

Detection of enteroviral sequence in endomyocardial tissues from patients with cardiac diseases in Northern Brazil

Detecção de seqüências de enterovirus em tecido miocárdico de pacientes com doenças cardíacas no norte do Brasil

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Resumo No presente trabalho, são mostrados resultados de um estudo piloto direcionado à detecção de seqüências de enterovirus em tecido cardíaco obtido a partir de biópsias endomiocárdicas de pacientes com doenças cardíacas na região Amazônica. Seis amostras coletadas de três pacientes foram analisadas por RT-PCR obtendo-se três espécimes positivos e três negativos. Esses achados preliminares sugerem a participação dos enterovirus na etiologia de doenças cardíacas, principalmente miocardites, e justificam estudos mais amplos nesse assunto.

Palavras-chaves: Doenças cardíacas. Enterovirus. RT-PCR.

Abstract In the present report we describe the results from a pilot study aimed at detecting enterovirus sequence in cardiac tissues, obtained through endomyocardial biopsies, from patients suffering from cardiac diseases in the Amazon region. Six samples that were collected from three patients were analysed by RT-PCR showing 3 positive and 3 negative results. These preliminary findings suggest the participation of enteroviruses in the etiology of cardiac diseases, mainly myocarditis, and warrant further and broader local studies on this subject.

Key-words: Cardiac diseases. Enterovirus. RT-PCR.

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Research supported by an agreement nº 022/ 97 between Fundo Estadual de Ciência e Tecnologia/ Secretaria de Estado de Ciência, Tecnologia e Meio Ambiente (FUNTEC/ SECTAM) and Fundação Luiz Décourt.

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Recebido para publicação em 15/10/97.

Cardiac diseases, in particular myocarditis, are often associated with the Picornaviridae enterovirus (EV) Coxsackie B⁶. This association has been suggested by several authors^{3 7 18} after isolation of the virus from patients yielding positive, specific serology. The first studies suggesting this association were done using the classic methodology which includes isolation of the virus and serology^{3 7 18}. Detection of EV sequence in cardiac tissues, particularly myocardial cells, was reported in the following years^{2 13}. In addition, other studies characterised the nucleotide sequence of the EV CB1 type¹⁰. Certainly those studies were greatly facilitated by the early description of the primary structure of the polioviruses¹⁴.

The genome of enteroviruses consists of one single-stranded, plus sense RNA molecule of 7.2 to 8.5kb. Besides of the conserved untranslated region (UTR) at the 5' end, (600-1200nt), there is a shorter untranslated region at the 3' extremity (3' UTR) (50-100nt) and a coding region for single polyprotein of 2100 to 2400 amino acids in the middle of the genome. Both ends of the genome are modified: the 5' end by VPg (23 amino acids) and the 3' end by poly(A)⁴.

The conserved sequence of the EV genome has been currently used for PCR amplification of the majority of the EVs. More recently, other sequences derive from several human picornaviruses have been used as probes for diagnostic purposes. The utilization of these probes for *in situ* hybridization²¹ and gene amplification by the PCR^{9 11 20 23} has helped to elucidate the etiology of myocardites. More recently, Hyypiä⁸ analysed samples from 276 myocardites and cardiomyopathy patients and detected enterovirus RNA in 56 myocardial biopsies and explanted hearts, using dot blot, *in situ* hybridization and PCR methods. The association between the viral infection and cardiomyopathies¹⁹ appears to be confirmed by the presence of the EV genome in persistently infected patients²³. These findings have suggested the possibility of the EV infection being related to the dilated cardiomyopathy^{1 16}.

Experimental studies that have been conducted to detect EV sequences in cardiac tissues^{12 15 17} have used generic and specific primers²².

In Brazil, studies on the association between EV and cardiac diseases are scarce and were based so far on the use of viral, isolation and serologic studies⁵. This study was undertaken to detect EV in cardiac biopsies from patients who

live in northern Brazil, using virological molecular procedures.

