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Efficient DNA Extraction Protocol for Single Nucleotide Polymorphisms Genotyping in Down Syndrome

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Abstract

The collection of buccal epithelial cells derived from saliva has been an alternative source to obtain human DNA, especially from newborns and patients with mental disabilities. Healthy volunteers without history of genetic or neurological disease were included as control to establish adequate conditions for the DNA extraction, storage and genotyping. The study group consisted of 100 Down syndrome (DS) children, 50 with oral motor difficulties. A buccal swab and/or mouthwash procedure before teeth brushing were used for obtaining high-quality genomic DNA for screening gene polymorphisms by conventional and real-time PCR. Comparison of different methods showed that average DNA yield from mouthwash was 343 ng/µl (range 233- 468 ng/µl) and from swabs was 94 ng/µL (range 32-260 ng/µL). Optimized protocol produced high-quality samples that allowed analysis of MTHFR c.677C>T and MTRR c.66A>G polymorphisms by both conventional and Real-time PCR. Extraction of genomic DNA from buccal epithelial cells proved to be a reliable, simple, inexpensive and noninvasive strategy for routine evaluation of genetic variations in DS patients.

Keywords: DNA extraction; Mouthwash; swab; Down syndrome; Protocol

Introduction

Down syndrome (DS) is the most frequent genetic form of intellectual disability. Nondisjunction is the leading cause of DS but molecular events underlying this mechanism remain unknown [1]. The identification of genetic alterations that may influence DS etiology is essential. Single nucleotide polymorphisms (SNPs) in folate metabolizing genes as maternal risk factors for DS have been studied in populations throughout the world [2-4].

Genetic studies require DNA of sufficient yield and good quality of immediate interest or to be stored for future research. A variety of methods have been established to isolate DNA [5,6]. An alternative source for obtaining human DNA is the collection of buccal epithelial cells derived from the saliva. Saliva has many advantages over blood collection: is usually painless and noninvasive and is considered more acceptable, especially for newborns and mentally retarded patients. Also, an advantage of saliva collection includes the possibility of storing samples at room temperature until DNA extraction [7-9] and does not require highly trained personnel making possible to obtain highly number of participants in a relatively short period of time.

Another non-invasive option for obtaining DNA is collecting buccal epithelial cells from oral swab. This collection mode is extremely useful for obtaining DNA samples from patients who have some kind of disability or who live in places where the collection and submission of the sample blood are not possible [10].

Despite the advantages presented and the existence of several kits for DNA extraction from saliva, there is ample debate upon adequate conditions required to collect and store the material in order to maintain a high quality sample for molecular studies [9]. The effect on storing samples for a period of time and the ideal temperature is always in question. Therefore, this article will present strategies of collecting and storing buccal cells aiming to obtain DNA, pointing out some variables that may interfere with the extraction process. DNA yield and quality by immediate sample processing or after storage was evaluated. Subsequently, genomic DNA extracted from buccal swabs and/or mouthwash of DS patients was amplified by conventional PCR and Real time PCR.

Materials and Methods

Population subjects

The study group consisted of 100 Down syndrome individuals attended at the Instituto de Puericultura e Pediatria Martagão Gesteira (IPPMG/ UFRJ) and five healthy volunteers without any recognizable genetic disease. This study was approved by the Ethics Committee (CEP-IPPMG) and written informed consent was obtained from the subjects.

Saliva collection

For the comparative study of storage conditions, the volunteers were asked to collect buccal cells with a saline solution. They thoroughly rinsed their mouth with 5 mL of a 0.14 M saline solution for 60 s and the rinsing fluid was used as mouthwash sample. This procedure was performed at least an hour after brushing teeth. In order to obtain a sufficient volume to perform the tests, collection was divided into three rinses, with an hour interval between each rinse. The product of all

these mouthwashes was homogenized and transferred to a unique 15 mL tube.

