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CHARACTERIZATION OF CLASSICAL AND ATYPICAL BSE IN GOAT: CLINICAL SIGNS, PATHOGENESIS AND DIAGNOSTIC ELEMENTS

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INDEX

INTRODUCTION

Prion diseases or Transmissible Spongiform Encephalopathies (TSEs) are a group of neurodegenerative diseases that affect both humans and animals. The natural hosts of TSEs are man, cow, sheep, goat, more rarely, the mouflon, and various species of deer, but the spectrum of affected species has become wider as a result of numerous cases of accidental transmissions.

TSEs are characterized by a long incubation period, neurological symptoms, a progressive and invariably fatal clinical course, degenerative lesions in the Central Nervous System (CNS) and by the absence of classical inflammatory lesions or immune reaction of the body.

It is also constantly present, especially in the brain, a pathological isoform PrPsc or PrPres of the physiological protein (called PrPc), which plays a crucial role in the pathogenesis of the disease and it is responsible of the development of degenerative lesions (Pocchiari 1994). The causative agent, called "prion" (from which PrP, or Prion Protein) is largely or wholly made from the protein, and it is also characterized by an extreme resistance to degradation made by different physical and chemical agents, able to inactivate a large variety of microorganisms.

The propagation of prions include direct protein-protein interaction between the host PrPc and inoculated PrPsc; the PrPsc would act to promote further conversion of PrPc into PrPSc through an autocatalytic process which proceeds as a cascade. Experimental transmission studies between different species (that codify PrPc with different primary structure) and *in vitro* conversion studies propose a model in which the propagation proceeds more efficiently when the interacting proteins have the same primary structure.

TSEs are experimentally transmissible in different ways (by injection, ingestion, intranasal) to a large number of mammals species, that are not naturally suffering from these diseases (Cardone et al., 1998). In nature, the TSEs transmission ways are still poorly understood: there are forms that can move from one host to another through ingestion of infected material (for example, during the epidemic of bovine spongiform encephalopathy, BSE) and other forms transmitted through percutaneous way (iatrogenic forms). Other routes of infection, such as transplacental passage or the exposure of mucous membranes to infected material have been suggested, but it is not clear their impact on the spread of these diseases.

Despite a lot of similarities between different TSEs, the knowledge of a form can not be automatically applicable to other forms of the disease. From an epidemiological point of view, for example, Creutzfeldt-Jakob disease (CJD) has spread around the world with an incidence of approximately 1-2 cases per million of people per year, while Scrapie of sheep and goat only a few flocks but all the animals of the flock are killed by the disease. The transmission from one individual to another can vary: human TSEs are not transmitted through vertical route (ie from mother to child), while this pathway is well documented for Scrapie (Cardone et al., 1998); regarding BSE, this type of transmission has never been clearly demonstrated (Wilesmith et al., 1997).

The appearance of BSE in the UK in the mid-eighties of the last century, and later in other European countries, has greatly accelerated the study of these diseases for the possibility that BSE could become a threat to public health, especially given the enormous consumption of beef products in industrialized countries. The appearance, always in the UK, ten years after the first cases of BSE, of a new form of human spongiform encephalopathy (called variant CJD, vCJD) in young people and with clinical and pathological aspects similar to the original form, has strengthened these fears, that have been finally confirmed by experimental studies which have

linked the cases of vCJD to BSE agent food exposure. Indeed, studies with western blotting showed that the characteristics of PrPres of vCJD differ from those of sporadic and iatrogenic CJD, but are similar to those of both natural and experimental BSE.

In humans, these forms of TSEs have been described to date:

• **Kuru**

Disease discovered in the Fore tribes of Papua New Guinea (1967), characterized by hyperexcitability, tremors, ataxia, uncontrolled laughter. It was linked to ritual cannibalism and today it has almost disappeared.

• **Creutzefeldt-Jacob Disease (MJD / CJD)**

It may be sporadic due to unknown hereditary mutation of the gene encoding the prion protein or, in rare cases of iatrogenic type. From the clinical point of view it is characterized by progressive dementia associated with at least two of the following clinical signs: myoclonus, visual disturbances or pyramidal or extrapyramidal cerebellar signs.

• **Gerstmann-Straussler-Scheinker Disease (GSS)**

Rare form of hereditary TSE, caused by a mutation in the gene encoding PrP. It is characterized by dementia, ataxia, dysphagia and atrophy.

• **Fatal Familial Insomnia (IFF)**

It is a rare inherited autosomal dominant form and it is characterized by insomnia, disorders of the autonomic nervous system, motor and cognitive disorders.

• **New variant of CJD (vCJD)**

This form differs from the classic form of CJD for the appearance even in young patients, for a longer duration of the disease and characteristic clinical symptoms represented by behavioral disorders, personality changes and depression. The majority of patients early develop cerebellar ataxia and with the progression of the disease, myoclonus may be preceded by choreic movements.

TSEs in animals are represented by:

• **Bovine Spongiform Encephalopathy (BSE)**

Fatal infectious disease of cattle, which appeared in 1986 in Great Britain. The main characteristic symptoms observed were hypersensitivity to external stimuli, incoordination of the hind limbs, with strong reactions of fear. It is a food infection, transmitted through meat and bone meal insufficiently treated to eliminate the causative agent.

• **Scrapie**

Endemic disease of sheep and goats. The name derived from the verb to scrape, a typical signs of affected animals. Affected animals scratch and bite themselves. It is an infectious disease that can be orally transmitted by ingestion of organic material infected, horizontally, and probably from mother to foetus.

• **Wild ungulates Encephalopathy**

It is an infectious disease similar to Scrapie, which was mainly observed in wild ungulates (oryx, antelope, nyala, kudu) bred in captivity and fed with contaminated feed.

• **Chronic wasting disease (CWD)**

It is a chronic disease of obscure origin, highlighted in the moose of the Rocky Mountains and in the mule-deer.

• **Transmissible mink encephalopathy (TME)**

It is a disease of farmed mink and it is food related form. The causative agent has the same physical, chemical and biological properties of Scrapie, it is not related to BSE and it is not transferable to different species from mink.

• **Feline spongiform encephalopathy (FSE)**

It was observed in cats and some other members of the cat family such as ocelots, cheetahs, pumas, etc.. The onset is associated with feeding the big cats in captivity with BSE-infected material.

TSEs AETIOLOGY

Structure and biosynthesis of PrP

PrPc is the normal cellular isoform of the prion protein, also called PrPsen because of its sensitivity to enzymatic treatment with proteinase K. It is a membrane glycoprotein of about 33-35 kDa, highly conserved in all species of animals and it is mainly expressed in the brain, especially in neurons and astrocytes, but also in many other organs and tissues, such as lung, spleen, testis, ovary, myocardium, intestinal mucosa and lymphocytes.

The gene encoding this protein is located on mouse chromosome 2 and human chromosome 20, which are considered homologous. All mammals have, probably, a gene for PrP and they are, therefore, exposed to the danger of developing these disorders. The PrP gene encodes a protein of about 250 amino acids consisting of an N-terminal portion (residues 20-120) and a C-terminal hydrophobic domain (residues 121-131) to which an anchor of glycosyl-Phosphatidyl -Inositolo (GPI) is bound. Within the N-terminal region there are a peptide of nine amino acids (PQGGGGWGQ, amino acids 51-59) and four repeated copies of a sequence of eight amino acids (PHGGGWGQ, amino acids 60-91). There are also, a disulfide bridge between cysteine 179 and 214 and two N-glycosylation sites at the level of asparagine 182 and 198. The protein may be present on the cell surface in a form monoglycosylated (25%), diglycosilated (70%) or non-glycosylated (5%); glycosylation occurs at asparagine residues present in the C-terminal portion in positions 181 and 198.

The use of Nuclear Magnetic Resonance (NMR) has allowed to determine the three-dimensional structure of the cellular protein: the C-terminal region forms a well structured globular domain

and characterized by three-helices and two short β-sheet, while the N-terminal region, which contains the repeated octapeptidic region, is not structured.

PrPc is synthesized in the endoplasmic reticulum, transferred to the Golgi apparatus and then transported, by means of secretory vesicles, to the outer surface of the cell membrane. The post-translational modifications consist in the removal of a residue of 22 amino acids at the N-terminal and a 23 amino acid residue COOH-terminal end; the latter is replaced by GPI, which allows the PrPc to anchor to the membrane surface, once transported outside the cell. The binding of GPI to the membrane occurs at the level of microdomains rich in cholesterol, sphingolipids and glycolipids. During normal conformational maturation process, PrPc shows an inherent tendency to adopt the conformation pathological isoform, but the presence of chains of glycans in N-terminal prevents such modification.

The abnormal isoform of PrP is called PrPres because of its characteristics of insolubility to detergents and its partial resistance to proteinase K: Treatment with the proteinase transforms the protein in a smaller one of approximately 27 to 30 kDa.

The two isoforms have identical amino acid structure and the same post-translational modifications, but they differ in the secondary structure: the secondary structure of PrPc is characterized by a high content of α-helices (42%) compared to that in β-sheet (3 %); on the contrary, the pathological protein is characterized by a greater amount of β-sheet (43%) and decrease of the content of α-helices (32%) (Figure 1).

Furthermore PrPc is rapidly synthesized and can be detected on the cell surface after about 20 minutes, then disappear rapidly (half-life of approximately 6 hours); on the contrary, the PrPres is very slowly synthesized and it is stable for a period of 24-48 hours. Moreover, the link with the cell surface, constituted by GPI, is sensitive to treatment with phosphatidylinositol-specific

phospholipase C (PI-PLC) in PrPc, while the PrPsc resists to this enzyme and tends to remain anchored to the cell surface by means of its stable core, consisting of residues 90-231.

The pathogenetic mechanism underlying the TSEs is described as a process of amplification cascade triggered by the post-translational conversion of the precursor PrPc in the pathological isoform PrPsc. The main steps of this process are the acquisition of resistance to phosphatidylinositol-specific phospholipase C, which allows the PrPsc to remain anchored to the cell surface and the acquisition of the characteristic insolubility to detergents, at the base of the tendency to aggregation of PrPsc molecules and their resistance to proteases.

Over the years, several theories have been formulated to explain the conformational changes on the basis of the conversion process.

The Prion theory (Prusiner, 1982), currently the most reliable, argues that a key role is played by PrPsc and it comprehensively explains some unconventional features of these infectious agents, such as the absence of inflammatory or immune response in the host, the unusual resistance to chemical and physical procedures that inactivate classical virus and resistance to treatments that destroy nucleic acids (UV and γ). The replication of prion would occur through the direct interaction between a molecule of PrPsc and PrPc, which assume β-sheets pathological conformation.

PrPc has in fact a structure rich in α-helices, while the PrPsc has a high content of β-sheets . It is assumed that the transformation of two chains α-helix of PrPc in β-sheets gives rise to the formation of PrPsc.

This conversion would trigger an autocatalytic mechanism responsible for the production of PrPsc. According to this theory, the first molecule of PrPsc can have exogenous origin (natural

or experimental infection) or may result from the spontaneous conversion of one molecule of endogenous PrPc.

The weak point of the prion theory is given by the existence of different strains of Scrapie. The various strains have been isolated and experimentally characterized by inoculation in different lines of mice of brain tissue from Scrapie affected sheep.

From a clinical point of view, the discriminating criterion is the incubation time of the disease, which remains constant for each isolated strain. Depending on the strain of scrapie, in addition, the histopathological lesions vary in severity and distribution. The analysis of the nine areas of the gray matter and three of the white allows to build a profile of brain injury that is typical of each strain of Scrapie and it is considered independent from the infectious dose. The presence of different infectious agent strains would be easily explained by the viral theory, although this theory has been almost completely abandoned. The viral theory refers to virus with special features compared to other pathogens and therefore called unconventional slow viruses. Slow refers to the long latency time that elapses from the time of infection to the onset of clinical symptoms.

As part of the viral theory it is assumed that the virus binds to PrPc and that as a result of this interaction it is modified in the protein PrPsc. Mutations and polymorphisms of the PrP increase the binding affinity between PrPc and the virus or the efficiency of the conformational change of PrPc into PrPsc. In both cases, the result will be a greater chance of developing the disease.

The viral particles produced inside the cell would fold tenaciously through hydrophobic interactions to PrPsc, protecting the virus from inactivation procedures. The absence of immunogenicity and inflammatory phenomena may be explained by the small size of the virus,

which would replicate inside the cells without activating them (Diringer et al., 1994; Pocchiari 1994).

A third theory, which is between viral and prion theory, is the theory of "virino." The virino would be constituted by the assembly of a molecule of PrPsc and from a nucleic acid not encoding exogenous PrPsc; it would bind to PrPc allowing the penetration of virino within the cell; at this point the nucleic acid has several replication cycles going to complexing one molecule of endogenous PrPc, determining the conformational conversion (Cardone et al., 1998). Overall, it remains unclear, however, the fundamental question concerning the role of PrPsc if that itself represents the causative agent of TSEs, as postulated by the "theory of prion" (Prusiner, 1982), or was simply a product of pathological 'infection, as the "viral theory" (Czub et al., 1986; Manuelidis et al., 1987). A variant of this hypothesis (Oloprion theory) also suggests that the nucleic acid has cellular origin: the infectious particle (oloprion) would consist of PrPsc (apoprion) and nucleic acid (coprion) (C. Weissmann 1991). Regardless of the nature of the causative agent, most important the pathogenic factor is the prion protein which undergoes in the endoplasmic reticulum and Golgi apparatus to a series of post-translational modifications. The protein is fixed on the outer surface of the cell membrane by an anchor of glicolisil-phosphatidyl-inositol (GPI). At this point the conversion of PrPc into PrPsc happens. PrPc is secreted or rapidly metabolized (half-life is approximately 6 hours) within the lysosomes, while the PrPsc is resistant to degradation by lysosomal enzymes, it accumulates inside the cell causing death. Following cell lysis, the PrPsc protein is deposited in the intercellular space, giving rise to the deposition of amyloid substance, sometimes organizing into plaques (SIM).

