Ribonucleic Acid Extraction From Archival Formalin Fixed Paraffin Embedded Myocardial Tissues for Gene Expression and Pathogen Detection

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Introduction: Archival tissue samples preserved in formalin are a great source of treasure for biomedical research and diagnostics. Formalin, though is a good preservative, causes the modification of nucleic acid limiting the application of fixed tissues. The present study evaluated three methods of RNA extraction for constitutive gene expression and pathogen detection. **Material and methods:** Sixteen archival formalin-fixed paraffin-embedded (FFPE) myocardial tissues were subjected to RNA extraction by Trizol, SDS, and RNeasy FFPE kit followed by RT-PCR and Taqman Real-Time PCR to study the expression of housekeeping genes. **Results:** RNA was extracted from all 16 myocardial tissues (100%) by RNeasy FFPE kit, as compared to 14/16 by Trizol and 8/10 by SDS methods. The expression of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was observed in RNA extracted by RNeasy FFPE kit and

Trizol. High yield of RNA was obtained by RNeasy FFPE kit than Trizol ($P = 0.002$) and SDS ($P = 0.012$). Of the three methods, RNeasy FFPE kit was evaluated for Enterovirus RNA detection in 16 other histopathologically confirmed FFPE tissues of dilated cardiomyopathy (DCM) cases and Enterovirus genome was detected in 4/16 (25%) FFPE tissues of DCM cases. The enteroviral sequences of the viral isolates revealed 99% homology with Human coxsackievirus B5. **Conclusion:** The Qiagen RNeasy FFPE kit resulted in significantly high reproducibility of RNA from FFPE myocardial tissues, which are suitable for amplification by Taq-Man Real-Time and RT-PCR. Thus, the results show that these FFPE tissues can be used for gene expression, pathogen detection, and epidemiological studies. J. Clin. Lab. Anal. 26:279–285, 2012. © 2012 Wiley Periodicals, Inc.

Key words: RNA extraction; archival tissues; formalin-fixed paraffin-embedded; gene expression; virus detection

INTRODUCTION

Archival tissue samples are a great source of treasure for biomedical research. With the advent of sophisticated molecular techniques such as reverse transcriptase polymerase chain reaction (RT-PCR), Real-Time PCR, and microarray, the interest has generated to explore the utility of archival samples in disease identification, diagnosis, and development of novel therapeutic strategies (1–3). Archival tissue samples are usually present in formalin or embedded in paraffin after formalin fixation. Formalin, though acts as a good preservative to maintain the tissue

integrity, causes the degradation and modification of nucleic acid leading to poor recovery of nucleic acid from preserved tissues (4, 5). Since the sensitivity of the molecular techniques depends upon the yield and quality of

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nucleic acid, the standardization of nucleic acid isolation from preserved archival tissues becomes highly essential.

Presently, various commercially available kits are available for isolation of nucleic acid from formalin-fixed paraffin-embedded (FFPE) tissue blocks, whereas Trizol and Sodium Dodecyl Sulfate (SDS) methods are still widely used in many molecular laboratories (6, 7). The present study therefore compared the Trizol (Tri Reagent, GIBCO, California, USA) and SDS (Amresco, Solon, US) methods with commercially available RNeasy FFPE kit (Qiagen, Hilden, Germany) for extraction of RNA, its quantitation, qualitative assessment, and study of expression of various constitutive genes by RT-PCR and Taqman Real-Time RT-PCR.

FFPE tissue has an added advantage for application in retrospective studies. Thus, in this study, we evaluated the optimized-RNA extraction protocol of RNeasy FFPE kit (Qiagen) for enterovirus genome (the most common etiological agent of viral myocarditis) detection in FFPE myocardial tissues of dilated cardiomyopathy (DCM) patients.

MATERIALS AND METHODS

Tissue Samples

Sixteen FFPE myocardial tissue blocks (3–10 years old) were randomly selected from the archives of Institute's pathology department. All the tissues were fixed in 10% buffered formalin before paraffin embedment. Each block contained one piece of myocardial tissue measuring at least 1.5×1.5 cm in area. A histological section of 10- μ m thickness was cut from each block using conventional microtome. A separate sterile blade was used for each block to prevent carryover contamination. All the reactions were carried out in RNase-free environment after cleaning instruments, autopipettes, and working bench top with RNaseZap (Ambion, Inc., Austin, TX). Sixteen histopathologically confirmed FFPE tissues of DCM cases were also collected from the archives for enterovirus detection. The study confirmed to Institution Ethics Committee of Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India.

