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PATHOLOGY

Use of immunohistochemistry and prion protein gene genotyping for detection of Scrapie in sheep in the state of Santa Catarina, Brazil

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ABSTRACT: Scrapie is a contagious disease of sheep and goats caused by prions (PrP^{Sc}). This study described an outbreak of Scrapie in sheep in the state of Santa Catarina, Brazil. An 1-year and 3-month-old sheep developed clinical signs characterized by motor incoordination of the pelvic limbs, pruritus and alopecia for three days. The 38 sheep from the flock that were over 1 year of age underwent biopsies of the third eyelid and rectal mucosa, in addition to anti-PrPsc immunohistochemistry (IHC). Blood containing EDTA was collected for PRNP gene genotyping from these sheep. Of the 38, 16 (42.10%) had immunostaining against PrP^{Sc}. IHC-positive animals were euthanized and necropsied, as well as lambs from positive mothers. Different organs of the 19 necropsied animals were collected in 10% buffered formalin for histopathological examination and anti-PrP^{Sc} IHC of the obex. The histopathology of the obex of the female with neurological signs presented discrete multifocal vacuolization of the cytoplasm of neurons and neuropil. The anti-PrP^{Sc} IHC showed that two out of the 19 obex samples had cytoplasmic immunostaining in neurons. The genotypes reported were ARQ/ARQ in 47.36%, ARR/ARQ in 36.84%, ARQ/VRQ in 10.52% and ARQ/VRR in 5.28%. The genotyping helps to identify susceptible animals and select animals more resistant to the development of Scrapie. The anti-PrP^{Sc} IHC from lymphoid biopsies, and genotyping demonstrated the high number of positive sheep classified in susceptible group.
Key words: enzootic paraplexy, prion disease, small ruminants.

Uso da imuno-histoquímica e genotipagem do gene da proteína priônica para detecção de Scrapie em ovinos no estado de Santa Catarina, Brasil

RESUMO: Scrapie é uma doença contagiosa de ovinos e caprinos causada por príons (PrP^{Sc}). O objetivo desse estudo é descrever um surto de Scrapie em ovinos no estado de Santa Catarina, Brasil. Uma ovelha de 1 ano e 3 meses desenvolveu sinais clínicos caracterizados por incoordenação motora dos membros pélvicos, prurido e alopecia durante três dias. Os 38 ovinos do rebanho que tinham idade acima de 1 ano foram submetidos a biópsias de terceira pálpebra e mucosa retal, além de imuno-histoquímica (IHQ) anti-PrP^{sc}. Coletou-se sangue contendo EDTA para genotipagem do gene prnp destes ovinos. Dos 38 ovinos, 16 (42,10%) apresentaram imunomarcação na avaliação IHQ anti-PrP^{sc}. Os animais positivos na IHQ foram eutanasiados e necropsiados, bem como os cordeiros das mães positivas. Diferentes órgãos dos 19 animais necropsiados foram coletados em formalina tamponada a 10% para exame histopatológico e IHQ anti-PrP^{sc} do óbex. Na histopatologia do óbex da fêmea com sinal neurológico havia vacuolização do citoplasma de neurônios e neurópilo multifocal discreta. Na IHQ anti-PrP^{sc} das 19 amostras de óbex, dois apresentaram imunomarcação citoplasmática em neurônios. Os genótipos encontrados foram ARQ/ARQ em 47,36%, ARR/ARQ em 36,84%, ARQ/VRQ em 10,52% e ARQ/VRR em 5,28%. A genotipagem auxilia a identificar os animais susceptíveis e seleciona animais mais resistentes ao desenvolvimento do Scrapie. A IHQ anti-PrP^{sc} de biópsias de tecidos linfoides e a genotipagem demonstram o elevado número de ovinos positivos classificados no grupo susceptível.
Palavras-chave: paraplexia enzoótica, doença priônica, pequenos ruminantes.

