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Prion protein genotypes of Italian sheep breeds with lysine-171 and phenylalanine-141
detection

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Abstract

Amino acid polymorphisms of the prion protein gene influence sheep susceptibility to classical and atypical scrapie. Substitutions at codons 136, 154 and 171 play an important role in classical scrapie. Codon 141 leucine to phenylalanine mutation (AFRQ) has been recognized as an increased risk factor for atypical scrapie. In addition a rare allele with lysine at codon 171 (ARK) has been detected in Mediterranean sheep breeds. The presence of ARK poses two problems: the determination of its frequency and its possible interference with genotyping output of routine methods lacking specific detection capacity for ARK. The aim of our work was the development of a routine genotyping method with the capacity to identify ARK and AFRQ in addition to the normally detected alleles and to determine the frequencies of all these alleles in 5 main Italian breeds: Sarda (n=2494), Bergamasca (n=2686), Appenninica (n=297), Comisana (n=361) and Massese (n=402). A multiplex primer extension assay targeting the 6 single nucleotide polymorphisms of interest was developed. Allele frequencies revealed a very low level of ARK in Bergamasca (6.91%) as opposed to the other breeds, very diverse levels of AFRQ ranging from absence in Comisana to (10.70%) in Massese and a restricted presence of ARK. This allele has only been detected in Bargamasca with a significant 3.67% and marginally in Appenninica (0.34%). These results underline the need for adequate routine methods for genotyping of breeds with alleles that can interfere with typing of important codons such as the case of ARK for codon 171.

Keywords: genotyping, PrP polymorphism, scrapie, sheep, 141-phenylalanine, 171-lysine

1. Introduction

Scrapie is a fatal neurodegenerative pathology of sheep and goat belonging to the group of prion diseases of animals and humans. Likewise the other prion diseases it is characterized by the accumulation of an abnormal isoform (PrP^{Sc}) of the host encoded cellular prion protein (PrP^C). Symptoms of classical scrapie include various neurological disorders followed by progressive wasting and death, the onset of the disease usually occurs not earlier than 18 months of age and susceptibility varies depending on the strain of the agent, the breed and the genotype of the animal (Goldman et al., 1994; O'Rourke et al. 1997). With regard to the genotype effects on scrapie, amino acid polymorphisms in different positions of the ovine prion protein are known which condition the susceptibility of sheep to the disease. In particular amino acid positions 136, 154 and 171 play a fundamental role and several alleles have been identified with reference to these positions (Hunter, 1997). These alleles are commonly reported using a three letters code (e.g. ARQ for alanine136/arginine154/glutamine171). Some of them are more commonly encountered and widespread among breeds of diverse origin, specifically alleles ARQ, ARR, AHQ, ARH and VRQ (Baylis and Goldman, 2004) , however more rarely encountered polymorphisms have been described in these positions: ARK (Guo et al., 2003) , TRQ (Billinis, et al., 2004), and ALQ (Alvarez et al., 2006). Evidence have accumulated indicating that the ARR allele is associated with minimum susceptibility particularly in case of the homozygous genotype ARR/ARR while ARQ and VRQ confer the highest susceptibility, an intermediate effect is attributed to the other common alleles (Belt et al., 1995; Clouscard et al., 1995; Hunter et al., 1996; Hunter et al., 1997). However, recently 2 cases of natural scrapie were reported by Groschup, et al. (2007) in sheep with ARR/ARR genotype. The biochemical and transmission characteristics of these 2 cases made them referable to classical scrapie albeit symptoms were not well defined, they were single cases in their flocks of origin and showed reduced proteinase-K resistance of PrP^{Sc}. This has led

these authors to conclude that the agent they described could be a particular scrapie strain responsible for extremely rare infections.

Alongside classical scrapie, atypical forms of the disease have been identified which show different epidemiology, clinical signs and distinct biochemical characters (Benestad et al., 2003). Furthermore prion protein genotypes condition atypical scrapie in a very different way compared to classical scrapie. Specifically, alleles AHQ and AF141RQ (hereafter AFRQ), an ARQ variant with L to F mutation at codon 141, have been associated with increased risk while the ARR allele gives no protection (Moum et al., 2005; Arsac et al., 2007; Lühken et al., 2007; Benestad et al., 2008).

In this context over the past decade selection programs have been implemented in European Union countries and elsewhere with the goal of increasing ARR allele frequency in order to protect sheep populations from classical scrapie, but we still have limited knowledge about the frequency of alleles that are not detected in routine genotyping for selection programs of ovine breeds. Among these are AFRQ and alleles confined to particular breeds such as ARK in some Italian breeds. The precise knowledge of these frequencies may contribute to the revaluation of selection programs for classical scrapie and give insights into susceptibility of the different breeds to atypical scrapie.

