UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

GENOTIPAGEM DE OVINOS PARA A DETERMINAÇÃO DA SUSCETIBILIDADE A SCRAPIE

Caroline Pinto de Andrade

Porto Alegre, novembro/2013

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GENOTIPAGEM DE OVINOS PARA A DETERMINAÇÃO

DA SUSCETIBILIDADE A SCRAPIE

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RESUMO

Scrapie é uma doença neurodegenerativa infecciosa que afeta ovinos e caprinos, o qual está relacionada a uma alteração conformacional da proteína priônica, que leva a deposição e agregação da proteína no sistema nervoso central. A predisposição a infecção pelo agente príon está associado a polimorfismos de nucleotídeos únicos no gene da proteína priônica. Os principais polimorfismos relacionados à infecção estão presentes nos códons 136, 154 e 171, sendo o genótipo VRQ o mais suscetível e o ARR o genótipo mais resistente. O presente estudo teve como objetivo identificar os polimorfismos de nucleotídeos únicos em ovinos das raças Suffolk, Dorper e Santa Inês, provenientes de surtos de scrapie clássico e de rebanhos livre de scrapie, os quais os proprietários estavam dispostos a colaborar com o projeto. O primeiro trabalho analisou polimorfismos de nucleotídeos únicos em 15 códons do gene da proteína priônica em um rebanho Suffolk afetado com scrapie clássico no Brasil. Dos 15 códons analisados, 3 apresentaram polimorfismos (136, 143 e 171). O códon 171 apresentou o maior número de polimorfismos, os quais foram encontradas todas as formas alélicas. Quando avaliado os grupos de risco, cerca de 96% do rebanho pertenceu aos grupos 1 a 3 (risco muito baxi a moderado). O segundo trabalho relatou o desenvolvimento da técnica de PCR em tempo real, baseado em sondas TaqMan, para a identificação de polimorfismos nos códons 136, 154 e 171 e sua aplicabilidade em rebanhos brasileiros. Um total de 142 amostras foram analisadas por PCR em tempo real. Ao comparar os resultados do PCR em tempo real com o sequenciamento, 100% das amostras foram idênticas. Para o códon 136, a maioria dos ovinos apresentou o genótipo AA. Para o códon 154, o genótipo RR foi o mais frequente, e para o códon 171, os genótipos mais frequentes foram QQ e QR. O terceiro trabalho descreve a caracterização de três surtos de scrapie clássico em ovinos da raça Dorper, em diferentes regiões do sul do Brasil. Os surtos ocorreram nos anos de 2011 a 2013, sendo que no segundo e no terceiro foram identificados ovinos do primeiro caso. Além disso, foi analisada a associação de scrapie com a genotipagem do gene da proteína priônica em ovinos presentes nos três rebanhos. No total, 22 ovinos foram positivos no teste de imuno-histoquímica, sendo que 4 deles apresentaram sinais clínicos da doença. Em todos os estudos, presentes as três raças analisadas, foi possível evidenciar a presença de ovinos, na sua maioria, geneticamente suscetíveis a infecção, pois a maioria pertenceu ao grupo de risco 3, considerado moderado.

ABSTRACT

Scrapie is an infectious neurodegenerative disease affecting sheep and goats. This is related to an altered conformational of the prion protein (PrP^{Sc}) that leads to the deposition and aggregation of protein in the central nervous system. The predisposition to prion infection agent is associated with single nucleotide polymorphisms in the prion protein gene. The mostly polymorphisms related with infection are present in codons 136, 154 and 171, being more susceptible genotype VRQ and ARR more resistant genotype. This study aimed to identify single nucleotide polymorphisms in sheep breeds Suffolk, Dorper and Santa Ines, from outbreaks of classical scrapie flocks and free scrapie flocks, which the owners were willing to collaborate with the project. The first article analyzed single nucleotide polymorphisms in 15 codons of the prion protein gene in a Suffolk sheep affected with classical scrapie in Brazil. Of the 15 codons analyzed, 3 showed polymorphisms (136, 143 and 171). Codon 171 showed the greatest number of polymorphisms, which were found all allelic forms. When assessed risk groups, about 96% of the herd belonged to groups 1 to 3 (very baxi to moderate risk). The second article reported the development of PCR real time based on TaqMan probes for the identification of polymorphisms in codons 136, 154 and 171 and their applicability in Brazilian herds. A total of 142 samples were analyzed by real-time PCR. When comparing the results of real time PCR with the sequencing of the samples were 100% identical. For codon 136, the majority of the sheep had the AA genotype. For codon 154, the RR genotype was the most frequent, and the codon 171, the most common genotypes were QQ and QR. The third article describes the characterization of three outbreaks of classical scrapie in Dorper sheep, in different regions of southern Brazil. The outbreaks occurred in the years 2011-2013, and the second and third were identified sheep of the first case. Furthermore, it was analyzed its association with genotyping prion protein gene in sheep present the three herds. In total, 22 sheep were positive in immunohistochemical testing, and 4 of them showed clinical signs of disease. In all studies, presents three races analyzed, it was possible to demonstrate the presence of sheep, mostly genetically susceptible to infection because the majority belonged to the risk group 3, considered moderate.

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LISTA DE ABREVIATURAS

ARCO: Associação Brasileira de Criadores de Ovinos

ARH: alelo do gene PRNP com os animoácidos alanina, arginina e histidina presentes nos códons 136, 154 e 171, respectivamente.

ARQ: alelo do gene PRNP com os animoácidos alanina, arginina e glutamina presentes nos códons 136, 154 e 171, respectivamente.

ARR: alelo do gene PRNP com os animoácidos alanina, arginina e arginina presentes nos códons 136, 154 e 171, respectivamente.

BSE: Encefalopatia Espongiforme Bovina

DAB: diaminobenzidina

DNA: Ácido desoxirribonucleico

dNTP: deoxirribonucleotídeos trifosfatados

EDTA: ácido etilenodiaminotetracético

EET: Encefalopatia Espongiforme Transmissível

IBGE: Instituto Brasileiro de Geografia e Estatístca

IHQ: Imuno-histoquímica

LANAGRO: Laboratório Nacional Agropecuário

MAPA: Ministério da Agricultura, Pecuária e Abastecimento

Nor98: cepa descrita na Noroega em 1998, referente a forma atípica de scrapie.

ORF: *Open reading frame*

pb: pares de base

PBS: solução salina fosfatada

PCR: reação em cadeia pela DNA polimerase

PNCRH: Programa Nacional de Controle da Raiva dos Herbívoros

PRNP: gene da proteína priônica

PrP: proteína priônica

PrP^{C}: proteína priônica celular (normal)

PrP^{Sc}: proteína priônica isoforma anormal

SECEX: Secretaria de Comércio Exterior

SNC: Sistema Nervoso Central

SNP: Polimorfismo de Nucleotídeo Único

SPV: Setor de Patologia Veterinária

UFRGS: Universidade Federal do Rio Grande do Sul

VRQ: alelo do gene PRNP com os animoácidos valina, arginina e glutamina presentes nos códons 136, 154 e 171, respectivamente.

1. INTRODUÇÃO

O Brasil possui aproximadamente 17,4 milhões de ovinos, os quais estão distribuídos praticamente nas regiões Nordeste e Sul. Essas duas regiões representam quase 85% do rebanho do país, distribuídos em mais de 400 mil estabelecimentos agropecuários, sendo que 56,7% destas propriedades encontram-se no Nordeste e outros 28,1% na Região Sul do Brasil (IBGE, 2013), sendo o Rio Grande do Sul o estado com o maior número de animais. Os dois principais municípios pertencem a este estado são: Santana do Livramento e Alegrete. Dentre os 20 municípios com os maiores efetivos, 11 estão no Rio Grande do Sul e os demais entre cidades da Região Nordeste, localizadas nos Estados da Bahia, do Ceará e de Pernambuco.

A ovinocultura brasileira apresenta um grande potencial de crescimento. Segundo dados da Associação Brasileira de Criadores de Ovinos (ARCO) e da Câmara Setorial da Cadeia Produtiva de Caprinos e Ovinos, há um défcit anual de mais de 32 mil toneladas de carne ao mercado interno, anualmente. Em 2007, foram importados 2,8 mil toneladas de carne ovina *in natura* (SECEX, 2008). Além de haver déficit na produção, há também um hábito de consumo ainda pouco explorado pelo marketing do setor. No Brasil, o consumo anual do produto é de apenas 0,7 quilos por habitante, menos da metade da Argentina, por exemplo, com 1,5 quilos *per capita*, e 56 vezes menos que os neozelandeses, maiores consumidores do mundo, com 39,7 quilos por habitante.

Apesar do aumento de crescimento no setor, existem alguns entraves para a produção de carne de ovinos como, por exemplo, os problemas sanitários e de competitividade com a carne importada, restringindo a carne de ovino nacional ao comércio regional. Entre os aspectos sanitários que podem potencialmente prejudicar a ovinocultura encontra-se a

paraplexia enzoótica dos ovinos (*scrapie*), que é alvo das ações da Secretaria de Defesa Sanitária, no âmbito do Programa Nacional de Controle da Raiva dos Herbívoros - PNCRH e das ações para a prevenção e o controle das Encefalopatias Espongiformes Transmissíveis – EETs.

A paraplexia enzoótica dos ovinos ou *scrapie* é uma doença pertencente ao grupo das Encefalopatias Espongiformes Transmissíveis – EETs, de caráter neurodegenerativo, progressivo e fatal, que acomete ovinos e caprinos e pode estar relacionada com a Encefalopatia Espongiforme Bovina (BSE-Bovine Spongiform Encephalopathy). Conhecida na Europa desde 1732, é caracterizada principalmente pelo acúmulo de príon (do inglês, *proteinaceus infectious particle*), agente transmissível composto de proteína priônica (PrP^{c}), na isoforma anormal PrP^{Sc} (Sc=*scrapie*), resultante de uma alteração conformacional da molécula normal codificado pelo hospedeiro (Prusiner, 1982; Foster et al., 2001). A célula nervosa não consegue liberar os príons produzidos, que se acumulam, levando a uma lesão degenerativa no cérebro dos ovinos e caprinos infectados (Kimberlin, 1990).

No Brasil o diagnóstico confirmatório é feito através de exame laboratorial para a detecção do príon, pela técnica da imuno-histoquímica (IHQ). Pode ser realizado no animal vivo, através da colheita de tonsilas e tecidos linfoides na terceira pálpebra e mucosa reto-anal (O'Rourke et al., 2002; Espenes et al., 2006). Após a morte do animal, é realizado o teste em amostras de tecidos do Sistema Nervoso Central.

Um método utilizado em vários países para controle do *scrapie* é o melhoramento genético baseado na seleção de animais com maior resistência à apresentação clínica da doença, com o objetivo de aliar métodos de diagnóstico da doença *in vivo* à seleção genética (Houston et al., 2002; Hickford et al., 2008).

Apesar dos recentes estudos, no Brasil ainda não existe nenhum programa de melhoramento genético que permita selecionar rebanhos resistentes à forma clássica de *scrapie*. O que existem são algumas iniciativas como a criação de um programa de certificação voluntária de rebanhos e de testes de suscetibilidade genética à *scrapie*, mas estas ainda estão bastante incipientes.

2. REVISÃO BIBLIOGRÁFICA

2.1 Scrapie

Scrapie ou paraplexia enzoótica dos ovinos é uma doença do grupo das Encefalopatias Espongiformes Transmissíveis – EETs, de caráter neurodegenerativo, progressivo e fatal, que acomete ovinos e caprinos e pode estar relacionada com a BSE dos bovinos. O nome *Scrapie* vem da expressão inglesa *"to scrape against something*", que significa "esfregar-se contra alguma coisa". Conhecida na Europa desde 1732, é caracterizada principalmente pelo acúmulo no sistema nervoso central de príon (do inglês, *proteinaceus infectious particle*), agente transmissível composto de proteína priônica (PrP^{C}), na isoforma anormal PrP^{Sc} (Sc= *Scrapie*). Tal acúmulo é resultante de uma alteração de conformação da molécula normal, o qual é codificado pelo próprio hospedeiro (Foster et al., 1996; Prusiner, 1982). Além disso, foi a primeira doença do grupo das EETs identificada em animais.

No mundo, a ocorrência dessa enfermidade é relatada desde o século XVIII (Watts et al., 2006), e vem sendo notificada em diversos países. Apenas Austrália e Nova Zelândia são países reconhecidos como livres da *scrapie*, sendo que o Brasil atualmente só permite a importação de ovinos desses países.

O *príon* apresenta resistência incomum à inativação pelo calor e pela radiação ultravioleta, e é capaz de estimular a sua produção no interior da célula infectada, através da conversão da forma celular normal na forma infecciosa. A célula nervosa acumula os *príons* produzidos, levando a uma lesão degenerativa no cérebro dos ovinos e caprinos infectados, os quais não conseguem produzir resposta imune ou reação inflamatória para combater a infecção (Kimberlin, 1990).

Ovinos e caprinos, em geral com dois a quatro anos de idade, usualmente contraem o agente infeccioso por ingestão de placenta ou fluídos contaminados, sendo a transmissão horizontal o principal modo de disseminação do agente, apresentando também um período de incubação variável, de 1 a 7 anos (McKinley et al., 1983; Kimberlin 1990; Foster et al., 1996). Porém, sabe-se que há casos de *scrapie* onde a transmissão vertical foi confirmada. Também já foram demonstrados casos em que o solo estava contaminado, onde dados experimentais mostram que o príon pode permanecer no solo por mais de 6 meses, prolongando o risco de infecção por um período maior de tempo (Nagaoka et al., 2010).

Os sinais da doença normalmente aparecem 2 a 5 anos após contato com o agente, e, até o momento, não existe vacina ou tratamento, pois a infecção não induz resposta humoral. Os principais sinais clínicos, em ovinos são prurido, hiperexcitabilidade, ranger de dentes, incoordenação motora e morte. A evolução da doença é lenta, levando o animal ao estado de caquexia, paralisia, movimento excessivo ou estresse ao manejo, podendo tremer ou cair em estado convulsivo. No cérebro ocorre perda neuronal, devido a uma intensa vacuolização, ocorrendo a degeneração de tecidos de Sistema Nervoso Central (Kimberlin, 1990).

