# Single nucleotide polymorphisms at 15 codons of the prion protein gene from a scrapie-affected herd of Suffolk sheep in Brazil[1]

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**ABSTRACT.-** Andrade C.P. de, Almeida L.L., Castro L.A., Leal J.S., Silva S.C. & Driemeier D. 2011. **Single nucleotide polymorphisms at 15 codons of the prion protein gene from a *scrapie*-affected herd of *Suffolk* sheep in Brazil.** *Pesquisa Veterinária Brasileira 31(10):893-898.* Setor de Patologia Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. E-mail: davetpat@ufrgs.br

*Scrapie* is a transmissible spongiform encephalopathy of sheeps and goats, associated with the deposition of a isoform of the prion protein ($PrP^{sc}$). This isoform presents an altered conformation that leads to aggregation in the host's central nervous and lymphoreticular systems. Predisposition to the prion agent infection can be influenced by specific genotypes related to mutations in amino acids of the $PrP^{sc}$ gene. The most characterized mutations occur at codons 136, 154 and 171, with genotypes VRQ being the most susceptible and ARR the most resistant. In this study we have analyzed polymorphisms in 15 different codons of the $PrP^{sc}$ gene in sheeps from a *Suffolk* herd from Brazil affected by an outbreak of classical *scrapie*. Amplicons from the $PrP^{sc}$ gene, encompassing the most relevant altered codons in the protein, were sequenced in order to determine each animal's genotype. We have found polymorphisms at 3 of the 15 analyzed codons (136, 143 and 171). The most variable codon was 171, where all described alleles were identified. A rare polymorphism was found at the 143 codon in 4% of the samples analyzed, which has been described as increasing *scrapie* resistance in otherwise susceptible animals. No other polymorphisms were detected in the remaining 12 analyzed codons, all of them corresponding to the wild-type prion protein. Regarding the risk degree of developing *scrapie*, most of the animals (96%) had genotypes corresponding to risk groups 1 to 3 (very low to moderate), with only 4% in the higher risks group. Our data is discussed in relation to preventive measures involving genotyping and positive selection to control the disease.

INDEX TERMS: Spongiform encephalopathy, Prion protein, *Suffolk*, genetics and DNA sequencing.

**RESUMO.-** [**Polimorfismos de nucleotídeos únicos em 15 códons do gene da proteína priônica em um rebanho *Suffolk* afetado com *scrapie* no Brasil.**] *Scrapie* é uma encefalopatia espongiforme transmissível de ovinos e caprinos, associado a deposição da isoforma da proteína priônica ($PrP^{sc}$). Essa isoforma apresenta uma alteração conformacional que leva ao acúmulo da proteína no sistema nervoso central e linforeticular do hospedeiro. A predisposição a infecção pelo agente priônico pode ser influenciado por genótipos específicos relacionados a mutações na sequência de aminoácidos do gene $PrP^{sc}$. As principais mutações caracterizadas ocorrem nos códons 136, 154 e 171, sendo o genótipo VRQ o mais suscetível e o genótipo ARR o mais resistente. Nesse estudo nós analisamos os polimorfismos de 15 códons diferentes da gene $PrP^{sc}$ em ovinos de um rebanho da raça *Suffolk* no Brasil afetado com *scrapie* clássico. Os amplicons do gene da $PrP^{sc}$, que contem os códons mais frequentemente encontrados foram sequenciados para determinar o genótipo de cada ani-

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mal. Nós encontramos 3 polimorfismos do 15 códons analisados (136, 143 e 171). O códon que mais teve variações foi o códon 171, onde todos os alelos foram identificados. Um polimorfismo raro foi encontrado no códon 143, em 4% das amostras analisadas, o qual tem sido descrito por aumentar a resistência a *scrapie* em animais suscetíveis. Nenhum outro polimorfismo foi detectado nos 12 códons restantes, todos então, correspondendo à proteína priônica selvagem. De acordo com a grau de risco a desenvolver *scrapie*, a maioria dos animais (96%) tiveram genótipo correspondentes aos grupos de risco 1 a 3 (muito baixo a moderado), e somente 4% no grupo de risco alto. Nossos dados discutem a relação das medidas de prevenção envolvendo a genotipagem e a seleção positiva para o controle da doença.

TERMO DE INDEXAÇÃO: Encefalopatia espongiforme, proteína priônica, *Suffolk*, genética e sequenciamento de DNA.

