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Development of immunohistochemstry for the detection of PrP^{SC} in bovine brain tissues

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Abstract

The aim of this study was to develop the immunohistochemical method for detection of the Bovine Spongiform Encephalopathy (BSE) specific protein (PrP^{SC}) in bovine brain tissue that is free from cross reactions. A slaughterhouse surveillance was conducted in two districts (Dhaka and Mymensingh). Around 1000 brain samples were collected from 30 months old cows, slaughtered for human consumption. The brainstem (obex), pyriform lobe, pieces of cerebellum and cerebrum were used to detect BSE specific protein, using an anti PrP^{SC} monoclonal antibody (prionic AG, Switzerland). A labelled Streptavidin Biotin (LSAB) method of immunohistochemical staining for the detection of BSE specific protein (PrP^{SC}) in paraffin embedded positive tissue sections was developed successfully. PrP^{SC} was detected in the cytoplasm of neurons of positive brain tissues. Some of the brain tissues collected from field showed some gliosis and inflammatory lesions but none of the lesions were consistent with BSE histopathologically and all of the samples were negative for the PrP^{SC} protein by immunohistochemical method. The developed protocol may be used as tool for the diagnosis of BSE.

Keywords: Bovine spongiform encephalopathy, PrP^{SC}, Immunohistochemical method, Bovine brain tissues

Introduction

Bovine Spongiform Encephalopathy (BSE) is commonly known as mad cow disease and is a fatal neurodegenerative infectious disease of cattle. The popular name mad cow disease refers to the symptoms of infected cattle: "staggering, drooling, signs of fear, grinding of teeth, aggression towards other animals" (Kimberlin, 1992; Rampton and Stauber, 1997). BSE belongs to the family of diseases known as "Transmissible Spongiform Encephalopathies". It has a causal link with an old disease "Scrapie", which are prevalent in sheep for over 200 years (Almond and Pattison, 1997; Chowdhury, 2005; Moore *et al.,* 2005). The origin of the BSE remains unknown, but most probably it is originated from adaptation and recycling of the sheep scrapie agent in the form of meat and bone meal (MBM) supplied to cattle as feed (Wilesmith *et al.,* 1991; Cutlip *et al.,* 2001;). The principal component of the infectious agent responsible for the disease appears to be an abnormal isoform of the host encoded prion protein (PrP), designated "PrP^{SC}" (Almond and Pattison, 1997; Chowdhury, 2005).

Different countries, particularly developed countries have banned importation of feeds and live animals from countries that had experienced with mad cow disease. Mad cow disease free countries are facing troubles to export bovine origin materials (feeds, gelatin, tallow etc) to developed countries as the developed countries want to know the status of mad cow disease surveillance in the exporting country (Prusiner, 2000; Vanopdenbosch and Roels, 2004; Chowdhury, 2005). Though Bangladesh is still free from BSE, but due to lack of surveillance, it is facing troubles to export bovine origin materials, particularly gelatin (Chowdhury, 2005).

Confirmatory diagnosis of BSE is based on postmortem histopathological examination of brain tissue from an animal where common hallmarks include vacuolation, gliosis and detection of PrP^{SC} by immunohistochemistry (Kubler *et al.,* 2003; Vanopdenbosch and Roels, 2004; Chowdhury, 2005). But if an animal remains in pre-incubation period of BSE infection in that case spongiosis and vacuolation are not observed during histopathological examination and ultimately histopathology gives no confirmed result. An animal in this phase of infection poses the same risk to the consumer like a visibly ill animal. So to avoid the transmission of this dormant stage of infection to human, at first it needs to confirm diagnosis and in this case immunohistochemistry is the only sensitive method. Immunohistochemistry is a technique used to assess the presence of a specific protein or antigen in cells by use of a specific antibody (monoclonal or polyclonal antibody). This sensitive diagnostic tool not only detects the presence of PrP^{SC} in BSE infected brain but also detects the pattern of distribution of PrP^{SC} in brain tissues. (Bolton *et al.* ,1982; Lorette and Javois, 1999; Chowdhury, 2005). The present study was carried out to develop the immunohistochemical method for detection of the BSE specific protein (PrP^{SC}) in bovine brain tissues, which would be free from cross reactions.

Materials and Methods

Antibody: Anti PrP^{SC} monoclonal antibody (mAb 6H4) a mouse IgG1 subtype antibody: Light chain: K subtype (Prionic AG, Switzerland).

