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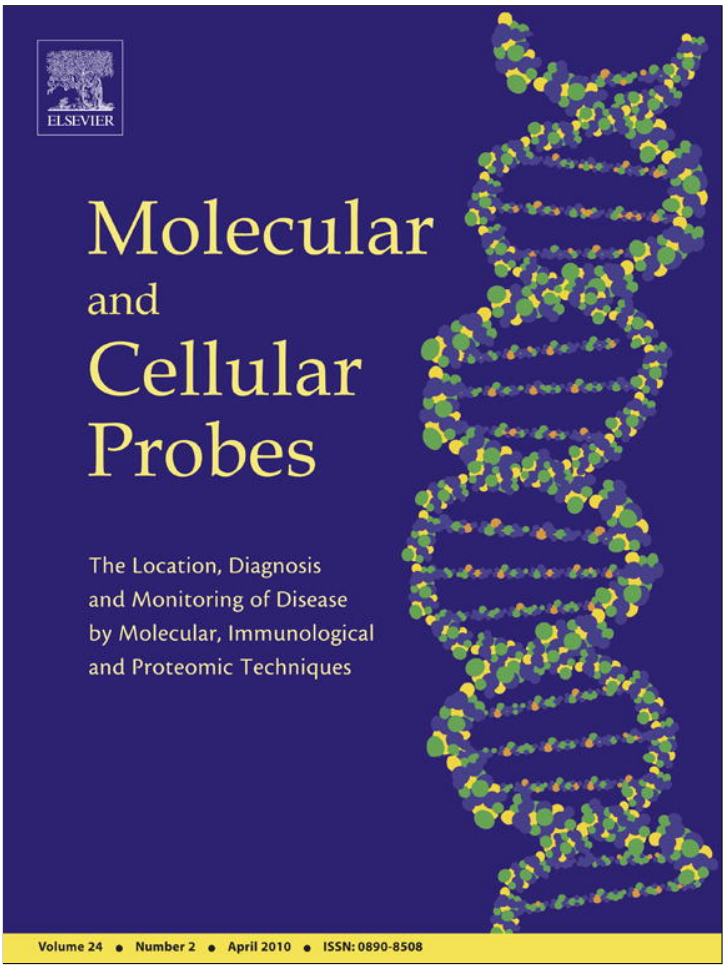

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A comparison of six methods for genomic DNA extraction suitable for PCR-based genotyping applications using ovine milk samples

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ABSTRACT

Isolation of amplifiable genomic DNA is a prerequisite for the genetic assessment of diseases and disease susceptibility in farm animals. Milk somatic cells are a practical, animal friendly and cost-effective source of genomic DNA in milking ruminants. In this study, six different DNA extraction methods were optimized, evaluated and compared for the isolation of DNA from ovine milk samples. Methods 1 and 2 were direct applications of two commercial kits, Nucleospin® Blood and Nucleospin® Tissue, respectively. Methods 3 and 4 were based on modified protocols of methods 1 and 2, respectively, aiming at increasing DNA recovery and integrity, and eliminating PCR inhibitors. Method 5 was a standard Phenol–Chloroform protocol application and method 6 was based on an in-house developed protocol using silica as the affinity matrix. Spectrophotometer, gel electrophoresis and real-time PCR measurements were used as criteria for evaluating quantity and quality of the extracted DNA. Processing time, intensity of labor and cost for each method were also evaluated. Results suggested that methods 1–4 were considered suitable for molecular downstream applications and performed better than methods 5 and 6. Modifications of protocols 3 and 4 increased the quantity and quality of the extracted DNA from ovine milk samples. Method 3 was proved to be highly efficient and robust for large scale use as demonstrated by its successful application to 1000 individual ovine milk and 50 bulk milk samples.

1. Introduction

Molecular techniques, such as micro-arrays and real-time PCR, are increasingly being used in scientific studies of animal health and production, addressing quantitative trait loci (QTL) identification, marker assisted selection, genome mapping, food traceability, diagnosis of genetic diseases and disease susceptibility [1]. Isolation of a sufficient amount of high quality DNA is a prerequisite to such applications and the selection of an appropriate DNA extraction method plays a pivotal role in this regard. Many different methods can be applied for DNA extraction from diverse sources of samples, with modifications depending on the selected tissue. In animals, peripheral blood leukocytes are the usual source of genomic DNA. However, blood collection is stressful to animals, fraught with technical difficulties, and requires trained personnel. Milk somatic cells may provide an alternative source of genomic DNA.

