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## **Low Level Myocardial Parvovirus B19 Persistence is a Frequent Finding in Patients with Heart Disease but Unrelated to Ongoing Myocardial Injury**

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**Low Level Myocardial Parvovirus B19 Persistence is a Frequent Finding in Patients with Heart Disease but Unrelated to Ongoing Myocardial Injury**

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6 **Patients with Heart Disease but Unrelated to Ongoing Myocardial Injury**  
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Running Title: Parvovirus B19 Genome in Heart Disease

## Abstract

While myocardial parvovirus B19 (B19V), aside from enteroviruses (EV) and adenoviruses (ADV), has recently been found often in patients with myocarditis and idiopathic dilated cardiomyopathy (IDC), the pathogenetic significance of B19V genomes in those patients has not yet been sufficiently elucidated. In the present study, left ventricular endomyocardial biopsies from 24 patients with left ventricular ejection fraction (LVEF) below 55% due to IDC, and tissue from the right atrial appendage of 10 control patients undergoing bypass surgery with normal LVEF (>55%) were investigated for B19V, ADV and EV genomes by specific nested polymerase chain reaction (PCR), by real time PCR or by reverse-transcription PCR, respectively. The myocardial tissue samples from the 10 controls were analyzed each in three different virological laboratories for B19V. In the IDC group, the frequency of the myocardial virus genomes found in 54% (13/24) of the patients was as follows: B19V: 50% (12/24), EV: 8% (2/24), including one patient with B19V and EV, and ADV: 0% (0/24). For comparison, the prevalence of B19V genomes was between 30 and 60% in the control group as detected in three different laboratories, but all these control subjects were EV- and ADV-negative. The number of B19V gene copies, however, was very low and similar both in the IDC and control group. In the majority of patients myocardial B19V persistence was associated with a low virus load irrespective of the underlying heart disease so that it may be of no importance in the pathogenesis of IDC.

**KEY WORDS:** parvovirus B19; dilated cardiomyopathy; pathogenetic significance

## INTRODUCTION

Parvovirus B19 (B19V) is associated with a broad spectrum of clinical disease manifestations [Heegaard and Brown, 2002; Young and Brown, 2004]. According to a seroprevalence of approximately 72% in healthy adults [Röhrer et al., 2008] and the lifelong persistence of B19V genomes in human tissues after an infection in the childhood as recently shown by Norja and co-workers [Norja et al., 2006], B19V is to our knowledge the most frequently detected human pathogenic virus. Erythema infectiosum is the typical disease manifestation of B19V in immunocompetent individuals [Heegaard and Brown, 2002; Young and Brown, 2004].

Recent investigations revealed that B19V is also the most frequent virus found in the myocardium of immunocompetent adults with myocarditis or idiopathic dilated cardiomyopathy (IDC) besides enterovirus (EV) and adenovirus (ADV) [Bowles et al., 1986; Kandolf et al., 1987; Why et al., 1994; Figulla et al., 1995; Pauschinger et al., 1999; Pankuweit et al., 2000; Pankuweit et al., 2003; Lotze et al., 2004; Köhl et al., 2005; Andersson et al., 2008].

So far, the frequency of B19V genome found in endomyocardial biopsies of subjects with and without heart failure due to left ventricular systolic dysfunction has only been described qualitatively [Pankuweit et al., 2003; Lotze et al., 2004; Andersson et al., 2008], thus the significance of the myocardial B19V virus load is not yet known. The purpose of the present study was to investigate the prevalence of B19V, EV, and ADV genomes in patients with IDC and control subjects with normal LVEF undergoing coronary artery bypass surgery and to assess the number of gene copies of B19V in order to elucidate the pathogenetic role of myocardial B19V infection in IDC.

## MATERIALS AND METHODS

### Patients

Between March 2001 and May 2002, 24 patients with LVEF below 55%, as assessed by left ventricular angiography [Dogde and Sheehan, 1983], due to IDC were enrolled in the study. After exclusion of hypertensive, coronary or hemodynamically significant valvular heart disease, IDC was diagnosed according to the criteria of the World Health Organization / International Society and Federation of Cardiology Task Force on the Definition and Classification of Cardiomyopathies from 1995 [Richardson et al., 1996]. Patients with acute myocarditis or chronic myocardial inflammation, according to histological [Dallas] and immunohistological criteria, respectively [Aretz et al., 1987; Richardson et al., 1996; Noutsias et al., 1999; Feeley et al., 2000], were not included in this investigation.

In order to compare the prevalence of intracardiac viral genomes in IDC patients with that in individuals without heart failure due to left ventricular systolic or diastolic dysfunction, 10 consecutive patients with normal LVEF (>55%) undergoing coronary artery bypass surgery were enrolled between August 2006 and September 2008 as control subjects with respect to the detection of myocardial B19V, EV, and ADV genomes. The study design was approved by the local ethic committee, and the informed written consent was obtained from all patients and control subjects participating in this investigation.

