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# Detection of Coxsackievirus B3 RNA in Myocardial Tissues by the Polymerase Chain Reaction

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***Coxsackievirus B3 is a possible etiologic agent in some forms of myocarditis and idiopathic dilated cardiomyopathy. A method for the detection of coxsackievirus B3 RNA was developed using the polymerase chain reaction based on the amplification of a cDNA copy of the positive-strand viral RNA. The fidelity of the method was established in two murine models for coxsackie B3 myocarditis. All cardiac specimens with adequate RNA for study from coxsackie B3-infected mice contained detectable viral RNA, in contrast to none of control specimens from noninfected mice. The sensitivity of the technique was established at approximately 1 to 100 plaque-forming units of virus per gram of tissue, and the specificity was established as limited to the coxsackievirus B3 serotype among nine viruses tested. In patients with myocarditis, one of five specimens contained detectable viral RNA, whereas none of 11 specimens from patients with idiopathic dilated cardiomyopathy or 21 myocardial specimens from patients with a wide variety of other cardiac disorders contained detectable coxsackie B3 viral RNA. The results show that the polymerase chain reaction is a useful means for detecting coxsackie viral RNA and its application should help in the evaluation of hypotheses concerning the infectious etiology of human myocarditis and idiopathic dilated cardiomyopathy. (Am J Pathol 1991, 138:497–503)***

Myocarditis is defined as a pathologic process characterized by an inflammatory infiltrate of the myocardium with necrosis and/or degeneration of adjacent myocytes not typical of the ischemic damage associated with coronary artery disease.[1] It can be caused by allergic reactions, drug effects, and various infectious agents but, in most cases, the cause is unknown. In many cases, the disease is presumed to have a viral etiology, sometimes supported by the results of serologic studies, although viruses are cultured only rarely from pathologic tissues. Coxsackie B viruses are thought to be the most common viral agent of myocarditis,[2] and, in fact, a coxsackievirus B3-infected mouse model represents one of the most commonly studied animal models for the disease.[3]

Idiopathic dilated cardiomyopathy is a pathologic process characterized by marked myocyte hypertrophy with myocytes containing large, bizarre nuclei, extensive interstitial fibrosis, focal replacement fibrosis, and endocardial thickening.[1,4] Foci of inflammatory cells are often seen,[5] and the distinction of this disease from myocarditis is difficult and controversial. By definition, idiopathic dilated cardiomyopathy has no known etiology, although the possibility that it represents an end stage in the evolution of myocarditis has been considered by a number of investigators.[6–8]

The detection of enteroviral-specific RNA sequences in myocardial biopsy samples from patients with myocarditis and dilated cardiomyopathy was reported.[9] Positive hybridization signals were obtained using a coxsackie B cDNA probe in slot-blot hybridization studies in 9 of 17 samples from patients with histologic evidence of active or healing myocarditis or dilated cardiomyopathy with inflammatory changes. However a large number of control tissues were not similarly studied.

Recently the polymerase chain reaction was used as a specific and highly sensitive method for the identification of infectious agents.[10–15] In the current study, we used this methodology for the identification of coxsackievirus B3 in tissue specimens in an effort to examine more precisely the role of coxsackievirus B3 in human myocarditis and idiopathic dilated cardiomyopathy.

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## Methods

### Specimens

Samples were studied from two animal models, viral-infected cell lysates, and biopsies obtained from human patients from a variety of centers. In one animal model (specimens donated by Charles J. Gauntt, PhD, San Antonio, TX),[16] adolescent CD-1 mice were killed 3 days after inoculation with the virus. The hearts were removed, washed in Dulbecco's phosphate-buffered saline, flash frozen in liquid nitrogen, and stored at −20°C until use. The strain of Coxsackievirus B3 inoculated, CVB3m, was derived from the experimental passage of the Nancy strain by E. D. Kilbourne and J. F. Woodruff at Cornell University Medical College, New York, NY. In the second animal model (specimens donated by Ahvie Herskowitz, MD, Baltimore, MD),[17] 14-day-old A.SW mice were killed 5 to 7 days after inoculation with the virus, and samples from the heart and pancreas were removed. The strain of coxsackievirus B3 inoculated in this model was derived from the stock of the Nancy strain maintained by A. M. Lerner at Wayne State University, Detroit, MI. For both models, uninfected control animals also were studied. For production of infected cell lysates, cultured HeLa cells were challenged with 100 to 200 plaque-forming units (pfu) of echoviruses 2, 4, 6, or 22, coxsackie viruses A21, B4, or B6, or encephalomyocarditis D. The uninfected cell line also was studied as a negative control.