## INTRODUCTION

*Scrapie*, a disease related to bovine spongiform encephalopathy (BSE) in bovines and Creutzfeldt–Jakob disease in humans, is a neurodegenerative and fatal disease that affects sheep and goat and is caused by an altered isoform of the normal host-encoded cellular prion protein. The main clinical signals are locomotion difficulty, prurits, hyperthermia, ataxia and death. The disease evolution is slow, leading to cachexia and excessive movement. Moreover, affected animals show trembling and convulsive state. In the brain this disease causes neuronal losses in the absence of immunological system response. The central nervous system tissue degeneration is due to the deposition of an altered prion protein isoform $PrP^{sc}$ (Foster et al. 2001). In its classic form the ovine *scrapie* is characterized by a broadly consistent disease phenotype, with variations in clinical signs and neuropathology being attributed to prion strain differences, with host genotype heavily influencing disease susceptibility (Mitchell et al. 2010).

There is a well-established association between sheep prion protein (PrP) genotype and the risk of death from *scrapie*. Certain genotypes are clearly associated with susceptibility to the disease and others to resistance (Baylis et al. 2004). In the classical form, *scrapie* susceptibility is highly related to changes in specific amino acids that leads to an altered form of the prion protein ($PrP^{sc}$). Polymorphisms in the host-encoded prion gene (*PRNP*) are major determinants of susceptibility to classic *scrapie*, with variations at codons 136, 154, and 171 conveying variable degrees of resistance (Baylis et al. 2002). There are over 15 polymorphisms reported in PRNP (DeSilva et al. 2003). Of these, only three codons (codon 136, 154 and 171) have been reported to affect the susceptibility to the disease. Susceptibility to ovine *scrapie* is also determined by the infective *scrapie* strain (O'Rourke et al. 1997). Two strains of *scrapie* have been defined. Type A produces the disease in sheep that are either homozygous or heterozygous for a valine at codon 136 while type C causes disease in sheep that are homozygous for a glutamine at codon 171.

The codon 136, valine (V) is associated with high *scrapie* susceptibility while alanine (A) is associated with low susceptibility, although this might depend on the strain of *scrapie* agent (Goldmann 1994). At codon 154, arginine (R) is associated with susceptibility while histidine (H) is associated with partial resistance. At codon 171, glutamine (Q) and histidine (H) are associated with susceptibility while arginine (R) is associated with resistance (Baylis et al. 2004). Codon variants at positions other than 136, 154, and 171 are also associated with *scrapie* resistance. An M112T variant on the ARQ haplotype has been associated with *scrapie* resistance in orally-inoculated *Suffolk* sheep in the U.S. (Laegreid et al. 2008). M137T and N176K variants on the ARQ haplotype have been associated with *scrapie* resistance in intercranially-inoculated, orally-inoculated, and naturally-infected Italian Sarda breed sheep (Vaccari et al. 2007; Vaccari et al. 2009). Variations at codon 141 can be related to the atypical *scrapie* form Nor98 (Mazza et al. 2010).

In several countries a *scrapie* control and eradication system associated with animal genotyping has been applied, based on the selection of animals carrying codons known to be associated with *scrapie* infection resistance. Through this genetic screening, it was possible to gradually eradicate the disorder, keeping the animals with ARR/ARR alleles (Acin et al. 2004).

Also important is the fact that sheep with the ARQ haplotype are not uniformly susceptible to *scrapie*, with implications for *scrapie* eradication programs, where ARQ sheep have previously been considered as a homogenous group, leading to losses of economically important sheep germplasm (Laegreid et al. 2008).

The first case report of *scrapie* in Brazil has been in a Hampshire Down sheep in the Rio Grande do Sul state (Fernandes et al. 1978). Since then, some studies have analyzed variation in codons 136, 154 and 171 of the prion protein (Passos et al. 2008, Lima et al. 2007, Sotomaior et al. 2008), but other codon variations in the PrP gene from Brazilian ovine and goat breeds have not been extensively analyzed.

The purpose of this study was to analyze the genetic polymorphisms found in the prion protein gene from a Brazilian *Suffolk* breed herd where an affected animal with the classical form of *scrapie* had been diagnosed. We have analyzed polymorphisms distributed amongst 15 codons of the prion protein gene which could be of importance for susceptibility and resistance to the classical form of the disease. Moreover, animals presenting variations in the three major codons (136, 154 and 171) were classified in risk groups using the haplotypes combination proposed by Dawson et al. (2008).

## MATERIALS AND METHODS

### Animals, sample collection and DNA preparation

A total of 93 animals from a herd of 811 *Suffolk* sheeps were selected. One animal from this herd had been previously diagnosed for the classical form of *scrapie* and was unavailable for sample collection, because has died from the disease.

