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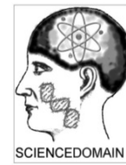

DNA from Oral Rinse- A Comparison of Three Protocols for Amplification of VDR, FTO and Beta-Globin Genes

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Authors' contributions

This work was carried out in collaboration between both authors. Authors MHL and SB designed the study. Author MHL collected the samples, did the bench work, wrote the protocol and wrote the first draft of the manuscript. Author SB managed the literature searches and finalized the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of the study was to evaluate the efficacy of three different protocols for DNA extraction from oral rinse on the basis of their quantity and quality of DNA, simplicity, cost effectiveness and rapidity, as well as its efficiency for amplification of vitamin D receptor (VDR), Fat mass and obesity (FTO) and β-globin genes.
Methodologies: The three methods included; Method 1 (organic solvent extraction), Method 2 (spin column) and Method 3 (ion-exchange extraction). DNA extracted from oral rinse was used for PCR amplification of β-globin, VDR-FOK1 and FTO gene.
Results: The amplified products of 268bp (β-globin), 265bp (VDR-Fok1) and 182bp (FTO) were observed on HeroLab Gel doc system (Germany). Method 2 provided the average highest DNA yield (9.54±1.85 ng/µl) compared to 6.66±1.14 ng/µl and 7.57±0.96 ng/µl by method 1 and 3. Method 2 was found to have a better performance in terms of DNA quantity and quality, however, method 3 was the fastest and method 1 was the most cost effective methods but the PCR amplification from DNA from all three methods was the same.

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Conclusion: Oral rinse was found one of the alternative non-invasive sources for DNA extraction and is sufficient for good quality and quantity of DNA extracted by three different methods. It may be concluded that method 1 can be employed for large scale epidemiological and molecular biological studies.

Keywords: Polymerase chain reaction; Chelex®-100resin; VDR-FOK1; FTO gene rs9939609 and Qubit 2.0.

1. INTRODUCTION

The isolation of high-purity genomic DNA is necessary for genomic analysis such as PCR, gene cloning, sequencing and fingerprinting [1]. The purpose of DNA extraction is to obtain DNA in a relatively purified form which can be used for further investigation. DNA extraction involves three main steps: cell disruption, DNA extraction and DNA purification. Genomic DNA is usually extracted with a special extraction buffer and is further purified by phenol/chloroform extraction followed by isopropanol or ethanol precipitation [2]. While DNA composition is more or less universal in all species, contaminants such as RNA and proteins and their relative amounts differ considerably while developing or selecting a cell lysis method [3]. Organic extraction samples were usually subjected to a detergent such as sodium dodecyl sulfate (SDS) and proteinase K, to disrupt cellular and nuclear membranes, and to lyse large proteins [3].

Organic extraction involves the use of phenol-chloroform for separation of protein into the organic phase and nucleic acid into the aqueous phase. Emulsification of organic and aqueous phase is a critical step in organic extraction to obtain the desired molecular weight of DNA. On the other hand, inorganic extraction methods include salt precipitation, absorption to silica surfaces, and ion-exchange protocols [4]. Precipitation and centrifugation steps are required to remove contaminating proteins prior to ethanol DNA precipitation [5]. Silica gel particles have become a popular method for DNA extraction. The basic principal of salica gel solid support spin column in the presence of high concentrations of chaotrophic salts, contaminants are washed away and DNA is eluted from the silica membrane in water or low-salt buffer. The silica is bound to a solid support, which eliminates the problem of glass-bead contamination of DNA samples. Vogelstein and Gillespie [6] provides a quick, convenient, nontoxic method and can produce high yields of pure DNA. Chelex-100 is one of the examples of Ion-exchange method. Chelex is a chelating resin that has a high affinity for polyvalent metal ions. Singer-Sam et al. [7] postulated that boiling a sample in the presence of Chelex-100 prevents the degradation of DNA. Apparently metal ions, which can act as catalyst in DNA breakdown at high temperatures in low ionic strength solutions, are chelated and inhibited from this action. Walsh et al. reported the use of Chelex-100 as a mean of extracting DNA from forensic samples containing whole blood, bloodstains, seminal stains, saliva, hair and post-coital samples [8]. Chelex-100 resin removes impurities from solution and the alkaline pH disrupts the cell membranes resulting in release of DNA [9]. Saliva contains exfoliated oral epithelial cells and other cells found in oral cavity, in which genomic DNA, mRNA, RNA can be sourced and extracted [10]. It also contains microbial genomic materials from normal flora of bacteria and under pathological conditions; particular opportunistic bacteria and viruses that invade and multiply in oral mucosa [11].