The human cardiac specimens consisted of either endomyocardial biopsies, portions of tissue taken at the time of allograft transplantation, or tissue obtained at autopsy (one case). The specimens were obtained fresh and frozen at −20°C at Stanford University Medical Center or were obtained from the frozen-tissue repository of the Myocarditis Treatment Trial following approval by the Immunology Committee. There were five cases of acute myocarditis using the Dallas criteria[1] for diagnosis (initial biopsies only studied), 11 cases of idiopathic dilated cardiomyopathy, 7 cases of coronary artery disease, 5 cases from normal hearts biopsied at the time of heart–lung transplantation, 3 cases from patients biopsied to rule out myocarditis with no myocarditis evident on histologic examination, 3 cases of allograft rejection, 1 case of congenital heart disease, 1 case of valvular disease, and 1 case from a patient with a normal heart. Four of the myocarditis samples were endomyocardial biopsy specimens, while one sample derived from tissue obtained at autopsy. The durations of symptoms were 1 week, 3 weeks, 4 weeks, and 26 weeks in the four cases of myocarditis in which this information was available. All 11 cases of idiopathic dilated cardiomyopathy were obtained at the time of transplantation. The durations of symptoms were 1 month, 1 month, 2 months, 2 months, 7 months, 8 months, 12 months, 36 months, 56 months, and 168 months, respectively, in the 10 cases of cardiomyopathy in which this information was available.

### Oligonucleotides

All oligonucleotides were purchased from Operon Technologies Inc. (Alameda, CA). The primers used for the coxsackie B3 polymerase chain reaction studies were the 24-base oligonucleotides: CB1, 5′-CAT ACA GTT CAA GTC CAA ATG CCG-3′, and CB2, 5′-TGT CTA GCG AGT ATC TGA CCT GTG-3′. The detection probe for the reaction products was the 35-base oligonucleotide: CB3, 5′-GTT TCC AGT GTA GAT TTT GTA CCA CCC ATG GCT GC-3′. All three oligonucleotides were constructed based on the sequence of the coxsackie B3 viral genome as reported by Lindberg et al.[18]

The primers used for the myoglobin gene polymerase chain reaction studies were the 20-base oligonucleotides: 5′-TGG GCA GGA AGT CCT CAT CA-3′ and 5′-AGC TCC AGG GCC TTG TTC AT-3′. The detection probe for the reaction products was the 30-base oligonucleotide: 5′-GAC GAG ATG AAG GCA TCT GAG GAC TTA AAG-3′. The primers were derived from exons 1 and 3, whereas the probe was derived from exon 2 of the human myoglobin gene nucleotide sequence published by Weller et al.[19] The primers and probe used for the c-abl gene polymerase chain reaction were as previously described.[20]

### Polymerase Chain Reaction Procedures

RNA was extracted from specimens by standard methods.[21] Two micrograms of purified cellular RNA were converted to single-stranded cDNA using the CB2 oligonucleotide, Moloney-MLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), and reaction conditions as described previously.[20] The cDNA homologous to the coxsackievirus positive-strand RNA was subjected to 30 cycles of amplification using an automated thermal cycler, Taq polymerase, the CB1 and CB2 primers, and other reagents obtained commercially (Perkin-Elmer-Cetus, Norwalk, CT). Cycle conditions included a 55°C annealing step for 2 minutes, a 72°C extension step for 3 minutes, and a 94°C denaturation step for 1 minute. One fifth of the reaction products were analyzed by Southern blot hybridization using the CB3 oligonucleotide end radiolabeled with gamma-$^{32}$P-adenosine triphosphate using hybridization and wash conditions described previously.[22] A 580-basepair DNA fragment corresponding to the P2-C region of the viral genome was detected under these conditions.

