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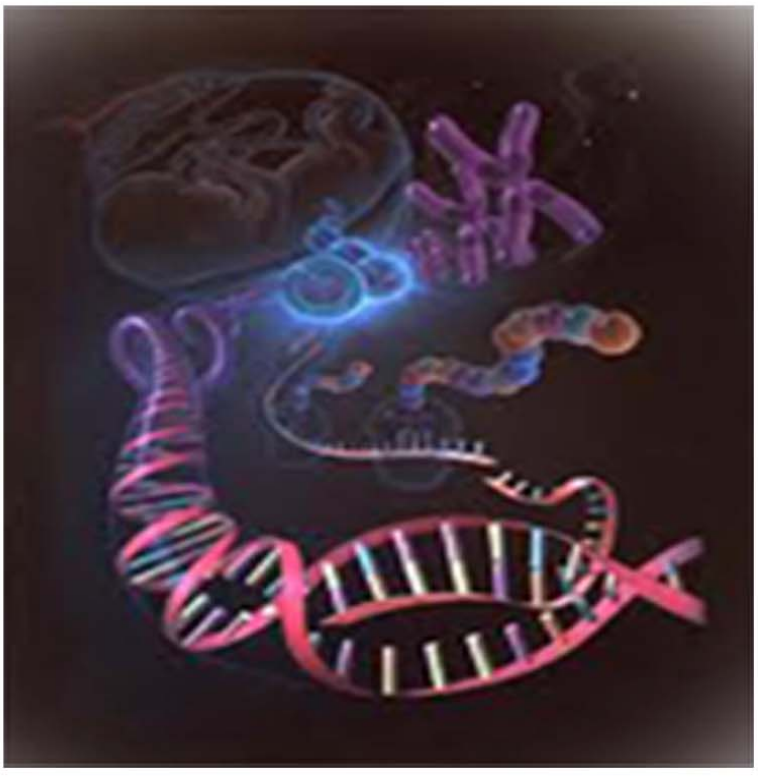

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Research Paper

EVALUATION OF SALT-OUT METHOD FOR THE ISOLATION OF DNA FROM WHOLE BLOOD: A PATHOLOGICAL APPROACH OF DNA BASED DIAGNOSIS

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A number of methods have been described for the isolation of genomic DNA from whole blood. However, these described methods are either unreliable or too expensive to be used routinely in small diagnostic laboratories of developing countries. We have evaluated the slightly modified salt-out method for DNA isolation so that it can be applied on whole blood samples using a three step procedure: (1) lysis of RBC; (2) removal of protein; and (3) precipitation of DNA. We compared the yield of DNA per mL of blood using our modified salt-out DNA extraction method, (40.8 ± 4.3 μg) with Phenol-chloroform extraction (38.5 ±7.3 μg) or QIAamp DNA Mini kits (35. 3± 5.4 μg). The described method allows simple, fast and cost efficient DNA extraction with qualitative parameters maintained and comparable to those of other extraction method described.

Keywords: RBC, Red blood cells, Ethylene diaminetetraacetic acid (EDTA), White blood cells (WBC), Polymerase chain reaction (PCR)

INTRODUCTION

The isolation of human genomic DNA is a fundamental step in molecular biology technique involved in genetic identity analysis. The isolation of DNA from biological samples can be tidious, time consuming and involve multiple steps for obtaining the high quality DNA. Many commercial and in-house methods exist for the extraction of mammalian DNA from peripheral blood. However, in our opinion none of the published methods meet the composite criteria for cost effective, safety, rapidity, reliability, yield, and, purity, to be used routinely in a small laboratory. Currently used techniques for DNA isolation lead to singnificant dilution of the sample or require the precipitaion, making the isolation of nucleic acids from small amount of biological sample difficult. A major goal of nucleic acid isolation is the removal of proteins. Seperation of nucleic acids from protein is accomplished due to their different chemical properties. In particular, the highly charged phosphate backbone makes the nucleic

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acids rather hydrophilic as compared to protein. Most nucleic acid isolation protocol involve a cell lysis step, enzymatic treatment, differential solubility and precipitaion. We have slightly modified the standard salt-out method to extract DNA from fresh as well as frozen blood. In this study we evaluated the quality and cost effectiveness of DNA isolation from peripheral blood by the Salt-out (Miller *et al*., 1988), Phenol-Chloroform extraction (Sambrook *et al*., 1989) and QIAamp DNA Blood Mini Kits methods.

