Coextraction and PCR Based Analysis of Nucleic Acids From Formalin-Fixed Paraffin-Embedded Specimens

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Background: Retrospective studies of archived human specimens, with known clinical follow-up, are used to identify predictive and prognostic molecular markers of disease. Due to biochemical differences, however, formalin-fixed paraffin embedded (FFPE) DNA and RNA have generally been extracted separately from either different tissue sections or from the same section by dividing the digested tissue. Our optimized co-extraction approach provides the option of collecting DNA, which would otherwise be discarded or degraded, for additional or subsequent studies because of the high importance and less availability of clinical FFPE specimen. **Methods:** Coextraction of DNA and RNA from a single gastric cancer FFPE specimen was optimized by using TRIzol and purifying DNA from the lower aqueous and RNA from the upper organic phases.

The protocol involves modification of incubation period for 30 min with proteinase K in glycine-tris-ethylenediamine tetra acetic acid buffer before adding TRIzol. **Results:** All samples tested successfully performed semiquantitative gene expression by reverse transcriptase PCR. The quantity and quality of DNA from FFPE samples was high which resulted in successful PCR amplification. The isolated DNA also aided in detection of *Helicobacter pylori* by amplifying the ribosomal 16S gene in a multiplex PCR reaction along with *cagA*. **Conclusion:** These results show that the RNA/DNA isolated by this method can be used for easy clinical diagnosis of disease-related gene expression as well as mutation and pathogen detection from a homogenous population of tumor cells. J. Clin. Lab. Anal. 29:485–492, 2015. © 2014 Wiley Periodicals, Inc.

Key words: archived samples; *Helicobacter pylori*; mitochondrial DNA; nucleic acid co-extraction; PCR diagnosis; reverse transcription

INTRODUCTION

Formaldehyde (HCHO), a principal ingredient of most commonly used tissue fixatives, does not physically degrade nucleic acids, but it leads to the generation of DNA-protein (1, 2) and RNA-protein (3) cross-linkages. Archived human specimens, with known clinical follow-up, represent a valuable resource for retrospective molecular studies and identification of biological markers that might be useful for risk prediction of disease or prognosis (4). Genomic DNA recovered from archived specimens, while degraded, can be analyzed by polymerase chain reaction (PCR) (5, 6), gene sequencing (7), and methylation assays (8–10). Contrastingly, RNA molecules recovered from paraffin-embedded samples display a large extent of degradation, and thus many studies aimed at representing their suitability for molecular analyses and specific protocols have been established for reverse transcription

PCR (RT-PCR) (5, 11), high-throughput gene expression (4, 12–14), and sequencing (15, 16).

Currently, different kit based methods have been developed for extracting DNA and RNA from paraffin-embedded samples (17, 18). Some methods are available to isolate DNA and RNA separately from the same sample (19). However, we were unable to find a protocol that allows simultaneous isolation of DNA and RNA from paraffin-embedded samples by a conventional and cost effective method. Instead, DNA and RNA are usually

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isolated from the same cell type but from adjacent tissue sections, thus possibly introducing errors if one is interested in determining how genomic changes correlate with gene expression changes. A method allowing simultaneous DNA and RNA extraction from the same population of cells would overcome this type of limitation, as well as address three additional issues: (1) limited availability of clinical tissues for research purposes; (2) time taken for separate DNA and RNA isolation protocols, and (3) the high cost of kit based methods.

For mitochondrial DNA (mtDNA) mutation and gastric cancer pathogen such as *Helicobacter pylori* genotyping studies, relatively good quality of DNA is required for PCR analysis (20, 21). *H. pylori* infection in gastric specimens can be demonstrated through the use of culture, histological examination, urease activity, and PCR assays (22, 23). It is well known that the PCR assay is highly reliable in the detection of *H. pylori*.

In this study, we wanted to determine if genomic DNA and total RNA could be effectively coextracted from paraffin-embedded specimens within a single reaction. Therefore, we optimized a coextraction method using TRIzol, which is the most trusted reagent for total RNA extraction from fresh tissues, because it allows DNA/RNA phase separation and recovery from fresh tissues (24). Then, using five human archived gastric cancer specimens, we quantitatively showed the PCR amplification of mitochondrial and *H. pylori* genes and expression of nuclear genes from the isolated total RNA.