Samples from the same volunteer were divided into six aliquots of equal volume and subjected to different storage conditions: saliva collected with saline and 3 mL of TNE Buffer (17 mM Tris/HCl (pH 8.0), 50 mM NaCl and 7 mM EDTA) and extracted fresh (condition 1); saliva kept at room temperature for seven days (condition 2); saliva with addition of 3 mL of 70% ethanol kept at room temperature for seven days (condition 3); saliva with addition of 3 mL of TNE at room temperature for seven days (condition 4); saliva kept frozen at −20°C for 7 days (condition 5); saliva with addition of 3 mL of TNE kept frozen for seven days (condition 6).

After these initial tests to check the different conditions for storage and DNA extraction, the condition which provided better yield and quality of DNA was selected for further studies. Fifty samples from DS patients were collected to evaluate the amplification efficiency of the material obtained in molecular analyses. TNE buffer was added in all samples and DNA extraction was performed on the same day of collection.

Collection swab

The collection of buccal cells by oral swab was also another variable analyzed (condition 7). Fifty samples of DS patients who had some inability to perform rinsing with saline solution were collected. Samples were collected using two swabs per individual. Scraping was performed in the entire length of inner cheeks with sterile swabs, about 5 times in a circular motion. The swabs were placed in a 15 mL tube containing 3 mL of TNE and samples were immediately processed.

Extraction and quantification of DNA

Isolation of DNA from mouthwashes and buccal swabs followed a method proposed by Aidar and Line [11] with some modifications. We used a 0.14 M saline solution instead of 3% sucrose. In all DS samples we added only TNE buffer (and not diluted in 66% ethanol). The DNA obtained in each condition was re-suspended in 100 µl of TE buffer [10mM Tris (pH 7.8) and 1mM EDTA] and quantified by spectrophotometry using NanoVue Plus Spectrophotometer (GE Healthcare Life Science). The ratio of 260nm/280nm was determined to evaluate DNA purity and further tested for polymorphism genotyping by conventional PCR and Real time PCR.

Genotyping by PCR and real time PCR

The quality of DNA was tested for polymorphism genotyping by conventional PCR and Real time PCR. Genotyping of the MTHFR c. 677C> T gene was performed by RFLP (Restriction fragment length polymorphism). A set of primers (forward 5'-GAAGCAGGGAGCTTTGAGGCTGACCT-3' and reverse 5'-AGTGATGCCCATGT CGGTGCATGCCT-3') were used in amplification reaction. The reaction mixture was prepared for a final total volume of 15 µL, containing 2 µl DNA (50 ng/µL); 10X Standard Reaction Buffer (Biotools), 2 mM $MgCl_2$; 200 µM dNTP's; 0.5 µM each primer, 1U Taq DNA Polymerase (BioTools). The PCR cycling consisted of a denaturation step at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 30 s; with a final extension of 72°C for 5 min. After amplification, the reaction products were resolved in 1.5% agarose gel and visualized under UV light using GelRedTM DNA intercalating dye (Biotium).

For Real-Time PCR we have employed the CFX 96 Real-Time System (BioRad). Genotyping for MTRR c.66A>G polymorphism was performed by allelic discrimination assay using 10 ng of DNA, Taqman® Universal PCR Master Mix kit and TaqMan® SNP Genotyping Assays (Applied Biosystems). The probe (C_3068176_10, Applied Biosystems) was labeled with the fluorophore VIC® to detect wild-type allele "A" and the fluorophore FAM® to detect polymorphic allele "G".

Results

The results obtained after DNA extraction of the five volunteers in different conditions of collection and storage are presented in Table 1. Conditions that provided better DNA yield and better purity were the samples in which 3 mL of TNE solution were added (conditions 1 and 6). The storage of sample with TNE for 7 days frozen at -20°C, did not affect significantly DNA yield (303,3 ng/µl; range 222.0 to 373,5) and PCR performance. However, average DNA yield obtained from swabs (condition 7) were significantly lower (94.0 ng/µl).

