

## Variation of mass (volume) taken with the calibrated syringe and of the results provided by the Bio-Rad Platelia™ BSE test upon storage of brainstem samples at –20 °C

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**Abstract** — The real mass obtained by sampling with the calibrated syringe provided by the supplier, and the degradation through cold storage was evaluated in view of the measured optical density of suspected bovine BSE brainstem material in the quick Platelia™ test from Bio-Rad. For this, 50 negative BSE cases and 2 positive BSE cases were tested. The brainstems were stored at –20 °C and the weight of each sample was taken with a calibrated syringe at an interval of 1 week. Moreover, the final optical density obtained with the quick Platelia™ ELISA test of each case was evaluated. This was done three times for each case (brainstem), including the primary sampling of the fresh brainstem tissue taken immediately after slaughter. The results indicate that storage at –20 °C gives a significant decrease in weight of the mass taken with the calibrated syringe ( $P = 0.0121$ ) and in optical density ( $P < 0.001$ ) between the fresh and 1 week old sample for the same volume. This decrease did not seem to continue significantly after this first week of storage. Furthermore, no significant correlation could be noted between the decrease in the weight of the mass (volume) taken between the fresh sample and the sample taken after 1-week storage and the decrease in optical density of the respective negative samples. Our results therefore suggest that there is a significant decrease in optical density after 1-week cold storage, but that this could not be associated with the concurrent decrease in weight of the mass (volume) taken by the calibrated syringe. However, this decrease in optical density did not influence the final diagnosis of BSE, using the ELISA (Platelia™) test. Additionally, this study also implicated the reliability of the calibrated syringe provided in the test kit.

**mass / calibrated syringe / cold storage / BSE / Platelia™**

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**Résumé — Utilisation du test rapide Platelia™ (Bio-Rad) pour la détection de l'encéphalopathie spongiforme bovine : influence de la masse (volume) de l'échantillon cérébral et du stockage à -20 °C.** La masse réelle d'échantillon cérébral prélevé avec la seringue calibrée fournie par le distributeur du test rapide Platelia™ de Bio-Rad et la dégradation du tissu après congélation ont été évaluées en vue de déterminer leur influence sur la densité optique des échantillons soumis à un test de diagnostic rapide d'encéphalopathie spongiforme bovine (ESB). Au total, 50 échantillons négatifs à l'ESB, et deux échantillons positifs ont été testés. Le poids de chaque échantillon prélevé avec la seringue calibrée ainsi que la densité optique finale obtenue avec le test rapide Bio-Rad ont été déterminés à l'abattage, puis 1 et 2 semaines plus tard après stockage des tronc cérébraux à -20 °C. Pour chaque prélèvement, les mesures ont été répétées trois fois. Les résultats indiquent que le stockage des tronc cérébraux à -20 °C a réduit significativement le poids de tissu cérébral prélevé ( $P = 0,0121$ ) ainsi que sa valeur de densité optique ( $P < 0,001$ ), à volume égal. Cette diminution s'observe lors la première semaine de stockage, et ne paraît pas se prolonger la semaine suivante. Enfin, il n'apparaît aucune corrélation significative entre la diminution du poids de tissu cérébral prélevé (volume) et la réduction des densités optiques des différents échantillons négatifs. Ces résultats suggèrent que bien qu'il y ait eu une diminution significative de la densité optique après 1 semaine de stockage à -20 °C, cette diminution n'est pas associée à une réduction simultanée et significative du poids de l'échantillon prélevé à l'aide de la seringue calibrée. De plus, la diminution des valeurs de densité optique n'a pas eu d'influence sur le diagnostic final de l'ESB lors de l'utilisation du test ELISA (Platelia™). Notre étude prouve la fiabilité de la seringue de calibration fournie dans le kit Bio-Rad.

