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## UNIVERSITÀ DEGLI STUDI DI TORINO

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## **Ten years of BSE surveillance in Italy: Neuropathological findings in clinically suspected cases**

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### **ABSTRACT**

Between 2001 and 2010, 244 clinically suspected cases of bovine spongiform encephalopathy (BSE) were reported in Italy. This report summarizes the neuropathological findings in cattle displaying clinical signs consistent with a diagnosis of BSE. All animal specimens were submitted for confirmatory testing; samples testing negative underwent neuropathological examination to establish the differential diagnosis. Immunohistochemistry for scrapie prion protein (PrP<sup>Sc</sup>) at the level of frontal cortex was carried out to exclude atypical BSE.

Neuropathological changes were detected in 34.9% of cases; no histological lesions were found in 52.3% of subjects; 12.8% of samples were found unsuitable for analysis. BSE was detected in one case, but no cases of atypical BSE were observed.

This study identified the diseases most commonly encountered in the differential diagnosis of BSE; furthermore, it demonstrated that the surveillance system is necessary for monitoring neuropathological disease in cattle and for the detection of BSE cases.

### **KEYWORDS**

Bovine; Brain; BSE; Neuropathology; Immunohistochemistry; Surveillance

### **INTRODUCTION**

Bovine spongiform encephalopathy (BSE), a transmissible neurodegenerative disease with a progressive and fatal course in adult cattle, is characterized clinically by alterations in behavior, ataxia and wasting. Neuropathological changes include vacuolation, gliosis, and mild neuronal degeneration without inflammatory reaction (Wells et al., 1987). BSE was first described in the UK in 1986 (Wells et al., 1987) after changes in meat and bone meal processing resulted in the exposure of cattle to feed contaminated with the prionic agent (Wilesmith et al., 1988).

BSE is classified among transmissible spongiform encephalopathies (TSE), a group of diseases characterized by the accumulation in the brain of scrapie prion protein (PrP<sup>Sc</sup>), an abnormal isoform of cellular prion protein (PrP<sup>C</sup>), normally found in host tissues. TSE can affect both animals and humans. The human forms include Creutzfeld–Jakob disease (CJD), Kuru, and the Gerstmann–Sträussler–Scheinker syndrome. Cases of a new form of CJD, called new variant CJD (nv-CJD) and caused by the BSE agent, were reported in the UK in 1996 (Will et al., 1996). Since then, because of its zoonotic potential, BSE has become a public health problem.

During the BSE epidemic, the work of veterinary services was pivotal in overcoming the crisis. Subsequently, the basis was laid for a massive surveillance system which has provided data to better understand the disease's epidemiology and to minimize its spread in livestock. This was achieved with an enormous investment of economic and professional resources.

Passive surveillance, which relies on the reporting of animals with neurological signs, was the principal means of detection of BSE cases prior to 2001, when active surveillance, with the introduction of rapid tests, became obligatory for all EU Member States. But even with the introduction of rapid tests, the European Commission has continued to stress the importance of clinical identification of BSE cases. Active surveillance systems are notoriously costly and cannot continue indefinitely; as such, they are intended for monitoring only during a growing epidemic because their efficiency diminishes as an epidemic declines.

In accordance with the 2008/908/CE Decision of January 2009, the age limit for the rapid testing of slaughtered, dead or euthanized cattle has been raised to 48 months in Italy. To date, 144 cases of BSE have been detected in Italy, only one of which was identified through passive surveillance. As *in vivo* diagnostic tests for TSE are not available, clinical examination is the first step in the diagnosis of the disease.

With the enactment of EC Regulation 999/2001, all clinically suspected BSE cases must be submitted to confirmatory testing by histologic, immunohistochemical and immunobiochemical analyses. If the brainstem tests negative for BSE, the OIE manual recommends that in countries with a low incidence of BSE clinically suspect cases be subjected to a standard neuropathological approach in which the whole brain is sampled, and a range of representative areas examined. Neuropathological examination of the entire brain from animals with nervous symptoms is a useful diagnostic practice, not only if rapid tests prove negative, but in confirmed cases to establish whether or not the pathology is typical.

It was using this approach in a study to assess the molecular and neuropathological characteristics of Italian BSE cases, that the first variant form, bovine amyloidotic spongiform encephalopathy (BASE), was identified (Casalone et al., 2004).

Since then, other cases of atypical BSE displaying molecular characteristics of PrP<sup>Sc</sup> unlike those of the classic form have been reported around Europe. A distinctive feature of BASE is the prevalence of PrP<sup>Sc</sup> in the more rostral portions of the brain rather than in the brain stem, as occurs in classic BSE.