Patients. The clinical and laboratory features of the patients studied are described as follows. The common finding in all of them was the abrupt onset of the cardiac disease. Patient 1 was a 35-year old black woman with history of fatigue starting at the 25th day of puerperium. She presented with congestive heart failure due to peripartum cardiomyopathy characterized as class III according to The New York Heart Association. When she was admitted to the cardiology ward, the ECG showed sinus rhythm, heart rate of 98bpm, left atrium enlargement, isolated ventricular premature beats and diffuse T-wave changes. Chest X-ray showed bilateral pulmonary congestion and moderate left ventricle enlargement. Patient 2, a 28-year old black woman, presented with syncope, fatigue and palpitations. The ECG showed *sinus* rhythm and frequent ventricular premature beats. Patient 3, a 74-year old white man, was admitted because of congestive heart failure due to dilated cardiomyopathy of recent onset characterized as class IV on the basis of The New York Heart Association. The ECG showed left bundle branch blockage. The chest X-ray showed pulmonary congestion and severe enlargement of the cardiac area. It should be pointed out that endomyocardial biopsies were done only when strictly indicated by cardiologists. In these cases, patients were fully informed on the procedure and written consents were routinely obtained from them.

PCR. The methodology was essentially the same used in previous studies^{1 19 23}. Two endomyocardial biopsy fragments were taken from the left ventricle by the myocardial biopsies and kept on -70°C or in formalin until they were analysed. Control samples were obtained at autopsy from patients with noncardiac diseases (belonging to the Unit of Molecular Virology of Pasteur Institut) The first procedure consisted of RNA extraction: the samples were homogenised in RNazol (guanidium/phenol) and chloroform. Extracted RNA was precipitated with isopropanol, centrifuged, washed in ethanol and vacuum dried. The pellet was dissolved in sterile water and reprecipitated by ethanol in presence of NaCl and kept at -20°C overnight. After a new centrifugation, the pellet was vacuum dried, dissolved in sterile water and submitted to RT-PCR assays. Initially the cDNA synthesis and the first PCR were performed in the same

reaction: 3µl of the extracted RNA and 47µl of the assay mixture were used. This was composed of transcription buffer (250mM Tris-HCl, pH 8.4, 50mM MgCl₂, 350mM KCl, 15mM dithiothreitol), RNAsin (40U), dNTPs mix (2mM), reverse transcriptase AMV (10U), primer 2, primer 3 (10pmole each), Taq polymerase (2.5U) and water. DNA amplification was carried out in a Cyclogene thermocycler PCH-3 for 30 cycles (denaturation 94°C, one min.; annealing 42°C, one min.; extension 60°C, one min.). In the second PCR were used 3µl of the product from the first PCR and 97µl of the reaction mixture: Taq buffer, dNTP, primer 2, primer 10, Taq polymerase and water. The amplification products were analyzed by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining (Figure 1).

Primers used. The primers used in this study were chosen in the 5' non coding region of poliovirus type 1 according to the following sequence: primer 2: 5'-CAAGCACTTCTGTTTCCCCGG-3'; primer 3: 5'-ATTGTCACCATAAGCAGCCA-3'; primer 10: 5'-ACGGACACCCAAAGTAGTCG-3'.

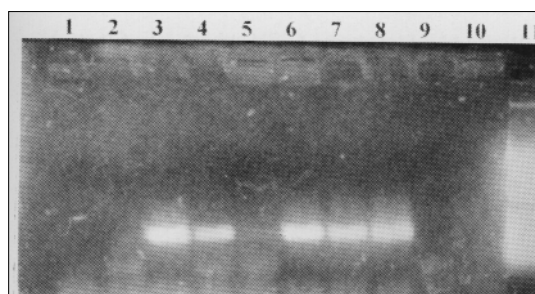


Figure 1 - RT-PCR amplification of EV nucleotide sequences from biopsies samples taken from cardiac patients inhabiting the Amazon region.

Lane 1, 2 and 3: samples stored at -70°C

Lane 4, 5 and 6: samples stored in formalin

Lane 7 and 8: positive control

Lane 9 and 10: negative control

Lane 11: molecular weight markers

Lane 1 and 4: patient 1, inconclusive

Lane 2 and 5: patient 2, negative

Lane 3 and 6: patient 3, positive

Of the three studied cases, one was positive (patient 3), one negative (patient 2) and one gave inconclusive results (patient 1) (i. e. the fragment stored in formalin was positive while that stored at -70°C was negative). Although a few number of specimens have been examined,

the detection of EV sequences in the formalin-fixed sample rather than in that stored at -70°C, may indicate the former storage procedure as being suitable for studies like ours. Another possible explanation for this would be the uneven distribution of the viral genome in the myocardial tissue. The demonstration of enterovirus sequence in myocardial biopsies taken from some patients suffering from cardiomyopathy suggests the possibility of Coxsackie B virus being associated to its pathogenesis. Although preliminary, these results lead us to postulate that the EVs can be associated with the cardiomyopathies in northern of Brazil and warrant further and broader investigations on the occurrence of cardiomyopathies of possible viral aetiology in this region

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