One of the several advantages of using saliva as diagnostic sample is its being the most accessible bio-fluid in our body. Currently by far the most common source of DNA for both clinical and research purpose is from blood lymphocytes [12]. Compared with blood samples which require venipuncture by a phlebologist or medically trained personnel, collection of oral rinse samples is non-invasive, painless and can be self-performed when an appropriate collection container is available [13]. With the use of oral rinse, the most accessible and non-invasive bio-fluid in our body, it is hoped that it can further supplement the use of blood as a diagnostic sample, a much less invasive, an easy-to-collect sample facilities. DNA purity can fundamentally affect successful DNA analysis by methods such as PCR. The aim of the study was to evaluate the efficacy of three different protocols for DNA extraction from oral rinse samples on the basis of their quantity and quality of DNA, simplicity, cost effectiveness and rapidity, as well as its efficiency for amplification of VDR, FTO and β-globin genes.

2. MATERIALS AND METHODS

The study was conducted at the Ziauddin University Research Laboratory, Clifton Karachi, Pakistan. This study was approved by Ethics Review Committee Ziauddin University. 150 oral rinse samples were collected from individuals after taking the informed consent. Subjects with age group 18-35 years and who agreed and signed the inform consent were included in this study and those who did not signed the inform consent were excluded from this study.

2.1 Sample Collection

2.1.1 Oral rinse sample collection

Oral rinse samples were collected from each subject at least 60 minutes of fasting by simple spitting method after rinsing mouth with distilled water. Distilled water was provided to each subject, who after swishing it for 30 seconds (monitored by a stop watch) spit into the labeled collection tubes (15 ml corning tubes). To get good collection of DNA a toothpick with a small bristle like dental floss was given to the subject to swipe inside the oral cavity to capture the oral epithelium and the toothpick was left in the oral rinse till further processing . A total of 10ml oral rinse was collected from each subject. The oral rinse was centrifuged for 15 min at 10,000 x g. Supernatant was discarded, leaving 1ml at the bottom and added 4ml of 1X PBS, which were then aliquots into 1.5 ml tubes. The samples were stored at-80ºC.

2.1.2 Method 1

2.1.2.1 Organic extraction method (organic solvent)

2.1.2.1.1 Isolation of DNA from oral rinse (Lucky MH et al. 2013)

DNA was extracted from 5 ml of the sample, centrifuged at 4000 xg for 10 minutes at room for cell sedimentation. Supernatant was discarded leaving approximately 200 µl at the bottom. This was transferred to 1.5 ml microcentrifuge tube and 500 µl lysis buffer was added, vortexed for 30 seconds and then incubated at 60℃ for 30 minutes in heat block. The tubes were sonicated for 30 second. The tubes were centrifuge for 15 minutes at 12500 xg. The supernatant were transferred into another 1.5 ml microcentrifuge tubes, 500 µl Isopropanol and 200 µl 3M sodium acetate were added and tubes were incubated for 2 minutes at room temperature, vortex for 30 seconds and then centrifuged for 15 minutes at 12500xg. Supernatant was discarded without disturbing the pellet and 1 ml of freshly prepared 70% ethanol was added. The tubes were then centrifuged for 5 minutes at 12500xg. The supernatant was discarded without disturbing the pellet. The tubes were placed vertically upside down on filter paper. The pellet was eluted in 50 µl TE buffer (Tris-EDTA).