INTRODUCTION

Scrapie is a neurodegenerative, progressive, and fatal transmissible spongiform encephalopathy, which naturally affects sheep and goats with progressive neuronal loss and a long incubation period (PRUSINER, 1991). The causative agent of the disease is a prion, which corresponds to an altered form of the normal prion protein (PrP^{c}) (PRUSINER, 1982). The abnormal isoform of cellular prion protein (PrP^{sc}) is characterized by deposition in several cell types of the host, mainly in the central nervous system and lymphoid tissues, forming protein aggregates, which are responsible for neurodegenerative disorders (ANDREOLETTI et al., 2000).

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The first diagnosis of the disease in Brazil occurred in Rio Grande do Sul in 1978 in a Hampshire Down sheep (FERNANDES et al., 1978). Since then, the disease has been reported in Mato Grosso do Sul, São Paulo, Paraná, Bahia, and Santa Catarina (OIE-WAHIS, 2021). The first cases in the State of Santa Catarina were diagnosed in 2011, followed by four outbreaks in 2012, 2017, and 2019 (OIE-WAHIS, 2021).

The degree of genetic susceptibility to the development of scrapie is considered important in the selection of animals resistant to the disease. The classification can vary from 1 to 5, where group 1 is considered extreme resistance to scrapie and 5 is high susceptibility (USDA-APHIS, 2010). Dorper, White Dorper and animals crossed with these breeds are associated with the highest genetic susceptibility (ANDRADE et al., 2015). Other breeds that are associated with genetic susceptibility are the Santa Inês, that is the most bred breed in Brazil (MCMANUS et al., 2010; IANELLA et al. 2012), Suffolk (HAMIR et al., 2005), Cheviot and Poll Dorset (HOUSTON et al., 2015).

Monitoring the disease and also susceptible genotypes are important available tools to prevent the prion spread (DAWSON et al., 2008). The diagnosis of the pre-clinical disease is performed by collecting a biopsy of the third eyelid or rectal mucosa because these places present lymphoid follicles where the prion protein accumulates and; therefore, an early diagnosis can be obtained (LEAL et al., 2012).

This study aimed to describe an outbreak of scrapie in sheep in the state of Santa Catarina, Brazil, detected through ante-mortem and post-mortem diagnoses, in addition to categorizing sheep according to the degree of susceptibility and resistance through genotyping of the prion protein gene (PRNP).

MATERIALS AND METHODS

A technical visit was made to a property located in the municipality of Pouso Redondo, with a flock of approximately 60 Dorper and crossbred sheep, requested veterinary care in July 2017 after noticing neurological signs three days before in a female of the flock.

Biopsies were collected from the third eyelid and rectal mucosa for *ante mortem* diagnosis due to suspected scrapie 38 sheep over one year old. The animals were restrained and subjected to a local anesthetic block to obtain the samples. In addition, blood was collected by venipuncture using Vacutainer® tubes containing EDTA. The third eyelid and rectal mucosa fragments were placed in 10% buffered formalin and the whole blood was refrigerated. The samples were sent to the Setor de Patologia Veterinária, Universidade Federal do Rio Grande do Sul (SPV-UFRGS), where they were submitted to anti-PrP^{Sc} immunohistochemistry (IHC) examination (LEAL et al., 2012). Euthanasia and necropsy were performed on positive animals, in addition to lambs from positive mothers.

Sheep over one year old positive in immunohistochemistry lambs from positive mothers underwent genotyping of the prnp gene. Genetic polymorphism analysis was performed by determining the allelic frequency of codons 136, 154, and 171 of the prion protein gene (ANDRADE et al., 2011), which allowed classifying the animals according to the degree of susceptibility and resistance to scrapie according to the National Scrapie Surveillance Plan table (USDA-APHIS, 2010).

