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sheep

Monoclonal and polyclonal antibodies for *ante-* and *post-mortem* detection of PrP^Sc in sheep

Utilização de anticorpos monoclonais e policlonais para diagnóstico *ante-* e *post-mortem* do scrapie

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Abstract

Scrapie is a disease that affects sheep and goats and is characterized by the accumulation of an abnormal isoform (PrP^Sc) of the cellular prion protein, PrP^C, in the central nervous system (CNS) and in lymphoid tissues. Detection of PrP^Sc in these tissues can be attempted by a variety of techniques, including immunohistochemistry (IHC) and western blotting (WB), for which a wide range of monoclonal and polyclonal antibodies are commercially available. The objective of this study was to test and compare the efficacy of monoclonal antibodiesF89/160.1.5, F99/97.6.1, and P4 and polyclonal antibodies M52 and R486 in the detection of PrP^Sc in lymphoid and CNS tissue samples by using IHC. Positive and negative control samples of sheep brain and tonsils were provided by the Animal Health and Veterinary Laboratories Agency (AHVLA, UK). The IHC examination of CNS samples with both monoclonal and polyclonal antibodies confirmed the granular deposition of PrP^Sc in the neurons of the positive control tissues. However, while the monoclonal antibodies did not produce positive reactions in the negative controls, the polyclonal antibodies showed some non-specific staining. The testing of positive control tonsil samples with polyclonal and monoclonal antibodies identified positive control-specific reactions, whereas the negative control tissues were IHC-negative with all antibodies, although P4 and the polyclonal antibodies produced some background staining. In summary, although the polyclonal antibodies may be more accessible, in this study their use is not advisable because of possible false positive reactions. The polyclonal antibody M52 was able to identify PrP^C in brain and spleen samples by WB but other lymphoid tissues were negative.

Key words: Scrapie, diagnosis, immunohistochemistry, antibody

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Resumo

Scrapie é uma doença que afeta ovinos e caprinos, sendo definida pelo acúmulo de uma isoforma anormal (PrP^{Sc}) da proteína priônica celular (PrP^{C}) no Sistema Nervoso Central (SNC) e em tecidos linfoides. Para as técnicas de eleição do diagnóstico de scrapie, imunohistoquímica (IHQ) e western blotting (WB), podem ser utilizados uma vasta gama de anticorpos monoclonais e policlonais comercialmente disponíveis. O objetivo deste estudo foi testar e comparar a eficácia dos anticorpos monoclonais disponíveis comercialmente, F89/160.1.5, F99/97.6.1 e P4, e os anticorpos policlonais R486 e M52, para a identificação da presença da PrP^{Sc} em amostras de tecido linfoide e SNC através da técnica de IHQ. Foram utilizadas nas avaliações de IHQ amostras positivas e negativas de cérebro e tonsila palatina de ovinos, cedidas pelo Animal Health Veterinary Laboratory Agency (AHVLA), Reino Unido. Para WB, foram utilizadas amostras de encéfalo, baço, linfonodo, terceira pálpebra e mucosa retal de ovino. As análises IHQ utilizando anticorpos monoclonais e policlonais em amostras positivas de cérebro confirmaram a deposição da PrP^{Sc} em neurônios, caracterizada por marcações de aspecto granular intraneural. Na amostra negativa de cérebro, os anticorpos monoclonais não identificaram marcações positivas, o que foi possível verificar ao utilizar os anticorpos policlonais. Testando a amostra positiva de tonsila palatina com os anticorpos monoclonais e policlonais, identificaram-se marcações positivas e, com a amostra negativa, não se observaram marcações com nenhum dos anticorpos, porém observou-se *backgroud* nos anticorpos monoclonal P4 e policlonais. Apesar dos anticorpos policlonais possuírem valores mais acessíveis, o presente estudo revelou que a utilização dos anticorpos policlonais é inviável, pois a análise pode gerar um resultado falso positivo.

Palavras-chave: Scrapie, diagnóstico, imunohistoquímica, anticorpos

Introduction

Scrapie or Enzootic Paraplexia is a progressive and fatal neurodegenerative disease naturally occurring in sheep and goats. It was the first transmissible spongiform encephalopathy (TSE) to be identified in animals (PRUSINER, 1982) and has been reported in most sheep rearing countries with the notable exceptions of Australia and New Zealand (DAWSON; MOORE; BISHOP, 2008; OIE, 2009). The disease is associated with the accumulation of the abnormal isoform (PrP^{Sc}) of the cellular prion protein, PrP^{C}, in the central nervous system (CNS) and in the lymphoreticular system (ANDRÉOLETTI et al., 2000).

