

Nested Polymerase Chain Reaction for High-Sensitivity Detection of Enteroviral RNA in Biological Samples

GIOVANNI MARIA SEVERINI,¹ LUISA MESTRONI,^{1,2} ARTURO FALASCHI,¹ FULVIO CAMERINI,²
 AND MAURO GIACCA^{1*}

International Centre for Genetic Engineering and Biotechnology, AREA Science Park, Padriciano 99, 34012 Trieste,¹ and Department of Cardiology, Ospedale Maggiore and University, 34100 Trieste,² Italy

Received 6 November 1992/Accepted 2 February 1993

A method based on nested polymerase chain reaction was developed for the detection of enteroviral genomes in biological samples. By taking advantage of the conserved 5' noncoding region of the enteroviral RNA, two sets of primers were utilized, enabling the detection either of a broad range of enteroviruses or of group B coxsackieviruses only. The sensitivity of the method is close to the detection of single molecules of viral RNA in as much as 1 mg of tissue sample. A preliminary study showed the usefulness of this technique for the analysis of endomyocardial biopsy samples from patients with idiopathic dilated cardiomyopathy and myocarditis.

In the picornavirus family, several members of the enterovirus (EV) genus (including polio-, echo-, and coxsackieviruses) are important pathogens for humans (18). Diagnosis of EV infection usually requires propagation of virus in cell cultures followed by neutralization typing with antiserum pools; this procedure, however, is laborious and time-consuming and often appears to have inadequate sensitivity (22), especially in diseases such as idiopathic myocarditis and idiopathic dilated cardiomyopathy, for which epidemiological and experimental data suggest a coxsackievirus infection but viral isolation usually fails (20).

Taking advantage of the availability of the complete nucleotide sequences of over 30 human picornavirus strains (reviewed by Stanway [28]), we have developed a method based on nested polymerase chain reaction (PCR) with two different sets of oligonucleotides able to recognize either all the EVs or specifically group B coxsackieviruses. This method does not require an internal hybridization step and has a sensitivity close to the detection of a single molecule of viral RNA present in 1 mg of tissue sample.

Nested PCR amplification for EV-specific and coxsackievirus group B-specific detection. Seven different primers were synthesized for reverse transcription-PCR amplification of EV genomes (see Fig. 1A for localization and 1B for primer sequences). All the primers are from the 5' noncoding region, which contains sequence homology boxes highly conserved among different EVs, some of which have been used by different authors for hybridization (2, 10, 23, 24) and PCR studies (5, 7, 9, 13, 21, 22, 31).

Nested PCR amplification for EV detection entails reverse transcription and a first step of amplification, followed by dilution of the sample and a second step of amplification using two primers internal to the first amplification product. This is one of the methods for obtaining maximum sensitivity from PCR without loss of specificity of amplification. Two different sets of primers were synthesized in order to obtain amplification either for a broad range of EVs (primer set B, Fig. 1C) or specifically for coxsackieviruses group B (primer set A). Set A includes primers CX4 and CX7 for the first amplification and primers CX2 and CX3 for the nested amplification. Since the sequence of primer CX4 is present in

all group B coxsackieviruses sequenced so far but not in the other EVs (Fig. 2), amplification with primer set A is able to recognize only the group B coxsackieviruses. It should be observed, however, that since the current criteria for classifying the EVs do not necessarily reflect the overall genetic relationships among the various members (4), this set of