The aims of our work were two. Firstly, the development of a robust and accurate routine PrP genotyping assay for codons 136, 141, 154 and 171 with added capacity to identify 171-lysine (ARK), already demonstrated by Acutis et al. (2004) in the Italian breed Biellese. Secondly, the determination of allele frequencies of AFRQ and ARK alongside the common alleles, in 5 main Italian breeds.

2. Materials and methods

2.1. *Animals*

6240 sheep were included in the study, they belonged to 5 Italian breeds: Sarda (n=2494, from 50 flocks), Bergamasca (n=2686, from 13 flocks), Appenninica (n=297, from 12 flocks), Comisana (n=361, from 5 flocks) and Massese (n=402, from 13 flocks). The animals were sampled within the genotyping program for scrapie resistance of Emilia-Romagna Region and the selective culling of scrapie outbreaks from Emilia-Romagna and Lombardy Regions. In outbreaks, all animals were sampled for the study and this accounted for 1205 samples from two outbreaks for Sarda and for 2479 samples from seven outbreaks for Bergamasca. No outbreaks involved Appenninica, Comisana and Massese. Concerning the genotyping program of Emilia-Romagna, all animals submitted to the program by individual breeders were included in the study. These subjects were selected on the basis of their productive performance and for potential use in reproduction within each flock. This preliminary selection process was not linked to scrapie resistance; therefore it was not prone to bias PrP allele frequencies in the study population. The differences in the number of samples and flocks per breed reflect the ovine population structure in Emilia-Romagna with the exception of Bergamasca; most samples of this breed came from Lombardy, its Region of origin. All samples were collected from March 2006 to May 2008. Resampling occurred for some flocks over this period. In case of resampling in a flock, only animals fulfilling the following criteria were included in the study: *a)* no resampling of the same animal occurred; *b)* animals were not born after that males of known genotype had become available to the breeder for genotype-directed mating; *c)* animals born after that females of the same flock had been genotyped were included in the study provided that no mating groups of selected females were created in the flock.

2.2. *Samples collection and DNA extraction*

Peripheral blood samples were collected using vacuum vials with EDTA anticoagulant and stored frozen until DNA extraction.

Genomic DNA was extracted from thawed whole blood using NucleoSpin 96 Blood (Macherey-Nagel) on a Hamilton StarLet robotic workstation (Hamilton Robotics); the extraction protocol of the manufacturer was modified as follows: 20 µl of blood were added to 180 µl of phosphate buffered saline prior to proteinase-K digestion.

2.3. Genotyping

The segment of ovine PrP coding region from nucleotide 306 to 650 was amplified by PCR from genomic DNA with the following primers: 5'-GAACAGCCCAGTAAGCCA-3' (forward), 5'-CACATTTGCTCCACCACTC-3' (reverse). The reaction volume of 10 µl contained 2 mM MgCl₂, 100 mM dNTPs, 0.5 U FastStart Taq DNA Polymerase (Roche), 0.5 mM of each primer and 2 µl of genomic DNA (corresponding to 10-20 ng). The amplification was carried out in a Mastercycler gradient 5330 (Eppendorf) with initial denaturation at 95 °C for 6 min followed by 35 cycles of 30 s at 95 °C, 30 s at 57 °C, 30 s at 72 °C and a final step at 72 °C for 7 min. Post-PCR clean-up was performed by adding 3 U of shrimp alkaline phosphatase (SAP) and 1.5 U of exonuclease I (EXO) (both from GE Healthcare) to the PCR tubes and incubating them for 60 min at 37 °C followed by 15 min at 75 °C for enzyme inactivation.

A multiplex primer extension reaction was performed in 10 µl volume containing 2 µl of purified PCR product, 4 µl of SNPStarter Kit (Beckman Coulter) and 4 µl of interrogation primers mix. The primers used were designed to be compatible in a multiplex reaction, 5'-polyT tails were added to obtain adequate length spacing of primers in order not to overlap in the subsequent electropherogram. The concentrations of primers were optimized to produce equilibrated signals. Sequence, position and final concentration of each primer are

reported in table 1. The reaction was performed in a Mastercycler gradient 5330 (Eppendorf) with the following thermal protocol: 25 cycles of 30 s at 95 °C, 30 s at 57 °C, 30 s at 72 °C. Unincorporated dyes terminators were removed incubating the reaction tubes with 1 U of SAP (GE Healthcare) for 30 min at 37 °C followed by 15 min at 75 °C for enzyme inactivation. Two µl of SAP treated reaction product were mixed with 20 µl of sample loading solution and 0.3 µl of Size Standard 80 (Beckman Coulter) and subjected to capillary electrophoresis on a Beckman Coulter CEQ 8000 automated sequencer. Raw traces were analyzed and peaks identified using the “Fragments” package of CEQ 8000 software.

