Applied Biological Research 21(2): 114-120; (2019)
DOI: 10.5958/0974-4517.2019.00017.X

EFFECT OF BLOOD STORAGE CONDITIONS ON THE INTEGRITY OF HUMAN DNA

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(Received 15 September, 2018; accepted 18 January, 2019)

ABSTRACT

A study was performed in blood samples, collected from 15 healthy individuals during July to December 2017, to evaluate the integrity of DNA by preserving blood samples at three temperatures i.e. 4, -20 and -80ºC as against the fresh sample (control) for an incubation period of 30 days. DNA was extracted by standard protocol of phenol-chloroform and salting out method. The effect of temperature on DNA quality and quantity was analyzed spectrophotometerically, followed by amplification of Cytochrome p450 1A1 (*CYP1A1)* gene. In this prognosis, the study revealed that higher purity was attained at 4ºC, yield and concentration at -20ºC on extraction by phenol-chloroform. On contrary, in salting out method maximal yield and concentration was acquired at 4ºC and purity at -20ºC. Comparison of methods showed that phenol-chloroform method gave best result. DNA obtained from preserved blood samples is appropriate for amplification and further studies.

Key words: *CYP1A1*, DNA analysis, PCR, phenol-chloroform, salting out

INTRODUCTION

New perspectives in the diagnosis, prognosis and therapeutic treatment have been elevated by recent molecular procedures (Malentacchi *et al*., 2015). DNA is vital for genomic studies related to the detection of an inherited disease, its carrier state and for advances in personalized medicine development. For meaningful conclusions, it is essential to analysis a large number of DNA samples and their maintenance conditions (Kim *et al*., 2011). Extraction and purification of DNA from various sources such as tissue samples, hair and whole blood is the preliminary step in various molecular techniques (Thomas *et al*., 1989). In biochemical, hematological and genetic studies as well as for the prevention of serious human disorders blood samples are pre-requisite. Large population studies and bio-banks have become more popular and are being recognized as a useful tool for genomic research. Rapid developments have been made in the extraction of samples for efficient separation of biomolecules in performing various assays. Whole blood is one of the available sources for procuring genomic DNA, meant for biochemical studies and human science research (Shams *et al*., 2011; Chacon-Cortes and Griffiths, 2012; Gong and Li, 2014; Huang *et al*., 2017). Various clinical studies involving gene polymorphism, DNA fingerprinting, gene

sequencing, pharmacogenomics, epidemiology, etc. employ a number of molecular techniques such as RFLP, RT/PCR, Sanger sequencing, microarrays, etc. wherein genomic DNA extraction from specimen serves as a basic key step (Qamar *et al*., 2017).

The scope of human genetic studies and technical advances in genotype has substantially increased with essentially unlimited sequence variation information. Now, it is possible to examine many polymorphism sequences in high outputs using less DNA per assay (Haque *et al*., 2003). To keep the DNA features intact, it is essential to maintain appropriate near-physiological conditions so as to prevent the degradation of molecules such as proteins, RNA, DNA, etc. which is likely to be affected under extreme conditions like high temperature, extreme pH, etc. and in presence of organic solvents (Schuurman *et al*., 2007). A large number of specimens are dispatched from peripheral centres to the centralized laboratories over a long distance and the delay of a few days or even a few hours may affect the sample integrity. Hence, there is need to preserve the sample so as to prevent any sample degradation. Preservation method, time period and extraction techniques are the pre-analytical variables which influence the quality and quantity of isolated DNA and affect the analytical performances. Improper storage and delay could lead to indefinite, inaccurate and unreliable result which unfavourably influences clinical decisions at the end (Malentacchi *et al*., 2015; Wu *et al*., 2017). Cytochrome p450 (CYPs) family encodes *CYP1A1* gene which belongs to phase-I detoxifying enzyme and catalyzes the conversion of environmental procarcinogens to reactive carcinogenic intermediate that produce carcinogenic effects (Shimada and Fujii-Kuriyama, 2004). Thus, the present study was aimed to evaluate the influence of storing blood samples at variable temperatures and durations on the integrity of DNA as well as to analyze its effects on PCR products by amplifying the *CYP1A1* gene.

