THAI AGRICULTURAL STANDARD

TAS 10000-2006

DIAGNOSIS OF BOVINE SPONGIFORM ENCEPHALOPATHY IN CATTLE

National Bureau of Agricultural Commodity and Food Standards
Ministry of Agriculture and Cooperatives
UNOFFICIAL TRANSLATION

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National Bureau of Agricultural Commodity and Food Standards
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Thai Agricultural Standard (TAS) on Diagnosis of Bovine Spongiform Encephalopathy in Cattle was established for certifying the free status of Bovine Spongiform Encephalopathy in cattle and to be used as standard manual in diagnostic techniques in laboratory. The laboratory diagnostic techniques are including Pathological method, Immunohistochemical method. The BSE certification will promote production of cattle in terms of safety and quality for human consumption both domestically and internationally.

The establishment of this standard is based on the information of the following documents:


Remark:

The standard title has been revised from “Thai Agricultural Commodity and Food Standard (TACFS)” to “Thai Agricultural Standard (TAS)” in accordance with the enforcement of The Agricultural Standards Act B.E. 2551 (2008).
The resolution of the 1/2549 session of the National Committee on Agricultural Commodity and Food Standards dated 8 June B.E.2549 (2006), was approved on the establishment of the Thai Agricultural Commodity and Food Standard entitled Diagnosis of Bovine Spongiform Encephalopathy in Cattle. This standard would be of benefits for facilitating trade and protecting consumers.

By virtue of the Cabinet Resolution on Appointment and Authorization of the National committee on Agricultural Commodity and Food Standards dated 19 November B.E.2545 (2002), the Notification on Thai Agricultural Commodity and Food Standard entitled Good aquaculture practices for disease free marine shrimp hatchery is hereby issued as voluntary standard, the details of which are attach herewith.

Notified on 10 August B.E.2549 (2006)

Mr. Somsak Brisananuntakul

Minister of Agriculture and Cooperatives
Chairperson of the National Committee on Agriculture Commodity and Food Standards
THAI AGRICULTURAL STANDARD
DIAGNOSIS OF BOVINE SPONGIFORM ENCEPHALOPATHY
IN CATTLE

1 SCOPE

This Standard applied to provide for the essential details of laboratory diagnosis of bovine spongiform encephalopathy in cattle by histopathology and immunohistochemistry methods.

2 DEFINITIONS

For the purpose of this standard:

2.1 Cattle means Bos taurus and Bos indicus, both male or female, living individual or in herd and raising for the human consumption.
2.2 Mad cow disease or bovine spongiform encephalopathy or BSE means disease occurring in cattle which causes brain atrophy or porous brain.
2.3 Diagnosis means investigation of the etiology of illness or death caused by disease.
2.4 Prion protein means normal small protein particles (cellular PrP^c).
2.5 Prion protein scrapies (PrP^sc) means small mutated prion protein particles causing neurological signs in animal such as scrapies in sheep or mad cow disease in cattle.
2.6 Obex means the upper wall of the ventricles or brain stem.
2.7 Foramen magnum means the opening of the brain through cervical spinal cord.
2.8 Screening test or rapid test means the diagnostic methods which are convenient and rapid such as histopathology and serology etc.
2.9 Confirmation test means the diagnostic methods which can confirm the disease and is accepted for high specificity and sensitivity.
2.10 World Organisation for Animal Health or Office International des Epizooties (OIE) means the international organization established by the multinational agreement on January, 1924 for the collaboration of controlling the important animal diseases.

3 DIAGNOSIS

There are several laboratory methods for the diagnosis of mad cow disease such as histopathology, immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), Western blot analysis etc. These methods have different specificity and sensitivity. After getting the results from the laboratory, the veterinarians or experts shall consider clinical signs, epidemiology and policy on prohibiting the usage of meat and bone meal for animal feed (annex A) for efficient prevention and controlling the disease.

The suggestive diagnostic method for the diagnosis of mad cow disease is the immunohistochemistry method due to the high specificity and sensitivity of this test. For the disease screening purpose, the other methods that have lower sensitivity and specificity, such as histopathology and ELISA, can be used.
3.1 SPECIMEN COLLECTION
According to the research results, the infected cattle had PrP^sc accumulation in high level at the obex of brain stem. For this reason, obex is the target tissue for the diagnosis of mad cow disease. The right collection of specimen is the first crucial step for the successful diagnosis of mad cow disease.

