

BSE RISK IN CANADA, PART 2: CURRENT METHODS OF TESTING FOR BOVINE SPONGIFORM ENCEPHALOPATHY (BSE)

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A Web-Essay by

Michael Tyshenko

Ph.D. Candidate, Department of Biology, Queen's University, and
Postdoctoral Fellow,

Research Chair Program in Risk Communication & Public Policy

Haskayne School of Business, University of Calgary and

McLaughlin Centre for Population Health Risk Assessment, University of Ottawa

tyshenko@biology.queensu.ca

Telephone (613) 562-5800, x2116

There are widespread calls for increased surveillance to more accurately detect the incidence of bovine spongiform encephalopathy (BSE) in North American cattle, following the discovery of two cases of BSE in 2003. The need for improved surveillance and testing to determine the prevalence of BSE arises from a number of factors, including the long latent incubation times for this disease (4-6 years) and the fact that the exact world-wide distribution of BSE in national cattle herds cannot yet be established with sufficient certainty (http://europa.eu.int/comm/food/fs/bse/bse21_en.html; Brown et al. 2001). In this context there is some debate about how much, and what kind, of BSE testing in Canada is appropriate or necessary. This paper is intended to contribute to that debate by giving an overview of the currently-available testing methods.

Prions that cause BSE in cattle and nvCJD in humans are transmitted through infected food products. Research has shown that after ingestion prions are absorbed in the intestine and can replicate in the lymphoid tissue. Later, prions invade nerve tissue,

migrating at the rate of about 1 mm per day, finally reaching the spinal cord and brain where lesions appear (http://www.alpha-technologies.info/featured_products.htm). While the incubation period of the disease is quite long, the onset of symptoms leads to extreme neurological debilitation within a few months.

All animals possess normal prion proteins, which are known by a variety of acronyms in the scientific literature, e.g., PrP, PrP^C or PrP^{Sc}. PrP^C are found on the surface of many cell types, including nerves, lymphocytes, and macrophages. All proteins “fold” themselves into specific three-dimensional shapes. Sometimes normal prion proteins become abnormally folded. Then the abnormal prions can “infect” normal ones by physical contact, in which the abnormally-folded prion causes the normal one also to become misfolded, thus spreading the diseased state.

Since nervous tissue does not renew itself rapidly or continuously, infected animals possess increasingly higher levels of the abnormal prion protein (abbreviated PrP^{Sc} or PrP^{Res}), which are localized to the animal’s brain stem and central nervous system (<http://www.gnsbio.com/prion/prionproteins.html>). The presence of the abnormal protein form can be identified in post-mortem bovine tissue samples by sampling through the occipital foramen – the opening at the back of the skull through which the spinal cord passes (http://www.bah.state.mn.us/diseases/cwd/brain_removal.htm).

At present, there is no test available capable of detecting BSE in living animals. The ability to detect bovine spongiform encephalopathy pathogens in post-mortem samples requires the identification of the abnormal prion form in a sample of tissue. Since the first description of BSE, the detection and diagnosis of this neurodegenerative disease has evolved, with several techniques now available. Screening of the sample uses techniques that fall into two categories, either *non-immunochemical* or *immunochemical* methods. Non-

immunochemical methods include visual monitoring prior to slaughter and post-mortem histology. Immunochemical methods include immunohistochemistry (IHC), mouse bioassay, western blotting, ELISA double-sandwich immunoassay, and conformation-dependent immunoassay (CDI). Each of these will be discussed briefly below.

Non-immunochemical methods:

Visual inspection.

Cattle too sick to walk or other suspect cattle with gross motor dysfunction that are shipped to slaughterhouses are known as “downers”. Within this category inspectors look for classic signs of BSE, such as staggering, rolling of eyes, and vibrating head. Cows suspected of BSE are segregated and the head is sent for post-mortem testing.

The downer cows that do not show overt signs of BSE, while usually rejected for human consumption in Canada, are rendered into feed for chickens pigs, and horses, as well as cats and dogs, all of which are species not thought to be susceptible to BSE. Due to the long incubation and rapid onset of symptoms for BSE, some argue that visual inspection is not a reliable method for monitoring BSE infection of cattle intended for human consumption (<http://www.organicconsumers.org/madcow/step53003.cfm>).

Histology.

BSE was first reported in England in 1986, with the diagnosis initially made using histology. Using a light microscope, brain tissue sections are stained with dye and visually examined for the appearance of a number of small holes within specific areas of the brainstem (pathological vacuolation) indicative of BSE infection (Wells et al. 1987). Infected brain samples were also homogenized and visually inspected for diagnostic scrapie-associated fibrils (SAFs) using a much more powerful electron microscope (Merz et al. 1981; Scott et al. 1990). Histology is useful to screen for a broad spectrum of different

neurological diseases and can establish the presence of tissue abnormalities associated with BSE, but it cannot distinguish between other diseases that may cause similar brain degeneration (Debeer et al. 2001).

Immunochemical methods:

All immunochemical methods rely on the use of antibodies made against purified prion protein. To make antibodies a host animal (usually rabbit, mouse or donkey) is injected under the skin or into muscles with a large amount of purified prion protein and re-injected several more times to ensure the production of high amounts of antibody. The host immune system recognizes small regions of the foreign protein (epitopes) and mounts an immune response, making antibodies that will bind the non-host protein. The antibodies from the blood serum are purified from the host animal and used for detecting BSE by their ability to bind specifically to prion proteins when used in various diagnostic tests.

Once a stock of antibody is prepared from a host it can be stored for use in testing as needed. A single host usually provides enough antibody for testing several thousand tissue samples. Antibodies prepared in this way are said to be polyclonal due to their ability to recognize multiple sites on the target protein. Monoclonal antibodies recognize and bind to only a single site on the target protein and are made by creating hybridomas (mouse spleen cells fused with myeloma cells) from which the IgG secreting positive cells are selected (<http://www.absea-antibody.com/faq.htm>).

The prion testing systems that rely on a so-called “double-sandwich” work by making two different antibodies for PrPc, as described above, that recognize and bind to different regions of the prion protein. A variation on the double sandwich is to use one of the anti-prion antibodies as the primary antibody, and a secondary antibody that is made

in a different host against the first host's immunoglobulin chain (IgG). For example, if a rabbit was used to make anti-PrPc primary antibodies, the secondary antibodies would be made in mouse and would be a "mouse-anti-rabbit IgG secondary antibody." One of the antibodies (usually the secondary) is bound to a reporter that can give off colour, light (luminescence) or fluorescence that can be detected, depending on which detection system is chosen.

Immunohistochemistry (IHC).

A significant breakthrough for BSE diagnostics was the development of specific antibodies directed against the resistant prion protein (Farquhar et al. 1989; Demart et al. 1999). Internationally, the "gold standard" for confirming BSE from suspected samples is the presence of PrPres detected by immunohistochemistry (IHC). There are several steps involved in this technique but they can be summarized into three main groups: tissue collection; tissue preparation and blocking nonspecific sites with subsequent antibody binding; and visualization with dye.

First, brain tissue is collected and a small piece is fixed with a preservative chemical (formalin), embedded into paraffin wax for stability and sliced into very thin layers. The paper-thin layers of tissue are then mounted onto glass microscope slides and prepared for antibody detection. Antibodies made to PrPres are allowed to bind to the tissue on the slide, which is then stained with dyes to locate the antibody. Since immunohistochemistry involves a specific antigen-antibody reaction, it has an advantage over traditional histology staining techniques that identify only a limited number of proteins, enzymes and tissue structures. The location of positive staining to PrPres protein by the antibody, in the appropriate regions of the brain (e.g., the obex), confirms the presence and diagnosis of BSE infection.

Mouse bioassay.

Animal bioassays have been used in BSE research for diagnostic testing but such bioassays have severe disadvantages, including the length of time required to obtain results and the cross-species barrier effect. Despite the disadvantages animal bioassays remain the only “direct” method to measure the infectious agent and are the most sensitive assay available for the detection of infectious prions. Initially, sheep and goat animal bioassays were first used for BSE research, but bioassays improved dramatically with the development of rodent-adapted scrapie strains (mice and hamsters). Research tested the sensitivity of wild-type mice to BSE by comparing them to cattle. Serial dilutions of infected brain from cattle were used to infect both cattle and mice by direct brain injection. Mice appear to be around 500 times less sensitive to BSE than cattle.

The detection of prions by bioassay was greatly improved by genetic engineering, through the creation of PrP-deficient (“knockout”), mutant, and transgenic mice that are much more sensitive strains. Transgenic mice expressing high levels of heterologous PrP^c (human or bovine) on an otherwise PrP-null background are a useful diagnostic tool for the detection of prions. Transgenic mice expressing high levels of bovine PrP^c are about 10 times more sensitive than cattle and nearly 1000 times more sensitive than are normal wild-type mice to infection with BSE prions. While sensitive for detecting BSE, the mouse bioassay system is highly impractical for food safety diagnostics, since long incubation times (200-400 days) are required to obtain results (Kubler et al. 2003).

Western blotting.

The brain tissue to be tested by western blotting is first homogenized and then incubated with a non-specific, protein-degrading enzyme called proteinase K. When digested with proteinase K enzyme, PrP^c is completely digested while PrP^{res} has only the first 67 amino acids removed, yielding a smaller intact fragment that still retains its

infectivity. It has been established that PrPres accumulates in the brain during infection and attains concentrations of 10–100 times greater than PrPc. The remaining undigested resistant prion proteins are denatured by the addition of a strong, negatively-charged soap molecule (SDS). The soap molecules cover the surface of the proteins, unfolding them and allowing the proteins to be separated according to their size (molecular weight) on a polyacrylamide gel.

After size separation, proteins are transferred out of the gel using an electric current onto a positively charged solid support, usually a piece of nylon membrane. After transfer the membrane has all non-specific binding sites blocked, and PrPc antibody (primary antibody) is incubated with the membrane. The primary antibody binds only where PrPc protein is located. A second antibody made to the first host's immunoglobulin (a part of the antibody protein) is added and it, in turn, only binds to the first antibody.

The secondary antibody is usually conjugated or bound to some type of dye or chemical that when reacted give off either a dark colour or light particles, depending on which type of detection system is used. In this way positive PrPres reactions can be detected directly on the membrane (chemical detection) or by exposure to x-ray film to detect light emissions (chemiluminescence). The positive reaction for PrPres gives an intense band of colour on the membrane where all of the PrPres protein has migrated to the same spot, or alternatively a dark exposed band on film which is easily observed by eye after developing. Western blotting has the advantage of being sensitive with the ability to detect small amounts of protein in the pico- to fempto-gram range (1×10^{-12} to 1×10^{-15} g), or a trillionth to a quadrillionth of a gram. The western blot approach, however, is a low-throughput technique, with a single membrane blot able to test only about a dozen samples. The minimum time to complete the test is seven to eight hours.

ELISA immunoassay technique.

The enzyme-linked immunoadsorbent assay (ELISA) measures macromolecules such as antigens and antibodies and is used for diagnosing the presence of infectious disease and immunoglobulins. The technique works on an immunochemical strategy similar to western blotting, but is carried out in plastic well-plates rather than nylon membranes. This allows for many samples to be tested, since multiple plates can be processed at the same time (allowing hundreds of samples to be tested per day by a single technician). For BSE testing the ELISA double sandwich technique is used.

The sandwich or double antibody technique begins with a prion-specific antibody bound to the bottoms of the 96-well plate. As in western blotting, the samples are pretreated with the non-specific protein degrading enzyme, proteinase K, before being added to the 96-well plate. The sensitive prions are completely digested while the resistant prions remain mostly undigested (only the first 67 amino acids are removed). The plate bound prion specific antibody captures the undigested prion proteins in the sample.

A second antibody is added that binds to a different region of the prion protein which has already been captured. The second antibody is linked to horse radish peroxidase (an enzyme) for detection. When luminol (substrate) and peroxide (catalyst) are added, the horse radish peroxidase reacts with the luminol, creating an excited intermediate that decays and gives off light particles. The light particles can be detected by a plate reader set to detect the light emissions. Positives for BSE are easily detected.

Conformation-dependent immunoassay.

Conformation-dependent immunoassay (CDI) is the most recently approved European Union BSE test for post-mortem samples and is based on the ELISA immunoassay. Unlike the other types of immunochemical tests (ELISA and western blot)

that may report false positives, CDI does not rely on protein digestion to degrade all of the normal prion protein and then test for any undigested (infectious) prions. Rather, it uses the conformation of the resistant protein to detect its presence with antibodies.

The test can detect much lower levels of the infectious prion protein than can the other approved immunological procedures. The CDI approach involves revealing a region of the protein that is usually exposed in the normal PrP^c but is buried in infectious PrP^{Sc} based on its distinct three-dimensional folded shape (Hileman 2003).

The technique begins with a brain sample to be tested that is homogenized and divided into 2 aliquots. The first sample is left untreated and the prion proteins remain folded in their native conformation (as they would exist normally inside the brain). The second half of the sample is denatured by chemicals and/or heat (unfolding all of the proteins). Both samples are put onto ELISA 96-well plates pretreated with an immobilized capture antibody called recFab Hum-P, which recognizes several species' prion proteins. This selectively binds only prion proteins to the plate; unbound proteins are washed away.

A second detection antibody bound to a fluorescent marker dye is added. The second antibody binds to the captured prion proteins already on the plate. In the untreated sample the native prion has the antibody binding site exposed, while the resistant prion has this binding site hidden (buried inside the protein due to its different protein folding). Thus the native sample represents all of the normal protein. Counting this untreated sample for fluorescence emission establishes a background level of prions. The denatured sample with all proteins unfolded allows the antibody to capture both sensitive and resistant prion proteins. When the denatured sample is counted for fluorescence emissions, there are two possible outcomes:

(1) The levels of emission are measured to be equal to or less than the native sample, since unfolding did not expose any new binding sites (the sample is free of resistant prions);

(2) The denatured signal is measured to be significantly higher, because new binding sites were exposed (the sample contains resistant BSE prions).

<http://www.aventisbehring.com/ABCMSPRDI/n35241pr25599/AventisBehringPresen.htm>)

The CDI test has many advantages due to its accuracy and sensitivity and may be a suitable test for live animals. In lab tests, researchers have already used the CDI test to detect prions in the muscles of living mice under controlled conditions (Safar et al. 2002). It is hoped that these results can be duplicated for testing live cattle tissue or fluids such as muscle, urine or blood. Moreover, the test is rapid and produces results in about five hours. Like the ELISA test, CDI assays use 96-well plates and large-scale testing can be automated by robotics for high throughput. This would allow for ongoing monitoring of BSE within cattle stocks suspected of BSE infection

http://www.cbc.ca/stories/2003/09/07/bse_test030907).

The immunoassay testing kits go by various names: Prionics – Check Western (western blot, Prionics AG); Platelia test (ELISA, Biorad Inc.); Enfer test (ELISA, Enfer); Prionics – Check LIA (Elisa, Prionics, AG). More detailed information about them can be found in: E. Kubler, B. Oesch and A.J. Raeber. 2003. “Diagnosis of prion diseases.” *British Medical Bulletin*. 66: 267-279.

A comparative analysis of the approved kits can be found in: European Commission, “The evaluation of tests for the diagnosis of transmissible spongiform encephalopathy in bovines.” http://europa.eu.int/comm/food/fs/bse/bse12_en.html.

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