In the present study, we examined the brains collected from cattle presenting with neurological signs and reported through BSE passive surveillance between January 2001 and December 2010. The study was carried out at the National Reference Center for Transmissible Spongiform Encephalopathy (CEA), Turin, Italy. All samples were submitted to further testing to determine the presence of other neurological diseases.

## MATERIALS AND METHODS

Between January 2001 and December 2010, 244 cattle (age range, 1–17 years) both male and female and of different breeds, were reported as clinically suspected cases of BSE in Italy. A standard data collection form was filled in for each animal. Clinical and epidemiological data were entered into a database. Histological, immunohistochemical and Western blot analyses for detecting PrP<sup>Sc</sup> in the brainstem were performed.

Brain samples of animals negative at confirmatory testing underwent further analysis to establish the differential diagnosis. The brains were collected and then divided by paramedian cut: the larger portion was fixed in 10% buffered formaldehyde solution for histological examination; the smaller portion was frozen at –20 °C for microbiological analyses. For histological examination, coronal sections of selected brain regions (telencephalon, diencephalon, mesencephalon, pons, cerebellum, obex) were stained with hematoxylin and eosin. Immunohistochemical analysis of the frontal cortex to exclude BASE cases was also carried out. The samples were deparaffinized, rehydrated, pretreated with 98% formic acid and autoclaved at 121 °C for 30 min. The sections were then incubated overnight at 4 °C with monoclonal primary antibody F99/97.6.1 (diluted 1:1000). Subsequent antibody detection was carried out using biotinylated goat anti-mouse secondary antibody (diluted 1:200) for 20 min at room temperature, followed by the avidin–biotin–peroxidase complex (Vectastain ABC kit, Vector Laboratories). Immunoreactivity was visualized using 3-3' diaminobenzidine (DAB) as chromogen, and the sections were counterstained with Mayer's hemalum.

In the histological sections with neuropathological features attributable to listeriosis, immunohistochemical analysis for *Listeria* spp. was performed using polyclonal rabbit antibody (diluted 1:250) (Virostat, Portland, ME, USA) applied overnight at 4 °C (Vectastain ABC kit). Cytokeratin (diluted 1:50) (Dako Carpinteria, CA, USA) and GFAP (diluted 1:1000) (DakoCytomation Denmark) immunohistochemical analysis of neoplastic lesions was performed to differentiate between ependymoma and carcinoma.

Samples from cases with neuropathological changes ascribable to non-suppurative meningo/encephalitis were submitted to molecular analysis. Frozen brain biopsies were individually macerated in a laminar flow bench and total nucleic acids were purified using a NucliSENS® easyMAG® system (bioMérieux Inc., Durham, NC, USA) according to the manufacturer's instructions. All tissues were tested for cellular adequacy and absence of PCR-inhibitors by PCR amplification of b-actin gene DNA (for DNA extraction) or cDNA (for RNA extraction) as previously described (Salata et al., 2009).

Real-time RT-PCR was carried out to detect West Nile Virus (WNV) and enterovirus-RNA using the oligonucleotide primers and TaqMan probe targeting the WNV E gene (Lanciotti et al., 2000) or the enteroviral genome 5' untranslated region (Donaldson et al., 2002). In this procedure, the nucleic acid (about 60 ng) was combined with Superscript® One Step RT-PCR System reagents (Invitrogen Ltd., Paisley, UK), primers and probe, reaching a total reaction volume of 20 µl, and amplified in a LightCycler® 2.0 real-time PCR System (Roche Diagnostics, Monza, Italy).

Real-time PCR assays were used to check for the presence of *Toxoplasma gondii* (Lin et al., 2000), *Borrelia burgdorferi* (Exner and Lewinski, 2003), and *Chlamydia* spp. (Yang et al., 2006) as previously reported with some modifications. Briefly, the extracted nucleic acid (about 60 ng) was assayed with a sequence detector system ABI PRISM 7700 (Applied Biosystems) in 25 µl of a PCR mixture containing 12.5 µl TaqMan universal master mix, 15 pmol of each primer, and 10 pmol of the probe under standard amplification conditions.

The end-point PCR assays were used to assay all samples for Borna virus (Cotto et al., 2003), tick-borne encephalitis virus (TBEV) (Puchhammer-Stoeckl et al., 1995), herpesvirus (Rose et al., 1997), and fungi (Rajeshwari Sutar et al., 2004). Reverse transcription, if necessary, was performed as described by Bergonzini et al., 2009.