Milk sampling is animal friendly, inexpensive and practical. More importantly, it is built into routine, large scale monitoring and recording of milking ruminant populations.

Furthermore, bulk milk can be used as a source of DNA for massive genotyping at flock or vat (milk tank) level. In order for DNA isolation to be representative of the flock or vat, high quantity of milk (2–5 samples of 50 ml) should be processed at different time intervals. Bulk milk sampling is an important tool, not only for scientific studies but also for public hygiene purposes, since increasingly strict regulations on food safety require new, practical, animal friendly methods for large scale implementation. For example, bulk milk samples can be useful for assessing the small ruminant transmissible spongiform encephalopathy (scrapie) risk in milk and its products at flock level, thereby avoiding individual animal genotyping and saving time and money [2]. Other applications can include selective genotyping in marker assisted selection (MAS) studies, QTL analysis within a daughter or granddaughter design, and QTL mapping projects [3,4].

Several studies have developed methods to extract DNA from epithelial somatic cells of human, bovine and caprine milk [5–9]. However, there is scarcity of literature on DNA extraction from ovine milk samples and with no reports on simultaneous comparisons of different extraction procedures. Ovine milk contains higher concentration of fat and other solids [10] compared to other ruminant species. These milk components can interfere with the isolation process rendering ovine milk a relatively difficult

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medium for extracting high quality DNA, suitable for PCR downstream applications [11].

The objective of the present work was to evaluate six different methods for extraction of genomic DNA from ovine milk samples in terms of DNA quantity, purity and PCR suitability, as well as utility and applicability.

2. Materials and methods

2.1. Samples

At first, individual milk samples were taken from 15 ewes of the Chios dairy breed raised in 5 different flocks. These samples were used to evaluate the DNA extraction methods described next. Milk samples were collected in 50 ml tubes in the milking parlor under aseptic conditions and were immediately placed in isothermic boxes and transferred to the laboratory where they were centrifuged for 10 min at 1500 g. Milk fat was removed using a sterile spatula and the liquid was discarded. The somatic cell pellet was picked up with different buffers depending on the DNA extraction method followed.

In addition, 1000 individual milk samples and 50 bulk milk samples were collected from Chios pure bred ewes raised in 23 flocks. These samples were used for a large scale application of the final method of choice.

2.2. DNA extraction methods

Six different DNA extraction methods, subsequently being referred to as methods 1–6, were evaluated using somatic cell pellets obtained from the individual milk samples of the first 15 ewes.

Methods 1 and 2 were direct applications of two commercially available kits, Nucleospin® Blood and Nucleospin® Tissue (Macherey-Nagel, Düren, Germany). The somatic cells were re-suspended in 200 μl PBS and extraction was performed according to the manufacturer's instructions.

In methods 3 and 4, the two commercial kits of methods 1 and 2, respectively, were modified to increase DNA recovery and eliminate inhibiting substances such as milk lipids and other solids from the samples. Milk pellets were treated with 200 μl TE [1 mM EDTA, 10 mM Tris–HCl (PH = 7.6)] and 300 μl 0.5 M EDTA (PH = 8) to dissolve milk casein. Somatic cells and casein micelles were re-suspended by rotating the 50 ml tubes for 45 min and transferred to a new 2 ml tube followed by centrifugation at 3000 g for 10 min. The supernatant was discarded and somatic cells were re-suspended in 200 μl PBS. Proteinase K and lysis buffers from each kit were added respectively and the mixture was incubated at 70 °C for 15 min. Subsequently, 700 μl of chloroform were added and the mixture was vigorously rotated for 5 min. After centrifugation at 14,000 g for 10 min, the aqueous phase was transferred to a tube containing 210 μl of ethanol. The mixture was then applied to nucleospin columns and DNA was absorbed onto the nucleospin silica gel membrane during one centrifugation at 11,000 g for 1 min. The silica was washed once using 500 μl of a guanidine containing buffer (BW) and then twice using 400 μl of an ethanol containing buffer (B5). The purified DNA was eluted from the nucleospin column in a 100 μl elution buffer (EB).