PATHOGENESIS

Experimental models studies have shown that the pathogenesis of TSEs varies depending on the route of infection.

The oral infection is one of the most important from an epidemiological point of view and data obtained from natural and experimental scrapie and BSE (Groschup et al., 1996; Van Keulen et al., 1999,2000; Baldauf et al. 1997 Beekes et al., 1998, 2000, Groshup et al., 1999, Kimberlin et al., 1983) show the involvement of the peripheral nervous system (PNS) and the enteric nervous system (ENS) in the spread of the infectious agent to the CNS.

A study conducted by VanKeulen et al., 2000 in Scrapie affected sheep and sacrificed at 5, 10, 14, 17, 21 and 26 months of age, the initial highlighting of PrPsc in ENS indicates that this is the door input of the agent (Figure 2). The subsequent spread to the CNS and spinal cord following a retrograde fashion through the sympathetic and parasympathetic efferent (motor) nerves (Figure 3). In particular, two ways have been recognized: the first along the vagus nerve and the second along the splanchnic nerves. Along the splanchnic nerves, the PrPsc was early observed at the level of enteric and abdominal ganglia (GCMC); from here PrPsc spreads into the spinal cord at the level of the intermediolateral column (IML). In the course of the vagus nerve, the first PrPsc deposits were observed at the level of the dorsal motor nucleus of the vagus (NMDV) and nucleus of the solitary tract (SN):

Hence the PrPsc invades the remaining areas of the brain and spinal cord going into cranial and caudal directions. The accumulation in the basal ganglia was assessed after that PrPsc has already accumulated in the CNS and is probably due to a spin diffusion of the agent through the afferent fibers of the nerves. In the path of the splanchnic nerves the dorsal motor ganglion

(GRD) is concerned, while nubby ganglia in vagus nerve (NG) (McBride et al., 2001; VanKeulen et al., 2000). PrPsc spread along the peripheral nerves through a mechanism of axonal transport.

The low levels of infectivity found at the terminal stage of the disease in the vagus nerve than those found in the brain, indicate that in nerve fibers there are not processes of replication, but they have only transit function.

It is still likely that in most natural infections, the involvement of lymphoid organs is necessary.

Long incubation period of TSEs is likely due to the slow and silent propagation of prions in "reservoirs", organs in which the replication does not cause detectable lesions (Mabbot et al., 2001); the hypothesis that the immune system and in particular the lymphoreticular system (LRS) act as a deposit and site of PrPsc replication before it reaches the CNS is well supported by experimental data. In fact, after experimental infection of peripheral hamster with Scrapie, the prion protein accumulates rapidly in all lymphoid tissues, starting from the Peyer's patches and mesenteric lymph nodes, where PrPsc replicate, after a passage through the gastrointestinal mucosa (Andreoletti et al., 2000; Beekes et al., 2000; Heggeb et al., 2000).

The transition from lymphoid cells to nerve cells occurs due to the existence of a communication system between the two cell lines: there is a close association between the ends of the autonomic nervous system fibers and certain cells, including macrophages and lymphocytes.

The transport of the prion is closely related to the presence of cells with a long half-life: neuronal cells and follicular dendritic cells (FDC). The first form the final seat of replication of the pathological protein, while the FDC are located into stromal portion of the spleen and on the surface of dendritic processes they express high amounts of PrPc in healthy animals and PrPsc in Scrapie affected animals (McBride et al. 1992).

The cortex of lymphoid follicles, innervated by unmyelinated fibers, which are more easily "traversable" by PrPsc than myelinated fibers, could be the point of entry of prions in the SNP.

Different strains of pathological prion protein may be characterized by different propagation ways: for example, the RML strain (Rocky Mountain Laboratory), inoculated into mice, is propagated by means of B lymphocytes and of FDC, while it does not happen with ME7 strain. Lymphocytes T do not play an important role in the pathogenesis of TSEs: in fact, in thymectomized neonatal mice subjected to experimental infection, the incubation period of the disease is unchanged; this finding was confirmed in mice deficient T lymphocytes function (Mabbot et al., 2001).

The role of lymphocytes B was studied in SCID mice (Severe Combined immunodeficient), carriers of a mutation that results in blocking the production of immunoglobulins, functional alterations of T cells and, at the same time, the maturation of FDC (Klein et al. , 1997; Lasmezas et al., 1996). These mice are less susceptible to peripheral infection respect to the immunocompetent ones, but they develop the disease if the infection occurs by the intracerebral route.

Resistance to peripheral infection could be related to the inability of the lymphoid tissues of SCID mice to support the replication of PrPsc protein. In fact, the grafting of bone marrow or fetal liver tissue from immunocompetent donor in SCID mice, which is able to restore the functionality of B and T lymphocytes and to facilitate the maturation of the FDC, allows the SCID mice to replicate the PrPsc in their lymphoid tissues, and thus become fully susceptible to infection by peripheral route (Klein et al., 1998).

This result would appear to emphasize the dependence of the infection by the presence of B cells and strengthening the hypothesis according to which the lymphocytes would be able to induce the maturation of cells that accumulate PrPsc in the spleen (Klein et al., 1998; Klein et al., 1997).

The discovery of PrPSc in splenic lymphocytes would seem to indicate that also the circulating lymphocytes may be involved in the transport of prions: however, in experimental models studied up to now, the PrPsc has never been detected on lymphocytes outside the LRS and, however, it is conceivable that there is no passage of lymphocytes through the blood-brain barrier, that transport or not PrPsc. So, it may exist in the spleen a subpopulation of lymphocytes sensitive to infection that are not able to reach the vascular system, or such cells may be directly eliminated by macrophages of the spleen without being able to spread over the prion protein (Race et al. , 2000).

The rate of diffusion of the protein along the nerve fibers (0.5-2 mm / day) is compatible with the hypothesis that the pathological protein goes trough fibers with a slow axonal transport (McBride et al., 2001).

The target cell is the neuron; the first effects caused by the infection are due to the activation of microglia. Activated microglia accumulates around PrPsc deposits. The activation and accumulation of microglia in infected brain areas precede neuronal death; a consequence of the activation of microglia is the induction of an astrocyte proliferation.

In conjunction to astrocytes hyperplasia and hypertrophy there is the accumulation of amyloid fibrils (SAF Scrapie Associated fibrils), which arise from the deposition and the subsequent organization of the pathological protein (Oberdieck et al., 1994; Piccardo et al. 1990; Forloni et al., 1994).

TSEs STRAINS

TSE strains variability and species barrier

Although the knowledge on TSEs pathogenesis have substantially grown in recent years, however, the phenomenon of strains variability remains one of the most complex and controversial topics of prion diseases research . The traditional approach of microbiology to understand the variability of strain - closely linked to the existence of variability in the nucleic acids that transmit genetic information of organisms - appears inadequate in the case of prion diseases, agents apparently devoid of such "informational "molecules and that are purely protein. The variability of the clinical-pathological phenotype of an infectious disease it would not be linked to genetic traits of the agent, in the case of prions, but it comes from the same stable epigenetic information transmitted by proteins. Results of some studies carried out in different lines of in bred-mice expressing a prion protein with the same primary structure, they seem to confirm that different prion strains can be propagated within these lines of mice and some of them can be reisolated unchanged even after some intermediate steps in guests who express a different cellular prion protein, thus demonstrating that the variability of strains does not necessarily depend on the primary sequence encoded by host PRNP gene.

Conversely, as several lines of research seem to suggest, necessary information to identify and define a "strain" would exclusively reside in the pathological prion protein by itself. In the case of transmissible mink encephalopathy (TME), for example, it is known the existence of two distinct strains: "hyper" (HY) and "drowsy" (DY) transmissible to hamster and distinguishable on the basis of the phenotype of the disease and for PrPsc physico-chemical properties accumulated in diseased animals brains (Hill, 2003). In these animals, a result of a limited

proteolysis, the examination by Western blotting performed on brain homogenate, reveals the existence of a PrPsc characterized by a immunobiochemical pattern related to a specific strain. This different electrophoretic mobility is attributable to the fact that proteolytic enzymes attack and cut the protein at the level of amino acid sites that change according to the strain-specific conformations assumed by the protein itself. This appears quite similar as observed in human cases of CJD in which the different phenotypic characteristics and biochemical properties can be transmitted in transgenic mice expressing the human PrP.

Experiments carried out already in 1960 have shown that the transmission of prion diseases from one species to another is less efficient than transmissions within the same species, a phenomenon which takes the name of "species barrier". The species barrier is manifested by a) a lengthening of the average incubation period, b) the appearance of atypical clinical and histological signs in the inoculated species, and c) the decrease in the number of animals that succumb to the disease. When, as a result of transmission from one species to the other one, the brain of a diseased animal is inoculated into an animal of the same species, the incubation period tends to decrease and become more consistent. This marked difference between the transmission parameters between the first and the second passage are diagnostic against the species barrier (Hill, 2003).

Several parameters are able to influence the transmissibility of prion strains between different species and thus well within the same species. Among these we consider a) the presence of polymorphisms at the level of the gene coding for the prion protein capable of determining differences in the primary structure between the cellular protein of the donor and the host, b) the type of prion protein, c) the route of inoculation (intracerebral versus intraperitoneal), d) the dose. All these factors help to determine and to adjust the species barrier.

Usually the appearance of a strong barrier is able to obstruct the occurrence of a disease in the clinical form when a prion strain is transmitted from one mammalian species to another. However, there are some cases in which the transmission is extremely efficient: it is the case for example of what happened to the BSE where the disease was transmitted both naturally and experimentally in many different species (Bruce, 1997, 2003).

Among the factors involved in modulating the transmission of various forms of prion disease between one species and another is necessary to take into account differences more closely linked to the prion strain rather than structural differences between donor and host cell protein . In fact it was observed that transgenic mice deprived of the gene coding for the murine PrP but capable of expressing human PrP show no species barrier against CJD but they are highly resistant against the vCJD, even if the human donor (PRNP 129 MM) express a prion protein with identical primary structure. From here it follows that concerning prion diseases it could be more correct to speak of "transmission barrier" referring with this term to the complex relationship between species, the primary protein structure and types of prion strain, factors which all contribute in determining the pathological picture.

Characterization of TSEs strains

Although it is not quite correct to speak of "characterization" referring to agents whose nature remains uncertain, however this term is used to indicate the identification of TSE strains responsible of the disease in the natural host. There are two possible approaches to the characterization of prion strains:

- The study of the strain biological properties by inoculation in different mice lines

- The study of the biochemical properties of the pathological prion protein.

Which relationship exists between the two approaches is not yet clear, conversely it is certain that the two methods are perfectly complementary (Ryder, 2002; Bruce, 2003)

The study of the strains by transmission in wild-type mice is based on the principle that the agent or agents present in sheep and / or cattle can be isolated by inoculation of brain homogenate from diseased animals into mice with different genotypes. Subsequently, serial passages in other mice help to purify the agent which can be characterized by studying the behavior in a panel of mice with two different genotypes and sometimes in some heterozygous mice. Ultimately, a strain of TSE is defined as an agent that following a series of steps in mice developed stable characteristics.

The characteristics used to define a strain are:

- *Incubation period*: it can be “absolute” if it is referred to a strain that is passed in a particular line of in-bred mice or “relative” if it passed in in-bred lines of mice with different genotypes.

- *Lesion profile*: refers to the study of the distribution of the lesions at the level of 9 defined areas of gray matter and 3 defined areas of white matter. Depending on the degree of vacuolation, a score ranging from 0 to 5 is assigned to each area.

Other features that may be considered are the ratio between the number of animals that showed clinical signs of disease and the number of inoculated animals, the clinical symptoms, the pattern of deposition of pathological prion protein in the brain and the biochemical profile of prion protein itself (Ryder, 2002; Bruce, 2003).

This system incontrovertibly proves that there is a variability of the agents of prion diseases. However, several practical problems are reported:

- Some ovine isolates can not be transmitted into mice and therefore it is not possible to make any characterization.

- The system requires a great deal of time: primary transmission can take up to 3 years and at least 6 months for each successive passage.

- The process is extremely expensive both in economic terms and in terms of animals used; it also requires highly skilled staff.

The biochemical approach to the study of the strains is based on the fact that PrPsc electrophoresis highlights three protein bands corresponding to forms di-, mono-and non-glycosylated protein (PrP is a glycoprotein, a protein that is with two different sites of anchorage of carbohydrate chains). The analysis of the molecular weight of the fragments generated by enzymatic degradation of PrPsc and the quantitative ratio between its three differently glycosylated forms, have indicated the existence of different strains, in particular as regards the human prion diseases and they certainly constitute a strains study instrument more efficient respect to mice transmission studies (Nonno, 2003; Thuring, 2004).

Unfortunately, the study of the correlations between PrPsc strains and PrPsc structural characteristics is still limited to the indirect characterization of the aggregates structure of PrPsc, because of the extreme difficulty in crystallizing or solubilize the PrPsc in order to determine directly, via X-rays or magnetic resonance nuclear power, the three-dimensional structure.

Using the techniques previously discussed, several strains of TSE have been identified in different animal species: in addition to the already mentioned strains in the hamster, the most important example is undoubtedly that more than twenty strains were identified in ovine species by transmission in mice, but the total number has not yet been precisely determined.

The passage of the BSE disease in mice gave rise to two strains: the 301V and 301C.

To date, atypical BSE and Scrapie passages to ovicaprine species have a particular interest in the field of prion diseases research.

Atypical cases of BSE

In 2004, as part of a detailed study of the neuropathological and molecular characteristics of Italian cases of BSE, the entire brain of twelve BSE positive cattle has been subjected to an immunobiochemical examination. During the investigation, two out of twelve examined brains, belonging to a Piemontese and Bruna Alpina cattle breed respectively, aged 15 and 11 years, showed absolutely new and unexpected aspects. In particular, both the molecular profile of the prion protein and immunohistochemical pattern results clearly different from other cattle with classical BSE. By Western blot examination, the glicotype (in percentage ratio between the band di-and monoglycosylated of pathological prion protein) presented a proportion of glycoforms strongly represented by the monoglycosylated band, different from classic cases of BSE, characterized by a stronger expression of diglycosilated band. In addition there was a different electrophoretic mobility of PrPsc migration bands: the non-glycosylated band has migrated faster due to a lower molecular weight compared to that of classical BSE. On the basis of this characteristic, two samples were defined Low-type (L-type).

