NOTAS CIENTÍFICAS

18S and GAPDH housekeeping genes seem to be equally useful in saliva

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RESUMEN.-

La saliva tiene potencial para ser un instrumento de diagnóstico, asimismo se espera que su uso como herramienta de diagnóstico aumente de manera exponencial en los próximos años. Nuestro objetivo principal fue estudiar el perfil de expresión génica de dos de los genes constitutivos más comunes en saliva, gliceraldehído-3-fosfato deshidrogenasa (GAPDH) y 18S, en muestras de quince voluntarios sanos. Comparamos el valor CT, que se define como la intersección entre una curva de amplificación y una línea de umbral. De acuerdo con el análisis realizado, no hay ninguna diferencia estadísticamente significativa entre ambos genes.

PALABRAS CLAVES: expresión génica, genes constitutivos, gliceraldehído-3-fosfato deshidrogenasa (GAPDH), saliva, 18S ARNr

ABSTRACT.-

Saliva has a potential to be a diagnostic instrument, also it is expected that its use as a diagnostic tool will increase exponentially in the next few years. Our main aim was to study the gene expression profile of two of the most common constitutive genes in saliva, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA, in samples of 15 healthy subjects. We compared the CT value, which is defined as the intersection among an amplification curve and a threshold line. According to the analysis, there is no statistically significant difference between the two genes.

KEYWORDS: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), gene expression, house-keeping genes, saliva, 18S rRNA

INTRODUCTION

Nowadays, the potential of saliva as a disease diagnostic tool and health surveillance is rising owing to its noninvasive accessibility, a specially useful quality for individuals with limited training and without special equipment (Pfaffe *et al.,* 2011), as well as cost-effectiveness advantages. (Lee *et al.,* 2012). As a clinical specimen, in terms of collection, storage, shipping, and voluminous sampling; saliva processes can be carried out very economically compared with serum or urine (Sreedevi *et al*., 2012).

Saliva is composed of water, electrolytes, hormones, microorganisms, mucins, enzymes, proteins, immunoglobulins and the nucleic acids, DNA and RNA (Nelson *et al*., 2003). Furthermore, saliva contains buccal epithelial cells with a valuable and wide range of genetic data that can be used for genomic research (Jerjes *et al.,* 2012). However, recent developments in salivary diagnostics tools that have been accomplished using genomic, transcriptomic, proteomic and metabolomic approach (Ahn *et al*., 2012), had given a source of unprecedentedly rich genetic information, in consequence, there is an increasingly world-wide growing interest in this research area (Chocolatewala *et al*., 2010).

It has been reported the presence of many markers in whole saliva, which might be related to the periodontal disease status in type 2 diabetes patients. Also, other studies have shown the utility of salivary mRNAs for detection of oral cancer, breast cancer and Sjögren syndrome (Lee *et al*., 2012).

Quantitative real-time PCR (qPCR) gives an insight into gene expression. In this regard, two procedures for analyzing data could be used, absolute quantification and relative quantification based on constitutive genes (Marsh 2006). In this line, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is one of the most common housekeeping genes, and is often used to normalize gene expression data. However, the levels of GAPDH mRNA expression vary among different tissues. Some of these differences may reflect the role of GAPDH in the cell. For example, GAPDH is one of 10 enzymes that catalyze reactions in the glycolytic pathway and its expression may be higher in tissues with high energy demands (Katz *et al.,* 2011), and might be affected by nutrition status (Groeger *et al*., 2011). Meanwhile, the 18S ribosomal RNA (18S rRNA), is frequently used in phylogenetic studies and has been reported as the most appropriate gene to be used as reference gene (Bostanci and Belibasakis 2012).

The aim of this study was to evaluate the GAPDH and 18S RNA gene expression profile in saliva to determinate if there are differences in their Cycle Threshold (CT) values.

MATERIAL AND METHODS

Patients.-
Fifteen healthy volunteers provided two saliva samples each one.

