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Nested RT-PCR to detect mRNA in formalin-fixed human cardiac tissue

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Introduction

Reverse Transcriptase-Polymerase Chain reaction (RT-PCR) recently has been applied to detecting viral RNA (e.g., HCV: Hepatitis C Virus (reviewed in Refs. 1 and 2) or HIV: human Immunodeficiency Virus) from paraffin-embedded or N2-fixed tissues to detect such RNA viruses. Because RNA is very susceptible to degradation, special care is required when isolating it from N2-fixed material. The strong denaturants used to inhibit endogenous RNase can degrade the RNA during the isolation. In addition, the paraffinization and deparaffinization procedures can contribute to RNA degradation (reviewed in Ref. 3). Because formalin solution is a good RNase inhibitor, RNAs can be preserved in formalin-fixed tissues for a long time. Our protocol is the first attempt to detect mRNA from formalin-fixed human cardiac tissue by using nested RT-PCR in which RT reaction and sequential twice PCR are carried out. In this method, the first-round primer pairs are designed on target mRNA, and the second-round primers are done on first-round amplified PCR products.

Protocol

1. Formalin-fixed tissue (100 mg) was stored at -80 ºC for 2 days. Frozen tissue was covered with aluminum foil then broken into pieces. We transferred the pieces of frozen tissue to a 10 ml centrifuge tube and added 5 ml TRIZOL reagent, which contains guanidinium thiocyanate, phenol, and an unspecified lysis agent. The mixture was homogenized with a Polytron at 4,000 rpm for 90 sec. The mixture incubated at room temperature for 10 min, then we added 1.0 ml chloroform and vortexed for the tube for 15 sec to extract RNAs. After 3 min at room temperature, the tube was centrifuged at 4 ºC for 15 min at 15,000 rpm, and the aqueous phase was transferred to four 1.5 ml

microcentrifuge tubes; 625 µl isopropanol was added to each tube. The tubes incubated at room temperature for 10 min then were centrifuged at 4 ºC for 15 min at 15,000 rpm. The supernatant was decanted, and RNA-containing pellets were rinsed in 200 µl 75% ethanol. These four pellets were transferred to a single tube, which was centrifuged at 4 ºC for 10 min at 15,000 rpm. The supernatant was removed, and the pellet, containing the RNA, was dried briefly. The RNA pellet was resuspended in 30 µl DEPC-treated water and stored at -80 ºC until required.

2. The reverse transcription followed the RNA extraction. The reaction volume was 20 µl, which contained 2 µl total RNA and 140 pmol random hexa-deoxyribonucleotides; Oligo-dT primers could not be used because formalin solution degrades the poly-A tail of mRNA. Conditions for the cDNA transcription were as follows: preannealing at 25 ºC for 5 min, extension at 42 ºC for 55 min, and RT inactivation at 95 ºC for 5 min. AMV reverse transcriptase, which has 3' to 5' exonuclease activity, was used in the reaction. The resulting cDNA was stored at -20 ºC until required.

