DETECTION OF PrP COMPLEX GENOTYPES IN SHEEP: AN IN VITRO APPROACH

Detectie van PrP-complexe genotypen bij het schaap: een in vitro *benadering*

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ABSTRACT

The PrP genotyping of sheep for selection from the population of individuals with scrapie resistant genotypes has become a very important component of the scrapie eradication strategy.

However, a limited number of sheep did not seem to possess a standard "two allele" genotype, but rather three or more PrP (prion protein) alleles (complex genotypes). One hypothesis is that these animals are blood chimerisms, due to exchange of blood with their co-twin during gestation. In this study, blood from sheep with different genotypes was deliberately mixed (mimicking chimerism in vitro) and genotyped using real-time Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism – Denaturing Gradient Gel Electrophoresis (RFLP-DGGE). At certain mixture ratios, complex genotypes were detected. This experiment shows that if the complex genotypes diagnosed in vivo are due to blood chimerism, they can to a certain extent be detected by these techniques.

SAMENVATTING

PrP-genotypering bij schapen vormt een zeer belangrijke bijdrage in de strategie voor de eradicatie van scrapie en de selectie naar scrapieresistente genotypen in de populatie. Bij een beperkte groep schapen werd niet het standaard "twee allelen" genotype gevonden, maar een genotype dat bestaat uit drie of meer PrP-allelen (= complex genotype). Een mogelijke verklaring hiervoor is dat bloedchimeren (d.w.z. bloeduitwisseling tijdens de dracht met de co-tweeling) aan de basis liggen van deze complexe genotypen. In deze studie werd bloed van schapen met een verschillend genotype gemengd (= nabootsen van chimeren *in vitro*) en gegenotypeerd met realtime polymerase chain reaction (RT-PCR) en restriction fragment length polymorphism – denaturating gradient gel electrophoresis (RFLP-DGGE). Bij bepaalde ratio's van het gemengde bloed werden complexe genotypen gedetecteerd. Dit experiment toont aan dat, indien de *in vivo* gediagnosticeerde complexe genotypen te wijten zijn aan bloedchimerisme, deze kunnen aangetoond worden met de gebruikte technieken tot een bepaald niveau.

INTRODUCTION

Scrapie is the oldest known transmissible spongiform encephalopathy (TSE), having been described in the literature since two and one-half centuries ago. It is a fatal neurodegenerative disease that occurs naturally in sheep and goats. Currently, the etiology is considered to be an infectious disease with a maternal and horizontal transmission, where host genetic factors play a central role (Dickinson *et al.,* 1974; Hunter *et al.,* 1993; Belt *et al.,* 1995; Smits *et al.,* 1997). Scrapie susceptibility in sheep

is associated with prion protein gene polymorphisms (Hunter, 1997), in particular regarding the codons 136 (valine (V) – alanine (A)), 154 (arginine (R) – histidine (H)) and 171 (arginine (R) – glutamine (Q) – histidine (H)). The genotyping of sheep has become very important for the "classical scrapie" eradication strategy and the breeding program aimed at producing TSE resistance via selection of the ARR alleles (European Commission, 2003). The TSE Regulation 999/2001 (European Commission, 2001) requires active surveillance of sheep and goats by one of the approved rapid screening tests for TSE. Additio-

nally, screening of the PrP genotype distribution of the different breeds in the EU Member States was also suggested in order to set up a global breeding scheme with the main goal of eliminating TSE in the European sheep population (European Commission, 2003). A breeding program is compulsory in all European member states.

Surveys in Great Britain on polymorphisms at codons 136, 154 and 171 revealed that in a small proportion of these tested genotypes (0.08 %) it has not been possible to report a standard "two allele" genotype (Dawson *et al.*, 2003; McKeown *et al.*, 2004). In fact, it appears that certain animals carry three or more PrP alleles, so that results such as ARR/ARR/ARQ or ARR/ARH/VRQ/VRQ, etc. could be reported. These genotypes have been described as "complex genotypes" (Dawson *et al.*, 2003; McKeown *et al.*, 2004). These genotypes are not caused by contamination of one blood sample with blood from another sheep at the moment of sampling. When the sheep concerned were subject to additional sampling, the re-samples produced results identical to those of the original samples. Till now, no other reports on these "complex genotypes" have been described in literature.

