



Original contribution

# Histopathologic, immunohistochemical, and polymerase chain reaction assays in the study of cases with fatal sporadic myocarditis

Jeannette Guarner MD<sup>a</sup>, Julu Bhatnagar PhD<sup>a,\*</sup>, Wun-Ju Shieh MD, PhD<sup>a</sup>, Kurt B. Nolte MD<sup>b</sup>, Dennis Klein MD<sup>b</sup>, Michelle S. Gookin BS<sup>c</sup>, Silvia Peñaranda BS<sup>c</sup>, M. Steven Oberste PhD<sup>c</sup>, Tara Jones MA, HTL (ASCP)<sup>a</sup>, Chalanda Smith BS<sup>a</sup>, Mark A. Pallansch PhD<sup>c</sup>, Sherif R. Zaki MD, PhD<sup>a</sup>

<sup>a</sup>Infectious Disease Pathology Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA

<sup>b</sup>Office of the Medical Investigator, University of New Mexico School of Medicine, Albuquerque, NM 87131, USA

<sup>c</sup>Polio and Picornavirus Laboratory Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA

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**Summary** Paraffin tissue blocks from 27 cases with sporadic myocarditis were collected during a 12-year period at a single medical examiner's office. Blocks were studied by using histopathology; immunohistochemistry for viruses (adenovirus, enterovirus, influenza A and B, and human herpes types 4 and 5), bacteria (*Neisseria meningitidis*, *Ehlichia* sp, spotted fever group *Rickettsia*) and parasites (*Toxoplasma gondii* and *Trypanosoma cruzi*); and polymerase chain reaction (PCR)/RT-PCR for adenovirus and enterovirus. We identified enterovirus in 5 (18.5%) cases and *Sarcocystis* in a 36-year-old woman who had focal inflammation and myocyte necrosis. Immunohistochemical evidence of enteroviruses was found in the myocytes of 2 patients less than 6 months old who had diffuse mononuclear myocardial inflammation, interstitial pneumonitis; one also had encephalitis. In these 2 patients, the presence of enterovirus was confirmed by RT-PCR targeting the 5' nontranslated region and was serotyped as coxsackievirus B2 by sequencing the VP1 capsid region. In another 3 cases (ages 12, 47, and 54), enterovirus was detected by the 5' nontranslated region region; VP1 sequencing identified these as echoviruses 6, 13, and 7, respectively. Accurately identifying an infectious agent is the foundation for clinical and public health interventions. Despite using multiple diagnostic methods, an organism could only be detected in a small proportion of sporadic myocarditis cases.

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## 1. Introduction

Postmortem studies have shown that myocarditis is a major cause of sudden, unexpected death in children and adults less than 40 years of age [1,2]. Outbreaks of myocarditis usually occur in young children, but sporadic

\* Corresponding author. Infectious Disease Pathology Activity, Centers for Disease Control and Prevention, Mailstop G32, 1600 Clifton Rd, NE, Atlanta, GA 30333.

E-mail address: jbhatnagar@cdc.gov (J. Bhatnagar).

cases are seen in older children and adults. The diagnosis of myocarditis is based on the histopathologic presence of inflammatory cells in the heart and can be classified by the Dallas or Marburg criteria [3-7]. These criteria define active or definitive myocarditis by the amount of inflammatory infiltrate in the myocardium, whereas cases with fibrosis accompanied by lesser degrees of inflammation are considered chronic, borderline, persistent (ongoing), and healing (resolving) myocarditis. The list of potential causes of myocarditis is extensive and includes viruses, bacteria, parasites, toxins, drugs, allergens, and autoimmune diseases [1-3]. Defining the specific etiologic agent that causes sporadic or outbreak cases of myocarditis is important for developing or implementing prevention and treatment strategies. A variety of specimens and methods (serology, cultures, immunohistochemistry [IHC], and polymerase chain reaction [PCR]) have been used to detect infectious agents in cases of myocarditis, but identification of microorganisms in formalin-fixed myocardial samples, often the only specimen available, and correlation with histopathologic features have been inconsistent [8]. By using histopathology, IHC, and PCR assays, we studied formalin-fixed, paraffin-embedded tissues from cases with sporadic myocarditis in an effort to detect infectious causes.