### Cardiac Catheterization and Echocardiographic Evaluation

In all patients, standard echocardiographic parameters such as left ventricular enddiastolic diameter and LVEF according to Simpson [Morrissey et al., 1994] were recorded before left heart catheterization and coronary angiography. Patients with a history of hypertension, systemic disorders or a known infection were excluded from the study. Left ventricular and coronary angiography and Swan-Ganz catheterization were performed according to standard methods [Figulla et al., 1985].

### Endomyocardial Biopsy Samples

A total of eight endomyocardial biopsy samples from the lateral free wall of the left ventricle were taken from each patient using a long sheath biptome (Signus, Alzenau, Germany). Two of the specimens were subjected to routine histopathological analysis, histomorphometric evaluations as well as immunohistological investigations.

Molecular virological analyses for B19V, EV and ADV genomes were performed on the remaining six endomyocardial biopsies as well as on the myocardial tissue samples of the right atrial appendage obtained from the control patients during the surgical procedure, according to the previously described protocol [Lotze et al., 2004].

### Histology and Immunohistology

Histopathological analysis, histomorphometric measurements and immunohistological investigations were performed according to standard methods as described elsewhere [Aretz et al., 1987; Figulla et al., 1995; Lotze et al., 2004]. In brief, histopathological examinations were done according to the Dallas criteria [Aretz et al., 1987], and histomorphometric evaluations included the assessment of the myocyte diameter and the volume fraction of fibrosis in the myocardium. Mononuclear cell infiltrates in the myocardium were characterized using monoclonal antibodies directed to CD2 (pan T cell marker), CD4 (T helper cells), CD8 (T suppressor cells), CD14 (macrophages) and CD45RO (activated T lymphocytes). In addition, the expression of the major histocompatibility complex antigens of class I (HLA-A, B, C) and class II (HLA-DR, DP, DQ). According to the criteria of the World Health Organization / International Society and Federation of Cardiology Task Force on the Definition and Classification of Cardiomyopathies from 1995 inflammation of the myocardium was diagnosed, if mononuclear infiltrates with  $>14$  leukocytes/mm<sup>2</sup> (CD45RO<sup>+</sup> activated lymphocytes and/or CD 14<sup>+</sup> macrophages) in the endomyocardial biopsies were

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4 detectable immunohistologically in combination with increased expression of HLA class II  
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6 antigens [Richardson et al., 1996; Noutsias et al., 1999; Feeley et al., 2000; Lotze et al., 2004].  
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### 10 11 **Detection of Parvoviral, Enteroviral and Adenoviral Genomes** 12

13 Both total RNA [Chomczynski and Sacchi, 1987] and DNA were extracted simultaneously  
14 from deparaffinized myocardial tissue samples of the IDC or control patients after proteinase  
15 K digestion with phenol/chloroform and quantified spectrophotometrically. Detection of  
16 B19V, EV and ADV genomes was performed using nested polymerase chain reaction (PCR)  
17 or reverse transcription-polymerase chain reaction (RT-PCR), respectively [Lotze et al.,  
18 2004]. Successful isolation of intact viral DNA and total RNA was proved by amplifying the  
19 housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase in a nested PCR [Bültmann et  
20 al., 2003].  
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32 For detection of B19V DNA, a one-step amplification method was performed using  
33 previously described primers PVB19/3: 5'-AGC ATG TGG AGT GAG GGG GC-3' and  
34 PVB19/4: 5'-GAA AGC ATC AGG AGC TAT ACT TCC-3', producing a 290-bp fragment  
35 [Salimans et al., 1989]. The PCR was performed in a final volume of 50µl, containing 5µl of  
36 10x Qiagen PCR buffer, 10 µl Q-solution, 8 µl dNTP-mix (1.25mM Promega), 1.5 µl 25 mM  
37 MgCl<sub>2</sub>, 1µl upstream primer PVB19/3 (10µM), 1µl of downstream primer PVB19/4 (10µM),  
38 1.25 U of Qiagen HotStarTaq DNA polymerase and 5 µl of template DNA. An initial  
39 denaturation step of 15 min at 95°C was followed by 50 cycles, including at 94°C for 50 s,  
40 55°C for 50 s, and 72°C for 1 min on a GeneAmp PCR system 9700 (Applied Biosystems,  
41 Weiterstadt, Germany). As confirmation of the specificity of the PCR results, especially of the  
42 positive B19V samples, automatic DNA sequencing of the B19V amplification products was  
43 performed [Smith et al., 1986]. Differentiation of the three B19V genotypes was performed by  
44 identification of the genotypes 1 (prototype), 2 (LaLi-like) and 3 (V9-like) as described  
45 previously [Servant et al., 2002].  
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4 To assess the interobserver variability in the detection of myocardial B19V genomes the  
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6 cardiac tissue from the 10 control patients was analyzed in addition to the Institute of Virology  
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8 and Antiviral Therapy of the University Hospital of Jena [Lotze et al., 2004] in two other  
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10 virological laboratories: 1. in the Institute of Pathology, Department of Molecular Pathology,  
11  
12 of the University Hospital of Tübingen (Germany), and 2. in the Institute of Virology of the  
13  
14 Medical School of Hannover (Germany).  
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18 In Tübingen, for the detection of B19V DNA by nested PCR, 35-cycle programs consisting  
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20 of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at  
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22 72°C for 45 seconds were performed on a GeneAmp PCR System 9600 (Perkin Elmer,  
23  
24 Boston, MA, USA) using primers from the VP1/VP2 coding sequence (GenBank Acc. No.  
25  
26 U385094). Primers PVB1 (5-AGC ATG TGG AGT GAG GGG GC-3) and PVB2 (5-AAA  
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28 GCA TCA GGA GCT ATA CTT CC-3) were used for the first round of reaction, resulting in  
29  
30 a PCR amplicon of 290 bp. A 173-bp B19V-specific amplicon was obtained with the nested  
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32 PCR primers PVB3 (5-GCT AAC TCT GTA ACT TGT AC-3) and PVB4 (5-AAA TAT CTC  
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34 CAT GGG GTT GAG-3). The specificity of the PCR products was as well confirmed by  
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36 automatic DNA sequencing [Bültmann et al., 2003]. In the virological laboratory in Hannover,  
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38 the myocardial tissue samples from the control subjects were investigated only quantitatively.  
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#### Quantification of Parvovirus B19