Infection normally occurs by oral transmission when sheep and goats ingest the residue of contaminated fetal membranes from other animals shortly after their birth (ANDRÉOLETTI et al., 2000; ANDRÉOLETTI et al., 2002). Oral infection can also be caused by the ingestion of milk from infected animals (KONOLD et al., 2008, 2013).

Traditionally, confirmation of disease or infection could only be achieved *post-mortem* but more recently, *ante-mortem* diagnostic techniques have been developed (GONZÁLEZ et al., 2008a). Methods using tonsil (JEFFREY et al., 2001) and third eyelid (O'ROURKE et al., 1998) biopsies were initially proposed. However, these procedures are not easily performed in the field and do not allow for repeated resampling. The description of constitutive lymphoid tissue in the sheep rectum (ALEKSANDERSEN; NICANDER; LANDSVERK, 1991) prompted the use of rectal mucosa samples for the *post-mortem* diagnosis of scrapie (GONZÁLEZ et al., 2006), since accumulation of PrP^{Sc} occurs in such tissue. Moreover, samples from the rectal mucosa can also be obtained using a simple and rapid biopsy technique that does not require specific instruments, sedation, or general anesthesia and that can be performed several times without any detrimental effects on the animal. The rectal biopsies can be examined by immunohistochemical methods (GONZÁLEZ et al., 2008a) or by rapid tests, such as an enzyme immunoassay (GONZÁLEZ et al., 2008b), and have been proven to be efficient in the *ante-mortem* diagnosis of scrapie both in the clinical and in the pre-clinical phases of the disease.

For the different techniques used in scrapie diagnosis, such as immunohistochemistry (IHC), western blotting (WB), or enzyme-based immunoassays (ELISA or EIA), a wide range of commercial monoclonal and polyclonal antibodies are available (GAVIER WIDÉN et al., 2005). Monoclonal antibodies derive from a single B lymphocyte that is artificially selected and replicated several times as a clone; therefore, they bind to only one epitope in a unique manner. Polyclonal antibodies consist of a mixture of different clones originating from distinct B lymphocytes, so that can bind to many epitopes of a single antigen (HARLOW; LANE, 1988).

In Brazil, several monoclonal antibodies are commercially available for the diagnosis of scrapie; however, they are all produced in another country, which makes their acquisition difficult. This also results in a more expensive diagnosis for the disease. In this study, five antibodies were compared to each other to evaluate the possibility of using a nationally approved antibody to diagnose scrapie. Using IHC, we therefore aimed to test and compare the efficacy of those five antibodies. Three of these antibodies are commercial monoclonals, one is a polyclonal that has already been established and is being used by researchers in the UK and other countries and one more is a nationally produced polyclonal antibody (Laboratory of Neurobiology of the Federal University of Paraná – UFPR), utilized for the identification of PrP^C in mouse brains by WB. We also aimed to test the efficacy of the nationally approved antibody for the accurate identification of the PrP^C present in different sheep tissues, using WB.

Materials and Methods

Immunohistochemistry – IHC

Samples

The tissue samples used in the IHC evaluation of the different antibodies (ovine brain and lymphoid tissues) were provided by the *Animal Health and Veterinary Laboratories Agency (AHVLA, UK)* and had already been tested in this laboratory by IHC. The samples used in the present study included six that had tested positive by IHC (one from thalamus, three from midbrain, one from medulla oblongata at the obex and one from palatine tonsils) and two that had tested negative (one from midbrain and one from palatine tonsils).

Antibodies

Five different antibodies were used in our analyses the commercially available monoclonals, F99/97.6.1, F89/160.1.5 and P4, and the polyclonal antibodies R486 and M52.

The F99/97.6.1 antibody (VRMD, Pullman, USA) is a monoclonal that recognizes the amino acid sequence 200-225 of ovine PrP, while F89/160.1.5 (VRMD, Pullman, USA) and P4 (R-Biopharm, AG) recognize sequences 142-145, 89-104 (HARDT; BARON; GROSCHUP, 2000), respectively.

