Detection and Quantification of *PrP* Alleles Based on Flock Bulk Milk of Dairy Ewes Using Gap-A Ligase Chain Reaction

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Introduction

Scrapie is a transmissible spongiform encephalopathy affecting sheep and goats. It is a fatal neurodegenerative disease caused by proteinaceus infectious particles (prions). Scrapie in sheep appears to be controlled by genetic factors, with polymorphisms at codons 136 (A or V), 154 (Q or R) and 171 (Q, R or H) of the open reading frame (ORF) of the gene locus coding for the *PrP* protein being the determining parameters (Hunter, 1996).

Peripheral blood leukocytes are the usual source of genomic DNA in animals for the identification of individual *PrP* genotypes. However, increasingly strict regulation on food safety and public hygiene require new, practical, animal friendly methods for large scale implementation. Milk somatic cells may provide an alternative source of genomic DNA in milking ruminants. Furthermore, bulk milk can be used as a source of DNA for massive genotyping at flock or vat (milk tank) level.

The aim of this study was to develop a quick, easy and accurate method for assessing the prevalence of *PrP* polymorphisms within a flock of sheep using bulk milk samples. The method described below was developed and evaluated for the detection and quantification of the most undesirable polymorphism, valine at codon 136 (V_{136}).

Material and methods

Sample collection and preparation of V_{136} standards. Sixty individual milk samples were taken in 50ml tubes from as many ewes of the Chios dairy breed raised in Greece. Genomic DNA was isolated from these milk samples using a commercial kit, Nucleospin® Blood (Mackerey-Nagel, Düren, Germany), modified properly for the milk conditions (Psifidi *et al*, 2009). All samples were genotyped for the *PrP* gene using a modified restriction fragment length polymorphism analysis protocol (Lünken *et al,* 2004). Standard samples containing different frequencies of V_{136} (50 %, 16 %, 8 %, 4 %, 2 %, 1 %, 0.5 %, 0.25%, 0.0625% and 0%) were created by mixing genomic DNA extracts from a homozygous V_{136} (mutant-type) and homozygous for alanine at codon 136 (A_{136}, wild-type) animals.

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All standard samples were created by mixing equimolar DNA extracts. In addition, eight artificial bulk milk samples containing 2-fold dilution series of V_{136} to A_{136} allele from 32% to 0.25% were created by mixing raw milk from individual ewes with known genotypes. Finally, twenty natural bulk milk samples were collected directly from the milk-tank at a flock where a heterozygous V_{136} ewe had been identified and was being milked. The milking strategy of the flock was adapted so that milk of a heterozygous V_{136} ewe was naturally mixed with milk of a total of 32, 64, 96, 128 and 160 other ewes from this flock.

Real-time PCR amplification and treatment with Antarctic Phosphatase SAP and Exonuclease ExoI. A real-time polymerase chain reaction (real-time PCR) targeting a *PrP* genomic region was developed to produce the template for real-time gap-A ligase chain reaction (gap-A LCR). One µl of extracted DNA was added to a 20 µl PCR reaction mixture. The PCR reactions were optimized for standard cycling conditions with an initial denaturation step at 95 ºC (3 min), followed by 40 cycles of denaturation at 94 ºC (30 s) and annealing at 62 ºC (1 min) using the Mj Mini™ personal thermal cycler (Biorad, Milan, Italy). Optimal reaction conditions for PCR were determined as follows; 0.5 units of Platinum® *Taq* DNA polymerase (Invitrogen, The Netherlands), 2 µl PCR buffer (10X), 200 µM each dATP, dCTP, dGTP, and dTTP, 2 mM $MgCl_2$, 1 µl DNA-specific fluorescent dye EvaGreen™ (Biotium, Hayward, CA, USA) (20X), 0.2 mM DGP8 and 0.2 mM CtDo2 *PrP* gene specific primers (Table 1), and water up to 20 µl. All samples were run in triplicates. Real-time PCR product was treated with 2 units of Exonuclease I (ExoI, New England Biolabs), 15 units of Antarctic Phosphatase (SAP, New England Biolabs) and 2.5 µl of Antarctic Phosphatase buffer (10X) in order to digest primers and single stranded DNA amplicons and to dephosphorylate the remaining dNTPs. The enzymatic treatment was performed at 37 ºC for 70 min followed by incubation at 68 ºC for 45 min to deactivate the enzymes.

Table 1: Oligonucleotides used in real-time PCR and gap-A LCR

Oligonucleotide	Sequence
DGP8	5´-CACAGTCATGCACAAAGTTGTTCTGG-3
CtDo2	5´-CATGAAGCATGTGGCAGGAGCTG-3´
LCPR1*	5´-**P**-CACTTCCCAGCATGTAGCCAC-3´
LCPR2x	5´-GTGGCTACATGCTCGGAAGTGT-3´
LCPR3x	5´-CAAAATGTATAAGAGGCCTGCTCATG-3´
LCPR4*	5´-**P**-CATGAGCAGGCCTCTTATACATTTTG-3´

* 5' phosphorylated oligonucleotide

Gap-A LCR assay. DNA template for gap-A LCR was a 1/100 dilution in TE (10mM Tris-HCL pH=7.4) of the real-time PCR product. Two µl of template - DNA was added to a 30 µl gap-A LCR-reaction mixture. The gap-A LCR reactions were optimized for standard cycling conditions with an initial denaturation step at 94 ºC (30 s), followed by 30 cycles of denaturation at 85 ºC (30 s), ligation at 62 ºC (10 s) and fluorescence measurement at 73 ºC (30 s), using the Mj Mini™ personal thermal cycler (Biorad, Milan, Italy). Optimal reaction conditions for real-time LCR were determined as follows: 10 units of *Taq* DNA ligase (New

England Biolabs), 1.8 units of Platinum® *Taq* DNA polymerase (Invitrogen, The Netherlands), 3 µl PCR buffer (10X), 0.525µl Ligase buffer (10X), 0.125 mM NAD^+, 1.5 µl DNA-specific fluorescent dye EvaGreen™ (Biotium, Hayward, CA, USA; 20X), 0.1mM adenine (A) nucleotide, 0.4 µM of "LCPR1", "LCPR2x", "LCPR3x" and "LCPR4" specific oligonucleotides (Table 1), and water up to 30µl. All individual samples were run in triplicates while water blanks containing everything except DNA were included as an additional negative control.