MATERIALS AND METHODS

Sample collection and processing

Paraffin embedded human gastric cancer tissue block of patients, with or without family history of cancer (Age: 35–63 years), receiving treatment at Mizoram State Cancer Institute, Aizawl, India were collected between April, 2013 and June 2013. Medical records were reviewed using a standard protocol to obtain information on cancer treatment, clinical stage, and cancer characteristics, extent of damage in mucosal part of stomach tissue. Senior pathologists reviewed the pathology slides to confirm the diagnosis of stomach cancer and their stage. Detailed information on demographic factors, dietary habits, previous disease history, physical activity, tobacco and alcohol use, weight history, and family history of cancer was collected during an in person interview by trained study interviewers using a structured questionnaire. The study protocol was approved by the Institutional Review Board of all institutes involved in the study. All patients participated in this study with their full consent. From each block 5 μ m sections were cut on a standard microtome (Leica-Microsystems) after treating the blade with DEPC,

placed individually in DEPC treated eppendorf tube, and stored at 4°C until extraction.

RNA/DNA coextraction from the tissue samples

Deparaffinization was carried out by adding 1 ml of xylene to the tissue section in each microfuge tube followed by vigorous vortexing for 10 min. The mixture was centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and the deparaffinization steps were repeated once again, followed by rehydration through subsequent washings with 100%, 90%, and 70% absolute ethanol diluted in RNase free DEPC treated water, respectively. The remaining tissues were collected after centrifugation at 12,000 rpm for 5 min after each step. After a 70% ethanol wash, the sample was suspended in 10 ml glycine-tris-ethylenediamine tetra-acetic acid buffer (100 mM glycine, 10 mM Tris-HCl - pH 8.0, 1 mM EDTA) and incubated at 55°C in a shaking water bath for 30 min with 20 μ l of proteinase K (10 mg/ml) and DTT (2 mM). The supernatant was discarded and the tissue pellet dried, followed by the addition of 1 ml of TRIzol solution. The tissue was homogenized, by a glass rod, with the TRIzol solution and mixed with TRIzol solution by roto-spin (Tarson, India) at room temperature for at least 5 min to dissociate nucleoprotein complexes. A 0.2 ml of chloroform was added and the tubes were vortexed vigorously for 15 s at room temperature for 2 to 3 min followed by centrifugation at 12,000 rpm for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and the lower phase was kept at –20°C for further DNA isolation. RNA was precipitated by adding 10 μ g of glycogen to 0.6 ml of isopropyl alcohol with the aqueous phase followed by incubation at –20°C for at least 1 h and centrifuged at 12,000 rpm for 10 min at 2–8°C. The pellet was washed in 1 ml of 100% ethanol, air-dried at room temperature, and dissolved in RNase-free water after DNase treatment. RNA integrity checked by electrophoresis on 1.8% agarose gels stained with ethidium bromide. The yield and quality [260/280 optical density (OD) ratios] of RNA product were measured by a spectrophotometer (Cary-UV, Agilent technology, Germany).

DNA was precipitated by adding 1,000 μ l of ethanol and 30 μ l of sodium acetate, in the lower phase which was kept in –20°C followed by incubation at room temperature for 3 min and centrifugation at 16,000 rpm for 30 min at 4°C. The DNA pellet was washed with 100% ethanol, air-dried at 50°C, resuspended in 600 μ l of cell lysis buffer (500 mM Tris pH 9.0; 20 mM EDTA; 100 mM NaCl) with 10 μ l of *Proteinase K* (10 mg/ml) and 20 μ l of 100 mM *dithiothritol* (DTT) followed by incubation at 56°C for 1 h. The sample was soaked in TE9 buffer (500 mM Tris pH 9.0; 20 mM EDTA; 100 mM NaCl) at 37°C for 2 h, with single buffer change. Tissues

were then minced thoroughly and 1 ml lysis buffer plus [50 µl 20% SDS, 0.5% TritonX100, 15 µl dithiothreitol (8 mg/ml)], and 10 µl proteinase K (10 mg/ml) were added and incubated at 55°C in a water bath for 1 h. Further, 20 µl each of SDS and proteinase K were added and incubated for 1 h at 65°C in a water bath. Centrifugation was performed at 10,000 rpm for 15 min, the supernatant was taken in a separate 2 ml tube and DNA was extracted twice with phenol, chloroform, *Isoamyl* alcohol (25:24:1), and once with chloroform washing. DNA was precipitated by adding double the amount of ice cold isopropyl alcohol and 3 M sodium acetate (1/10 of total volume) to the supernatant followed by 2 h precipitation at -20°C. The solution was centrifuged at 10,000 rpm for 10 min at 4°C to pellet out the DNA which was subsequently washed with freshly prepared 70% ethanol, air dried, re-suspended in 80 µl 1X TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA) and the purity as well as the quantification of DNA was assessed (25).