R486 (AHVLA, UK) is a rabbit polyclonal recognizing bovine PrP amino acid residues 217-231 (GONZÁLEZ et al., 2002). The polyclonal anti-body M52 was produced in the Laboratory of Neurobiology of the UFPR. As shown by Zanata et al. (2002), knockout mice for the gene that encodes the cellular prion protein (*PRNP* gene) were immunized with the murine recombinant prion protein (6His-PrP^C) expressed in the *E. coli* heterologous system. Four rounds of immunization were performed, spaced by 15 days, with a dose of 15 μg of the recombinant protein, 50% (v/v) Freund adjuvant (Sigma-Aldrich) (complete in the first immunization and incomplete in the other three), and 16% of aluminum hydroxide each time, with the volume adjusted to 100 μL, using a saline solution buffered with phosphate (PBS, 140 mM NaCl; 2,7 mM KCl; 10 mM Na_2HPO_4; 1,8 mM KH_2PO_4). Eleven days after the last immunization, the serum was obtained and tested by ELISA using recombinant PrP and by WB using mouse brain as respective substrate.

Immunohistochemistry

Tissue sections from positive and negative control sheep were subjected to IHC according to the protocol used by the Federal University of Rio Grande do Sul, as follows. Firstly, the slides were dried for 16 hours at 37°C, cooled down at room temperature, and labeled with a hydrophobic Dako pen (Dako®) to delimitate the slice edges. The slices were deparaffinized and treated with 10% hydrogen peroxide in methanol for 20 minutes to block the endogenous peroxidase. After washing in TBST (150 mM NaCl; 50 mM Tris-HCl pH 7.4; 0.05% Tween 20) for five minutes the sections were treated with 95% formic acid at room temperature for five minutes and then washed three times in TBST and treated with proteinase K (DAKO, Ready-to-use) diluted in distilled water (1:1) for one minute. After washing again to block the protein and to reduce the nonspecific ligations, the sections were treated with 5% skimmed milk (Molico, Néstle) for 30 min and washed in TBST once more. Tests with and without the protein blocking solution were performed for the palatine tonsil sections. Primary antibodies, mono- and poly-clonal were all used at 1:500 dilution and the sections incubated in a humidity chamber for 16 hours at 4°C. Later, the sections were treated with a biotinylated secondary antibody DAKO LSAB 2 kit® (DAKO Corp., Carpinteria, USA) for 20 minutes, and washed and treated with a streptavidin-peroxidase conjugate (DAKO Corp., Carpinteria, USA) for another 20 minutes. The samples were then placed in the 3,3′-diaminobenzidine (DAB) chromogen stain diluted in distilled water (1:1) until the appearance of the brown color. After this, the slides were briefly washed in distilled water. Counterstaining was performed with hematoxylin for 15 minutes, followed by washing in tap water, dehydration in ethanol, clearing in xylene, and mounting on synthetic balsam. For each of the eight tissue samples, five slides were prepared, one for each antibody, for a total of 40 slides examined.

Western Blotting – WB

Samples

Samples of rectal mucosa, third eyelid, lymph nodes, spleen, and brain were obtained from a male, Texel crossbreed, 60-day-old lamb, which belonged to the Sheep Breeding Department of the Pontifical Catholic University of Paraná (PUCPR). The lamb was subjected to barbiturate overdose (xylazine 0.10 mg/kg I.V. and sodium thiopental 15mg/kg I.V) followed by exsanguination.

Protein extract preparation

Samples from the different tissues were placed in individual homogenization tubes filled with cold lysis buffer (50 mM Tris-HCl pH 7.4; 1% NP40; 0.2% sodium deoxycholate) in a volume five times greater than that occupied by the tissue. The buffer also contained the protease inhibitors PMSF and NEM, along with EDTA (1 mM final concentration). Lysis was carried out for 40 minutes in a cold-water bath. Every 10 minutes, a maceration round (10-15 times) was performed with a glass pistil. After lysis, the samples were centrifuged at 4°C for 30 minutes at 19000 g. The supernatant was set aside, and the sediment was subjected to another round of lysis (with half the initial volume of the lysis buffer), followed by centrifugation. The resulting supernatant was combined with that obtained after the first centrifugation. After determining its protein concentration using the Bradford (1976) method, the extract was used for a WB reaction.

