Ministry of Agriculture,
Livestock and Food Supply

Secretariat of Animal and Plant Health and Inspection
Animal Health Department

Procedures for the Diagnosis
of the diseases of the Central Nervous System of Cattle

BSE
Bovine Spongiform Encephalopathy

"Prevention is our work"

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MINISTRY OF AGRICULTURE, LIVESTOCK AND FOOD SUPPLY
SECRETARIAT OF ANIMAL AND PLANT HEALTH AND INSPECTION
ANIMAL HEALTH DEPARTMENT

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Brasília-DF, Brazil

2003

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In source cataloguing
Coordenação Geral de Informação Documental Agrícola - CENAGRI

Coordination and Planning of Marketing and Production
PeterPublisher & Associados

Art Direction
Américo de Brito

Printing
Lid Gráfica Editora Ltda.

Barros, Claudio Severo Lombardo.

Procedures for the diagnosis of the diseases of the central nervous system of cattle / Claudio Severo Lombardo Barros and Guilherme Henrique Figueiredo Marques. - Brasília : MAPA/SDA/DDA, 2003.
50 p. : il. color.

1. Mad Cow Disease. 2. Animal disease - EEB. 3. Animal disease - EEB. 4. Diagnosis. 5. Prevention. 6. Control. 7. Mad Cow Disease - Manual. I. Marques, Guilherme Henrique Figueiredo. II. Secretariat of Animal and Plant Health and Inspection. Animal Health Department. III. Ministry of Agriculture, Livestock and Food Supply. IV. Title.

AGRIS 5212; L73; L70
CDU 619 : 636.2

Presentation

With the steady growth of market globalization, it has become crucial to mantain the health status of the herds in order to preserve the agricultural health of countries such as Brazil. Currently international trade standards establish stringent sanitary demands that constitute larger hurdles than the tariff barriers themselves. In this context, if we do not have an efficient animal health program, one that is able to attest the quality of our products, and acts as a partner to the essential exporting segments, private initiative will not be able to compete in a globalized market which offers good quality products.

In the last two decades the epidemics of bovine spongiform encephalopathy (BSE) reported from the United Kingdom and, subsequently, from other European countries, caused huge losses to the cattle industry, mainly due to the public's perception of the public health risk, which resulted in a marked reduction of beef consumption in the European continent.

Although the risk of BSE introduction in Brazil is extremely small, it is absolutely essential that the country adopt sanitary measures to prevent it, because if BSE is introduced, the losses to the Brazilian cattle industry will be immeasurable. And, of course, the potential risk to human health will have huge social and economic repercussions as well.

Thus, since the emergence of BSE in the United Kingdom, Brazilian health authorities have been very concerned in preventing the introduction of the disease into the country. This concern was reflected in the adoption of sanitary measures which included restrictions to the imports of susceptible animals and their products when the origin were countries considered at risk for BSE; the tracing of live cattle previously imported from such countries; and the prohibition of animal protein in ruminant feed.

International credibility is essential for trade. It is imperative that our Brazilian animal health defense services continue to monitor in the most transparent way the quality improvement and certification of our herds. These services should involve motivated professionals who are well informed and periodically evaluated. At the least, fundamental activities of the animal health services should include:

- constant and permanent gathering of information referring to the issue under consideration and the processing of this information in a systematic and opportune fashion;
- the analysis and elaboration of informative documents supporting the system;
- the storage of data in predetermined local and central levels;
- the feedback to the surveillance and prevention system, considering all the social participants involved.

In this context, the Brazilian Ministry of Agriculture, Animal Industry and Supply (Ministério da Agricultura, Pecuária e Abastecimento) based on specific legislation, introduced the National Surveillance System for the Transmissible Spongiform Encephalopathies (EETs). This handbook is now added to this system. The handbook is designed to standardize procedures and provide technical information helpful in the diagnosis of the neurological syndromes in cattle. Both official and private veterinarians participating in animal health programs can benefit from the information in this handbook which will help to safeguard the health of our herds. We consider that the handbook will constitute an valuable asset for keeping the Brazilian status as free from BSE.

João Crisostomo Mauad Cavalléro

Director of the Department of Animal Defense

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BOVINE SPONGIFORM ENCEPHALOPATHY: UNDERSTANDING THIS DISEASE

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BOVINE SPONGIFORM ENCEPHALOPATHY: UNDERSTANDING THIS DISEASE

1 - Definition

Bovine spongiform encephalopathy (BSE) widely known as "mad cow disease", is a chronic transmissible neurodegenerative disease which affects the central nervous system (CNS) of cattle. It is characterized clinically by nervousness and aggression, overreaction to external stimuli and disturbances in locomotion, mainly related to the pelvic limbs[6].

The disease is named "spongiform" due to the spongy changes found during histologic examination of brains from affected cattle. The agent of BSE is extremely resistant to heat, to conventional sterilization processes and does no elicit immune or inflammatory reaction from the host. BSE has been reported in cattle from approximately 20 countries, although more than 90% of the cases are reported from Great Britain. BSE is part of a complex group of diseases that affect human and animals, know as transmissible spongiform encephalopathies (TSEs). The main characteristics of TSEs are listed on Table 1. The main TSEs from humans and animals are respectively listed in Tables 2 and 3.

2 - The cause of BSE and other TSEs

During the research aimed to elucidated the etiology of TSEs, homogenized brains of scrapie-infected hamsters were fraccionated into several components. Of those componentes the most infectious fraction was found to contain large amounts of a particular protein that was not destroyed by proteases (enzymes that normally break down proteins). This particular protein, named prion (***pro****tei-naceous* ***in****fectious particle*, with "**i**" changing position wityh "**o**" for linguistics purposes)[7], was not foun in the brains of normal hamsters. The PrP aminoacid sequence was identical to that of a normal protein found in equal amounts in infected and non-infected brains; however, this normal protein, unlike the PrP, could be broken down by proteolytic enzymes. Thus, there was two forms (structural presentations) of the same protein. The form found only in brains of infected animals was subsequentely named PrP^{sc} (where sc stands for scrapie, the disease prototype for EETs). The form found in both infected and non-infected brains alike was named PrP^{c} (where c stands for cellular, i.e, belonging to normal cells)[1]. Several evidences accumulated to this date indicate that the PrP^{sc} is the etiologic agent for the EETs, although other theories still claim for a virus or a virino as the etiologic agent.

PrP^c is a normal glicoprotein in the cellular plasma membrane; it is found in most of the cells but predominantly in the cells of the central nervous system (CNS). PrPc is transformed in its abnormal isoform PrP^{sc} which accumulates in te CNS inducing the disease

The etiologic agent of EETs is extremely resistant to heat inactivation (360° C dry heat for 1 hour), ultraviolet radiation and chemical substances.

Studies on the pathogenesis of the EETs done with experimental scrapie indicate that, when peripheral routes of infection are used the agent inicially replicates in the spleen and lymph nodes and reachs the brain probably via the the fibers of the visceral sympathetic nerves which are connected to the midportion of the thoracic spinal cord[14]. From the spinal cord the agent proceeds to the brain in a rate of 1 mm/day. Once in the CNS it can go centrifugally to peripheral tissues.

The presence of the agent of EETs in tissues is determined by innocculating mice with the tissue to be tested; this is a time consuming task which could take 700 days. BSE has been experimentally transmitted to several species. In naturally infected cattle the agent was found in the brain, spinal cord and retina. In experimentally infected calves the agent was additionally found in distal ileum, bone marrow, trigeminal ganglion and dorsal root ganglia of spinal nerves. No evidence of infectivity was found in millk or meat (muscle tissue) of cattle infected either experimentally or naturally.

3 - Epidemiology

BSE was first diagnosed in 1986 in Great Britain[10], but later, the reviewing of epidemiological data and filed histological preparations indicated the occurrence of cases in 1985[13] and some studies suggest that the disease could have been present already in the 70's. The vast majority of cases of cattle with BSE are from Great Britain herds. Officially there were 180,000 BSE cases in Great Britain since 1986; these are distributed among 35.000 herds, averaging 1-2 cases per affected herd.

Outside Great Britain, BSE was confirmed in a relatively small numbers of cattle (around 2,800) from Austria, Belgium, Canada, Czech Republic, Denmark, Finland, France, Germany, Greece, Ireland, Israel, Italy, Japan, Liechtenstein, Luxembourg, Netherlands, Poland, Portugal, Slovakia, Slovenia, Spain, and Switzerland. With the exception of a few cases, all of the 2,800 are native to these countries. In some countries or territories, like Falkland Islands and Oman, there were cases reported only in imported cattle. Updated information on the distribution of the disease can be obtained from the World Organization for Animal Health (OIE) website (www.oie.int).

Although there is no predilection for breed or sex, due to animal husbandry methods the disease in Great Britain occurs mainly in 3-6-year-old dairy cows.

BSE was transmitted to cattle by feeding them meat and bone meal rations contaminated by the etiological agent. The source for the infection could have been scrapie infected sheep or cattle with a sporadic form o the disease, that was, up to then, unknown. Of course, the entrance of the early cases of cattle with BSE in the food chain magnified the epidemics. Changes in the meat and bone meal manufacturing methods, occurring in the late 70's and early 80's might have contributed to the appearance of the BSE[13]. The ingestion of less than one gram of an infected brain is what it takes to induce the disease. There is no evidence that BSE can be disseminated horizontally, but it is suggested that maternal transmission may occur; however in very low levels, which would not maintain the epidemics[13].

4 - Clinical signs

The BSE incubation period (the time elapsed since the animal is infected up to the time it shows the first clinical signs) is 2-8 years (mean 5 years), although longer incubation periods have been reported. The disease has invariably a fatal outcome after a clinical course of three weeks to 6 months. Affected BSE cattle have a progressive degeneration of the central nervous system and may show disturbances in behavior, sensitivity and locomotion[2,8,9,12]. General clinical signs include drop in milk yield and loss of weight, despite continued appetite.

a. *Disturbances in behavior.*

Those include nervousness, fearfulness or agression, abnormal posture, incoordination and difficulty in rising. BSE affected cattle are usually very nervous, alert, and hypersensible; disturbances in behavior such as fearfulness and nervousness are more clearly pronounced when the animal is excited.
Other clinical signs include increased salivation and a frightened look. Sometimes the animal will grind its teeth. Frequent licking of the muzzle and wrinkling of the nose occur. Some affected cows also exhibit nervous ear movements. In the terminal stages of BSE the animal has difficulty getting up or it may assume a permanent recumbency.

b. *Disturbances in sensitivity.*

BSE affected cattle commonly display disturbances in sensitivity and overreact to touch (more commonly), to sound and to light.

c. *Disturbances in locomotion.*

Stiff gait, incoordination, hypermetria and generalized ataxia are frequent clinical signs in BSE affected cattle. Hypermetria is more pronounced in the hindlimbs which induces high step action resembling that seen in stringhalt in horses. From severe ataxia the disease progresses to falls and terminally to posterior paresis, recumbency and death.