## Results

A reverse polymerase chain reaction (PCR) procedure specific for coxsackie B3 viral positive-strand RNA was used for these studies. This procedure consisted of converting viral positive-strand RNA to single-strand DNA by reverse transcription and subsequent amplification of the complementary DNA by 30 cycles of PCR (Figure 1). The amplified DNA then was analyzed by Southern blot hybridization using a radiolabeled internal oligonucleotide as probe. Hybridization analysis ensured that even low levels of viral RNA in the examined tissues would be detected. The amplified segment of the viral genome corresponded to the P2-C region, which is highly conserved among different B3 strains.

To establish the fidelity of the PCR procedure based on amplification of coxsackie B3 viral RNA, tissues from two mouse strains, CD1 and A.SW, which are models for coxsackievirus B3-induced myocarditis, were examined. Analyses of these tissues showed amplification products of the correct size (580 basepairs) that hybridized with the coxsackievirus B3-specific oligonucleotide in six of seven infected mice (Figure 2, lanes 1 to 7, upper panel). The data confirmed the presence of coxsackie B3 viral RNA within tissues of the infected mice, five from the CD-1 strain and one from the A.SW strain. The origin of a second, slightly smaller band homologous to the CB3 probe is not clear but may have resulted from amplification of alternatively processed coxsackeviral mRNAs or deletions incurred during the PCR. Unexpectedly one specimen from an infected animal did not show detectable amplification products (Figure 2, lane 7, upper panel). This probably resulted from RNA degradation in this specimen because parallel amplifications of the myoglobin RNA showed no detectable products for this ubiquitous transcript (Figure 2, lane 7, lower panel). The latter served as a control in analyses of all RNA specimens to evaluate the quality of RNA extracted from the biopsy tissues. Tissues from uninfected control animals, three from the CD-1 strain and one from the A.SW strain, showed no detectable viral RNA because they had no

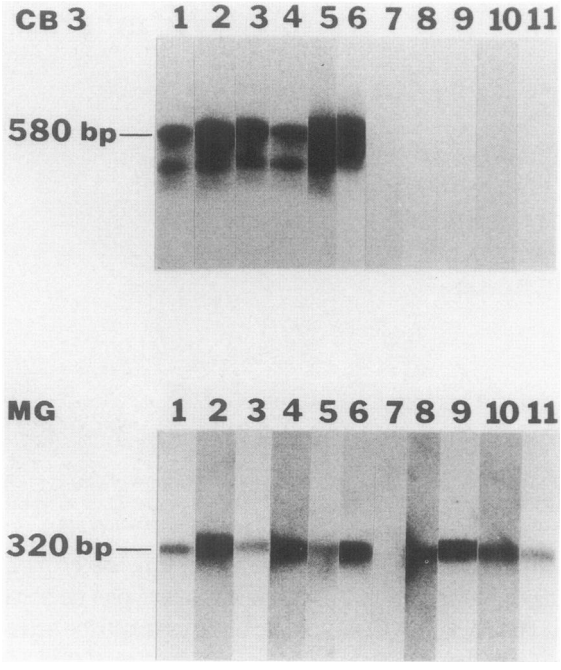
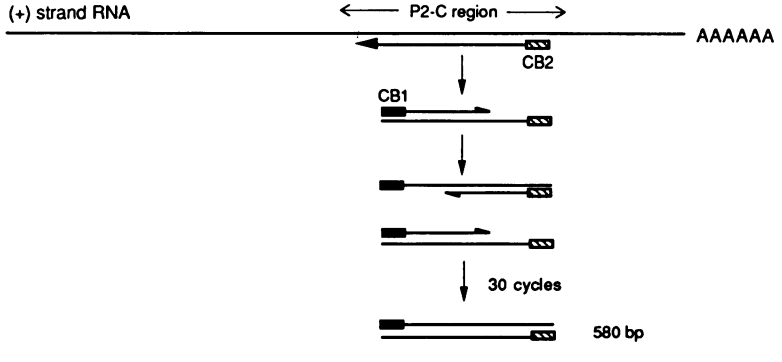