2.1.3 Method 2

2.1.3.1 Inorganic extraction method (Spincolumn)

2.1.3.1.1 PureLink® genomic DNA kit (Invitrogen, life technologies, USA)

Oral rinse sample of 500 µl was transferred into 1.5 ml tube containing 200 µl of genomic lysis buffer. Incubated the tube at 55℃ for 10 minutes, then added 96% ethanol, vortexed and after a short spin placed the lysate into spin column. The spin column was placed in centrifuged machine for one minute at 10,000 x g. The collection tube was discarded and the spin column was placed in a clean collection tube. 500 µl of wash buffer 1 prepared with ethanol was added to column and was placed in the centrifuge machine for one minute at 10,000 x g at room temperature. The collection tube was discarded and the spin column was placed into a clean collection tube and 500 µl Wash buffer2 prepared with ethanol to the column was added. The column tube was placed in centrifuge machine for three minute at 14,000 x g at room temperature, after centrifugation the collection tube was discarded and then the spin column was placed in a sterile 1.5 ml microcentrifuge tube, 100 µl of genomic elution buffer was added to the column, incubated for one minute at room temperature. The column tube was centrifuged at 14,000 x g for one minute at room temperature. The column was removed and discarded and the tube collected purified genomic DNA. The purified DNA was stored at -20℃ or at 4℃ for immediate use.

2.1.4 Method 3

2.1.4.1 Inorganic extraction method (Ion-exchange)

2.1.4.1.1 Chelex®-100 resin DNA extraction method (Bio-Rad, USA)

According to (D Sweet et al. 1996) [14] 200 µl of 5% Chelex-100 were added to 1.5 µl

microcentrifuge tube containing 500 µl aliquots of oral rinse. These samples were incubated at 56℃ for 30 minutes before vortexed at high speed for 5 to 10 seconds. The tubes were then again incubated at 100℃ for 8 minutes. The tubes were then vortexed at high speed for 5 to 10 seconds. The tubes were then centrifuged for 2 to 3 minutes at 10,000 to 15,000 x g. carefully transferred the supernatant into another 1.5 ml microcentrifuge tube and discard the pellet containing debris. The sample is then stored -20℃ or 4℃ for immediate use.

2.2 Analysis of Extracted DNA for Quality Control

Qualitative and quantitative analysis of extracted DNA was performed by Qubit 2.0 measurement, PCR and agarose gel electrophoresis.

2.3 Determining the Quality of DNA by Agarose Gel Electrophoresis

The integrity of DNA extracted by each method was assessed by gel electrophoresis. 5ul µlof each DNA extract was analyzed in a 1.5% agarose gel containing 0.5% ethidium bromide and was visualized by U.V. illumination. The presence of high molecular weight DNA with no smearing on the gel suggests that the DNA is of high quality [15].

2.4 Determining the Quantity of DNA by Qubit Measurements

Quantity of DNA extracted by the different methods was assessed using Qubit 2.0 fluorometer (Invitrogen, Life technologies). The qubit fluorometer calculates concentration based on the fluorescence of a dye which binds to double stranded (dsDNA). The Qubit fluorometer picks up this fluorescence signal and converts into a DNA concentration measurements using DNA standards of known concentration. Qubit dsDNA BR assay kit was used for the DNA quantification. Based on DNA concentration derived from the Qubit measurements and the volume of the DNA extract, total DNA yield was calculated with a simple multiplication [16,17].

2.5 PCR

The extracted DNA was amplified by using primers (GeneLink, USA). Sequence of primers is given Table 1. The primer used were representing β-globin gene (268bp) present on chromosome 11; of VDR-FOK1 gene (265bp) present on Exon 2 (rs2228572) of chromosomes 12 and of FTO gene rs9939609 (182bp) present on intron 1 of chromosome 16.All PCR were performed in thermal cycler (XP cycler, Bioer).

2.5.1 PCR protocol for β- globin gene

According to (Lucky MH et al, 2013) [16] The PCR reaction was carried out in 25 µl volume, containing 12.5 µl of GoTaq® Green master mix (GoTaq® DNA Polymerase is supplied in 2X Green GoTaq® Reaction Buffer pH 8.5, 400 µMdATP, 400 µMdGTP, 400 µMdCTP, 400 µMdTTP and 3 mM MgCl2, Promega, USA) 2.5 µl of 1 µM forward primer (GH20), 2.5 µl of 1 µM reverse primers (PCO4), 5 µl of DNA template and 2.5 µl of PCR graded water. The PCR program were first stage followed by pre-denaturation step at 95ºC for 5 minutes, second stage followed by 30 cycles of denaturation at 95ºC for 30 seconds annealing at 51ºC for 30 seconds and extension at 74ºC for 30 seconds, third stage followed by the final extension at 74ºC for 3 minutes.