All steps from PCR to electrophoresis were carried out with Hamilton automation.

2.4. Primer extension cross-validation

Direct sequencing was used as the gold standard to validate the newly developed primer extension assay. Ninety-seven samples were sequenced after they had been genotyped by primer extension. They represented 20 genotypes and all the alleles reported in this study. Sequencing and primer extension were carried out starting from the same purified PCR product in order to keep to a minimum the sources of variation between the 2 protocols. Sequencing of both strands of the amplicons from nucleotide 306 to nucleotide 613 of the coding region (encompassing the SNPs to validate), was done on a Beckman Coulter CEQ 8000 automated sequencer with DTCS Quick start kit (Beckman Coulter) according to manufacturer’s instructions, with primer sequence 5’-GAACAGCCCAGTAAGCC-3’ for the forward strand, and 5’-CAGTTTCGGTGAAGTTCTCC-3’ for the reverse strand.

2.5. Field trial

The primer extension assay presented in this study was used in routine conditions for the genotyping of over 7000 samples in 2 years.

3. Results

3.1. Genotyping assay

The developed primer extension reaction produced clearly interpretable electropherograms with balanced peak heights. This allowed for reliable identification of the PrP genotypes corresponding to the 6 single nucleotide polymorphisms (SNPs) interrogated by the corresponding primers. All known alleles corresponding to the interrogated SNPs have been detected in this study with the exception of ALQ which was reported by Alvarez et al. (2006) in Spanish Churra breed. The Cross-validation experiment gave concordant results for all 97 sequenced samples. During the field trial approximately 1% of the samples needed retesting. The main reasons for retesting were two: *a)* poor genomic DNA quality or quantity, resulting in insufficient or null amplification, *b)* incomplete enzymatic clean-up of PCR with consequent confusing extra peaks in electropherograms. The first case was easily identified by lack of sample peaks in presence of size standard peaks; re-extraction of DNA was the routine remedy. The second case appeared as a sequence-like trace and repetition of PCR clean-up systematically removed the problem. Centrifugation of PCR plates before adding EXO-SAP prevented the problem.

3.2. Genotype and allele frequencies

Genotype and allele frequencies are shown in table 2 and 3 respectively. Concerning the allele frequencies reported for Sarda breed, we evidence that the contribution of 1205 samples out of 2494 by only two flocks (outbreaks) does not bias the overall figures for this breed. In fact the frequencies calculated for those two flocks only are similar to those calculated for the whole set of Sarda samples, reported in table 3. The frequencies in the two flocks are: 55.31% (ARQ), 40.08% (ARR), 2.07% (AHQ), 0.17% (ARH) and 2.37% (AFRQ). The resulting picture shows significant differences among the investigated breeds, in particular with regard to ARR, VRQ, AFRQ and ARK frequencies. Bergamasca is

characterized by the lowest ARR frequency accompanied by the highest VRQ and ARK frequency. In addition this breed shows the highest PrP genetic variability with 7 alleles detected, distributed in 25 genotypes. On the opposite side Comisana possesses only 3 alleles and 5 genotypes. ARK is almost exclusively restricted to Bergamasca with a limited frequency in Appenninica (0.34%). AFRQ is present in all breeds except Comisana.

4. Discussion

In 2003 the genetic strategy for scrapie control was established in the European Union (European Commission Decision 2003/100/EC). However, technical or practical limitations of commonly used genotyping methods have prevented extended screening for both AFRQ and unusual alleles. The primer extension method that we developed has made it possible to detect AFRQ and ARK alleles with no extra costs and extra time during routine genotyping. This is particularly significant in breeds where ARK is present, such as Bergamasca. In fact, in these breeds two problems would arise without ARK detection capacity: *a)* no estimate of ARK frequency would be possible and *b)* a wrong estimate of ARQ frequency would be produced in primer extension since, without a specific primer for the first base of codon 171, ARK would be misclassified as ARQ. The outcome would be even worse using methods based on allele-specific probes. In this case, without ARK-specific probe, heterozygous animals with ARK/XXX genotypes would be misclassified as homozygous XXX/XXX with detrimental consequences (e.g. in case of ARK/ARR wrongly typed as ARR/ARR).

