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# Role of Virus-Induced Myocardial Affections in Sudden Infant Death Syndrome: A Prospective Postmortem Study

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## ABSTRACT

The cause of sudden infant death syndrome (SIDS) is an unresolved problem of high relevance. Previous studies indicate a role of infections. In our prospective study, we investigated the frequency of virus-induced myocardial affections in SIDS. Postmortem samples from SIDS victims and control subjects were investigated prospectively. Pediatric cases of unnatural death served as controls. Samples were studied for enteroviruses, adenoviruses, parvovirus B19, and Epstein-Barr virus applying PCR. Immunohistochemical investigations for inflammatory cells, the necrosis marker C5b-$9_{(m)}$ complement complex, and the enteroviral capsid protein VP1 were performed. Overall, 62 SIDS victims were studied. As controls, 11 infants were enrolled. Enteroviruses were detected in 14 (22.5%), adenoviruses in 2 (3.2%), Epstein-Barr viruses in 3 (4.8%), and parvovirus B19 in 7 (11.2%) cases of SIDS. Control group samples were completely virus negative. Compared with controls, immunohistochemical investigations partially revealed a significant increase in the number of T lymphocytes in SIDS myocardial samples ($p < 0.05$). Furthermore, cases with elevated numbers of leukocytes and macrophages, microfocal C5b-$9_{(m)}^{+}$ necroses, and enteroviral VP1 capsid protein within the myocardium were detected. Applying a comprehensive combination of molecular and immunohistochemical techniques, our results demonstrate a clearly higher prevalence of viral myocardial affections in SIDS. Our results emphasize the importance of PCR-based diagnosis of viral myocardial affections. We suggest preliminary criteria for cellular immunohistochemical diagnosis of viral myocardial affections derived from our findings. For future investigations in SIDS, we suggest a comprehensive approach that includes PCR and immunohistochemistry. Our results offer novel strategies for diagnosis of pediatric myocardial viral affections. **(*Pediatr Res* 55: 947–952, 2004)**

**Abbreviations**

**AV,** adenovirus
**C5b-$9_{(m)}$,** complement complex C5b-$9_{(m)}$
**CVB3,** coxsackievirus B3
**EBV,** Epstein-Barr virus
**EV,** enterovirus
**HHSV6,** human herpes simplex virus type 6
**HPF,** high-power field
**PVB19,** parvovirus B19
**RT-PCR,** reverse transcriptase–PCR
**SIDS,** sudden infant death syndrome
**VP-1,** viral protein 1

The diagnosis of sudden infant death syndrome (SIDS) is established by comprehensive exclusion of all other possible causes of death in the age group. Myocarditis is a widely known explanation for sudden death in cases of suspected SIDS as well as in older children (1). Recent clinical studies and anecdotal communications reported on different viruses in such cases applying molecular techniques to detect the genome sequences of enteroviruses (EV) (2), adenoviruses (AV) (3), Epstein-Barr virus (EBV) (4), and parvovirus B19 (PVB19) (5) in clinical and autopsy samples, respectively. Until recently, a maximum percentage of up to 17% of cases of sudden unexpected death in infancy showing—if at all—histologic hints of viral myocardial affections was assumed (6). The aim of our study was to determine the incidence of virus-induced myocardial affections in cases of SIDS with modern immunohistochemical and molecular pathologic methods.

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## METHODS

***Postmortem samples.*** In this prospective study, postmortem tissue samples were obtained in from 1996 to 2001. Initially, 74 cases were studied; in 12 cases, a plausible

cause of death [*e.g.* pneumonia, meningitis, myocarditis according to the Dallas criteria (7)] was revealed by macroscopic or conventional histologic investigations. All slides were investigated by two independent blinded investigators. Subsequently, 62 SIDS cases (study group) without any relevant pathologic findings and 11 cases of unnatural deaths—traffic accident ($n = 4$), home accident ($n = 3$), stabbing ($n = 2$), and drowning ($n = 2$)—aged 2 to 11 mo (control group) were enrolled (Table 1). Partially, investigations were conducted to verify the cause of death upon instruction by the prosecution authorities; in all other cases, informed consent was obtained from the relatives.