The most plausible hypothesis for explaining these imbalanced profiles may be that some degree of blood chimerism occurs due to twin-twin transfusion in utero (McKeown *et al.*, 2004). Chimeras are animals which are composed of two or more different populations of genetically distinct cells that originated in different zygotes. It is known that chimerism can occur naturally, though rarely, in twin sheep (Jönsson and Gustavsson, 1969; Szatkowska and Switonski, 1996) and can lead to the development of freemartins. Freemartins arise when vascular connections form between the placentas of developing heterosexual twin fetuses, with resulting XX/XY chimerism and masculinization of female lambs (Padula, 2005). The purpose of this paper is to test whether "artificial chimerism" achieved by mixing blood from sheep with different genotypes will result in the detection of "complex genotypes" in vitro. This in vitro test will be necessary in order to set a threshold for such cases for the purpose of differentiating the actual PrP genotype of the animal from the interfering PrP genotype.

MATERIALS AND METHODS

Non-coagulated blood samples of sheep with different genotypes (previously confirmed both in blood and in brain) were mixed in different proportions (Tables 1 and 2). In total, 21 different concentrations were analyzed for each mixture (Tables 1 and 2). The genotypes of these mixtures were analyzed using a combination of Restriction Fragment Length Polymorphism and Denaturing Gradient Gel Electrophoresis (RFLP-DGGE) (Bossers *et al.*, 1996) supplemented with real-time Polymerase Chain Reaction (PCR) (Renard *et al*, 2005). All sheep involved originated from the TSE epidemiosurveillance and were all TSE negative according to both rapid (TeSeE – Biorad) and confirmatory testing (Roels *et al.*, 2004).

RESULTS

The composition of the mixtures with increasing concentration of the ARR haplotype is given in Table 1. The DGGE analysis of the corresponding mixtures is shown in Figure 1. These results demonstrate that the ARQ haplotype is solely detected in lanes 2 to 4. The intensity of the ARR band, which appears in lane 5, gradually increases until it becomes equal to the intensity of the ARQ band. From this point on, the ARQ band slowly decreases in intensity until only the ARR band remains detectable.

The same mixtures were analyzed using real-time PCR (Figure 2). The results confirmed that the probes were indeed allele-specific. For example, in sample 1 (0% ARR; Table 1) no fluorescent signal could be obtained with the mutant probe but an amplification plot with the steepest slope and the highest value of relative fluorescence units (RFU) was detected with the wild type probe, while the opposite was observed for sample 21 (100% ARR). Samples with an intermediate percentage of ARR and ARQ generate amplification plots with smaller slopes and lower RFU values, in direct relation with their concentration in haplotype. Figure 3 shows the combination between fluorescent signals produced with the wild type probe and the mutant probe for an increasing of ARR concentration. We observe a progressive increase of the fluorescent signal with the mutant/FAM probe and a decrease of wildtype probe/VIC signal. The results of the DGGE analysis of a more complex haplotype mixture, namely ARR/ARH – ARQ/AHQ, are shown in Figure 4. From lanes 2 to 22 the concentration of the DNA extracted from blood of genotype ARQ/AHQ increases with decreasing concentration of the ARR/ARH genotype. The composition of the mixtures is given in Table 2. Lanes 2 and 22 represent pure ARR/ARH and ARQ/AHQ genotypes, respectively. The haplotypes ARQ and AHQ, which become detectable from lane 4, correspond to 10% of ARQ/AHQ DNA added to the mixture. In lane 9, corresponding to 35% ARQ/AHQ, the intensity of the different bands is almost equal. The bands corresponding to the ARR/ ARH haplotypes were no longer detectable from lane 14. The results of the real-time PCR analysis of these mixtures are shown in Figure 5. In contrast to sample 147, sample 127 (50% ARR – 50% ARH; Table 2) has no fluorescent signal for the WT 171probe,

Table 1. Mixtures of two non-coagulated blood samples of sheep with different genotypes: ARR/ARR and ARQ/ARQ.

