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A Simple And Modified Protocol Of DNA Isolation From Whole Blood Cells In A Multiple Hereditary Exostoses Affected Proband.

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ABSTRACT

Multiple Hereditary Exostoses (MHE) is a very rare kind of disorder having occurrence as 1/50,000 individuals. MHE is a medical condition in which multiple bony spurs or lumps develop on the bones of affected patient. Exostosin protein is found to be mutated in such affected patients. This exostosin locus has been identified at three different chromosomes i.e. *ext1* at chromosome 8q23-24, 5; *ext2* to chromosome 11p11-p12, 6 and *ext3* to chromosome 19p. This study was an attempt to standardize the protocol required for isolation of the DNA from such affected patients, further which could be used for PCR mutation detection studies. In the present study three different protocols were used for isolation of DNA from whole blood of MHE patients and normal person. The DNA obtained was further quantified spectrophotometrically.

KEY WORDS: Multiple Hereditary Exostoses, Osteochondroma, Exostosin, *ext1, ext2,* and *ext3*

INTRODUCTION:

In the United States, the terms “exostoses” and “hereditary multiple exostoses” have been used to denote the growths and the disorder, but the World Health Organization (WHO) has selected the nomenclature “osteochondromas” for exostoses and “multiple osteochondromas” for the disorder [2]. Hereditary Multiple Exostoses (HME) is a rare medical condition in which multiple bony spurs or lumps (also known as exostoses, or Osteochondromas) develop on the bones of affected patient. This disorder has an estimated prevalence of 1/50,000 making it one of the most frequent skeletal dysplasias [28]. Osteochondromas are rarely present at birth, but in more than 80% of the patients they develop gradually during the first decade of life and increase in size until closure of the growth plates at the end of puberty [9-16]. Although osteochondromas are benign, they can cause several secondary complications by exerting pressure on neighboring tissues, osteochondromas cause pain, nerve compression, and disturbance of the blood circulation as a result of blood vessel compression [16 & 18]. Hereditary Multiple Exostoses is caused by mutations in the exostosin protein family. Multiple hereditary Exostoses are genetically heterogeneous and at present, two genes, *EXT1* and *EXT2* located on 8q24 and 11p11–p12 respectively, has been cloned [3-8]. MHE is a heterogeneous skeletal disorder in which the penetrance is from 96% to 100% [1]. It is characterized by multiple outgrowing bony tumors capped by cartilage, mostly affecting the metaphyses, but also the juxta-metaphyses of the long bones of the upper and lower limbs [26-28]. The number of osteochondromas that develop in an affected person varies widely even within families. Involvement is usually symmetric. Most commonly involved bones are the femur (30%), radius and ulna (13%), tibia (20%), and fibula (13%). Hand deformity resulting from shortened metacarpals is common. Abnormal bone remodeling may result in shortening and bowing with widened metaphyses [23]. Hip dysplasia may result from osteochondromas of the proximal femur and from coxa valga. Decreased center-edge angles and increased uncovering of the femoral heads may lead to early thigh pain and abductor weakness and late arthritis [1]. The most serious complication of MHE is sarcomatous degeneration of an osteochondroma. Axial sites, such as the pelvis, scapula, ribs, and spine, are more commonly the location of degeneration of osteochondromas to chondrosarcoma [23].

About 200 mutations in the *ext1* gene have been identified in people with hereditary multiple exostoses type 1. Most of these mutations are known as "loss-of-function" mutations, as they prevent any functional exostosin-1 protein from being made. The loss of exostosin-1 protein function prevents it from forming a complex with the exostosin-2 protein and adding heparan sulfate to proteins. It is unclear how this impairment leads to the signs and symptoms of hereditary multiple exostoses. To understand about the impairment, the primary step involves successful isolation of DNA from peripheral blood cells of a MHE affected proband. The availability of adequate high quality genomic DNA is essential to succeed in various molecular biological techniques such as sequencing, cDNA synthesis and cloning, RNA transcription, nucleic acid labeling random primer labeling etc [23]. Hence, extraction of high quality DNA with minimum time and cost is always of interest in molecular genetic studies. To meet these criteria many DNA isolation procedures have been