## 2. Materials and methods

### 2.1. Cases

A search of the electronic data base of the New Mexico Office of the Medical Investigator (OMI), the statewide centralized medical examiner agency for New Mexico, identified 42 cases of fatal myocarditis during the years 1986 to 1998. From each case, hematoxylin and eosin (H&E)-stained heart sections were reexamined for the presence of myocyte necrosis or degeneration associated with inflammation. Only patients with active myocarditis [3-7] were included in this study (N = 27). For these cases, demographic and epidemiologic data were obtained, and IHC and PCR tests were performed. IHC and PCR assays were performed in 2 heart paraffin blocks that showed inflammation. In addition, lung paraffin blocks were tested with adenovirus and influenza viruses, whereas spleen or lymph node paraffin blocks were tested with the herpes viruses (Epstein-Barr virus and cytomegalovirus).

### 2.2. Immunohistochemistry

IHC assays used a previously described, indirect immunoalkaline phosphatase technique for infectious agents [9,10]. Briefly, IHC was performed on 3- $\mu$ m sections of formalin-fixed, paraffin-embedded tissues that were deparaffinized, rehydrated, and placed in a Dako Autostainer (Dako, Carpinteria, CA). Sections were incubated for 1 hour using the primary antibodies. Table 1 presents the antibodies used in the IHC assays with their reactivity spectrum, for

example, the enterovirus antibody reacts against formalin-fixed, paraffin-embedded enterovirus-infected cells (including representative viruses for each serotype), similarly the adenovirus antibody reacts against all adenovirus species. Optimal dilutions of the antibodies had been determined by previous experiments on positive control tissue samples. After incubation with the primary antibody, slides were washed, and the LSAB2 Universal alkaline phosphatase system (Dako) was used for colorimetric detection. Sections were then counterstained in Mayer's hematoxylin (Fisher Scientific International Inc, Hampton, NH).

For each test, positive controls included tissue sections of formalin-fixed, paraffin-embedded cultures or cases known to contain the respective parasite, bacteria, or virus. Negative controls consisted of a sequential tissue section from each block incubated with nonimmune serum or ascitic fluid of the species from which the primary antibody was derived (Table 1).

### 2.3. RNA extraction and RT-PCR assays for enterovirus

For each formalin-fixed, paraffin-embedded block that contained heart, one 10- $\mu$ m section was deparaffinized in xylene and rinsed in two 100% ethanol washes to remove residual xylene. Ethanol was aspirated, and the tissue pellet was air-dried for 10 to 15 minutes. The dried tissue pellet was resuspended in 105  $\mu$ L of proteinase K (1:20) digestion buffer cocktail and incubated at 45°C overnight. Before RNA extraction, the sample was incubated at 99°C for 7 minutes to inactivate proteinase K. The subsequent steps for RNA extraction were as described in the Paraffin Block RNA Isolation Kit's protocol (Ambion Inc, Austin, TX). RNA was resuspended in 15  $\mu$ L of RNA storage solution and stored at -80°C.

Primer sequences, expected sizes of PCR products, and annealing temperatures have been previously published and are summarized in Table 2 [11-16]. All samples were tested by enterovirus-specific and seminested reverse transcription (RT)-PCR assays targeting the 5' nontranslated region (5' NTR), which is a region present in all enteroviruses [11,12]. Enterovirus RT-PCR-positive cases were then typed by using a nested RT-PCR assay targeting the VP1 gene as previously described [13] and sequencing of the amplicons. To monitor the quality of extraction and presence of PCR inhibitors, each sample was also tested for the amplification of 18S rRNA by RT-PCR [14]. Each run included one positive control (either in vitro-transcribed RNA from enterovirus 68 [13] or RNA extracted from enterovirus-infected cells) and 2 no-template negative controls.

The RT-PCR assays were performed using the QIAGEN OneStep RT-PCR kit (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. Amplification was carried out on a GeneAmp-PCR System 9700 Thermocycler (Applied Biosystems, Foster City, CA). For each primer set, annealing temperature was adjusted accordingly (Table 2).