Method 5 was a standard Phenol–Chloroform based extraction. Briefly, 1 ml of a cell lysis reagent SLB [10 mM Tris–HCl (PH = 7.5), 1 mM EDTA, 50 mM NaCl, 0.2% SDS] and 1 mg of proteinase K were used to digest milk somatic cell pellets overnight at 42 °C. The lysate was extracted twice with 1 ml of phenol:chloroform (1:1, v/v). The aqueous phase was transferred and the DNA was precipitated at −20 °C for 3 hours after the addition of 2.5 volumes of ethanol and 0.1 volume of sodium acetate 3 M (PH = 5.2). The DNA was recovered after centrifugation at 12,000 g for 20 min, the supernatant was discarded and the DNA pellet was washed with 70% ethanol. After a final centrifugation the DNA pellet was dried and finally re-suspended in 100 μl elution buffer (10 mM Tris–HCl, PH = 8.0).

Method 6 was an in-house developed protocol based on the lysing and nuclease-inactivating properties of a chaotropic agent, guanidinium hydrochloride (GuHCl), together with the use of silica particles as the affinity matrix. In brief, 700 μl lysis buffer [8 M GuHCl, 25 mM EDTA, 25 mM sodium citrate, 50 mM Tris–HCl (PH = 6.6), 1% Sarcosyl and 2% Triton® X-100] were added to the milk somatic cell pellets and samples were incubated at 70 °C for 10 min. The lysate solution was added to a 2 ml tube containing 300 μl chloroform. The mixture was vigorously rotated for 5 min. After centrifugation at 16,000 g for 10 min, 400 μl of the aqueous phase was transferred to a tube containing 200 μl ethanol and the mixture was then applied to a homemade silica column [12]. The DNA bound to the silica was washed sequentially by centrifugation, once using 500 μl of wash-1 buffer (50% ethanol, 4 M GuHCl, 25 mM Tris–HCl, PH = 6.6) and twice using wash-2 buffer (80% Ethanol, 100 mM NaCl, 10 mM Tris–HCl, PH = 7). The purified DNA was eluted from silica column in 100 μl elution buffer (10 mM Tris–HCl, PH = 8.0).

2.3. Spectrophotometer measurements

Quality of DNA extracted by the different methods was assessed using an Eppendorf Biophotometer. The ratio of absorbance at 260 nm and 280 nm was used to assess protein contamination while the ratio of absorbance at 260 nm and 230 nm was calculated to assess guanidine contamination. Both spectrophotometer measurements constituted criteria for DNA quality assessment with higher values associated with better DNA quantity and purity.

2.4. Gel electrophoresis

The quantity and integrity of DNA extracted by each method was assessed by gel electrophoresis [13,14]. Specifically, 5 μl of each DNA extract was analyzed in a 1.5% agarose gel containing 0.5% ethidium bromide and was visualized by U.V. illumination. The relative intensity of the genomic DNA bands was assessed using the Gel-Pro Analyzer™ version 3.0 software (Media Cybernetics®, Maryland, USA). High values for this criterion were considered desirable as they related with enhanced DNA quantity and integrity.

2.5. Real-time PCR

A real-time PCR targeting the ovine prion protein gene (*PRNP*) was developed to assess presence of amplifiable DNA in extracts of milk samples. A set of two primers, LCPR2 (5′-GTG GCT ACA TGC TGG GAA GTG-3′) and CTDO2 (5′-CAC AGT CAT GCA CAA AGT TGT TCT GG-3′), amplifying a 167 base pair (bp) *PRNP* genomic region were designed. Amplification reactions were run in a total volume of 20 μl using 2 μl of extracted DNA. The real-time PCR reactions were optimized using the Mj Mini™ personal thermal cycler (Biorad, Milan, Italy). Standard cycling conditions included an initial denaturation step at 95 °C (3 min), followed by 40 cycles of denaturation at 95 °C (30 s), annealing at 64 °C (30 s) and extension at 72 °C (15 s). Fluorescence levels were measured at the end of each cycle. Optimal reaction conditions for real-time PCR were determined as follows; 0.5 units of Platinum® *Taq* DNA polymerase (Invitrogen, The Netherlands), 1X PCR buffer, 200 μM each dATP, dCTP, dGTP, and dTTP, 2 mM $MgCl_2$, 1X DNA-specific fluorescent dye

EvaGreen™ (Biotium, Hayward, CA, USA), 0.3 μM of "LCPR2" primer and 0.1 μM of "CTDO2" primer. Following amplification, a melting curve analysis was performed to verify the correct product by its specific melting temperature. All samples were run in triplicates. Dilutions of a control DNA were used as a standard to determine the linearity and amplification efficiency of the real-time PCR assay. This was an 840 bp amplicon of the *PRNP* gene produced by a standard PCR assay using genomic ovine DNA and two *PRNP* specific primers; 5′-GAT TCT TAC GTG GGC ATT TGA TGC TG-3′ and 5′-ACC ACT ACA GGG CTG CAG GTA GAC-3′. The amplicon was recovered by gel electrophoresis and purification, followed by spectrophotometric quantification. Ct values obtained in the process were used to assess the amount of amplifiable DNA obtained. Smaller values are desirable in this regard.

