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CASE REPORT
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Classical Scrapie Diagnosis in ARR/ARR Sheep in Brazil

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ABSTRACT

***Background*:** Scrapie is a transmissible spongiform encephalopathy (TSE) that affects sheep flocks and goat herds. The transfer of animals or groups of these between sheep farms is associated with increased numbers of infected animals and with the susceptibility or the resistance to natural or classical scrapie form. Although several aspects linked to the etiology of the natural form of this infection remain unclarified, the role of an important genetic control in scrapie incidence has been proposed. Polymorphisms of the PrP gene (prion protein, or simply prion), mainly in codons 136, 154, and 171, have been associated with the risk of scrapie.

***Case*:** One animal from a group of 292 sheep was diagnosed positive for scrapie in the municipality of Valparaíso, state of São Paulo, Brazil. The group was part of a flock of 811 free-range, mixed-breed Suffolk sheep of the two genders and ages between 2 and 7 years from different Brazilian regions. Blood was collected for genotyping (for codons 136, 141, 154 and 171), and the third lid and rectal mucosa were sampled for immunohistochemistry (IHC) for scrapie, from all 292 animals of the group. IHC revealed that seven (2.4%) animals were positive for the disease. Collection of samples was repeated for 90 animals, among which the seven individuals diagnosed positive and 83 other animals that had some degree of kinship with those. These 90 sheep were sacrificed and necropsied, when samples of brain (obex), cerebellum, third eyelid, rectal mucosa, mesenteric lymph node, palatine tonsil, and spleen were collected for IHC. The results of IHC analyses carried out after necropsy of the seven positive animals submitted to the second collection of lymphoreticular tissue and of the 83 animals with some degree of kinship with them confirmed the positive diagnosis obtained in the first analysis, and revealed that three other sheep were also positive for scrapie. Samples of 80 animals (89%) were negative for the disease in all organs and tissues analyzed. In turn, 10 sheep (11%) were positive, presenting immunoreactivity in one or more tissues. Genotyping revealed the presence of four of the five alleles of the PrP gene commonly detected in sheep: ARR, ARQ, VRQ and ARH. These allele combinations formed six haplotypes: ARR/ARR, ARR/ARQ, ARH/ARH, ARQ/ARH, ARQ/ARQ and ARQ/VRQ. Animals were classified according to susceptibility to scrapie, when 8.9% of the genotyped sheep were classified into risk group R1 (more resistant, with no restriction to breeding). In turn, 40% of the animals tested ranked in groups R4 and R5 (genetically very susceptible, cannot be used for breeding purposes).

***Discussion*:** The susceptibility of sheep flocks depends on the genetic pattern of animals and is determined by the sequence of the gene that codifies protein PrP. Additionally, numerous prion strains are differentiated based on pathological and biochemical characteristics, and may affect animals differently, depending on each individual's genotype. Most epidemiologic data published to date indicate that animals that carry the ARR/ARR genotype are less susceptible to classical scrapie. However, in the present study, the fact that two scrapie-positive sheep presented the haplotype ARR/ARR indicates that this genotype cannot always be considered an indicator of resistance to the causal agent of the classical manifestation of the disease. The coexistence in the same environment of several crossbred animals from different flocks and farms, which characterizes a new heterogeneous flock, may have promoted a favorable scenario to spread the disease, infecting animals in the most resistant group.

Keywords: biopsy, scrapie, TSEs, immunohistochemistry.

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INTRODUCTION

Scrapie, also called epizootic tremor, is a transmissible spongiform encephalopathy (TSE) that affects sheep flocks and goat herds [44]. The relocation of animals to and from sheep farms has been associated with increased numbers of infected animals [28, 39]. Once it is introduced in a flock, the disease may be transmitted both vertically, from ewe to lamb, and horizontally, across animals [15, 39, 49]. Many aspects surrounding the etiology of the natural form of this infection remain to be clarified, though the existence of an important genetic control has been proposed to explain the disease's incidence [24]. The analysis of the gene PrP (prion protein, or simply prion) in ovine of different breeds has drawn attention to the interaction between host genotype polymorphisms and susceptibility to the infectious agent of scrapie [10, 21-23, 31].

Single nucleotide polymorphisms (SNT) have been linked to susceptibility or resistance to classical scrapie. These polymorphisms occur at codons 136 (A or V, alanine or valine), 154 (R or H, arginine or histidine) and 171 (R, Q or H, arginine, glutamine or histidine) [16]. The diagnosis of the classical form in sheep with haplotype A136R154R171 is rare [24]. Under natural exposure conditions, this genotype (ARR/ARR) has been acknowledged as having the lowest risk for the classical form [16]. This case report describes the occurrence of an outbreak in a flock of mixed Suffolk sheep of varied origins in the state of São Paulo, southeastern Brazil, when the disease was diagnosed in two animals carrying the genotype ARR/ARR, compatible with classical scrapie.

