Journal of Medical Virology 80:1756–1761 (2008)

First Report on Fatal Myocarditis Associated With Adenovirus Infection in Cuba

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Myocarditis is caused frequently by viral infections of the myocardium. In the past, enteroviruses (EV) were considered the most common cause of myocarditis in all age groups. Other viruses that cause myocarditis are adenovirus and influenza viruses. Parvovirus B19 infection is associated sometimes with myocarditis. Members of the Herpesviridae family, cytomegalovirus (CMV), and human herpesvirus 6 (HHV-6) have been associated occasionally with myocarditis. During an atypical outbreak of acute febrile syndrome, eight children, with ages from 5 months to 15 years, died in cardiogenic shock due to myocarditis in July–August 2005, in the city of Havana, Cuba. Nested polymerase chain reaction (nPCR) and nested reverse transcription-PCR (nRT-PCR) were carried out on fresh heart muscle and lung tissue to analyze the genomic sequences of adenovirus, CMV, HHV-6, herpes simplex virus, Epstein–Barr virus (EBV), varizella zoster virus, influenza virus A, B, C, respiratory syncytial virus (RSV) A and B, parainfluenza viruses, rhinoviruses, coronavirus, flaviruses and enteroviruses. Evidence was for the presence of the adenovirus genome in 6 (75%) of the children. Phylogenetic analyses of a conserved hexon gene fragment in four cases showed serotype 5 as the causal agent. No others viruses were detected. Histological examination was undertaken to detect myocardial inflammation. After exclusion of other possible causes of death, the results indicated that viral myocarditis was the cause of death in patients with adenovirus infection. ***J. Med. Virol. 80:1756–1761, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: myocarditis; polymerase chain reaction; adenovirus

INTRODUCTION

Myocarditis is an inflammatory disease of the cardiac muscle which is caused by intramyocardial infiltration by immunocompetent cells [Richardson et al., 1996]. Etiologically, the relevant factors are the direct or indirect influence of infectious pathogens or toxic, chemical or physical agents, allergic–hyperergic reactions and myocardial inflammatory events in the

Grant sponsor: Cuban National Program of Surveillance and Control of Acute Respiratory Infection (partial support).

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Accepted 2 June 2008
DOI 10.1002/jmv.21274
Published online in Wiley InterScience (www.interscience.wiley.com)

context of systemic diseases [Feldman and McNamara, 2000]. An infectious cause of myocarditis is usually suspected when unexplained heart failure or arrhythmia occurs in a person with a febrile illness or upper respiratory tract infection. Acute myocarditis is typically sporadic, although clusters have been reported during outbreaks of viral disease [Helin et al., 1968; Woodruff, 1980].

Viral myocarditis shows different clinical features, depending on the age of the patient. In pediatric patients, viral myocarditis can present as acute heart failure and cardiogenic shock, and in older patients, it presents often as chronic, slowly progressive heart failure and dilated cardiomyopathy [Peter et al., 2000]. Myocardial biopsy specimens used for pathological examination, the conventional standard for diagnosis, have been considered difficult to collect in nonfatal cases [Fowles and Mason, 1982; Billingham, 1990]. Developments in molecular biology techniques have helped to establish a viral etiology in patients with myocarditis. In situ hybridization and PCR remain the most reliable diagnostic tools for confirming viral heart disease, and PCR is ideal to confirm viral persistence in the myocardium of patients with myocarditis [Baboonian and Treasure, 1997].

The commonest viral causative agents of human myocarditis include *Coxsackieirus B group* and adenoviruses [Peter et al., 2000]. *Adenoviruses* are important human pathogens infecting a wide range of tissues. They represent a large family, currently comprising 52 different types, which is divided into 7 different subgroups named from A to G, based on different oncogenic, hemagglutinating, morphological, and phylogenetics. The prevalence of adenovirus infection is high, as revealed by serological studies [Wigand et al., 1964; Peter, 2000; Shenk and Horwitz, 2001].

In Cuba, there is a previous report of a myocarditis outbreak in infants in which Coxsackie virus was the etiological agent [Mendiondo et al., 1972].

The purpose of this study was to identify the etiological agent involved in the cause of death of eight children who died from cardiogenic shock due to myocarditis.

MATERIALS AND METHODS

Patients

In July 2005, eight patients between 5 months and 15 years old were admitted to three Pediatric Hospitals of the city of Havana, Cuba, within a 3-week period. The clinical presentation suggested myocarditis which was confirmed by electrocardiogram and echocardiogram. In addition, an expert pathology panel who reviewed the autopsied hearts according to the Dallas Criteria provided histopathological findings in postmortem diagnosis of myocarditis [Aretz, 1986].