In the virological laboratory in Jena, B19V was quantified according to an in-house  
LightCycler protocol and hybridization format using the QuantiTect Probe PCR Kit (Qiagen,  
Hilden, Germany) with 0.2  $\mu$ M of the fluorescein-labeled probe, 0.4  $\mu$ M of the Red 640-  
labeled probe and two primers PVL-1 and PVL-2, which amplified a 168 bp amplicon out of  
the VP1-region of the virus [Lotze et al., 2004]. In Tübingen, the B19V quantitative PCR  
(Taqman 7000; Applied Biosystems, Weiterstadt, Germany) was performed as described  
previously [Tschöpe et al., 2005]. Reactions and cycling were performed as recommended by

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3 the manufacturer's instructions. Alternatively, in Hannover, all B19V-positive samples were  
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5 quantitatively analyzed by LightCycler (LC 1.5) technology using the RealArt Parvo B 19 LC  
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7 PCR Kit (Qiagen, Hilden, Germany). The myocardial B19V DNA levels were determined per  
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9 cell with help of a second real-time PCR protocol for the human CRP gene [Wandinger et al.,  
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11 2000]. All samples were investigated at a minimum in duplicate. The results of the B19V  
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13 quantification were calculated as copies/ $\mu$ g total DNA.  
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### 21 **Serological Analysis**

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23 In order to diagnose an acute or past B19V infection, the prevalence of B19V  
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25 immunoglobulin (Ig) G and IgM antibodies was analysed retrospectively using a recombinant  
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27 antigen-based enzyme immunoassay including immunoblotting for detection of IgG and IgM  
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29 antibodies to human B19V (recomLine parvovirus B19 IgG and recomLine parvovirus IgM,  
30  
31 MIKROGEN GmbH, Neuried, Germany) according to the manufacturer's instructions.  
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### 38 **Statistical Analysis**

39  
40 Categorical data are presented as relative frequencies, and continuous data as mean  $\pm$   
41  
42 standard deviation. Comparisons between groups were made using chi-square or Fisher's  
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44 exact test, where appropriate, for categorical variables, and unpaired Student's t-test or Mann-  
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46 Whitney test for continuous data. A p-value  $<0.05$  was accepted as statistically significant.  
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## 53 **RESULTS**

### 54 **Study Population**

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56 The study group consisted of 21 men and 3 women (mean age  $\pm$  standard deviation [SD]:  
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58 51.5  $\pm$  10.7 years; range, 21-66 years) with IDC. Clinical baseline data and echocardiographic  
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60 and hemodynamic findings are summarized in Table I. The duration of symptoms ranged  
between 15 days and 181 months (mean  $\pm$  SD: 22.0  $\pm$  42.6 months). In 54% (13/24) of the  
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4 IDC patients chronic congestive heart failure corresponding to New York Heart Association  
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6 functional class II was diagnosed, and 46% (11/24) of the patients were in New York Heart  
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8 Association functional class III.  
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10  
11 The baseline data, including the extent of coronary artery disease of the 10 control patients  
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13 with a LVEF above 55% undergoing coronary artery bypass surgery, are given in Table II.  
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### 16 17 18 **Endomyocardial Biopsy** 19