Together with routine autopsy samples from all internal organs, eight samples were taken from standardized locations of each heart (right ventricle anterior and posterior, ventricular septum cranial and caudal, left ventricle anterior wall and posterior wall, cranial and caudal) and from liver and spleen. As fixative neutral phosphate-buffered formaldehyde (pH 7.0) was used, the samples were fixed for up to 48 h. Sections (5 $\mu$m) from all samples were stained with hemalaun-eosin.

***Sample preparation and PCR procedures.*** Sections from myocardial, liver, and spleen samples were investigated for enteroviral RNA including Coxsackie virus B3 RNA (Fig. 1) and several viral DNA [AV, EBV (Fig. 2), PVB19] by (semi-) nested reverse transcriptase–PCR (RT-PCR) and PCR, respectively. Nucleic acids were isolated from paraffin-embedded samples applying techniques published elsewhere (8). Control PCR amplification to verify the presence of amplifiable nucleic acid extracted from each sample was performed using modified cyclophilin primers (9). Primers and thermal conditions used for amplification of virus-specific sequences and the housekeeping gene cyclophilin are shown in Tables 2 and 3. Amplified PCR products were analyzed on 8% polyacrylamide gels; DNA fragments were detected by silver staining as published previously (8). For avoiding false-positive results from contamination, preventive measures were followed (10) and negative controls were performed in all experiments. For each assay, known positive controls, derived from infected viral cells, were added. Purified PCR products were sequenced on an automated ABI 310 sequencer. Sequence comparison was performed by BLAST search of National Center of Biotechnology Information GenBank database.

***Immunohistochemistry.*** The methods applied for immunohistochemical stainings and antibodies used have been described in detail elsewhere (8, 11). For visualization of all antibodies, a labeled streptavidin-biotin technique was used; 3-amino-9-ethylcarbazol was applied as chromogen. The sections were counterstained with Mayer's hemalaun. In all tests, a negative control was performed and the primary antibody was replaced with diluent only. Human tonsil tissue was used for positive controls.

**Table 1.** *Epidemiologic data of investigated groups of autopsy cases*

| | SIDS group ($n = 62$) | Control group ($n = 11$) |
|---|---|---|
| Age (mo) | 1–11 | 2–11 |
| Sex | | |
| female | 27 | 4 |
| male | 35 | 7 |
| Cause of sudden death | unknown/SIDS | nonnatural |

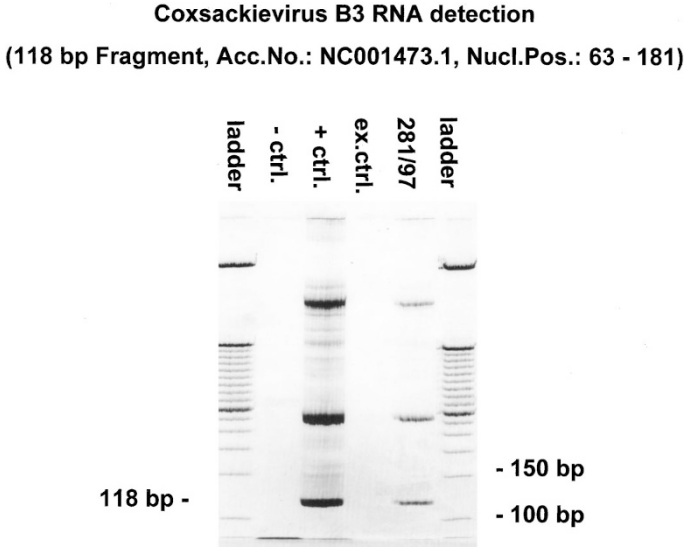


**Figure 1.** Detection of coxsackievirus B3 by nested RT-PCR in myocardium (left ventricle anterior wall cranial, 20 wk, male). −ctrl., negative control; +ctrl., positive control; ex.ctrl., extraction control (case no. 287/01).