All animals that were euthanized and necropsied had brain, spinal cord, pharyngeal tonsil, lung, heart, liver, mesenteric lymph node, ileum, kidney and spleen collected in 10% buffered formalin for routine histopathological examination and hematoxylin and eosin (HE) staining. In addition, anti-PrPSc IHC was performed for the brain stem (obex) of all necropsied sheep.

RESULTS

The female sheep was examined, and the suspicion of scrapie was based on the neurological evaluation. Therefore, the property was quarantine by the official state agency, Companhia Integrada de Desenvolvimento Agrícola de Santa Catarina (CIDASC).

The sheep that developed a neurological condition was an 1-year and 3-month-old female, four months pregnant. The physical examination showed a lean animal that ate and drank water normally; however, had difficulty staying in the standing position, incoordination in the pelvic limbs, and remaining in sternal recumbency, in addition to pruritus and areas of bilateral alopecia on the lateral face of the thigh, remaining with these signs for 11 days until slaughter (Figure 1A). Other five sheep of flock had pruritus and areas of alopecia in the lateral region of the thorax and bilateral abdomen (Figure 1B). These animals were positive in the test of manually scratching that responded with “nibble” reflex, totaling six animals with these clinical signs. The outbreak occurred during the calving period and there was no maternity pen. Therefore, several positive animals

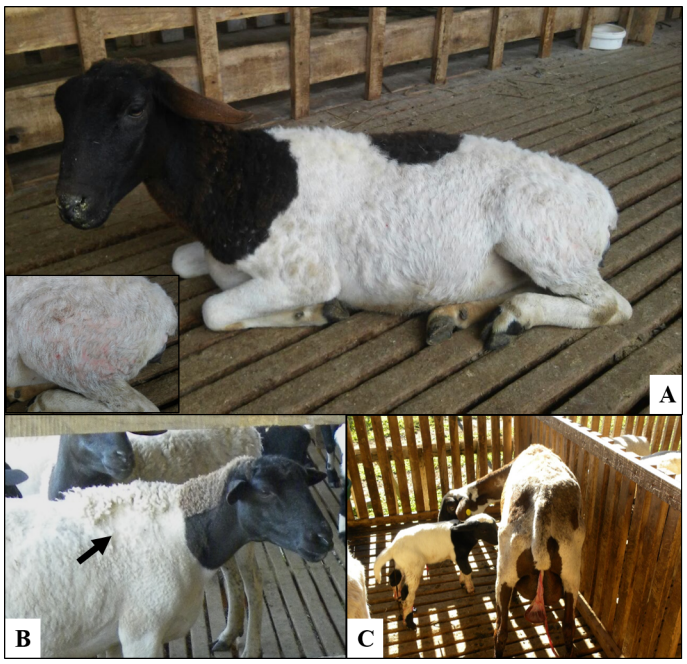

Figure 1 - Scrapie outbreak in sheep. (A) One-year and three-month-old crossbred Dorper female sheep in sternal recumbency. The detail shows an area of alopecia on the lateral face of the thigh. (B) Adult crossbred Dorper female sheep with areas of alopecia in the lateral region of the thorax (arrow). (C) Adult Dorper crossbred female sheep positive for scrapie with the placenta and uterine secretions in contact with other sheep.

gave birth, and the others came into contact with the placenta and uterine secretions (Figure 1C).

The 38 evaluated animals ranged in age from one to six years, consisting of 35 females (92.10%) and three males (7.89%). Sixteen sheep (42.10%) were immunostained with anti-PrP^{Sc}, with 14 (87.5%) showing staining in at least three lymphoid follicles of the third eyelid and rectal mucosa and two (12.5%) only on the third eyelid (Figure 2). Six out of 14 positive animals in the anti-PrP^{Sc} IHC assessment of the three lymphoid follicles of the third eyelid and rectal mucosa for scrapie developed these characteristic signs of pruritus and wool loss. The female with neurological signs also showed immunostaining and was euthanized on the 11th day of disease evolution.

