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Comparison of Rapid DNA Extraction Techniques for Conventional PCR-RFLP Analysis from Mammalian Whole Blood Cells

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Abstract: Clean, high molecular weight DNA is pre-requisite for DNA markers. The amount and quality of DNA is a crucial point for all further analysis. A unique advantage of these PCR techniques is the rapid DNA analysis of many animal samples using small quantities of DNA. Thus, a simple and rapid DNA extraction method is needed for studies such as genetic analysis that require large populations. Several methods for minimizing the DNA extraction steps have been reported but they require a large amount of animal tissues. In addition, bleeding and management of sampling and storage of the blood sample in freezers is often difficult due to space constraints. To overcome these problems, some techniques developed a DNA extraction method using the milk or hair root or semen. Researchers compared 4 methods of rapid DNA extraction with isolations of mammalian whole blood samples. DNA extraction methods included boiling, salting out, phenol-chloroform and silica gel procedures. Spectrophotometry and gel monitoring evaluated the DNA yield and purity for the 4 methods. The silica gel and phenol-chloroform methods yielded significantly purity and higher concentration of extracted DNA compared with other DNA extraction methods.

Key words: DNA extraction, DNA fingerprint, PCR, RFLP, target gene, Iran

INTRODUCTION

Generally, DNA quality is indicated by the absence of contaminating RNA, protein, lipids and other cellular constituents that may interfere with restriction enzymes, ligases and thermostable DNA polymerases (Berthomieu and Meyer, 1991; Williams *et al*., 1990). The large size of mammalian genomic DNA also requires that the isolation method be gentle enough to minimize mechanical shear stress which would fragment the large genomic DNA during the course of purification (Jeanpierre, 1987; Merante *et al*., 1999; Reymond, 1987). Commonly used procedures employ a buffer containing one or several detergents for example, SDS, NP-40 or Triton X-100.

This detergent lyses cells and assist in the removal of proteins from the DNA. More through deproteinization is achieved by the use of proteinase K in the lysis buffer. This enzyme is active in the presence of SDS and remains functional at elevated temperature (56-65). Under these conditions denatured or partially denatured proteins are more easily digested by proteinase K. In contrast, most other enzymes (e.g., DNases) are denatured under these conditions. Pure DNA can simply be obtained by a single precipitation with isopropanol following proteinase K treatments. Residual protein and contaminating lipids can be effectively removed by extraction with phenol and chloroform.

Treating the sample with DNase-free. RNase followed by a chloroform extraction to remove the RNase can easily degrade contaminating RNA. By the addition of B-mercaptoethanol to a final concentration of 0.2% and by the addition of hydroxyauinolinto a final concentration of 0.1%. The shelf life of phenol can be extended by storage at hydroxyquinoline also imparts a light orange color to the phenol, enabling easier phase recognition during extraction. DNases are dependent on Mg^{2+} and Ca^{2+} for activity. EDTA (Ca^{2+} least 2 mm) included in the DNA extraction buffer chalets these cations and thereby prevents the degradation and random nicking of high molecular weight DNA by Dnases.

Washing the cells with Phosphate-Buffered Saline (PBS) without Mg^{2+} and Ca^{2+} minimizes the activities of DNases for the same reason. Isoamyl alcohol is added to

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the chloroform to prevent foaming. In this study, comparison 4 rapid DNA extraction techniques from mammalian whole blood are described (Berthomieu and Meyer, 1991; Boom *et al*., 1990; Chikuni *et al*., 1997; Jeanpierre, 1987).

MATERIALS AND METHODS

Blood samples were randomly collected from 10 Iranian Sarabi cattle (*Bos taurus*). Authorized veterinarian collected blood samples for DNA genotyping from jugular. Blood was collected on K_2EDTA and stored at -20°C for few weeks or -70°C up to several months.

Boiling DNA extraction: This protocol was carried out according to the procedure described by Jeanpierre (1987). About 100 μL bloods collected into 1.5 mL of microcentrifuge tube. To the above tube 500 μL of lysis buffer (Sucrose: 0.32 M, Tris-HCl: 10 mM, $MgCl_2$: 5 mM, Triton×100: 1%) was added, vortex and centrifuged (12000×g) for 1 min and washing was repeated once more. The above pellet was suspended with 100 μL (NaOH 50 mM) and was incubated at 100°C for 20 min then adds 20 μL Tris-Cl (pH = 7.5) for concentration of DNA. Then inverts and centrifuged at 20 sec and protein was precipitated and upper layer transmitted for other tube.