2.6. Statistical analysis

Model 1 was used to assess the 6 different DNA extraction methods. Each DNA evaluation criterion (two spectrophotometer measurements, gel electrophoresis score, real-time PCR results) was assessed separately.

$$\mathbf{Y_{ij}} = \mu + \mathbf{p_i} + \mathbf{e_{ij}} \quad \text{(Model 1)}$$

where $\mathbf{Y_{ij}}$ = DNA score by evaluation criterion for the $\mathbf{j^{th}}$ sample of the $\mathbf{i^{th}}$ extraction method, μ = overall mean, $\mathbf{p_i}$ = effect of $\mathbf{i^{th}}$ DNA extraction method ($\mathbf{i} = 1,\ldots,6$), $\mathbf{e_{ij}}$ = random residual

Post-hoc analyses, based on Least Squares Differences and a Bonferroni adjustment for multiple testing, were used to compare different DNA extraction methods for each evaluation criterion. Statistical significance level was set at 0.05. All analyses were performed using the statistical package SPSS.

3. Results

3.1. Spectrophotometer measurements

Marginal means of spectrophotometer measurement ratios for each extraction method and comparisons between methods derived from the statistical analysis are shown in Table 1. According to the 260/280 nm absorbance ratio results, the Modified Blood Kit method extracted the purest DNA, followed by Modified Tissue Kit and the two commercial kits. Although differences between these four methods were not significantly different from zero ($P > 0.05$), they all led to significantly ($P < 0.05$) purer DNA compared to the standard Phenol–Chloroform and the in-house developed protocols.

According to results for 260/230 nm ratio, the Phenol–Chloroform protocol, which was free from guanidine contamination, along with the Modified blood Kit method were associated with the highest (most desirable) values. These two methods did not differ significantly from each other ($P > 0.05$) with regards to this criterion.

3.2. Gel electrophoresis score

Quantity and integrity of extracted DNA were assessed by agarose gel electrophoresis (Fig. 1). All extractions were scored using the Gel-Pro Analyzer™ software. Marginal means for each extraction method are shown in Table 1. Gel electrophoresis revealed that the Modified Blood and Tissue Kit methods yielded higher quantity of and more intact DNA compared to the respective non-modified methods. The introduction of modifications attained statistical significance in performance in the case of Nucleospin® Blood kit. Lowest quantity and slight degradation of DNA were observed when the standard Phenol–Chloroform and the in-house developed protocols were used. Moreover, four samples (out of 15) extracted by Phenol–Chloroform protocol yielded no detectable by gel electrophoresis DNA (Fig. 1). Based on the statistical analysis results, the highest DNA yielding protocol (Modified Blood Kit) was significantly better than the standard Phenol–Chloroform ($P = 0.002$), the in-house developed protocol ($P = 0.000$) and the Nucleospin® Blood kit ($P = 0.046$). Furthermore, the superiority of the Modified Blood Kit over the Nucleospin® Tissue kit was just marginally non-significant ($P = 0.068$).

3.3. Real-time PCR

The linear range of real-time PCR detection was from 2×10^8 to 2000 DNA copies, with an amplification efficiency of 108% (Fig. 2). A single product peak at ~82 °C was observed, representing the specific melting temperature of the 167-bp PCR product. Marginal means for Ct values obtained by each extraction are shown at Table 1. Lower Ct values are considered more desirable since they are associated with larger amounts of amplifiable DNA. The Modified Blood Kit method gave the lowest mean Ct value, which was statistically better only when compared to the standard Phenol–Chloroform protocol ($P = 0.011$). Some samples extracted by the standard Phenol–Chloroform protocol had unexpectedly high Ct values although their gel electrophoresis scores were high as well. No amplification was detected in the non-template controls.