**Table 1** Primary antibodies used in the immunohistochemical assays and the tissues tested

Antibody against	Source	Type (species)	Comments	Tissues tested
Adenovirus	Centers for Disease Control and Prevention (CDC)	Monoclonal (mouse)	Reacts against representative serotypes from each adenovirus species (A, Ad31; B, Ad7; C, Ad5; D, Ad8; E, Ad4; F, Ad41)	Heart and lung
Enterovirus	Chemicon (catalogue no. 39125)	Monoclonal (mouse)	Reacts against enterovirus 71, coxsackie A7, A16, A21, coxsackie B1, B2, B3, B4, B5, and B6, echovirus 7, 11, and 12, and poliovirus, rhinovirus and some adenovirus	Heart
Influenza A virus	CDC	Monoclonal (mouse)	Reacts with the nuclear protein of influenza A virus	Heart and lung
Influenza B virus	CDC	Monoclonal (mouse)		Heart and lung
Human herpes virus type 4 (Epstein-Barr virus)	Dako (catalogue no. M0897)	Monoclonal (mouse)	Reacts against the latent membrane protein of Epstein-Barr virus	Heart, spleen, or lymph node
Human herpes virus type 5 (cytomegalovirus)	Dako (catalogue no. M0854)	Monoclonal (mouse)	Combination of 2 antibodies against cytomegalovirus early and intermediate early nuclear proteins (p52 and 76KDa)	Heart, spleen, or lymph node
<i>Neisseria meningitidis</i>	CDC	Polyclonal (horse)	Reacts against all <i>N meningitidis</i> serogroups	Heart
<i>Ehrlichia</i> sp.	CDC	Polyclonal (dog)	Reacts against <i>Ehrlichia chaffeensis</i> and <i>Ehrlichia canis</i>	Heart
Spotted fever group <i>Rickettsia</i>	CDC	Polyclonal (rabbit)	Reacts against <i>Rickettsia rickettsii</i> , <i>Rickettsia akari</i> , <i>Rickettsia parkeri</i> , <i>Rickettsia africae</i> , and <i>Rickettsia conorii</i>	Heart
<i>T gondii</i>	Dako (catalogue no. 123BC)	Polyclonal (rabbit)		Heart
<i>T cruzi</i>	CDC	Polyclonal (rabbit)		Heart

The amplification products were analyzed by electrophoresis in a 1.8% agarose gel (or 10% polyacrylamide gel for small amplicons) and visualized by ethidium bromide staining and UV light transillumination.

#### 2.4. DNA extraction and PCR assay for adenovirus

DNA extraction of heart and other tissues was performed with the QIAamp DNA mini kit (Qiagen) after deparaffinization with xylene and ethanol wash. The dried tissue pellet was resuspended in 180  $\mu$ L of lysis buffer ATL and 20  $\mu$ L proteinase K and incubated at 56°C overnight. The rest of the procedure was followed as described in the manufacturer's protocol. DNA was eluted in 100  $\mu$ L of AE buffer and stored at -20°C.

Primers used in the assay detect all adenoviruses [15] and are described in Table 2. All samples were tested by the adenovirus group-specific heminested PCR assay. To monitor the quality of extraction and presence of PCR inhibitors, each sample was also tested for the amplification of housekeeping gene  $\beta$ -globin by PCR [16]. Each run included 1 positive control (DNA extracted from adenovi-

rus-infected cells) and 2 no-template negative controls. The PCR assay was performed with the High Fidelity PCR kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.

#### 2.5. Sequencing and analysis of PCR enterovirus amplification products

Amplification products were extracted from a 1.8% agarose gel by using QIAquick gel extraction kit (Qiagen) and sequenced with a Big Dye Terminator 1.1 ready reaction cycle sequencing kit on a Prism 3100 automated sequencer (Applied Biosystems). Amplicon sequences were compared with the VP1 sequences of enteroviruses reference strains, including at least one representative of each recognized serotype, by script-driven sequential pairwise comparison using the program Gap (Wisconsin Sequence Analysis Package, version 10.3, Accelrys, Inc, San Diego, CA), as described previously [17]. In cases where the result was not unequivocal (highest score less than 75% or second-highest score greater than 70%), deduced amino acid sequences were compared using a similar method [13,17].