Sample number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Blood genotype																					
ARQ/ARQ (µl)	200	190	180	170	160	150	140	130	120	110	100	90	80	70	60	50	40	30	20	10	0
Blood genotype																					
ARR/ARR (µl)	0	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200
% ARQ	100	95	90	85	80	75	70	65	60	55	50	45	40	35	30	25	20	15	10	5	0
% ARR	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
Lane DGGE (Figure 1)	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
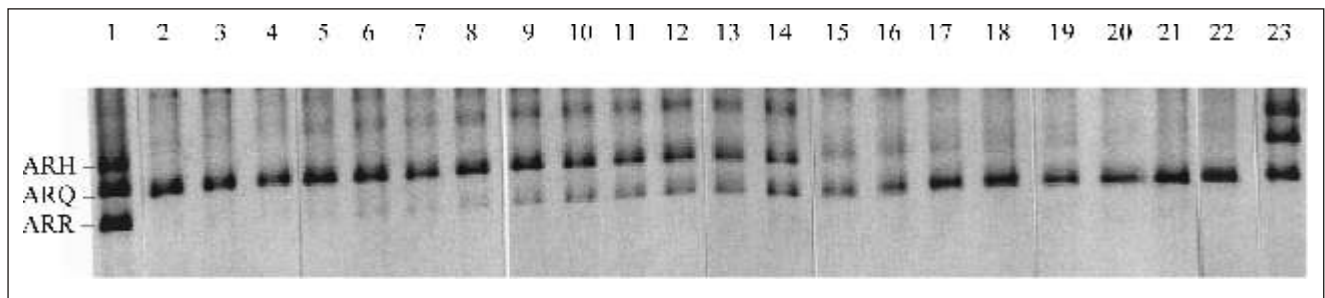
Figure 1. DGGE analysis of PCR-amplified DNA. Mixtures of two non-coagulated blood samples of sheep with different genotypes: ARR/ARR and ARQ/ARQ. Lanes 1 and 23 are control lanes that contain three combined allelic variants (mixture of three plasmids: ARR/ARQ/ARH). Lanes 2 to 22 are DGGE patterns obtained from the different mixtures (see Table 1).
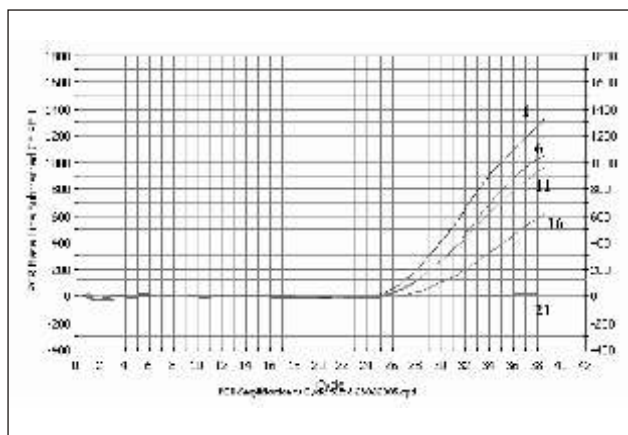

**Figure 2a. Fluorescent signals for the WT probe/VIC (Q / codon 171).**
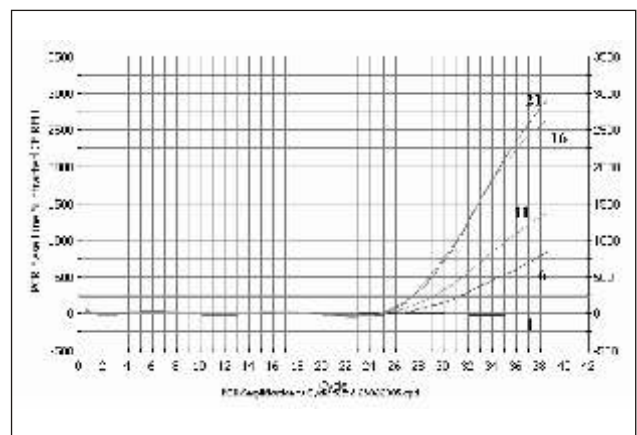
Figure 2b. Fluorescent signals for the mutant probe/FAM (R / codon 171).