Primer extension for ovine PrP genotyping has already been used by others (Zsonlai et al., 2003; Vaccari et al., 2004; Alvarez et al., 2006; Benkel et al, 2007) who demonstrated its reliability. Our validation experiment confirmed the accuracy of the technique. In addition, this study is the first example of use of primer extension for the detection of three consecutive SNPs in the ovine PrP gene, the three polymorphic bases of codon 171. This is

made possible by use of two overlapping forward primers for the first two bases of the codon. No interference occurred between the two primers. Of course, when the first base of the codon was mutated (ARK) the second base of the same allele was not detectable by the primer directed to the second base, because of the mismatch corresponding to the 3' end of the primer. Anyway the situation was easily interpretable given the contemporary detection of the first base mutation. The third base of codon 171 was interrogated by a reverse primer that did not interfere with the two forward primers.

The results of our survey indicate that considerable genetic difference exists among the investigated breeds. ARR frequency appears significantly low in Bergamasca (6.91%) compared to the other breeds where it ranges from 37.81% of Comisana to 46.97% of Appenninica. On the contrary VRQ shows higher frequency in Bergamasca (4.11%) than the others. In particular its presence can be considered negligible in Sarda where only 1 animal out of 2494 resulted VRQ carrier. AFRQ is remarkably variable: it has been detected in all breeds except Comisana, it is scarcely present in Appenninica (0.34%), moderately represented in Sarda (2.53%) and reaches 5.01% and 10.70% in Bergamasca and Massese respectively. These two breeds also show the highest AHQ frequencies. High AFRQ and AHQ levels render Massese and Bergamasca more genetically prone to atypical scrapie. ARK has been detected only in Bergamasca and Appenninica. Furthermore Appenninica possesses this allele with a very low frequency (0.34%). Consequently Bergamasca is the only breed of this study with high ARK level (3.67%), indeed the highest reported so far. In fact, ARK has been detected with very low frequencies in other breeds from different countries, specifically in Mongolian Sheep (Gombojav et al., 2003), in Oklahoma sheep (DeSilva et al., 2003) in Greek breeds (Billinis et al., 2004). Acutis et al. (2004) reported 2.5% of ARK in Italian Biellese breed. The same authors speculated that this allele could be present in other breeds but the use of common methods prevented its identification during routine genotyping. The results of our study confirm the need for

methods with ARK detection capacity when working with ARK positive breeds such as Bergamasca. On the other hand our results do not evidence a widespread diffusion of ARK among different breeds. The role of this allele in scrapie susceptibility is still scarcely understood. The finding of a scrapie positive sheep with ARK/ARH genotype by Acutis et al. (2006) rules out a strong protective effect of this allele but the same authors reported that the positive animal they found had no symptoms and showed only weak positivity in the obex without involvement of any other nervous or linforeticular tissue. This pathologic behaviour does not allow ultimate conclusions on the effects of ARK on susceptibility to the disease. Experiments addressing this issue are underway.

5. Conclusion

The genotyping method developed accurately identifies AFRQ and ARK in addition to common alleles. Our survey indicates a good level of genetic resistance to classical scrapie due to high ARR frequencies in all investigated breeds with the exception of Bergamasca. AFRQ is very differently distributed ranging from absence to over 10%. Massese and Bergamasca appear the most susceptible to atypical scrapie based on their higher AFRQ and AHQ frequencies. ARK is restricted to two breeds: Bergamasca, where it reaches the highest frequency ever reported and Appenninica with a very low level. The exact role of ARK in scrapie susceptibility needs to be investigated.

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Table 1

Interrogation primers used for primer extension reaction at reported codons

| Codon | SNP ^a | Amino acid | Interrogation primer (5'-3') ^b | Concentration (μM) |
|---------|--------------------------|------------|---|--------------------|
| 136 | <u>GCC</u> <u>GTC</u> | A V | (15T)GGTGGCTACATGCTGGGAAGTG | 0.040 |
| 141 | <u>CTT</u> <u>TTT</u> | L F | (32T)GGAAGTGCCATGAGCAGGCCT | 0.040 |
| 154 | <u>CGT</u> <u>CAT</u> | R H | (9T)GGGGTAACGGTACATGTTTTA ^c | 0.020 |
| 171-I | <u>CAG</u> <u>AAG</u> | Q K | (38T)CCAAGTGACTACAGACCAGTGGAT | 0.016 |
| 171-II | <u>CAG</u> <u>CGG</u> | Q R | CAA GTGTACTACAGACCAGTGGATC | 0.040 |
| 171-III | <u>CAG</u> <u>CAT</u> | Q H | (19T)GCACAAAGTTGTTCTGGTTACTATA ^c | 0.012 |