MATERIAL AND METHOD

Collection of Blood

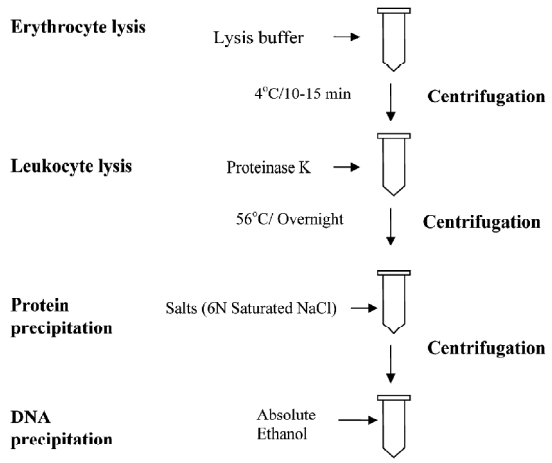
The fresh blood 2-5 mL was collected in EDTA (as anticoagulant) containing 15 mL polypropylene tube. Samples were preferably processed same day for the isolation of DNA or kept at –20 °C for future use. This study was conducted after the informed consent was obtained from all participants and the ethical approval of institute at (Infectious Diseases Research Laboratory), Institute of Medical Sciences, Banaras Hindu University, Varanasi and Kala-Azar Medical Research Centre (KMRC), Muzaffarpur, Bihar, India.

DNA ISOLATION

Salt-Out Method

The fresh or frozen blood, after thawing, was resuspended in nine volume of Buffer A (109.5 g sucrose, 10 mL 1 M Tris HCl (pH 7.6), 5 mL 1 M $MgCl_2$,1% Triton-X-100) and kept on ice for 10-15 min followed by 10 min centrifuge at 10,000 rpm at 4 °C. The supernatant was discarded and nuclei pellets were resuspended in 1.8 mL of Buffer B (25 mM EDTA (pH 8.0), 75 mM NaCl, 20 µg/mL pancreatic Ribonuclease A) and 150 µl of 10% SDS, and 20 µl Proteinase K (20 mg/mL); a serine protease from *Tritirachium album* (Ebeling *et al*., 1974; Cabral *et al*., 2000). The suspension was incubated overnight at 56 °C on low speed rocker or orbiter shaker in water bath. Saturated NaCl (700 µL) was added and the sample was vigorously mixed for 15 min followed by spining at 10,000 rpm at 4 °C for 10 min. The supernatant was transfered to another 15 mL polypropylene tube, and the pellets were discarded. The DNA was precipitated by mixing supernatant in two volumes of ice cold absolute ethanol (scheme 1). The extracted DNA threads were collected with the help of cut tips and washed three times with 70% ethanol. The threads were air-dried for 30 min and dissolved in TE buffer (10 mM Tris HCl [pH 7.5], 1 mM sterile EDTA).

Scheme 1: Isolation of DNA from Whole Blood by Salting-out Method



Phenol – Chloroform Method

DNA was also isolated by the phenol–chloroform method following standard protocol (Sambrook *et al.,* 1989). Fresh and frozen blood samples after thawing were treated with RBC lysis buffer ([pH 7.4]; 155 mM NH_4Cl, 10 mM Potassium Biocarbonate, 0.1 mM EDTA). After centrifugation the White Blood Cells (WBC) pellets were

suspended in extraction buffer (10 mM Tris HCl [pH 8.0], 0.1 mM EDTA, 20 µg/mL pancreatic Ribonuclease A, and 0.5% SDS). Proteinase K (200 µg/mL) was used in both cases. DNA was extracted by phenol-chloroform extraction and ethanol precipitation. DNA threads were washed with 70% ethanol and air-dried. DNA was dissolved in TE buffer.

The QIAamp DNA Blood Mini Kits

QIAamp DNA Blood Mini kits (Qiagen Inc. USA) was designed for rapid purification of an average of 6-8 µg of total DNA from 200 µL of whole blood. The procedure is suitable for use with whole blood treated with citrate, heparin or EDTA. Sample tested were either fresh or frozen.

ANALYSIS OF GENOMIC DNA

Spectrophotometric data for the genomic DNA isolated from blood samples by Salt-out, Phenol-chloroform and QIAamp DNA Mini kits methods is given in Table 1. Genomic DNA was analyzed by agarose gel electrophoresis (Figure 1). Undigested DNA was resolved on a 1% agarose gel and visualized by ethidium bromide staining.

Table 1: Human Genomic DNA Yields from Different Methods and its Purity

Method	Sample	Yield/ml of Blood (µg/mL)	A_{260}	A_{280}	A_{260}/A_{280}
Salting Out	Whole Blood	40.8±4.3µg/mL	0.353±0.051	0.192±0.013	1.90±0.07
Phenol-chloroform	,,	38.5±7.3µg/mL	0.315±0.040	0.180±0.015	1.78±0.04
QIAamp DNA Blood Mini kits	,,	35.3±5.4µg/mL	0.245±0.010	0.134±0.004	1.82±0.08

Figure 1: DNA Extracted Using Different Isolation Procedures A) Lane 1, λDNA (2 µg), Lanes 2-8, DNA Extracted from Phenol-Chloroform Method B) Lane 1, λDNA (2 µg), Lanes 2-8, DNA Extracted from Salt-out Method

