Molecular and Cellular Probes 23 (2009) 122–125

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Short Communication

A new genotyping strategy for efficient scoring of closely positioned SNPs in the ovine prion protein gene

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ARTICLE INFO

Article history:
Received 6 October 2008
Accepted 8 January 2009
Available online 20 January 2009

Keywords:
Genotyping
Primer extension
Prion protein
Sheep
Single-nucleotide polymorphism

ABSTRACT

Amino-acid polymorphisms of the ovine prion protein have been known to influence susceptibility to scrapie for many years. Recently, a role in both classical and atypical scrapie was assigned to new mutations, increasing the overall number of polymorphisms of interest for breeding plans. Besides, the high number and density of polymorphisms in the prion protein gene (*PrP*) and the presence of unusual mutations in some breeds hampers genotyping methods, making multiplexing difficult and sometimes compromising analytical results. We developed a multiplex genotyping method for the ovine *PrP* that overcomes the limitations posed by the high number and density of the polymorphisms to interrogate. Nine primers were designed to be compatible in a single primer-extension reaction developed for routine genotyping, with the capacity to identify the following polymorphisms: A136V, M137T, L141F, I142K, R154H, Q171R, Q171H, Q171K and N176K. Site-specific mutations were inserted in primer sequences in order to prevent extension of reciprocally complementary primers. Complete accuracy and repeatability of the assay was assessed with reference to 97 sequenced samples.

The presented method constitutes an improved tool for ovine *PrP* genotyping and a general strategy for the use of primer extension in a genetic context of high density of polymorphisms.

1. Introduction

Many single-nucleotide polymorphisms (SNPs) have been described in the ovine prion protein gene (*PrP*) and some of them have been known to affect sheep susceptibility to scrapie for many years [1–5]. Polymorphisms at codons 136, 154 and 171 are the most important for scrapie susceptibility despite differences among breeds and with respect to the strain of the agent. In particular, allele $A^{136}R^{154}R^{171}$ (amino-acids are indicated by single-letter code: alanine at codon 136 and arginine at codons 154 and 171) has been shown to confer high resistance [6,7]. On this basis, many countries have implemented breeding plans aiming at increasing the ARR frequency in their sheep populations. These plans are based on routine genotyping of the *PrP* gene in order to identify homozygous ARR rams suitable for reproduction. But new elements have changed the picture of scrapie genetics over the past few years. Firstly, a leucine to phenylalanine mutation at codon 141 has been associated to increased risk for the atypical form of scrapie [8–10]. Secondly, the demonstration in some breeds of unusual mutations that can interfere with the correct identification of *PrP* alleles, if such mutations are near the SNPs of interest. This problem can arise with some genotyping methods routinely used (e.g. real-time PCR with hydrolysis probes), unless such methods can correctly interrogate SNPs closely positioned. An example of closely positioned SNPs is the case of the ARK allele (Q171K mutation) detected in some Mediterranean breeds [11,12]. This allele is a consequence of a cytosine to adenine mutation of the first position of codon 171 which is adjacent to the polymorphic second and third position of the same codon; these last two polymorphisms respectively originate the common alleles ARR and ARH. Thirdly, protective effect was recently associated to previously not investigated alleles [13]. The availability of resistance alleles in addition to ARR, the only positively selected allele in the ongoing breeding plans, would help to attenuate the loss of variability of the *PrP* locus and limit the inbreeding risk in breeds with very low ARR frequency. Therefore, confirmation of the protective role of these alleles and the estimate of their frequencies would be useful for the improvement of selection programmes. This scenario would benefit from routine genotyping methods with typing capacity extended beyond the five routinely detected alleles, i.e. ARQ, ARR, AHQ, ARH and VRQ. Nonetheless, the unusually high density of SNPs in the ovine *PrP* gene, with some of them next to one another or separated by only a few bases, generally complicates multiplexing, essential to save

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doi:10.1016/j.mcp.2009.01.002

Table 1
Interrogation primers used for primer-extension reaction at reported loci

Locus	SNP[a]	Amino-acid	Interrogation primer (5′–3′)[b]	Concentration (μM)
136	G**C**C	A	*T15*GGTGGCTACATGCTGGGAAGTG	0.040
	G**T**C	V		
137	A**T**G	M	*T27*AAAATGTAG(T)AAGAGGCCTGCTC[c]	0.006
	A**C**G	T		
141	**C**TT	L	*T20GT5*CCTCATAGTCATTGCCAAAATG	0.002
	TTT	F	TATAA[c]	
142	A**T**A	I	GCCATTG(GA)GCAGGCCTCTTA	0.008
	A**A**A	K		
154	C**G**T	R	*T9*GGGGTAACGGTACATGTTTTA[c]	0.020
	C**A**T	H		
171.I	**C**AG	Q	*T35CT2*CCAAGTGTACTACAGACCAGTG	0.014
	AAG	K	GAT	
171.II	C**A**G	Q	CAA GTGTACTACAGACCAGTGGATC	0.008
	C**G**G	R		
171.III	CA**G**	Q	*T19*GCACAAAGTTGGA(TT)CTGGTTACT	0.014
	CA**T**	H	ATA[c]	
176	G**C**C	A	*T45*CAGTGGATCAGCG(TA)TAGTAACCA	0.024
	G**T**C	V	GAA	