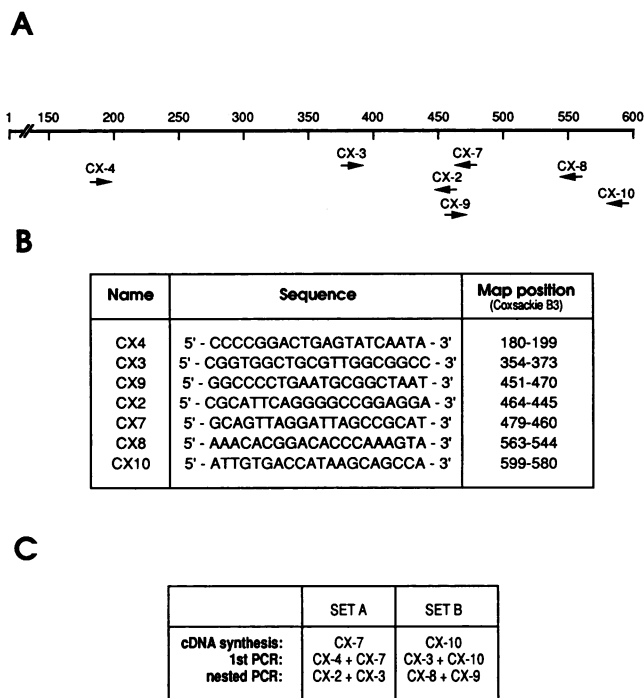


FIG. 1. Sequence and localization of oligonucleotides used in this study. (A) Schematic representation of the positions of the oligonucleotides on the first 600 5' nucleotides of the EV RNA. Arrows indicate the 5'-to-3' orientations. (B) Nucleotide sequences of the primers and localization on the coxsackievirus B3 sequence (16). All the oligonucleotides utilized in this work were synthesized by the ICGEB Oligonucleotide Synthesis Service with an Applied Biosystems AB 394-B synthesizer. (C) Primer choice for reverse transcription and nested PCR amplification for EV-specific (primer set B) and coxsackievirus-specific (primer set A) detection.

* Corresponding author.

CX-4 (179-199)		CX-3 (354-373)		CX-9 (451-470)		CX-2 (464-445)	
5'-CCCCGGACTGAGTATCAATA-3'		5'-CGGTGGCTGCGTTGGCGGCC-3'		5'-GGCCCCGTAATGCGGCTAAT-3'		5'-CGCATTGAGGGCCGGAGGA-3'	
Polio 1	TT**CCGG**TGTGCT**	TCCA*****	*****	*****	*****	*****	*****
Polio 2	T**CCGG**GC*GT**	TCCA*****	*****	*****	*****	*****	*****
Polio 3	TT**CCGG**GCCGC**	TCCA*****	*****	*****	*****	*****	*****
CAV-9	*****	***C*****C***T***	*****	*****	*****	*****	*****
CAV-21	TT**CCGG**AATCAT**	*CCA*****	*****	*****	*****	*****	*****
CXB-1	*****	*****	*****	*****	*****	*****	*****
CXB-3	*****	*****	*****	*****	*****	*****	*****
CXB-4	*****	*****	*****	*****	*****	*****	*****
EV-70	TT**CCGG**AGT*GC**	TCCA*****	*****	*****	*****	*****	*****
CX-7 (479-460)		CX-8 (563-544)		CX-10 (599-580)			
5'-GCAGTTAGGATTAGCCGCAT-3'		5'-AAACACGGACACCCAAAGTA-3'		5'-ATTGTCACCATAAGCAGCCA-3'			
Polio 1	*AG***G*****	*****	*****	*****	*****	*****	*****
Polio 2	*TG*****	*****	*****	*****	*****	*****	*****
Polio 3	ATG*****	*****	*****	*****	*****	*****	*****
CAV-9	*****G*****	*****	*****	*****	*****	*****	*****
CAV-21	*TG*****	*****	*****	*****	*****	*****	*****
CXB-1	*****	*****	*****	*****	*****	*****	*****
CXB-3	*****	*****	*****	*****	*****	*****	*****
CXB-4	*****	*****	*****	*****	*****	*****	*****
EV-70	*****	*****	*****	*****	*****	*****	*****

FIG. 2. Homology of oligonucleotide sequences with the available sequences of EV genomes. Sequences used for comparative purposes were for poliovirus (Polio) type 1 (Mahoney) (14), poliovirus type 2 (Lansing) (15), poliovirus type 3 (Leon/37) (29), coxsackievirus A9 (CAV-9) (4), coxsackievirus A21 (8), coxsackievirus B1 (CXB-1) (11), coxsackievirus B3 (16), coxsackievirus B4 (12), and EV-70 (25). All the sequences were obtained from GenBank (release 92.2.0). Alignments were obtained starting from the 3' end of each oligonucleotide, because pairing in this region is considered crucial in priming DNA polymerization in PCR experiments.