**Figure 2.** *At top are the results of Southern blot hybridization of the products of the coxsackievirus B3 PCR, while at bottom are the results of Southern blot hybridization of the products of the myoglobin PCR. Lanes 1 to 5 represent cardiac tissues from infected CD-1 strain mice. Lanes 6 and 7 represent pancreatic and cardiac tissues, respectively, from A.SW mice. Lanes 8 to 10 represent cardiac tissues from uninfected CD-1 strain mice, while lane 11 represents the analysis of cardiac tissue from an uninfected A.SW mouse.*

observable amplification products on hybridization analysis, despite the fact that they contained high-quality RNA as judged from the myoglobin amplification (Figure 2, lanes 8 to 11). These data confirmed that the CB3-specific PCR procedure was performing properly and capable of detecting the CB3 genome in RNA isolated from tissues affected by acute myocarditis.

To assess the sensitivity of the technique, we performed reconstitution experiments using six 10-fold serial dilutions of two of the positive CD-1 samples and addition of uninfected RNA (Figure 3). Hybridization signals were seen in all but the $10^6$ dilution with both specimens. Because it is known that there is about $1 \times 10^5$ to $1 \times 10^7$

**Figure 1.** *Strategy for the PCR analysis.*



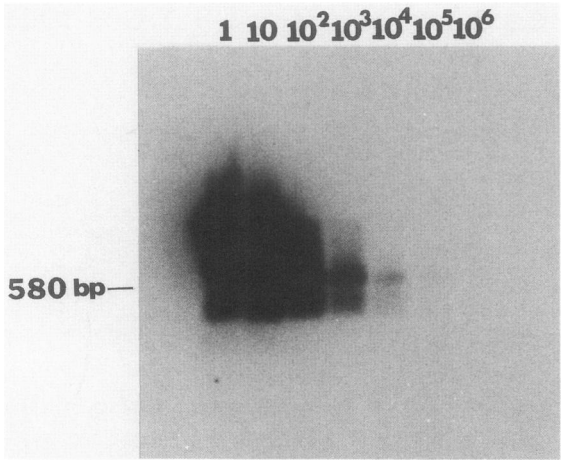



**Figure 3.** *Results of a Southern blot hybridization of the products of coxsackievirus B3 PCR in serial dilution studies of coxsackievirus B3-infected CD-1 heart tissue (24-hour autoradiogram).*

pfu of virus per gram of tissue in CD-1 murine hearts 3 days after inoculation (Dr. Charles Gauntt, oral personal communication, 1990), these studies establish the sensitivity of the technique at approximately 1 to 100 pfu of virus per gram of tissue.

To provide an assessment of the specificity of the technique, analyses were performed on RNA extracted from infected cell lysates of HeLa cells challenged with a variety of enteroviruses, including echoviruses 2, 4, 6, and 22, coxsackieviruses A21, B4, and B6 or EMCV (Figure 4). None of these specimens were positive using the coxsackievirus B3 primers, although all specimens showed hybridization using the c-abl primers and probe as a positive control for the adequacy of the samples.

