Detection of Enterovirus RNA in Myocardial Biopsies From Patients With Myocarditis and Cardiomyopathy Using Gene Amplification by Polymerase Chain Reaction

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Recent molecular studies have suggested that viral myocarditis frequently underlies human congestive cardiomyopathy; however, only moderately sensitive and specific techniques were used. Polymerase chain reaction (PCR) gene amplification is a sensitive, specific technique ideally suited for the diagnosis of viral disease in small tissue samples where low copy numbers of the viral genome may be present. Using PCR and high stringency condition, we screened biopsies taken from 48 patients with clinically suspected myocarditis or dilated cardiomyopathy. Five patients demonstrated positive enteroviral signals by PCR; two of them had myocarditis by pathology, whereas the other three had changes consistent with cardiomyopathy. Four other patients had myocarditis diagnosed by pathology from 3 months to 1 year earlier but were now negative by both PCR and pathology. Both pathology and PCR were negative for active myocarditis in all other patients. Ventricular samples taken from left ventricular myectomy in four additional patients with hypertrophic cardiomyopathy, normal human ventricle samples, and uninfected monkey kidney cells were also negative by PCR. This study supports a link between viral infection and dilated cardiomyopathy in some patients. PCR gene amplification provides a new diagnostic approach to patients with suspected myocarditis.

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Enteroviruses, particularly the coxsackieviruses, are believed to be the most common viral agents for the pathogenesis of human myocarditis.1 Although serological studies have indicated an association of coxsackievirus B viral infection with myocarditis,2-5 viral cultures of myocardial tissue obtained by endomyocardial biopsies from patients with biopsy-proven myocarditis or dilated cardiomyopathy are almost always negative, even when the clinical history or serological studies indicate recent viral infection.6,7 In addition, immuno-cytochemical studies of myocardial tissue have repeatedly failed to demonstrate the presence of viral antigens in patients with myocarditis and chronic dilated cardiomyopathy.8

Recombinant DNA technology holds great promise for the study of virus-associated myocarditis by detecting the presence of the viral genome in endomyocardial biopsy tissue. The molecular genetics of the enteroviruses, particularly coxsackievirus, and poliovirus, are well documented. The sequences of coxsackievirus B1, B3, and B4 (CVB1, CVB2, and CVB3) and poliovirus 1, 2, and 3 (PV1, PV2, and PV3) have been reported.9-12 Using coxsackievirus B complementary DNA (cDNA) probes, two groups have reported the presence of CVB2 and coxsackievirus B4 (CVB4) signals in myocardial biopsies from patients with myocarditis or dilated cardiomyopathy using slot-blot and in situ hybridization techniques.13,14 In these studies, nonspecific hybridization was a concern.

Polymerase chain reaction (PCR) gene amplification is a technique that allows rapid and substantive amplification of specific DNA sequences. This sensi-
tive and specific technique is ideally suited to the diagnosis of viral disease where low copy numbers of the viral genome may be present. In this study, we report the application of PCR gene amplification to the diagnosis of viral myocarditis using cardiac biopsies taken from 48 patients with clinically suspected myocarditis or dilated cardiomyopathy.

**Methods**

**Preparation of Coxsackievirus B RNA**

African Green monkey kidney cell culture in a concentration of $10^6$ cells/ml and suspended in medium 199 containing 10% fetal calf serum and antibiotics was supplied by Connaught Laboratories, Toronto, Ontario. This cell suspension was seeded in 25-cm² tissue culture flasks and incubated at 37°C for 24 hours. The medium was then replaced with minimum essential medium (MEM) supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml). The cell culture flasks were further incubated at 37°C until a confluent monolayer of cells was observed before viral inoculation.