RNA extraction.-
After 3 minutes of oral rinse, subjects were asked to spit 5 ml of saliva in eppendorf tubes, which were centrifuged 10000 x g for 10 minutes at 4º C, supernatant was collected and TRIzol® reagent (Invitrogen Life Technologies) was used to deliver high-quality total RNA. Cells were lysed with 500 µl of TRIzol® reagent, subsequently of five minutes incubation, for separation phase, 100 µl of chloroform were incorporated. Subsequently, the tubes were capped and incubated for 15 minutes.

In order to precipitate RNA, the tubes were centrifuged at 12000 x g for 15 minutes; the aqueous phase was transferred to a fresh tube. 250 µl of isopropanol were added and subsequently the tubes were vortexed at moderate speed for 10 seconds and incubated at room temperature for 5 minutes. The samples were centrifuged at 12 000 x g for 8 minutes; the supernatant was removed leaving the pellet aside.

Pellets were washed with 1 ml of 75% ethanol and centrifuged at 7500 x g for five minutes. Finally, the ethanol was eliminated without

disturbing the pellets and RNA was dissolved in 50 µl of nuclease-free water. With the purpose of assessing RNA quality and purity a spectrophotometer was used, and only samples with high purity were analyzed. To avoid RNA degradation it was stored at -70º C.

Reverse transcription.-
cDNA was synthesized using the high capacity RNA to cDNA kit (Invitrogen™) protocol. Briefly, aliquots of 20 µl were prepared on ice as follows: 10 µl 2X reverse transcriptase buffer, 1 µl 20X reverse transcriptase enzymes, 50 ng/µl RNA and up to 9 µl of nuclease free H_2O. The tubes were sealed and centrifuged; the reactions were loaded into the thermal cycler. The cDNA was produced after a three steps program; firstly, the temperature was set at 37º C for 60 min, secondly the temperature was increased up to 95º C for 5 minutes, and successively, the temperature was decreased to 4º C. The resulting cDNA was used in qPCR reaction.

qPCR
All samples were processed by TaqMan PCR assay (7500 Fast Real-Time PCR System, Applied Biosystems, USA). The 20 µl reactions were prepared in 96-well assay plate, containing 10 µl of TaqMan Universal Master Mix II (Applied Biosystems), 2 µl of cDNA, 1 µl of TaqMan assay and 7 µl of RNase-free water. The 7500 amplification conditions used were as follows: 10 minutes at 95º C, 15 seconds at 95º C and 1 minute at 60º C (Table 1).

Table 1. Description of reference genes and probes

GENE SYMBOL	GENE NAME	AB Assay	Ref Seq
18S rRNA	*18S ribosomal RNA*	*Eukaryotic 18S rRNA Endogenous Control (VIC®/ MGB Probe, Primer Limited)*	X03205.1
GAPDH	*Glyceraldehyde 3-phosphate dehydrogenase*	Human GAPD (GAPDH) Endogenous Control (FAM™/MGB Probe, Non-Primer Limited)	NM_002046.3

Ethics.-
The research project was done in accordance with institutional and national ethical guidelines and following the principles of Helsinki Declaration.

Statistical analysis.-
Statistical analysis was done using the Statistical Package for Social Science (SPSS) software, version 20 (SPSS Inc., Chicago, United States). Differences between genes were assessed by Mann-Whitney-U-test, considering a $p \leq 0.05$ as statistically significant.

RESULTS AND DISCUSSION

The CT value of 30 samples was analyzed, discarding four of them due to biological degradation. With the final N of 26 samples from 13 subjects (9 women and 4 men, mean age 23.9 ± 2.9), the mean CT values were of 29.6 and 26.7 for GAPDH and 18S, respectively; both of them fulfilled the condition of a desirable CT value, between 8 and 35.

