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Original Research

A comparative evaluation of four DNA extraction protocols from whole blood sample

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Abstract: All organisms have Deoxyribonucleic acid (DNA) within their cells. DNA is a complex molecule that contains all of the information necessary to build and maintain an organism. DNA extraction is one of the most basic and essential techniques in the study of DNA that allow huge advances in molecular biology, biotechnology and bioinformatics laboratories. Whole blood samples are one of the main sources used to obtain DNA and there are many different protocols available in this issue. In current research, compared four DNA extraction protocols from blood samples; include modified phenol-chloroform protocol, two salting-out and enzyme free method and from commercial kit. The extracted DNAs by these protocols were analyzed according to their time demands, quality and quantity, toxicity and functionality in PCR method. Also the quality and quantity of the extracted DNA were surveyed by gel electrophoresis and Nanodrop spectrophotometry methods. It was observed that there are not significantly differences between these methods about DNA Purity (A260/A280), but the DNA yield (ng DNA/µl) of phenol/chloroform method was higher than other methods. In addition, phenol/chloroform was the most toxic method and it takes more time than other methods. Roche diagnostics GmbH kit was the most expensive among the four methods but the least extraction time was required and it was the safest method.

Key words: DNA extraction, Whole blood, Phenol/chloroform method, Salting out.

Introduction

All living things have Deoxyribonucleic acid (DNA) within their cells. DNA is a complex molecule that contains all of the information necessary to build and maintain an organism. DNA is made of nucleotides that made of three parts: a phosphate group, a sugar group and one of four types of nitrogen bases [adenine (A), thymine (T), guanine (G) and cytosine (C)]. To form a strand of DNA, nucleotides are linked into chains, with the phosphate and sugar groups alternating (1, 2, 3).

The DNA extraction method, as a common and routine work in the diagnostic clinical, forensic and molecular laboratories, is the main step that affecting technology of molecular DNA. Also, pure extracted DNA from animal and plant biological samples is needed for various molecular techniques such as Polymerase Chain Reaction (PCR) and PCR based methods, to detect mutation, genotyping, restriction enzyme analysis (4, 5). DNA extraction method allows scientists to identify genetic disorders or diseases in medical sciences, to detect bio threat agents in environmental and forensic samples, to study how human DNA may be destroyed by certain types of electromagnetic waves at certain frequencies (6, 7). In addition, DNA extraction is useful to create genetically engineered organisms that can produce beneficial products such as insulin, antibiotics, and hormones, to produce DNA fingerprints of individuals, need for gene therapy and used in agriculture to develop transgenic (8, 9, 10). Genetic characterization of livestock and their diversity were studied using molecular genetics tools. At present, an array of molecular techniques is available to detect diversity at DNA level for the overall genetic characterization and breed differentiation.

For the first time, DNA extraction was performed in 1869 by Friedrich Miescher (11). Today, there are many different available protocols for DNA extraction. Most DNA extraction protocols consist of two parts: 1: lyse the cells gently and solubilize the DNA. 2: enzymatic or chemical methods to remove contaminating proteins, RNA, or macromolecules. But these protocols are different in cost of consumables, laboratory facilities, the quality and quantity of obtained DNA, toxicity and time demands (12, 13). DNA extraction methods from human whole blood samples are salting out methods, organic solvent/chaotropes methods (include phenol–chloroform method and its modifications), glass milk/silica resin methods, anion exchange methods and magnetic beads methods. In addition some commercial kits are available for this purpose (8). Commercial kits for DNA extraction are simple, rapid and safe but these kits are very expensive with low yield, so is not suitable for DNA extraction in large scale (14).

Phenol-chloroform method that usually used has advantages and disadvantages. Advantages are yields relatively pure with high molecular weight DNA, DNA is double stranded and good for RFLP (restriction fragment length polymorphism). But this method is time

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consuming, requires sample to be transferred to multiple tubes (increases risk of contamination) and Involves use of toxic chemicals (15).