Cell cultures were inoculated with CVB3 at a titer of $10^6$ particles or 50% tissue culture infection dose ($10^5$ TCID₉₀). After a 1-hour incubation at room temperature, the infected cell culture was washed twice with MEM and then incubated in 5.0 ml MEM containing antibiotics without fetal calf serum at 37°C. The infected cell culture monolayer was examined daily for 25–50% cytopathic effect. The cells were then suspended in 0.5 ml of 8 M guanidine-HCl (pH 5.0), passed twice through a 25-gauge needle, and precipitated with one-half volume of ethanol at −20°C overnight. The precipitate was dissolved in autoclaved H₂O, and the RNA was quantified by ultraviolet spectrophotometry.

**Myocardial Biopsies and Histopathology**

Right ventricular endomyocardial biopsy samples were obtained from 48 patients with a Stanford biotome by the internal jugular approach. Five samples were usually taken from each patient, and four were fixed in 10% formalin, embedded in paraffin, and cut into 4–5-μm sections; one section (usually less than 1 mg) was instantly frozen in liquid nitrogen for molecular diagnosis.

Multiple sections of the biopsy were stained with hematoxylin and eosin and examined by light microscopy. Each section was studied in detail, and the diagnosis of myocarditis was based on the Dallas criteria.

Myocardial biopsy samples for study by PCR were homogenized with 0.5 ml of 8 M guanidine-HCl (pH 5.0) in a small homogenizer and spun down to remove tissue debris. Total nucleic acids were then precipitated with ethanol, dissolved in 15 μl of autoclaved H₂O, and prepared for further assay.

**Blood DNA Isolation**

The procedure for rapid DNA isolation from blood was a modification of the method described by Lindblom et al. Blood samples (5 ml) were lysed with cold sucrose buffer (10 ml, pH 7.5, 1% Triton X-100 in 320 mM sucrose, 1 mM Tris-HCl, and 5 mM MgCl₂). After centrifugation (2,000 g at 4°C for 20 minutes), the pellet was resuspended in the same buffer and incubated with proteinase K (500 μg/ml, fresh stock solution: 1 mg/ml in 1% sodium dodecyl sulfate [SDS] 2 mM EDTA [pH 8.0]). Proteins were extracted by the addition of the same volume of Tris-EDTA–saturated phenol:chloroform:isoamyl alcohol (25:24:1). After centrifugation (10,000 g at 4°C for 10 minutes), the upper aqueous phase was transferred to a new tube, and DNA was precipitated by adding 2 vol of absolute ethanol (20°C for 2 hours or longer). The DNA was dissolved in sterilized H₂O and quantitated by ultraviolet spectrophotometry.

**Oligonucleotide Synthesis and Labeling**

Oligonucleotides (20–60 base pairs) were prepared by a solid-phase phosphotriester method and labeled at the 5' end with phosphorus-32-β-ATP (specific activity, 3,000 Ci/mmol, New England Nuclear, Boston, Mass.). Oligonucleotide (0.6–1 μg) was incubated in standard kinase buffer plus 150 μCi 32P-β-ATP and 10 units polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 50 μl total volume for 1 hour at 37°C. Labeled oligonucleotide was separated from unincorporated ATP by passage through a G-50 column (Pharmacia, Sweden) in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA. The specific activity of the probes was 7–8×10⁵ cpm/μg.

**Viral cDNA Synthesis and PCR Gene Amplification**

**First strand cDNA synthesis.** We synthesized the first strand cDNA using a virus-specific primer (see Figure 1). Four microliters (0.5 μg) of kidney cell RNA and 15 μl of biopsy RNA samples (RNA heated to 70°C for 5 minutes before synthesis) were added to 40 μl of 500 mM Tris-HCl (pH 8.3), 500 mM KCl, 100 mM MgCl₂, 10 mM 1,4-dithiothreitol (DTT), 10 mM EDTA, 100 μg/ml bovine serum albumin (BSA), 80 mM sodium pyrophosphate, 10 mM spermidine-HCl, 10 mM deoxynucleotide triphosphates (dNTP), and 100 μg/ml oligo d(T), 12–18 1 μM of each primer, and 1,000 units/ml avian murine virus (AMV) reverse transcriptase. These mixtures were incubated at 42°C for 50 minutes and then at 65°C for 10 minutes to denature the AMV reverse transcriptase. The samples were then ready for PCR gene amplification.