Tissues: The paraffin embedded, Bovine Spongiform Encephalopathy (BSE) positive tissues (BSE positive tissue –1 and BSE positive tissue- 4) collected from Institute of Veterinary Pathology, University of Zurich, Switzerland were used as the positive control for the development of immunohistochemical method.

Autoclaving: Tissues were autoclaved for 30 minutes at 121^0C. (optimal autoclaving time was selected from a series of autoclaving time like (10min, 15min, 20min, 25min and 30min).
Pretreatment: Tissues were pretreated for 15 minutes in proteinase K at room temperature (RT). (Optimal time was set by a series of pretreatment time using proteinase K (8 min, 10 min, 12 min and 15 min) and serial variations of proteolytic pretreatment time were set by using protease (8 min, 10 min, 12 min and 15 min).

Incubation with antibody: After over night incubation at room temperature with the monoclonal anti- PrP^{SC} antibody (mAb 6H4) a mouse IgG1 subtype antibody: Light chain: K subtype with a dilution of 1:800 the reaction was visualized using the DAKO ChemMate™ detection kit (no. K 5003), DAKO A/S,produktionsvej 42,DK-2600 Glostrep, Denmark according to DAKO instruction, where substrate 3- amino 9-ethylcarbazole(AEC) was used. The dilutions of the primary antibody tested were1:200. 1:400, 1:600 and 1:800. At the end, it was counterstained with Mayer's hematoxylin.

1000 brain samples were collected during January 2005 to June 2006, from cattle older than 30 months of age, slaughtered for human consumption in the district slaughterhouses. Tissue samples from the brainstems (obex), pyriform lobe, pieces of cerebellum and cerebrum were preserved in 10% neutral buffered formalin. 1000 sections were stained with H & E and histopathological examinations were performed. The hundred samples that showed some lesions were used for immunohistochemistry reactions.

Results and Discussion

BSE immunohistochemistry was developed successfully. Two BSE positive controls and one negative control were run in each time. The negative control sections were treated with PBS instead of primary antibody. All of the collected field samples and the negative control were found negative for PrP^{SC} (Fig. 1). For the development of this method several trials were attempted and finally a clear positive reaction almost free from nonspecific reactions was found (Fig. 4).The positive immunohistochemical reactions were indicated by the presence of red colored granular staining specifically found in gray matter nuclei as intra-nuclear pattern (Fig. 4). Accumulation of PrPsc was observed mainly in the brainstem. In the medulla oblongata, the largest accumulations of PrP were in the nucleus tractus spinalis nervi trigemini. Large accumulations of PrP were in the nucleus dorsalis nervi vagi, nucleus tractus solitarii, nucleus olivaris and gray matter of the formatio reticularis. Red colored staining around the vacuoles were considered positive (Fig 3). The infected neurons were usually found as clusters, while neurons of other area, were found clearly negative for PrP^{SC}.

Histopathological lesions observed in the brain sections collected from fields included microgliosis, oligodendrogliosis, hemorrhage, congestion, encephalitis, neuronal degeneration etc. Representative sections showing lesions are shown in (Fig. 5, 6 and 7). But none of the brain samples has revealed spongiform lesions like the mad cow disease and therefore, none of the lesions were consistent with BSE histopathologically. Those samples that showed some gliosis and inflammatory lesions were selected for immunohistochemical method and all the samples were found negative for the PrP^{SC} protein by immunohistochemistry.

A standard protocol of LSAB method of immunohistochemistry was followed on the basis of available literature, where some modifications were done until a suitable standard effective protocol was obtained. The optimum dilution of the primary antibody was selected empirically. On the basis of suitable balance between specific and background staining a dilution of 1:800 was selected. Incubation of the positive tissue sections with the primary antibody was performed overnight and this was performed by Stephane *et al.,* (2003). However, to reduce the background reactions, few other modifications, e.g. pre-treatment with proteinase K, autoclaving of tissue sections, were also practiced.