The salting out methods for DNA Isolation involve the addition of salts then precipitation of DNA from the protein in a subsequent step with isopropanol or ethanol and pelleted by spinning with a centrifuge and the supernatant removed. Usually, the salting out methods are laborious and time consuming but this method is safer than phenol- chloroform method and cheaper than commercial kits (13, 8, 16).

In this research, four DNA extraction protocols were compared from blood samples based on quality and quantity of extracted DNA. These DNA isolation procedures include one modified phenol-chloroform protocol (15), two salting-out and enzyme free method (17, 18) and one commercial kit (Roche diagnostics GmbH kit).

Materials and Methods

DNA extraction methods

The blood sample was collected from healthy volunteer, samples were collected in tubes and samples were treated by Ethylene diamine tetra acetic acid (EDTA). Blood samples were stored at -70^{0}C till further use. DNA was extracted from the samples with the following four protocols. All required materials were purchased from the Merk Company (Germany).
Isolation of DNA from blood involves four major steps

1. Lysis of cells using a detergent such as SDS
2. Digestion of proteins, released from cell lysis, with proteinase K.
3. Remove the proteins with phenol
4. Precipitation of DNA with alcohol

Method 1: (Traditional phenol/chloroform method)

In this protocol, DNA was extracted as described by Sambrook *et al*., 2001 (15). In this method Tris base, Triton X-100, $MgCl_2$, Sucrose, NaCl, EDTA, Sodium dodecyl sulphate (SDS), Sodium acetate, Chloroform, Isoamyl alcohol, Ethanol and Proteinase k were used for DNA extraction. Buffers were prepared according to the protocol (15). After that DNA extracted by this protocol:

i. 1 ml of distilled water and 1 ml of blood are transferred into 2 ml micro-tube and mix. Then centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded. This step was repeated 2 or 3 times.
ii. 1 ml of Lysis I buffer were added to the pellet and vortex the tube. Then tube centrifuged at 4000 rpm for 5 minutes.
iii. The supernatant was discarded. 800 µl of Lysis II buffer and 100 µl of SDS 10% and 25 µl of Proteinase k were added to the pellet and tube incubated in 56°C for 2 hours.
iv. Then 400 µl of phenol were added to the tubes and shaked the tube. After that tube centrifuged at 4000 rpm for 5 minutes.
v. The upper phases transferred in to a new tube, then 400 µl of Chloroform- Isoamyl alcohol (24:1) and 50 µl of Sodium acetate were added to the tubes, after that shaking the tubes and centrifuged at 4000 rpm for 5 minutes.
vi. Then we added cold absolute Ethanol three times the resulting solution of previous stage and the tubes were shaked slightly and centrifuged at 3000 rpm for 3 minutes.
vii. The supernatant was discarded and 1 ml of ethanol 70% was added and centrifuged at 3000 rpm for 3 minutes.
viii. The supernatant was discarded and DNA air-dried. Then 100 µl of ddH2O were added to the pellet and stored on -20°C.

Method 2: (combination of salting-out and boiling methods)

In this protocol, DNA was extracted as described by Moradi *et al*., 2014 (17). In this method, NaCl, Tris base, Triton X-100, Na2EDTA, $MgCl_2$, Sucrose and Chloroform were used for DNA extraction. Buffers were prepared according to the protocol (17). After that, DNA extracted by this protocol:

i. 1 ml of distilled water and 1 ml of blood are transferred into 2 ml micro-tube and mix for 30 sec. Then centrifuged at 7000 rpm for 5 minutes. The supernatant was discarded. This step was repeated 2 times.
ii. 1 ml of buffer added to the pellet, then mixed and vortex. After that, the tubes centrifuged at 7000 rpm for 5 minutes and supernatant was discarded.
iii. 1 ml of buffer B and 100 µl of SDS 10% were added and vortex the tube. Then tube incubated in 65 °C for 15 minutes.
iv. Then tube was placed on ice for 3 min and 400 µl chloroform and 400 µl of saturated NaCl were added and shaken for 30 sec and centrifuged at 5000 rpm for 10 minutes at 4°C
v. The upper phases transferred in to a new tube and equal volume of cold ethanol was added and shaked the tube. Then the tubes centrifuged at 13000 rpm for 10 minutes at 4°C and supernatant was discarded.
vi. 1 ml of ethanol 70% was added and centrifuged at 10000 rpm for 10 minutes.
vii. The supernatant was discarded and DNA air-dried. Then 50-100 µl ddH2O was added and stored on -20°C.

Method 3: (Non enzymatic salting out method)

Nucleic acids were extracted as described by Suguna *et al*., 2014 (18). In this method, Tris-HCl, Potassium Chloride, $MgCl_2$, EDTA, NaCl, Sodium dodecyl sulphate (SDS), Isopropanol, Ethanol and Triton-X were used for DNA extraction. Buffers were prepared according to the protocol (18). After that DNA extracted by this protocol:

i. 900 µl of TKM 1 and 50 µl of 1x Triton-X were added to 300 µl of blood and incubated at 37°C for 5 minutes.
ii. Eppendorf were centrifuged at 8000 rpm for 3 minutes and the supernatant was discarded (This step was repeated 2-3 times).
iii. 300 µl of TKM 2 and 40 µl of 10% SDS were added to the eppendorf. Then mixed and incubated at 37°C for 5 minutes.
iv. 100 µl of 6M NaCl was added and vortexes. Then cells were centrifuged at 8000 rpm for 5 minutes.
v. The supernatant was transferred into a new tube and 300 µl of isopropanol was added to them. Then

tubes were centrifuged at 8000 rpm for 10 minutes.
vi. Supernatant was discarded, 70% ethanol was added and mixed slowly. Finally the tubes were centrifuged at 8000 rpm for 5 minutes.
vii. Supernatant was discarded and DNA air-dried. After that, 50 µl of TE buffer was added to DNA.

Method 4: (commercial kit)

High pure PCR template preparation kit (Roche). In this protocol DNA was extracted as described by Roche diagnostics GmbH. In this method binding buffer, proteinase K, isopropanol, inhibitor removal buffer, wash buffer and elution buffer were used for DNA extraction.

Quantity and quality assessment of the extracted DNA

Concentration and purity of samples were assayed by NanoDrop (Thermo) by measuring the 260/230 and 260/280 nm absorbance ratios (A260/230 and A260/280). The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of one corresponds to approximately 50µg/ml for double-stranded oligonucleotide. The standard conversions of spectrophotometric readings i.e., OD to nucleic acid concentration are given below:

Spectrophotometric Conversions

1A260 unit of double-stranded DNA =50µg/ml
1A260 unit of single-stranded DNA =33µg/ml

In order to examine the samples for the presence of fractures or DNA degradation, gel electrophoresis was carried out by loading 5 µl of extracted DNA on 1% agarose gel.

Gene amplification

PCR reaction was performed to check the intactness of the genomic DNA and to determine whether any inhibitory material was interfering with the polymerase enzyme and reaction. So, GPX (Glutathione peroxidase) gene was amplified and the PCR products electrophoresis on 2% agarose gel.

Statistical analysis

In this experiment, each method of extracting DNA consisted of 7 replications. The obtained data were analyzed with SAS (version 9.1) and Excel (2010) software. Mean values were compared according to Duncan test at ($p < 0.05$ and 0.01). The concentration of DNA samples were estimated using the following formula:
DNA concentration (ng/µl) = OD_{260} × (dilution factor) × 50 µg/ml.

Results

In this study, three methods and a commercially kit for isolating DNA from human blood samples were compared and analyzed for the following perspectives: yield and purity of DNA, time demands, toxicity, functionality in PCR method and absorption in 260, 280 and 230 nm.

