

Extraction and purification of RNA from human carious dentine: an approach to enable bacterial gene expression studies

Extração e purificação de RNA proveniente de dentina humana cariada: uma abordagem para viabilizar estudos de expressão gênica

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Abstract

Background: RNA isolation from bacteria within dentine caries lesions could be difficult due to reduced amount of collectable biomass and high mRNA instability. Attempting to overcome this challenge we describe one protocol developed to extract and purify total RNA from dentine lesions. **Objective:** customize a bacterial RNA extraction and purification method from human carious dentine. **Methods:** quantity and purity of extracted RNA were measured with a microvolume UV-VIS spectrophotometer, RNA integrity was assessed by standard denaturing agarose gel electrophoresis and images were captured under ultraviolet light with camera and analyzed. DNase treatment removed genomic DNA and an additional step of purification was carried out in silica spin column. **Results:** final yield (ng/ μ l) was 67.01 ± 22.33 , absorbance ratio A260/A280 = 2.0 ± 0.07 and RNA integrity were obtained. The purified samples were reversely transcribed and the expression of atpD and fabM gene from *Streptococcus mutans* analyzed by quantitative real-time PCR. **Conclusion:** the extraction methodology developed produced high-quality RNA from dentine microbiota for transcriptional analysis.

Key words: Dentine Caries. RNA Extraction. Gene Expression. *Streptococcus mutans*.

Resumo

Introdução: o isolamento de RNA de bactérias dentro de lesões de dentina cariada pode ser difícil devido à quantidade reduzida de biomassa e alta instabilidade de mRNA. Na tentativa de superar esse desafio, descrevemos um protocolo desenvolvido para extrair e purificar o RNA total das lesões dentinárias. **Objetivo:** personalizar um método de extração e purificação de RNA bacteriano a partir da dentina cariada humana. **Métodos:** a quantidade e a pureza do RNA extraído foram medidas com um espectrofotômetro UV-VIS de microvolume, a integridade do RNA foi avaliada por eletroforese em gel de agarose desnaturante padrão e as imagens foram capturadas sob luz ultravioleta e analisadas. O tratamento com DNase removeu o DNA genômico e uma etapa adicional de purificação foi realizada em coluna de spin de sílica. **Resultados:** o rendimento final (ng / μ l) foi de $67,01 \pm 22,33$, a razão de absorvância A260 / A280 = $2,0 \pm 0,07$ e a integridade do RNA foram obtidas. As amostras purificadas foram transcritas reversamente e a expressão do gene atpD e fabM de *Streptococcus mutans* analisadas por PCR quantitativo em tempo real (RT-qPCR). **Conclusão:** a metodologia de extração desenvolvida produziu RNA de alta qualidade da microbiota dentinária para análises transcricionais.

Palavras-chave: Cárie Dentinária. Extração de RNA. Expressão Gênica. *Streptococcus mutans*.

INTRODUCTION

Dental caries is a progressive disorder that begins with demineralization of tooth tissues associated with dysbiosis of the colonizing microbiota¹. To have a better understanding about microbial roles in dental caries process, several molecular methods of investigation have been adopted in the last years. Among them, RNA-based analysis focus in detection of metabolically active microbial members and identification of

genes expressed under different circumstances^{2,3}. The majority of RNA-based investigations of oral microbiota analyzed only planktonic cells⁴, in vitro biofilms^{5,6,7} or in vivo biofilms from rodent animals^{8,9}. Although the first transcriptomic studies of human dental biofilm^{10,11} and dentine^{12,13} were carried out, just a few articles showed is given about technical procedures, RNA yield, purity and integrity from these human samples

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Conflict of interest: There are no conflicts of interest in this research.

Received: 1 Mar 2019; Revised: 10 Mar 2019; 20 Mar 2019; Accepted: 22 Mar 2019

studies^{13,14}. Besides, more attention has been requested about transcriptional studies in human dentinal caries, which can reflect the behavior of cariogenic bacteria in loco².