The specimen collection method of the obex of brain stem through foramen magnum:

1) Remove the head from the body by cutting between the atlas vertebra and the occipital condyles of the skull.
2) Turn ventral surface of the head uppermost to locate foramen magnum.
3) Using spoon-shaped instrument with sharp edges that has approximately 18 cm of handle or index finger inserted through the foramen magnum between the dura mater and the ventral/dorsal aspect of the medulla and advanced rostrally, keeping the convexity of the bowl of the instrument applied to the bone of the skull and moving with a side-to-side rotational action. This severs the cranial nerve roots without damaging the brain stem.
4) Pass this instrument rostrally for approximately 12-15 cm in this way and then angled sharply toward the dorsal/ventral aspect of the brainstem to cut and separate the brainstem from the rest of the brain. The instrument is kept in the angled position and withdrawn from the skull to eject the brainstem through the foramen magnum. The brain stem is on cutting board (Annex B, Figure B1 and B2) and cut the brain stem as in Figure B2.

3.2 HISTOPATHOLOGICAL METHOD
The principle of this method is to evaluate disease at the tissue level by staining the preserved tissue using hematoxylin and eosin dyes and examining the tissue by light microscope.

3.2.1 Method
Process part of the brain and spinal cord from step 3.1 according to standard histopathological procedure for sectioning the tissue

1) Dehydrate tissue using 95% and 100% ethanol, respectively
2) Remove ethanol by xylene
3) Impregnate tissue by paraffin
4) Embed tissue in paraffin box
5) Sectioning tissue
6) Stain with hematoxylin and eosin
   6.1) Melt paraffin-sections at 60 °C for 30 min and place tissue sections in xylene
   6.2) Rehydrate tissue sections in 100% and 95% ethanol, respectively
   6.3) Wash tissue sections for five min using tap water
   6.4) Stain tissue sections with Mayer’s hematoxylin for 5-7 min
   6.5) Wash tissue sections using tap water for 15-30 s
   6.6) Stain tissue sections with eosin for 30-60 s
   6.7) Dehydrate tissue sections using 95% and 100% ethanol, respectively
   6.8) Remove ethanol by immerse tissue sections in xylene
   6.9) Cover tissue sections with coverslip
3.2.2 Results
When examined the specimen under the light microscope, the lesion was the vacuolation in the neuropil and neurons (Annex B, Figure B3 and B4). However, the vacuolation in the brain can occur due to several factors as follows:

A. Improper tissue conditions due to
   - Post-mortem changes and autolysis
   - Frozen tissue before fixing in proper fixative
   - Inadequate fixation both for time and volume
   - Too high temperature paraffin
   - Fixing brain sample in 70% alcohol more than 48 h

B. Pathological conditions due to
   - Genetic disease due to abnormal amino acid metabolism
   - Other abnormal metabolism such as hepatoencephalopathy
   - Toxic substances such as ammonium hexachloroprene

3.3 IMMUNOHISTOCHEMISTRY METHOD (IHC)

The principle of this method is to examine the disease at the tissue level by staining with specific antibody. Tissue sections are digested with proteinase K and are processed through immunohistochemistry protocol for the detection of PrP\textsuperscript{sc} with specific antibody against PrP. Finally, tissue sections were observed under the light microscope looking for the positive stained cells (Annex B, Figure B5). Immunohistochemistry technique is the gold standard technique, established by World Organisation for Animal Health (OIE), due to highly specificity and sensitivity, inexpensive, and convenient. However, this technique is time consuming comparing to other techniques.

3.3.1 Method

1) Tissue preparation
   1.1) Process 10% formalin-fixed obex for 48 h using standard histopathological procedure until getting paraffin block
   1.2) Cut paraffin block at three to four µm thickness and place on coated slide or positive charge slide
2) Deparaffinization and rehydration
   2.1) Incubate tissue sections at 60 °C for 30 min
   2.2) Immerse tissue sections in xylene three times for two min each
   2.3) Immerse tissue sections in 100% ethanol three times for two min each
   2.4) Immerse tissue sections in 95% ethanol three times for two min each
   2.5) Wash tissue sections using distilled water for five min
   2.6) Wash tissue sections using phosphate buffer saline (PBS) three times five min each
3) Digest PrP\textsuperscript{sc} using proteinase K at 37 °C for 15 min
4) Unmasking epitope with autoclave
   4.1) Put tissue section in the stainless box and pour boiling water into stainless box until cover the tissue sections and close the stainless box
   4.2) Autoclave stainless box at 121 °C, one kPa for 20 min
   4.3) Wash tissue sections with PBS three times for five min each
5) Quenching of endogenous peroxidase
   5.1) Incubate tissue sections with 0.9% H\textsubscript{2}O\textsubscript{2} in methanol at room temperature
   5.2) Wash tissue section with PBS three times for five min each
6) Block non-specific reaction using 5% normal swine serum at room temperature for 30 min