Five specimens from patients with a histologic diagnosis of myocarditis using the Dallas criteria[1] had adequate RNA for analysis, as assessed by PCR studies for myoglobin expression (Figure 5, lanes 1 to 4). Of these five specimens, one had detectable coxsackieviral genomes by PCR studies. This patient presented with chest pain and peripheral edema and had a history of a prodromal viral syndrome (upper respiratory tract infection/flulike symptoms) 6 months earlier. In contrast, none of 11 specimens from patients with a pathologic diagnosis of idiopathic dilated cardiomyopathy and containing adequate RNA as assessed by studies for myoglobin expression had evidence of coxsackieviral genomes (Figure 5, lanes 5 to 15). In addition, 21 myocardial specimens from patients with a wide variety of histologic diagnoses not thought to be infectious in etiology (see Methods) and having adequate RNA as assessed by PCR for the myoglobin gene showed no evidence of coxsackieviral genomes by similar studies (data not shown).

## Discussion

In the past few years, the polymerase chain reaction was shown to be a specific and highly sensitive method for the diagnosis of infectious agents whose nucleotide sequence is known. Theoretically, and in practical use, this technique can detect specific nucleic acid sequences present in a single cell.[23] Some of the viruses for which the polymerase chain reaction has been used already for diagnosis include cytomegalovirus, herpes simplex I, human papilloma virus, human T cell lymphotrophic virus type 1, human immunodeficiency virus type 1, and hepatitis B virus.[10–15]

In the current study, we applied the polymerase chain reaction technique for the identification of coxsackievirus B3-specific RNA in tissue specimens. The sensitivity of technique was established at approximately 1 to 10 pfu/g of tissue in reconstitution experiments. The fidelity of the technique was established by studying tissues obtained from experimentally infected mice. To resolve the possibility of false-negative results due to variations in different strains of virus, two animal model systems were studied. Analysis of murine tissues infected with two different isolates of the Nancy strain of coxsackievirus B3 confirmed that any differences in these isolates would not result in false-negative analyses due to variation in nucleotide sequence. We also attempted to minimize this possibility by selecting our primers from the P2-C gene of the coxsackievirus B3 genome. This gene codes for a protein that is proposed to participate in replication of the genome[24] and appears to be highly conserved.[18]

The fact that the P2-C gene is highly conserved between different picornaviruses raises the possibility that primers derived from the coxsackievirus B3 genomes might also detect other, closely related viral genomes. For example, the set of primers used to identify coxsackievirus B3 genomes have 88% homology with similar nucleotide sequences of the coxsackievirus B4 genome,[25] suggesting that these primers may potentially identify nucleic acid sequences from the coxsackievirus B4 genome as well as the B3 genome. However analyses of several other viruses, including coxsackievirus B4, showed no evidence of cross-reactivity with our technique.

Only one of five specimens of myocarditis and none of 11 specimens of idiopathic dilated cardiomyopathy had evidence of coxsackievirus B3 genomes by our methods. There are several possible interpretations of this data. First the virus may have been present within the hearts, but not sampled. However *in situ* hybridization studies applied to athymic mice persistently infected with coxsackievirus B3 as a model system have shown that the myocardium is affected in a disseminated, multifocal manner.[26] In fact, viral RNA was found not only within histologically abnormal areas but also in isolated myocytes in apparently normal myocardial tissue. Given the exquisite sensitivity of the polymerase chain reaction, it would seem unlikely that this viral RNA would not be detected. Second it is possible that a viral infection that re-