5 - Necropsy fidings and histopathology

There are no gross lesions directly related to the disease, but microscopic lesions associated with BSE are highly specific. They are symmetrical and bilateral degenerative lesions and are distributed in certain parts of the gray matter of the brain stem[11,12]. These changes are characterized by vacuoles which impart a spongiform aspect to the neuropil.

Careful interpretation of histopathological signs should be done since there are some potentially misleading incidental findings. For example, vacuoles in the perikaryon of red nucleus neurons in the mesencephalon are common incidental findings in bovine brains, being reported in 64% of normal cattle[4]. When present only in this location they should not be considered as indication of BSE[11,12]. Nonspecific non-suppurative inflammation (perivascular cuffings) are found in approximately 30% of the brain of normal adult cattle[4] and may result from subclinical or latent infections. Also the occurrence of intracytoplasmic granules of ceroid-lipofuscin are common in neurons of normal old cattle; these latter two changes appear to have no clinical importance[12] either and should also be considered as incidental findings.

6 - Diagnosis

Currently, there is no available test to diagnose BSE in the live animal. BSE can be confirmed by histological examination of brain tissue or by detecting the abnormal form of prion protein (PrP^{sc}) in brain samples. The latter can be done by electron microscopy or immunoligical methods. When proprely prepared samples from brains affected with TSEs are examined under the electron microscope, PrP^{sc} will show up as rods named SAFs (***s**crapie **a**ssociated **f**ibrils*). Immunoligical methods include the detection of PrP^{sc} by immunohistochemistry or Western immunoblotting[3] and by the so called rapid tests based on ELISA or immunoblotting[5].

Currently, in Brazil the diagnosis is carried out by histological examination of selected sections of the brain stem and by immunohistochemistry. For both tests the brain samples should be sent as specified in this handbook (*see* Sampling of Cattle Brain for Laboratory Examination).

7 - Control, prophylaxis and treatment

There is no treatment or vaccine that will halt or prevent the disease. Measures to prevent the introduction of cases include 1) restriction of import of ruminant and ruminant products from countries considered at risk for BSE, 2) do not feed ruminants with protein of animal source, chicken litter or leftovers from swine raising facilities and 3) collect and destroy all the carcasses in the field.

8 - The Brazilian BSE surveillance program

The dairy and beef production in Brazil almost exclusively uses cattle that is raised at pasture. The supplementation of feed, when it occurs, is done with plant derived protein; thus the occurrence of BSE in the country is unlikely.

Nonetheless, immediately after BSE emerged in the United Kingdom (UK), the Brazilian agriculture and health officials, aiming to protect the cattle industry and human health, took measures to safeguard the country against the introduction of BSE. This measures include prohibition and/or restrictions on imports of susceptible animals and their products from countries with risk of BSE; tracing of cattle already imported from such countries and enforce restrictions on the manufacturing of feed destined to ruminant feeding.

A great number of products for human use are obtained from tissues of several ruminant species. These animal-derived substances go into the production of drugs, cosmetics, biological products and other items for human use. Several sanitary measures were adopted by the National Agency for Health Surveillance (Agência Nacional de Vigilância Sanitária ANVISA, website: www.anvisa.gov.br/vacalouca.htm), aiming to safeguard the health of Brazilian people, making sure that the products and services offered to the population were safe.

Brazil did not import meat and bone meal from countries considered at risk for BSE. The only possible source of risk are the cattle imported in the past from such countries; however up to this date there were no clinical signs compatible with BSE detected in such cattle. Considering that the imports of live cattle occurred 10-23 years ago (time considerably longer than the average incubation period for BSE), the possibility that these animals will develop BSE is remote. Nonetheless, all these imported cattle and their descendents are being followed up, including with laboratory examination. Table 4 lists the bovine categories from which brains are being examined in the Brazilian BSE surveillance system. During 2001-2002 5,894 brains were examined histologically and 1,361 were from cattle with clinical signs of neurological disturbances. These numbers are by far greater than the ones recommended by OIE, i.e., that 433 brains form cattle with neurological signs be examined each year in a country having a cattle population the size as Brazil has.

Countries that import animal products from Brazil consider that both the measures adopted by the Brazilian government in relation to cattle imports into this country in the past and our surveillance system are adequate and safe, and classify the BSE risk of importing Brazilian animal products and cattle as negligible.

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ANNEX 1

Table 1. Characteristics of the transmissible spongiform encephalopathies (TSEs)

Extended incubation periods (month to years)
Progressive and always fatal neurological disease
Pathological changes restricted to the central nervous system (CNS)
Spongiform changes in the CNS
Transmissible (naturally or experimentally)
Absence of immune or inflammatory response
Occur in human beings and animals (see Tables 2 and 3)

Table 2. Transmissible spongiform encephalopathies (TSEs) in humans

Sporadic disease (no known antecedent events)	Creutzfeldt-Jakob disease (CJD). Occurs worldwide with incidence of about 1 in a million.
Acquired disease (acquired by contamination with infectious agent)	Kuru. Epidemic amongst the Foré people of Papua New Guinea Iatrogenic CJD New variant CJD. Thought to result from eating food contaminated with BSE agent.
Familial disease (genetically inherited)	Familiar CJD. Accounts for about 10-15% of all CJD cases. Gerstmann-Sträussler-Scheinker syndrome. Incidence about 1 in 10 million Fatal familial insomnia Atypical prion disease. Does not easily fit the various diagnostic criteria for prion disease.

From Baker HF, Ridley RM: Fatal protein. The story of CJD, BSE and other prion diseases, p. 3. Oxford University Press, Oxford, England, 1998.

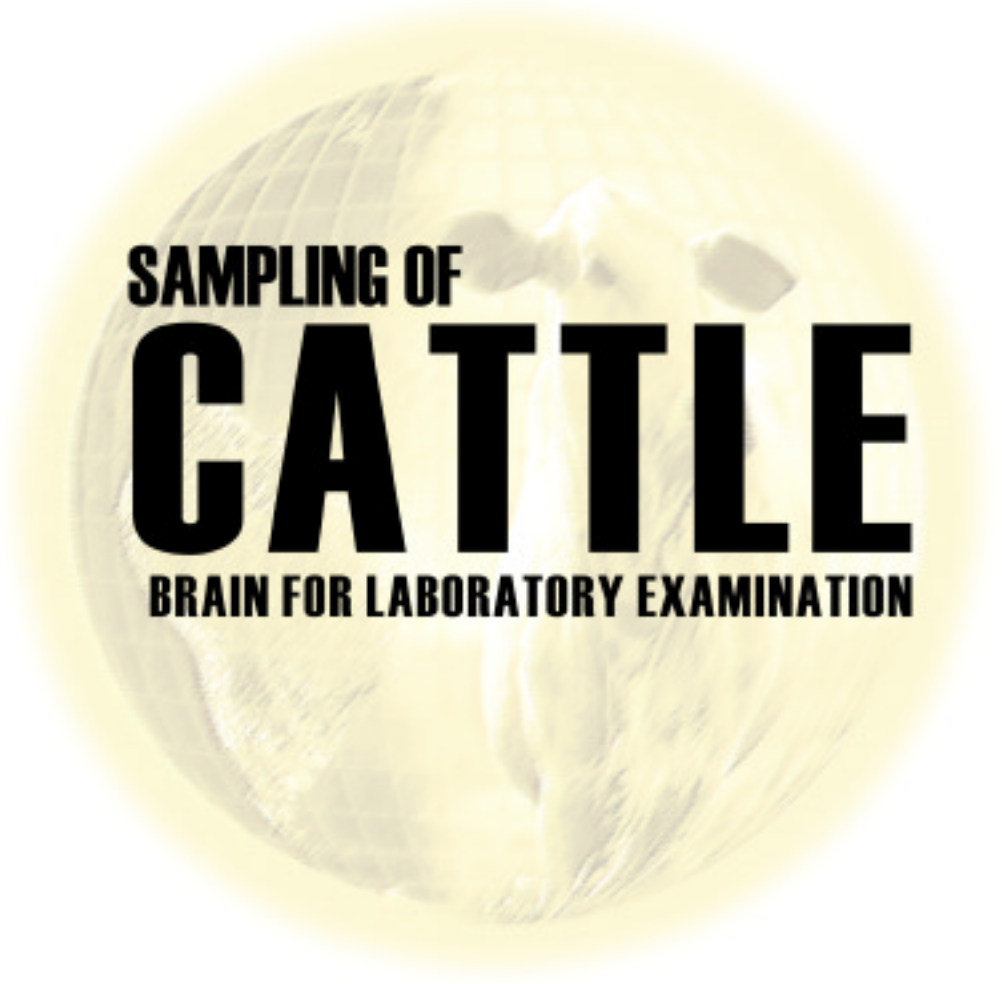
Table 3. Transmissible spongiform encephalopathies (TSEs) in animals

Scrapie	A rare endemic brain disease of sheep and goats. Considered to be the prototype of EETs.
Transmissible mink encephalopathy (TME)	A disease of farmed mink, probably caused by feeding scrapie-contaminated meat.
Chronic wasting disease (CWD)	A scrapie-like disease, of obscure origin, in wild and captive mule deer and Rocky Mountain elk.
Bovine spongiform encephalopathy (BSE)	Endemic disease in dairy cows, mainly in the UK, caused by feeding cows with feedstuffs containing rendered remains of scrapie-affected sheep and BSE-affected cattle.
Feline spongiform encephalopathy (FSE)	Disease seen in domestic cat, and in a few other cats, including puma, cheetah, and ocelot. Assumed to be caused by feeding BSE-infected material.
Spongiform encephalopathy of other species	Identified in a number of zoo animals; for example, kudu, gemsbock, nyala, eland, Arabian oryx, and scimitar. Assumed to be caused by feeding BSE-infected material.