**masse / seringue calibrée / stockage à -20 °C / ESB / Platelia™**

## 1. INTRODUCTION

Bovine spongiform encephalopathy (BSE) is a fatal, progressive, transmissible neurodegenerative disease of adult cattle. Since its first detection in the United Kingdom in 1986 [11], numerous countries have found the disease in their own cattle population.

The transmissibility of the agent of BSE to man causing the variant Creutzfeldt-Jakob disease, is now generally accepted but the extent of a possible future epidemic in man cannot be defined at present.

The EU has developed a method to assess the geographical BSE risk (GBR), defining a GBR level, which is based on the qualitative analysis of (1) the likelihood that the BSE agent was introduced into a country and if so, when and to what extent; (2) the potential of it being recycled or eliminated; (3) the likelihood that today one or more cattle could be infected. All EU countries are situated between classes II and IV, with class I being the lowest, meaning that it is considered highly unlikely that

one or more cattle are clinically or pre-clinically infected [10]. However, a number of EU countries did not detect any cases of BSE before 01/01/2001 despite their rather high GBR level. Therefore, and also to protect consumer health, the EC has imposed the application of rapid BSE tests on all cattle when slaughtered animals and animals found dead on the farm ("fallen stock"), are sent to the rendering plants.

For this purpose, the European Commission evaluated 4 tests in 2000 [7]. The results of this survey indicate that the Prionics immunoblotting, the Enfer chemiluminescent ELISA and the CEA sandwich ELISA have excellent potential for detecting or confirming clinical BSE for diagnostic purposes or for screening dead or slaughtered animals for such cases, particularly casualty animals or carcasses sent for rendering. Since January 1st 2001, Belgium has selected the CEA test, which is commercialised by Bio-Rad, to be applied as its rapid routine test [2]. This test has proven to be the most sensitive of the three [7, 8] being capable of detecting previously undiagnosed

BSE – affected animals which could consequently be eliminated from the food chain. Moreover it allows tighter epidemiological monitoring of the BSE epidemic [3]. Recently, the efficacy of the Bio-Rad test was evaluated in order to detect PrP<sup>res</sup> in central nervous system (CNS) tissue for the post-mortem diagnosis of BSE at various times throughout the disease course [4]. This study indicates that by using the Bio-Rad test, animals in the late stages of incubation or pre-clinical stages could also be detected, namely up to 3 months before the appearance of the clinical signs.

The principle of this quick test, or Platelia™ Bio-Rad test is based on the detection of PrP<sup>res</sup> and its aggregates (Scrapie Associated Fibrils) in the brain of BSE infected animals, based on a process that can be divided in a selective patented purification step followed by “sandwich” ELISA, using two different monoclonal antibodies [2].

Before starting the purification step, a sample of the obex region must be taken using a calibrated syringe, provided by the manufacturer and included in the test kit, resulting in samples of  $350 \text{ mg} \pm 40 \text{ mg}$  for a standardised volume. This quantity is necessary to guaranty an optimal reaction of the prepared proteinase K that must be added in a further step, since the quantity of prepared proteinase K is calculated for this precise quantity of brainstem material [1]. When, however, this precaution is not met, some PrP<sup>c</sup> or normal PrP that must be destroyed by the proteinase K will remain and give rise to a false increase of the measured optical density. This could result in false positive BSE cases.

Pilot studies performed in our lab have proven that this guarantee is not always given by using the calibrated syringe. Our impression was that sometimes the freshness could influence the mass of the brainstem material taken by the calibrated syringe.

In order to obtain additional validation data for this test, we started this study in order to find out if there was really a significant difference in brainstem weight taken by using the calibrated syringe and, if so, if it influenced the final optical densities significantly. Additionally, we tried to find out if this difference could be due to a transformation of the tissue during cold storage, and if so, how this degradation evolved during time.