About brain sections examined by immunohistochemical analysis, they showed a PrPsc deposition pattern characterized by amyloid plaques similar to Kuru disease and granular deposits into glial and extracellular level. The plaques appeared as dense structures, unicentric or more rarely multicentric, generally with a round shape and with a diameter of 25 µm, with a clear core surrounded by a darker border. Concerning the distribution, it was found that the

presence of PrPsc was mainly in the more rostral portions of the brain, related to the olfactory system, rather than in the brainstem, most affected in the course of classical BSE.

In light of these results, this different PrPsc phenotype was called BASE (Amyloidotic Bovine Spongiform Encephalopathy) (Casalone, 2004).

Also in 2004, another atypical case of BSE different from BASE have been identified and described in France (Biacabe, 2004) and later in other European countries. This second new variant, according to the immunobiochemical characteristics, was defined High-type (H-type), characterized by an higher molecular weight compared to BSE. The H-type cases showed no significant differences from the classical BSE about the glicotype. An immunohistochemical study performed at the level of the brainstem of two H-type cases, identified respectively in the Netherlands and Germany, has shown that in such cases the prion protein predominantly presented a pattern of intraneuronal and intraglial type, contrary to what happens in the course of BSE, where the pattern most representative is the granular type (Buschmann, 2006).

Classical and atypical TSEs in goats

There are overwhelming evidences that different TSEs agents circulate in goat populations in Europe:

- **Classical Scrapie**

Concerning typing studies by bioassay in rodent models, only limited information on the characteristics of goat isolates is available. In particular, the study of Vaccari et al., 2006, reports that all scrapie cases investigated showed the same profile of pK-resistant PrPSc, different from BSE and indistinguishable from that reported in Italian sheep scrapie cases (Nonno et al., 2003).

The transmission characteristics of a Scrapie isolate from an Italian goat to bank voles, C57Bl mice and hamsters were reported by Piening et al. in 2006, and appear to be very similar to those observed with an Italian sheep isolate (Agrimi et al, 2008). They both transmitted with short survival time to bank voles but very inefficiently or not at all to wild type mice.As far as studies in the natural host are concerned, Sofianidis et al. (2006) reported that histopathological and immunohistochemical examinations of several goats from two Greek Scrapie affected herds showed diverse lesion profiles and PrPSc distribution patterns. In this case, the variability might be influenced by PrP genotype, age and clinical stage. Another study from Sofianidis et al. (2008) describes asymptomatic goats from scrapie-affected herds in which PrPSc deposition was mainly confined to cortex and rostral brain areas. PrPSc was found by ELISA at the level of the obex only in a limited number of animals and was never associated with immunoreactivity in the dorsal vagal nucleus. Finally, molecular analyses of goat isolates from France, UK and Cyprus suggest that the recently recognised ovine "CH1641-like" isolates (Baron et al., 2008), are also present in goats.

- **Atypical Scrapie**

Atypical caprine Scrapie have been described in different states of Europe such as Switzerland, France, Spain and Italy.

Information on atypical goat Scrapie is limited, but there is clear evidence of similarities with the distinctive features observed in sheep. In particular, the molecular pattern of pK resistant PrPSc obtained in most goat cases is very similar to that observed in Nor98 in sheep (Moum et al., 2005). In a single Atypical case reported in a goat in Switzerland, differences in the distribution of histopathological lesions and PrPSc deposition compared to Atypical sheep scrapie were described but the meaning of such differences is unknown (Seuberlich et al., 2007).

- **Classical BSE**

Two confirmed case of natural goat BSE was discovered in France in 2002 and in UK in 2011 through active surveillance and retrospective examination (Eloit et al., 2005, Spiropoulos et al., 2011).

The identification of two cases of BSE in goats associated with the results obtained from experimental studies carried out by inoculating the classical BSE in sheep with different genotypes showed that the passage of BSE in sheep and goats represents a serious public health problem. In particular, some aspects are particularly important:

- The observed symptoms do not substantially differ from natural Scrapie. In fact, the clinical symptoms observed are weakness in the hindquarters, ataxia, pruritus and dullness.

- Significant similarities were also found regarding neuropathology. The spongiosis is very intense in the medulla oblongata where the dorsal motor nucleus of the vagus is clearly impressed. The cuneate and olivary nuclei appear affected. An intense spongiosis is present at the level of the reticular substance as well as in the raphe, while it is less pronounced at the level of the pons nuclei. The midbrain is affected mainly at the level of the gray matter. Within the diencephalon, hypothalamus appears occasionally hit, while the spongiosis is variable in the thalamic nuclei.

- The distribution of the BSE agent in orally infected sheep was much wider than in cattle and the transmission mode is similar to Scrapie that is both horizontal and vertical.

On the basis of these elements high threshold of attention to TSEs in sheep and goats is essential and it is important to discriminate between different strains of TSEs in sheep and goat, in particular to detect the presence of BSE.

To this end, currently, the biological test remains the method of choice; experimental infection of mice with strains of the infectious agent of bovine BSE, ovine BSE and Scrapie allows to identify symptoms, lesions in various organs and spread absolutely indistinguishable for the three diseases. However, this test requires a very long time (about two years) and it is laborious and difficult to perform on a large scale. For this reason, some authors have studied and standardized immunobiochemical methods for the molecular characterization of PrPsc in order to recognize in a short time BSE in caprine population (Baron et al., 2000; Stack et al., 2002).

- **Atypical BSE in sheep and goats**

Two recent studies reported the experimental transmission of L-type BSE in sheep. Nonno et al. described that homozygous A_{136} R_{154} Q_{171} sheep experimentally infected via intracerebral route showed PrPSc accumulation in the brain, whereas Matsuura et al.(2013) described the pathology in a Cheviot ewe with an incubation period of 1,562 days with PrPSc distributed throughout the brain and peripheral nervous tissues, such as trigeminal ganglia and dorsal root ganglion, and adrenal gland, but absent in lymphoid tissues.

There are no data at the moment concerning L-type BSE in goats.

AIMS OF THE STUDY

This project has as objectives the study of different aspects of classical and atypical BSE in small ruminant. In particular, we want to determine goat susceptibility to L-type BSE infection, to determine clinical aspects of classical BSE and L-type BSE in goats, to characterize L-type strain in goats (lesion profile, PrPSc distribution pattern and immunobiochemical profile), to analyze PrPSc distribution in peripheral tissues and biological fluids and finally, to extend the knowledges about typical and atypical BSE pathophysiology in small ruminants. For these reasons, the experimental transmission of BSE and BASE in goats by intracranial and oral infection has been carried out in this study. The choice of this animal model is due to the fact that caprine species has considerable interest in the field of public health for its proven susceptibility to classical BSE. There are no available knowledges to date on the susceptibility of goats to atypical variants of BSE. However, it is not inconceivable that, in the past and in natural conditions the caprine species has been exposed to this type of infection.

MATERIALS AND METHODS

Animals selection

In order to identify a sufficient number of animals for the experiment, 50 Saanen goats aged between 4 and 6 months were subjected to clinical examination. From each animal, a blood sample for genetic analysis was taken in order to exclude subjects carrying mutations that could confer resistance against BSE: L168 and M142 (Goldmann W., 1996, 2006) or Scrapie (I142M , N146S / D, R154H, R211Q, Q222K).

Preparation and characterization of the inocula

Inocula were prepared from pools of brains tissues of two positive cattle for BSE and characterized by a PrPSc electrophoretic pattern attributable to classical BSE (16819/06) the first one, and to L-type BSE or BASE (141387/02) the second one. It has not been necessary to proceed with a phenotypic characterization of the two animals chosen for the preparation of inocula since immunohistochemical and immunobiochemical characteristics of these two animals have been investigated in previous research projects and they have been widely published (Casalone C., 2004).

Animals' inoculation

The inoculation of animals was carried out in September 2011. All the animals were challenged at the age of 6-7 months. Goats challenged by intracerebral route were subjected to general anesthesia with xylazine (0.22 mg / kg, im) in association with ketamine (11 mg / kg, im), with

local anesthesia (xylocaine 2%) . The dose injected at mesencephalic level was 0.5 ml / animal. Concerning the oral group, the inoculum was equal to 20 ml / head.

Clinical evaluation of animals

The animals were subjected to daily clinical observation carried out by the staff of the animal care with an assessment of general health on a weekly basis by an experienced veterinarian. The neurological examination was performed monthly by a neurologist vet. During the neurological examination, a standardized protocol previously used was used (D'Angelo A., 2007). This protocol included the evaluation of mental state of the animal, posture, gait, postural reactions, cranial nerves, spinal reflexes and sensitivity (by evaluating the forward and posterior flexor reflexes). Responsiveness to external stimuli was assessed by the response to luminous and acoustic stimulations, as described for classical BSE (Braun U., 1997); an animal was considered hyperactive in the presence of an exaggerated response to the stimulus for at least three consecutive times. The nibble reflex is defined positive in the case where, after being stimulated at the level of the withers and the lumbosacral area, it showed head and neck hyperextension and chewing reflection associated with head and tongue movements. An animal was considered symptomatic in the presence of at least two of the following clinical signs: changes of the fleece, mental state and behavioural changes, abnormal gait, postural reaction / altered proprioception and / or positive nibble reflex. Neurological visits have been intensified in the presence of symptomatic animals. Upon completion of the clinical evaluation, in conjunction with the neurologic examination, blood samples were taken from all animals for hematochemical investigations. The animals that simultaneously showed the presence of several clinical signs and

/ or a noticeable impairment of the welfare state, were humanely killed with Enbutramide / Mebenzonio iodide / Tetracaine hydrochloride (Tanax®, Intervet) intravenously administered.

Tissues and organs sampling

From each dead animal, brain, spinal cord, lymphoreticular system, different organs and peripheral tissues were sampled. A complete list of the tissues sampled is shown in Table 1. From each sampling, a portion was snap-shot frozen at -80 ° C for immunobiochemical investigation and the remaining part was fixed in 4% formaldehyde for histopathological analysis.

Samples preparation from central nervous system

For histopathological analysis, after fixation in 4% formaldehyde for at least seven days, the brain and the brainstem of each goat were coronally cut at the level of: obex, medulla, pons, cerebellum, midbrain, diencephalon and telencephalon. The sections were then processed and embedded in paraffin. After the cut made with the microtome to a thickness of 5 μm, the slices were stained with hematoxylin-eosin. In seven macro areas (brainstem, medulla, pons, cerebellum, midbrain, diencephalon and telencephalon) further 23 sub-areas were selected and they are shown in Table 2. At the level of each sub-area severity and distribution of vacuolar lesions were assessed through semiquantitative analysis with optical microscope, and they were blinded performed by two different operators. In the areas where the presence of vacuolization has been detected, a scale from 0 (no vacuolation) to 4 was rated on (extensive vacuolation) in

relation to the intensity of the vacuolation, as already described by Ligios et al. (2002) (Figure 4).

Deposition patterns of prion protein (PrPSc)

The same 23 areas assessed for the intensity of vacuolation were also subjected to immunohistochemistry evaluation of PrPSc deposition patterns. Sections were processed according to the methods already described (Casalone C., 2004): Briefly, after rehydration the slices have been exposed with formic acid to 99% for 20 minutes at room temperature, followed by autoclaving at 121 ° C for 30 minutes. After washing, they were incubated overnight with the monoclonal antibody anti-PrPSc F99 / 97.6.1, diluted 1: 1000. They were then incubated with anti-mouse biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1: 200 for 20 minutes and with avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories). Immunoreactivity was visualized using the 3,3_diaminobenzidina as chromogen. PrPSc patterns in each area were described and a score was assigned to each one depending on the intensity of the presence of PrPSc (0 absent, 1 = very weak, 2 mild, 3 moderate, 4 very intense) (Figure 5).

PrPSc peripheral distribution

All the organs and peripheral tissues taken from each goat have been tested by immunohistochemical examination with antibody F99 / 97.6.1 (Table 1). In this case a score related to presence / absence of prion protein was assigned.

Western blot analysis

Western Blot (WB) has confirmed the diagnosis of transmissible spongiform encephalopathy in slaughtered goats; then we proceeded to highlight pathological prion protein at the level of neural and extraneural tissues and to the molecular characterization of PrPSc. The analyses were conducted on frozen organs and tissues collected from culled goats. These analyses have included positive control samples. The study of molecular characteristics of PrPSc was based on the evaluation of the following parameters:

- electrophoretic mobility of non-glycosylated band

- relation between di-and mono-glycosylated bands

- different immunodetection by 6H4 and P4 monoclonal antibodies.

According to literature, it is possible to distinguish PrPSc extracted from BSE-infected cattle samples to sheep BSE and Scrapie samples. In particular, it is known that ovine BSE is characterized by a molecular weight of the non-glycosylated band lower than Scrapie one, while bovine BSE shows an intermediate molecular weight between Scrapie and sheep BSE. In addition, Scrapie could also be discriminated from ovine and cattle BSE for its strong affinity to P4 antibody instead that it does not react with BSE. The relationship between glycoforms (glicotype) can be useful to distinguish between BSE and sheep Scrapie rather than cattle BSE (Stack MJ., 2002, Nonno R., 2003, Acutis PL., 2006).

Extraneural tissues pre-treatment

Extraneural tissues, with the exception of the spleen, before being subjected to prion protein extraction, were finely chopped with a scalpel and incubated over night at room temperature with

a very slow agitation in 10 % of trypsin solution dissolved with PBS, in order to facilitate tissue disintegration. After this treatment, samples were rinsed with ultrapure water and subjected to the next phase of protein extraction.

