Journal of Scientific and Innovative Research 2014; 3(6): 552-557
Available online at: www.jsirjournal.com

Research Article

ISSN 2320-4818
JSIR 2014; 3(6): 552-557

Received: 10-11-2014
Accepted: 29-12-2014

Gayathri N.
PG and Research Department of Biotechnology, Kongunadu Arts and Science College Coimbatore-641029, TN, India

R. Parvatham
Department of Biochemistry, Avinashilingam University for Women Coimbatore-641043, TN, India

B. R. Lakshmi
Department of Biochemistry, Avinashilingam University for Women Coimbatore-641043, TN, India

M. Shakthivel Murugan
Department of Biochemistry, Avinashilingam University for Women Coimbatore-641043, TN, India

R. Subashkumar
PG and Research Department of Biotechnology, Kongunadu Arts and Science College Coimbatore-641029, TN, India

Correspondence:
R. Subashkumar
PG and Research Department of Biotechnology, Kongunadu Arts and Science College Coimbatore-641029, TN, India
E-mail: rsubashkumar@gmail.com

Non-invasive genetic diagnosis of Duchenne Muscular Dystrophy probands using salivary DNA

*Gayathri N., R. Parvatham, B. R. Lakshmi, M. Shakthivel Murugan, R. Subashkumar**

Abstract

Duchenne Muscular Dystrophy (DMD) is the most common fatal X-linked recessive disorder, which means that the disease is carried by mother but usually appears in boys. Since muscle wasting occurs in these children, it is difficult to draw blood from them for the DNA test and also it is an invasive technique. The present study is to bring out the non-invasive method of DNA test for the children. Saliva also harbors a wide spectrum of genetic data that can be used for genetic research and clinical diagnostic applications. Saliva samples from several DMD patients and non DMD patients (as control) were collected and the total DNA was extracted by the modified method and their quality were also determined by spectroscopic method. The resultant DNA was of better quality and good for amplification of the DMD exons by PCR, both uniplex and multiplex. But only bands of less intensity were seen on the gel which may be due to PCR inhibitors and this was further confirmed by PCR method. DNA isolated by this method does not have PCR inhibitors and this was shown by the fact that both multiplex and uniplex PCR's showed a significant level of amplicons. This study has shown that saliva could be used as a source of genomic DNA for use in PCR based genetic analysis. If standardized, this could be cost effective and less invasive and will benefit the children and old patient too for the diagnosis of all genetic disorders. Thus, this study may be used for the analysis of disease diagnosis and genetic disorder using saliva DNA.

Keywords: Muscular Dystrophy, Salivary DNA.

Introduction

Muscular Dystrophy (MD) is a genetic disorder that gradually weakens the body muscles. It's caused by incorrect or missing genetic information that prevents the body from making the proteins needed to build and maintain healthy muscles. Blood samples are examined for mutations in some of the genes that cause different types of MD. Duchenne Muscular Dystrophy (DMD) is the most common fatal X-linked recessive disorder, which means that the disease is carried by mother but usually appears in boys. Since muscle wasting occurs in these children, it is difficult to draw blood from them for the DNA test and also it is an invasive technique. The present study is to bring out the non-invasive method of DNA test for the children. Saliva also harbors a wide spectrum of genetic data that can be used for genetic research and clinical diagnostic applications.

Methodology

Selection of the subjects for sample collection

The saliva samples were collected from patients clinically diagnosed to have Duchenne Muscular Dystrophy. The samples were collected from the patients visiting Molecular Diagnostic Counseling Care and Research Centre, Avinashilingam Deemed University, Participants of the DMD conducted for DMD affected kids by the facility during February 2011 at Black Thunder Resort, Mettupalayam and from patients admitted at Myopathy Kappagam a rehabilitation centre for neurological problems in the district of Tirunelveli,

Tamil Nadu. The control samples were collected from the staffs working at MDCRC. The samples were collected after explaining the objective and purpose of the study and written consent was obtained from every participant.