Western blotting

Tissue extract samples were used for one-dimension gel electrophoresis (12% SDS-PAGE – SDS- *polyacrylamide gel electrophoresis*) (SAMBROOK; RUSSEL, 2001). The samples were electro-transferred from the gel to a nitrocellulose

membrane (Hybond-C Extra Amersham Biosciences – GE Healthcare) for one hour, at 100 V in a cold transfer buffer (192 mM glycine; 25 mM Tris; 20% methanol). The membrane was blocked for two hours in TBST (150 mM NaCl; 50 mM Tris-HCl pH 7.4; 0.05% Tween 20) containing 5% skim milk (Molico, Nestlé), and incubated with the primary antibody M52 at a 1:500 dilution in the blocking buffer, for 16 hours at 4°C with constant shaking. After incubation, the membrane was washed for three minutes in TBTS, before adding the mouse secondary antibody, anti-immunoglobulin, conjugated to HRP (*horseradish peroxidase*) (BD Bioscience) at a 1:4000 dilution. The membrane was kept in this incubation solution for one hour at room temperature. A new washing round was performed before incubating the membrane with the substrate appropriate for the chemo-luminescent reaction, using the West Pico kit (Pierce Co.). Autoradiogram films (Amersham Hyperfilm™ ECL – GE Healthcare) were exposed to the membrane until the visualization of the expected bands.

Results and Discussion

Immunohistochemistry

The immunohistochemical examinations using monoclonal antibodies on the five positive encephalon samples confirmed the deposition of PrP^{Sc} in neurons (Figure 1), characterized by intra-cytoplasmic signals with a granular appearance, as also described by Keulen et al. (1995), Thorgeirsdottir et al. (2002), and González et al. (2002). With the F99 antibody (Figure 1A), the immunolabelling was stronger than in slides incubated with the F89 (Figure 1B) and P4 (Figure 1C) antibodies.

Slides incubated with the polyclonal antibodies also showed intra-neuronal positivity but with a more accentuated *background* when the R486 antibody was used (Figure 1D). In this experiment, the same dilution (1:500) was used for all antibodies, which might have caused the increased background observed for the polyclonal antibodies. Working with the R486 polyclonal antibody at a 1:10,000 dilution, González et al. (2002) reported evident positive labeling, without any *background* staining. In addition to the positive intraneuronal signals, Thorgeirsdottir et al. (2002) and Martins et al. (2012) observed labelling in the neuropil and O'Rourke et al. (1998) described the appearance of granules in the neuropil and around the intraneuronal vacuoles, as well as PrP^{Sc} aggregates around the glial cells.

The negative control brain samples tested with the monoclonal antibodies did not show any labeling and only showed some *background* staining. When they were tested with the polyclonal antibodies, intra-neural signals reminiscent of those in the positive samples were identified (Figure 2). It would appear therefore that R486 and M52 can produce some non-specific labelling and could give rise to some false positive diagnosis. The M52 polyclonal antibody was previously tested only for use in WB to identify the PrP^{C} in murine brain (COSTA et al., 2009) but the R486 anti-body was successfully used by González et al. (2002), González, Martin and Jeffrey (2003) and Lezmi et al. (2004) to detect PrP^{Sc} and the same authors also obtained good results with other polyclonal antibodies, such as 521 and 505 antibodies.