PrP extraction

Homogenates from nervous and extraneural tissues were prepared at 10% (w / v) using lysis buffer constituted by 10% Sarkosyl diluted in TBS (pH 7.4). After incubation at room temperature for 20-30 minutes, in order to eliminate cell debris, homogenates were centrifuged for 20 minutes at 10 ° C at 23 000 rpm (Ultracentrifuge Optima TLX, TLA 55 rotor, Beckman Coulter). Aliquots of 1 ml of protein extracts obtained were subsequently digested with the proteinase K enzyme at a concentration of 40 µg / ml (pK; Sigma Aldrich) for 1 hour at 37 ° C with constant shaking at 1200 rpm, in order to eliminate all the proteins present in the sample, including cellular prion protein. The obtained extracts from extraneural samples, before pK treatment to remove nucleic acids in the sample, were incubated with Benzonase enzyme at a concentration of 50 meq / ml (Novagen) for 30 minutes at 750 rpm at 37 ° C. After pK digestion, samples were centrifuged for 1 hour at 10 ° C at 63000 rpm (Optima TLX Ultracentrifuge, Rotor TLA 110, Beckman Coulter). The obtained pellets were resuspended in 25 µl of distilled water and 25 µl of reduction buffer, Laemmli Buffer (2x), and subsequently incubated at 99 ° C for 10 minutes.

Deglycosylation

Samples digested with proteinase K were deglycosylated with recombinant peptide N-glycosidase F (PNGase F) according to the instructions provided by the supplier (Boehringer Mannheim).

Electrophoresis, WB and PrPSc immunological detection

Samples, after reduction, were loaded onto a polyacrylamide minigel 4-12% Bis-Tris and subjected to SDS-PAGE electrophoresis. Subsequently, the proteins were transferred on a PVDF membrane (Immobilon P, Millipore) to 150 Vk for 50-60 minutes using a wet-blot. The membranes, after saturation in TBS-5% BSA for 1 hour at room temperature, were incubated simultaneously over night at 4 ° C with two different monoclonal antibodies, P4 (1: 10000 in TBS-BSA; R-Biopharm AG, Darmstadt, Germany) and 6H4 (diluted 1: 15 000 in TBST; Prionics AG, Switzerland), respectively with the epitope between amino acid residues 93-99 and 156-164. To detect pathological prion protein presence in analyzed samples, the membranes were incubated for 30 minutes at room temperature with a secondary antibody consisting of an anti-mouse serum conjugated with alkaline phosphatase enzyme (AP). After a series of washes with TBS solution containing 0.1% Tween-20, to remove excess of secondary antibody, the membranes were coated with a chemiluminescent substrate (Immun-Star, Bio-Rad) for 5 minutes to room temperature. PrPSc signals were visualized on autoradiographic plates (Hyperfilm ECL, GE-Healthcare) and quantified using a specific software (UVI Prochemi, Uvitec, Cambridge, UK). In order to evaluate the distribution of PrPSc in the brain, the glicotype (percentage ratio of di-and monoglycosylated bands), the reactivity of the two mAbs against prion protein of the samples and the optical densities obtained were then processed using Microsoft Excel.

RESULTS

Animals selection

Goats with mutations at codons 142, 211 and 222 were excluded from the study because these mutations are known in goat for their involvement in the modulation of TSE resistance. Concerning mutation at codon 142, in fact, studies of experimental infection with BSE and Scrapie strains with CH1641, SSBP / 1 and ME7 made in the UK by Goldmann (1996), showed a prolongation of incubation period after inoculation by intracerebral route of goats carrying this polymorphism . In Spain, Acin et al. (2004) have described a case of natural Scrapie in an heterozygous I142M Saanen goat, suggesting that the presence of methionine may reduce the susceptibility, increasing the incubation period, but it does not confer resistance. Concerning codon 211, studies carried out in France suggest a protective role; despite having found the mutation in affected animals, the analysis by Chi-square test will show a different statistically significant distribution, between cases and controls (Barillet F., 2009), and this protective role has also been suggested by Greek studies (Bouzalas IG., 2010). Italian studies attribute to the lysine at codon 222 a role of resistance: this mutation is not ever been observed in goats with scrapie (Acutis PL., 2006, Vaccari G., 2006); further European research, noting this mutation in affected animals, have confirmed its protective role (Barillet F., 2009, Bouzalas IG., 2010). Concerning codon 240 polymorphism, it was never observed any significant association with the disease; a possible explanation could be the cleavage of the C-terminal portion of the protein, including that codon, as a result of post-translational modifications, as demonstrated in rodents (Stahl N., 1990). For this reason, goats with proline allele at codon 240 were considered eligible for the study. Therefore, only 29 out of 50 examined goats showed a wild-type genotype suitable

for the present research; in this group, 20 female goats have been selected, aged between 5 and 12 months, and they were transferred to CISRA, the Interdepartmental Centre Admissions Service Animals, in the Faculty of Veterinary Medicine of Turin, recognized as a producer of goat enclosure [authorization number 2.9 of 22 July 2002 issued by the City of Grugliasco - number of breeding: 120TO025 (ASL Collegno 3)] where the animals were identified by ear tags and personal microchips and an homogeneous group was created. The animals were also monitored and followed up by the Centre according to the UNI EN ISO 9001: 2008 in order to exclude the presence of pathogens that could interfere with the experiment. For this purpose, the animals were subjected to parasites treatments and vaccinated for clostridia and pastorellosis.

At the end of this first phase, the animals were transferred to isolation stables of Institute Zooprofilattico of Lombardia ed Emilia Romagna, in Brescia, where, at the end of an acclimatization period of 15-30 days, they were divided into experimental groups to proceed with prion inoculation. The experimental groups of animals were formed: a first group of 5 goats and a control for intracranial inoculation with classical BSE; a second group of 6 goats and 2 control for intracranial inoculation with BASE, and a third group consisting of 5 goats and a control for oral inoculation with BASE. The controls are useful to highlight any side contamination from infected animals during the experiment (Tables 2, 3, 4).

Clinical evaluation of animals

At 36 months post inoculation (mpi) all the goats of BSE ic group and two goats belonging to the BASE i.c group were culled at the terminal stage of the disease. The negative control of BSE i.c. group was also culled, although it did not present any clinical signs. The animals of BSE i.c.

group showed incubation times of disease shorter (17-19 mpi) than those of BASE ic goats (21-27 m.p.i.).

Concerning BSE ic group, a goat (81553) began to show the first clinical signs at 3-5 mpi with ataxic gait, facial mimic deficit (unilateral) and absent menace response (unilateral), but injuries were exclusively due to a prosencefalic lesion on the right side caused by an incorrect operation of inoculation. Concerning remaining goats of this group, at the onset of the disease, they have showed areas of hair loss with broken hair at the level of head neck and loins, then spread to the hips and tail base. Sometimes over-reactivity to touch was present, characterized by an exaggerated response when they were touched for neurological tests. Later, towards the final stage of disease, animals showed dullness, characterised by a low head carriage when undisturbed and inappetence, fear, and ataxic and hypermetric gait, proprioception deficits, cranial nerve deficit and positive scratch test, grade 2 (Table 5).

Concerning two culled goats belonging to BASE i.c. group, the disease occurred at 21 and 27 mpi respectively, with hair loss, that was intermittent in one case (81557). Then, the sintomatology evolved in both cases with a manifestation of lameness in the limbs, tremors and proprioceptive deficits. In the final phase, the animals showed dullness, disorientation, difficulty in rising, teeth grinding and positive scratch test (Table 5, Figure 6 ABCDEF).

Among remaining live goats, the symptomatic are: two belonging to BASE ic group that show intermittent hair loss, intermittent pruritus and generalized tremors; and three belonging to BASE os group that show intermittent fleece changes associated in one case with intermittent tremors. To date, scrarch test is negative.

Lesion profile

Lesion profiles were performed on CNS of all the goats belonging to BSE ic group and the first two goats belonging to BASE i.c group.

Concerning BSE ic group, the greater degrees of vacuolation were detected at the level of the thalamus and basal nuclei (in particular in the nucleus caudatus and putamen). Even the midbrain, brainstem (n. Trigeminal nerve) and the first sections of medulla (the ventral horns) appear affected, albeit with a lesser extent (Graph 1).

As regards BASE i.c. group however, the major degrees of vacuolation were detected at the level of thalamus and brainstem (trigeminal n.). Instead, the first section of the cervical spinal cord presents a lower degree of vacuolization (Graph 2).

Evaluation of PrPSc deposition patterns

Intense immunolabelling was detected in all brain areas of goats belonging to BSE i.c. group (Figure 7), and occurred over a wide range of PrPSc types. PrPSc accumulations consisted of marked deposits in neuronal and glial cell cytoplasm, and extracellular PrPSc labelling patterns, such as:

-Granular: granular and ovoid scattered deposits of PrPSc;

-Glial: intense reactivity of PrPSc in the cytoplasm of glial cells.

-Linear: Very thin thread-like deposits of PrPSc scattered in the neuropil

-Intraglial: Intense granular and ovoid deposits of PrPSc scattered in the cytoplasm of glial cells, often in the proximity of the nucleus.

-Intraneuronal: granular PrPSc deposits in the pericarion of neurons, surrounding the nucleus.

-Perineuronal: PrPSc deposits placed around the pericarion neurons and neurites.

-Small Aggregates: Accumulations of larger PrPSc deposits.

In particular, prominent glial and granular patterns were found in telencephalon, diencephalon, midbrain, cerebellum, pons and medulla, whereas prominent extracellular deposits such as small aggregates were mainly present at the level of telencephalon, midbrain and medulla.

Concerning two goats belonging to BASE group, immunohistochemistry revealed moderate amounts of granular and particulate PrPSc deposition in olfactory areas, talami nuclei including the lateral and medial genicolate nuclei, the inferior colliculus of midbrain and the spinal cord. Small aggregates sometimes were detected at the level of cerebellum. Very low levels of PrPSc immunolabelling were generally observed in the cerebral cortices and basal ganglia (Figure 8).

Peripheral distribution of PrPSc

By immunohistochemistry, concerning BSE ic group, a widespread distribution of prion protein in Gasser ganglion, pituitary gland, tonsils, ileocecal valve, parotid lymph node, retro pharyngeal and submandibular lymph nodes, spleen and kidney was found, while all other tissues and organs were negative. Regarding BASE ic group, the positivity was found only at the level of central nervous system. Western Blot analysis on these tissues is on going

Western blot analysis

WB analysis at the level of frozen brainstems taken from all the culled goats, showed the presence of PrPSc, thus confirming in these animals PrPSc positive findings with other diagnostic tests (IDEXX, immunohistochemical examination and histological examination). Immunoblotting investigations carried out on frozen brain of a goat belonging to the BSE i.c. group have revealed, both with P4 antibody and with 6H4, the presence of PrPSc in other brain areas, with the exception of temporal cortex, parietal cortex and thalamus (Figure 9).Quantitative analysis conducted by special software also showed a different intensity of the PrPSc signals among different brain areas: signals of greater intensity were evident in particular at the level of the brainstem and basal ganglia. Intense signals of PrPSc were also present at the level of the different sections of medulla (cervical, thoracic and lumbar parts) (Figure 10).

However, the screening of BSE and BASE goats brains revealed PrPSc, that is better recognised by P4, rather than 6H4.

PrPSc molecular investigations performed on BSE ic goat and two positive controls showed an electrophoretic pattern characterized by three bands with different molecular weights, corresponding to three glycoforms of the protein (di, mono-and not-glycosylated). In particular, the comparison of not-glycosylated band, immunorevealed with the antibody 6H4, showed a minor electrophoretic mobility of this band in both samples of goat BSE ic, compared to samples of bovine BSE and Scrapie (Figure 11). All analyzed samples (goat BSE ic 81533, caprine Scrapie and bovine BSE) have been recognized by both antibodies (6H4 and P4); as it was expected, only the bovine BSE has not been revealed by P4. The ratio of the intensities of PrPSc signals simultaneously immunorevealed with 6H4 and P4 antibodies (6H4 / P4) gave a decreasing scale of values in the following order: BSE bovine> goat 81533> caprine Scrapie.

Ratio obtained from quantitative analysis of PrPSc glycosylated bands (diglycosylated / monoglycosylated) showed, in all analyzed samples, an higher value for diglycosylated band, but in particular the obtained glycotype from both samples of nervous tissue of the goat 81553 was very similar to that obtained from the sample of BSE (Table 6).

Comparing the reactivity to 6H4 and P4 antibodies of goats belonging to BSE ic and BASE i.c groups, to cattle BSE and BASE (Figures 12A and B), it is evident the reactivity of goats of BSE and BASE groups for both antibodies, although a better signal is with P4 (epitope localization in the N-terminus). Different reactivity of bovine BSE sample that well reacts with both P4 and 6H4 instead of bovine BASE that well reacts with 6H4 but not with P4. Interestingly, even as BASE ic goats, with 6H4 antibody, they have similar phenotype to bovine BASE because monoglycosylated band is more evident, while with P4 antibody a phenotype very similar to Scrapie is present , with no difference between di and monoglycosylated bands. With deglycosylation (C) however, PrPSc of both BSE and BASE goats appears to be in lower levels compared to cattle BSE and BASE samples.