## 2. MATERIALS AND METHODS

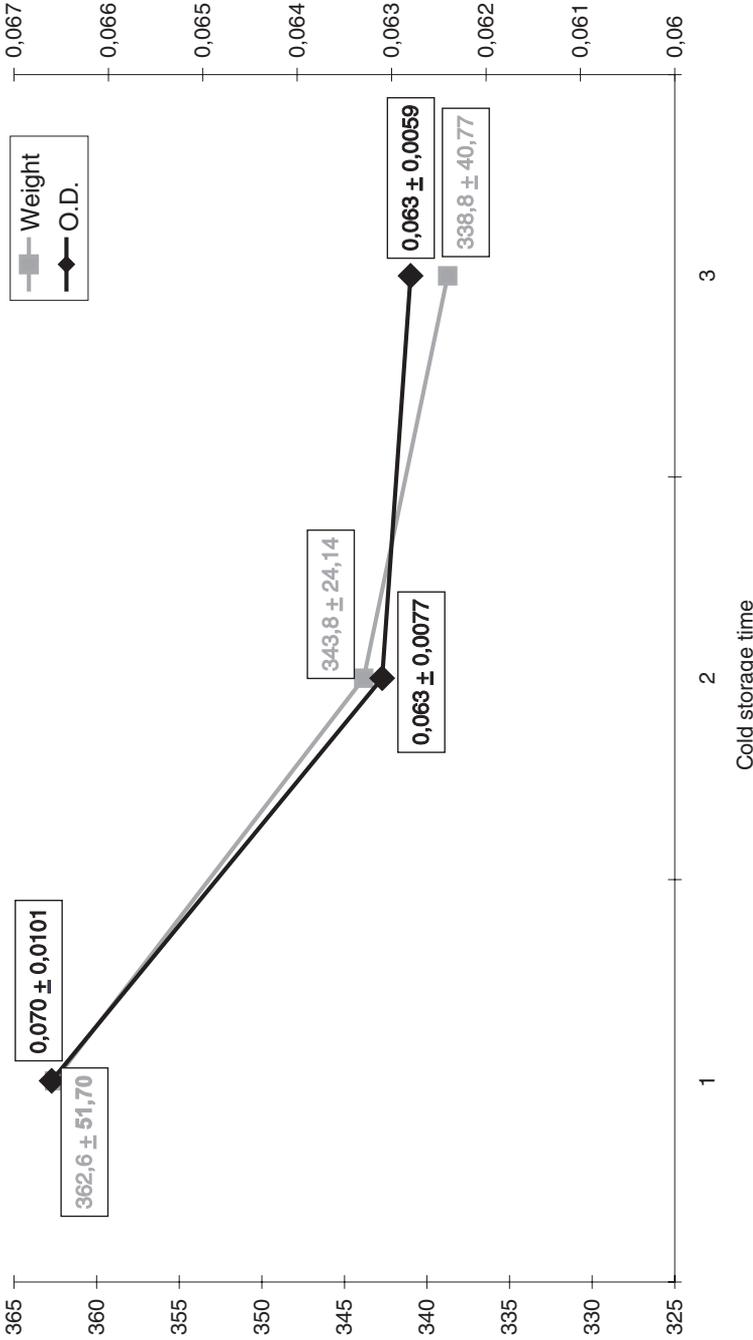
### 2.1. Materials and sampling

We selected 50 samples of brainstem material of bovines originating from screenings of positive BSE farms. These consisted of whole brainstems originating from freshly culled animals that were sent in directly after sampling. Primarily, the ELISA test was done on a fresh sample from the obex region using the calibrated syringe provided by the manufacturer (Bio-Rad). Subsequently, the sample was put in the freezer at a temperature of  $-20 \text{ }^{\circ}\text{C}$  and this during a week. After that week, the brainstem tissue was sampled again and a second ELISA test was performed. This protocol was repeated again after another week in the same freezer.

After each sampling of the standardised volume with the calibrated syringe, the weight of the sample used for the ELISA test was measured using a calibrated analytical balance (AB 204, Mettler Toledo, Switzerland).

As the positive control, two fresh brainstem samples of positive BSE cases originating from the active epidemiological surveillance slaughterhouse were also included.

