(ii) The ban on feeding ruminants with meat and bone meal and greaves derived from ruminants has not been effectively enforced for eight years;

2. OR where the last indigenous case has been reported less than seven years ago, the BSE incidence rate, calculated on the basis of indigenous cases, has been less than one case per million during each of the last four consecutive twelve-month periods within the bovine animal population over 24 months of age in the country or region or - when in a country or a region the bovine animal population over 24 months of age is less than 1 million animals - one case per real number of this population (calculated on the basis of Eurostat statistics), and where:

(i) The ban on feeding ruminants with meat and bone meal and greaves derived from ruminants has been effectively enforced for at least eight years;
(ii) The criteria in points (b) to (e) have been complied with for at least seven years;
(iii) The affected bovine animals as well as:
- If these are females, their last progeny born within two years prior to, or after, clinical onset of the disease;

- All bovine animals from the cohort are killed and completely destroyed if they are still alive in the country or region concerned.

For this classification account may be taken, by way of derogation from point (iii), of the existence of other measures offering an equivalent level of protection in relation to the killing of animals at risk.

1.2.6.4 CATEGORY 4: Country or region with low incidence of BSE

Any country or region where:

1. The criteria listed above and complied with the BSE incidence rate, calculated over the past 12 months, has been greater than or equal to one indigenous case per million and less than or equal to one hundred cases per million within the bovine animal population over 24 months of age in the country or region; or

2. The criteria listed above are complied with and the BSE incidence rate, calculated as specified in point 1 has been less than one indigenous case per million for less than four consecutive 12 month periods and the affected cattle as well as:

- If these are females, their last progeny born within two years prior to, or after the first clinical signs of onset of the disease,
- All bovine animals from the cohort, if alive in the country or region, are killed and completely destroyed.

For this classification account may be taken, by the way of derogation from this point, of the existence of other measures offering an equivalent level of protection in relation to the killing of animals at risk. Countries or regions where the BSE incidence rate, calculated over the past 12 months, has been less than one indigenous case per million within the cattle population over 24 months of age in the country or region, but where a risk analysis as described has been conducted which demonstrates that at least one of the criteria enabling the country or region to be classified in category 2 or 3 is not complied with, must be regarded as countries or regions belonging to category 4.

1.2.6.5 CATEGORY 5: Country or region with high incidence of BSE

Any country or region where:
1. The criteria listed above are complied with, and the BSE incidence rate, calculated over the past 12 months, has been greater than one hundred cases per million within the bovine animal population over 24 months of age in the country or region; or

2. The BSE incidence rate, calculated over the past 12 months, has been greater than or equal to one case per million and less than or equal to one hundred cases per million within the bovine animal population over 24 months of age in the country or region, and at least one of the criteria listed and is not complied with.

1.2.7 Prohibitions concerning animal feeding

The feeding of protein derived from mammals is prohibited to ruminants.

Member States, or regions thereof, in category 5 shall not be permitted to export or store feed intended for farmed animals which contains protein derived from mammals or feed intended for mammals, except for dogs and cats, which contains processed protein derived from mammals.

Third countries, or regions thereof, in category 5 shall not be permitted to export to the Community feed intended for livestock which contains protein derived from mammals or feed intended for mammals, except for dogs and cats, which contains processed protein derived from mammals.

1.2.8 Standards for certain products of animal origin derived from or containing ruminant material

The use of ruminant material for the production of the following products of animal origin is prohibited:

(a) Mechanically recovered meat;

(b) Dicalcium phosphate intended as feeding stuffs for livestock;

(c) Gelatine, unless it is produced from ruminant hides;

(d) Derivatives made from rendered ruminant fat;

(e) Rendered ruminant fat, unless it was produced from:

(i) Discrete adipose tissue declared fit for human consumption;

HYPOTHESIS :

Slovenia is expecting to come from 4th category as BSE contaminated country to 3rd category. Cases of sporadic Creutzfeldt-Jakob disease (sCJD) and iatrogenic Creutzfeldt-Jakob disease (iCJD) will probably remain rare. Variant Creutzfeldt-Jakob disease (vCJD) has not been detected yet in Slovenia.

PURPOSE OF THE RESEARCH IN MASTER THESIS

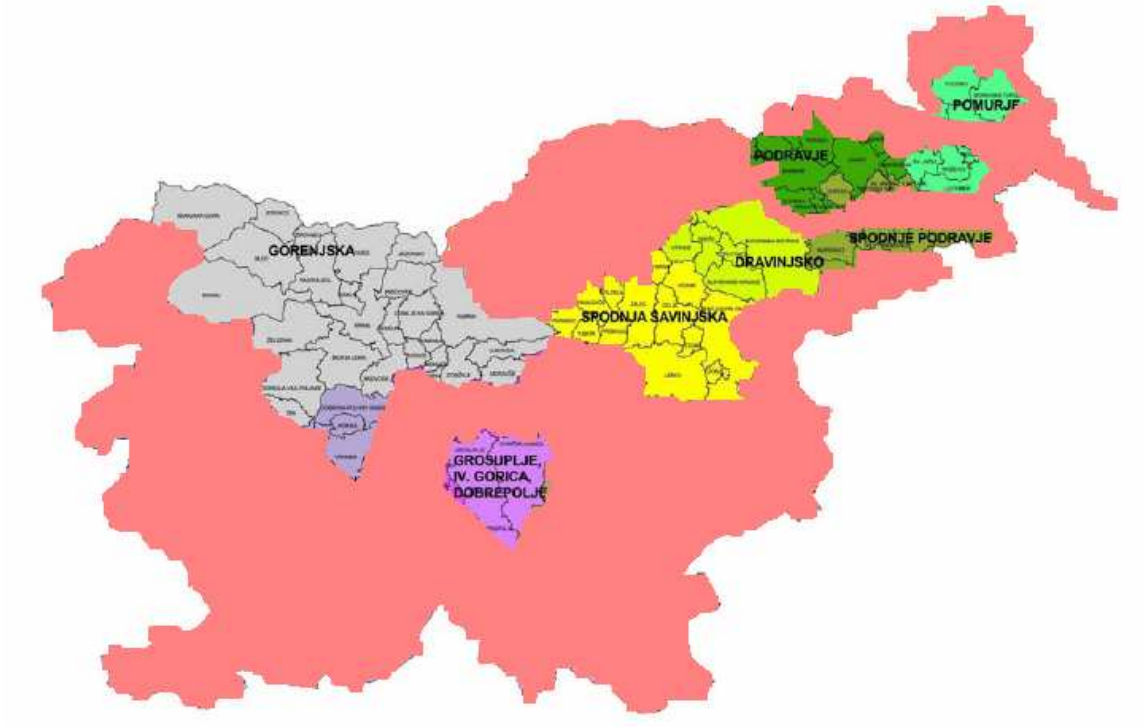
As well known, the use of MBM in cattle food was responsible for the dissemination of BSE in European countries. Thesis of my Master thesis should also include the necessary data for its production and import to Slovenia. There is only a single producer in the country. It appears absolutely to investigate its methods of production, storage, delivery and/or incineration. The time and temperature of autoclaving, use of organic solvents, testing of final product for the presence of ruminant protein with exact method for this testing and to enable the denomination the MBM as ruminant protein free as well. What happened with MBM if not immediately incinerated? Might it be used for other animals? The imported MBM particularly between 1995-2000, how it was controled? Was it requested (to remember, its use was banned for cattles from 1996). Which animals were intended? Where are documents showing that the imported MBM from 1995-2000 was free of ruminant protein? On one side, it use for ruminants was banned in 1996, but on the other side its import was allowed. These data are extremely important for BSE cases in Slovenia.

2 EXPERIMENTAL

2.1 Questionnaire about dairycow fodder

The questionnaire about dairycow fodder was performed in all big milk farms (at least 50 cows) in Gorenjska region (upper and lower region), some parts near Ljubljana (Grosuplje) and in some Stajer region and Prekmurje as presented in Figure 8. In Questionnaire was included 100 farmers.

Figure 8 *: Regions included in questionnaire about dairycow fodder*



1. What were the ingredients of the fodder rate for your cows from the year 1995 until now?
    - Maize and rest of cereals
    - Soya beans
    - Potatoes, beetroot
    - Mowing and hay-making
    - Sowing of grassy-clover mixtures
    - Pasture
    - Vitamine-mineral additions
    - Meat-Bone Meal

    (If you know the country of import, please add or encircle following countries)

    Austria,Croatia,Italy,.....

    - Other

2. What were the ingredients of the fodder rate for your cows before the year 1995?
    - Maize and rest of cereals
    - Soya beans
    - Potatoes, beetroot
    - Mowing and hay-making
    - Sowing of grassy-clover mixtures
    - Pasture
    - Vitamine-mineral additions
    - Meat-bone meal

    (If you know the country of import, please add or encircle following countries)

    Austria,Croatia,Italy,.....

    - Other

.

2.2 The inquiry in the production of Meat and Bone Meal

In Slovenia there is only one facility for production of Meat Bone Meal.

1. Do you have or you have had only your own production of meat-bone meal (MBM)? Have you ever imported MBM?

    - Own production and import
    - Own production
    - Import

    If the answer is import, please write down until which year you have imported and the country of import.

    .....

Have anybody else beside you (if you have imported it) imported MBM?

Yes No

If the answer is yes, please write also the name of the company.

.....

2. What is now the purpose of using MBM out of your plant?
    - Animal fodder (pigs, poultry,...)
    - Manure
    - Goes and combustion
    - Biodiesel
    - Other.....

3. If MBM goes into combustion, do you have the restored control of transportation from your plant to combustion, respectively, have you introduced the HACCP system on this field of work?

Yes No

If the answer is yes, please write down the way of control.
.....
.....

4. Have you ever controled or do you control now the presence of ruminant proteins in MBM?

Yes No

If the answer is yes, please write down which tests have you used or which test you are using for control.
.....
.....
.....

5. Have you ever or are you carrying out now special measures to prevent contamination of MBM ? (measurment of T and p, HACCP system,...)

Yes No

If you encircled yes, please write down the way you use.
.....
.....

6. Please describe shortly the procedure of getting MBM in your plant!

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.....

.....

2.3 The inquiry in manufactures of animal fodder

The inquiry was performed in all 8 manufactures for animal fodder in Slovenia.

1. When did you use Meat and Bone Meal (MBM) for the last time?

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2. When did you use the animal fat for the production for the last time?