DISCUSSION AND CONCLUSIONS

TSEs are multifactorial diseases in which molecular, genetic and environmental aspects determine different pathological and clinical phenotypes. The present research project, using a multidisciplinary approach, had as objectives to deepen and clarify where possible, some of the mechanisms at the basis of prion diseases and in particular of atypical cattle TSE known as L-type atypical forms or BASE. In this experimental transmission of BSE and BASE in goats, at 36 months post inoculation, all the goats belonging to BSE ic group and two of BASE i.c group showed clinical symptoms attributable to TSEs and therefore, they were culled. This data confirm uppermost the transmissibility of BASE to goats and consequently, the fact that BSE and BASE are indeed two distinct strains characterized by different incubation periods and different attack rates. In fact, considering data related to clinical symptomatology of currently alive goats and data relating to culled goats, clearly appears that caprine species is more sensitive to classical BSE rather than to the atypical forms, unlike other animal species where L-type strain shows more aggressiveness (Lombardi G., 2008, Capobianco R., 2007, Béringue V., 2007). In fact, in BSE ic group, the onset occurred earlier than in BASE ic group, presenting an evolutive framework, characterized in most cases by hair loss and ataxic gait and hesitated in a short time in the death of all the subjects of the group. Ataxia is the predominant sign in the experimental BSE goats of this study; moreover, deficient menace response, that is rare in cases of Scrapie, is also frequently displayed in these goats. Therefore, results obtained up to now about the incubation period of BSE ic group are in line with what is described by Foster et al. (2001) but they diverge from Konold et al. (2010) that in a similar experiment he has reported a longer incubation period (20.5 -29.5 mpi) of experimental BSE goats. Therefore, symptoms observed are in agreement with Konold et al. (2010): BSE goats present with high incidence

areas of alopecia with broken hair not associated with pruritus, which confirms the existence of a "non-pruritic" form of BSE in goats as described by Konold et al. (2010). In the central nervous system, intense immunolabelling was detected in all brain areas but with particular intensity at the level of basal ganglia, thalamus, midbrain, and medulla, confirming previous data about experimental BSE in goats (Foster 2001)

Different from classical BSE, the analyses on two BASE goats have revealed incubation periods quite similar (21-27mpi) to what described by Nonno about ARQ/ARQ sheep intracerebrally inoculated with BASE (29±3 mpi), a later onset of the disease compared to Scrapie and BSE in sheep. The symptoms of two goats were mainly characterized by intermittent hair loss sometimes with pruritus and proprioceptive deficits hesitating at terminal stage of disease in the death of animals. In the central nervous system, a greater level of vacuolation in caudal areas such as thalamus, brainstem and medulla has been highlighted with more PrPSc deposition with predominantly intraneuronal and granular patterns, quite similar to what described by Matsuura (2013) in BASE sheep with conventional immunohistochemistry. Only with TSA method he has been able to highlight particulate deposits of PrPSC also in the neuropil of the cerebral cortex and basal ganglia, areas that in BASE goats of this study were less involved. In both cases, however, plaques have not been highlighted.

Regarding the study by immunohistochemistry on PrPSc peripheral distribution of BSE in goats, the investigations showed that the positivity was not limited only to the central nervous system but that it was also present in the peripheral tissues within the lymphatic system. This result suggests a possible involvement of the lymphatic system in goats with BSE as already described in ARQ Cheviot sheep inoculated intracerebrally with C-type BSE, similar to Scrapie (Martin S., 2005); PrPSc accumulation in these tissues might occur by centrifugal propagation after accumulation in central nervous tissues. This characteristic of BASE goats has importance

because the positive tissues can be easily reached during a biopsy for *in vita* diagnosis. The absence of PrPSc accumulation in the lymphoid tissues of BASE goats was identical to previous observations in sheep (Nonno R., 2008, Matsuura Y., 2013). By contrast, PrPSc accumulation in the lymphoid tissues has not been reported in naturally-occurring cases of BSE in cattle. These results suggest that the species of ruminant is far more important, although prion strain may have a role in the accumulation of PrPSc in ruminants.

The results of the study of molecular characteristics of PrPSc in BSE goats reveal the presence of a different prion protein from the inoculum and from caprine Scrapie. The lower electrophoretic mobility of the unglycosylated band of PrPSc clearly reveals a different cleavage site by proteinase K from BSE and Scrapie samples. The molecular weight of the unglycosylated band (~ 18 kDa) and PrPSc glycotype would suggest a pathological protein with molecular characteristics quite similar to those observed in sheep infected with BSE (Nonno R., 2008). Similarly, the lesion profile and PrP deposition patterns studies have allowed us to build up a neuropathological “picture” with different characteristics from those of both classical and atypical Scrapie, but rather comparable to other experimental BSE goats.

The results of the study of the molecular characteristics of PrPSc in BASE goats, revealed that with 6H4 antibody, caprine BASE show molecular phenotype similar to bovine BASE inoculum for the main monoglycosylated band, while it is interesting to note that with P4 antibody BASE goats present a very similar phenotype to Scrapie, as there are no differences between di- and mono glycosylated bands.

In deglycosylation analysis, PrPSc of BSE and BASE goats appears to be at a lower level compared to cattle BSE and BASE samples and this would mean that pK, in goats samples, cut an area farther from the N-terminal portion of the protein compared to BSE and BASE samples.

In addition, the study of PrPSc peripheral distribution showed no deposits of prion protein in peripheral tissues, confirming data of Nonno R. (2008) and Matsuura Y. (2013).

In conclusion, this is the first study that documents the susceptibility of goats to atypical L-type BSE, although goat appears to be more resistant to this form rather than to the classical BSE. The topographical PrPSc distribution and PrPSc types in caprine L-type BSE were found to differ from those in ovine and caprine C-type BSE. Therefore, our findings show that BASE behaves as a distinct prion strain in goats, different from classical BSE based on disease phenotype, but its peculiar characteristic to show a molecular phenotype similar to Scrapie has an extreme importance concerning the identification of the disease in caprine species with confirmation routine test, because it may be misdiagnosed as Scrapie, with important implications for public health and security. Characterizing atypical BSE isolates in ruminants using other interspecies transmission studies may help to reveal the origins and pathogenetic mechanisms of this disease.

FIGURES, TABLES AND GRAPHICS

Figure 1. A model of the transition of the PrP^{C} non-toxic form that has been solved with NMR left, to the PrP^{Sc} hypothetic fibril forming right (http://cmpharm.ucsf.edu/cohen/)

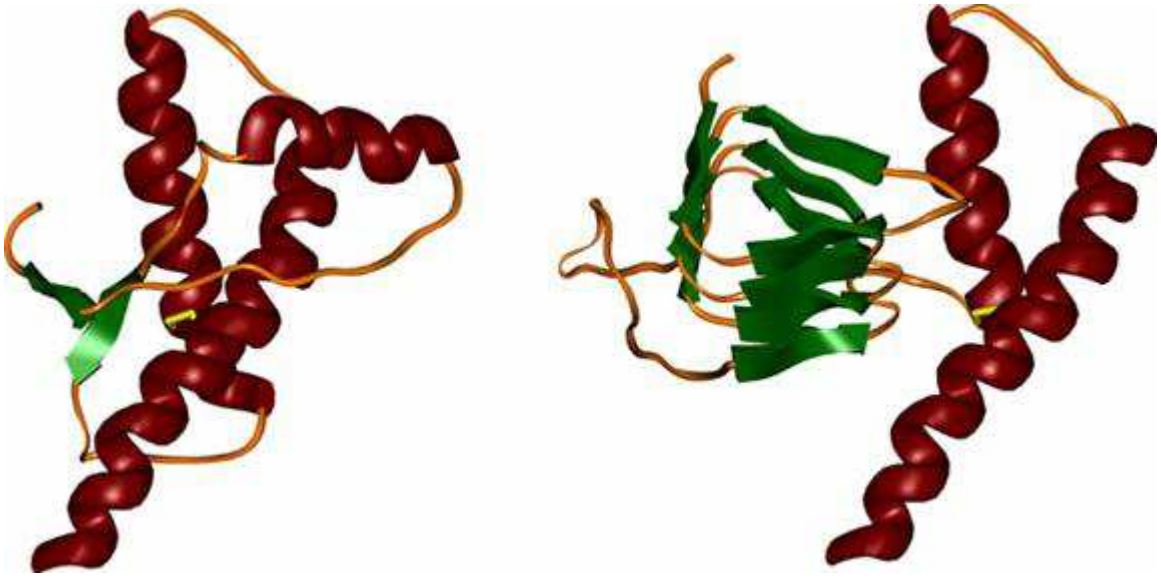


Figure 2. Schematic representation of the possible ways of intestinal epithelial crossing and subsequent invasion of the ENS (Van Keulen, 2000).

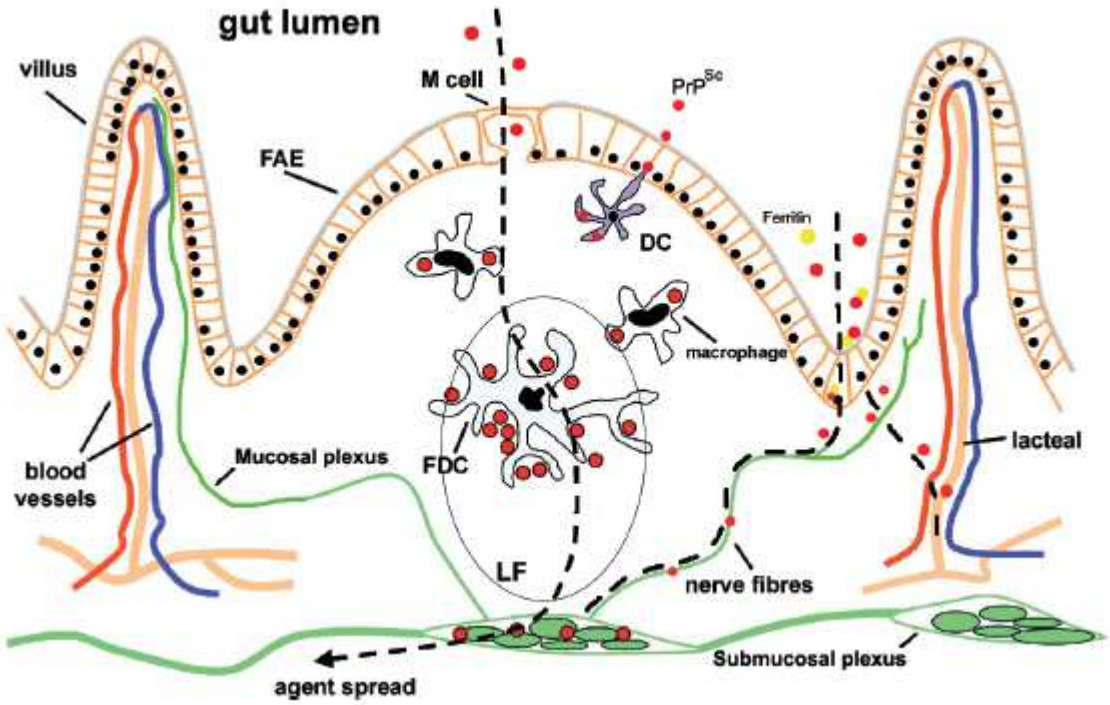


Figure 3. Schematic representation of the parasympathetic (shown on the left) and sympathetic (shown on the right) innervation of the ENS (Van Keulen, 2000).

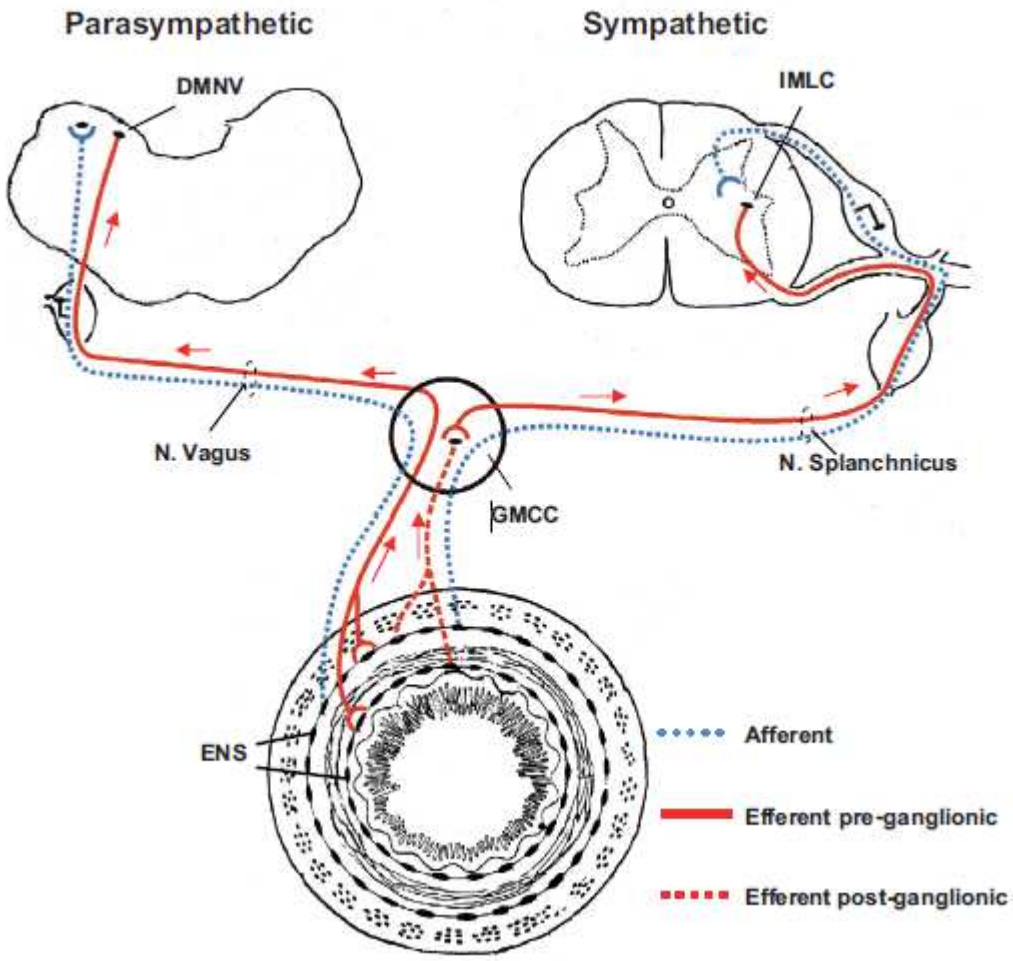


Figure 4. Example of lesion profile evaluation, HE: A. score 1, 10x; B. score 2, 10x; C. score 3, 10x; D. score 4, 10x.