primers should presumably also amplify genomes from other EVs that in molecular terms are very similar to the coxsackievirus B group, like coxsackievirus A9, which shares a perfect match with primer CX4 (see Fig. 2 for sequence comparison) (4). Primer set B includes primers CX3 and CX10 for the first amplification and primers CX8 and CX9 for the nested amplification. The sequences of all the four primers are conserved in all the human EVs (Fig. 2) and rhinoviruses (28) whose sequences have been determined so far. These primers were also designed both to allow high temperatures of annealing, in order to ensure high specificity to the amplification reaction, and to obtain short amplification products (112 and 113 bp with primer sets A and B, respectively), thus enabling amplification even if the quality of the RNA extracted from the biological samples is not optimal.

cDNA synthesis was carried out with primers CX7 and CX10 for sets A and B, respectively. Total RNA from infected cells (100 ng, corresponding to 1 μ l) was reverse transcribed in 50 mM Tris (pH 8.3)–75 mM KCl, 3 mM MgCl₂–10 mM dithiothreitol–20 U of RNasin (Promega, Madison, Wis.)–1 mM each deoxynucleoside triphosphate (dNTP)–20 pmol of primer–200 U of murine leukemia virus reverse transcriptase (GIBCO BRL, Paisley, United Kingdom) (final reaction volume, 20 μ l). The reaction mixture was overlaid with 60 μ l of mineral oil (Sigma, St. Louis, Mo.), and the reaction was allowed to proceed for 45 min at 42°C. The reaction was stopped by heating at 95°C for 5 min.

Eighty microliters of a solution containing 250 μ M each dNTP, 10 pmol of the primer used for cDNA synthesis, 30 pmol of the other primer, 2.5 U of *Taq* polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk, Conn.), 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, and 0.01% gelatin were directly added to the 20 μ l used for cDNA synthesis.

Reaction mixtures (100 μ l) were subjected to 40 cycles of amplification in a programmable thermal cycler (Perkin-Elmer Cetus) by using the following sequence: 94°C for 40 s, 53°C for 1 min, and 72°C for 1 min, plus a final extension step at 72°C for 7 min.

Two microliters of the first amplification reaction was further amplified with the nested pair of primers in 100 μ l of a reaction mixture containing 250 μ M each dNTP, 30 pmol of each primer, 2.5 U of *Taq* polymerase, 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, and 0.01% gelatin. Reaction mixtures were overlaid with 60 μ l of mineral oil and subjected to 30 cycles of amplification by using the following sequence: 94°C for 40 s, 55°C for 1 min, and 72°C for 1 min, plus a final extension step at 72°C for 7 min.

Amplification products were visualized by ethidium bromide staining after electrophoresis in 6% acrylamide gels.

All precautions were taken in order to avoid carryover of amplification products, including physical separation of pre- and post-PCR procedures, UV irradiation of nondisposable devices, use of aerosol-resistant tips (Continental Laboratory Products, San Diego, Calif.), and assembling of master mix solutions to reduce manipulation of samples. Furthermore, several negative controls were included in each reverse transcription-PCR experiment. Amplification of rat myocardial RNA, total RNA from uninfected cell lines (HeLa, BGM [African green monkey kidney], and H9), and water consistently produced negative results, without the appearance of nonspecific amplification products or primer dimer artifacts.