The significant levels for measured traits, such as DNA purity, DNA yield and 260/230 absorption are shown in Table 1. The analysis of variance showed significant difference between the treatments (different protocols for DNA extraction) for DNA yield and A260/A230 absorption, but there is no significant difference between these methods about DNA Purity (A260/A280).

Yield, purity, A260/A230 and time demands of the extracted DNA by the four methods were listed in Table 2. The result shows that the Moradi *et al* (17) protocol as optimized salting out method and phenol/chloroform protocols gave good DNA yield (Table 2 and Fig. 1a).

Pure preparation of DNA has an OD 260/OD 280 values ranging from of 1.8 and 2.0 (19, 20). Observed that all of protocols gave good DNA purity and there are not significantly different between these methods about DNA Purity (Table 2 and Fig. 1b). High 260/280 purity ratios are not indicative of an issue, although low A260/A280 ratio may be caused by remaining phenol or other reagent by samples.

Absorbance at 230 nm is accepted as being the result of other impurity, expected 260/230 values are gene-

Table 1. Mean square for DNA quantity and quality of four DNA extraction methods.

		Mean square		
Source	**Df**	**A260/A230**	**DNA Purity (A260/A280)**	**DNA yield (ng DNA/µl)**
Method	3	3.373**	0.013^{ns}	233041.518**
Replication	6	0.081^{ns}	0.089^{ns}	21469.225^{ns}
Error	18	0.166	0.057	21530.63
CV%		25.35	12.82	81.99

ns: non-significant; **: Significant differences (P< 0.01), Df (Degree of freedom).

Table 2. Mean comparison of different DNA extraction methods based on DNA quantity and quality

Method	**A260/A230**	**DNA Purity (A260/A280)**	**DNA yield (ng /µl)**	**Time (hr)**
Method 1 Phenol/chloroform method	1.871^{a}	1.826^{a}	302.00^{a}	3hr, 30 min
Method 2 Moradi *et al*, (2014)	2.185^{a}	1.842^{a}	368.43^{a}	1hr, 40 min
Method 3 Suguna *et al* (2014)	0.600^{b}	1.928^{a}	21.40^{b}	1hr,10min
Method 4 Roche diagnostics GmbH kit	1.771^{a}	1.857^{a}	24.00^{b}	35min

Mean values within a column with same letter are not significantly different based at P < 0.05.

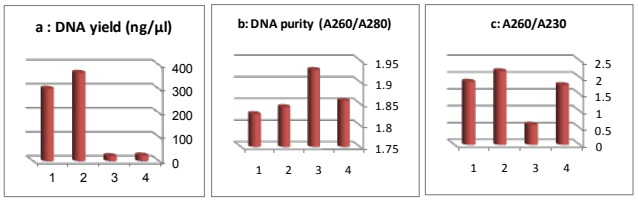

Figure 1. Comparison of methods for DNA yield (a), purity (b), and A260/A230 (c). Horizontal curves indicating the methods of DNA extraction, where, 1: phenol/chloroform method, 2: Moradi *et al*, (2014), 3: Suguna *et al* (2014) and 4: Roche diagnostics GmbH kit.

rally in the range of 2.0-2.2. Abnormal 260/230 values may demonstrate a problem with the sample or with the extraction method (21). Observed that Moradi *et al* (17) method was the only acceptable method about 260/230 absorption (1, 6). A low A260/A230 ratio in other methods may be the result of residual phenol or guanidine (often used in column based kit) in extracted DNA samples (Table 2 and Fig. 1c).

The quality of extracted DNA was observed on 1% gel electrophoresis (Fig. 2). Sharp molecular DNA band without smear and sign of degraded DNA were observed. Finally, compared that PCR amplification of GPX gene using DNA extracts by different protocols. Figure 3 shows a representative agarose gel containing a 314-bp fragment of glutathione peroxidase human enzyme that was PCR amplified from DNA that extracted by four different protocols. In PCR reaction 300 ng of extracted DNA was used as a template. The extracted DNA samples by all of these methods were suitable enough for PCR and PCR based methods.