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3. For what sort of animals, these fodders were intended for?

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4. What was the per cent of uncleanliness present at used fat?

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.....

5. Who was the producer of meat-bone meal and animal fat?

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.....

6. Do you still use the animal fat?

Yes No

If the answer is yes, from whose producer?
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.....
.....

7. Have you done for instance in the year 2000, when the use of MBM was still allowed at some determined sorts of ruminants, in mixer-house any kind of measures, which could prevent the cross contamination during the process?

Yes No

If the answer is yes, what measures have you carried out?
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8. Has anykind of maesure been carried out to prevent the cross contamination of fodder during the transportation?

Yes No

If the answer was yes, which were the measures that were carried out?

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.....

2.4 The inquiry in the slaughterhouse

The enquiry was performed in all 6 Slovenian slaughterhouses that are slaughtering cattle.

1. How do you cut cow or cattle in first phase of slaughter?
    - Via head
    - Via trunk
    - Other

      …….

2. What do you do with instruments (knives, axe) after the slaughter? Please define the procedure!

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3. When do you do quick test for BSE after or before slaughter?

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4. What do you do to assure the traceability of meat after slaughter ?

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5. What do you do with instruments for slaughter if the result of quick test for BSE is positive?

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6. How do you perform the desinfection of working area if the quick test for BSE is positive? Who is performing this for you?

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7. What is the average of slaughter cows in your slaughterhouse per day? What is the time difference bettwen one to another slaughter during the working time?

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8. Which personal protection do you use during slaughter in working time?

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2.5 The inquiry at the Veterinary Administration board of Republic Slovenia (VARS)

1. Which process do you execute to prevent the spreding of prion diseases?

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2. Which institution carries out these measures and which one controls them?

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3. Which institution educates performers and controllers that perform measures? How often is this carried out and in what form?

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4. Has Meat Bone Meal (MBM) ever been imported?

Yes No

If the answer is yes, please write down the country of import, the year of import and the quantity of imported MBM.

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5. Who carries out diagnostic tests of possible presence of BSE in slaughter houses?

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6. The last three cows infected with BSE were discovered in slaughter house. What tests are carried out in slaughter houses?

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7. How many tests were carried out in slaughter house from the year 2000 until today?

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8. What are the procedures in the case of at the positive test on BSE in the slaughter houses?

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9. Who has an obligation to deal with these procedures?

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3 RESULTS AND DISCUSSION

3.1 Questionnaire about dairycow fodder

***Table 9** : Results of the questionnaire about dairycow fodder*

Type of fodder for dairycow in farms	Before 1995	After 1995 untill today
Maize and rest of cereals	88 %	91 %
Soya beans	20 %	35 %
Potatoes, beetroot	13 %	6 %
Mowing and hay-making	88 %	93 %
Sowing of grassy-clover mixtures	50 %	53 %
Vitamine-mineral additives	59 %	82 %

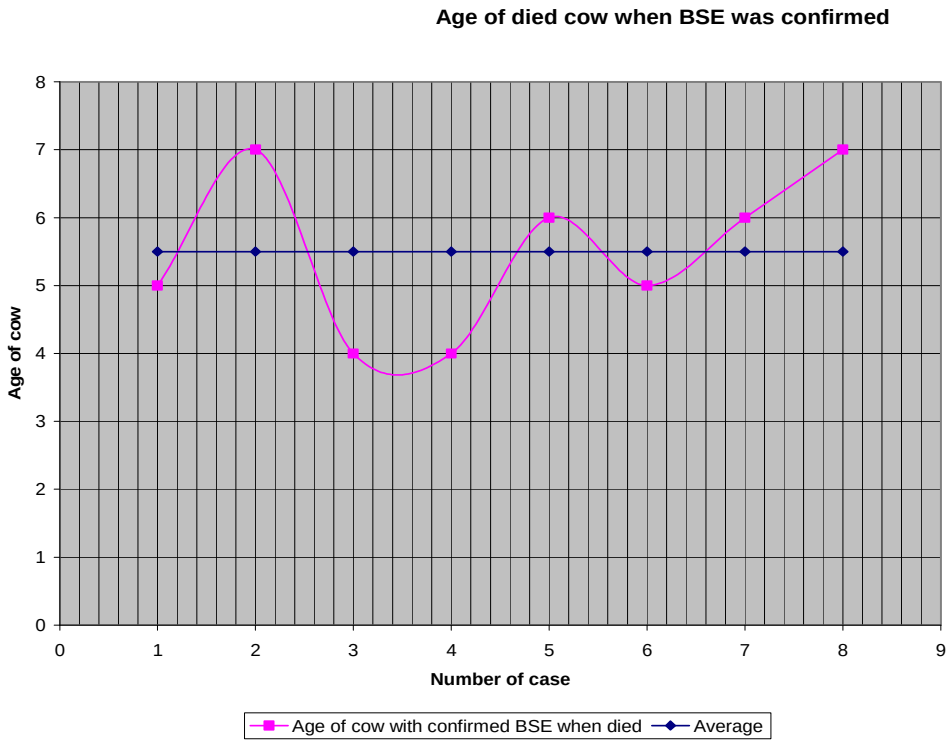
Type of fodder for dairycow in farms	Before 1995	After 1995 untill today
MBM	3 %	0 %
Other	1 %	2 %

Why was questionnaire about dairycow fodder in process? Cows that are used for milk production live much longer than cows that are raised for meat. Here appears very important data about dairycow fodder, which is that farmers from 1995 untill now did not use MBM for fodder. But is this true? They could buy fodder for pigs and poultry , which had as a content MBM (untill the end of 2000), and MBM was also in strong fodders. If farmers did not mix fodder for pigs and poultry with a reason there can happen unintentional crosscontamination, if the farmer is not carefull enough. VARS went to producers of fodder, to farmers, to object that are mixing fodder and to warehouses for fodder and check fodder for ruminants. In year 2006 (data from Congres of Veterinary workers in 2008) in one test of fodder for ruminants in one farm there was disovered residues of fish meat meal because of unintentional crosscontamination with fodder for poultry and fodder for ruminants in warehouse. If this fodder was infected with PrP^{Sc} Slovenian cows can still be at risk to have prion disease BSE. If farmers did not use fodder for pigs and poultry for dairycows than everything is under control, and Slovenia is on good way to become the country with BSE in 3rd cathegory or even better in 2nd cathegory according to Regulation (EC) No. 999/2001 of the European Parliament. In addition we can see, that over 40% of dairycows are in pasture as presented in Table 9. This is very important data, because we know that there can be infected grass or infected soil with PrP^{Sc} and the cow can with oral transsmition pass the infected particles to its own body. How can infected particles PrP^{Sc} come on grass or in soil? There are two ways: 1) via infected MBM used as fertilizer, or 2) if sheeps were there to pasture and they have had prion disease scrapie. Very important data is also that untill 1995 59 % farmers did use mineral additives and that now they are using even more mineral additives 82%. Farmers must be very careful not to use additives that contain Ca^{2+} from bones. We know that this is not in use anymore in European Union,

but we do not know for the other countries, so veterinary inspection must be very carefull what is on declaration, or even more important, what is not on declaration.

The average age of dead cow diagnosted as BSE positive is 5,5 years. The oldest cow with confirmed BSE was in age of 7 and youngest dead cow with confirmed BSE was 4 years old as presented in Figure 9.

***Figure 9**: Age of deceased cow in Slovenia when BSE was confirmed*



3.2 Inquiry in the production of Meat-Bone Meal

It appears of importance to perform testing of Slovenian cases to see whether the types are identical and if so, whether they correspond to types examined in countries, exporting the MBM to Slovenia. The BSE testing in slaughter houses has considerably

decreased in general. What we can do is to control its production to avoid ruminant proteins or to follow the incineration. In Slovenia, however, the only MBM producer had no interest on cooperation. The veterinary administration should clarify this problem.

3.3 Inquiry in manufacturers of animal fodder

1. When did you use Meat-Bone Meal (MBM) for the last time?

- 75 % answers was until the end of 2000,
- 25% until the end of 1999.

2. When did you use the animal fat for the production for the last time?

- 25 % did answer until 1999,
- 50 % until 2001,
- and 25 % until the end of 2003.

3. For what sort of animals, these fodders were intended for?

- 12,5 % answered for pigs and poultry,
- and 25 % did answer that they used only for poultry.
- 62,5 % did not give me any answer.

4. What was the percent of uncleanliness present at used fat?

- 25% of answers were that uncleanliness present at used fat was less than 1 %,
- 25% of answers were that uncleanliness present at used fat was less than 5 %,
- and uncleanliness present at used fat up to 8% was in 25 % cases,
- but 25 % of producers did not use any uncleanliness.

5. Who was the producer of meat-bone meal and animal fat?

- 100 % of answers was the same, they did cooperate only with one factory that produced MBM.

6. Do you still use the animal fat?

- 100% of answers was No.

7. Have you done for instance in the year 2000, when the use of MBM was still allowed at some determined sorts of ruminants, in mixer-house any kind of measures, which could prevent the cross contamination during the process?

- 25 % did answer Yes. They put attention on order of mixing fodder. First they produce fodder for poultry, than for pigs and in the end for cattle. This order is important because as in this order the fodder contained less and less MBM, for cattle was forbiden, so they did this mixing in the end.
- 75% did not do anything to prevent cross contamination.

8. Has any kind of measure been carried out to prevent the cross-contamination of fodder during the transportation?

- 100% of producers of fodder did not do any kind of prevention of cross contamination during the transport.

3.4 The inquiry in the slaughterhouse

1. How do you cut cow or cattle in first phase of slaughter?

- 100% of answers were that they do the slaughter through trunk.

2.What do you do with instruments (knives, axes) after the slaughter?

- 83, 3 % did answer that they use cold and than hot water and for finalization they use sterilazer.

- 16, 7 % of slaughterhouses use only mechanical cleaning (physical method) and desinfector.

3.When do you do quick test for BSE, after or before slaughter?

- In all slaughterhouses the quick test is used after the slaughter (post-mortem test).

4.What do you do to assure the traceability of meat after slaughter ?

- They all use (100% of them) the number in the ear and LOT number that is on every piece of meat from the slaughtered animal.

5.What do you do with instruments for slaughter if the result of quick test for BSE is positive?