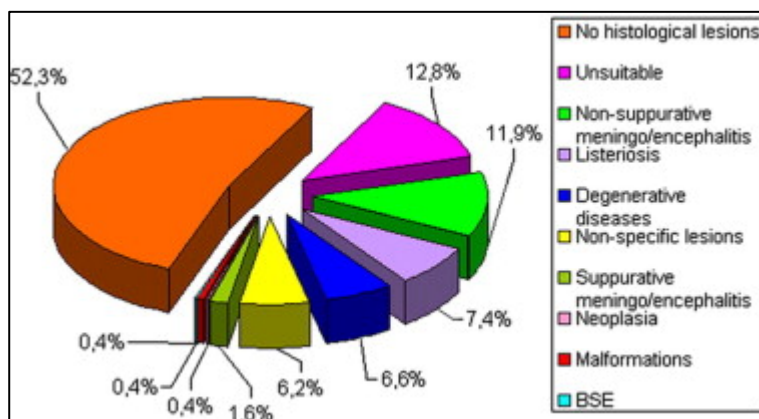
## RESULTS

The clinical signs recorded by the veterinary practitioners were grouped into six main categories: abnormal posture, altered mental status/behavior, gait deficits, cranial nerve involvement, downer cow, and seizures (Table 1). If an animal was recumbent, but did not display altered mental status, it was classified in the “downer cow” category, in agreement with the literature definition of the syndrome. Seizures made up a separate category distinct from the altered mental status/behavior category.

**Table 1. Frequency of main clinical signs in cattle reported as clinically suspected cases of BSE.**

Clinical signs	No. of animals
Posture abnormality	209
Altered mental status/behavior	206
Gait deficits	170
Cranial nerve involvement	47
Downer cow	21
Seizures	8

Table 1 reports the distribution of clinical signs. Diagnoses from examination of brain samples of cattle reported as suspected cases of BSE are shown in the diagram (Fig. 1).



**Fig. 1. Diagram. Graphic representation of the diagnoses from examination of brain samples of cattle reported as suspected cases of BSE.**

Histological lesions were detected in 34.9% of the samples; only one case of BSE was detected; no neuropathological changes were observed in 52.3% of subjects, although they had presented with neurological signs; and 12.8% of the cases were unsuitable for analysis.

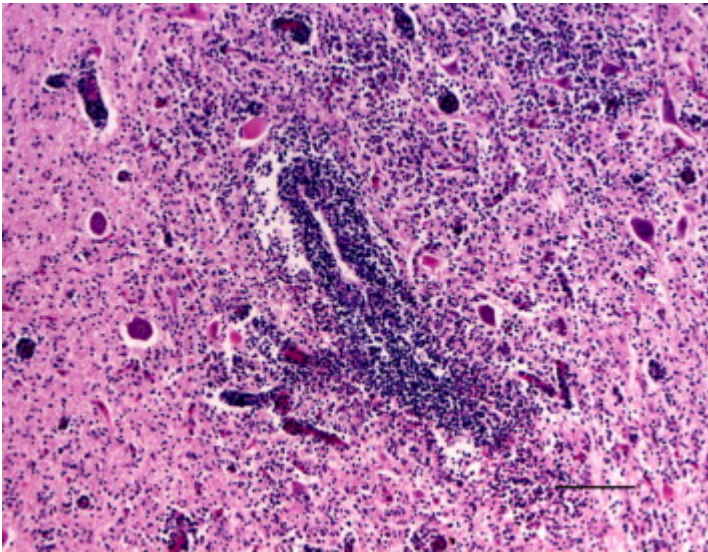
### NON-SUPPURATIVE MENINGO/ENCEPHALITIS

In 29 animals, the neuropathological examination revealed a mild to very severe non-suppurative meningo/encephalitis. The microscopic hallmark was perivascular cuffs composed of single or

multiple layers of mononuclear cells, predominantly lymphocytes and macrophages, occasionally plasma cells, and moderate or severe gliosis, depending on lesion severity. Histological changes often involved both the gray and white matter.

In the cases with severe lesions, distension of the perivascular space by an inflammatory exudate was observed and the surrounding nervous tissue showed diffuse gliosis or isolated glial nodules in the areas immediately adjacent to the vascular lesions. Edema was seen predominantly in the cortex, accompanied by neuronal necrosis, satellitosis and neuronophagia. On the basis of lesion distribution and severity, three cases were classified as sporadic encephalitis.