O controle em um rebanho infectado inclui abate de animais com sinais clínicos e de seus parentes maternos, procedimento que exige identificação confiável e registros detalhados por várias gerações (Sargison, 1995). Com diferentes áreas de parição em pastagem durante anos subsequentes, pode-se reduzir a contaminação. Com a utilização de carneiros jovens como reprodutores também pode ser indicada, após um período de dois a três anos, quando a prevalência de *scrapie* nos seus ascendentes for determinada (Sargison, 1995).

Além da forma clássica de scrapie, em alguns países ocorrem também os casos atípicos

de *scrapie*. As formas atípicas de *scrapie* foram diagnosticadas pela primeira vez na Noruega em 1998 (Nor98), e desde então, vários países europeus, incluindo Portugal, confirmaram a sua existência. Estes casos diferem do *scrapie* clássico na distribuição anatômica da PrP^{Sc} no SNC, onde é possível verificar um maior acúmulo no cerebelo. Além disso, o perfil eletroforético difere da forma clássica e ocorre uma maior sensibilidade da PrP^{Sc} à proteinase K (Soto, 2004; Gavier-Wieden et al., 2005; Benestad et al., 2008).

A acumulação de PrP^{Sc} foi demonstrada durante a fase de pré tremor epizoótico nos linfonodos periféricos e em folículos linfoides de tonsilas (Schreuder et al., 1998). Estudos têm sido realizados para encontrar um meio diagnóstico das EETs em animais vivos. Schreuder et al., (1998) sugeriram que a imuno-histoquímica de biópsias de tonsilas poderia ser utilizada com base em resultados obtidos sob circunstâncias experimentais. A PrP^{Sc} também foi identificada em folículos linfoides de terceira pálpebra e mucosa retal , com a sensibilidade do teste por imuno-histoquímica variando entre 85 e 90% (O'Rourke et al., 2002). Além disso, a coleta do tecido é vantajosa, devido à localização superficial, facilidade de acesso e possibilidade à anestesia local.

2.2 Proteína priônica

A proteína priônica apresenta cerca de 250 aminoácidos, com massa molecular de 33-35 kDa. Encontra-se na membrana externa das células, ancorada por glicofosfatidilinositol (GPI). A estrutura da proteína priônica normal (PrP^{C}) é formada por α-hélice, já a isoforma anormal da proteína (PrP^{Sc}) é formada principalmente por estrutra β folha (Figura 1), (Hunter, 1997). A proteína priônica normal (PrP^{C}) é considerada uma glicoproteína, o qual está associada a complexos moleculares na membrana, e também

possui diversas funções em diferentes compartimentos celulares, dependendo de sua localização (Lee et al., 2003).

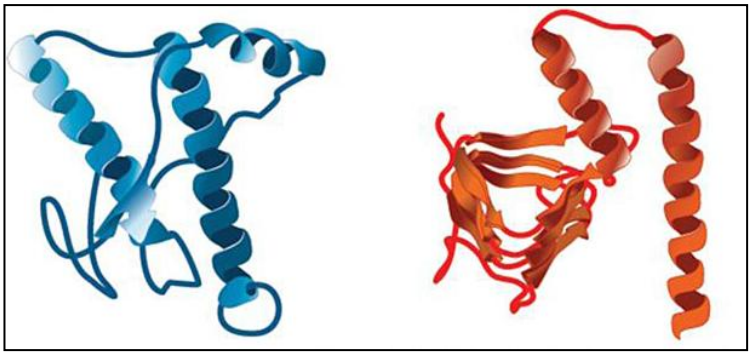

Figura 1: Estrutura da proteína priônica normal PrP^{C} (em azul) com predomínio de estrutura α-hélice. Em vermelho, a estrutura da proteína priônica em sua isoforma anormal PrP^{Sc} com predomínio de estrutura β folha (Lee et al., 2012).

A isoforma anormal da proteína priônica é glicosilada, hidrofóbica e resistente à proteinase. Quando ocorre a ação da proteinase a porção N-terminal da proteína é degradada, gerando uma proteína de 27 a 30 kDa (Figura 2), (Prusiner et al., 1998). Ao contrário da proteína normal, sua estrutura é rica em β folha e, essa mudança conformacional de estrutura gera a sua forma infecciosa. Além disso, por ser uma proteína hidrofóbica, acumula-se nos tecidos nervoso e linfoide, formando estruturas conhecidas como amiloides (Watts et al, 2006).

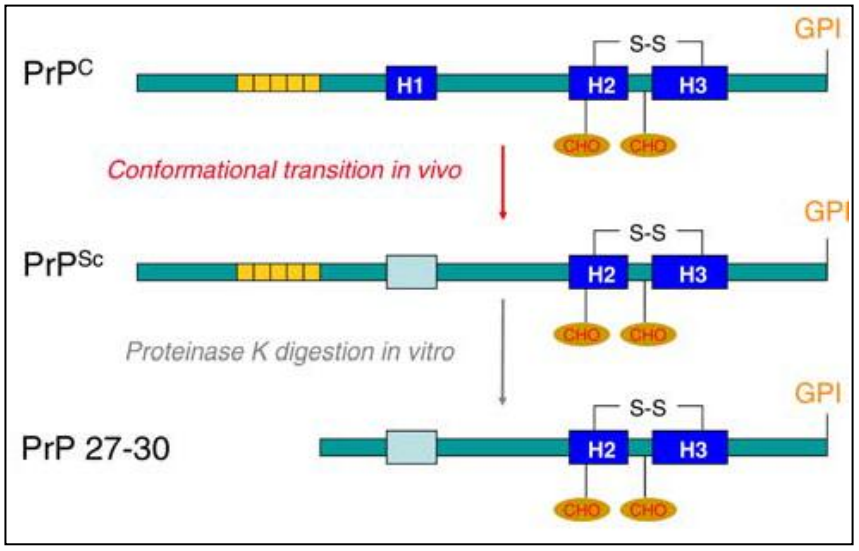

Figura 2: Representação linear da proteína priônica normal e infecciosa. Porção N-terminal é degradada pela proteinase K. Mudança conformacional no centro da molécula é ilustrada pela alteração da cor da α-hélice (Watts et al., 2006).

O gene PRNP em ovinos está localizado no cromossomo 13, o qual é constituído por dois exons não codificantes e um exon que contém a ORF (*open reading frame*), constituída por 236 códons (Lee et al., 1998). O gene é constitutivo, onde é expresso em praticamente todos os tecidos, com predomínio em tecidos neuronais. O fato da proteína ser expressa em grandes quantidades em neurônios sugere sua importância nestas células podendo estar relacionada com propagação do impulso nervoso por meio dos axônios, tanto em impulsos pré como pós-sinápticos (Herms et al., 1999).

2.3 Diagnóstico

No Brasil o diagnóstico confirmatório é feito através de exame laboratorial para a detecção do *príon*, pela técnica da imuno-histoquímica (IHQ). O teste pode ser realizado no animal vivo, através da colheita de tonsilas e tecidos linfoides na terceira pálpebra e mucosa reto-anal (O'Rourke et al., 2002; Espenes et al., 2006). Após a morte do animal, é realizado o

teste em amostras de tecidos do Sistema Nervoso Central. O teste rápido de *western blot* também é passível de ser realizado em amostras de cérebro de animais com imuno-histoquímica positiva dos tecidos linfoides encaminhados para necropsia. Este exame é importante na comprovação da diferenciação entre amostras de *scrapie* e BSE (Soto, 2004; Gavier-Widén, 2005), pois já foi constatada transmissão experimental da BSE aos ovinos, e casos de *scrapie* ocorrem no Brasil. Quando BSE é inoculada em ovinos há predominância de antígeno em tecido linfoide de forma semelhante ao que ocorre quando apresentam *scrapie* (Foster et al., 2001). Apesar de *scrapie,* ao contrário da BSE em bovinos, não provocar a doença em humanos, a comprovação de que a doença é *scrapie* e não BSE é necessária, visto que também pode ocorrer em ovelhas (Foster et al., 2001; Soto, 2004).

Há mais de 20 cepas diferentes de *scrapie,* às vezes até duas cepas podem estar presentes em um mesmo animal. Atualmente, o grande desafio é diferenciar BSE de *scrapie* em animais através de testes diagnósticos simples (Hadlow, 1999; Soto, 2004; Gavier-Widén et al., 2005). Sabe-se que mesmo o *príon* de BSE não se desenvolve em ovinos geneticamente resistentes para *scrapie* (Bradley et al., 2002; Martin et al., 2005). Há cepas que afetam animais geneticamente resistentes detectadas em países europeus, mas com prevalência baixa. Entre as cepas que afetam genótipos resistentes já detectadas, a mais característica é denominada Nor98 em referência a sua descrição na Noruega em 1998 e atualmente foi observada em diversos países, ocorrendo nos genótipos mais resistentes e não nos genótipos considerados os mais suscetíveis. Esta cepa apresenta um perfil glicoprotéico diferente das outras cepas quando analisado por testes de *Western blot* e distribuição de antígenos pelo cérebro de forma diferente do convencional na imuno-histoquímica (Soto, 2004; Gavier-Widén et al., 2005; Benestad et al., 2008).

2.4 Genotipagem

Através da biologia molecular, inúmeras pesquisas têm sido feitas visando detectar animais predispostos a sofrerem infecção. Existem predisposições genéticas resultantes da sequência dos genes da proteína priônica (PRNP) do hospedeiro, que os favorece a expressar a PrP^{C} e ter a doença na sua forma clássica, excetuando-se a cepa Nor 98 (Wemheuer et al., 2009). Sabe-se que a sequência dos aminoácidos nos códons 136, 154 e 171 da proteína PrP^{c} tem relação com a manifestação de *scrapie* (Gavier-Widén et al., 2005). As possibilidades de combinação dependem dos três códons do gene da proteína PRNP: códon 136: Alanina (A) ou Valina (V); códon 154: Arginina (R) ou Histidina (H); códon 171: Glutamina (Q), Arginina (R) ou Histidina (H). Em algumas raças, a suscetibilidade a *scrapie* é maior nos genótipos $V_{136}R_{154}Q_{171}$/ $V_{136}R_{154}Q_{171.}$ Em raças tais como Hampshire Down e Suffolk, que não apresentam a valina no códon 136, o maior risco de ter *scrapie* está relacionado com o genótipo ARQ/ARQ. Ovinos com genótipos ARR/ARR são considerados os mais resistentes (Dawson et al., 1998). No Brasil os casos de *scrapie* pertenceram as raças Hampshire Down, Suffolk, Dorper e Santa Inês, e nestas raças (ditas "cara-negra") os alelos predominantes são ARQ e ARR e em pequena quantidade casos ARH (Passos et al., 2008; Ianella et al., 2012). Desta forma, os polimorfismos de nucleotídeos únicos (SNPs) no códon 171 são os mais importantes para determinar a suscetibilidade nestas raças. De acordo com Dawson e colaboradores em 2008, foi possível estabelecer uma classificação de risco de acordo com a combinação de haplótipos dos três códons principais (136/154/171). Os grupos de risco são classificados de R1 (baixo risco) a R5 (risco elevado) para as diferentes raças (Tabela 1).

Tabela 1: Classificação dos haplótipos do gene PRNP (códons 136, 154 e 171) referente aos grupos de risco, segundo o Plano Nacional de *Scrapie* em combate à *scrapie*, estabelecido pela Grã-Bretanha em 2001.

Haplótipo	Classificação no Plano Nacional de *Scrapie*	Risco associado à *Scrapie*
ARR/ARR	R1	Muito baixo
ARR/AHQ ARR/ARH ARR/ARQ	R2	Baixo
AHQ/AHQ AHQ/ARH AHQ/ARQ ARH/ARH ARH/ARQ ARQ/ARQ	R3	Moderado, especialmente em ARQ/ARQ
ARR/VRQ	R4	Moderado
VRQ/AHQ VRQ/ARH VRQ/ARQ VRQ/VRQ	R5	Elevado

Fonte: DAWSON et al., 2008.

Esta classificação visa estratégias de cruzamento com a finalidade de eliminar genótipos suscetíveis. Através de estudos de simulação, utilizando-se machos ARR/ARR é possível tornar o rebanho praticamente livre de alelos de suscetibilidade na 6ª até 9ª geração (De Vries, 2004).

Em estudo de genotipagem feito em 58 ovinos da raça Santa Inês no Brasil, foi observado que 20,7% apresentam haplótipo ARQ/ARQ, 1,7% o haplótipo VRQ/VRQ e outros haplótipos com suscetibilidade intermediária constituindo 50% dos animais com potencial de suscetibilidade a *scrapie* (Lima et al., 2007).

Análises de polimorfismo no códon 171 em diferentes raças do Brasil demonstraram que de 172 ovinos Hampshire Down 37,21% tinham genótipo suscetível para o códon 171

(Q/Q) e de 129 ovinos Suffolk 48,84% eram animais altamente suscetíveis (Passos et al., 2008). Segundo Ianella e colaboradores (2012), as raças mais suscetíveis presentes no Brasil são: Dorper, Ile de France e Santa Inês, com o número maior de ovinos com o haplótipo correspondente ao grupo R5 (risco elevado). Já Sotomaior e colaboradores em 2008, verificaram que as raças mais suscetíveis foram: Dorper, Santa Inês e Texel.

Entretanto, é sabido que para a ocorrência de *scrapie é* necessário que o animal tenha contato com o agente infeccioso, não tendo, portanto, a doença um caráter exclusivamente genético. Assim sendo, faz-se necessário a adoção de medidas preventivas nos rebanhos de diversas raças, tais como genotipagem seletiva e a não inclusão de alelos potencialmente suscetíveis *à scrapie,* de modo a manter afastada esta enfermidade dos plantéis ovinos.

Em 2003, a Comissão da União Europeia determinou que os Estados-Membros deveriam estabelecer programas visando à seleção de animais geneticamente resistentes às Encefalopatias Espongiformes Transmissíveis em todas as raças europeias de ovinos e caprinos, durante o corrente ano. O objetivo era "aumentar a frequência do alelo ARR no efetivo de ovinos e eliminar a frequência do alelo VRQ". Esta eliminação era voluntária, e a partir de 2005 se tornou obrigatória. Neste sentido, estava explicitamente determinado que "é obrigatório proceder à determinação do genótipo de todos os carneiros destinados à reprodução, antes de serem utilizados", havendo depois diferentes estratégias de seleção, mas em que no mínimo, o abate de machos portadores do alelo VRQ é obrigatório (Dawson et al., 2008).