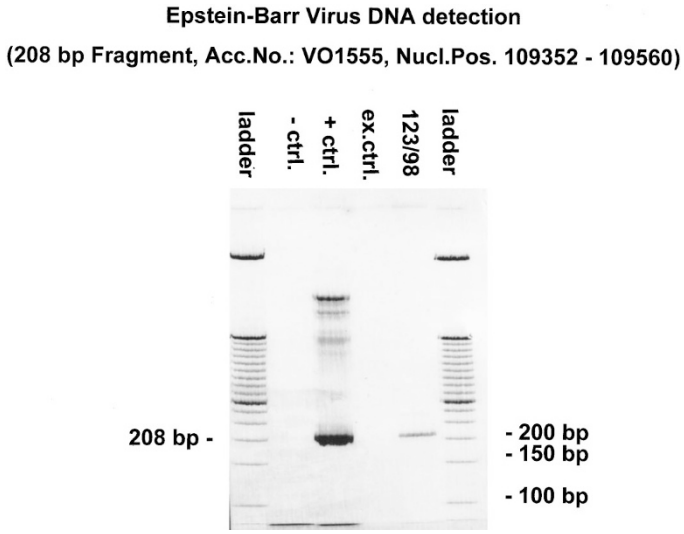


**Figure 2.** Detection of EBV genome by nested PCR in myocardium (left ventricle posterior wall cranial, 26 wk, male). −ctrl., negative control; +ctrl.; positive control; ex.ctrl., extraction control (case no. 123/98).

Leukocytes, T lymphocytes, and macrophages were counted in 20 randomized high-power fields (HPF; magnification ×400), and the mean value was calculated. In contrast to criteria for adults (12, 13), more rigorous criteria were applied for the diagnosis of myocardial affections in infants, discussed in detail below.

A lymphocytic infiltration, *i.e.* myocarditis, was defined as a mean value of >10 lymphocytes/HPF or a leukocytic infiltration of >15 leukocytes/HPF. Mean values of 5–9 T lymphocytes and >10 macrophages/HPF were regarded as "suspicious."

Statistical analysis was performed with Wilcoxon's and *t* tests (SPSS version 10.0). A $p < 0.05$ was deemed significant.

**Table 2.** *Details of PCR primers used to amplify viral genes: EV (27); CVB3 (28), AV (3), EBV (29), PVB19 (30), and cyc (9)*

| Primer | Nucleotide sequences (5′ to 3′) | Product size (bp) |
|---|---|---|
| EV1b | CAA TTG TCA CCA TAA GCA GCC A | 150 (EV1b/EV2a; first round) |
| EV2a | TCC GGC CCC TGA ATG | 110 (EV2a/EV2b; second round) |
| EV2b | ACA CGG ACA CCC AAA GTA GT | |
| CoxF | CGG TAC CTT TGT GCG CCT GT | 316 (CoxF/CoxR; first round) |
| CoxR | CAG GCC GCC AAC GCA GCC AC | 118 (CoxF/CoxB3; second round) |
| Cox B3 | GGT AAC AGA AGT GCT TGA TC | |
| P1 | TTC TTT TCA GCT TTT TAG G | 200 (P1/P2; first round) |
| P2 | TGA ATT GCA TGG TCT TCA TG | |
| P3 | TAT AAG TTT CCT CCA GTG CC | 120 (P3/P4; second round) |
| P4 | TTT GGG TCA CCT CCT AAT GT | |
| AVF | GCC GCA GTG GTC TTA CAT GCA CAT | 300 bp (AVF/AVR; first round) |
| AVR | CAG CAC GCC GCG GAT GTC AAA GT | |
| AV3 | AGA CGT ACT TCA GCC TGA A | 130 bp (AV3/AV4; second round) |
| AV4 | CCT TGT ACG AGT ACG CAG TA | |
| EBV1 | AAG GAG GGT GGT TTG GAA AG | 309 bp (EBV1/EBV2; first round) |
| EBV2 | AAC AGA CAA TGG ACT CCC TTA | |
| EBV3 | ATC GTG GTC AAG GAG GTT CC | 208 bp (EBV3/EBV4; second round) |
| EBV4 | ACT CAA TGG TGT AAG ACG AC | |
| cycF | CGTC CAG CAT TTG CCA TGG A | 180 bp |
| cycR | GAC AAG GTC CCA AAG ACA G | |