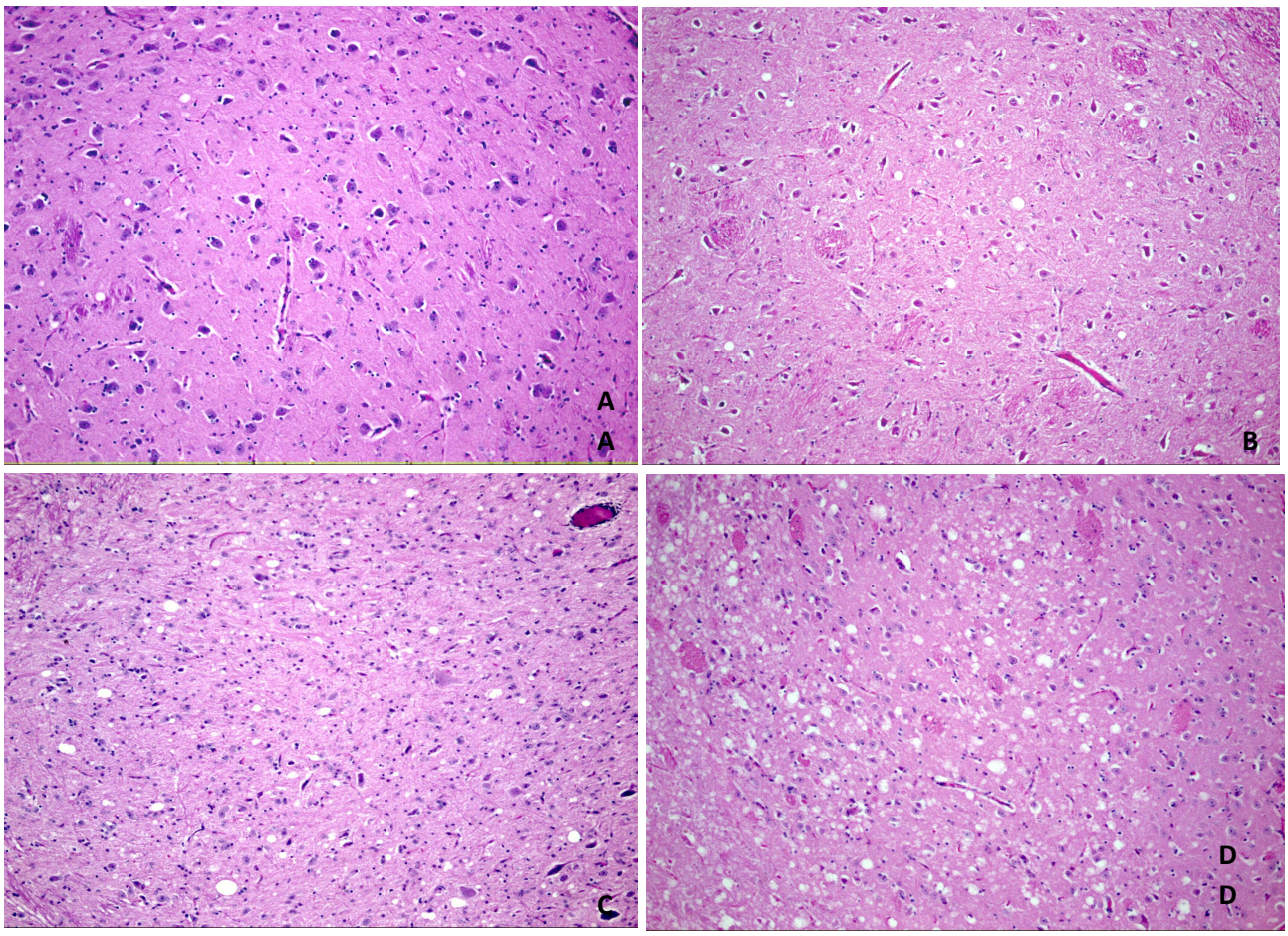


Figure 5. Evaluation of the intensity of PrPSc, IHC: A score of 0, 10x; B. 1 score, 10x; C. score 2-3, 10x; D. score 4, 10x.

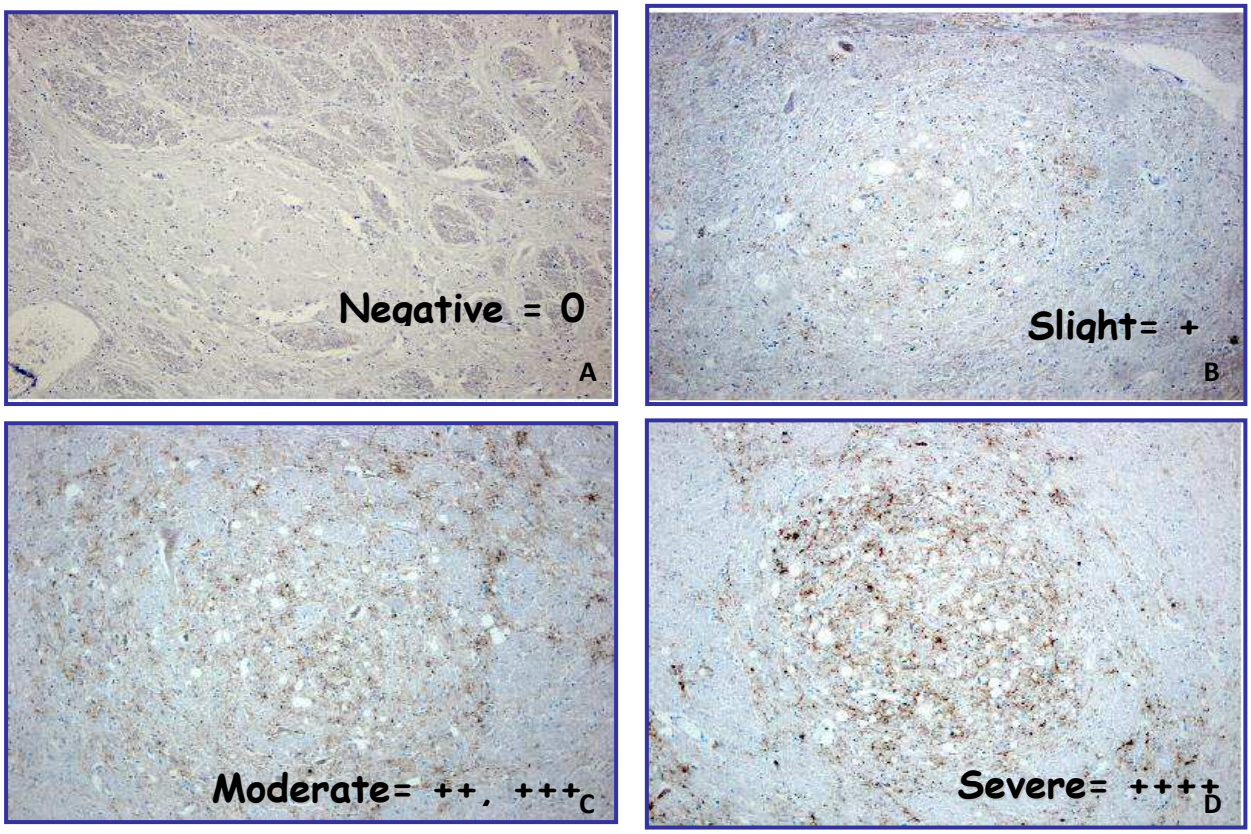


Figure 6. A-B-C-D-E-F. A-B-C: Goats belonging to BSE i.c. group. Alopecia and broken hair at the level of the head (A), loins (B) and tail base / hind limbs (C). D-E-F: Goats belonging to BASE i.c group. Alopecia and broken hair at neck base (D) and tail base (EF).

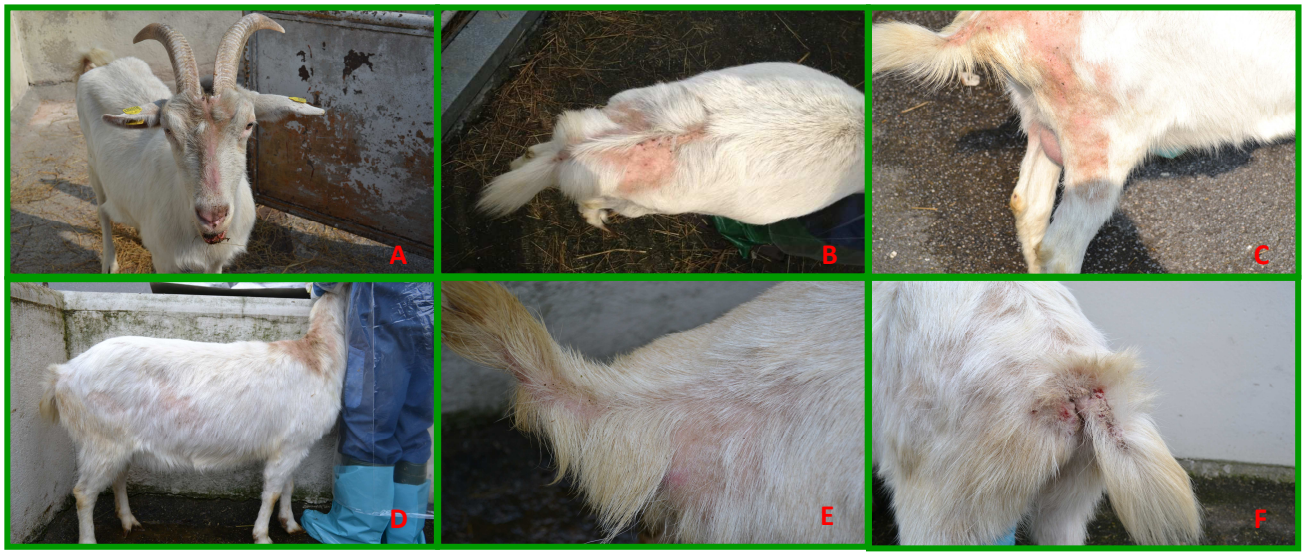


Figure 7. BSE-goats IHC patterns of PrPSc deposition (10X): 1. Hypothalamus, glial 2. Medulla, granular 3. Medulla, intraneuronal 4. Putamen, perineuronal 5.Thalamus, linear 6. Thalamus, small aggregates.

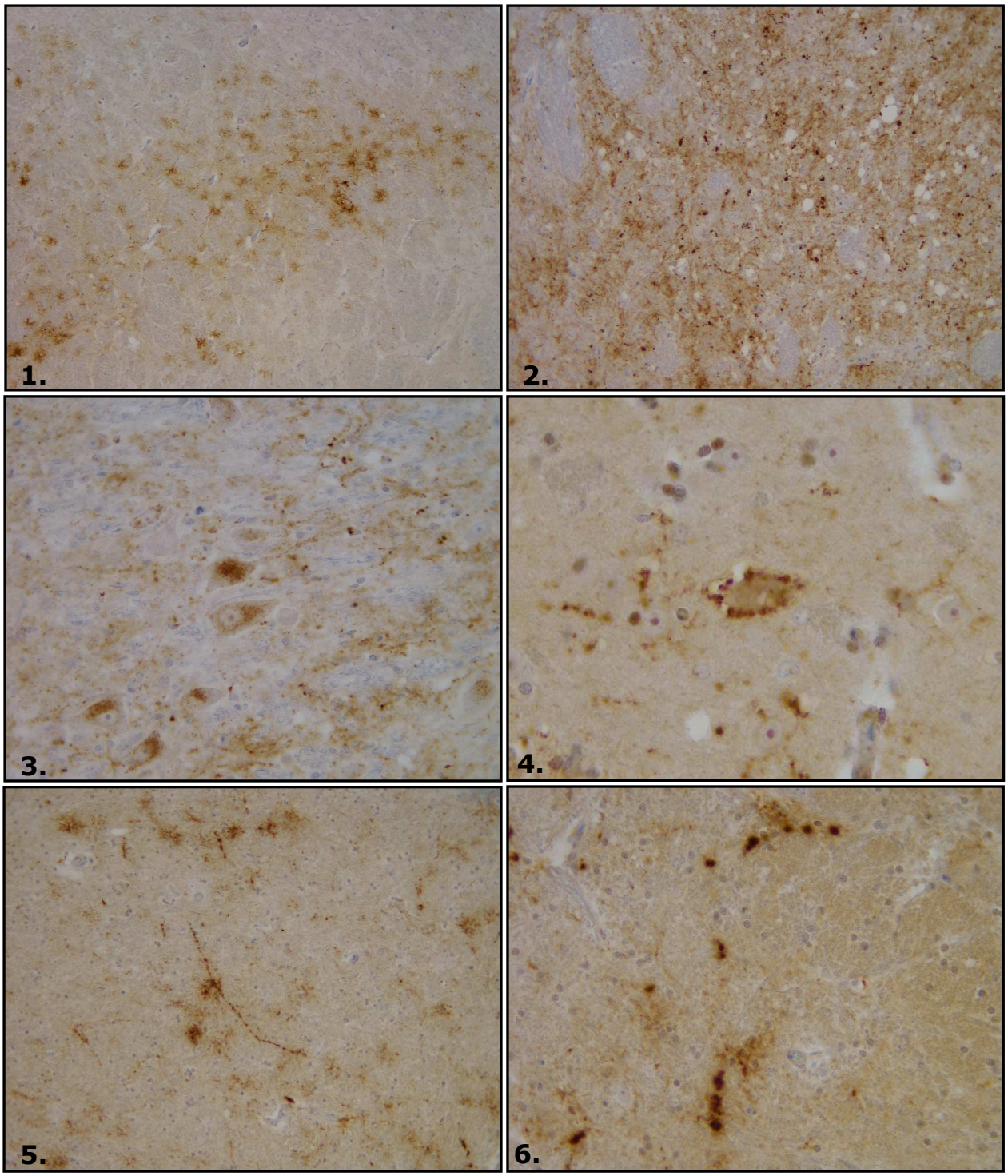


Figure 8. BASE-goats IHC, PrPSc deposition (10X): 1. Frontal cortex 2. Thalamus 3. Pons 4. Cerebellum 5.Brainstem, Trigeminal n. 6. Medulla, dorsal horn.