**PCR gene amplification.** Two microliters (0.025 μg) of both uninfected and infected cell samples and 20 μl of biopsy samples containing the first strand cDNA as described above were added to 40 μl of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% (wt/vol) gelatin, 200 μM each deoxynucleo-
tide triphosphates, and 1 μM of each primer (Perkin-Elmer-Cetus protocol). After 10 minutes at 94°C, 5 units of Taq polymerase (Stratagene, La Jolla, Calif.) and 80 μl mineral oil were added, and PCR cycles were started (denaturation: 1 minute at 90°C; annealing: 2 minutes at 52°C; elongation: 3 minutes at 72°C).

**Blot Hybridization**

After PCR gene amplification, the products (40 μl each) were added to 120 μl of a solution of 6.15 M formaldehyde and 10× SSC (same as cDNA control: 2 μl of uninfected cell, and infected cell and 20 μl of biopsy cDNA, respectively, with dH2O added to 40 μl). These mixtures were incubated for 15 minutes at 65°C. The denatured samples were loaded onto a Schleicher and Schuell Minifold II Slot Blot apparatus (containing a nitrocellulose filter that had been soaked in water) with suction. Each sample well was washed with 200 μl of 10× SSC. The nitrocellulose sheets were vacuum-dried for 2 hours at 80°C. Filters were prehybridized in 6× SSC, 5× Denhardt’s solution (0.1% Ficoll, 0.1% BSA, 0.1% polyvinylpyrrolidone, 0.5% SDS, and 100 μg/ml sheared salmon sperm DNA) at 55°C for at least 2 hours. After prehybridization, a second hybridization was performed with the prehybridization solution except that approximately 1×10⁹ cpmp of radiolabeled probe was added. After 8–16 hours at 55°C, filters were rinsed in 6× SSC and 0.1% SDS at room temperature, washed in 3× SSC and 0.1% SDS for 15 minutes at 55°C twice and 65°C for 5–10 minutes, and autoradiographed at −70°C for various times with intensifying screens.

**Results**

Enteroviruses exhibit areas of extensive nucleotide sequence homology even between disparate members such as the coxsackievirus and polioviruses. Other regions are virus specific. Using virus-specific primers (see Figure 1) to synthesize specific regions of cDNA, we could select primer pairs that would amplify sequences of a broad range of enteroviruses. The regions between these pairs could then be identified by probes exhibiting homologies to specific viruses or to the broad family. The sequence of synthetic oligonucleotide primers and probes and their relation to the target enterovirus genomic RNA region.

"FIGURE 1. Sequences of synthetic oligonucleotide primers and probes and their relation to the target enterovirus genomic RNA (positive strand) region. Primers A and C are complementary to the negative strand, and primers B and D are complementary to the positive strand. Probes 1 and 2 are complementary to the coxsackievirus genomic RNA."
In our initial experiments, we did not use PCR gene amplification but instead used a conventional method in which synthetic oligonucleotides derived from coxsackievirus sequences served as diagnostic probes. Figure 3 shows the results of slot-blot analysis of four biopsy samples by the conventional method. Biopsy sample A was from a patient whose clinical diagnosis indicated myocarditis with severe left ventricle dysfunction and who had myocarditis and pericarditis by pathology. Biopsy B was from a patient who had ventricular tachyarrhythmias after myocardial infarction a few weeks earlier but showed no evidence of coronary disease by coronary angiography and pathology. Biopsies C and D were from two patients whose clinical diagnoses indicated dilated cardiomyopathy and whose pathology indicated changes compatible with congestive cardiomyopathy but showed no evidence of myocarditis. The cell debris was not removed from biopsies B, C, and D after guanidine-HCl homogenation, whereas the cell debris from sample A was removed. After RNA precipitation, all four samples were dissolved in 30 µl autoclaved H2O and subjected to slot-blot analysis. From these experiments, it is clear that cross-hybridization exists between viral RNA and human DNA and RNA, leading to false-positive results under low stringency conditions and negative results under higher stringency conditions. To improve both the sensitivity and specificity, we used PCR gene-amplification methodology in subsequent studies.