MATERIALS AND METHODS

The study was conducted on the blood samples collected randomly from fifteen healthy individuals aged between 20 to 50 years within an identical ethnic population, irrespective of their sex. The study was performed in the Cytogenetic Laboratory, Department of Biochemistry, Pt. J.N.M. Medical College, Raipur (India) from July to December, 2017. Two millilitres of blood samples were collected by venipuncture method and immediately subject to storage at three temperatures *viz*., 4, -20 and -80ºC for a period of 30 days. Written informal consent was obtained from all the subjects as well as from the Ethical Committee of the University. DNA was extracted from the preserved blood samples by phenol-chloroform (Ghaheri *et al*., 2016) and salting out (Suguna *et al*., 2014) methods. The effect of different temperatures on purity, yield, concentration and amplification was studied by using fresh blood samples (taken as control).

Phenol-chloroform method: For extraction of DNA by this traditional method the standard protocol of Ghaheri *et al*. (2016) was followed. In a small tube, 1 mL distilled water and 1 mL blood was poured and centrifuged (REMI, Vasai India) at 4000 rpm for 10 min. The supernatant was discarded. In settled pellet, 1 mL lysis buffer I was added and vortexed to dissolve the pellet, followed by centrifugation at 4000 rpm for 5 min. Supernatant was discarded followed by the addition of 800 µL lysis buffer II, 100 µL 10% SDS (SRL, India) and 25 µL proteinase K (Hi-media, India) and incubated at 56°C for 2 h. After incubation, 400 µL phenol was mixed and centrifuged at 4000 rpm for 5 min. After centrifugation, the upper phase was transferred to a new tube and 400 µL chloroform-iso-amyl alcohol (24:1) and 50 µL sodium acetate added. After gentle shaking the tubes were centrifuged at 4000 rpm for 5 min. Then three equal volumes of absolute chilled ethanol were added through shaking tubes and centrifuged at 3000 rpm for 3 min. The supernatant was discarded and 1 mL 70% ethanol added. DNA pellet was air-dried and stored at respective temperature in 100 µL TE buffer.

Salting-out method: DNA extraction was performed as per the method of Suguna *et al.* (2014). For this, 900 µL low salt buffer, 50 µL triton-X (Sigma-Aldrich, USA) and 300 µL blood sample were mixed in a 2 mL centrifuge tube, followed by incubation at 37ºC for 5 min. for the lysis of RBC. The tubes were centrifuged at 8000 rpm and supernatant discarded. These steps were repeated 3 times till RBC was completely lysed and a white pellet obtained. In pellet, 300 µL high salt buffers and 40 µL 10% SDS were added, mixed properly and incubated at 37ºC for 5 min. After centrifugation, 100 µL 6 M NaCl was added and vortex leading to centrifugation at 8000 rpm for 5 min. The supernatant was then transferred to a new tube containing 300 µL isopropanol and precipitated by inverting the tube. Again, the tubes were centrifuged at 8000 rpm for 10 min. to settle down the pellet. The supernatant was discarded, 70% ethanol added and slowly mixed. The tubes were centrifuged at 8000 rpm for 5 min. to obtain pellets. Supernatant was discarded and tubes air-dried, followed by the addition of 50 µL TE buffer to dissolve the DNA and stored at respective temperatures.

The DNA purity and yield were estimated by taking absorbance ratios at 260/230 and 260/280 nm, respectively, in double beam UV-visible spectrophotometer (ELICO, India). Readings were noted at 260 nm to calculate DNA concentration in a sample (Ghaheri *et al*., 2016) using the formula:

$$\text{DNA concentration (ng } \mu L^{-1}) = OD_{260} \times \text{(dilution factor)} \times 50 \text{ µg } mL^{-1}$$

$$\text{DNA yield (µg)} = \text{DNA concentration} \times \text{total sample volume (mL)}$$

Analyzing the effect of preservation of blood samples, the amplification was carried out by subjecting the DNA to polymerase chain reaction (PCR) in a 2720 thermal cycler (Applied Biosystems, Singapore) as per Jain *et al*. (2017). The PCR primers (Sigma-Aldrich, India) for cytochrome p450 1A1 gene *CYP1A1* used in analysis were as follows:

F-5'ACTCACCCTGAACCCCATTC-3'
R-5'GGCCCCAACTACTCAGAGGCT-3'

