Parvovirus B19: diagnosis, distribution and disease associations

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PARVOVIRUS B19

DIAGNOSIS DISTRIBUTION AND DISEASE ASSOCIATIONS

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Diagnosis, Distribution and Disease Associations

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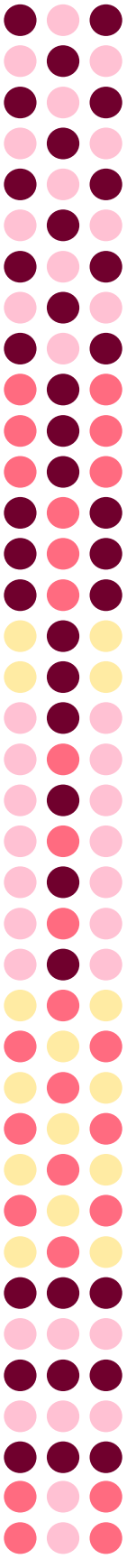

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General introduction

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STRUCTURE AND TAXONOMY

Human parvovirus B19 (B19V) is a member of the family of *Parvoviridae.* This family consists of two well established subfamilies; *Parvovirinae* and *Densovirinae*. The two subfamilies are distinguished by their ability to infect vertebrate animals and invertebrates, respectively [1]. Recently, a third subfamily *Hamaparvovirinae* has been proposed that can infect both vertebrates and invertebrates (figure 1) [1]. Members of the family *Parvoviridae* are small, non-enveloped viruses with linear, single-stranded DNA genomes of 4 - 6 Kb. Due to their small genomes, which do not encode their own replicative enzymes, most *Parvoviridae* require actively dividing host cells for replication and some viruses require helper viruses from other families.

The subfamily of *Parvovirinae* contains ten genera, which include 75 species as described in a taxonomy revision in 2019 [1]. *Parvovirinae* infect a wide range of vertebrates (birds, reptiles, mammals), although the tropism of each *Parvovirinae* species appears to be restricted to a single host species and is often tissue-specific. Viruses that infect humans are present in several genera. B19V belongs to the genus *Erythroparvovirus*, which contains seven species (B19V, seal parvovirus, simian parvovirus, rhesus macaque parvovirus, pig-tailed macaque parvovirus, chipmunk parvovirus and bovine parvovirus 3). In this genus, B19V is the only species to infect humans [1, 2].

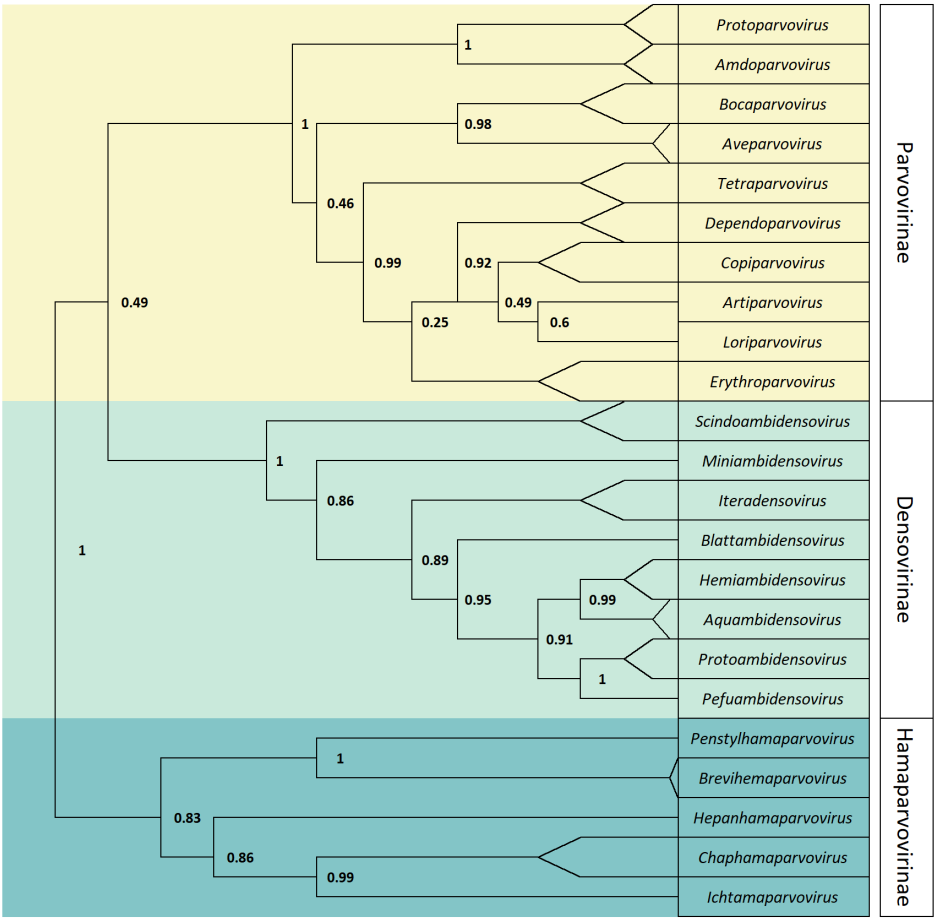


Figure 1: *Phylogenetic relationship of the Parvoviridae at the genus level, based on Bayesian inference of the tripartite helicase domain (167 amino-acids), which is conserved throughout the family Parvoviridae. The size of the last branching (most right) represents the distance from the most basal node in the genus to the branch peaks for each genus, which comprises multiple species (adapted from Pénzes et al [2])*

B19V harbors a linear, single-stranded DNA genome of 5596 nucleotides. The genome is packaged in a small (~25 nm diameter) stable capsid with an icosahedral structure and no surrounding envelope. The genome codes for 3 major proteins: Viral Protein (VP) 1, VP2 and non-structural protein 1 (NS1). VP2 is the major structural protein and accounts for 96% of the capsid structure. VP1 is the minor capsid protein. VP 1 and VP2 have overlapping sequences, but the VP1 protein differs

from VP2 by an additional 227 amino acids that make up the VP1 unique region (VP1u) (figure 2). NS1 is the major regulatory protein, which regulates the entire viral replication cycle and is capable of inducing apoptosis in infected cells. Apart from these major proteins, a 11 kDa protein is involved in DNA replication and apoptosis and a 7.5 kDa protein is expressed, of which the function is still unknown [3].

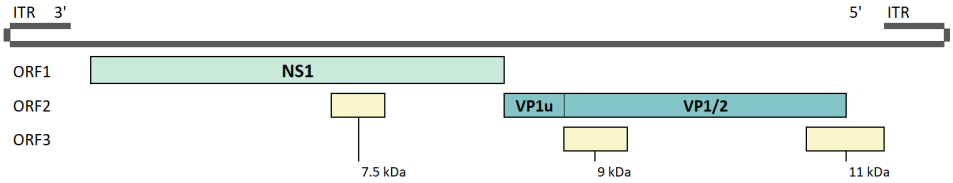


***Figure 2**: Genome organization of B19V*

B19V has 3 genotypes, which are distinct with ~10% divergence on the whole genome [4]. Within genotypes, variation is restricted to 1 to 4% and further subtypes have been described [5, 6]. Genotype 1 circulates worldwide and is now dominant in Northern Europe. Genotype 2 co-circulated with genotype 1 until half a century ago and then disappeared from circulation. As B19V DNA is able to persist in tissue life-long (a topic which will be explored further in this introduction), genotype 2 genomes are now only detected in individuals born before 1973 [7]. Genotype 3 co-circulates with genotype 1 in Africa, South-America and Asia and is occasionally detected in other regions [8-10]. Despite genome variation, capsid protein sequences are similar among genotypes which results in antibody cross-neutralization [11, 12].

CELL ENTRY AND REPLICATION

In natural infection, the virus is transmitted by respiratory secretions. The virus is subsequently carried to the bone marrow where it infects erythroid progenitor cells (EPCs). Productive infection of EPCs is dependent on the combination of the expression of specific receptors and co-receptors on the cell surface and the presence of essential cellular factors for replication. It has been shown that only certain types of EPCs in relatively late stages of differentiation are susceptible to productive infection [13-16]. Historically, globoside, or P-antigen, was regarded as the main receptor for B19V-binding and uptake, with a possible role for co-receptors, in which Ku80

and alfa5-beta-integrin have been implicated [17, 18]. More recent evidence has shown that viral uptake by the cell is mediated by interaction of VP1u with a yet unknown erythroid-specific receptor, and globoside is involved, but dispensable for uptake [19, 20]. After uptake, the presence of globoside does seem essential for endocytic trafficking. In the absence of globoside, incoming viruses are retained in the endocytic compartment and the infection is aborted [21, 22]. It has been observed that persons with a rare mutation in the globoside synthase gene who do not express globoside seem naturally resistant to infection [23]. Globoside is found on erythroblasts, but its distribution is much wider than B19V's narrow tropism, indicating that other factors are also essential for productive infection [24]. In addition to the interaction of VP1u and a cell receptor, antibody-dependent uptake has also been shown as a mechanism by which B19V is internalized in monocytes, endothelial cells and B-cells, and has been proposed as a mechanism to explain the sustained presence of B19V DNA in cells other than erythroid progenitor cells [25-27]. The exact mechanism by which respiratory epithelium is infected has not yet been elucidated, but this mechanism differs from the uptake by EPCs [28].

With regard to productive infection, the presence of erythropoietin (EPO) seems essential for B19V replication, as it is for the maturation of erythroid progenitor cells. It has been demonstrated in vitro that B19V replication does not take place in erythroid progenitor cells in the absence of EPO [13, 15]. Hypoxia has also been shown to promote viral replication [29-31]. Furthermore, it has been observed *in vitro* that parvoviruses also need actively replicating host cells for their own replication, as they depend on host enzymes for viral replication [32].

Infection of EPCs leads to cell cycle arrest and apoptosis of the cell, leading to depletion of the EPC pool in the bone marrow, until the infection is cleared. This leads to a disappearance of reticulocytes from the peripheral blood, as these are the immediate precursors of erythrocytes. This temporary halt in erythropoiesis impacts different patient populations differently. It is not clinically significant in otherwise healthy hosts, but can cause severe anemia in fetal infection or in those with underlying predisposing conditions. This will be discussed in the paragraph on disease associations.

DIAGNOSIS OF PARVOVIRUS B19

Figure 3 provides an overview of the different courses of clinical and laboratory parameters of B19V infection in different patient populations.

Serology: Availability of viral antigens has been a limiting factor for specific serology, until the development of baculovirus expression systems for this purpose [33]. Parvovirus B19 IgM and IgG testing is used as the gold standard for diagnosing recent and past infection, although it has been suggested that a combination of methods should preferably be used for most accurate diagnosis [34, 35]. In immunocompetent persons, serological profiles follow the classic pattern in which IgM becomes detectable around 10-12 days after infection and persists for several weeks up to 3 months, followed or accompanied by IgG which usually becomes detectable around two weeks after infection and remains detectable life-long [36, 37]. In severely immunocompromised individuals such as transplant patients, serologic responses may be weak and protective immunity may develop late or only after tapering of immunosuppressive treatment [38-41]. Once natural protective immunity has developed, immunity is considered to be life-long.

PCR in blood: Replicative infection is characterized by viremia with high viral levels up to 10^{14} IU/mL. In many individuals, the viral load decreases after the acute phase of infection to around 10^4 IU mL, and such levels of viral DNA may remain detectable for months. This observation was for the first time reported in 1993 when PCR remained positive during 9 months with low B19V DNA loads after infection in the presence of antibodies and has been observed many times over (42-45). This particular course with waning levels also suggests that low DNA loads can be used as indicators for late stages of infection or past infection, which underscores the need for accurate viral load quantification [35].

PCR on bodily fluids: In pregnant women, intrauterine B19V infection can be diagnosed by PCR on amniotic fluid (as a replacement for fetal blood) [46]. Saliva has been proposed as a non-invasive alternative for diagnosis of acute B19V infections, but due to its suboptimal sensitivity its use should be considered with care [47].

PCR in tissue: In tissue, B19V DNA may remain detectable by PCR for years and probably life-long. In figure 4, an overview of studies on B19V DNA persistence in different tissues (excluding cardiac tissue; which is discussed elsewhere) is presented [27, 48-91]. This figure shows that B19V DNA persistence has been unequivocally and consistently demonstrated in a very broad range of tissue types. The observed PCR positivity rates vary between studies, which can probably be explained by heterogeneous populations with differing seroprevalences (not always reported in the studies) and by differing methodologies. The prolonged detection of B19V DNA typically is not associated with any apparent disease activity and the

ability of B19V to reactivate from a latent state, as is common in Herpesviridae, has never been demonstrated. This has sparked many debates about the clinical interpretation of the tissue detection of B19V DNA. Although detected in many different tissues, it remains largely unknown what type of cells contain B19V DNA and in what form B19V DNA occurs in these cells. A crucial question in the prolonged detection of B19V DNA in both blood and tissue is whether such presence of B19V DNA originates from active replicative infection or whether the presence of B19V DNA consists of viral remnants (of a past infection), which may have been passively absorbed in a variety of tissues.

(Metagenomic) next-generation sequencing (mNGS): in recent years, the fast developments in (m)NGS assays have also contributed to the identification of B19V DNA in compartments and situations, while not primarily searched for. Examples are the presence of B19V DNA detected by mNGS in serum in (fatal) liver failure, viral encephalitis and in cardiomyopathy [92-94]. Due to the ability of B19V to persist in practically all compartments of the body, interpretation of these findings in (m)NGS only seems possible when these data are accompanied by at least reliable quantification of mNGS data, reliable evidence of viral gene expression or additional proof of any presumed disease-causing activity.

Cell culture: Due to its tropism for human erythrocyte progenitor cells, propagation in standard tissue culture is difficult. In the earliest attempts, cell culture was only possible on freshly harvested human bone marrow [13, 14, 95]. Later, certain well-established leukemic or megakaryoblastoid cell lines have been shown to support B19V replication, but these are only semi-permissive [96]. More recently, the development and expansion of erythroid progenitor cells from human hematopoietic stem cells has improved *in vitro* culture of B19V, but this culture model still requires strict *in vitro* circumstances to attain productive infection [15, 30, 97, 98]. The demanding and specialized techniques required for these cell cultures underscore the need for reliable, easily applicable methods to determine viral replicative activity.

Other methods: other diagnostic techniques have been applied to detect or visualize B19V. In acute infection, electron microscopy (EM) can show viral particles in blood and bone marrow and diseased tissue such as infected fetuses or placentas [99, 100]. Immunohistochemical staining can show viral inclusions in erythroblasts [101] and in situ-hybridization has shown B19V DNA in fetal tissue [102, 103]. These methods are not employed in routine diagnostic settings.

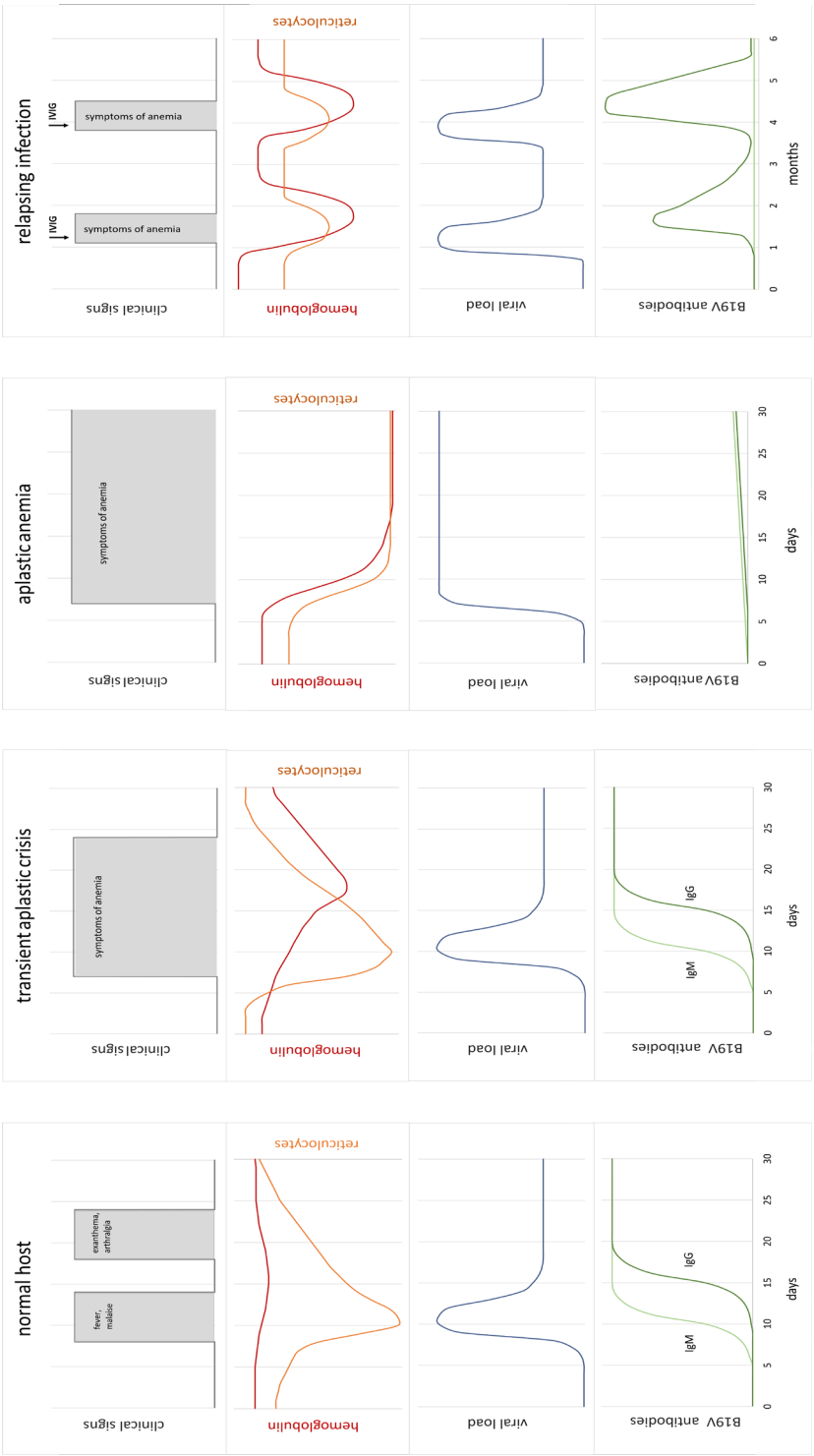


***Figure 3**: Overview of different clinical manifestations and course of laboratory parameters of B19V infections in different patient populations, from left to right: normal host; transient aplastic crisis in patients with underlying red blood cell disorders; aplastic anemia in immunocompromised patients; relapsing infection in immunocompromised patients after IVIG treatment*

TRANSMISSION DYNAMICS AND SEROPREVALENCE

Natural transmission and seroprevalence

Human-to-human transmission in the community occurs via respiratory secretions. Most B19V infections occur in childhood, when cases may be recognized as classical 'fifth disease' (see below), but infections can also pass undiagnosed as non-specific febrile illness. This is apparent from a seroprevalence of 35 to 58% in children at age ten in certain European countries, which increases to 57-73% at the age of 30 years [104]. Furthermore, seroprevalence is slightly, but significantly higher in day care workers [105]. B19V infections peak yearly in late spring and have been reported to occur in regular four-year cycles (oscillations), although divergent superimposed cycles have also been observed [106-110].

Vertical transmission

When a non-immune woman is infected in pregnancy, vertical transmission may occur. The incidence of B19V infection in seronegative pregnant women is estimated to be 1 to 5%. This may increase to 10% in epidemic periods [111, 112]. Vertical transmission occurs in 40 to 51% of pregnancies in which maternal infection occurs [113, 114]. The severity of fetal symptoms largely depends on the stage of pregnancy at infection, which will be discussed in the paragraph on disease associations.

Iatrogenic transmission: transfusion-transmitted B19V infection

Due to the persistence of B19V DNA in plasma, B19V has raised potential concerns in blood banking. Prevalence of B19V DNA in blood donations is estimated between zero and 1%, with varying DNA loads [115]. B19V transmission by blood products has been observed, but transfusion-transmitted-B19V (TT-B19V) disease primarily occurs after transfusion of high-load blood donations from acutely infected donors [116-119]. Blood donations that contain low levels of B19V DNA are not considered to be infectious and European guidelines for blood banking accept blood donations with DNA loads not exceeding 10^5 IU/mL for the production of certain plasma products [116, 120, 121]. It is unknown why low-level DNAemic blood donations are not infectious. It has been suggested that simultaneously with infectious virus, neutralizing antibodies from the donor are transmitted. Alternatively, it may be that the presence of viral DNA does not indicate that infectious viral particles are present. It could also be that TT-B19V disease may be overlooked due to a mild clinical course

[115, 116, 120, 122]. It clearly is an issue why low-level DNAemic samples do not transfer infection.

Iatrogenic transmission: transplantation-associated B19V infection

Due to their immunocompromised state, transplant patients are at risk of developing severe anemia when they become infected with B19V (see paragraph on clinical manifestations). Obviously, infection after transplantation may occur through natural transmission but proven or very probable donor-transmitted infection has been reported in case reports and case series [38, 123]. Of all solid organ transplantation (SOT) patients, B19V infection in kidney transplantation (KTx) patients has been studied most extensively. Of all symptomatic B19V infections in KTx, it is estimated that two thirds of episodes occur in the first three months following transplantation [40]. It is unknown whether this follows from a high percentage of donor-transmitted infection shortly after transplantation, or whether transplant patients are more at risk for severe B19V disease in the first months after transplantation, due to a stringent immunosuppressive regimen and often pre-existent anemia as a consequence of renal failure. The prevalence of B19V DNAemia in the first year after kidney transplantation is estimated at 7.6% (CI 3.7%-15.0%), but not all patients are symptomatic [124]. In patients with anemia, the incidence rate of B19V DNAemia is estimated at 27.4% [124]. Conversely, of B19V DNAemic patients, anemia (as a proxy for symptomatic B19V disease) can be estimated to occur in 37% at the time of B19V detection [123-126]. It is remarkable that not all severely immunosuppressed patients in which (early) B19V DNAemia after transplantation is identified, develop symptoms of B19V-disease. In addition, true donor-transmitted B19V infection seems a relatively rare event although PCR-positivity rate of kidney tissue is not rare (fig 2) and no measures are currently being taken in transplant medicine to match serostatus for donors and recipients for B19V and thus prevention of transplantation-associated B19V. These findings raise the question how to interpret B19V DNAemia after SOT.

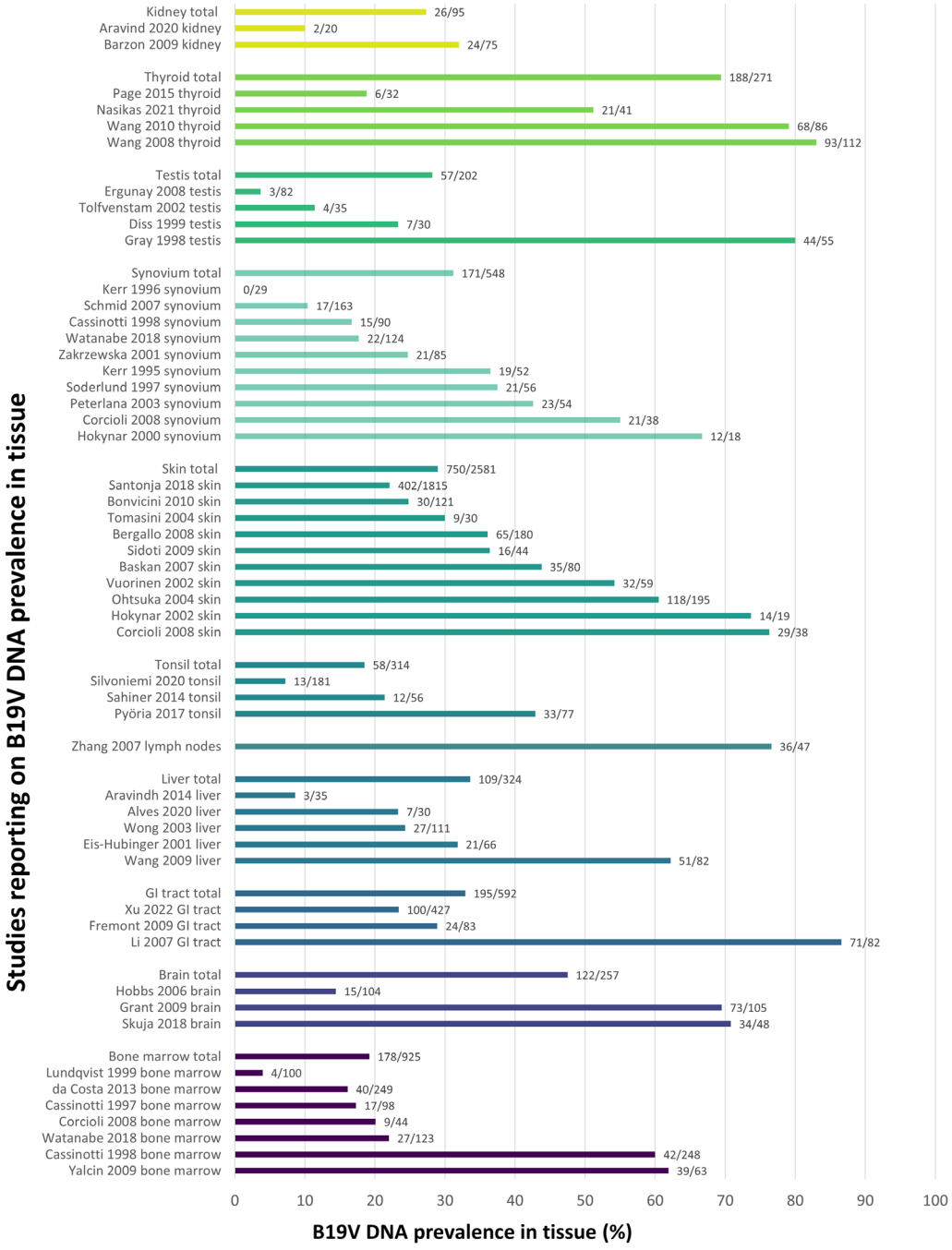


***Figure 4**: PCR positivity rates of B19V DNA in various human tissues. Numerator/denominator is displayed at the end of the bar. Studies are listed by first author and year of publication.*

PARVOVIRUS B19 AND DISEASE ASSOCIATIONS

B19V was discovered in 1975 by the Australian virologist Yvonne Cossart and her co-workers. In a comparative analysis of methods to detect hepatitis B surface antigen in sera from patients and healthy blood donors, the researchers identified viral particles that were similar to animal parvoviruses when examined by electron microscopy. The designation 'B19' originates from the coding of the microtiter plates that were used for the experiments; one of the virus-containing wells was sample number '19' in panel B (99).

In the years hereafter, B19V 'found its disease'. It was identified in a common childhood illness known as 'fifth disease' [127], and subsequently in a set of clinical syndromes that all result from the tropism of the virus for erythrocyte progenitor cells. Its manifestations and their severity are highly dependent on the immunological status of the host or whether an underlying red blood cell disorder is present. These clinical syndromes will now be briefly discussed in different patient categories.

Infection in the healthy host

As B19V is a ubiquitous pathogen, contact often occurs in early life and cases of infection usually manifest themselves in childhood. The classical clinical syndrome in an immunocompetent child is known as the historical exanthematous 'fifth disease' or erythema infectiosum and consists of a febrile illness in combination with erythema of the cheeks ('slapped cheeks'), followed by a rash on trunk and limbs with a lacy and reticular pattern. Although rare in children, adults may experience arthropathy after B19V-infection. This is estimated to occur in 30-50% of adults and is more frequent in women [128]. Arthropathy mainly manifests as symmetric joint involvement, usually of the hands and occasionally of ankles, knees and wrists. Arthropathy usually resolves spontaneously within a few weeks and joint destruction does not occur. It is believed that both rash and arthropathy are immunecomplex-mediated symptoms of the infection [129].

Infection in pregnancy

Infection of the healthy pregnant woman may run a mild course or may even go unnoticed, but when the infection is vertically transmitted to the fetus, serious fetal complications may occur. Transplacental infection of the fetus may result in a very severe anemia, followed by a high-output cardiac failure, which in turn leads

to fetal hydrops. The high output cardiac failure of fetal hydrops can be demonstrated non-invasively by measuring the middle cerebral artery blood flow (MCA flow), already in very early stages. Severity of the symptoms in the fetus strongly depends on the stage of pregnancy at infection. Before 20 weeks of gestation, the risk of fetal hydrops is 3.9 to 11.9%, but after 20 weeks of gestation, fetal hydrops hardly occurs after B19V infection [113, 114, 130]. Severe fetal hydrops is managed with intrauterine erythrocyte transfusions (IUT), which have improved survival rates considerably in the past decennia [130-132]. It is thought that long-term consequences of intrauterine B19V infection, when successfully managed with IUT, are minimal. However, this is mostly based on population studies [133-135]. More detailed evaluation in small study groups shows that neurologic sequelae might occur more frequently than previously thought [136, 137]. Further studies are needed to gain insight into the consequences of severe intrauterine infection. In addition, the true birth prevalence of B19V infection is unknown, as there are no national screening programs for B19V and infection in the healthy pregnant woman may go unnoticed. It is therefore also unknown how many fetuses suffer mild, undetected intrauterine infection with B19V and whether long-term consequences of mild infections occur and what they may consist of. Diagnosis of intrauterine B19V infection is now performed with invasive techniques during pregnancy (PCR on fetal plasma/amniotic fluid); the first step towards estimation of disease burden is development of a suitable non-invasive screening tool.

Infection in the host with underlying red blood cell disorders

The special tropism for erythrocyte progenitor cells results in a special category of patients that are vulnerable to the effects of B19V infection. In patients who suffer from either decreased red blood cell production or an increased red blood cell turnover, the halt in erythropoiesis leads to an immediate and profound drop in hemoglobulin levels, leading to the condition known as ‘transient aplastic crisis’ (TAC). In case of decreased red blood cell production, this can be attributed to the lack of resting progenitor cells that could be recruited to compensate for the consequences of the elimination of active cells by the wave of B19V infection. In case of an increased red blood cell turnover, the half-life of existing erythrocytes is too short to compensate for the halt in erythropoiesis. Disorders among the patients susceptible to this complication, as a non-exhaustive list, include sickle cell disease, thalassemia, spherocytosis, erythrocyte enzyme deficiencies and even malaria [129]. As

these patients are usually capable of mounting adequate immune responses, aplastic crises resolve spontaneously in a few weeks, although good supportive treatment is essential for full clinical recovery.

Infection in the immunocompromised host

In immunocompetent persons, the dip in reticulocytes is too transient to affect erythrocyte levels in a clinically relevant way. In contrast, in persons with impaired immunity, a robust immune response does not occur, resulting in prolonged infection of EPCs, leading to clinically relevant anemia (figure 3). This so-called 'pure red cell aplasia' (PRCA) has been reported to occur in patients with severe congenital or acquired immunodeficiencies, such as transplant patients and AIDS-patients [40, 129, 138-140]. In such patients, B19V infection only resolves when neutralizing antibodies become present, either by intrinsic production or external administration in the form of intravenous immunoglobulins (IVIG). This is evident from transplant patients in whom viremia and anemia resolve after treatment with IVIG, but who relapse when IVIG has been cleared from the circulation after approximately 3-4 months [141]. Lasting immunity is only obtained when the immunocompromised state improves, e.g. by tapering immunosuppressive treatment and the development of subsequent natural immunity. Although neutralizing antibodies are considered to be crucial in the clearance of infection, there is evidence for T-cell responses also playing a role in controlling infection [142-145].