From Baker HF, Ridley RM: Fatal protein. The story of CJD, BSE and other prion diseases, p. 8. Oxford University Press, Oxford, England, 1998.

Tabela 4. Cattle categories from which brains are examined in the Brazilian surveillance program for BSE

Cattle which tested negative for rabies in the officiallly accredited lab for rabies diagnosis
Cattle with neurological clinical signs
Cattle imported from countries where native cases of BSE were reported
Dairy cattle over 30-month-old with high milk yield slaugthered in Federal inspected abbattoirs
Cattle from emergency slaughter in Federal inspected abbattoirs
Cattle over 30-month-old with any wasting disease
Fallen stock over 30-month-old



SAMPLING OF CATTLE BRAIN FOR LABORATORY EXAMINATION

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SAMPLING OF CATTLE BRAIN FOR LABORATORY EXAMINATION

1 - Introduction

Bovine spongiform encephgalopathy (BSE) widely known as "mad cow disease" is a chronic progressive degenerative disease of the central nervous system of cattle. It was first recognized in Great Britain in 1986[12] and caused a great economic impact in the cattle industry of United Kingdom. Later, the disease was also confirmed in native cattle from Austria, Belgium, Canada, Czech Republic, Denmark, Finland, France, Germany, Greece, Ireland, Israel, Italy, Japan, Liechtenstein, Luxembourg, Netherlands, Poland, Portugal, Slovakia, Slovenia, Spain, and Switzerland. The possibility that BSE could be a risk for human health was a concern since the start of the epidemics in England. This concern reached extremely high levels in March 1996 when the English Ministry of Health, in a communication to the English Parliament, stated that the occurrence of a new variant of the neurological disease of humans Creutzfeldt Jakob (CJD) was probably related to BSE[1,2]. This widespread concern resulted in greater demands from the international communitiy on the beef exporting countries. This demands included the presentation of evidences that the exporting country was free of BSE. In other words countries exporting cattle or cattle products need to have a surveillance system capable to attest that their cattle herd is free from BSE, *capable to identify the diseases which affect the central nervous system of catlle in the country and capable to recognize cases of BSE should they occur.*

The aim, of this section of the handbook is to supply guidelines for sampling of brain tissue for the microscopic diagnosis of BSE and other diseases of the central nervous system of cattle. Although BSE does not occur in Brazil, it is necessary tohave in place an efficient

have in place an efficient surveillance system for the disease. Instructions regarding the preliminary exam of the central nervous system, sampling of tissues and shipment of the tissues to the laboratory are included in this section. The diseases that directly or indirectly affect the nervous system of cattle in the State of Rio Grande do Sul Brazil were reviewed recently[10] and it is presumed that at least a similar distribution of these diseases occurs in other states, with perhaps differences occurring only in regard to prevalence. These diseases should be considered when sampling tissues for laboratory examination.

In order that a significant number of cattle brain samples are examined, it is expected that samples received by the official laboratories that carry out herbivore rabies diagnosis also be included in the BSE surveillance system. For that reason, we tried to include in this section, sampling instructions that are adequate for both the diagnosis of both rabies and BSE. This type of sampling will also allow the diagnosis other diseases that commonly affect the central nervous system of Brazilian cattle[10].

2 - General instructions

2.1 The diseases of the central nervous system are usually not obvious at gross examination. For that reason the veterinary pathologist that examine the mailed in samples in his laboratory depends on a properly taken clinical history and on reliable clinical data in order to orient himself on the nature of neurological disease[9]. A form with the main data referring to the affected animal(s), and the main clinical, epidemiological and necropsy findings should be filled (Annex 1). Removing and sampling tissues from the central nervous system demands time and a great deal of work. Thus, it is necessary to apply some criteria when deciding to undertake this task. If there are neither history nor clinical signs of neurological disease it is unlikely that the histological examination of the nervous system will reveal any significant lesion. When the clinical history is poor, absent or inaccurate or when the animals death occurred without premonitory signs, the histological examination of the brain is advised.

2.2 The following data should be informed: date and time of the death of the animal, the time elapsed between the death and the necropsy and any delay occurring in sampling and fixing the neural tissues. These data are important to the interpretation of the neuropathological exam. Some central nervous diseases of animal could be a risk for human health (e.g., rabies, listeriosis); they should be considered before neuropathological examination and proper care should be taken. The use of gloves and goggles during the opening of the calvarium is recommended.

2.3 It is extremely important that the handling of unfixed neural tissue sampled for histological examination be minimal. Handling of fresh (unfixed) neural tissue induces artifacts that hamper the histological evaluation of lesions. Thus, detailed gross examination should be carried out after fixation.

2.4 As much as possible, a systematic gross examination should be done on the brain (fixation hardens tissues). This make easier selecting the proper brain slices for the diagnosis of specific diseases and allows determining the distribution of lesions. The distribution of lesions in the central nervous system (i.e., bilateral, symmetrical, focal, multifocal, white matter, gray matter) is characteristic for several diseases and should be recorded. Many times the whole brain can not be fixed as would be desirable, because unfixed parts of the organ are needed for virological or bacteriological examination.

2.5 Do not mix tissues from different animals even if they represent cases of the same disease. Tissues of each individual animal should be clearly identified.

3 - Removal of the brain

Undiscriminated sampling of several parts of the unfixed brain may prevent good neuropathological examination in the laboratory. When there are several animals for necropsy in an outbreak of a neurological disease, the time involved in removing one brain can be a factor limiting how many brains would be possible to remove. In this case, select some animals from which the brain should be submitted to neuropathological examination. Remove the brain trying to eliminate or extremely minimize any mechanical damage to the nervous tissue.

3.1 Using a ventral approach remove the head, cutting the atlantooccipital joint. At this point examine the capsular surface of the joints and the physical aspect of cerebrospinal fluid (CSF) that oozes when the dura is cut. In cases of recent death a non-contaminated sample of CSF can be obtained before sectioning the dura.

3.2 Remove the skin and the muscles of the head. Open the cranium following the lines shown in Figure 1. This can be done with a hand saw or with an axe. The brain is then exposed with the intact dura.

3.3 Using scissors, remove the dura-mater cutting the *falx cerebri* and the *tentorium cerebelli* (Figure 2). Tilt the head of the animal and remove the brain by cutting the cranial nerves. Without sectioning these structures it is not possible to remove the brain intact. In order to avoid artifacts when removing the brain, try, as much as possible, minimize handling, pressing or squeezing the neural tissue.

3.4 The ganglium of the trigeminal nerve (Gasserian ganglium) and the carotid rete mirabile should be sampled with the hypophysis (Figure 3). The histopathological examination of this pair of ganglia of the fifth cranial nerve is important for the diagnosis of such diseases as rabies and bovine meningoencephalitis caused by bovine herpesvirus 5 (BHV-5). In these two diseases inflammation (ganglioneuritis) of the trigeminal nerve and ganglium is a frequent finding. In cases of malignant catarrhal fever[11], the vessels of the *rete mirabile* show a characteristic vascular lesion (vasculitis).

3.5 Examine the brain for any gross lesions looking for any asimmetry (i.e, structures in one side of the midline more prominent than the correspondent structure on the other side), areas of discoloration (e.g., hyperemia of the meninges, congestion of the cortex in cases of cerebral babesiosis [*Babesia bovis* infection], tan-yellow cortex in cases of polioencephalomalacia).

4 - Selection of brain samples

Samples for virology and bacteriology should be taken before fixing the brain. For some virological methods (e.g., FA for rabies) brain tissue can be frozen. However freezing will turn sample inadequate for histopathology. Since in many cases the three types of exams (bacteriological, virological and histopayhological) will be required a compromise is necessary.

4.1 Sampling for bacteriology and virology

4.1.1 First remove the cerebellum by cutting at the level of cerebellar peduncles. From the caudal aspect of the cerebellum, insert the blade in the fourth ventricle (Figure 4). Cut rostrally and horizontally the cerebellar peduncles in both sides separating the cerebellum from the brain stem. When this is done the cerebellum will be completely separated from the rest of the brain (Figure 5).

4.1.2 Cut at the level of the thalamus, separating the brain stem from the remaining of the cerebrum (Figure 6). When this step is completed you will obtain the following three parts a) brain stem, b) cerebellum c) cerebrum (Figure 7).

4.1.3 To obtain sample 1, make a sagital slice (about de 0,5 cm thick) on the cerebellar vermis (Figure 8).

4.1.4 To obtain sample 2, take a 2,5 cm segment of the cervical spinal cord (Figure 9).

4.1.5 To obtain sample 3, take a slice (about de 1 cm thick) of the thalamus (Figure 10).

4.1.6 To obtain sample 4, divide one of the cerebral hemispheres at the level of the optic chiasm, separating the caudal portion (Figure 11).

4.1.7 At this point the four samples to be sent to virology or bacteriology were obtained (Figure 12). The selected fragments are adequate for rabies diagnosis[3,8] and for the diagnosis of other diseases induced in the bovine central nervous system by bacteria and other viruses[9-11]. These four samples should be shipped refrigerated (4 C). If the time between sampling and shipment to the laboratory is greater than 24 hours, freezing is advisable; however these samples should never be fixed.

4.1.8 The remaining of the brain (Figure 13) should be fixed in 10% formalin according instructions in item 4.2, since it is destined for histopathological examination. The block of tissues shown in Figure 3 (*rete mirabile*, trigeminal ganglia and nerve) should be also fixed in 10% formalin and shipped for histopathology along the tissues shown in Figure 13.

4.2 Sampling and fixation of tissues for histopathology

4.2.1 A 10% formalin solution is indicated to fix the brain. In order to prepare one liter of these solution, use 100 ml of formaldehyde (35%-40%) and 900 ml of tap water. There is a frequent confusion between formaldehyde and commercial formalin. Formaldehyde is a gas from which an 35%-40% acquous solution is prepared. This solution constitute what is known as common commercial formalin.

Thus a 10% formalin solution represents a solution prepared by mixing 10 ml of commercial formalin (35%-40% formaldehyde) and 90 ml of water4. Better quality slides are obtained by the fixation of tissues with buffered 10% formalin solution. The following instructions are to prepare one liter of buffered 10% formalin solution7.