developed. Literally hundreds of procedures for DNA preparation from various sources of tissue have been published over the last few decades.[20] Many modified versions of the conventional phenol / chloroform extraction methods are still in use as they produce reliable high quality DNA [19,20,21 & 23]. One of the obstacles encountered when extracting DNA from a large number of samples is the cumbersome method of deproteinizing cell digests with the hazardous organic solvents phenol and isochloroform. Several other non-toxic extraction procedures have been published, but require either extensive dialysis or the use of filters [22]. These proteins and RNA obtained interferes later for various other techniques and causes lots of hinderance. Most important in this study was to obtain maximum yield of DNA without containing any proteins digests or RNA residues. The three different protocols used in the study are commonly used for DNA isolation. This research would guide us in a number of ways; such as, choice for the protocol selection and follow the step to yield maximum DNA and also to check the purity of isolated DNA obtained using the different protocols.

MATERIALS AND METHODS

Multiple Hereditary Exostoses affected patients are identified and their consent form is filled up as per the format and guidelines given by Medical Council of India. The ethical clearance for the research study is obtained from the Institutional Ethical Committee. Blood Sample of the identified patients wascollected with the help of a certified lab technician. The blood sample was collected in BD Vacutainer (Sodium heparin 68 USP units). The sample size was six in numbers and volume was 4 ml. DNA extraction process was carried out from the obtained blood samples using three different protocols. The three protocols used in the study are very commonly used for DNA isolation; hence a comparative analysis of these protocols would be useful selection method for DNA extraction process.

Protocol I- Extraction of genomic DNA from whole blood taken from Protocol's Reference Book, Laura-Lee Boodram, Nov2004.

Protocol II- Rapid Isolation of Mammalian DNA referred from J. Sambrook, E.F. Fritsch, and T. Maniatis, Molecular Cloning: a laboratory manual– 2nd ed. (1989)[17]

Protocol III- DNA Preparation from Blood taken from DNA Preparation from Blood (2005), Section of Cancer Genomics, Genetics Branch, NCI, National Institutes of Health.

The DNA yield obtained from these three different protocols is further quantified using UV-VIS Spectrophotometer Model No. UV-1800 based on the principle that nucleic acids absorb ultraviolet light in a specific pattern. In a spectrophotometer, a sample is exposed to ultraviolet light at 260 nm, and a photo-detector measures the light that passes through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample.

DNA EXTRACTION:

The blood sample obtained was used for extraction of DNA. Three different methods were followed for extraction of DNA. The methods used in the present study are as follows: 1. Extraction of genomic DNA from whole blood. 2. Rapid Isolation of Mammalian DNA 3. DNA Preparation from Blood

Extraction of genomic DNA from whole blood: (Protocol I)

The protocol is simple and fairly rapid. It does not require the use of organic solvents but rather utilizes salt extraction to precipitate contaminating proteins. High quality DNA is obtained suitable for immediate PCR applications. The approximately yield was 100-200 µg of DNA from 4-8 ml of fresh or frozen whole blood.

