Histol Histopathol (2012) 27: 1227-1230
DOI: 10.14670/HH-27.1227

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Domestic microwave processing for rapid immunohistochemical diagnosis of bovine rabies

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Summary. The present study describes the use of a microwave processing protocol for the rapid histopathological and immunohistochemical diagnosis of bovine rabies. Immunohistochemistry has been used for rabies diagnosis in formalin-fixed tissue with satisfactory results, although the time to diagnosis is considerably longer than that with direct immunofluorescence. The protocol provided a provisory histopathological rabies diagnosis in approximately three and half hours and the immunohistochemical diagnosis was available after six hours. The protocol achieved 100% correlation with direct immunofluorescence and is a promising method, particularly in situations in which only material in formalin is available for diagnosis or when the refrigeration or transportation of biological material is difficult.

Key words: Cattle, Histopathology, Microwave histoprocessing, Microwave fixation, Rabies virus

Introduction

Rabies is an infectious disease caused by a virus of the genus *Lyssavirus* that affects mammals and is considered to be one of the greatest economic problems related to livestock and public health in South America (Barros et al., 2006). Between 1987 and 2010, 66,135 herbivores, including 58,932 cattle, were infected by rabies virus in Brazil (Brasil, 2011). Although these data are significant, the real numbers could be even higher, as the disease is under-reported (Teixeira et al., 2008).

Immunohistochemistry (IHC) has been used as an alternative to direct immunofluorescence test (DIF) to detect rabies in formalin-fixed tissues from different animal species and humans (Jackson et al., 1999; Jogai et al., 2000, 2002; Bagó et al., 2005; Pedroso et al., 2008; Tobiume et al., 2009; Stein et al., 2010). Microwave irradiation of tissues for fixation and histological processing has reduced the time required to obtain slice sections and has offered the advantage of preserving antigens and nucleic acids (Mayers, 1970; Boon et al., 1986; Morales et al., 2002; Hafajee and Leong, 2004; Suri et al., 2006). The rapid diagnosis of bovine rabies in formalin-fixed tissues may also reduce the risks inherent to the handling and sending of material contaminated with the virus (Rodriguez et al., 2007).

The aim of the present study was to assess an alternative protocol for rapid rabies diagnosis in cases inadvertently shipped in formalin or from regions with limitations regarding the storage and transport of cooled biological material. In such situations, it is unfeasible to carry out DIF, which may hinder the adoption of control and preventive measures for the disease.

Materials and methods

Thirteen cases of bovine rabies were evaluated in southern Minas Gerais state (Brazil) from July 2010 to May 2011. Twelve necropsies were performed by the staff of Veterinary Pathology (VP) from Federal University of Lavras (UFLA, Brazil) and one was performed by an autonomous veterinarian who forwarded the refrigerated and formalin-fixed material to the VP. Fragments from spinal cord, cerebellum, brainstem, hippocampus and cerebral cortex of cattle were frozen and sent to the *Instituto Mineiro de Agropecuária* (IMA) for DIF examination and inoculation in mice test.

Fragments of cerebellum (13/13) and medulla oblongata at the level of obex (8/13) (approximate

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thickness: 3 mm) were collected in a 10% buffered formalin solution. Selected areas were based on previous studies (Pedroso et al., 2008). Fragments remained in formalin from one to two hours, which was the time between collection and the beggining of microwave processing. One of the 13 brains had already been fixed upon arrival to the VP and it wasn't possible to determine the duration of fixation in this case. Fragments were cleaved into 1 mm slices and irradiated in the same fixing solution for one minute at full power (100%), followed by one minute and a half at minimum power (10%) in a domestic microwave oven (NN-S65B, 1000W, Panasonic®) (Kahveci et al., 1997).

After fixation, tissues were dehydrated in the microwave in absolute ethyl alcohol at medium power (50%) for four minutes and diaphanized in isopropyl alcohol at medium power (50%) for four minutes, followed by immersion in paraffin and irradiation for seven minutes at close to full power (80%), with another container inside the microwave containing 200 mL of water (Boon et al., 1986; Suri et al., 2006). Cuts of 5 μm thickness were taken from blocks and placed on slides with gelatin and Silane® (Sigma-Aldrich, USA) for hematoxylin and eosin staining and IHC, respectively.