**Table 3.** *Details of PCR conditions used to amplify viral genes; EV (27), CVB3 (28), AV (3), EBV (29), PVB19 (30), and cyc (9)*

| Primer | Denaturation (°C) | Annealing (°C) | Extension (°C) | No. of cycles |
|---|---|---|---|---|
| EV1b | 94 (30 s) | 58 (30 s) | 72 (60 s) | 30 (first round) |
| EV2a | | | | 20 (second round) |
| EV2b | | | | |
| CoxF | 94 (30 s) | 58 (30 s) | 72 (60 s) | 30 (first round) |
| CoxR | | | | 20 (second round) |
| CoxB3 | | | | |
| P1 | 94 (30 s) | 50 (30 s; first round) | 72 (60 s) | 30 (first round) |
| P2 | | 60 (30 s; second round) | | 20 (second round) |
| P3 | | | | |
| P4 | | | | |
| AVF | 94 (60 s) | 58 (60 s) | 72 (60 s) | 30 (first round) |
| AVR | | | | 20 (second round) |
| AV3 | | | | |
| AV4 | | | | |
| EBV1 | 94 (60 s) | 58 (60 s) | 72 (60 s) | 30 (first round) |
| EBV2 | | | | 20 (second round) |
| EBV3 | | | | |
| EBV4 | | | | |
| cycF | 94 (60 s) | 56 (60 s) | 72 (60 s) | 55 |
| cycR | | | | |

***Toxicologic investigations.*** The autopsies were complemented with toxicologic analyses for ethanol and medical and illicit drugs in all cases.

## RESULTS

***Molecular investigations.*** The results of the molecular investigations are listed in Table 4. One case with immunohistochemically detectable marked cellular infiltration and negative results for the viruses investigated so far was diagnosed as human herpes simplex virus type 6 (HHSV6)-induced myocarditis by external investigations. In total, myocardial samples from 27 (43.5%) of 62 SIDS cases were virus positive for one of the viruses investigated. In these cases, viral genome was detectable in some but not all eight myocardial samples taken at autopsy. No cases of double infection were found. All myocardial samples from the control group were found to be virus negative. Moreover, all liver and spleen samples from both groups investigated were found to be virus negative, indicating an isolated myocardial localization of the viruses detected.

***Immunohistochemistry.*** In myocardial slides investigated, leukocytes, T lymphocytes, and macrophages were found in diffuse distribution patterns and occasionally as microfocal accumulations (Fig. 3). Within the study group, four cases exhibited microfocal C5b-$9_{(m)}^{+}$ myocardial necroses. Microfocal expression of enteroviral VP1 capsid protein was detected in six SIDS cases (Fig. 4). Neither C5b-$9_{(m)}^{+}$ myocardial necroses nor enteroviral VP1 capsid protein were found in myocardial samples of the control group.

Twelve cases showed immunohistochemical signs of inflammation but negative PCR results for the viruses investigated. Seven PCR-diagnosed virus-positive cases with ac-

**Table 4.** *Results of molecular pathologic investigations*

| | SIDS group ($n = 62$) | Control group ($n = 11$) |
|---|---|---|
| EV | $n = 14$ | 0 |
| CVB3 | $n = 3$ | 0 |
| AV | $n = 2$ | 0 |
| EBV | $n = 3$ | 0 |
| PVB19 | $n = 7$ | 0 |
| HHSV6 | $n = 1$ | – |

companying immunohistochemical signs of inflammation were found. Five EV cases were diagnosed; four of these EV cases showed T-lymphocytes counted as "suspicious," and one case exhibited >10 T-lymphocytes and was diagnosed as "sign of active myocarditis" by immunohistochemistry. In addition, one HHSV6 and one PVB19 case were found; both were assessed as "suspicious" because of their elevated T-lymphocyte count. A third PVB19-positive case was diagnosed as "macrophage-rich inflammatory process." A fourth one presented >15 leukocytes/HPF.