CASE

In 2011, one ovine head from a group of 292 animals was diagnosed with the classical form of scrapie. These sheep were part of a larger flock of 811 free-range animals of both genders and between 2 and 7 years of age that were brought from southern, southeastern and midwestern Brazil. Since the animal died, and diagnosis was carried out after the death, a decision was made to collect blood samples from all 292 animals of the group, for sequencing and genotyping (for codons 136, 141, 154 and 171). In addition, the third eyelid and the rectal mucosa of all 292 animals were biopsied for immunohistochemistry (IHC). After IHC, a new collection was conducted in 90 animals (approximately 30% of the original group). These included the animals with positive diagnosis in the first collection, and those that had some degree of kinship with scrapie-positive sheep in the original group. These animals were sacrificed and necropsied to collect brain tissue (obex), cerebellum, third eyelid, rectal mucosa, mesenteric lymph node, palatine tonsil, and spleen used in the IHC analyses.

Tissue samples were collected and processed for histology and IHC for PrP^{Sc} following the methodology proposed by O'Rourke *et al*. [43]. Rectal biopsy samples were collected and processed according to Espenes *et al*. [17]. Anti-prion[1] monoclonal antibodies F89/160.1.5 and F99/97.6.1 were diluted to a 1:500 solution and added to samples, which were then incubated in a humid chamber at 4ºC for 12 h [34].

Blood was collected by punction of the jugular vein using EDTA as anticoagulant and stored at -20ºC for subsequent processing. Genomic DNA of sheep was extracted using 500 µL whole blood and the QIAmp™ DNA Blood Kit[2] according to the manufacturer's instructions. PCR was carried out using the DNA sample, 15 pmol each primer, 1X PCR buffer (Tris-HCl pH 8.4, 50 mM KCl)[3], $MgCl_2$ 1.5 mM, dNTP[4] 200 µM, and 1U Platinum™ enzyme Taq DNA Polymerase[3] according to the following cycles: 95ºC for 5 min, 35 cycles at 95ºC for 30 s and at 58ºC for 30 s, and 72ºC for 30 s. PCR was performed using a forward primer flanking the 136 codon position (5'-ATGAAGCATGTGGCAGGAGC-3') and a reverse primer flanking the 171 codon position (5'-GGTGACTGTGTGTTGCTTGACTG-3'). A 245-bp fragment was generated, which contains the regions of the main codons analyzed for susceptibility to scrapie [36].

The PCR product was purified and quantified using the commercial products Purelink™[5] and Qubit™[5], respectively, following the manufacturers' instructions. Sequencing was performed with 3 ng DNA and 3.2 pmol each primer, using the BigDye Terminator v.1.1 Cycle Sequencing kit[6] in the ABI PRISM 3110 Genetic Analyzer[6].

Of the 292 mixed Suffolk sheep whose lymphoreticular tissues of the third eyelid were analyzed by IHC, seven (2.4%) were positive for scrapie in the first sample collection.

The IHC results of the second samples collected from these seven sheep after necropsy and of the samples collected from the other 83 animals with some degree of kinship with them confirmed the

positive diagnosis obtained initially, and revealed that three other animals were also positive for the scrapie. The samples of all organs and tissues of 80 animals (89%) were negative, while those of 10 sheep (11%) were positive, with immunoreactivity in one or more tissues.

At least three lymphoid follicles were analyzed by IHC in all samples obtained from necropsied animals. No animal was positive in all samples collected, but different organs and tissues showed immunoreactivity. The third eyelid (Figure 1) and the palatine tonsil were the tissues with the highest percentage of immunoreactive samples (90%, 9/10). The lymphoid tissue of the rectal mucosa (Figure 2) showed immunoreactivity in only one animal (10%, 1/10). No immunoreactivity was observed in mesenteric lymph node, spleen and obex samples.

Genotyping of codon 141 showed homozygosis for lysine (L141L or L/L) in all 90 animals investigated. The genotypes and frequencies of alleles for codons 136, 154 and 171 of these sheep (10 positive and 80 related) are shown in Table 1.

Four of the five alleles of the PrP gene commonly detected in ovine were found: ARR, ARQ, VRQ and ARH. The allele AHQ was not detected in any sample. Of the 15 possibilities, these allele combinations formed six haplotypes: ARR/ARR, ARR/ARQ, ARH/ARH, ARQ/ARH, ARQ/ARQ and ARQ/VRQ.