Figure 4 shows the sensitivity and specificity of probe 1, primer A, and primer B after 30 PCR cycles. It indicates that virus-specific primer was better than oligo d(T) for synthesizing specific cDNA in 5’ region. Because the CVB3 genome is 7.5 kb in length, it will be difficult to synthesize a full-length cDNA. Figure 5 shows the results of agarose gel electrophoresis and oligonucleotide hybridization analysis of PCR-amplified DNA samples from monkey kidney cells. A single band is present, migrating at 184 bp in DNA from infected cells but not from uninfected cells. This result also demonstrates the sensitivity and specificity of the PCR gene-amplification method.

Figure 6 shows a typical PCR analysis of Enterovirus RNA from endomyocardial biopsy samples using probe 1, primer C, and primer D. Like other patients whose data are presented in Table 1, the cell debris was removed before slot-blot analysis. Negative and positive controls were used for each experiment. Unlike conventional slot-blot analysis, PCR analysis does not reveal a continuum of hybridization signal intensity. Signals were either positive or absent.
in patient samples 1–7, 11a, 14, 15a, and 16. Probe 2 and primers C and D were used in all other patients, including 11b, 15b, and 15c. Five patients (14–16, 29, and 42) contained enterovirus RNA in myocardial tissue as shown by PCR gene amplification. A biopsy from one of these patients (patient 15) 3 months later continued to be positive. However, a third biopsy 4 months later was negative. The results derived from PCR analysis were correlated to the pathological results (see Table 1). In two patients (15 and 42), both PCR analysis and pathology indicated the presence of myocarditis. Three patients (14, 16, and 29) with congestive cardiomyopathy but not active myocarditis as indicated by pathology exhibited definite viral signals by PCR. Four patients (1, 8, 11, and 19) with myocarditis by pathology 3 months to 1 year earlier had a negative PCR result and no evidence of myocarditis by pathology when examined during this study. PCR analysis was performed using blood from one of these patients (15a), but no viral RNA was indicated. Samples were taken from the left ventricle in four additional patients with hypertrophic obstructive cardiomyopathy, from normal human ventricles, and from uninfected monkey kidney cells (not shown). No viral RNA was indicated by PCR analysis in these tissues.

**Discussion**

The genomic sequence relation of the enteroviruses such as CVB1, CVB3, CVB4, PV1, PV2, and PV3\(^9\text{--}^{12}\) allow the introduction of methods using synthetic oli-
gonucleotides, which are CVB- and Enterovirus-specific for the detection and analysis of viral genomic RNA sequence. These probes, used in conjunction with PCR technology, are particularly well suited to the diagnosis of viral infections of heart tissue because myocardial biopsies are of limited size and the viral RNA copy number in cardiac cells is probably very low.

PCR is a unique in vitro gene-amplification method that can produce a greater than $10^5$-fold increase in the amount of target sequence, permitting analysis of as little as 1 ng of genomic DNA. The enteroviral genome is a single-stranded, positive-sense RNA molecule. Available sequence data allowed us to choose a viral region that would exhibit homology to a broad range of enteroviruses in a sensitive and specific manner. We screened 48 patients with clinically suspected myocarditis and cardiomyopathy with this method; only five patients exhibited an enteroviral genome in their myocardium.

One of our patients presented with a postpartum cardiomyopathy; however, her endomyocardial biopsy revealed active myocarditis by pathological examination. It is of interest to note that when this patient was examined 3 months later, pathology was negative for myocarditis, but the PCR result was still positive. Four months later, both pathology and PCR produced negative results. This case demonstrates that viral myocarditis can be the underlying etiology of some cases of postpartum cardiomyopathy.