The required standard temperature for initial denaturation was 95°C for 5 min., followed by 35 cycles of denaturation at 95°C for 40 sec., annealing at 61°C for 40 sec. and extension at 72°C for 40 sec. This was followed by the final extension for 7 min. at 72°C. The whole experiment was performed in triplicates. The data was statistically analyzed by using SPSS software 16.0. and data expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

The study comprised of random blood sampling from 15 healthy subjects which were stored at three temperatures to assess the effect of temperature on purity, yield and concentration of DNA. DNA extraction by phenol-chloroform method from stored blood samples exhibited maximum purity at 4°C (1.439 ± 0.165) [Table 1]. The ideal DNA purity i.e. 260/280 ratio is said to be 1.8-2.0. A lower ratio represents contamination of phenols and proteins. Thus, the purity at 4°C was highest, though all the samples contained contamination. The highest yield (159.63 ± 0.054 µg mL^{-1}) and concentration (1596.3 ± 782.9 ng μL^{-1}) was observed at -20°C.

DNA retrieved from stored blood by salting out method displayed maximum yield 150.46 ± 0.194 µg mL^{-1} and concentration 1504.06 ± 645.3 ng μL^{-1} at 4°C while highest purity 1.071 ± 0.4115 ng mL^{-1} was observed in the samples stored at -20°C (Table 1). We did not find any result at -80°C temperature preservation. If immediate extraction of DNA cannot be done then blood preservation and storage must be appropriate for further analysis. The results suggested that 4 and -20°C are suitable temperatures for storing the whole blood for a month and exhibit good yield, concentration with two methods of DNA extraction. The effects of different temperature on purity, yield and concentration have also been studied by various researchers. Cushwa and Medarno (1993)

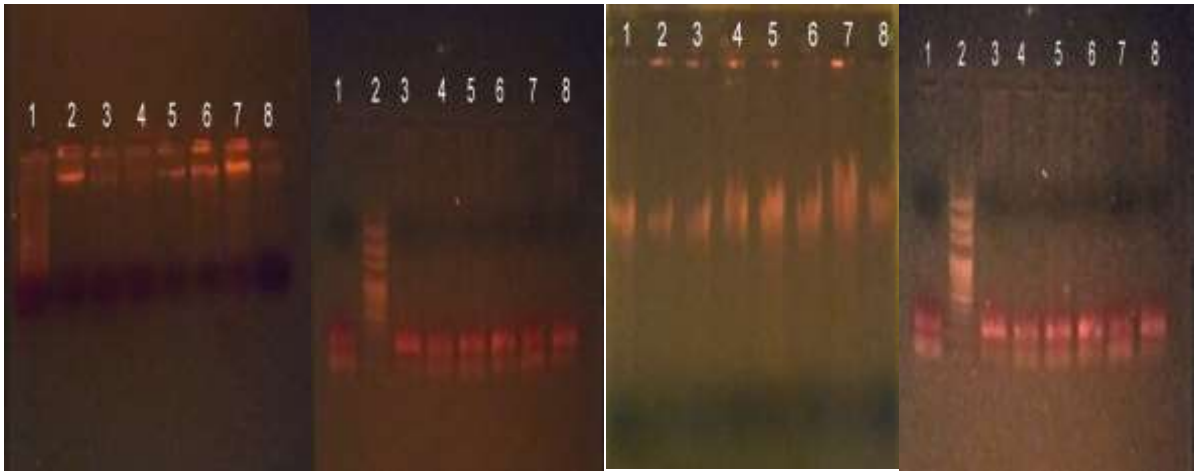
Table 1: Mean comparison of purity, yield and concentration of extracted DNA

Methods	Temperature (˚C)	Purity	Yield (µg mL^{-1})	Concentration (ng μL^{-1})
Phenol-chloroform	4	1. 439 ± 0.165	128.52 ± 0.030	1285.2 ± 698.8
	-20	1. 244 ± 0.182	159.63 ± 0.054	1596.3 ± 782.9
	-80	1. 265 ± 0.294	122.00 ± 0.165	1220.0 ± 853.2
Salting out	4	0. 959 ± 0.479	150.46 ± 0.194	1504.1 ± 645.3
	-20	1. 071 ± 0.411	139.80 ± 0.069	1398.1 ± 713.9
	-80	-	-	-

reported that DNA stored at 4°C gave good quality when isolated from a bovine blood sample and stored for a month. AIRokayan (2000) demonstrated that fine quantity, quality and intact DNA can be obtained from blood samples stored at 4°C and -20°C yield. Storage of blood samples at 4°C with different incubation periods can be an alternative source for further molecular analysis (Richardson *et al*., 2006; Bulla *et al*., 2016).