Além dos códons principais, diversos estudos descrevem a importância de identificar polimorfismos em outros códons existentes no gene da proteína priônica, que podem estar envolvidos na resistência ou suscetibilidade a infecção. Por exemplo, o alelo heterozigoto do

códon 143(HR) tem sido sugerido estar associado a uma parcial proteção em cabras infectadas naturalmente. Tal estudo concluiu que a proporção de cabras afetadas como genótipo homozigoto era maior quando comparado ao genótipo heterozigoto (Billinis et al., 2002). Outro exemplo é polimorfismo no códon 142, o qual está associado ao aumento no período de incubação da doença em ovinos e caprinos afetados com *scrapie* (Goldmann et al., 1996). Além desses, outros polimorfimos foram identificados com ou sem função conhecida, como o códon 141 (*scrapie* atípico), códons 112, 127, 137, 138, 143, 172 e 176 (DeSilva et al., 2003; Acin et al., 2004; Lima et al., 2007; Benestad et al., 2008; Vaccari et al., 2009; Maestrale et al., 2009).

No Brasil não existe programa de melhoramento genético para a prevenção de scrapie, embora existam algumas iniciativas como a criação de um programa de certificação voluntária de rebanhos e de testes de suscetibilidade genética à *scrapie*, mas estas ainda estão bastante incipientes. A genotipagem permitirá criar rebanhos resistentes a forma clássica de *scrapie*.

2.4.1 Situação da doença no Brasil

A doença foi diagnosticada pela primeira vez no Brasil em 1978, após a introdução de ovinos Hampshire Down importados (Fernandes et al, 1978). Posteriormente, o primeiro caso oficial foi notificado em ovinos importados do Reino Unido no ano de 1985 (Ribeiro, 1993). Nesse caso os ovinos foram para a quarentena, e a medida preventiva foi aplicada, onde todos os ovinos positivos foram sacrificados, além de seus descedentes, bem como os ovinos considerados expostos.

Segundo o MAPA, foram notificadas duas ocorrências em ovinos importados dos Estados Unidos, uma no ano de 1996 e outra em 2000, adotando-se as medidas condizentes à

contenção da doença. O primeiro caso autócne no Brasil ocorreu em fevereiro de 2003, sendo o animal acometido descendente de ovino importado também dos Estados Unidos. Em 2004 a 2006 foram notificados mais três casos, e em 2007, houve a primeira notificação em ovino Santa Inês no Estado do Mato Grosso com diagnóstico pré-clinico em tecido linfoide. Em 2008, foi diagnosticado *scrapie* também em ovinos da Raça Santa Inês no Estado de São Paulo (Leal, 2009), e um surto descrito em ovinos da raça Suffolk. Além desses, desde 2010 foram notificados três casos de *scrapie* em ovinos da raça Dorper, todos provenientes da região sul do Brasil. No ano de 2013 foi notificado mais um surto em ovinos Dorper na Bahia (http://www.farmpoint.com.br/cadeia-produtiva/giro-de-noticias/adab-constata-caso-de-scrapie-na-bahia-85488n.aspx).

No Brasil a doença é de notificação obrigatória, e um programa sanitário específico, adequado à realidade do país, está em elaboração. O programa visa evitar a disseminação da doença no rebanho nacional, adotando medidas sanitárias condizentes às investigações epidemiológicas promovidas nos focos. Mas até o momento, as únicas medidas sanitárias adotadas em caso de ocorrência de *scrapie* estão baseadas nas seguintes legislações:

- Decreto º 24.548, de 03 de julho de 1934,

- Portaria nº 516, de 09 de dezembro de 1997.

- Instrução Normativa nº 15, de 02 de abril de 2008.

Existem ainda, poucas iniciativas de criação de um programa de certificação voluntária de rebanhos e até o momento não foi implantado no país um programa de melhoramento genético, visando eliminar os ovinos suscetíveis dos rebanhos. A principal medida sanitária para prevenir e controlar a *scrapie* em um país é a proibição da importação de ovinos e caprinos, ou outros produtos de risco para a doença, de países onde a doença é enzoótica. No

caso de animais com suspeita clínica, o serviço de defesa sanitária animal deve ser comunicado, para que a adoção de ações específicas e necessárias (MAPA).

Entre os anos de 2005 e 2013 o Setor de Patologia Veterinária da UFRGS realizou atividades de diagnóstico em oito surtos de *scrapie* em ovinos em conjunto com a Secretaria de Agricultura do Estado do Rio Grande do Sul e do Paraná, além do Ministério da Agricultura Pecuária e Abastecimento do Brasil (MAPA). Um total de 762 animais foram analisados para o diagnóstico pré-clínico por imuno-histoquímica através da coleta de tecidos linfoides por biópsia, como por exemplo, terceira pálpebra e mucosa do reto. Destes casos, 35 ovinos foram diagnosticados com *scrapie*, alguns apresentando sinais clínicos característicos da doença. Neste período, três, dos oito surtos ocorreram no Estado do Rio Grande do Sul, os demais foram provenientes do Sul e Sudeste do Brasil.

3. OBJETIVOS

3.1 Gerais

- Avaliar o genótipo, através do gene da proteína priônica (PRNP), de raças de ovinos no Brasil a fim de determinar a resistência ou susceptibilidade à forma clássica de scrapie.

3.2 Específicos

- Desenvolver uma metodologia, baseada em PCR em Tempo Real por sondas TaqMan, para genotipagem de ovinos relacionada a resistência ou susceptibilidade a scrapie.
- Analisar o genótipo, por PCR em tempo real, de diversas raças de importância econômica no Brasil, através dos principais códons descritos para resistência ou susceptibilidade a scrapie: códons 136, 154 e 171.

- Analisar o genótipo de ovinos provenientes de surtos notificados pelo Ministério da Agricultura (MAPA).
- Analisar a presença de polimorfismos menos frequentes em outros códons, através da técnica de sequenciamento dos ovinos estudados.

4. METODOLOGIA

O trabalho genoipagem e imuno-histoquímica foram executados no Laboratório de Biologia Molecular Aplicada, vinculado ao Setor de Patologia Veterinária, na Faculdade de Veterinária/UFRGS. As amostras foram coletadas por veterinários do laboratório, de propriedades que por voluntariedade aceitaram a participar do projeto. Também foram recebidas amostras de animais, atreavés do MAPA, o qual o Setor de Patologia foi credenciado para o diagnóstico, durante o período de 2004 a 2012, através do teste de imuno-histoquímica.

O número de amostras coletadas em cada rebanho foi baseado no cálculo de amostragem de população infinita, com um intervalo de confiança de 95% e prevalência de 10% da doença, se existir. Quando existiram casos de *scrapie* na propriedade foi realizado um levantamento epidemiológico para determinar animais expostos à doença com prioridade de diagnóstico. No caso em que não ocorreram surtos da doença na propriedade, o número amostral foi baseado em 30-50% dos animais do rebanho.

Amostras de sangue total de todos os ovinos foram coletadas através de tubos vacutainer com EDTA e armazenadas a -20°C para futuras análises. O DNA genômico dos ovinos foi extraído a partir de 200 µL de sangue total ou soro, utilizando o QIAmp Mini Kit e seguindo as instruções do fabricante (Qiagen).

Os primers e sondas para o PCR em tempo real foram projetados utilizando o software PrimerExpress 3.0 (Applied Biosystems). As sondas foram projetadas nas regiões dos três principais códons descritos para resistência ou suscetibilidade ao agente: códon 136, 154 e 171. A padronização e as análises por PCR em tempo real foram realizada no equipamento StepOnePlus (Applied Biosystems). Para a padronização da técnica foram testadas diferentes concentrações de primer, sonda, cloreto de magnésio, além de um gradiente de temperatura para primers e hibridização das sondas. As amostras utilizadas para a padronização eram previamente genotipadas por sequenciamento de DNA.

Para a reação de sequenciamento foram utilizados o primer direto que flanqueia a região do códon 136 (5'-ATGAAGCATGTGGCAGGAGC-3') e o primer reverso que flanqueia a região do códon 171 (5'-GGTGACTGTGTGTTGCTTGACTG-3'), para amplificação de um fragmento de 245 bp que contem as regiões dos principais códons analisados para suscetibilidade à *scrapie* (L´Homme et al., 2008). O produto de PCR foi purificado utilizando o kit Purelink® (Invitrogen™) e quantificado com o kit Qubit®, (Invitrogen™) de acordo com instruções do fabricante. Cada amostra foi sequenciada independentemente usando o conjunto de primers direto e reverso. Os cromatogramas e suas respectivas sequências de consenso foram determinadas usando o pacote de softwares Staden package version 1.7.0 (Staden et al., 2003), associado ao programa Phred (Ewing & Green, 1998; Ewing et al., 1998), e o software novoSNP versão 3.0.1 (Weckx et al., 2005).

A frequência genotípica de cada rebanho foi calculada, baseado na fórmula (fij=n_{ij}/N), onde f_{ij} corresponde a frequência do genótipo *ij*, n_{ij} corresponde ao número de animais que apresentarem o genótipo *ij* e N corresponde ao número total de animais analisados. A frequência alélica (p_i) foi calculada usando a fórmula ($p_i=(2f_{ij}+\sum f_{ij})/2$) (Sotomaior et al.,

2008). Além disso, o rebanho analisado foi classificado de acordo com os grupos de risco descrito por Dawson et al. (2008).

5. RESULTADOS

Os resultados estão apresentados nos artigos colocados a seguir. O artigo intitulado "*Single nucleotide polymorphisms at 15 codons of the prion protein gene from a scrapie-affected herd of Suffolk sheep in Brazil*" foi publicado na revista Pesquisa Veterinária Brasileira em outubro de 2011. O artigo descreve a genotipagem de ovinos da raça Suffolk provenientes de uma propriedade onde um ovino foi diagnosticado com a doença. Através da análise de sequenciamento de DNA, foi possível identificar polimorfismos de nucleotídeos únicos (SNPs) de 15 códons pertencentes ao gene da proteína priônica, com importância para a suscetibilidade e resistência a forma clássica de *scrapie*.

O artigo intitulado "*Development of a real-time polymerase chain reaction assay for single nucleotide polymorphism genotyping codons 136, 154, and 171 of the PRNP gene and application to Brazilian sheep herds*" foi publicado como *Short Communication* na *Journal Veterinary Diagnostic Investigation* em janeiro de 2013. O trabalho teve como objetivo validar a técnica de PCR em tempo real baseado em sondas Taqman para identificar polimorfismos de nucleotídeos únicos (SNPs) dos três principais códons do gene da proteína priônica associados a resistência e/ou suscetibilidade a *scrapie*, 136/154/171. Essa técnica permitirá, devido a sua rapidez, acurácia e repetibilidade, ampliar os estudos de genotipagem em rebanhos de ovinos com importância econômica no Brasil, além de auxiliar na complementação do diagnóstico em casos de rebanhos com surto.

O terceiro artigo intitulado "*First report of an outbreak of classical scrapie in Dorper sheep and the associated prion protein gene polymorphisms in affected flocks*" foi

submetido a BMC Veterinary Research, e está na segunda etapa de correção. O estudo descreve pela primeira vez a análise de três surtos de *scrapie*, os quais possuem relação entre eles, em ovinos da raça Dorper, todos localizados no Sul do Brasil. Além da genotipagem dos três rebanhos foi realizado o levantamento epidemiológico e testes para o diagnóstico pré-clínico em amostras de biopsia nos ovinos considerados expostos aos animais positivos para a doença.

5.1 Artigo 1

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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*
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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 al- tered conformation that leads to aggregation in the host's central nervous and lymphore-ticular 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 sus- ceptible 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 re- levant 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 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 do 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 de 15 códons analisados (136, 154 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 genotypes 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 lowsusceptibility, 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, 200uM dNTPs, 1x Platinum Taq buffer, 1 U Platinum Taq DNA Polymerase (Invitrogen) and 1uL of genomic DNA in a final volume of 25uL. 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 30s and 72ºC for 30s, and a final step at 72 ºC 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 $p=(2f+\sum f)/2$.

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.

form the remaining samples had a Phred score bellow 20 for the consensus sequence at this region, making it impos-

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).

sible 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 program 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 program 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 affects 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 analysed (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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5.2 Artigo 2

Development of a real-time polymerase chain reaction assay for single nucleotide polymorphism genotyping codons 136, 154, and 171 of the *prnp* gene and application to Brazilian sheep herds

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Abstract. Scrapie is a transmissible spongiform encephalopathy of sheep and goats and is associated with the deposition of an abnormal isoform of prion protein (PrP^{sc}). This isoform presents an altered conformation that leads to its aggregation in the host's central nervous and lymphoreticular systems. A predisposition to the prion-agent infection can be influenced by specific genotypes that are related to polymorphisms in the ovine *prnp* gene. The most characterized polymorphisms occur at codons 136, 154, and 171, with genotype VRQ being the most susceptible and ARR the most resistant. In the current study, a real-time quantitative polymerase chain reaction (qPCR) technique based on allele-specific TaqMan probes was developed to identify single nucleotide polymorphisms in the *prnp* gene from Brazilian herds. Specific primers and TaqMan probes were designed for all 3 codons of interest. Samples from a total of 142 animals were analyzed by qPCR, followed by DNA sequencing of the amplicons. All of the genotypes determined by qPCR were in agreement with the data determined by DNA sequencing. In all 3 of the analyzed breeds, the majority of the animals were AA homozygous for the 136 codon. The most frequent genotype for codon 154 was RR, and genotypes QQ and QR were the most frequent for codon 171. The results are discussed in relation to establishing scrapie control measures and breeding programs for Brazilian herds.

Key words: Genotyping; prion protein polymorphism; scrapie; TaqMan real-time polymerase chain reaction.