- 66,7 % of slaughterhouses do the sterilization.
- 33,3 % of slaughterhouses do the sterilization by the instruction of VARS (they had the expirience with positive BSE case).

6.How do you perform the desinfection of working area if the quick test for BSE is positive? Who is performing this for you?

- For 66,7 % of slaughterhouses VARS or Institut of public health is performing desinfection of working area.
- 33,3 % of slauhgterhouses do desinfection by themselves (usually in small slaughterhouses).

7.What is the average of slaughtered cows in your slaughterhouse per day? What is the time difference between one to another slaughter during the working time?

- Up to 10 cow per day – 50 % of slaughterhouses – small slaughterhouses.
- Up to 20 cow per day - 16,7 % of slaughterhouses- medium slaughterhouses.
- More than 100 cow per day – 33,3 % of slaughterhouses- big slaughterhouses.

8.Which personal protection do you use during slaughter in working time?

- 66,7 % of workers in slaughterhouses did answer that they used gloves (iron gloves and rubber gloves), rubber apron, rubber boots and head gear.
- 33,3 % of workers in slaughterhouse do use gloves (iron gloves and rubber gloves), rubber apron, rubber boots and helmet (16,7% said that this helmet has a glass to cover eyes).

Routine desinfectants are not recommended for prion decontamination because of inefficiency. Physical methods for inactivation prions are also ineffective. There is no performing of prion decontamination in all slaughterhouses. If we would like to prevent crosscontamination during the slaughter or after slaughter (next day) workers in slaughterhouses should know what is prion decontamination and how should be performed to work properly and that results are as we expect - which means that instruments are free of prions.

One thing in this inquiry suprised me. Workers in slaughterhouses do not use any kind of protection for there eyes. What is the reason for such behaviour? There are two reasons why they do not use eye protection. One is that they are not trained enough why they should protect their eyes, and the other is, that conductors responsible for personal protection, do not give them protection or they do not do enough control how personal protection is used. Eyes are very sensitive, and during the slaugher there is a lot of blood (red cells units are highly infective, if there are prions in the blood), that can come into worker's eyes.

3.5 The inquiry at the Veterinary Administration board of Republic Slovenia (VARS)

1. Which process do you execute to prevent the spreading of prion diseases?

Laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (TSEs) they perform practices according to directive 999/2001 and they are :

- Prohibition of using animal ruminant's proteins in fodder.
- Performing programs of taking control over TSE in different animal species (cattle, sheep, goat and deer).
- They are excreting risk materials (SRM) from food chain.
- They have measures how to act when there is suspicion of TSE in animals or if there is confirmed case of TSE in animals.
- They perform genetypisation for sheep with purpose to discover gene resistant sheep and to eliminate those with unresistant genes for TSE.

2. Which institution carries out these measures and which one controles them ?

These measures are performed by VARS, and also farm holders of activity (farmers as owners of animals and food manufacturers) and National Veterinary Institute (NVI) at Veterinary Faculty and veterinary organization. Official surveillance is performed by VARS.

3. Which institution educates, performes and controlls these measures? How often is this carried out and in what form?

The education is performed in several levels. They do education with:

- animals owners,
- manufacturers of food,
- official veterinary workers,
- NVI workers, and
- veterinary workers in organizations.

The performers of education are VARS, Veterinary Faculty, Veterinary Chamber and Chamber for Agriculture.

4. Has meat bone meal (MBM) ever been imported?

Yes, MBM was imported untill the end of 2000, with the severely determined import requirements from countries without confirmed BSE case. From 02.12.2000 the import of MBM, meat meal, bone meal, fat, fat from animal residues and cadavers and all the fodder from animal cadavers and all fodder with previously mentioned substances was prohibited by directive 111/00. From 2.12.2000 onward there was no import of MBM in Slovenia. From 01.04.2004 they are importing it from 3rd countries by the severely determined import requirements, determined by EU. Results are presented in Table 10.

***Table 10**: Import of MBM in Slovenia*

Year	Import of MBM in tones / Country of import
1999	41 Croatia
2000	97 Croatia
2000	47 Slovakia

The prohibition of using MBM in fodder for cattle is from 1996, and for other animals, that are used for human food from 1.1.2001.

5. Who carries out diagnostical tests for possible presence of BSE in slaughter houses?

Samples in slaughterhouses are taken by official veterinary workers, who are performing the official surveillance. The tests are performed by locensed laboratory of National Veterinary Institute at Veterinary Faculty in Veterinary public health and diagnostic division- diagnostic sector. They are performing tests by directive ES 999/2001 Annex X.

6. The last three cows infected with BSE were discovered in slaughter house. What tests are carried out in slaughter houses?

Official veterinary workers are sampling all healthy cattle ment for human food older than 30 months, and unhealthy cattle that were slaughtered urgently and are older than 24 months. They are sempling obex in cattle,in small ruminants they sample obex and part of cerebellum. All samples are tested by quick test. In the case of positive results they do perform confirmaltory test-pathological and immunohistochemical test.

7. How many tests were carried out in slaughter house from the year 2000 until today?

Untill 2007 were performed **309.780** tests on cattle as presented in Table 11. Number of positive cases was 8.

***Table 11**: Number of BSE tests performed on cattle and number of positive tests in Slovenia*

Year	**Number of tested cattles**	**Positive cases**
2001	32.616	1
2002	64.496	1
2003	66.167	1
2004	45.666	2
2005	36.784	1
2006	32.667	1
2007	31.384	1
Total	**309.780**	**8**

8. What are the procedures in the case of at the positive test on BSE in the slaughter houses?

Measures in case of suspection and then in case of positive BSE case (quick test and confirmatory test) is performed by directive ES 999/2001 and are written in Measurments of procedures in the case of positive BSE case.

The measurements are performed by VARS, manufacturers for food (slaughterhouses), farmers (owners of animals) and by National Veterinary Institute NVI. Production of MBM in Slovenia in tones in presented in Table 12.

***Table 12**: Production of Meat and Bone Meal in Slovenia*

Years*	Quantity of MBM in tones
1995	11 636
1996	13 325
1997	13 868
1998	14 598
1999	15 174
2000	14 940

**there is no confidental data before 1995*

Source : Data from Slovenian custom administration / Slovenian Television

***Table 13**: Import and export of MBM from Slovenia*

Data from Slovenian custom administration / Slovenian Television		
Year	Export of MBM in tones	Import of MBM in tones / Country of import
1990	272	429 Austria,Italy
1991	1839	135 Italy, Germany
1992	1643	216 Croatia, Austria, Italy
1993	1582	57 Croatia, Austria
1994	670	299 Austria, Croatia
1995	480	474 Austria, Croatia, Italy

Data from Slovenian custom administration / Slovenian Television		
Year	Export of MBM in tones	Import of MBM in tones / Country of import
1996	394	555 Austria, Croatia, Italy
1997	912	302 Austria, Croatia
1998	1321	432 Austria, Croatia
1999	1527	122 Austria, Croatia
2000	2044	454 Austria, Croatia

There is no doubt that Slovenian BSE cases must have been infected with contaminated MBM in the cattle feed (MBM), either from the own production or from abroad. BSE cases in countries exporting MBM to Slovenia as presented in Table 13 were Slovakia (23 BSE cases) 5, 6, 2, 7, 3 from 2001-2005; Italy (127 BSE cases) 48,38,29,7,8,7 from 2001-2006; Austria (5 BSE cases) 1,2,2 2001, 2005-2006.

3.6 Studies on Meat and Bone Meal (MBM)

The use of MBM as cattle or other animal food has been banned in most European countries as well as in USA, Canada and Japan. This ban has also been valid for Slovenia. Theoretically, its use as cattle food would be possible, if it is PrP^{Sc} free. How difficult is this and not very safe, appears evident from the Table in Annex 1. That is indicating how much research, how complicated and how high priced the methodology appeared. It has not been well standardized and automatised for mutliple, simultaneous testing of MBM batches produced by rendering companies. Tests for the PrP^{Sc} at slaughter houses have already been automatised and available for multiple testing using the best available method (Western blot), but the results have been frequently false negative in asymptomatic animals, although infectious. The number of such testing has significantly decreased recently, particularly in Switzerland. Some rendering companies have supplied PrP^{Sc} negative batches of the MBM , particularly when hyperbaric steam

was used (Taylor, 1997b). But this has not always been true and the control of each MBM batch cannot be avoided. This does not seem to be possible because the batches of MBM should be small. The use of the MBM for swines and poultry appears possible from the side lateral animals, but side infections with cattles could be expected as documented in Germany and France. The possibility of human infections has also been evaluated. The extreme infectivity of the PrP^{Sc} (10^9 per ml) must be taken into account (one cattle brain infects for more than 100.000 animals).

Other possibilities how to use the MBM have been recently studied. Incinerated MBM may be used as fertilizer (only incinerated; see prions in soil) or as source of calcium, the raw MBM can be used for the production of the bio-diesel (confirmed to be free of PrP^{Sc}) etc. Much work has to be done in the near future to answer such questions. It should not be forgotten that BSE in cattle will exist for many years resulting from 10% conatally transmitted infections, which are, by now for the diagnostics in asymptomatic cases. In any case the current public health measures have intended to exclude high risk bovine tissue from the animal food chains.

A summary for future investigations was published by Brown (2006). Future studies have to be undertaken in countries with large national herds but without orally acquired infections (Argentina 50 millions cattle, Australia 30, USA over 100 millions). Millions of molecular-biology tests will have to be performed, but financial supports have not yet been available.

The future and success of the prion research is not clear in spite of over 10 thousands scientific articles published, and several hundreds millions US $ invested internationally. The virus theory has been still discussed by serious scientists. A successful therapeutic facility does not exist at all. The 17 millions European meat and bone meal produced have been still open for discussion.