In order to test the specificity of amplification with the two different sets of primers, nested PCR was performed from RNA extracted from BGM cell monolayers infected with all six group B coxsackieviruses, the three polioviruses, and echoviruses 9, 19, and 31, obtained either from the Center

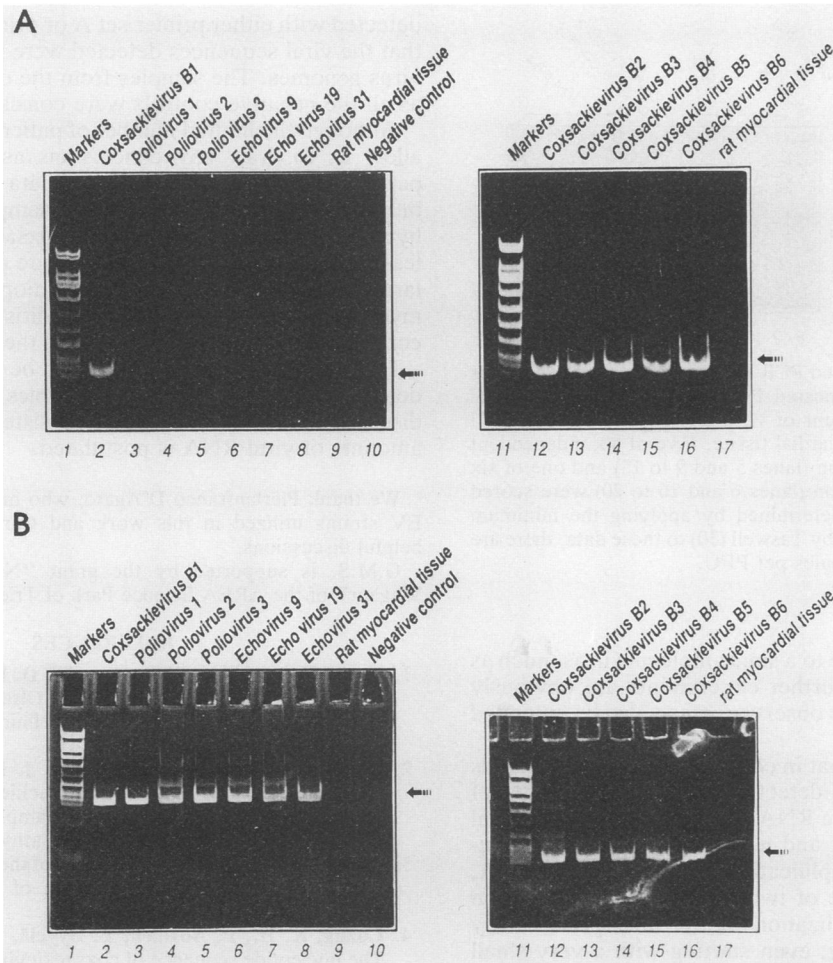


FIG. 3. Nested PCR amplification of selected EV strains. Total RNA from BGM cells infected with the indicated viral types was reverse transcribed and subjected to nested PCR amplification; amplification products were resolved by polyacrylamide gel electrophoresis and stained with ethidium bromide. Uninfected rat myocardial RNA (100 ng) and water were included in the experiments as negative controls. The arrows indicate the positions of the amplification products (112 bp for primer set A [A] and 113 bp for primer set B [B]).

for Public Health (London, United Kingdom) or from a clinical virology laboratory (Istituto di Igiene, Ospedale Burlo Garofolo, Trieste, Italy). Total RNA was extracted 20 h postinfection (6), reverse transcribed, and amplified by nested PCR with primer sets A and B (Fig. 3A and B, respectively). Amplification was obtained for all the EV strains with primer set B but only for group B coxsackieviruses (B1 to B6) with primer set A. These results are clearly in agreement with those expected from the primer sequence homologies reported in Fig. 2.

To further determine the specificity of nested amplification, the amplification products obtained from EV-infected cell lines by using primer set B were sequenced by the dideoxy-chain-termination method of Sanger et al. (26) with primer CX9 and the T7 sequencing kit (Pharmacia LKB, Uppsala, Sweden). Sequence analysis revealed complete consistency, with only a few nucleotide changes (among almost 80 nucleotides sequenced), between our EV sequences and the sequences present in the data banks (data not shown).