All parents gave written informed consent for virological testing to determine a possible etiological agent in systemic infection known cardiac involvement.

Clinical features were evaluated retrospectively using both the hospital records and all other clinical information available.

Samples

Samples of lung and heart tissue (right anterior and posterior ventricle, ventricular septum and left anterior ventricle wall) from eight myocarditis cases were received at the Department of Virology ("Pedro Kourí" Tropical Medicine Institute, Havana, Cuba) for virological study. All specimens were collected in 5 ml of virus transport medium (MEM, Gibco-BRL, Life Technologies, Paisley, Scotland; penicillin 200 U/ml, and streptomycin 200 μg/ml, BioWhittaker, Germany; mycostatin 200 U/ml, Sigma Chemical Co., St. Louis, MO; bovine albumin 0.25%, Merck, Darmstadt, Germany). Specimens were aliquoted in duplicate; one of which was immediately processed and the rest stored at -80°.

Nucleic Acid Extraction

Total viral RNA/DNA extracted from 200 μl aliquots from tissue samples were first homogenized using the guanidinium thiocyanate method as described previously by Casas et al. [1995]. The lysis buffer included 100 copies of the cloned, amplified product of the internal control described by Coiras et al. [2003]. It was used for checking the extraction process, the amplification efficiency, and the presence of inhibitors in the clinical specimens. After processing, the dried pellet was resuspended in 15 ml of RNAse-free sterile water. Negative controls, consisting of RNAse-free sterile water (Sigma Chemical Co.) and heart tissue sections from five different persons with no evidence of heart disease were treated following the same procedure. For each assay, known positive controls, derived from infected viral cells, were added.

Detection of Viral Genomes

Lung and myocardial samples were examined for adenovirus, *influenza viruses A*, *B*, and *C*, RSV A and B, *Parainfluenza viruses*, *Coronaviruses*, *Rhinoviruses*, and EV, CMV, HHV-6, EBV, *Herpes Simplex 1* and *2*, *Varizella Zoster virus*, and *Flaviruses* genomes using nPCR and nRT-PCR. The protocols used have been previously published [Tenorio et al., 1993; Coiras, 2003; Coiras et al., 2004; Sanchez-Seco et al., 2005]. The products of each reaction were analyzed by agarose gel electrophoresis containing 0.5 mg/ml ethidium bromide (Sigma Chemical Co.), and the DNA product was visualized by UV transillumination.

In all cases, clinical samples were defined as positive when the nPCR or nRT-PCR assays were positive in two different aliquots tested at different times. In addition, they were confirmed by DNA sequencing.

The procedures for preparing and amplifying specimens included all conventional recommendations to avoid contamination with PCR amplification products or positive control [Kowk and Higushi, 1989].

DNA Sequencing

Positive specimens for ADV were processed by two independent nested reactions as described previously [Casas et al., 2005]. The PCR products were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany), and the sequence was determined to confirm the identity of the virus detected by the PCR reaction. Sequences were obtained by an automatic DNA sequencer (ABI Prism 3700; Applied Biosystems, Foster City, CA) using Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems). Nucleotide sequences obtained were compared and aligned with previously published sequences using CLUSTAL X (version 1.83) program.

Nucleotide sequences accession numbers. The GenBank accession numbers of the nucleotide presented in this study are EU179786 to EU179791.

RESULTS

Patients

Patients (n = 8) with myocarditis from ages 5 months to 15 years. The clinical characteristics of all patients are summarized in Table I.

Clinical Presentation

A review of all medical records of patients with a diagnosis of myocarditis at all three pediatric hospitals in the city of Havana was conducted and it was found that the initial symptoms included fever (100%), anorexia (100%), headache (75%), oliguria, vomiting and diarrhea (25%), malaise (37.5%), and cough (62.5%). Parents referred a previously flulike illness (75%) and gastrointestinal symptoms (25%).

Signs of diminished cardiac output such as tachycardia, weak peripheral pulse, distal cyanosis, cool limbs, decreased capillary refill, and pale skin were present in all patients. All cases showed cardiogenic shock due to myocarditis at the final stage.

The chest radiography showed slight cardiomegaly in all cases. Electrocardiographic abnormalities were common and included low-voltage QRS (<5 mm through the limb leads), sinusal tachycardia, depression of the ST segment, inversion of the T wave, short PR segment and prolonged QT interval. Echocardiograms obtained on most patients uniformly showed a poorly contractile globular left ventricle with low output (ejection fraction <60%).