3.6.1 The future of Meat and Bone Meal

The future of MBM is not clear. It is actually banned from the food and feed chain because of possible contamination with prions. As the consequence of the BSE epidemics in UK, the ban on feeding ruminant proteins to ruminants was introduced in 1988, and another ban on using materials from cattle tissue, most likely to be infective (brain, spinal cord, intestines) for human food, and another ban in 1991 on using such materials for feeding poultry and pigs. In 1994 the EU banned the use of all mammalian proteins in ruminant feed (EU decision 1994/381): since October 2000 specific risk material (SRM) has to be removed from the food and feed chain Europewide (EU decision 2000/418). Due to the BSE crisis in France and Germany, a 6 months ban on feeding animal MBM for livestock (EU decision 2000/1234), was introduced. Germany and some other countries forbade the use of animal fat in feed. The rendering industries has been forced to find the possibilities for the use of 16 million tons of animal byproducts and animal carcasses including MBM. Animal MBM will be combusted almost completely in power stations and cement works (actually under protect from local authorities; (Delo March 8, 2008) . Animal fat was mostly used in the chemical industry or burnt. A more economic use is the production of biodiesel, based on fatty acid methyl esther (FAME) by transestrification of the extracted animal fat (analogous to the production of biodiesel from vegetable oils (purely or blended with conventional diesel fuel in diesel fuel in diesel engines)). The dificulty is the potential presence of prions in raw material. The high resistence of prions to decontamination procedures has been well known. A study has been performed to analyse whether this biodiesel is safe for humans and environment. The highly sensitive Western Blot was used to analyze the manufactured biodiesel (Siedel, 2006) from prion-contaminated animal fat. The results confirmed that the biodiesel produced was safe under the tested process conditions (each single step of the process leads to a significant reduction of the concentration of prions (PrP^{Sc})). However, it cannot be generalized, because of different process controls, using other conditions but it is clear, that a bio diesel production represents a more economic usage that than the combustion of such material.

As previously noted the MBM production must be safely disposed. Specific incineration remains an alternative offering the oportunity to achieve both thermal valorization and solid waste recovery as ashes or calcium phosphate rich material. Studies were also performed (Deydier, 2007) to analyze ashes efficiency for in situ remediation of lead contaminated aqueous solutions and soils and to asses the bioavailability of lead. No toxic or genotoxic effects of ashes were observed with concentrations of 0,1-5 g of ashes/L. Only 100 mg of ashes/L neutralized lead toxicity even with lead concentrations up to 10 ppm. Ashes do not only neutralize lead toxicity but also act as a fertilizer. Another study (Mouchet, 2007) confirmed the toxicity and genotoxicty of lead in the aquatic compartment suggesting the potential utility of the MBM combust residues for use in remediation. Ashes from specific incineration (laboratory) and co-incineration (industrial process) were also studied (Coutand, 2007). Three of the four MBM ashes were mainly composed of calcium phosphates. The major compositions were in the range of natural phosphate rocks. Trace elements, including heavy metals were below 0,6%, but industrial ashes contained much more heavy metals. According to EU classification the laboratory ash can be classified as an inert waste and two industrial ashes were also mostly inert. Only one was higly leachable. The results considered to give some economic value to these ashes.

3.7 Rendering processes and controls in Slovenia

It is well known that specific risk material (SRM) residues from BSE infected cattle may contaminate the sewage in slaughterhouses (Yamamoto, 2006). British experience in the course of BSE epidemic revealed a significantly higher contaminating probability in small, private slaughterhouses in comparison with modern, technically well equipped locations. A proportion of the sludge, discharged from wastewater treatment, has been historically used to produce fertilizer. Instalation of satisfactory drains to the wastewater may significantly reduce the contamination of sewage. Tissues of, mostly clinically inapparent BSE animals, may infect slaughter works and personal. This possibility is

real and it was confirmed by appropriate studies. In this cases, also, the contamination probability appears greater in small, private localities. How was this problem handled by veterinary administration : with disinfectants. Widely used in the routine practice, may be good disinfactants, according to normal microbiological criteria, but they are ineffective for prions. How are the slaughtering instruments decontaminated? The best chemical for this purpose has been sodium hydroxide and it also appears acceptable for the environment. Which concentration should be used? How long? How is the chemical decontamination performed in private stalls? This is praticular important for stalls with animal cadavers. How is this regulated? How is to handle instruments and equipments? Remember the iatrogenic CJD infections in hospitals, and the succesfull transmission, by instruments used, to another surgery candidate. The term »prion decontamination« has been misused by now, and this should be considered for the future. A study compared also risk of spreading disease by slaughtering in France and Great Britain. A higher risk was detected in France (Jacob, 2007).

4 CONCLUSIONS

Slovenia has an education programme for veterinarians, breeders and those who transport, trade and slaughter bovine animals and also for farmers, which seeks to encourage them to report all cases of neurological manifestations in adult bovine animals.

Slovenia is also cooperating in the compulsory reporting and examination of all bovine animals showing clinical signs of BSE.

A system in Slovenia has continuous surveillance and monitoring of BSE and CJD with particular reference to the risks, taking account of the guidelines or in accordance with the appropriate international standards; reports on the number of examinations carried

out and results are kept for at least seven years. According to Table 11 this is performed and they are working according to directive EU 999/2001.

Laboratory workers do the examination in an approved laboratory from VARS (National Veterinary Institute (NVI)) of samples of encephala or other tissues collected under the surveillance system taken by veterinary workers.

For this classification, the existence of other measures should be taken into consideration offering an equal level of protection in relation to the killing of animals at risk.

Slovenia is following criteria by the EU directive 999/2001. If Slovenia will continue to following the rules, it can be put from the 4th category into 3rd of BSE status in the near future (around the year 2014); if there will be no cases of BSE in next five years.

5 SUMMARY

First case of severe neurological disease in cattle, resembling scrapie in sheep, was published in 1986. Shortly after the appearance of BSE in the UK, the causative responsibility of Meat and Bone Meal (MBM) was significantly confirmed. It was first assumed, that the aetiological agent, named prion (proteinaceous infections particle) present in the MBM, originated from sheep scrapie, which slaughter offal's were also used for cattle feed. Clinical and laboratory methods confirmed the similarity of both diseases. Recent studies indicated that scrapie prions are able to persist in the soil for years, without loosing their infectivity. Scientific data have also confirmed the molecular-biology similarity of the pathological prion (PrP^{Sc}) in the natural scrapie sheep and BSE linked diseases.

Actually available molecular-biology methods have enabled the detection and evaluation of many prion strains in humans and animals. For the transmission experiments transgenic mice with bovine or human prion genes (mo^{bo}, mo^{hu}) were assayed to avoid species barrier. The most important study characteristics were incubation, molecular mass with glycosylation, transmissibility, conformation and protease resistence. It appears of extreme importance that they are retained during serial passages and are not linked to the infecting prions only. The strains have been detected in all human and animal prion diseases.

The use of MBM as foodstuff for poultry and pigs has been actually discussed in several countries, although we know, that the safe control of it for the presence of prions is practically not possible (17 milions tons per year produced in EU). What to do with it?

Slovenia needs a good prion laboratory to study the development of prion strains. Such laboratory already exists in Slovenia, so the final diagnostic should be done in this laboratory to avoid sending materials abroad. The bioassay must also be possible. A close cooperation between human and veterinary medicine must be forced; the results of such uncooperation, as happened in the UK, are well known. Animal and, particularly human prion diseases will probably continue to exist for a long period of time.

The use of MBM for foodstuff is now prohibited. Use of MBM as cattle food would be possible, if proven that is PrP^{Sc} free. Incinerated MBM may be used as fertilizer or as source of calcium, the raw MBM can be used for the production of the bio-diesel.

Slovenia is following all criteria by the EU directive 999/2001. If Slovenia will continue to following the rules, it can be put from the 4th category (country or region with low incidence of BSE) into 3rd (BSE provisionally free country or region where at least one indigenous case has been reported) of BSE status in near future (around the year 2014), if there will be no cases of BSE in next five years.

A new BSE epidemic should not happen again but this can not be stated with certainty.

5 POVZETEK

Prvi primer številnih nevroloških boleznih pri govedu, povzeto po praskavcu pri ovcah, je bil opisan leta 1986.Vzročna krivda za epidemijo BSE, je bila kmalu po začetku epidemije dokazana v uporabi mesno-kostne moke (MKM). Prva predvidevanja o povzročitelju bolezni,imenovanem prion, ki naj bi bil v MKM, so izvirala iz s praskavcem okuženih trupel ovc, ki naj bi se uporabljala za krmo goved. Klinične in laboratorijske metode so potrdile podobnost obeh bolezni. Nedavne raziskave so pokazale, da praskavčevi prioni lahko ohranjajo svojo infektivnost v zemlji, tudi do nekaj let, brez izgube le-te. Znanstveni dokazi so potrdili tudi molekularno-biološke podobnosti patološkega priona (PrP^{Sc}) v naravno pojavljajočem praskavcu in z BSE povezanimi boleznimi.

Dostopne molekularno-biološke metode so omogočile detekcijo in ocenitev tako imenovanih prionskih tipov pri človeku in živalih. Da bi se izognili medvrstni barieri, so naredili tako imenovane transgene miške z govejimi in človeškimi prionskimi geni (mo^{bo}, mo^{hu}). Najbolj pomembne študije karakteristik bolezni so določevanje inkubacijske dobe, molekulske mase z glikolizacijo, prenosljivost, konformne oblike in rezistence na proteinazo. Pokazalo se je za zelo pomembno, da ti znaki med pasažami pri enakem tipu ostanejo enaki in niso vezani le na infektivnost prionov. Tipe bolezni so zasledili pri vseh človeških in živalskih prionskih boleznih.

O uporabi MKM, kot krme za piščance in prašiče se je razpravljalo v večih državah, čeprav vemo, da je varnostna kontrola le-te na prisotnost prionov praktično nemogoča, če vemo da v Europi letno pridelajo 17 milijonov ton MKM. Kaj naj naredimo z njo?

Slovenija potrebuje dober prionski laboratorij, v katerem se bo razvilo ločevanje tipov prionskih bolezni. Tak laboratorij v Sloveniji že obstaja, torej bi lahko vso potrditveno diagnostiko izvajali pri nas, hkrati pa bi se izognili pošiljajnu materiala v druge referenčne laboratorije v Europi na potrditev diagnostike. Potrditev bolezni mora biti mogoča tudi z testnimi živalmi. Nagibati moramo k tesnemu sodelovanju medicinske in

veterinarske stroke, saj po izkušnjah v Veliki Britaniji dobro vemo, kam nesodelovanje med tema dvema vejama znanosti pripelje. Človeške in predvsem živalske prionske bolezni bodo verjetno obstajale še dolgo vrsto let.

Uporaba mesno-kostne moke kot krme za živali je zdaj prepovedana. Uporaba MKM za goveda, bi bila možna če bi dokazano bila prosta PrP^{Sc}. Sežgana MKM se lahko uporablja kot gnojilo ali kot vir kalcija, surova MKM pa je lahko uporabljena v proizvodnji bio-diesla.