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21 Histological evaluations revealed that the histological characteristics such as hypertrophy  
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23 of myocytes or fibrosis, respectively, of the myocardial tissue samples in all 24 patients were  
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25 compatible with an underlying IDC. According to the Dallas and immunohistological criteria  
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27 [Aretz et al., 1987; Richardson et al., 1996; Noutsias et al., 1999; Feeley et al., 2000], no  
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29 patient had active or borderline myocarditis or chronic myocardial inflammation, respectively.  
30  
31 On the basis of the PCR analysis, 46% (11/24) of the patients were virus-negative and 54%  
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33 (13/24) virus-positive: B19V genomes were detected in 50% (12/24), EV genomes in 8%  
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35 (2/24), of whom one patient was positive for both B19V and EV genomes, and ADV genomes  
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37 in none (0/24) of the endomyocardial biopsy samples. In figure 1, examples of the  
38  
39 quantification of B19V genomes by LigthCycler real-time PCR are shown. The numbers of  
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41 gene copies in the B19V-positive patients were low between 50 and 150 copies/ $\mu$ g total DNA  
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43 and were confirmed by duplicate analysis. The determination of the three B19V genotypes  
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45 revealed that the genotypes 1 and 2 were detectable in 33% and 67% of the 12 B19V-positive  
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47 IDC patients (including one patient with both myocardial B19V and EV genomes),  
48  
49 respectively, whereas genotype 3 was not found. B19V genotype 1 was found in patients born  
50  
51 between 1938 and 1968, and B19V genotype 2 was detected in IDC patients born between  
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53 1939 and 1960. In the control hearts as well, B19V genotype 2 was by far the dominant virus  
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55 type as compared to genotype 1 (Table III).  
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3 All endomyocardial biopsy samples of the control subjects were negative for EV or ADV  
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5 genomes, respectively.  
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8 No significant differences were found among virus-positive, especially with respect to  
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10 B19V, and virus-negative groups regarding the myocyte diameter and the volume fraction of  
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12 interstitial fibrosis ( $30.3\mu\text{m} \pm 5.8\mu\text{m}$  vs.  $28.8\mu\text{m} \pm 5.0\mu\text{m}$ ,  $P= 0.52$  and  $4.0\% \pm 3.3\%$  vs.  $3.3\%$   
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14  $\pm 3.0\%$ ,  $P= 0.62$ , respectively, for B19V positive- and virus-negative patients).  
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### 18 19 20 **Interobserver Variability** 21

22 The prevalence of the myocardial B19V genomes in the 10 control patients varied between  
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24 30, 50 and 60% in the three different virological laboratories. There was a complete  
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26 conformity in 60% of the positive and negative results between the three virological  
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28 laboratories and an interobserver variability in 40% of the cases with a factor of 1.2 to 2. All  
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30 B19V-positive control patients had quantitative results within the range of 1 log (30 to 184  
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32 gene copies/ $\mu\text{g}$  total DNA) in all three laboratories. All results were close to (or even under)  
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34 the lower limit of the quantification range and close to the sensitivity limit. Furthermore, there  
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36 was only a good correspondence between two of the three virological laboratories with respect  
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38 to the number of gene copies/ $\mu\text{g}$  total DNA in the individual control patient (Table III).  
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### 48 **Serological findings** 49

50 Since the serological investigations were performed retrospectively, sera from only two  
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52 thirds of the IDC patients and no control patient were available. Thus, IgG and IgM antibodies  
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54 to B19V could be determined in only 16 of the 24 IDC patients (eight B19V-positive and  
55  
56 eight B19V-negative patients). Although IgG antibodies were found in 13 of the 16 (81%)  
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58 analysed sera, there were no IgM antibodies detected in any of the samples. In addition, IgG  
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60 antibodies to B19V were found in all eight B19V-positive patients. On the other hand, all

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3 three seronegative patients did not show B19V genomes in their myocardial tissues, and in the  
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5 remaining five B19V-negative IDC patients IgG antibodies to B19V were detected.  
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## 10 11 **DISCUSSION**

### 12 13 **Frequency of B19V Genome in the IDC Patients**

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15 According to previous reports, B19V in addition to EV [Petitjean et al., 1992; Weiss et al.,  
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17 1992; Kämmerer et al., 1994; Why et al., 1994; Figulla et al., 1995; Grumbach et al., 1999;  
18  
19 Pauschinger et al., 1999] and ADV [Pauschinger et al., 1999; Pankuweit et al., 2000] belongs  
20  
21 to the most common cardiotropic viruses in children [Schowengerdt et al., 1997; Enders et al.,  
22  
23 1998; Dina et al., 2008; Tavora et al., 2008] and adults [Heegaard et al., 1998; Bock et al.,  
24  
25 2005]. The B19V genome in cardiac tissue samples has recently been found in patients with  
26  
27 IDC at a rather high prevalence rate [Lotze et al., 2004; Kühl et al., 2005; Andersson et al.,  
28  
29 2008; Kühl et al., 2008]. In this investigation, the presence of B19V DNA in endomyocardial  
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31 biopsies obtained from patients with IDC was demonstrated in 12 out of 24 (50%) cases  
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33 including one patient who was positive for both B19V and EV genomes.  
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41 This finding was in line with our previous results regarding the detection of parvoviral  
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43 genomes in 42% of endomyocardial specimens in a study group consisting of 52 patients with  
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45 IDC [Lotze et al., 2004] and data from Kühl and coworkers who could also detect B19V in 51  
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47 % of patients with idiopathic left ventricular systolic dysfunction [Kühl et al., 2005].  
48  
49 Altogether, the present results regarding the prevalence of myocardial B19V genomes are  
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51 within the range of 17-71% found in similar previous studies with IDC patients [Pankuweit et  
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53 al., 2003; Lotze et al., 2004; Kühl et al. 2005; Andersson et al., 2008; Kühl et al., 2008]. As  
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55 far as control groups are concerned in these investigations, only a few authors enrolled  
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57 patients with normal left ventricular systolic function and arterial hypertension [Pankuweit et  
58  
59 al., 2003] or undergoing artery bypass surgery [Lotze et al., 2004], and donor hearts  
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[Andersson et al., 2008] as controls. The prevalence of myocardial B19V DNA in these