The success of any transcriptional analysis depends not only on the amount of RNA extracted, but mainly on RNA quality, because the purity and integrity of this molecule can impact the accuracy of techniques such as RT-qPCR and microarrays^{15,16}. Therefore, an absorbance ratio A260/A280 \approx 2.0 and detection of 16S and 23S rRNA bands with integrity and without smearing should be observed to consider a "high quality" intact RNA¹⁷.

Despite the carious dentine being a site of a diversity of bacteria, such as *Streptococcus mutans*, *Lactobacillus*, *Bifidobacterium* and *Veillonella*¹⁸, some particular limiting factors can hamper the obtaining of adequate quantity and quality of RNA, such as low amount of carious biomass per sample⁵ and low proportion of bacteria when compared to other in vivo samples (e.g., oral biofilm)^{19,20}. Furthermore, bacterial characteristics can also interfere with the adequate messenger RNA (mRNA) acquisition: (1) the high percentage of ribosomal RNA (rRNA) accounting for over 90 percent of total RNA, especially those with positive metabolic activity; (2) the relatively short half-life of mRNA; (3) rigid and thick cell walls of Gram-positive²¹. Besides, RNA concentration and quality can be definitely influenced by adequate and well-conducted extraction and purification techniques based on the nature of the samples²¹.

In an effort to solve possible challenges in RNA extraction from carious dentine and to establish an approach to enable bacterial transcriptional studies, the objective of this research was to customize a bacterial RNA extraction and purification method from human carious dentine.

MATERIAL AND METHODS

Study population and ethical statements

In order to collect the sample, one hundred eighty-one children from 48 to 71-month-old were examined in three public schools in Fortaleza, Ceará, Brazil, by a previously calibrated examiner (intraexaminer weighted kappa = 0.72). Of these, fourteen children were diagnosed with Early Childhood Caries (ECC) and met the inclusion criteria: the children had one or more primary teeth with at least one carious surface with distinct cavity and visible dentine without pulpal disease (confirmed by clinical and radiographic examination); the parent or guardian were willing to consent to the child's clinical examination and dentine sampling. The study excluded children that were sick or that had a bad behavior at the moment of the dental examination; and that had used antibiotics within the last 3 months. The study design was explained to the child's parent or guardian before the dental examination, from whom informed consent was obtained if they were disposed to participate with their child. The Ethics Committee of the Federal University of Ceará, protocol number 548.405, approved the study design, protocol, and informed consent.

Sample Collection

One tooth with visible dentinal caries selected from each child was cleaned with pumice and local anesthesia was delivered prior to sample collection. The carious teeth were isolated with rubber dam cleaned with 2% chlorhexidine and the carious dentine from cavities was removed using individual, sterilized and RNase-free spoon excavators (S.S. White Duflex, Rio de Janeiro, RJ, Brazil), whose size was determined by the dimension of the lesion. These instruments were submitted previously to autoclaving sterilization and heated at 200 °C for 18 hours to eliminate RNases²². During excavation, dentine hardness was checked and caries removal was considered completed when hard dentine was detected with a probe (S.S. White Duflex). Dentine was considered hard when, at applying a firm pressure, the probe was not able to penetrate into the tissue²³. It is important to note that the biofilm layer found over the lesions in contact with the oral environment was detached before sample collection. The samples were immediately transferred to sterile RNase-free microtubes (Axygen, Union City, CA, USA) containing RNA stabilizer solution (RNAlaterTM - Ambion Inc., Austin, TX, USA) and stored at 4 °C for 18 hours. After this period, the tubes were transferred to freezer (-80 °C) for storage until RNA extraction.

Cavities were lined and restored with a suitable restorative material and dental appointments were scheduled for continuing treatment if these were needed. In addition, the children and their parent or guardian were instructed about caries prevention.