Slovenija sledi vsem kriterijem za preprečitev prionskih bolezni skladno z EU direktivo 999/2001. Če bo Slovenija še naprej dosledno upoštevala pravila, bo lahko prešla iz 4 kategorije države okužene z BSE (dežela z nizko incidenco BSE) v 3 kategorijo (dežela brez BSE, kjer pa je bil prijavljen v preteklosti domači primer bolezni) v bližni prihodnosti (okoli leta 2014), če v naslednjih 5 letih ne bo zabeleženega nobenega primera BSE.

Nova epidemija BSE naj se ne bi ponovila, žal pa tega ne moremo trditi z gotovostjo.

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ANNEX 1: Detection of ruminant proteins in foodstuffs details

***Table a**: Studies to detect ruminant (sheep and cattle) proteins in human and animal foodstuffs details*

Material	Methods	Comments	Authors
Rendering mass (MBM)	**Bioassay in laboratory animals**	**The only test confirming the prion infectivity**	**Taylor, 1997b**
Animal stuffs with less than 0,125% of MBM	PCR for bovine derived meat and MBM	Results specific, rapid and sensitive	Tartaglia, 1998
Brain or spinal cord in meat	ELISA for glial fibrillary acidic protein (GFAP)	Valid and repeatable	Schmidt, 1999
Cooked sausages	Immunohistochemistry	High specificity of immunoreactions	Wenisch, 1999
Beef carcasses, CNS tissues	ELISA for glial fibrillary acidic protein (GFAP)	Presence of normal sausage ingredients did not affect the detection of GFAP	Schmidt, 2001
Bovine materials in cattle feedstuffs	PCR with specific primers for bovine specific DNA	Detection of 0,125 % of bovine derived material	Krcmar, 2001
Meat, blood samples different species	165rRNA gene mitochondrial DNA	Detection limit 0,0625 %	Bottero, 2003
Bovine CNS in meat and meat products	Reverse transcription PCR coupled with GFAP	0,5 % brain homogenate successful	Seybold, 2003
Cattle food (MBM)	PCR targeting Subunit 8 of the ATP synthesis gene	Effectful from 0,1 to 0,01%	Kusama, 2004
Cattle food (MBM)	Real-time fluorescent PCR	Sensitivity, specificity 100% at 0,005%	Rensen, 2005

Material	Methods	Comments	Authors
Complete cattle feed	PCR using DNA forensic kit for DNA extraction	Sensitivity 0,006% at 16 hours incubation	Yancy, 2005
Cattle feed	Lateral-flow test kits. Neogen corp. FDA comparable	Detection level 2%	Myers, 2005
Raw and heat treated cattle meals	PCR. DNA sequence 12SrRNA (val) - 165rRNA gene	Detection limit 0,05% available for heat treated	Ha, 2006
Meat products, CNS tissues	F (fluorescent) EIA, R(Ridascreen) EIA, IHC (Immunohistochemistry)	(F) EIA the best	Hossner, 2006
CNS + or - meat	(R) Ridascreen EIA targeting GFA protein	Sensitivity 97,9 Specifity 97,9	Bozzetta, 2006
Bovine meat and MBM	PCR primers 1t 134-154 and 233-213	Contamination level 0,1%	Henk, 2006
Bovine blood in feed	Sandwich ELISA with monoclonal antibodies	Sensitive 0,05%-0,5%	Ofori, 2007
MBM in feed	Bovine mitochondrial DNA in MBM PCR with cattle specific primers	Pos. with 0,1% added MBM	Toyoda, 2004
Bovine DNA in cattle feeds	Fluorescent PCR	Sensitivity 1% with commercial Neogen	Sawyer, 2004
Bovine & other animals	PCR for animals mtDNA (canine, feline, bovine, ovine, porcine or poultry)	Bovine mtDNA in 27 or 37 samples	Myers, 2004
Rendered material	DNA forensic kit	Bovine accuracy 98,9%	Myers, 2006

ANNEX 2: Diagnosis details

Pathohistology and immunocytochemistry

Pathohistology is one of the first methods for confirmation of Prion diseases. Degenerative changes were demonstrated histologically in certain brain stem gray matter locations with neuronal degeneration and loss of neurones with discretes ovoid or spherical vacuoles or microcavities. Balooned neurons with a narrow rim of cytoplasm are responsible for a spongiform appearance. A mild gliosis accompanied degenerative changes. Scrapie associated fibrils as observed in scrapie, were also found. The method has still been used in the majority of pathologic laboratories worldwide, but it cannot be used on a large scale simultaneously.

Immunocytochemistry has been, by now, accepted as a safe diagnostic method. The approval based on the UK five centre consensus report. Laboratory produced monoclonal antibodies (see Table a) ; mostly from mice, reacted with pathologic prions in the tissue section (mostly brain). After appropriate incubation a secondary antibody (mouse anti-globulin), labelled with a visualizing agent, is added and counterstained. The original, very carefully developed method, as showed in the Table b is very time consuming and is used for special purposes. In the beginning of performing diagnostic tests the normal cellular protein (PrP^c) had to be destroyed to avoid false positive reactions. mAbs were then prepared to avoid reaction with the normal PrP^c. The Table a shows the partial sequences of residues (amino acids) generated as antigens for the immunization of mice. There are many more by now.

***Table a** : Monoclonal antibodies / Immunogens / PrP sequences (Residues)*

23-40 23-85 54-60 58-71 79-92 89-104 90-155 95-108 95-110 106-126 109-112 139-150 140-180 141-159 142-160 144-152 **144-156** 145-163 151-165 167-179 200-231 **214-226** 221-234

Antibodies to the prion protein (PrP) have also been studied in the last years. Andrievskaia (2006) tried to develop antibodies against bovine recombinant prion protein (brecPrP) in four strains of mice. Immunization of antoimmunity- prone mice NZB/NZW F (1) was tried, for the first time, to overcome the self tolerance against PrP. Several monoclonal antibodies recognizing continuous and discontinuous epitopes were established. BALB/c mice produced antibodies titering 1:1000 to 1:1500, by Enzyme immunoassay, the NZB/NZW F(1) mice responded with titers 1:7000 to 1:11000. A panel of sera recognized continuous epitopes. In Ljubljana (Cernilec, 2007) mice lacking the prion gene /PRNP/ were used for immunization. Immune response was achived immunizing the BALB mice with bovine recombinant PrP (brecPrP). Antibody E12/2 reacted specifically with bovine and human PrP but not with PrP from other mammals. It epitope was located at the C terminus and of helix 1; residue 155 (His) was crucial for binding structural differences mice-bovine PrP. They are sufficient to provoke a prominent humoral response. In Japan (Furuoka, 2007) carried analyses on brain sections of cattle with BSE, sheep with scrapie, mice infected with scrapie and humans with CJD or GSS. Immunoreactivity varied probably because of differences in the aminoacid sequences of the PrP in various species. Some mouse recombinant PrP (morecPrP) reacted strongly with bovine, ovine and murine PrP^{Sc} even though the aminoacid sequences, determined by the antibody epitope were not fully identical with aminoacid sequences proper to the species. In the region 134-159 of moPrP. Not all epitopes play an important role in antigen-antibody reaction. The presence of the core epitope is of vital interest to understand the antibody binding ability.

In 2004 a monoclonal antibody (V5B2), raised against the peptide (214-226) from the C-terminal part of the prion protein, recognized an epitope specific for the pathologic form of it (PrP^{Sc}) i.e. sequences 214-226 used for immunization. Recently another such monoclonal antiserum was produced. They enable the performance of the test before previously destroying the competitive normal cellular form of it (PrP^{c}).

Table b summerizes details of the immunocytochemistry technique.

***Table b**: Consensus protocol for PrP^{CJD} Immunocytochemistry*

Step	
1	5μm sections floated on to Vectabond-coated slides
2	Sections to water
3	Picric acid 15 min
4	Water
5	3% hydrogen peroxide 30 min
6	Water
7	Hydrated autoclaving (121^{o}C for 10 min in distilled water)
8	Water
9	96% formic acid for 5 min
10	Water
11	4 M quanidine thiocyanate for 2 h at 4 ^{o}C
12	Water then Tris buffered saline
13	Blocking serum for 20 min
14	Exposure to primary PrP antibody
15	Tris buffered saline
16	Exposure to secondary antibody
17	This buffered saline
18	ABC kit
19	Tris buffered saline
20	Visualizing agent
21	Water
22	Haematoxylin counterstain
23	Dehydration, clearing and mount in Pertex
24	Dried, mounted slides may be decontaminated again by immersion in 96 % formic acid for 5 min before labelling. This is regarded as a useful additional precaution as the slides leave a possibly contaminated laboratory.

Source : Bell, 1997

Antibodies for the PrP^{Sc} may be polyclonal or, most frequently by now, monoclonal, produced mostly in mice. The immunocytochemisrty method was described in details. Formic acid, guanidin thiocyanate and hydrated autoclaving pre-treatment in conjunction with monoclonal PrP^{Sc} antibody (purchased) produced the clearest results. Diaminobenzidene was used as visuolizing agent. However, the method could not, in all cases, distinguish clearly, between PrP^{C} and PrP^{Sc}. Guanidin thiocyanate served also as decontaminating agent. The method gave satisfactory results, in most cases, with experienced examiners, but does not appear useful for large-scale testing. It was recomended to store the pathologic material at - 70 ^{o}C or, at least, at -20 ^{o}C. Details on the technique are from Bell (1997).

Western blot (WB)

Western blot (WB), known since 1979 has been widely used for the diagnostic of human infections with the Human Immunodelficiency Virus (HIV). It appears, by now, as the most sensitive and specific laboratory method for the diagnostic of human and animal prion disease including BSE. The only superior diagnostic method is the bioassay on animals (mostly mice), but it takes weeks or months for final interpretation of the results. WB analysis appears relatively simple when molecular bands are strong and easily interpreTable. The method was also automated and used for simultaneous testing of cattle material from slaughterhouses. The test presents the molecular mass and glycosylation state; it has also been used for the strain differentiation, that become considerably more important in the last few years, particularly to verify the BSE types as for instance, the recently discovered BASE types (sporadic BSE ?).