Figure 2. Real-time PCR analysis. Mixtures of two non-coagulated blood samples of sheep with different genotypes: ARR/ARR and ARQ/ARQ. Only the results of five mixtures are shown (0, 25, 50, 75, 100 % ARR). The numbers on the graph correspond to the mixture numbers (see Table 1).
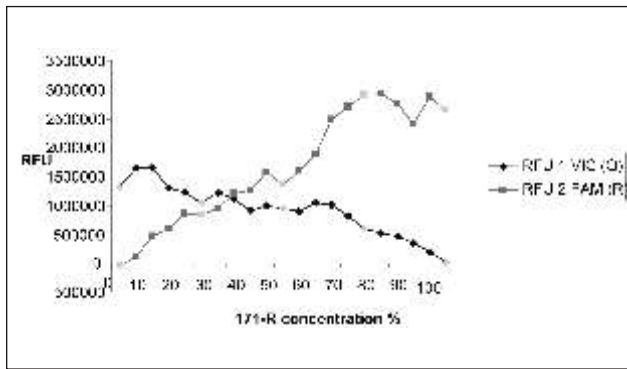
Figure 3. Combination between fluorescent signals of wild type probe and mutant probe for increasing ARR concentration. Mixtures of two non-coagulated blood samples of sheep with different genotypes: ARR/ARR and ARQ/ARQ. They are mixed in different proportions, varying from 0 (= 0%) to 200 μl (= 100%), with ratios of 10 μl (= 5%) (see Table 1).
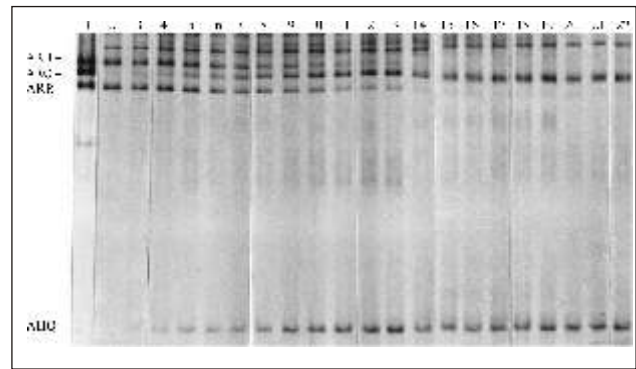
Figure 4. DGGE analysis of PCR-amplified DNA. Mixtures of two non-coagulated blood samples of sheep with different genotypes: ARR/ARH and ARQ/AHQ. Lane 1 is a control lane that contains three combined allelic variants (mixture of three plasmids: ARR/ARQ/ARH). Lanes 2 to 22 are DGGE patterns obtained from the different mixtures (see Table 2).

Table 2. Mixtures of two non-coagulated blood samples of sheep with different genotypes: ARR/ARH and ARQ/AHQ.

Sample number	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147
Blood genotype																					
ARR/ARH (μl)	200	190	180	170	160	150	140	130	120	110	100	90	80	70	60	50	40	30	20	10	0
Blood genotype																					
ARQ/AHQ (μl)	0	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200
% ARR	50	47,5	45	42,5	40	37,5	35	32,5	30	27,5	25	22,5	20	17,5	15	12,5	10	7,5	5	2,5	0
% ARH	50	47,5	45	42,5	40	37,5	35	32,5	30	27,5	25	22,5	20	17,5	15	12,5	10	7,5	5	2,5	0
% ARQ	0	2,5	5	7,5	10	12,5	15	17,5	20	22,5	25	27,5	30	32,5	35	37,5	40	42,5	45	47,5	50
% AHQ	0	2,5	5	7,5	10	12,5	15	17,5	20	22,5	25	27,5	30	32,5	35	37,5	40	42,5	45	47,5	50
Lane DGGE (Figure 4)	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22

but it does have an amplification plot with the steepest slope and the highest RFU value for the mutant 171 probes. It also demonstrates a signal for the WT 154 probe, but no signal for the mutant 154 probe. Sample 147 shows an intermediate intensity with both 154 probes (50% ARQ – 50% AHQ). We also observed a strong fluorescent signal for codon 171 with the WT probe, but no (or a very low) signal with the mutant probes. The remaining mixtures demonstrate a signal in direct relation with their concentration in haplotype ARR, ARH, ARQ and AHQ. Figures 6a and b show the combination between

fluorescent signals of wild type probes and mutant probes for an increasing of ARQ/AHQ concentration for codons 171 and 154. We observed a progressive increase of the fluorescent signal of the WT/VIC probe and a decrease for two mutants probes (Figure 6a).