**Table 2** Oligonucleotide primers used in the RT-PCR assays

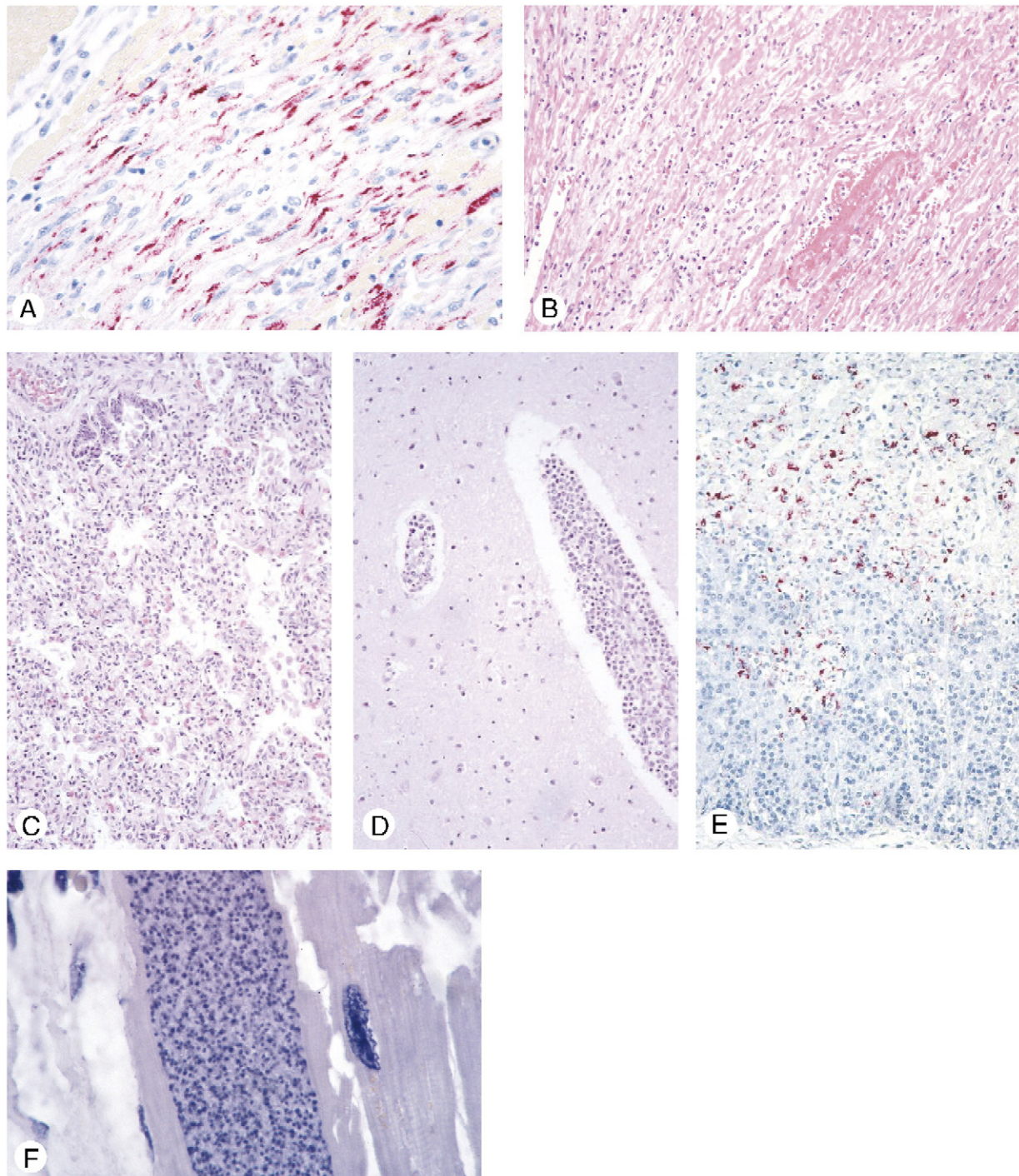
Virus/Control	Primer	Sequence (5' -3')	Location	Product size (bp)	Annealing temperature (°C)	Reference
All enteroviruses	E4KB-F	AAG GTG YGA AGA GYC TAT TGA GCT A	5' NTR	231	57	[11]
	E1-R	CAC CGG ATG GCC AAT CCA	5' NTR			
All enteroviruses (seminested)	MB-EV2	ATT GTC ACC ATA AGC AGC CA	5' NTR	152	56	[12]
	MB-EV1	CTC CGG CCC CTG AAT GCG	5' NTR	115	56	
	EV-PCR1	ACA CGG ACA CCC AAA GTA GTC GGT TCC	5' NTR			
All enteroviruses (nested)	224	GCI ATG YTI GGI ACI CAY RT	VP3	993	42	[13]
	222	CIC CIG GIG GIA YRW ACA T	VP1			
	AN89	CCA GCA CTG ACA GCA GYN GAR AYN GG	VP1	376	60	
	AN88	TAC TGG ACC ACC TGG NGG NAY RWA CAT	VP1			
All adenoviruses (seminested)	ADNEST-F	TTC CCC ATG GCI CAY AAC AC	Hexon	482	56	[15]
	ADGENE-R	CCC TGG TAK CCR ATR TTG TA	Hexon	442	56	
	ADNEST-R	AGG AAC CAR TCY TTR GTC AT	Hexon			
RNA internal control	18S-F	GTA ACC CGT TGA ACC CCA TT	18SrRNA	151	58	[14]
	18S-R	CCA TCC AAT CGG TAG TAG CG	18SrRNA			
DNA internal control	$\beta$ -globin-F	CAG GTA CGG CTG TCA TCA CTT AGA	$\beta$ -globin	184	55	[16]
	$\beta$ -globin-R	CAT GGT GTC TGT TTG AGG TTG CTA	$\beta$ -globin			