2.5.2 PCR protocol for VDR-FOK1 gene (rs2228572)

According to (Lucky MH et al, 2014) [17]The PCR reaction was carried out in 25 µl volume, containing 12 µl of GoTaq® Green master mix (GoTaq® DNA Polymerase is supplied in 2X Green GoTaq® Reaction Buffer pH 8.5, 400 µMdATP, 400 µMdGTP, 400 µMdCTP, 400 µMdTTP and 3 mM MgCl2,Promega, USA) 2.5 µl of 1 µM of each primer (Genelink, USA), 5 µl (100-200 ng) of DNA template and 2.5 µl of PCR graded water (Promega, USA). DNA samples were amplified with cycling parameters as follows: Initial denaturation at 94℃ for 5 minutes followed by 35 cycle of 94℃ for 45 seconds, 58℃ for 45 seconds, followed by 74℃ for 45 seconds, and a final extension at 74℃ for 3 minutes.

2.5.3 PCR protocol for FTO gene (rs9939609)

According to (Lucky MH et al. [18]) The PCR reaction was carried out in 25 µl volume, containing 12 µl of GoTaq® Green master mix (GoTaq® DNA Polymerase is supplied in 2X Green GoTaq® Reaction Buffer pH 8.5, 400 µMdATP, 400 µMdGTP, 400 µMdCTP, 400 µMdTTP and 3 mM MgCl2, Promega, USA) 2.5 µl of 1 µM of each primer (Genelink, USA), 5 µl (100-200 ng) of DNA template and 2.5 µl of PCR graded water (Promega, USA).

Table 1. Primer sequences of genomic markers

Gene	Primer sequence	PCR product
β-globin	Forward GH20 5'-CAACTTCATCCACGTTCACC-3'	268bp
	Reverse PCO4 5'-GAAGAGCCAAGGACAGGTAC-3'.	
VDR-FOK1	Forward 5'AGCTGGCCCTGGCACTGACTCTGCTCT-3'	265bp
(rs2228572)	Reverse 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'	
FTO gene rs9939609	Forward 5'-AACTGGCTCTTGAATGAAATAGGATTCAGA-3'	182bp
	Reverse 5'AGAGTAACAGAGACTATCCAAGTGCAGTAC-3'	

DNA samples were amplified with cycling parameters as follows: Initial denaturation at 95℃ for 10 minutes followed by 35 cycle of 95℃ for 30 seconds, 57℃ for 30seconds, followed by 74℃ for 30 seconds, and a final extension at 74℃ for 5 minutes.

2.6 Gel Electrophoresis and Image Documentation

The PCR products were analyzed by running on 2% agarose gel stained with ethidium bromide and visualized under UV transilluminator. All gel images were captured on Hero Lab (Germany) Gel Doc Imaging system.

3. RESULTS

We compared three different methods of DNA extraction form oral rinse samples. The PCR product of 268bp of beta-globin gene shown in Fig. 3. The PCR product of 265bp of VDR-FOK1 gene shown in Fig. 4. The PCR product of 182bp of FTO gene variant rs9939609 shown in Fig. 5. Table 2. Shows the comparison of all three DNA extraction methodologies.

4. DISCUSSION

Three decades ago a non-invasive source of DNA was a vision now it is a reality in the form of saliva. Now that the researchers are looking for biomarkers in saliva for various conditions they also want a fast cost effective method for genomic studies especially in low income countries where there is scarcity of such baseline studies. Oral cells are an excellent source of DNA for diagnosis and large scale molecular epidemiological studies. Since the earliest report for extraction of DNA from oral cells was published in 1988 by lench et al. [19]. Several protocols have been developed to obtain DNA from oral cells. In this study, it was noted that the procedure devised to get more of DNA was a use of toothpick with a small bristle on the other end used for dental floss which by swiping over oral mucosa gathers more epithelial cells. In this study, after collection of oral rinse, a gentle brushing all around inside the oral cavity was done with the help of a small brush at the other end of dental floss which is very economical and widely available. The dental floss was left in the oral rinse till DNA extraction. This gave a good quantity of mucosal cells and ultimately good DNA yield. When samples were collected without brushing the DNA, yield was compromised. DNA extraction is a multi-step procedure involving cell lysis by treatment with lytic enzymes and detergents, DNA extraction with organic solvents, and DNA recovery by alcohol precipitation [3]. The yield and purity of the extracted DNA is essential for subsequent analysis including PCR-based diagnostics of pathogens and different diseases. It was found that the higher quantity of DNA was extracted from oral rinse samples from different extraction methods. Although quantification was possible from all the samples subjected for analysis, it was found that there was an insignificant difference in the quantity of DNA extracted from organic and inorganic methodologies. The Qubit assay is the method of choice for accurate estimation of DNA quantity. Qubit platform provides a rapid, sensitive and accurate method for dsDNA quantification with minimal interference from RNA, protein, single stranded DNA (primers) or other common contaminants that affect UV absorbance [20].