RNA extraction and purification

The dentine samples were thawed and centrifuged (11,000 \times g/ 1 min/ 4°C). The RNAlater TM solution was removed using an automatic pipettor without disturbing the pellet. The samples were carefully transferred to cryogenic tubes containing 0.16 g of 0.1 mm diameter zirconium beads. The mechanical disruption of bacterial cells was made by Mini-Beadbeater (Biospec Products Inc., Bartlesville, OK, USA) at maximum power (2 cycles of 60 s with 1 min rest on ice). Then, 850 μ l of RLT buffer (Qiagen, Valencia, CA, USA) with 10% of β -mercaptoethanol (β -ME) was added and the suspension homogenized by vortexing. After centrifugation (11,000 \times g/ 2 min/ 4 °C), 2 aliquots of 350 μ l of the supernatant were transferred to microtubes with 250 μ l of pure ethanol, and vortexed. Next, the solutions were transferred to silica spin columns from RNeasy MinikitTM (Qiagen, Valencia, CA, USA) and centrifuged (11,000 \times g/ 30 sec/ 20 °C) to allow RNA binding to the silica. After discarded the flow-through, 700 μ l of RW1 buffer was added on it column, centrifuged (11,000 \times g/ 30 s/ 20°C) and discarded. Then, 500 μ l of RPE buffer was added, centrifuged (11,000 \times g/ 30 s/ 20°C) and discarded. This operation was repeated. After additional centrifugation (11,000 \times g/ 2 min/ 20°C) for removal of residual RPE buffer, each column was positioned in a new tube and 40 μ l of RNase-free water was dispensed on the center of it. After centrifugation (11,000 \times g/ 1 min/ 20 °C), the RNA through the

column was collected, and immediately stored at -80 °C freezer.

RNA concentration (ng/ µl) and purity (absorbance ratio A260/A280) were verified by Nanodrop 2000c microvolume spectrophotometer (Thermo Scientific, Wilmington, DE) and the integrity was verified by the detection of the 16S and 23S bands of rRNA in 1.2% formaldehyde agarose gel stained with ethidium bromide. Digital images were captured under ultraviolet light camera and analyzed by Gel Logic 200 Imaging System software (Eastman Kodak Co, Rochester, NY, USA).

DNase treatment and additional purification

The two aliquots of RNA obtained from each sample was joined and submitted to treatment with Turbo™ DNase kit (Applied Biosystems, Ambion, Austin, TX, USA) to removal genomic DNA. Five µl of enzyme and 9 µl of buffer from kit were added to RNA sample and incubated at 37 °C for 15 min. Then, an additional purification step was executed by addition of 300 µl of RLT and 250 µl of pure ethanol to the sample. This solution was vortexed and transferred to silica spin columns from RNeasy MiniElute™ CleanUp kit (Qiagen, Dus, Bundesland, Germany). The purification steps previously described, including addition of RW1 and RPE buffers were repeated. Next, to elute the ultra-purified RNA, 40 µl of RNase-free water were added in the center of the silica column and centrifuged (11,000 ×g/ 1 min/ 4°C). Concentration and integrity of the RNA were checked.

cDNA synthesis

The cDNA was synthesized from the RNA using iScript™ cDNA Synthesis Kit (Bio-rad, Hercules, CA, USA). Reverse transcription reactions were prepared with 6 µl of 5x iScript reaction mix, 1 µl of iScript reverse transcriptase, 1 µg of purified RNA and RNase-free water in sufficient amount for final volume of 30 µl. The prepared solution was homogenized by vortexing for 5 s and incubated at 25 °C for 5 min, heating at 42 °C for 2 h and incubated at 85 °C for 5 min in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Afterwards, the concentration of cDNA from all samples was adjusted to 10 ng/µl.