Significant differences were found with regard to the counted mean values of T-lymphocytes in the study group in comparison with the control group ($p < 0.05$)

***Conventional histologic analysis and toxicologic investigations.*** In tissue sample slides from internal organs, no relevant pathologic findings were found. Fourteen cases from the study group showed slight bronchitis, all cases with pulmonary edema and hepatic congestion. Cases from the control group also revealed no relevant pathologic findings. Medicolegal toxicologic investigations were completely negative in both groups.

## DISCUSSION

The present study for the first time proves evidence of a more than doubled incidence of virus-induced myocardial affections in SIDS than originally assumed (43.5% *versus* 16.8%). These important findings should have a marked impact on the quality of future pediatric-pediopathologic and medicolegal investigations in such cases. Most important, the results presented and the investigation techniques and criteria suggested here will enable physicians to clarify the cause of death in an increased number of cases of suspected SIDS.

Up to now, only case series and small morphologic studies reported on inflammatory myocardial processes in SIDS victims applying molecular pathologic and immunohistochemical methods to detect viral genome (8, 14, 15). The results of these studies already pointed toward a possible myocardial affection as cause of death, most probably as a result of viral infection. In accordance with these findings, EV were found to be the most common causal agents of myocarditis with seasonal accumulation. Further evidence came from reports of elevated incidence of sudden cardiac death during enterovirus epidemics, particularly CVB3 (16, 17).

Moreover, in adults, virus detection by RT-PCR and *in situ* hybridization as well as serologic studies detecting viral antibodies revealed an association between enterovirus infection, especially the cardiotropic Coxsackie group B viruses and myocarditis (2). Coxsackie B viruses are one of the most frequently identified infectious agents in acute myocardial infections. Recently, a cDNA clone that encodes the common Coxsackie-adenoviral-receptor was discovered (18). Intriguing is that this receptor has been shown to be down-regulated after birth. The Coxsackie-adenoviral-receptor protein is not detectable in adolescents and adults but was found to be expressed in cases of chronic inflammatory cardiomyopathies in adults. Therefore, this observation offers a feasible molecular basis for the explanation of the observed predominance of the abovementioned viruses in the heart.

Although there are reports about the myocytopathic effect of enteroviruses *in vitro* and in animal models (19), the underlying pathomechanism of myocardial damage in humans has remained partially unsolved up to now. It was demonstrated *in vitro* that enteroviral protease 2A cleaves and therefore functionally impairs dystrophin, a cytoskeletal protein of cardiomyocytes. During infection with Coxsackie B3 viruses, the dystrophin-glycoprotein complex therefore becomes disrupted and the sarcolemmal integrity is lost (20). To us, these results lead to the conclusion that the detection of enteroviral genome within the myocardium is itself a pathologic finding.

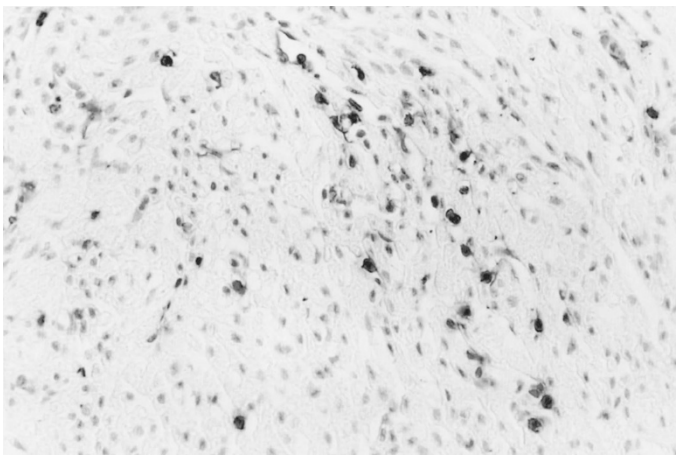

**Figure 3.** Representative micrograph of microfocal accumulation of $CD45^+$ T-lymphocytes in myocardial sample (interventricular septum cranial, 14 wk, female). Magnification: ×400.