3.4. Time, labor and cost analysis

Comparison of the six protocols for labor intensity, throughput time and material cost per sample is reported in Table 2. The most rapid extraction methods were the two commercial kits while the most time-consuming was the Phenol–Chloroform protocol. The in-house developed protocol was the cheapest but not as easy to apply as the commercial kits. The Modified Blood and Tissue Kit methods, which yielded DNA of highest quality and quantity

Table 1
Comparison of six extraction methods using two spectrophotometer measurements (260/280 and 260/230 nm), an electrophoresis score and real-time PCR results (Ct value); standard errors of marginal means are indicated in parentheses.

Extraction method	260/280 nm[1]	260/230 nm[1]	Electrophoresis score[1]	Ct value[2]
Nucleospin® Blood Kit	1.73 (0.03)^{a}	1.67 (0.14)^{a}	45.0 (10.1)^{b}	24.3 (0.40)^{a,b}
Nucleospin® Tissue Kit	1.71 (0.03)^{a}	1.67 (0.14)^{a}	48.0 (9.6)^{a,b}	23.7 (0.41)^{a,b}
Modified Blood Kit	1.80 (0.03)^{a}	2.10 (0.14)^{a,b}	89.1 (10.0)^{a}	22.8 (0.46)^{a}
Modified Tissue Kit	1.74 (0.04)^{a}	1.85 (0.14)^{a}	54.5 (10.1)^{a,b}	23.4 (0.41)^{a,b}
Phenol–Chloroform protocol	1.55 (0.03)^{b}	2.71 (0.14)^{b}	28.5 (10.52)^{b}	25.0 (0.39)^{b}
In-house protocol	1.43 (0.04)^{b}	1.43 (0.18)^{a}	11.5 (12.5)^{b}	24.1 (0.49)^{a,b}

[1]Higher values are desirable.
[2]Lower values are desirable.
^{a,b}Comparison of values within each column; values with the same superscript are not statistically different ($P > 0.05$) from each other but they differ significantly ($P < 0.05$) from values with different superscript.

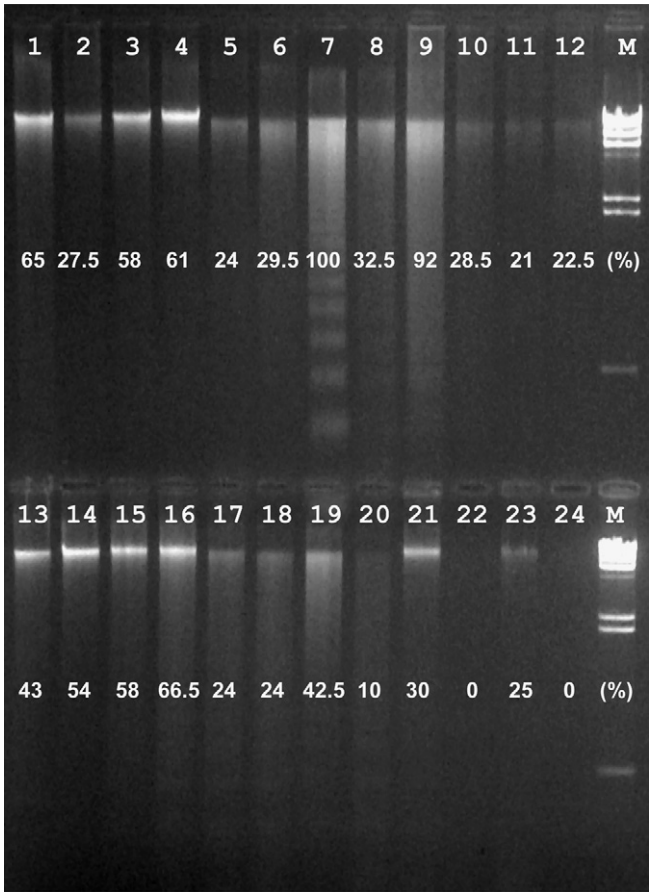

Fig. 1. Representative results from gel electrophoresis analysis of genomic DNA from four different ovine milk samples extracted by six methods. Relative amounts of chromosomal DNA measured by Gel-Pro Analyzer™ software are indicated below each genomic DNA band. Modified Tissue Kit (lanes 1–4), Nucleospin® Tissue Kit (lanes 5–8), in-house protocol (lanes 9–12), Modified Blood Kit (lanes 13–16), Nucleospin® Blood Kit (lanes 17–20), Phenol–Chloroform protocol (lanes 21–24); M = molecular weight marker λ DNA/Hind III digest.

amongst the six methods, had intermediate time requirements and were only slightly more expensive than the others.