7) Apply primary antibody
   7.1) Place tissue sections in moisture chamber
   7.2) Incubate tissue sections with 1:800 rabbit IgG anti-PrP<sub>c</sub> antibody at 37 °C for four h or 1:1000 rabbit IgG anti-PrP<sub>c</sub> antibody at four °C for overnight
   7.3) Wash tissue sections with PBS three times for five min each

8) Apply secondary antibody
   8.1) Place tissue sections in moisture chamber
   8.2) Incubate Biotinylated anti-rabbit IgG antibody (bottle A) at room temperature for 10 min
   8.3) Wash tissue sections with PBS three times for five min each

9) Apply peroxidase conjugated streptavidin
   9.1) Place tissue sections in moisture chamber
   9.2) Incubate peroxidase conjugated streptavidin (Bottle B) at room temperature for 10 min
   9.3) Wash tissue sections with PBS three times for five min each
   9.4) Wash tissue sections with distilled water for two times

10) Coloration
    10.1) Place tissue sections in moisture chamber
    10.2) Incubate tissue sections with amino ethyl carbazol (AEC) (Bottle C) at room temperature for five min
    10.3) Wash tissue sections with distilled water for two times

11) Examine under light microscope
    11.1) Mount slide using two to three drops of glycergel and then cover tissue sections with coverslip
    11.2) Examine slide under light microscope and comparing with positive and negative control slides

3.3.2 Interpretation

It is necessary to compare the specific and nonspecific staining patterns between the tested tissue sections and positive and negative control.

Reminder for the positive staining
- If tissue section is positive for PrP<sup>c</sup>, PrP<sup>c</sup> will be stained with red color (Annex B, Figure 5).
- The positive PrP<sup>c</sup> staining will be stained in grey matter.
- The positive PrP<sup>c</sup> staining can be seen both focal and diffuse and the staining patterns can be longitudinal stained along the axon (linear form) or granular stained (granular form) around the neuron.

3.4 Diagnosis with diagnostic test kit

There are several rapid diagnostic methods for the diagnosis of mad cow disease such as Western blot analysis (Annex D), ELISA etc. At the moment, the commercial test kit is available, however the choices of test kit shall be approved by World Organisation for Animal Health (OIE) and if the result is suspected, the immunohistochemistry method shall be confirmed.
ANNEX A
EPIDEMIOLOGY, PATHOGENESIS AND CLINICAL SIGNS OF MAD COW DISEASE

A1 EPIDEMIOLOGY
Mad cow disease or so-called Brain Atrophy disease or Bovine spongiform encephalopathy (BSE) belongs to the group of transmissible spongiform encephalopathy (TSE). It is a highly virulent neurological infectious disease and a cause of death in cattle especially in adult. It is in the notifiable list of World Organisation for Animal Health (OIE). Mad cow disease is a zoonotic disease and related to variant Creutzfeldt-Jacob’s disease (vCJD) in human. Mad cow disease was firstly reported in United Kingdom in 1986. Later on, it has been reported in several countries in Europe, Canada, Japan, United State of America and Sweden. Thailand has several acts to prevent occurring of mad cow disease since 1990.

The etiology of mad cow disease is to feed cattle with PrP\textsuperscript{sc}-contaminated. PrP\textsuperscript{sc} is a small branch protein particles (Annex B, Figure B.6) with 12-16 nm in diameter and has molecular weight of 32 to 35 kDa. It is resistant to heat at cooking level, ultraviolet, frozen, pasteurization, sterilization and digestion with protease. However, it can be destroyed by heat at 133 °C and atmospheric pressure at three kPa (43.3 psi) for 18 min or 2N sodium hydroxide (NaOH) or Sodium hypochlorite (NaHClO\textsubscript{4}) for at least 1 h.

A2 PATHOGENESIS
When PrP\textsuperscript{sc} gets into the body of cattle, it attacks the neuron and induces conformational change of the PrP\textsuperscript{c} on cell membrane to PrP\textsuperscript{sc}. The newly change PrP\textsuperscript{sc} causes chain reaction of conformational changes of PrP\textsuperscript{c} to PrP\textsuperscript{sc} and finally the affected neuron is destroyed and spongiform is appear in neuropil. The incubation period of PrP\textsuperscript{sc} is approximately two to eight years. Thus the infected cattle usually show signs of infection at more than 24 months of age. However, there are some reports that the youngest infected cattle is 19 months and the oldest infected cattle is 15 years. The infected cattle usually die.