As confirmatory tests, histopathology, immunohistochemistry and a transmission electronmicroscopical control in the presence of scrapie associated fibrils (SAFs)



**Figure 1.** The evolution in the mean weight (mg) of the mass (volume) taken by the calibrated syringe and mean optical density ( $\pm$  standard deviation) of the 50 negative BSE cases.  
Axis X: 1 = fresh samples / 2: after one week cold storage ( $-20\text{ }^{\circ}\text{C}$ ) / 3: after two weeks cold storage ( $-20\text{ }^{\circ}\text{C}$ ).  
The mean weight evolution varied from 362.55 mg  $\pm$  7.31 (Standard error of the mean) for the fresh sample, over 343.83 mg  $\pm$  3.41 (SEM) after 1 week cold storage, to 338.75 mg  $\pm$  5.77 (SEM) after two weeks cold storage.  
The mean optical density evolution varied from 0.0695  $\pm$  0.0143 (SEM) for fresh samples, over 0.0631  $\pm$  0.0110 after 1-week cold storage, to 0.0628  $\pm$  0.00840 (SEM) after two weeks storage.

were used on all samples, including the positive controls [10].

## 2.2. Statistical evaluation

The measured masses taken from each case at the time of sampling as well as the optical densities provided by each sample were compared and evaluated using STATISTIX software (Version 1.0).

One-way ANOVA analysis was used to compare the different measured masses and optical densities. The Pearson correlation test was used to evaluate the correlation between the masses and optical densities. Statistical significance was fixed at  $P = 0.05$ .

## 3. RESULTS

The optical density of the positive samples remained above the threshold of 4 for each of the three ELISA tests performed. All samples were negative for BSE except for the positive controls, which remained strongly positive during all three ELISA tests. The confirmatory tests proved to be negative for all 50 negatives included. The two positives were confirmed by immunohistochemistry and the presence of SAF.

One-way ANOVA revealed a significant decrease between the weight of the masses (volumes) taken using the calibrated syringe on a fresh sample and a sample taken from the same case after a 1-week storage at  $-20\text{ }^{\circ}\text{C}$  ( $P = 0.0121$ ). There was also a significant decrease comparing the average weight of the mass (volume) of the fresh samples and the samples after two weeks of storage at  $-20\text{ }^{\circ}\text{C}$  ( $P = 0.0243$ ). However, no significant decrease could be noted between the average weight of the mass (volume) of the samples taken after one week and the sample taken after two weeks of cold storage (Fig. 1). A similar significant decrease could be noted in the average of optical densities. In fact, the decrease was

significant between the fresh and first week ( $P < 0.001$ ) and the fresh and second week storage ( $P < 0.001$ ) for the same volume. There was no significant difference between the optical densities obtained after one or two week storage at  $-20\text{ }^{\circ}\text{C}$  (Fig. 1).

Using the Pearson correlation test, no significant correlation could be noted between the decrease in weight of the mass (volume) of fresh and 1 week stored samples and the decrease of the respective optical densities.

## 4. DISCUSSION

For routine diagnosis in countries with a high incidence of BSE, histopathological examination confined to the medulla oblongata is the laboratory investigational method used to handle the large number of suspect cases. A demonstration of typical changes provides a definitive diagnosis. When the result of the histopathological examination is inconclusive or negative or the brain material taken post-mortem is unsuitable for the histological examination because of autolysis or damage, it is important that the disease-specific diagnostic criterion of the detection of abnormal PrP accumulation be applied. Immunohistochemistry to detect PrP<sup>res</sup> accumulation in formalin-fixed, paraffin-embedded material can be used in conjunction with or even as an alternative to, the histopathological evaluation of the medulla sections. Another method includes the detection of PrP<sup>res</sup> by electrophoresis separation and immunoblotting techniques (Western Blot) carried out on fresh (unfixed) or frozen brain or spinal cord material. Automated Western blot and enzyme-linked immunosorbent assay (ELISA) techniques have been developed allowing screening of large numbers of brain samples. Such techniques are easy and rapid to perform and are potentially more sensitive than the histopathological evaluation [9].