Validation of gap-A LCR. The sensitivity of the assay for V_{136} detection was determined by a standard curve that had been generated by plotting the threshold cycle values (Ct-values) versus the logarithm of the V_{136} frequency in the equimolar standard DNA samples (Figure 1). Although **gap-A** LCR conditions were optimized for discrimination of mutant V_{136} allele, there was still a delayed amplification of the wild type A_{136} templates; these signals were used for the determination of the limit of detection (LOD). According to Schwarz *et al.* (2004), a practical LOD can be set at the concentration that gives a signal equal to three times the standard deviation of the wild type sample measurements. Similarly, limit of quantification (LOQ) was set at ten times the standard deviation of the wild type sample measurements. To assess the reproducibility of the assay, the intra- and inter-assay coefficient of variation (CV %) was evaluated. For intra-assay CV % calculation, standard samples were measured five-fold at each concentration level of the V_{136} allele on one plate. For inter-assay CV % calculation, standard samples were tested in five different assay runs. The different assay runs were performed on different days using freshly prepared reagents.

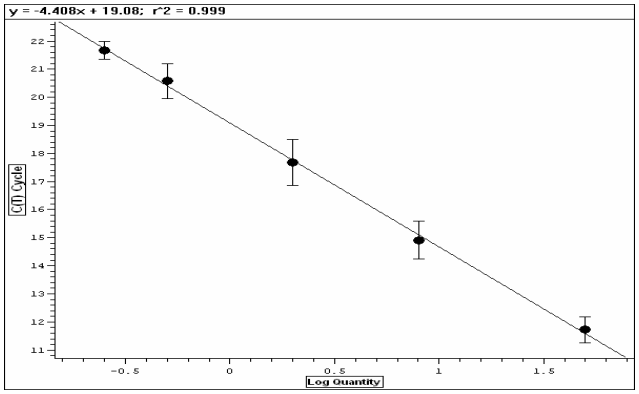

Figure 1: Standard curve generated by dilutions representing V_{136} frequencies from 50% to 0.25%.

Results and discussion

Calibration curve slopes showed that the gap-A LCR system was linear within the dynamic range defined by the Ct values for the highest and lowest target concentrations, as indicated by a square correlation coefficient after linear regression > 0.99 (Figure 1). These results demonstrated the high correlation of the original number of mutant allele copies (V_{136}) and the Ct values obtained by amplification of mutant alleles in the standard samples based on

equimolar DNA extracts. The lowest frequency of V_{136} in the standard samples that was consistently detected and quantified was 0.12 % (LOD) and 0.26 % (LOQ), respectively. These LOD and LOQ results are comparable to or better than others from highly sensitive assays suitable for quantifying single nucleotide polymorphisms in pool samples (Yu *et al.*, 2006), attesting to the utility of gap-A LCR. Moreover, good reproducibility of results, which is another essential requirement of quantitative assays, was attained by this method as attested to by its low experimental intra- and inter-assay variability. The former was 20.9%, 8.9% and 15.3% for V_{136} frequencies of 50%, 8% and 0.25%, respectively, while the inter-assay coefficient of variation was 23.8%, 20.14% and 29%, respectively. Application of the method to artificial and natural bulk milk samples verified that the assay was also very reliable as V_{136} was clearly detected at a frequency as low as 0.25% and 0.31%, respectively. Future experiments should be contacted to account for the potential bias in quantification results obtained with natural samples that may be due to different somatic cell contribution by each ewe to the bulk milk, especially in presence of sub-clinical mastitis. This problem would be alleviated if individual somatic cell count is measured in a routine milk recording scheme and is accounted for in the analysis.

Conclusion

Results clearly show that the gap-A LCR method is a useful tool for a practical and quick screening of flocks for undesirable *PrP* polymorphisms such as V_{136}. Although the method was described using this particular allele, its principle applies to any polymorphism in the *PrP* gene. For the DNA isolation to be representative of the flock, bulk milk sampling could take place on the same date as milk and SCC recording. When individual SCC is not available, possible quantification bias could be overcome by averaging repeat bulk milk sample results obtained at different time intervals during lactation. Monthly tests during the first 4-5 months of lactation would probably suffice in this regard. The proposed method could be also applied for the determination of "scrapie risk" in milk and milk products produced by a flock. This can enable potential labeling and marketing of "scrapie free" dairy sheep products.

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The aim of this study was to develop a novel method for assessing the prevalence of *PrP* polymorphisms within a flock using bulk milk samples. The method was evaluated for the detection and quantification of the undesirable *PrP* polymorphism V_{136}. DNA was extracted from milk somatic cells and part of the gene was amplified with real-time PCR. The amplicons were used as targets in the advanced real-time gap-A Ligase Chain Reaction. This assay detected and quantified V_{136} at a frequency as low as 0.12 % and 0.26 %, respectively. Application of the method to bulk milk samples in flocks with animals with sub-clinical mastitis should account for elevated somatic cell counts. In order to alleviate this bias individual somatic cells count should be available in a routine milk recording scheme. The proposed method could be used for assessing the scrapie risk in sheep flocks as a pre-screening test.