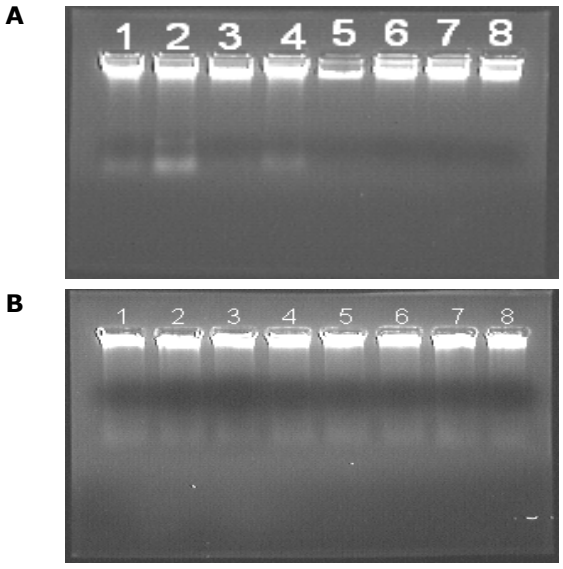
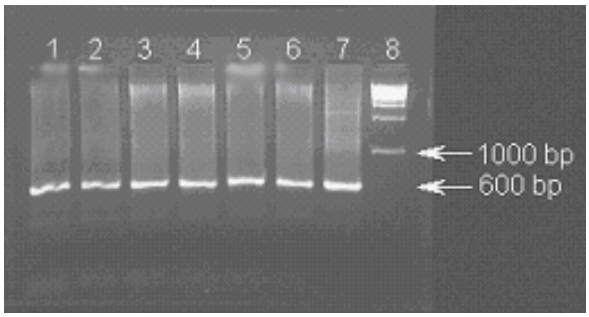


Figure 2: Lane 8 Marker (1 kb), Lanes 1-7 PCR product of the kinetoplast DNA of L. donovani. DNA was isolated from whole blood of Kala-azar patients by salting out method. Ld1 primers are designed from the kDNA of the minicircle gene. The forward primer is 5'-AAATCGGCTCCGAGGCGGGAAAC-3'. The reverse primer is GTACACTCTATCAGTAGCAC-3'. The PCRs were performed with 100-200 ng of genomic DNA in 50 µL of PCR reaction , 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCI, 50 ng each primer, 200 µM of each of the four deoxynucleotides (dNTP), and 1.25 U Taq polymerase (all from Banglore Genie India). The reaction conditions were 94°C for 2 min for first denaturation, followed by 40 cycles (94°C for 1 min, 45°C for 1 min, 72°C for 2 min) and then a 3 min final extension at 72°C



The isolated DNA was further analyzed by PCR amplification using Ld 1 primer pairs designed to amplify a ~ 600 bp region corresponding to mini circle of kinetoplast DNA of *Leishmania donovani*; causative agent of Kala-Azar. In this analysis 100-200 ng of patient DNA was used as template for amplification by Taq DNA polymerase. Amplification product were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining (Figure 2). A unique amplification product of the approximate size was detected; demonstrating the excellent performance of the isolated DNA for amplification based diagnostic methodologies.

DISCUSSION

In order to accelerate and economize the standard salt-out method (Miller *et al.,* 1988), red cell were lysed instead of white blood cell separation (Sambrook *et al.* 1989; Johns and Paulus-Thomas, 1989; and Grimberg *et al.*, 1989) incubated the WBC with Proteinase K and deproteinization with a saturated NaCl. DNA was precipitated by absolute ethanol. In this study, we measured yield per mL of blood (Salting out method, 40.8±4.3 µg; Phenol-chloroform, 38.5± 7.3 µg; QIAamp DNA Mini kits, 35. 3± 5.4 µg). We compared the Salt-out, Phenol-chloroform and QIAamp DNA Mini kits by electrophoresis and OD ratio 260/280. There was no significant difference between the quality (1.90±0.07; 1.78±0.04; 1.82 ± 0.08). DNA was not degraded and it did not inhibit PCR with sequence specific primers Ld 1 (Maurya *et al.*, 2005). During two years of use for urgent clinical samples, this method had less than 1% failure rate and the cost of reagents were $ ~0.2 for 5 mL blood sample. Therefore, this method is economical and with equal yield of DNA both in term of quality and quantity. Furthermore it is a useful technique makes efficient DNA extraction possible even in small laboratories.

CONCLUSION

The method described here has had less than 1% failure rate during two years of use for diagnosis of disease Indian Kala-azar, and the cost of reagents were $ ~0.2/sample. The described method allows simple, fast and cost efficient DNA extraction with qualitative parameters maintained and comparable to those of other extraction method. Furthermore, it is a useful technique makes efficient DNA extraction in small diagnostic laboratories.

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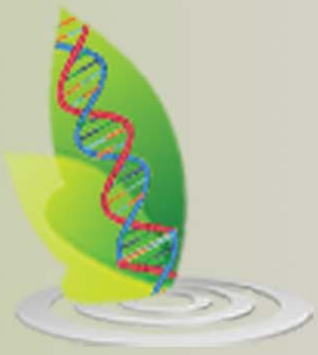

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