In immunohistochemistry, the main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections (Javois, 1999). A very sensitive LSAB kit together with sequenza system was used in this study that minimized the background reactions. The Streptavidin, derived from *streptococcus avidini*, is a recent innovation for substitution of avidin. This Streptavidin does not contain carbohydrate groups which might to bind to tissue lectins, resulting in some background staining (Polak and Nordon., 1997). The possible causes of high background signal in BSE immunohistochemistry was due to cellular PrP (PrPc) since PrPc and PrP^{SC} originates from the same single-copy host gene (Oesch, 1985). PrP^{C} is heat labile but PrP^{SC} is heat stable. PrP^{SC} is a protein with high resistance to inactivation by irradiation, heat and harsh chemical treatments and can survive the "autoclave" sterilization process and used for most surgical instruments (Plum, 1997; Collinge, 2001). Optimum autoclaving time 30 min reduced almost all non-specific reactions. Autoclaving of tissue section to reduce non-specific reaction by PrP^{C} has also been found effective in another investigation by Debeer *et al.,* (2001).

The positive staining appeared as dark red granular precipitate at the site of BSE specific antigen localization. The antigen was found to be localized in the cytoplasm of neurons. The pattern of staining of the positive tissues was similar to those observed by Kimura *et al.,* (2002) and Castilla *et al.,* (2004).

In Hematoxylin and Eosin staining of the field samples, though some gliosis and inflammatory lesions were observed but all the samples were found negative for PrP^{SC} by BSE immunohistochemistry and it was similar to those observed by Koo *et al.,* 2001.

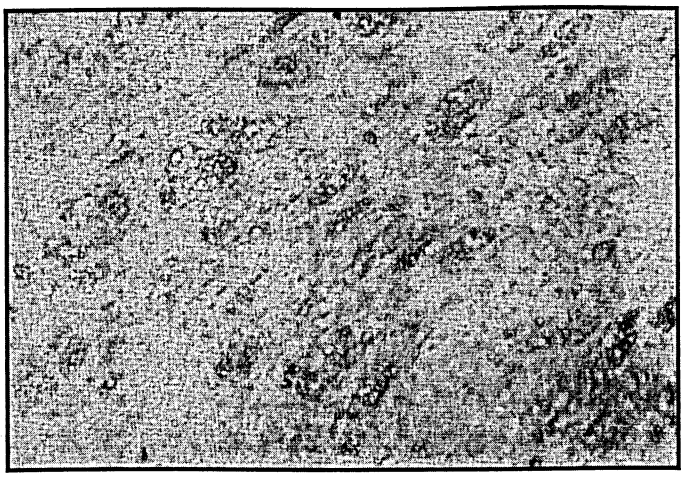

Fig. 1. PrP^{SC} negative control where no positive reactions are labeled (X 82.5)

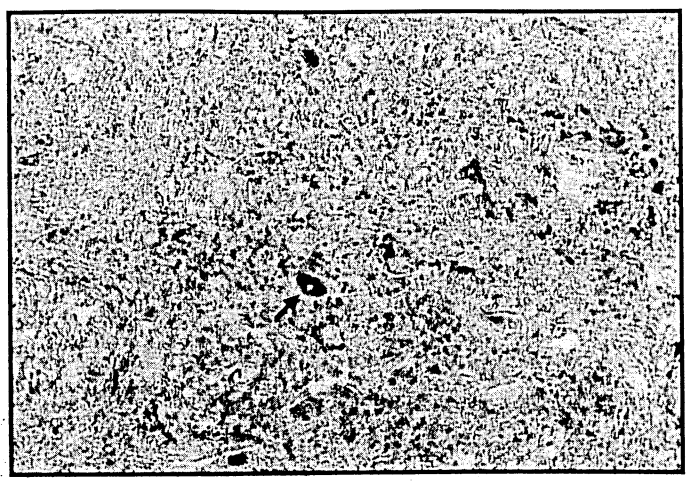

Fig. 2. PrP^{SC} positive control showing red colored granular precipitates in the neurons and presence of strong background reactions outside the neurons (LSAB method of immunohistochemical staining, AEC substrate, Mayer's hematoxylin counterstain, X 82.5)