Sanger sequencing

Six cDNA samples were submitted to conventional PCR with primers for genes from *Streptococcus mutans* (SM) related to their tolerance acid: *atpD* (5'-TGTTGATGGTCTGGGTGAAA-3' and 5'-TTTGACGGTCTCCGATAACC-3') (Xu et al., 2011) and *fabM* (5'-CTGATTAATGCCAATGGGAAAGTC and 5'-TGCGAACAAAGAGATTGTACATCATC-3') 21 and subsequently to Sanger sequencing. The objective was to investigate specificity of the primers for SM genes in human samples, since these primers were used only in vitro studies. PCR assays were executed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA) and the reaction mix included 1 µl of Buffer PCR 10x, 1 µl of dNTPs 10mM, 2.5 µl of MgCl₂, 3 µl of each primer F/R 10 µM, 0.25 µl of Taq polymerase 5 U/µl, 50 mM 1 µl of

template cDNA (10 ng/µl) and 37.25 µl of nuclease-free water. The thermal-cycling consisted in 5 min at 95°C; 35 cycles of 15 s at 95 °C, 60 s at 60 °C and 20 s at 72 °C; 4 min at 60°C. Their products were purified with commercial kit (QIAquick® PCR Purification Kit, QUIAGEN, USA) and the sequencing reactions were performed using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) and submitted to thermal-cycling (20 s at 95 °C; 40 cycles of 15 s at 96 °C, 15 s at 50 °C and 4 min at 60 °C; 60s at 60 °C). At the end, the products were precipitated, dried, resuspended in 10 µl of formamide and denatured for 5 min at 95°C. Sanger sequencing occurred in a 3500 Series Genetic Analyzer (8-capillary) (Applied Biosystems, Foster City, CA, USA). The sequencing data obtained was submitted to BioEdit software, version 7.2.5, to obtain contigs sequences, The BLAST (Basic Local Alignment Search Tool; <http://estexplorer.biolinfo.org/hsd>) program was used to analyze sequences similarity.

Reverse Transcription – Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

In order to get DNA from *S. mutans* UA159 to obtain standards curves, this strain was cultured in broth for 24 h as recommended by Bergey's Manual of Determinative Bacteriology²⁵. After centrifugation and washing in sterile saline solution (sodium chloride 0.9%), the quality and purity of bacterial cultures were checked by Gram staining and their DNA was recovered using an organic extraction protocol based on phenol/chloroform purification and alcohol precipitation²⁶. Serial dilutions starting from 600 ng to 0.0006 ng (10-fold) of *S. mutans* DNA concentrations were used as standards and positive controls for relative quantification of the targeted bacteria. A standard amplification curve and a melting-point product curve were obtained for each primer set.

The RT-qPCR assays were performed to test the functionality of RNA extracted in gene expression studies using a StepOne™ Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The reactions were performed using MicroAmpFast Optical 48-Well Reaction Plate (Applied Biosystems, Foster City, CA, USA) covered with Optical Adhesive Film (Applied Biosystems). A mixture of 5 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 2.4 µl of nuclease-free water, 0.3 µl of each primer F/R 10 µM and 2 µl of cDNA (20 ng) was added in each spot of a 48-well plate. Assays were carried out in duplicate, and the final analyses were based on the mean of the two reactions. Negative control included reactions without template. The standard curves were used to transform the quantification cycle (Cq) values to the mass of cDNA amplified. The normalization of gene expression was made using the ratio between final mass of cDNA detected and cDNA amount initially inserted in each reaction.

RESULTS

A total of 14 dentine caries samples was collected and yielded 25.5±9.7 mg average of dentine carious tissue

(Values: maximum= 39,4 mg and minimum= 10,3 mg). After applying the customized method of total RNA extraction and purification from this substrate, we obtained adequate RNA yield (67.01 ± 22.33 ng/ μ l - values: maximum= 130,3 ng/ μ l and minimum= 46,05 ng/ μ l), purity ($A_{260}/A_{280} = 2.00 \pm 0.07$) and

integrity (Table 1 and Figure 1). Results of atpD and fabM gene expression for Streptococcus mutans were also shown in table 1. Amplification plot, standard curve and melt curve for atpD and fabM gene expression were presented in Figure 02a and Figure 2b, respectively.

Table 1. RNA yield before and after DNase treatment, purity and gene expression values of S. mutans from dentine lesions (n=14). Values are mean \pm standard deviation. cDNA mass used to begin the RT-qPCR (20 ng) was used for normalization of gene expression.