According to Qubit measurements, the method 1 (In-house Lucky MH et al protocol) extracted sufficient DNA quantity for use in PCR application. Moreover, method 2 and 3 (PureLink® and chelex® methods) have higher concentration of DNA obtained from oral rinse. In this study, the PCR results from oral rinse had shown successful results. The (Figs. 2, 3 and 4) Amplification of the β-globin, VDR-FOK1 and FTO gene detected by ethidium bromide staining of electrophoresed PCR products was the end point measure in this study (Lucky MH et al. 2014). Organic solvents extraction, PureLink® extraction and Chelex®-100resin was successful in all samples. In Fig.1 the DNA extracted by PureLink® extraction method found the average DNA concentration was 9.54±1.85 which is at

least higher when compared with the organic solvents method which was 6.66±1.14 and Ion-exchange extraction methods which was 7.57±0.96. The yield and purity of isolated DNA are also dependent on the researcher's handling procedures. A decreased in DNA quality and quantity was observed when the oral rinse sample was not processed immediately or the sample was stored for longer time without added PBS.

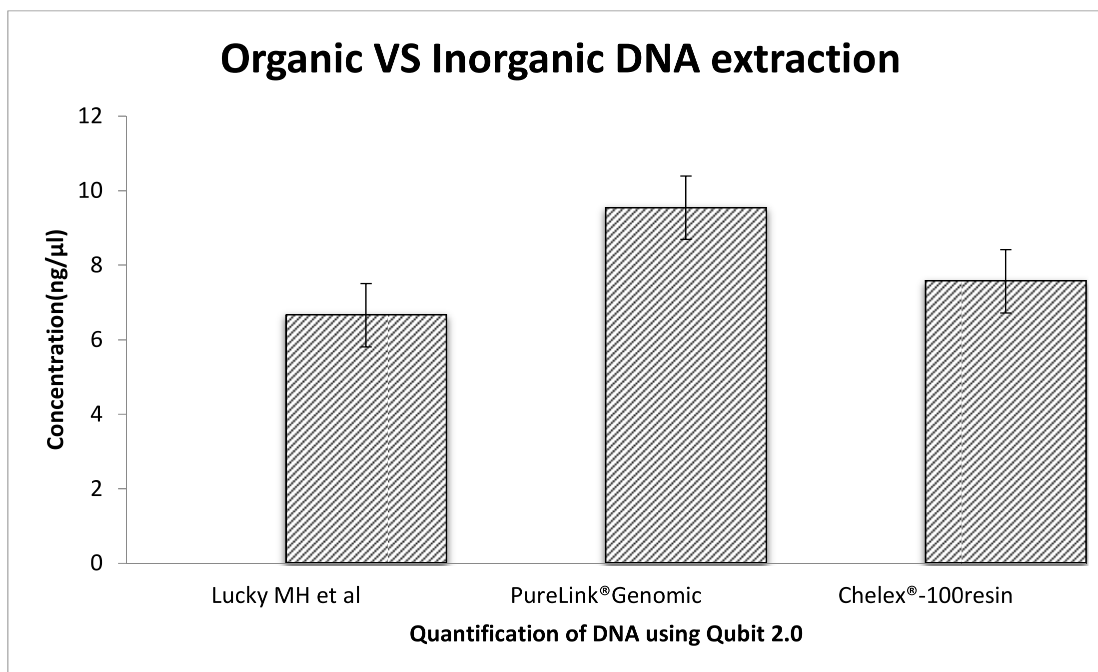

Fig. 1. DNA extraction from three different methods

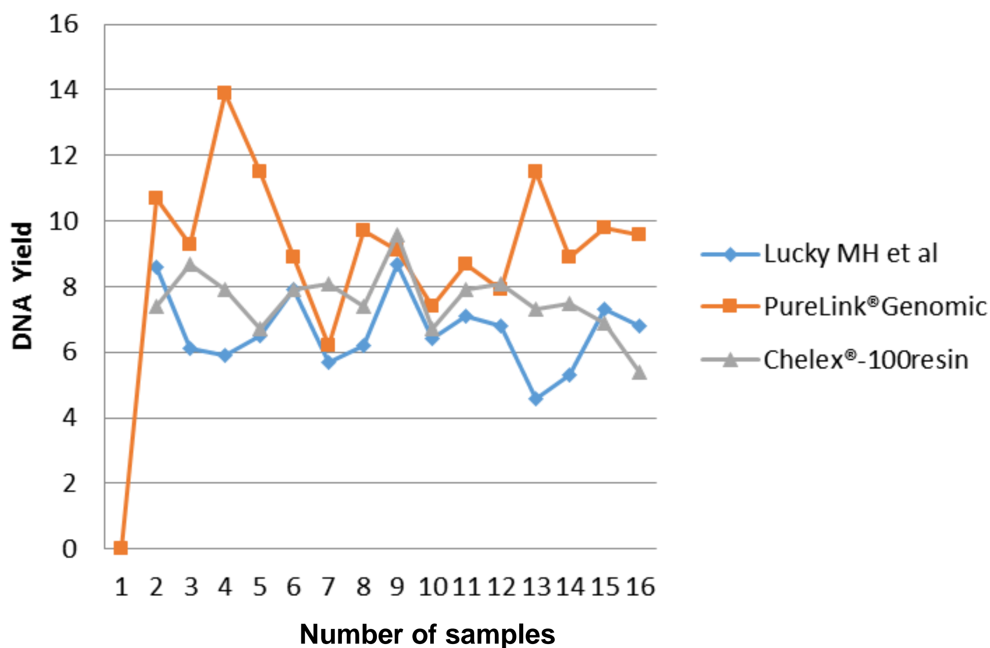

Fig. 2. DNA yield obtained from three different methods from sixteen samples

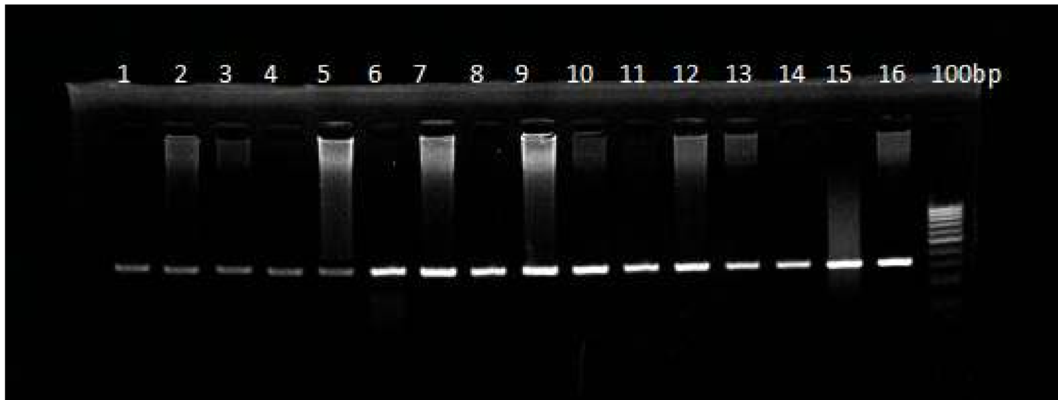

Fig. 3. Gel image of β-globin gene

Lane 1-5 were the PCR products of DNA obtained from method 2, Lane 6-12 were the PCR products of DNA obtained from method 1 and Lane 13-16 were the PCR products of DNA obtained from method 3