Reagents

Formaldehyde (de 35-40% solution)	100ml
Distilled water	900ml
Sodium monophosphate	4g
Sodium diphosphate	6,5g

Procedures

Even though the formalin solution is buffered, as time goes by the pH will decrease, and the formation of formalin pigment (acid hematin) will occur in congested tissues (tissues with large amount of blood).

Observations

Mesmo que essa solução de formol seja tamponada, com o tempo o pH vai baixar, provocando o aparecimento de pigmentos de hematina ácida em tecidos congestos (que têm muito sangue).

4.2.2 The volume of fixing solution should be at least 10 times greater than the volume of tissue (brain) to be fixed. Thus, 6 liters of the formalin solution will be necessary to fix a whole adult cattle brain (600 cm^3) the minimal time needed for fixation varies depending on the size of the brain to be fixed. A mouse brain will take 24 hours, a sheep brain approximately four days and the adult bovine brain will take a week to be fixed. After fixation a considerably smaller amount of formalin would be necessary to maintain fixation; this would facilitate the shipment of sample.

When fixing the brain, avoid mixing it with other material which may compress and damage the neural tissue.

5 - Shipment of samples to the laboratory

5.1 Each brain should be shipped in a a non-deformable container (e.g., a hard plastic jar) instead of being enclosed in cotton or gauze. The container should be completely filled with the formalin solution in order to exclude air bubbles, allowing a cushion effect for the shaking movements that occur during shipment. In case there is urgency in the diagnosis the tissues may be transported unfixed, but in this case, information about the time of fixation should be included.

5.2 It is important that the container with the brain tissue (both fixed and unfixed) to be sent to the laboratory be tightly closed in order to avoid leakage and exposition of personnel handling the package. Any submission should be accompanied by the filled form shown in Annex 1.

6 - References

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7 - Glossary

Arachnoid: The brain and spinal cord are surrounded by a series of three closely associated sheet-like connective membranes (called meninges) which are from the outermost inwardly: *dura mater*, *arachnoid* and *pia mater*. Arachnoid is the middle one of theses sheet-like coverings.

BSE: Bovine spongiform encephalopathy.

CJD: Creutzfeldt Jakob disease. Neurological disease of humans caused by prion with lesions similar to those of BSE.

Dura mater: The brain and spinal cord are surrounded by a series of three closely associated sheet-like connective membranes (called meninges) which are from the outermost inwardly: *dura mater*, *arachnoid* and *pia mater*. Dura mater is the outermost and the thickest (also called pachymeninge) of these sheet-like coverings.

Falx cerebri: projection of the dura mater which extends ventrally within the median longitudinal fissure which separates the two brain hemispheres[6].

Ganglium: aggregations of neurons outside the central nervous system (plural = *ganglia*).

Leptomeninge: (*Lepto* meaning thin) are the membrane formed by arachnoid and the pia mater together.

Pachymeninge = dura mater

Pia mater: The brain and spinal cord are surrounded by a series of three closely associated sheet-like connective membranes (called meninges) which are from the outermost inwardly: *dura mater*, *arachnoid* and *pia mater*. Pia mater is the innermost and most delicate (*pia* = tender) layer of the meninges.

Prion: protein molecule considered as the etiological agent of the transmissible spongiforme encephalopathies such as BSE. This protein is central to the development of these diseases. In healthy individuals it is broken down by proteases (enzymes which break down proteins). In affected individuals this protein undergoes a post-translational change and becomes resistant to the proteases. The name prion was constructed from ***pro***teinaceous ***in***fectious particle. It should logically be called *proin*, but prion *triped* better from the mouth[1].

Rete mirabile: complex network of blood vessels intercalated in the course of an artery[4]. The carotid rete is located on each side of hypophysis, between this gland and the trigeminal ganglium.

Tentrorium cerebelli: A connective septum which is an extension from the dura-mater. It is oriented in a diagonal plane within the transverse fissure between the cerebellum and the occipital lobes of telencephalon[6].

Brain stem: The cephalic continuation of the spinal cord into the cranial cavity. It is composed of the medulla oblongata, pons, midbrain and diencephalon. Therefore the brain stem can be exposed by removal of the cerebral hemispheres and the cerebellum[6].

ANNEX 1

Sample Submission Form

Sample no._____County_____State_____
Submitting veterinarian_____CRMV no._____
Address_____Phone ( )_____
E-mail_____Fax ( )_____

This box should be filled only in case of imported cattle
Animal name_____Animal no. _____Country of origin_____
Had neurological signs? [ ] yes [ ] no For indemnity? [ ] yes [ ] no

Owner_____Premises_____
Address_____County_____State___
E-mail_____Phone ( )_____Fax ( )_____

Species: [ ] bovine [ ] ovine [ ] caprine Breed_____Age (months)_____
Were there other animal species affected [ ] yes [ ] no Sex [ ] male [ ] female
Please fill the number of animals in the spaces beteen brackets
In the herd there were (_____) animals of which (_____) were affected and (_____) died.
The animal of this sample was vaccinate against [ ] rabies [ ] clostridial diseases [ ] other

Date when the dfisease/outbreak started____/__/____
Type of clinical signs

Sudden death ☐	Blindness ☐	Circling ☐	Flaccid paralysis of thoracic Limbs ☐
Depression ☐	Incoordination ☐	Convulsions ☐	Flaccid paralysis of pelvic limbs ☐
Ataxia ☐	Tetany ☐	Dysmetria ☐	With paralysis but still bright ☐
Opisthotonus ☐	Aggression ☐	Trembling ☐	Nistagmus ☐

Clinical course (time from the first clinical signs to death):
Were there animals which recovered from the clinical signs? [ ] yes [ ] no
If so which percentage recovered? _____%.

Date of death ____/____/____ Time of death_____
Time from death to sampling of tissue:_____hours___minutes
Time from sampling to fixation of tissue:_____ hours___minutes
Tissue preserved in:_____

Veterinarian sampling the tissues_____CRMV no._____
Address_____Phone ( )_____
E-mail_____Fax ( )_____

Observations:

City/Date: _____, __/__/_____

ANNEX 2

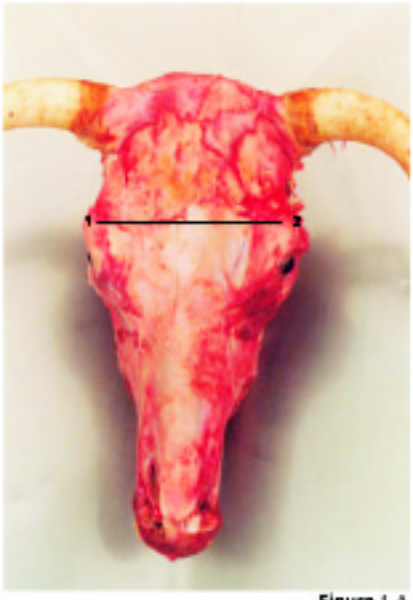

Figure 1 A

Figure 1 B

Figure 1 C

Figure 1. Removal of the brain. Dark lines show the trajects where the cranium should be cut for removal of the brain.. **A.** The first line connects two points (1 e 2) immediately cranial to the orbits . **B.** The second line connects the points 1 e 2 to the occipital foramen. **C.** The line shown in B can be visualized in the posterior portion of the head.

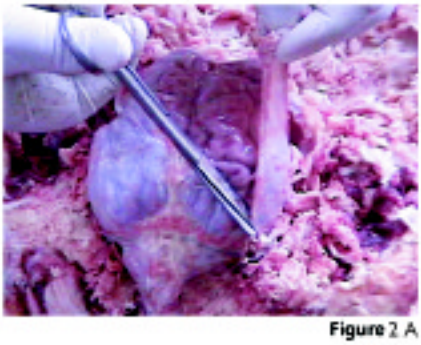

Figure 2 A

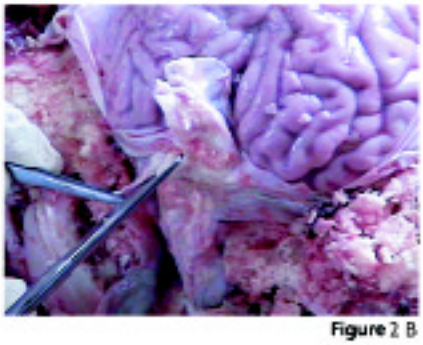

Figure 2 B

Figure 2. Using scissors, cut the falx cerebri and remove the dura mater (**A**) and the tentorium cerebelli (**B**).

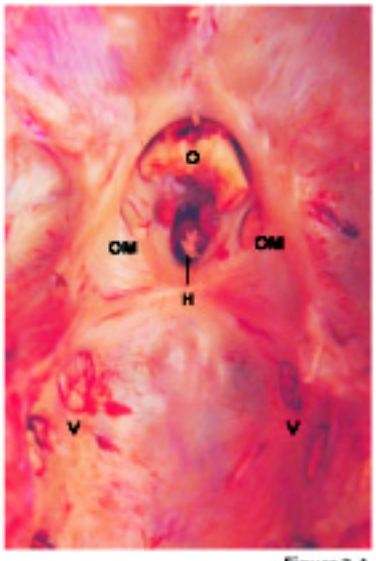

Figure 3 A

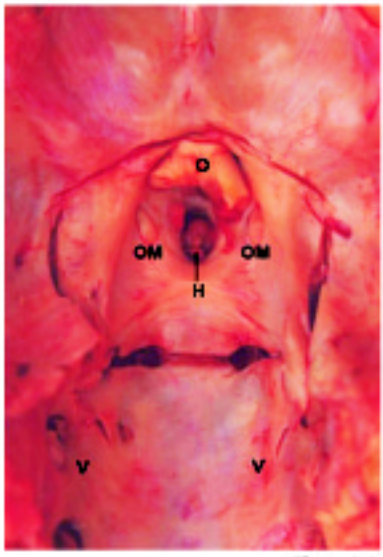

Figure 3 B

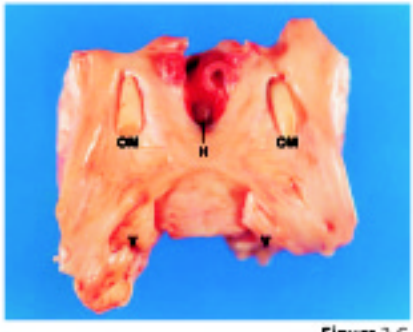

Figure 3 C

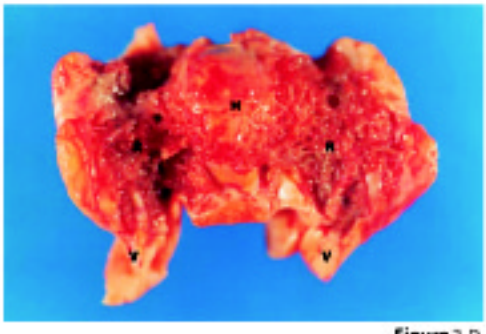

Figure 3 D

Figure 3. These three structures: ganglium of trigeminal nerve (V cranial nerve, Gasserian ganglium), carotid rete mirabile and hypophysis should be removed as a single piece (block 1). **A.** Floor of the cranial vault showing the anatomical references for the removal of block 1. Trigeminal nerve (V), oculomotor nerve (OM), optic nerve (O), and site of hypophysis (H). **B.** The figure shows the line of cut which should be done with the scalpel for the removal of block 1. The following structures are identified: Trigeminal nerve (V), oculomotor nerve (OM), optic nerve (O), and site of hypophysis (H). **C.** Dorsal view of block 1; the following structures are identified: Trigeminal nerve (V), oculomotor nerve (OM), and site of hypophysis (H). **D.** Ventral view of block 1; the following structures are identified: Trigeminal nerve (V), ganglium of trigeminal nerve (G) oculomotor nerve (OM), rete mirabile (R), and hypophysis (H). This block should be fixed in 10% formalin for histopathology.