RNA Extraction

Three different protocols—Trizol (Tri Reagent), SDS (Amresco) and RNeasy FFPE kit (Qiagen, Hilden, Germany) with minor modification—were used in parallel for the extraction of RNA from FFPE myocardial tissues. The protocol included deparaffinization, proteinase K digestion, extraction, elution or hydration procedures, and DNase treatment to obtain DNase free RNA (Table 1).

RNA Analysis

RNA was quantified by reading absorbance at 260 nm using spectrophotometer (Analytic, Jena, Germany). The purity of extracted RNA was calculated from ratio of absorbance at 260 nm: 280 nm.

Expression of Housekeeping Genes

The RNA extracted by different methods was tested for expression of two different housekeeping genes: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and RnaseP.

Expression of GAPDH by RT-PCR

Expression of GAPDH was studied by RT-PCR. RNA extracted from the FFPE tissues by all the three methods was reverse transcribed to cDNA using random hexamer and murine Moloney Leukemia Virus (mMULV) reverse transcriptase (MBI Fermentas, Hanover, USA) following manufacturer's instructions and stored at -20°C . The cDNA was then subjected to GAPDH RT-PCR using the protocol and primers supplied by MBI Fermentas.

Forward: 5'CAAGGTCATCCATGACAACCTTTG 3'

Reverse: 5'GTCCACCACCCTGTTGCTGTAG 3'

The thermal profile of the reaction was as follows: initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 45 sec. The PCR product of 496 bp was analyzed by 2% agarose gel electrophoresis.

Expression of RNaseP by real-time RT-PCR

To assess the quality of RNA extracted from FFPE tissues and its applicability in gene expression studies, the expression of common housekeeping gene *RNase P* was studied by Real-Time PCR using Taqman primers and probe (ABI, Foster City, CA) (8). Real-time PCR was performed using Light Cycler 480 II (Roche). The reaction mixture contained $1 \times$ reaction master mix, $0.5 \mu\text{l}$ primer mix, $1.0 \mu\text{l}$ enzyme mix, and 500 ng RNA input. The thermal profile used was as following: reverse transcription at 50°C for 30 min and Taq inhibitor inactivation at 95°C for 10 min followed by 45 cycles each of PCR amplification at 95°C for 15 sec and 55°C for 32 sec.

Enterovirus genome detection by RT-PCR

RNA extracted using RNeasy FFPE kit (Qiagen) was evaluated for enterovirus genome detection in myocardial FFPE tissues. RNA was reverse transcribed using Revert-Aid cDNA synthesis kit (MBI Fermentas) following manufacturer's instructions. Five microliter of

TABLE 1. Comparison of different methods of RNA extraction from archival tissue samples

	Trizol (Tri Reagent, GIBCO)	SDS (Amresco)	RNeasy FFPE kit (Qiagen)
Deparaffinization	Xylene: 37°C for 30 min	Xylene: 37°C for 30 min	Xylene: RT for 15 sec and centrifuged at 10,000 rpm for 2 min
	Ethanol (100%, 70%, and 50%) washes: 37°C for 15 min	Ethanol (100%, 70%, and 50%) washes: 37°C for 15 min	Ethanol (100%): 15 sec and centrifuged at 10,000 rpm for 2 min
Proteinase K digestion	Trizol 750 µl + Proteinase K (20 mg/ml) (Sigma, St. Louis, Missouri) 60 µl + RNase Inhibitor (MBI Fermentas, USA) 2 µl 60°C overnight	lysis buffer (10 mmol/l Tris, 0.1 mmol/l EDTA, 2% SDS) 540 µl + Proteinase K (20 mg/ml) (Sigma) 60 µl + RNase Inhibitor (MBI Fermentas, USA) 2 µl 60°C for 20 hr/till tissue dissolves completely	PKD buffer 150 µl + Proteinase K 60 µl 55°C for 15 min 80°C for 15 min
RNA isolation	Extraction: Chloroform 300 µl at 20,000 rpm for 20 min at 4°C Precipitation: isopropanol 800 µl -20°C overnight Washing: 75% ethanol	Extraction: Phenol: Chloroform (70%:30%) at 20,000 rpm for 20 min at 4°C Precipitation: isopropanol 1 vol and sodium acetate 0.1 vol -20°C overnight Washing: 75% ethanol	Binding: PBC buffer 320 µl Undigested tissue removal: gDNA eliminator column RNA isolation: Elute spin column Washing: PE Buffer 500 µl
Elution	20 µl RNase free water	20 µl RNase free water	20 µl RNase free water
DNase treatment	2 µg RNA + 0.5 µl DNase I (10 KU) incubation at 37°C for 30 min 2 µl of 25 mM EDTA incubation 65°C for 5 min	2 µg RNA + 0.5 µl DNase I (10 KU) incubation at 37°C for 30 min 2 µl of 25 mM EDTA incubation 65°C for 5 min	2 µg RNA + 0.5 µl DNase I (10 KU) incubation at 37°C for 30 min 2 µl of 25 mM EDTA incubation 65°C for 5 min