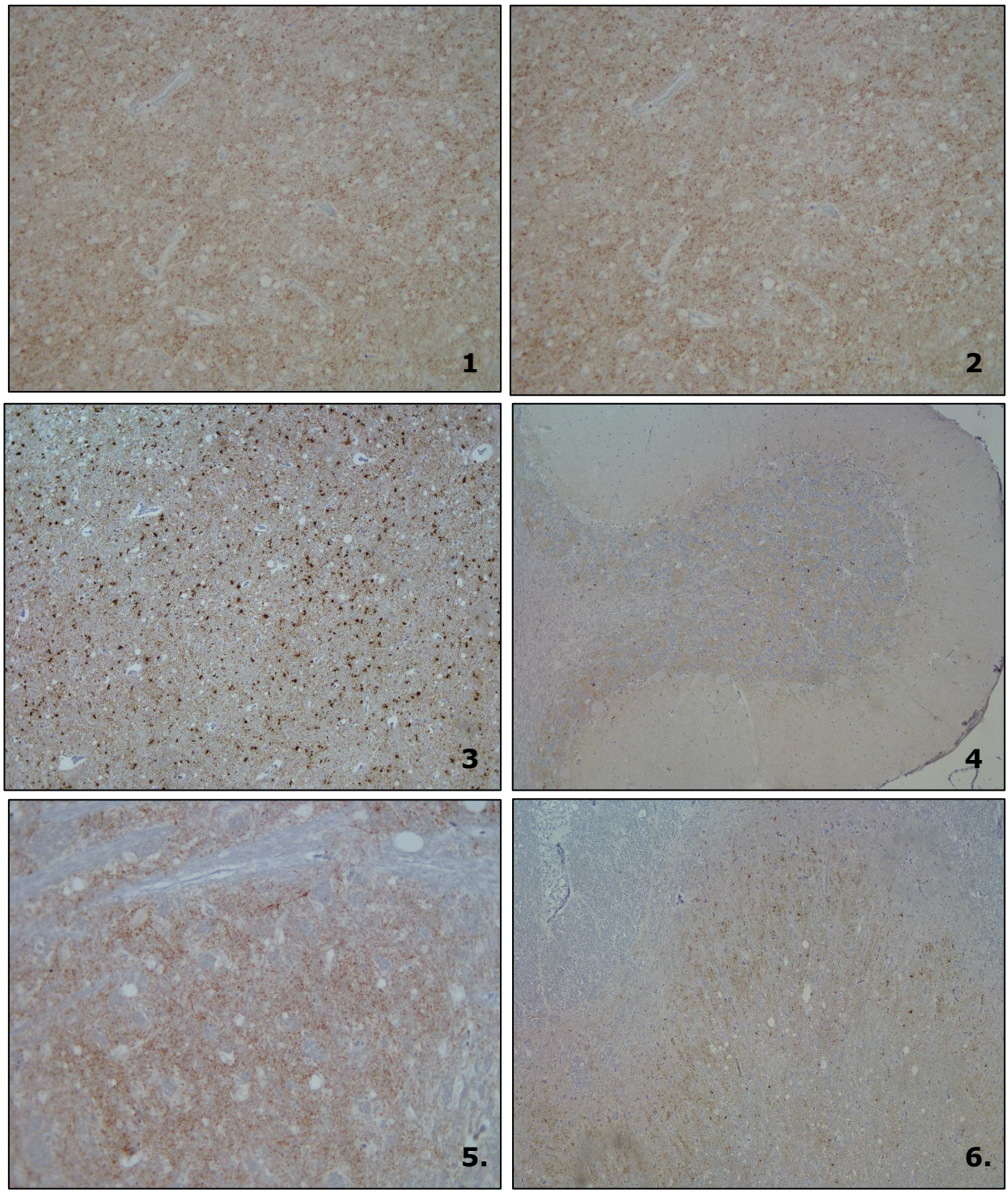


Figure 9. Western Blot analysis on the goat 81553 brain. Immunodetection with 6H4 and P4 antibodies. Scr. = Goat scrapie positive; BSE = bovine 128204; C: goat scrapie negative; c.f. = c. front; n.b. = basal ganglia; c.t. = temporal cortex ; c.p. = parietal cortex; tal. = thalamus; L.P. = piriform lobe; cvt = cerebellum; c.o. = occipital cortex; tr. = brainstem

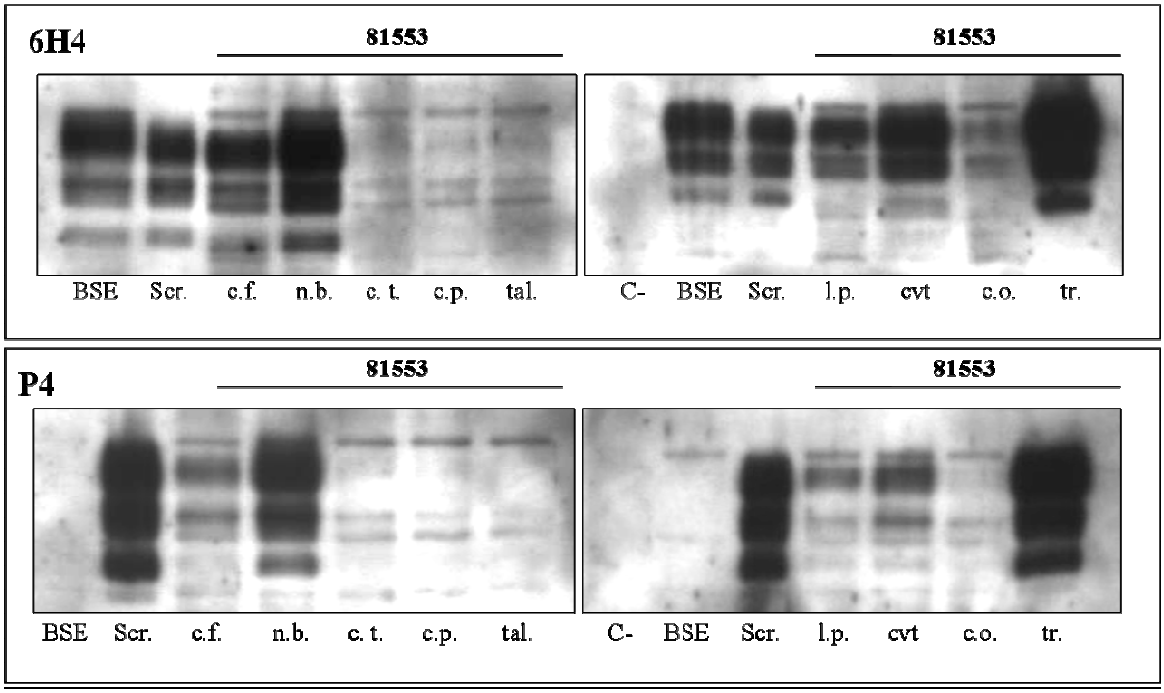


Figure 10. Western Blot Analysis of goat 81553 medulla. Immunodetection with 6H4 and P4 antibodies. Scr. = Goat Scrapie positive; BSE = bovine 128204; 1 = lumbar section; 2 = cervical section 2; 3 = cervical section 4; 4 = thoracic section 2; 5 = thoracic section 4.

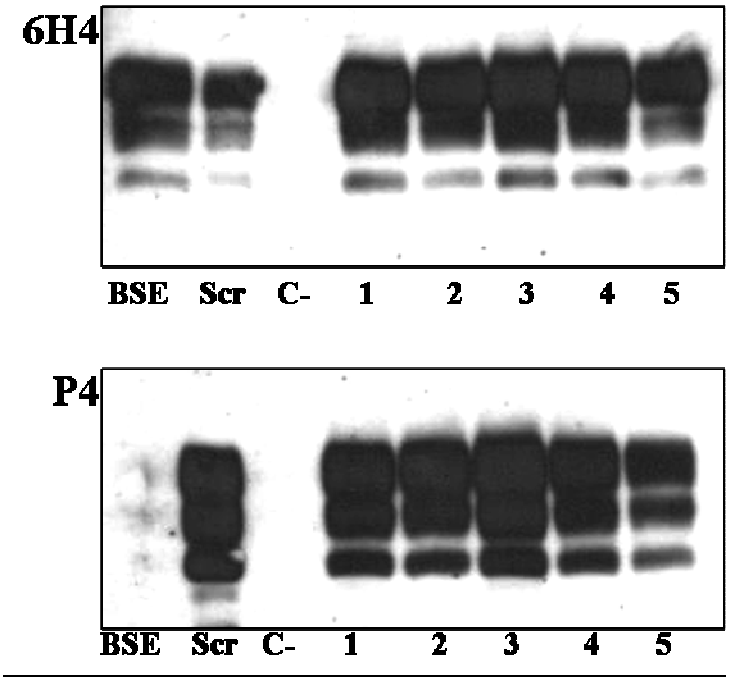


Figure 11. Electrophoretic mobility comparison and PrPSc reactivity. Scr. = Goat Scrapie positive; BSE = bovine 128204; C1 = 81553, brainstem; C2 = 81533, medulla -thoracic section.

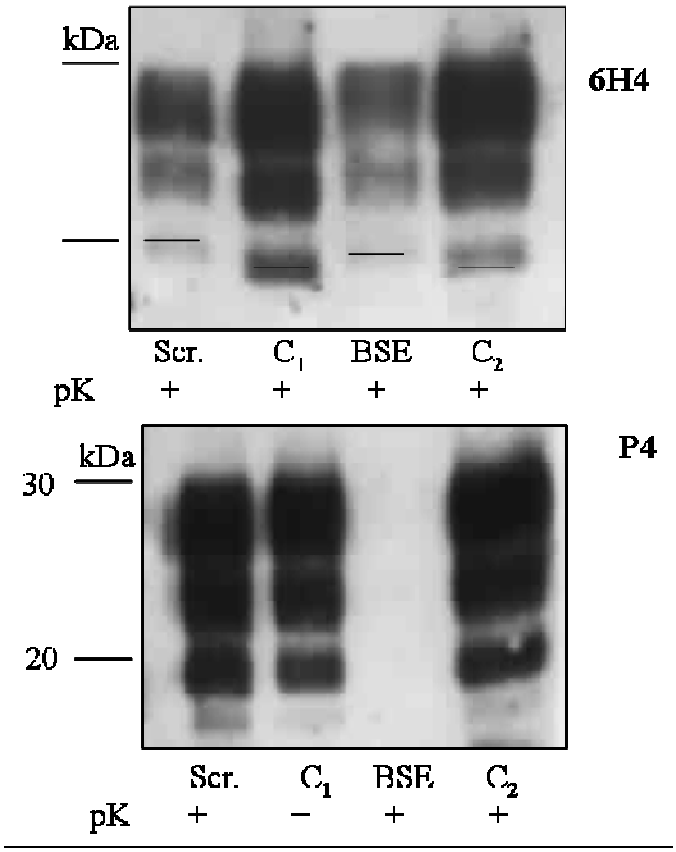


Figure 12 A-B. Western blot analysis of PrPSc extracts from brainstem of two BSE ic (G1, G2) and BASE i.c. (G3, G4) goats after treatment with proteinase K (A and B) and enzymatic deglycosylation (C). MW indicates the molecular mass markers. The antibodies used are P4 and 6H4.

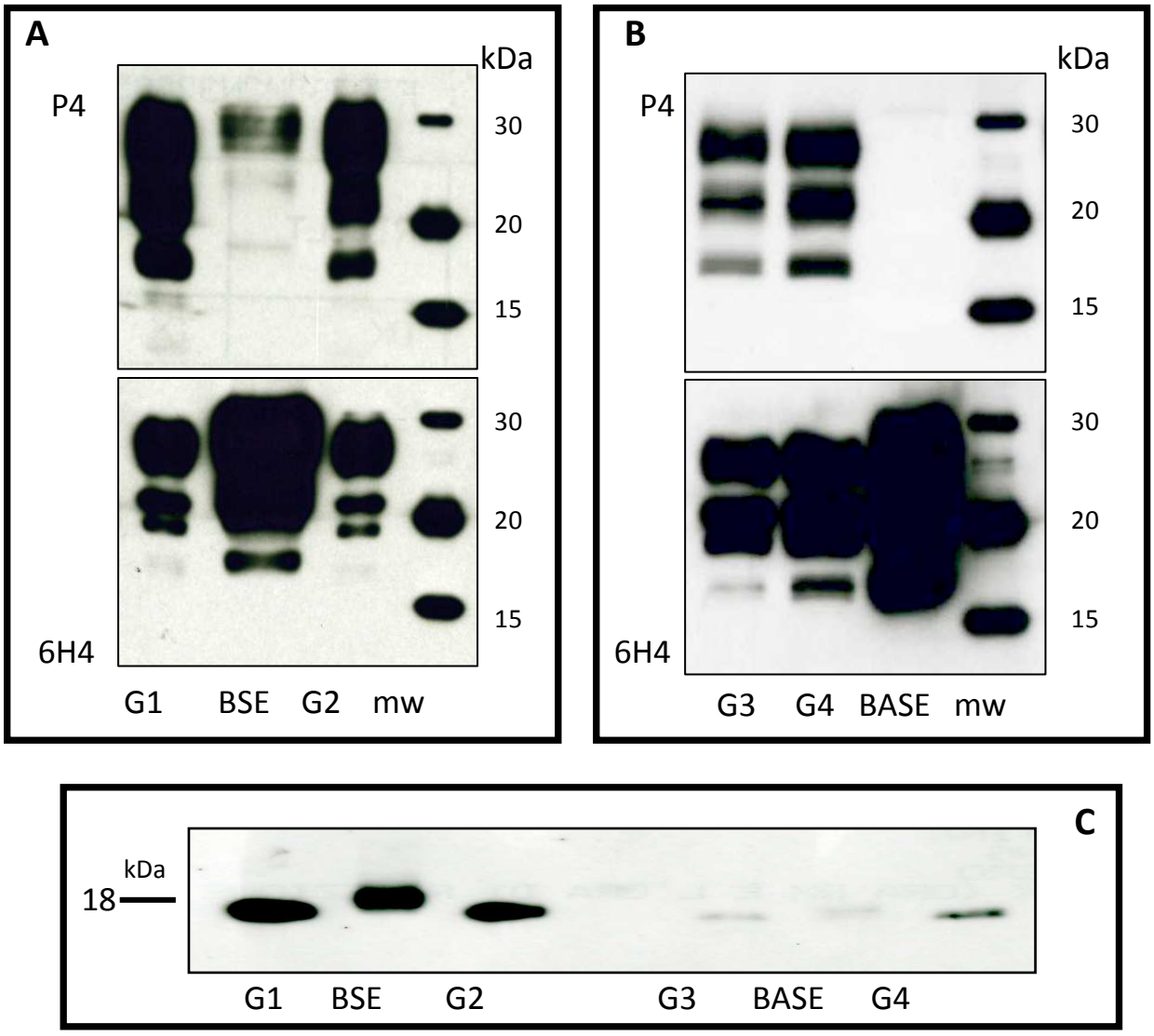


Table 1. Sampled tissues during necropsy.