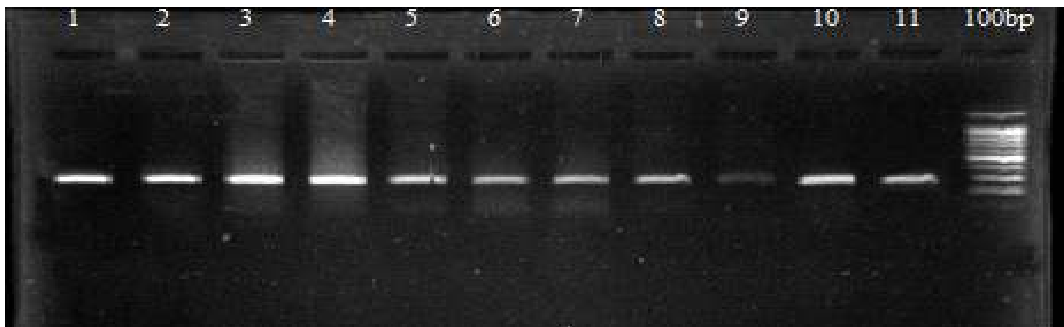

Fig. 4. Gel image of VDR-FOK1 gene

Lane 1-4 were the PCR products of DNA obtained from method 1, Lane 5-8 were the PCR products of DNA obtained from method3 and Lane 9-11 were the PCR products of DNA obtained from method 1

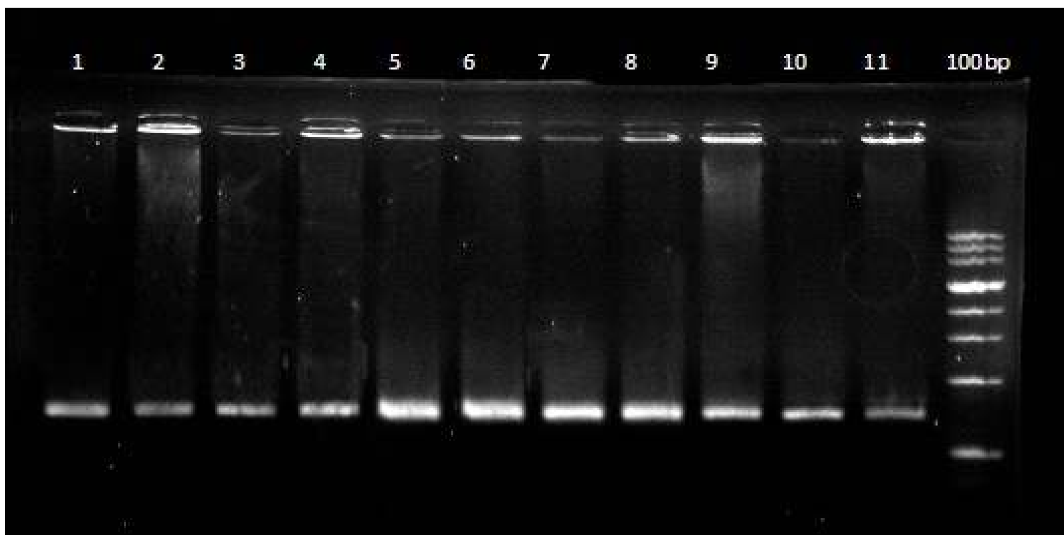

Fig. 5. Gel image of FTO gene (rs9939609)

Lane 1-4 were the PCR products of DNA obtained from method 2, Lane 5-8 were the PCR products of DNA obtained from method 1 and Lane 9-11 were the PCR products of DNA obtained from method 3

Table 2. Comparison of three different protocols for DNA extraction from oral rinse

Purification system	Format	Sample (input)	Elution volume	Processed time	DNA concentration (ng/µl)	B-globin PCR	VDR-Fok1 PCR	FTO rs9939609 PCR
Lucky MH et al.	Organic solvent	5 ml	50 µl	1 hour 30 minutes	6.66±1.14	++++	+++	+++
PureLink® genomic mini DNA kit	Cellulose spin column	500 µl	100 µl	30 minutes	9.54±1.85	++++	++++	++++
Chelex®-100 resin	Ion exchange resin	500 µl	--------	45 minutes	7.57±0.96	++++	++++	+++

Inorganic extraction is fast and easy method, uses non-toxic and nonhazardous materials and produces high quality DNA while Organic extraction require toxic and hazardous material and it is time consuming in process. The present study did not exhibit any significant difference between the extracted DNA PCR amplification products from oral cells immediately after sample collection or from frozen samples at -80℃. Thus, the isolation of DNA from oral rinse is an attractive, non-invasive method for obtaining relatively large amounts of DNA. Oral rinse sample saves resources compared to phlebotomy, allow access to patients who are difficult to reach and also increases willingness to participate in studies.