PCR and RFLP of mtDNA control region and *H. pylori* gene amplification

PCR of the extracted total DNA was performed for three genes and their conditions are listed in Table 1 (25, 26). Restriction fragment-length polymorphism (RFLP) of the amplified mtDNA D-loop region was performed to assess the contamination of the extracted DNA, if any, by digesting with two different restriction enzymes, *HaeIII* and *AluI*. The digested PCR products were subjected to electrophoresis on a 12% polyacrylamide gel (27). Multiplex PCR amplification for Hp1-Hp2 and CagAF-CagAR primer sets was performed for amplification of *H. pylori* 16SrRNA and CagA genes, respectively (Table 1) (28). The PCR products were electrophoresized with 1.5% agarose gel.

Reverse transcription and polymerase chain reaction

For semiquantitative RT-PCR analysis, RNA was reverse transcribed using superscript II reverse transcriptase (Invitrogen) and oligo d(T) primers and then cDNA was used in PCR according to manufacturer's instructions (Invitrogen). With superscript II reverse transcriptase, 20 µl reaction tube containing 0.5 µg of oligo d(T) primers, 0.4 mM of each dNTP, and 1 µg of RNA were set following the instructions. The reaction conditions were as follows: 5 min at 65°C, 52 min at 37°C, and 15 min at 70°C, using vepa-protect Mastercycler (Eppendorf, Germany). Two microliters of cDNA was then used in a 25 µl PCR reaction, with 0.4 mM dNTPs, 1.5 mM MgCl₂, 0.25 µM primers and 1 U of Taq DNA polymerase. The primers used were Cdh1-F (5'-CCCTTTCTGATCCCAGGTCT-

3'), and Cdh1-R (5'-GCCTGGAGTTGCTAGGGTCT-3'), specific for epithelial cadherin1 (E-cadherin1), with an expected product size of 111 bp. The 334 bp fragments of a housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA were amplified using the following primers: GAPDH FP (5'-GGC TGGGCAAGGTCATCC-3') and GAPDH RP (5'-TCCACCACCCTGTTGCTGTA-3'), the concentrations of components being the same as stated above for CDH1 PCR. The reaction conditions for both primer pairs using oligo d(T) primers for the RT step were as follows: primary denaturation 94°C for 7 min; 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min (35 cycles); and final extension at 72°C for 10 min. The PCR products were analyzed on 2% agarose gel for a better resolution.

RESULTS

In this study, we performed coextraction of nucleic acids from five gastric cancer tissue blocks after deparaffinization. We used xylene and three rinses in different concentrated ethanol simultaneously to deparaffinize the sections. DNA was successfully extracted from all the samples (Fig. 1A), but one sample did not yield good RNA (Fig. 2, Table 2). Using TRIzol method, the yield of RNA was better than DNA in case of coextraction (Table 1) and the sole recovery of FFPE-DNA only provided higher yields than the coextraction methods. RNA and DNA quality was checked by spectrophotometric analysis and gel electrophoresis (Figs. 1A and 2).

PCR products with three primers showed satisfactory results for the extracted DNA from archival FFPE tissues. Generally, the PCR amplification of FF tissues was successful for smaller size fragments due to fragmentation of the genomic DNA (29)), but in this study, 1,030 bp of mitochondrial D-Loop region was amplified successfully (Fig. 1B). PCR-RFLP band patterns were better in the case of FFPE tissue DNA (Fig. 1C and D). After digesting the D-loop product with *RsaI* enzyme, we observed 20 and 30 bp small fragments. The amplification of *H. pylori* 16SrRNA (109 bp) and CagA (400 bp) region also showed satisfactory results by multiplex PCR (Fig. 3). We got positive result of *H. pylori* presence in three samples. For the efficient genotyping cagA and 16SrRNA oligonucleotide primers were designed in a way that allowed the simultaneous analysis of different subtypes by multiplex PCR. We selected primers that had the same annealing temperature (60°C) but produced PCR amplification products of different sizes (Table 1), so that the primers specific for the different genomic virulence determinants could all be used in a single multiplex PCR assay and their products distinguished by gel electrophoresis. Multiplex PCR assays produced reliable, distinct amplification products of

TABLE 1. Amplification Conditions of Mitochondrial D-Loop and *H. pylori* Genes From the Extracted Total DNA