Sensitivity of nested PCR. The sensitivity of the nested PCR method described above was assessed by a limiting dilution experiment. Serial 10-fold dilutions of the superna-

tant of coxsackievirus B1-infected BGM cells were used to infect BGM monolayers in order to determine the virus titer by the plaque assay technique (19). The amount of virus corresponding to a single PFU was further serially diluted, and aliquots containing from 10^{-1} to 10^{-4} PFU were mixed with about 1 mg of rat myocardial tissue. From each sample, total RNA was extracted, reverse transcribed, and amplified with the broad-range-detection primer set B. As shown in Fig. 4, an amplification product is clearly visible up to the dilution corresponding to 10^{-2} PFU. Five of six independent samples of the 10^{-3} dilution gave a positive result (Fig. 4B, lanes 5 and 9 to 13), while five of six samples of the 10^{-4} dilution were negative (lanes 6 and 16 to 20). From these data, the number of amplifiable genomes per PFU was estimated according to the minimum chi-square method described by Taswell (30), with the assumption that the distribution of molecules in the limiting dilution experiment could be described by the Poisson probability distribution. By this analysis, it was determined that about 1,800 single RNA molecules per PFU could be detected. Since it has been estimated that there is a particle/infectivity ratio of between 100 and 1,000 for the EVs (18, 22), we conclude that the nested PCR method employed was able to detect an

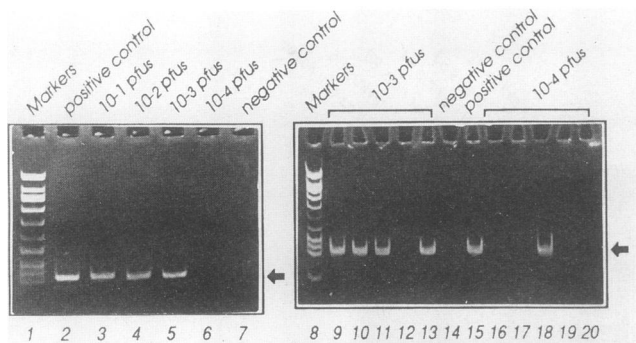


FIG. 4. Sensitivity of nested PCR amplification. Total RNA was extracted and subjected to nested PCR amplification from serial 10-fold dilutions of the amount of virus corresponding to 1 PFU mixed with 1 mg of rat myocardial tissue. Five of six independent samples from the 10^{-3} dilution (lanes 5 and 9 to 13) and one of six samples from the 10^{-4} dilution (lanes 6 and 16 to 20) were scored positive by the assay. As determined by applying the minimum chi-square method described by Taswell (30) to these data, there are about 1,800 detectable molecules per PFU.

amount of viral RNA close to a single molecule in as much as 1 mg of tissue sample. Further experiments are obviously needed to see whether this observation can also be extended to other EV serotypes.

These results indicate that in contrast to the PCR methods developed so far for the detection of EVs, consisting of reverse transcription of the RNA extracted from the clinical samples followed by PCR and hybridization of an oligonucleotide internal to the amplification product (5, 7, 9, 13, 21, 22, 32), the combined use of two subsequent amplification steps allows direct visualization of the final products by ethidium bromide staining, even starting with a very small amount of initial template. In addition, nested PCR should also have some advantages over PCR plus hybridization even in terms of specificity. In fact, the hybridization step demands almost perfect homology between the probe and the amplification product, which is often not predictable because of the high sequence variability of EVs. In contrast, nested PCR requires primer-template complementarity only for a few nucleotides at the 3' end of the primers (27). As a consequence, it can be predicted that viral isolates detectable by nested PCR could not always be recognized by PCR plus hybridization if they carry point mutations in the region complementary to the internal primer.

Detection of enteroviral RNA in endomyocardial biopsy samples. To determine the applicability of this technique to clinical samples, primer sets A and B were used to detect the presence of EV sequences in left ventricular endomyocardial biopsy samples (about 1 mg each) of 10 patients with idiopathic dilated cardiomyopathy (diagnosed on the basis of reduced left ventricular pump function in the absence of any known etiologic factor, according to World Health Organization criteria [3]) and 1 patient with idiopathic myocarditis (diagnosed according to the "Dallas" criteria [1]). The hypothesis of a viral origin of these diseases in humans has long been considered (20), and recent molecular studies suggest that EV persistence (in particular, group B coxsackieviruses) could play a pathogenetic role (for a review, see reference 17).