DISCUSSION

Freemartinism is a common form of intersexuality found in cattle and sheep (Rejduch, 2001). It occurs in twins of opposite sex and results from the exchange of

Figure 5. Real-time PCR analysis. Mixtures of two non-coagulated blood samples of sheep with different genotypes: ARR/ARH and ARQ/AHQ. Only the results of five mixtures are shown (0, 12.5, 25, 35, 50 % ARQ). The numbers on the graph correspond to mixture numbers (see Table 2).
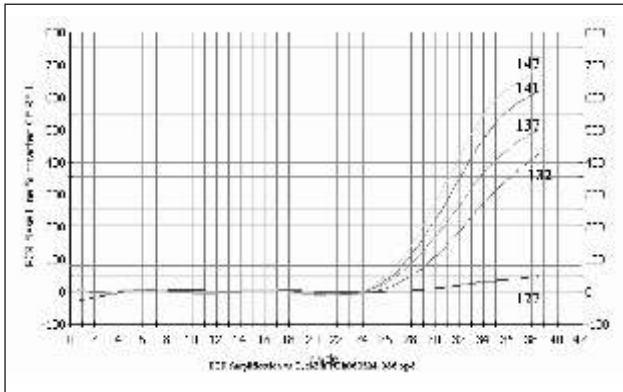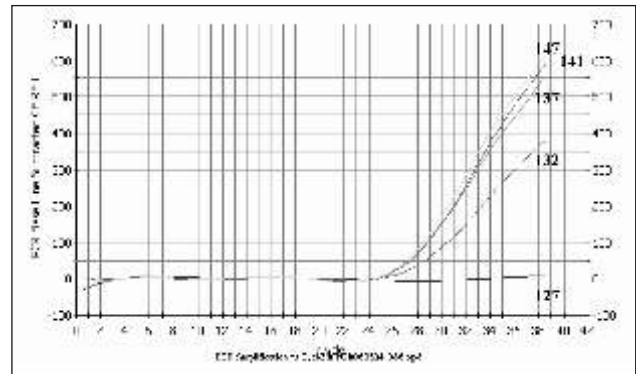

Figure 5a. Fluorescent signals for the WT probe/VIC (Q/codon 171).
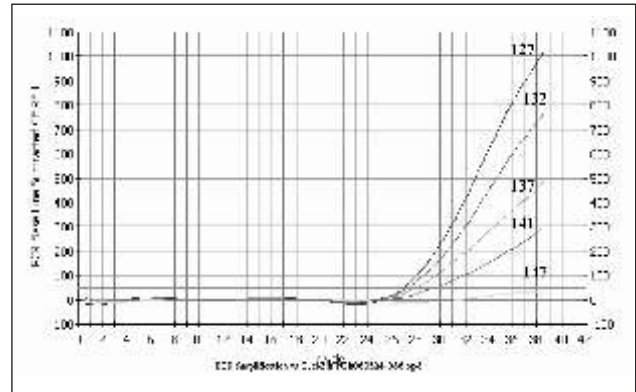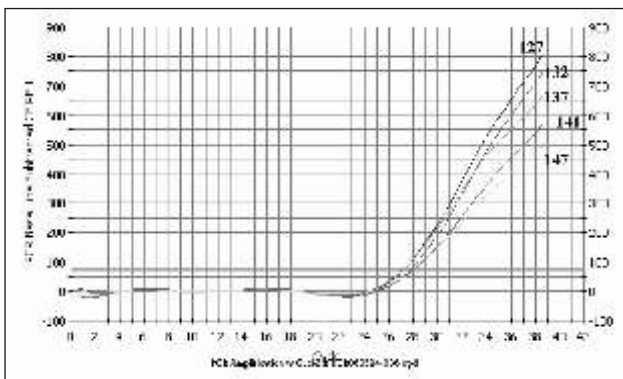