Three other patients had biopsies that were positive by PCR analysis. These patients had clinically manifested cardiomyopathy and exhibited negative
pathology by the Dallas criteria but had histories suggestive of myocarditis. Although the pathology indicated no evidence of active myocarditis, there was a significant amount of fibrosis and myocyte hypertrophy present, suggesting considerable myocardial damage due possibly to myocarditis. In contrast to the PCR gene-amplification results, conventional slot-blot hybridization failed to exhibit a viral signal with coxsackievirus probes under stringent conditions in each of these four patients. Thus, in these patients, the level of viral RNA was less than the limit of conventional detection or the viral genome was incomplete, possibly accounting for the failure to isolate infectious progeny virus or to demonstrate the presence of virus-specific antigens from biopsy samples in previous studies.

These results suggest that residual viral genome may be present in myocardial cells in the absence of acute, pathologically manifest myocarditis and may predispose such individuals to ensuing dilated cardiomyopathy. It is also possible that the difference between pathology and PCR evaluation is a manifestation of sampling heterogeneity and that the biopsy samples analyzed by hybridization may have exhibited pathologically evident myocarditis had they been subjected to microscopy. One of our patients was an 18-year-old woman who had symptoms of myocarditis and a positive pathological diagnosis on her first biopsy that showed evidence of an inflammatory infiltrate and scattered muscle fiber necrosis. Unfortunately, her initial biopsy sample was not available for PCR. Six months later when a tissue sample was

FIGURE 6. Examples of polymerase chain reaction (PCR) analysis of Enterovirus RNA in endomyocardial biopsy samples.

cDNA, complementary DNA; CVB3, coxsackievirus B3.
available for analysis by PCR, it was negative. Although pathology revealed no active myocarditis, there was myocyte destruction and fibrosis consistent with clinical congestive cardiomyopathy. This patient went for a heart transplant in October 1988 due to severe dilated congestive cardiomyopathy but subsequently died. Her myopathic heart was available for detailed hybridization study with different enteroviral primers and probes, but although pathology exhibited areas of myocarditis, PCR analysis was consistently negative. One possible explanation was that the free viral RNA in the myocardium had degraded despite the relatively rapid RNA isolation because the heart was not immediately frozen.

Another patient, a 50-year-old woman, who had a sudden onset of pulmonary edema after the onset of herpes zoster infection 1 week earlier, showed active myocarditis by pathology but no enteroviral signal by PCR. Myocarditis in this case may have been due to infection by herpes virus rather than an enterovirus. Three other patients had a definite history and biopsy-derived evidence of myocarditis; however, when biopsied 3 months to 1 year later, both pathology and PCR analyses were negative. The negative PCR and pathological data in these cases could be due to sampling heterogeneity. Furthermore, although we used primers and probes designed to capture most enteroviruses, there are other viral families that can cause viral myocarditis.

### Table 1. Detection of Enteroviral RNA Sequences in Myocarditic Human Heart Biopsies by Polymerase Chain Reaction Technique