5. CONCLUSION

Genetic variation in human genome is an emerging source for studying cancer and other complex diseases. Oral rinse is one of the alternative non-invasive sources for DNA extraction and has sufficient amount of good quality and quantity of DNA. The researcher has the choice to select from the three different methods discussed above.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Cheng H, Jiang N. Extremely rapid extraction of DNA from bacteria and Yeasts. Biotechnology Letters. 2006;28(1): 55-59.
2. Fredricks D, Relman D. Improved amplification of microbial DNA from blood cultures by removal of the PCR inhibitor sodium polyanethole sulfonate. J. Clin. Microbiol. 1998;36:2810-2816.
3. Moore E, Arnscheidt A, Kruger A, Strompl C, Mau M. Simplified protocols for the preparation of genomic DNA from bacterial cultures. Molecular Microbial Ecology Manual, Second Edition 1. 2004;01:3-18.
4. Siun Chee Tan, Chin Eng Ong, Yuen Kah Hay, Beow Chin Yiap. Cellulose and its application in biomolecules purification. International Research Journal of Applied and Basic Sciences. 2013;7(5):267-276.
5. Bogner P, Killeen A. Extraction of nucleic acids. In: Coleman WB, Tsongalis GJ, editors. Molecular diagnostics for the clinical laboratorian. 2nd ed. New Jersey: Humana Press. 2006;25–30.
6. Vogelstein B, Gillespie D. Preparative and analytical purification of DNA from agarose. Proceedings of the National Academy of Sciences. 1979;76(2):615-619.
7. Singer-Sam J, Tanguay RL, Rjggs AO. Use of Chelex to improve PCR signal from a srnall number of cells. Amplifications: A Forum for PCR Users. 1989;11.
8. Walsh PS, Metzger DA, Higuchi R. Chelex®100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques. 1991;10:506–513.
9. Sweet D, Lorente M, Valenzuela A, Lorente J, Alvarez J. Increasing DNA extraction yield from saliva stains with a modified Chelex method. Forensic Science International. 1996;83(3):167-177.
10. Kupfer D, White V, Jenkins M, Burian D. Examining smoking-induced differential gene expression changes in buccal

mucosa. BMC Med Genomics. 2010;3(1): 24.
11. Zhang L, Xiao H, Wong D. Salivary biomarkers for clinical applications. Mol Diag Ther. 2009;13(4):245-259.
12. Neidhart J, Gochnour D, Roach R, Young D, Steinberg J. A comparative trial of mitoxantrone and doxorubicin in patients with minimally pretreated breast cancer. Semin Oncol. 1984;11:11-14.
13. Sun F, Reichenberger E. Saliva as a source of genomic DNA for genetic studies: Review of current methods and applications. Oral Health Dent management. 2014;13(2):217-222.
14. Quinque D, Kittler R, Kayser M, Stoneking M, Nasidze I. Evaluation of saliva as a source of human DNA for population and association studies. Analytical Biochemistry. 2006;353(2):272-277.
15. Alex L, Sayhean L, Chahil K, Jagdish NH, Velapsamy S, et al. Evaluation of quality of DNA extracted from buccal swabs for microarray based genotyping. Ind J Clin Biochem. 2011;27(1):28-33.
16. Lucky MH, Baig S. Isolation of DNA from Oral rinse from HPV positive patients. J Coll Physicians Surg Pak. 2013;23(7):455-458.
17. Lucky MH, Baig S. (VDR) gene polymorphism-rapid and non-invasive oral detection method. Pak J Med Dent. 2014; 3(3):13-18.
18. Lucky MH, Baig S. Association of re9939609 FTO gene variant with obesity among Karachi adolescent. EJBB. 2014; 2(6):20-24.
19. Lench N. Simple non-invasive method to obtain DNA for gene analysis. The Lancet. 1988;331(8599):1356-1358.
20. O'Neil J. Gender role conflict research 30 years later: An evidence-based diagnostic schema to assess boys and men in counseling. Journal of Counseling & Development. 2013;91(4):490-498.

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