4. Discussion

Milk sampling, compared to blood sampling, is easier and less stressful to animals because it does not require capture, handling and venipuncture. In the early nineties, classical Phenol–Chloroform and Chelex resin protocols were used for the extraction of genomic DNA from bovine and caprine milk [5, 7]. Nevertheless, none of these methods was found suitable for large scale genotyping projects because consistent quantifiable amounts of good quality DNA were not obtainable from milk somatic cells. In recent years, new extraction techniques have been adopted with some modifications for milk somatic cells. A rapid simple salting-out method for DNA extraction from caprine milk was proposed [1] but only 75% of tested samples were suitable as a substrate for PCR-RFLP genotyping. In another study, a solid phase absorption commercial kit (Wizard DNA clean up kit – Promega) was tested in ruminant's milk with reliable results [9]. In that case though, low milk volumes were used and overnight incubation of samples with proteinase K was required.

Because of the increasing usefulness of milk pooling experiments, there is a renewed interest in DNA extraction methods for milk somatic cells [3]. Application projects of bulk milk include

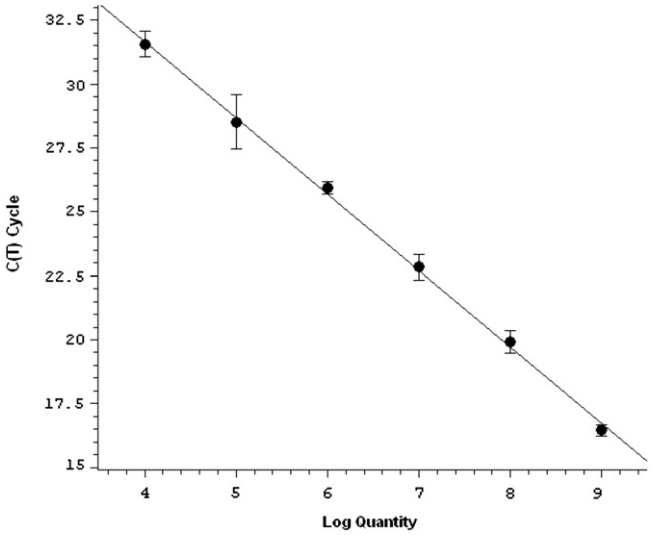

Fig. 2. Standard curve generated from a ten-fold dilution series of *PRNP* gene PCR amplicon. From left to right the curves represent DNA from 2×10^8 to 2000 copies per reaction done in 3 replicates. Ct values are plotted against copy number to construct the standard curve, $y = -3.14x + 43.63$, $r^2 = -0.999$, Efficiency = 108%.

population screening for disease susceptibility, monitoring and eradicating genetic diseases, and detection of milk from other species in dairy products. In addition, bulk milk samples can be used for clinical diagnosis of infectious pathogens present in the milk somatic cells such as retroviruses. For example, small ruminant lentivirus proviral DNA can be detected in macrophages and epithelial cells in ovine milk [15]. However, bulk milk applications require large volumes of samples and this may affect the purity and quality of the extracted DNA.

In the present study, six different DNA extraction protocols were evaluated using large volumes of ovine milk samples. The evaluation was based on spectrophotometer measurements, gel electrophoresis scorings and real-time PCR Ct values. The two spectrophotometer measurements (ratios 260/280 and 260/230 nm) are frequently used for the evaluation of DNA purity [16–18]. A 260/280 nm ratio of approximately 1.8 is considered standard for pure DNA. A 260/230 nm ratio lower than 2 is indicative of contamination with guanidine carried over during the washing steps of the silica columns. According to these ratios, only Modified Blood Kit, followed by the Modified Tissue Kit, extracted pure DNA in the present study. The 260/230 nm ratio differences between the modified and the commercial kits should be attributed to the additional washing step of the silica column.

Gel electrophoresis is a commonly used criterion for estimating DNA quantity and integrity. Fat, proteinases and high concentrations of Ca^{+2} have been proposed as potential inhibitors of PCR [11]. The two modifications introduced to the commercial kits in the present study consisted of sample pretreatment to eliminate these

Table 2
Assessment of cost and duration of six DNA extraction methods; cost includes only material.