Figure 1. Immunohistochemical photomicrographs of the medulla oblongata at the obex from an ovine positive for PrP^{Sc}. (A) F99 antibody showing neurons with granular deposits characteristic of PrP^{Sc} (arrow). (B) F89 antibody, showing intra-neuronal labelling. (C) P4 antibody showing discrete positive immunolabeling (arrows). (D and E) R486 and M52 polyclonal antibodies, respectively, showing some neurons with positive signal (arrows), but with some *background* noise (*). All scale bars: 100 μm.
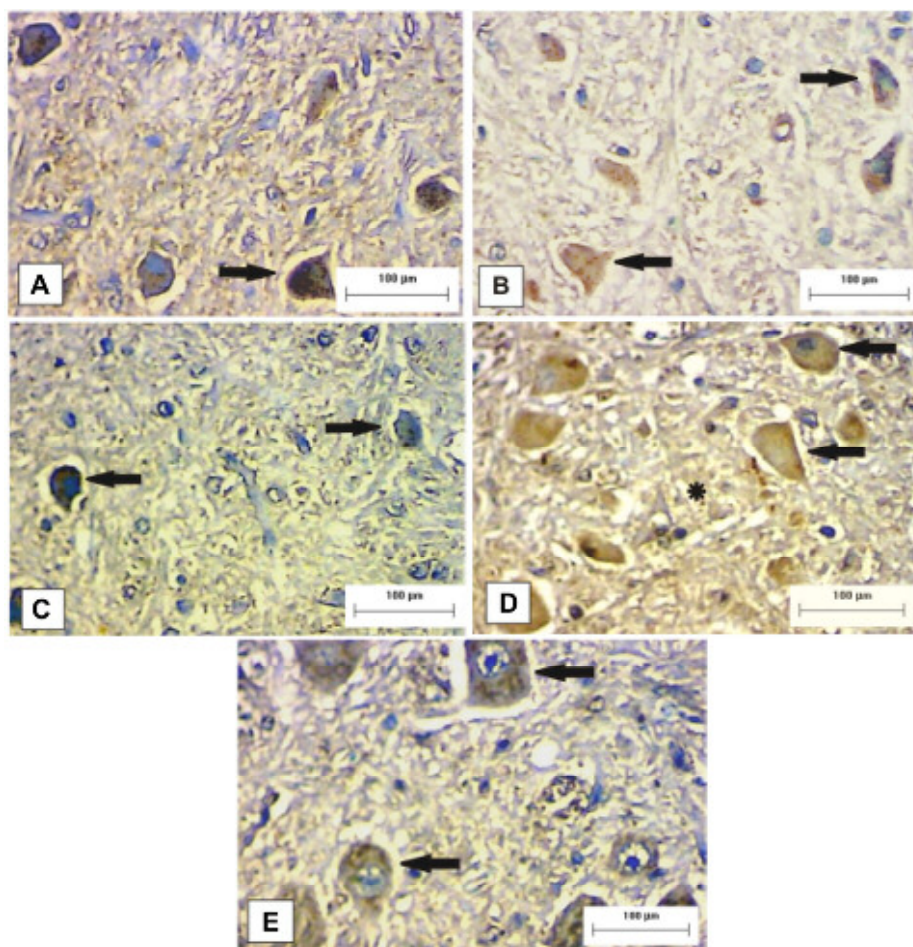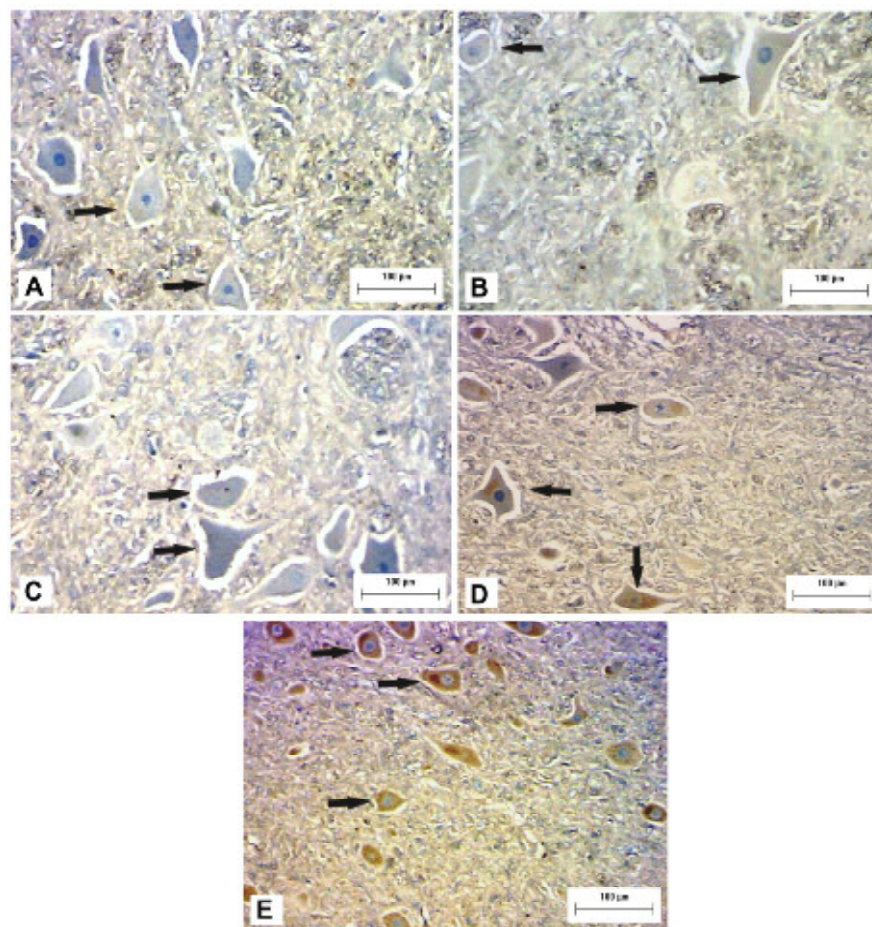

Source: Elaboration of the authors.