Add 1 volume of buffer A to 1 volume of blood and 2 volumes of cold, sterile, distilled, deionised water. Vortex gently or invert tube 6-8 times and leave to incubate on ice for 2-3 minutes. Spin at 3500 rpm for 15 minutes at 4^{o}C. Discard supernatant into 2.5% bleach solution and re-suspend pellet (vortex for 30 seconds at medium speed) in 2 ml of buffer A and 6 ml of water. Spin at 3500 rpm for 15 minutes at 4^{o}C. The pellet should be white to cream in color. If pellet is significantly red, repeat washing step again. Add 5 ml of Buffer B and 500 µl of 10% SDS to pellet. Re-suspend pellet by vortexing vigorously for 30-60 seconds. Then add 50 µl of Proteinase K solution (20mg/ml). The Proteinase K solution should be made fresh and refrigerated prior to use. The Tubes are incubated for two hours at 55^{o}C in a water bath. Remove samples and leave to cool at room temperature (or leave for 2-3 minutes on ice). Add 4 ml of 5.3 M NaCl solution. Vortex gently for 15 seconds. Spin at 4500 rpm for 15-20 minutes at 4^{o}C. Pour off supernatant into a fresh tube. Take care not to dislodge pellet. Add an equal volume of cold isopropanol (stored at -20^{o}C). Invert 5-6 times gently to precipitate DNA. Remove DNA with a wide bore tip and transfer to a microfuge tube. Wash with 1 ml of 70% ethanol. The DNA pellets is kept for drying for 15-20 minutes at 37^{o}C and re-suspend the pellet in 300-400 µl of Tris HCl, pH 8.5 to re-dissolve overnight at room temperature. DNA can be safely refrigerated for up to a year. Long-term storage may involve ethanol at 70^{o}C.

Extraction of genomic DNA from whole blood: (Protocol II)

The Mammalian DNA prepared from blood or tissues as described in this protocol are 20-50 kb in size and suitable for use as a template in PCRs. The yields of DNA vary between 0.5 and 3.0 µg/mg tissue or 5 and 15 µg per 300 µl of whole blood.

- Transfer 300-µl aliquots of whole blood to each of two microfuge tubes. Add 900 µl of 20 mM Tris-Cl (pH 7.6) to each tube and invert the capped tubes to mix the contents. Incubate the solution at room temperature for 10 minutes, occasionally inverting the tubes.
- Centrifuge the tubes at maximum speed for 20 seconds at room temperature in a microfuge.
- Discard the supernatant not fully but keep about 20 µl of the solution.
- Resuspend the pellet of white cells in the small amount of supernatant left in each tube. Combine the suspended cell pellets in a single tube.

Transfer the minced tissue or the resuspended white blood cell pellets to a microfuge tube containing 600 µl of ice-cold cell lysis buffer. Homogenize the suspension quickly with 30-50 strokes of a microfuge pestle.

(The SDS will precipitate from the ice-cold cell lysis buffer producing a cloudy solution. This precipitation will not affect isolation of DNA). (Optional) Add 3 µl of proteinase K solution to the lysate to increase the yield of genomic DNA. Incubate the digest for at least 3 hours but no more than 16 hours at 55°C. Allow the digest to cool to room temperature and then add 3 µl of 4 mg/ml DNase-free RNase. Incubate the digest for 15-60 minutes at 37°C. Allow the sample to cool to room temperature. Add 200 µl of potassium acetate solution and mix the contents of the tube by vortexing vigorously for 20 seconds. Pellet the precipitated protein/SDS complex by centrifugation at maximum speed for 3 minutes at 4°C in a microfuge. (A pellet of protein should be visible at the bottom of the microfuge tube after centrifugation. If not, incubate the lysate for 5 minutes on ice and repeat the centrifugation step.) Transfer the supernatant to a fresh microfuge tube containing 600 µl of isopropanol. Mix the solution well and then recover the precipitate of DNA by centrifuging the tube at maximum speed for 1 minute at room temperature in a microfuge. Remove the supernatant by aspiration and add 600 µl of 70% ethanol to the DNA pellet. Invert the tube several times and centrifuge the tube at maximum speed for 1 minute at room temperature in a microfuge. Carefully remove the supernatant by aspiration and allow the DNA pellet to dry in air for 15 minutes. Redissolve the pellet of DNA in 100 µl of TE (pH7.6). The solubilization of the genomic DNA pellet can be facilitated by incubation for 16 hours at room temperature or for 1 hour at 65°C.