It is known without doubt that qPCR is an extremely sensitive tool, very useful in order to recognize the amount of a defined sequence or a gene expression. Nonetheless, it is expected that the expression of a housekeeping gene can go up and down under different experimental conditions. Furthermore, the stability of a reference gene is a fundamental prerequisite for internal standardization of a target gene. Basically, the variability of a housekeeping gene

expression depends on experimental conditions and the CT of each sample must be adjusted with the threshold of the qPCR reaction (Masunaga *et al.,* 2010).

Despite the fact that there was not statistically significant difference between the analyzed genes (p=0.457), in this initial approach, the curves varied in the point at which the amplification begins, with the 18S rRNA line being expressed first and with a better defined amplification curve. The literature shows different conclusions in relation to the housekeeping genes, for example, Vaiphei ST *et al.*, concluded that, in blood, GAPDH, either alone or in combination with 18S, was found to be the most suitable endogenous control gene (Vaiphei *et al*., 2014). On the contrary, Moreno LI *et al.*, published that beta-2 microglobulin exhibited the highest expression for all body fluids in forensic applications (Moreno *et al.,* 2012).

According to our results, the gene that had less deviation in CT was the 18S; thus, this might be an enhanced constitutive gene compared to GAPDH that shows a wider CT data variation (Figure 1).

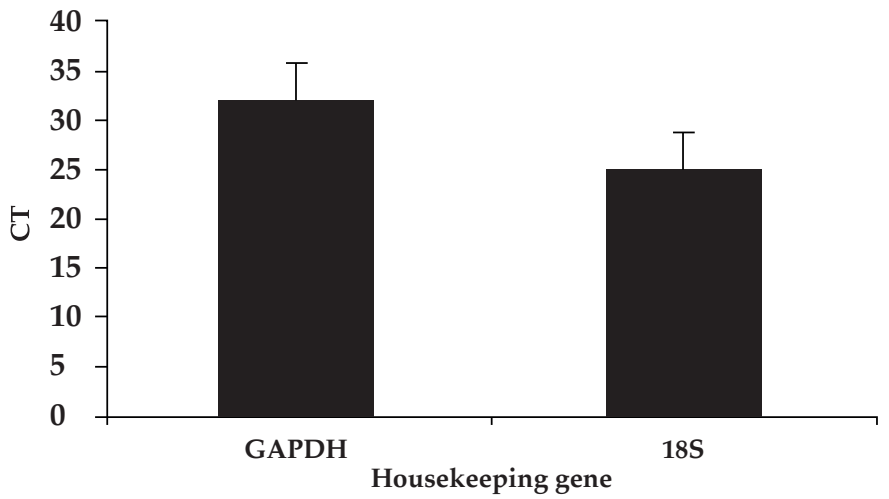

Figure 1. Threshold cycle (Ct) value of the GAPDH and 18S housekeeping genes

Notwithstanding, the lack of significant statistical difference of the above mentioned results, could be an underestimation due to the low number of cases.

In the qPCR studies it is crucial the correct election of a housekeeping gene. While GAPDH has been widely used as an internal control gene, in data derived from a variety of cells and tissues (Katz *et al.,* 2011), its selection has to be based on the experimental circumstances. By comparison, the 18S RNA has been described in the literature as a more stable gene (Bostanci and Belibasakis 2012). Recent studies suggested that expression of these genes vary significantly under different environmental conditions (Tenorio *et al.,* 2011). Our results demonstrated a lack of any statistical difference between both genes, but it has to be considered that our number of cases was limited.

CONCLUSION

Although GAPDH and 18S RNA genes are suitable and equally useful in saliva, the choice of one of them should be based on the conditions of the study that will be performed. Further research increasing the sample number is needed to evaluate differences between other possible control genes such as 25S ribosomal RNA (25S rRNA), ubiquitin (UBQ), or β-actin (ACTB), under different experimental conditions and must be compared to GAPDH and 18S rRNA. We agree with Schafer CA *et al.*, in that saliva offers a considerable leap forward in

health care evaluation (Schafer *et al.*, 2014), but as a first approach, it has to be clarified the best housekeeping genes for this corporal fluid to be used in qPCR.

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