For the analyses of lymphoid tissues, the positive sample of palatine tonsil subjected to immunohistochemistry with monoclonal and polyclonal antibodies showed evident labeling in the germinal center of the lymphoid follicles, as described by Leal et al. (2012), with a higher intensity in the light than in the dark zone, as reported by Gomez et al. (2007). With the F99 antibody, the signals were more intense in several areas of the follicles and this anti-body was the one providing the best overall results. With the P4 (Figure 3C) and R486 antibodies (Figure 3D), the labeling was also very evident and sufficient to characterize the sample as positive, as observed by Lezmi et al. (2004). With the antibodies F89 (Figure 3B) and M52 (Figure 3E), the signals were less obvious. O'Rourke et al. (2002) observed the presence of PrP^{Sc} in lymphocytes, macrophages, and dendritic cells in preclinical samples of ovine third eyelid, using a mixture of the F99 and F89 antibodies.

Figure 2. Negative sample of ovine mesencephalon subjected to immunohistochemistry. (A, B and C) All three monoclonal antibodies (F99, F89 and P4, respectively) fail to detect any PrP signal in neurons (arrows). (D and E) R486 and M52 show some intraneuronal signal, which could be confused with those found in positive samples (arrows). All scale bars: 100 µm.
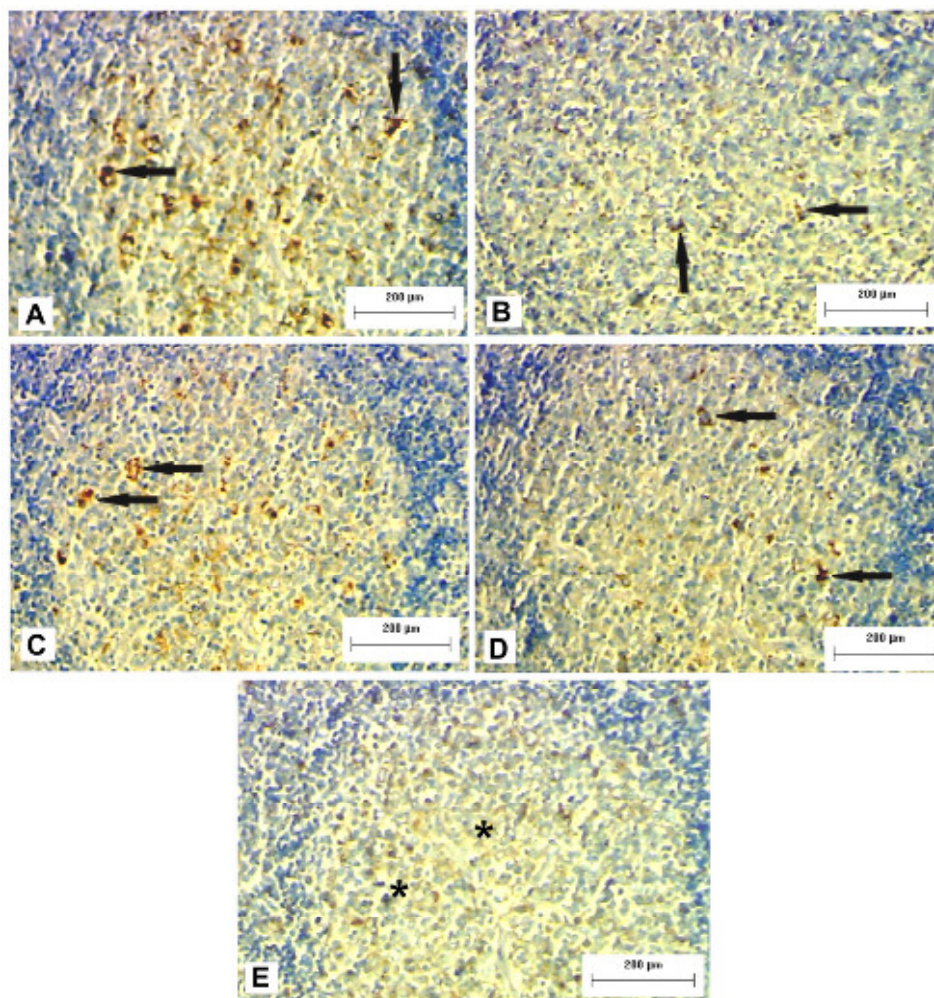

Source: Elaboration of the authors.

The negative palatine tonsil sample tested with the monoclonal antibodies F99 (Figure 4A) and F89 (Figure 4B) showed no immunolabelling and no background, as observed by O'Rourke et al. (2000) in a sample negative for PrP^Sc. The monoclonal antibody P4 (Figure 4C), and the polyclonal antibodies R486 (Figure 4D) and M52 (Figure 4E) also showed the appearance of a negative sample, but with strong *background* staining.