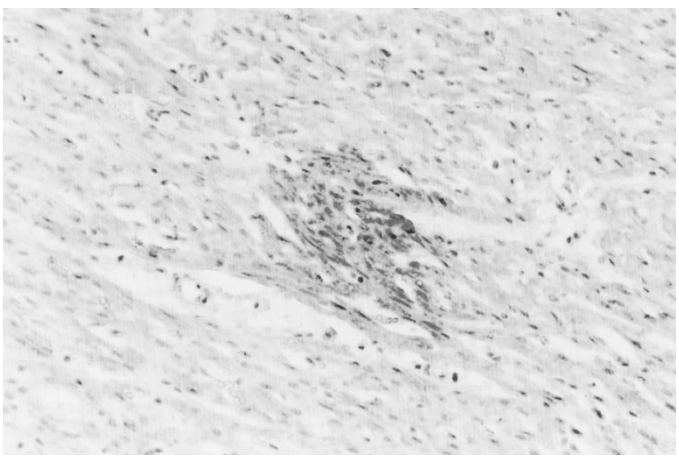

**Figure 4.** Representative micrograph of immunohistochemical detection of enteroviral VP1 capsid protein in myocardial sample (left ventricle posterior wall cranial, 16 wk, male). Magnification: ×200.

However, for the other viruses investigated in our study, the pathomechanisms still remain elusive and are a matter of great scientific interest. In clinical practice, however, it is widely recognized that EBV and HHSV, for example, are without doubt cardiopathogenic agents when detected in endomyocardial biopsy samples. Furthermore, recent studies have demonstrated a correlation between outcome of a coxsackievirus infection and immune status of the host. SIDS victims are often premature infants and mainly die between 8 and 16 wk of age, as the level of maternal antibodies declines. Although the clinical relevance of PVB19 has not been elucidated completely yet, there is an increasing amount of reports on PVB19-induced lethal myocarditis in children (5, 21).

As a matter of fact, all viruses investigated in our prospective study were detected exclusively in myocardial samples, whereas spleen and liver samples were found to be virus negative without exception, indicating an isolated affection of the myocardium in SIDS victims. This is underlined by the fact that all tissue samples from the control group were completely virus negative. Previous studies with histologic investigation of myocardial lesions in cases of SIDS based on conventional stainings revealed only unspecific findings (6), and diagnosis often is not reliable because of considerable interobserver variability (22).

In our prospective study, we performed molecular investigations in combination with immunohistochemistry and conventional light microscopy, offering a new and comprehensive approach to virus detection in myocardial and tissue samples, respectively. As a first step, a defined spectrum of viruses was investigated by PCR, which have been shown to cause myocarditis in infants and adults (3, 5, 15).

With regard to the time-dependent course of viral myocarditis, as studied in a mouse model, early virus-induced myocardial damage already takes place before histologic and immunohistochemical signs of myocarditis defined by the Dallas criteria can be observed (23, 24). These early-phase-dependent viral lesions can be detected only *via* electron microscopy; they also occur before immunohistochemical signs of myocarditis (19). This is confirmed by our findings, as only four cases of the study group were showed microfocal necroses applying the early necrosis marker C5b-$9_{(m)}$. Six cases of SIDS were stained positive for the enteroviral capsid protein VP1; most intriguing, these all were confirmed as virus positive by RT-PCR for EV RNA. However, according to our observations, an infiltration of leukocytes can be observed by immunohistochemistry before myocardial necroses develop.