A3 CLINICAL SIGNS
Clinical signs of mad cow disease can be divided into two groups as follows
A3.1) typical signs group: Infected cattle show abnormal behavior such as aggression, kick, isolate from herd, hypersensitive to sound, light and contact, and abnormal gait (incoordination, weakness of hind limbs) etc.

A3.2) Atypical signs group: Infected cattle show loss of weight, thin and dead with unknown cause etc.

Presently, there is no cure and vaccine for this disease. Thus, the best way to control is to destroy infected cattle and destroyed contaminated or suspicious products. Thus, mad cow disease causes severe economic loss. Moreover, mad cow disease can affect the cattle trade both domestically and internationally. Mad cow disease becomes the important issue for the World Trade Organization (WTO). Presently, there is a limitation for the diagnosis of mad cow disease due to the need of the brain tissue which can be examined only after the animal die. There is no other accepted method for diagnosis of mad cow disease in live animal.
A4 SURVEILLANCE PROTOCOL

1990-2000 Diagnosis of mad cow disease by differential diagnosis using histopathology of submitted brain tissues from all over the country at every laboratory of the Department of Livestock Development.

2001-2002 National Institute of Animal Health, Department of Livestock Development and Bureau of Disease Control and Veterinary Services established the pilot project examining 515 specimens of both dairy and beef cattle older than 23 months form abattoirs for mad cow disease. Histopathology and immunohistochemistry specific for PrP<sup>sc</sup> were employed. The result of this pilot project was negative.

2003-2004 launched survey project with definitive target in Thailand by collection of specimen in each part of country using epidemiological principle. Target cattle were dairy cattle older than 23 months, beef cattle from farms using concentrate feed sending to abattoir, cattle having neurological signs, cattle immediately sent to abattoir and culling cattle. The sample size was indicated by OIE. The diagnostic methods were histopathology and immunohistochemistry. Project of 2003 has already done and the number of the survey specimens was 950. All of them were negative. Presently, this project is still continuing.

A5 PREVENTION PROTOCOL

The Department of Livestock Development has the protocol for the prevention of mad cow disease by issuing several ministerial regulations and notifications of Department of livestock development under Animal Epidemics Act 1956 as follows:

1990 reduction of imported live cattle, beef and related products from United Kingdom
1995 reduction of imported live cattle, beef and related products from United Kingdom and other BSE-reported countries
1996 prohibition of imported animal feed containing meat and bone meal that originated from United Kingdom
1997 prohibition of imported animal feed containing meat and bone meal for feeding ruminant
2000 prohibition of imported animal feed containing meat and bone meal that originated from the European Union and other BSE-reported countries
ANNEX B
FIGURES FOR THE DIAGNOSIS OF MAD COW DISEASE

Figure B1: Method of bringing obex out of the skull via foramen magnum (from OIE, 2004)

Figure B2: Illustration for sectioning of obex at position 1, 2 and 3 (from OIE, 2004)
Figure B3: Vacuolation in neuropil of the brain. (x40, Hematoxylin & Eosin, Courtesy by Dr. Jira Kongklong)

Figure B4: Vacuolation in neurons. (x100, Hematoxylin & Eosin, Courtesy by Assoc. Prof. Dr. Ajchariya Sailasuta)
Figure B5: PrP$^c$ in the brain section stained red color of ABC. (x40, immunohistochemistry, Courtesy by Dr. Jira Kongklong)

Figure B6: Electron micrograph of brain tissue illustrated SAF Tubulofilamentous particle of PrP$^c$ (from Stack et al., 1996).
ANNEX C
MATERIALS AND PREPARATION OF SOLUTION USING IN IMMUNOHISTOCHEMISTRY METHOD

C.1 MATERIALS AND CHEMICALS

C1.1 Moisture chamber
C1.2 Chemicals
- Xylene
- 95% ethanol
- Absolute ethanol
- Hydrogen peroxide
- Methanol
- Proteinase K
- Phosphate buffer saline (PBS)
- 1 M tris/HCl, pH 8.0
- 0.1 M CaCl$_2$
- 5% normal swine serum
- Polyclonal rabbit anti-bovine PrP primary antiserum (Neuro-center, Berne, Switzerland)
- Glycergel
C1.3 Coated slide or positive charge slide and cover glass
C1.4 Dako kit ChemMate™ detection kit peroxidase/AEC composed of
- Bottle A biotinylated anti-rabbit IgG secondary antiserum
- Bottle B peroxidase conjugated streptavidin
- Bottle C substrate (amino ethyl carbozol, AEC)
C1.5 Water bath
C1.6 Autoclave
C1.7 Light microscope