Salting out DNA extraction: This protocol was carried out according to the procedure described by Sambrook *et al*. (2000). About 100 μL bloods collected into 1.5 mL of microcentrifuge tube. About 500 μL of lysis buffer (Sucrose: 0.32 M, Tris-Hcl: 10 mM, $MgCl_2$: 5 mM,Triton×100: 1%) was added, vortex and centrifuged (12000×g) for 1 min and washing was repeated once more then add 250 μL of P-buffer (100 mM EDTA, 10 mM Tris-Cl, 20 mM NaCl, 1% SDS). And the mix was incubated at 55°C for 1 h. Following proteinase K digestion, 100 μL of 5 M NaCl added to the samples. This mixture was vortexed vigorously for 15 sec and centrifuged for 5 min.

Supernatant was transferred to new eppendorf tube and the DNA precipitated using 1 mL of ice-cold 100% ethanol. The precipitated mixture was kept on ice or at -21°C for 5 min prior to centrifugation at 4°C. The DNA was washed once with 70% ethanol, dried and suspended in 20 μL of distilled H_2O.

Phenol-chloroform DNA extraction: This protocol was carried out according to the procedure described by John *et al*. (1991). About 100 mL blood collected into 1.5 mL of microcentrifuge tube. About 500 μL of lysis buffer (Sucrose: 0.32 M, Tris-Hcl: 10 mM, $MgCl_2$: 5 mM,Triton×100: 1%) was added, vortex and centrifuged (12000×g) for 1 min and washing was repeated once more then 250 μL of P-buffer (100 mM EDTA, 10 mM Tris-Cl, 20 mM NaCl, 1% SDS). Following proteinase K digestion, samples were extracted with saturated phenol: chloroform (1:1) followed by 2 extractions with chloroform. Transfer upper phase to a clean tube. The DNA was washed once with 70% ethanol, The DNA was precipitated with 2 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and precipitated by centrifugation (15 min at 12,000×g). The DNA was washed once with 70% ethanol dried and suspended in 20 μL of distilled H_2O.

Silica gel DNA extraction: This protocol was carried out according to the procedure described by Boom *et al*. (1990). About 100 mL blood collected into 1.5 mL of microcentrifuge tube. About 400 μL of lysis reagent (Guanidin Thiocyanate: 20 mM, EDTA: 20 mM, Tris-Cl: 10 mM,TritonX_{100}: 40 g L^{-1}, DTT: 10 g L^{-1}) was added, vortex once more then was heated in 65°C for 5 min, added 20 μL of Nucleose solution (silica gel: 4 g, Guanidin solution: 100 mL) into tube and was mixed for 10 min and centrifugation (10 sec at 12,000×g) and then was added 200 μL from Lysis reagent again. About 400 μL Saline Buffer (1 M NaCl, 10 mM Tris-Cl, 1 M KCl, 20 mM EDTA) was added and was repeated once more then and centrifugation (10 sec at 5,000×g).

The DNA was precipitated with 45-55 μL from extra gene (Ion Exchange Resin (chelex): 10%, Orange G color: 0.02%, TritonX_{100}: 0.01%) and was incubated in 65°C at 3-5 min centrifugation (3 min at 1000×g) protein was precipitated and upper layer transmitted for other tube.

Calculation of DNA concentration: DNA concentrations were calculated by spectrophotometry by taking the Optical Density (OD) at a wavelength of 260 nm. The concentrations of extracted DNA in each sample was determined with the formula μg mL^{-1} of DNA = A260×50 where 50 is a double-stranded DNA factor. The DNA was estimated from the ratio of absorbance at 260/280 nm with the mean of ten spectrophotometric readings.

Statistical analysis: Mean (±standard deviation) extracted DNA concentrations and sample purities (A260: 280) for the 4 methods were compared analysis of variation samples with purity value <1.6 were considered to potentially have DNA value less than the calculated value and the Duncan test was used for post hoc identification of the methods with highest DNA yield by SPSS software.

PCR-RFLP: Amplified region is located in the intron between 2 exons of leptin. The genomic. The sequence of

the forward and reverse primers, respectively were: Forward primer: 5'-TGGAGTGGCTTGTTATTTTCTTCT-3', Reverse primer 3'-GTCCCCGCTTCTGGCTACCTAACT-3' PCR condition were 2.25 mm $MgCl_2$, 200 μm dNTP, 1 μm of each primer, 50-100 ng of genomic DNA and 0.2 Taq DNA polymerase.

The first cycle of PCR was 3 min at 94°C, 1 min at 55°C and 1 min at 72°C followed by 34 cycle of 45 sec at 94°C 1 min at 55 °C, 1 min at 72°C and ending with a 10 min extension phase at 72°C PCR product for each sample was digested with 10 unit of Sau3AI at 37°C for overnight gels were stained with 0.5 mg L^{-1} ethidium bromide for 20 min. Distained and photographed.