IHC was performed following a pre-established protocol (Pedroso et al., 2008; Stein et al., 2010). Goat anti-rabies polyclonal antibody was used as the primary antibody (Millipore, 5199) at a dilution of 1:500. IHC was processed with and without antigen retrieval. A fragment of the central nervous system (CNS) of a rabid bovine was used as the positive control, processed in the conventional manner for histopathology and confirmed by DIF and IHC. A fragment of CNS from a bovine euthanized due to trauma was used as the negative control, with a negative result for rabies in DIF, mice inoculation and IHC.

Results

Microwave tissue processing allowed a histopathological evaluation in approximately three and half hours after collection at the necropsy and one and half hours after receiving the formalin-fixed material sent to VP-UFLA. Tissue morphology was relatively

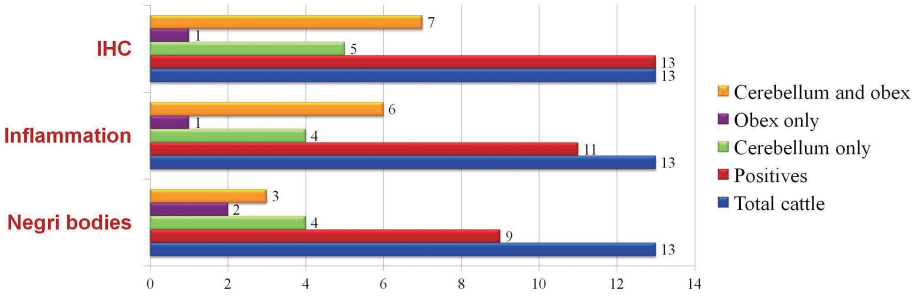

Fig. 1. Bovine rabies. Results of histopathology and immunohistochemistry of tissues processed in microwave.

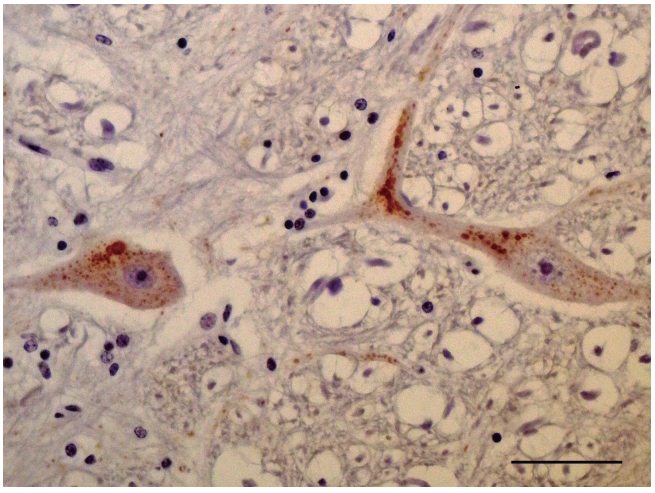

Fig. 2. Bovine rabies. Immunohistochemistry of medulla oblongata at the level of obex processed in microwave, revealed in diaminobenzidine peroxidase substrate (Dako, Carpenteria, CA). Bar: 50 μm.

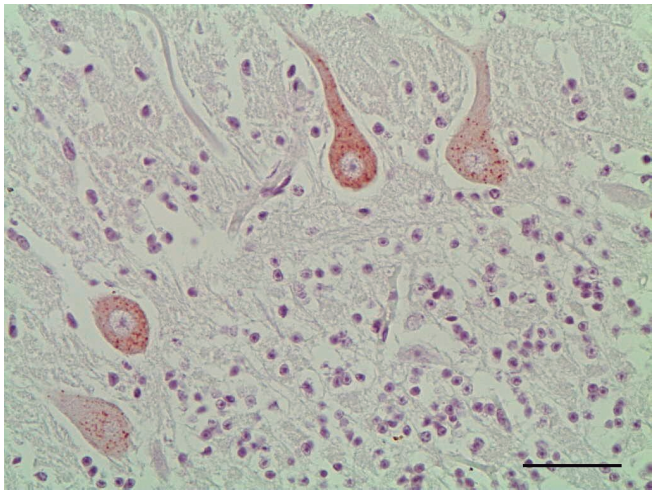

Fig. 3. Bovine rabies. Immunohistochemistry of cerebellum processed in microwave, revealed in Vector® Nova*RED*™ substrate kit for peroxidase. Bar: 50 μm.

well preserved, particularly in the cerebellum, with Negri bodies visualized in nine cases (69.2%): four in cerebellum, two in medulla oblongata at the level of obex and three in both. Nonsuppurative meningoencephalitis, characterized by a predominant lymphocytic infiltration, was found in 11 cases (84.6%): four in the cerebellum, one in medulla oblongata and six in both (Fig. 1).