### 3. Results

Forty-two potential myocarditis cases were identified during this 12-year period from the electronic database of

the OMI. Active myocarditis was present in 27 (65%) cases, of which 13 had diffuse inflammation, whereas 14 had focal inflammation. The remaining cases were excluded from further studies because histopathologic examination



**Fig. 1** Myocarditis caused by Coxsackievirus B2. A, Enterovirus antigens inside myocytes detected using immunohistochemical assays (original magnification,  $\times 80$ ). B, Mononuclear inflammatory infiltrate in the heart with myocyte necrosis, edema, and hemorrhage (H&E, original magnification,  $\times 50$ ). C, Interstitial pneumonitis (H&E stain, original magnification,  $\times 50$ ). D, Encephalitis in patients with Coxsackie B2 virus infection (H&E stain, original magnification,  $\times 50$ ). E, Enterovirus antigens in the medulla of the adrenal gland detected using immunohistochemical assays (original magnification,  $\times 50$ ). F, *Sarcocystis* in myocardium of patient with myocarditis (Hematoxylin stain, original magnification,  $\times 250$ ).

revealed pericarditis (3 cases), endocarditis (4 cases), and noninflammatory (fibrosis and hypertrophy) heart pathology (8 cases). The mean age of the cases with definitive myocarditis was 28 years (range, 2 months to 57 years); there were 6 children less than 10 years of age. There were 16 males and 11 females. There were 16 white patients, 7 Hispanic, 3 Native American, and 1 black. Cases did not occur during a particular season or in a geographic location.

In 25 (93%) cases, IHC failed to provide evidence for the presence of the tested infectious agents. Enteroviruses were detected in myocytes (Fig. 1A) of 2 male patients aged less than 6 months who had diffuse mononuclear myocardial inflammation (Fig. 1B). Both patients had interstitial pneumonitis (Fig. 1C); however, viral antigens were not detected in the lung. One patient had encephalitis (Fig. 1D), and enterovirus antigens were observed in neurons in the central nervous system and in the adrenal medulla (Fig. 1E). Enterovirus antigens were also detected in occasional acinar pancreatic cells in the patient without encephalitis. These patients lived in 2 widely separated towns in New Mexico, and their deaths occurred 4 years apart.