In one animal with mild brain lesions (Fig. 2), PCR detected infection with *Chlamydia* spp.; all other cases tested negative for the pathogens investigated.



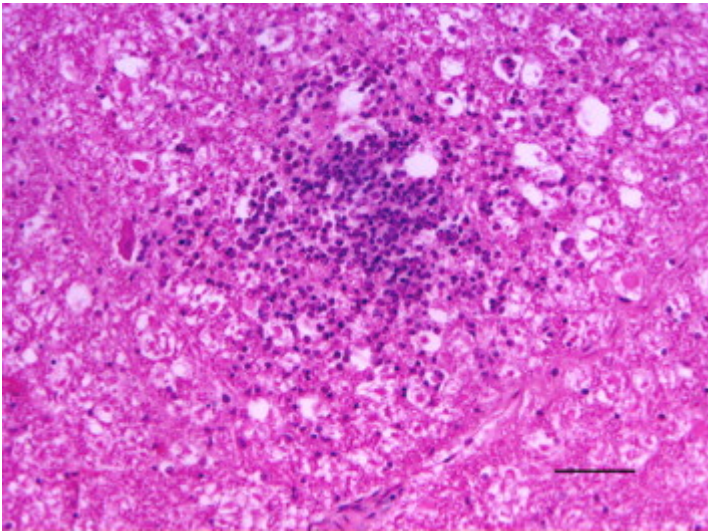
**Fig. 2. Brain; cow. Non-suppurative meningo/encephalitis. Mild lesion** – prominent perivascular cuffs composed of lymphocytes, macrophage and plasma cells, inflammatory infiltrate in the parenchyma, gliosis, neuronal necrosis and neuronophagia. HE. Bar, 100  $\mu$ m.

Only 55% of the animals with non-suppurative meningo/encephalopathy showed altered posture or mental status/behavior or gait deficits.

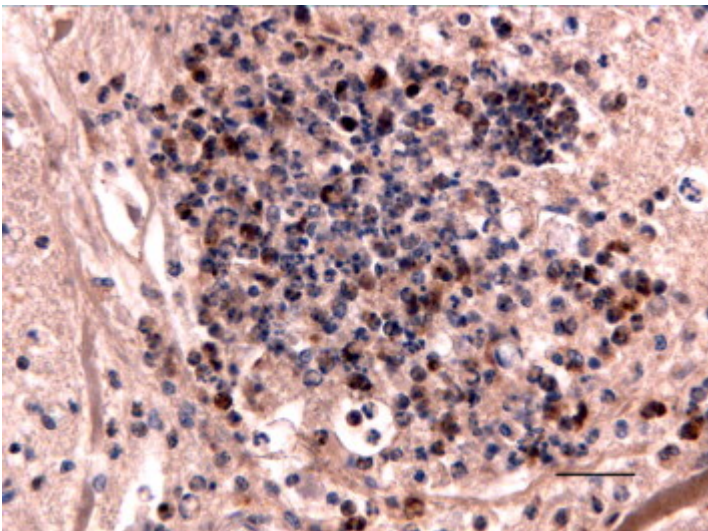
### ***LISTERIOSIS***

In 18 animals, the lesions were attributable to listeriosis. Histology was characterized by disseminated microabscesses (Fig. 3) associated with prominent perivascular cuffs of mononuclear cells. The lesions were centered in the caudal brainstem and mesencephalon; a mononuclear infiltrate was also noted in the meninges. Immunohistochemical analysis demonstrated *Listeria monocytogenes* antigen in the microabscesses and necrotic foci, predominantly within the cytoplasm of the polymorphonuclear leukocytes (Fig. 4) and occasionally in the extracellular environment. In rare cases the antigen was detected in the perivascular cuffs.





**Fig. 3. Brain; cow. Listeriosis** – microabscesses in the reticular formation at the level of the obex. HE. Bar, 50  $\mu$ m.



**Fig. 4. Brain; cow. Listeriosis** – expression of *L. monocytogenes* antigen, predominantly in the cytoplasm of polymorphonuclear leukocytes, in a microabscess. IHC. Bar, 25  $\mu$ m.

Clinically, 56% of the animals with listeriosis showed cranial nerve involvement, in addition to altered mental status/behavior, abnormal posture, and gait deficits.