In 2001 the sensitivity of WB was significantly improved. The proteins (BSE brain material) were first digested with the Proteinase K to eliminate PrP^{c}. After a sucrose density gradient the material was electrophoresetically separated on 16% tris-glycine polyacrylamide gel and electroblotted on to polivinylidene fluoride membrane and then cut into strips. Blots were inoculated with a monoclonal antibody to PrP^{Sc} (mouse) and,

finally, treated with goat antibody to mouse IgG conjugated with alcaline phospharase. The higher sensitivity of the method depends on the use of Sodium Phosphotungstic Acid (NaPTA), that selectively precipitate PrP^{Sc} but not PrP^{C}.

The WB also enabled (partially) to correlate with the diagnostic sensitivity of the bioassay (mostly performed with mice). Transgenic mice (Tg mo) expresing bovine (bo) prion protein (PrP) serially propagate BSE prions and there is no species barriere for tansmission from BSE cattle to Tg mo expresing PrP. These mice were also highly susceptible to BSE and variant CJD prions. The incubation time and neuropathology were also indistinguishable. These two characters are specific for all prion strains and they are retained by serial passages as well.

Enzyme Immunoassay (EIA), Enzyme Linked Immunosorbent Assay (ELISA)

The base of the test is the fact, that one molecule of an enzyme can split many molecules of a substrate thus giving rise to, and an accumulation of a large number of end-product molecules. The breakdown product of the substrate can be identified visually, microscopically or radiometrically, colorimetrically, fluorometrically or by luminescence. Among a number of enzymes alkaline phosphatase or horseradish peroxidase appeared as most versatile and applicabile for diagnostic microbiology. They may be used as a label for imunoreactant (IgG, IgM, IgA). The method uses a solid support (usually polystirene) in form of microplates (usually with 96 wells or indentations). The BSE brain preparation to investigate for PrP^{Sc} presence is first fixed to this support. Some test modifications first destroy the PrP^{c} to not participate in the test itself. In the next step PrP^{Sc} Ab (monoclonal, mouse) is added. In positive cases it will bind to the for PrP^{Sc} antigen and form an immunocomplex-specific binding of Ag and Ab. Finally, an enzyme labeled mouse immunoglobulin is added which react, in positive cases, with the mouse globulins in the complex and a colour change develops. It appears

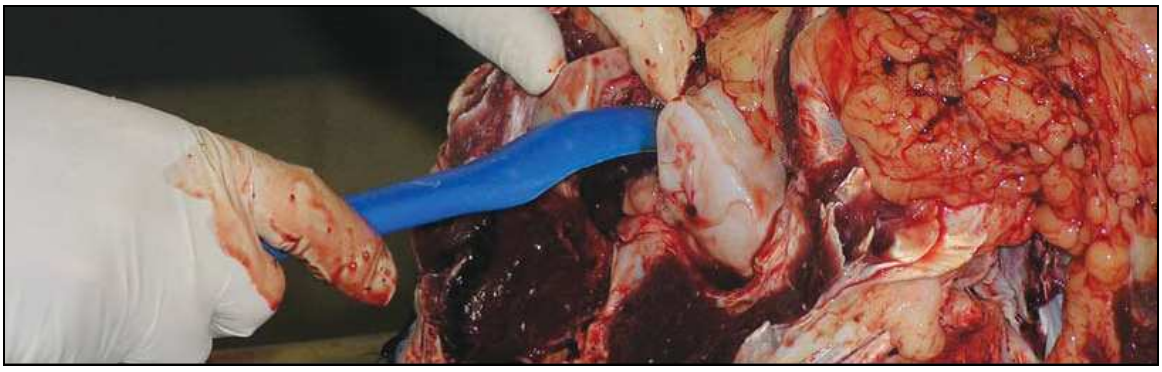
readily visible, but it should be read by photometer to determine the optical density which determins the result.

The EIA tests are useful for mass-testing of a large number of previously prepared cattle brain materials from the slaughterhouses, for the presence of pathologic PrP^{Sc}. It also can be used for the detection of ruminant proteins in the Meat and Bone Meal (MBM): The sensitivity and specificity of EIA compares with that from WB but does not give molecular-biological informations conserning the molecular mass and glycosylation, used for the strain typing. This has become considerably more important in the last years.

Enzyme Imunoassay (EIA, ELISA) for BSE diagnostic-direct ELISA

1. Adsorbtion of treated brain material (with or without BSE for PrP^{Sc}) to the plate wells wall.
2. Addition of mouse monoclonal anti- for PrP^{Sc} antibody. If positive, formation of an immunocomplex.
3. Addition of an enzyme-labelled mouse antiglobulin
4. Addition of a substrate for colour development.

Figure a: *Taking sample of obex for testing on BSE*



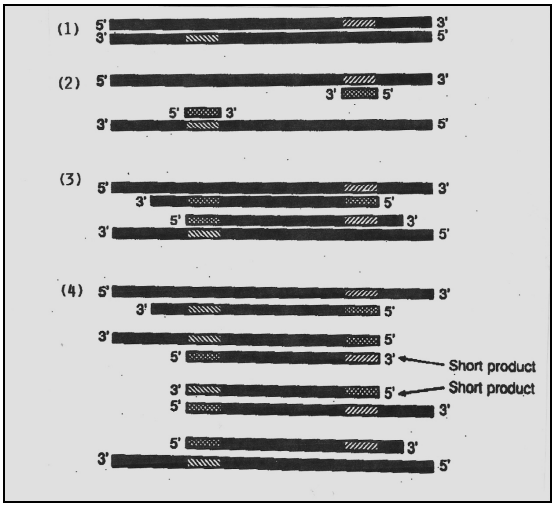
Source : Yearly report of VARS (2006)

Polymerase chain reaction (PCR)

PCR is a higly sensitive and rapid method for amplifying and detecting small quanties of specific nucleic acids in clinical specimens (Mullis, 1987) by increasing the quantity of specific nucleotide sequence of the suspected infectious agent. This appears possible by the directed DNA synthesis. Primers corresponding to a single gene sequence must be known.

In the first cycle (1) the two DNA strands are seperated by heat denaturation and the two synthetic oligonucleotide primers, 20-25 nucleotides long (an automated DNA synthesizer are commercially available) anneal (2) to their respective recognition sequence in the 5' → 3' orientation. (3) A termostabile DNA polymerase (Klenow fragment of Escherichia coli DNA polymerase, U.S. Biochemical Corp., Cleveland, Ohio) initiates synthesis at 3' ends of the primers. Extension of the primers results in new primer binding sites. The result after one round are two copies of the original target. In the second cycle (4) each of the four DNA strands anneal to primers (present in excess) to initiate a new round of DNA synthesis, of the eight single stranded products, two are of a lenght defined by the distance between and including the primary annealing sites; this » short product« accumulates exponentially in subsequent cycles.

***Figure b** : Schema of the polymerase chain reaction (PCR)*



Within a few cycles the predominant product of PCR becomes a double-stranded DNA sequence (sum of lenghts of two primers plus the intervening target DNA) As few as 20 cycles would yeald one million times amount of target DNA initially present. However, problems may arise hindering the introduction of in-house developed PCR. This problem should be discussed elsewhere.

In prion diseases the PCR has been used for the genetic studies including genetic manipulations of the prion protein gene and cloning experiments. The studies have been intensified in the past few years in order to produce cattle, which are prion-free and unsusceptible to the Bovine spongiform encephalopathy infection. Its diagnostic use is minimal, if any, excepting some controls of the cattle food.

ANNEX 3: Prion strain gene typing in animal's details

Experimental transmission of Transmissible mink Encephalopathy (TME) to golden hamsters resulted in two different syndromes, Hyper (HY) and Drowsy (DY) differing by the clinical signs, incubation, brain infectivity titers, lesion profiles and pathogenicity. HY incubation period was 65 +/- days with hyperaesthesia and cerebellar ataxia. DY incubation was 168 +/- 2 days (lethargy was representative for the DY strain). Brain titers were $10^{9.5}$ and $10^{7.4}$ LD_{50}/g respectively. DY agent was the major mink pathogen and HY a minor (Bessen, 1992a). The strains differed in resistance to proteinase K and migration in polyacrilamyde gels. DY strain was more sensitive to proteases with specific differences in immunoreactivity in the region 89-103.The strains differed in composition, conformation or both (Bessen, 1992b). Further studies (Bessen, 1994) showed that PrP^{Sc} conformation could play a role in targeting TME strains to different neuronal populations in which strain scpecific formation occurrs. HY and DY strains are cleaved at different aminoterminal sites by Proteinase K (Bessen, 1995). The self propagation of PrP^{Sc} with distinc tridimensional structure could be the molecular basis of scrapie strains.

In Italy (Casalone, 2004) an active surveillance system on BSE in cattle was started in 2001. Whole brains of eight cattles, by Western blot (WB) BSE positive were studied. In two cattle the PrP^{Sc} glycotype was different from the known PrP^{Sc} molecule with widespread, eosinophilic PrP^{Sc} amyloid plaques. This PrP^{Sc} type and the PrP^{Sc} type associated with the sporadic human CJD (sCJD) showed a convergent molecular signature. The strain was denoted as BASE (Bovine Amyloid Spongiform Encephalopathy). The frequency of this strain type has not been known; it appear important to perform strain typing in BSE cases with deposition of amyloid in the brain. In these cases there is a different pattern of regional distribution and topology. The protease resistant fragment was of lower molecular mass. In summarising strictly the molecular signature was similar to that encountered in a distinct subtype in sporadic human CJD. The protease resistant fragment was also of faster electrophohoretic

mobility. Further monitoring however, showed that the pattern of immunopathology was identical in Italy and the United Kingdom (Casalone, 2006).

The existence of atypical molecular phenotypes among cattle with BSE was demonstrated in France (Biacabe, 2004). Three cases showed unusual features of the WB electrophoretic profile of PrP^{Sc}. The molecular mass was higher as compared with 55 typical BSE cases. This was confirmed by further studies (Baron, 2006). Intracerebral inoculation of such isolated into C57BL/6 mice produced a disease with presentation of PrP^{Sc} molecular features distinct from known BSE strains.