PCR Analysis

All samples were positive for the presence of internal control, indicating the successful isolation of nucleic acid (Fig. 1). Of the 16 samples tested, PCR amplified viral genome of adenovirus in ten samples (62.5%) of eight patients (Table II). None of the samples was positive for the rest of the viruses investigated. None of the control samples were positive for any virus. Sequencing of the amplified fragments and comparison with the published sequences confirmed the specificity of the PCR and the identity of the amplimers for all positive samples.

A BLAST search of the GenBank sequence database (NCBI, Bethesda, MD) demonstrated that all 10 adenovirus-positive samples had between 98% and 100% nucleotides identity with the hexon gene of adenovirus type 5.

DISCUSSION

Many etiological causes of myocarditis have been described, including the infectious, rheumatological and allergic factors as well as the use of medications and toxins. Defining the specific etiologic agent that causes sporadic or outbreak cases of myocarditis is important for developing or implementing prevention and treatment strategies.

Enteroviruses have been considered responsible for up to 50% of cases of acute myocarditis [Bowles et al., 1989; Jin et al., 1990]. However, some reports have shown that adenoviral infection is at least as common as enterovirus infection [McCarthy et al., 1997; Liu and Opavsky, 2000; Bowles et al., 2003]. Generally, one-third of the patients recover the full cardiac function, one-third of the patients develop chronic heart failure, and one-third either require cardiac transplantation or die [Wheeler and Kooy, 2003]. The true incidence of pediatric viral myocarditis is unknown because many cases are asymptomatic initially and, therefore, unrecognized.

TABLE I. Characteristics of Patients With Fatal Myocarditis

Patient	Age	Gender	Previous disease(s) or immucompromising condition(s)	Duration of symptoms in days
1. 1609	5 months	F	None	One day
2. 1645	12 years	M	Dilated cardiomyopathy	Three days
3. 1729	1 years	F	None	One day
4. 1762	5 years	M	Splenectomy	One day
5. 1589	15 years	M	Sick cell anemia	Four days
6. 1682	10 months	M	B-Talhasemia	Three days
7. 1473	9 years	M	Epilepsy	One day
8. 1617	2 years	F	None	Two days

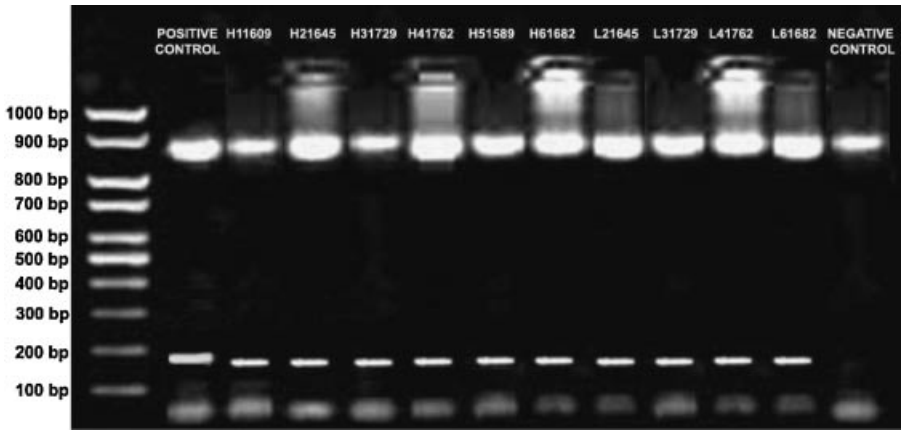

Fig. 1. Detection of adenoviral genomic DNA by nPCR with primers designed to hexon region. A 100-bp ladder is shown in first lane. Adenovirus-positive control PCR is seen as 181-bp amplimer in second lane (from left to right). From lanes 3 to 12 are patient samples indicated by sample number and kind of tissue (H-hearth and L-lung) and negative control in the last lane.

Frequency and identification of infectious agents in cases with myocarditis have varied widely from 10% to 100% [Feldman and McNamara, 2000; Vare et al., 2000; Bowles et al., 2003; Huhn et al., 2005]. The data presented in this demonstrate that in children with acute myocarditis that progressed to death, adenoviral DNA can be detected in a significant proportion of cases (six of eight, 75%). However, this is not surprising; there are similar reports from pediatric patients with myocarditis in whom adenoviral DNA have been detected [Bowles et al., 1999, 2002, 2003; Vare et al., 2000].

The inability to detect infectious agents in patients with myocarditis is not infrequent and has generated several hypotheses [Peters and Poole-Wilson, 1991; Feldman and McNamara, 2000], including antigen mimicry, efficient clearing of the organism by the time of testing, autoimmune reactions, methods used to detect the infectious agent, confirmation of results, correlation with clinical and histopathological findings, and the time when sample was obtained during the illness. It is possible that in this study the two cases with PCR-negative myocarditis might be explained by noninfectious in nature or by a causative role of less common cardiotropic viruses that were not investigated. However, it should be noted that in Cuba, the last reports on mumps and rubella cases were in 1993 and 2004, respectively [PAHO, 2007].