B19V and its association with miscellaneous syndromes

The above-mentioned clinical syndromes are historically well-described and have a profound evidence-based pathogenetic etiology. Apart from this set of well-described clinical syndromes, B19V has also been implicated in a myriad of clinical manifestations for which the evidence is still debated. Many of these associations are only reported in case studies or case series, often solely basing the association on the detection of B19V DNA in certain tissues and lacking additional support for the association.

A presumed role of B19V in causing myocarditis or cardiomyopathy has been particularly confusing and will be discussed in some detail. Soon after PCR assays became available on a large scale, it was observed that a large proportion of both diseased and non-diseased hearts contained B19V DNA in cardiac tissues. Figure 5 presents an overview of the studies which report on the prevalence of detection

of B19V DNA in both diseased and non-diseased cardiac tissue [52, 146-181]. Most studies have looked at the prevalence of B19V DNA in diseased hearts, in particular patients with myocarditis (MC) or dilated cardiomyopathy (DCM). The distribution of PCR positivity in both diseased and non-diseased cardiac tissue has a very wide range, but suggests that positivity rates are comparable. This is confirmed by a recent meta-analysis, which shows that the relative risk ratio of B19V DNA positivity in cardiac tissue is not significantly higher in patients with cardiac disease compared to control groups [182]. Obviously, the comparable presence of viral DNA in diseased and normal hearts casts doubt on the significance of this finding as a cause of cardiac disease.

Subsequently, it has been suggested that to fully understand the capability of B19V to cause cardiac disease, other parameters such as height of viral load and measures of viral activity should be taken into account [183]. A number of studies found that DCM was associated with higher cardiac B19V viral loads [148, 176, 180], but several other studies have found no association between viral load and pathology, even reporting viral loads in non-diseased hearts that are higher than those in DCM [146, 160, 164, 179]. Other methods including detection of mRNA, immunohistochemistry, in-situ hybridization, electron microscopy and specific B19V T-cell responses have also been employed to understand the significance of the presence of B19V DNA in cardiac tissue, but these studies are often characterized by methodological shortcomings and also report conflicting outcomes [152, 169, 180, 184-186]. These divergent results and shortcomings of diagnostic techniques do not support a clear role for B19V in myocarditis and cardiomyopathies, but above all underline the importance of reliable methods to evaluate the nature of the B19V DNA detected in such syndromes.

Based on the detection of B19V DNA, B19V has also been implicated in hepatitis, skin diseases, thyroid diseases, rheumatoid disease and vasculitis [72, 82, 91, 187, 188], while other studies have failed to confirm a causal relationship in all these syndromes [62, 73, 77, 189].

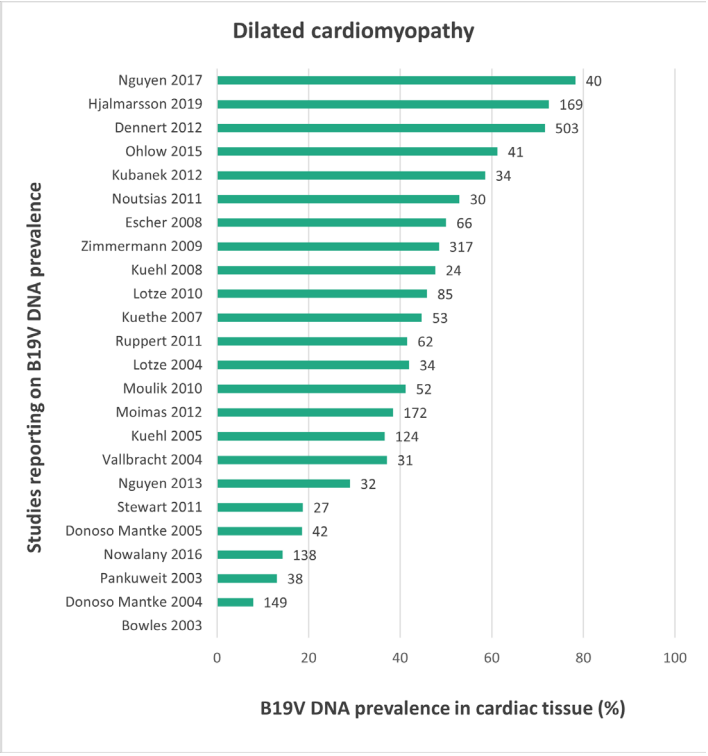
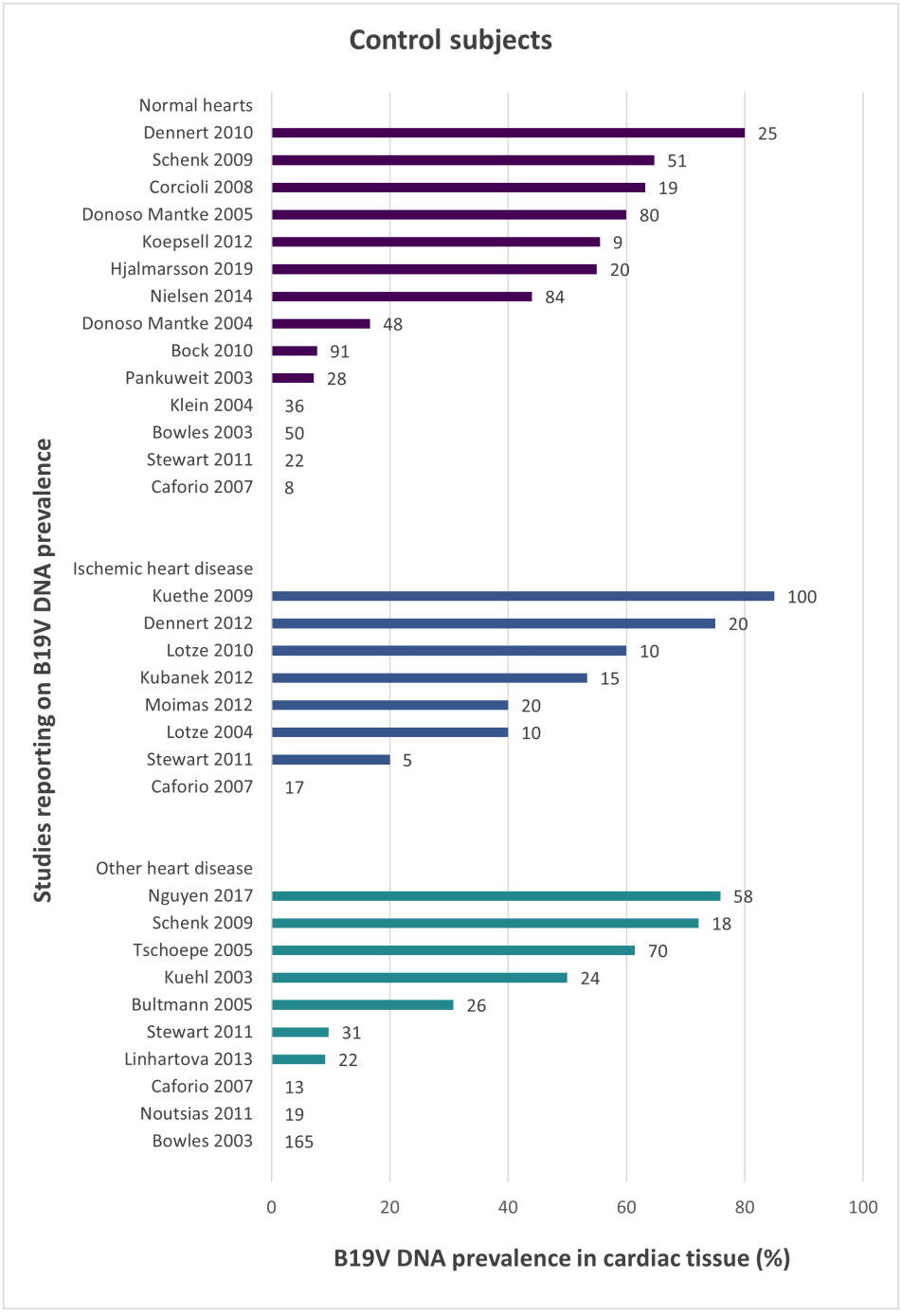


Figure 5: *Overview of prevalence of B19V DNA in cardiac tissue in various clinical conditions. End of border notes denominators. Studies are listed by first author and year of publication. Adapted from [183], supplemented with recent literature.*


Control subjects
Studies reporting on B19V DNA prevalence
Normal hearts
Dennert 2010 25
Schenk 2009 51
Corcioli 2008 19
Donoso Mantke 2005 80
Koepsell 2012 9
Hjalmarsson 2019 20
Nielsen 2014 84
Donoso Mantke 2004 48
Bock 2010 91
Pankuweit 2003 28
Klein 2004 36
Bowles 2003 50
Stewart 2011 22
Caforio 2007 8
Ischemic heart disease
Kuethe 2009 100
Dennert 2012 20
Lotze 2010 10
Kubanek 2012 15
Moimas 2012 20
Lotze 2004 10
Stewart 2011 5
Caforio 2007 17
Other heart disease
Nguyen 2017 58
Schenk 2009 18
Tschoepe 2005 70
Kuehl 2003 24
Bultmann 2005 26
Stewart 2011 31
Linhartova 2013 22
Caforio 2007 13
Noutsias 2011 19
Bowles 2003 165
0 20 40 60 80 100
B19V DNA prevalence in cardiac tissue (%)

TREATMENT

In persistent or relapsing B19V infection with clinically relevant anemia, mainly among immunocompromised patients, treatment with intravenous immunoglobulins is now standard practice [141, 190]. Treatment with IVIG reduces B19V viral load and leads to improvement of anemia [141]. However, relapse after IVIG is seen in about 30% of patients, and these patients require a second course or even multiple courses of IVIG [39, 40, 191, 192]. As relapsing infection after IVIG may occur, while B19V DNA can also be present in blood for months after infection without causing disease, it is essential to differentiate between active infection (and thus, the need for repeated treatment) and past infection.

IVIG has also been applied in other supposed manifestations of B19V disease. A randomized, double-blind, placebo-controlled trial using IVIG in idiopathic chronic cardiomyopathy with B19V persistence in cardiac tissue did not show a beneficial effect on left ventricular ejection fraction (LVEF) or other outcomes such as functional capacity, quality of life or cardiac B19V DNA load [193].

Currently, targeted antiviral treatment is not available for B19V infection. In the search for compounds inhibiting B19V replication, multiple compounds including known antiviral agents such as nucleotide analogues (e.g. cidofovir), foscarnet and the S-phase inhibitor hydroxyurea have been evaluated. At present, observed in-vitro effects and data from case reports or small case series have not yet shown convincing efficacy or clear directions for use in clinical care [194, 195]. The very limited availability of specific viral enzymatic targets obviously limits the development of targeted antiviral therapy.

AIMS AND OUTLINE OF THIS THESIS

The main aim of this thesis is to investigate new approaches and techniques to diagnose B19V infection and to determine the significance of detection of the infection in different patient populations. This would lead to a better understanding of the pathogenetic nature of B19V, its clinical impact and disease burden.

In **chapter 2**, epidemiological trends for B19V over the last 33 years are analyzed. The changes that occurred during and after the COVID-19 pandemic are described and their clinical impact is investigated by analyzing trends in intrauterine erythrocyte transfusions, performed for B19V-related fetal hydrops.

Chapter 3 investigates the clinical impact of the B19V epidemic in 2024, showing the increase in intrauterine erythrocyte transfusions during the epidemic and reporting on the outcome of pregnancies after intrauterine erythrocyte transfusion.

Chapter 4 investigates the persistence of B19V DNA in postnatal blood of newborns with intrauterine B19V infection. The suitability of postnatal blood samples as potential screening samples for intrauterine B19V is investigated, to improve insight in the real prevalence of this infection.

Chapter 5 describes the development and application of a new tool, an endonuclease-based assay, to differentiate between the presence of intact B19V viral particles and DNA remnants in blood. This could improve the interpretation of detection of B19V viral DNA in various clinical conditions, which is currently challenging, as described before.

In **chapter 6**, the application of the endonuclease-assay in a cohort of patients with presumed B19V-induced cardiomyopathy is evaluated.

Chapter 7 determines the prevalence of B19V DNAemia after kidney transplantation in a large Dutch teaching hospital and investigates the relevance of this finding, in relation to the presence of active replicative infection.

In **chapter 8**, mNGS is used to determine viral loads of 6 different DNA-viruses, including B19V. The relationship between viral load, clinical course of the disease and identification of clinically relevant cut-off points or thresholds for intervention is analyzed.

In **chapter 9**, intra-host viral evolution and potential humoral evasion are explored, analyzing longitudinal samples of relapsing B19V infections in immunocompromised patients by whole genome sequencing and protein modeling.

Chapter 10 contains the general discussion, in which the implications of the findings in this thesis are discussed and in which remaining areas for future research are identified.

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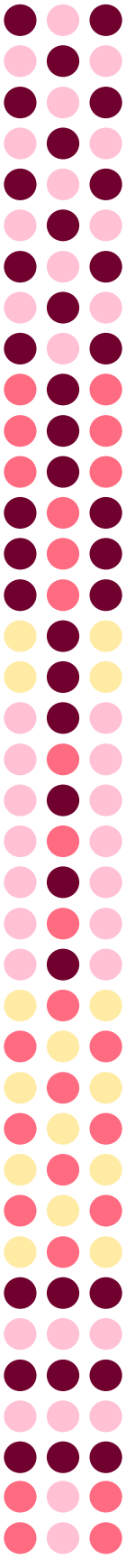

2

Changing epidemiology of parvovirus B19 in the Netherlands since 1990, including its reemergence after the COVID-19 pandemic

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ABSTRACT

Parvovirus B19V (B19V) infection during pregnancy can be complicated by potentially life-threatening fetal hydrops, which can be managed by intrauterine transfusion (IUT). This study investigates the long-term temporal patterns in the epidemiology of B19V and evaluates the impact on fetal hydrops, by combining data on B19V infections from the Dutch Sentinel Surveillance system in the period 1990 to 2023; Dutch blood banking data and hospital data on fetal hydrops. Using wavelet analysis, we identified annual epidemic cycles in the Netherlands in the period 1990-2019 and we identified superimposed multiannual cycles in the period 1990-2009. After 2009, no multiannual cycle could be identified, although the incidence fluctuated and correlates with number of IUT performed. As of 2020, weekly reports of B19V infection demonstrated a historically low incidence and B19V-DNA positive blood donors were nearly absent. From May 2020 to May 2023, no IUT for B19V-related hydrops was performed. In the spring of 2023, B19V infections re-emerged, reaching pre-pandemic epidemic levels. Due to the changes in B19V epidemiology over the last 30 years and the near-absence of B19V during the COVID-19 pandemic, the resulting low immunity levels may lead to rebound outbreaks. Alertness to severe complications such as fetal hydrops is warranted.

INTRODUCTION

Parvovirus B19 (B19V) is best known as the pathogen involved in fifth disease, a self-limiting febrile erythematous illness in children. B19V infects erythrocyte progenitor cells, causing an arrest in erythropoiesis. In normal hosts, this arrest is temporary and does not lead to clinically significant anemia. However, the arrest of erythropoiesis may lead to severe anemia in susceptible persons such as immunocompromised hosts or individuals with a short half-life of red blood cells. When non-immune women acquire B19V infection during pregnancy, the virus can be transmitted to the fetus and fetal infection can lead to severe fetal anemia which in turn may lead to potentially fatal fetal hydrops. Fetal hydrops is managed by intrauterine erythrocyte transfusion (IUT), a highly specialized and costly procedure, which is only performed in specialized prenatal care hospitals. Insight in B19V epidemiology is relevant in view of the serious consequences of the infection in vulnerable hosts, in particular unborn children.

B19V infections occur according to a seasonal cycle with annual epidemics of varying size occurring in the spring [1, 2]. Larger epidemics have been shown to occur once every four years, indicated as 'oscillation' [1-4]. In the Netherlands, this pattern was last described in 2002, covering the period 1990-2002 [1]. Data from other countries indicate that this pattern may vary; for example a six-year epidemic cycle is reported in Ireland [5]. Recently, it became clear that COVID-19 restrictions had a profound impact on the occurrence and seasonal variation of many viruses transmitted by the respiratory route, such as influenza virus and RSV [6]. The transmission dynamics of B19V also appeared to be affected by COVID-19 restrictions. B19V infections among blood donors fell sharply and became a rare finding in blood banks since the spring of 2020 [7, 8].

The aim of this study was to describe the regular seasonal variation of B19V infections in the Netherlands over the past three decades, and to analyse the impact of the COVID-19 pandemic on B19V epidemiology, also considering possible clinical consequences such as fetal hydrops. To gain a comprehensive insight, we used data from the Dutch Sentinel Surveillance system, which receives data from laboratories on laboratory-confirmed B19V infection, combined with data from the Dutch national blood bank and hospital data on severe fetal B19V infection. Using wavelet analysis, an advanced technique to uncover periodicities in a time series, we show that the earlier described oscillation has become disturbed over the past decade, possibly due to a decreasing birth rate. After near-absence of

B19V during the COVID-19 pandemic, B19V is now reemerging, which requires vigilance for severe complications in fetuses or other hosts susceptible to severe infection.

RESULTS

Seasonal pattern and periodicity

Figure 1 (panel A) shows the 9-week running mean of the number of B19V infections based on national Sentinel Surveillance data for the period January 1st, 1990, to December 31st, 2023. For the period up to 2020, annual peaks are present, although the annual peaks are less pronounced in 2015 and 2016. Wavelet analysis confirmed this annual peak and showed that in the period 2020-2022, the annual peaks had disappeared (figure 1, panel B). For the period up to 2009, a 4-year periodic pattern is present in addition to the annual peaks. After 2009, a multiannual periodicity could not be identified by wavelet analysis. However, the incidence did fluctuate in the period 2010-2019, with the lowest number of infections reported in 2016 (n=94) and the highest in 2010 (n=221). Figure 2 shows that the annual peaks occur mostly in spring, with most infections being reported in May.

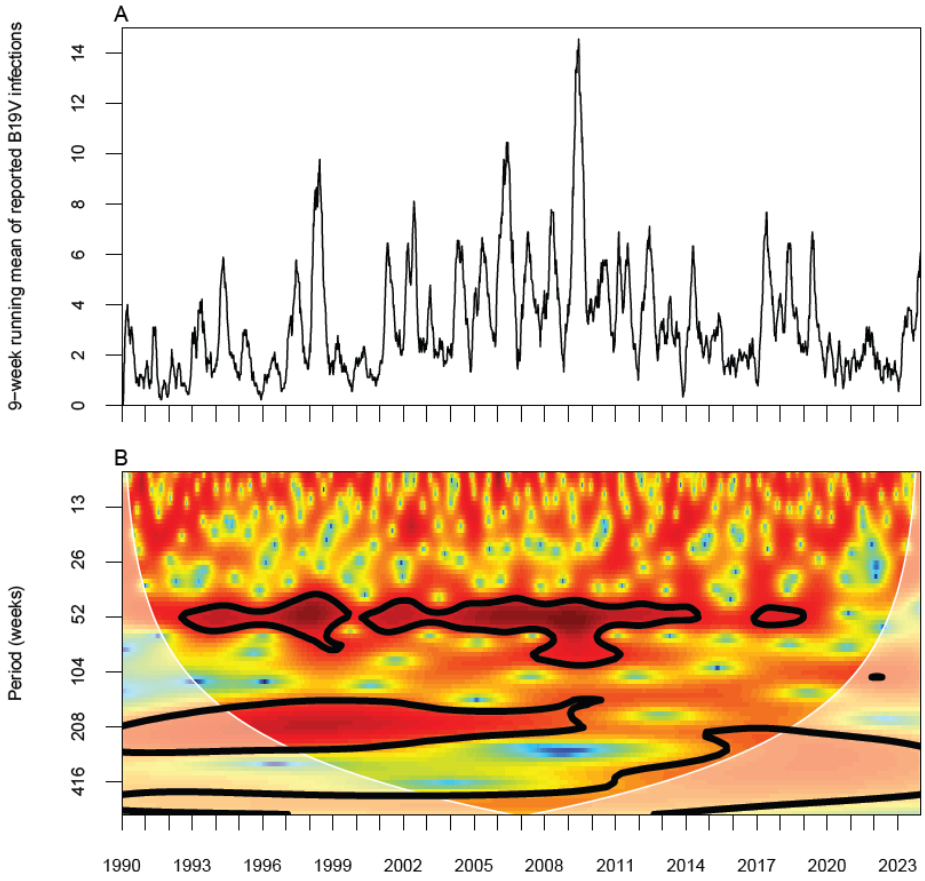

Figure 1: B19V epidemiology over a period of 33 years*: Panel A: 9-week running mean of B19V-infections reported to the Dutch weekly Sentinel Surveillance system for the period Jan 1st, 1990 - Dec 31st, 2023. Panel B: Wavelet power spectrum of the Sentinel Surveillance data of B19V infections for the period Jan 1st, 1990 – Dec 31st, 2023. Colours indicate wavelet power (from low power in blue to high power in red). Black contour lines enclose significant areas (95% confidence) where the power is higher than the power of red noise with the same autocorrelation coefficient as the data. The shaded area represents the cone of influence, which is a region where edge effects become important. Significant periodicities are visible around 52 weeks (annual peaks) and around 208 weeks (four-year cycle) up to 2009.*

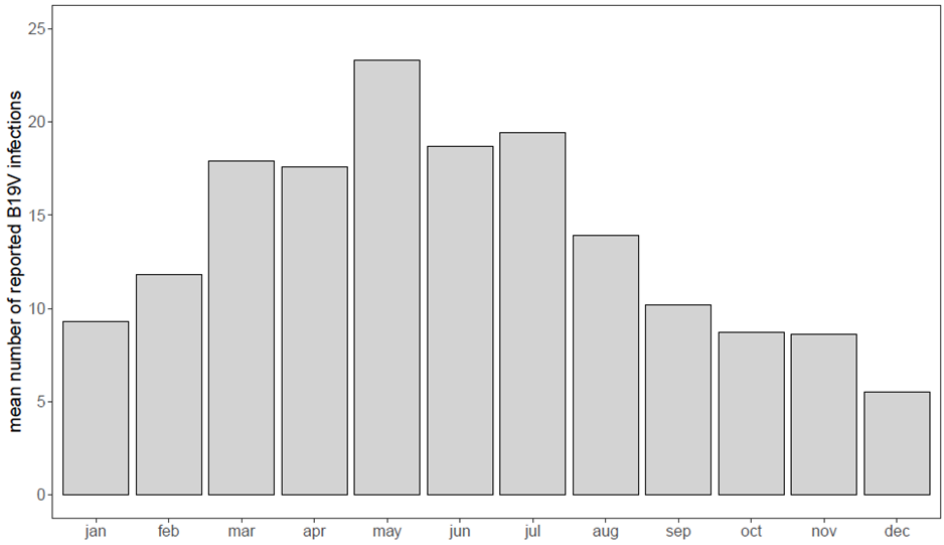

Figure 2: Seasonal variation in B19V incidence*: Mean number of B19V infections per week reported to the national Sentinel Surveillance system for the period Jan 1st, 2002 – Dec 31st, 2023*

Data from the Dutch blood bank on the incidence of high load viremic donations showed a similar pattern to Sentinel Surveillance data, with annual peaks in viremic donations in spring (figure 3). For the period 2013-2019, the minimum incidence of viremic donations was 0.18 per 10.000 donations (2015) and the maximum incidence was 0.60 per 10.000 donations (2019). Figure 4 (panel A) shows the annual number of IUT performed in the Netherlands for B19V-related fetal anemia in the period Jan 1st, 2002- Dec 31st, 2023. The number varies from 0 IUT per year to a maximum of 9 IUT per year.

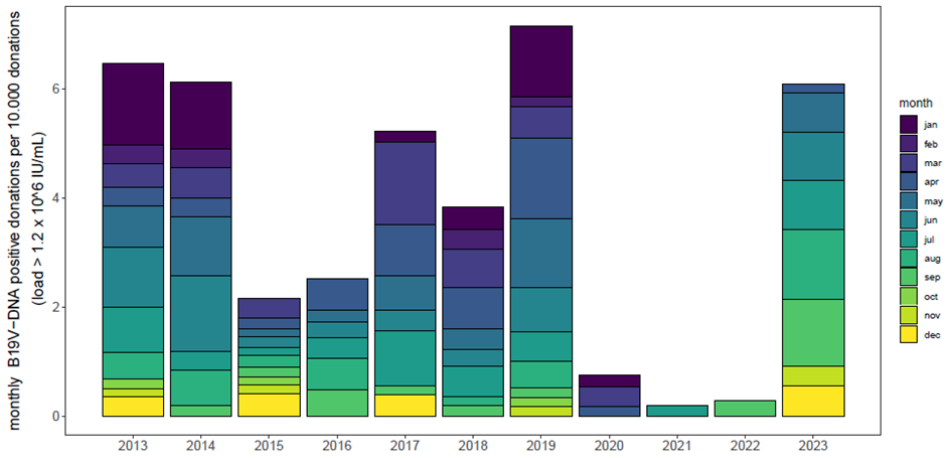

Figure 3: B19V positive blood donations*: Number of monthly high-load B19V-DNA positive donations per 10.000 donations at the Dutch national blood bank for the period Jan 1st, 2013 – Dec 31st, 2023*

Cross-correlations between different time-series

Figure 4 (panel B) shows the over-all correlation between Sentinel Surveillance data and IUT. Analyses of cross-correlations between Sentinel Surveillance data and IUTs at different lags showed significant correlations at different lags, with maximum lag occurring at 1 week where Sentinel Surveillance data precedes IUT data (data not shown). Cross-wavelet analysis showed similar results (data not shown).

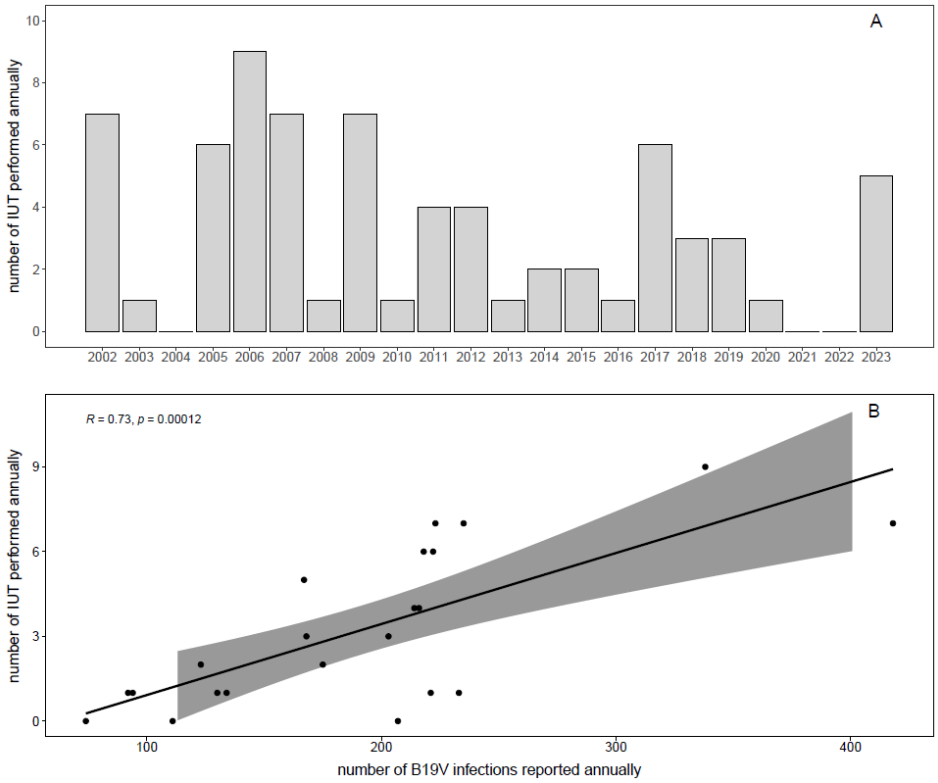

***Figure 4: Number of annually performed IUT and correlation with reported B19V infections**: Panel A; absolute number of IUT performed annually for B19V-related fetal hydrops in the period Jan 1st, 2002 - Dec 31st, 2023, in the Netherlands. Panel B; correlation between number of annually reported B19V infections by the Sentinel Surveillance and number of IUT performed annually. Gray lines indicate the confidence interval of the Pearson correlation coefficient at 95%. The p-value is the significance level of the t-test.*

Near absence of B19V infections since the start of the COVID-19 pandemic and re-emergence

In the Sentinel Surveillance data, the mean annual number of reported infections was 173 in the period 1990-2019. In the period 2020-2022 the mean annual number of reports was much lower at 92 reports per year. Data from the Dutch blood bank show that high-load viremic donors were virtually absent during the COVID-19 pandemic (figure 3). The incidence of viremic donations dropped to 0.01 per 10.000 donations in 2021. Also, no IUT for B19V-related fetal hydrops was performed in the period 2020-2022. In the spring of 2023, Sentinel Surveillance data

showed an increasing number of B19V infections (figure 1), indicating the re-emergence of B19V that continues into the winter of 2023. In week 52, the 9-week running mean was 6.1. This is the highest number of reported infections since the autumn of 2019. In addition, the incidence of viremic donations increased from 0.17 per 10.000 donations in April to 1.28 per 10.000 donations in August, a level which is at least comparable with previous endemic years. In the last months of 2023, the number of B19V-positive donations is again increasing (figure 3). As of May 2023, 5 IUTs have been performed in 2023, of which 4 were performed in the period September – December 2023.

DISCUSSION

To our knowledge, our analysis considers the longest period of B19V epidemiology world-wide. Based on Sentinel Surveillance data from the Netherlands, our analyses revealed a strong 1-year periodicity of B19V epidemics over the period 1990-2014, and a weaker 4-year period of increased epidemic circulation superimposed on the annual epidemics over the period 1990-2009 (figure 1). This pattern of 1- and 4- year cycles has been described previously in various countries in Europe and Australia [1-4]. Interestingly, our analyses also show that these patterns have become less pronounced over the past decade, particularly the 4-year periodicity. In the pre-vaccination era, recurrent epidemics of childhood diseases such as measles, mumps, and whooping cough were also characterized by a pattern of annual epidemics and more pronounced epidemics every 2 to 5 years. These patterns have been explained by the gradual build-up of population susceptibility by demographic turnover, in particular births. A decrease in population susceptibility leads to shifts in cyclical outbreak dynamics [9-11]. This could also apply to the dynamics of B19V infections. A slowly decreasing demographic turnover as observed in the Netherlands, where the number of births has decreased from approximately 200.000 per year in 2000 to approximately 170.000 per year in the 2020s, could have contributed to the disturbance of a 4-year pattern [12].

It is remarkable that in the blood bank data and the IUT data, B19V infections were virtually absent in the period 2020-2022, while the Sentinel Surveillance system still reported infections at low levels. The Sentinel Surveillance system receives data on diagnosed infections from a network of laboratories. B19V is not a reportable disease and symptoms are generally mild; in most cases of B19V infec-

tion there is no need for laboratory diagnosis. Therefore, the amount of laboratory-proven infections will represent only a small part of all B19V-infections in the population. The definition of diagnosed B19V infection is left to the discretion of the laboratory. In practice, laboratories diagnose and report B19V infection based on positive serology and/or PCR-proven high viral loads in blood in combination with the appropriate symptomatology, if such clinical information is available. The availability of clinical information may vary between laboratories. In this system, occasional occurrence of false-positive IgM findings, leading to incorrect reports of infection, cannot be excluded. False-positive IgM would not influence trends, but could cause a slight overestimation of infections, especially during the COVID-19 pandemic. In addition, we used data on different populations, which may have differed in timing of presentation. The Sentinel Surveillance system includes only laboratory-proven infections and thus represents symptomatic individuals presenting to healthcare providers. Data from the national blood bank report only on subclinical infections (the health check before donation will exclude symptomatic patients). IUTs are performed in symptomatic fetuses when primary maternal infection will have occurred weeks before. This may create different time lags between start of circulation of (subclinical) infection and reported infections. Nonetheless, the same trends as well as the near-absence of B19V during and after the COVID-19 pandemic are confirmed by all different sources. By making use of diverse sources of population and patient data, we have attained a complete and reliable insight into B19V epidemiology.