The whole peripheral blood samples were collected in EDTA vacutainer tubes for PCR, sequencing and genotyping. Genomic DNA was extracted from 500µL whole blood using the QIAamp DNA Blood Mini Kit (Qiagen) following the manufacturer's ins-

tructions. DNA was recovered in 100 µl elution buffer and stored under refrigeration for further analyses.

**Primers and PCR conditions**

PCR reactions were carried out using a forward primer flanking the 136 codon position (primer 136F: 5'-ATGAAGCATGTGGCAGGAGC-3') and a reverse primer flanking the 171 codon position (primer 171R: 5'-GGTGACTGTGTGTTGCTTGACTG-3'), generating a 245 bp amplicon (L'Homme et al. 2008). PCR reactions contained 15 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1x Platinum Taq buffer, 1 U Platinum Taq DNA Polymerase (Invitrogen) and 1 µL of genomic DNA in a final volume of 25 µL. PCR reactions were performed on an ABI Veriti automated DNA thermal cycler (Applied Biosystems) using the following parameters: 95ºC for 5 min followed by 35 cycles at 95ºC for 30 s, 58ºC for 30 s and 72ºC for 30 s, and a final step at 72ºC for 10 min.

**DNA sequencing and analysis**

PCR products were purified using PureLink™ PCR Purification Kit (Invitrogen), and quantified using a Qubit fluorescence quantification system (Invitrogen) according with manufacturer's instructions. Sequencing of the PCR products was carried out using the BigDye Terminator version 3.1 Cycle Sequencing kit in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Each sample was sequenced independently using both forward and reverse primers, until a Phred quality score of at least 20 (Ewing and Green. 1998; Ewing et al. 1998) was obtained for each individual base in the consensus sequence. The resulting chromatograms were analyzed using the Staden package version 1.7.0 programs (Staden et al., 2003) and novoSNP (Weckx et al. 2005).

Sequence data from the amplicons, encompassing a region containing 15 different codons of interest (Table 1) were used to derive genotype frequencies using the formula $f_{ij}=n_{ij}/N$, where $f_{ij}$ corresponds to the *ij* genotype frequency, $n_{ij}$ corresponds to the number of animals presenting the genotype *ij* and N corresponds to the total number of animals analyzed. The allelic frequencies ($p_i$) were calculated using the formula $p_i=(2f_{ij}+\sum f_{ij})/2$.

**Table 1. Genotype frequencies determined for the 15 different codons from the PrP gene**

| Codon | Genotype | n | Frequency |
|---|---|---|---|
| 112 | M/M | 38[a] | 1.00 |
| 116 | A/A | 93 | 1.00 |
| 127 | G/G | 93 | 1.00 |
| 136 | A/A | 88 | 0.95 |
| | A/V | 5 | 0.05 |
| 137 | M/M | 93 | 1.00 |
| 138 | S/S | 93 | 1.00 |
| 141 | L/L | 93 | 1.00 |
| 143 | H/H | 89 | 0.96 |
| | H/R | 4 | 0.04 |
| 151 | R/R | 93 | 1.00 |
| 154 | R/R | 93 | 1.00 |
| 167 | R/R | 93 | 1.00 |
| 168 | P/P | 93 | 1.00 |
| 171 | Q/R | 42 | 0.45 |
| | Q/Q | 32 | 0.34 |
| | Q/H | 9 | 0.10 |
| | R/R | 9 | 0.10 |
| | H/H | 1 | 0.01 |
| 176 | N/N | 93 | 1.00 |
| 180 | H/H | 93 | 1.00 |

[a] Only 38 samples had consensus sequences with Phred score ≥20 and could be analyzed.

## RESULTS

We have analyzed a Brazilian *Suffolk* breed herd where one animal with the classical form of *scrapie* had been previously diagnosed. DNA from 93 animals was purified from whole blood samples, submitted to PCR and the amplicons' sequence were determined by Sanger sequencing in order to establish each animal's genotype for the 15 codons analyzed.

The genotype frequencies for the 93 animals are presented in Table 1. In Table 2 is presented a list of polymorphisms and respective degrees of *scrapie* resistance described in the literature for the codons we have analyzed, together with the calculated allelic frequencies.