Scrapie, a neurodegenerative disease affecting sheep and goats, is one of several transmissible spongiform encephalopathies or prion diseases. The disease is characterized by the accumulation of an abnormal isoform (PrP^{sc}) of a host-encoded cellular prion protein PrP^{c} in the central nervous system.[13] Although scrapie is an infectious disease, the susceptibility of sheep is strongly influenced by polymorphisms of the prion protein gene (*prnp*).[4] The combined detection of polymorphisms at codons 136, 154, and 171 suggests that VRQ (valine, arginine, glutamine) and ARR (alanine, arginine, arginine) are antagonistic in determining the susceptibility to the disease: VRQ is associated with a high incidence of natural scrapie, and ARR is associated with a low incidence of natural scrapie.[6]

Programs for selecting sheep with PRNP genotypes that appear to confer resistance to the development of classical scrapie has been used for several years in an attempt to eradicate the disease.[14] However, in Brazil, there is no selection program in operation related to scrapie, even though research in sheep genetics and breeding has increased significantly in recent years. Such studies include research on the characterization, breeding, and crossing of sheep using new technologies that incorporate both classical quantitative and molecular genetics.[19] Since 2007, some studies have analyzed the PRNP genotypes in Brazilian sheep herds.[2,15,17,21,22] However, all of this research is based on sequencing and/or restriction fragment length polymorphism (RFLP) and has been applied to a limited number of animals. Several PRNP genotyping methodologies for detecting single nucleotide polymorphisms (SNPs) have been described, including sequencing, RFLP, mass spectrometry, quantitative polymerase chain reaction (qPCR), and amplicon melting temperature analysis.[1,3,12,18]

Direct sequencing accurately determines the genotype of the samples with respect to the SNPs in codons 136, 154, and 171 and will reliably identify any additional polymorphisms in neighboring codons. However, SNP genotyping technologies, such as sequencing and RFLP, are costly for the analysis of

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a large number of samples.[9] TaqMan[a] SNP genotyping is a well-proven technique and has been successfully used to determine PRNP genotypes in sheep.[24] Moreover, a careful design of the probes used should prevent cross-reactions with a mismatched probe and the generation of a nonspecific signal.[14] The main benefit of sequence-specific detection is that it allows the unambiguous detection of target sequences without the contaminating signals that arise from primer dimers and other nonspecific PCR events. This is due to the low likelihood that a nonspecific PCR will generate a hybridization site for an oligonucleotide probe to bind and generate a signal.[10,11,25] Furthermore, TaqMan probes are the most widely used technology for qPCR. The aim of the present study was to develop and apply a qPCR TaqMan assay for SNP genotyping that would reliably identify alleles A/V in the 136 codon, alleles R/H in the 154 codon, and alleles Q/R/H in the 171 codon of the sheep *prnp* gene.

Whole peripheral blood samples were collected using ethylenediamine tetra-acetic acid evacuated blood collection tubes[b] from 142 animals belonging to the Suffolk breed (100 animals), Dorper/White Dorper breed (19 animals), and Santa Inês breed (23 animals). All of the animals were from herds originating in the southern and southeastern regions of Brazil. The genomic DNA was extracted from 500 µl of whole blood using a commercially available kit,[c] following the manufacturer's instructions. The DNA was recovered in 200 µl of elution buffer and stored under refrigeration for further analyses. Every genomic DNA sample was quantified using a spectrophotometer[d] and diluted to 20 ng/µl final concentration for use in the PCR reactions.

Each sequence-specific probe, representing alternate alleles of the *prnp* gene, was designed with a different fluorescent label, and both were combined in a reaction with the same set of primers. The probes and primers were designed for the *Ovis aries prnp* gene (GenBank accession no. M31313) using commercial software.[e] The primers and probes sequences used for the allelic discrimination assays at the 136 codon were: forward (5′-GGGCCTTGGTGGCT ACATG-3′), reverse (5′-TCCTCATAGTCATTGCCAAAA TGTAT-3′), 136A probe (5′-6FAM-TGGGAAGTGCCA TGAG-3′), and 136V probe (5′-VIC-TGGGAAGTGTCA TGAG-3′). Primers and probes used for codon 154 were: forward (5′-GGCCTCTTATACATTTTGGCAATG-3′), reverse (5′-ATCCACTGGTCTGTAGTACACTTGGT-3′), 154R probe (5′-6FAM-ACCGTTACTATCGTGAAA-3′),and154H(5′-VIC-ACCGTTACTATCATGAAA-3′). Primers and probes used for codon 171 were: forward (5′-GTTACCCCAACCAA GTGTACTACAGA-3′), reverse (5′-TGTTGACACAGTCA TGCACAAAG-3′), 171Q probe (5′-6FAM-CAGTGGATCA GTATAGTAA-3′), 171R probe (5′-VIC-CAGTGGATCGG TATAG-3′), and 171H probe (5′-NED-CAGTGGATCATTA TAG-3′). All probes contained MGBNFQ (minor groove binding nonfluorescent quencher) at the 3′-end. The PCR conditions were previously optimized for the temperature and concentrations of the probe, primer, and magnesium chloride. All of the optimizations were performed using samples of known genotypes. The reaction efficiencies were determined using calibration curves for each set of primers and probes. The primers were tested at 10, 15, 20, and 25 pmol, and the probes were tested at 120, 200, and 300 nM. The hybridization temperatures were tested in a gradient ranging from 58ºC to 66ºC (2ºC step). The magnesium chloride concentration ranged from 1 to 3 mM. For codon 171, a triplex qPCR was tested that included probes 171Q, 171R, and 171H without success; reactions could only be optimized for 2 duplex reactions: 171Q/171R and 171Q/171H.

The PCR reactions contained 20 pmol each primer, 200 µM of deoxyribonucleotide triphosphate (dNTPs), 1× Taq buffer,[f] 1 U Taq DNA polymerase,[f] and 1 µl of genomic DNA (20 ng) in a final volume of 25 µl. The concentrations of magnesium chloride and probes, and the hybridization temperatures, varied in the 4 reaction sets. The reactions were performed using a real-time PCR system,[e] and the PCR reaction for each sample was performed in duplicate. A negative control in duplicate was included for every 96-well plate run.

The PCR reactions for obtaining amplicons for sequencing were performed using a forward primer flanking the 136 codon position and a reverse primer flanking the 171 codon position, generating a 245-bp amplicon.[16] The PCR reactions contained 15 pmol of each primer, 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 1× Taq buffer,[f] 1 U of Taq DNA polymerase,[f] and 1 µl of genomic DNA in a final volume of 25 µl. The PCR reactions were performed using a automated DNA thermal cycler[e] with the following parameters: 95ºC for 5 min; followed by 35 cycles at 95ºC for 30 sec, 58ºC for 30 sec, and 72ºC for 30 sec; and a final step at 72ºC for 10 min.

The PCR products were purified using a commercial kit[f] and quantified using a fluorescence quantification system[f] according to the manufacturer's instructions. The sequencing of the PCR products was performed using Sanger sequencing.[e] Each sample was sequenced independently using both forward and reverse primers until a Phred quality score of at least 20 was obtained for each individual base in the consensus sequence.[7,8] The resulting chromatograms were analyzed using the Staden package version 1.7.0 program[23] and novoSNP version 3.0.1.[26]

Initially, the standardization of the qPCR was accomplished using samples that were previously sequenced and with a known genotype. For each set of reactions, different concentrations of the primers, probes, and magnesium chloride were tested, as were the temperature gradients. Four different reaction sets were established for SNP genotyping the variations at codons 136 (A/V), 154 (R/H), and 171 (Q/R and Q/H). The optimum hybridization temperatures were 66ºC (136 codon reaction set), 60ºC (154 codon reaction set), and 62ºC (both 171 codon reaction sets). All of the reaction sets contained 1 mM of $MgCl_2$, except the 136 reaction set for which a concentration below 3 mM led to a low specificity. The optimum probe concentration was determined to be 120 nM, except for probes 136V and 171H for which a

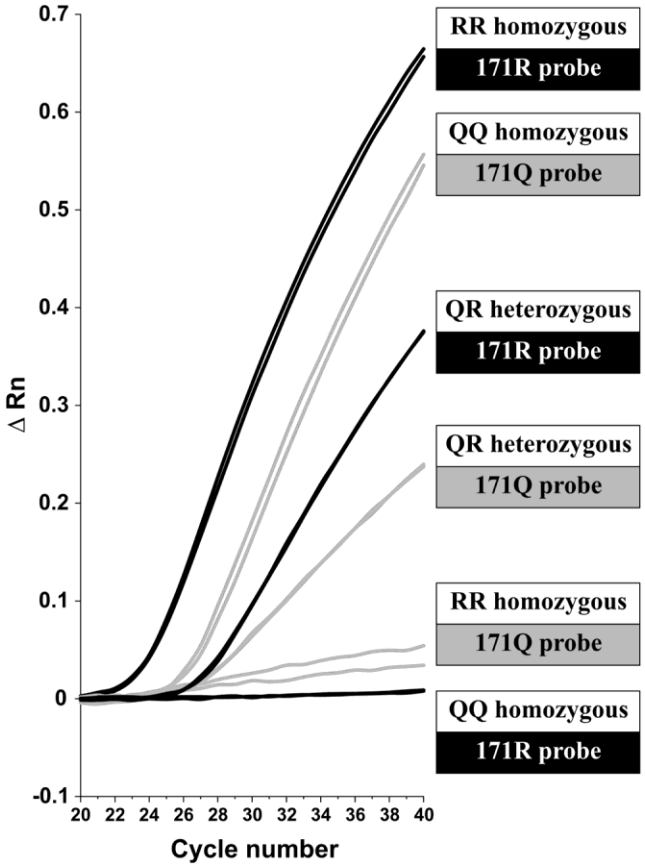

Figure 1. Representative amplification plots for quantitative polymerase chain reaction (qPCR) using TaqMan probes 171Q and 171R. The DNA from 3 animals with genotypes RR, QQ, and QR for codon 171 was subjected to a duplex qPCR using probes 171Q and 171R. Each sample was analyzed in a duplicate reaction.

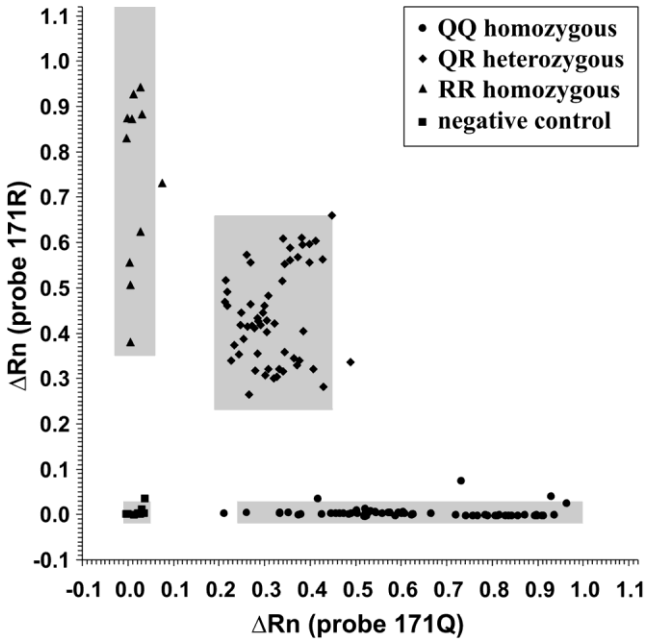

Figure 2. ΔRn values for quantitative polymerase chain reaction using TaqMan probes 171Q and 171R. Each point represents the mean ΔRn value for the duplicates. The gray areas indicate the standard deviation.

concentration below 300 nM produced a very weak fluorescence signal.

A representative amplification plot for probes 171Q and 171R is shown in Figure 1. Both the amplification curves and the ΔRn at the final cycle are diverse enough to allow differentiation of the 3 possible genotypes. For both of the probes, the ΔRn values for the samples from heterozygote animals are almost half the value when compared to those from the homozygous animals. In addition, the curves for the duplicates of each sample are almost identical, and the samples from homozygous animals have a ΔRn close to zero with the mismatch probe.

To establish the cutoffs to assign the possible genotypes to each sample, the mean ΔRn values for each sample duplicate obtained for each probe pair were compared. In Figure 2, the plot obtained for probes 171Q and 171R is shown applied to 138 of the samples. All of the samples from the homozygous animals (QQ and RR) presented very low fluorescence with the mismatch probe, and the samples from the heterozygous animals presented intermediate fluorescence with both of the probes. The mean ΔRn values (endpoint) for qPCR using TaqMan probes 171Q were 0.62 (± 0.19), 0.32 (± 0.07), and 0.02 (± 0.02) for genotypes QQ, QR, and RR, respectively. The mean ΔRn values (endpoint) for qPCR using TaqMan probes 171R were 0.74 (± 0.19), 0.44 (± 0.11) and 0.00 (± 0.01) for genotypes RR, QR, and QQ, respectively.

The amplicons from all 142 of the samples were sequenced to validate the real-time PCR results, and no discrepancies were found. Duplicate independent amplicons were obtained and sequenced for those animals with genotypes considered to be of a low frequency (136AV, 136VV, 154RH, 171RR, 171QH, and 171HH).

A previous study concluded that the real-time PCR method is superior to RFLP because of the increased repeatability of the assays, the simple one-step assay protocol, and the clarity of the results obtained compared to RFLP in which an incomplete digestion of the PCR products can produce ambiguous results.[10] In the current study, the results showed that the assay of SNP genotyping using real-time PCR for the detection of SNPs in the prion protein gene is efficient due to its accuracy, rapidity, and the specificity of the results; furthermore, the technique did not require post-PCR manipulations.

The genotype distribution for SNPs at the *prnp* gene found among the analyzed herds is shown in Figure 3. All known allelic forms at the 136 codon were found in the present study. For the 154 codon, the homozygous allele for histidine (HH) was not found and, for codon 171, none of the animals presented the heterozygous allele RH.

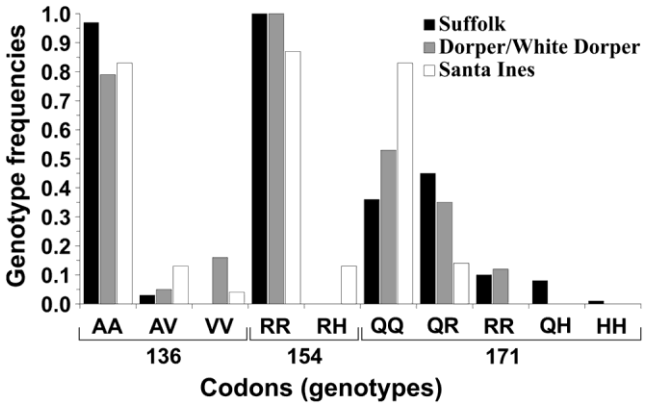

Figure 3. Genotype frequencies for single nucleotide polymorphisms at codons 136, 154, and 171 of the *prnp* gene. Only the genotypes found among the analyzed Suffolk, Dorper/White Dorper, and Santa Inês sheep are shown.