The annual epidemic cycle for B19V has been absent for three consecutive years. Observational data show that the epidemiology of different respiratory-transmitted viruses is differentially impacted by the COVID-19 pandemic. In the first year after COVID-19 restrictions were active in most European countries, RSV epidemics showed a lag of approximately 6 months compared to previous epidemic cycles in the first year of the pandemic [6, 13-15]. Influenza followed its regular epidemic pattern, although with flattened seasonal peaks in 2021 and 2022, after an initial absence of a year [6]. B19V differs from most other respiratory-transmitted viruses such as RSV and influenza by inducing long-lasting immunity, whereas reinfections by RSV and influenza occur frequently [16, 17]. For B19V, this may result in a less susceptible population when reintroduction into the population occurs. Also, it could be hypothesized that B19V must be partially reintroduced into the population from other (transcontinental) regions, in which case it would be very interesting to

monitor genotype distribution after re-emergence of the infection. For RSV, it has been observed recently that following relaxation of COVID-19 restrictions in Australia, there has been a major collapse in circulating genomic lineages, with only 2 dominant RSV-A clades now circulating in large, distinct parts of Australia [18]. For B19V, the global genotype distribution differs with genotype 1 almost exclusively occurring in Europe and North America and genotype 3 being common in Africa, next to genotype 1 [19, 20].

In this study, we found a clear association between epidemic years and the incidence of severe intrauterine infections. This is consistent with previous studies, in which the epidemic cycles of B19V infections were reflected in the incidence of intrauterine complications, such as intrauterine death or fetal hydrops. The risk of intrauterine death was estimated at 12 per 100.000 pregnancies in nonepidemic years to 48 in epidemic years [1, 21]. Due to the absence of B19V in recent years, the number of susceptible individuals will have increased substantially. This implies that when B19V re-enters the population on a large scale, an epidemic amongst children could result in a sizeable increase in infections during pregnancy. Currently, the seasonal spring peak in 2023 as observed in this study and recently described in France and Israel, clearly indicates that the return of large-scale viral circulation has commenced [22, 23]. At the time of conclusion of this study, an ongoing rise in B19V circulation is being observed, even in winter. Awareness of the potential for B19V outbreaks in the near future, requires prompt diagnostics and monitoring, to detect infections as early as possible to prevent fetal morbidity and mortality in pregnancy. This also applies to other populations susceptible to severe B19V-infection, including all persons with underlying disorders of erythropoiesis and transplant patients.

METHODS

Data collection: Sentinel Surveillance system: national data on laboratory-proven B19V infections were obtained from the Dutch weekly Sentinel Surveillance system for the period January 1st, 1990, to December 31st, 2023. The Sentinel Surveillance system retrieves their data from a network of 21 Dutch clinical microbiological laboratories that report the absolute number of laboratory-proven B19V infections weekly to the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands). Each week, the participating laboratories extract the number of patients from their records that were diagnosed with B19V-

infection. Individual diagnosis can be made with serology, PCR (in case of high viral loads) or a combination of both. Because of this method, the Sentinel Surveillance system generates a representative view of the epidemiology of a given infection, but does not report on absolute incidence [24,25].

Dutch national blood bank (Sanquin): In compliance with European blood banking guidelines, the Sanquin Blood Supply Foundation routinely screens donated plasma for B19V DNA to exclude donations with a high B19V load before fractionation, using a procedure as described previously that did not change during the period under study [7,26]. In short, regular donations were tested in pools of 480, and donations used for production of solvent-detergent-treated plasma were screened in pools of 96. When a pool tested positive, the pool was deconstructed to identify the donation(s) containing a high load of B19V DNA (> 1.2 x10^6 IU/mL). For the period January 1st, 2013, to December 31st, 2023, the monthly number of high-load B19V DNA-containing donations and the total number of donations for each month were extracted from the Sanquin records.

IUT: All patients for whom IUT is indicated in the Netherlands are referred to the Leiden University Medical Center (LUMC). IUTs performed for fetal hydrops due to intrauterine B19V infection, as diagnosed by a positive B19V PCR on fetal blood and/or amniotic fluid, in the period January 1st, 2002, to December 31st, 2023, were included. Data on the annual number of IUT and the week in which they were performed were retracted from the obstetric records of the LUMC.

Statistical analysis: Periodicity of the data from the Sentinel Surveillance system was studied using wavelet analysis [27,28]. Main output of the analysis is a contour plot of the relative importance of periodicities (wavelet power) in the time-periodicity plane. Before analysis, we transformed the data using a Box-Cox transformation with optimized scaling parameter, to homogenize the variance and to approximate a normal distribution [29]. Throughout, we used the Morlet wavelet [30]. Significance of the periodicities was assessed using a χ^2 significance test [27]. Specifically, statistical significance was assessed by testing against the null hypothesis that observed periodicities are generated by a red noise process with the same autocorrelation coefficient as the data. The Pearson correlation coefficient for the Sentinel Surveillance data and IUT was calculated using the functions cor. test and ggscatter in the R package ggpubr. Cross-correlation between Sentinel Surveillance data and IUT was studied using the cross-correlation function (CCF). Our analyses of the cross-correlation and wavelets were implemented

in R (version 4.3.1) using the package EnvStats to estimate the optimal parameter of the Box–Cox transformation (version 2.7.0) and the package biwavelet (version 0.20.21) to perform wavelet analysis [31,32]. Data and code are available in the supplement.

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Author contributions: AR, AV, AK: conceptualisation and study design. AR, JV, MMB, HZ data collection and analysis. MB, EB: wavelet analysis. AR, MB: writing – original draft. EB, JV, MMB, HZ, AV, AK; writing - critically reviewing and editing. AV and AK; study supervision.

Data availability: Input data (S1) and code of the wavelet analysis (S2) are made available as supplementary information and can be accessed online: https://www.nature.com/articles/s41598-024-59582-7#Sec12

Conflict of interest: none.

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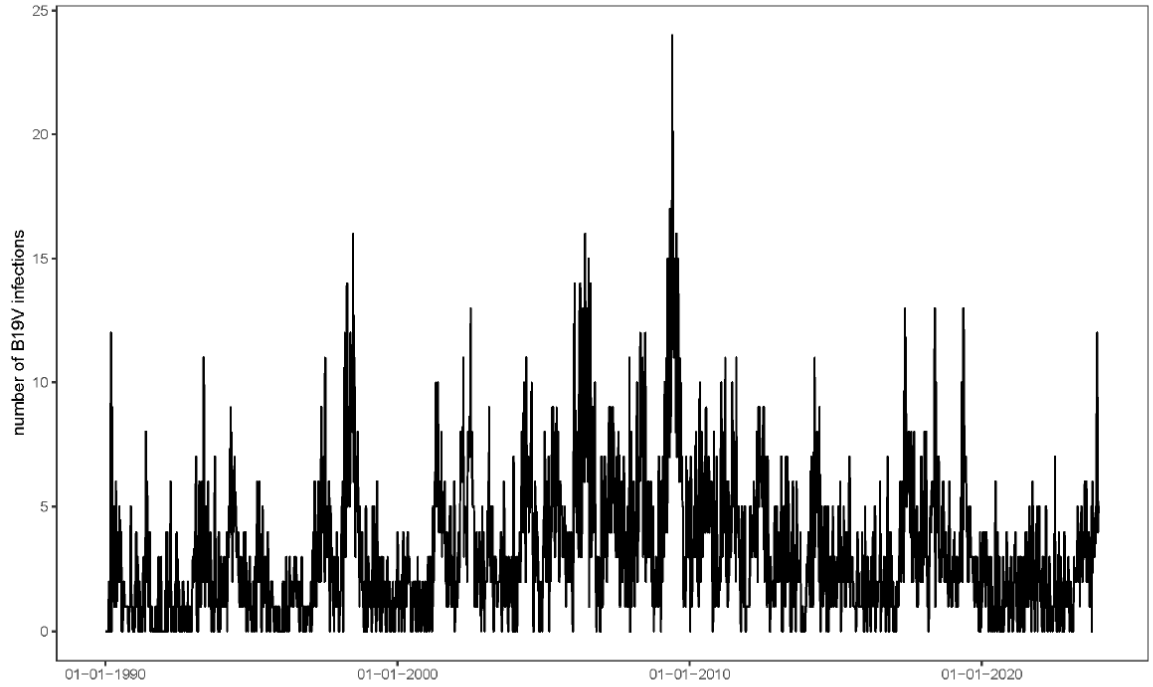
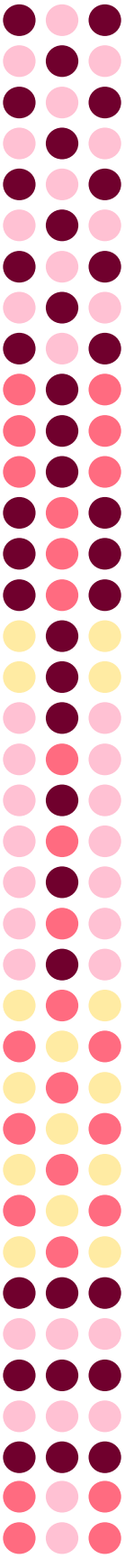
Supplementary information



Supplementary figure S1: absolute number of weekly reported B19V infections to the Sentinel Surveillance system for the period Jan 1st 1990 – Dec 31st 2023



3

Extreme upsurge of parvovirus B19 resulting in severe fetal morbidity and mortality

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At present, there is an unprecedented upsurge of cases of severe intrauterine infection with parvovirus B19 in northwestern Europe. Parvovirus B19 is well known as the causative agent of erythema infectiosum (also known as fifth disease), a common and usually self-limiting infection in school-aged children. However, when non-immune people acquire the infection during pregnancy, vertical transmission occurs in 33–51% of individuals, and this can lead to severe fetal anaemia and subsequently to life-threatening fetal hydrops [1]. When diagnosed promptly, parvovirus B19 infection in pregnancy can be managed by close monitoring of the fetus using ultrasound to look for signs of fetal anaemia. When anaemia or hydrops occurs, intrauterine transfusion can substantially reduce the risk of fetal death. Therefore, the survival rate after intrauterine transfusion for parvovirus B19 is 67–84% as opposed to 30–50% who do not receive intrauterine transfusion [2]. Intrauterine transfusion for parvovirus B19 requires specific expertise and is mostly performed in national referral centers as these fetuses are deeply anaemic or hydropic and often also suffer from thrombocytopaenia. Timely diagnosis is challenging, as parvovirus B19 infection often is asymptomatic in pregnant people (in 30–50% of cases) or might present as a non-specific febrile illness, which is accompanied by a rash or arthropathy in 30–40% of cases [3].

We report the number of first intrauterine transfusions for fetal anaemia due to proven parvovirus B19 performed in a large part of Northwestern Europe (Leiden, Netherlands; Leuven, Belgium; and Paris, France) since 2010 (figure A). These referral centers for intrauterine transfusion account for at least two thirds of all intrauterine transfusion in these countries, and therefore are a good reflection of intrauterine transfusion activity in this part of Europe. The total number of intrauterine transfusion in the first months of 2024 has already surpassed the annual maximum amount of intrauterine transfusion ever performed in all participating referral centers. Figure B shows the monthly amount of intrauterine transfusion from June 1, 2023 to June 1, 2024. In total, 59 fetuses in this region have been treated with at least one intrauterine transfusion during the current upsurge since Jan 1, 2023, with 44 fetuses receiving intrauterine transfusion since Sept 1, 2023. Of these 59 fetuses, 21 (36%) had adverse outcomes (13 [22%] perinatal death, four [7%] termination of pregnancy due to severe fetal anomalies, and four [7%] ongoing pregnancies with persistent hydrops or severe cerebral anomalies), while the definite outcome is not yet known for 19 pregnancies. Gestational age at first intrauterine transfusion did not differ significantly for the period of Jan 1, 2023, to

June 1, 2024 (mean 21.5 weeks, SD 3.2) compared to Jan 1, 2010, to Dec 31, 2022 (22.4, 2.9; p=0·087). However, gestational age at first intrauterine transfusion was significantly higher in the group with adverse outcomes (22.8, 3.5) compared with the group without adverse outcomes (20.8, 2.8; p=0·010) in the period of Jan 1, 2023, to June 1, 2024.

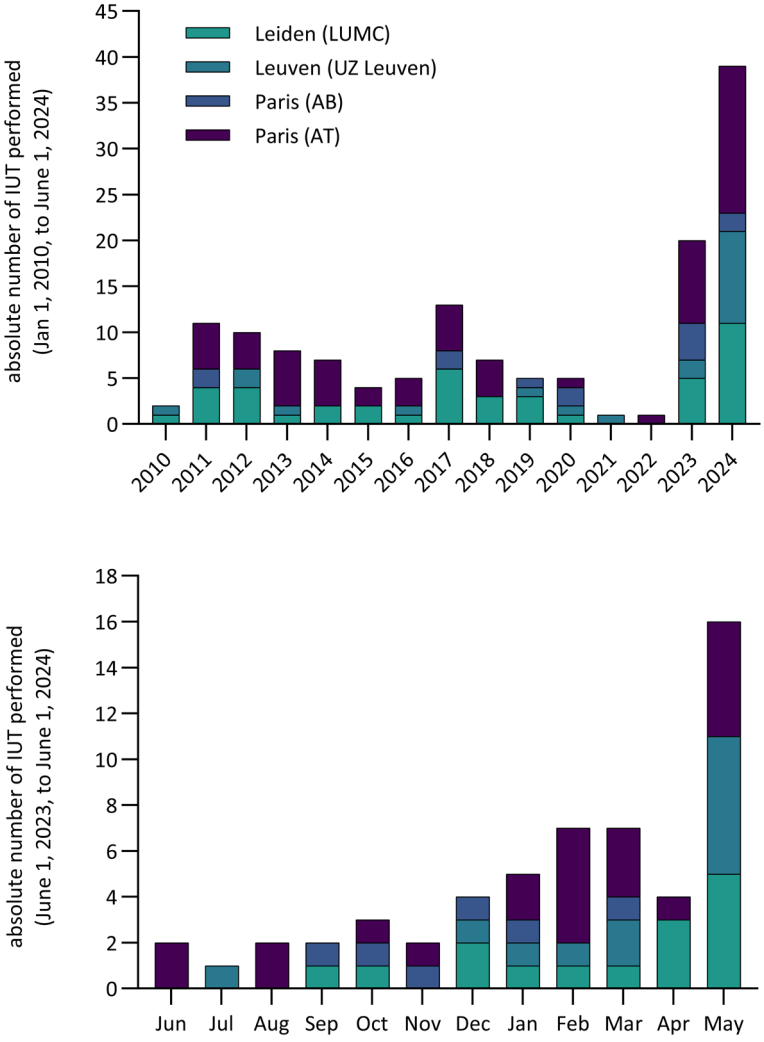

Figure 1: Intrauterine transfusion in Northwestern Europe*. Data are up to June 1, 2024. (A) Annual amount of intrauterine transfusion for parvovirus B19 infection in Leiden, Netherlands; Leuven, Belgium; and Paris, France. The chosen areas represent approximately two-thirds of all intrauterine transfusion activity combined in these countries. (B) Monthly amount of intrauterine transfusion from June 1, 2023, to June 1, 2024. AT=Armand-Trousseau hospital. AB=Antoine Béclère hospital. LUMC=Leiden University Medical Center. UZ Leuven=University Hospitals Leuven.*

Recent research [4] shows that the traditional pattern of annual parvovirus B19 epidemics in late spring with superimposed multi-annual epidemics has become disturbed, with local disappearance of multi-annual and annual epidemics since 2014 and the virtual absence of parvovirus B19 infections during and after the COVID-19 pandemic [4]. The resulting increase in susceptible host populations will have led to the large-scale parvovirus B19 epidemic we witness at present, a rebound effect similar to post-pandemic surges of other, also primarily respiratory transmitted, infectious diseases. The proportion of parvovirus B19 fetal cases with adverse outcomes is larger than expected based on existing literature and previous experiences. At present, genotyping of circulating strains does not suggest altered strains with altered virulence [5]. Although gestational age at first intrauterine transfusion did not differ between 2010–22 and the current epidemic in 2023–24, we observed that gestational age at first intrauterine transfusion was significantly higher in the group with adverse outcomes. The considerable proportion of adverse outcomes therefore might be explained by suboptimal awareness for parvovirus B19 infections in general health care or obstetric care, possibly due to its prolonged absence, resulting in late referral and increased risk of adverse outcomes. Considering the ongoing epidemic, we urgently recommend vigilance for parvovirus B19 infections in pregnant people. At present, the threshold of performing a serological test for parvovirus B19 should be low, including (1) pregnant people with febrile illness, exanthema, or painful or swollen joints; (2) pregnant people who have been in contact with an individual with parvovirus B19, and (3) pregnant people with reduced fetal movements or fetal hydrops at routine ultrasound examination. Whether national screening programmes for parvovirus B19 in pregnancy are warranted, particularly in severe epidemic years, is a matter for further study. As pregnant people might present in different stages of parvovirus B19 infection, interpretation of diagnostic tests should be done with care together with a virologist. Early consultation with a fetal medicine specialist is recommended when parvovirus B19 infection in pregnancy is suspected. In the case of a confirmed parvovirus B19 infection, referral to a fetal medicine specialist is indicated for advanced ultrasound examination and frequent follow-up.

We declare no competing interests.

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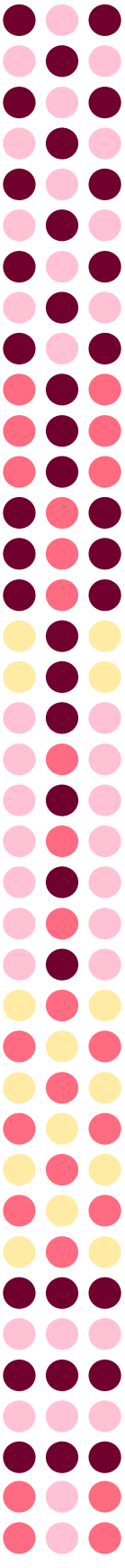

4

Diagnosis of intrauterine parvovirus B19 infection at birth – value of DNA detection in neonatal blood and dried blood spots

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ABSTRACT

Background: Diagnosis of congenital viral infection at birth is generally attempted by direct detection of the virus by PCR in various neonatal materials. How to reliably diagnose intrauterine infection with parvovirus B19 (B19V) at birth is unknown.

Objectives: To evaluate the performance of B19V DNA detection in cord blood (CB) or neonatal dried blood spots (DBS) in diagnosing fetal infection.

Study design: Two cohorts of children diagnosed prenatally with an intrauterine B19V infection were included in this study. CB samples of intrauterine B19V infections that were sent to a reference laboratory for congenital infections in Stuttgart, Germany in the period 1995-2014 were tested in triplicate for B19V DNA by quantitative PCR. DBS from children with intrauterine B19V infection that underwent IUT at the LUMC, Leiden, the Netherlands in the period 2009-2014 were tested for B19V DNA by quantitative B19V PCR in triplicate.

Results: Fourteen of twenty (70%) CB samples tested positive for B19V DNA. The positivity rate was 40% (4/10) in those with a prenatal diagnosis <20 weeks gestation. When intrauterine B19V infection was diagnosed thereafter, 100% (10/10) samples were B19V DNA positive. Of the thirteen available DBS, twelve (92 %) tested positive. Viral load in CB and DBS corresponded inversely with time from fetal diagnosis to birth.

Conclusion: B19V DNA can be detected in neonatal blood samples of children following intrauterine B19V infection, although the possibility of false-negatives, even in severe infections, should be considered. B19V viral load at birth correlates with timing of infection.

BACKGROUND

Parvovirus B19 (B19V) is a ubiquitous human pathogen, evident from the seroprevalence of 50-70% in adults [1,2]. B19V infects erythrocyte progenitor cells and generally causes only a mild illness in immunocompetent individuals. In pregnancy however, infection can lead to fetal hydrops and fetal death due to severe fetal anemia. Vertical transmission occurs in 40-51% of infections in pregnancy [3,4] and risk of fetal hydrops in maternal infection is estimated at 3.9-11.9% with a peak incidence between 17-24 weeks of gestation [3-5]. The overall risk of fetal death in maternal infection is 6.4-6.9% [3-5], where fetal death is a rarity when infection occurs after 20 weeks of gestation. Over the past decades, correction of fetal anemia in hydropic foetuses by intrauterine erythrocyte transfusion (IUT) has considerably improved the fetal survival rate, from an estimated 50% or less to 75-84.6% [5-8]. It is estimated that the incidence of B19V infection during pregnancy is 1% in endemic periods and may increase up to 10% in epidemic periods, which occur every 4-5 years [9]. However, actual data on how many fetuses experience intrauterine B19V infection are lacking. Maternal infection may go unnoticed. Severe cases of fetal anemia may be diagnosed at the 20-week ultrasound, offered to all pregnant women in many European countries. Less severe or subclinical cases might be missed, and even mild fetal hydrops can resolve spontaneously [4].

Until recently, long term outcome after IUT was thought to be unaffected [10-12]. More recent data however show that neurodevelopmental impairment occurs more frequently in children with intrauterine B19V infection compared with the general population [7,13]. Being able to diagnose an intrauterine B19V infection after birth might become valuable in children with developmental disorders or in assessing disease burden of B19V in an epidemiological context.

PCR techniques have been used previously to determine B19V prevalence in neonatal blood. The reported vertical transmission rates of 40-51% are partly based on the results of PCR on neonatal blood [3,4]. Also, the role of B19V in other disorders has been researched with the use of neonatal DBS [14,15]. The assumption that neonatal blood is suitable for the postnatal diagnosis of intrauterine infection is reasonable, as B19V DNA can be commonly detected in blood for months after infection in children and adults [16-21] and cytomegalovirus can also be reliably detected in postnatal blood in case of congenital infection [22], but its suitability has not yet been evaluated.

Objectives: This study researched the value of CB and DBS as means to diagnose intrauterine infection with B19V after birth.

STUDY DESIGN

Study populations

Postnatal material was collected from 2 cohorts with intrauterine B19V infection, proven by PCR on fetal blood or amniotic fluid. One cohort consisted of patients who underwent invasive prenatal diagnosis (amniocentesis and/or fetal blood sampling) in various fetal medicine centers in Germany in the period 1995-2014. Cord blood, collected shortly after birth, was sent to the laboratory in Stuttgart, Germany. The other cohort consisted of patients who underwent IUT in the period 2009-2014 at the Leiden University Medical Center (LUMC), the Netherlands. Informed consent was obtained from parents to retrieve the DBS, collected in the first week after birth as part of the national screening programme for metabolic and endocrine disease. DBS were subsequently obtained from the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands) where they are stored for five years after birth. To test performance in the general population, for every DBS of a child with proven infection, 5 control DBS from Dutch children out of the general population were requested from the RIVM, matched for year and region of birth.

DNA extraction and B19V quantitative PCR

CB and DBS: All CB and DBS samples were tested at the LUMC, Leiden, the Netherlands. For CB, automated DNA extraction was performed on a MagNA Pure 96 instrument (Roche Diagnostics, Rotkreuz, Switzerland) using the MagNA Pure 96 DNA and viral NA small volume kit, according to the manufacturer's instructions. Extraction efficiency and PCR inhibition was controlled internally by adding a fixed concentration of an internal control (phocine herpesvirus DNA) to the lysis buffer. Samples were subsequently tested with an in-house PCR in triplicate as described below.

DNA extraction from DBS was performed by spin columns as described previously [15]. Briefly; one entire DBS was punched, corresponding with approximately 50 µL of whole blood. The individual DBS punch was subsequently incubated with lysis buffer and proteinase K and an internal control DNA (phocine herpes virus) was added. After adding ethanol 96-100% and after centrifugation, the

supernatant was applied to a spin column (QIAamp DNA mini blood kit, Qiagen, Hilden, Germany) and DNA extraction was performed according to the manufacturer's instructions. The eluate was directly used for real-time PCR. Quantitative B19V PCR on CB and DBS was performed by means of an internally controlled in-house real-time PCR targeting the NS region of the genome as described by Knoester [23]. Quantification was performed using the WHO international standard (code 99/800, NIBSC, Potters Bar, UK). For optimal sensitivity, PCR was performed in triplicate for each eluate of CB and DBS as most viral loads were expected to be low.

Fetal blood samples: Due to small volumes, fetal plasma at time of IUT was not available for quantitative re-testing to assess exact viral load at time of fetal diagnosis. For CB, viral loads as measured at time of fetal diagnosis are reported. For DBS, viral loads in fetal blood were estimated based on PCR cycle threshold (Ct) values measured at time of diagnosis, using the standard curve of the WHO international standard.

B19V Viral load calculation in CB and DBS:

If only one triplicate tested positive, PCR was repeated. It was only considered truly positive if repeat PCR was also positive. If one or two out of the three measurements in the triplicate were positive we assumed B19V DNA was present in the entire triplicate however with such a low viral load that it could not consistently be detected, as values would probably lie around the limit of detection. To not underestimate the mean viral load of the DBS, we used a method to correct for negatives around the limit of detection. Mean viral loads in DBS were thus calculated as described by Croghan et al [24], with minor modifications. Firstly, limit of detection (LOD) of the particular triplicate was defined as follows: if two out of three measurements were positive then LOD was considered the mean of the two. If only one triplicate was positive, this value was considered the LOD. Secondly, the negative value(s) were imputed by substituting the negative value with $LOD/\sqrt{2}$. In case of 2 negative measurements within a triplicate, the mean of the entire triplicate was calculated as $(LOD/\sqrt{2} + LOD/\sqrt{2} + LOD)/3$. In case of 1 negative measurement within a triplicate the mean of the triplicate was calculated as $(LOD/\sqrt{2} + LOD + LOD)/3$.

Statistical analysis: The data were analysed using IBM SPSS Statistics 25. Confidence-intervals for sensitivity and specificity were calculated using the Clopper-Pearson method. Correlation between lapse of time and viral load was calculated using the Pearson correlation coefficient. Positivity rates were compared using Fisher's exact test.

RESULTS

CB and DBS: Table 1 describes the characteristics of the German and Dutch cohorts and the result of the quantitative B19V PCR on CB and DBS respectively. In the German cohort invasive prenatal diagnosis and/or IUT's were performed at a median gestational age (GA) of 20 weeks (range 14-33) and median hemoglobulin level was 3.8 g/dL (range: 1.7-12.1). Seventy percent (14/20) of CB samples tested positive. The positivity rate was 40% (4/10) in those with a prenatal B19V diagnosis at <20 weeks gestation. When intrauterine B19V infection was diagnosed thereafter, all CB samples (10/10) were B19V DNA positive.

Table 1. Clinical characteristics and test results in umbilical cord blood (CB) and dried blood spots (DBS) at birth of B19V-positive fetuses

child	GA* at PD† (weeks)	GA at birth‡ (weeks)	Prenatal diagnosis				IUT**	Number of positive triplicates	Mean VL (IU/mL) in triplicate	Clinical interpretation of test result
			Sample type§	VL ¶ (IU/mL)	Hb¦ (g/dL)	Abnormal ultrasound/Doppler findings				
CB										
1	14	42	AF	$> 10^7$	unknown	none	no	1	2.0×10^2	neg††
2	15	39	AF	10^6	unknown	unknown	no	0	0	neg
3	16	40	AF	$>10^7$	unknown	MCA‡‡ with increased PSV§§	no	3	5.1×10^2	pos
4	16	37	AF	10^6	unknown	increased NT¶¶	no	0	0	neg
5	17	38	FB	$>10^7$	2.8	MCA with increased PSV	yes	3	2.5×10^2	pos
6	17	38	FB	$>10^7$	10.6	MCA with increased PSV	no	2	1.5×10^2	pos
7	18	40	FB	$>10^7$	9	MCA with increased PSV	yes	0	0	neg
8	19	40	FB	$>10^7$	4.8	MCA with increased PSV	yes	0	0	neg
9	19	42	FB	10^5	2.8	fetal hydrops	yes	2	3.2×10^2	pos
10	19	42	FB	$>10^7$	2.4	fetal hydrops	yes	0	0	neg
11	21	40	FB	$>10^7$	4	fetal hydrops	yes	2	1.5×10^2	pos
12	21	38	FB	$>10^7$	3.5	fetal hydrops	yes	3	2.4×10^2	pos
13	23	36	AF	$>10^7$	1.7	fetal hydrops	yes	3	2.9×10^3	pos
14	24	37	AF	10^6	unknown	none	no	2	5.4×10^2	pos
15	25	38	AF	10^5	unknown	pericardial effusion	no	3	3.3×10^2	pos
16	26	38	AF	10^5	unknown	none	no	3	1.7×10^3	pos
17	26	40	FB	10^5	unknown	none	no	3	3.4×10^2	pos
18	28	38	FB	$>10^7$	unknown	fetal hydrops	yes	3	1.8×10^3	pos
19	32	38	AF	10^4	unknown	unknown	no	3	1.3×10^5	pos
20	33	38	FB	10^5	12.1	MCA with increased PSV	no	3	3.4×10^2	pos

DBS										
1	19	40	AF	10^6	3.2	fetal hydrops	yes	2	1.5×10^1	pos
2	20	39	unknown	unknown	1.9	fetal hydrops	yes	1	1.0×10^1	pos
3	20	41	AF	10^6	4.0	fetal hydrops	yes	2	2.6×10^2	pos
4	20	39	FB	10^9	2.3	fetal hydrops	yes	1	2.2×10^2	neg††
5	20	37	AF	10^8	5.0	fetal hydrops	yes	3	9.2×10^2	pos
6	20	41	FB	10^9	1.8	fetal hydrops	yes	2	2.5×10^2	pos
7	21	39	FB	10^9	2.4	fetal hydrops	yes	3	1.0×10^3	pos
8‖	21, 22	36	AF	10^6	2.6, 4.0	fetal hydrops	yes	3	9.9×10^3	pos
9	21	37	FB	10^{10}	3.9	fetal hydrops	yes	3	3.3×10^3	pos
10	22	41	AF	10^6	2.7	fetal hydrops	yes	2	2.0×10^2	pos
11	23	37	AF	10^6	6.1	fetal hydrops	yes	3	1.5×10^3	pos
12	27	38	AF	10^6	1.9	fetal hydrops	yes	3	2.7×10^5	pos
13‖	29, 30	38	FB	10^{10}	4.3, 8.0	fetal hydrops	yes	3	2.8×10^5	pos

*Legend to table 1: *GA = gestational age, †PD = prenatal diagnosis, ‡birth is taken as indicator of time of CB/DBS sampling, §AF = amniotic fluid, FB = fetal blood, ¶ VL = viral load, ¦Hb = hemoglobulin, **IUT = intrauterine transfusion, †† the triplicate is considered negative as repeat analysis could not confirm the positive finding (see methods), ‡‡MCA = middle cerebral artery, §§ PSV = peak systolic velocity, ¶¶ NT = nuchal translucency, ‖ these children received a second IUT*

Of 21 children who underwent IUT at the LUMC, informed consent was obtained to test the DBS of 13 children. Two children received two IUT's. First IUT's were performed at a median GA of 21 weeks (range: 20-29 weeks) and fetuses had a median hemoglobulin level of 3.0 g/dL (range: 1.8-6.1) at the time of IUT. Ninety-two percent (12/13) DBS tested positive for B19V.