[a] Single-nucleotide polymorphism; wild type sequences in first line, mutants in second line. Bold-underlined letters indicate polymorphic bases.
[b] The number and type of bases added to create 5′ tails of primers is reported in italics; bases not corresponding to template DNA (inserted mutations) are underlined and followed by corresponding template bases (in parenthesis).
[c] Primer complementary to the reverse strand.

time and money, and negatively affects methods based on SNP-specific probes, since the behaviour of probes is conditioned by very close polymorphisms, determining confusing or wrong results. To overcome the limitations posed by the high number and density of the SNPs to interrogate, we developed and validated a multiplex primer extension for contemporary detection of the following amino-acid mutations: A136V, M137T, L141F, I142K, R154H, Q171R, Q171H, Q171K and N176K. Mutations M137T, I142K and N176K were included given their presence and protective effect in Italian most important breed, Sarda, as recently reported [13]; Q171K was included since it is present in some Mediterranean breeds [11,12]. We chose primer extension for its multiplexing capacity and its specificity for the targeted SNPs.

2. Materials and methods

2.1. Genotyping

Genomic DNA was extracted from whole blood treated with EDTA using NucleoSpin 96 Blood (Macherey-Nagel), blood samples were diluted 1:10 in phosphate buffered saline, immediately before proteinase-K digestion.

The *PrP* coding sequence from nucleotide 306 to nucleotide 650 was amplified by PCR in 10 μl of reaction volume containing 2 μl of genomic DNA (corresponding to 10–20 ng), 2 mM $MgCl_2$, 100 mM dNTPs, 0.5 U FastStart *Taq* DNA Polymerase (Roche) and 0.5 mM each of the following primers: 5′-GAACAGCCCAGTA AGCCA-3′ (forward), 5′-CACATTTGCTCCACCACTC-3′ (reverse). The following thermal protocol was used: 95 °C for 6 min followed by 35 cycles of 30 s at 95 °C, 30 s at 64 °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 were designed to be compatible in a multiplex reaction and with 5′ non-complementary tails to confer adequate length differentiation in order to obtain well-resolved peaks in the subsequent electropherogram. Site-specific mutations were inserted to prevent spurious primer extensions due to reciprocal or self-annealing of primers; concentrations were optimized to generate equilibrated signals. Position, sequence, inserted mutations and final concentration of each primer are reported in Table 1. Thermal protocol was: 35 cycles of 10 s at 90 °C, 20 s at 45 °C. Unincorporated dye-terminators were dephosphorylated incubating reaction tubes with 1 U of SAP (GE Healthcare) for 30 min at 37 °C followed by 15 min at 65 °C for enzyme inactivation. Two microlitres of SAP-treated reaction product was 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 DNA extraction to electrophoresis set-up were carried out on a Hamilton StarLet robotic workstation (Hamilton Robotics) in 96 well plates for high throughput.

2.2. Primer-extension validation

Ninety-seven sequenced samples were tested by the newly developed assay. They represented 20 genotypes and all known polymorphisms for the investigated SNPs with the exception of R154L detected in Spanish Churra breed [14]. Twenty-four of these samples were tested four times to assess repeatability of the assay.

2.3. Field trial

The primer-extension assay presented in this study was used in routine conditions for the genotyping of 1324 samples.

3. Results

3.1. Genotyping assay

The primer-extension reaction that we developed produced well-resolved electropherograms, simply interpretable by one-sight visual observation of the single trace corresponding to each sample. This allowed the quick and reliable identification of the *PrP* genotypes determined by the nine SNPs interrogated by the corresponding primers. Some example traces are presented in Fig. 1. During reaction development two technical problems occasionally appeared: *a)* poor genomic DNA quality or quantity, resulting in insufficient or absent 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. Careful DNA extraction and centrifugation of PCR plates before adding EXO-SAP prevented the problems. The validation experiment gave concordant results for all 97 sequenced samples and equal results were obtained from all replicates.

3.2. Field trial

Six samples out of 1324 needed retesting, which constitute an approximate 0.5%. The cause of retesting was the insufficient or absent amplification as a consequence of deficient DNA extraction.