During the European evaluation [8] of four rapid BSE detection tests, it was proven that three of them could be used with a sensitivity and specificity of 100% using material of clinical BSE cases as reference.

However, the report also mentions that the sandwich ELISA of Bio-Rad (Platelia™) proved to be the one that could still detect BSE at a dilution level with a titre of  $10^{-2.5}$ , and this in contrast to the two others which were limited till  $10^{-1.5}$  (Enfer) and  $10^{-1}$  (Prionics). Based on these data, we could conclude that the sandwich ELISA is the most sensitive of the three, including a chemiluminescent ELISA (Enfer) and an immunoblotting (Prionics).

Nevertheless, despite its simplicity and convenience, in practice ELISA can be fraught with difficulties. A common problem in ELISA is non-specific, or specific, undesirable binding of different serum or other proteins. This can be reduced by the addition of proteins such as bovine serum albumin (BSA), animal sera with or without Tween 20, or casein hydrolysate to buffers but unfortunately the choice of additives is not universally suitable for all assays. Another problem is the specific interactions due to cross-reactivity or low levels of natural antibodies present in the reagents used. Another impact is the temperature variation, which can be minimised by incubating in a controlled environment, avoiding stacking of a large number of plates, placing on heated blocks and selecting incubation times which are sufficient in duration to allow the content in the wells to gain the appropriate temperature. Proper control and storage of laboratory reagents makes it possible to develop robust ELISA tests giving good reproducibility and precision [5, 6].

The specific ELISA technique for the rapid detection of BSE provided by the manufacturer includes a calibrated syringe in order to guaranty a correct standardised volume sampling of the brainstem, which

may vary in weight between 310 mg and 390 mg. This specific volume guaranties the correct correlation with the reagents, which are used in the process of purification, more precisely the correct ratio between the tissue and the prepared proteinase K. This provides an optimal reaction of the proteinase K so that the normal PrP present can be destroyed without interfering with the abnormal PrP, namely PrP<sup>Pres</sup>. So the reliability of the sampling using this syringe is very important for the final evaluation. Therefore we controlled the exact samples taken by using the calibrated syringe and this in order to find out if this can influence the optical density. Additionally, we also controlled the evolution of this mass (volume) in view of cold storage at  $-20\text{ }^{\circ}\text{C}$  and its influence on the final optical density. This last control was done because little is known concerning the concentration of PrP<sup>Pres</sup> in older material stored in the freezer.

Our data indicate that there is a decrease in the weight of the mass (volume) taken by the calibrated syringe when stored for 1 week at  $-20\text{ }^{\circ}\text{C}$ . This decrease was also noted for the optical densities, but the decrease in weight of the mass (volume) was not correlated with the decrease in optical density. When the same was done after two weeks storage in the freezer, both the mass and optical density decrease was still there when compared to the fresh sampling, but not when compared to the sampling after 1-week storage. This suggested that after 1-week storage at  $-20\text{ }^{\circ}\text{C}$ , a lower plateau is reached which remains afterwards.

The absence of a correlation between the decrease of the weight of the mass (volume) taken by the calibrated syringe and the optical density suggests that the optical density decrease is linked to processes independent of the sampled mass. This could give rise to some controversy when one looks at the very comparable evolution of the two parameters as a group (see Fig. 1). However, when one compares every sample to itself,

this absence of correlation can be explained by comparing several samples having a lower volume associated with a higher optical density at all three intervals. Nevertheless, these effects do not influence the final BSE diagnosis by using the Platelia™ BSE test on negative and positive samples, proving the stability of the test and reliability of the calibrated syringe involved. In the future, studies are needed that implicate an important number of bovine BSE samples with optical densities at the cut-off values, in order to evaluate if in such samples, the same statement can be formulated.

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