A necropsy was performed on 14 positive animals, as two of them had died naturally between the collection period and euthanasia, in addition to five lambs from positive mothers. No significant changes were observed during the macroscopic evaluation, only body condition varying from regular to poor and areas of alopecia in the thoracic and abdominal regions in six (42.85%). Histologically, the only finding was observed in the obex of the female that developed clinical sign, characterized by discrete multifocal vacuolization of the neuropil (Figure 3A).

The anti-PrP^{Sc} IHC of the 19 brain stem (obex) samples collected post-mortem showed that two animals had moderately immunostaining in the cytoplasm of neurons in the region (Figure 3B), one of them consisting of the female with neurological signal. In addition, these two IHC-positive animals were also reported to have ARQ/ARQ and ARQ/VRQ genotypes. These genotypes are associated with mild resistance and high susceptibility to scrapie development, respectively. Table 1 shows the genotypic analyses of PRNP.

DISCUSSION

Sheep and goats older than 12 months of age that present neurological signs for more than 15 days are considered to be animals with clinical suspicion

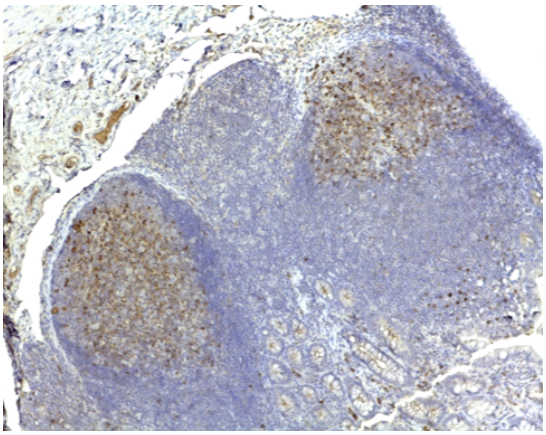

Figure 2 - Scrapie outbreak in sheep: One-year and three-month-old crossbred Dorper female sheep. Rectal mucosal biopsy with moderate immunostaining in lymphoid follicles for PrP^{Sc}. Obj. 10.

of scrapie (MAPA, 2008). In the present outbreak, the sheep had only three days of neurological signs at the time of clinical evaluation and interdiction of the property. Considering the well-founded clinical suspicion, the property must be quarantine to prevent the entry and exit of sheep and consequently, prion propagation (MAPA, 2008). One of the hallmarks of the disease that can assist in the diagnosis is the test of manually scratching the back of the animal, which will respond with a "nibbling" reflex. Sheep usually lose weight, present pruritus, and wool loss (MARTINS et al., 2012). Six positive animals in the anti-PrP^{Sc} IHC developed these characteristic signs. One sheep developed clinical neurological signs and pruritus and other five sheep showed only pruritus and areas of alopecia. The scrapie incubation period in most cases is higher than one year and cases occur between 2 and 5 years of age (OIE, 2018) and may also be correlated with the amount of PrP^{Sc} deposition and last from 10 days to months (CASSMANN et al., 2019).

The prion is believed to be transmitted horizontally and orally to animals. Furthermore, the placenta harbors the prion, thus becoming a potential risk of dissemination (HUNTER, 2003). Spread via uterine discharge leads to contamination of the environment where the lamb is more than once in a prolonged period of clinical latency. The age incidence for the development of scrapie is 2 to 4 years, but a minority of cases occur in older sheep (DAWSON et al., 2008). In this outbreak, the contact of the sheep with the placental remains was remarkable due to the absence of maternity pens, thus contributing to the prion propagation and the development of the vast majority of cases in young animals (under 2 years of age).