Amplification was obtained with samples from two patients (one with idiopathic dilated cardiomyopathy and one with idiopathic myocarditis); both positive results were

detected with either primer set A or primer set B, suggesting that the viral sequences detected were related to coxsackievirus genomes. The samples from the other patients as well as all the negative controls were consistently negative.

Although the limited number of patients analyzed does not allow us to draw any conclusions as to the role of EV persistence in these diseases, the data confirm the applicability of this technique to clinical samples and support the hypothesis of the persistence of coxsackievirus RNA in at least a fraction of the patients. We are presently analyzing a larger population of patients with idiopathic dilated cardiomyopathy and idiopathic myocarditis as well as clinical controls, in order to further extend these observations.

In conclusion, nested PCR can be very useful for the detection of EVs in clinical samples from patients with diseases for which chronic persistence of very small amounts of viral RNA is postulated.

We thank Pierlanfranco D'Agaro, who made available to us the EV strains utilized in this work and Carlo Monti-Bragadin for helpful discussions.

G.M.S. is supported by the grant "Nerina Vera Kreilshein Wagner" of the AREA Science Park of Trieste.

REFERENCES

1. Aretz, H. T., M. E. Billingham, W. D. Edwards, S. M. Factor, J. T. Fallon, J. J. Fenoglio, E. G. J. Olsen, and F. Schoen. 1986. Myocarditis. A histopathologic definition and classification. *Am. J. Cardiovasc. Pathol.* 1:3-14.
2. Bowles, N. E., E. G. J. Olsen, P. J. Richardson, and L. C. Archard. 1986. Detection of coxsackie-B-virus-specific RNA sequences in myocardial biopsy samples from patients with myocarditis and dilated cardiomyopathy. *Lancet* i:1120-1123.
3. *British Heart Journal*. 1980. Report of the WHO/ISFC task force on the definition and classification of cardiomyopathies. *Br. Heart J.* 44:672-673.
4. Chang, K. H., P. Auvinen, T. Hyypää, and G. Stanway. 1989. The nucleotide sequence of coxsackievirus A9; implications for receptor binding and enterovirus classification. *J. Gen. Virol.* 70:3269-3280.
5. Chapman, N. M., S. Tracy, C. J. Gauntt, and U. Fortmueller. 1990. Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridization. *J. Clin. Microbiol.* 28:843-850.
6. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
7. Gow, J. W., W. M. H. Behan, G. B. Clements, C. Woodall, M. Riding, and P. O. Behan. 1991. Enteroviral RNA sequences detected by polymerase chain reaction in muscle of patients with postviral fatigue syndrome. *Br. Med. J.* 302:692-696.
8. Hughes, P. J., C. North, P. D. Minor, and G. Stanway. 1989. The complete nucleotide sequence of coxsackievirus A21. *J. Gen. Virol.* 70:2943-2952.
9. Hyypää, T., P. Auvinen, and M. Maaronen. 1989. Polymerase chain reaction for human picornaviruses. *J. Gen. Virol.* 70:3261-3268.
10. Hyypää, T., P. Stålhandske, R. Vainionpää, and U. Pettersson. 1984. Detection of enteroviruses by spot hybridization. *J. Clin. Microbiol.* 19:436-438.
11. Iizuka, N., S. Kuge, and A. Nomoto. 1987. Complete nucleotide sequence of the genome of the coxsackievirus B1. *Virology* 156:64-73.
12. Jenkins, O., J. D. Booth, P. D. Minor, and J. W. Almond. 1987. The complete nucleotide sequence of coxsackie B4 and its comparison to other members of the Picornaviridae. *J. Gen. Virol.* 68:1835-1848.
13. Jin, O., M. J. Sole, J. W. Butany, W.-K. Chia, P. R. McLaughlin, P. Liu, and C.-C. Liew. 1990. Detection of enterovirus RNA in myocardial biopsies from patients with myocarditis and cardiomyopathy using gene amplification by polymerase chain