Figure 3. Sample of ovine palatine tonsil from scrapie positive controls incubated with (A) F99 antibody, showing evident brown-colored granular labelling in the follicle (arrows), (B) F89 antibody showing discrete signals (arrows) and (C) P4 antibody showing intense granular labelling (arrows), (D) R486 antibody, showing moderate signals (arrows) and (E) M52 antibody showing intense *background* (*), hindering the identification of specific positive signals. All scale bars: 200 μm.
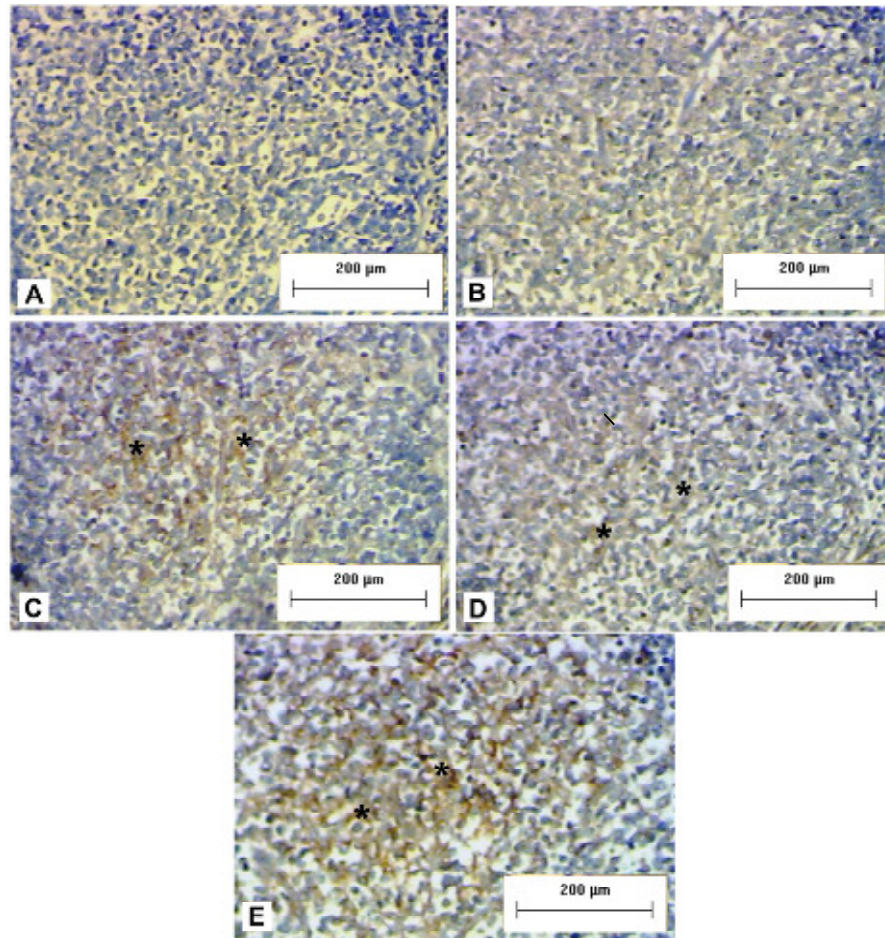

Source: Elaboration of the authors.

The identification of the affected animals in the preclinical period is essential to control scrapie, allowing the elimination of sheep that could be potentially infectious for other animals in the same herd or in other herds (LEAL et al., 2012). Detection of PrP^{Sc} in lymphoid tissue is useful to identify the affected ovine in the preclinical stage of the disease (GONZÁLEZ et al., 2008a). The tonsils, the lymphoid tissues of the third eyelid and the rectal mucosa, as well as the retropharyngeal lymph nodes accumulate significant amounts of PrP^{Sc} that are detectable by IHC at the initial infection stage (O'ROURKE et al., 1998, 2000).

Figure 4. Sample of palatine tonsil from a scrapie negative control sheep. Specific immunolabelling is not detected with any of the antibodies but only those labelled with F99 (A) or F89 (B) are devoid of background, while those incubated with P4 (C), R486 (D) and M52 (E) show different levels of non-specific background (*). All scale bars: 200 μm.

Source: Elaboration of the authors.