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3 control groups varied from 7% [Pankuweit et al., 2003] to 40% [Lotze et al., 2004] or 57%  
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5 [Andersson et al., 2008], respectively. In our control patients, myocardial B19V genome was  
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7 found in 30-60% which is comparable to our previous results [Lotze et al., 2004] and the data  
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9 from Andersson and co-workers [Andersson et al., 2008]. Two recent investigations in  
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11 patients undergoing open-heart surgery [Kuethe et al., 2009] and in subjects postmortem  
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13 [Schenk et al., 2009], each of them without evidence of IDC or myocarditis, demonstrated  
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15 B19V genomes in 85% and 67% of the heart tissue samples, respectively. Thus, the wide  
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17 range of the prevalence of myocardial B19V genome both in the patient and control groups  
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19 may be, on the one hand due to the varying sensitivity limit of the PCR influenced by different  
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21 primers used in the several investigations [Pankuweit et al., 2003; Lotze et al., 2004, Kühl et  
22  
23 al. 2005; Andersson et al., 2008; Kühl et al., 2008; Kuethe et al., 2009; Schenk et al., 2009]  
24  
25 or, on the other hand based on a sampling error in investigating myocardial tissue for B19V  
26  
27 genomes in the present and some previous studies mentioned above, given a lifelong  
28  
29 persistence of B19V in the heart and a high B19V seropositivity even in subjects not suffering  
30  
31 from myocarditis or IDC [Kuethe et al., 2009; Schenk et al., 2009]. The differentiation of the  
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33 three B19V genotypes in our IDC and control patients demonstrating genotype 2 in the  
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35 majority of cases as compared to genotype 1, and genotype 3 neither in the IDC nor in the  
36  
37 control group is in line with the distribution of B19V genotypes in the myocardium as found  
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39 in two recent investigations [Kühl et al., 2008; Kuethe et al., 2009].  
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### 51 52 **B19V Serology and the Detection of Myocardial B19V Genome**

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54 Serological testing of the 16 available sera out of the 24 IDC patients revealed that 13 (81%)  
55  
56 patients had suffered a past B19V, but that no patient had an acute B19V infection. This high  
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58 prevalence rate of B19V IgG antibodies measured herein corresponds to the seroprevalence of  
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60 72%, 85% and 70%, recently demonstrated in constitutionally healthy adults [Röhrer et al.,  
2008], patients not suffering from IDC or myocarditis who underwent open-heart surgery

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3 [Kuethe et al., 2009] or in postmortem blood samples from subjects without IDC or  
4  
5 myocarditis [Schenk et al., 2009], respectively. The detection of IgG antibodies to B19V in all  
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7 eight available sera of the PVB19-positive IDC patients suggests a prior exposure to B19V  
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9 during their lives. Accordingly, the three seronegative IDC patients lacked contact to B19V  
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11 and hence the presence of a myocardial B19V genome. Furthermore, considering a lifelong  
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13 persistence of the B19V genome as recently described in the myocardium [Kuethe et al., 2009;  
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15 Schenk et al., 2009] and other tissues [Norja et al., 2006], the absence of myocardial B19V  
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17 genomes in five IDC patients despite the occurrence of B19V IgG antibodies, and thus a past  
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19 B19V infection, indicates a possible sampling error in detecting cardiac B19V DNA or a  
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21 problem with respect to the sensitivity limit of the PCR in some of these cases.  
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### 30 **Methodological Aspects**

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32 The varying results in the control patients indicate an interlaboratory variability in 40% of  
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34 the cases associated with differences in quantification of up to 50%. However, it should be  
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36 kept in mind that all control samples had only low B19V DNA copy numbers that were close  
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38 to and even beyond the lower limit of the linear range and even close to the sensitivity  
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40 threshold of the B19V PCR methods. This can explain the imprecise quantification and the  
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42 high interlaboratory variability of detection. Nevertheless, the high frequency of myocardial  
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44 B19V genome detected both in IDC and normal LVEF by means of PCR, immediately raises  
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46 the question of a possible contamination leading to false positive results in this extremely  
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48 specific and sensitive PCR method as discussed previously [Hornsleth et al., 1994]. However,  
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50 nucleotide variability in the VP1 region of the parvoviral genome was shown by sequence  
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52 analysis of all B19V-positive endomyocardial biopsies from the IDC patients. Furthermore, all  
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54 B19V-positive samples were subjected to repeated analyses. Therefore, the frequent detection  
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56 rate of B19V genome in patients with IDC and in the control group was not based on  
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4 contamination, but due to the high sensitivity of the PCR method that is capable of detecting a  
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6 few parvoviral genomes, e.g. less than 50 copies/ $\mu$ g total DNA.  
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### 10 11 **Pathogenetic Significance of Myocardial B19V Persistence in Heart Disease**