Extraction of genomic DNA from whole blood: (Protocol III)

About 10 ml whole blood (EDTA, heparin, and citrate) then add 30 ml lysis buffer, shake gently, incubate for 30 min on ice, and centrifuge at 1200 rpm for 10 min at 4^{o} C. Remove supernatant , and then add 10 ml lysis buffer, resuspend the pellet, and centrifuge for 10 min at 4°C (1200 rpm). Discard supernatant and to the pellet add 5 ml SE-buffer, resuspend the pellet, and centrifuge for 10 min at 4°C (1200 rpm) discard supernatant. It is possible to store the pellet at -80°C, so add 1 ml SE-buffer and resuspend the pellet. Use a cryo-tube and centrifuge at 1200 rpm for 10 min at 4°C. Remove the supernatant and freeze the pellet. Add 5 ml SE-buffer and resuspend the pellet, add 40 µl proteinase K (10 mg/ml) and 250 µl 20% SDS shake gently, and incubate overnight at 37°C in a water bath. Add 5 ml SE-buffer and 10 ml phenol shake gently by hand for 10 min, and centrifuge at 3000 rpm for 5 min at 10°C. Transfer the supernatant into a new tube, add 10 ml Phenol/chloroform/isoamyl alcohol (25:24:1), gently mix with hand for 10 min, and centrifuge at 3000 rpm for 5 min at 10°C. Again transfer the supernatant into a new tube, add 10 ml chloroform/isoamylalcohol (24:1), shake gently by hand for 10 min, and centrifuge at 3000 rpm for 5 min at 10°C. Transfer the supernatant into a new tube, add 300 µl 3 M sodium acetate (pH 5.2) and 10 ml isopropanol, shake gently until the DNA precipitated, use a glass pipette, make a hook over a bunsen burner, and capture the DNA. Wash the DNA in 70% ethanol and dissolve the DNA in 0.5-1 ml TE-buffer overnight at 4°C on a rotating shaker. (If the DNA is not dissolved leave it longer at

4°C on the rotating shaker). Measure the DNA concentration in a spectrophotometer (Pharmacia, GeneQuant) and separate the DNA (200 ng) on a 1% agarose gel. The DNA yield obtained from these three different protocols will be measured and the protocol which gives maximum yields will be used for extraction of DNA for further reference.

RESULTS AND DISCUSSION

In order to perform PCR for detection of mutation in *ext1* exon 2, DNA is isolated from patient blood sample. For comparison of mutation, sample was also isolated from normal persons. Isolation was carried out by three different protocols as detailed in materials and method. The isolated DNA was dissolved in 100 µl TE buffer and quantified by measuring the absorbance at 260 nm on a UV-visible spectrophotometer. The purity of the DNA isolated was estimated by comparing the ratio of Absorbance of 260/280 nm.

Using the Beer Lambert Law the amount of light absorbed is related to the concentration of the absorbing molecule. At a wavelength of 260 nm, the average extinction coefficient for double-stranded DNA is 0.020 $(\mu g/ml)^{-1}$ cm^{-1}, for single-stranded DNA it is 0.027 $(\mu g/ml)^{-1}$ cm^{-1}, for single-stranded RNA it is 0.025 $(\mu g/ml)^{-1}$ cm^{-1} and for short single-stranded oligonucleotides it is dependent on the length and base composition (estimation 0.030 $(\mu g/ml)^{-1}$ cm^{-1}). Thus, an optical density (or "OD") of 1 corresponds to a concentration of 50 µg/ml for double-stranded DNA.

The DNA concentration will be calculated using the following formula:
DNA concentration (µg/ml) = (OD_{260}) x (dilution factor) x (50 µg DNA/ml)/ (OD_{260} unit)
Where,
OD_{260} Unit = 50ug/ml for double-stranded DNA,
OD_{260} Unit = 40ug/ml for single-stranded RNA,
OD_{260} Unit = 40ug/ml for single-stranded DNA,
OD_{260} Unit = 20ug/ml for single-stranded oligonucleotides.