Extraction method	Process duration	Cost per sample (€)
Nucleospin® Blood Kit	1.15 h	2.7
Nucleospin® Tissue Kit	1.15 h	2.9
Modified Blood Kit	2.15 h	2.9
Modified Tissue Kit	2 .15 h	3.1
Phenol–Chloroform protocol	1.5 days	2.0
In-house protocol	2.15 h	1.5

PCR inhibitors and thus increase DNA yield. The first modification was the addition of a TE-EDTA solution to dissolve milk casein. The latter is an undesirable substance for DNA extraction because it interferes with the digestion by proteinase K, of cellular enzymes which are responsible for degradation of the DNA [8]. Milk casein contains calcium and EDTA chelates calcium. Thus, calcium is removed from casein and casein is dissolved. The second modification was the addition of chloroform after proteinase K incubation. Chloroform removed milk lipids, which are potential PCR inhibitors, and improved the quality of DNA. According to gel electrophoresis results, the highest quantity of DNA was obtained after the incorporation of these modifications whereas the standard Phenol–Chloroform and the in-house protocols yielded lower quantity of DNA. The low level of DNA recovery using the standard Phenol–Chloroform method suggested that DNA was more susceptible to accidental loss during this procedure when compared to the other techniques. The low quantity and the slightly degraded DNA extracted by the in-house developed protocol could be attributed to the lack of proteinase K treatment.

In recent years, real-time PCR has become a reliable tool for assessing DNA quantity and quality for downstream applications [19–23]. The reason is that Ct values directly reflect the utility of the extracted sample for molecular analysis. According to real-time PCR measurements, the two modified kits studied here were the most sufficient DNA extraction methods. Although the Ct value differences between the modified and commercial kits did not attain statistical significance, they are considered substantial, since one cycle difference in the Ct value is associated with double quantity of DNA (PCR efficiency $\geq$ 100%). Some samples extracted by the standard Phenol–Chloroform protocol showed high gel scores, indicating high DNA quantities. However, based on the poor real-time PCR performance (high Ct values), the presence of inhibitors in the extracted DNA was revealed.

The six DNA extraction methods were also compared for labor intensity, throughput time and cost per sample. The two commercial kits were the fastest and simplest to perform. However, the modified protocols were only associated with an increased cost of 0.20 euros per sample and a longer throughput time of one hour, compared to the commercial kits. The in-house developed protocol was the cheapest but required additional labor for the construction of the homemade silica columns and preparation of the lysis and washing buffers. The standard Phenol–Chloroform protocol was the most time-consuming and technically difficult to perform, and required the use of highly toxic phenol.

Based on the overall evaluation by all criteria, the standard Phenol–Chloroform and the in-house developed protocol didn't perform as well as the other four protocols. The two commercial kits and the two modified versions extracted good quality of amplifiable genomic DNA. Nevertheless, the Modified Blood and Tissue Kits gave less degraded DNA and most desirable values in all criteria, compared to the respective not modified Nucleospin® Blood and Nucleospin® Tissue kits. It is expected that these modifications would be advantageous in cases where higher volumes of milk samples, containing higher quantities of lipids and PCR inhibitors, have to be processed. On the other hand, commercial kits without modifications are less time-consuming procedures. Therefore, the method of choice for DNA extraction from ovine milk samples depends on the requirements of sample volume and downstream applications following the extraction.

The method that overall performed best in the present study (Modified Blood Kit) was further tested in order to assess its large scale applicability. The method was used to extract genomic DNA from 1000 individual ovine milk samples and 50 bulk milk samples. The extracted DNA was examined for its suitability as a template for PCR-RFLP genotyping of the *PRNP* gene [24]. All samples tested gave amplifiable DNA showing no signs of PCR inhibition. Gel electrophoresis of PCR products from individual and bulk milk samples revealed specific clear bands with adequate amplicon quantity, suitable for RFLP analysis. Amplifiable DNA was also obtained from animals with sub-clinical mastitis (38 samples) indicating the method's ability to perform well with problematic samples that contain higher concentrations of total proteins, fat and Ca^{+2} [25,26].

5. Conclusions

This study identified an improved DNA extraction method, which can be used reliably for large scale genotyping based on individual or pooled ovine milk samples. Results may be generalized to other dairy species and/or downstream applications such as selective genotyping for marker-QTL association studies, monitoring and eradicating genetic diseases, and population screening for disease susceptibility.

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