Sample collection

The participants were made to rinse their mouth twice with clean drinking water. For the next 5 min, they were requested to pool saliva in their mouth. The saliva samples were then collected in a sterile 50 mL centrifuge tubes. For samples collected at MDCRC, DNA extraction was taken up the same day. For the samples collected at Black Thunder Resort, they were transported to the laboratory in a thermo coal box filled with dry ice and the DNA extraction was carried out within 48 hrs. For the samples collected at Myopathy Kappagam (Tirunelveli), they were again transported to the laboratory in a thermo coal box filled with dry ice and DNA extraction was carried out within 48 hrs.

DNA extraction

All DNA extraction procedures involve cell lysis, protein removal, and recovery of DNA. When DNA is isolated from whole blood, for example, non nucleated red cells are first lysed to separate them from white cells. The white cells are then lysed using an anionic detergent, which solubilizes the cellular components. This is done in the presence of a DNA preservative that limits the activity of DNases. Cytoplasmic and nuclear proteins are removed by salt precipitation. Genomic DNA is precipitated with alcohol and dissolved in TE buffer. A portion of the DNA is diluted to a specified concentration, and the stock and diluted samples are stored in small aliquots at –70ºC. This avoids repeated freezing and thawing of a large sample, which can lead to precipitate formation. Water is not used for storage because of the risk of acid hydrolysis of DNA. Any contaminants in the water may also cause DNA degradation.

In this study, the manual method of DNA extraction from saliva made use of the same methodology as blood, except the erythrocyte lysing step using ELB was omitted.

Pre- processing

The next set of samples was washed with PBS to remove all fatty materials. This step is repeated for more than 5 times for a thorough wash.

Storage condition

Due to logistic, financial, practical reasons in large studies, it is often necessary to store saliva samples prior to the extraction of DNA. Thus, in this study, we sought to determine whether different storage conditions (time and temperature) of saliva samples collected had an impact on the quality of DNA extracted.

Kit method of DNA extraction

Genomic DNA was extracted from saliva using 2 kit methods - Oragene Kit method and Qiagen Kit method

Quantification of DNA for PCR reactions

The quantification of DNA was done using the Biophotometer at 260nm. About 4 µl of DNA was taken and 96 µl of TE buffer was added to give a 1:25 dilution. It was then quantified. The reading gives the amount of DNA in µg/ml. The stock concentration and yield were also calculated as follows,

Stock concentration= Amount of DNA (µg/ml) X Dilution factor

Yield = Amount of DNA (µg/ml) X

Dilution factor X Volume of TE buffer added to dissolve the extracted saliva DNA

Polymerase chain reaction (PCR)

PCR is an in vitro method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A respective series of cycles involving denaturation of DNA (90°**C)**, primer annealing (60°**C)** and extension of annealed primers (65°**C)** by DNA polymerase results in exponential accumulation of specific fragments, whose termini are defined by the 5’ ends of the primers. The extension product synthesized in one cycle serves as template. In our study to pick mutations in the DNA extracted, select exons in hot spot region of dystrophin gene was chosen and PCR was conducted using Mastercycler (eppendorf).

Primer sequences for exons of the Dystrophin gene, their annealing temperature and the product size:

Exon	Primer Sequence
Exon 43 F	5’- GAACATGTCAAAGTCACTGGACTTCATGG- 3’
Exon 43 R	5’- ATATATGTGTTACCTACCCTTGTCGGTCC- 3’
Exon 44 F	5’- CTTGATCCATATGCTTTTACCTGCA- 3’
Exon 44 R	5’- TCCATCACCCTTCAGAACCTGATCT- 3’
Exon 45 F	5’- AAACATGGAACATCCTTGTGGGGAC- 3’
Exon 45 R	5’- CATTCCTATTAGATCTGTCGCCCTAC- 3’
Exon 46 F	5’- GCTAGAAGAACAAAAGAATATCTTGTC- 3’
Exon 46 R	5’- CTTGACTTGCTCAAGCTTTTCTTTTAG- 3’
Exon 47 F	5’- CGTTGTTGCATTTGTCTGTTTCAGTTAC- 3’
Exon 47 R	5’- GTCTAACCTTTATCCACTGGAGATTTG- 3’
Exon 49 F	5’- GTGCCCTTATGTACCAGGCAGAAATTG- 3’
Exon 49 R	5’- GCAATGACTCGTTAATAGCCTTAAGATC- 3’