Atypical BSE cases were also recovered in Germany (Buschmann, 2006), resembling atypical cases detected in France / Italy; they were two types either as H-type, characterized by significantly higher molecular size but a conventional glycosylation or the L-type with only slightly lower molecular size but distincly different glycosylation. These atypical cases suggested the possible of sporadic BSE cases in bovines. It appears feasible that UK epidemic had also been initiated from a sporadic BSE case. Atypical BSE case was also detected in Japan (Hagiwara, 2007) with predominant deposition of monoglycosylated PrP^{Sc}, thus resembling the type 2 human sporadic CJD. First BSE case was detected in Sweden 2006 (Gavier-Widen, 2008). The molecular characteristics were in accordance with previous descriptions of H-type BSE (higher susceptibility for proteinase K, higher molecular bands, peculiar banding pattern and an aditional band at 14 kD position). Affinaty to the N-terminus PrP antibodies were also found.

ANNEX 4: Scrapie

The molecular biology knowledge on prions based predominantly on the results of studies with scrapie, a disease of sheep and goats, occurring worldwide until recently and known for more than 200 years. The prion gene in sheep was primarily understood to be responsible for the incubation time from infections to the beginning of disease symptoms (Sinc gene from Scrapie Incubation). Numerous studies of this gene revealed a homozygous genotype ARR/ARR (Alanine, Arginine) that was considered to be resistant for transmissible spongiform encephalopathies (Scrapie and BSE). Animals with this genotype were selected to eradicate scrapie from sheep flocks and to protect the human food chain from small ruminant BSE risks (Andreleotti, 2006). Some other genotypes were found to be susceptible for infections. EU financially supported such studies and the practical performance. Further studies have suggested that there was a strain variation in natural scrapie and that the spectrum of strains may have changed over the past 20 years (Bruce, 1997). Some new prion resistant strains have also been found in the meantime.

It was a matter of the debate whether natural scrapie is a genetic disease arising from certain gene alleles (Ridley, 1996) or whether they only control the susceptibility to an infectious agens. Finally (Hunter, 1997) the evidence was presented, that scrapie is not solely a genetic disease, as scrapie associated alleles, responsible for the acceptance of the infection, are present in sheep from Australia and New Zealand; both regions have been entirely free of scrapie. Other form of infectious, oral and congenital scrapie transmissions have been observed from over 40 years ago until recently.

ANNEX 5: Human prion diseases

Degenerative parenchym processes in the gray zone of the brain were critically observed and studied in Germany about 1916. Three of hitherto unknown clinical cases were presetented at the Conference of German neurologists in Leipzig, 1920 by Jakob, and, shortly thereafter published by Creutzfeldt (Creutzfeldt, 1920). By histopathology the focal degeneration of neurones was typical with neuronophagia by glia cells. Surprisingly there was a total absence of any inflammatory reaction with cellular exudation. Infective or intoxication aetiology did not appear as probable. Psychological and progressive psychomotor symptoms were clearly evident. The focal appearance of neuronal lesions was indicated by Creutzfeldt. The loss of neurones was particularly evident in the thalamic region. The duration of the disease was six months to one year. Considering our recent with cases reported in Germany in 1920 correspond significantly with the actual knowledge. To denote the disease as the Creutzfeldt-Jakob one appear justified.

Human prion diseases include Cretuzfeldt-Jakob disease (CJD) forms as hereditary acquired, genetic (gCJD), sporadic (sCJD), iatrogenic (iCJD) or variant (vCJD) and the group of Gerstmann-Sträussler-Schenker syndrome (GSS) and Fatal familiar insomnia (FFI). The last member of this group is Kuru, transmitted by ritual kanibalism at Fore people (New Guinea) which is disappearing by now. Iatrogenic methods of transmission include the use of unproperly decontaminated surgical instruments in neurosurgery, corneal transplantation, by the medical application of hypophisal hormones from deceased persons (growth hormones, gonadotropine) corneal transplantation and transfusion of blood products. Extensive literature on these iatrogenic forms were described by Pana (2005). The most frequently encountered form of the CJD is the sporadic CJD (sCJD), responsible for 85-90 percent of all CJD cases worldwide, the newest form called variant CJD (vCJD) described by Will (1996) resulting from the consumation of infected meat (BSE). Human prion diseases are presented in Table a.

***Table a**: Syndrome of human transmissible prion diseases*

Genetic forms	Number of forms	Point mutations in /PRNP/
CJD familiar GSS syndrome Fatal familiar insomnia	34	P102L P105L G114V A117V G131Y G142S Y145X R148H Q160X N171S D178N V180X V180I T183A H187R T188A T188K T188R T193I E196K F198S E200K D202N V203I R208H V210I E211Q Q212P Q217R Q218K E219K M232T M232R P238S
INDEL	8	Insertion (base pairs) 24 ,48, 96, 120, 144, 168, 192, 216 in /PRNP/
	2	Deletion (base pairs) 24 and 48 in /PRNP/
Non-genetic forms	Number of forms	
CJD sporadic CJD iatrogen CJD variant Fatal sporadic insomnia	4	Polymorphism at codon 129 m/m (98%)

(A) Alanine, (R) Arginine, (N) Asparagine, (D) Asparagine acid, (C) Cysteine, (Q) Glutamine, (G) Glycine, (E) Glutamic acid, (H) Histidine, (I) Isoleucine, (L) Leucine, (K) Lysine, (M) Methionine, (P) Proline (Fenil alanine), (S) Serine, (T) Threonine, (W) Tryptophan, (Y) Tyrosine, (V) Valine

Source : Pana and Jung (2005) actualized 2007

Criteria for diagnosis of CJD

Sporadic CJD

- Rapidly progressive dementia
- Myoclonus
- Visual or cerebral problems
- Pyramidal or extrapyramidal features
- Akynetic mutism
- Typical EEG
- Duration of disease-6 monnths
- Age mostly over 60 years

Familial (genetic) forms

- Definite or probable CJD in a first degree
- Family relative or neuropsychiatric disorder
- Plus /PRNP/ mutation

Iatrogenic CJD

- Progressive cerebellar syndrome with dementia and a recognized previous exposure risk

Human genetic prion diseases

Human genetic prion diseases have been intensively studied in the past decades. Laboratory techniques for the sequencing of base pairs in the genome were available by chemical or enzymatic methods, but also not too much complicated, practically available only to specialized laboratories. An enormous succes was the construction of specialized machines performing the sequencing automatically, thus being available to small laboratories as well resulting in quick and precise results. The sequencing has not been used for the genetic studies exclusively, but for the forensic and specialized genetic

medicine as well. The sequencing for the scientific purposes has been widely accepted. In prion diseases diagnostic might be also critical investigating clinically inaparent members of a family with a known clinical case. A positive diagnostic in such case does not necessarly mean that the person will also be developing the disease. The diagnostic must therefore be carefully investigated.

One of the most compelling transgenic evidence for the transmission of BSE prions to humans was published by Scott (1999) and the Prusiner group in San Francisco, California. For the experimental transfer they used transgenic mice expressing bovine prion protein (Tg(BoPrP)) mice. There was no species barriers for transmission from BSE cattle being in this way highly suspected for infection. Incubation, neuropathology and PrP^{Sc} were indistinguishable from BSE and differed drastically from natural scrapie. This was the most compelling evidence to date that prions from cattle with BSE have infected humans (vCJD). Identical results with transgenic mice were obtained by Hill (1997). The clinical course in infected mice was much longer that in transmission of typical CJD, vCJD was also associated with long clinical duration. Some vCJD inoculated mice persistently walked backwords, never observed in transmission of typical CJD.

It has been generally accepted that prions are mammalian pathogens composed of conformational isomers of a host encoded glycoprotein which appears to lack nucleic acids. Much attention has been paid on the molecular bases of prion propagation and the species barriers that controls the cross-species transmission. This has been intimately linked how multiple prion strains are encoded. Recent advantages suggested that prion themselves are not directly neurotoxic, but their propagation involves propagation of toxic species (Collinge, 2007). Although distinct strains of conventional pathogens can be explained by differences in their nucleic acid genome (i.e. residue 42 serine) in the viral protein of Influenza virus plays a critical role in the pathologenicity of the new Avian Influenza virus (Jiao, 2008). It has been less clear how a polypeptide chain could encode multiple disease phenotypes. The molecular basis of strain diversiry, recognizable in scientific experiments and full reproduction in transmission tests has not yet fully been understood.

Polymorphism at the codon 129 of the /PRNP/, appears responsible for the susceptibility to variant CJD. Point mutaitions of a single base pair in the /PRNP/ appears responsible for several forms of the CJD and they are clinically and pathologically well documented. Insertion or deletion of octapeptides in the N-region of the prion protein (PrP). Genetic prion diseases are responsible for 10 to 20 percent of all human prion diseases. Scientific details on prion genetics and relevant problems are presented on the next pages; they are based on review articles published by Jung (2003).

***Table b**: Amino acids and nucleotide bases*

Alanine	(A)	Ala
Arginine	(R)	Arg
Asparagine	(N)	Asn
Asparagine acid	(D)	Asp
Cysteine	(C)	Cys
Glutamine	(Q)	Gln
Glycine	(G)	Gly
Glutamic acid	(E)	Glu
Histidine	(H)	His
Isoleucine	(I)	Ile
Leucine	(L)	Leu
Lysine	(K)	Lys
Methionine	(M)	Met
Proline	(P)	Pro
Phenylalanine	(F)	Phe
Serine	(S)	Ser
Threonine	(T)	Thr
Triptophane	(W)	Trp
Tyrosine	(Y)	Tyr

Valine	(V)	Val
Nucleotide (base pairs AT/ GC)		
Purines	Pyrimidines	
Adenine (A)	Thymine (T)	
Guanine (G)	Cytosine (C)	
	*Uracile (U)	

**instead of thymine in the RNA*

***Table c**: Point, Missens* mutations in the prion protein gene*

Codon normal	Mutation
P102L	Proline-Leucine (Pro-Leu)
P105L	Proline-Leucine (Pro-Leu)
G114V	Glycine-Valine (Gly-Val)
A117V	Alanine-Valine (Ala-Val)
G131Y	Glycine-Thyrosine(Gly-Tyr)
A133V	Alanine-Valine(Ala-Val)
G142S	Glycine-Serine(Gly-Ser)
Y145X	Thyrosine-Stop codon (Tyr-X)
R148H	Arginine-Histidine (Arg-His)
Q160X	Glutamine-Stop codon (Gln-X)
N171S	Asparagine-Serine(Asn-Ser)
D178N	Asparagine acid-Asparagine (Asp-Asn)
V180I	Valine-Isoleucine (Val-Ile)
T183A	Threonine-Alanine (Thr-Ala)
H187R	Histidine-Arginine (His-Arg)
T188A	Threonine-Alanine (Thr-Ala)
T188K	Threonine-Lysine(Thr-Lys)
T188R	Threonine-Arginine (Thr-Arg)
T193I	Treonine-Isoleucine (Thr-Ile)
E196K	Glutamic acid-Lysine (Glu-Lys)