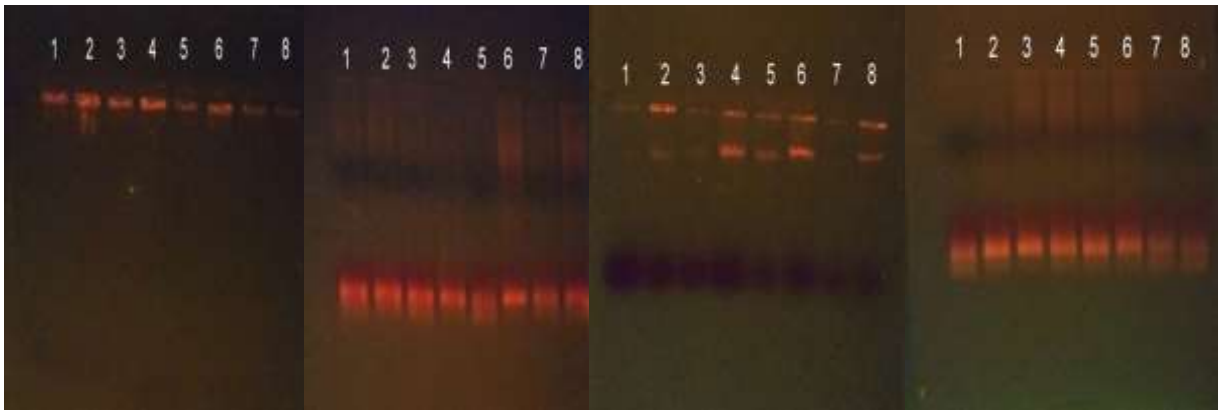
For comparison of two isolation procedures a fresh sample was used. The methods were quite efficient but phenol-chloroform method gave higher yield and concentration at -20°C and higher DNA purity at 4°C. The results of mean comparison between the methods were quite prolific. Chacon-Cortes and Griffiths (2012), Richardson *et al*. (2006) and Eslami *et al*. (2016) also reported excellent quality DNA in terms of higher purity, yield and concentration by salting out and phenol-chloroform methods. The proficient amount and purity of DNA were obtained by these methods (Jelassi *et al*., 2016). Salting out method is one of the simplest methods, less time consuming, cost-effective and gives a better concentration of DNA required for genotype studies with large sample size (Suguna *et al*., 2014). The phenol-chloroform method is routinely practiced to remove proteins and lipids from cellular digest. The phenol inactivates potentially infectious agents and permits storage and transportation, regardless of the viability of an appropriate environment, needed in extensive field testing aimed for clinically relevant genetic and infectious diseases. The use of phenol is considered a major drawback as it can cause severe burns while chloroform is a hepatotoxic and carcinogenic agent (Thomas *et al*., 1989; Albirno and Romanowski, 1994).

The extracted DNA and PCR products of fresh blood were examined by 1% agarose gel electrophoresis. A sharp orange DNA band was found under UV-transilluminator. PCR amplification of *CYP1A1* gene was done by using extracted DNA samples (Fig. 1). The result showed an agarose gel having a 194 bp band. The extracted DNA after preservation was found suitable for further