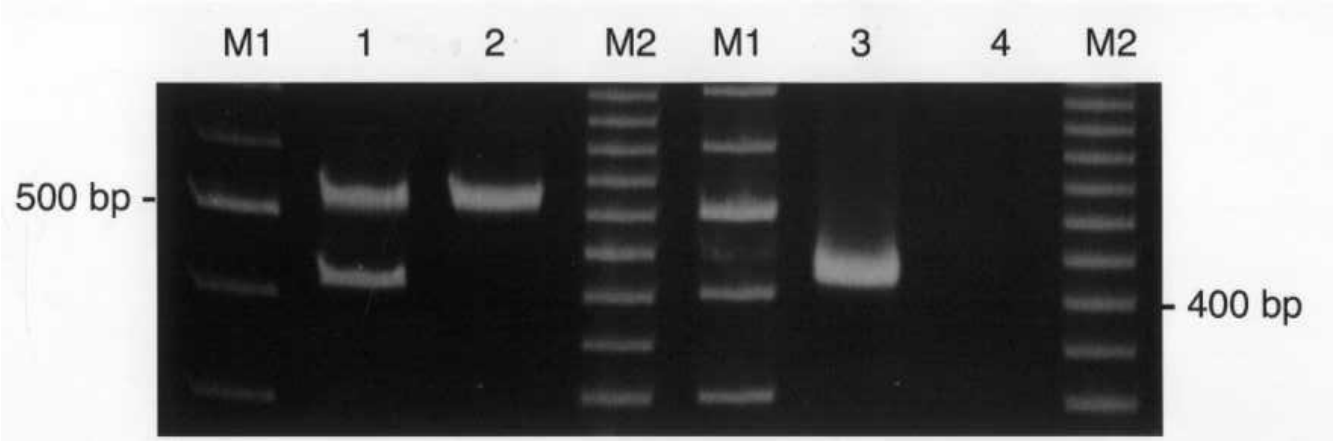


Figure 1. Nested RT-PCR Product was electrophoresced by 8% denaturing-polyacrylamide gel containing 10% glycerol and stained with EtBr. PCR reaction was performed with 'Hot Start' method and produced the expected 424-bp product. M1: 100 bp ladder marker; M2: 50 bp ladder marker; Lane 1 and 3, RTase (+) RT-PCR product; Lane 2 and 4, RTase (-) RT-PCR product.

3. The PCR reaction was performed according to the manufacturers (QIAGEN) recommendations with modifications. For the first round of amplification, ß-Myosin heavy chain (MYH7) mRNA sense primer was 5'-CGC AAGT CAG AGA AGG AGC GGC TAG AAG-3', and the MYH7 mRNA antisense primer was 5'-ACT GGT TTT CTC TGT CTG TGT CAG CAT-3'; these reagents yield an expected product of 487 bp. The first round of nested RT-PCR was carried out in a 50 µl reaction volume containing 5 µl 10x PCR buffer [Tris-HCl, KCl, (NH4)2SO4, 1.5 mM MgCl2; pH 8.7 (20ºC)]; 10 µl 5x Q-solution, 1.5 µl 25 mM MgCl2, 1 µl 10 mM dNTP, 1.5 U HotStarTaq DNA polymerase (QIAGEN), and 0.5 µl cDNA. The reaction mixture was denatured at 95 ºC for 15 min immediately before cycling; the conditions for the 35 cycles were 95 ºC for 15 sec, 56 ºC for 30 sec, and 72 ºC for 1 min. The second round of amplification used the same sense primer as in the first round but with the antisense primer 5'-AGA TGG AGA AGA TGT GGG GCG GGG CCT C-3; these primers lead to a 424 bp product. The template for the second round of nested RT-PCR was 1 µl of the amplified first reaction mixture, and the reaction components were the same as in the first round, except the concentration of MgCl2 was 15 mM. The second reaction mixture was denatured 95 ºC for 15 min before amplification; the conditions for the 40 cycles were 95 ºC for 15 sec, 68 ºC for 30 sec, and 72 ºC for 1 min.

The ethidium bromide-stained nested RT-PCR product (Fig. 1) was electrophoresed in an 8% denaturing polyacrylamide gel containing 10% glycerol. Myosin heavy chain mRNA (424 bp) was detected clearly because HotStarTaq DNA polymerase was used in our protocol. However contaminating genomic DNA product occurred as well. MYH7 mRNA mutations were related with a type of hypertrophic cardiomyopathy that triggers cardiac sudden death (reviewed in Ref. 4).

Relevance to Forensic Medicine

In the practice of a forensic medicine, formalin-fixed organs are always preserved for the purpose of a routine histopathological examination and/or other retrospective ones. RNAs in these organs will be decomposed or degenerated, so we can not always detect mRNA using RT-PCR method easily. Because nested RT-PCR method is more sensitive and gene-specific, we think it is very useful method for detection of mRNA from formalin-fixed organs.

References

1. L Cao, M Duchrow, U Windhovel, P Kujath, H-P Bruch and R Broll : Expression of MDR1 mRNA and encoding P-glycoprotein in archival formalin-fixed paraffin-embedded gall bladder cancer tissues. Eur. J. Cancer. 34. 1612-1617, 1998.
2. C Mies : A simple, rapid method for isolating RNA from paraffin-embedded tissues for reverse transcription-polymerase chain reaction (RT-PCR). J. Histochem. Cytochem. 42. 811-813, 1994.
3. RD Foss, NG Thakurta, RM Conran, P Gutman : Effects of fixative and fixation time on the extraction and polymerase chain reaction amplification of RNA from paraffin-embedded tissue. Diagn. Mol. Pathol. 3. 148-155, 1994.
4. G Bonne, L Carrier, P Richard, B Hainque, K Schwarts : Familial hypertrophic cardiomyopathy from mutations to function defects. Circ. Res. 1998. 83. 580-593, 1998.