When all postnatal samples are pooled together, sensitivity of B19V DNA detection for diagnosing intrauterine B19V infection was 79% (95 CI 61.0 - 91.0%). Positivity rate in samples from children with an IUT performed before 20 weeks gestation was 45% (5/11), in cases with an IUT after 20 weeks positivity rate was 95% (21/22; p-value 0.003). Fetuses not treated with IUT had a positivity rate of 73% (8/11) in postnatal samples, fetuses who underwent IUT had a positivity rate of 78% (18/22; p-value 0.66). The inverse correlation of the time-span between diagnosis of intrauterine infection and postnatal sampling on the one hand and the postnatal B19V viral load on the other is depicted in figure 1 (R-squared: 0.51, p-value < 0.001).

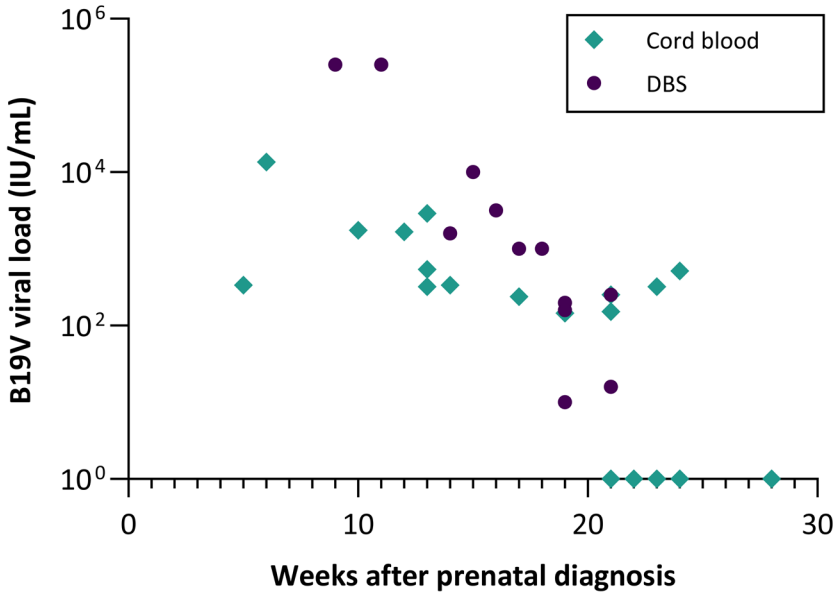

***Figure 1**. Viral load in CB and DBS according to weeks after prenatal diagnosis. Green diamonds represent cord blood, purple dots represent dried blood spots.*

Sixty-three out of 65 DBS from the general Dutch population tested negative in all triplicates for B19V. One child tested positive in all three triplicates (mean viral load 2.2 x 10^2 IU/mL) and one child tested positive in one triplicate, but negative in all three triplicates upon repeated testing. When this one is considered false-positive, specificity of the PCR is 98.4% (95% CI 91.7-100%).

Fetal plasma: For five fetuses in the DBS cohort, fetal plasma viral load was estimated by applying the WHO international standard curve to previous Ct-values. The estimated mean fetal plasma load at time of IUT was 7.2 x 10^{10} IU/mL (range: 2.0 x10^9 – 7.9 x 10^{10}). Viral load in DBS is dependent on height of viral load in fetal blood samples and on lapse of time since IUT, with an estimated detectable B19V DNA half-life of five days as demonstrated in figure 2.

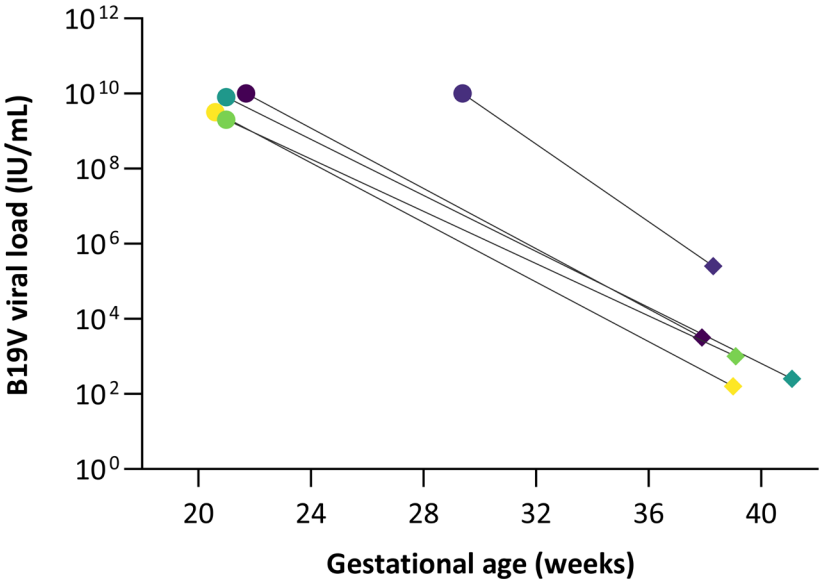

Figure 2. *Viral load in fetal plasma and DBS according to gestational age. Dots represent loads in fetal plasma; the newborn's corresponding load in DBS is indicated by diamonds in the corresponding colour.*

DISCUSSION

In this study we demonstrate the feasibility of detecting an intrauterine B19V infection after birth by real-time PCR in postnatal blood samples, both in CB and DBS. Viral loads in neonatal blood were low and often around the limit of detection, as demonstrated by the fact that triplicates were not consistently positive in the PCR. The low viral loads and inconsistent positivity of triplicates might make distinction from false positives, e.g. from PCR contamination, difficult. A first application in the general population already yielded 3% (2/65) positive DBS, of which one can probably be interpreted as a true positive considering its consistent positivity in all triplicates, but the other DBS is more difficult to interpret. As DBS from the general population were tested anonymously it was not possible to verify clinical information on these cases. This study shows that validation before implementation is important as high sensitivity cannot be assumed and false negatives or weak positives do occur. Furthermore, positive results may also be found in a control population.

In adults, B19V DNA persists in peripheral blood for months to even years after infection [16-19]. It is remarkable that samples of proven fetal infections – even in cases with severe hydrops - have such low viral loads, or remain negative, 5 to 6 months after infection. For eventual clearance of parvovirus B19 infection, immunity is essential. From the second trimester onwards, the immune system of the fetus is functioning but it is still immature. Even with the addition of vertically transmitted maternal IgG, which is considered to be most important in clearance of B19V infection [25], it is unlikely that fetuses clear B19V infection more adequately than otherwise healthy adults. It should be taken into account that the absolute blood volume (as gross indicator of the number of erythrocytes) of a 4-kg newborn is around 300 mL, while adults have a mean blood volume of 5 L [26]. Also, neonatal erythrocytes of term infants have a life span of 60 to 90 days compared to 120 days in adults [27]. Time to clearance of DNA in peripheral blood is affected both by the lower blood volume and the lower half-life; possibly resulting in faster clearance in fetuses and newborns.

The postnatal detection of intrauterine B19V infection may serve two goals. Retrospective diagnosis of infection may be used in case of postnatal clinical suspicion of an intrauterine infection. Recent research has shown that developmental delay might occur in children with intrauterine B19 infection [8,9,28]. However, it is still unclear whether this is the result of the B19V infection itself or the result

of severe fetal anemia. Furthermore, the number of children with an intrauterine infection without adverse fetal outcome may be considerable compared to those with adverse outcome. Therefore, it will be challenging to interpret a PCR-positive result in neonatal blood in children with developmental delay. Another goal of diagnosing intrauterine B19V infection after birth is the assessment of the prevalence and the disease burden of fetal infection with B19V. As mentioned previously, there are no data yet on the number of intrauterine infections.

Our study has limitations. Many children in our study underwent IUT. The effect of IUT on fetal viral load is unknown. In our limited dataset, IUT did not influence the positivity rate of postnatal diagnosis. However, supplementing erythrocytes causes a temporary decrease in erythropoiesis [29], possibly resulting in a decrease in viral replication as less erythrocyte progenitor cells are available to the virus and thus, possibly resulting in a reduction of the postnatal viral load. Consequently, higher postnatal viral loads might occur in patients with no or mild anemia who are not treated with IUT. On the other hand, children with severe anemia might be expected to represent the population with highest viral loads, although previous research in transfused children did not establish a relation between viral load and severity of anemia [30].

In conclusion, we demonstrated the feasibility of diagnosing intrauterine B19V infection after birth by testing CB and DBS from newborns. This new application of B19V PCR creates a widened timeframe for the diagnosis of intrauterine B19V infection in the individual and it opens possibilities for epidemiological studies to improve insight in the prevalence and disease burden of intrauterine B19V infection. Nevertheless, sensitivity is rather low when infection takes place in early pregnancy. The possibility of false negative results in postnatal samples, even in severe infection, should be considered.

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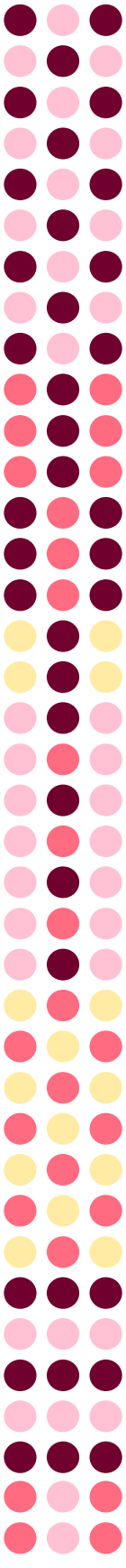

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Detection of parvovirus B19 DNA in blood: viruses or DNA remnants?

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ABSTRACT

Background: Parvovirus B19 (B19V) DNA can be detected in blood over a long period after acute infection. Several reports associate the presence of B19V DNA with disease, irrespective of timing of the initial B19V infection.

Objectives: This study aims to analyze the properties of B19V DNA in blood, in order to differentiate between bare, non-infectious strands of DNA and B19V DNA in viable virions.

Study design*:* Ten blood donors with asymptomatic acute B19V infection were followed and sampled up to 22 months after infection. The samples were treated with and without an endonuclease and tested for B19V DNA, to distinguish between DNA in virions and naked DNA.

Findings*:* In the acute phase of infection, high levels of B19V DNA (> 10^6 IU/mL) were detected, concurrent with B19V IgM antibodies. B19V DNA apparently was encapsidated, as indicated by resistance to endonuclease degradation. Subsequently, B19V DNA was detectable for more than one year in all donors at low levels (< 10^5 IU/mL). Approximately 150 days after infection B19V DNA became degradable by an endonuclease, indicating that this concerned naked DNA. In some donors a second endonuclease-resistant peak occurred.

Discussion*:* Detection of B19V DNA in blood by PCR does not necessarily imply that B19V replication takes place and that infectious B19V virions are present. We propose that remnant B19V DNA strands can be released from tissues without active replication. This finding urges to reconsider an assumed role of B19V infection based on B19V DNA detection in blood, a much debated subject in clinical syndromes such as myocarditis and arthritis.

1. BACKGROUND

Parvovirus B19 (B19V) is a small, nonenveloped, single-stranded DNA virus, replicating primarily in erythroid progenitor cells in the bone marrow. B19V infections are widespread in the human population and transmission predominantly takes place by respiratory droplets. During acute B19V infection a short-lived exceptionally high peak of B19V DNA occurs in peripheral blood (> 10^{12} IU/mL) [1]. During a much longer period following acute infection low levels of B19V DNA (10^{1}-10^{4} IU/mL) may be detected in the blood of immunocompetent individuals, lasting for months or years, long after the anti-B19V IgG response has developed [2-4]. B19V DNA is lifelong detectable in various tissues of immunocompetent adults testing positive for B19V antibodies, such as skin, synovia, tonsils, liver and heart and even in 70-year-old bone remnants [5-7].

Since normally no B19V DNA is detectable in blood, considerable attention was given in the last two decades to the frequent detection of parvovirus B19V DNA in blood and tissues. This has triggered interest in the role of B19V in several clinical conditions, particularly in a number of cardiac disorders, but also in chronic arthropathy and liver failure [8,9]. In many reports, a causal relationship between the detection of viral DNA in blood or tissue and the clinical disorder was considered likely [10, 11]. In this way, parvovirus is often classified among the causes of myocarditis and cardiomyopathy [11-13]. On the other hand, based on the absence of further support by serology, clinical signs and epidemiological evidence, several reports remained cautious about the presumed causal relationship [14-17]. In addition, the persistence of viral DNA on the tissue level was considered an alternative source for the release of viral DNA, suggesting that tissue damage could lead to passive release of B19V DNA [6,18,19]. Most likely, in some cases passive release of viral DNA explains its occurrence in blood, while in other cases active viral infection is responsible. Since no further analysis is available which could differentiate between these conditions, divergent views on the relevance of B19V viremia still coexist in literature.

To establish the nature of parvovirus DNA in a patient is a matter of theoretical and practical importance. Passively released viral DNA can be interpreted as causally irrelevant and non-infectious. Viral DNA produced by viral replication indicates active infection and infectivity. This differentiation is relevant for transfusion safety, transplantation purposes and disease management. The availability of a simple test determining the nature of B19V DNA in various conditions would be valuable to interpret the presence of B19V DNA correctly.

2. OBJECTIVES

This study explored potential differences in the properties of parvovirus DNA as detected in different settings: immediately during an established acute infection, and in later stages. The differentiation of B19V DNA as present in infectious viral particles and B19V DNA as bare fragments, released from various cell types, is attempted by determining the effects of a nuclease treatment on samples.

3. STUDY DESIGN

3.1. Blood samples

Ten series of follow-up samples during and after acute B19V infection were obtained as follows. Dutch plasmapheresis donors, who donate frequently, are routinely screened for B19V DNA. Ten plasmapheresis donors, who tested strongly positive for B19V DNA (> 10^6 IU/mL), were selected. Follow-up samples from their routine blood donations were obtained from the blood bank repository. All donors agreed that (a part of) their donation could be used for research. The follow-up samples cover a period of 11-22 months after the index donation. In addition, a sample just before the index donation was obtained from the repository. The ten series of donor samples cover an average period of 18 months, including on average 10 serial samples, with an average inter-donation interval of 62 days. In addition to the 10 donor series, 18 serial samples from a technician in the laboratory who experienced B19V infection were available, covering a period of 25 months.

3.2 Endonuclease treatment and dual target B19V PCR

To investigate whether detected B19V DNA concerns packaged DNA in B19V virions, or whether it is naked, free B19V DNA, a treatment step using Benzonase® (Sigma-Aldrich, The Netherlands) was developed. Benzonase® is an endonuclease which can degrade all forms of DNA and RNA, including 'naked', unpacked B19V DNA, but not the B19V DNA in virions [20,21]. The optimized treatment for EDTA plasma samples was as follows: To 210 µL EDTA-plasma 3.2 µL 1M $MgCl_2$ was added to obtain a final concentration of 15 mM $MgCl_2$. This extra $MgCl_2$ is necessary for the endonuclease to have an optimal activity in EDTA-plasma. Subsequently, the sample was split in 2x 100 µL, and to one of the 100 µL samples 250 Units endonuclease was added. The other 100 µL sample was not treated with endonuclease, to determine the effect of the endonuclease treatment. Both samples were incubated in a shaking incubator at 37°C at 120 rpm for 1 hour. Next the samples

were allowed to cool to room temperature and B19V DNA PCR procedure was performed (see below). In each experiment a negative control and 2 positive controls (naked, extracted B19V DNA and B19V DNA in virions, obtained from an acute B19V infection [load 2 x 10^{13} IU/mL without B19V IgM and IgG]) were tested with and without endonuclease to determine the efficiency of the endonuclease treatment and to control the B19V PCR. B19V DNA was extracted from the samples and controls and the extracts were used for dual target NS1-VP2 B19V PCR, this entire procedure was described previously[22].

3.3. IgM and IgG antibodies to B19V

All serial samples were tested for B19V IgM and IgG antibodies using the Biotrin Parvovirus B19 IgM and IgG Enzyme Immunoassays (DiaSorin, Ireland), following the manufacturer's instructions, with the adaptation that IgM values >1.5 were considered positive and IgM values between 0.9 and 1.5 were considered equivocal.

3.4. Estimation of viremic and IgM positive period

An estimate of the viremic and IgM positive period was made as described previously[23]. Briefly, the median proven duration of viremia (endonuclease resistance) was calculated from all blood donors with more than one B19 endonuclease resistant donation. To this calculated interval 0.5 times the median interval between the last negative and first positive donation and 0.5 times the median duration between last positive and first negative donation were added. In addition this was also performed for proven IgM-positive period with donors which had more than 1 positive IgM time point.

RESULTS

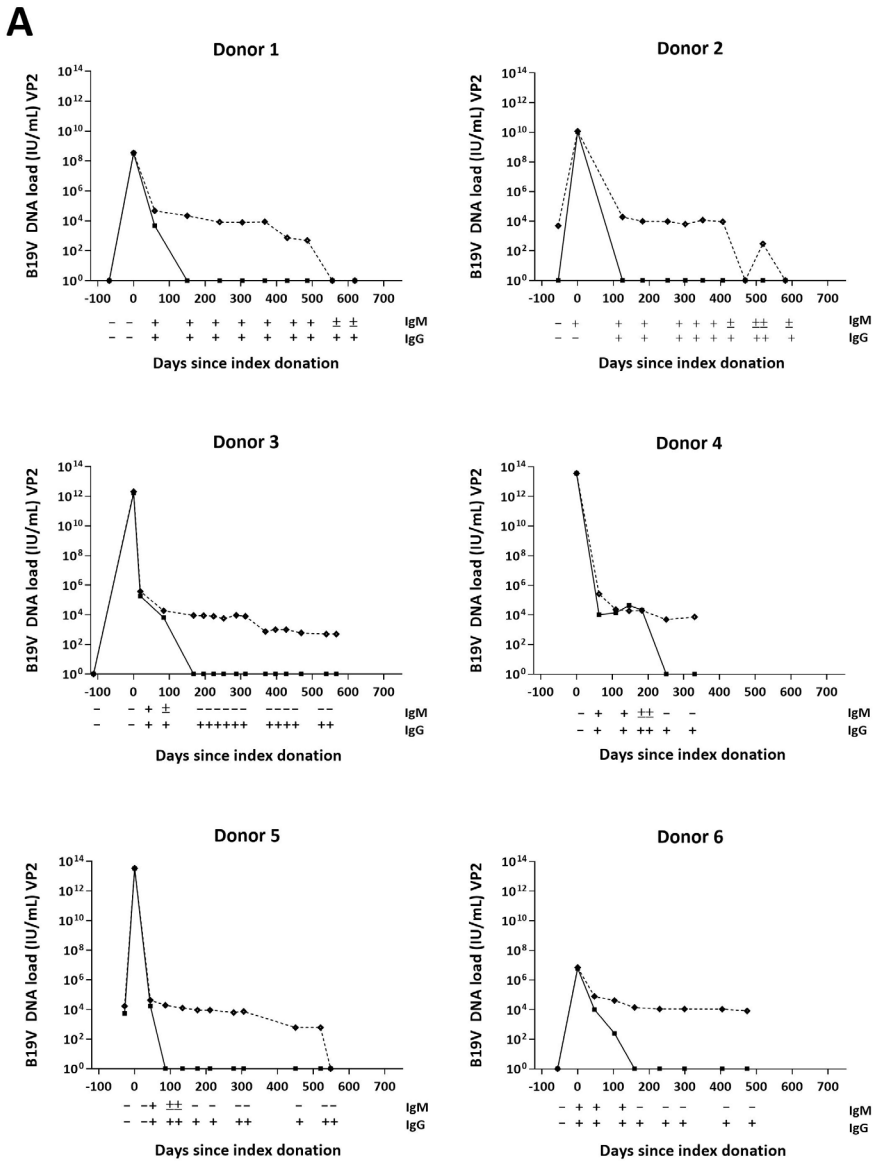
4.1. Effect of endonuclease treatment

There was no reduction observed by endonuclease treatment in any of the 11 high level peaks of acute B19V infections. However, there was a strong reduction, of minimal 4 log10, of B19V DNA levels during later phases of infection and in naked DNA controls.

4.2. Course of B19V DNA levels

The serial samples obtained from 10 B19V infected plasma donors and the B19V infected technician showed the following pattern (see Fig. 1):

1) In the 10 donors the donation before the 'acute' index donation tested negative for B19V DNA and for B19V IgM and IgG antibodies.
2) The index donations showed high B19V DNA levels (>1x10^6 IU/mL) and often these were still negative for B19V IgM (7/10) and B19V IgG (9/10). In the index donations the B19V DNA level was not sensitive to endonuclease treatment, which is compatible with an encapsidated state of B19V DNA in viral particles.
3) After the index donation the samples showed significant lower levels of B19V DNA (10^3-10^4 IU/mL) during several months. Most donors (7/10) were still B19V DNA positive in the last available sample. However, 150 days following the index donation most samples were 100% sensitive to endonuclease, suggesting that B19V DNA in these samples is no longer encapsidated, but 'naked', non-infectious B19V DNA. The median B19V viremic (endonuclease resistant) period was estimated at 137 days. The median B19V DNAemic period could not be estimated since most donors were still positive for B19V DNA in their last donation.
4) All samples following the index donation tested B19V IgG positive, and IgM antibodies gradually disappeared in most donors. The disappearance of B19V IgM seemed to correlate with the transition from B19V virions to naked B19V DNA. The median time of IgM presence was estimated at 142 days. In two donors no IgM seroconversion was detected. This was probably due to the fact that the time between the index donation and subsequent donation was too long (68 and 122 days) to detect IgM.
5) Strikingly, three donors and the technician showed a second endonuclease-resistant B19V DNA peak, on average 10 months after acute infection (see Fig. 1B). This second peak seemed to reflect a temporary return of B19V virions. The presence of an endonuclease-inhibiting factor was ruled out by adding Parvovirus 4 (PARV4) DNA extract in these samples, which was subsequently degraded by endonuclease. In addition, adding a higher concentration of endonuclease (1250 and 2500 units) did not degrade the B19V DNA in the second peak either.


A
Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
Donor 6
B19V DNA load (IU/mL) VP2
Days since index donation
IgM
IgG

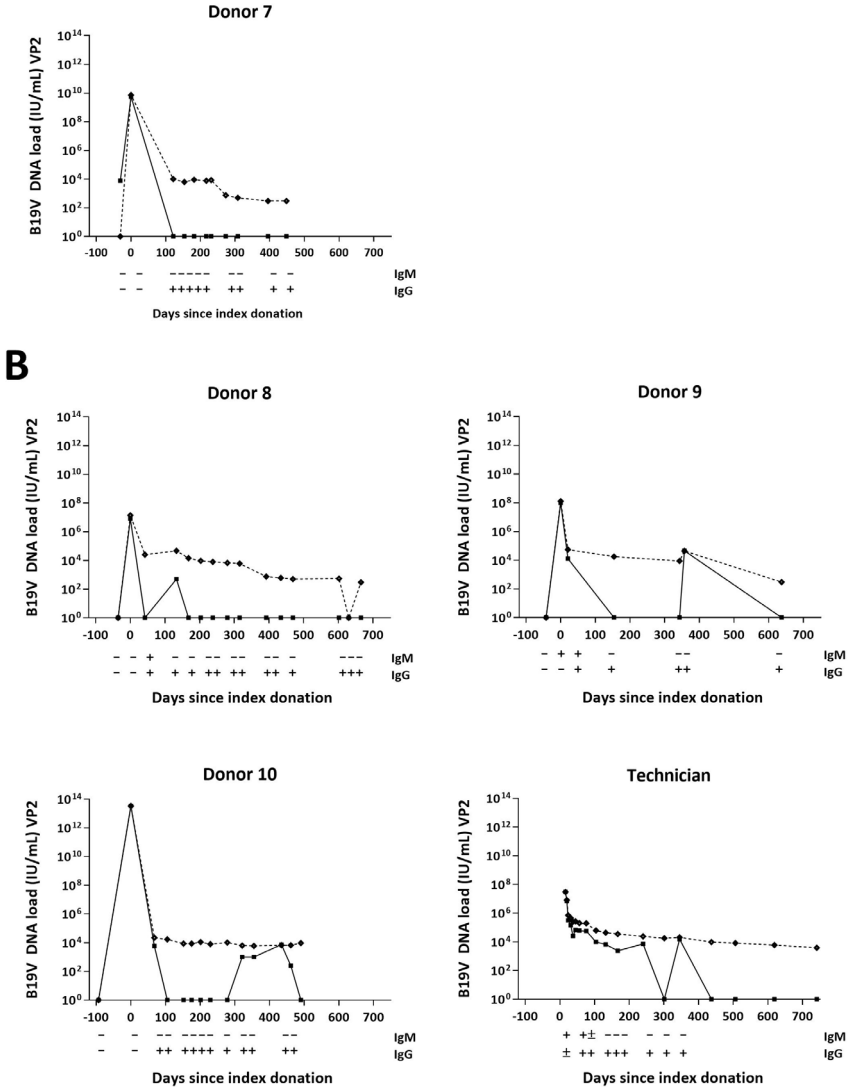


***Figure 1**: Follow-up of B19V DNA levels and B19V serology after acute B19V infection in 11 persons. The B19V DNA level was determined without (diamonds) and with (squares) endonuclease treatment. Panel A shows the predominant pattern: initially B19V DNA is endonuclease-resistant, indicative of encapsidated viral DNA. Subsequently a longer episode of endonuclease-degradable B19V DNA follows, indicating the presence of bare B19V DNA strands. Panel B shows 4 persons with a second endonuclease-resistant peak, suggestive of a second episode of virus replication.*

DISCUSSION

This study provides evidence that the presence of B19V DNA in blood not necessarily correlates with active B19V replication. The serial samples from acutely B19V-infected, asymptomatic blood donors demonstrated that B19V DNA can be detected for a long period after acute B19V infection in accordance with previous studies [2,3,24]. Endonuclease treatment demonstrated that only in the first months after acute B19V infection presence of B19V DNA is indicative of the presence of B19V virions. Five months after the acute peak almost all B19V DNA could be degraded by endonuclease, indicating that predominantly naked B19V DNA was present. The transition from virions to naked DNA correlated with the disappearance of B19V IgM in most cases. Naked B19V DNA was detected when B19V DNA levels decreased below 10^5 IU/mL. This finding is in agreement with the observation that after acute infection, blood donors with B19V levels below 10^4 IU/mL do not transmit B19V infection to recipients of blood and blood products [25,26]. Until now it was assumed that this lack of infectivity is due to concurrent neutralising B19V antibodies, or an inadequate amount of infused virions [24]. We hypothesize that such low 'viremic' blood donors simply do not carry B19V virions anymore.

Interestingly in 4 out of 11 persons a second temporary endonuclease-resistant B19V DNA peak occurred between 6 and 12 months after primary infection. Spiking of one of these samples with naked PARV4 DNA showed that this was not due to endonuclease-inhibiting factors, since naked PARV4 DNA was degraded. The possibility of a second phase of replication is confirmed by Lindblom et al, who reported that 1 of 5 patients with B19V infection showed increasing B19V DNA levels 50 weeks after infection [27]. Why this second peak arises and whether it has clinical consequences remains to be elucidated.

The endonuclease treatment has some limitations. The interpretation of serial samples is straightforward, but the interpretation of a single sample might be more challenging, if only partial reduction by endonuclease occurs. In addition, multiple freeze-thawing cycles of samples could damage the B19V capsid and cause overestimation of naked B19V DNA.

After the DNA peak, donors show a short rapid decline in B19V DNA to 10^4–10^5 IU/mL. This rapid decline coincides with B19V IgG production and might reflect efficient neutralization of the virus by IgG. This is in accordance with the results of Lindblom et al. which show a rapid decline in B19V DNA, when B19V IgG antibodies

appear. In addition, this coincides with resolution of acute clinical symptoms [27]. After this sharp decline, B19V DNA levels remain stable at 10^4IU/mL and only slowly decrease over years. The B19V DNA becomes endonuclease sensitive after the sharp decline and this seems to correlate to the disappearance of IgM. In addition the clearance rates of virions (endonuclease resistance) and IgM were quite comparable, 137 versus 142 days respectively.

The finding of bare B19V DNA in plasma months or years after acute B19V infection thus seems unrelated to active viral replication and probably has a different origin. During acute infection B19V infects erythrocyte progenitors via the globoside receptor and via co-receptors α5β1 integrins and Ku80 [28,29]. After cell entry, B19V can replicate only when the cell is in late S-phase. Recently, von Kietzell et al reported that B19V can also enter cells via enhanced antibody-mediated endocytosis involving heat-sensitive complement factor C1q [30]. However, antibody-mediated endocytosis does not seem to initiate a productive infection cycle [30]. It is tempting to speculate that antibody-mediated endocytosis leads to accumulation of B19V DNA in several types of cells, which are non-permissive for B19V infection. B19V may not replicate due to the presence of antibodies, or because these cells fail to provide the correct proteins for B19V replication, e.g. are not in S-phase. In addition, the extreme high levels of B19V viremia during acute infection may cause B19V to enter cell types and biocompartments via otherwise inefficient, non-specific receptors. Once in a non-permissive cell, B19V DNA will persist episomally, since cells have no mechanism to degrade DNA. This is confirmed by the fact that many tissues which are non-permissive for B19V have been reported to contain B19V DNA [5,7]. When these cells are damaged by any disease process or when renewed (normal cell turnover), naked B19V DNA will be released into the circulation. For long-lived cells this may occur years or even decades after initial B19V infection. Many acute B19V infections appear to be followed by long-term, slowly declining levels of B19V DNA. As different cell types have different renewal rates, this might cause a stochastic release of B19V DNA, explaining the slowly declining 'viremia' after acute infection. In addition to apoptosis and necrosis, the release of viral DNA via exocytosis might contribute to the presence of naked B19V DNA in plasma [31].

Small amounts of B19V DNA can be detected because PCR assays are very sensitive. However, they do not discriminate between naked and virion-associated DNA. The method presented in this study could prove useful to determine whether

B19V DNA is infectious and linked to replication or not. This question is particularly urgent in myocarditis or cardiomyopathies, often found to be associated with detectable levels of B19V DNA. If indeed B19V DNA in myocarditis patients is not encapsidated, this would suggest that B19V DNA is released from damaged cardiomyocytes years after infection, and that B19V is not the cause of the myocarditis but only a marker. During the entire human life it has been estimated that only 39% of cardiomyocytes is renewed [32]. Thus, they may store B19V DNA passively for decades. Several authors have considered this passive role of released B19V DNA as a mere marker of tissue damage the most likely explanation [14-18]. When B19V indeed is not replicating, antiviral treatment with IVIG, a proposed treatment in B19V myocarditis [33], will be ineffective.

In summary, this study demonstrated that after infection, levels of B19V DNA below 10^5 IU/mL were not based on active B19V replication. Pre-treatment of blood samples with endonuclease enabled discrimination between naked B19V DNA (unrelated to replication) and B19V DNA encapsidated in virions. This method can be useful to settle longstanding controversies on the nature of B19V DNA in various conditions, whether it is the cause of pathology or whether it is just a consequence, an innocent bystander.

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6

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.

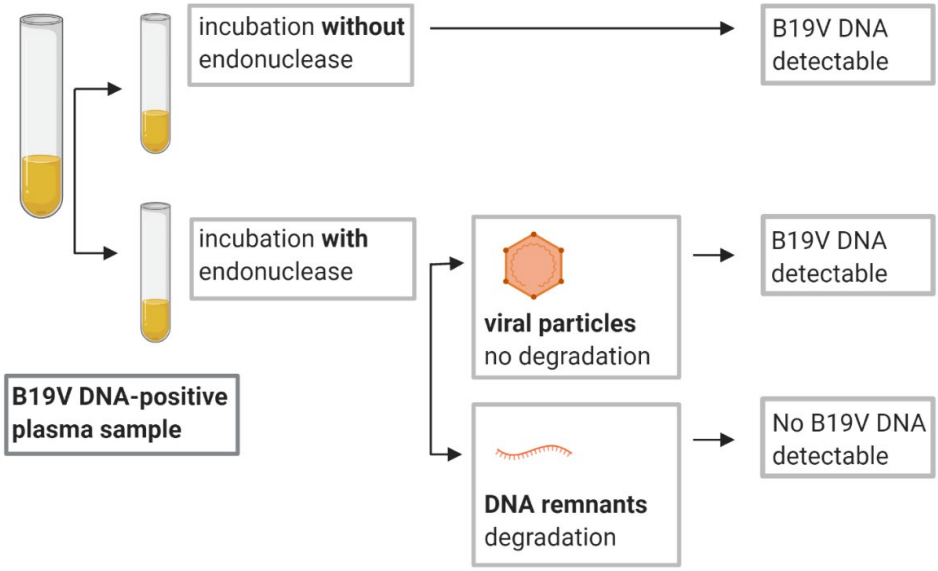

Figure 1: the principle of the endonuclease test. *DNA is degraded by endonuclease when only bare DNA strands are present. When intact viral particles are present, DNA remains intact as it is protected within the viral particle. Incubation without endonuclease acts as positive control. Plasma is used 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*

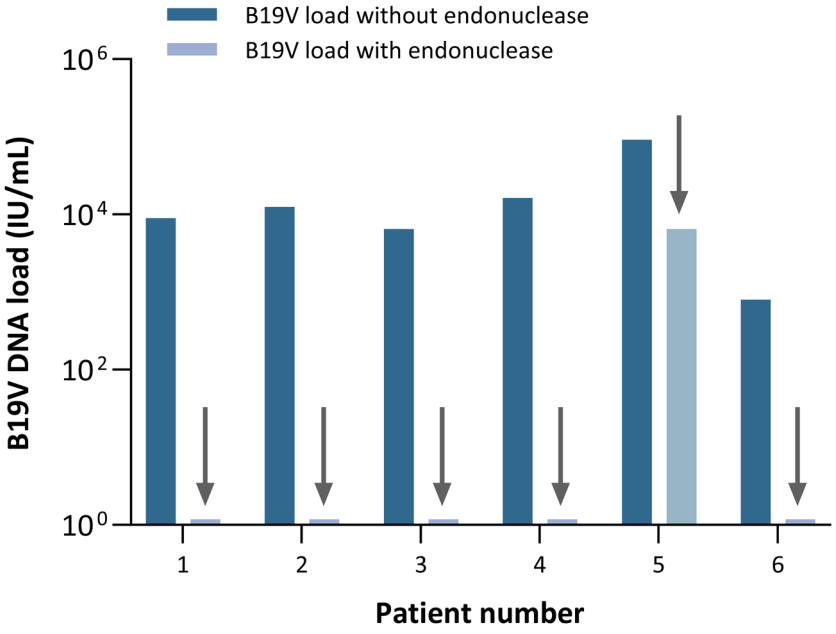

Figure 2: B19V DNA absent or degradable in blood of patients with DCM. *Blood samples of DCM patients with and without endonuclease treatment. Dark bars indicate DNA load without endonuclease treatment, light bars and/or arrows indicate DNA load with endonuclease treatment (DNA load absent after endonuclease 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 ($p = .000$).

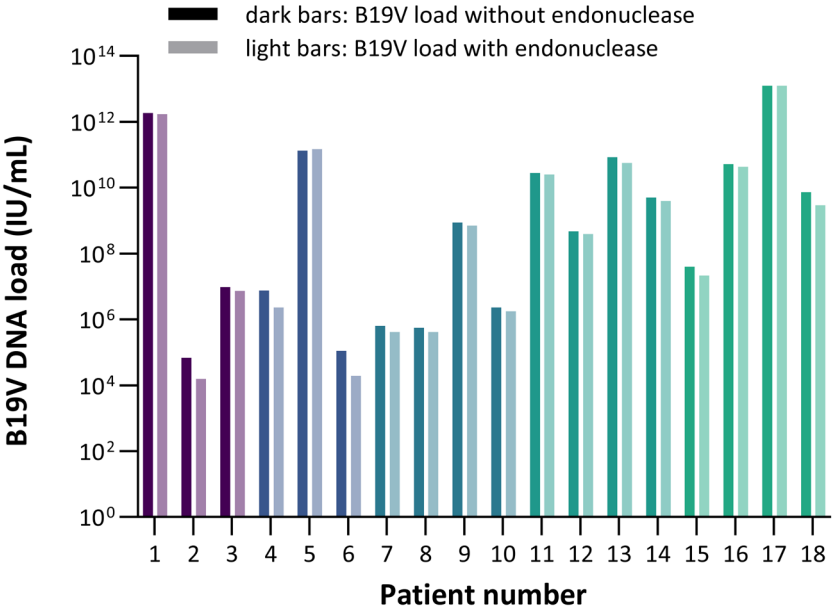

Figure 3: B19V DNA not degradable in patients with active B19V infection. *Blood samples of patients with recent infection with and without endonuclease treatment. Different colors indicate different patient groups. For each patient, the dark bar indicates DNA load without endonuclease treatment, while 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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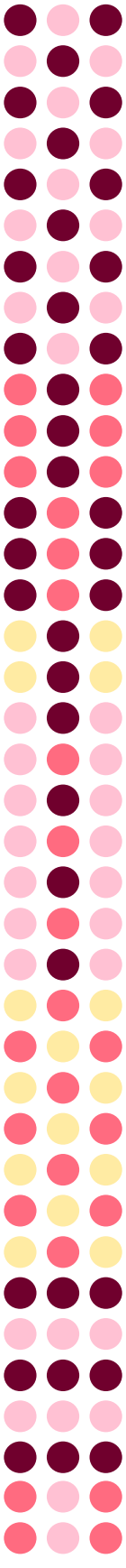

7

Transient parvovirus B19 DNAemia after kidney transplantation: a two-sided story

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ABSTRACT

Parvovirus B19 (B19V) DNAemia appears a relatively common finding after kidney transplantation. However, not all DNAemia signifies active infection with replicating virus. This study screened 134 patients post-transplantation for B19V DNAemia and identified two cases in which viral DNA was present after transplantation, with the donor kidney as probable source of the DNA. In both cases intact viral particles could not be detected using an endonuclease method, indicating the presence of non-infectious DNA remnants.

BACKGROUND

Parvovirus B19 (B19V) is a ubiquitous human pathogen with a unique predisposition to infect erythroid progenitor cells (EPC), causing viremia with extremely high viral loads up to 10^{14} IU/ml during acute infection [1]. In immunocompromised patients such as transplant patients, this may lead to severe, and often relapsing, anemia [2-4]. Apart from EPC, B19V DNA appears to enter multiple cell types using mechanisms other than cell-receptor specific entry during acute infection [5, 6]. This results in lifelong detectability of B19V DNA in some tissues, including renal tissue, without apparent disease activity [7-9]. Also in blood, B19V DNA remains detectable for months after infection [10]. Of all transplant patients, attention for B19V infection in kidney transplantation is particularly relevant as kidney transplant patients often suffer from anemia for various reasons, of which B19V infection may be one [11]. Detection of B19V DNA (DNAemia) in blood after kidney transplantation is not uncommon; shortly after transplantation, the incidence is 0.5 to 1.4% but the overall incidence of B19V DNAemia is estimated at 7.6% in the year after kidney transplantation [12, 13]. However, due to the unique characteristic of B19V DNA to persist in tissue lifelong, the clinical relevance of DNAemia after kidney transplantation should be carefully considered. True B19V infection by kidney transplantation appears to be rare, as it supposes (semi)acute infection in the donor combined with a susceptible recipient. Due to an adult seroprevalence of 60-70% many recipients will not be susceptible [13]. Detectable DNA may originate from intact viral particles, causing true infection, or from DNA remnants released from previously infected cells [10, 14-16]. Application of an endonuclease based assay can differentiate between these two scenarios [10]. Endonuclease treatment degrades DNA remnants but not DNA in intact viral particles, which is protected by the viral capsid. This study investigates DNAemia after kidney transplantation and presents two cases where DNAemia after kidney transplantation should not be mistaken for active B19V infection.

METHODS

Patient screening: One hundred ninety-two patients received a living kidney transplantation at the Leiden University Medical Center in the period January 2013 – June 2016. Of these, blood samples were available from 134 patients in the period 30-90 days after transplantation. For each patient, one blood sample in that period was

screened with qualitative B19V PCR for the presence of B19V DNA. If positive, additional donor and longitudinal patient blood samples were tested with quantitative B19V PCR. PCR was also performed on kidney biopsies from the time of transplantation. To determine viral activity, blood samples were also tested using endonuclease pretreatment.

B19V PCR and sequencing: DNA extraction was performed by MagNAPure (Roche Diagnostics) with 200 µl of plasma or serum as input for the PCR, according to the manufacturer's instructions. Subsequently, eluates were tested with an in-house B19V PCR targeting the VP1/VP2 region of all known 3 genotypes as described by Knoester et al [17]. For kidney biopsies, 5 sections of 10 µm were deparaffinized and homogenized with a pre-treatment as used for routine tissue diagnostic procedures in our laboratory. Subsequently, DNA extraction and PCR were performed as described above in duplicate. An in-house PCR for the housekeeping gene beta-globin was performed in duplicate on the kidney biopsies to determine cell count, as described previously, to express DNA load in kidney biopsies as IU per cell [18]. Near whole-genome sequencing on the kidney biopsy and plasma samples of patient 1 was performed as described previously [19].

B19V serology: IgM and IgG serology were performed on blood samples using the Liaison Biotrin Parvovirus IgM/IgG assay (Diasorin) according to the manufacturer's protocol.

Endonuclease (Benzonase) assay: If B19V DNA positive, a blood sample was retested applying endonuclease treatment as described by Molenaar et al [10]. In short; each plasma sample was split in two 80-100 µl aliquots, the exact amount depending on the availability of sufficient material. To one of the aliquots 250 units of Benzonase (Sigma- Aldrich, the Netherlands) were added. Subsequently both aliquots were incubated for one hour at 37°C in a shaking incubator at 120 rpm. After cooling to room temperature, DNA extraction and quantitative dual target NS-VP2 PCR were performed as described previously [20]. Negative, low-positive and high-positive controls were included in the endonuclease assay.

Histochemical staining of kidney biopsies: Staining of paraffin slides (10 micrometer) was performed using monoclonal anti-Parvovirus B19 antibody, targeted against amino-acid 328-344 of the VP2 capsid protein, clone R9276 (Sigma-Aldrich). A positive control stain was included in the analysis.

Ethics: The investigation was done in accordance with the principles outlined in the declaration of Helsinki. The ethical committee of the LUMC approved the

research protocol. As the study was not subject to the Medical Research Involving Human Subjects Act (WMO) and patient data cannot be traced back to the individual patient, informed consent was waived.

RESULTS

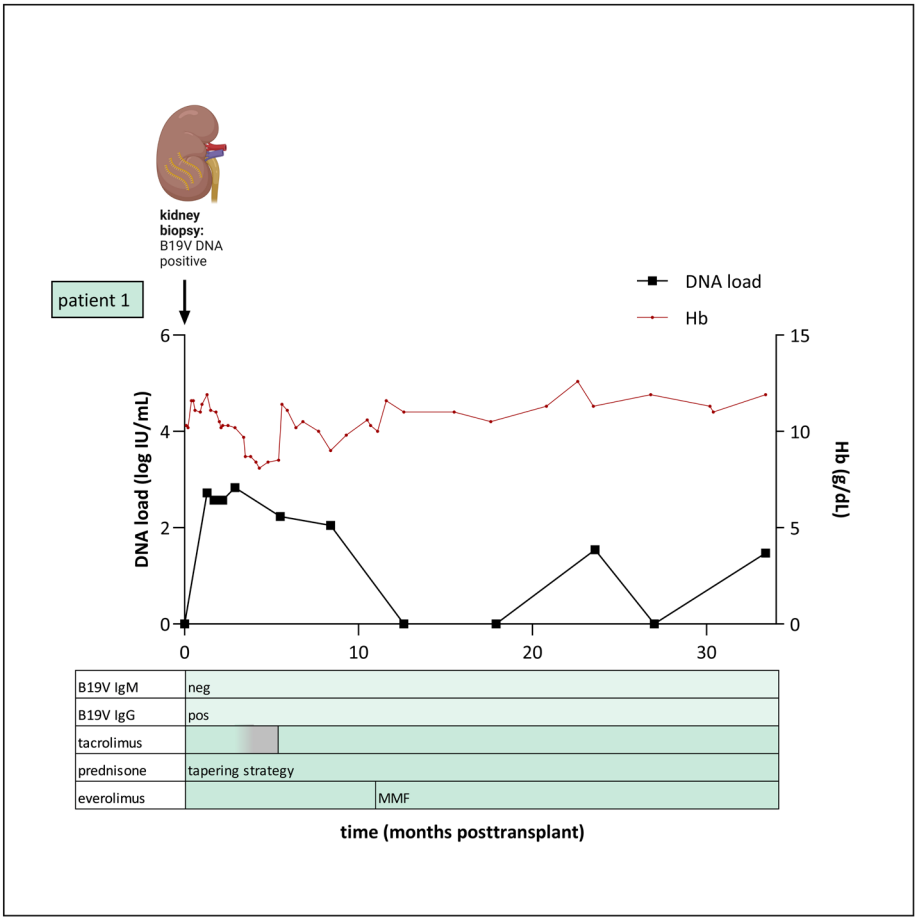
A total of 134 kidney transplant patients were screened for B19V DNAemia 30 to 90 days after kidney transplantation. Clinical characteristics are shown in Table 1.

Table 1. Patient characteristics

Patients screened for B19V DNA	n = 134
Age, years, mean (SD)	51 (14)
Male sex, n (%)	83 (61.9)
B19V IgG-positive at baseline, n (%)	93 (69.4)*
Time to screening sample, days after transplantation, mean (SD)	65 (14)
Positive B19V PCR-result, n	2

* 1 IgG status not available

Two of the 134 patients tested positive for B19V DNA in the screening, after which additional donor kidney and recipient blood samples were tested. The longitudinal B19V DNAemia profiles of both patients are shown in Figure 1, accompanied by clinical information and the course of hemoglobulin (Hb) levels.


kidney biopsy: B19V DNA positive
patient 1
DNA load
Hb
DNA load (log IU/mL)
Hb (g/dL)
0
2
4
6
0
5
10
15
0
10
20
30
B19V IgM
neg
B19V IgG
pos
tacrolimus
prednisone
tapering strategy
everolimus
MMF
time (months posttransplant)

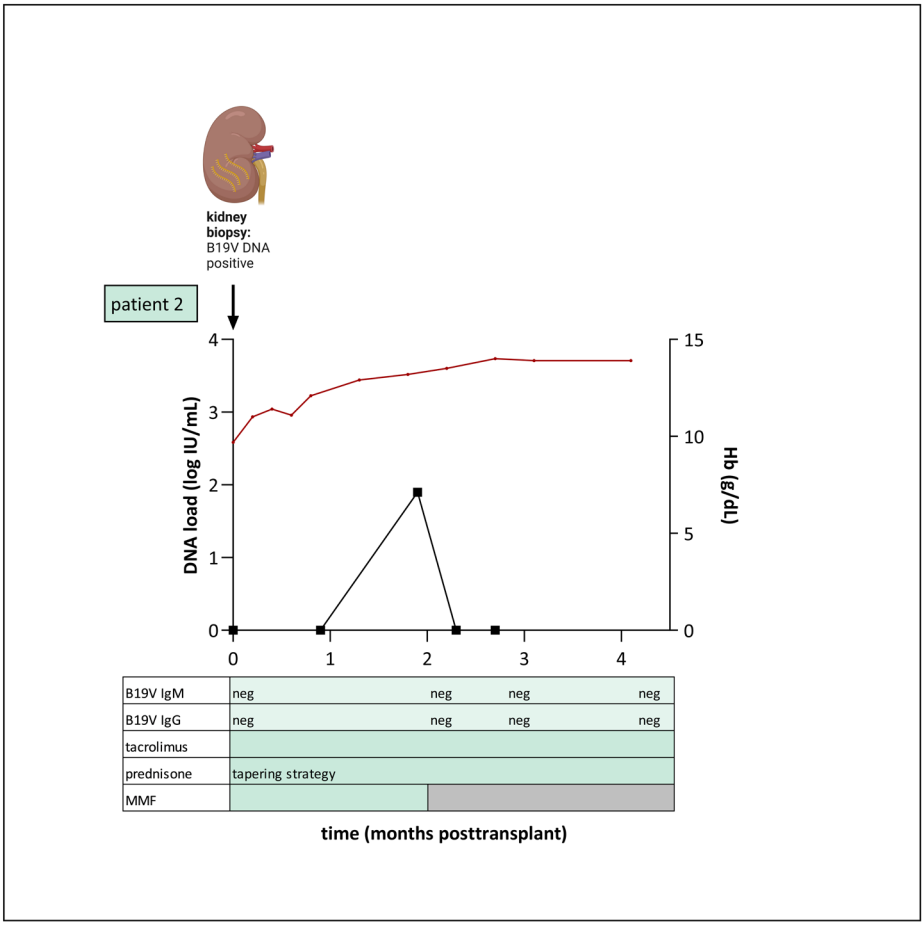


Figure 1: *Longitudinal course of B19V DNAemia in 2 kidney transplant recipients, in relation to hemoglobin levels and information regarding seroconversion and immunosuppressive treatment. MMF, mycophenolate mofetil. Dark green bars represent active treatment; gray bars represent no treatment. (Figure partly created with Biorender.com)*

Patient 1, a 49-year-old male, was B19V IgG-seropositive before transplantation and B19V DNA-negative in blood. He received a living-related kidney transplant. The kidney biopsy at the time of transplantation tested positive for B19V DNA (200 IU/cell). After transplantation, patient 1 tested consistently positive for B19V DNA in blood for more than 10 months, with a gradual decrease in DNA load. Endonuclease testing of all DNA-positive blood samples showed 100% degradability of the detected DNA. Patient 1 showed no obvious symptoms of active B19V infection after transplantation. The patient suffered from anemia 4 months post-transplantation with a nadir hemoglobulin (Hb) level of 8.0 g/dL (see figure), which recovered in the following months. The presence of B19V DNAemia was unknown to clinicians in the years after transplantation and he received no specific B19V treatment such as IVIG. His immunosuppressive regimen was moderated by a temporary stop of tacrolimus due to a concomitant BK-nephropathy (see figure), but this did not lead to clearance of B19V DNA, whereas his Hb-levels did improve.

Patient 2, a 54-year-old male, was B19V IgG-seronegative and B19V DNA-negative before transplantation. The patient received a living-related kidney transplant from a B19V-seropositive donor. The kidney biopsy at the time of transplantation tested weak-positive in 1 of 2 duplicates for B19V DNA, indicating a DNA load around the limit of detection. After transplantation, patient 2 tested positive at one timepoint with a low B19V DNA-load in blood of 79 IU/mL. A false-positive finding was ruled out by confirmation with a different dual-target PCR-method in a different laboratory. The sample was 100% degradable in the endonuclease assay. Clinically, the patient showed no signs of a B19V infection with a stable Hb-level of around 13 g/dL in the months post-transplantation. His immunosuppressive regimen was also moderated due to a concomitant BK-nephropathy, but in the months following the positive sample, the patient remained B19V IgM and IgG-negative.

DISCUSSION

This study describes 2 patients testing positive for B19V DNA in blood after kidney transplantation, which was found to be unrelated to B19V replication, and could be attributed to 'passive' B19V DNA remnants, most likely originating from the donor kidney. This interpretation was supported by the degradation of the B19V DNA in an endonuclease test; by the fact that the B19V load did not decrease with tapering of immunosuppression; by the relatively low DNA loads in blood as opposed to the high viral loads commonly seen in active infection; by the absence of

seroconversion; and by the lack of symptoms consistent with B19V infection. Patient 1 did suffer from anemia, but anemia occurs in 20-51% of kidney transplant patients at various time points after transplantation and the improving course of anemia does not correspond to the persistent detection of B19V DNA [11].

In previous studies, it was already observed that low-level B19V DNAemia after transplantation does not correspond to symptomatic infection. Barzon et al describe a pediatric cohort of 75 transplantations in which 24 of kidney biopsies test positive for B19V DNA. Twenty recipients become DNA-positive in the year after transplantation, of which 10 are B19V-seronegative before transplantation. However, only 4 patients are reported to develop symptoms of B19V infection after transplantation. Data regarding seroconversion are not mentioned [21]. A similar observation was made recently by Rezahosseini et al [22]. In their study, 6 previously B19V IgG-negative SOT recipients were identified with B19V DNAemia after transplantation. Seroconversion was documented in 4 patients but in the other 2 patients, DNAemia was low-level and transient and IgG was negative one year after transplantation. This illustrates that post-transplant B19V DNAemia can be caused by two mechanisms: either true B19V infection with viral replication; or clinically irrelevant presence of inactive B19V DNA fragments (i.e. remnants from earlier infection). Although the above-mentioned studies by Barzon et al and Rezahosseini et al report on patients in which passive transfer of clinically irrelevant DNA seems probable, they do not distinguish between these two manifestations by additional laboratory testing and do not comment on the possible different interpretations of DNAemia. The relative proportions of true infection and clinically irrelevant DNAemia can still be considered unknown. The endonuclease assay used in this study appears to be a useful and reliable tool to distinguish between active B19V infection and presence of inactive B19V DNA remnants, which can be applied in research settings, for example, to determine proportions of true infection vs irrelevant DNAemia, and in clinical practice to guide patient management.

This study also showed that B19V DNAemia can arise from the graft after kidney transplantation. Patient 1 was B19V IgG-seropositive before transplantation, so B19V DNA may have been derived from a previous infection in the recipient. However, the course and properties of DNAemia in patient 1 strongly suggested that DNAemia originated from the donor kidney, which was retrospectively found to be positive with for B19V DNA. In patient 2, it is also likely that the B19V DNA-

positive kidney is the source of the B19V DNA transmission, as the patient had no evidence of a previous B19V infection. Unfortunately, it was not possible to investigate the genetic relationship between donor and recipient B19V DNA, because the viral loads proved too low for reliable sequencing (data not shown).

It is remarkable that B19V DNAemia is reported in many patients with evident kidney damage, e.g. kidneys from deceased donors or concomitant BK-reactivation [23]. Our 2 patients also showed BK-reactivation. It can be hypothesized that B19V DNA present in tissue, will be released into the bloodstream during tissue damage, for example, induced by BK-reactivation. This mechanism has been described for donor-derived cell-free DNA, which is used as a biomarker for tissue injury [24]. This mechanism could also explain clinically irrelevant B19V DNAemia after transplantation. Immunohistochemical staining for B19V capsid antigen in kidney biopsies was negative (data not shown), which is in line with persistence of DNA in kidney tissue but not persistence of intact viral particles.

In summary, this study showed that B19V DNAemia after kidney transplantation is not necessarily associated with active infection. The nature and origin of B19V DNAemia should be carefully considered before it is assumed to have clinical relevance, to avoid erroneous treatment decisions.

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8

Longitudinal Monitoring of DNA Viral Loads in Transplant Patients Using Quantitative Metagenomic Next-Generation Sequencing

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ABSTRACT

Introduction: Immunocompromised patients are prone to reactivations and (re-)infections of multiple DNA viruses. Viral load monitoring by single-target quantitative PCRs (qPCR) is the current cornerstone for virus quantification. In this study, a metagenomic next-generation sequencing (mNGS) approach was used for the identification and load monitoring of transplantation-related DNA viruses. Methods: Longitudinal plasma samples from six patients that were qPCR-positive for cytomegalovirus (CMV), Epstein-Barr virus (EBV), BK polyomavirus (BKV), adenovirus (ADV), parvovirus B19 (B19V), and torque teno virus (TTV) were sequenced using the quantitative metagenomic Galileo Viral Panel Solution (Arc Bio, LLC) reagents and bioinformatics pipeline combination. Qualitative and quantitative performance was analysed with a focus on viral load ranges relevant for clinical decision making. Results: All pathogens identified by qPCR were also identified by mNGS. BKV, CMV, and HHV6B were additionally detected by mNGS, and could be confirmed by qPCR or auxiliary bioinformatic analysis. Viral loads determined by mNGS correlated with the qPCR results, with inter-method differences in viral load per virus ranging from 0.19 $\log_{10}$ IU/mL for EBV to 0.90 $\log_{10}$ copies/mL for ADV. TTV, analysed by mNGS in a semi-quantitative way, demonstrated a mean difference of 3.0 $\log_{10}$ copies/mL. Trends over time in viral load determined by mNGS and qPCR were comparable, and clinical thresholds for initiation of treatment were equally identified by mNGS. Conclusion: The Galileo Viral Panel for quantitative mNGS performed comparably to qPCR concerning detection and viral load determination, within clinically relevant ranges of patient management algorithms.

1. INTRODUCTION

Opportunistic viral infections frequently occur after solid organ or hematopoietic cell transplantation, with associated morbidity and mortality of up to 40% [1]. Successful prevention and early detection of viral infections including reactivations are the cornerstones of transplant patient management. For effective preemptive and therapeutic treatment strategies, accurate viral load quantification is essential. Typically, in immunocompromised hosts, multiple viruses can reactivate simultaneously, which makes comprehensive identification of replicating pathogenic viruses essential. Currently, the monitoring of opportunistic viral infections in transplant patients is most frequently performed by multiple singleplex quantitative PCRs.

Metagenomic next-generation sequencing (mNGS) is increasingly being applied for the identification of pathogens in undiagnosed cases suspected of infection [2–4]. Quantification of viral loads utilising mNGS remains a challenge [5–8]. Complicating factors are the varying amount of background sequences from the host and from bacterial origin, technical bias affecting target sequence depth, unselective attribution of reads, and the number of calibration curves that are needed simultaneously when using untargeted sequencing for viral load calculations. Reports comparing mNGS with qPCR demonstrated a correlation with normalised sequence read counts but never as accurate as qPCR for viral load prediction [5]. Other previous research concerning the quantification of shotgun sequence read counts focused mainly on differential expression of RNA [9–12].

Recently, the Galileo Viral Panel (Arc Bio, LLC) has been designed as a quantitative mNGS approach for ten transplant-related DNA viruses [13,14]. This all-inclusive approach encompasses the library preparation kit, controls, calibration reagents, and cloud-based user-friendly software for bioinformatic analysis. Previous data on the performance of this mNGS approach demonstrated that the analytical performance was comparable to qPCR results with regard to the limits of detection, limits of quantification, and inter-assay variation [13,14].

In this study, we analysed the performance of the Galileo Viral Panel for viral load quantification in transplant patients over time. Subsequent samples from six transplant patients with proven infections or reactivations with transplantation-related DNA viruses (adenovirus, ADV; BK polyomavirus, BKV; cytomegalovirus, CMV; Epstein-Barr virus, EBV; human herpesvirus type 6A, HHV-6A; human herpesvirus type 6B, HHV-6B; herpes simplex type 1, HSV-1; herpes simplex type 2, HSV-

2; JC polyomavirus, JCV; varicella-zoster virus, VZV; parvovirus B19, B19V; and torque teno virus, TTV) were analysed in comparison with qPCR. Accuracy of viral load quantification by mNGS was studied in relation to thresholds that had been used for the initiation of treatment or tapering of immunosuppression. Furthermore, we investigated the additional detection of DNA viruses identified by the broad mNGS approach, for which no targeted qPCR had initially been ordered.

2. METHODS

2.1. Patients and Sample Selection

Six adult immunocompromised patients (one allogeneic stem cell transplant patient, four kidney transplant patients, and one patient with hematological malignancy) were retrospectively selected based on available follow-up EDTA plasma samples that previously tested positive for one or more transplantation-related DNA viruses. Samples had previously (July 2008–December 2019) been sent to the Clinical Microbiological Laboratory (CML) of the Leiden University Medical Center (LUMC, The Netherlands) for viral load monitoring as part of routine patient care. Routine patient diagnostics consisted of several collection points, resulting in positive qPCR's with a wide range of viral loads. CMV/EBV were routinely screened for in plasma post transplantation. BKV was screened in urine post renal transplantation; when positive it was also screened for in plasma. ADV and B19V were not routinely screened for but ordered at the discretion of the treating physician based on symptomatology. TTV viral load had been tested retrospectively by qPCR in the context of a different study. Patient plasma samples were stored at −80 °C until mNGS analysis.

2.2. Ethical Approval

Approval was obtained from the ethical committee from the LUMC (P11.165 NL 37682.058.11, and Biobank Infectious Diseases protocol 2020-03 & 2020-04 B20.002).

2.3. Extraction of Nucleic Acids; Internal Controls

Patient plasma samples were spiked with an internal control (baculovirus, Arc Bio, LLC) before extraction. Nucleic acids were extracted from 200 µL plasma using the MagNApure 96 DNA and Viral NA Small volume extraction kit on the MagNAPure 96 system (Roche Diagnostics, Almere, The Netherlands) with 100 µL output eluate.

The eluate was concentrated using vacuum centrifugation by a SpeedVac vacuum concentrator (Thermo Scientific) to a volume of 26 µL.

2.4. Library Preparation and Sequencing

Sequence libraries were prepared using the Galileo Viral Panel sequencing kit (Arc Bio, LLC., Cambridge, MA, USA) according to the manufacturer's instructions. The protocol was based on enzymatic fragmentation at 37 °C for 5 min, followed by end repair and A-tailing at 65 °C for 30 min. Subsequently, fragments were ligated using unique dual-index adapters (ArcBio) at 20 °C for 15 min and purified using magnetic Kapa Pure Beads (Roche). No RNase treatment was included in the procedure, and human DNA was depleted using human depletion reagents at 45 °C for 2 h followed by 45 °C for 15 min, after which libraries were amplified using library amplification primers for 45 °C for 30 s, by 14 cycles of 98 °C for 10 s and 65 °C for 75 s and 65 °C for 5 min. The final library preparation products were purified using magnetic Kapa Pure Beads (Roche) and quantified using a Qubit fluorometer (Thermo Fisher) followed by equally pooling using the Arc Bio calculation pooling tool. After a final quantity and quality check using a Bioanalyser (Agilent), samples were sequenced using the NovaSeq 6000 sequencing system (Illumina, San Diego, CA, USA) at GenomeScan B.V. (Leiden, the Netherlands). For sequencing, S4 flowcells were used and samples were sequenced in two runs, where each pool consisted of around 12% of the lane capacity. Ten million reads per library were aimed for; the total reads per sample can be found in Supplementary Table 1.

2.5. Calibration Samples

Initial calibration runs were performed testing the multi-analyte mixture (MAM) of whole-virus particles at viral loads of 0, 1000, 5000, 10,000, and 100,000 copies/mL or IU/mL plasma, in quintuple (Arc Bio, LLC) for the following 10 viruses: hADV-C1, BKV, CMV, EBV, HHV-6A, HHV6B, HSV-1, HSV-2, JCV, and VZV. For TTV and B19V, no Arc Bio calibrator panels were available, and therefore the Galileo Signal values were plotted against the calibrator plot of other viruses that demonstrated optimal agreement with the viral load (JCV and VZV, respectively), representing a semi-quantitative result.

2.6. Bioinformatic Analysis

After demultiplexing of the sequence reads using bcl2fastq (version 2.2.0)(Illumina, San Diego, CA, USA), FASTQ files were uploaded to the Galileo Analytics web application [13,15] which automatically processes data for quality assessment and pathogen detection using a custom database of DNA viruses involved in transplant-associated infections: ADV, CMV, EBV, HHV-6A, HHV-6B, HSV-1, HSV-2, JCV, VZV, B19V, and TTV. Human reads were removed before uploading the fastq files to the web application after mapping them to the human reference genome GRCh38 with Bowtie2 version 2.3.4 [6]. The analytics web application aligns sequence reads to the genomes of the DNA viruses in their calibration kit, scores these read alignments based on complexity, uniqueness, and alignment scores, and reports this in a signal value. The signal value is normalised for read counts across libraries, correcting for differences in genome lengths and technical bias, based on the spiked-in normalisation controls. The signals reported are related to the genomic depth and the observed amount of viral DNA being present in a sample, belonging to non-confounding genomic regions [13]. The sample signals were visualised in linear calibration curves (Supplementary Figure 1).

2.7. Analysis of Performance and Additional Findings

Performance of the metagenomic Galileo Viral Panel assay was assessed in comparison with routine qPCR, analysing both qualitative and quantitative detection. Additional findings by mNGS were confirmed by additional qPCR analysis. In case no remaining sample was available, the Galileo Analytics software results were compared with results from the analysis using alternative bioinformatic tools: metagenomic taxonomic classifier Centrifuge (1.0.4-beta) [16] and de novo assembly-based viral metagenomic analysis software Genome Detective [17].

3. RESULTS

3.1. Calibration Curves

After metagenomic sequencing, the viral loads were calculated for each virus by the Galileo Analytics web application. Signals of both the calibrators and patient plasma samples were plotted in load graphs (Supplementary Figure 1) and the corresponding viral load of the patient samples was extrapolated. As no calibrator panels for B19V and TTV virus were available, these signals were plotted against other calibration curves of viruses that demonstrated the optimal agreement with

the known viral load for semi-quantitative detection. All calibration sample signals correlated well with the titre (R^2 range 0.84–0.92).

3.2. Viral Load by mNGS Versus qPCR

In total, six patients were tested by qPCR and mNGS for quantification of different viruses at subsequent time points. The agreement between the methods for qualitative detection was 100% for the viruses targeted by PCR. Quantitative results per patient are shown in Table 1, and Figure 1 depicts viral loads by mNGS versus qPCR per target virus. CMV and EBV viral loads demonstrated the highest agreement, with a maximum difference in viral load of 0.70 $\log_{10}$ IU/mL. Mean differences in viral loads were 0.43 for CMV and 0.19 $\log_{10}$ IU/mL for EBV. Genotyping had not been performed for ADV (patient 1) and TTV (patient 4) in the context of routine care but resulted in the human adenovirus 1 and TTV-like mini virus, respectively, using mNGS data (based on de novo genome assembly followed by blastn). Viral loads were higher when quantified with mNGS with a mean difference of 0.90 $\log_{10}$ c/mL. For BKV, viral loads by mNGS were lower in comparison with qPCR, with a mean difference of 1.32 $\log_{10}$ c/mL. When taking into account viral loads measured above the limit of quantification of 2.5 $\log_{10}$ c/mL, as applied in our diagnostic qPCR for BKV, the mean difference is 0.62 $\log_{10}$ c/mL and a trend towards a better agreement with higher viral loads could be observed. Semi-quantitative detection of B19V and TTV viruses by mNGS resulted in mean differences of, respectively, 0.39 $\log_{10}$ IU/mL and 3.0 $\log_{10}$ c/mL in comparison with qPCR.

Table 1. Viral load quantification by qPCR and mNGS per patient sample.

Patient-sample	Viral Load qPCR	Viral Load qPCR (log_{10})	Viral Load mNGS	Viral Load mNGS (log_{10})	ΔqPCR-mNGS (log_{10})
Virus: ADV					
P1-S1	675 c/mL	2.83 c/mL	1,277 c/mL	3.11 c/mL	0.28 c/mL
P1-S2	4,517	3.65	66,273	4.82	1.17
P1-S3	34,740	4.54	287,844	5.46	0.92
P1-S4	136,900	5.14	1,435,130	6.16	1.02
P1-S5	60,540	4.78	777,172	5.89	1.11
Virus: BKV					
P2-S1	796 c/mL	2.90 c/mL	3 c/mL	0.48 c/mL	−2.42 c/mL
P2-S2	614	2.79	3	0.48	−2.31
P2-S3	233,700	5.37	9,011	3.95	−1.41
P2-S4	2,401,000	6.38	1,857,785	6.27	−0.11
P2-S5	71,480	4.85	32,321	4.51	−0.34
Virus: CMV					
P3-S1	2,370 IU/mL	3.37 IU/mL	6,246 IU/mL	3.80 IU/mL	0.42 IU/mL
P3-S2	122,800	5.09	275,657	5.44	0.35
P3-S3	10,680	4.03	22,242	4.35	0.32
P3-S4	4,915	3.69	11,366	4.06	0.36
P3-S5	9,156	3.96	46,231	4.66	0.70
Virus: EBV					
P3-S1	2,083 IU/mL	3.32 IU/mL	4,581 IU/mL	3.66 IU/mL	0.34 IU/mL
P3-S2	12,970	4.11	1,573	4.20	0.09
P3-S3	17,710	4.25	14,549	4.16	−0.09
P3-S4	10,500	4.02	15,077	4.18	0.16
P3-S5	7,723	3.89	14,844	4.17	0.28
Virus: TTV*					
P4-S1	140 c/mL	2.15 c/mL	4 c/mL	0.60 c/mL	−1.54 c/mL
P4-S2	2,400,000	6.38	5,142	3.71	-2.67
P4-S3	5.7E+09	9.76	319,074	5.50	−4.25
P4-S4	2.4E+08	8.38	46,261	4.67	−3.71
Virus: B19V*					
P5-S1	1.34 *10^{11} IU/mL	11.13 IU/mL	2.07 *10^{11} IU/mL	11.32 IU/mL	0.19 IU/mL
P5-S2	1,407,365	6.15	1,235,416	6.09	−0.06
P5-S3	45846	4.66	41,787	4.62	−0.04
Virus B19V*					
P6-S1	4.07 *10^{10} IU/mL	10.61 IU/mL	4.37 *10^{11} IU/mL	11.64 IU/mL	1.03 IU/mL
P6-S2	5,309,308	6.73	9,376,953	6.97	0.25
P6-S3	8,569	3.93	49,601	4.70	0.76

*B19V and TTV results were considered semi-quantitative since no Arc Bio calibration samples were available for these targets.

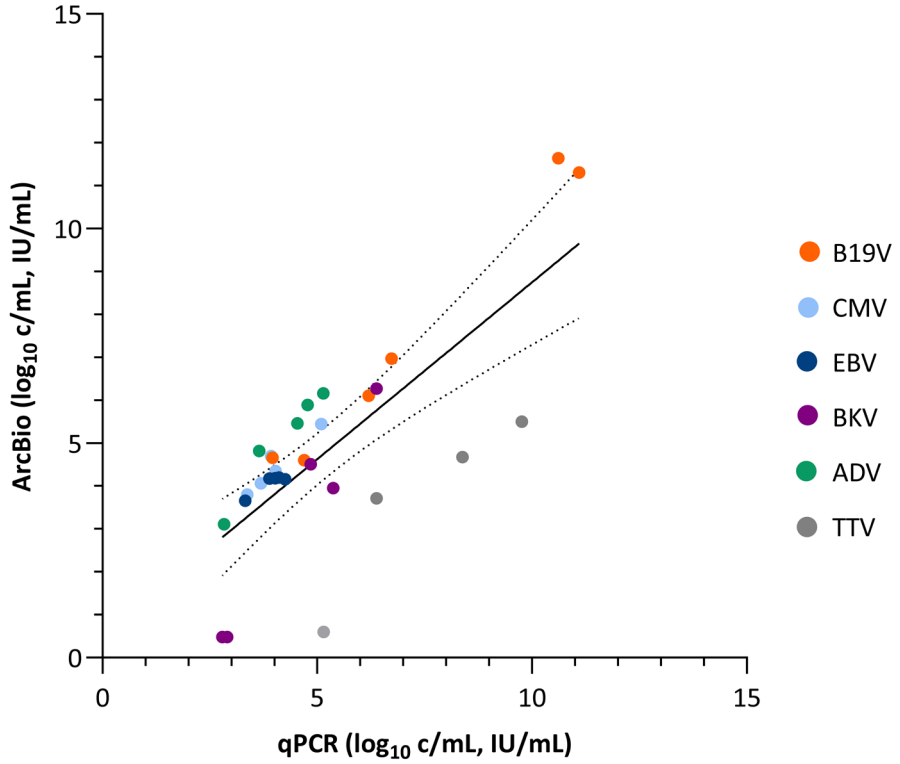


Figure 1. *Viral loads as predicted by Galileo Viral Panel mNGS versus qPCR (copies/mL for ADV, BK, and TTV, and IU/mL for CMV, EBV, and B19V). B19V and TTV results were considered semi-quantitative since no Galileo calibration panels were available for these targets.*

3.3. Longitudinal Patient Follow-Up and Clinical Decision Making

Table 2 gives an outline of patient characteristics and provides clinical information on underlying conditions and complications during the sampling period. Furthermore, for each patient, the viral loads over time were plotted in graphs with clinical information, symptomatology, relevant laboratory parameters, and treatment (Figure 2). For CMV, EBV, and BKV, in our clinical practice, specific viral load thresholds are used to decide whether immunosuppression should be tapered and/or antiviral therapy should be administered. Viral load quantification around these thresholds demonstrated good agreement in identifying these clin-

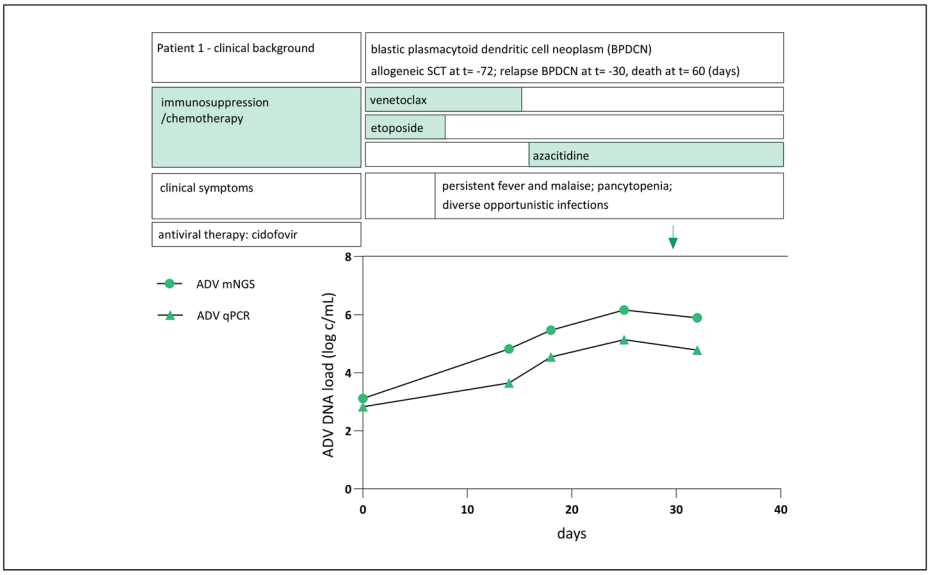
ical decision-making breakpoints. In Patient 3, the antiviral treatment with Foscarnet was started for CMV-reactivation when viral load measured by qPCR exceeded 4.0 log_{10} IU/mL. By mNGS, this critical threshold for treatment initiation was correctly identified with a viral load by mNGS of 5.44 log_{10} IU/mL. In the same patient, rituximab was administered when the EBV load by qPCR was repeatedly above the threshold of 4.0 log_{10} IU/mL, consistently quantified thrice above 4.0 log_{10} IU/mL before administration of rituximab, both by qPCR and mNGS.

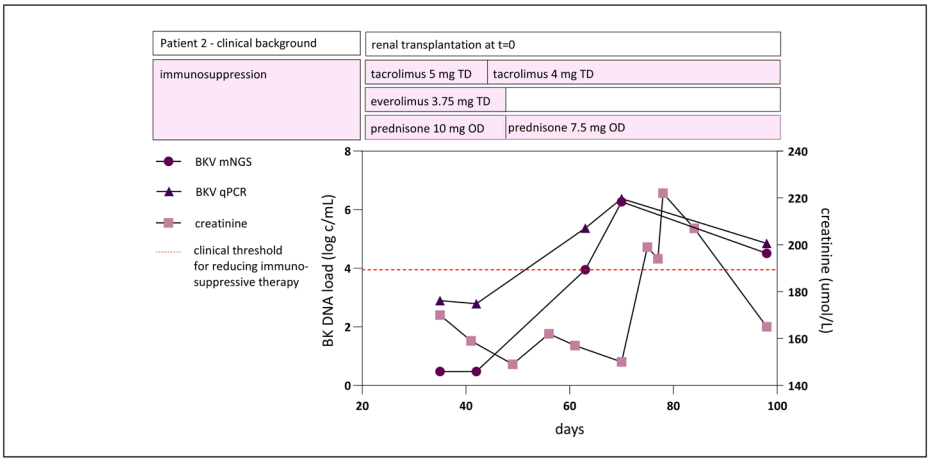
For B19V, ADV, and TTV, no predefined thresholds were used for changing the treatment regimen. For all viruses, the observed trends in load over time in each patient were comparable for qPCR and mNGS, despite the semi-quantitative nature of the B19V mNGS assay. Effect of treatment (anti-viral drugs, immunoglobulins, and/or tapering of immunosuppressive drugs) in patients was estimated by follow-up of viral loads by qPCR. For B19V in Patients 5 and 6, the effect of intravenous immunoglobulins (IVIG) could be assessed by the decreasing viral load in the weeks after administration, as also observed by mNGS. For ADV, in patient 1, antiviral therapy with cidofovir was started when a consistent increase in viral load was detected, both by qPCR and mNGS.

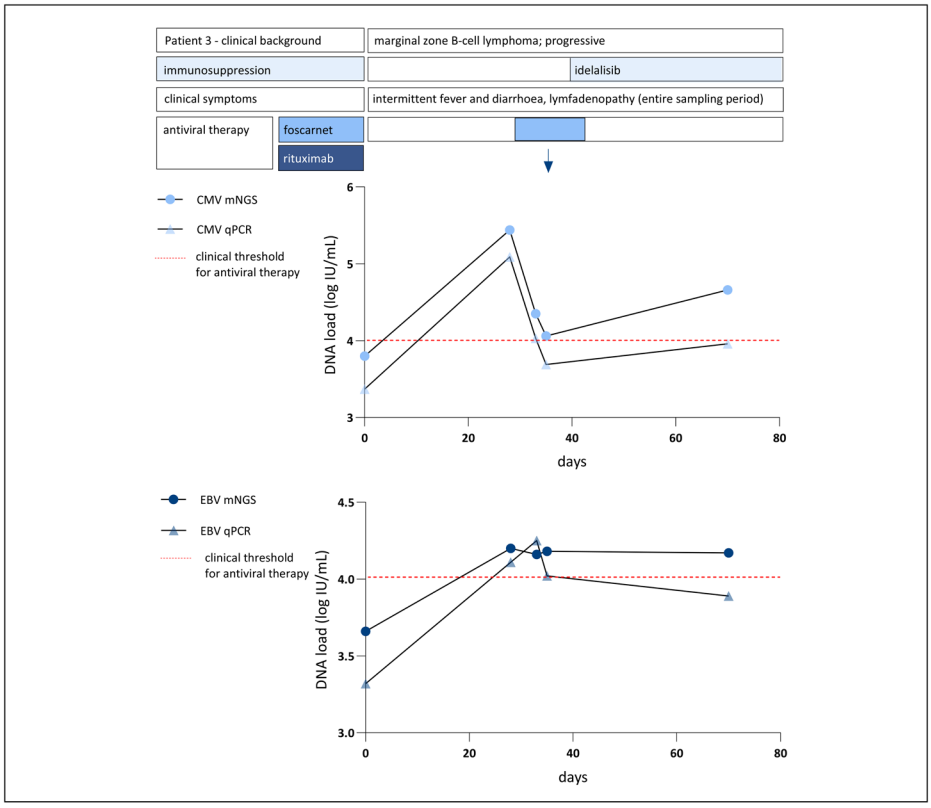
Table 2. Patient characteristics and clinical background at start of longitudinal follow-up.

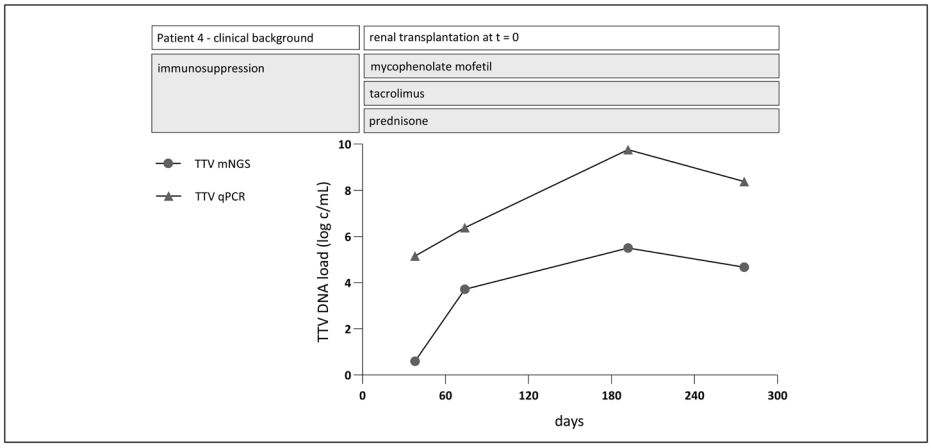
Patient Number	Virus	Age Range	Sex	Underlying Condition	Conditioning Regimen	Transplantation	Other Known Infectious Complications During Sampling Period
1	ADV	60–79	V	Blastic plasmacytoid dendritic cell neoplasm (BPDCN)	Two failed remission-induction regimens; followed by t* = −3: COPADM† t= −2: COPADM	t = 0: Non-myeloablative allogeneic stem cell transplant from unrelated donor; t = 1: relapse BPDCN	1. Probable pulmonal aspergillosis 2. CMV reactivation treated with foscarnet (week before sampling period) 3. *Enterococcus faecalis* UTI‡
2	BKV	20–39	M	Chronic renal insufficiency due to TIN¥, as an extraintestinal manifestation of known colitis ulcerosa or medicine-induced	Alemtuzumab	Pre-emptive living-related renal transplant	1. CMV reactivation
3	CMV, EBV	60–79	V	Marginal zone B-cell lymphoma; established 4 years previously, now progressive	Recent chemotherapy: t = −6: CHOP¦	Not applicable	1. *Escherichia coli* UTI 2. rhinovirus RTI¶
4	TTV	40–59	V	IgA nephropathy	Basiliximab	Living-related renal transplant	1. *Escherichia coli* UTI
5	B19V	40–59	M	IgA nephropathy	Basiliximab	pre-emptive living-unrelated renal transplant	
6	B19V	40–59	M	Focal segmental glomerulosclerosis (FSGS)	Not applicable	Non-heart beating renal transplant 4 years previously; 15 years previously living-related renal transplant	

*t = time in months; †COPADM = cyclophosphamide, oncovin (vincristine), prednisone, Adriamycin (doxorubicin), methotrexate; ‡UTI = urinary tract infection; ¥TIN = tubulointerstitial nefritis; ¦CHOP = cyclophosphamide, oncovin (vincristine), Adriamycin, prednisone ¶RTI = respiratory tract infection. For a complementary longitudinal overview of symptomatology, including laboratory parameters and treatment, see Figure 2.


Patient 1 - clinical background
blastic plasmacytoid dendritic cell neoplasm (BPDCN)
allogeneic SCT at t= -72; relapse BPDCN at t= -30, death at t= 60 (days)
immunosuppression /chemotherapy
venetoclax
etoposide
azacitidine
clinical symptoms
persistent fever and malaise; pancytopenia; diverse opportunistic infections
antiviral therapy: cidofovir
ADV mNGS
ADV qPCR
ADV DNA load (log c/mL)
days


Patient 2 - clinical background
renal transplantation at t=0
immunosuppression
tacrolimus 5 mg TD
tacrolimus 4 mg TD
everolimus 3.75 mg TD
prednisone 10 mg OD
prednisone 7.5 mg OD
BKV mNGS
BKV qPCR
creatinine
clinical threshold for reducing immuno-suppressive therapy
BK DNA load (log c/mL)
creatinine (umol/L)
days


Patient 3 - clinical background
marginal zone B-cell lymphoma; progressive
immunosuppression
idelalisib
clinical symptoms
intermittent fever and diarrhoea, lymfadenopathy (entire sampling period)
antiviral therapy
foscarnet
rituximab
CMV mNGS
CMV qPCR
clinical threshold for antiviral therapy
DNA load (log IU/mL)
days
EBV mNGS
EBV qPCR
clinical threshold for antiviral therapy
DNA load (log IU/mL)
days


Patient 4 - clinical background
renal transplantation at t = 0
immunosuppression
mycophenolate mofetil
tacrolimus
prednisone
TTV mNGS
TTV qPCR
TTV DNA load (log c/mL)
days

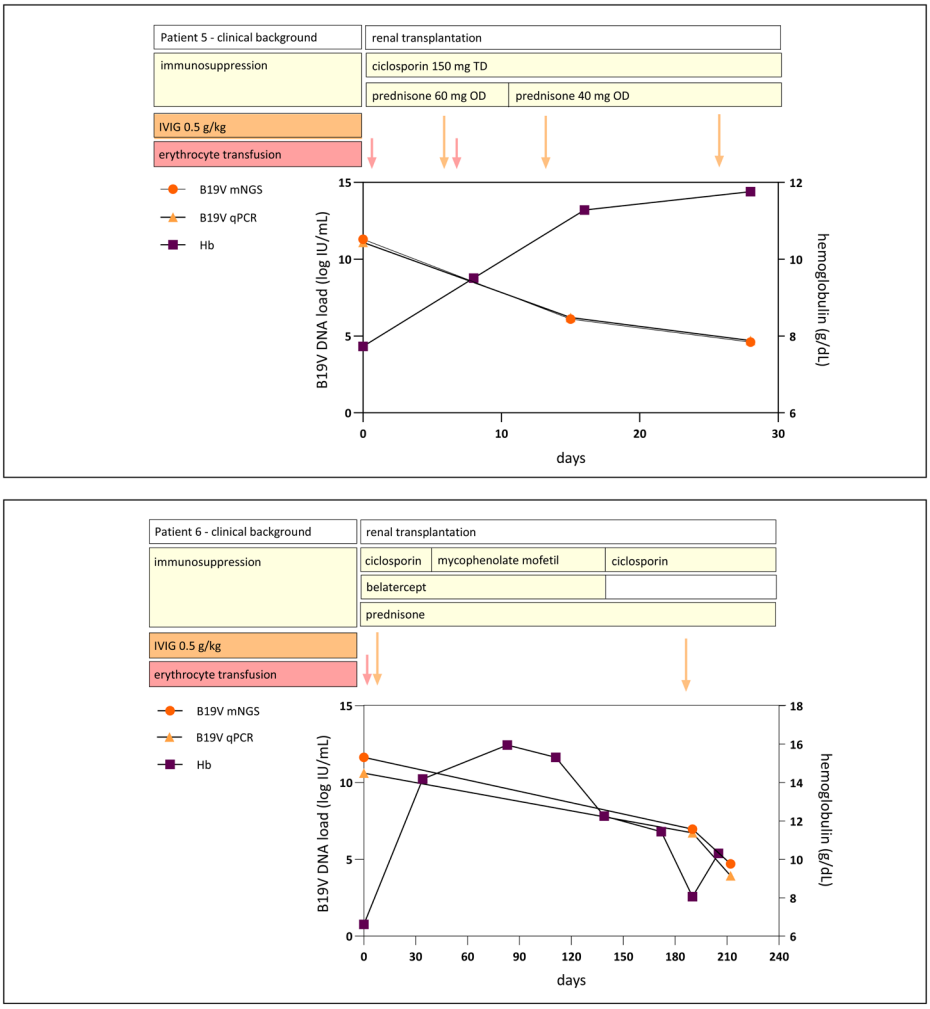


Figure 2. *Longitudinal follow-up of DNA viral loads in immunosuppressed patients over time, as predicted by mNGS (Galileo Viral Panel, Arc Bio) versus qPCR. Clinical information and therapeutic agents are included.*

3.4. Additional Findings

For some samples, additional viral reads were detected in the pathogenic mNGS reports that were not initially tested for by qPCR (Supplementary Table 1). Most additional findings were supported by a secondary bioinformatic analysis using the Centrifuge and Genome Detective: BK (1 patient), CMV (1 patient), HHV-6B (1 patient), and TTV (4 patients, torque teno virus was the deepest level of classification obtained, using mNGS data, with lower than 100% genome coverage). In a few cases, additional findings were not confirmed by a second analysis, leaving some low mNGS signals for CMV, EBV, and HSV. JCV was detected by mNGS in a sample with a high concentration of BKV, which possibly indicated forced alignment contamination due to high sequence homology between JCV and BKV [13,14].

4. DISCUSSION

In this study, the performance of a quantitative mNGS assay for the longitudinal follow-up of DNA viral loads was analysed in six immunocompromised patients. Viral loads determined by mNGS were comparable with loads determined by qPCR, and differed less than 1 $\log_{10}$ for DNA viruses with calibration panels available, in line with previous studies [13,14]. In the current study, the performance of viral loads assessed by mNGS was also evaluated with regard to clinical decision making. In the management of reactivating viruses in immunocompromised patients, local and international guidelines use viral load breakpoints to decide whether antiviral therapy should be administered or whether immunosuppression should be tapered [18–22]. Viral loads under investigation in this study were determined by qPCR as part of routine patient care. When local clinical breakpoints were considered for each virus, mNGS performed comparably to qPCR to identify the clinically relevant breakpoints. B19V is not considered to be a reactivating virus, but quantification may be helpful to distinguish clinically relevant replicative infection from merely DNA remnants [23]. In the range of these breakpoints, viral loads were adequately determined by mNGS to guide clinical decision making. Additionally, the longitudinal trend was similar in comparison with qPCR, indicating precision of mNGS for clinical quantification and reliable indication of the trend in viral load. Clinical decision making is often guided by follow-up of viral load trends, in addition to the cross-sectional viral load measurements for viral infections without available thresholds. In the future, more research is desired to

analyse the performance in the lower ranges to map the limit of quantification (LOQ) of mNGS procedures. It is anticipated that the LOQ is somewhat higher than the LOQ of qPCR, given the generally higher limit of detection in combination with the variability of mNGS, mainly resulting from the varying amounts of background sequences.

The principle of a quantitative catchall approach to detect all transplantation-related viruses in a single run is an attractive feature in the clinical follow-up of the immunocompromised host. Simultaneous reactivation of persistent viruses during immunocompromised episodes is common. Co-infection rates of up to 32% have been described using PCR and, importantly, were associated with higher rates of acute rejection or graft dysfunction [24]. Co-infections may be missed when ordering targeted PCRs, while the catchall approach of mNGS could guarantee that active infections are not overlooked. Indeed, our approach demonstrated a complementary yield of seven reactivating viruses in five patients, which had not been identified earlier by qPCR. Some of these unnoticed viruses are not considered pathogenic, such as TTV. However, the role of TTV in clinical management is still developing, as recent and ongoing research suggests its potential as marker of functional immunity, with an inverse correlation between TTV-load and risk of rejection. Clinical trials exploring its role as a marker for balancing immunosuppressive treatment, with a focus on tacrolimus, are currently being conducted (e.g., ClinicalTrials.gov NCT04198506) [25–28]. ADV, generally, is not systematically screened for in the severely immunosuppressed adult population. In our patient, although actively diagnosed, ADV-loads were rapidly increasing and a catchall approach could guarantee that such less common infections are not overlooked, especially in the absence of localizing symptoms.

A significant complementary virus identification yield by mNGS in transplant patients of 31/49 plasma samples was also reported by Sam et al. [14], with the majority, being viruses, considered pathogenic. These findings demonstrate that mNGS could improve pathogen detection in clinical practice.

Another advantage of mNGS would be its capacity to genotype viruses and detect mutations associated with antiviral resistance, without the need for additional, time-consuming, target-specific ‘wet’ lab procedures that could delay diagnosis and treatment. As an example, Patient 3 in our study was treated with Foscarnet for persistent CMV reactivation pending the results of mutational analysis

after clinical failure of valganciclovir treatment. If the results of mutational analysis had been immediately available, resorting to second-line treatment may have been avoided.

Widespread implementation of mNGS approaches in clinical diagnostic settings has been limited by several factors. The 'wet' lab protocols can be time-consuming, costly, and have a relatively long turnaround time, mainly due to the time required for sequencing. With various sequencing techniques still rapidly evolving, the costs and sequencing turnaround time of such protocols are expected to improve considerably in the future [29]. Furthermore, bioinformatic skills are generally needed for validation and implementation as a diagnostic assay. User-friendly, all-in-one mNGS data analysis software packages for cloud-based and automated analysis enable use in laboratories with minimal bioinformatic knowledge and allow access to high-performance computing capacity.

Limitations in this current study are the relatively low number of samples and viruses when considering a metagenomic approach, including two viruses without calibration panels available. This small-scale study provides a proof-of-principle demonstration in a retrospective design demonstrating that the current version of the Research Use Only Galileo Viral Panel enables longitudinal viral load monitoring by mNGS. It is expected that, after these initial studies, indicating high performance in terms of limit of detection and quantification, inter-run precision, and prospective viral load monitoring, the kit and software will be expanded to include more viruses, calibration samples, and potentially fit for different sample types. Furthermore, technical and bioinformatic features might be evolved in future versions of the assay.

Overall, viral metagenomic sequencing is a promising approach not only for DNA virus detection and identification, but also for reliable estimation of the viral load in a clinical setting, and potentially mutational typing for drug sensitivity analysis. Several milestones essential for implementation in diagnostic settings have been met by the specific assay used in this study: the limits of detection, the limits of quantification, precision, and overall technical performance, which were comparable with qPCR assays. Precise quantification was accomplished by read normalisation based on a designed control. These accomplishments pave the way for further developments and optimisation of quantitative metagenomic sequencing for longitudinal viral load monitoring and beyond.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/pathogens11020236/s1, Figure S1: Calibration graphs of the six viruses in six patients in this study with associated slope, intercepts and R^2 values; Table S1: Additional findings of the metagenomic Galileo Viral Panel compared to Centrifuge and Genome Detective software.

Author Contributions: Conceptualisation: J.J.C.d.V., E.C.C., and A.C.M.K.; methodology: J.J.C.d.V.; investigation: M.E.M.K. and C.S.d.B.; data analysis: E.C.C., M.E.M.K., and I.A.S.; writing of original draft: E.C.C. and A.R.; visualisation: A.R.; review and editing: J.J.C.d.V., E.C.J.C., A.C.M.K., and M.C.W.F.

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Ethical Approval: Approval for this study involving patient material was obtained from the ethical committee from the LUMC (P11.165 NL 37682.058.11, and Biobank Infectious Diseases protocol 2020-03 & 2020-04 B20.002).

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Conflicts of Interest: None.

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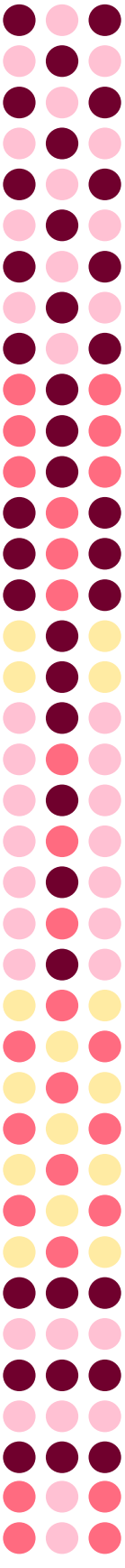

9

Intra-host evolution during relapsing parvovirus B19 infection in immunocompromised patients

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Submitted

Introduction

Parvovirus B19 (B19V) can cause severe relapsing episodes of anemia in immunocompromised individuals, which are commonly treated with intravenous immunoglobulins (IVIG). Few data is available on B19V intra-host evolution and the role of humoral immune selection. We studied the dynamics of genomic mutations and subsequent protein changes during relapsing infection.

Methods

Longitudinal plasma samples from immunocompromised patients with relapsing B19V infection in the period 2011-2019 were analyzed using whole genome sequencing to evaluate intra-host evolution. The impact of mutations on the 3D viral protein structure was determined by computational modeling.

Results

Of the three immunocompromised patients with relapsing infections for 3 to 9 months, one patient developed two consecutive nonsynonymous mutations in the VP1/2 region: T372S/T145S, and Q422L/Q195L. The first mutation was detected in multiple B19V IgG seropositive follow-up samples, and resolved after IgG seroreversion. Computational prediction of the VP1 3D structure of this mutant showed a conformational change in proximity of the antibody binding domain. No conformational changes were predicted for the other mutations detected.

Discussion

Analysis of relapsing B19V infections showed mutational changes occurring over time. Resulting amino acid changes were predicted to lead to a conformational capsid protein change in an IgG-seropositive patient. The impact of humoral response and IVIG treatment on B19V infections should be further investigated to understand viral evolution and potential immune escape.

INTRODUCTION

Parvovirus B19 (B19V) is a 5.6 kb, single-stranded DNA virus. The viral genome codes for three major proteins: the viral capsid consists of a VP1 and VP2 protein, which share part of their coding sequences, while the non-structural (NS) protein is involved in replication and cellular processes [1]. Most infections occur in childhood and their presentation in children with fever and rash is known as erythema infectiosum or 'fifth disease', a mild self-limiting disease. However, due to the unique tropism of B19V for erythroid progenitor cells, B19V may cause severe anemia in certain susceptible populations. In severely immunocompromised individuals such as stem-cell or solid organ transplantation patients, this may lead to the clinical syndrome of 'pure red cell aplasia' (PRCA). PRCA can be treated by administering of intravenous immunoglobulins (IVIG), which usually leads to a reduction of viral load and alleviation of symptoms [2]. Because this effect of IVIG disappears with decreasing IVIG titer, a pattern of recurrent infections may occur, each episode managed by IVIG until the patient's own immunity is restored [3].

In immunocompromised individuals, the inability to clear common self-limiting infections can lead to long term persistence of viruses, creating a reservoir in which mutants may arise. Immune escape mutants have been observed during prolonged infections with a variety of viral pathogens, such as SARS-CoV-2 and influenza virus, as well as latent DNA viruses including cytomegalovirus (CMV), during antiviral treatment [4-7]. Due to the relapsing nature of B19V in immunocompromised individuals, we hypothesize that new viral variants may also emerge in B19V infections over time.

Previous studies showed some genetic drift in prolonged B19V infection in patients treated with IVIG; small numbers of point mutations have been reported in single case reports or small case series [8-11]. These studies used conventional Sanger sequencing for analysis and mostly looked at partial genomes, focusing on capsid proteins. Over the past decade, the rapid development of whole-genome sequencing (WGS), has enabled more detailed evaluation of intra-host evolution of viral genomes [12, 13].

Here, we report on the intra-host evolution of B19V during relapsing infection in three immunocompromised patients. We determined genome-wide mutations in the B19V genome from plasma of these patients, and investigate *in silico* the potential conformational changes in the mutated viral proteins. Additionally, we provide a literature overview on intra-host and inter-host B19V genome evolution.

METHODS

Patients: All patients with a known relapsing or prolonged B19V infection were selected from the laboratory records of the Leiden University Medical Centre (LUMC) in the period 2011-2020. For each patient, four to six B19V DNA-positive serial samples from the laboratory archives were selected for WGS. Samples had previously been sent to the Clinical Microbiology Laboratory (CML) as part of routine patient care for B19V diagnostics, at the discretion of the treating physician. Plasma samples were stored at -80 ˚C until WGS analysis.

Ethics statement: This study has been approved by the Medical Ethics Committee of the LUMC (B20.002, Biobank Infectious Diseases 2020-03 and B20.014, 2020-04).

B19V PCR and whole genome sequencing: Viral loads were determined by quantitative PCR as previously described (14). Whole genome sequencing was performed using the Arc Bio Galileo Pathogen Solution kit, a complete kit and protocol for quantitative metagenomic detection of several DNA viruses in blood of immunocompromised patients, as previously described (15). In short, patient samples were spiked with an internal baculovirus control before extraction. Nucleic acids were extracted from plasma using the DNA and Viral NA small volume extraction kit on the MagNAPure 96 system (Roche diagnostics, Almere, The Netherlands). After concentration, library preparation was performed with the Galileo Viral Panel sequencing kit (Arc Bio (present: Cantata Bio), LLC, Cambridge, MA, USA) according to the manufacturer's instructions. Samples were sequenced using the NovaSeq 6000 platform (Illumina, San Diego, CA, USA) at GenomeScan B. V. (Leiden, the Netherlands).

Bioinformatic analysis: Sequence reads were demultiplexed using bcl2fastq (version 2.2.0) (Illumina, San Diego, CA, USA), resulting in FASTQ files. *De novo* assembly was performed using SPAdes (version 3.11.1). Contigs were mapped against B19V reference genome NC_000883.2. Threshold for nucleotide consensus was set at > 50%. FASTA files were uploaded in Geneious version 2024.0.3 for further comparative and phylogenetic analysis.

Phylogenetic analysis: All available (near-) complete genome sequences (4800 to 5596 bp) of taxonomy ID 10798 ('parvovirus B19') and taxonomy ID 344889 ('unclassified erythrovirus') were downloaded from the NCBI database. Clonal and artificial sequences were excluded. Alignments were created with Geneious (version 2024.0.3) A Neighbour-Joining phylogenetic tree (Jukes-Cantor model) was constructed. A second tree was constructed using the NS1-VP1/VP2 section of the genomes (≥4280 bp) in relation to European GenBank genotype 1 submissions.

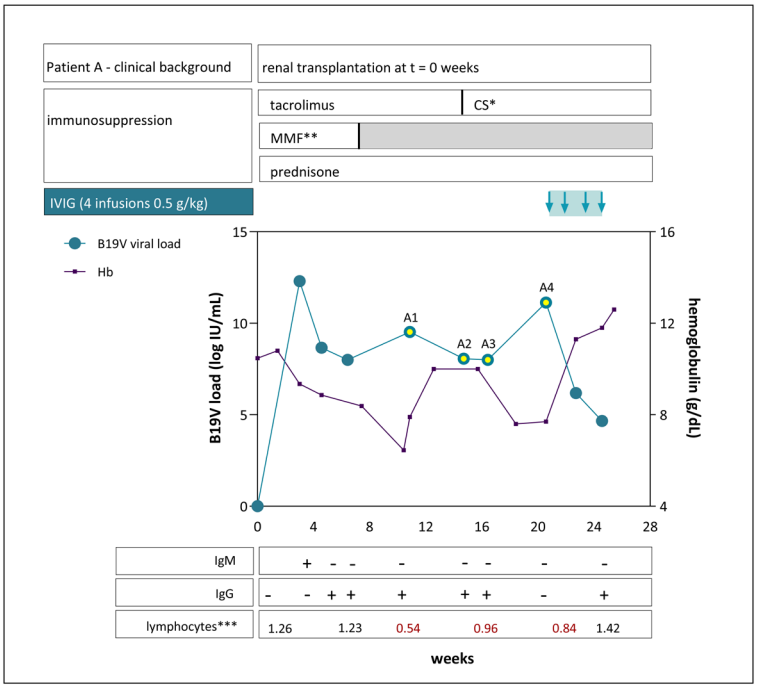
Protein structural modeling: Protein sequences with and without the mutations were subjected to protein structure prediction using Alphafold [16]. Mol* was used to visualize the predicted structures and map the predicted local distance difference test (pLDDT) values as heat color on the structure [17]. pLDDT is a per-residue measure of local confidence as predicted by AlphaFold with higher scores indicating a more accurate prediction. In addition to the structure predicted by AlphaFold, the computed pLDDT values reflect the molecular dynamic of the predicted structure [18]. Default parameters in AlphaFold were used after updating all required databases as of June 2023. The evaluation focused on the relaxed models.

RESULTS

In the period 2011-2020, three patients were identified with relapsing B19V infection. Clinical background, the course of viremia, hemoglobulin levels and immune parameters, including the presence of antibodies and lymphocyte counts, are shown in Figure 1. Patient A and B received a kidney transplantation at t = 0 weeks, at which time both patients tested negative for B19V viremia. Patient C received a stem cell transplantation (SCT) for Non-Hodgkin lymphoma (NHL) 5 months previously, and t = 0 represents the latest time-point before B19V viremia. During most of the sampling period, patients A and B were lymphopenic due to immunosuppressive treatment. While stable donor chimerism was initially achieved after SCT in patient C, lymphocyte function can be considered progressively impaired due to underlying progressive Non-Hodgkin lymphoma (NHL) after SCT from t = 0 onwards. This patient received donor lymphocyte infusions (DLI) as part of NHL treatment during the sample period. Patient A and B were B19V seronegative before transplantation. Patient C was B19V seropositive before transplantation, but the SCT donor was B19V seronegative. Patient A first became IgM-positive, then IgG-positive from 3 weeks after transplantation, but IgG reversion occurred between 20 and 24 weeks after transplantation. Patient B became IgG-positive after IVIG treatment at 12 weeks after transplantation. Patient C first became IgM-positive, then IgG-positive from t = 7 weeks.

In patients B and C, viral genomes remained unaltered over a course of 3 months and 6 months of follow-up, respectively: no (non-)synonymous mutations were detected by whole genome sequencing. In patient A, several mutations were identified over a course of 3 months. The first mutation, C3742G, occurred at 14

weeks of follow-up and remained present at 16 weeks of follow-up, while B19V IgG was positive, and was no longer detected in the sample at 20 weeks of follow-up when B19V IgG was negative (Figure 1). At 20 weeks of follow-up, mutation A3982T was detected. From this time point onwards, IVIG was administered and the patient started clearing the infection; no samples with sufficiently high loads were available for sequencing. These mutations were not detected at any time point in patients B and C. Table 1 shows an overview of the detected mutations and the corresponding amino acid changes in patient A.



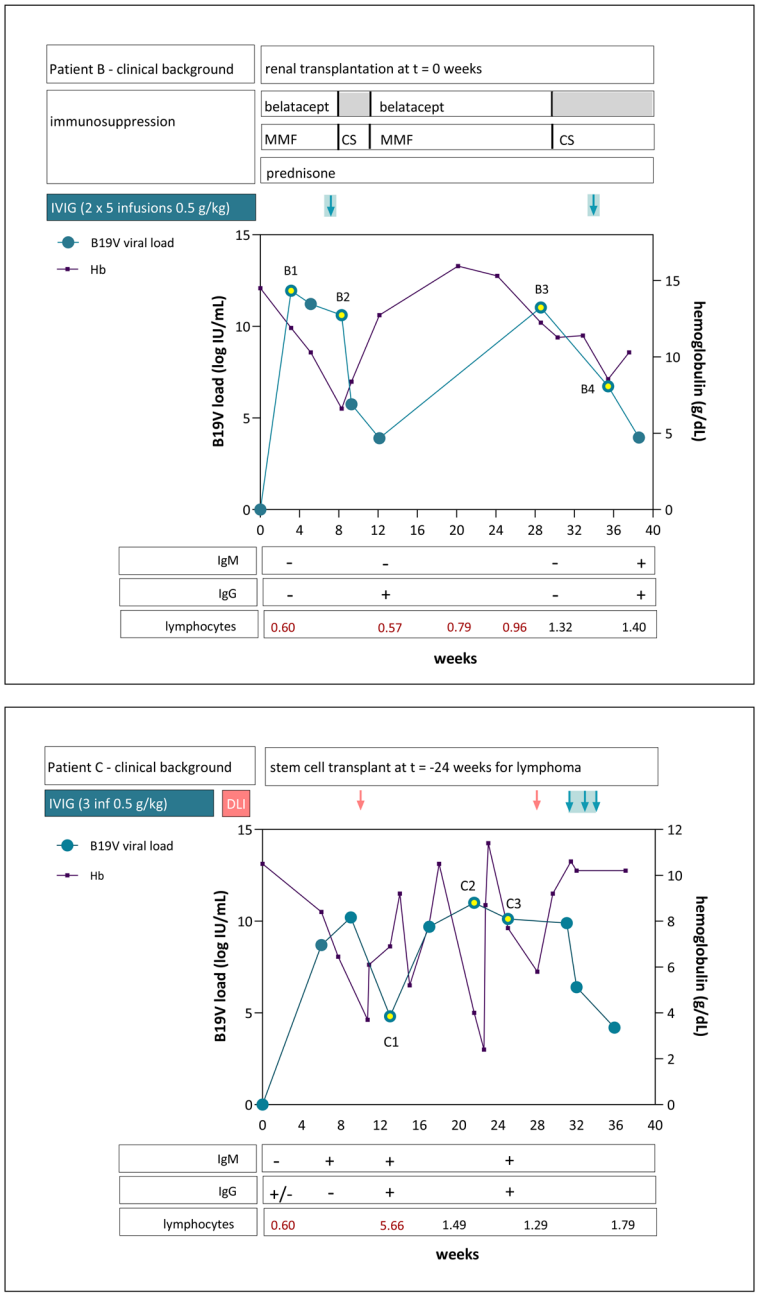


***Figure 1**: Clinical background, longitudinal course of laboratory parameters and treatment of 3 patients with relapsing B19V infection. *CS = ciclosporin; **MMF = mycophenolate mofetil; ***lymphocytes (x10E9/L), reference values 1-3.5*

Table 1: overview of nucleotide substitutions and associated amino-acid substitutions from a relapsing infection in patient A

		Nt substitution	AA substitution	AA substitution	AA substitution
	t		VP1	VP2	NS
Patient A	0 weeks				
	10 weeks	-	-	-	-
	14 weeks	C3742G	T372S	T145S	na*
	16 weeks	C3742G	T372S	T145S	na
	20 weeks	A3892T	Q422L	Q195L	na

Nt = nucleotide; AA = amino acid; Na = not applicable. No synonymous Nt substitutions were detected in patients A-C (whole genome).

To assess the impact of amino acid changes on the structure of the virus, protein structural modeling was performed on the mutated VP1 and VP2 proteins of B19V as detected in patient A. The C3742G mutation (patient A) translates into a T145S substitution in VP2 which had no predicted impact on its structure. Likewise, the A3892T mutation resulting in a Q422L/Q195L substitution was predicted not to impact VP1/VP2 structure. Notably, the C3742G mutation and associated amino-acid substitution T372S resulted in a predicted conformational change in VP1 – whilst modeling indicated no effect of the same mutation on VP2 conformation. Figure 2 shows the modelled protein structure of the baseline VP1/2 and the modelled protein structure with the amino-acid substitution T372S. The T372S structure shows an additional conformational loop in VP1. The high pLDDT-values resulting in the loop indicate that the T372S structure has changed from a static to a dynamic structure. The predicted additional loop appears adjacent to the primary antibody binding site (VP1u), suggesting that possible reduced antibody binding may be caused by the conformational changes and the resulting steric hindrance.

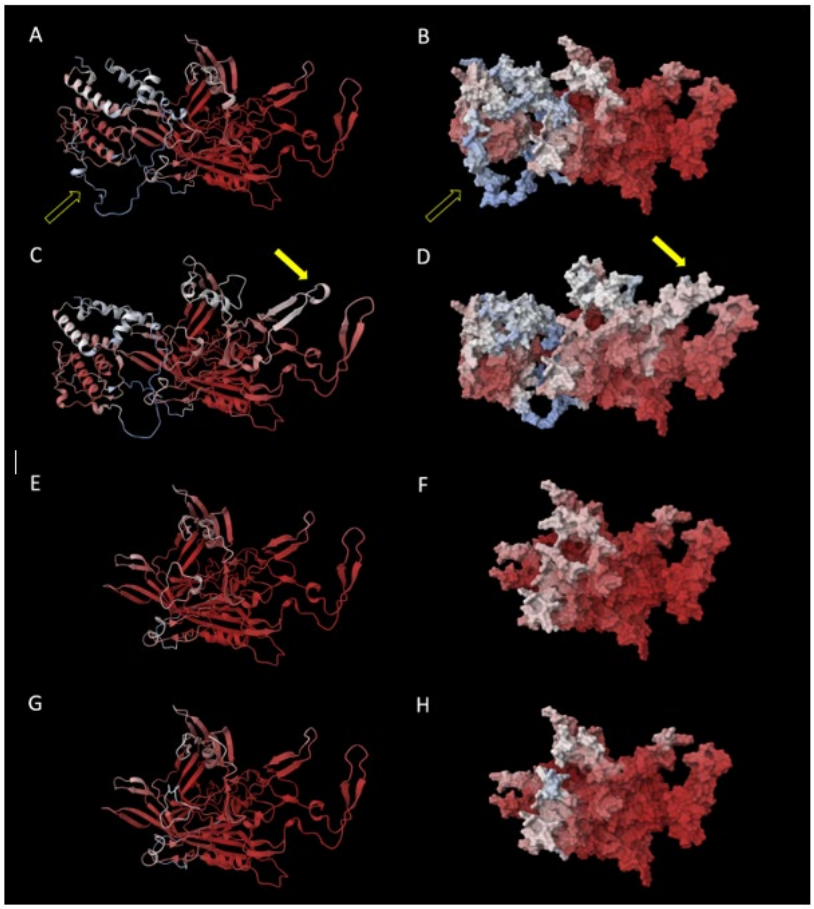


Figure 2: Protein structural modeling of the mutant VP1 and VP2 AA sequence from patient A*. 2A: baseline protein structure of VP1 as modelled from the AA sequence for time point A1 (cartoon representation), open arrow indicates VP1u-region (for a view of the entire VP1u-region, see supplementary figure S1); 2B: surface representation of 2A; 2C: protein structure of VP1 as modelled from the AA sequence with T372S substitution (timepoint A2), resulting in an additional loop marked by the closed yellow arrow. 2D: surface representation of 2C; 2E: baseline protein structure of VP2 as modelled from the AA sequence for time point A1 (cartoon representation); 2F: surface representation of 2E; 2G: protein structure of VP2 as modelled from the AA sequence with T372S substitution (timepoint A2), not resulting in structural changes; 2H: surface representation of 2G. The heat color indicate pLDDT values (see methods); red indicates high values.*

We also constructed a phylogenetic tree using (near) whole genome sequences from GenBank to assess divergence from other published B19V genotype 1-3 viruses (Figure 3). The viruses from patient A (2019) and B (2015) clustered with genotype 1a, while the virus from patient C (2011) clustered with genotype 3a. The viruses from patient A clustered most closely with relatively recent strains from Serbia (2011) and other recent viruses from France (2017). The viruses from patient B were relatively distant from patient A, but still clustered with genotype 1a. Patient C was infected with genotype 3, which is uncommon in Europe and it is unclear how this patient got infected with a genotype 3 strain.

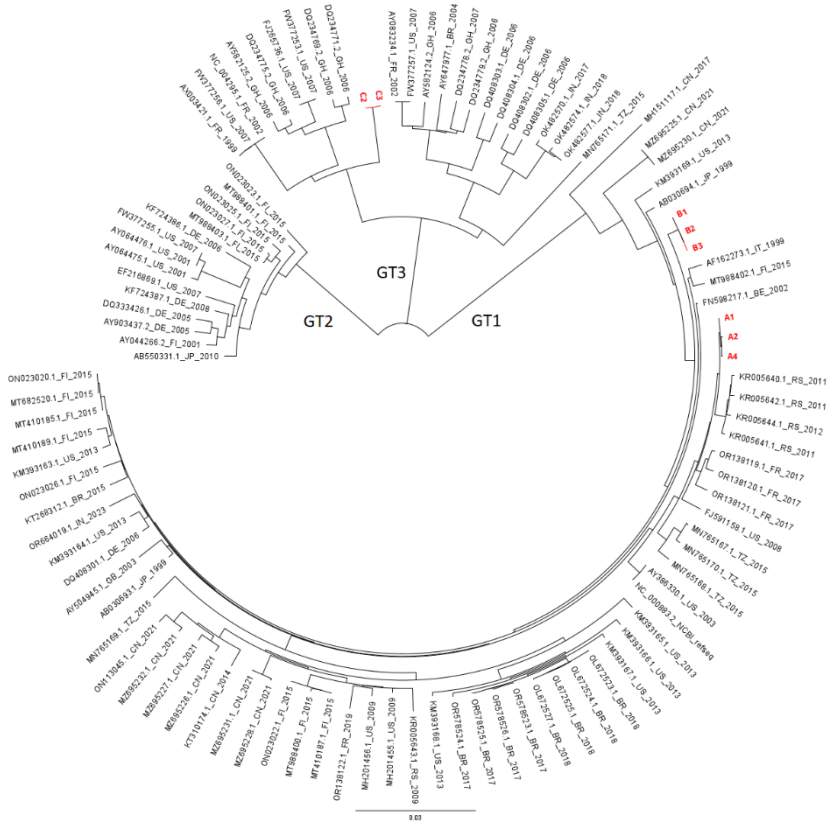


Figure 3: Phylogenetic analysis of patient's A and B sequences and global B19V GenBank strains. *Selected GenBank strains > 4800 bp of genotype 1, 2 and 3. GenBank strains are denoted by GenBank entries followed by country (2-digit code according to ISO-3166-1 alpha 2 country codes) and year of isolation.*

To assess the relation to other local strains, a separate tree was constructed using European GenBank sequences, analyzing a subregion of the genome (4280 bp) which includes the complete NS1-VP1/2 fragments. The viruses from patient A and B clustered closely with other national strains within genotype 1a (supplemental figure S2). As there were very few European GenBank entries for genotype 3, patient C is not included in this analysis.

DISCUSSION

In this study, we investigated the intra-host evolution of B19V in immunocompromised patients with relapsing B19V infection. In two patients, the viral genome remained stable over several months, despite multiple episodes of intense replication and treatment with IVIG during the sample period in one of the patients. In one patient, two non-synonymous/missense mutations were detected in the VP1/VP2 region, resulting in AA substitutions in 4 locations. These mutations have not previously been described in the literature (table 1) and were not present in GenBank sequences used in our study. Modeling of protein folding predicted that one of these mutations (C3742G) would result in a conformational change in VP1.

The B19V capsid is composed of VP1 (5%) and VP2 (95%), while the NS protein is not expressed on the surface. Therefore, VP1 and VP2 are considered the most antigenic, which has also been shown in immunization assays [19, 20]. The main antigenic domain is thought to be the 'VP1 unique region' (VP1u), a protein structure consisting of 227 amino acids on the B19V capsid involved in receptor binding (see figure 2 and supplemental figure S1) [21]. We hypothesized that humoral immune pressure and specifically IVIG might be drivers for B19V evolution and we expected that changes would most likely occur in the VP1u-region. Although we did observe mutations under humoral immune pressure, these mutations did not occur in the region coding for VP1u. Also, modeling of protein folding predicted a conformational change in VP1 but not at the site of VP1u (figure 2). However, the conformational change indicates a change from a static to a dynamic structure, and it is not yet clear how this may affect the interaction with surrounding molecules. To assess the possible role of the predicted conformational change in humoral immune evasion, its position in relation to the complete capsid structure should be evaluated. Future research should therefore firstly focus on changes in capsid structure. Subsequently, molecular docking studies, in depth molecular dynamic simulations and

in vitro binding studies would be needed to understand the impact of the predicted molecular changes on e.g. antibody binding.

In patient A the first mutations emerged when the patient was B19V IgG-seropositive. The serological profile of patient A indicated a pattern most compatible with natural immunity, the appearance (and disappearance) of IgM followed by prolonged detection of strong-positive signals of IgG. Remarkably, IgG-seroreversion occurred shortly after the appearance of mutations (fig 1). The patient had not yet received IVIG or other blood products that may explain transient IgG-positivity, so most likely this indicates a loss of natural immunity, which may be considered rare. In patient B, who was sampled during IVIG treatment, no mutations occurred. Patient C was not sampled during IVIG-treatment but did receive DLI during the sampling period. This might have resulted in a decrease in viral load (although the SCT donor was B19V seronegative) (Figure 1). Notably, this primarily T-cell mediated treatment did apparently not lead to selection of variants. These results suggest that natural humoral immune pressure might play a more important role in intra-host evolution than IVIG and T-cell-mediated immunity.

Previous studies on B19V intra-host evolution have shown varying results. Table 2 shows an overview of available literature on B19V intra-host evolution [8-11, 22]. One study on intra-host evolution found a relatively high mutation rate compared to other studies [9]. In this study by Hung et al, B19V longstanding infection was studied in three AIDS patients and a large number of single nucleotide polymorphisms (SNPs) were observed during a maximum follow-up of 11 months. Remarkably, the large number of mutations only occurred in the two patients treated with HAART. Only one mutation was found to appear under IVIG treatment. Of note, none of the new strains became dominant. In our study, we observed a disappearance of the nonsynonymous substitution after seroreversion at 18 weeks of follow-up. Combined with the other studies in which relatively few changes in the viral genome were detected in long-term infection and reversion of these changes in the course of follow-up, these studies suggest a relatively high level of genetic stability. As we only found mutations occurring while natural immunity was developing, it could be hypothesized that humoral immune selection is stronger with natural immunity than with IVIG treatment. As we investigated a limited patient population, further studies with larger patient populations would be required to investigate this hypothesis.

Table 2: literature overview of studies on intra-host evolution of B19V

First author	Year	Study population	B19V treatment	Method	Nucleotide region sequenced	Follow-up period	results
Gallinella	1996	1 patient; chronic anemia	nm*	Sanger	2400-3400	16 months	No changes in viral genome
Plentz	2004	1 patient; bone marrow transplantation	IVIG	nm	nm	8 months	3 lasting changes after temporary variations: T3463C C4852G T4867C
Hung	2006	3 patients; AIDS	IVIG (n=3); HAART (n=2)	Sanger	436 – 2431; 3125- 4283	11 months	Pt 1: A3271C Pt 2: 18 SNP (14 N**) Pt 3: 15 SNP (9 N)
Suzuki	2014	1 pediatric patient; cord blood transplantation	IVIG	Sanger	602-5014	29 months	6 SNP T/C941T T/C1037T A1048A/G (N) T1112C T1118T/C A1266A/G (N)
Jain	2018	13 pediatric hematological malignancy patients followed up; 3 patients with at least one mutation detected	nm	Sanger	1747-2691	6 months	Pt 1: G579A Pt 2: C577G Pt 3: C672G

*nm = not mentioned; **N = nonsynonymous mutation

Whole-genome phylogenetic analysis on the viruses of our patients showed that our viral sequences clustered with other circulating viral strains, suggesting these are representative strains for these regions and related to other Dutch and European strains. Studies on inter-host evolution fairly consistently report a substitution rate of ~1 x 10^{-4} substitutions per site per year (s/s/y) for B19V, with highest s/s/y reported for the capsid sequences VP1 and VP2 (for an overview of studies, see supplementary table 1) (8, 23-28, 31). This is a relatively high substitution rate for DNA viruses, as substitution rates for other DNA viruses are estimated at 10^{-5} to 10^{-9} s/s/y, although with higher s/s/y for single-stranded DNA viruses (such as B19V) [29]. However, these studies on B19V are based on a relatively short period of observance which may cause an overestimation of mutation rate [30]. From an evolutionary point-of-view, B19V intra-host genomic stability is plausible considering the long-term history that B19V and mankind share, as B19V was already demonstrated in 7000-year old human bone samples [27]. In these studies investigating ancient DNA, substitution rates between 1.02-1.22 x 10^{-5} s/s/y were reported [27, 31].

Despite the observed relative genetic stability on population level, much remains unknown on intra-host viral evolution. B19V relapsing infections are a relatively new phenomenon. It is only in the last decades that the circumstances in which B19V may relapse have become present; the development of transplantation medicine has created the existence of consistently severely immunocompromised hosts that may form a reservoir for viral persistence and evolution. In addition, specific treatments such as IVIG may still add to selection pressure and data on intra-host evolution are still scarce for B19V. We recommend to monitor genome changes and their structural impact on the virus in larger series to increase our understanding of viral evolution under these relatively new circumstances.

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Conflict of interest: none reported.