Only 38 animals could have their genotypes determined for codon 112, all of them being M/M. Sequences derived

**Table 2. Allele frequencies determined for the 15 different codons from the PrP gene**

| Codon | Aminoacid variations[a] | Allele frequencies | *Scrapie* resistance |
|---|---|---|---|
| 112 | Methionine (M) | 1.00 | T > M[b] |
| | Threonine (T) | 0 | |
| 116 | Alanine (A) | 1.00 | Unknown |
| | Proline (P) | 0 | |
| 127 | Glycine (G) | 1.00 | Unknown |
| | Valine (V) | 0 | |
| | Serine (S) | 0 | |
| 136 | Alanine (A) | 0.97 | A > V[c] |
| | Valine (V) | 0.03 | |
| 137 | Methionine (M) | 1.00 | T > M[d] |
| | Threonine (T) | 0 | |
| 138 | Serine (S) | 1.00 | N > S[e] |
| | Asparagine (N) | 0 | |
| 141 | Leucine (L) | 1.00 | L > F/F (Nor 98)[f] |
| | Phenylalanine (F) | 0 | |
| 143 | Histidine (H) | 0.98 | R > H[g] |
| | Arginina (R) | 0.02 | |
| 151 | Arginina (R) | 1.00 | C > R[e] |
| | Cysteine (C) | 0 | |
| 154 | Arginine (R) | 1.00 | H > R[c] |
| | Histidine (H) | 0 | |
| 167 | Arginine (R) | 1.00 | Unknown |
| | Serine (S) | 0 | |
| 168 | Proline (P) | 1.00 | L > P[h] |
| | Leucine (L) | 0 | |
| 171 | Arginine (R) | 0.32 | R > H > Q[c] |
| | Glutamine (Q) | 0.62 | |
| | Histidine (H) | 0.06 | |
| 176 | Asparagine (N) | 1.00 | K > N[i] |
| | Lysine (K) | 0 | |
| 180 | Histidine (H) | 1.00 | Unknown |
| | Tyrosine (Y) | 0 | |

[a] The amino acids present in the wild type PRNP protein are listed first for each codon (Goldmann et al., 1990, GenBank accession M31313).
[b] Laegreid et al. (2008); [c] Heaton et al. (2010); [d] Vaccari et al. (2009); [e] Thorgeirsdottir et al. (1999); [f] Benestad et al. (2008); [g] Vaccari et al. (2006); [h] Goldmann et al. (2006); [i] Maestrale et al. (2009).

form the remaining samples had a Phred score bellow 20 for the consensus sequence at this region, making it impossible to determine the genotype with accuracy.

Codons 116, 127, 137, 138, 141, 151, 167, 168, 176 and 180 all had genotypes corresponding to the wild type PrP gene (GenBank accession M31313, Tables 1 and 2).

A rare polymorphism was detected at codon 143, were 4 animals out of 93 presented the heterozygous genotype H/R (Table 1). The R allele, which has been reported as conferring a higher degree of *scrapie* resistance than the H allele (Colussi et al. 2010), had a frequency of 0.02 in the analyzed population (Table 2).

The most frequent genotype found for the 136 codon was A/A, with only 5% of the animals presenting the A/V heterozygous genotype (Table 1), corresponding to allele frequencies of 0.97 (A) and 0.03 (V) (Table 2).

All analyzed animals were R/R homozygous for the 154 codon (Table 1).

Regarding the 171 codon, all the most frequent literature described alleles were found amongst the animals we have analyzed. The most frequent genotypes found were Q/R and Q/Q, representing 79% of the population (Table 1). Some of the rare genotypes for the *Suffolk* breed (Q/H and H/H, Passos et al. 2008) were also found, but at a reduced frequency as expected (Table 1). The frequency for the genotype R/R, considered wild type for the 171 codon, was only 0.10 (Table 1). The Q allele was the most frequent, with a frequency of 0.62, followed by the R allele, with a frequency of 0.32 (Table 2).

Regarding the risk group classification which evaluates the degree of resistance to *scrapie* according to genotypes at codons 136, 154 and 171 (Dawson et al. 2008), most of the animals (96%) were classified in the first three risk groups (R1, R2 and R3). Only 4% of the genotypes were classified in the risk group R4 and R5 (moderate to high risk) (Table 3).

**Table 3. Distribution of animals in risk groups according to genotypes determined for codons 136/154/171**

| NSP[a] risk group | Genotype | n | Frequency |
|---|---|---|---|
| R1 (very low) | ARR/ARR | 9 | 0.097 |
| R2 (low) | ARR/ARQ | 39 | 0.419 |
| R3 (moderate) | ARH/ARH | 1 | 0.441 |
| | ARH/ARQ | 9 | |
| | ARQ/ARQ | 31 | |
| R4 (moderate) | ARR/VRQ | 3 | 0.032 |
| R5 (high) | ARQ/VRQ | 1 | 0.011 |

[a] NSP, National *Scrapie* Plan (Dawson et al. 2008).

## DISCUSSION

In this study we have evaluated the genetic polymorphisms found in the prion protein gene from a Brazilian *Suffolk* breed herd where an affected animal with the classical form of *scrapie* had been diagnosed.