Immunohistochemical diagnosis required a processing time of approximately six hours from collection at necropsy and approximately four hours after receiving the formalin-fixed material. All 13 cases were positive for rabies (Fig. 1), with labeling in all eight fragments of medulla oblongata at the level of obex (100%) (Fig. 2) and 12 of cerebellum fragments (92.3%) (Fig. 3). The bovine without positive labeling in the cerebellum displayed weak labeling at the obex level and was the only one in the present study euthanized with barbiturates. Positive labeling in IHC for rabies, with or without antigen retrieval, was obtained in all 13 cases of microwave processing. The results of this technique achieved 100% positive correlation with the DIF performed by IMA.

Discussion

The protocol used for microwave processing proved efficient for rapid immunohistochemical diagnosis of bovine rabies. Histological cuts were obtained approximately three and half hours after collection, which led to a rapid provisional diagnosis in 84.6% of cases, based on the presence of nonsuppurative meningoencephalitis and/or Negri bodies (Summers et al., 1994). However, IHC, performed in a six-hour period from the time of necropsy, provided greater specificity and sensitivity. The efficiency of IHC, to detect rabies virus' antigens, in animals and humans, has been demonstrated in a great number of studies (Jackson et al., 1999; Jogai et al., 2000, 2002; Bagó et al., 2005; Pedroso et al., 2008; Tobiume et al., 2009; Stein et al., 2010). The time required to complete the immunohistochemical diagnosis was similar to that required for DIF (Dean and Abelseth, 1973). Rapid diagnosis is crucial to adoption of preventive measures in the herd, as well as to humans who had contact with infected animals.

Although this protocol has been used in a limited number of cases, it does look promising, particularly in situations in which only tissue in formalin is available for confirmation of the diagnosis. This technique could be useful in regions with transportation limitations and it also avoids problems with the storage and shipping of fresh biological material. Handling of formalin-fixed tissues is less risky than cooled or frozen material in terms of contamination, since the rabies virus is sensitive to formalin (Rodriguez et al., 2007). Furthermore, domestic microwave processing and IHC require a simpler and less expensive laboratory structure (Suri et al., 2006).

IHC was essential for rabies diagnosis in formalin-fixed tissues, particularly for cases in which no compatible lesions were found on histopathologic examination. The weak labeling in medulla oblongata and absence of labeling in cerebellum of the euthanized bovine were probably associated with lesser dissemination of rabies virus in CNS (Barros et al., 2006) due to the interruption of the disease's course.

Immunohistochemical labeling was achieved even without antigen retrieval, which reduced the processing time by 15 minutes. Nevertheless, retrieval may be important in samples with prolonged fixation (Stein et al., 2010).

Formaldehyde-fixative solutions contain little formaldehyde: instead, they contain mainly methylene glycol, formed by the reaction between formaldehyde and water. Fixation occurs in three steps: first, diffusion of methylene glycol into the tissue; second, formation of formaldehyde by dehydration of methylene glycol into the tissue; and third, binding of formaldehyde to the proteins by cross-linking. At microwave irradiation, all three steps are accelerated (Kahveci et al., 1997; Kok and Boon, 2003), contributing to the faster fixation achieved in the present study.

The material that remained in formalin at room temperature for about two hours, before microwave processing, exhibited better morphology than that stored for a short time. This better morphology of tissues was likely favored by initial penetration of methylene glicol. Microwave irradiation of fresh tissue, not previously immersed in formalin at room temperature, causes sponginess in the center of the tissue and lysis of erythrocytes (Kok and Boon, 2003). This sponginess was visualized in some cuts of medulla oblongata which were kept in formalin for less time before irradiation.

Moreover, this technique also led to a considerable reduction in the use of xylene, which was completely excluded from the tissue processing and only used in the mounting of slides (Suri et al., 2006).

Processing tissues in a domestic microwave oven for a rapid immunohistochemical diagnosis of rabies was efficient in the cases presented here.

Acknowledgements. The authors are grateful to Professor David Driemeier for donation of primary antibody; veterinarian Denis Lucio Cardoso of the IMA for providing access to the cases from Perdões, MG, Brazil; the Animal Health Laboratory, IMA, Belo Horizonte, MG, Brazil, for DIF results of materials sent; and to Brazilian fostering agencies CAPES for Master's grant and FAPEMIG for assistance in funding this study.

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Accepted April 9, 2012