IMPLEMENTATION OF DOT-BLOT IN RAPID DIAGNOSIS OF BSE

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Abstract

The article describes the preliminary results from the implementation of dot-blot technique in the diagnosis of bovine spongiform encephalopathy (BSE). Simple methodology, low equipment requirements and fast procedure coupled with small volume of liquid waste prompted us to use this technique for the detection of pathological, proteolysis resistant form of prion protein (PrP\textsuperscript{res}). The method was assessed with respect to sensitivity and specificity and it was compared with already approved rapid test for BSE diagnosis. Ability to detect all positive cases used in the study, lack of non-specific signal from negative samples, short time of testing procedure and higher sensitivity in comparison to the commercial test show that the method may be used in surveillance programs for BSE.

Key words: bovine spongiform encephalopathy, diagnosis, dot-blot, PrP\textsuperscript{res}.

Transmissible spongiform encephalapathies (TSEs) including variant Creutzfeldt-Jakob disease (vCJD) in humans, BSE in cattle and scrapie in sheep form a group of fatal neurodegenerative diseases of uncertain and yet not fully elucidated etiology (7). Among many theories aiming at resolving the nature of the causative agent of TSEs, prion theory proposed by Prusiner is the one most accepted (2, 3). According to his hypothesis, cellular prion protein (PrP\textsuperscript{C}) is posttranslationally modified to a pathological form – PrP\textsuperscript{Sc}, which, among other distinct features, is protease-resistant. At the moment PrP\textsuperscript{Sc} is regarded as the molecular marker of prion diseases and its detection is equivalent with positive diagnosis of any TSE.

All diagnostic tests for BSE are for post-mortem analysis only (6). It is due to the fact that both spongiosis and PrP\textsuperscript{Sc} location are limited to the central nervous system. Good diagnostic test should be reliable, sensitive, specific, easy to perform and of course rapid with automated procedures enabling high throughput. At the moment EU accepts eleven rapid tests for BSE and commercial test, reagents provided for the latter test were used for dot-blot too. Homogenization of 0.5-0.75 g brainstem samples in homogenization buffer (Prionics) was done for 1 minute with an Omni homogenizer at 20 000 rpm using plastic tips. Digestion with proteinase K (PK at 1 mg/ml) (Prionics) was done at 48°C for 40 min. The reaction was stopped with digestion stop solution (Prionics). After digestion, the homogenate was centrifuged for 5 min at 5 000 x g. To meet dilution factor of commercial test, 1.3 volumes of resultant supernatant was mixed with one volume of TBS buffer (25 mM Tric/HCl, 150 mM NaCl, 2.5m M KCl, pH=7.4). It was further diluted 1:10 in TBS and 100 µl of this dilution was applied. 96-well plates with PVDF membrane at the bottom (MultiScreen IP,
Millipore) were used with MultiScreen Vacuum Manifold (Millipore) and vacuum pump. Before use, PVDF membrane was pretreated with 70% ethanol and washed with distilled water and then with TBS. Positive and negative samples were applied in triplicates. For the analysis of the sensitivity of dot-blot, positive BSE sample was applied in triplicates after being diluted serially in twofold steps up to 1:128. Undigested negative sample was also diluted serially in twofold steps up to 1:128 but it was applied in duplicates. For background signal analysis homogenization buffer diluted 1:10 was applied. Incubation was done for 30 min. at room temperature (all the incubations were done at room temperature). Applying vacuum to the manifold enabled the removal of any content of the wells during testing. Single wash was done with 200 µl of TBST (TBS with 0.05% Tween 20). All the following washes were done with same volume of TBST applied per well. Next, 200 µl of 3 M guanidine thiocyanate was applied and incubated for 10 min. After two washings the membrane was blocked for 30 min. with 5% solution of bovine serum albumin (Sigma) in TBST. Primary antibody 6H4 (Prionics) was added at 1:5 000 dilution in TBST and the membrane was incubated for 1 h. Next, the membrane was washed 3 times and secondary antibody conjugated with alkaline phosphatase (Prionics-Check) was used at 1:5 000 dilution in TBST. Thirty minute incubation was followed by five washes. Chemiluminescence technique was used to detect the signal. First the membrane was incubated in luminescence buffer (Prionics-Check) for 5 min. Next, 20 µl of substrate (CDP-Star from Sigma) was applied per well and incubated for 5 min. Then the plate was sealed with foil and placed on x-ray film. Exposure times were in a range of 10 s and 5 min. Dot-blot results in the form of developed x-ray films were analysed visually and by means of the computer software (“One D-scan” from Scanalytics) after scanning the film to get the digital input file. Optical density (OD) values for the tested samples were calculated as mean values from three wells each.