Peripheral Nervous System	Linforeticular Tissues	Respiratory Tract	Gastrointestinal Tract	Muscles	Miscellaneous
Gasser ganglion Pituitary gland Brachial plexus Ischial N. Cervical medulla Toracic medulla Lumbar Medulla Sacral medulla	Spleen Tonsils Mediastinal L. Mesenteric L. Retropharyngeal L. Third eyelid Pre scapular L.	Lung Etmoidal Volutes	VIC Rumen Duodenum Jejunum Reticulum Esophagus Ciecum Pancreas Liver Stomach Rectal mucosa	Masseter Tongue Extensor carpi Trapezius Intercostals Flexor carpi Ulnar flexor carpi Biceps femori Pectoral Semitendinosus Peroneus Longissimus dorsi	Bladder Kidney Eye Adrenal gland Ovary Aorta Uterus Heart Salivary G. Parotid G. Submandibular G.

Table 2. Id number and genotypes of goats belonging to BSE ic group

BSE i.c.		
GOAT ID	**BREED**	**GENOTYPE**
81553	Saanen	ARQ/ARQ 240 S/P
81556	Saanen	ARQ/ARQ 240 P/P
81559	Saanen	ARQ/ARQ 240 P/P
69557	Saanen	ARQ/ARQ 240 P/P
69556	Saanen	ARQ/ARQ 240 P/P
69538	**Saanen**	**ARQ/ARQ 240 P/P**

Table 3. Id number and genotypes of goats belonging to BASE ic group

BASE i.c.		
GOAT ID	**BREED**	**GENOTYPE**
81560	Saanen	ARQ/ARQ 240 P/P
81554	Saanen	ARQ/ARQ 240 P/P
81562	Saanen	ARQ/ARQ 240 P/P
81557	Saanen	ARQ/ARQ 240 P/P
69540	Saanen	ARQ/ARQ 240 P/P
69548	Saanen	ARQ/ARQ 240 S/P
69547	Saanen	ARQ/ARQ 240 P/P
81563	**Saanen**	**ARQ/ARQ 240 P/P**

Table 4. Id number and genotypes of goats belonging to BASE os group

BASE os		
GOAT ID	**BREED**	**GENOTYPE**
69558	Saanen	ARQ/ARQ 240 P/P
69536	Saanen	ARQ/ARQ 240 P/P
69561	Saanen	ARQ/ARQ 240 S/P
68175	Meticcia	ARQ/ARQ 240 P/P
69559	Saanen	ARQ/ARQ 240 S/P
69560 (ctrl)	**Saanen**	**ARQ/ARQ 240 P/P**

Table 5. Timeline of clinical manifestations of the goats belonging to BSE i.c. group and BASE ic. group. Each column corresponds to the timepoint (mpi) where a specific clinical symptom appeared.

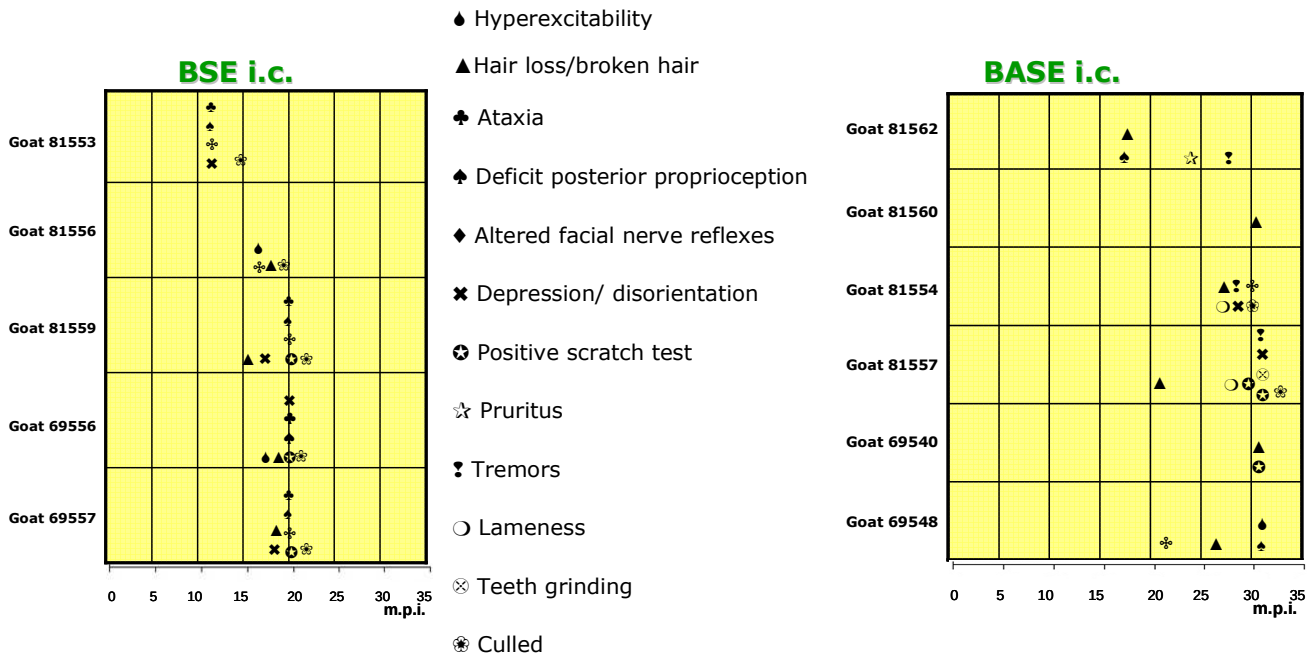
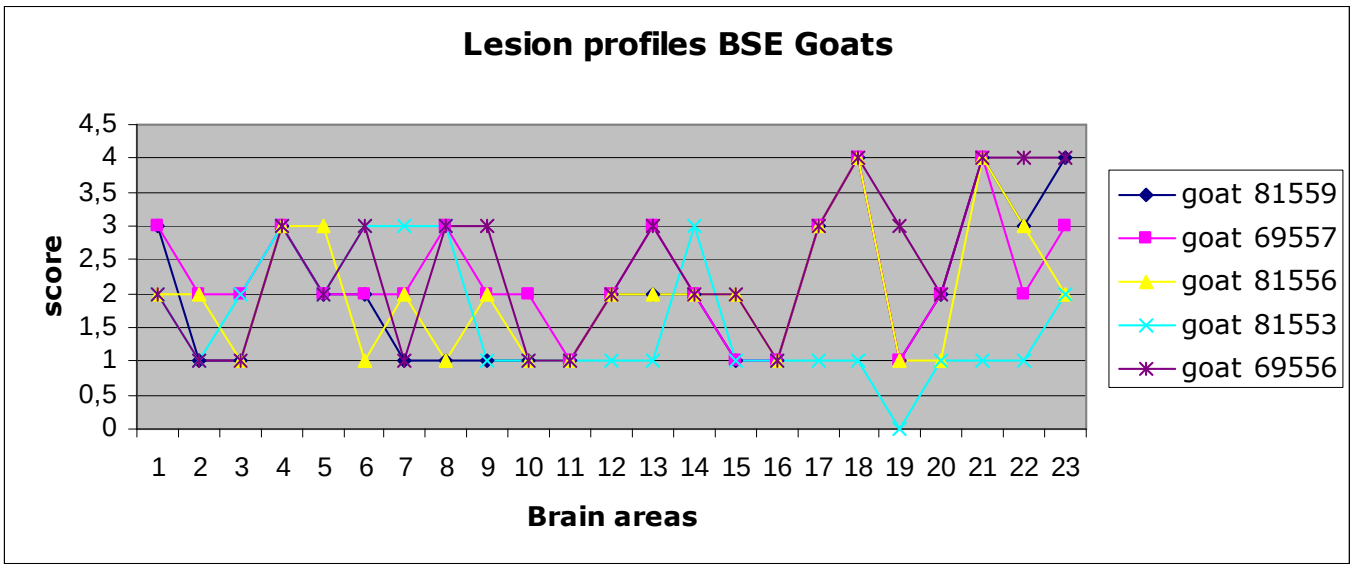


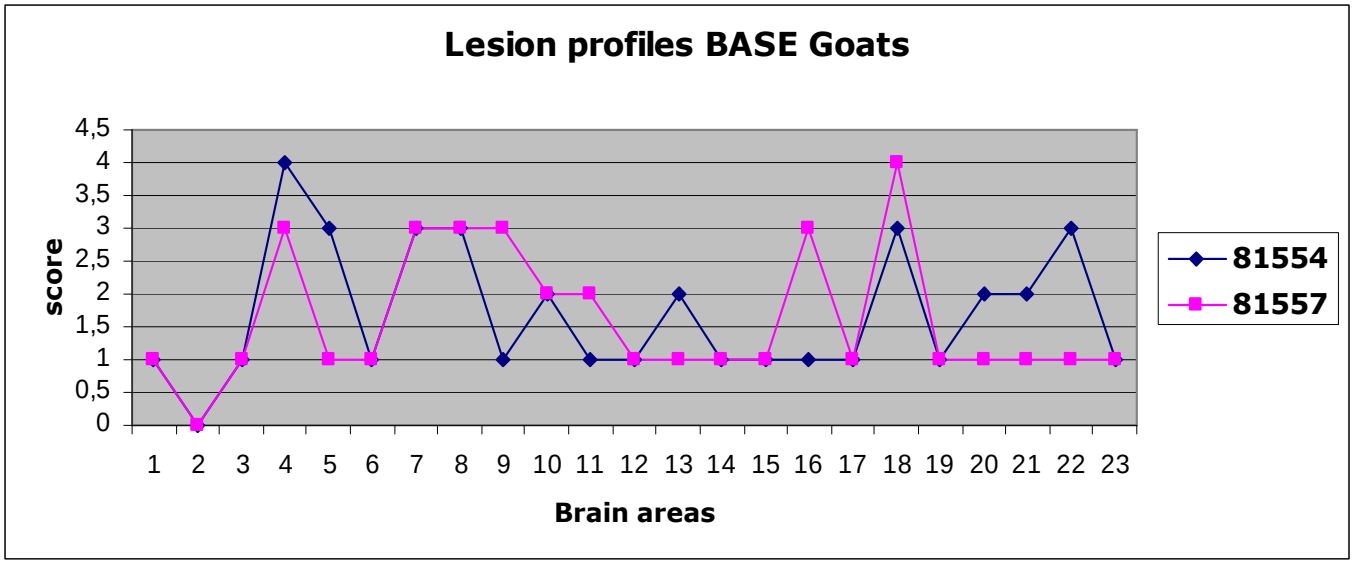
Table 6. PrPSc glycotype with 6H4 antibody

Sample	Diglycosylated %	Monoglycosylated %	Nonglycosylated %
Scrapie	58.20	33.83	7.96
BSE 128204	63.14	27.91	8.93
81553 Brainstem	61.72	28.04	10.22
81553 Thoracic Medulla	72.31	20.66	7.02

Graph 1. Lesion profile of BSE ic goats: 1 Obex, vagus nerve dorsal N. 2 Obex, hypoglossal N. 3 Obex, olivar N. 4 Obex, trigeminal n 5 Obex, cuneate n, 6 Obex, Reticular formation 7 Medulla, dorsal horns, 8 Medulla, ventral horns 9 Pons, vestibular complex n. 10 Cerebellum, granular layer 11 Cerebellum, molecular layer, 12 Midbrain, gray substance 13 Midbrain, superior colliculi 14 Midbrain, substantia nigra 15 Midbrain, feet 16 Diencephalon, parietal cortex 17 Diencephalon, hypothalamic area 18 Diencephalon, N. thalamic 19 Diencephalon, hippocampus 20 Telencephalon, frontal cortex 21 Telencephalus , caudate, n. 22 Telencephalus N. accumbens 23 Telencephalus, putamen.



Graph 2. Lesion profile of BASE ic goats: 1 Obex, vagus nerve dorsal N. 2 Obex, hypoglossal N. 3 Obex, olivar N. 4 Obex, trigeminal n 5 Obex, cuneate n, 6 Obex, Reticular formation 7 Medulla, dorsal horns, 8 Medulla, ventral horns 9 Pons, vestibular complex n. 10 Cerebellum, granular layer 11 Cerebellum, molecular layer, 12 Midbrain, gray substance 13 Midbrain, superior colliculi 14 Midbrain, substantia nigra 15 Midbrain, feet 16 Diencephalon, parietal cortex 17 Diencephalon, hypothalamic area 18 Diencephalon, N. thalamic 19 Diencephalon, hippocampus 20 Telencephalon, frontal cortex 21 Telencephalus , caudate, n. 22 Telencephalus N. accumbens 23 Telencephalus, putamen.



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