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13 Considering the previous and the present study data, the question arises whether  
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15 myocardial occurrence of the B19V genome is merely an epiphenomenon or an indication of a  
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17 latent persistence of the virus which can be activated in some individuals by hitherto unknown  
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19 factors. B19V infection is known to be facilitated by the presence of the erythrocyte-P antigen  
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21 [Brown et al., 1993; Brown et al., 1994) also found on endothelial cells. However, B19V  
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23 replicates only in red blood cell precursors, not in endothelial cells, but might be able to enter  
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25 the cell and cause damage also without active replication [Söderlund-Venermo et al., 2002].  
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27 Thus, acute myocarditis could be caused by B19V affecting intracardiac endothelial cells  
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29 possibly associated with endothelial dysfunction, deterioration of myocardial microcirculation  
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31 and occurrence of inflammatory infiltrates in the myocardium, as reported recently [Bültmann  
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33 et al., 2003; Bock et al., 2005]. Except for mitotically active host cells [Young, 1996], the  
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35 circumstances under which B19V infection leads to myocardial damage or not are not known  
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37 to date. The rather favourable prognosis of IDC with intracardiac B19V DNA in  
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39 immunocompetent adults [Lotze et al., 2004] suggests that in many cases, B19V infection is  
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41 characterized by a mild course probably representing a latent long-standing virus persistence  
42  
43 associated with a low number of gene copies and without viral activity. Thus, the mere  
44  
45 presence of myocardial B19V genomes does not prove that B19V is the etiological agent for  
46  
47 the currently underlying heart disease [Söderlund-Venermo et al., 2002]. On the other hand,  
48  
49 there have been also cases of potentially fatal acute myocarditis in children and adults [Enders  
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51 et al., 1998; Bültmann et al., 2003; Dina et al., 2008; Tavora et al., 2008] obviously caused by  
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53 myocardial B19V infection indicating that, in these individuals, B19V may cause moderate to  
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55 severe damage to the heart due to a higher virus load and active viral replication [Bock,  
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4 personal communication]. The fact that the myocardial number of gene copies of B19V in the  
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6 IDC patients without an inflamed myocardium was as low as in our control patients indicates  
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8 a low virulence or even absent significance of B19V in IDC, if the intracardiac virus load is  
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10 low. By contrast, in the case of a higher virus load of >500 viral gene copies/ $\mu$ g total DNA,  
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12 which is regularly associated with cardiac inflammation and ongoing myocardial injury, B19V  
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14 could definitely play a significant role in the development of inflammatory heart disease as  
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16 found in patients with acute (n=25) or chronic (n=297) myocarditis [Bock, personal  
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18 communication] and recently described in a patient with acute fatal myocarditis [Bültmann et  
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20 al., 2003]. In patients with IDC (n=175), the number of myocardial B19V gene copies/ $\mu$ g  
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22 total DNA is significantly lower than in those patients with acute or chronic myocarditis ( $392$   
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24  $\pm 317$  vs.  $3.2 \pm 8.2 \times 10^5$  and  $709 \pm 828$  gene copies/ $\mu$ g total DNA, respectively,  $p < 0.0001$ )  
25  
26 [Bock, personal communication]. Thus, the etiopathogenetic role of B19V in myocarditis and  
27  
28 IDC may primarily depend on the number of gene copies found in the myocardium and a  
29  
30 genetically determined predisposition [Pankuweit et al., 2009]. Whether the B19V genotype1  
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32 possibly plays a more important role than the genotype 2 in the etiology of myocardial injury  
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34 in IDC [Kühl et al., 2008], could not be elucidated in present study due to the small number of  
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36 the IDC patients and requires further investigation. Finally, taking into consideration the low  
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38 numbers of gene copies in both our IDC patients and the controls (50-150 vs. 30-184 gene  
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40 copies/ $\mu$ g total DNA), one cannot assume a significance of B19V in the development of  
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42 cardiac damage in these IDC patients.  
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#### 55 **Prevalence of Enteroviral and Adenoviral Genomes in the IDC Patients**

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57 Although the prevalence of enteroviral RNA in the IDC patients (including one patient  
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59 who was positive for both EV and B19V) appeared to be rather low (8%), as compared to  
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prevalence rates (13-67%) in many previous studies [Petitjean et al., 1992; Weiss et al., 1992;  
Kämmerer et al., 1994; Figulla et al., 1995; Grumbach et al., 1999; Pauschinger et al., 1999;

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Fujioka et al., 2000], it is within the range between 3% and 53 % as reported by Pankuweit and co-workers [Pankuweit et al., 2000]. With respect to the interpretation of the low frequency obtained for the EV genome, the heterogenic nature of the study groups as well as the various conditions under which RT-PCR was performed including the different primers have to be taken into consideration [Petitjean et al., 1992; Weiss et al., 1992; Kämmerer et al., 1994; Grumbach et al., 1999; Pauschinger et al., 1999; Fujioka et al., 2000].