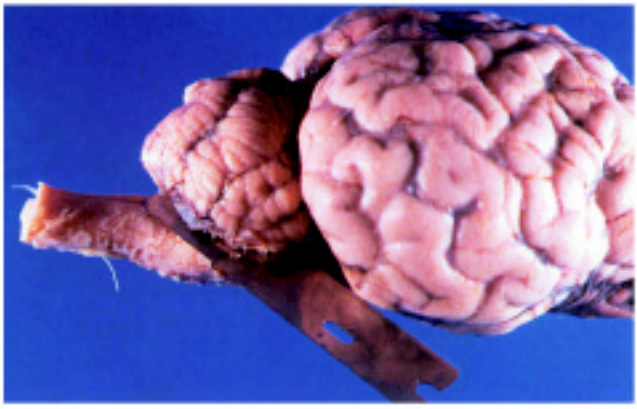

Figure 4. Removal of the cerebellum. From the caudal aspect of the cerebellum, insert the blade in the fourth ventricle. Cut the cerebellar peduncles rostrally and horizontally on both sides separating the cerebellum from the brain stem.

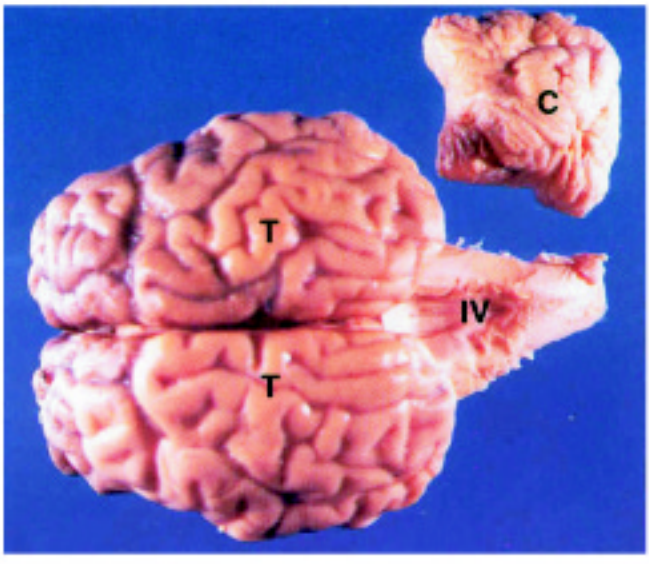

Figure 5. When the step described in Figure 4 is completed, the cerebellum will be fully separated from the rest of the brain. Telencephalic hemispheres (T), fourth ventricle (IV) and cerebellum (C) are identified in the figure.

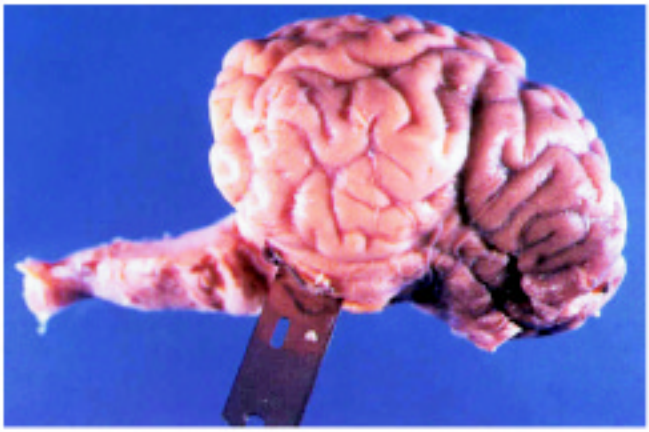

Figure 6. Cut at the level of the thalamus on both sides, separating the brain stem from the remaining of the cerebrum.

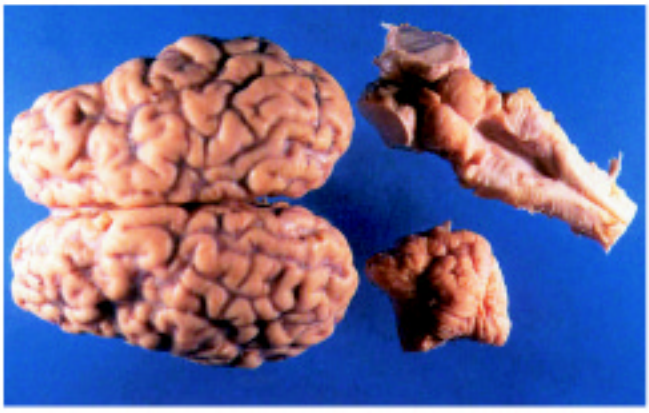

Figure 7. When the step described in Figure 6 is completed the following three parts will be obtained: the brain stem (at the top, to the right), the cerebellum (at the bottom to the right) and the telencephalic hemispheres.

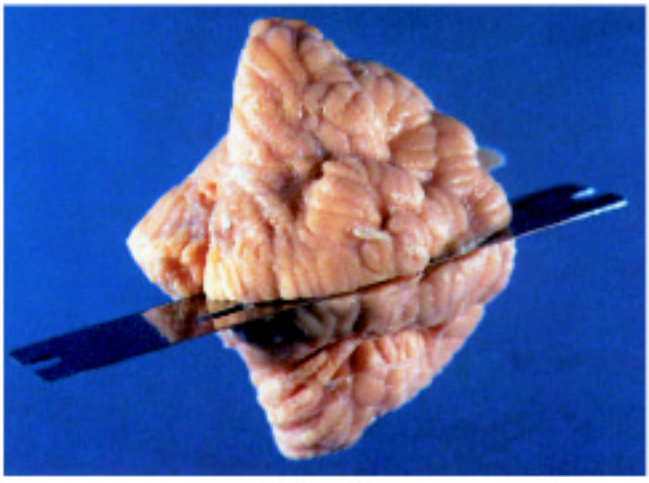

Figure 8 A

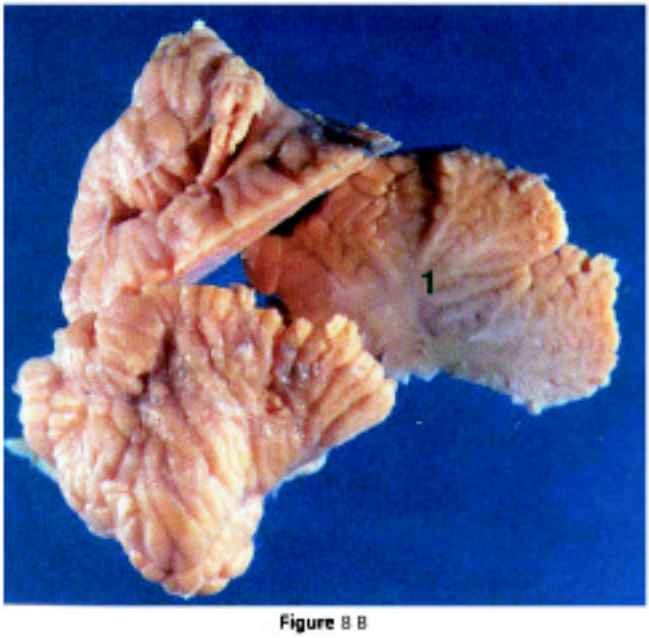

Figure 8 B

Figure 8. A. To obtain sample 1, to be sent to virological and/or bacteriological examination, make a sagital slice (about de 0,5 cm thick) on the cerebellar vermis B. This slice (1) should be refrigerated or frozen.

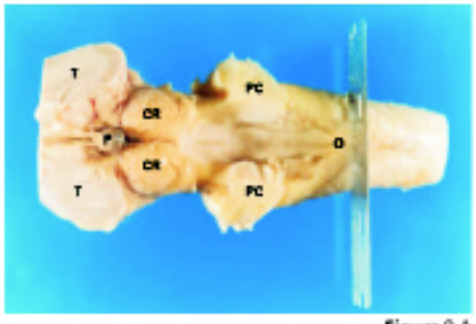

Figure 9 A

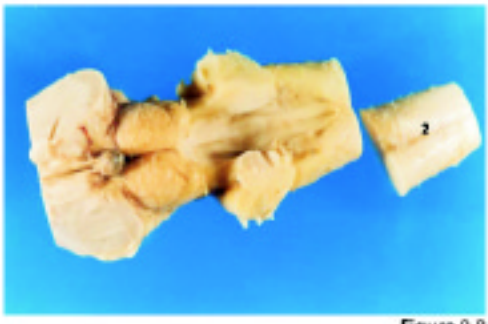

Figure 9 B

Figure 9 A. To obtain sample 2, take a 2,5 cm segment of the cervical spinal cord cutting just caudal to the border of brain brain stem. Obex (O), cerebellar peduncles (PC), rostral colliculi (CR), pineal body (P) and thalamus (T). **B**. This segment (2) should be refrigerated or frozen.