A polymorphism at codon 143 has been described by De Silva et al. (2003) in *Suffolk* breeds, where an arginine (R) is substituted for a histidine (H). Later, Vaccari et al. (2006) suggested a possible increase in *scrapie* resistance of animals with this H143R variation even in animals presenting susceptible genotypes for codons 136/154/171. We have found four animals H/R heterozygous for the 143 codon, three of them with genotypes ARR/ARQ (risk group R2) for the 136/154/171 codons. One animal presented the genotype ARQ/ARQ, corresponding to risk group R3 (moderate). This last animal, despite having a susceptible genotype regarding the 136/154/171 codons, could be considered less susceptible to *scrapie* development when taken into consideration the genotype for the 143 codon.

Another codon in the $PrP^{sc}$ gene that has been described has increasing *scrapie* resistance in *Suffolk* sheep is the 112 codon. According to Laegreid et al. (2008), animals homozygous for threonine at the 112 codon ([T/T]ARQ) did not develop the disease when orally challenged, ,whereas heterozygous animals for the same codon ([M/T]ARQ) developed the disease. These findings reinforced the suggestion that different haplotypes associations are important to determine the resistance to developing *scrapie*.

Heaton et al. (2010) have shown that genotype frequencies for ARQ/ARQ in the *Suffolk* breed are higher than other breeds. They also described that 51% of the animals with genotype ARQ/ARQ also presented a high frequency of the T/T or M/T genotype for the 112 codon, which correlates to *scrapie* resistance. From the 38 samples we have determined the genotype for the 112 codon, all of them were of the M/M homozygous genotype indicating a possible increase in susceptibility to *scrapie* in this population.

The genotype frequencies we have found were comparable to the ones described by Passos et al. (2008) for another herd of *Suffolk* breed in southern Brazil. They have analyzed polymorphisms in the 136 and 171 codons and found that approximately 49% of the animals (in a herd of 129 sheeps) had the highly susceptible genotype QQ for the 171 codon, whereas we have found 34% of the animals presenting this genotype.

The atypical *scrapie* form Nor98 (Benestad et al. 2008), associated with the variation L141F, has not been described in Brazil. In our work we have found all animals to be of the L/L homozygous genotype.

Maestrale et al. (2009) and (Vaccari et al. 2007) have described that animals presenting the susceptible genotype ARQ/ARQ would display a decrease in the risk of developing the disease, both in natural or experimental infection, when the rare variants M137T and N176K were present. None of the animals analyzed in our work had any of these rare variants.

In The Netherlands an eradication programme of susceptible animals has been enforced since 2002 with a corresponding downward trend in the prevalence of *scrapie* in country (Melchior et al. 2010; Hagenaars et al. 2010).

In the United Kingdom a National *Scrapie* Plan (NSP) has been implemented using a voluntary programme with Ram Genotyping Scheme (RGS) since 2001. Each of the 15 possible PrP genotypes for codons 136, 154, and 171 have been assigned to one of five groups, according to the risk of disease in the individual and in first generation progeny. The plan requires negative selection of the VRQ allele

and encourages positive selection for the ARR allele. The use of rams in risk groups R1, R2 and R3 is not restricted. Rams in risk groups R4 and R5 are required to be culled or castrated. Recently, Dawson et al (2008) published an analysis of the plan implementation. A comparison of allele frequencies between years 2002 and 2006 showed a 36.5% increase in allele ARR and a 60% decrease in allele VRQ. No adverse effects were identified in other performance traits of the selected animals. Also, the PrP selection had no impact on inbreeding and genetic diversity. In conclusion, a industry-wide selection on PrP genotype is feasible and a voluntary Ram Genotyping Scheme has attracted participation from the majority of ram producers. With the help of the genotyping and selective culling programmes applied to affected flocks, it appears that *scrapie* is being brought under control.

In the herd we have analyzed only about 4% of the animals were in risk groups R4 and R5, needing to be culled or castrated. However, about 44% of the animals were in risk group R3 (moderate risk), showing a clear advantage of implementing a genetic selection programme in Brazilian herds in order to reduce the frequency of susceptible alleles.

Some other factors should also be taken into consideration when planning any programme for positively selecting animals for *scrapie* resistance. Despite data showing that several alterations outside the 136/154/171 codons, the relationship between the alteration and *scrapie* resistance could be specific to the breed analyzed (Colussi et al. 2010; McManus et al. 2010). Therefore, it is vital to determine genotype for codons other than the classical ones associated with *scrapie* resistance and susceptibility for the different ovine breeds, establishing guidelines for genetic programmes aiming at *scrapie* epidemic risk reduction.

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