C.2 PREPARATION OF SOLUTION FOR IMMUNOHISTOCHEMISTRY METHOD

C2.1 Proteinase K (150 mg)
1 M tris/HCl pH8.0 7.5 ml
0.1 M CaCl$_2$ 2.25 ml
Proteinase K (14.7mg/ml Roche 1373196 51.0 µl
Distilled water 140.2 ml

Method
1) Pre-warmed water bath at 37 °C
2) Mixed tris/HCl, CaCl2 and distilled in coffin jar
3) Warm coffin jar at 37 °C
4) Added proteinase K into coffin jar and incubated for 15 min

C2.2 0.9% H$_2$O$_2$ in methanol
Methanol 150 ml
H$_2$O$_2$ (30%) 4.5 ml

C2.3 normal swine serum 5%
PBS 4 ml
Normal swine serum 200 µl

C2.4 polyclonal rabbit anti-bovine PrP primary antiserum
Dilution 1:800
PBS 50 ml
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<td>Primary antiserum</td>
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ANNEX D
WESTERN BLOT TECHNIQUE

WESTERN BLOT TECHNIQUE*

D1. METHOD

1) Weigh fresh obex for approximately 500 mg (450-700 mg)
2) Add homogenized buffer at the ratio of 1:10 (500 mg of brain : five ml of homogenized buffer)
3) Homogenize obex with homogenizer and pipette 1 ml of homogenate into master plate and keep at -20 °C until use
4) Add 10 µl of digestion buffer and 100 µl of tissue homogenate into each well of 96-well plate
5) Add 10 µl of proteinase K into each well, mix by pipetting and heat at 48 °C for 40 min
6) Add 10 µl of digestion stop reagent into each well of digestive plate
7) Add 100 µl of PAGE sample buffer, mix by pipetting up and down and heat at 96 °C for five min
8) Assembling gel electrophoresis insert 12% NuPAGE® gel into electrophoresis set
9) Add SDS-MOPS running buffer at cover the lower rim of the gel
10) Add 10 µl of control sample in lane 1 and 10 µl of samples (from step 7) into the next well (2 wells for each sample)
11) Add SDS-MOPS running buffer in both outer chamber and inner chamber and add 500 µl of antioxidant into inner chamber
12) Turn on the power supply at 200 V for 30 min until the purple band above the lower rim of the gel for one to two cm
13) Incubate PVDF membrane in methanol for two to three sec and follow by incubating in transfer buffer for 10 min

*Reference on Prionics® – Check Western of Prionic Company in which the test kit is certified by the OIE

14) Place PVDF membrane on filter paper that lie over the sponge of the cassette frame of transfer unit
15) Cut off stacking gel and the lower part of gel under the purple band and place gel on the membrane in step 14
16) Bring gel (step 12) lie over the sponge of the cassette frame
17) Insert cassette frame into transfer unit by setting gel on the cathode site (black color wire). Protein is moving from cathode site to anode site
18) Add cold transfer buffer (4 °C) into transfer unit until cover the cassette frame
19) Turn on power supply using 150 V for 1 h at 4 °C
20) Separate membrane from the cassette frame, place membrane on the staining unit, mark the control lane and stain membrane for two min on shaker using 1 X Ponceau S
21) Wash membrane by Tris buffered saline mixing with Tween 20 (TBST) until no stain
22) Incubate membrane with 50 ml of PVDF blocking buffer for 30 min at room temperature on shaker
23) Incubate membrane with 50 ml of TBST containing 10 µl of monoclonal antibody 6H4 (1:5000) at room temperature for 1 h
24) Wash membrane with TBST three times for five min each
25) Incubate membrane with 50 ml of TBST containing 10 µl of goat anti-mouse conjugated alkaline phosphatase at room temperature for 30 min
26) Wash membrane with TBST three times for five min each
27) Incubate membrane with luminescence buffer for five min
28) Place membrane on a piece of glass, pour CDP-Star® onto the membrane and wrap membrane with Saran wrap
29) Expose ECL hyperfilm with membrane in the film cassette for 5 to 20 min, develop film and see the result

**D2. INTERPRETATION**
Positive specimen shall be shown on the following results
   - A band of PrP^{sc} after digested with proteinase K
   - Molecular weight of the protease-resistant PrP^{sc} shall decrease to 27 to 30 kDa
   - Found glycosylation pattern of PrP^{sc}
ANNEX E
UNIT

The units and symbols used in this Standard and the units of SI (International System of units or *Le Système International d’Unités*) recognized to be used are as follows.

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