Table 1:- Optimization of DNA by three different protocol.

DNA ISOLATION METHOD	DONOR	Absorbance at		Measured DNA concentration (µg/µl)	Ratio of (260/280)
		260 nm	280 nm		
PROTOCOL I	NORMAL	0.587	0.325	0.029	1.81
	PATIENT	0.33	0.184	0.0165	1.793
PROTOCOL II	NORMAL	0.328	0.175	0.0164	1.874
	PATIENT	0.299	0.164	0.01495	1.823
PROTOCOL III	NORMAL	2.277	1.252	0.11385	1.82
	PATIENT	1.23	0.675	0.0615	1.822

The amount of DNA using protocol I, II, III are seen in table 1 above. From the result obtained it can be observed that protocol III yielded highest amount of DNA compare to other two protocols. The amount of DNA was found using following formula:

DNA concentration (µg/ml) = (OD $_{260}$) x (dilution factor) x (50 µg DNA/ml)/ (OD_{260} unit)

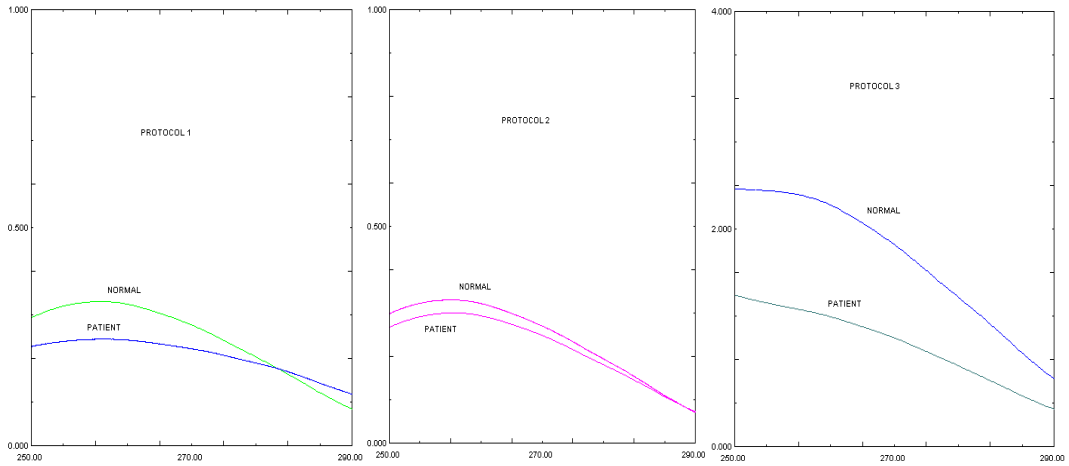

Fig 1:- The yield of DNA Sample obtained from normal and affected patient's blood sample using Protocol I, Protocol II & Protocol III.

Based on the calculations performed it was observed that protocol III yielded 113850µg of DNA in normal patients sample while 61500µg of DNA in affected patients. These results when compare with results obtained in protocol I, II were found to be much higher. Ratio of absorbance at 260/280 is an indication of the purity of the isolated DNA. Pure DNA has a 260/280 ratio of around 1.8. In general all the three tested methods gave a roughly good purity of the DNA (Abs 260/280 between 1.79 to 1.87). Protocol III gave the highest yield of DNA compared to the other two protocols. Although the amount of DNA in protocol III was higher, the sample may have organic contamination of reagent such as Chloroform, Ethanol, Isoamyl alcohol & isopropanol. Protocol III was considered superior because of high yield of DNA obtained.

CONCLUSION

The Present study concluded that DNA isolation from blood will be carried out using protocol III because it yield high amount of pure DNA compared to that of other two protocols.

Also, the chemicals involved in protocol III are cheaper and commonly used chemicals in laboratories. Hence the protocol is less cost effective and time consuming.

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