Figure 5b. Fluorescent signals for the mutant probe/FAM (R/codon 171).
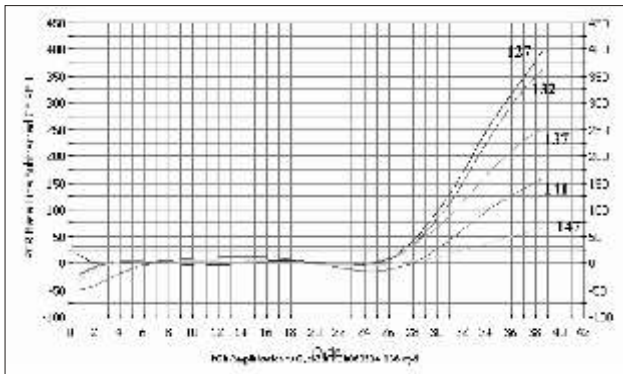
Figure 5c. Fluorescent signals for the other mutant probe/ VIC (H/codon 171).

Figure 5d. Fluorescent signals for the other mutant probe/VIC (H/codon 154).

Figure 5e. Fluorescent signals for the other WT probe/ FAM (R/codon 154).

hematopoietic tissue cells that takes place through ana-stomosed vessels of the fetal membranes that are formed in the early postimplantation stage of intrauterine life after a gestation period of 30-40 days (Lillie, 1917; Jönsson and Gustavsson, 1969; Zhang *et al.*, 1994). In the case of he-terosexual twins, the sexual development of a female co-twin is altered due to the action of the male hormones that are produced by the male co-twin (Szatkowska and Switonski, 1996). An exchange of hematopoietic stem

cells between these two fetuses also takes place (Szat-kowska and Switonski, 1996), an exchange which is stable and results in long-term postnatal hematopoietic chimerism (Zanjani *et al.*, 2005). This results in stem cells from one twin being present in the other and vice versa (Szatkowska *et al.,* 1998; Keszka *et al.,* 2001; Ver-berckmoes *et al.,* 2002), which is evidenced by leukocyte sex chromosome (XX/XY) chimerism. Several cases of intersexes in sheep have been described (Jönsson and Gustavsson,1969). There are even reports suggesting the hereditary tendency of certain breeds to develop placen-tal anastomoses between co-twins in sheep (Gill and Da-vies, 1991; Szatkowska and Switonski, 1996).

Nevertheless, the chorions of twin sheep commonly fuse, but anastomosis of blood vessels is rare (Jönsson and Gustavsson, 1969). Although freemartinism is likely to occur in only 0.03 % of the total sheep population (Smith *et al.,* 1998), recent studies indicate that the preva-lence of freemartinism in sheep is increasing (Parkinson *et al.,* 2001).

Till now, no studies on the degree of blood cell ex-change in such cases have been published. Questions
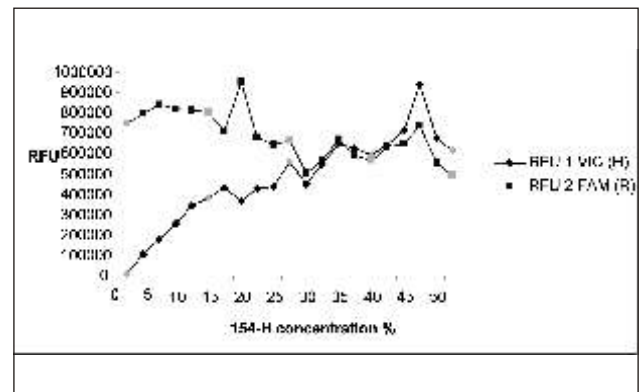
Figure 6. Combination between fluorescent signals of wild type probe and mutant probes for increasing ARQ/AHQ concentration. Mixtures of two non-coagulated blood samples of sheep with different genotypes: ARR/ARH and ARQ/AHQ. They are mixed in different proportions, varying from 0 (= 0%) to 200 µl (= 100%), with ratios of 10 µl (= 5%) (see Table 2).
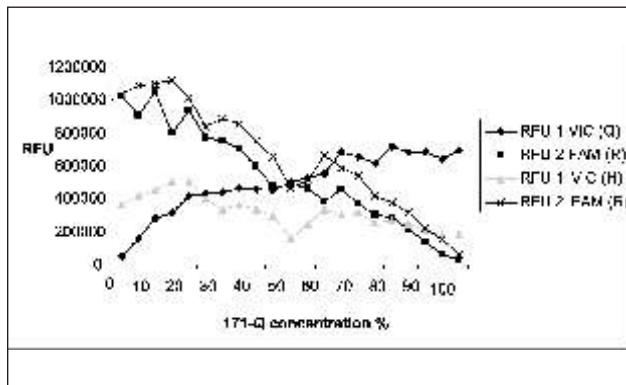
Figure 6a. Combination of fluorescent signals of 171 probes.