Exon 50 F	5'- CACCAAATGGATTAAGATGTTCATGAAT- 3'
Exon 50 R	5'- TCTCTCTCACCCAGTCATCACTTCATAG- 3'
Exon 51 F	5'- GAAATTGGCTCTTTAGCTTGTGTTTC- 3'
Exon 51 R	5'- GGAGAGTAAAGTGATTGGTGGAAAATC- 3'

Electrophoresis of PCR product

After 20 min when the gel gets solidified, the trough was placed in electrophoresis tank containing 1X TBE Buffer. About 15µl of amplified DNA product was mixed with 10µl of bromo phenol blue and loaded on to the wells of 2% agarose gel. The electrophoresis was run at 210V for 20 min.

The gel image was captured by gel documentation system (BioRad, USA) using Quality one software. UV was used to visualize ethidium bromide staining.

Result and Discussion

The sample for this study was collected from the children affected by Duchenne muscular dystrophy. A total of 30 samples were collected at the camp site at fun weekend organized for the children. DNA was extracted by manual DNA extraction method without any modification. The DNA Samples were run in 1% agarose gel to check their quality and it was observed that the quality of DNA varied widely among the samples (fig.1). It can be seen that in samples that are comparatively good, for example G1, G2, G4, G5, G7, G9, G15, G16, G20, G23 and G24, there is a crisp DNA band followed by thick shearing DNA and another thick band at the end of the run. This could be due to shearing and mechanical damage to the DNA.

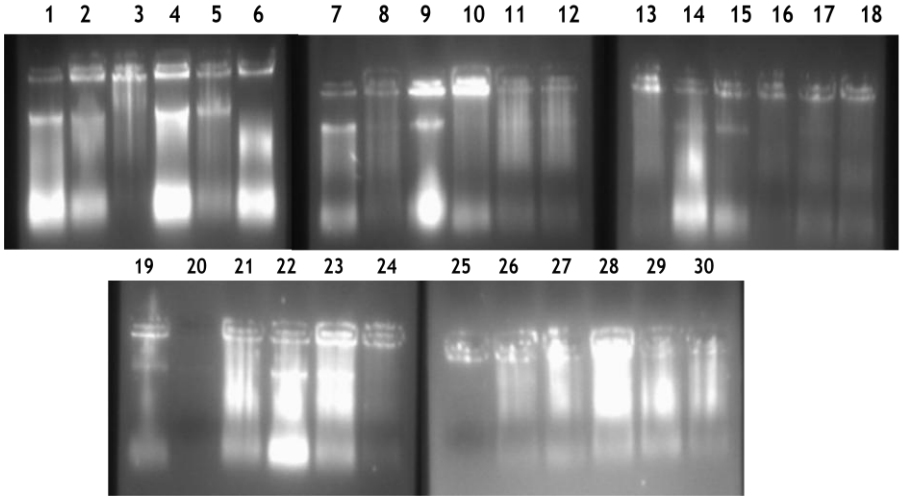

Figure 1: DNA extraction using manual method

Further, it was observed that contamination of saliva with mucus (as in the case of sputum) renders DNA extraction by this manual method difficult resulting in poor yield and quality. This could mainly be due to the presence of mucin in the mucus which is protein whose lysis will hinder the lysis of cell wall with the lysis reagents added in the protocol. So, it is essential that the saliva collection procedure is explained well to the subjects so that only saliva is collected by them, and not sputum.

We observed that the general quality of DNA was not good, and it could be attributed to the presence of cell debris, mucus and proteins in the DNA isolated. To get rid of the mucin and other dissolved proteins and enzymes in saliva, we tried a modified protocol of manual extraction was undertake, samples were washed with PBS (pH 7.4) before cell lysis by SDS and proteinase K.