The haplotype ARR/ARQ was detected in 39 samples (43.3%) and was the most frequent, followed by haplotypes ARQ/ARQ, detected in 34 (37.7%), ARR/ARR, present in eight (8.9%), and ARQ/ARH, observed in five samples (5.6%). Haplotypes ARH/ARH and ARQ/VRQ were detected in two samples each (2.2%). The classification of animals according to the susceptibility criteria described by Dawson *et al*. [13] placed 8.9% of the total number of genotyped animals in scrapie risk group R1, which includes more resistant animals that are not subject to reproduction restrictions. A significant percentage of animals (43.3%) was in risk group R2, which requires careful selection for breeding. In addition, 7.8% of animals were in group R3 (intermediate risk), while 40% were in groups R4 and R5 (highly susceptible animals that should not be included in reproduction programs).

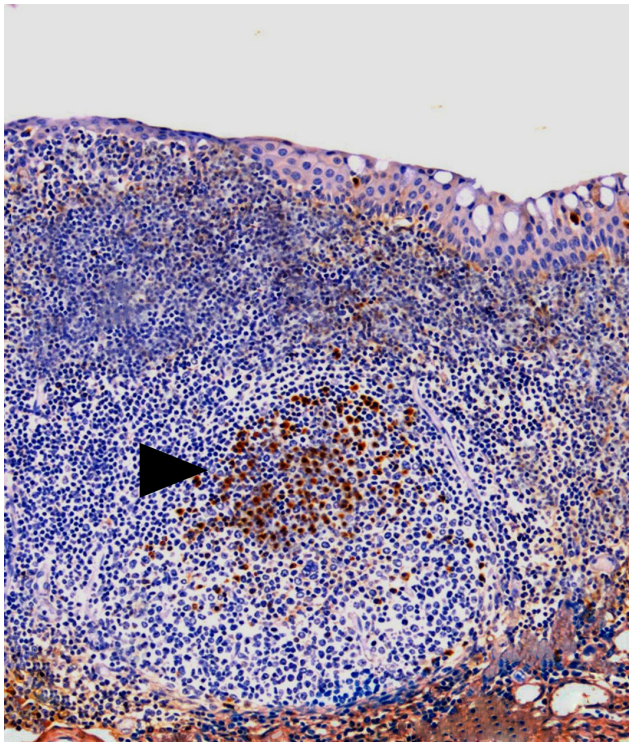

Figure 1. Immunohistochemistry to diagnose scrapie in a histologic section of the third eyelid of a sheep. Lymphoid follicle with immunoreactivity for PrPSc in the germinative center (arrow head). [Magnification: 400x].

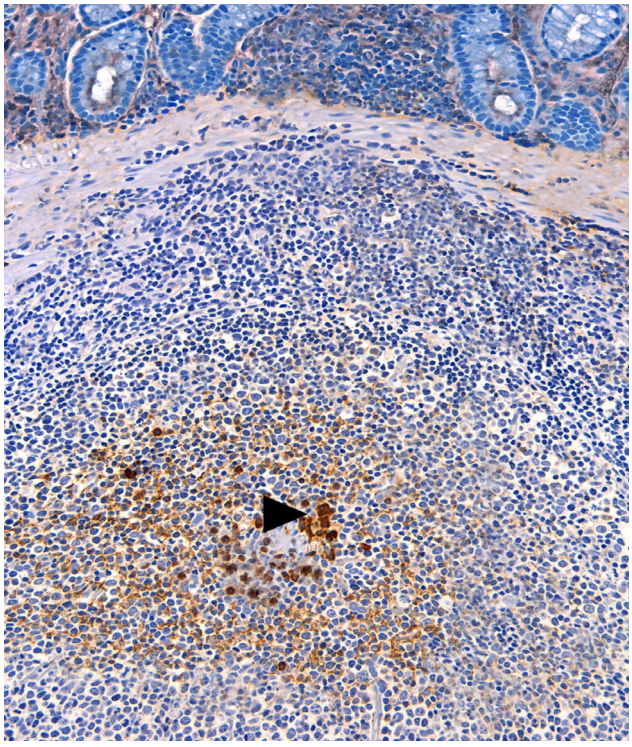

Figure 2. Immunohistochemistry to diagnose scrapie in a histologic section of the rectal mucosa of a sheep. Lymphoid follicle with immunoreactivity for PrPSc in the germinative center (arrow head). [Magnification: 400x].

Table 1. Immunohistochemistry results for different genotypes of codons 136, 154 and 171, and scrapie risk category of 90 Suffolk sheep

Genotype	Animals n	Risk	Immunohistochemistry	
			Positive n (%)	Negative n (%)
ARR/ARR	8	R1	2 (2.2%)	6 (6.7%)
ARR/ARQ	39	R2	2 (2.2%)	37 (41.1%)
ARH/ARH	2	R3	1 (1.1%)	1 (1.1%)
ARQ/ARH	5	R3	0 -	5 (5.6%)
ARQ/ARQ	34	R4	4 (4.4%)	30 (33.3%)
ARQ/VRQ	2	R5	1 (1.1%)	1 (1.1%)
Total	90	-	10 (11.1%)	80 (88.9%)

DISCUSSION

The susceptibility of sheep flocks to scrapie depends largely on the genetic pattern of the animal, and is determined mainly by the sequence of the gene that codifies the PrP protein, since there are several polymorphisms that affect the conversion of the cell protein PrP^{C} to its pathological form, PrP^{Sc} [8, 9]. Nevertheless, it is not possible to consider the occurrence of only one form of ovine prion, since there are numerous prion strains with different pathological and biochemical characteristics that may affect animals distinctively, depending on their genotypes [1, 30].