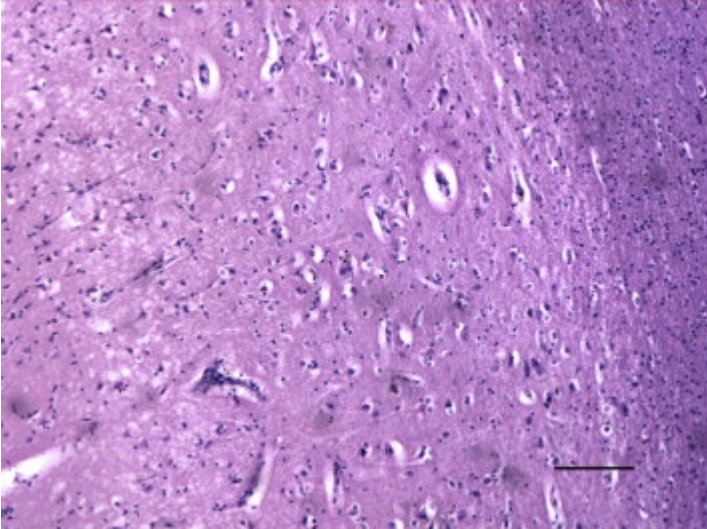
### ***NEURODEGENERATIVE LESIONS***

In 14 subjects, the histological lesions were characterized by edema, focal or generalized spongiosis of the white matter, gliosis, neuronal degeneration, and vascular proliferation of variable intensity. The lesions were diffusely distributed with variable intensity throughout the medulla oblongata, cerebellum, diencephalon, telencephalon. In 1/14 subjects, histology revealed bilateral symmetric intense spongiosis in the white matter and in the transition regions between the white and gray matter. Small groups of type II Alzheimer cells were found in the cerebral cortex. The lesions were consistent with a toxic or metabolic etiology.

In two brain samples, the histological features were ascribable to polioencephalomalacia. Neuropathological examination revealed laminar necrosis of the deep layer of the cerebral cortex



adjacent to the white matter. The lesions were distributed symmetrically and bilaterally in the frontal and parietal cortex. Degenerative-necrotic lesions of the neurons were observable in the cortical layer, with contracted ischemic neurons, eosinophilic cytoplasm, and pyknosis. Variable degrees of hypertrophy and hyperplasia of the vascular endothelium were also observable. The picture was consistent with cerebro-cortical necrosis caused by thiamine deficiency (Fig. 5).



**Fig. 5. Brain; cow. Degenerative lesion** – cerebro-cortical necrosis of the frontal cortex. HE. Bar, 100  $\mu$ m.

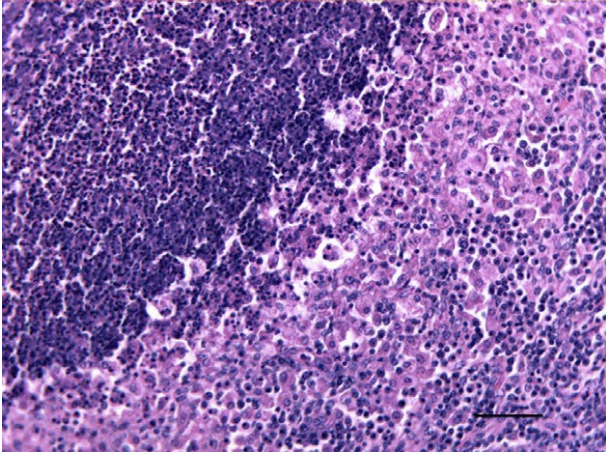
Clinically, 50% of the animals with neurodegenerative lesions presented with altered mental status/behavior, posture and gait deficits.

#### ***NON-SPECIFIC LESIONS***

In 15 animals, histology revealed astrocytosis and moderate perivascular cuffs. On clinical examination, these subjects showed mild neurologic signs.

#### ***SUPPURATIVE MENINGO/ENCEPHALITIS.***

In four animals, histology was consistent with suppurative meningo/encephalitis, in two of which the microscopic changes showed small multiple abscesses foci disseminated throughout the cerebral parenchyma. In the brain of a third animal there was an isolated abscess nodule with a necrotic center and a fibrous capsule due to fibroblastic reaction of the surrounding tissue. Outside this area there were astrocytes and a granulocytic perivascular infiltrate. The fourth animal showed an abscess in the mesencephalon (Fig. 6); in the adjacent areas and in the medulla oblongata, focal hemorrhaging and lymphohistiocytic perivascular cuffs with eosinophils were also observed. Clinically, three of the four animals showed altered mental status/behavior, posture and gait deficits.