It was observed that the DNA precipitated in granular form and extraction was done by centrifugation, hence, extracting cell debris and other cellular components along with the DNA. This was also reflected in the insoluble deposit that collected at the bottom tubes. The gel run from this supernatant showed good crisp DNA bands, which can be seen in fig 2. These results suggest that the pre-processing of saliva with PBS in the modified manual method is a very important step in saliva DNA extraction by manual method.

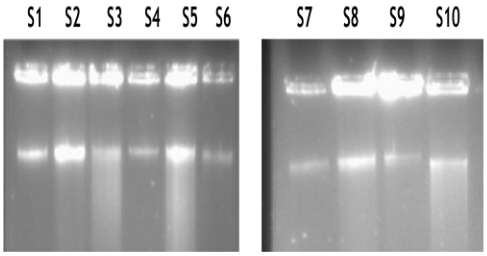

Figure 2: DNA extraction using modified manual method

DNA was extracted from the different aliquots by using modified manual, Oragene kit method and Qiagen blood midi kit method. It was observed that all the three methods gave good quality of DNA. The gel results for all these samples showed similar results confirming that the modified manual method

works well (fig 3). However, DNA degradation in these samples, extracted by modified manual method, after long storage needs to be tested. The DNA extracted by kit methods has already proven stable by the kit manufacturers.

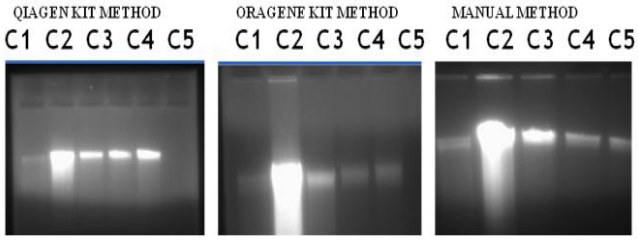

Figure 3: DNA extraction using kit method

Table 1: Selection of samples for PCR reaction

S No.	Sample	Concentration (µg/ml)	A260/ 280	Result
1.	G1	800	1.94	Exon 44 deleted
2.	G4	650	2.01	Exons 46-52 deleted
3.	G7	125	1.89	Exons 46-50 deleted
4.	G10	850	1.77	Exons 46-50 deleted
5.	G16	100	2.04	No deletion
6.	S1	375	2.12	No deletion
7.	S2	450	2.10	No deletion
8.	S3	175	2.08	Exons 49-50 deleted
9.	S5	225	1.95	No deletion
9.	S7	125	2.09	Exons 48-50 deleted
10.	S8	300	2.14	Exons 48-52 deleted
11.	S10	425	1.99	Exons 50-52 deleted

The results of the uniplex and multiplex PCR are shown in fig 4, fig 5 and 6. It can be observed that the multiplex PCR had worked well for all the samples except G10. While a few samples (C3, S8, S10 and G10) show bright bands, the other samples (say G4) show faint bands. The deleted exons are not amplified and the exons which are not deleted are amplified and seen as a band. Negative control shows no bands for any of the exons.

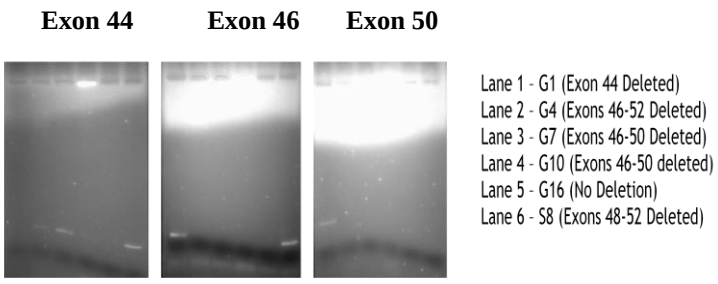

Figure 4: Uniplex PCR results for exons 44, 46 and 50 for samples extracted by modified manual method

Note that all samples except G16 have worked and shown the right result

With good DNA samples been extracted, samples were checked for the PCR quality of the DNA and the Usefulness of this DNA in the diagnosis of DMD gene deletions.