Sequence analysis of enterovirus-specific RT-PCR assays targeting the 5' NTR and the VP1 capsid region using RNA extracted from formalin-fixed, paraffin-embedded tissue samples confirmed the presence of enterovirus in the 2 cases with IHC staining and 3 others (Table 3). The IHC-negative, enterovirus RT-PCR-positive cases included a 12-year-old boy with diffuse myocarditis, a 54-year-old man with focal myocarditis and vasculitis, and a 47-year-old man with focal polymorphonuclear and mononuclear myocardial inflammation. Further evaluation of the serotype by sequencing the amplicon of the VP1 RT-PCR assay identified coxsackievirus B2 in the 2 IHC-positive cases and echoviruses 6, 13, and 7 in the 3 IHC-negative, enterovirus RT-PCR-positive cases. Adenovirus-specific heminested RT-PCR assays using DNA extracted from formalin-fixed, paraffin-embedded samples were negative in all cases. Housekeeping genes for RNA and DNA extractions confirmed the presence of amplifiable nucleic acid in all cases.

In deeper heart sections that were used for IHC testing, one case showed a smooth, nonseptated, thin-walled cyst with small zoites (Fig. 1F). The cyst did not show IHC evidence of *Trypanosoma cruzi* or *Toxoplasma gondii*. Based on morphology and lack of IHC staining, the cyst was diagnosed as a

*Sarcocystis*. This patient was a 36-year-old white woman with focal inflammation and myocyte necrosis.

#### 4. Discussion

In a cohort of patients with sporadic active myocarditis from New Mexico, we identified microorganisms from formalin-fixed, paraffin-embedded heart tissue samples in 22% of cases by using histopathology, IHC, and RT-PCR assays. This study was unique in the diagnostic approach because positive results were confirmed using a variety of techniques. Frequency and identification of infectious agents in cases with myocarditis have varied widely from 10% to 100% [1,18-21]. The large variation between studies derives from several factors including the sample studied (serum, heart tissue), methods used to detect the infectious agent, confirmation of results, correlation with clinical and histopathologic findings, and time during the illness when sample was obtained (active versus healing myocarditis).

In this series of cases with sporadic fatal myocarditis, formalin-fixed, paraffin-embedded tissue blocks were the only consistently available specimen, and histology, IHC, and PCR were the methods that could be performed to detect infectious agents. In our study, we only tested cases with acute disease, which may have created a selection bias for infectious agents that cause severe pathology; however, some studies have demonstrated organisms when the tissues show minimal or no clinical or pathological evidence of disease. For example, a study that compared the presence of viruses in heart donors and recipients demonstrated evidence of enteroviruses and adenoviruses in 60% of donors compared with 47% of heart recipients [19]. Another study that used in situ PCR demonstrated a microbiologic agent in endothelium, myocytes, and inflammatory cells for each case tested [20]. Finding an infectious agent in cases without pathology or in a cell that is not the usual target suggests specimen contamination or detection of persistent, noninfectious virus; thus, positive results need to be confirmed.

False-positive results can occur with PCR-based assays or IHC assays. For example, we have interpreted staining of endothelial and inflammatory cells when applying the antibody used by Zhang et al [22] to be nonspecific because it has been present in these same cells in cases known to have other infections. Thus, a positive result needs to be confirmed by other techniques such as serology, culture, special stains, PCR assays targeting other genes or regions of the virus, or IHC using an antibody directed toward another epitope. In this study, the RT-PCR assays were more sensitive than the IHC assay for detection of enteroviruses, and positive results with the highly conserved 5' NTR region were confirmed by sequencing the amplicon obtained with the type-specific VP1 region.

In this series, concordant diagnosis of myocarditis by the OMI and secondary review of specimens occurred in 64%

**Table 3** Results of enterovirus-positive cases by IHC and RT-PCR assays

Age/Sex	IHC-positive tissues	Sequence identity
6 mo/male	Heart, brain, adrenal	Coxsackievirus B2
1 mo/ male	Heart, pancreas	Coxsackievirus B2
12 y/male	None	Echovirus 6
47 y/male	None	Echovirus 13
54 y/male	None	Echovirus 7

All 5 cases were RT-PCR-positive with assays that target the enterovirus 5' NTR and VP1 regions.