The ante-mortem diagnosis of scrapie through PrP^{Sc} detection by IHC in lymphoid tissues is considered effective to detect positive sheep, as clinical manifestations may not occur in carrier animals (LEAL et al., 2012). The use of lymphoid tissue from the third eyelid has a high specificity and sensitivity for preclinical tests (O'ROURKE et al., 2000). However, the suspicion of the disease is not eliminated when the result is negative, making

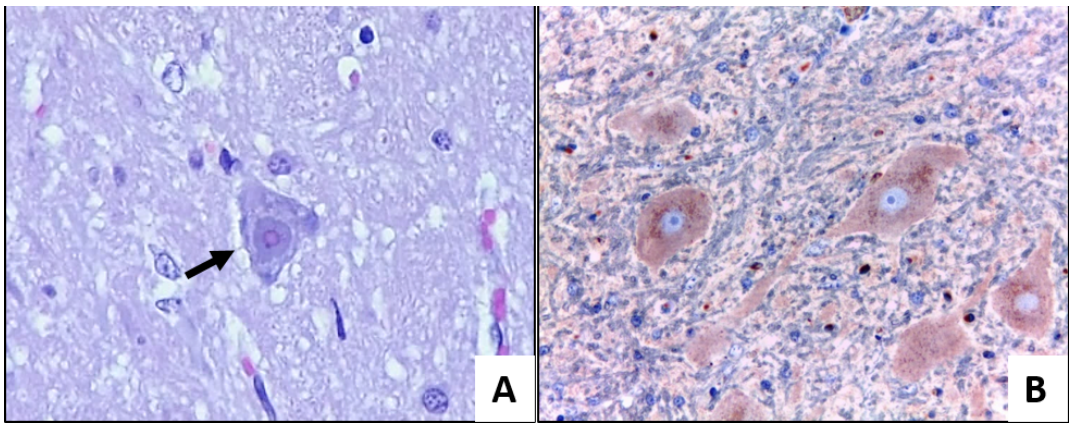

Figure 3 - Scrapie outbreak in sheep: Brain stem (obex) of one-year and three-month-old crossbred Dorper female sheep. (A) discrete multifocal vacuolization of the neuropil (arrow). HE Obj. 40. (B) moderate immunostaining for PrP^{Sc} in the cytoplasm of neurons and neuropil. Obj. 40.

Table 1 - Scrapie outbreak in sheep in the state of Santa Catarina. Distribution and classification of PRNP gene genotypes according to the National Scrapie Surveillance Plan (USDA), 2010.

Associed scrapie risk		R1	R2	R3	R4	R5	Total
Genotype		-	ARR/ARQ	ARQ/ARQ	ARQ/VRR	ARQ/VRQ	-
Number of animals (%)		-	7 (36.8%)	9 (47.4%)	1 (5.3%)	2 (10.5%)	19 (100%)
Age	0-11 m	-	2	3	-	-	5
	12-23 m	-	1	1	-	-	2
	24-35 m	-	2	2	-	1	5
	36-47 m	-	2	1	1	1	5
	> 47 m	-	-	2	-	-	2
Total		0	7	9	1	2	19

m = months.

periodic monitoring of suspected animals necessary due to the long incubation period. A wide disease spread was demonstrated in the presented outbreak when the evaluation by anti-PrP^{Sc} IHC was carried out, with almost half of the flock affected, and 87.5% of the positive animals had immunostaining in the lymphoid tissue of the third eyelid and rectal mucosa. An outbreak recorded in the state of Rio Grande do Sul in a flock of 318 sheep showed a positivity of 5.98% for PrP^{Sc} IHC in the third eyelid, with no animals positive in the lymphoid tissue of the rectal mucosa (LEAL et al., 2012).

The studied property had no sanitary control of the purchase and sale of animals nor is the genotyping of the acquired animals requested. The ewes of this outbreak were not separated into paddocks or farrowing pens, and the excretion of the prion in the environment might have occurred through placental remains, which have a large PrP^{Sc} accumulation, being an important factor to be considered in the spread of scrapie to animals that have high susceptibility (SCHNEIDER et al., 2015).