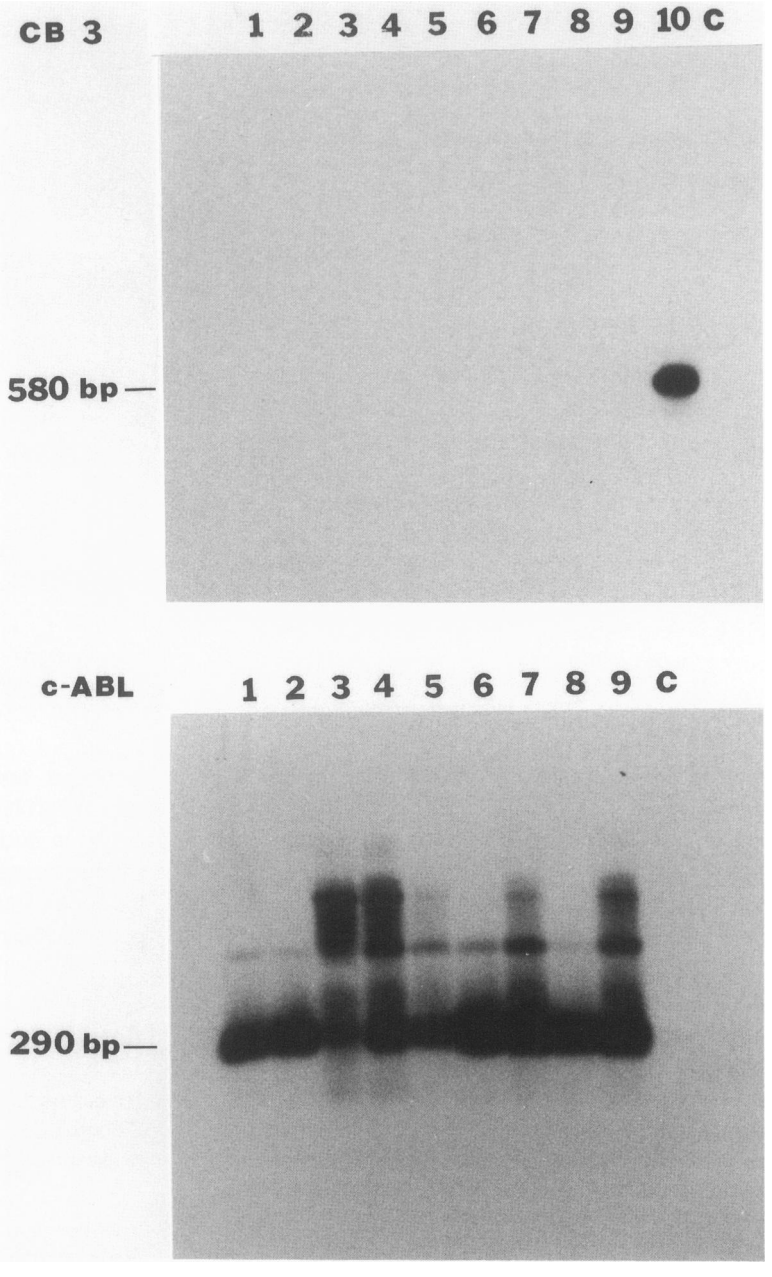



**Figure 4.** *At top are the results of Southern blot hybridization of the products of the coxsackievirus B3 PCR, while at bottom are the results of Southern blot hybridization of the products of the c-abl PCR. Lanes 1 through 8 represent cell lysates infected with echovirus (EV) 2, EV4, EV6, EV22, coxsackievirus A21, coxsackievirus B4, coxsackievirus B6, and encephalomyocarditis virus D, while lane 9 represents uninfected HeLa cells. Lane C represents an $H_2O$ control. Lane 10 above represents coxsackievirus B3-infected cardiac tissue from an A.SW mouse.*

solved set off an autoimmune reaction that led to the clinical and histologic findings of myocarditis. Biopsy during this latter stage might not identify the virus that was present previously. We tried to minimize this possibility by limiting our study to initial biopsy specimens from patients with myocarditis. Mouse models have shown persistence of virus for many weeks after initial infection[26]; however their relevance to the timing of human infection is not clear.

Although enteroviruses may be an important cause of myocarditis and idiopathic dilated cardiomyopathy, specific pathogens could include a wide range of viruses, of which a large proportion may not have been recognized using the primers to coxsackievirus B3. This may explain why others using cDNA probes in slot-blot hybridization studies identified a higher proportion of cases of myocarditis and idiopathic dilated cardiomyopathy to be associated with viruses than the current study.[9] However the potential for nonspecific hybridization generally is higher with slot-blot hybridization analyses than in polymerase chain reaction analyses using carefully chosen primers. Thus it is possible that coxsackievirus B3 may only be an occasional cause of myocarditis in humans and a rare cause of idiopathic dilated cardiomyopathy. Recently another group reported their experience using the polymerase chain reaction to detect enterovirus RNA in myocardial biopsies from patients with myocarditis and cardiomyopathy.[27] Using primers selected to allow de-

