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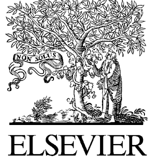

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Original contribution

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

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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 menigitidis*, *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

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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-μm 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-μm 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 μL 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 μL 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).

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