Experimental condition*	DNA concentration (range)*	OD Ratio 260/280
1. Fresh mouthwash; TNE buffer	343.3 (233 - 468.5)	1.85
2. Saliva; 7 days RT storage	185.6 (69.5 - 304)	1,95
3. Saliva; 70% ethanol; 7 days RT storage	77.0 (60.0 - 106.5)	1.95
4. Saliva; TNE buffer; 7 days RT storage	178.5 (108.5 - 261.5)	1.84
5. Saliva; 7 days storage at -20°C	253.3 (113.5 - 404.5)	1.89
6. Saliva; TNE buffer; 7 days storage at -20°C	303.3 (222 - 373.5)	1.9
7. Fresh oral swab; TNE buffer	94 (32.5 - 260.2)	2.1

Table 1: Effect of distinct conditions upon DNA concentration (expressed in *ηg/µL) and quality. RT: Room Temperature, *O.D: Optical Density.

Samples of DS patients were collected in the first condition and the average DNA yield obtained after extraction of the samples was 416.1 ng/µl (range 275.2 to 594.0 ng/µl); and the average ratio 260/280 was 1.9. Both samples obtained from mouthwash samples as well as from swabs allowed sufficient DNA quantity and quality for molecular analyses. All the samples were amplified using standard PCR and real time system. PCR results are illustrated in Figure 1 for MTHFR polymorphism 677C>T.

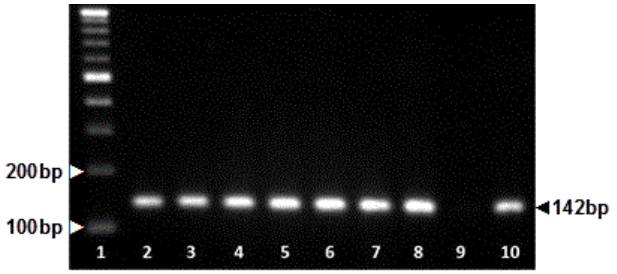

Figure 1: Verification of PCR products for MTHFR 677C>T polymorphism on 1.5% agarose gel. Line 1: 100 bp ladder, Lines 2 to 7: DNA extracted from mouthwash samples; Line 8: DNA extracted from oral swab; Line 9: Negative control (all PCR reagents except DNA, substituted by pure water); Line 10: Positive control (PCR reaction using a previously evaluated DNA template). bp=base pairs.

The real time PCR technique with allele-specific assay (TaqMan probe) was also performed. It was possible to genotype all 100 individuals. Figure 2 shows the graphic obtained of an individual heterozygous for MTRR c.66A>G polymorphism.

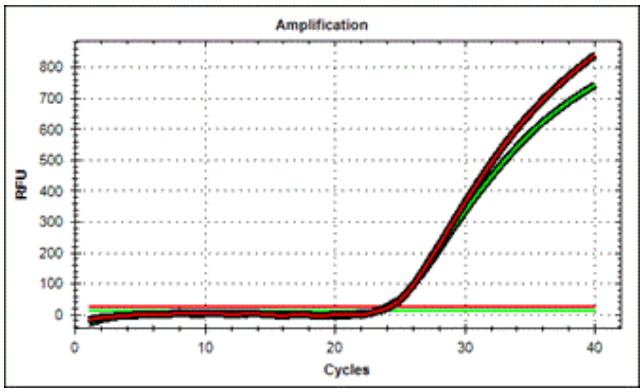

Figure 2: Allelic discrimination is achieved by the selective annealing of TaqMan® probes. This sample represents an individual heterozygous for MTRR c.66A>G polymorphism as fluorescence is observed for both allele-specific probes (VIC=A allele - red; FAM=G allele - green).

Discussion

Current technical molecular developments are valuable contribution for clinical applications especially in genetic diseases. The quality and quantity of DNA obtained from biological samples depend on several factors; including the extraction methodology and storage conditions. Although blood is the traditional source for DNA isolation, its collection has inherent limitations. The main constraints include: invasive and painful collection; potential risk of disease transmission, special care in collection and trained professionals to perform the procedure [5,8,12,13].