The allele frequencies estimated for the Suffolk breed found in the current study at codons 136 and 154 were similar to the frequencies reported by other studies analyzing herds from Brazil (Sotomaior CS: 2007, Polimorfismo do gene da proteína prion celular (PrPC) e a suscetibilidade/ resistência ao scrapie em ovinos no Estado do Paraná [Polymorphism of the cellular prion protein gene (PrPC) and susceptibility/resistance to scrapie in sheep in the State of Paraná]. Doctoral thesis in Animal Health, Faculty of Veterinary Sciences, Universidade Federal do Paraná, Curitiba, PR, Brazil).[2,21] However, at codon 171, a higher variety of genotypes was found when compared to these previous studies (Fig. 3). The present study found Q171R to be the most frequent (44.5%) genotype in Suffolk animals, in accordance with previous studies including German and Irish Suffolk breeds (40–42%).[5,15,20] However, some other previous studies in Brazil found higher frequencies for homozygosity at 171Q.[21,22]

Studies including Dorper and White Dorper herds from Brazil and elsewhere in the world showed genotype frequencies similar at codon 154 (Fig. 3).[12,15,22] For codon 136, it was found that allele V was homozygous with a 15.8% frequency and that genotype A136V had a 5.3% frequency (Fig. 3). Previous studies reported a higher frequency in A136V (20–25%).[15,22] Homozygous genotype 171Q was the most frequent in Dorper and White Dorper (52.9%; Fig. 3), but other authors found frequencies as high as 75–80%.[15,22]

For the Santa Inês breed, genotype frequencies at codon 136 and 154 were found to be quite similar to previous works with Brazilian herds of approximately the same size, varying from 0.82 to 0.89 (Fig. 3).[17,22] However, another study with different herds also from Brazil, but with a larger number of animals (421), found frequencies as low as 0.68 for the homozygous 154R genotype.[15] Finally, at codon 171, frequencies of 0.83 and 0.17 were found for genotypes 171Q and Q171R, respectively, differing from previous studies with Brazilian herds that found frequencies of 0.60 and 0.40 for these genotypes.[15,22]

Taken together, the results of the current study indicate that the herds of Santa Inês and Dorper/White Dorper present a high frequency of animals with scrapie-susceptible genotypes. The extension of the present work to include a larger number of animals from different herds, regions, and breeds from Brazil will allow for a clearer overview of the strategies that should be applied in order to reduce the harmful impact of classical scrapie on the sheep production system in Brazil. The authors plan to broaden the study in order to analyze other polymorphisms in the *prnp* gene related to atypical scrapie, such as the polymorphism at the 141 codon.

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Declaration of conflicting interests

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Sources and manufacturers

a. F. Hoffmann-La Roche Ltd., Basel, Switzerland.
b. Vacutainer, BD, Franklin Lakes, NJ.
c. QIAamp DNA blood mini kit, Qiagen Biotecnologia Brasil Ltda, São Paulo, Brazil.
d. GeneQuant 1300, GE Healthcare Technologies, Piscataway, NJ.
e. Primer Express version 3.0 software, StepOnePlus real-time PCR system, Veriti automated DNA thermal cycler, BigDye terminator cycle sequencing kit version 3.1 (in an ABI PRISM 3130 Genetic Analyzer); Applied Biosystems, Life Technologies do Brasil, São Paulo, Brazil.
f. Platinum buffers, PureLink PCR purification kit, Qubit fluorescence quantification system; Invitrogen Brasil Ltda, Sao Paulo, Brazil.

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5.3 Artigo 3

First report of an outbreak of classical scrapie in Dorper sheep and the associated prion protein gene polymorphisms in affected flocks

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Abstract

Background

Scrapie is an infectious neurodegenerative disease affecting sheep and goats. This is related to an altered conformational isoform of the prion protein (PrP^{Sc}) that leads to the deposition and aggregation of protein in the central nervous system. Disease susceptibility is influenced by host genetic factors. In sheep, this is associated with a single nucleotide polymorphism in the prion gene. This study is the first to report three scrapie-affected Dorper flocks located at three different farms in Brazil. From these flocks, 192 sheep were selected for third eyelid and rectal mucosal immunohistochemistry and 308 sheep had blood samples taken for genotyping using real-time polymerase chain reaction (PCR).

Results

A total of twenty-two scrapie-affected sheep were positive by immunohistochemistry, with the majoritary ARQ/ARQ and ARQ/ARR haplotypes. Of these, four presented with clinical signs and had scrapie immunoreactivity in the obex. The major haplotypes found on the farms involved in these outbreaks were ARQ/ARQ, ARQ/ARR, and ARQ/VRQ for codons 136, 154 and 171. Most of the sheep on these farms had susceptible genotypes and were considered to be at moderate to high risk for developing scrapie.

Conclusions

Our data demonstrate the importance of preclinical scrapie diagnosis in Brazilian sheep, as most of the affected sheep showed no clinical signals despite being positive via immunohistochemistry. In addition, this also emphasizes the importance of genotyping for future breeding programs to reduce the number of susceptible animals.

Keywords: Outbreak, Dorper, scrapie, prion, immunohistochemistry, real time PCR.

Introduction

Scrapie is a naturally occurring transmissible spongiform encephalopathy in sheep and goats that causes progressive neurological deterioration and death [1]. It is characterized by central nervous system (CNS) accumulation of an abnormal isoform (PrP^{Sc}) of a host-encoded cellular prion protein (PrP^{c}) [2].

Scrapie can be transmitted from infected ewes to lambs [3,4] and laterally transmitted to other sheep [5]. Detection of PrP^{Sc} in placental tissues supports the idea that the disease is transmitted *in utero* [6,7]. Studies have demonstrated that the host genotype plays a role in disease transmission, as PrP^{Sc} accumulating cells were identified in the placentas of fetuses with susceptible genotypes from sheep naturally exposed to scrapie, demonstrating the role of the fetal genotype in placental PrP^{Sc} accumulation [6].

During the incubation period, genetically susceptible ewes can accumulate massive amounts of PrP^{Sc} and disseminate the agent in the environment through the placenta as early as the first gestation and long before the onset of clinical disease [8]. The introduction of animals with resistant alleles in infected flocks reduces the incidence of the disease and prevents the environmental spread of the scrapie agent from infected ewes by the placental route [9].

The major polymorphisms of the prion gene (PRNP) associated with susceptibility or resistance to the infection are at codons 136 (A or V), 154 (R or H), and 171 (R, Q or H) [10,11]. The PRNP genotypes VRQ/VRQ, ARQ/VRQ and ARQ/ARQ are associated with high scrapie susceptibility, while the ARR/ARR genotype exhibits high resistance to natural and experimental infections with classical scrapie [2,11,12].

Polymorphisms at other codons can also be involved in infection resistance or susceptibility. A study observed a polymorphism in scrapie-affected ovines at codon 143

[13]. In addition, a polymorphism at codon 142 has been described in sheep and is associated with a longer disease incubation period [14]. Other codon alterations were identified, with or without defined functions, include codons 141 (atypical scrapie), 112, 127, 137, 142, 143, and 176 [13,15–19].

The first case of classical scrapie in Brazil was diagnosed in 1978 [20]. Since then, a total of 16 cases of scrapie have been reported from 1978 to 2010 [21]. Our previous work described a classical form of the disease in Suffolk sheep where one animal presented with clinical signals of infection [22]. Until now, no cases of scrapie have been reported in Dorper and White Dorper sheep. Additionally, while some work has been done to characterize the PRNP polymorphisms in Brazilian flocks, few studies report these polymorphisms in association with scrapie outbreaks [22-28]. The present study describes for the first time three cases of the classical scrapie occurring in Dorper and White Dorper herds at three different rural properties in Brazil. To characterize these outbreaks, we analyzed PRNP polymorphisms at codons 136, 154 and 171, and performed immunohistochemical analysis of obex and lymphoid tissues of exposed and affected animals.

Materials and Methods

Epidemiological background for the outbreaks

On the first property (hereafter referred to as ‘outbreak 1’) located in southern Brazil, one sheep were diagnosed with the classical form of scrapie based on immunohistochemical analysis, clinical signs, and histopathological findings (index cases). Subsequently, an epidemiologic survey of this rural property was performed by the Brazilian Ministry of Agriculture and Livestock (MAPA) and Parana State Department of Agriculture (SEAB) to identify other animals that may have been exposed directly or indirectly to the affected sheep. This rural property produced pure Dorper and White Dorper sheep; it was also home

to female Santa Inês, Suffolk and Texels, which were used as receptors in embryo transfer.

The sheep selected for testing had potential contact with the affected sheep and included animals born in the same stall or to the same receptor animal as the affected animal. Data collected for each animal included sex, age, registry number and degree of purity.

On the second property (called 'Outbreak 2'), one Dorper sheep was diagnosed with clinical signals of scrapie. This occurred approximately a year after the first outbreak at a rural property in southern Brazil, 280 km distant from outbreak 1. The diagnosis was confirmed by histopathology and immunohistochemistry. An epidemiologic survey of this rural property was performed, which had found that sheep that had been shipped the property from the first outbreak, after it has occurred. There had been exchange of animals between the rural properties where outbreaks 1 and 2 occurred.

Two years after the first outbreak, a third outbreak ('outbreak 3') was identified. This farm is also located in the southern part of Brazil, 543 and 513 km from the first and second outbreaks, respectively. Three animals at this property had been obtained from the site of the first outbreak, after it has occurred. All Dorper sheep over a year of age were considered exposed; one of these showed clinical signs.

Animals, sample collection and DNA preparation

After outbreak 1, samples from the third eyelid and rectal mucosa were taken for antemortem scrapie diagnosis from 75 animals (71 females and 4 males) from a herd of 335 Dorper/White Dorper sheep. Blood samples were also collected from 181 animals for real-time PCR genotyping. Samples of the third eyelid, rectal mucosa and blood were collected from all 74 animals in the herd involved in outbreak 2, which consisted of crossbred Dorper sheep. Blood samples from the entire herd (53 Dorper sheep) and 43 samples of third eyelid and rectal mucosa were collected from the farm involved in outbreak 3. Fewer samples of

third eyelid and rectal mucosa tissue were collected because 10 of the animals were less than one year old, and immunohistochemistry is less likely to detect PrP^{Sc} in the lymphoid tissues of young animals, and according to normative of the country is only collected from animals over one year.

Rectal biopsies were collected using a vaginal speculum and application of 1% lidocaine hydrochloride using a syringe ogf1mL. Third eyelid collection was performed using 1% tetracaine hydrochloride 0.1% phenylephrine drops as previously described [29]. For histological examination, 3µm sections were stained with hematoxylin and eosin (HE). Lymphoid follicles in each tissue section were counted via light microscopic examination and animals whose samples did not contain adequate follicles (4 or more) were rebiopsied.

Animals with scrapie immunoreactivity on biopsy samples were euthanized and the obex collected for later HE and immunohistochemical examination. Clinical signs were determined by questioning owners or attending veterinarians at the time of sample collection.

Peripheral blood samples were collected for PCR, sequencing, and genotyping using EDTA vacutainer tubes. Genomic DNA was extracted from 500 µL of whole blood using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) per the manufacturer's instructions. DNA was recovered in 100 µL of elution buffer and refrigerated until further use.

Immunohistochemistry

After paraffin embedding, tissue sections were dried for 12 hours at 60°C, deparaffinized, and treated with 10% hydrogen peroxide in methanol for 20 minutes to reduce endogenous peroxidase activity. Sections were then incubated with formic acid (Merck, Darmstadt, Germany) for 5 minutes and washed in distilled water, TBS (Tris-buffered saline), and 0.1% Tween in TBS for 1 minute each, followed by proteinase K

treatment for 40 seconds. PrP^{Sc} immunohistochemistry was performed using the primary anti-*PrP* monoclonal antibody mAb F99/97.6.1 (VMRD Inc., Pullman, WA), which recognizes an epitope at the 220–225 amino acid residues of the PRNP sequence. The antibody was diluted 1:500 in phosphate buffered saline (PBS) prior to use. Sections were incubated with the primary antibody for 18 hours at 4°C, treated with a biotinylated secondary antibody (DAKO, California, USA) for 20 minutes, and then treated with peroxidase-conjugated streptavidin (DAKO, California, USA) for 20 minutes. Tissues were exposed to the chromogen diaminobenzidine (DAB – DAKO, California, USA) for 1 minute or 3-amino-9-ethylcarbazole (AEC – DAKO, Ventana) counterstained with hematoxylin for 30 seconds. Finally, coverslips were mounted using Entellan® (Merck, Darmstadt, Germany) aqueous solution. Each experiment included control sections of obex and lymphoid tissues of scrapie-affected and healthy control sheep.

Primers, PCR and sequencing conditions

Real-time PCR for PRNP codons 136, 154 and 171 genotyping was performed as described previously [30]. The prion genes of sheep with less common genotypes (VV for codon 136, RH or HH for codon 154, and QH, RH, and HH for codon 171), animals with polymorphisms not been previously identified in our laboratory, and animals that were positive by immunohistochemistry had their prion genes sequenced for genotype confirmation as described below. In total, 43 samples from the first farm, 21 samples from the second, and 18 samples from the third were sequenced.

PCR reactions were carried out using a forward primer flanking codon 136 (primer 136F: 5'-ATGAAGCATGTGGCAGGAGC-3') and a reverse primer flanking codon 171 (primer 171R: 5'-GGTGACTGTGTGTTGCTTGACTG-3'), generating a 245-bp amplicon [31]. 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, Carlsbad, CA, USA),

and 1 μL of genomic DNA, and water to achieve a final volume of 25 μL. PCR reactions were performed on an ABI Veriti automated DNA thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following parameters: 95°C for 5 min followed by 35 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 30 s, and a final step at 72°C for 10 min.