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical diagnosis</th>
<th>Pathology result</th>
<th>PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Myocarditis</td>
<td>Dilated CMP (myocarditis by biopsy 1 year earlier)</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Myocarditis</td>
<td>Interstitial vessels show luminal stenosis</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Myocarditis</td>
<td>Mild interstitial fibrosis</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Myocarditis</td>
<td>Mild, focal interstitial fibrosis (myocarditis by biopsy 4 months earlier)</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Myocarditis</td>
<td>Right ventricular dysplasia</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Myocarditis fibrosis</td>
<td>Mild interstitial</td>
<td>-</td>
</tr>
<tr>
<td>11a</td>
<td>Myocarditis, CMP</td>
<td>Congestive CMP (myocarditis by biopsy 4 months earlier)</td>
<td>-</td>
</tr>
<tr>
<td>11b</td>
<td>Myocarditis, CMP</td>
<td>Myocarditis</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>CMP</td>
<td>Pompe's disease</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Myocarditis, CMP</td>
<td>Congestive CMP</td>
<td>+</td>
</tr>
<tr>
<td>15a</td>
<td>Postpartum CMP</td>
<td>Active myocarditis</td>
<td>+</td>
</tr>
<tr>
<td>15b</td>
<td>Postpartum CMP</td>
<td>No evidence of active myocarditis</td>
<td>+</td>
</tr>
<tr>
<td>15c</td>
<td>Postpartum CMP</td>
<td>No evidence of active myocarditis</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>Myocarditis, CMP</td>
<td>Congestive CMP</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>Myocarditis</td>
<td>Mild, focal interstitial fibrosis (myocarditis by biopsy 1 year earlier)</td>
<td>-</td>
</tr>
<tr>
<td>29</td>
<td>Myocarditis</td>
<td>Dilated CMP (moderately severe interstitial fibrosis; focal mild increase in interstitial mononuclear cells)</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>Myocarditis</td>
<td>Increased adipose tissue, possible right ventricular dysplasia</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>Myocarditis</td>
<td>Active myocarditis (1 week after onset of herpes zoster)</td>
<td>-</td>
</tr>
<tr>
<td>32</td>
<td>Myocarditis</td>
<td>Hypertrophic CMP (Becter's muscular dystrophy)</td>
<td>-</td>
</tr>
<tr>
<td>39</td>
<td>Myocarditis</td>
<td>Mild subendocardial fibrosis</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>Myocarditis</td>
<td>Patchy mild interstitial fibrosis</td>
<td>-</td>
</tr>
<tr>
<td>41</td>
<td>Myocarditis</td>
<td>Patchy muscle fiber hypertrophy</td>
<td>-</td>
</tr>
<tr>
<td>42</td>
<td>Myocarditis</td>
<td>Focal myocarditis</td>
<td>+</td>
</tr>
<tr>
<td>43</td>
<td>Myocarditis</td>
<td>Presence of amyloid (on special stains)</td>
<td>-</td>
</tr>
<tr>
<td>46</td>
<td>Myocarditis</td>
<td>Tissue inadequate for diagnosis</td>
<td>-</td>
</tr>
<tr>
<td>47</td>
<td>Myocarditis</td>
<td>Patchy endocardial and interstitial fibrosis</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>Myocarditis</td>
<td>Significant iron overload</td>
<td>-</td>
</tr>
<tr>
<td>17, 20, 21, 22, 23, 26, 27, 34, 35</td>
<td>Myocarditis</td>
<td>Normal</td>
<td>-</td>
</tr>
<tr>
<td>2, 4, 6, 7, 13, 18, 24, 25, 28, 33, 36, 37, 38, 44, 45</td>
<td>Myocarditis</td>
<td>Dilated CMP</td>
<td>-</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction; CMP, cardiomyopathy; -, negative; +, positive.
We also took blood samples from 14 patients to determine the usefulness of PCR analysis during theoretical viremia. All blood studies were negative for viral signals, including a sample from one patient (15) whose heart biopsies were positive by both PCR and pathology.

This study supports a link between Enterovirus infection and congestive cardiomyopathy in some patients. However, the frequency of Enterovirus-like RNA sequences in clinically suspected myocarditis and cardiomyopathy samples is not as high as reported by others using slot-blot hybridization without PCR or in situ hybridization. Sampling has been a major impediment to the pathological diagnosis of myocarditis from biopsy analysis. It is estimated that 17.2 samples are required per patient for 79% diagnostic sensitivity; such sample numbers are clinically unattainable. Although sampling heterogeneity may account for differences between PCR studies and those using standard hybridization techniques, it is unlikely to account for a difference of this magnitude. It would seem more likely that homologies between human DNA and RNA and viral sequences led to false-positive results under conditions that were not highly stringent. In conclusion, this study supports a link between viral infection and dilated cardiomyopathy in some patients. It appears that the number of viral RNA copies present in myocardial cells from human myocarditis hearts is very low; however, the PCR gene-amplification method can provide a very sensitive and specific means for the study of such tissue.

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