In the past, using conventional histologic stainings, a mean value of >5 T lymphocytes/HPF has been regarded as a sign of active myocarditis in adults (13). Other authors suggested an upper normal limit of at least >10 lymphocytes and macrophages per HPF for this diagnosis (12, 25). In our prospective study, we observed great differences in the number of the cells that might reflect normal interindividual variability in infancy. Given the abovementioned discussion on a diagnostic criterion for myocarditis based on the number of inflammatory cells per HPF, we would like to suggest the following preliminary criteria for cellular immunohistochemical diagnosis of viral myocardial affections. In contrast to criteria for analysis of adult samples, we have chosen more rigorous criteria to ensure the quality of diagnosis in infants. These suggestions are derived from our findings, especially with respect to the analysis of the control group. More than 10 T-lymphocytes/HPF (as observed in 6 of 62 SIDS cases) should be interpreted as a reliable sign of *active myocarditis*. Furthermore, cases that show >15 T lymphocytes and macrophages in summation should also be diagnosed as "*active myocarditis*" (6 of 62 SIDS cases). Cases with 5 to 10 T-lymphocytes/HPF (11 of 62 SIDS cases) should be regarded as "*suspicious*." In addition, we found five cases with >10 $CD68^+$ macrophages/HPF accompanied by <5 T-lymphocytes, resulting in a total of <15 cells as postulated above. This phenomenon remains unclear at the moment; however, it may indicate a late inflammatory process (*e.g.* resolving myocarditis). Therefore, we would like to suggest the preliminary term "*macrophage-rich inflammatory process*." Cases that present with <5 T-lymphocytes or 10 macrophages should be assessed as "*without pathologic findings*" (as observed in all samples of the control group).

As a matter of fact, 17 of 27 virus-positive cases detected by PCR were observed without any immunohistochemical signs of inflammation according to our new criteria (Table 5). Given the time course of myocarditis development discussed above, these cases might represent a very early and clinical inapparent phase with death preceding any inflammatory cellular reaction. Twelve cases exhibited immunohistochemical signs of inflammation but negative PCR results for the viruses investigated (Table 5). It has to be mentioned here that the total spectrum of cardiotropic viruses includes a larger number than assessed in our study.

The question of whether early viral infection can indeed cause lethal myocardial damage has been an issue of scientific debate in the past. Depending on the localization, a virus-induced inflammatory process that affects the myocardial con-

**Table 5.** *Immunohistochemical quantification and molecular pathologic virus detection in the SIDS group (per investigated case, more than one immunohistochemical diagnosis could be established)*

| | Virus | | | | | |
|---|---|---|---|---|---|---|
| Immunohistochemical diagnosis | EV ($n$ = 14) | AV ($n$ = 2) | EBV ($n$ = 3) | PVB19 ($n$ = 7) | HHSV6 ($n$ = 1) | Unknown virus or virus-free ($n$ = 35) |
| *T-lymphocytes > 10/HPF* | 1 | | | 1 | 1 | 3 |
| *T-lymphocytes 5–10/HPF* | 4 | | | 1 | | 6 |
| *Leukocytes >15/HPF* | | | | 1 | 1 | 4 |
| *Macrophages >10/HPF* | | | | 1 | | 4 |
| *Inconspicuous immunohistochemistry* | 9 | 2 | 3 | 3 | | 26 |

duction system already at an early stage must be considered as a possible cause of sudden death not only in infants. With regard to that issue, clinical studies as well as case reports describe enteroviral, adenoviral, and EBV-induced myocarditis characterized by a fulminant clinical course with malignant arrhythmias (17).

## CONCLUSION

In synopsis, the results from our prospective study on the role of virus-induced myocardial affections in unexpected SIDS indicate that the incidence of potentially lethal viral myocardial affections is more than twice than assumed up to now. However, our results should be regarded as a part of numerous findings pointing toward an underlying inflammatory process in cases of SIDS. After exclusion of other possible causes of death, our results indicate that viral myocardial affection is the cause of death in EV, AV, EBV, and HHSV6-positive SIDS cases; moreover, PVB19 seems to play a more critical role than assumed so far.

Our results prove the importance of combined investigations using molecular pathologic techniques and immunohistochemical methods. We suggest new accurate sampling standards as well as novel diagnostic criteria for future investigations. These standards and criteria should enable pathologists to establish more reliable diagnoses of myocarditis in pediatric fatalities and therefore derive possible future clinical therapeutic strategies (26).

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