PCR products were purified using the PureLink™ PCR Purification Kit (Invitrogen, Carlsbad, CA, USA) and quantified using a Qubit fluorescence quantification system (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions. PCR products were sequenced using the BigDye Terminator version 3.1 Cycle Sequencing kit in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Each sample was sequenced independently using both forward and reverse primers until a Phred quality score of at least 20 [33,34] was obtained for each individual base in the consensus sequence. The resulting chromatograms were analyzed using the Staden package version 1.7.0 programs [34] and novoSNP [35].

Data analysis

To establish cutoffs for assigning possible genotypes to each sample, we compared the mean ΔRn values obtained via real time PCR for each sample duplicate and each probe pair. The amplification curves and the ΔRn at the final cycle are diverse enough to allow differentiation of all genotypes. The real-time PCR data from sheep samples 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 with the genotype *ij*, and N corresponds to the total number of animals analyzed. These results were used to classify sheep according to the risk groups described by Dawson et al. [36].

Results

Immunohistochemistry identified 22 sheep with biopsy samples that had positive scrapie immunoreactivity (see Table 1). Animals that presented with clinical signs

compatible with scrapie (pruritus, difficulty in locomotion, and movement disturbances) also had scrapie immunoreactivity in the obex (Figures 1 and 2).

Analysis of Outbreak 1

Immunohistochemistry identified 4 positive animals out of 75 sheep tested. A total of seven animals were sacrificed; these included the four sheep positive via immunohistochemistry and three additional that were considered high risk owing to a relationship or history of exposure to positive animals (Table 1). Only one animal showed clinical signs of disease (pruritus and locomotion difficulty). Histopathologically, this animal had intraneuronal vacuoles in the obex that distended the perikaryon, giving the neurons a rounded appearance (Figure 1, female positive 2.7, according Table 1).

Immunohistochemistry revealed PrP^{Sc} in the neuronal cytoplasm (Figures 2A-female positive number 2.7, according Table 1; and 2B-female positive 3.2, according Table 1).

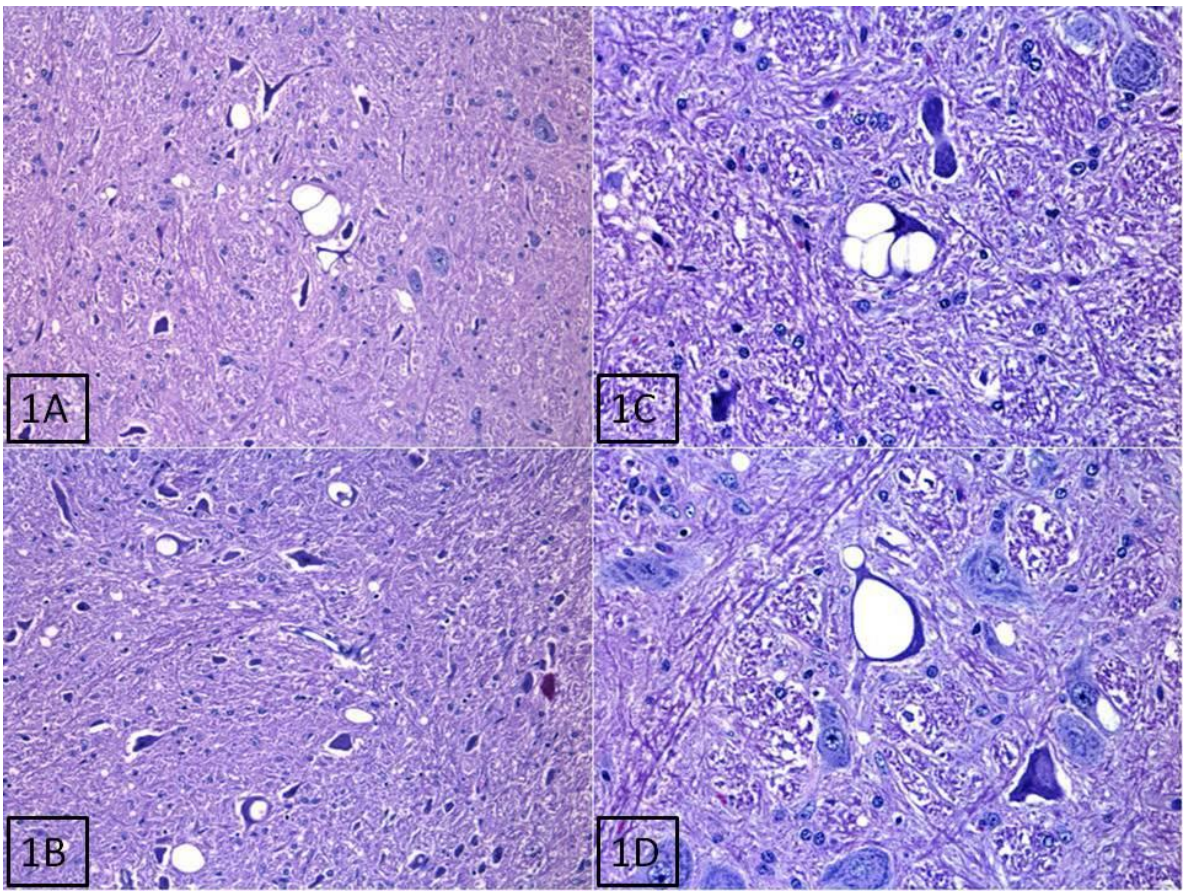

Figure 1: Representative histopathology of the obex of a sheep with scrapie, with vacuolization adjacent to neurons (case 2.7 in Table 1). Magnification: 1A and 1B, 200x; 1C and 1D, 400x. Staining with hematoxylin and eosin.

Table 1: List of positive sheep samples described in three outbreaks in Dorper sheep. (a): Born at the same time as the animal with clinical signs; (b): Lambed in the same bay the animal with clinical signs was born; (c,d): Obtained from the property in outbreak 1.

Animal number	Sex	Age	Pure or Cross	IHC Results	Tissue PrP^{Sc} Immunoreactivity	Genotypes 136	154	171	127	142	143	Haplotypes	Clinical signs	Risk group
1.1 (a)	Female	2 years	Purebred	**Positive**	Third eyelid	AA	RR	QQ	GG	II	HH	ARQ/ARQ	-	3
1.2 (b)	Female	4 years	Crossbreed	**Positive**	Third eyelid	VV	RR	QQ	GG	IT	HH	VRQ/VRQ	-	5
1.3	Female	4 years	Crossbreed	**Positive**	Rectal mucosa	AA	RR	QQ	GG	II	HH	ARQ/ARQ	-	3
1.4	Male	2 years	Purebred	**Positive**	Obex	AA	RR	QH	GG	II	HH	ARQ/ARH	+	3
2.1	Female	2 years	Crossbreed	**Positive**	Rectal mucosa	AA	RR	QQ	GS	II	HH	ARQ/ARQ	-	3
2.2	Female	2 years	Crossbreed	**Positive**	Rectal mucosa	AA	RR	RR	GG	II	HH	ARR/ARR	-	1
2.3	Female	2 years	Crossbreed	**Positive**	Third eyelid and rectal mucosa	AA	RR	QQ	GG	II	HH	ARQ/ARQ	-	3
2.4	Female	2 years	Crossbreed	**Positive**	Rectal mucosa	AA	RR	QQ	GG	II	HH	ARQ/ARQ	-	3
2.5	Female	2 years	Crossbreed	**Positive**	Rectal mucosa	AA	RR	QQ	GG	II	HH	ARQ/ARQ	-	3
2.6	Female	2 years	Cossbreed	**Positive**	Third eyelid and rectal mucosa	AA	RR	QR	GG	II	HH	ARQ/ARR	-	2
2.7 (c)	Female	2 years	Purebred	**Positive**	Obex	AA	RR	QQ	GG	II	HH	ARQ/ARQ	+	3
2.8	Female	2 years	Purebred	**Positive**	Obex	AA	RR	QQ	GG	II	HH	ARQ/ARQ	+	3
3.1	Female	> 1 year	Purebred	**Positive**	Third eyelid and rectal mucosa	AV	RR	QQ	GG	II	HH	ARQ/VRQ	-	5
3.2 (d)	Female	> 1 year	Purebred	**Positive**	Third eyelid, rectal mucosa, obex	AA	RR	QQ	GG	II	HH	ARQ/ARQ	+	3
3.3	Female	> 1 year	Purebred	**Positive**	Rectal mucosa	AA	RR	QR	GG	II	HH	ARQ/ARR	-	2
3.4	Female	> 1 year	Crossbreed	**Positive**	Third eyelid and rectal mucosa	AA	RR	QR	GG	II	HH	ARQ/ARR	-	2
3.5	Female	> 1 year	Purebred	**Positive**	Rectal mucosa	AA	RR	QQ	GG	II	HH	ARQ/ARQ	-	3
3.6	Female	> 1 year	Purebred	**Positive**	Third eyelid and rectal mucosa	AA	RR	QQ	GG	II	HH	ARQ/ARQ	-	3
3.7	Female	> 1 year	Crossbreed	**Positive**	Rectal mucosa	AA	RR	QR	GG	II	HH	ARQ/ARR	-	2
3.8	Female	> 1 year	Purebred	**Positive**	Third eyelid and rectal mucosa	AA	RR	QR	GG	II	HH	ARQ/ARR	-	2
3.9	Female	> 1 year	Purebred	**Positive**	Rectal mucosa	AA	RR	QQ	GG	II	HH	ARQ/ARQ	-	3
3.10	Female	> 1 year	Purebred	**Positive**	Rectal mucosa	AA	RR	QR	GG	II	HH	ARQ/ARR	-	2

The other three sheep showed no clinical signs and had no histologic changes in the obex. The three additional animals were 3-4 years old, with one male and three females and two Dorper and two Dorper crossbred sheep. The diagnosis was confirmed via PrP^{Sc} immunolabeling of lymphoid follicles of the third eyelid and/or rectal mucosa (Figure 2C, female positive 1.2, according Table1; and 2D-female positive 3.10, according Table 1).

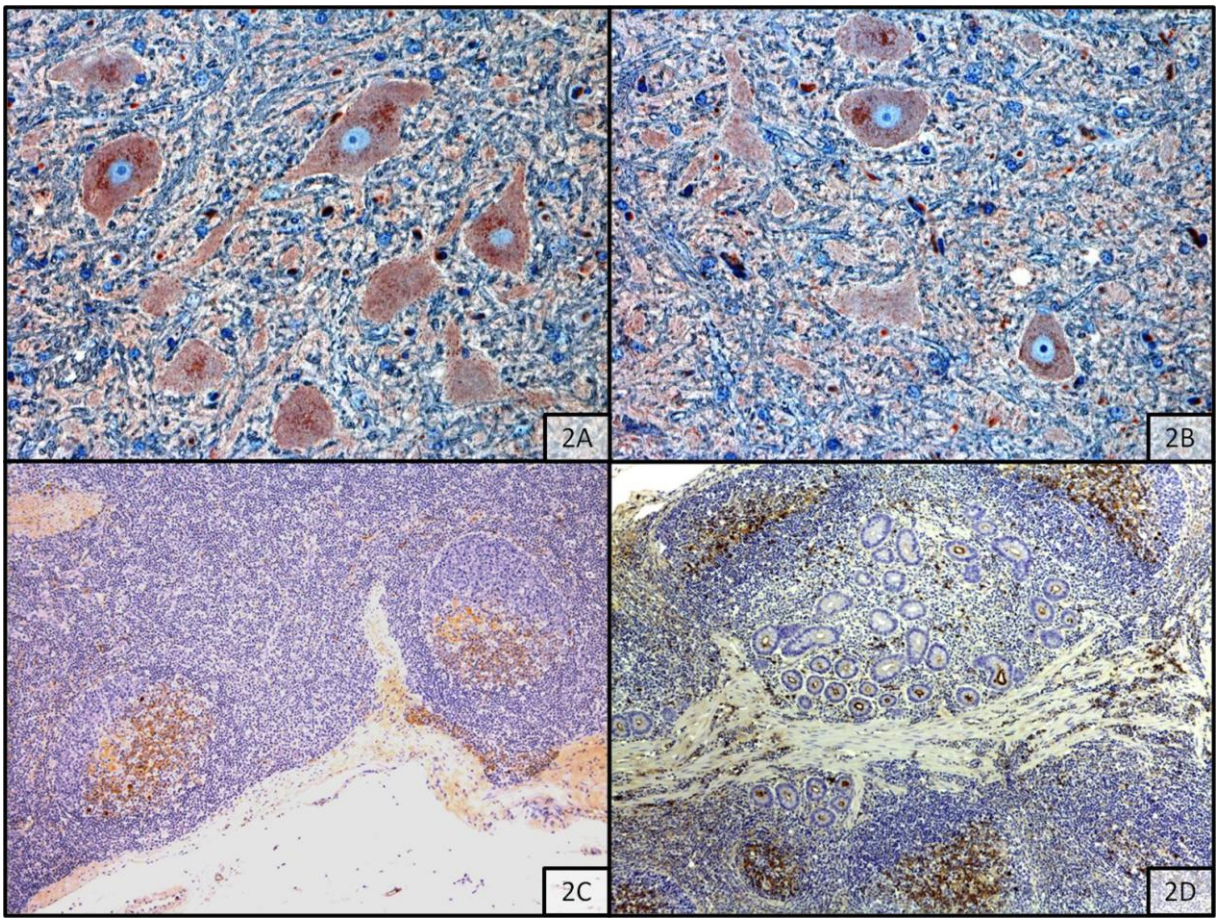

Figure 2: Representative immunohistochemistry of the obex of a sheep with scrapie (A: case 2.7 and B: case 3.2 in Table 1) and lymphoid follicles from the rectal mucosa (C: case 1.2 and D: case 3.10 in Table 1). PrP^{Sc} is detected in the cytoplasm of neurons using AEC as a chromogen. A, B, and D are at 400× magnification and C is at 200×.

While one animal had no known interactions with the sheep showing clinical signs,

the majority of animals that tested positive did. One positive female lambed in the same stall in which the clinically affected animal was born. Two of the positive sheep were born at approximately the same time near the stall of the clinically affected animal. The other sacrificed animals were related to the immunohistochemically positive female.