Patient DNA samples extracted from saliva were used to set multiplex PCR for DMD gene exons. This was set based on the blood DNA results (Table 1) and exons were selected such that there was one control exon which was not deleted in any of the samples tested and other exons which were deleted in most of the samples.

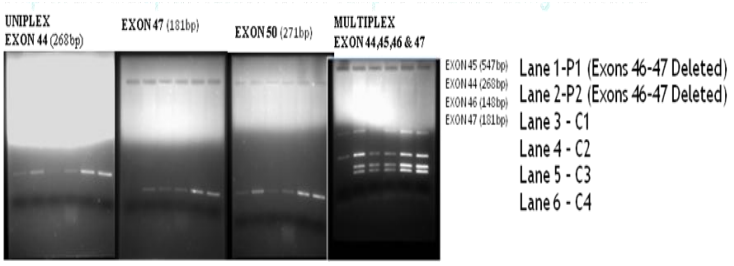

Figure 5: Uniplex and multiplex reaction for the samples extracted using kit method

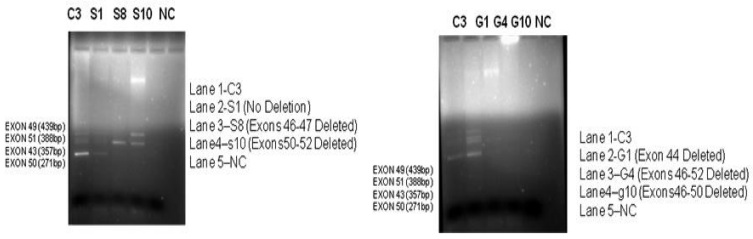

Figure 6: Multiplex PCR results of one control and 3 patient samples extracted by the modified manual method for exons 43, 49, 50 and 51

Dull bands in some of the samples and the lack of any amplification in sample G10 may be due to PCR inhibitors in the

sample. PCR inhibitors usually affect PCR through interaction with DNA or interference with the DNA polymerase. Inhibitors can escape removal during the DNA purification procedure by binding directly to single or double-stranded DNA. Alternatively, by reducing the availability of cofactors (such as Mg^{2+}) or otherwise interfering with their interaction with the DNA polymerase, PCR is inhibited. In a multiplex PCR reaction, it is possible for the different inhibition effects to different extents, leading to disparity in their relative amplifications. Inhibitors may be present in the original sample, such as blood, fabrics, tissues and soil but may also be added as a result of the sample processing and DNA extraction techniques used. Excess salts including KCl and NaCl, ionic detergents such as sodium deocycholate, sarkosyl and SDS, ethanol, isopropanol and phenol among others, all contribute via various inhibitory mechanisms, to the reduction of PCR efficiency. Since the method of DNA extraction from saliva involved salt extraction and also in many samples DNA did not form firm strands but formed granules which made cell debris to be isolated along with the DNA. Though the reason why DNA precipitated as granules and not as thread needs to be identified and this could solve the problem of contamination with cell debris and organic and inorganic salts. It may also be noted that DNA samples separated by centrifugation from the cell debris after isolation were amplified in PCR, which strongly suggests that inhibitors may be isolated along with the DNA in the modified manual protocol. However, it has been proved by the above PCRs and the following experiments that DNA isolated from saliva is a good source of genomic DNA and can be used for the molecular diagnosis of DMD yielding the same results of blood DNA.