of cases, although each group reviewed slides prepared from the same tissue blocks. Diagnostic discrepancies appeared to be related to location of the inflammatory process (endocardium versus pericardium versus myocardium) and cases with fibrosis and hypertrophy with minimal inflammation that could correspond to healing myocarditis. Both the Dallas and Marburg criteria were developed to help standardize and establish the diagnosis of myocarditis in endomyocardial biopsies [3,4,6]; however, interobserver variability continues to occur and can be explained by potential discordance between clinical and histologic diagnosis and the focal nature of myocarditis [23,24]. Even when using larger tissue fragments obtained during autopsies, concordance of the diagnosis of myocarditis can be as low as 32%, largely due to overdiagnosis in patients who have died suddenly or are found dead [25].

In our study, 78% of cases of fatal myocarditis remained unexplained, this is a higher percentage than that found in a recent study of formalin-fixed heart specimens using PCR assays for enteroviruses and adenoviruses [18]. The inability to detect infectious agents in patients with myocarditis is not infrequent and has generated several hypotheses [1,3] including antigen mimicry, efficient clearing of the organism by the time of testing, and autoimmune reactions. It can be speculated that some of the microorganism-negative cases, particularly in adults, are noninfectious in nature and are due to drugs and toxins. Lastly, limitations of the assays might also be responsible for the inability to make an organism-specific diagnosis in every case of myocarditis. In our study, we may not have tested for microorganisms that could have been the cause of the myocarditis or the number of microorganisms might have been so low that the IHC assay could not detect them.

In an effort to identify infectious agents in our study, we tested a variety of tissues besides the heart, particularly for those infectious agents with primary noncardiac tissue targets. For example, influenza A and B viruses were tested in samples from the heart and lungs, since previous IHC studies have detected influenza virus in the lung of patients with myocarditis but failed to demonstrate the virus in the heart [9,26]. Similarly, for cytomegalovirus and Epstein-Barr virus, we tested heart and lymphoid organs. In systemic diseases that are accompanied by myocarditis such as Rocky Mountain spotted fever, ehrlichiosis, and meningococcemia, IHC assays have been used to detect organisms in the heart; thus, testing of other tissues was not necessary [10,27].

Using IHC, we detected coxsackievirus B2 in the hearts of 2 patients aged less than 6 months with active myocarditis, interstitial pneumonitis, and encephalitis. The histopathologic findings were consistent with a systemic viral infection, and enterovirus antigens were present in well-known target cells including myocytes and neurons. Echoviruses were identified in 3 IHC-negative, enterovirus RT-PCR-positive cases, aged more than 12 years, and showed varying degrees of myocardial inflammation. Echoviruses 6 and 7 are consistently among those most

commonly isolated in the United States, but echovirus 13 was relatively rare until it emerged as a major cause of aseptic meningitis in 2001 [28,29]. Echoviruses have not been previously associated with myocarditis. Finding coxsackieviruses and echoviruses in different age groups and showing different IHC reactions suggest that not all enterovirus serotypes have the same pathogenic mechanisms when causing myocarditis. Our results emphasize the importance of serotype identification in the virologic investigation of myocarditis cases.

In this cohort, we found 1 patient with *Sarcocystis* accompanied by myocarditis. *Sarcocystis* has been described as a possible cause of myocarditis when a cyst disintegrates and elicits a localized inflammatory reaction [30]. Morphologically, it may be difficult to distinguish tissue cysts of *Sarcocystis* from *T gondii* or *T cruzi*. Some *Sarcocystis* species have thick cyst walls and internal septations that can help in the diagnosis, but others do not and may pose a diagnostic problem that can only be solved using IHC or PCR assays. In our case, *Sarcocystis* was only detected after deeper sections had been studied indicating a low parasite burden.

In summary, we used a unique combination of histopathology, IHC, and PCR assays in formalin-fixed, paraffin-embedded tissues to identify microorganisms and serotype enteroviruses in cases with sporadic myocarditis. Detection limits for each test vary; thus, cautious interpretation of results is required not to over- or undercall potential etiologic agents. Better, well-validated, microorganism-specific techniques that will aid in the diagnosis of causative agents of cases with myocarditis are needed.

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