The non-specific immunostaining obtained with R486 and M52 polyclonal antibodies in negative brain samples and the *background* signal observed in the negative control palatine tonsil with the same antibodies would advice against their use in the diagnosis of scrapie in sheep and goats under the conditions presented in this study. Although monoclonal antibodies are more expensive than polyclonal antibodies, the former assures diagnosis reliability. False positive results caused by the use of low-efficiency antibodies may result in great losses for the producers, because, according to the NORMATIVE INSTRUCTION No. 15 OF APRIL 2nd, 2008 (BRASIL, 2008), the animals whose samples are positive for scrapie must be sanitarily sacrificed, and production must be halted until further actions carried out in the facilities are concluded.

Western blotting

In the brain and lymphoid tissue samples (spleen, lymph nodes, rectal mucosa, and third eyelid gland) subjected to WB, the M52 antibody was able to recognize the PrP^C present in high amounts in the brain (Figure 5), as reported by Prusiner (1998).

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The antibody in question recognized the antigen in its diglycosylated (35 kDa), monoglycosylated, and non-glycosylated forms. A more tenuous signal was observed in the spleen samples. In the third eyelid and lymph node samples, no labeling could be visualized. The absence of a signal in these samples may result from the low amount of the target protein, which could be outside the detection limit of the WB technique. The presence of PrP^C in these tissues has already been reported (WECHSELBERGER et al., 2002). In the rectal mucosa samples, a band corresponding to ≈50 kDa was detected. Priola et al. (1995) showed the presence of a 60 kDa PrP^C in neuroblastoma cells that expressed the *PrP* gene of hamsters. PrP^C, with a molecular mass of 60 kDa, corresponds to the formation of 30 kDa covalently ligated PrP^C dimers. According to the authors, the PrP^C dimers present characteristics of both protein isoforms, the cellular and the scrapie forms, and may be crucial for the conversion of the cellular isoform into the proteinase K-resistant isoform. Therefore, the results observed in the rectal mucosa samples need to be investigated further.

Figure 5. Samples of ovine cerebral cortex (1), spleen (2), rectal mucosa (3), third eyelid (4), and submandibular lymph node (5) analyzed by western blotting using the PrP M52 primary polyclonal antibody at a 1:500 dilution, and the mouse anti-IgG secondary antibody at a 1:4000 dilution. The band corresponding to the rectal mucosa showed a molecular size of approximately 50 kDa, and the bands corresponding to the spleen and encephalon approx. 35 kDa. The lymph node and third eyelid gland samples were not detected.
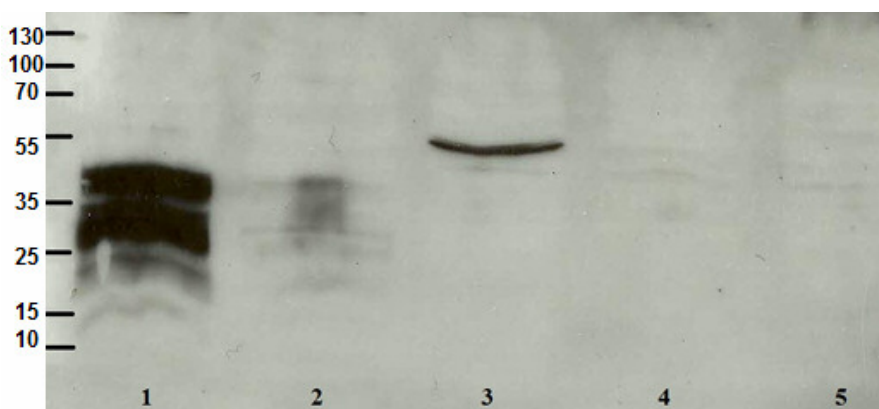
Source: Elaboration of the authors.

Several authors have reported the use of WB to identify the PrP^{Sc} in samples of ovine brain (O'ROURKE et al., 1998; GONZÁLEZ et al., 2006; BILHEUDE et al., 2007). However, for *ante-mortem* diagnostics using samples of lymphoid tissues, this method presents a few limitations, owing to the fact that the harvested sample shows a low amount of lymphoid follicles, thus reducing the chance of obtaining detectable amounts of PrP^{Sc}, and increasing the probability of false negative results (LEAL et al., 2012). Such a situation may have occurred in this study, where no PrP^C was detected in the third eyelid and lymph node samples.

Despite the polyclonal antibodies presenting a more affordable solution than the monoclonal antibodies, and despite the fact that the M52 antibody is being produced in Brazil, one can assert that under the conditions of this work, the use of polyclonal antibodies would not be suitable, because the corresponding analyses led to non-reliable results. The use of the M52 antibody to identify the PrP^C in encephalon and spleen samples using WB proved to be efficient, but it could not be detected in some tissues of the lymphoid system.

Aknowledgements

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