cDNA was amplified in 50 µl of reaction mixture according to the method described by Paushinger et al., 1999 (9). The reaction contained 50 mmol/l KCl and 10 mmol/l Tris-HCl, 1.5 mmol/l MgCl₂, 0.3 µM each primer, and 2.5 U Taq-polymerase. The thermal profile of the amplification was 30 cycles of denaturation at 94°C for 45 sec, annealing at 61°C for 45 sec, and extension at 72°C for 90 sec. Cocksackievirus B (CVB) infected tissue culture supernatant was used as positive control. Ten microliters of PCR product was analyzed by 2% agarose gel electrophoresis and then was visualized and documented by UV transilluminator using the gel documentation system.

Sequencing

The specific bands for enteroviral genomes were purified by Gel Extraction kit (Qiagen) according to the manufacturer's instructions and the purified DNA was subjected to nucleotide sequencing by Genetic Analyzer 310, Applied Biosystems Inc., USA, using an ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (PE Applied Biosystems Inc., Foster City, CA). The sequence product was confirmed by searching the database using BLAST program available at NCBI web site.

Statistical Analysis

The means of RNA quantity extracted by three different protocols were compared by Wilcoxon Signed Rank test using SPSS version 18.0. The confidence intervals were set at 95%. Statistical significance was concluded if the *P* value was < 0.05.

RESULTS

Comparison of Different Methods for RNA Extraction

RNA could be extracted from all 16 myocardial tissues (100% positivity) by RNeasy FFPE kit (Qiagen) whereas the same was positive in 87.5% (14/16) and 80% (8/10) in samples extracted by Trizol method (Tri Reagent, GIBCO), and SDS method (Amresco) respectively. The quantity of RNA extracted by RNeasy FFPE kit (Qiagen) was significantly higher as compared to Trizol (TriReagent GIBCO) method (*p* = 0.002) and SDS method (*p* = 0.012). The comparison of three different methods is shown in Table 2.

TABLE 2. Comparative analysis of RNA extracted by three different methods from FFPE tissues

S. No	Trizol method		SDS method		RNeasy FFPE kit kit (Qiagen)	
	RNA qty	Ct	RNA qty	Ct	RNA qty	Ct
1	28 µg/ml	33	60 µg/ml	Undetected	144 µg/ml	32
2	8 µg/ml	30	50 µg/ml	33	310 µg/ml	30
3	54 µg/ml	40	54 µg/ml	Undetected	174 µg/ml	34
4	4 µg/ml	40	NIL	Undetected	508 µg/ml	40
5	NIL	Undetected	NIL	Undetected	398 µg/ml	32
6	38 µg/ml	40	24 µg/ml	Undetected	358 µg/ml	35
7	48 µg/ml	38	38 µg/ml	Undetected	232 µg/ml	31
8	10 µg/ml	Undetected	50 µg/ml	Undetected	238 µg/ml	32
9	8 µg/ml	35	32 µg/ml	Undetected	226 µg/ml	31
10	226 µg/ml	33	34 µg/ml	Undetected	588 µg/ml	30
11	128 µg/ml	32	NIL	Undetected	502 µg/ml	28
12	182 µg/ml	31	NIL	Undetected	314 µg/ml	28
13	NIL	31	NIL	Undetected	290 µg/ml	29
14	162 µg/ml	37	NIL	Undetected	158 µg/ml	31
15	84 µg/ml	33	NIL	Undetected	282 µg/ml	30
16	296 µg/ml	31	NIL	Undetected	290 µg/ml	30

Expression of GAPDH by RT-PCR

mRNA of GAPDH was detected in RNA of all the 16 samples (100%) extracted by RNeasy FFPE kit (Qiagen), 12/16 RNA samples extracted by Trizol method (Tri Reagent, GIBCO), and in none of the RNA sample extracted by SDS method (Amresco) (Fig. 1).

Expression of RNase P by real-time PCR

RNase P was detected in all the 16 RNA samples extracted by RNeasy FFPE kit (Qiagen) and consistently higher levels of *RNase P* expression was observed as shown by lower C_t values compared to that of Trizol (Tri Reagent, GIBCO) and SDS (Amresco) methods. *RNase*

Lane-1: 100bp Molecular Marker (Fermentas, USA); Lane-2: No template control of RT-PCR; Lane3 & 7: GAPDH expression in RNA extracted from myocardial tissue samples by RNeasy FFPE kit; Lane-4: Negative control of cDNA synthesis; Lane5 & 6: GAPDH expression in RNA extracted from myocardial tissue samples by Trizol method

Fig. 1. Agarose gel analysis of RT-PCR for expression of GAPDH in myocardial tissue samples.