Initial yield (ng/ μ l)	Final yield (ng/ μ l)	Ratio (A260/A280)	atpD gene expression	fabM gene expression
92.54 ± 27.15	67.01 ± 22.33	2.00 ± 0.07	0.007 ± 0.010	0.007 ± 0.012

a = atpD mass (ng)/ 20 ng cDNA; b= fabM mass (ng)/ 20 ng cDNA

Figure. 1. RNA integrity (16S and 23S bands) (n=14).

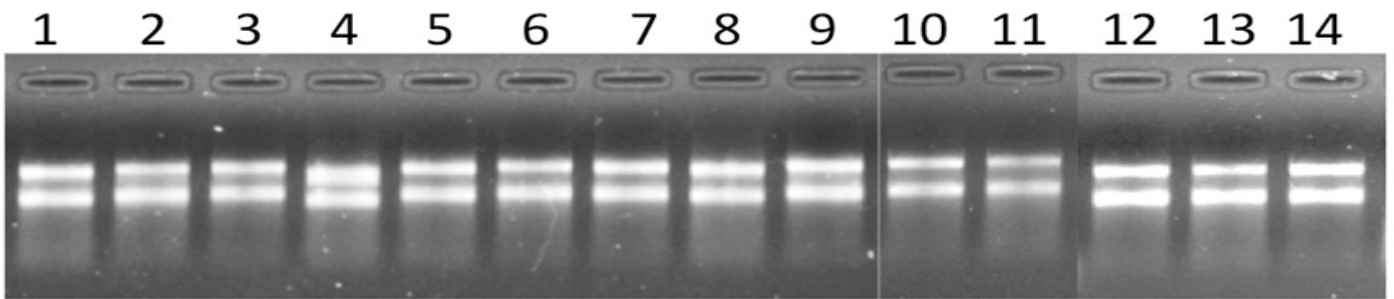


Figure 2a. RT-qPCR amplification plot and standard curve data for atpD gene from S. mutans. The values of expression detected for the tested samples (depicted in blue) are within the standard curve range (depicted in red).