Gene Name	Primer name	Primer sequences (5'–3') / (Expected product size)	PCR mix	PCR conditions
D-Loop	HMt-F	CACCATTAGCACCCAAAGCT	100 ng template DNA	95°C–60 s
	HMt-R	CTGTTAAAAGTGCATACCGCCA(1030 bp)	0.2 pM each primer 1X PCR buffer 1.5 mM MgCl ₂ 0.2 M dNTPs 1U Taq polymerase (Marck Biosciences Ltd.)	63°C–60 s 72°C–90 s (40 cycles)
16SrRNA (<i>H. pylori</i>)	Hp1	CTGGAGAGACTAAGCCCTCC	100 ng template DNA	95°C – 30 s
	Hp2	ATTACTGACGCTGATTGTGC (109 bp)	1X PCR buffer 50 mM Kcl	60°C – 30 s 72°C – 30 s
CagA (<i>H. pylori</i>)	CagA-F	AATACACCAACGCCTCCAAG	0.001% Gelatin	(35 cycles)
	CagA-R	TTGTTGCCGCTTTTGCTCTC (400 bp)	2.5 mM MgCl ₂ 0.2 pM each primer 0.2 M dNTPs 1U Taq polymerase (Marck Biosciences Ltd.)	

PCR consisted of 25 µl total reaction volume and condition for all the genes involves an initial denaturation at 95°C for 5 min and a final extension at 72°C for 10 min.

all specimens, making it a potentially useful diagnostic tool for routine pathology testing (Fig. 3).

The expression of the stomach cancer related and housekeeping genes, CDH1 and GAPDH, respectively, was investigated by RT-PCR analysis in FFPE tissue sample. Four samples examined were found to express GAPDH, while the CDH1 transcript was detected in three patients (Fig. 4).

DISCUSSION

It is known that denaturation or modification of macromolecules by formalin leads to precipitation and thereby minimizing the loss of nucleic acids from fixed tissue. But, the solubilization of RNA/DNA from FF specimens is negatively correlated with the duration of formalin treatment and the yield of RNA/DNA extraction may be significantly reduced. In this study, we optimized a TRIzol-based approach for coextraction of total genomic DNA

and RNA from archived specimens within a single reaction. Our approach was maximal coextraction of both nucleic acids without having to split the proteinase K digested FFPE tissue prior to nucleic acid recovery or having to use additional FFPE tissue to obtain sufficient amount of nucleic acid. We have developed a method that allows efficient purification of both DNA and RNA from a single population of biopsy cells. The purified DNA and RNA samples were found to be useful for genome and transcriptome profiling as judged by PCR-RFLP and RT-PCR technique. We demonstrated that DNA and RNA obtained by this method were of high quality and quantity. The method not only presents a more efficient and cost-effective way to use precious gastric cancer patient tissue samples, but it also provides an approach for providing stronger inferences about the relationship between genomic alterations and gene expression for cancer research. To our knowledge, this is the first report providing information to meet the technical challenge of obtaining

TABLE 2. Comparative Quantification of RNA and DNA Extracted by TRIzol –Phenol/Chloroform Conventional Coextraction From FFPE Tissues

S. No	Total RNA		Total DNA	
	Yield (ng/µl)	Purity (260/280)	Yield (ng/µl)	Purity (260/280)
1	144	1.88	82	1.78
2	128	1.79	113	1.72
3	226	1.93	107	1.83
4	358	1.79	325	1.68
5	–	1.34	264	1.78

Fig. 1. (A) DNA from five formalin fixed paraffin embedded cancer tissue samples. (B) PCR amplified products of mtDNA D-Loop (1,030 bp) region. PCR product digested with AluI (C) and RsaI (D) enzymes. Lane M, Low range Marker (100–3,000 bp); Lanes 1 to 5, cancer tissue samples.

Fig. 2. RNA integrity checked by electrophoresis on 1.8% agarose gels stained with ethidium bromide. Lanes 2–5 isolated RNA from cancer tissue samples.

Fig. 3. 16SrRNA (109 bp) and CagA (400 bp) region of *H. pylori* amplified by multiplex PCR using total DNA extracted from the gastric cancer tissue samples. S2 to S4 - *H. pylori* positive samples; S1 and S5 - positive and negative control, respectively, M - Low range Marker (100–3,000 bp).

DNA and RNA from the same cells obtained by gastric cancer archived tissue specimen by conventional method, yielding samples that are suitable for gene mutation study as well as gene expression study.