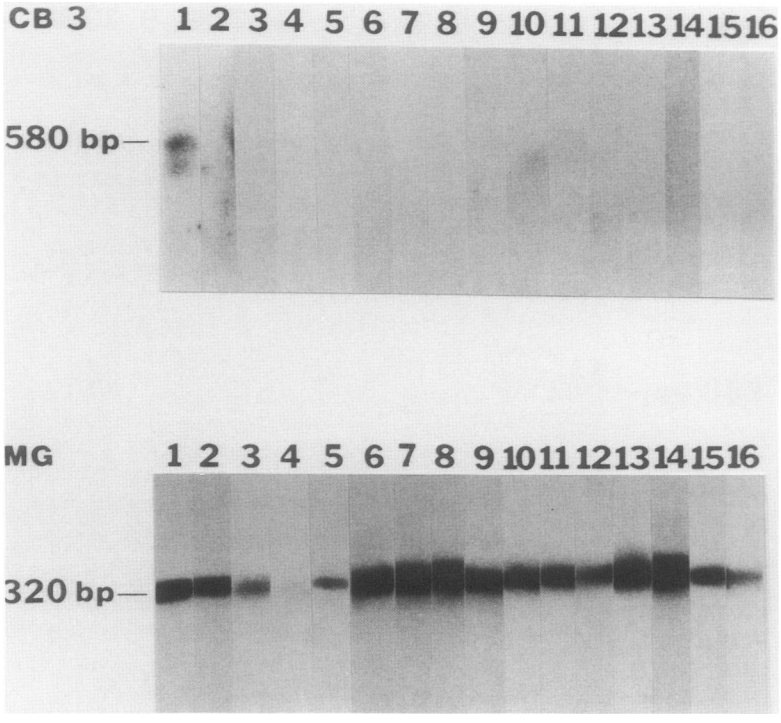



**Figure 5.** *At top are the results of Southern blot hybridization of the products of the coxsackievirus B3 PCR in 15 human cardiac specimens, while at bottom are the results of the Southern blot hybridization of the products of the myoglobin PCR. Lanes 1 to 5 represent cases of human myocarditis, while lanes 6 to 16 represent cases of idiopathic dilated cardiomyopathy.*

tection of a wide variety of enteroviruses, only 5 of 48 samples were found to have evidence of enteroviral genomes. Another report describing a polymerase chain reaction technique to identify enteroviruses also was published, but no data concerning analysis of human specimens were given.[28]

In the course of this study, one additional consideration became evident—that of sample requirements. The large majority of human myocardial samples taken at the time of surgery had sufficient amounts of RNA for study as assessed by polymerase chain reaction studies for myoglobin. However only a minority of specimens obtained by transcutaneous endomyocardial biopsy had adequate RNA for study (unpublished observations). Although size of the biopsy may be an important factor, it is also possible that the histologic findings were also crucial because specimens from patients with myocarditis comprised the large majority of the small transvascular endomyocardial biopsy specimens and an inordinate percentage of the insufficient specimens. One of the infected mouse myocardial specimens also contained inadequate amounts of RNA for study, suggesting that tissues harboring myocarditis may contain factors, possibly enzymes derived from inflammatory cells, that lead to the rapid degradation of the RNA within the tissues.

The methods described here should facilitate studies to evaluate critically the association of coxsackievirus B3 infection with human myocarditis and idiopathic dilated cardiomyopathy. Further studies are needed with more control tissues to determine the true specificity of the technique, and in particular, animals must be studied later in the chronic course of their disease after peak viremia. In addition, studies in the murine models are needed to determine the size of tissue necessary to make the appropriate diagnosis, both in the acute and chronic phase of murine myocarditis.

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