As described in other outbreaks in Brazil and the world, the disease occurred without an apparent macroscopic lesion. Loss of wool and weight can be related but are not characteristic of the disease (MARTINS et al., 2012; ESTEVES et al., 2021). Histological lesions in classic scrapie are at the brain stem (obex), characteristic of vacuolization of neurons and neuropil without inflammatory reaction, as described in this study. However, these lesions have also been described to a lesser extent in the midbrain, pons, medulla oblongata, and lateral and ventral horns of the spinal cord (MARTINS et al., 2012). There is no established pattern in the distribution of atypical scrapie lesions, but lesions in the cortex and cerebellum without changes in the obex have been described (GREENLEE, 2019).

Importantly, only one animal in this outbreak, had characteristic lesions in the obex. This intensity is uncommon, as usually the animals have very characteristic and evident neuronal or neuropil vacuolization in the obex in outbreaks, as observed in some studies in Brazil (MARTINS et al., 2012; ANDRADE et al., 2015). Fourteen sheep were positive by the IHC in the lymphoid tissues, however, only two animals that were positive for IHC in the obex. In a study carried out in Rio Grande do Sul sheep with immunostaining of the third eyelid did not show immunostaining in the central nervous system or the rectal mucosa and might be considered asymptomatic even with accumulation of PrP^{Sc} in the brain and other tissues (LEAL et al., 2012).

So far 15 haplotypes are known in sheep; the most resistant is the ARR allele whereas the most susceptible is the VRQ allele (USDA-APHIS, 2010). Two out of the 19 animals that underwent genetic analysis presented the ARQ/VRQ genotype, considered the most susceptible to scrapie development and no animal had the high resistance genotype. This fact must be considered of great importance, as it demonstrates that the genetic selection of these animals does not yet occur in Brazil. In Europe, selection practices by genotyping between 2002 and 2006 have been efficient in eliminating susceptible animals and increasing extremely resistant animals (DAWSON et al., 2008), which may help in the future to reduce animals with the disease.

The National Scrapie Plan for Great Britain is a voluntary program started in 2001 and

advocates the eradication of the VRQ allele and encourages positive selection for the ARR allele, while sheep with the VRQ allele are euthanized or castrated (DAWSON et al., 2008).

Dorper sheep are considered highly susceptible to scrapie in the South of Brazil (SOTOMAIOR et al., 2008; IANELLA et al., 2012; ANDRADE et al., 2015). In previous study reported the ARQ/ARR genotype to be the second most frequent (ANDRADE et al., 2015), as in the present study. The third most observed genotype was ARQ/VRQ, similar to that found by ANDRADE et al. (2015); however, unlike two other studies that observed an occurrence higher than 20% (IANELLA et al., 2012) and 22% (SOTOMAIOR et al., 2008). The ARQ/VRR genotype, considered rare (BARAITAREANU et al., 2013) was observed in 5.26% of the evaluated sheep, similar to that found by ANDRADE et al. (2015) and SOTOMAIOR et al. (2008). The largest number of genotypes were classified between the light and high resistance groups, as well as the data reported in other studies with animals of the same breed in Brazil (SOTOMAIOR et al., 2008; ANDRADE et al., 2015).

Genotyping allows identifying resistant animals, which is an extremely important measure to prevent this disease (ALARCON et al., 2021). It also allows the selection of males and females carrying at least one resistant allele and its use in crosses to increase the frequency of resistance alleles, as recommended by selection programs for genetically resistant animals, considering that they do not transmit the infectious prion (GUBBINS & RODEN, 2006; USDA-APHIS, 2019).

CONCLUSION

In this outbreak had a high number of animals positive for PrP^{Sc}, these sheep genotyped were classified in mild resistance to high susceptibility, showing the importance of surveillance of this disease. The clinical picture observed in a sheep associated with PrP^{Sc} IHC from lymphoid tissue biopsies were effective in confirming the scrapie diagnosis.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS’ CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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