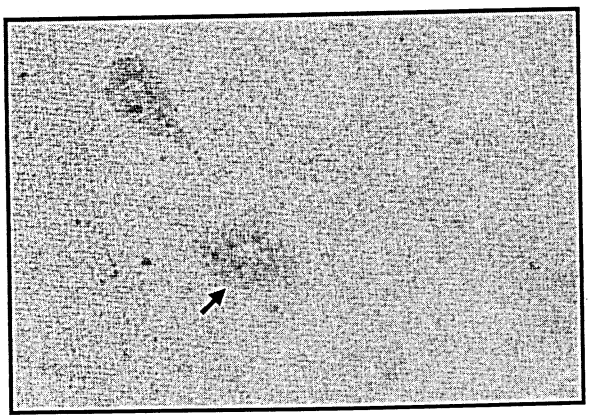

Fig. 3. PrP^{SC} positive control showing red colored granular precipitates in the neurons and presence of less nonspecific reactions (LSAB method of immunohistochemical staining, AEC substrate, Mayer's hematoxylin counterstain, X 330)

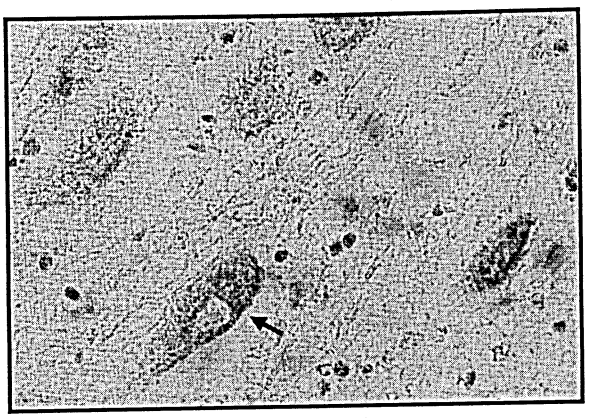

Fig. 4. PrP^{SC} positive control showing clear reaction (red colored distinct granular precipitates in the neurons) and almost free from nonspecific reactions (LSAB method of immunohistochemical staining, AEC substrate, Mayer's hematoxylin counterstain, X 330)

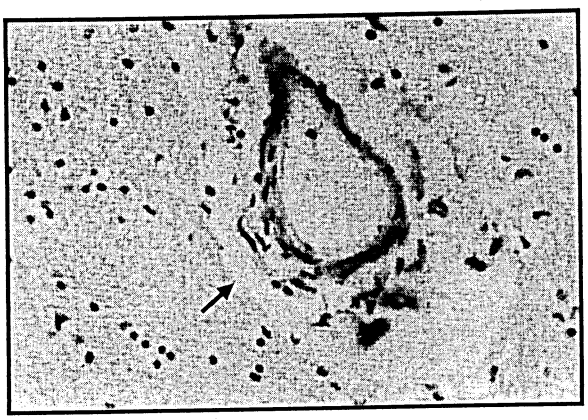

Fig 5. Microgliosis and perivascular edema (H & E, X 330)

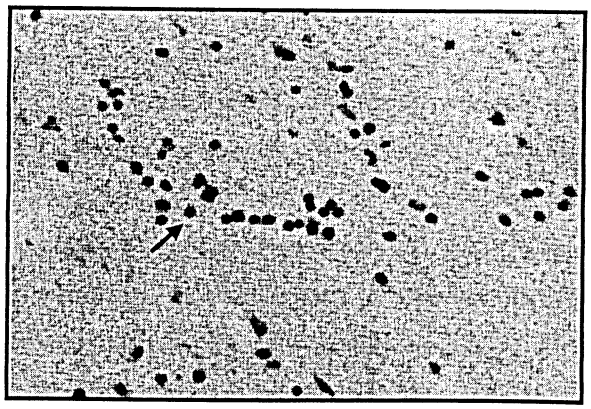

Fig 6. Microglial star (H & E, X 330)

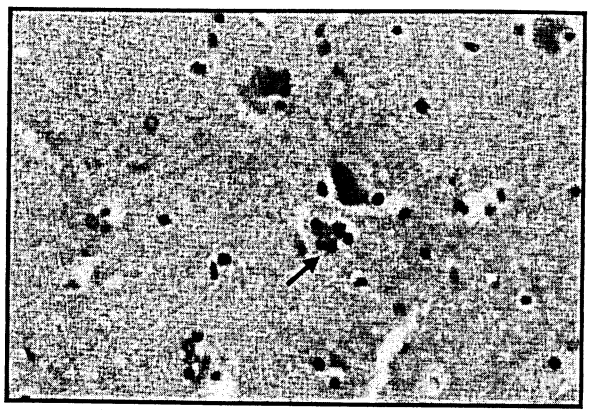

Fig. 7. Neuronal degeneration and satellitosis (H & E, X 330)

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