RESULTS AND DISCUSSION

This study demonstrated that silica gel and phenol-chloroform methods are effective technique for extracting DNA from whole blood samples. Disadvantages to boiling method include the degradable DNA with higher than protein contamination (Fig. 1-3). Silica gel method resulted in significantly greater DNA yield and less protein contamination compared with other

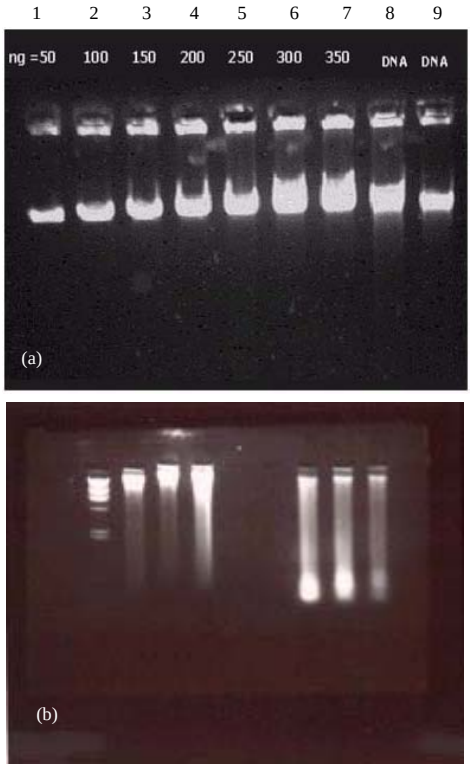

Fig. 1: Comparison of DNA extraction methods. Part (a) indicated silica gel methods and part (b) indicated phenol-chloroform, salting out and boiling methods, respectively

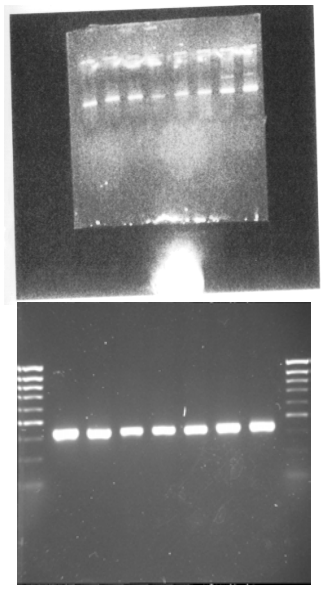

Fig. 2: The quality of PCR products that were extracted with different DNA extracted by different methods. PCR products with boiling extraction method (a) and PCR products with Silica gel extraction method (b)

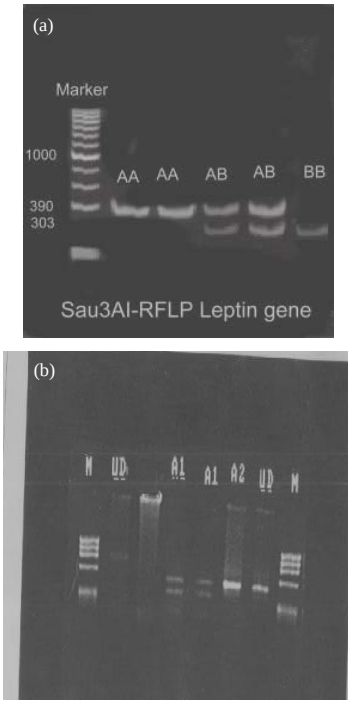

Fig. 3: The quality of digested PCR products that were extracted with different DNA extracted by different methods. RFLP fragments with boiling extraction method (a) and RFLP fragments with Silica gel extraction method (b)

Table 1: DNA concentration (μg mL^{-1}) and purity of DNA by 4 different methods

DNA extraction methods	Purity	Concentration
Boiling	0.88±0.16**	167±13*
Salting out	1.13±0.06**	214±15*
Phenol-chloroform	2.10±0.08**	218±9*
Silica gel	1.70±0.08**	346±22*

All methods were performed in replication of 10. Values given are mean±SD; *, **Indicate that mean is significantly different ($p<0.05$), ($p<0.01$), respectively

methods and also silica gel method is relatively simple and safe procedure that less time consuming. Although, all of methods have been shown to yield appropriately sized DNA produces for amplification fragments by PCR techniques. Mean±SD DNA concentrations and purities each DNA extraction methods are shown in Table 1.

CONCLUSION

In the study, all 4 methods yielded extraction products were successfully amplified by PCR for 422 bp from *leptin* gene subjectively, the PCR products were most abundant for sample extracted by silica gel and phenol-chloroform techniques.

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