The use of buccal cells as a source of DNA is highly relevant considering that the amount of epithelial cells 4.3 × 10^5/mL in saliva is comparable to the amount of nucleated cells peripheral blood (4.5 to 11 × 10^5/mL) [7]. Usually buccal epithelial cells are obtained with swabs or by pouring saliva directly into sterile tube. Protocols for DNA extraction are commonly based on the use of solutions or columns in commercial kits that facilitated the extraction process but are costly [14]. Besides presenting high sensitivity and stability of the formed product a methodology for clinical routine diagnosis should ideally be low-cost and lack toxicity [15,16]. Herein we present evidence that saliva collection from DS children using mouthwash solution with TNE buffer is a useful strategy to obtain high-quality and stable DNA samples from buccal epithelial cells. We tested several experimental conditions and observed that immediate DNA extraction provided high yield of DNA (343.3 ng/µl). Even after storage at -20°C for seven days thus indicating that addition of TNE affer after saliva collection improved the stability of genomic DNA and preserved the material (303 ng/µl). Such result may be related to the efficiency of TNE that reduced sample viscosity allowing efficient precipitation of buccal epithelial cells and EDTA preserving DNA integrity [11,17]. Hence, addition of 3 mL of TNE did not affect the integrity even after seven days storage at room temperature.

For the second and fifth conditions of this experiment, saliva was preserved only with saline, varying the storage condition. The average yield for the two conditions was not significantly different: 185.6 ng/µl and 253.7 ng/µl, respectively. Interestingly the use of ethanol (70%) affected DNA preservation and promoted DNA degradation. Our data indicated that 70% ethanol addition combined with room temperature storage provided the worst performance, with an average of 77 ng/µL DNA. This result contrasts with a previous report [18] showing no significant difference with the addition of 70% ethanol in different conditions. It is also important to emphasize that storage at low temperature had a beneficial effect on sample preservation, therefore indicating that besides the collection procedure, the temperature of storage is critical for maintenance of DNA integrity.

Our results showed that the best conditions for the extraction of DNA from saliva associated collection in saline solution with addition of 3 mL of TNE, with subsequent extraction of the sample still fresh (condition 1) or frozen for 7 days (condition 6). Thus 50 samples from DS children were collected, and subsequently DNA was extracted. The average yield was 416.1 ng/µl and the mean ratio of 260/280 was 1.97.

Beckett et al. [19] compared two methods of obtaining oral samples, including the use of oral swabs, however the DNA yield was low, requiring subsequent whole genome amplification. We have used the same protocol for both swab and saliva DNA extraction and obtained sufficient DNA mass for performing molecular techniques without further genome amplification. The extraction of saliva through mouthwash was more efficient, allowing a higher DNA recovery yield. Our findings contradict those observed by Carvalho et al. [12], who found no statistically significant differences between the two forms of biological sample collection. The 260/280 ratio of the samples had an average of 1.9, an adequate value, since pure nucleic acids typically have 260/280 ratio between 1.8 and 2.0. These values demonstrate e ficient removal of proteins by precipitation with ammonium acetate.

Indeed, the methodology provided enough DNA to perform molecular analysis to detect folate polymorphisms by RFLP for MTHFR c.677C>T and TaqMan® Assays by real-time PCR for MTRR 66A>G polymorphism in children with Down syndrome. The DNA obtained is perfectly applicable to sensitive genetic analyses that require a high degree of purity, a TaqMan® assay have a simple workflow and it is a powerful tool to genotype DNA samples. Thus,

buccal epithelial cells from saliva may be considered as a good alternative for providing large amount of high-quality DNA to be used for routine genetic screening aimed at identifying risk factors and diagnosis of genetic diseases, including Down syndrome.

Conclusion

Buccal epithelial cells obtained with mouthwash solution with TNE buffer or oral swabs produced high-quality and stable DNA samples. This strategy provided large amounts of DNA sufficient to perform molecular analysis to detect gene polymorphisms by both RFLP and real-time PCR in children with Down syndrome.

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