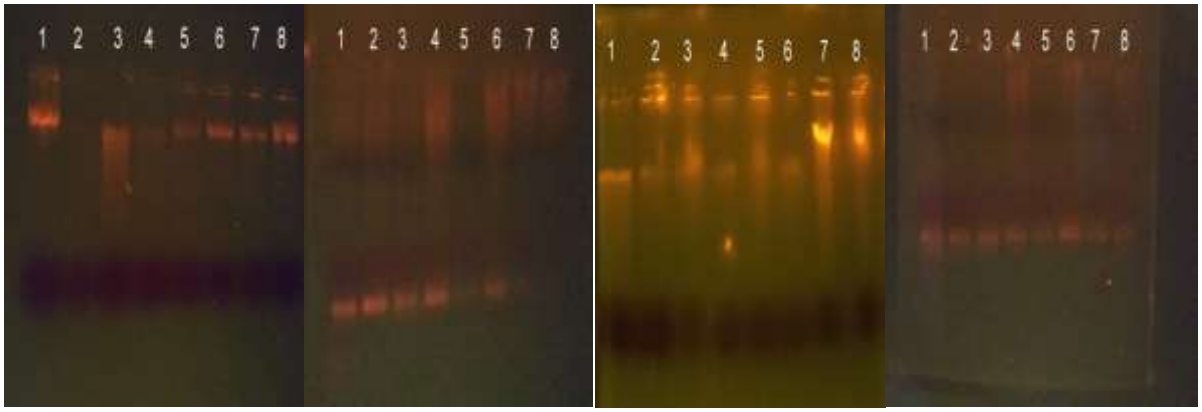
(a) (b) (c) (d)

Fig. 1: (a) Agarose gel electrophoresis of DNA extracted by phenol chloroform method; (b) PCR products of phenol chloroform method; (c) Agarose gel electrophoresis of DNA extracted by salting out method, and (d) PCR products of salting out method



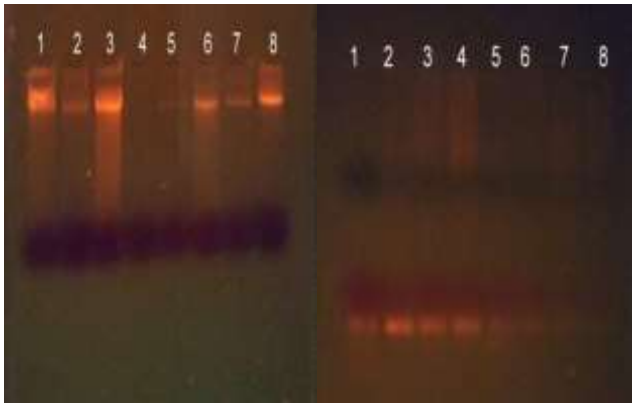
(a) (b) (c) (d)

Fig. 2: (a) Agarose gel electrophoresis of DNA extracted by phenol chloroform method stored at 4˚C; (b) PCR products of phenol chloroform method stored at 4˚C; (c) Agarose gel electrophoresis of DNA extracted by salting out method stored at 4˚C, and (d) PCR products of salting out method stored at 4˚C



(a) (b) (c) (d)

Fig. 3: (a) Agarose gel electrophoresis of DNA extracted by phenol chloroform method stored at -20˚C; (b) PCR products of phenol chloroform method stored at -20˚C; (c) Agarose gel electrophoresis of DNA extracted by salting out method stored at -20˚C, and (d) PCR products of salting out method stored at -20˚C



(a) (b)

Fig. 4: (a) Agarose gel electrophoresis of DNA extracted by phenol chloroform method stored at -80˚C; (b) PCR products of phenol chloroform method stored at -80˚C

analysis. PCR-based tests of blood samples are widely used for diagnostic and forensic analysis.

The assay studies demonstrated that both the methods gave upto the mark outputs. Keeping the blood samples for a month at three temperatures unveils that 4°C (Fig. 2) and -20°C (Fig. 3) are suitable temperatures for preservations with absorbance ratio of 1.8. This revealed that DNA is pure, hence sample storage at this temperature may be useful for further analytical studies. DNA extraction result at -80°C was only expressed in phenol-chloroform method (Fig. 4). This means that long time storage of blood at -80°C can hamper DNA yield, quality, concentration and purity.

Conclusions: Whole blood collection can be an important source of DNA, with the appropriate information of extraction methods, preservation time and temperature of the biological specimen. This study recommended that blood samples stored at 4°C and -20°C may be considered as a reliable and latent source for obtaining fine quality and quantity of DNA as well as PCR product for future genotyping studies and retrospective analysis.

Acknowledgements: The study was financially supported by Medical Biotechnology, Department of Biochemistry, Pt. J.N.M. Medical College, Raipur (C.G). We gratefully acknowledge their help in conducting the experiment, interpretation of results and preparation of manuscript.

Conflict of Interest: The authors declare no conflicts of interest.

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