^a Single nucleotide polymorphism; wild type sequence in first line, mutant in second line^b The number of T bases added to 5'-end of primers is reported in parenthesis^c Primer complementary to the reverse strand

Table 2

PrP genotypes and their frequencies (%)

| Genotype | Breeds (no. of flocks) | | | | | | | | | |
|--------------|------------------------|---------------|-----------------|---------------|------------------|---------------|--------------|---------------|--------------|---------------|
| | Sarda (50) | | Bergamasca (13) | | Appenninica (12) | | Massese (13) | | Comisana (5) | |
| | n | % | n | % | n | % | n | % | n | % |
| ARQ/ARQ | 688 | 27.59 | 1550 | 57.71 | 52 | 17.51 | 45 | 11.19 | 126 | 34.90 |
| ARQ/ARR | 1089 | 43.66 | 275 | 10.24 | 123 | 41.41 | 133 | 33.09 | 189 | 52.36 |
| ARR/ARR | 451 | 18.08 | 14 | 0.52 | 64 | 21.55 | 62 | 15.42 | 40 | 11.08 |
| ARQ/AHQ | 63 | 2.53 | 141 | 5.25 | 3 | 1.01 | 19 | 4.73 | | |
| ARR/AHQ | 64 | 2.57 | 10 | 0.37 | 5 | 1.68 | 29 | 7.21 | | |
| AHQ/AHQ | 3 | 0.12 | | | | | 2 | 0.50 | | |
| ARQ/ARH | 6 | 0.24 | 45 | 1.68 | 19 | 6.40 | 1 | 0.25 | | |
| ARR/ARH | 8 | 0.32 | | | 14 | 4.71 | 6 | 1.49 | | |
| AHQ/ARH | | | 1 | 0.04 | 3 | 1.01 | 3 | 0.75 | | |
| ARH/ARH | | | | | 2 | 0.67 | | | | |
| VRQ/ARQ | | | 174 | 6.48 | | | 16 | 3.98 | 2 | 0.55 |
| VRQ/ARR | | | 18 | 0.67 | 7 | 2.35 | 6 | 1.49 | 4 | 1.11 |
| VRQ/AHQ | | | 3 | 0.11 | | | | | | |
| VRQ/ARH | | | 2 | 0.08 | 1 | 0.34 | | | | |
| VRQ/VRQ | | | 4 | 0.16 | | | | | | |
| ARQ/ARK | | | 152 | 5.66 | 1 | 0.34 | | | | |
| ARR/ARK | | | 16 | 0.59 | 1 | 0.34 | | | | |
| AHQ/ARK | | | 3 | 0.11 | | | | | | |
| ARH/ARK | | | 1 | 0.04 | | | | | | |
| VRQ/ARK | | | 5 | 0.19 | | | | | | |
| ARK/ARK | | | 6 | 0.22 | | | | | | |
| ARK/ARQ | | | 8 | 0.29 | | | | | | |
| ARQ/AFRQ | 73 | 2.93 | 198 | 7.37 | 1 | 0.34 | 30 | 7.46 | | |
| ARR/AFRQ | 37 | 1.48 | 24 | 0.89 | 1 | 0.34 | 36 | 8.96 | | |
| AHQ/AFRQ | 7 | 0.28 | 12 | 0.44 | | | 5 | 1.24 | | |
| ARH/AFRQ | | | 10 | 0.37 | | | 3 | 0.75 | | |
| VRQ/AFRQ | 1 | 0.04 | 11 | 0.41 | | | | | | |
| AFRQ/AFRQ | 4 | 0.16 | 3 | 0.11 | | | 6 | 1.49 | | |
| Total | 2494 | 100.00 | 2686 | 100.00 | 297 | 100.00 | 402 | 100.00 | 361 | 100.00 |

Table 3

PrP alleles and their frequencies (%)

| | Breeds | | | | |
|--------------|---------------|---------------|---------------|---------------|---------------|
| | Sarda | Bergamasca | Appenninica | Massese | Comisana |
| Allele | % | % | % | % | % |
| ARQ | 52.26 | 76.04 | 42.25 | 35.94 | 61.36 |
| ARR | 42.10 | 6.91 | 46.97 | 41.54 | 37.81 |
| AHQ | 2.81 | 3.16 | 1.85 | 7.46 | |
| ARH | 0.28 | 1.10 | 6.90 | 1.62 | |
| VRQ | 0.02 | 4.11 | 1.35 | 2.74 | 0.83 |
| AFRQ | 2.53 | 5.01 | 0.34 | 10.70 | |
| ARK | | 3.67 | 0.34 | | |
| Total | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |