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ABSTRACT

The objective of this paper was to investigate the prion protein (PrP) genotype and haplotype frequencies in three Sicilian dairy sheep populations. The three populations were: (1) 1096 Valle del Belice animals, (2) 1143 Comisana animals, and (3) 1771 individuals from 5 flocks with scrapie outbreaks, in which the animals were crossbreds derived from indigenous Sicilian dairy breeds. PrP genotypes are described for the three codons 136 (Alanine or Valine; A, V), 154 (Histidine or Arginine; H, R), and 171 (Glutamine, Arginine or Histidine; Q, R, H) which represent polymorphisms known to be linked with scrapie susceptibility. The Valle del Belice haplotype frequencies were 32.3% ARR, 6.5% AHQ, 1.0% ARH, 58.8% ARQ, and 1.4% VRQ. The Comisana frequencies were 39.4% ARR, 2.9% AHQ, 2.9% ARH, 50.9% ARQ, and 3.9% VRQ. In the flocks with scrapie outbreaks the frequencies were 32.8% ARR, 2.4% AHQ, 1.7% ARH, 59.1% ARQ, and 3.9% VRQ. In all three populations ARQ and ARR were the most frequent haplotypes. Multiple generations of strong selection will be needed to fixate the most resistant ARR haplotype.

Key words: Haplotype frequency, Prion protein, Scrapie, Sheep.

RIASSUNTO

FREQUENZE GENICHE AL LOCUS DELLA PROTEINA PRIONICA IN TRE POPOLAZIONI OVINE DA LATTE SICILIANE

Lo scopo di questo lavoro è stato quello di stimare le frequenze alleliche e genotipiche al locus PrP in tre diverse popolazioni ovine Siciliane. Le tre popolazioni erano: 1) 1096 individui di razza Valle del Belice, 2) 1143 individui di razza Comisana, e 3) 1771 animali da incrocio provenienti da diversi allevamenti con focolai di scrapie. I genotipi al locus PrP sono stati descritti per i tre codoni 136 (Alanina o Valine; A, V),
Introduction

Transmissible Spongiform Encephalopathies (TSE) are fatal neurodegenerative diseases occurring in a number of mammalian species. Examples are the human Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease, Fatal Insomnia and Kuru, Bovine Spongiform Encephalopathy, Feline Spongiform Encephalopathy, Large Cat Feline Spongiform Encephalopathy, Chronic Wasting Disease affecting deer and elk, Transmissible Mink Encephalopathy, Exotic Ungulate Encephalopathy affecting antelopes, Zoo Primate Spongiform Encephalopathy and scrapie affecting mouflons, sheep and goats. Scrapie is characterised by the accumulation of an abnormal protease resistant isoform of a host-encoded prion protein (PrPsc) in the central nervous system and lymphoid tissues of the affected individuals, resulting in neurological impairment and eventually leading to death. The TSE diseases are widely spread geographically. In Italy the first case of scrapie was reported in 1977 (Cravero et al., 1977). The main risk of BSE in sheep lies in the possible exposure of sheep to the same risk factors (i.e. infected meat and bone meal) that caused the BSE epidemic in cattle. The effects of this would be huge due to the known effect of BSE causing variant Creutzfeldt-Jakob disease in humans. To avoid any risk of scrapie or BSE transmission from sheep to humans the EU decided that selection against susceptible PrP haplotypes should be implemented (Commission Regulation EC 999/2001 and Commission Decisions 2002/1003/EC and 2003/100/EC). The anti-scrapie selection as suggested by EU works through the system of 'stamping out', which means that all susceptible PrP alleles are eradicated from the sheep populations of participating countries. An initial step is the investigation of the PrP haplotype frequencies in various breeds.

It has been shown consistently, in various sheep breeds, that the level of scrapie risk is associated with PrP genotype (Laplancher et al., 1993; Hunter et al., 1994, 1997; Gonzalez et al., 2002). Therefore selection for scrapie resistance is a valuable tool in the control of scrapie. At least 25 polymorphic codons, resulting from 40 haplotypes, have been identified within the protein-coding region of the sheep PrP gene (Goldmann et al., 1990, 1991, 2005; Gonzalez et al., 2002). However, generally (e.g. Goldmann et al., 1990; Belt et al., 1995), the PrP genotypes are described for the three codons 136 (Alanine or Valine; A, V), 154 (Histidine or Arginine; H, R), and 171 (Glutamine, Arginine or Histidine; Q, R, H) which represent a polymorphism known to be linked with scrapie susceptibility. At these codons, named after the amino acids they encode, the following five haplotypes of
the 12 possible haplotypes are commonly distinguished: ARR, AHQ, ARH, ARQ, and VRQ. These are the five haplotypes mentioned in EU Commission Decision 2002/1003/EC on minimum requirements for a survey of prion protein genotypes of sheep breeds.

The ARQ and AHQ haplotypes have been found to be associated with different levels of susceptibility according to the breed and the country (Drögemüller et al., 2001; François et al., 2003; Baylis et al., 2004) and in Italian breeds these two alleles seem highly susceptible (Acutis et al., 2003). The frequency of the susceptible PrP haplotypes varies between different sheep breeds but is usually at a level which makes it impossible to remove these haplotypes in a single generation. Furthermore, strong selection for scrapie resistance would also result in a loss of genetic variability especially in smaller breeds and can have an effect on any traits which are by pleiotropy or by linkage associated with PrP genotypes. This implies that selection against susceptible haplotypes should be implemented using breed specific selection strategies in order to be cost-effective and maintain genetic variation. EU member states are required to undertake a breed genotype survey and subsequently introduce genotyping and breeding programs. In such programs, sires without any scrapie susceptible PrP haplotypes (ARR/ARR) should be preferred as reproducers, because this would help in decreasing the frequency of susceptible haplotypes in the following generations.

In Sicily, the Comisana and Valle del Belice breeds are the two most common sheep breeds. The Comisana breed is also the second largest sheep breed in Italy. The Valle del Belice dairy sheep originated in western Sicily (Italy) and consists of more than 60,000 mature ewes (Finocchiaro et al., 2005). Sicilian dairy sheep are reared in semi-extensive management with natural mating and without a formal breeding program. Milk production is aimed at cheese manufacturing.

In this context the aim of this paper is to present the results of the investigations on PrP genotype and haplotype frequencies in the Sicilian dairy sheep populations. For this purpose three Sicilian populations have been genotyped for the PrP gene. These populations were: (1) Valle del Belice, (2) Comisana, and (3) a number of Sicilian flocks with scrapie outbreaks, in which the animals were indigenous Sicilian dairy sheep, of the Comisana, Valle del Belice and Pinzirita breeds and cross-breeds between these breeds. The first two populations did not have any scrapie outbreaks. Together these three populations give a view of the actual Sicilian situation.

**Material and methods**

**Populations**

Data on both sexes were collected in three Sicilian dairy sheep populations. Dataset 1 is from eight flocks with pure-bred Valle del Belice sheep participating in a pilot study and contains PrP genotypes of 1096 animals. Dataset 2 is from three flocks with pure-bred Comisana sheep which together comprise 1143 animals with PrP genotypes. Dataset 3 is from five flocks where a scrapie outbreak occurred and consists of 1771 animals with PrP genotypes. These flocks contained indigenous Sicilian dairy sheep of the Comisana, Valle del Belice and Pinzirita breeds and cross-breeds between these breeds.

PrP haplotype and genotype frequencies

Genomic DNA was isolated from EDTA (ethylenediaminetetraacetic acid)-treated blood using a DNA isolation kit for mam-
malian blood (GenEluteTM-Mammalian Genomic DNA Purification Kit, SIGMA). PCR amplification of the PrP-gene was performed with 100 ng of DNA. The “Ovin PrP Gene Test Kit (Nuclear Laser Medicine)” using a reverse hybridization method has been used for the PrP haplotype determination. This is a rapid method developed to detect single nucleotide polymorphisms at codons 136, 154, and 171. At codon 136, presence of an Alanine versus a Valine codon was checked. At codon 154, Arginine versus Histidine. At codon 171, two checks were undertaken, one for Arginine versus Histidine and one for Arginine versus Glutamine.

Animals that mistakenly occurred multiple times in the datasets were only included in the analyses if their data was consistent between records and therefore could be used to complete a single record, otherwise they were excluded from the analysis. Furthermore, all animals with haplotype codes differing from the five common haplotypes ARR, AHQ, ARH, ARQ, and VRQ were removed from the dataset. No confirmative genotyping was undertaken; therefore it is unknown if the unusual haplotypes are true or false. From dataset 1, five ARQ/VHQ, one ARR/VHQ, and one ARQ/AHR were removed. From dataset 2, one ARR/AHR and one AHQ/AHR genotypes were removed. From dataset 3, five ARQ/VHQ and four ARR/VHQ genotypes were removed. The remaining size of the three datasets was 1089 (1), 1141 (2), and 1771 (3) genotypes.

Analyses

For all three datasets, haplotype and genotype frequencies were estimated over all flocks together. The Hardy-Weinberg equilibrium was verified using the exact probability test (Haldane, 1954) of the GENEPOP 3.4 software (Raymond and Rousset, 1995). Simulations were undertaken of one generation of segregation to obtain future (next generation) expected intervals of the haplotype frequencies. The simulations assume that the number of sires and dams from all animals in a flock were 8% and 60% of the flock size, respectively. Note that all animals together resulted from several mating seasons. Each sire contributed equally to the offspring, and similarly each dam had equal contribution, different from the sires. In the simulation there were no migration, no selection and no mutation affecting PrP haplotype frequencies. Therefore the distribution of the simulated haplotype frequencies depended only on drift, resulting from a limited number of parents producing a limited number of offspring. A total of 1 million repeats were undertaken including all flocks in each dataset.

Results and discussion

PrP haplotype frequencies

Table 1 shows the PrP haplotype frequencies found in each dataset. ARQ and secondly ARR were the most common haplotypes in Valle del Belice. The most susceptible haplotype, VRQ, appears to have a very low frequency and therefore can be eradicated from the population relatively fast. Codon 136 is still the only dimorphism associated with high susceptibility (Goldmann et al., 2005). Also ARH is at such a low frequency that it could be eradicated easily. However, the second most susceptible haplotype ARQ is the most frequently found. The higher the frequency of a haplotype, the more generations will be needed to eradicate it. The eradication of ARQ can be achieved by using only sires which are not carriers of ARQ (and of course not of ARH and VRQ either).

The second row with the 95% confidence intervals, obtained by simulation, indicates where the haplotype frequencies are expect-
ed to be in the next generation if no direct or indirect selection on PrP occurs. For example the VRQ haplotype is highly likely to remain at a low frequency in each flock because its frequency could only be substantially increased if several of the sires used are carriers. The chance of that occurring is low. In fact the probability of the VRQ haplotype disappearing due to drift from the five Valle del Belice flocks within one generation, as obtained by the simulation, is 0.3% in flock 2, 8.1% in flock 3, 65.8% in flock 5, 12.1% in flock 6, and 20.5% in flock 8 together resulting in a joint probability of 0.0022% for the five flocks with VRQ together. In two generations the joint probability is increased to 0.4374%. These joint probabilities follow from simulating all flocks together for two generations. Note that there were just 14 VRQ haplotypes in flock 2, 6 in flock 3, 1 in flock 5, 5 in flock 6, and 4 in flock 8 while the other three flocks were free of VRQ. Considering the assumptions, especially no migration, the intervals obtained from simulation should be considered conservative.

The Comisana breed showed the same two haplotypes, ARQ and ARR, as most common. The largest relative differences between both breeds are for the least common haplotypes. The AHQ haplotype frequency was half and the ARH and VRQ haplotype frequencies were twice as high compared to Valle del Belice. The infected indigenous animals also had the ARQ and ARR haplotypes as most common. The VRQ frequency was at 3.9% and the ARQ frequency was at 59.1%.

Palhière et al. (2003) reported PrP haplotype frequency estimates in 29 French breeds. In 27 of those breeds ARR and ARQ were the two most frequent haplotypes. The frequency of VRQ was at most 25%, but most often below 10%. Dawson (2003) reported PrP haplotype frequency estimates in nine British breeds. Eight of these breeds had ARR and ARQ as most frequent haplotypes. The VRQ haplotype frequency was always below 7%. In all seven Spanish breeds studied by Acín et al. (2004) ARR and ARQ were the two most frequent haplotypes. The frequency of VRQ was at most 13.5%, which was in a small sample. Hence the haplotype frequencies of the three investigated Sicilian populations fit within this common pattern.

### Table 1. Haplotype frequencies for the prion protein gene obtained in three Sicilian dairy sheep populations, with a simulated 95% confidence interval for the next generation.

| Population          | Number | Amino acid haplotype frequency (%) | ARR | AHQ | ARH | ARQ | VRQ |
|---------------------|--------|-----------------------------------|-----|-----|-----|-----|-----|
| Valle del Belice    | 1089   |                                   | 32.3| 6.5 | 1.1 | 58.8| 1.4 |
| Next generation     | 28-36  |                                   | 28  | 5-9 | 0-2 | 55-63| 0-2 |
| Comisana            | 1141   |                                   | 39.4| 2.9 | 2.9 | 50.9| 3.9 |
| Next generation     | 35-43  |                                   | 35  | 2-5 | 2-5 | 47-55| 3-6 |
| Infected indigenous | 1762   |                                   | 32.8| 2.4 | 1.7 | 59.1| 3.9 |
| Next generation     | 30-36  |                                   | 30  | 2-4 | 1-3 | 56-62| 3-5 |

*Number: number of animals genotyped.*
Agrimi et al. (2003) gave the following results for 111 Italian Comisana animals: ARR (41.4%), AHQ (4.1%), ARH (0%), ARQ (44.6%), and VRQ (9.9%). The precise origin of these animals is not mentioned. Our results on 1141 Comisana animals showed a presence of ARH and a lower VRQ frequency of 3.9%.

PrP genotype frequencies

Table 2 shows the PrP genotype frequencies found. The genotypes AHQ/VRQ, ARH/VRQ, ARQ/VRQ, and VRQ/VRQ are considered the highest susceptible genotypes, followed by ARR/VRQ. In total these genotypes include 2.7%, 7.7%, and 7.8% of the Valle del Belice, Comisana and infected indigenous populations respectively. The resistant animals are ARR/ARR, ARR/AHQ, ARR/ARH, and ARR/ARQ, which together comprise 54.3%, 60.1%, and 51.5% of the same populations. The remaining 43.1%, 32.2%, and 40.7% is considered susceptible to scrapie. In total 84.6%, 75.8%, and 82.9% of the animals carry at least one ARQ haplotype and 55.3%, 63.0%, and 54.4% carry an ARR haplotype. The flocks with infected indigenous animals seem to have somewhat higher frequencies of the ARQ and VRQ haplotypes as the non-affected flocks.

Verification of the Hardy-Weinberg equilibrium using the exact probability test (Haldane, 1954) resulted in a Chi-square of 24.0 with 16 df and a probability of 0.0889 across all flocks with Valle del Belice. In Comisana a high significance with an infinite Chi-square with 6 df was found. The results for Comisana resulted from too many ARH/ARH animals within one flock. At the same time there was a lack of ARR/ARH animals and it is therefore assumed that some genotyping errors occurred at codon 171. In the indigenous animals a Chi-square of 17.4 with 10 df and a probability of 0.0660 was found. These probabilities do not clearly indicate the presence or absence of the Hardy-Weinberg disequilibrium. Hence we can conclude that selection, which mainly aims at cheese milk production, so far does not strongly affect the PrP locus.

Conclusions

The patterns of PrP haplotype and genotype frequencies in the three investigated Sicilian populations are similar to
each other and to the patterns found in other European breeds by other authors. Undesired haplotypes and non-resistant genotypes have a high frequency. Therefore the eradication of the susceptible haplotypes can be achieved by using only ARR/ARR sires during several generations of selection. Indeed based on our survey results Comisana and Valle del Belice are both in the ARR<40% category of the National/Regional Breeding Plans for Scrapie resistance. Concerning the Comisana breed our results are in agreement with the survey carried out in 2002 for the National/Regional Breeding Plan for Scrapie resistance. The Valle del Belice breed was not investigated in the 2002 survey. Therefore our results on this breed are important because they give an indication on how to direct the selection for this breed. At this point, results presented in this paper clearly indicate the necessity to undertake a selection scheme which in a reasonable time will bring these two breeds above the ARR threshold of 40% as presented in the National/Regional Plan. In the future it might be needed to genotype other PrP codons as well, if other polymorphisms are found to be related with scrapie susceptibility. This is recently suggested for codon 141 (Goldmann et al., 2005).

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