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Parvovirus B19 DNA detectable in hearts of patients with dilated cardiomyopathy, but absent or inactive in blood

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ABSTRACT

Aims: Parvovirus B19 (B19V) is often assumed to be a cause of dilated cardiomyopathy (DCM), based on the quantification of B19V DNA in endomyocardial biopsies (EMB). Whether presence of B19V DNA correlates with active infection is still debated. Application of the enzyme endonuclease to blood samples results in degradation of B19V DNA remnants but leaves viral particles intact, which enables differentiation between active and past infection. In this study, the susceptibility to degradation by endonuclease of B19V DNA in blood was compared between DCM patients and a control group of recent B19V infections.

Methods and Results: Twenty blood samples from 20 adult patients with DCM, who previously tested positive for B19V DNA in EMB and/or blood, were tested with B19V PCR before and after application of endonuclease to the samples. Six blood samples tested positive for B19V DNA with a mean viral load of 2.3×10^4 IU/mL. In five samples, B19V DNA became undetectable after endonuclease (100% load reduction); in one sample DNA load showed a 23% log load reduction (viral load before endonuclease: 9.1×10^4 IU/mL; after: 6.5×10^3 IU/mL. Presence of cardiac inflammation did not differ between patients with B19V DNAemia (1/4) and patients without B19V DNAemia (6/14) (p-value = 1.0). In all 18 control samples of proven recent B19V-infections DNA remained detectable after application of endonuclease, showing only a mean log load reduction of 2.3% (mean viral load before endonuclease: 8.1×10^{11} IU/mL; after: 8.0×10^{11} IU/mL).). Load reduction differed significantly between the DCM group and the control group; indicating the presence of intact viral particles in the control group with proven active infection and the presence of DNA remnants in the DCM group (p-value = .000).

Conclusion: During recent B19V infection viral DNA levels in blood were unaffected by endonuclease. In contrast, B19V DNA in blood in patients with DCM, became undetectable or strongly reduced after application of endonuclease. Circulating viral DNA in this subset of patients with presumed parvovirus-associated DCM, does not consist of intact viral particles. Viral replicative activity cannot be assumed from demonstrating B19V DNA in cardiac tissue or in blood in DCM patients.

INTRODUCTION

Dilated cardiomyopathy (DCM) has a broad spectrum of aetiologies, with damage to cardiomyocytes by viral infection as a prominent cause [1]. For certain viruses, such as Coxsackie B virus, the causal relationship between presence of viral nucleic acid in cardiac tissue and cardiac disease is widely accepted, but in case of parvovirus B19 (B19V) this relationship is still debated. After initial infection by B19V, the presence of B19V DNA can be demonstrated in peripheral blood for months or even years, without apparent disease activity [2, 3]. In tissue, B19V DNA may remain detectable for life as has been demonstrated in various tissues, including heart, brain, bone, liver, kidney, skin and lymphoid tissue [4, 5]. Without additional clinical or laboratory signs of persistent infection, numerous reports remained cautious about assuming a causal relationship between the presence of B19V DNA in cardiac tissue or in blood, and cardiac disease [6-9]. Furthermore, several studies did not demonstrate a higher incidence of B19V DNA in endomyocardial biopsies (EMB) of DCM patients, as compared to control subjects at autopsy [10, 11]. On the other hand, evidence for a causal role of B19V in cardiac disorders is reported [12-14], particularly when the cardiac viral load is quantified [15]. Therefore, the exact role of B19V in cardiac pathology remains unclear. It is suggested that additional factors need to be considered to determine the pathogenicity of B19V, such as viral activity [16].

Previously, it was demonstrated that application of endonuclease to circulating parvoviral DNA leads to its degradation, except when DNA is packaged and thus protected in viral particles during an active viral infection [17, 18]. This enabled differentiation between active infection, where intact viral particles are present, and past infection, where mere parvoviral DNA remnants are released from persistence in cells. In this study, the susceptibility to degradation of B19V DNA in blood was compared between patients with confirmed DCM and a control group of well-defined recent B19V infections as a measure for viral activity.

METHODS

Blood samples of DCM patients: Twenty plasma samples were selected from 20 adult patients diagnosed with DCM from a cohort of inpatients and outpatients of the Maastricht University Medical Centre in the period 2006-2016. The cohort was part of a randomized controlled trial investigating intravenous immunoglobulins

(IVIG) in the treatment of B19V-positive patients with DCM (NCT:00659386). Sample selection was based on highest B19V viral loads in EMB as these patients were expected to have B19V detectable in blood. Eighteen patients previously tested positive for B19V DNA in EMB; 2 patients did not undergo EMB, but they had previously tested positive for B19V DNA in blood.

Blood samples of patients with recent B19V infection: Eighteen plasma samples from 18 B19V PCR-positive patients from different patient groups were selected. If plasma was not available, serum was used as the alternative. This selection consisted of patients from the Leiden University Medical Center with well-characterized, active B19V infection in the period 2011-2017. Patients were categorized by clinical presentation: recent B19V infection in a normal host; severe B19V infection due to underlying haematological pathology; patients presenting with arthropathy; hydropic foetuses and B19V infection in the severely immunocompromised.

Endonuclease (Benzonase®) assay and PCR: The test principle of the endonuclease assay is depicted in figure 1. Samples were tested as described by Molenaar-de Backer et al [17]. Both plasma and serum are suitable for B19V PCR and the endonuclease assay. In short; each plasma or serum sample was split in two 100 µl aliquots. In case of plasma, MgCl was added to oppose the chelating effects of EDTA on nucleases. To one of the aliquots 250 units of Benzonase® (Sigma- Aldrich, the Netherlands) were added. Subsequently both aliquots were incubated at 37°C in a shaking incubator at 120 rpm for one hour. After cooling to room temperature DNA extraction and dual target NS-VP2 PCR were performed as described previously [19]. Viral loads differed between samples of patients with recent B19V infection and patients with DCM. Therefore, the detection of B19V DNA with and without application of endonuclease was also performed on samples after standardizing the B19V DNA load to 10^4 IU/mL by adding B19V-negative plasma.

Statistical analysis: The data were analysed using IBM SPSS Statistics 26. Categorical data between unrelated groups was compared using Fisher's exact test. Numerical data between unrelated groups was compared using the Mann-Whitney U test. Results were considered statistically significant at the $p = 0.05$ level.

Ethics: The investigation conforms with the principles outlined in the declaration of Helsinki. All cardiomyopathy patients gave written informed consent as part of the Maastricht Cardiomyopathy Registry (for in- and exclusion criteria of the registry; see Verdonschot et al [20]). For control patients, samples were collected with patient's informed consent for the use of the samples for B19V diagnostics.

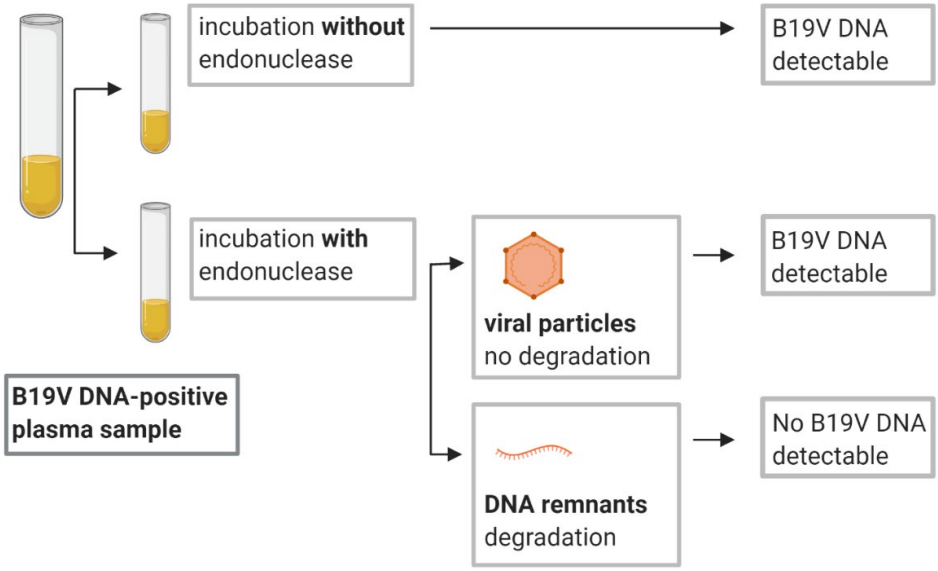

Figure 1: the principle of the endonuclease test. *DNA is degraded by endonuclease when only bare DNA strands are present. When intact viral particles are present, DNA remains intact as it is protected within the viral particle. Incubation without endonuclease acts as positive control. Plasma is used in the example; both serum and plasma are suitable. (Figure created with Biorender.com)*

RESULTS

DCM patients: Six of the 20 DCM patient blood samples tested positive for B19V DNA. Fourteen DCM patients with high cardiac viral loads had no detectable B19V DNA in their blood. Patient characteristics are described in table 1. Mean viral load was 2.3 x 10^4 IU/mL. In 5 patients B19V DNA became entirely undetectable after application of endonuclease; one remained detectable but showed a viral load reduction from 9.1 x 10^4 to 6.5 x 10^3 IU/mL, corresponding to a log reduction of 23% (see figure 2). In this patient, B19V DNA had already been detected with a higher load (5.9 x 10^5 IU/mL) in a blood sample taken 6 weeks previously which was not available for testing with endonuclease. Presence of cardiac inflammation did not differ between patients with B19V DNAemia (1/4) and patients without B19V DNAemia (6/14) (p-value = 1.0).

Table 1: Overview of DCM patients with and without B19V DNA detectable in blood

Patient	Clinical information	Laboratory parameters at time of EMB**			Immunohistochemical parameters in EMB (cells/mm²)						Cardiac inflammation	B19V load in EMB (c/µg DNA)	Time from diagnosis DCM to EMB (weeks)	B19V load in blood (IU/mL)	Time from EMB to blood sample (weeks)
		hsTnT (ng/L)	CK (U/L)	NTproBNP (pmol/L)	CD3	CD4	CD8	CD20	CD45	CD68					
1	Heart failure, diagnosed since approx. 9 months; declining VL* in blood	na††	517	8.3	0	0	6.5	0	7.2	2.4	no	2333	40	8.9×10^3	-3
2	DCM (EF† 35%); recently diagnosed	<0.01	70	286	20	7.4	11.5	0	23.1	4.6	yes	1025	3	1.3×10^4	0
3	DCM (EF 32%) after acute perimyocarditis (EF 10%)	33	63	401	2.7	5	0.8	0	6.1	4.6	no	601	1	6.5×10^3	0
4	DCM (EF 10%), recently diagnosed, improving to 45% with CRTD‡ treatment	na	302	344	1.7	1	1.4	0	5.2	2.4	no	312	3	1.6×10^4	0
5	Heart failure, diagnosed since 6 weeks, declining VL in blood	na	na	14	na	na	na	na	na	na	no EMB	na	na	9.1×10^4	-
6	Heart failure, diagnosed since 1 week, pre-existent hypertensive heart disease	0.02	84	3070	na	na	na	na	na	na	no EMB	na	na	2.9×10^3	-
7	DCM (EF 33%); dyssynchronization due to LBBB; improving to 51% with medication	na	na	na	11	7.5	3.3	0	12.2	5.4	yes	9948	3	neg	0
8	DCM (EF 32%)	12	111	49.5	3.7	1.6	2.1	0	8	2.7	no	7250	2	neg	-7
9	DCM (EF 10%); improving to EF 38% during IVIG trial; declining VL in EMB; possibly auto-immune component	120	94	1032	2.4	0.6	8.6	0	6.1	4.9	no	6889	9	neg	-1

10	DCM (EF 46%); recently diagnosed in work-up atrial fibrillation; improving to EF 54% during IVIG trial	5	232	2.4	3.3	0.4	1.4	0	4.5	1.4	no	5669	2 years	neg	0
11	DCM (EF 45%); diagnosis after cardiac arrest	na	176	107	9.9	4.4	5.5	0	29.7	19.8	yes	2806	0	neg	0
12	DCM (EF 20%); recently diagnosed; improving to 55% during IVIG trial	na	na	38.7	2.5	na	3.3	0	23	3.3	yes	2726	9	neg	16
13	DCM (EF 40%); improving to 52% during IVIG trial	na	na	16	2.5	10.7	0.5	0	6.1	1.5	no	2436	11	neg	-7
14	DCM (EF 45%); chronic since 7 years	<0.01	na	4	9.3	na	7.9	0	10	na	no	2379	7 years	neg	-8
15	DCM (EF 40%); recently diagnosed; possibly past myocarditis	<0.01	na	65	35.4	25.8	20.4	0	36	12.7	yes	2220	4	neg	-20
16	DCM (EF 35%); recently diagnosed; improving to 51% during IVIG trial	<0.01	866	278	na	0.6	0	0	1.2	1.5	no	2185	12	neg	-13
17	DCM (EF 20%); recently diagnosed; improving to 35% with medication	<0.01	92	77.3	1.8	na	0.7	0	3.3	na	no	2163	2	neg	-5
18	DCM (EF 44%) after cardiotoxic chemotherapy; recovery to previous 62%	na	na	10.1	25.4	21.2	21.5	1.4	42.3	22.6	yes	1829	8	neg	0
19	DCM (EF 29%); MRI: midmyocardial fibrosis, possibly past myocarditis	11	61	46.9	6.6	8.6	4.6	0	12.2	2.3	yes	875	3	neg	0
20	DCM (EF 30%); chronic since 2 years; improving to EF 51% with medication	18	24	17.7	3.2	1.6	1.6	0	3.7	2.4	no	691	3 years	neg	0

** VL = viral load; †EF = ejection fraction; ‡CRTD = cardiac resynchronizaton therapy defibrillator; **EMB = endomyocardial biopsy; ††na = not available. Cardiac inflammation is diagnosed according to the criteria of the European Society for Cardiology*

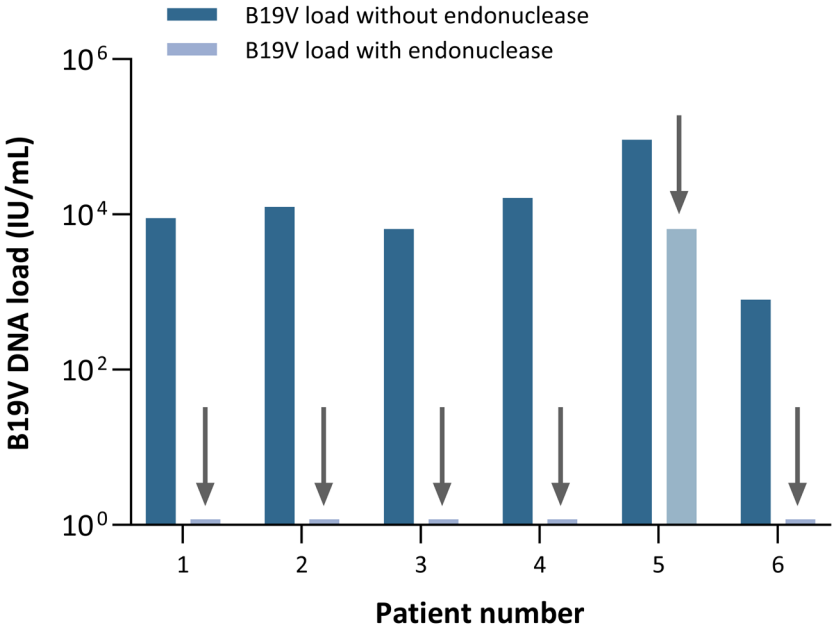

Figure 2: B19V DNA absent or degradable in blood of patients with DCM. *Blood samples of DCM patients with and without endonuclease treatment. Dark bars indicate DNA load without endonuclease treatment, light bars and/or arrows indicate DNA load with endonuclease treatment (DNA load absent after endonuclease in 5 out of 6 samples)*

Proven recent B19V patients: All 18 patients with recent B19V-infection tested positive with B19V PCR with a mean viral load higher than the DCM patients (8.1×10^{11} IU/mL). Table 2 shows background information for all 18 patients. Patients were grouped according to their clinical background (see methods). None of the control patients showed cardiological signs or symptoms. After endonuclease, all remained detectable with a mean viral load of 8.0×10^{11} IU/mL, corresponding to only a mean 2.3% log reduction of viral load. Figure 3 shows the results of testing with and without application of endonuclease for individual patients in different patient categories. Load reduction differed significantly between the DCM group and the control group ($p = .000$).

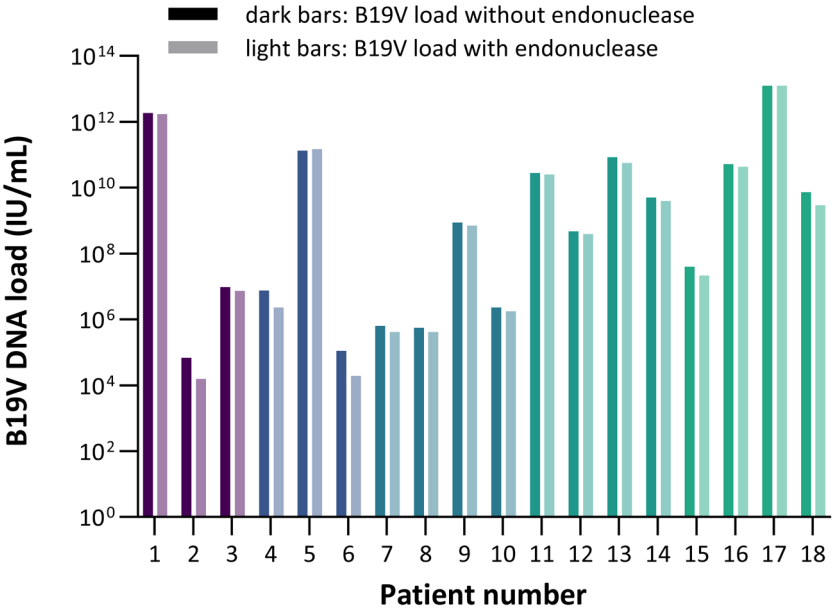

Figure 3: B19V DNA not degradable in patients with active B19V infection. *Blood samples of patients with recent infection with and without endonuclease treatment. Different colors indicate different patient groups. For each patient, the dark bar indicates DNA load without endonuclease treatment, while the light bar indicates DNA load with endonuclease treatment. Patients 1-3 represent viral loads in recent B19V infections in normal hosts; patients 4-6 represent viral loads in severe B19V infection due to underlying haematological pathology; patients 7-10 represent viral loads in patients presenting with arthropathy; patients 11-14 represent antenatal viral loads of hydropic foetuses and patients 15-18 represent viral loads in the severely immunocompromised.*

Standardized testing at 10^4 IU/mL. Samples with viral loads exceeding 10^4 IU/mL were standardized to 10^4 IU/mL by diluting them with B19V-negative plasma. Twelve samples were available for standardization in the control group. After standardizing, the mean log reduction in viral loads in the control group before and after endonuclease was 2.4%. Samples from cardiac patients were not diluted to 10^4 IU/mL as all six samples already had viral loads not exceeding 10^4 IU/mL.

DISCUSSION

Control patients with recent or acute B19V infections showed high B19V DNA levels in blood which were unaffected by endonuclease. In contrast, B19V DNA levels were low or absent in blood samples of patients with DCM. When B19V DNA was present, it became undetectable after application of endonuclease in the majority of patients. The degradability of circulating B19V DNA in DCM patients indicates that replicating viral particles are absent in blood, suggesting that the detected B19V DNA consists of DNA remnants. In concordance with this finding is the relatively low viral load in blood of these DCM patients. Similar loads were demonstrated in patients after acute infection who were no longer able to transmit infection [21]. Standardizing testing conditions by diluting samples with very high viral loads to viral loads of 10^4 IU/mL gave identical results in the patient group with recent infection, that is, application of endonuclease did not result in a significant reduction of viral load. Therefore, the effect of endonuclease does not depend on viral load in this range. Dilution of samples to viral loads lower than 10^4 IU/mL was not performed; further dilution could result in inaccurate measurements as 10^4 IU/mL is a relatively low viral load.

B19V DNA resulting from viral replication can be demonstrated in blood in nearly all patients with proven acute infection [22]. In patients with proven chronic symptomatic B19V infection, DNAemia is also invariably present in blood [23-25]. The absence of DNAemia is therefore suggestive of the absence of viral replication. In the cohort of DCM patients, the positivity rate in blood was only 33% (6/18), so most patients with B19V detectable in cardiac tissue did not demonstrate DNAemia. One cardiac patient with a positive B19V PCR in blood showed only partial degradation of B19V. Given that this patient had B19V detectable in an earlier sample and that the level of B19V DNA in blood was decreasing, this is suggestive of a recent infection. A positive IgM would confirm a recent B19V infection but unfortunately no additional serology could be performed in this case, due to insufficient material.

Viral infections are one of the causes of DCM. In addition to the direct effect of viral replication causing tissue damage, it has been proposed in the case of B19V and DCM that the virus elicits a chronic inflammatory process or that molecular mimicry plays a role [16, 26, 27]. IVIG are being studied in the treatment of presumed B19V-induced DCM. Dennert et al [28] treated DCM patients with relatively high cardiac B19V-loads with IVIG. Viral load in EMB decreased and cardiac functions improved significantly after IVIG treatment. However, the uncontrolled pilot

study included only 17 patients and the absolute improvement in cardiac function was modest. Therefore, a double-blinded randomized trial has been started to investigate any beneficial effects of IVIG in B19V-associated DCM (NCT:00659386). The results of the study are expected to be published in 2021. However, the exact mode of action of the beneficial effects in this application is unknown. If viable viral particles are indeed absent in presumed B19V-induced DCM patients, then a direct antiviral effect of IVIG will not be the primary mechanism. In addition, IVIG also has anti-inflammatory and immunomodulating properties, which could also explain its beneficial effect in DCM patients [29]. Determining viral activity will be an important factor to consider during the analysis and interpretation of the results of the randomised clinical trial.

Recently, it was observed that immunosuppressive, anti-inflammatory medication (prednisolone and azathioprine) to treat inflammatory cardiomyopathy (DCMi) did not have an adverse outcome in patients with cardiac B19V persistence but was equally beneficial for both DCMi patients with and without B19V DNA detectable in EMB (median viral load 80 c/µg DNA, range 1-5074) [30]. Similar observations were made in a recent Swedish cohort of DCM patients, where the frequent occurrence of B19V DNA in cardiac tissue in both patients with DCM and in healthy donor hearts was confirmed. Prognosis of patients with DCM did not differ between patients with or without B19V DNA in cardiac tissue in this study, without taking viral load into account [31]. This suggests that the mere presence of B19V DNA does not contribute to the disease process. In reaction, it has been suggested that methods of determining viral activity should be further investigated to understand the pathogenic role of B19V [27]. The endonuclease test could be a new and easy instrument to contribute to assessment of viral activity. Its application can be useful in the etiological work-up of DCM, but also in cases of acute myocarditis where B19V genomes are detected in EMB and blood and where a strong B19V-specific immune response is elicited [32].

There are limitations to this study. We have investigated the nature of B19V DNA in peripheral blood but not in cardiac tissue, as the endonuclease test can only be applied to blood samples. To expose intracellular viral particles or DNA in tissue to endonuclease, samples would need to be treated with a protease. This would already affect any intact viral particles present, degrading their capsid and therefore interfering with a subsequent endonuclease assay, that is, the application of protease would produce naked DNA. Future research could aim to develop

a specific endonuclease assay suitable for cardiac tissue. Another limitation is the difference in disease duration between DCM cases and controls with recent infection. The pathophysiological process in DCM often unfolds silently for a period of time before becoming clinically apparent, as opposed to acute infection with B19V. Although the results from this study do not point to continuing viral replication in the course of DCM, the relation between timing of initial B19V infection and the development of DCM cannot be specified. Therefore, a possible role of B19V in the induction of DCM remains to be elucidated. Also, our study focused on determining viral activity by the method of endonuclease. Additional methods may also be employed to prove the presence or absence of replicating, infectious virus. Viral culture can be considered a gold standard for viral activity, although viral culture in case of B19V is notoriously challenging and insensitive, partly due to its specialized tropism for erythroid progenitor cells [33]. Previous research also proposed the evaluation of mRNA intermediates as a biomarker for B19V activity [34, 35]. The use of mRNA intermediates can still be considered a pioneering technique and sensitivity and specificity are not yet established. It would however be valuable to employ complementary approaches such as mRNA intermediates to gain a complete understanding of the replicative status of B19V.

In conclusion, the results confirm the presence of circulating viral DNA in all cases with clinically proven active B19V-infection. This viral DNA cannot be degraded enzymatically, probably because it is contained in in protective viral particles. In patients suspected of chronic B19V-associated DCM, there is a much lower incidence of circulating viral DNA. When viral DNA is present in blood in such cases, it is degradable by endonuclease. These findings show that viral replicative activity cannot be assumed from demonstrating B19V in cardiac tissue or in blood. Multidisciplinary approaches are necessary to further investigate the role of B19V in the pathogenic mechanisms in the development of DCM.

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