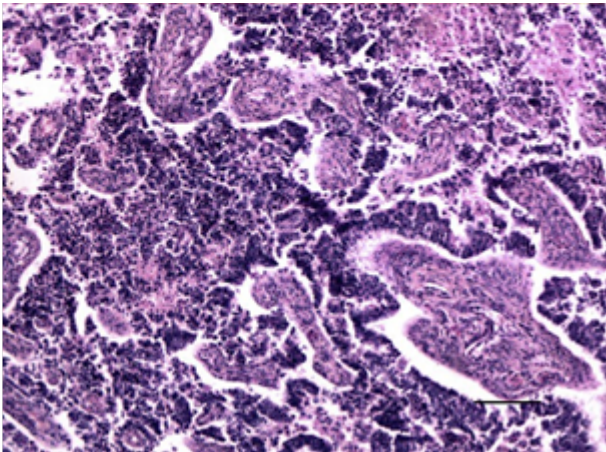
**Fig. 6. Brain; cow. Suppurative meningo/encephalitis** – abscess in the mesencephalon. HE. Bar, 50  $\mu$ m.

### ***MALFORMATIONS***

Cerebellar hypoplasia was macroscopically noted in one animal; histology demonstrated loss of Purkinje cells, rarefaction of the granular layer and demyelination of the white matter in both hemispheres.

### ***NEOPLASMS***

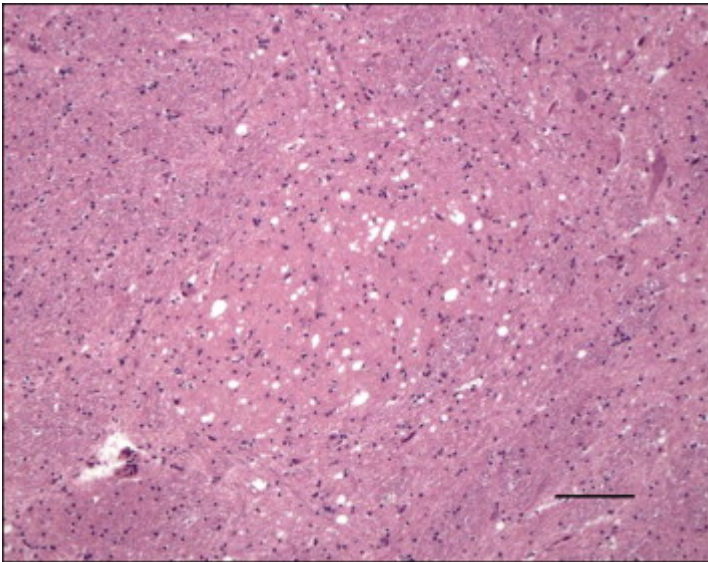
A neoplasm was detected in only one animal. Histology showed anaplastic features such as loss of papillary architecture, cellular atypia, and increased mitotic index (Fig. 7); the lesion extended also in the periventricular area. Immunohistochemically, neoplastic cells reacted positively with cytokeratin and negatively with GFAP, thus confirming the diagnosis of choroid plexus carcinoma.



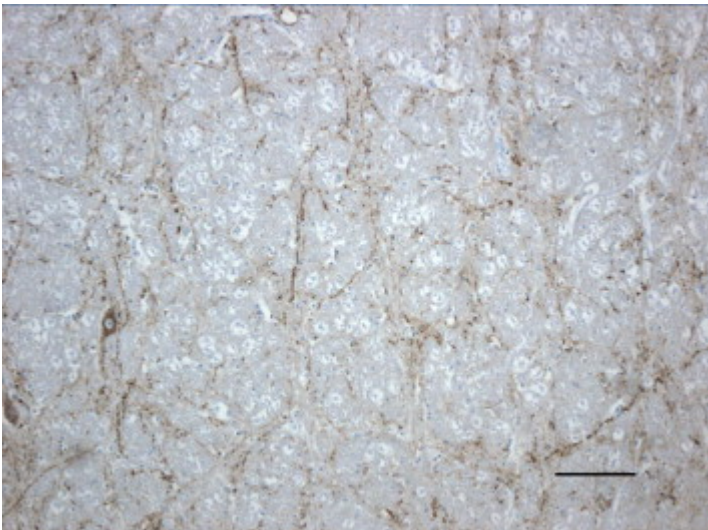
**Fig. 7. Brain; cow. Neoplasia. Choroid plexus carcinoma** – loss of papillary architecture and cellular atypia. HE. Bar, 100  $\mu$ m.