**Western blot.** To compare the sensitivity of dot-blot with commercial test (Prionics-Check Western), we performed the latter test utilizing the same positive BSE sample as the one used for dot-blot diluted serially in twofold steps up to 1:128 (TBS). Also the homogenate volume applied in both tests was the same. In commercial test, samples digested with proteinase K were subjected to electrophoresis and western blot according to manufacturer’s protocol. The following steps including incubations with antibodies and chemiluminescence detection were identical with the ones used in dot-blot test.

**Results**

All samples from confirmed cases of BSE tested in dot-blot had stronger signal when compared with negative samples (Figs 1, 2). Unambiguous classification of eight positive samples as positives was possible in visual examination.

![Fig. 1. Results of dot-blot testing for BSE.](image-url)

- columns 1, 2: negative sample undigested with PK tested in duplicate (diluted to 1:128)
- columns 3-5: positive sample digested with PK tested in triplicate (diluted to 1:128) – sensitivity of the test
- columns 6-8: eight Polish cases of BSE digested with PK tested in triplicate
- columns 9-11: rows A-C: three Polish cases of BSE digested with PK tested in triplicate
- columns 9-11: rows D-H: five negative samples digested with PK tested in triplicate
- columns 12: background signal from reagents used in dot-blot
For the remaining three samples, weaker signal was observed in single wells. However, digital analysis with “One D-scan” software showed that mean value for the weakest positive sample was higher than the sum of mean value of negative samples and two standard deviations (x+2SD), 0.184 and 0.078, respectively.

The analysis of dot-blot sensitivity enabled the detection of positive signal in all dilutions up to the highest 1:128 dilution (Figs 1, 2, 3). The strongest signal was obtained at 1:4 dilution (Fig. 2). Values for the highest dilutions (1:64 and 1:128) were comparable. Commercial test used for comparison showed positive signal up to 1:64 dilution (Fig. 3).

**Discussion**

Our results show that dot-blot technique can be successfully implemented in BSE diagnosis. All positive samples used in the study were clearly positive. Sensitivity analysis in comparison with commercial test showed that dot-blot was more sensitive. Amount of brainstem homogenate applied in dot-blot was equivalent to the amount used in commercial test. Detection limit for dot-blot could not be reached at the range of dilutions used in the study but still it was possible to compare its sensitivity with commercial test. Multiple sample loading could be used as an option to
improve test sensitivity. Other advantages of dot-blot technique confirmed in our work were: short time needed to run the test, simplicity of the procedure coupled with low equipment requirement and low volume of liquid waste produced during the testing. The same methodology was used by others to diagnose scrapie in sheep and chronic wasting disease (CWD) in mule deer (1, 5). However, those techniques required the drying of the membranes after application of brain homogenates which took up to 18 h. In our hands the whole protocol took 4 h.

When optimizing dot-blot for BSE diagnosis, different parameters were evaluated. The most significant one was the incubation of digested samples with chaotropic agent (guanidine thiocyanate - GT), which enabled clear distinction of positive and negative samples. Treatment of BSE positive brain homogenates with heat and guanidine thiocyanate to detect BSE-specific PrPSc was described by Meyer et al. (4). According to these authors the use of chaotropic agent exposed more epitopes of PrPSc which due to complex tertiary structure of prion protein are usually hidden. Although the authors used 10 times lower concentration of GT compared with our study they compensated this deficiency by heating the samples to increase the solubility of pathological form of PrP. In our study the use of higher concentration of GT enabled simplification of the whole procedure because additional step of heating of the samples was omitted. Optimal incubation time for the conjugate in terms of the strength of the signal in dot-blot was reached at 30 min and it was not improved after 1 h of incubation. Prolonged incubations with the conjugate increased the background signal, which hindered the reading of the results.

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References