Forensic Science International 262 (2016) 166–172

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Postmortem diagnosis of infectious heart diseases: A mystifying cause of Sudden Infant Death

Imed Gaaloul [a,b,c,d,*], Samira Riabi [a], Mark Evans [b], Timothy Hunter [c], Sally Huber [d], Mahjoub Aouni [a]

[a] Laboratory of Transmissible Diseases LR99-ES27, Faculty of Pharmacy, Avenue Avicenne 5000, Monastir, Tunisia
[b] University of Vermont, Department of Pathology, Division of Experimental Pathology, Burlington, USA
[c] University of Vermont, DNA Microarray Facility, 305 Health Science Research Facility, Burlington, USA
[d] University of Vermont, Department of Pathology, 208 South Park Drive, Suite #2, Colchester, VT 05446, USA

ARTICLE INFO

Article history:
Received 10 January 2016
Received in revised form 4 March 2016
Accepted 5 March 2016
Available online 12 March 2016

Keywords:
Sudden infant death
Coxsackievirus B
Histopathology
Immunohistochemistry
Molecular pathology

ABSTRACT

Sudden infant death (SID) is an unresolved problem of high relevance and previous studies have indicated a role of viral heart infections. The diagnosis remains difficult in clinical practice using routine diagnostic tests and must be substantially improved.

A prospective study based on post-mortem samples from SID victims whose heart disease was not clinically recognized was conducted for 4 years in a Tunisian University Hospital. Pediatric cases of unnatural death served as controls. Both SID victims and controls were investigated for possible coxsackievirus-B (CV-B) infection in heart tissue.

During the study period, 39 cases with a male predominance (77%) were reported. There was no positive family history of coronary artery disease among the victims. In 35 cases (90%), low birth weight and/or critical development period were reported. All SID victims had complained of mild fever and insomnia for a few days preceding death, which required infectious laboratory investigations marked with an elevated white blood cell count (WBC) and C-reactive protein (CRP). The cardiac biomarkers were also elevated. The histopathological investigations of the heart tissue samples revealed signs of myocardial and pericardial inflammation. Enterovirus was detected by immunohistochemistry (IHC) and PCR from myocardial samples from 6 cases (15.3%) having myocarditis and 3 cases (7.7%) having perimyocarditis.

The current study is of great interest and is aimed at urging health professionals to adopt systematically long intensive heart care in infants with underlying vulnerability as well as new diagnostic approaches including histopathology complemented with IHC and molecular pathology.

1. Introduction

Sudden death is mainly defined as rapid, unexpected and natural death of an individual who appears healthy but dies suddenly within a short time due to a pre-existing disease or a functional disorder. Most often, sudden unexpected death due to natural causes results from previously unknown cardiovascular diseases though extra cardiac causes should not be ruled out [1–3]. New medical research has reported that infants have a higher risk of sudden unexpected death [4]. Ruling out extra cardiac causes of death essentially cerebral, pulmonary and other factors such as prematurity and exogenous stressors, infants died suddenly due to silent cardiovascular infections [5,6]. Infectious heart diseases include a group of entities involving the heart wall, mainly myocarditis and pericarditis. Myocarditis is clinically defined as an inflammatory myocardial disease [7]. Patients who have suffered from a heart attack may develop pericarditis pathologically defined as an acute inflammation of the pericardium [8]. The coexistence of acute myocarditis and pericarditis is not uncommon since both are commonly caused by cardiotropic viruses. The two terms (perimyocarditis) and (myopericarditis) are used to describe the disease. While perimyocarditis implies predominant myocardial involvement and myopericarditis implies predominant pericardial involvement, both terms are used interchangeably [9,10]. Recent clinical studies and anecdotal

* Corresponding author at: Laboratory of Transmissible Diseases LR99-ES27, Faculty of Pharmacy, Avenue Avicenne 5000, Monastir, Tunisia.
Tel.: +216 73 466 950; fax: +216 73 465 754.
E-mail address: chbeli_imed@yahoo.fr (I. Gaaloul).

http://dx.doi.org/10.1016/j.forsciint.2016.03.002
0379-0738/

communications reported on different viruses. Among them, CV-B, a small nonenveloped single-stranded, positive-sense RNA virus in the Picornaviridae family and Enterovirus genus, has been implicated in 25% to 40% of acute myocarditis and dilated cardiomyopathy cases in infants and young adolescents [11].

In this framework, the current study is aimed at investigating into how accurately CV-B heart infection is involved in SID by using a multitude of tests: conventional histopathology for the detection of inflammatory infiltrates and necrosis sites, molecular pathology for the investigation of the enteroviral genomic RNA (conserved sequences and VP1-capsid-protein coding region) and modern IHC to reveal the enteroviral VP1-capsid-protein and immune inflammatory markers (CD3-T and CD19-B-lymphocytes).