By genotypic analysis determined that three of the immunohistochemically two positive sheep had haplotype ARQ/ARQ, one had haplotype ARQ/ARH and one with haplotype VRQ/VRQ. Both of these are associated with moderate susceptibility (Figure 3). Animals considered high risk owing to exposure to the infected animal had haplotype ARQ/ARQ. Only one sheep had haplotype ARQ/ARR, which is considered resistant to infection (Figure 3). The biological mother of the animal with clinical signs was negative by immunohistochemistry on biopsy samples, despite possessing a haplotype associated with a moderately high risk (ARQ/VRR).

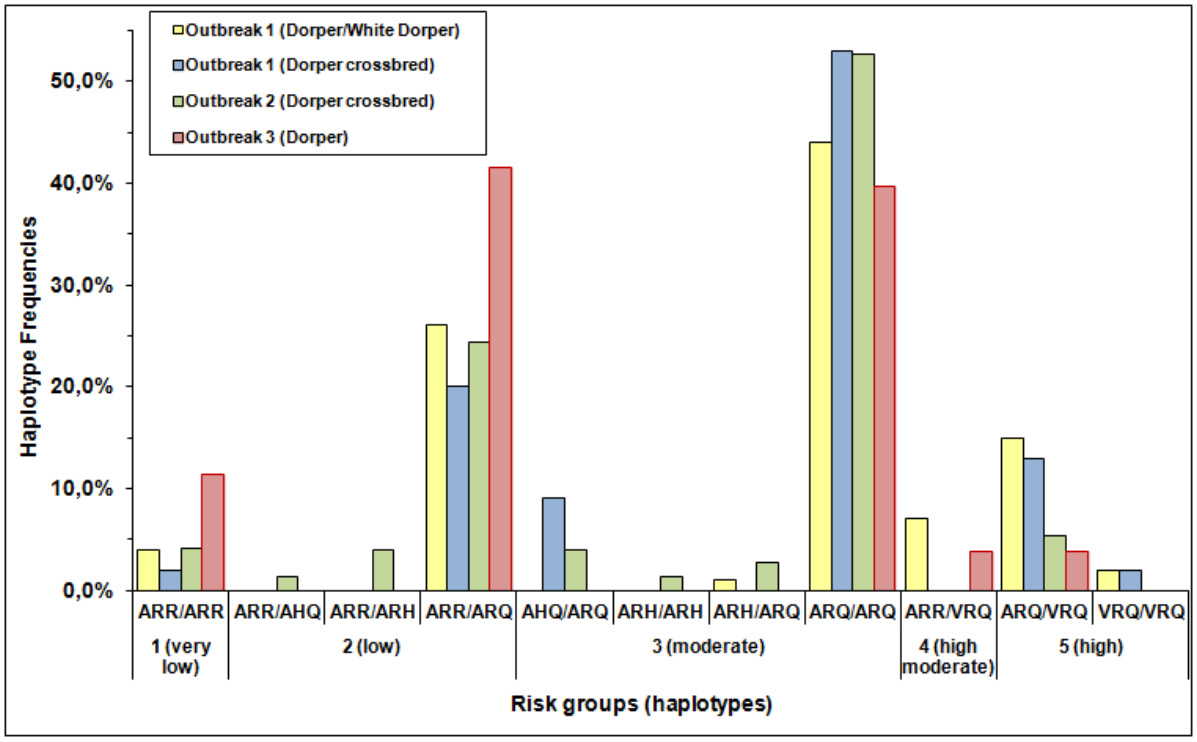

Figure 3: Distribution of animals within risk groups per Dawson et al. 2008, according to

genotypes determined for codons 136/154/171 in scrapie-affected and healthy sheep from three outbreaks (n=180, 74, and 53 for outbreaks 1–3, respectively). The risk groups correspond to 1 (very low risk), 2 (low risk), 3 (moderate risk), 4 (high moderate risk), and 5 (high risk).

The frequency of genotypes within the flock was also determined. For codon 136, AA was the most frequent genotype (76%), followed by heterozygous AV (22%). Only 2% of purebred animals had the susceptible genotype for codon 136 (VV). For codon 154, all animals had genotype RR, which is considered wild type. The flock had four different genotypes at codon 171; the most frequent was QQ (61%), followed by QR (34%), RR (4%), and QH, which was only detected in the sheep with clinical signs (Figure 4). Analysis of the genotypes of the crossbred sheep also indicated a high frequency (84.4%) of genotype AA for codon 136, followed by 13.3% for AV and 2.3% for the susceptible VV genotype. At codon 154, a change in the heterozygous allele (HR) was observed in 9% of the animals examined, while for codon 171 the most prevalent genotype was QQ at a frequency of 78%. Based on this analysis, both the Dorper/White Dorper and crossbred sheep appear to be susceptible to the disease due to the high frequency of susceptible genotypes in the flock.

Samples from positive animals and animals with less frequent genotypes were sequenced for confirmation and to identify single nucleotide polymorphisms at other codons. Codons 142 and 143 had the highest polymorphism frequency in crossbred sheep. At codon 142, the allelic frequencies were 73.3% (II), 23.3% (IT), and 3.4% (TT). One immunohistochemistry positive animal had changes in these codons, resulting in the substitution of an isoleucine for a threonine. Among the purebred Dorper sheep, only one had a codon 142 polymorphism (IT). Polymorphism at codon 143 was observed only in crossbred animals; the heterozygous form of the allele (HR) had a frequency of 10% (Figure

4). Furthermore, there is no polymorphism was identified at codon 141, and all the samples presented to the wild form of the codon.

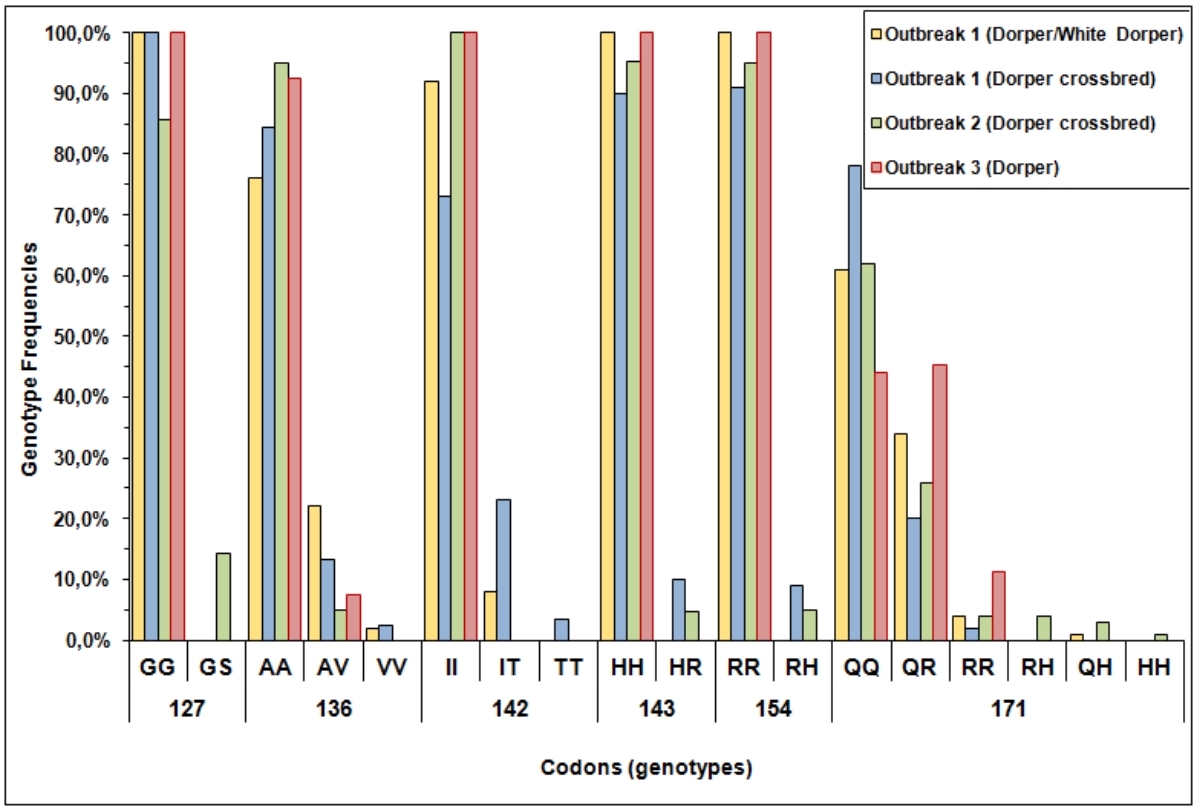

Figure 4: Genotypic frequencies of six different codons of the PRNP gene in scrapie-affected and healthy sheep from three outbreaks (n=180, 74, and 53 for outbreaks 1-3, respectively). Samples sequenced: 43, 21, and 15 for outbreaks 1–3, respectively, at codons 127, 142, and 143.

When we classified sheep into risk groups as described by Dawson et al. in 2008, 17.7% of purebreds and 15.5% of crossbred sheep were classified as risk group 5 (highly susceptible), 50–53% of the herd were classified as risk groups 3 and 4 (moderately susceptible), and 22.2–30% were risk groups 1 and 2 (resistant) (Figure 3).

Analysis of Outbreak 2

Of the 74 samples collected at the second farm, 8 were positive by

immunohistochemistry and were sacrificed (Table 1). All sheep tested were approximately 2-year-old crossbred Dorper females. Two positive animals showed clinical signs (disturbances in movement) and vacuolization of neurons in the obex consistent with scrapie. The other sheep had no clinical signs or histological lesions. The histopathologic lesions in these cases were similar to those in outbreak 1, with PrP^{Sc} immunolabeling in the neurons of the obex (Figures 2A and 2B), third eyelid lymphoid follicles, and rectal mucosal lymphoid tissue (Figures 2C and 2D).

The haplotype ARQ/ARQ (risk group 3) was found in six of the positive animals, including both animals with clinical signs. The remaining two positive animals had haplotypes ARQ/ARR and ARR/ARR. For codons 136 and 154, the allelic frequencies were 95% (AA and RR) and 5% (AV and RH). The herd had all allelic forms of codon 171, with frequencies of 62% (QQ), 26% (QR), 4% (RH), and 1% (HH).

Similar to outbreak 1, sequencing was performed on samples from immunohistochemistry positive animals and animals with less common genotypes. This identified a rare polymorphism at codon 127 that resulted in a substitution of a serine for a glycine, with an allelic frequency of 14% in ARQ/ARQ sheep (including one positive sheep) (Figure 4). No polymorphism were identified at codon 141.

Classification of these sheep into risk groups according to haplotype indicates that, in general, the flock is considered susceptible; 66.2% of animals are classified as moderate to high risk, and 33.8% is considered as low risk for infection (Figure 3).

Analysis of Outbreak 3

Of the 43 samples collected from the third property, 10 over 1 year old female Dorper sheep were positive by immunohistochemistry and were sacrificed (Table 1). Only one of these showed clinical signs (difficulty in locomotion), and this animal was originally

from the farm involved in outbreak 1. This sheep had similar histological and immunohistological findings as animals with clinical signs from the other two outbreaks.

Haplotype analysis found four positive animals with the ARQ/ARQ allele (risk group 3) and one with the ARQ/VRQ allele (risk group 5); all are considered susceptible. The other sheep on this farm had the ARQ/ARR allele, which is associated with low susceptibility to infection (Figure 3). Risk group analysis divided this flock into low-risk (54.7%) and moderate-risk (45.3%) groups (Figure 3). No polymorphisms were found in other codons by sequencing, including codon 141. In this group of animals, the allelic frequencies for codon 136 were 92.45% (AA) and 7.55% (AV), 100% (RR) for codon 154, and 45.28% (QR), 43.4% (QQ), and 11.32% (RR) for codon 171. No less frequent codon 171 alleles (QH, RH and HH) were found.

Discussion

This paper describes the first reported outbreak of classical scrapie in Dorper/White Dorper sheep. According to other publications and our data, these breeds are genetically susceptible to scrapie infection [27,37].

Identification of the possible routes of transmission and determination of the infectivity of each route via natural infection or experimental models is the first step in combating the disease [38]. The ante-mortem diagnosis results from outbreak 1 reported in this paper show that while the supposed biological mother and sisters of the infected animal were not infected, animals born in the same bay as the infected sheep tested positive. These data support the hypothesis that transmission of disease occurred not from dam to fetus but by postpartum transmission. Determination of the mechanism of maternal transmission will aid in understanding and could contribute to our knowledge of tissues that are infectious and the mechanisms behind horizontal transmission [39]. Other studies have demonstrated the

agent in placental tissues, supporting the hypothesis that disease transmission can occur soon after birth [6,7,40]. Preventing exposure to placental tissues could help to control the spread of the disease in susceptible herds. Thus, removing placentas from the environment is recommended to control scrapie transmission [41].

Horizontal transmission is also dependent on fetal genetics. Fetuses with resistant genotypes born to naturally infected dams did not develop disease, unlike susceptible fetuses [6]. During the incubation period of scrapie, genetically susceptible sheep may accumulate large amounts of PrP^{Sc}, which can be disseminated to the environment via the placenta [8]. Introduction of the resistant allele (ARR) into infected herds not only reduces disease incidence [9] but helps prevent agent spread to the environment. The use of genetically resistant sheep in infected herds both increases the long-term frequency of the resistance gene and contributes to exposure reduction [42].

In the three outbreaks described in this paper, we observed a large number of susceptible sheep regardless of breed. For example, at codon 171, considered the most relevant to disease resistance, approximately 90% of sheep at the three properties examined had genotype QR or QQ. This finding is consistent with data obtained by others [26,27] that found 90–100% of the Dorper sheep in Brazil were of susceptible genotypes. These data suggest that both Dorper/White Dorper and crossbred sheep are predisposed to infection owing to genetic susceptibility. When sheep were classified into risk groups, approximately 69% of animals in Dorper/White Dorper flocks from these three outbreaks belong to moderate to high risk groups. This is consistent with other studies, which showed that Dorper herds 70% [27] to 89% [26] moderate to high risk. However, the latter study identified 11% as low-risk, while in our work and others [27], 30% of Dorper/White Dorper sheep were low risk.