In the present study, the frequency of codon VRQ was very low (2.2%), confirming previous findings, which revealed that the alleles ARR and ARQ prevail in Suffolk sheep, and that the allele ARH sometimes is detected [12, 32]. The high sensitivity of homozygous VRQ carriers or of individuals with ARQ haplotypes has also been reported in the literature [24]. This condition raises concerns about susceptibility from the epidemiological perspective, since the allele VRQ, which is rare or absent in breeds like Suffolk, was present in two animals, one of which was positive for scrapie.

Most epidemiological and genetic data published indicate that sheep carrying the haplotype ARR/ARR are less susceptible to classical form, while animals with the haplotype VRQ in homozygosis or with ARQ haplotypes are highly susceptible [24]. This hypothesis is supported by genotyping data for thousands of sheep with the disease around the world. For example, a study carried out in Japan described a classical scrapie case in one ARR/ARR sheep [16]. Sensitivity of ARR/ARR sheep in a scenario of oral exposure to the disease has also been reported [3]. Atypical cases were observed in ARR/ARR animals [11, 42].

Polymorphisms at codon positions 136, 154 and 171 are not the only ones associated with resistance or susceptibility to scrapie [33]. An analysis of the variation of codon positions 136 and 171, for instance, showed that each has several adjacent polymorphic sites and may codify up to four amino acids [7, 50]. The atypical scrapie form, characterized by strain Nor98 [6], is more frequently detected in AHQ animals that carry a polymorphism in codon 141, and has not been described in Suffolk sheep in Brazil [2]. This atypical form expresses phenylalanine (F), instead of leucine (L) in the form L141F [6, 37, 46].

However, although it is generally acceptable that classical scrapie is an infectious and contagious disease [14], contagion with the atypical form is questionable in light of the fact that the specific marker for the atypical manifestation of the disease is detected outside the central nervous system [5, 20, 29], even in cases experimentally transmitted to transgenic mice [35] and sheep [47]. Several studies have demonstrated that susceptibility to the atypical form is consistently associated with PrP codons 141 (L/F) and 154 (R/H) [6, 42]. In fact, studies have proposed the hypothesis that this form may evolve when the animal is not exposed to the infectious agent [5, 18, 29, 48], given the limited knowledge of the physiopathology of this manifestation of the disease [19].

In the present study, two (2/8) positive animals presented the haplotype ARR/ARR, which is considered to be the least susceptible and therefore responsible for the lowest risk of scrapie. However, like all sheep that were genotyped, these animals did not present any change in lysine in codon position 141. This change (that is, when lysine is replaced by phenylalanine) has been associated with atypical scrapie in Suffolk sheep [6]. Therefore, these two ARR/ARR

sheep do not fit in the genotypic characteristics of sheep that may commonly present the atypical form. It is possible that the presence of several crossbred animals of different flocks and farms in the same environment, which characterizes an heterogeneous flock, has created the favorable conditions for the disease to evolve and spread, infecting the more susceptible animals.

The variation in the frequency of the PrP genotype between flocks has been identified as a real risk factor for the disease [4]. The introduction of adult sheep free of scrapie in contaminated flocks is believed to allow lateral transmission, even between adult animals with less susceptible genotypes [40, 45], although young sheep are more predisposed [43]. Other reasons behind differences in occurrence include the stress caused during husbandry and large population numbers [26]. Additionally, the lack of a defined epidemiological pattern and the different strains of the causal agent play an important role in inter-flock variability [40]. Several models were based on the assumption that outbreak duration is influenced by flock size and by the frequency of the PrP genotype in one flock [25, 26, 38, 51]. Commercial flocks with high genetic diversity, mainly in codons other than 136, 154 and 171, are more consistently affected. In these animals, the onset of clinical manifestations occurs at significantly different ages, with means varying from 2 to 5.7 years, due to noteworthy dissimilarities in age and PrP genotype profiles [40]. The purchase of infected animals has been pointed out as the main scrapie infection mechanism in flocks [27, 41].

The diagnosis of scrapie in two homozygous ARR/ARR sheep indicates that the resistance of this genotype to the classical form of the disease is debatable. Although scrapie in these animals is rare, the cases presented in this case report lend strength to the notion that its occurrence depends on a combination of infectious factors, including differences in biological and biochemical properties in the natural hosts to this prion.

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