2. Materials and methods

2.1. Postmortem samples

The present study reported 39 SID victims (study group) 30 males and 9 females aged 3–9 months. The postmortem samples, heart necropsies and – when present – pericardial fluids were obtained from 2010 to 2014. Some clinical information including low birth weight and critical development period were reported by the victims' families. All SID victims had complained of mild fever and insomnia for a few days prior to death requiring infectious laboratory investigations marked with an elevated WBC count and CRP. The cardiac biomarkers were also elevated. In terms of susceptibility, there was no positive family history of coronary artery disease and the maternal smoking factor was excluded. A total of 17 cases of unnatural death home accidents all males aged 2–11 months (control group) were used as controls (Table 1).

In this prospective study, the 39 SID victims were classified as follows: in 3 cases, myocarditis revealed histologically during autopsy was undoubtedly fatal (non-SIDS). The 36 remaining victims did not present any significant pathological changes and were categorized as SIDS.

An autopsy was performed at the Forensic Medicine Departments of Fattouma Bourguiba University Hospital (Monastir) and Farhat Hached University Hospital (Sousse). Five necropsies (1 cm^3) were taken from each of the three standardized heart locations (the right ventricle, the septum and the left ventricle, *i.e.*, fifteen necropsies from each victim's heart). The necropsies were divided into two categories: in each case, 9 of the 15 necropsies were fixed in formalin and embedded in paraffin for histopathology and IHC while the remaining 6 necropsies were frozen at −80 °C for enteroviral genome amplification. The postmortem samples (necropsies and pericardial fluids) were obtained by a forensic doctor 24 h after death and were sent to our Department to detect viral infectious agents. The research protocol related to the SID cases was referred to the Ethics and Medical Research Committees which gave their approval. As for the samples, they were taken in compliance with the Tunisian law (Act 91-22; March 25th 1991) pertaining to human organ removal and transplantation.

2.2. Histopathology: hematoxylin–eosin staining (H&E)

Neutral buffered formaldehyde 30% diluted to 1/10 was used as fixative. The first category of necropsies was fixed for 24 h and embedded in paraffin. The sections (5 μm) were cut from the paraffin-embedded tissues with a microtome. All sections were stained with hematoxylin eosin (Invitrogen: Vermont, USA) and the slides were investigated for myocarditis according to the Dallas criteria [6,9–11].

2.3. Molecular pathology

The viral RNA was extracted from pericardial fluids and frozen myocardial tissues (second category of necropsies) using TRIzol® Plus RNA Purification Kit (Invitrogen) according to the manufacturer's instructions. DNase treatment during RNA purification was adopted using PureLink™ DNase (Invitrogen) in order to obtain DNA-free total RNA. The total extracted RNA was investigated for enteroviral genome (conserved sequences and VP1-capsid-protein coding region).

For the conserved sequences, a fragment of 155 bp of the extracted RNA was amplified by one-step Reverse Transcriptase-PCR (RTPCR) (Invitrogen SuperScript™ One-Step RT-PCR with Platinum® Taq) using 006 and 007 primers [12] directed to the conserved sequences in the 5′-UTR of the enterovirus genome. The RT-PCR was performed on a mixture containing 25 μl 2X reaction mix (a buffer containing 0.4 mM of each dNTP, 2.4 mM MgSO4), 0.2 μM each of sense and anti-sense primers, 1 μl enzyme mix (RT/Platinum® Taq; invitrogen), and RNase free water to 50 μl. The reaction was conducted with an initial reverse transcription step at 42 °C for 30 min, followed by PCR activation at 94 °C for 5 min, 30 amplification cycles (94 °C, 30 s; 42 °C, 1 min; 72 °C, 2 min) and a final 10-min extension at 72 °C in an Eppendorf Mastercycler Thermal Cycler. The RT-PCR products were run on a 2% agarose gel stained with ethidium bromide and visualized under UV light.

Enterovirus PCR from VP1- capsid protein region was conducted in two stages as follows: (1) *first stage* – 10 μl of previously extracted RNA were reverse-transcribed into cDNA at 42 °C for 45 min using 200 units of Super-ScriptIII reverse transcriptase and 2.5 ng/μl of random primers (Invitrogen, Cergy Pontoise, France) in the presence of 10 units of RnaseOUT recombinant RNase inhibitor (Invitrogen, Cergy Pontoise, France) and (2) *second stage* – 5 μl of cDNA were amplified using 50 pmol of the 292 and 222 primers directed to the enteroviral VP1-capsid-protein coding region (Table 2) and 1.25 units of Platinum Taq DNA polymerase (Invitrogen, Cergy Pontoise, France) in 50 μl of reaction mixture according to the protocol [13]. A band of the expected size of 357 bp was observed after agarose gel electrophoresis.

Table 1
Clinical and epidemiological data of investigated groups.

Group	No. of subjects	Age (months)	Sex		No. of positive cases				
					Clinical diagnosis	Diagnostic approaches			
			Males	Females		Histology	Histopathology	RT-PCR	IHC
SID victims: (study group)	39	3–9	30	9	Perimyocarditis	3	3	3	3
					Myocarditis	0	0	6	6
Total						3	3	9	9
Unnatural deaths home accidents: (controls)	17	2–11	17	–					