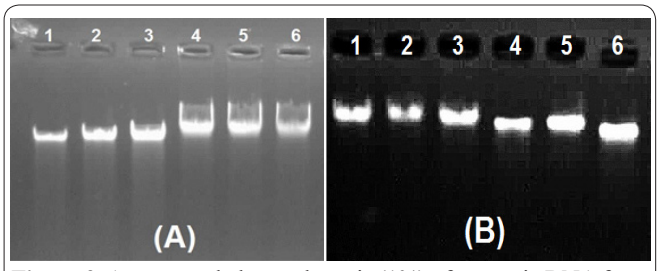

Figure 2. Agarose gel electrophoresis (1%) of genomic DNA from four methods of DNA extraction. [A: Lanes 1-3 (Roche diagnostics GmbH kit), 4-6 (phenol/chloroform method)]. [B: Lanes 1-3 (Suguna *et al* method), 4-6 (Moradi *et al* method)].

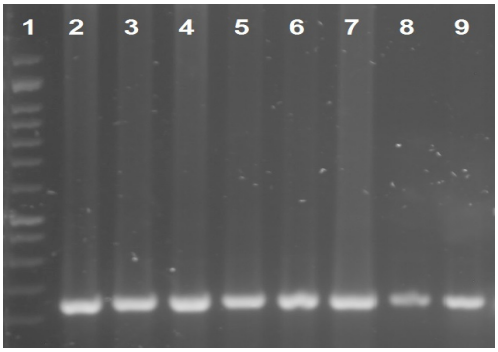

Figure 3. Agarose gel electrophoresis (2%) of GPX gene PCR product by using DNA templates extracted by the following methods; Lane 1: 100 bp DNA ladder, phenol/chloroform method (lanes 2-3), Moradi *et al* method (lanes 4-5), Suguna *et al* method (lanes 6-7), Roche diagnostics GmbH kit (lanes 8-9).

Discussion

Currently several modified protocols for DNA extraction from human blood are available, but it is important to choose a protocol that required low time and cost for DNA extraction (22-27).

Lee *et al*., 2010 used three kits for genomic DNA extraction and reported that there are no significantly different between these kits about DNA yield and all of them are low yielded that is agreement with this research (28).

In this study, Roche diagnostics GmbH kit was the most expensive among the four studied methods but the least extraction time was required and it was the safest method, but the DNA that extracted by this method is very pure with low yield (29).

In another study, Chacon-Cortes *et al*., 2012 (30) compared three different protocols from whole blood samples (a traditional salting out method, a modified salting out method and a commercial kit). They reported that there are no significantly different between these methods about quantity and quality of the obtained DNA, but there are very different about cost and time demanded.

In phenol/chloroform method Proteinase k were used for Lyse nuclear membrane and digest proteins that is need 2 hours for digest the proteins. So it required a long time for DNA extraction. In addition phenol/chloroform method is not safe due to the use of phenol and chloroform (toxic materials) to remove the proteins from DNA (15).

In this research, the DNA that extracted by suguna *et al* method (18), was low yield with low purity in comparison whit other method, but this method is safe, fast and economical. Moradi *et al* method (17) is safe, fast and easy on the pocket. The DNA that extracted by this method is very pure with high yield.

The results of this study suggest that phenol/chloroform and Moradi *et al* (17) methods were suitable for DNA extraction in large scale, but Moradi *et al*., 2014 method (17) was the best choice to extract total DNA from human blood samples because this method was found safe, simple, fast, Cheap enough and without the need for advanced laboratory equipment, with good purity and yield of DNA extracted. So this method is suitable for laboratories with time and cost friendly.

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