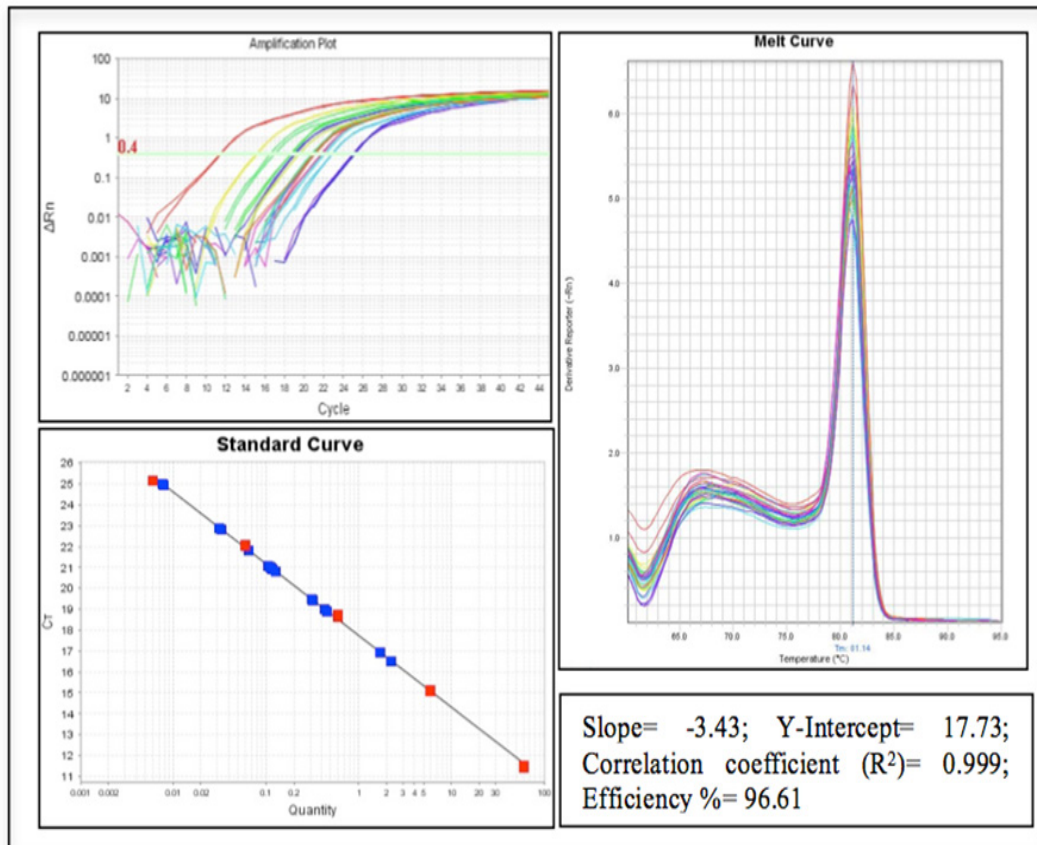
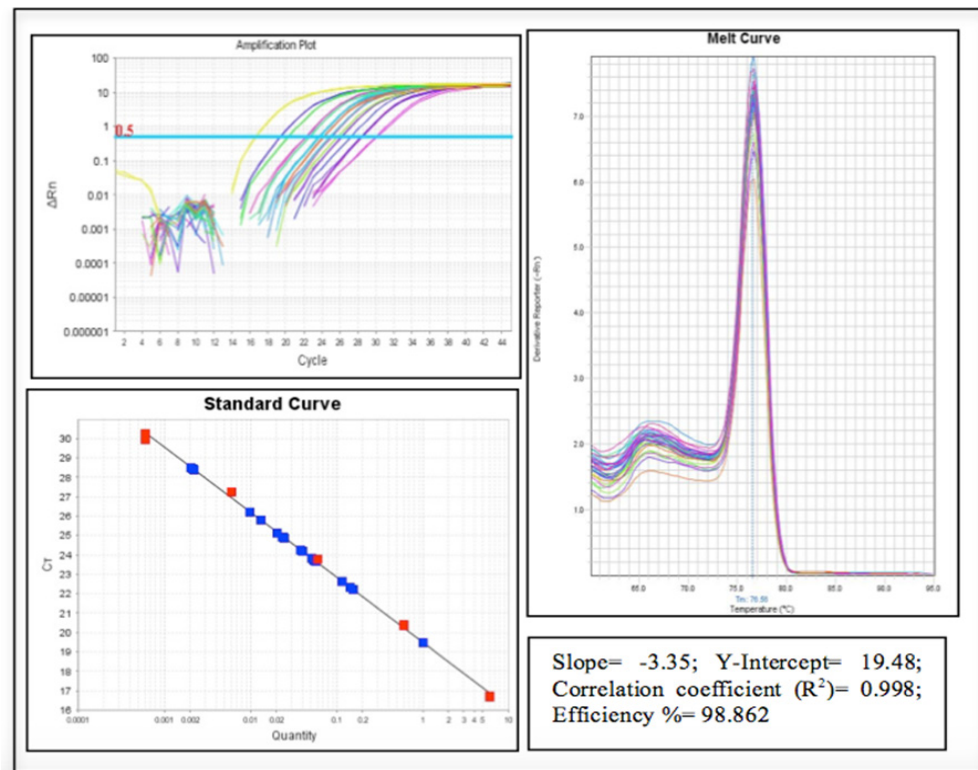


Figure 2b. RT-qPCR amplification plot and standard curve date for fabM gene from *S. mutans*. The values of expression detected for the tested samples (depicted in blue) are within the standard curve range (depicted in red)



DISCUSSION

Considering the importance of a better understanding of the bacterial behavior in carious sites², the current research established a methodology for RNA extraction and isolation to enable bacterial gene expression studies from human carious dentine samples. The majority of transcriptional studies of cariogenic bacteria was performed in planktonic cells in vitro and in vivo biofilms^{7,9,11}, substrates where higher bacterial concentration is found when compared to dentine caries lesions¹⁹.