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The prevalence of adenoviral genomes including the adenovirus serotypes 2, 5 and 8 in none of the patients in our study is in contrast to other investigations which described adenovirus DNA in endomyocardial biopsy samples in 13% (12/94) or 6% (52/860) of patients with IDC, respectively [Pauschinger et al., 1999; Pankuweit et al., 2000]. However, our results do correspond to data by Grumbach and co-workers who found no adenoviral genome in endomyocardial specimens from 16 patients with IDC using PCR technique [Grumbach et al., 1999]. This discrepancy may be due to the various enrolment criteria, the size of the patient collectives, and the different methods used for detection of the adenoviral genome, including the various adenovirus serotypes found in the underlying studies [Grumbach et al., 1999; Pauschinger et al., 1999; Pankuweit et al., 2000].

#### 47 48 49 50 51 52 53 54 55 56 57 58 59 60 **The Virus-negative IDC Patients**

In several of the 11 virus-negative IDC patients, a familial dilated cardiomyopathy (FDC) [Mestroni et al., 1999; Pankuweit et al., 2009] has as well to be taken into consideration which may be the etiology of up to 30% of IDC [Pankuweit et al., 2009]. Even in the 13 virus-positive IDC patients, a FDC cannot be completely excluded. However, this issue was neither addressed in the present study nor in previous investigations mentioned above [Pankuweit et al., 2003; Lotze et al., 2004; Köhl et al. 2005; Andersson et al., 2008; Köhl et al., 2008], but will be an interesting scientific subject in the future.

### Study Limitations

The important factor that the tissue samples were obtained from different intracardiac sites in the two study groups has to be considered as a limitation of the study, since inflammatory myocardial changes are known to be able to occur in a non-uniform manner and it could be possible that the use of the right atrial site rather than the left ventricular endocardium for the control subjects may have affected the results. Thus, sampling error in detecting the inflammatory response and the myocardial B19V genome as well in some cases is one of the major limiting factors of the present study. Finally, the small group size of both the IDC and control patients has also to be taken into account.

### Conclusions

In conclusion, the results of the present study favour the view that myocardial B19V persistence associated with a low virus load does not trigger chronic cardiac damage resulting in left ventricular systolic dysfunction. Therefore, detection of low B19V concentrations in the myocardium seems not to be related to the pathogenesis of IDC without myocardial inflammation. In addition, it should be also taken into consideration that there is obviously an interobserver variability in 40% of the cases in the detection of low number myocardial B19V genomes.

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### Conflict of Interest

There exists no conflict of interest.

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TABLE I. Clinical, Echocardiographic and Hemodynamic Data of the Patients With Idiopathic Dilated Cardiomyopathy

	All patients (n=24)	B19V-positive patients (n=11) 46%	EV-positive patients (n=1) 4%	B19V plus EV-pos. patients (n=1) 4%	Virus-negative patients (n=11) 46%
Age ± [SD] (years)	51.5 ± 10.7	52.1 ± 7.5	61	33	51.7 ± 12.8
Male (%)	21 (88)	10 (91)	1 (100)	1 (100)	9 (82)
Duration of symptoms (months)	22.9 ± 43.2	30.3 ± 55.0	17	1	18.1 ± 33.8
<b>NYHA functional class</b>					
<b>I (%)</b>	0 (0)	0 (0)	(0)	(0)	0 (0)
<b>II (%)</b>	13 (54)	6 (55)	1 (100)	1 (100)	5 (45)
<b>III (%)</b>	11 (46)	5 (45)	(0)	(0)	6 (55)
<b>IV (%)</b>	0 (0)	(0)	(0)	(0)	0 (0)
<b>Medication</b>					
<b>ACE-I (%)</b>	23 (96)	11 (100)	1(100)	1 (100)	10 (91)
<b>ARB (%)</b>	1 (4)	0 (0)	0 (0)	0 (0)	1 (9)
<b>diuretics (%)</b>	19 (79)	8 (73)	1 (100)	0 (0)	10 (91)
<b>spironolactone (%)</b>	15 (63)	6 (55)	1 (100)	0 (0)	8 (73)
<b>digitalis (%)</b>	11 (46)	5 (45)	0 (0)	0 (0)	6 (55)
<b>β-blocker (%)</b>	23 (96)	11 (100)	1 (100)	0 (0)	11 (100)
<b>Echo LVEF (%)</b>	28.9 ± 8.7	29.1 ± 9.6	43	40	26.5 ± 6.7
<b>Echo LVEDD (mm)</b>	65.3 ± 9.0	64.1 ± 6.9	57	56	69.3 ± 8.4
<b>Angio LVEF (%)</b>	30.7 ± 9.6	30.5 ± 6.7	32	54	28.5 ± 10.4
<b>Mean PCWP at rest (mmHg)</b>	21.6 ± 7.7	20.0 ± 7.6	22	19	23.5 ± 8.3

ACE-I = angiotensin-converting enzyme inhibitor; Angio = angiographic; ARB = angiotensin II-receptor blocker;

Echo = echocardiographic; EV = enterovirus; LVEDD = left ventricular end-diastolic diameter; LVEF = left ventricular ejection fraction;

NYHA = New York Heart Association; PCWP = pulmonary-capillary wedge pressure; B19V = parvovirus B19.

TABLE II. Baseline Characteristics of the Control Patients Undergoing CABG

Number (n)	10
Mean age $\pm$ [SD] (years)	73 $\pm$ 4
Male (%)	7 (70)
LVEF (%)	67 $\pm$ 5
Coronary artery bypass grafting (%)	10 (100)
Extent of coronary artery disease	
1-vessel disease (%)	0 (0)
2-vessel disease (%)	3 (30)
3-vessel disease (%)	7 (70)
Main stem disease (%)	1 (10)

CABG= coronary artery bypass grafting; LVEF= left ventricular ejection fraction; SD= standard deviation.

TABLE III. Interobserver Variability in the Detection and Quantification of the Myocardial Parvovirus B19 Genomes in the 10 Control Patients

Cardiac auricles Sample-No.	Institute of Virology and Antiviral Therapy Jena	Department of Molecular Pathology Tübingen	Institute of Virology Hannover
1/10	Negative	Negative	Positive 80 copies / µg total DNA*
2/10	Positive 120 copies/ µg total DNA B19V genotype 2	Negative	Negative
3/10	Positive 100 copies/ µg total DNA B19V genotype 2	Positive 184 copies/ µg total DNA B19V genotype 2	Positive 60 copies/ µg total DNA B19V genotype 2
4/10	Negative	Negative	Positive 40 copies/ µg total DNA*
5/10	Negative	Negative	Negative
6/10	Positive 120 copies/ µg total DNA B19V genotype 2	Positive 122 copies/ µg total DNA B19V genotype 2	Positive 60 copies/ µg total DNA B19V genotype 2
7/10	Negative	Negative	Negative
8/10	Positive 100 copies/ µg total DNA B19V genotype 1	Negative	Positive 130 copies/ µg total DNA B19V genotype 1
9/10	Positive 100 copies/ µg total DNA B19V genotype 2	Positive 91 copies/ µg total DNA B19V genotype 2	Positive 30 copies/ µg total DNA B19V genotype 2
10/10	Negative	Negative	Negative

B19V= parvovirus B19. \* Sequencing of B19V DNA not done.

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For Peer Review

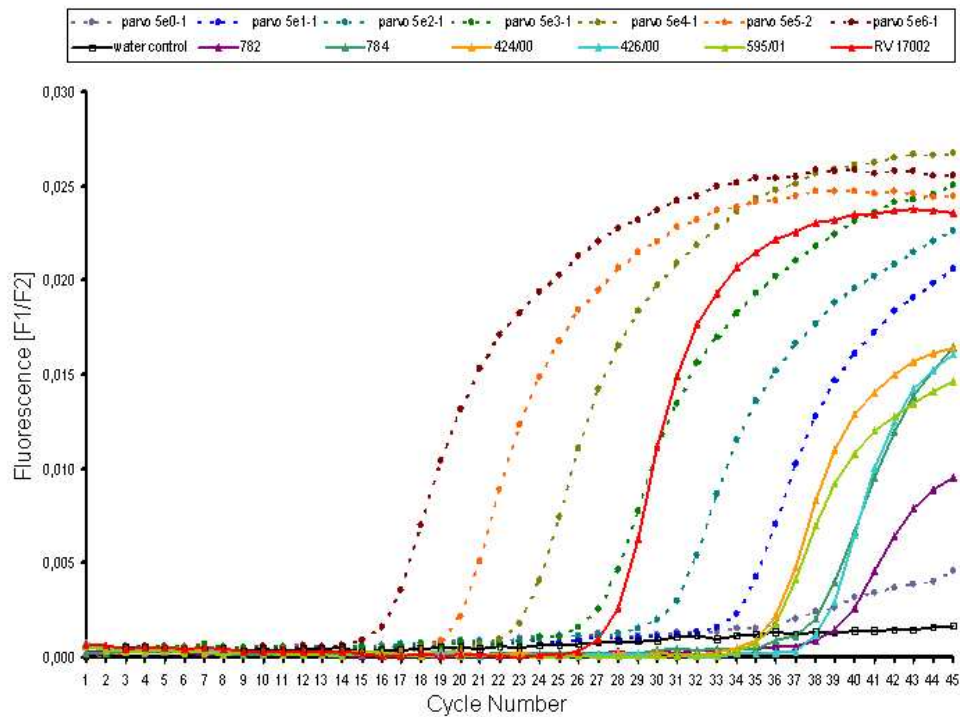


Figure 1: LighCycler real-time PCR for quantification of the PVB19 genomes

Results of the LighCycler real-time PCR showing the dotted standard curves of endomyocardial biopsy samples containing  $5 \times 10^0$  to  $5 \times 10^6$  copies of a B19V plasmid harbouring the amplified region of VP1, the red curve of the positive control (RV 17002) with 8500 copies and the curves of five IDC patients (violet: 5, green: 9, orange: 28, turquoise: 7, and light green: 23 copies/run).

IDC= idiopathic dilated cardiomyopathy; B19V= parvovirus B19; PCR= polymerase chain reaction; VP1= virus protein 1.

254x190mm (72 x 72 DPI)