### ***BSE***

Only one case of BSE was detected. Histological examination of the obex showed spongiosis in the solitary tract nucleus and in the spinal tract nucleus of the trigeminal nerve (Fig. 8). The diagnosis was confirmed by immunohistochemistry (Fig. 9) and Western blot analysis.



**Fig. 8. Brain; cow. BSE** – spongiosis in the solitary tract nucleus. HE. Bar, 50  $\mu$ m.



**Fig. 9. Brain; cow. BSE** - PrPSc deposition in the reticular formation of obex. IHC. Bar, 100  $\mu$ m.

### ***UNSUITABLE SAMPLES***

The brains of 31 clinically suspected animals were unsuitable for neuropathological examination because the samples were either in an advanced stage of autolysis or showed freezing artifacts.

Immunohistochemical analysis for PrPSc detection on the frontal cortex was negative in all samples.

### **DISCUSSION**

The present study summarizes findings from an analysis of the main neuropathological changes observed in cattle with nervous signs and reported to the CEA within the framework of BSE passive surveillance in Italy between 2001 and 2010. Our results are similar to previously reported data during the investigation of adult animals with suspected but unconfirmed BSE (Caramelli et al., 2000, Jeffrey, 1993 and McGill and Wells, 1993).



The brain appeared normal in 52.3% of cases, whereas non-specific lesions were observed in the brain of 6.2% of animals with nervous signs. This finding is substantially in line with published data (Caramelli et al., 2000 and McGill and Wells, 1993). The large percentage of animals without lesions at neuropathological examination can be explained by the fact that certain syndromes in cattle (e.g., ketosis, hypomagnesemia and acute lead poisoning), although they induce neurological signs, often do not cause alterations in the brain (Jeffrey, 1993).

On clinical examination, subjects were noted to have some neurological signs, including increased reactivity to stimuli, sensory depression and difficulty in ambulation, but these were generally attributable to pain from extra-neural systemic disorders, e.g., joint injuries, surgical problems (displaced abomasum, foreign body syndrome and circulatory deficits), metritis, mastitis and placental retention.

In this study, the most frequent findings were non-suppurative meningo/encephalitis and listeriosis: non-suppurative meningo/encephalitis was observed in 11.9% of cases presenting with different grades of lesion severity.

In three cases, the neuropathological findings were consistent with sporadic encephalitis, with a distribution similar to cases reported previously (Theil et al., 1998). First reported in Switzerland, sporadic encephalitis in cattle has a sporadic onset and follows a subacute to chronic course. Its etiology remains unclear (Theil et al., 1998); however, co-infection with Chlamydia and viruses has been reported. Interestingly, a chlamydial infection was detected only one case in our series, a 7-year-old cow in which the brain lesions differed from those observed in the three cases of classical sporadic encephalitis which tested negative on all tests. Moreover, the cow presented with less severe neuropathological changes than those described previously (Piercy et al., 1999 and Storz et al., 1971).

Listeriosis was observed in 7.4% of cases. *Listeria monocytogenes* has been reported in cattle with neurological signs but negative for BSE in 20% of cases in Germany (Miyashita et al., 2004), in 8% in the UK (McGill and Wells, 1993), in 11% in Scotland (Jeffrey, 1993) and in 36% in Denmark (Agerholm et al., 2002). These results underline the importance of including listeriosis in the differential diagnosis of BSE.

Suppurative lesions were observed only in 1.6% of cases. Notably, brain abscesses can develop in both young and adult animals alike and may often arise either through hematogenous metastasis or direct extension from local lesions. However, the disease is relatively infrequent in cattle (El-Khodery et al., 2008). The most commonly described etiologic agents isolated from brain abscesses in cattle are *Escherichia coli* and *Arcanobacterium pyogenes*.

Degenerative lesions were observed in 6.6% of cases. In 14 samples, they were attributable to toxic-metabolic disorders. The histology ranged from mild degenerative phenomena caused by brain hypoxia to more severe lesions associated with neuronal degeneration and diffuse spongiosis. The pathogenesis of the lesions was not definitively clarified, but the findings could be attributed to metabolic disorders or encephalopathies of renal or hepatic origin. According to previous studies, spongiform white matter degeneration in these disorders is often associated with axonal demyelination (Summer et al., 1995). Possible causes are generally congenital, toxic or metabolic. Congenital portosystemic shunt is the most common cause of encephalomyelopathy in adult cats and dogs, whereas in large animals the disease is associated with hepatotoxic conditions (Café Marçal et al., 2008 and D'Angelo et al., 2007). In hepatic encephalopathies, type II Alzheimer cells grouped in small clusters or isolated in the deep layers of the cerebral cortex can frequently be found. In humans and the horse, this finding is suggestive of hepatic encephalopathy or

hyperammonemia (Summer et al., 1995). In the present study, this type of cells was found in only one brain sample.