Ecological factors of dentin caries have been poorly studied, possibly due to the complex structural composition of this tissue²⁵. Besides that, other limitations factors, such as low amount of carious biomass per sample, mainly in deciduous teeth, due the proper low dimension of the teeth organ, could hinder the achievement of RNA extraction²⁷. Some studies used in vivo carious dentine tissue from extracted permanent teeth from adults with large occlusal soft and active carious lesions, which enables a higher quantity of carious dentinal tissue¹⁴.

Several studies have described different RNA extraction protocols for different tissues, however, each one showed particular difficulties for isolation of proper amounts and high quality of RNA, since the efficiency of each method is highly dependent of the tissue composition^{28,29}. In this study, the samples were collected from deciduous teeth and yielded an average weight of 25.5±9,7 mg of dentine per lesion, which is

considered a low amount, when compared to other substrates, such as biofilms from in vitro studies (≈100 mg dry weigh)³⁰. Moreover, a reduced number of bacteria within dentine in relation to in vivo biofilms¹⁹ and the reduced quantity of mRNA in metabolically active bacteria¹¹ can hamper the achievement of acceptable quantity and quality of RNA. However, despite the nature of the tissue, our results showed yield (67.01 ± 22.33 ng/ μl), purity (A260/ A280 = 2.00 ± 0.07) and integrity of RNA sufficient for RT-qPCR assays, revealing a successful isolation protocol for extraction of RNA for dentine carious tissue.

Our adequate results were acquired from a customized method based on mechanical cell disruption (bead-beating) and a commercial kit. Mechanical disruption has several advantages over the traditional methods of cell lysis (lysis buffer, manual disruption and liquid nitrogen) including reduction of work time, multiple sample extraction and a reduction of cross contamination risks³⁰. Moreover, this is a recognized method to obtaining better RNA yield and quality^{6,8,25}, due to its capacity of lysing any bacterial cell wall and release all nucleic acids from the cells without degrade them²⁷. Besides, we used a commercial extraction kit based on guanidine thiocyanate salts added of β-ME, which irreversibly denature RNases³⁰, reduces time-consuming and avoid contact with toxic products such as phenol/chloroform³⁰, occurring with previous techniques for RNA extraction from oral biofilms^{5,9,32}. Authors was found relating RNA extraction from human carious dentine, using lysis buffer associated with sonication or commercial kit singly or

associated to mechanical disruption, however no details about methodology steps, yield and quality of RNA extracted were demonstrated^{12,13,34}.

Additional RNA purification with RNeasy MiniElute™ CleanUp kit was performed in our research to guarantee the elimination of salt and other possible contaminants^{34,35}, as well as to improve RNA yield^{33,31}. The purified RNA samples showed good performance in reverse transcriptase reactions, since the cDNA templates were amplified with efficiency in RT-qPCR assays. These reactions were performed with *S. mutans* primers associated with bacterial virulence (atpD and fabM), responsible for its acidogenic and aciduric properties. Besides, even in low presence in the bacterial population from carious dentine as cited by some authors^{9,12,17}, *S. mutans* genes were detected in our RT-qPCR, confirming the efficiency of this customized RNA extraction method.

CONCLUSION

The cell mechanical disruption associated to the adapted use of commercial kits was considered an acceptable RNA extraction and purification method from carious dentine and, consequently, satisfactory for bacterial gene expression studies. With this finding, bacterial transcriptional researches in human dentinal caries can be well conducted and can promote new knowledge about behavior of bacteria in dentinal sites.

ACKNOWLEDGMENTS

This study was supported by CNPq (475346/2011-4 -MCT/CNPq 14/2011 Universal).

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How to cite this article/Como citar este artigo;

Bezerra DS, Neves BG, Guedes SFF, Regis WFM, Stipp RN, Rodrigues LKA. Extraction and purification of RNA from human carious dentine: an approach to enable bacterial gene expression studies. *J Health Biol Sci.* 2019 Abr-Jun; 7(2): 145-151.