TABLE II. PCR Results

	PCR for adenovirus	
Patient	Lung	Heart
1. 1609	Negative	Positive
2. 1645	Positive	Positive
3. 1729	Positive	Positive
4. 1762	Positive	Positive
5. 1589	Negative	Positive
6. 1682	Positive	Positive
7. 1473	Negative	Negative
8. 1617	Negative	Negative

The results presented show that the clinical presentation in the eight children with acute myocarditis was severe with acute cardiac decompensation that progressed to death. Five cases (62.5%) presented underlying medical conditions. With a diagnosis of myocarditis, it is important to consider whether there is an underlying condition causing myocarditis. The list of possible underlying conditions mentioned in various sources for myocarditis includes: bacterial infection, viral infection (enteroviruses, adenoviruses), autoimmune myocarditis, rheumatic fever, Q fever, scrub typhus, Chagas disease, Toxoplasmosis, radiotherapy, and certain medications. Adenovirus infections can range in severity from unapparent or mild clinical illness to severe, life-threatening disease. Thus, the majority of adenovirus infections are self-limited, with severe or disseminated disease occurring sporadically in immunocompromised patients (e.g., infants), patients with AIDS, transplant recipients, and those with underlying disease [Carrigan, 1997; Hong et al., 2001; La Rosa et al., 2001]. Since myocarditis is an inflammatory disease in which infectious agents and immunological disorders go hand in hand. The pathophysiological mechanisms represent a critical area for investigation. Individual genetic variation, specific viral properties, or both are accepted as candidate mechanisms [Liu et al., 2006].

The diagnosis of acute myocarditis in children is based on histological criteria. The clinical manifestations are very variable, with signs and symptoms ranging from viral syndrome to cardiac arrhythmia, heart failure, or death. Clinical suspicion is critical. The physical examination may reveal respiratory compromise or other signs of congestive heart failure. The management of acute viral myocarditis relies on early recognition to prevent deterioration further [Wheeler and Kooy, 2003].

The proof of a viral affliction of the myocardium requires analysis of endomyocardial biopsies with more sensitive molecular biology methods, such as in situ hybridization or PCR [Bowles et al., 1999, 2003]. Posi-

tive serological tests cannot prove viral infection of the myocardium. In this report, the polymerase chain reaction identified adenovirus as the unique agent in the myocardium of Cuban children with myocarditis.

The classification of adenoviruses into serotypes is based on virus isolation, followed by neutralization tests with type-specific sera, and may take several weeks, which limits the clinical value of this approach, especially in the management of immunocompromised patients. There is a growing clinical interest in serotype determination of clinical isolates since it is becoming increasingly clear that specific serotypes are associated with the manifestation and severity of the disease presentation [Takeuchi et al., 1999; Fujimoto et al., 2000; Gray et al., 2007; Jones et al., 2007]. Conversely, identification of adenovirus in a patient specimen by PCR, followed by characterization of the amplimer, allows the identification of the virus at the species or type level more expeditiously.

Direct sequencing was used to confirm positive results and to characterize the virus. Sequencing analysis of the PCR products showed that in all positive samples for adenovirus, the serotype detected was adenovirus type 5, a group C adenovirus commonly detected in patients with myocarditis. Adenovirus types 2 and 5 appeared to be cardiovirulent serotypes of adenovirus in adults and children [Pauschinger et al., 1999]. The cause and effect association between the detection of virus and the etiology of myocarditis has been widely accepted.

The results of this study demonstrate the importance of combined investigations using clinical diagnosis, molecular techniques with serotype identification in the virological investigation of myocarditis cases. Therefore, further research is required to clarify the involvement of others viruses in cardiovascular disorders.

ACKNOWLEDGMENTS

The nucleotide sequence was performed in the Instituto de Salud Carlos III, Madrid, Spain and was funded by the proposal MPY-1177/06 entitled Caracterización molecular de ADV detectados en muestras clínicas de pacientes con infección respiratoria. Authors wish to thank all of the physicians who provided samples and clinical data. Special thanks to Dr. Enrique Tabares López from Facultad de Medicina, Universidad Autónoma de Madrid for his collaboration in this study. We also acknowledge all technicians and researchers of the Microbiology Division of the Instituto de Medicina Tropical Pedro Kourí, la Habana, Cuba, who collaborated in this investigation.

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