Joint profiling of the genome and transcriptome of the same tumor can also be achieved by obtaining DNA and RNA from stomach cancer cells of adjacent tissue sections. However, cancer tissues are highly heterogeneous and there may be some inherent differences between adjacent sections that could introduce errors when comparing

Fig. 4. Semiquantitative RT-PCR analysis of (A) CDH1 and (B) GAPDH gene expression. Lane M: low range ruler plus, Lanes 1–5: RNA isolated from FFPE, lane 6: Negative control.

genome changes, such as associated SNP with gene expression (30, 31). In addition, two dissections may not match in cellular composition to each other perfectly, especially when large dissections are performed over a wide range of the tissue field. Thus, even if the quantity of tissue specimen is enough to support separate extraction of DNA and RNA of the same region of tissue but on two adjacent tissue sections may introduce processing bias. The method described here obtains DNA and RNA from the same population of cells. Thus, the relationship between genome and transcriptome profiles could be better approximated than if separate samples were interrogated. Besides the robustness of conventional RNA extraction process, the RNA yields also depends on changes in tissue conformation during sectioning, difference in postsurgery tissue handling, fixation procedure, and duration of tissue storage (32). Thus, large scale analysis on FFPE tissue is problematic due to limited yield of extracted RNA, but in case of our methods RNA and DNA can be extracted from FFPE tissues in significant amount and of better quality, which was further used for the mutation study or expression of various constitutive genes. In addition, this method saves time and cost when compared with traditional or kit-based methods to obtain DNA and RNA from separate tissue sections from FFPE specimen.

Quality and quantity of DNA and RNA are key elements for successful genome and transcriptome profiling. In this regard, we have also tested DNA and RNA isolated from gastric cancer tissue using TRIzol reagent. This is consistent with observations reported by the inventor of the TRIzol method that the digestion efficiency by restriction enzymes is reduced in DNA extracted by this method (33). But we successfully amplified the 1,030 bp mitochondrial gene by PCR, and digested with two different enzymes and distinct separations of restricted products were obtained in 12% polyacrylamide gel. It is reported that the quality of the DNA is not good enough for genome profiling after treating with TRIzol (34), but in our study we successfully amplified human

mitochondrial control region from FFPE sample by using TRIzol method.

We hypothesized that 30 min of proteinase K with glycine-tris-ethylenediamine tetra-acetic acid buffer treatment, designed for optimal FFPE-RNA recovery was insufficient for removing FFPE-DNA/protein cross-linkages and thus we subjected the DNA pellet to additional proteinase K treatment and extracted the FFPE-DNA using the conventional phenol-chloroform method. In the present study, the addition of glycine has quenched the fragmentation with the concomitant cross-linking of fragmented DNA by formaldehyde. DTT was added for the reduction of disulfide bonds of nuclease leading to the inhibition of nuclease activity. TritonX100 can disrupt the fixed phospholipids and lipoproteins, resulting in rapid isolation of DNA from FFPE tissues.

Another aim, of the present study, was to design a combined detection method for the *H. pylori* genotyping and virulence gene identification by means of PCR on FFPE gastric tissues. *H. pylori* are one of the most genetically diverse bacterial species and there are geographic genetic variations among *H. pylori* strains (35). Different types of *H. pylori* genotypes are associated with the gastric cancer phenomenon. Recently, the importance of sialic acid binding adhesin (sabA) has been increasingly clarified by researchers (36). When genotyping *H. pylori* DNA extracted from cultured bacteria it is possible that specific *H. pylori* strains may be favored, whereas genotyping of DNA extracted from paraffin wax embedded tissue eliminates the preselection of strains or the modification of virulence genes by in vitro culture (37). However, to determine which *H. pylori* genotypes are associated with different gastric diseases it is important to determine whether a given patient is infected with a single *H. pylori* strain. Although it is possible that patients can be infected with more than one *H. pylori* strain. Therefore, this method will be more efficient and cost-effective if both DNA and RNA from the same cells will be used in downstream applications.

In summary, we have demonstrated that, with certain modifications, such as incubate 30 min with proteinase K with glycine-tris-ethylenediamine tetra-acetic acid buffer before adding TRIzol method can be used for simultaneous extraction of DNA and RNA from FFPE specimen for genome and transcriptome profiling as well as pathogenic organism identification and genotyping. Based on this current study, we can simultaneously and easily detect mitochondrial DNA and nuclear gene mutations and *H. pylori* genotyping presence from a homogeneous population of cells, find the gene expression levels, and detect the presence of any RNA virus from same tissue population. The recovery of genomic DNA and total RNA from the same specimen has the benefit of analyzing matched nucleic acid segments, from the same cells,

which is extremely valuable for validations as well as for integrative studies. Maximizing DNA and RNA retrieval from a single specimen might also be very useful when using tissues that are available in small amounts.

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