Fig. 2. (A) Amplification curve of RNA extracted from FFPE tissues using RNeasy FFPE kit (Qiagen) in real-time PCR. (B) Amplification curve of RNA extracted from FFPE tissues using Trizol method in Real-Time PCR.

P was detected in 14 RNA samples that were extracted by Trizol (Tri Reagent, GIBCO) method whereas SDS (Amresco) method could detect one of ten samples tested (Figs. 2A and 2B).

Enterovirus Genome Detection in Myocardial Tissues

Enterovirus was detected in four of 16 (25%) myocardial tissue samples collected from DCM cases (Fig. 3).

Fig. 3. Agarose gel analysis of persistence of CVB genome in myocardial tissues.

The consensus enteroviral sequences of the noncoding 5' region of 314 nucleotides obtained from the viral isolates revealed 99% homology with Human coxsackievirus B5.

DISCUSSION

RNA analysis is usually done in fresh or snap-frozen tissues by RT-PCR, Real-Time PCR, northern blotting, and microarrays. However, gene expression studies and RNA extraction from archival samples has always been a challenge. Biopsies and surgical samples are fixed in formalin and paraffinized for routine pathological examination. Although these preservation methods have been optimized for histological and immunochemical analysis of FFPE samples but, are not considered suitable for RNA extraction due to poor RNA yield, small size of extracted fragments, and low levels of detectable RNA in the extracts (10, 11). Thus, optimization of RNA extraction from FFPE tissues is required for applicability in molecular studies.

All the three methods of RNA extraction from FFPE tissues included three steps: deparaffinization, digestion, and purification. While the deparaffinization and RNA purification steps were kept similar in Trizol (Tri Reagent, GIBCO) and SDS (Amresco) methods, the digestion step was modified in all the three methods. Proteinase K digestion has been found to be critical in RNA extraction protocols as it can degrade proteins covalently linked to RNA and also inactivates RNases thus, allowing efficient RNA extraction from FFPE tissues (12, 13). In Trizol method, lysis was done by Trizol (Tri Reagent, GIBCO) with a high concentration of 1.2 mg/ μ l of Proteinase K (Sigma) and addition of RNase Inhibitor (MBI Fermentas). This increased RNA stability and allowed complete breakage of RNA-protein complexes formed due to formalin fix-

ation and thus, led to more RNA yield. In RNeasy kit method, digestion step was modified by adding 1.2 mg/ μ l of Proteinase K, which possibly digested the tissue completely and released total RNA within 15 min of incubation. Further, incubation at 80°C for 15 min before purification denatures Proteinase K thus, avoiding damage to RNA. RNeasy kit uses the 100% ethanol for binding the sample to column filter cartridges provided in the kit, affecting the total RNA yield and release of longer RNA fragments from RNA-protein complexes (2). However, in SDS (Amresco) method, as reported earlier (14), digestion was done with lysis buffer (10 mmol/l Tris, 0.1 mmol/l EDTA, 2% SDS) supplemented with high concentration of Proteinase K (Sigma) and RNase Inhibitor (MBI Fermentas) for a longer time. This possibly resulted in lower RNA yield and shorter RNA fragments, which could not be detected by Real-Time PCR. The degradation of RNA could be due to use of strong lysis buffer in this method.

Besides the robustness of extraction process, the RNA yields also depends on changes in tissue conformation during sectioning, differences in postsurgery tissue handling, fixation procedures, and duration of tissue storage (2). Thus, large-scale analysis on FFPE tissues is problematic due to limited yield of extracted RNA (15, 16). However, RNA could be extracted from FFPE tissues in significant amount and of better quality, which was further used for the expression of various constitutive genes.

The modified protocol of RNeasy kit (Qiagen) was employed in other 16 histopathologically confirmed FFPE tissues of DCM cases for the detection of enterovirus genome where 25% of myocardial tissues of DCM cases were found to be positive for enterovirus genome, which were confirmed by sequencing.

CONCLUSION

The recovery and amplification of RNA from FFPE tissues is a growing concern in retrospective studies. In this study, RNA could be extracted from FFPE tissues using different protocols, however, higher yield was achieved with RNeasy kit with minor modification. RNA extracted from FFPE tissues was suitable for amplification by TaqMan Real-Time PCR and RT-PCR, and could successfully demonstrate enterovirus genome in these tissues of DCM cases. Thus, the results show that the archive of FFPE tissues is valuable in retrospective, molecular, and genomic studies.

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