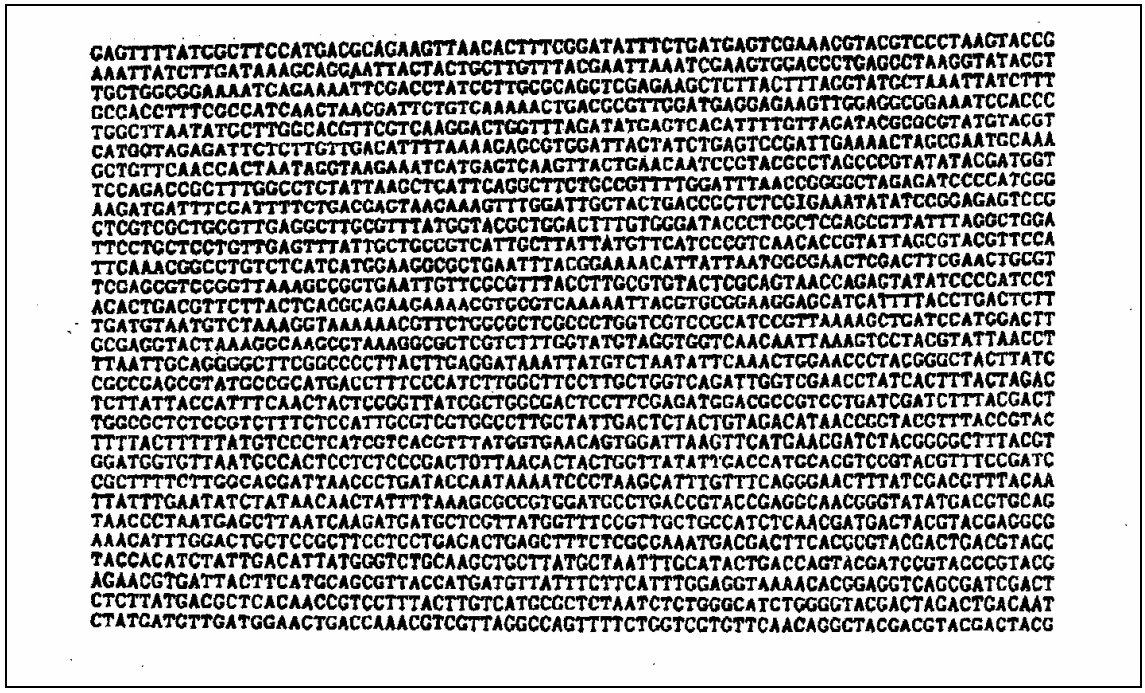
Codon normal	Mutation
E198S	Phenylalanine-Serine (Phe-Ser)
E200K	Glutamic acid-Lysine(Glu-Lys)
D202N	Asparagine acid-Asparagine (Asp-Asn)
V203I	Valine-Isoleucine (Val-Ile)
R208H	Arginine-Histidine (Arg-His)
V210I	Valine-Isoleucine (Val-Ile)
E211Q	Glutamic acid-Glutamine (Glu-Gln)
Q212P	Glutamine-Proline (Gln-Pro)
Q217R	Glutamine-Arginine (Gln-Arg)
E219K	Glumatic acid-Lysine (Glu-Lys)
M232T	Methionine-Threonine(Met-The)
M232R	Methionine-Arginine (Met-Arg)
P238S	Proline-Serine(Pro-Ser)

* *No change in the open-reading frame of /PRNP/*

***Figure a**: Types of mutation*

A	T	G	G	C	T	
T	A	C	C	G	A	Normal / Wild type
A	T	**T**	G	C	T	
T	A	**A**	C	G	A	Substitution
A	T	**G**	C	T		
T	A	**C**	G	A		Deletion
A	T	**A**	G	G	C	T
T	A	**T**	C	C	G	A Insertion

***Figure b**: Sequence of base pairs (2400) in a DNA double helix (complete genome a 7,1x 10^9 bp in each diploid cell)*



Source :Point mutations in a single bp change (Muench, 1988)

***Figure c**: Guanine/Adenine/Thymine/Cytosine. Sequence of base pairs in DNA double helix (complete genotype is 7,1 x 10^9)*

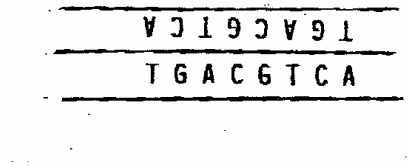


***Figure d** : Indel (not true*) mutations in the prion protein gene /PRNP/*

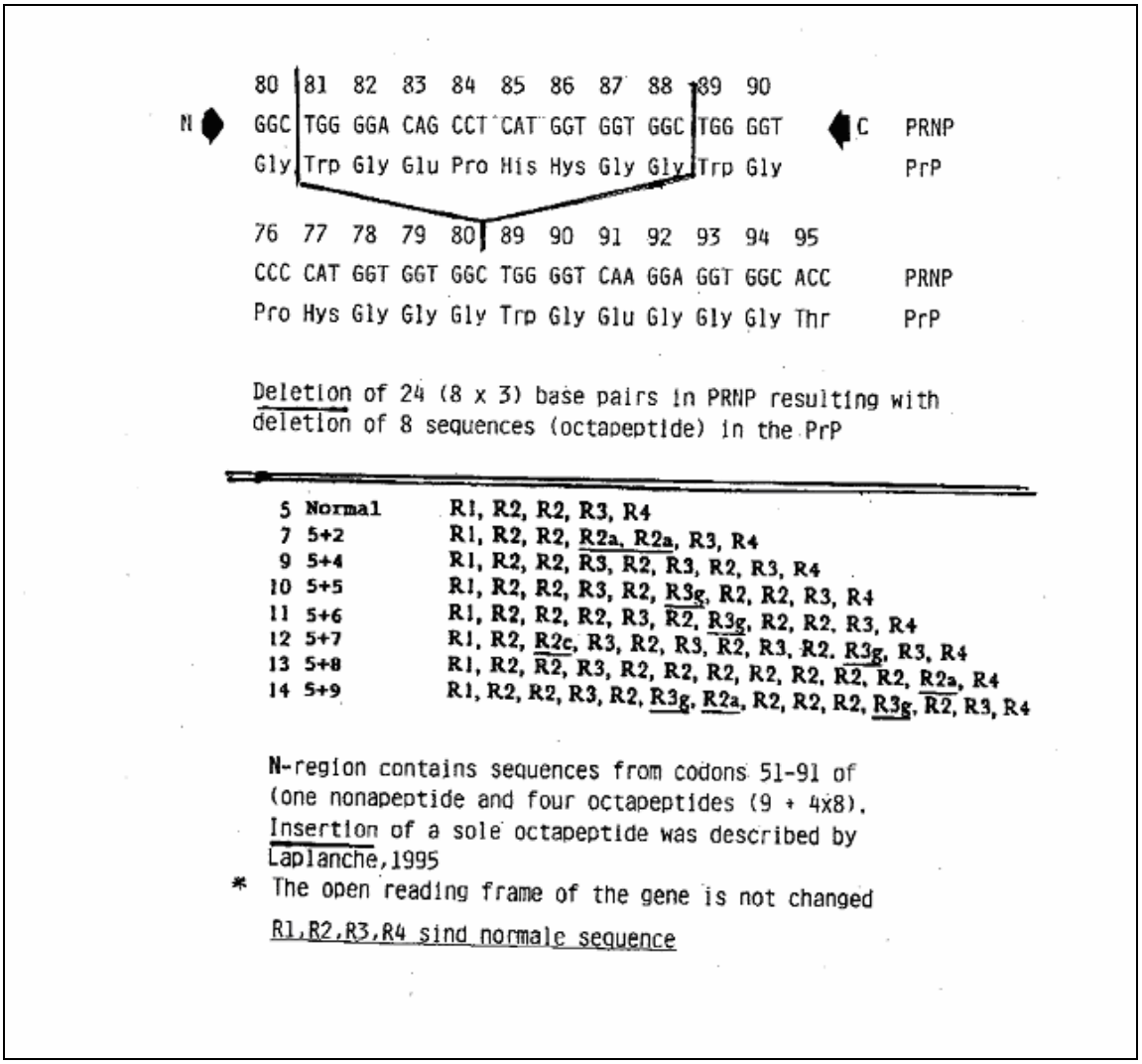


***Table d**: Human Iatrogenic Creutzfeldt-Jakob disease (iCJD) confirmed by molecular biology methods and in all cases by bioassays in laboratory animals*

Neurosurgery (brain electrodes)	Bernoulli (1977)*
Corneal transplantation	Duffy (1974)
Blood	Liewelyn (2004)
Dura mater grafting	Prichard (1987)

Pituitary growth hormone	Hintz (1985)
Pituitary gonadotropin	Cochius (1990)
Instrument contamination	Gibbs jr (1994)
Labor contaminated instruments	Zobeley (1999)

** by bioassay of conserved original material confirmed in monkey by Gibbs jr (1994)*

The incidence of Creutzfeldt-Jakob disease (CJD) is monitored in the UK by the National CJD Surveillance Unit (NCJDSU) based at the Western general Hospital in Edinburgh, Scotland. The Unit brings together a team of clinical neurologists, neuropathologists and scientists specialised in the investigation of this disease.

Bruce (1997) studied the strains of prion diseases; they were distinguishable by disease characteristics at infected animals, incubation periods and neuropathology. BSE characteristic pattern of disease, transmitted to mice, are retained after experimental passages through a variety of intermediate species. Bruce tested natural scrapie from live sheep, BSE from cattle, from two cats and from three cases of human variant CJD. She described that the BSE signature (incubation and pathology) is seen only in transmission from animals suspected or known that have been infected with BSE. Investigated was also the lesion profile, a semiquantitative method to measure the targeting of vacuolation in different brain regions. It appeared clear, that causative link between different species cannot be made on the bases of glycoform alone. It also appears feasible, that the infectious agent must interact with genetic factors as the host to control the timeing and neuropathology with such precision. The study of Bruce and co-workers was of enormous high costs. Their data provide strong evidence that the same strain is included in the both BSE and the variant CJD.