Figure 6b. Combination of fluorescent signals of 154 probes.

such as what percentage of the co-twin's blood is exchanged remain unanswered. When blood cell exchange is only limited, "complex genotypes" may not be detectable with the currently available techniques or they may only be detectable at a high ratio of the limited allele in the blood mixture. When hematopoietic stem cell exchange was abundant, the detection of the different alleles was possible at lower ratios of the blood mixture. In fact, a difference of 5% in the ratio is sufficient to detect the other allele in a blood mixture. The two techniques used make it possible to detect the presence of complex genotypes.

When curves are analyzed in real-time PCR, such as for example ARR/ARQ and ARQ/ARQ, the only difference is noted for probe 171. Probe 171-VIC (Q) has an amplification plot with the steepest slope and the highest RFU value for ARQ/ARQ (homozygote), but for ARR/ARQ (heterozygote) the slope becomes less steep and the intensity decreases. We can apply this observation to complex genotypes. The presence of a haplotype is easily detected and the fluorescent intensity and the slope of the curves determine the importance of this haplotype. A sharp slope and strong intensity indicate that large amounts of the probe are fixed and reveal the importance of the corresponding alleles. In Figure 2, the samples 1 (ARQ/ARQ) and 21 (ARR/ARR) are clearly identified as being homozygote, while sample 11 can be regarded as ARR/ARQ heterozygote. A combination of intermediate curves for different probes, always compared to non-complex homozygous and heterozygous control samples, can predict complex genotypes. For example, the curve profile for sample 6 is between that of a homozygote and a heterozygote for the WT probe. At the same time, its profile for the mutant probe is between non-detection and a heterozygote. This result is not possible for a simple genotype. It is indicative of a complex genotype with two different alleles. In Figure 5, a complex genotype

with 4 different alleles is shown (homozygote profiles are not represented in Figures 5b and 5c). For sample 132, curve profiles between non-detection and a heterozygote were found for all three probes. It is clear that, despite the fact that the profiles become more complicated, the detection of complex genotypes with more than two different alleles is easier.

Using the analysis in DGGE, the intensity of lanes gives an indication of importance of different alleles, but in this case the technique for determining a complex genotype is less powerful. Most techniques detect the different alleles only qualitatively, while quantitative techniques are necessary to reveal the presence of more than one of the same allele. This is especially the case in chimerism of genotypes consisting of only two different alleles (e.g. ARR/ARQ and ARR/ARR).

Currently, one of the most important issues is to determine the real genotype of the animal. Indeed, the real genotype determines the offspring genotype, which is the basis of the breeding program for TSE resistance via selection of the ARR alleles (E. C. Decision 1803/2002). "Chimerism" has little importance in this program and we are quite confident that the two techniques show real genotypes and indications on the presence of chimerism. To this end, the real-time PCR technique is more accurate, since we can compare fluorescence intensity and the slope of the curves. However, controls should always be used (homozygote, heterozygote for each probe) when making comparisons.

In conclusion, this study demonstrates that if the complex genotypes diagnosed in vivo are due to blood chimerism, they can to a certain extent be detected by these techniques. However, a real field study of certain sheep breeding lines and/or twins is needed to confirm this hypothesis and to quantify the incidence of the phenome-

non under natural conditions in different breeds and geographical areas. In the future, it would be interesting to work with a wild chimer, comparing blood and tissues to determine the real genotype in an effort to evaluate real complex and artificial chimerism.

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