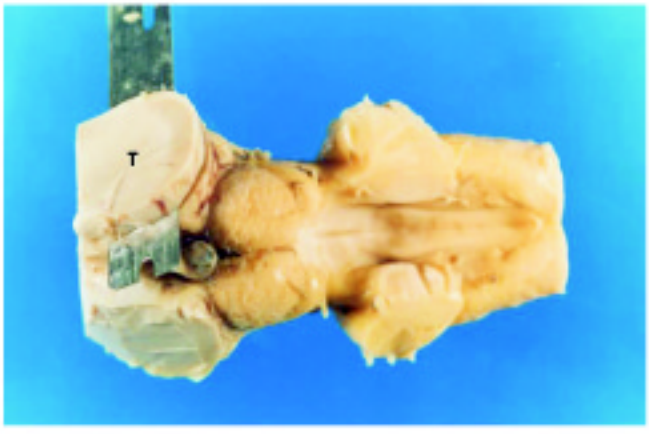

Figure 10. To obtain **sample 3**, take a slice (about de 1 cm thick) of the thalamus

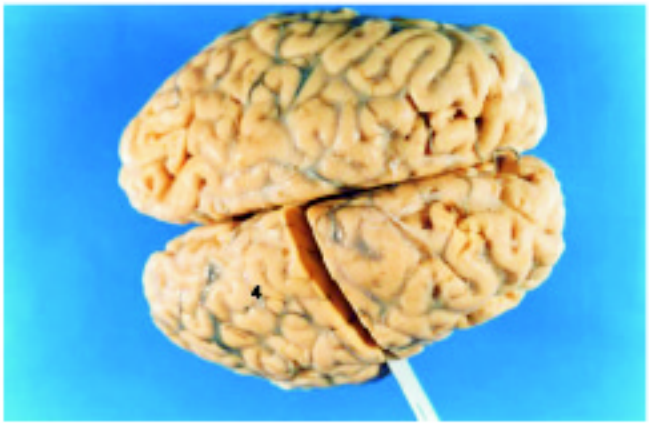

Figure 11. To obtain **sample 4**, divide one of the cerebral hemispheres at the level of the optic chiasm, separating the caudal portion (**4**) from the rest of the telencephalon.

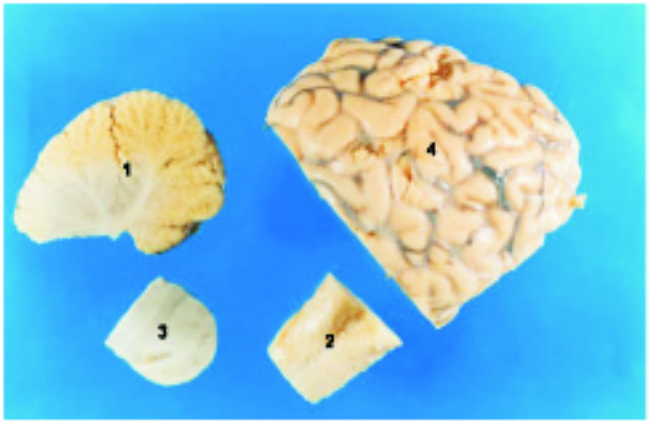

Figure 12. These are the four samples to be sent to virology or bacteriology. **1**, slice of cerebellum which was cut along the cerebellar vermis; **2**, segment of cervical spinal cord; **3**, slice of thalamus and **4**, caudal half of one telencephalic hemisphere. These four samples should be refrigerated or frozen.

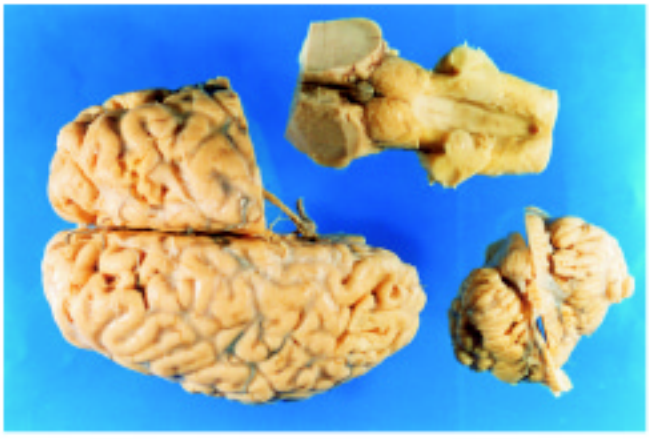

Figure 13. The samples shown in this figure are what is left after the removal of samples 1-4 (see Figure 12) for virology/bacteriology. The samples shown here consisted of the whole brain stem (at the top to the right), two parts of cerebellum (at the bottom to the right) an ¼ of telencephalic hemispheres. They are for histopathological examination and should be fixed in 10% formalin.

PROCESSING OF BRAIN FOR THE HISTOLOGICAL DIAGNOSIS OF BOVINE SPONGIFORM ENCEPHALOPATHY

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35 BSE

PROCESSING OF BRAIN FOR THE HISTOLOGICAL DIAGNOSIS OF BOVINE SPONGIFORM ENCEPHALOPATHY

1 - Introduction

This is the second part of the instructions on the procedures for the diagnosis of the diseases of the central nervous system of cattle. The first part (Sampling of Cattle Brain for Laboratory Examination) of this handbook was aimed to guide veterinarians removing the brain of cattle in the field and preparing and shipping the neural tissues samples to the laboratory. The objective of this second part is to provide the basic instructions for the diagnosis of bovine spongiform encephalopathy (BSE) in order to maintain a surveillance program for this disese in Brazil. However, it is our intention that the instructions provided here are also helpful in the diagnosis of other diseases of the central nervous system in cattle in Brazil. For this reason the first part includes instructions for the shipment of samples for virological (especially rabies diagnosis), bacteriology and histopathology.

The main diseases that direct or inderectly affect the nervous system of cattle in Brazil are briefly reviewed in order to orient veterinarians in the differential diagnosis. These diseases are dealt with briefly but the reader will find more details on each one of them in the publications listed on the reference list[1,4-5]. The instructions for the processing and histological examination of tissues samples are given assuming the neural tissue is received by the lab in accordance with which is described in previous section (Sampling of Cattle Brain for Laboratory Examination) of this handbook.

2 - Bovine spongiform encephalopathy (BSE)

2.1 Sections to be obtained for the histological diagnosis of BSE

The gross examination should be done on the fixed brain. Routinely the fixed brain is coronally sliced at 5 mm intervals. The sections used for the diagnosis of BSE are obtained from the brain stem (Figure 1). The processing of our sections are recommended:

1. Medulla oblongata at the level of obex (Figure 2)

2. Pons at the level of caudal cerebellar peduncles (Figure 3)

3. Mesencephalon including the caudal colliculi (Figure 4)

4. Mesencephalon including the rostral colliculi (Figure 5)

2.2 The histopathology of BSE

The microscopic changes in BSE are highly specific and considered pathognomonic[2,7-10]. They are degenerative, symmetrical and bilateral lesions distributed to certain regions of the brain stem gray matter[8-10]. Neuronal vacuolization occurs as two presentations. In the neuropil, 20-μm vacuoles are observed in neurites; this change is known as spongiform encephalopathy (Figure 6). The other presentation included larger (30-40-μm), single or multiple vacuoles in the neuronal perykaryon (Figure 7); these vacuoles distend the perykaryon resulting in ballooning neurons which maintain only a thin rim of cytoplasm (Figure 8). The presence o vacuoles in the gray matter neuropil and in the neuronal perykaria are the main criteria for a positive histological diagnosis of BSE[10].

Attention: vacuoles occur in the red nucleus of mesencephalon in the brain of normal cattle. They are considered a common normal incidental finding and have been found in 64% of mature or older normal cattle[3]. When present only in this location, they should not be consider as indicative of BSE [9,10].

The distribution of lesions is fairly regular[10]. They occur mainly in the nucleus of solitary tract, in the reticular formation in the medulla, in the periaqueductal gray matter of the mesencephalon, in the paraventricular area of the thalamus and in the thalamic septum. The vacuole density is greater in the medulla, mesencephalon and thalamus. Changes in the cerebellum, hippocampus, basal ganglia an cerebral cortex are minimal.

The mapping of lesions in the brain of 684 BSE affected cattle revealed that in 99.6% of the cases, the section of medulla (see Figure 2) show the characteristic histopatological changes of the disease, especially the spongiform changes in the nucleus of the solitary tract and in the tract of the trigeminal nerve (see Figure 6), indicating that this is the most important brain section for the diagnosis of BSE. Spheroids and individual neuronal necrosis occur occasionally, but there is no evidence of neuroniophagia. There is no inflammatory reaction, but a mild gliosis with gemistocytic astrocytes may be observed. In a small percentage of cases one can observe amyloidosis.

Non-suppurative non-specific inflammation (mononuclear perivascular cuffings) is found in approximately 30% of normal adult cattle[3] and may result from subclinical or latent infections. Other incidental findings with no clinical significance found the brain of symptomless cattle include intracytoplasmic granules of ceroid-lipofuscin in neurons[10].

3 - Differential diagnoses

The neurological disturbances in cattle may result from several causes[1,4-6]. In Brazil, the most frequently diagnosed neurological disease in cattle is rabies[6].

Clinical signs of rabies can be mistaken for those of any other disease of the central nervous system including BSE; however the clinical course of rabies is much shorter (2-7 days). Fluorescent antibody (FA) test in neural fresh unfixed tissue will definitively confirm rabies and settle the issue. Furthermore, the histological lesions of BSE are highly characteristic and differ by far from those of rabies. In the latter there is non-suppurative meninoencephalitis and, in aproximately 2/3 of the cases, one can observe intracytoplasmic acidophilic inclusion bodies - the Negri bodies.

A careful study of the clinical history, epidemiology and necropsy findings helps distinguishing among the several neurological diseases which affect cattle. For example, congenital diseases (inherited or otherwise) occur in newborn or very young animals, while BSE will affect only adult cattle (4-5 year-old). Additionally certain intoxications, as the one caused by the mycotoxin from the fungus *Claviceps paspali* occcur seasonally (usually in early autumn) when the parasitized grass (*Paspalum spp.*) is on seed. The main neurological diseases reported in cattle from Brazil are listed in Tables 1-4 where information is given on age and category of affected animals, main clinical signs and lesions found in each disease. Information given on these tables will only serve as a general guidance for the differential diagnoses. Additional information on each of theses diseases should be obtained from the publications provided in the reference list or in specialized textbooks.

4 - Additional sections

Regularly, additional sections which help in the differential diagnoses of the diseases of the central nervous system of cattle should be taken. These include:

1. A sagital section through the cerebellar vermis (Figure 9).

2. Coronal section of the frontal telencephalic cortex just rostrally to the optic chiasm (Figure 10).

3. Coronal section of the frontal telencephalic cortex at the level of mammilary bodies (Figure 11).

When the histological examination of the sections selected above are inconclusive, additional sections of the brain should be examine, up on discretion of the veterinary pathologist.

5 - References

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ANNEX I

Table 1. Some inherited/congenital neurological disturbances in cattle.

Disease	Age/category	Clinical signs	Lesions
Hydrocephalus	NB	Prolonged bellowing, unable to stand	Domed cranium, dilatation of cerebral ventricles
Cerebellar hypoplasia	NB	Unable to maintain balance, incoordination, visual deficits.	Cerebellum small or absent
Congenital hypomyelinogenesis	NB	Sporadic occurrence. Progressive ataxia, recumbency.	Histology: absence of myelin in the white matter
Cerebellar abiotrophy	Up to six-month-old	Sporadic occurrence. Ataxia, dysmetria, rithymic movements with the head	Histology: degeneration of cerebellar neurons

NB = newborn

Table 2. Some neurological disturbances of metabolic/nutritional causes in cattle.

Disease	Age/category	Clinical signs	Lesions
Polioencephalomalacia	Young animals/feedlot calves	Diarrhea, slowing of movements, nystagmus, recumbency, blindness, twitching of the ears, opisthotonus, terminal convulsions, coma	Necrosis (malacia) of the gray matter of the brain
Ketosis	High milk yield dairy cows with energetic deficit/ sudden deprivation of food in pregnat beef cows	Loss of weight, continuous licking, grinding of teeth, drooling of saliva, incoordination, circling, head pressing, muscle tremors and tetany	Fatty degeneration of the liver
Milk fever	Around parturition/dairy cows	Initial excitement, localized tetanic spasms that progress to sternal recumbency, depression of reflexes, mydriasis, lateral recumbency, loss of consciousness, coma and death	There are no lesions

Table 3. Some neurological disturbances of infecious causes in cattle.

Disease	Age/category	Clinical signs	Lesions
Rabies	All ages	Paralytic form most common. Continuous bellowing, mania, tenesmus, drooling, constipation and paralysis	Non-suppurative meningoencephalitis, intracytoplasmic acidophilic inclusion bodies (Negri bodies) in neurons
Meningoencephalitis caused by bovine herpesvirus 5 (BHV-5)	Mainly young animals	Fever, abdominal pain, head pressing, circling, flaccid paralysis of the tongue, depression	Non-suppurative meningoencephalitis, vasculitis, neuronal necrosis, intranuclear acidophilic inclusion bodies in astrocytes and neurons
Malignant catarrhal fever	Usually adults	Sporadic cases; less frequently in outbreaks. Associated to the presence of sheep, mainly in lambing time. Mucopurulent nasal discharge, fever, palpebral edema, corneal opacity, congestion of sclera vessels. Diarrhea or constipation. Mucosal hyperemia or ulceration, dermatitis. Marked depression, incoordination. Head pressing, convulsions and paralysis	Histology: Non-suppurative meningoencephalitis, intracytoplasmic, arteritis and fibrinous exsudate in leptomeninges, Disseminated proliferation of lymphoid cells, vasculitis and necrosis of the epithelial coverings
Listeriosis	All ages, but mainly adults	Dropped jaw, drooling of saliva , facial hypoalgesia, ptosis, dropped ear (generally unilateral), loss of palpebral menace reflex, circling, ataxia, hemiparesis.	Gross lesions may be absent or small tan foci (microabscesses) can be observed in brain stem cut surface. Histology: microabscesses and perivascular mononuclar cuffings in the brain stem. Listeria monocytogenes can be cultured from the unfixed fresh brain stem, but the culturing is capricious. Gram stain can reveal the organism in histological preparations.
Cerebral babesiosis (Babesia bovis)	All ages, but mainly adults	Fever, depression, prostration and convulsions	Cerebral and cerebellar cortex are cherry-red (pathognomonic). Histology: congestion and edema of cerebral cortex. In cortical brain smears, B. bovis is readly identifiable in red blood cells sequestered in capillaries.
Botulism	Adult animals	Flaccid paralysis	No lesions
Coccidiosis	Calves, mainly feedlot	Bloody diarrhea. Ataxia, muscle tremors, blindness, hyperestesia, tonicoclonic convulsions, nystagmus, opisthotonus	There are only lesions related to the Eimeria parasitism. No lesions in the central nervous system are seen. The clinical signs of the nervous form is apparently induced by a toxin produced by the intestinal coccidia

Table 4. Some neurological disturbances caused by toxins and neoplasia in cattle.

Disease	Age/category	Clinical signs	Lesions
Hepatic encephalopathy secondary to *Senecio* spp poisoning	Adults	Aggression, ataxia, circling, depression, tenesmus, diarrhea	Subcutaneous edema, cavitary edema, edema of abomasal folds. Histology: hepatic fibrosis and megalocytosis with billiary ducts hyperplasia. In the central nervous system (CNS) there is spongy degeneration of the white matter.
Atelela gaiazioviana poisoning	Various ages	Lethargy, blindness, staggering, dry feces, dropped ears. The disease is also associated with abortions, sudden death and clinical signs of right side congestive heart failure.	Necropsy: pale and firm areas in the myocardium. Nutmeg liver. In the CNS there is spongy degeneration (edema) of the white matter.
Solanum fastigiatum poisoning	Mainly adults	Periodic seizures. Extension of the neck, wide base stance, hypermetry, nystagmus, opistothonus, muscle tremors and falls.	Histology: vacuolization, degeneration and loss of the cerebellar Purkinje neurons.
Diplodia maydis poisoning (mycotoxicosis)	Several	Serous ocular discharge, drooling, muscle tremors, ataxia and dysmetria with exagerated flexion of limbs, difficulty in walking, paralysis, opisthotonus. When contaminated corn is withheld, affected animals recover in 7-10 days..	There are no specific lesions
Claviceps paspali poisoning (mycotoxicosis)	Several	Muscle tremors, ataxia. Occurs in the autumn	There are no specific lesions
Phalaris spp.poisoning	Mainly adults	Hyperexcitement, muscle tremors, horizontal movements with the head, incoordination, staggering	Necropsy: bluish or greenish discoloration, of the CNS. Histology: brown pigment in the cytoplasm of neurons.
Cynodum dactylon poisoning	Several	Alert . Muscle tremors which get worse when the animal is moved. Ataxia, hypermetric gait and falls	There are no lesions
Organophosphate/ Carbamate poisoning	All ages	Sporadic occurrence. Drooling, diarrhea, miosis, bradycardia, muscle tremors, tetania, sweat, ataxia, disorientation, seizures and coma.	Peripheral nerve and spinal cord tract degeneration.
Neoplasia			
Enzootic leukosis	Adults; more common in dairy cows	Incoordination of pelvic limbs and paralysis	White, soft tumor mass (lymphosarcoma) compressing the spinal cord. There are also similar neoplasms in other sites (lymph nodes, myocardium, abomasum, etc.)

ANNEX 2

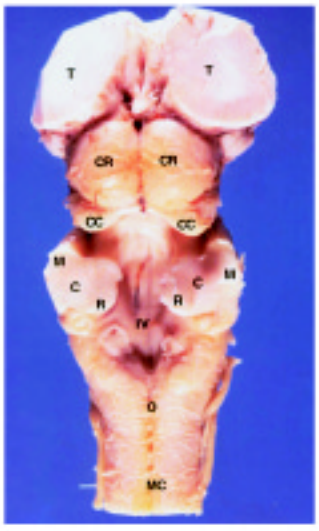

Figure 1. Dorsal view of the bovine brain stem. The cerebellum was excluded. Identified structures are rostral (CR) and caudal (CC) colliculi; middle (M), caudal (C) and rostral (R) cerebellar peduncles; floor of the fourth ventricle (IV), obex (O), and cervical spinal cord (MC).

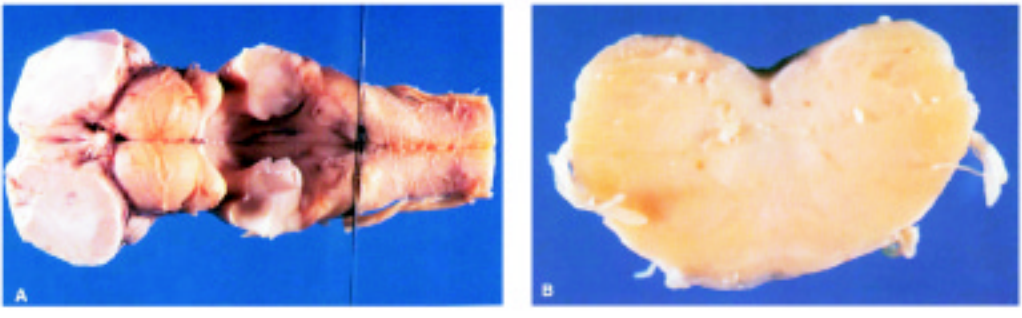

Figure 2. Localization of the four sections to be made in the brain stem for the diagnosis of BSE. **A**. Section 1, medulla at the level of obex. **B**. Fragment of tissue obtained by section 1 which should be processed for histopathology.

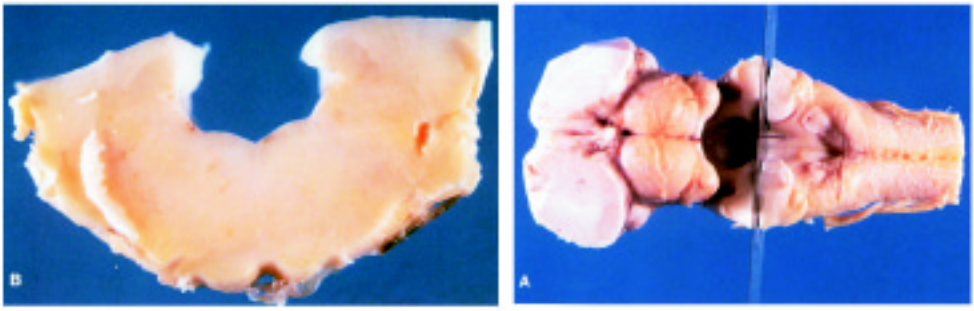

Figure 3. Localization of the four sections to be made in the brain stem for the diagnosis of BSE. **A.** Section 2, pons at the level of caudal (posterior) cerebellar peduncles. **B.** Fragment of tissue obtained by section 2 which should be processed for histopathology.

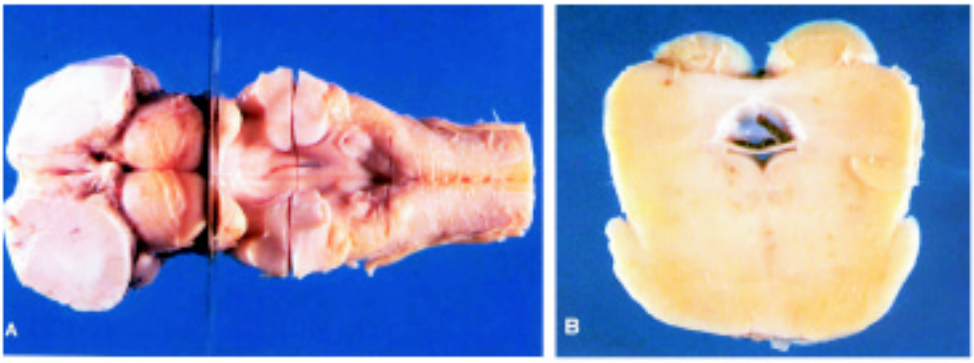

Figure 4. Localization of the four sections to be made in the brain stem for the diagnosis of BSE. **A.** Section 3, mesencephalon at the level of caudal colliculi. **B.** Fragment of tissue obtained by section 3 which should be processed for histopathology.

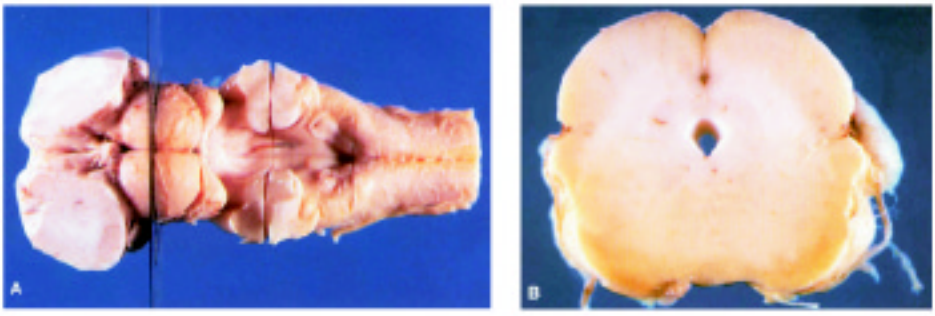

Figure 5. Localization of the four sections to be made in the brain stem for the diagnosis of BSE.. **A**. Section 4, mesencephalon including the caudal colliculi and red nucleus. **B**. Fragment of tissue obtained by section 4 which should be processed for histopathology.

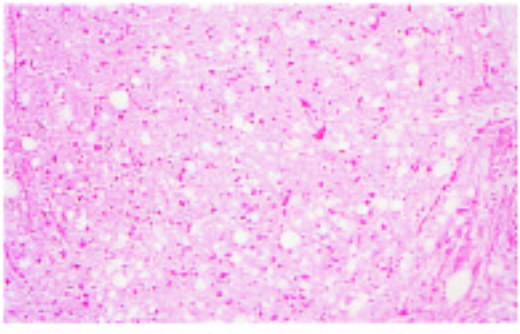

Figure 6. Bovine spongiform encephalopathy. Microcavitation (spongiform change) in the dorsal gray matter of medulla. Hematoxilin and eosin (Photographed from a glass slide courtesy of G.A.H. Wells).

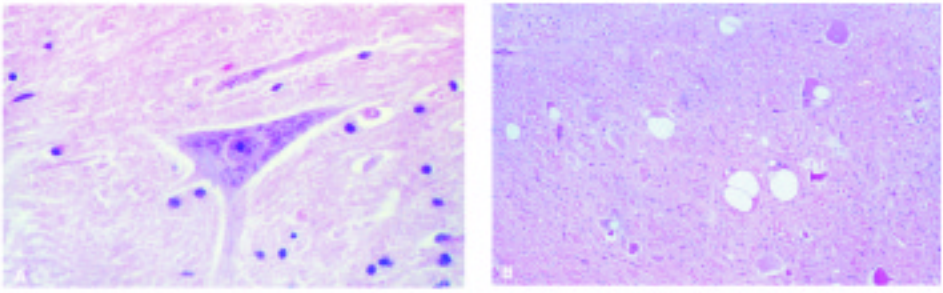

Figure 7. A. Normal neuron. The cytoplasm show the characteristic Nissl formed by blue granule. Hematoxilin and eosin. B. Brain stem from a BSE affected cow. Several neurons of medulla display single or multiple vacuoles in the perykarya. Hematoxilin and eosin (the lesion shown in B was photographed from a glass slide courtesy of G.A.H. Wells).

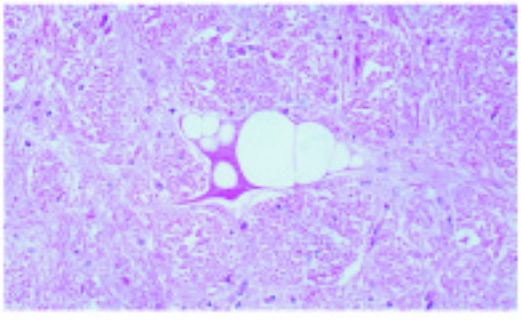

Figure 8. Medulla from a BSE affected cow. Multiple vacuoles in the cytoplasm of a neuron. These vacuoles distend the perykaryon imparting a ballooning aspect to the neuron which keeps only a narrow cytoplasmic rim. Hematoxilin and eosin. (Photographed from a glass slide courtesy of G.A.H. Wells).

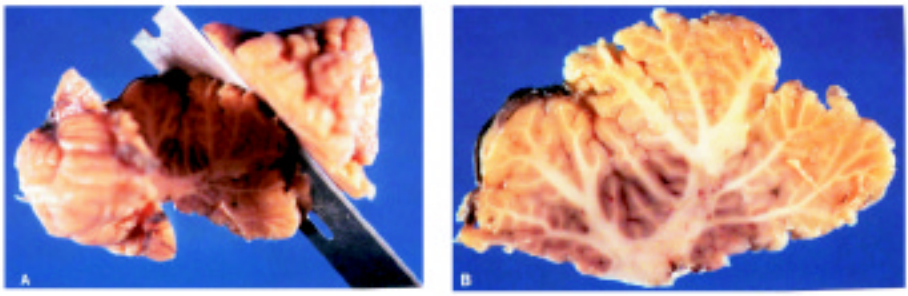

Figure 9. Additional sections. **A.** Cut through the cerebellar vermis. **B.** The section obtained which should be processed for histological examination.

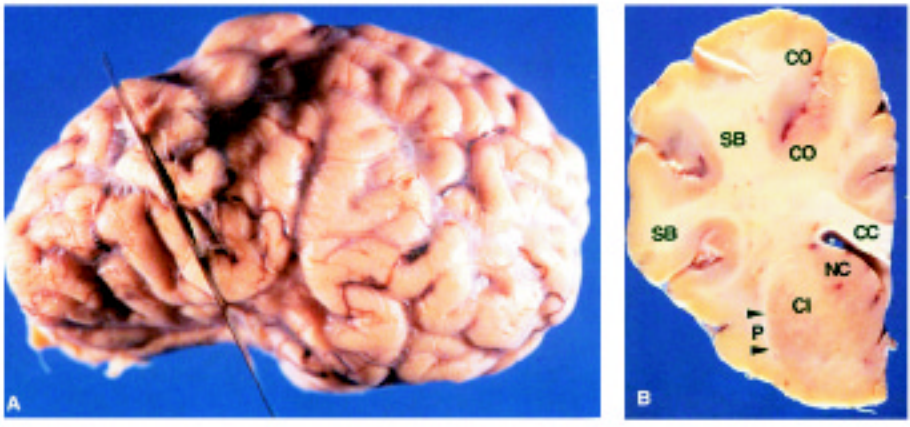

Figure 10. Additional sections. **A.** Coronal cerebral section through the frontal cortex rostral to the optic chiasm. **B.** The section obtained which should be processed for histological examination. If necessary, divide the fragment in two in order to fit in the cassette. Corpus callosum (CC), internal capsule (CI), caudate nucleus (NC), putamen (P), external capsule (arrowheads), subcortical white matter(SB), cortex (CO), lateral ventricle (asterisc).

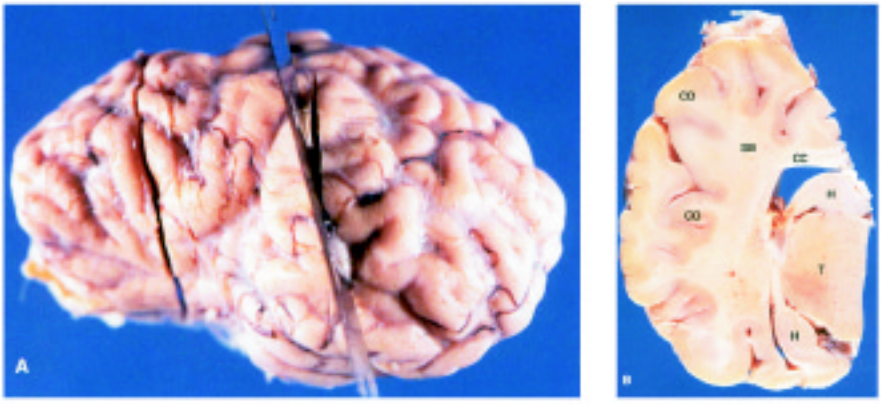

Figure 11. Additional sections. **A.** Coronal cerebral section through the parietal cortex at the level o mammillary bodies. **B.** The sectiopn obtained which should be processed for histological examination. If necessary, divide the fragment in two in order to fit in the cassette. Corpus callosum (cc), Hippocampus (h), thalamus (T), subcortical white matter (SB), cortex (CO).

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