- reaction. *Circulation* 82:8-16.
14. Kitamura, N., B. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. Van der Werf, C. W. Anderson, and E. Wimmer. 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. *Nature (London)* 291:547-553.
 15. La Monica, N., C. Meriam, and V. R. Racaniello. 1986. Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. *J. Virol.* 57:515-525.
 16. Lindberg, A. M., P. O. K. Stålhandske, and U. Pettersson. 1987. Genome of coxsackievirus B3. *Virology* 156:50-63.
 17. McManus, B., and R. Kandolf. 1992. Evolving concepts of cause, consequence, and control in myocarditis. *Curr. Opin. Cardiol.* 6:418-427.
 18. Melnick, J. L. 1990. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses, p. 549-605. *In* B. N. Fields and D. M. Knipe (ed.), *Virology*. Raven Press, New York.
 19. Minor, P. D. 1985. Growth, assay and purification of picornaviruses, p. 25-41. *In* B. W. J. Mahy (ed.), *Virology. A practical approach*. IRL Press, Oxford.
 20. O'Connell, J. B. 1983. Evidence linking viral myocarditis to dilated cardiomyopathy in humans, p. 93-108. *In* J. A. Robinson and J. B. O'Connell (ed.), *Myocarditis: precursor of cardiomyopathy*. The Collamore Press, Toronto.
 21. Olive, D. M., S. Almufti, W. Almulla, M. A. Khan, A. Pasca, G. Stanway, and W. Alnakib. 1990. Detection and differentiation of picornaviruses in clinical samples following genomic amplification. *J. Gen. Virol.* 71:2141-2147.
 22. Rotbart, H. A. 1990. Enzymatic RNA amplification of the enteroviruses. *J. Clin. Microbiol.* 38:438-442.
 23. Rotbart, H. A., S. Eastman, J. L. Ruth, K. K. Hirata, and M. J. Levin. 1988. Nonisotopic oligomeric probes for the human enteroviruses. *J. Clin. Microbiol.* 26:2669-2671.
 24. Rotbart, H. A., M. J. Levin, and L. P. Villarreal. 1984. Use of subgenomic poliovirus DNA hybridization probes to detect the major subgroups of enteroviruses. *J. Clin. Microbiol.* 20:1105-1108.
 25. Ryan, M. D., O. Jenkins, P. J. Hughes, A. Brown, N. J. Knowles, D. Booth, P. D. Minor, and J. W. Almond. 1990. The complete nucleotide sequence of enterovirus type-70—relationships with other members of the Picornaviridae. *J. Gen. Virol.* 71:2291-2299.
 26. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
 27. Sommer, R., and D. Tautz. 1989. Minimal homology requirements for PCR primers. *Nucleic Acids Res.* 17:6749.
 28. Stanway, G. 1990. Structure, function and evolution of picornaviruses. *J. Gen. Virol.* 71:2483-2501.
 29. Stanway, G., P. J. Hughes, R. C. Mountford, P. Reeve, P. D. Minor, G. C. Schild, and J. W. Almond. 1984. Comparison of the complete nucleotide sequences of the genomes of the neurovirulent poliovirus P3/Leon37 and its attenuated Sabin vaccine derivative P3/Leon12a1b. *Proc. Natl. Acad. Sci. USA* 81:1539-1543.
 30. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. *J. Immunol.* 126:1614-1619.
 31. Tracy, S., V. Wiegand, B. McManus, C. Gauntt, M. Pallansch, M. Beck, and N. Chapman. 1990. Molecular approaches to enteroviral diagnosis in idiopathic cardiomyopathy and myocarditis. *J. Am. Coll. Cardiol.* 15:1688-1694.
 32. Weiss, L. M., L. A. Movahed, M. E. Billingham, and M. L. Cleary. 1991. Detection of coxsackievirus B3 RNA in myocardial tissues by the polymerase chain reaction. *Am. J. Pathol.* 138:497-503.