Conclusion

Saliva as a source of genomic DNA for the diagnosis of DMD was studied. Saliva samples from several DMD patients and controls were extracted and the DNA quality was observed. It was observed that the manual method used for DNA extraction from blood did not give good quality DNA. Modification in the manual protocol with a PBS wash step prior to cell lysis was introduced. This modified manual protocol yielded good quality DNA which was confirmed by gel. However, DNA precipitated as granules and not as single thread, which forced us to isolate the DNA by centrifugation. This resulted in cell debris and other cellular and salt components to isolate along with the DNA, thus contaminating the DNA with nuclease and other PCR inhibitors. It is observed that these nucleases caused the DNA to degrade when stored over a period of time which was confirmed by gel and quantification methods. To prevent this, DNA was separated out from the cell debris by centrifugation. Effect of further storage of this DNA needs to be studied and the quality of the DNA needs to be assessed by more specific method for dsDNA (Eg. Picogreen method) to establish the contribution of bacterial DNA. The resultant DNA was of better quality and good for amplification of the DMD exons by PCR, both uniplex and multiplex. Again it was observed that the amplification was not good, and only bands of less intensity were seen on the gel. This was attributed to PCR inhibitors, which could have been in the isolated DNA due to inefficient extraction and purification protocols. This was further confirmed by conducting PCR experiments on DNA obtained from saliva using Qiagen blood midi kit. DNA isolated by this method does not have PCR inhibitors and this was shown by the fact that both multiplex and uniplex PCRs worked well. This study has shown that saliva could be used as a source of genomic DNA for use in PCR based genetic analysis. Saliva is a good source of DNA for genetic diagnosis of Duchenne Muscular Dystrophy by multiplex PCR. The modified manual method yielded DNA good enough to be used for uniplex and multiplex PCRs, but it requires further modifications to ensure complete removal of cell debris, nucleases and PCR inhibitors. If standardized, this could be cost effective and less invasive and will benefit the children and old patients too for the diagnosis of all genetic disorders. This study is the first of its kind in India and the first study on the use of saliva DNA for DMD diagnosis.

References

1. Streckfus, C. F., Bigler, L. R. Saliva as a diagnostic fluid. Oral Dis 2002; 8:69-76.

2. Biggar, W. D., Klamut, H. J., Demacio, P. C., Stevens, D. J., Ray, P. N. Duchenne Muscular Dystrophy: current knowledge, treatment and future prospects. Clin Orthop. Aug; 2002; 401:88-106.

3. Ng DP, Koh D, Choo S, Chia KS. Saliva as a viable alternative source of human genomic DNA in genetic epidemiology. Clin Chim Acta. 2006; 367(1-2):81-85.

4. Pink, P., Simek, J., Vondrakova, J., Faber, E., Michl, P., Pazdera, J and Indrak, K. Saliva as a diagnostic medium, Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2009; 153(2): 103-110.

5. Quinquez, P., Kittler, R., Kayser, M., Stoneking, M and Nasidze, I. Evaluation of saliva as a source of human DNA for population and association studies. Analytical Biochemistry 2006; 353(2): 272-277.

6. Walsh, D. J. *et al*. Isolation of deoxyribonucleic acid (DNA) from saliva and forensic science samples containing saliva. J. Forensic. Sci. 1992; 37:387.

7. Streckfus, C. F., Bigler, L. R. Saliva as a diagnostic fluid. Oral Dis 2002; 8:69-76.

8. Birnboim, H. C. DNA yield with Oragene DNA. DNA Genotek, PDWP-001, 2004.

9. Mitsouras , K., Faulhaber, A. E. Saliva as an alternative source of high yield canine genomic DNA for genotyping studies, BMC Research Notes, 2009; 2:219.

10. Mettules, T. Duchenne Muscular Dystrophy. RN, 2002; 65(10): 39-44.

11. Zeng, F., Ren, Z. R., Huang, S. Z., Kalf, M., Mommersteeg, M., Smit, M and White, S. Array-MLPA: Comprehensive detection and duplications and its application to DMD patients. Hum Mutat, 2008; 29(1):190-197.

12. Miller. S. A., Dykes, D. D and Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988; 16(3):1215.

13. www.dmd.nl

14. Basak, J., Dasgupta., *et al*. Analysis of dystrophin gene deletions by multiplex PCR in eastern India, Neurol India. 2006; 54(3):310-311.