In two samples with degenerative lesions, a polioencephalomalacia was described, and the histological features resembled those due to thiamine deficiency. The thiamine deficiency syndrome has been widely described in the past as disease with neurological signs and more often occurring in young animals kept under intensive stocking conditions (calves aged 4–8 months) fed with silage and feed (Cravero, 1983).

Its incidence is lower in adult animals in which thiamine deficiency may result from the administration of feed high in sulfates, which reduces the intestinal absorption of thiamine. Sulfite, an intermediate reduction product of sulfate, splits thiamine, thus reducing its bioavailability. The literature reports episodes of polioencephalomalacia in adult cattle given drinking water with elevated sodium sulfate levels (Hamlen et al., 1993). Polioencephalopathy may be observed in cases of acute lead poisoning, acute sodium chloride poisoning, and water deprivation (McGill and Wells, 1993). More recently, an association between type 5 bovine herpes virus (BHV-5) and cerebrocortical necrosis has been demonstrated (Pérez et al., 2003) but no correlation was found in our study.

One case of cerebellar hypoplasia was found in a 4-year-old Fresian cow. The animal had presented hyperexcitability to stimuli, nervousness, salivation, head tremors, opisthotonus, ataxia and hypermetry of gait involving all four limbs. The age at signs onset, history, and clinical signs were consistent with suspected BSE. The brain lesions were confined to the cerebellum and were more prominent in the cerebellar hemispheres than in the vermis. On the basis of published data, the bovine viral diarrhea (BVD) virus may cause cerebellar hypoplasia following uterine infection; at birth the animal may show clinical signs of cerebral damage which do not worsen with age. In this animal, however, no correlation was found (D'Angelo et al., 2008).

The only case of neoplasia reported in our investigation was choroid plexus carcinoma in a 4-year-old animal. Clinical examination revealed circling movements and head tilt, sialorrhea, generalized hypotony, and persistent recumbency. Choroid plexus tumors are rare neoplasms that derive from the epithelium of the choroid plexus and are rarely found in dogs, cats, horses or cattle. The recent World Health Organization (WHO) Classification of Tumors of the Nervous System in Domestic Animals lists neuraxis metastasis as a diagnostic criterion for choroid plexus carcinoma.

Immunohistochemical analysis to detect PrP<sup>Sc</sup> showed only one case of BSE and no cases of BASE among our samples. The BSE case showed behavioral changes characterized by fear, irritability, aggressiveness, hypersensitivity to stimuli and frequent ear movement. Other reported signs were tremors, wide based stance and cachexia. Clinical signs and neuropathological changes were similar to those reported previously (Wells et al., 1987).

In BASE, the pattern of the abnormal prion protein is characterized by the presence of PrP-amyloid plaque-like deposits, Kuru-like plaques, and granular extracellular and glial deposits. Since PrP<sup>Sc</sup> is prevalently found in the more rostral portions of the brain, correlated with the olfactory system, it is assumed that the prion does not spread by dietary intake. However, unless the pathogen propagates along the olfactory or other peripheral nervous pathways, BASE may be considered as a sporadic form of BSE in cattle (Casalone et al., 2004).

From research into experimental infection with BASE in cattle it was found that the disease manifests itself with nearly silent signs. The few clinically relevant changes (weight loss, mild alteration of behavior) are not consistent with the characteristic signs of BSE (Lombardi et al.,

2008). Because the bland signs may not be easily recognized, there is the increased risk that such cases may go unnoticed.

The high percentage (12.8%) of samples unsuitable for neuropathological examination confirms the need for continuing training of operators in the correct method of sampling, conservation, and transport of material for testing. Histopathological examination of suitable material is essential to establish a correct morphological and etiological diagnosis; moreover, histological and immunohistochemical analyses are fundamental for effective surveillance of symptomatic animals.

## **CONCLUSION**

This study summarizes findings from the monitoring of neurological diseases in adult cattle; however, further research is needed to broaden our understanding of bovine neuropathology. Passive surveillance retains a role of primary importance in the diagnosis of neurological diseases and is key to identify classical and atypical BSE cases.

## **CONFLICT OF INTEREST**

I disclose that there are no financial and (or) personal relationships with other people or organisations that could inappropriately influence my work.

## **ACKNOWLEDGMENTS**

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