One of the sheep positive for scrapie in this study possessed the haplotype ARR/ARR, which is associated with infection resistance [36, 46]. Few studies have described the occurrence of classical scrapie in sheep with this genotype; however, an accumulated protein considered to represent atypical scrapie in sheep has been demonstrated in ARR/ARR sheep, suggesting that it is possible for the infection to be transmitted to animals despite genetic resistance [43,44,45,46].

Some rare polymorphisms in codons 127, 142 and 143 were found by sequencing. The largest number of polymorphisms of codons 142, 143, 154 and 171 were found in crossbred animals, suggesting that crossbreeding may contribute to an increased rate of polymorphisms. One animal that was diagnosed positive had a polymorphism at codon 127. The effect of this change on scrapie susceptibility has not yet been defined. This polymorphism has been previously described in Mongolian [47] and Chinese sheep [48]. A change to serine at this codon was identified both in goats with and without scrapie, with allele frequencies of 10 and 11.5%, respectively, and is therefore unlikely to be related to infection.

One genetically highly susceptible (VRQ/VRQ) sheep that was both positive by immunohistochemistry had a polymorphism at codon 142 that resulted in the substitution of isoleucine for threonine. The effect of this substitution is not yet known but has been described in sheep in New Zealand [49]. In addition to these codons, another polymorphism, previously identified in other herds [50,51,52], was found at codon 143. Another study [16] also identified this polymorphism and suggested that the heterozygous allele (H143R) may offer some protection against infection in goats.

The data presented in this paper support the conclusion that Brazil should implement scrapie control measures, such as pre-clinical diagnosis via lymphoid tissue biopsy [52] and

breeding programs aimed at the selection of genetically resistant sheep in predisposed breeds [53,54].

Conflict of interest statement

All authors of this paper have disclosed financial and personal relationships with other people or organizations that could inappropriately influence this work. The author(s) declare that they have no competing interests.

Authors' contributions

CPA participated in the design of the study, carried out the genotyping study and immunoassays, including the analysis and interpretation of the data, and drafted the manuscript. JSL participated in the collection of samples for genotyping and immunoassays, and drafted the manuscript. GLFC participated in the collection of samples for genotyping and immunoassays, and drafted the manuscript. LLA participated in the design of the study, genotyping, and drafted the manuscript. LAC participated in the design of the study and the interpretation of the data. SCS participated in the design of the study and supervised the interpretation of the data. DD coordinated the study and supervised the drafting of the manuscript. All authors read and approved the final manuscript.

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5.4 Resultados e discussão sobre a análise do rebanho de ovinos Santa Inês

A genotipagem de um rebanho livre de scrapie da raça Santa Inês, proveniente da Paraíba, foi analizada para os códons 136, 154 e 171, através do PCR em tempo real e por sequenciamento. As amostras de sangue (n= 94) foram cedidas pelo propreítário que ajudou a colaborar com o projeto. Na figura 1 é possível observar a identificação de polimorfismos de nucleotídeos únicos em dez códons diferentes.

Alguns polimorfismos considerados “raros” foram identicados nesse rebanho, entre eles, nos códons 127, 138, 140, 141, 142, 143 e 172. Esses polimorfismos já foram identificados em seis desses códons, em outro rebanho de ovinos Santa Inês no Brasil (Lima et al., 2007). O códon 127 apresentou cinco formas de polimorfismos, com uma frequência de 70,2 %, 19,1 % e 8,5 % para os genótipos GG, GS e GA, respectivamente. Os códons 138, 140, 142 apresentaram polimorfismo na forma heterozigota dos alelos em uma proporção de 1,1 a 6,4 %. Uma frequência maior foi encontrada no genótipo HR do códon 143, com cerca de 10,6%. Além desses, foi identificado em um ovino (ARQ/ARQ) o polimorfismo no códon 141, com genótipo LF, o qual está associado à forma atípica de scrapie. Esse mesmo ovino apresentou um polimorfismo no códon 127. Outro estudo no Brasil também identificou o polimorfismo no códon 141 em ovinos Santa Inês (Ianella et al., 2012).

Das 94 amostras, cinco apresentaram polimorfismos no códon 172, na forma heterozigota. Dessas cinco amostras, duas continham o genótipo QQ para o códon 171 e o resultado foi idêntico tanto no PCR em tempo real, como no sequenciamento. Porém, três amostras apresentaram resultados diferentes no PCR em tempo real e sequenciamento para o códon 171. No real time o genótipo identificado foi o 171RR e no sequenciamento foi 171QR. Esse resultado foi inesperado, visto que, até o momento nenhuma amostra havia

sido discrepante ao comparar as técnicas. Entretanto, de acordo com a literatura, o polimorfismo no códon 172 pode interferir na hibridização de sondas projetadas para o códon 171, e assim gerar um resultado adverso, devido o polimorfismo estar presente na zona de reconhecimento da sonda (Traoré et al., 2008).

Ao analisar as amostras que apresentaram polimorfismos, verificou-se que nove continham dois polimorfismos em códons diferentes, sendo que oito apresentaram a alteração no códon 127. Já a análise dos genótipos nos três códons principais (136, 154 e 171), mostrou uma frequência de 96,8% para o genótipo AA e de 3,2% do genótipo AV no códon 136; frequência de 83% do genótipo HH e de 17% do genótipo RH no códon 154. Para o códon 171 a frequência foi de 62,8%, 25,6%, 7,4% e 4,2 para os respectivos genótipos: QQ, QR, RR e QH.

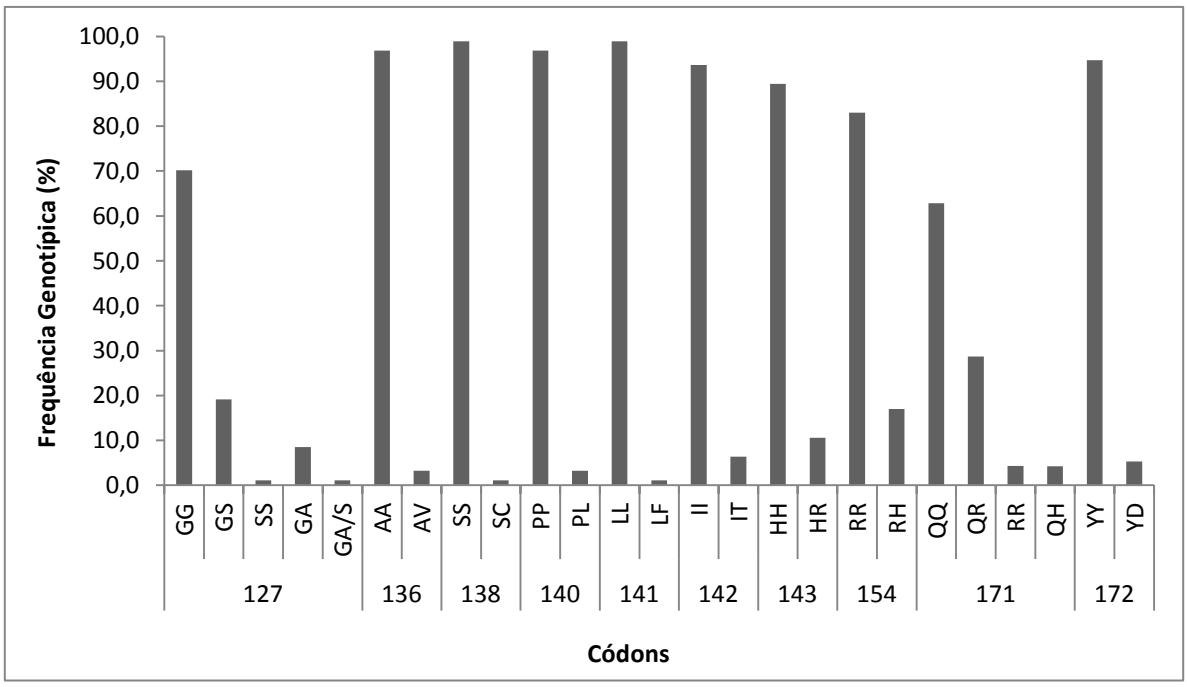

Figura 1: Frequência genotípica de 10 códons do gene PRNP em ovinos da raça Santa Inês (n=94 amostras). Amostras avaliadas por PCR em tempo real e sequenciamento.

Ao comparar os haplótipos quanto aos grupos de risco, descrito por Dawson e

colaboradores, pode-se verificar que cerca de 68% do rebanho foi considerado susceptivel de risco moderado (grupo de risco 3), sendo o haplótipo ARQ/ARQ o mais frequente (47,8%). Esse dados estão de acordo com outros trabalhos de genoptipagem de ovinos Santa Inês (Lima et al., 2007; Sotomaior et al., 2008; Ianella et al.,2012; Santos et al., 2012).

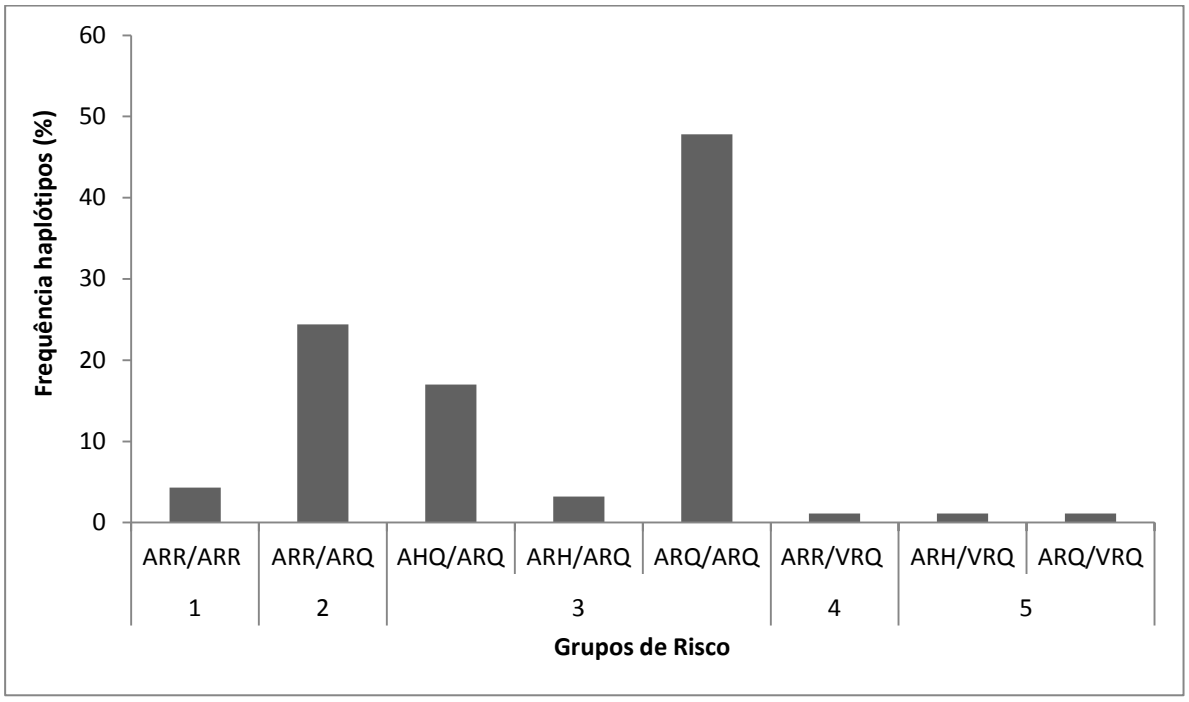

Figura 2: Frequência dos haplótipos (n=94 ovinos) em relação aos grupos de risco a suscetibilidade a *scrapie*, descritos por Dawson et al. (2008), em ovinos da raça Santa Inês. Grupo 1: risco muito baixo; Grupo 2: risco baixo; Grupo 3: risco moderado; Grupo 4: risco moderado alto; Grupo 5: risco elevado.

6. Conclusões

A técnica de PCR em tempo real desenvolvida foi de extrema importância para a execução do projeto. Os resultados obtidos de genotipagem foram idênticos ao analisado por sequenciamento, demonstrando assim que a técnica pode ser aplicada nos futuros rebanhos que serão testados, devido à confiabilidade e rapidez do teste.

Ao analisar o surto de scrapie clássico em ovinos da raça Suffolk, foi possível identifcar polimorfismos em 3 dos 15 códons avaliados. Já, de acordo com o estudo a maioria dos ovinos pertenceu ao grupo de risco 3, considerado moderado. Visto que, um dos polimorfismos encontrados estava presente no códon 143, e esse atribui umas proteções parciais em ovinos suscetíveis, sugere-se a importância da associação não só dos três códons principais, mas também dos outros códons para determinar a suscetibilidade a scrapie.

Como conclusão do trabalho, também podemos verificar que todos os rebanhos analisados, independente das raças estudadas, foram considerados geneticamente suscetíveis a infecção. A maioria dos ovinos apresentou o haplótipo ARQ/ARQ, considerado grupo de risco moderado. Nas raças Dorper e Santa Inês foram identificados polimorfismos raros em outros códons não considerados como os principais associados à suscetibilidade, como por exemplo, nos códons 127, 142, 143 e 172. Além disso, ao comparar ovinos Dorper de origem pura aos mestiços, foi possível verificar um maior polimorfismo em ovinos mestiços, sugerindo que cruzamento entre ovinos mestiços aumenta a frequência do mesmo.

Esse resultado é relevante para futuramente aprofundar os estudos e possivelmente associar o polimorfismo a uma função específica na suscetibilidade ou resistência à doença.

O trabalho descreveu os primeiros surtos de *scrapie* em ovinos da raça Dorper. O que podemos observar é que a raça pode ser considerada suscetível a infecção, além de provavelmente manter o agente presente durante um longo período de tempo, já que os três

surtos descritos possuem correlação e ocorreram em um intervalo de tempo de três anos. Podemos também concluir a importância da identificação dos ovinos, o qual permite a rastreabilidade dos mesmos que transitam no comércio, o que possibilita uma estratégia de controle mais eficaz no País.

E por fim, devido ao aumento de surtos de s*crapie*, acredita-se que o Brasil deveria se adequar a medidas preventivas para o controle da doença, associando o diagnóstico a genotipagem de ovinos. Assim seria possível determinar as raças de ovinos predispostas à doença, com o intuito de selecionar ovinos geneticamente resistentes.

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