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Supplementary information

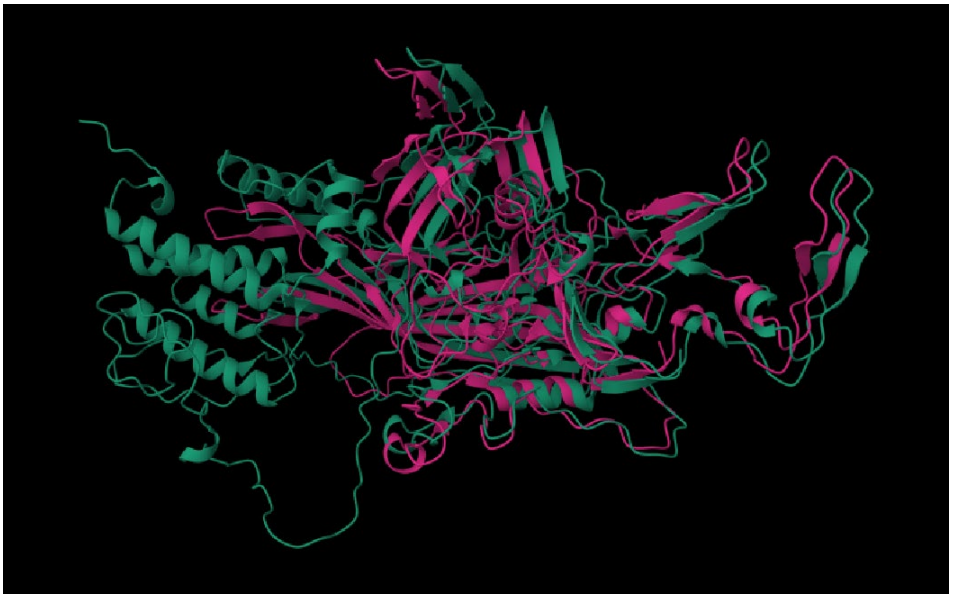


***Figure S1**: Protein structural modeling of VP1 (green) and VP2 (pink) in a superimposed view. The area of VP1 that does not overlap with VP2 is the VP1-unique region, which holds the receptor-binding domain.*

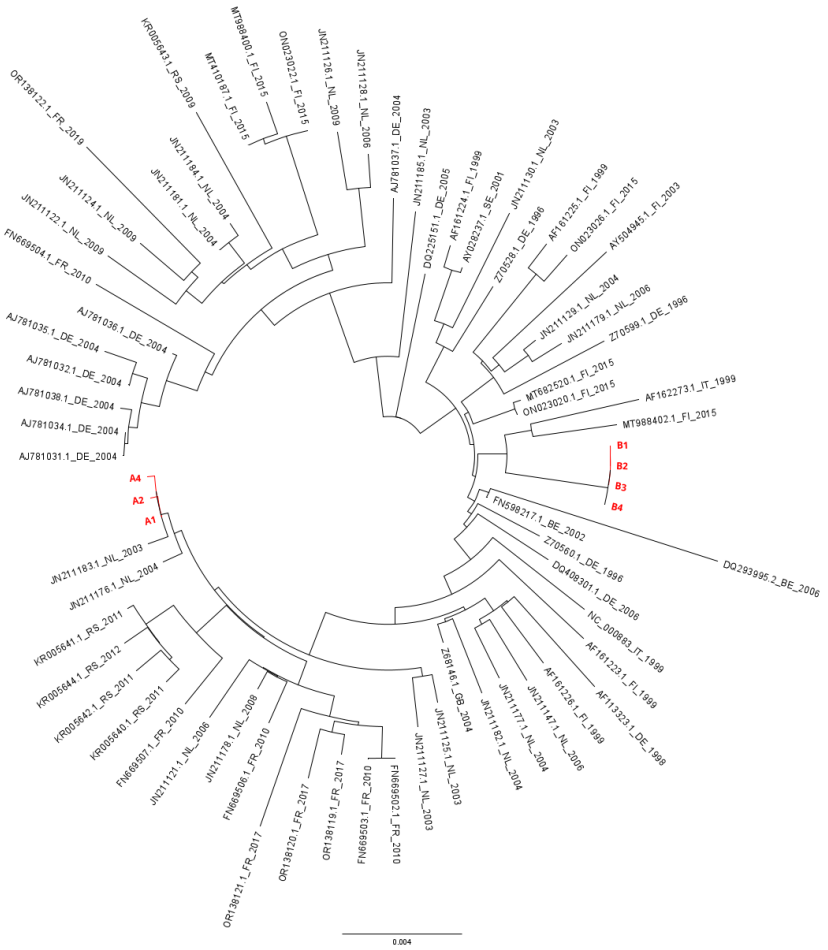
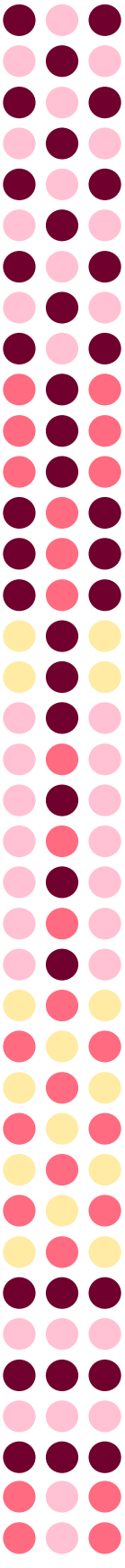


***Figure S2**: Phylogenetic analysis of NS1,VP1/2 (4280 bp) of study isolates in relation to other European genotype 1 strains. GenBank strains are denoted by GenBank entries followed by country (2-digit code according to ISO-3166-1 alpha 2 country codes) and year of isolation.*

Supplementary table 1: literature overview of studies on population evolution of B19V

First author	year	Study population	Geographical sampling location	Sampling period	Sequencing method	Results
Gallinella	1996	Blood samples from 7 known B19V patients; nt 2400-3400 (VP) sequenced	Italy	1989-1994	Sanger	No s/s/y* reported; divergence rate within geographic cluster similar to global divergence
Shackelton	2006	Data set B19V genotype 1: 38 VP1 sequences; 27 NS1-VP1 sequences	Europe (England, Germany, Finland, Ireland, Sweden); USA; Brazil; Japan; Korea; China	1973-2001	Gen Bank sequences	Both VP1 and NS1: ~1 x 10^{-4} nucleotide s/s/y
Norja	2008	63 blood or autopsy samples; nt 3242-4612 (VP) sequenced	Europe (Germany, Finland, UK)	1980-2007	Sanger	4 x 10^{-4} s/s/y
Suzuki	2008	104 NS1-VP1 junction sequences	Sapporo (Japan)	1980-2008	Sanger	No s/s/y reported; 2 distinct patterns observed in small region: 1. accumulation of SNP and 2. Sudden replacement of strains
Toppinen	2015	43 bone samples^; part of NS1/VP sequenced	Finland	WWII casualties	Sanger (?)	1.1-3.2 x 10^{-4} s/s/y
Stamenkovic	2016	137 Gen Bank sequences nt 665-4851	Europe; USA; Brazil; Japan; Vietnam	1973-2012	Gen Bank sequences	1.03 x10^{-4} s/s/y
Mühlemann	2018	10 samples of preserved ancient B19V from teeth (63.9 -99.7% coverage)	Eurasia; South-East Asia; Greenland	~4800 BC – 1000 AD	NGS	1.22 x 10^{-5} s/s/year (strict clock)
Guzman-Solís	2021	3 samples of preserved ancient B19V from teeth (92.4-99.1% coverage)	Mexico	16th- 18th century	NGS	1.03 x 10^{-5} s/s/y (strict clock)

*s/s/y = substitutions/site/year; ^bone material from WWII casualty graves



10

General discussion

GENERAL DISCUSSION

Although parvovirus B19 (B19V) has been proven to coexist with humans for over millennia, we are only aware of our relationship with this virus since 1975, when B19V was discovered by Yvonne Cossart and fellow scientists [1, 2]. In the years following its discovery, it became clear that B19V was responsible for an array of disease presentations, all related to its tropism for erythroid precursor cells. Although disease proved mostly to be mild (fifth disease), in unborn children the infection can prove lethal, and serious manifestations are also seen in other patient groups. In this thesis, B19V infections have been evaluated from a broad perspective. This discussion aims to describe the insights obtained from the research, its clinical consequences and to provide an outlook for future directions. The discussion will focus on four different aspects of this viral infection, which were studied in this thesis: B19V epidemiology and its relation to intrauterine infections (chapter 2, 3 and 4); the significance of B19V detection in blood (chapter 4, 5, 6 and 7); the role of next-generation sequencing in B19V diagnostics (chapter 8 and 9) and the role of B19V in immunocompromised individuals (chapter 7, 8 and 9). In addition, the future perspective is discussed.

B19V epidemiology and its relation to intrauterine infections

B19V is a ubiquitous virus and is widely transmitted in the general population, generally with little consequences. On the other hand, B19V has the potential to cause very severe, but treatable, disease. Therefore, insight into B19V epidemiology is essential to understand its disease burden, in particular the risk for unborn children. **Chapter 2** analyses B19V epidemiology, including the influence of the COVID-19 restrictions, but also taking into account the three preceding decades. It was shown that the incidence of B19V and its seasonality had decreased in the past decade up until the spring of 2023. A decrease in seroprevalence merits attention, because it may lead to an increased risk for primary infection at childbearing age, i.e. possibly during pregnancy, which may lead to an increase in intrauterine or congenital infections. For herpes simplex virus, both a decreasing seroprevalence and a higher incidence of neonatal herpes have been observed in the last decades in the Netherlands [3, 4]. A disturbance of the traditional epidemiological patterns of B19V infection, leading to a decrease in seroprevalence, especially at childbearing age, could have the same consequences. It is therefore advisable to regularly monitor B19V seroprevalence to assess the risk of B19V infection during pregnancy.

Chapter 2 also showed that the incidence of severe intrauterine B19V infection correlates with the incidence in the general population. It is therefore expected that extreme epidemic years will cause extreme fetal morbidity, which is also shown to occur in **chapter 3**. At the time of completion of this thesis, in the summer of 2024, the largest outbreak of B19V has been recorded since the beginning of monitoring by the Dutch Sentinel Surveillance, as predicted in **chapter 2** and demonstrated in **chapter 3**. In the first months of 2024, a record number of pregnant women with B19V infection were followed up at the Division of Fetal Therapy of the LUMC. Such outbreaks in the general population after the COVID-19 pandemic are also being recorded in other countries [5-8]. This has led to the National Institute of Public Health (RIVM) and the Division of Fetal Therapy at the LUMC issuing alerts to all Dutch prenatal care providers to be vigilant for B19V infection during pregnancy. However, as B19V infections may present with few or aspecific symptoms, many B19V infections can go unnoticed. Apart from severe presentations, including fetal hydrops, we are not well informed about the additional burden of disease, either in pregnancy or later in life. Considering B19V is such a ubiquitous virus, it would be advisable to perform more research in this area. The relative rarity of severe intrauterine infection has also prevented definite conclusions from being drawn on long-term consequences, i.e. neurodevelopmental outcome, of B19V infection. The COVID-19 restrictions created extraordinary circumstances for decreased virus circulation and the resulting upsurge of intrauterine B19V infection is unprecedented. It is not expected that such an epidemic will readily repeat itself. Therefore, it is strongly advised to study the long-term consequences of B19V infection in the newborns born in this epidemic, as this epidemic provides an opportunity for sufficiently powered research into the long-term consequences.

To study the epidemiology and disease burden, neonatal blood in the form of dried blood spots (DBS), as routinely obtained from all newborns, has been proven very useful for other intrauterine infections in particular congenital cytomegalovirus infection (cCMV) [9, 10]. **Chapter 4** showed that B19V PCR applied to DBS can be used to diagnose intrauterine infection, but it also showed this method lacked sensitivity, especially when infection occurred in the first half of pregnancy. This is comparable to the reported sensitivity of DBS testing for cCMV, but for screening purposes, high sensitivity testing is desirable [11]. In addition, DBS are stored at the National Institute of Public Health (RIVM) for a standard period of five years. Due to the

oscillating yet possibly decreasing incidence of B19V as described in **chapter 2**, this period may not be long enough and may not cover sufficient epidemic years to perform reliable retrospective studies on the incidence of B19V in the general population. This practical drawback, in combination with a suboptimal sensitivity of DBS, makes it interesting to explore other methods for diagnosing intrauterine infection.

Recently, there have been developments in screening maternal plasma samples for infection during pregnancy by NGS, next to prenatal screening for genetic abnormalities. In this way, high viral B19V loads were detected in non-invasive prenatal testing (NIPT)-samples [12, 13]. The obvious advantage of using maternal samples during pregnancy for diagnostics is the possibility to act on the diagnostic test in the interest of the individual patient, in addition to using the data for national prevalence studies. Although the need for prenatal universal screening is at present debatable in B19V infection due to the rarity of severe intrauterine infection and the relatively unknown long-term consequences, it should be considered a missed opportunity if data on active maternal B19V infection are present in NIPT-samples but not acted upon. This becomes especially urgent in the case of extreme epidemic years such as 2024, when intrauterine infections are not so rare at all. In addition, as NIPT-testing is offered free of cost to all pregnant women in the Netherlands since April 1st 2023, the opportunity for screening for B19V in pregnancy may be closer than we think.

Significance of B19V DNA detection in blood: the need and ability to differentiate between active and past infection

At the end of the 20th century, the development and introduction of PCR diagnostics in routine patient care sparked a true microbiological diagnostic revolution. PCR diagnostics resolved many diagnostic issues (e.g. detection of pathogens that were hardly detectable by conventional culture systems, including many viruses), but in the field of B19V a new diagnostic issue was created. It became clear that B19V DNA could be detected by PCR in many body compartments and in blood for a long time after the infection occurred. The presence of B19V DNA in certain diseased tissues has often been interpreted as the cause of the disease, for example in cardiomyopathy. On the other hand, the abundance of B19V DNA in non-diseased tissue contradicts a causal relationship [14]. Debates on the interpretation of such PCR-positive findings ensued and continue to this day. To contribute to this rather unique debate was one of the intentions of the work in this thesis.

One new approach that is introduced in this thesis is an enzymatic method, namely an endonuclease assay, to differentiate between the presence of intact viral particles and DNA remnants, which would be useful in the aforementioned debate. This technique is applied in chapters 5 and 6. In **chapter 5**, the application of endonuclease is evaluated in a 'proof of principle' study. It was shown that in the early phase of infection intact viral particles were present, while in a later phase of infection only DNA remnants were detectable in plasma. The viral load level corresponded with the presence of intact viral particles versus DNA remnants. This finding made it clear that low-load B19V positive blood donations are not infectious, because they do not contain intact viral particles. In addition, it followed that detection of B19V DNA in peripheral blood does not always signify active infection.

To investigate the role of B19V in patients suffering from cardiomyopathy, the nature of the B19V DNAemia that may be found in these patients is studied in **chapter 6**. Our results showed that all DNAemia in this study population is caused by DNA remnants, whereas all DNAemia in a control population with active infection was from viral particles. The commonly assumed causal relationship between B19V and cardiomyopathy was previously proven to be at least doubtful based on the occurrence of B19V in cardiac tissue of healthy controls; this research provided additional virological arguments to doubt this association. Furthermore, it is remarkable that a substantial percentage of cardiomyopathy patients in this study population (33%) showed DNAemia while the prevalence of DNAemia in a population of healthy blood donors is only around 1% [15-17]. Therefore, we hypothesized that B19V DNA detectable in blood is released from damaged cells, meaning cardiac cells in this population, and could be considered a marker for tissue damage, rather than the cause of the disease.

The identification of DNA remnants as opposed to viral particles was also shown in **chapter 7**, where we explored the course and significance of DNAemia in kidney transplant patients. B19V may cause clinically relevant anemia in kidney transplant patients, but it is important to identify those patients with true B19V-driven anemia and patients in whom only B19V DNA remnants are present. We first evaluated the prevalence of B19V DNAemia after kidney transplantation and then determined whether intact viral particles or mere DNA remnants were present. In our cohort, it was remarkable that both patients in whom B19V DNA remnants were detected also had concomitant BK reactivation. BK reactivation was also associated with B19V

DNAemia in a large study on B19V in renal transplant patients [18]. This would again be compatible with the hypothesis that B19V DNA, released by injured tissue may serve as a marker for tissue damage, in this case the transplanted kidney.

In these studies, the endonuclease assay proved to be a robust and very practical tool to evaluate viral intactness in patient samples. Endonuclease was previously been evaluated as a marker for viral activity, but these applications have been restricted to infectivity studies in the food safety area. For example, DNase and RNase treatments have been used before to determine the infectivity of norovirus or hepatitis A virus on food products [19, 20]. The studies described in this thesis provide opportunities to evaluate the application of these assays in clinical samples and patient management, which are not restricted to B19V research. An example of a different useful clinical application is the application of RNase on SARS-CoV-2 low-load positive samples, to differentiate between early, transmissible (intact virus) or late, non-transmissible infection (RNA remnants). An exploratory study showed the potential in differentiating between intact viral particles and viral remnants [21]. In epidemic or pandemic situations this distinction is of great importance for infection prevention strategies.

The role of next-generation sequencing in B19V diagnostics

After the revolution sparked by PCR diagnostics, a second molecular microbiological revolution was triggered by the development of next-generation sequencing (NGS). NGS has broadened and deepened the molecular playing field by its ability to provide a diagnostic catch-all approach and an in-depth analysis of microbial populations [22]. A present shortcoming of NGS however, is its limited ability to accurately determine the precise quantities of any target, like the viral load in samples [23]. In **chapter 8,** we evaluated an NGS method that could accurately measure viral load, not only for B19V but also for other relevant DNA viruses. In this way, a new diagnostic tool is now available to determine the significance of B19V DNA in a variety of clinical situations, including the aforementioned detection of viral DNA associated release from tissue damage: in these cases the viral load is significantly lower than in cases with ongoing viral replication.

Accurate viral quantification is particularly relevant since metagenomic NGS (mNGS) is increasingly being applied in clinical diagnostics; precisely because of its catch-all approach. Due to the abundance of B19V DNA in all humans who have

been infected with B19V at a certain point in life, it is likely that B19V DNA will manifest even more as a chance finding in the future [24]. Detection combined with quantification is essential to interpret the significance of such chance findings; when detection and quantification are performed simultaneously, diagnostic delays are prevented and this will contribute to clinically relevant application of NGS.

As mentioned before, NGS can provide much more in-depth sequencing than traditional Sanger sequencing and thus has the ability to analyze subpopulations or quasispecies. This has emerged as an important tool in the study of viral evolution, and NGS is progressively establishing itself as a new standard in diagnosis of viral drug resistance or immune escape for a variety of viruses [25-27]. In **chapter 9**, we studied intra-host evolution of B19V. In this study, we indeed observed the occurrence and subsequent disappearance of dominant genetic mutations under immune pressure, although we did not look into the appearance of (transient) subpopulations. For a complete understanding of evolutionary dynamics, analysis of subpopulations with NGS should be included in such studies.

B19V and its role in the immunocompromised host

An important patient group in which B19V has great clinical implications, are the severely immunocompromised, in particular solid organ or stem cell transplant patients. . This group is still growing in number, as the possibilities for transplantation and immunosuppressive treatment continue to develop. For example, the number of kidney transplantations worldwide has increased from ~73.000 in 2010 to ~102.000 in 2022 [28]. B19V may cause pure red cell aplasia (PRCA) in immunocompromised individuals, which can be managed by tapering of immunosuppressive treatment (if applicable) and treatment with IVIG. However, as long as natural immunity does not develop, B19V infection may lead to relapsing infection, as is also demonstrated in **chapter 7** and **8**.

Multiple aspects need to be considered around B19V detection in individual transplant patients. Firstly, this unique relapsing presentation of infection requires even more attention to the significance of B19V DNA detection in this group of patients. In otherwise healthy individuals, low viral loads can be interpreted as a sign of past B19V infection. In transplant patients, it should be considered that a low viral load may still be a foreboding of a relapse. The usefulness of accurate viral load

quantification was also demonstrated in **chapter 8**. Continuous virological and serological follow-up is necessary to monitor the development of natural immunity.

Secondly, it should be considered that together with the transplant organ, persisting B19V DNA may be transferred from the donor to the recipient and that this does not have to lead to productive infection (the viral DNA being viral remnants). In **chapter 7**, we demonstrate that the source of B19V DNA in a seronegative recipient is very likely the transplant kidney. Such persons should not be considered to be truly exposed to B19V infection when they were seronegative before transplantation, as these remnants do not trigger an immune response. Again, virological follow-up by means of viral load quantification, viral intactness assays and serology are necessary to be able to differentiate between active infection and past infection (of the donor, in the case of passive DNA transfer). In addition, it should be taken into account that the presence of B19V viral remnants in blood may indicate damage to the donor kidney, not necessarily caused by B19V, especially in seronegative patients, as also suggested in chapter 6 and supported by findings from literature [29].

Thirdly, the unique relapsing nature of B19V infections in these patients leads to a situation in which high-level viral replication repeatedly occurs under humoral immune pressure, likely due, at least in part, to the administration of IVIG. The viral genomic consequences of this situation are studied in **chapter 9,** where we witnessed genetic changes occurring after natural antibody production (and subsequent seroreversion). Relapsing B19V-infection thus resembles the situation in which immunocompromised patients fail to clear common self-limiting viruses. For SARS-CoV-2, this situation has been suggested to create a reservoir for the emergence of variants of concern [30]. As B19V is also a respiratory-transmitted, ubiquitous virus, studying intra-host evolution may be useful to track changes in pathogenicity or transmission routes. Although B19V genomes should probably be considered more stable than previously estimated and B19V genotypes have not been shown to differ in pathogenicity or transmission routes [31], relapsing infection is still a relatively new phenomenon occurring in an ever increasing group of immunocompromised individuals and it merits further monitoring.

Future perspectives

Although this thesis provided insight into the clinical relevance of B19V DNAemia, a knowledge gap remains regarding the exact nature of B19V persistence in tissue. Although multiple studies have shown that B19V uptake is possible in many cell

types, without subsequent viral replication, it is still uncertain where exactly B19V DNA hides [32]. Further studies should concentrate on elucidating the mechanism responsible for B19V persistence and the subsequent release of B19V DNA remnants into the bloodstream. Preferably, a study should be conducted comparing B19V activity in compartments of proven viral replication, e.g. bone marrow, with other compartments of interest, e.g. myocardial tissue. Several techniques can be used to determine and compare viral activity in tissue. These techniques could include a transcriptomics approach: recently progress has been made in in-situ polyadenylation assays, where viral activity has been visualized by in-situ mRNA sequencing analysis in myocardial tissue in experimental myocarditis in a murine model [33]. A different approach could be a visualization of processes in the cell. For SARS-CoV-2 it has been made possible to visualize the cellular processes involved in RNA synthesis and export to the cytosol [34]. The same type of research could possibly be employed for B19V, although an in vitro system with replicating B19V viral particles is required and in vivo application, i.e. visualizing replicative activity in tissues, would be very challenging.

Apart from the debate on the causal role of B19V in a number of disease presentations, a related debate still exists about the role of B19V as a trigger for auto-immune processes. The studies in this thesis have shown that the mere detection of B19V should not be confused with viral activity; however, much research has also focused on the role of the presence of persisting B19V DNA in human cells in triggering certain cellular auto-immune processes [14, 35-37]. Clear and consistent support for a role of B19V in autoimmunity is thus far lacking, but much remains unknown about possible pathophysiological processes in the cell that persisting B19V DNA may trigger. Elucidating the mechanism and form of persisting B19V DNA in the cell is a first step towards understanding the possible immune effects persisting B19V may have. Obviously, this is not exclusive to B19V, but understanding the interplay between viral persistence and immunological reactions should be considered a relevant study topic for the entire virome, which we are only beginning to grasp [38, 39].

Concluding remarks

The widespread occurrence of B19V infections in the general population, characterized by epidemic episodes, and their serious consequences in pregnancy, as well as in other patient groups, warrant due attention to B19V epidemics and their

consequences. However, as the infection can go unnoticed, there is a challenge in finding the balance between this widespread occurrence and the timely diagnosis of severe consequences of B19V infections in pregnancy, which remain relatively rare even during regular epidemics. In the future, the catch-all approach of NGS may aid in timely identification of B19V infections during pregnancy.

Interpreting B19V diagnostics has proven difficult in certain settings. This is evident from long-standing doubtful disease associations. Also in routine diagnostics, B19V DNA detection may be misinterpreted as active infection. The results described in this thesis have contributed to a deeper understanding of the significance of B19V DNA detection with very practical applications and implications. These findings may not only concern B19V but can be studied in other pathogens. The endonuclease assay contributes to a correct understanding of the clinical significance of B19V DNA detection. It is advisable that this easy-to-implement laboratory assay finds its way into the work-up of difficult-to-interpret B19V diagnostic results. This is especially advisable in kidney transplant patients, as PCR-positivity and disease burden of B19V appear to be highest in this particular group of transplant patients. At present, for complex cases, there is no single definitive test to differentiate between active infection or past infection and the interpretation of the various tools applied must be done in dialogue between the medical microbiologist and the clinician.

Despite these advances, challenges still exist in understanding the exact nature of B19V persistence. Once it becomes clear what form B19V DNA hides in, this will be an incremental step forward to understanding B19V host-pathogen interactions. In the past years, it has become clear that many more viruses apart from B19V are simply present in the host. Progress in understanding B19V persistence may ultimately also be helpful in understanding our coexistence with our entire virome.

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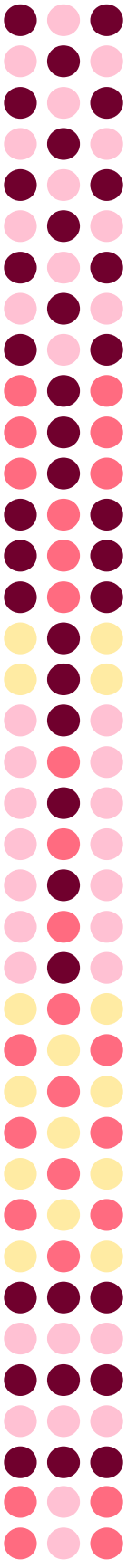

Addendum

NEDERLANDSE SAMENVATTING

Achtergrond

Parvovirus B19 (B19V) werd ontdekt in 1975. Het is naar virologische begrippen een klein virus, wat het naamsdeel 'parvo' verklaart ('parvus' betekent 'klein' in het Latijn). B19V werd als bijvangst ontdekt in de zoektocht naar een geschikte test voor hepatitis B virus, en is vernoemd naar het nummer van het testpanel waarin het virus aangetoond werd (nummer B19).

In latere jaren werd aangetoond dat B19V primair verantwoordelijk is voor het ziektebeeld 'vijfde ziekte', een koortsende ziekte op kinderleeftijd. Kinderen kunnen hierbij naast koorts ook kenmerkende vuurrode wangen (de zogenaamde 'slapped cheeks') en een fijnmazige uitslag op het lichaam ontwikkelen. Hierbij treedt spontaan herstel op. De infectie kan ook geheel zonder symptomen verlopen. Ongeveer 70% van de bevolking heeft op volwassen leeftijd B19V doorgemaakt. Na doorgemaakte infectie, is men levenslang beschermd tegen een nieuwe infectie met B19V.

Naast de vijfde ziekte, kan B19V in bepaalde patiëntgroepen ook ernstiger ziektebeelden veroorzaken. B19V infecteert de voorlopercellen van rode bloedcellen in het beenmerg. Rode bloedcellen zijn onderdeel van een cyclus waarbij voortdurend rode bloedcellen sterven en aangevuld worden vanuit nieuw aangemaakte cellen uit het beenmerg. Infectie met B19V zorgt ervoor dat de voorlopercellen sterven, en er dus tijdelijk geen nieuwe aanmaak plaatsvindt. In gezonde personen heeft dit geen merkbaar effect; er zijn genoeg gezonde rode bloedcellen om de tijdelijke stop in aanmaak op te vangen. Er zijn echter enkele patiëntgroepen waarbij de stop in aanmaak wel merkbaar en relevant kan zijn. Zij ontwikkelen dan een (ernstige) bloedarmoede. Dit zijn:

- Personen die om andere redenen al een gestoorde bloedaanmaak of een verhoogde bloedafbraak hebben (zoals patiënten met sikkelcelziekte of andere aangeboren stoornissen van de bloedaanmaak). Deze patiënten hebben geregeld een bloedtransfusie nodig om de bloedarmoede te bestrijden, maar zij herstellen uiteindelijk zelf.
- Personen met een ernstige stoornis in het immuunsysteem, waardoor zij de infectie niet zelf onder controle krijgen; de bestaande rode bloedcellen kunnen de stop dan niet langer opvangen. Deze patienten kunnen behandeld worden met intraveneuze immuunglobulines (IVIG), waarbij B19V

geremd wordt totdat de eigen afweer functioneert.
- Ongeboren kinderen die in de baarmoeder geïnfecteerd raken met B19V. Ongeboren kinderen kunnen voor hun bloedarmoede behandeld worden met een bloedtransfusie in de baarmoeder, een zogeheten intra-uteriene transfusie (IUT). Vervolgens herstellen de ongeboren kinderen vaak vanzelf weer.

Ondanks dat B19V voornamelijk deze voorlopercellen infecteert, kan het DNA van het virus aangetoond worden in vele verschillende weefsels waar dit DNA levenslang aantoonbaar blijft. Daarnaast blijft B19V DNA na doorgemaakte infectie ook maandenlang aantoonbaar in het bloed. De persisterende aanwezigheid van B19V DNA in bloed en weefsels kan leiden tot diagnostische dilemma's; is het DNA dat op een bepaald moment in een patiënt wordt aangetoond een oorzakelijk agens van actuele ziekte, of is het een restant van een oude infectie en daarmee bijvangst in de diagnostiek?

Dit proefschrift

Het belangrijkste doel van dit proefschrift was om meer inzicht te krijgen in de betekenis van B19V DNA-detectie in bloed of weefsel, in verschillende patiëntgroepen. Dit kan leiden tot een beter begrip van het pathogenetisch potentieel van B19V, de klinische impact van B19V en de ziektelast. Een aanvullend doel was om inzicht te krijgen in de epidemiologie van B19V en de samenhang hiervan met de klinische impact en ziektelast van het virus.

In Hoofdstuk 2 is de epidemiologie van B19V infecties in Nederland in de laatste decennia in kaart gebracht, en de impact daarvan op ongeboren kinderen. Hiertoe werden gegevens gebruikt van Sanquin (de Nederlandse bloedbank), de virologische weekstaten (wekelijkse rapportage van virusinfecties door laboratoria) en het aantal ongeboren kinderen in Nederland dat per jaar met een IUT behandeld werd in de afgelopen twintig jaar. Hierin werd aangetoond hoe B19V een jaarlijkse seizoenspiek beleeft met elke paar jaar een grotere verheffing, maar laat ook zien dat dit patroon het laatste decennium verstoord is geraakt. Ook werd beschreven hoe B19V infecties verdwenen gedurende de COVID-19 pandemie, en vervolgens weer terugkeerden. Tevens werd aangetoond dat er een relatie bestaat tussen het vóórkomen van B19V in de algemene bevolking op enig moment en het aantal ongeboren kinderen dat een IUT nodig heeft. In **hoofdstuk 3** werd de verheffing in IUT beschreven in 2024 in Nederland, België en Frankrijk, die

waargenomen werd na het eerdere verdwijnen van B19V gedurende de COVID-19 pandemie. In hoofdstuk 3 bleek ook dat er aanwijzingen zijn dat de uitkomst na IUT nadeliger was gedurende de recente epidemie ten opzichte van de jaren hiervoor. Er werd een associatie aangetoond met meer gevorderde amenorroeduur bij IUT en nadelige uitkomst. Een verklaring hiervoor zou kunnen zijn dat patiënten tijdens de recente epidemie relatief laat verwezen werden, mogelijk omdat alertheid op B19V infecties in de zwangerschap in de afgelopen jaren afgenomen is.

De diagnose intra-uteriene infectie met B19V wordt doorgaans tijdens de zwangerschap gesteld. In **hoofdstuk 4** werd onderzocht of bloed van pasgeborenen ook geschikt is om ná de geboorte een intra-uteriene infectie vast te stellen. Deze toepassing zou van waarde kunnen zijn bij epidemiologisch onderzoek naar het vóórkomen van intra-uteriene infecties met B19V, waarover we nu niet goed geïnformeerd zijn (behoudens de meest ernstige gevallen die zich presenteren voor IUT). In dit hoofdstuk werd aangetoond dat in hielprikbloed of navelstrengbloed de sensitiviteit van PCR 79% was, en dat de sensitiviteit het laagst was indien infectie vroeg in de zwangerschap had plaatsgevonden (bij een zwangerschapsduur van < 20 weken bij diagnose was de sensitiviteit slechts 45%). Met deze suboptimale sensitiviteit moet rekening gehouden worden indien deze diagnostiek wordt ingezet voor individuele patiëntenzorg of in epidemiologisch verband.

Hoofdstuk 5 beschreef de ontwikkeling en toepassing van een nieuwe methode om te differentiëren tussen de aanwezigheid van intacte B19V viruspartikels en de aanwezigheid van enkel virale overblijfselen, d.w.z. het 'naakte' DNA van het virus. In dit hoofdstuk werd het beloop van een acute B19V infectie gevolgd in 10 bloeddonoren. Bloedmonsters die met PCR-test positief waren getest voor B19V, werden onderworpen aan een vergelijkende test. Een deel van het monster werd geïncubeerd met endonuclease, een enzym dat 'naakt' DNA afbreekt maar het DNA in een viruspartikel niet kan bereiken, omdat dit beschermd wordt door de eiwitmantel van het virus. Na incubatie werd de PCR test herhaald; indien het DNA bestaat uit 'naakt' DNA is dit door endonuclease afgebroken en is virale load nu sterk gedaald of zelfs negatief, en was hier dus sprake van een 'DNAemie', en geen viremie. Indien het DNA beschermd is door een eiwitmantel (en het dus gaat om een intact viruspartikel), heeft endonuclease geen effect gehad en is de load ver-

gelijkbaar gebleven. Uit dit hoofdstuk bleek dat bij de acute infecties in 10 gezonde bloeddonoren, na gemiddeld 150 dagen het aanwezige B19V DNA geheel door endonuclease afbreekbaar was. Dit moment leek samen te vallen met de overgang van hoge virale loads naar lage virale loads rond 10^4 IU/ml. Hieruit blijkt dat na acute infectie, gedurende de maandenlange periode waarin B19V DNA in lage hoeveelheden aantoonbaar is in het bloed, het gaat om DNA overblijfselen van B19V en geen infectieus, replicerend virus.

De persisterende aanwezigheid van B19V DNA in bloed en weefsels heeft tot veel wetenschappelijk discours geleid. Dit geldt met name voor de betekenis van aantoonbaar B19V DNA in patiënten met cardiomyopathie. In het verleden is vaker gesteld dat B19V een belangrijke oorzaak van cardiomyopathie zou zijn, met name gebaseerd op de detectie van B19V DNA in cardiaal weefsel, terwijl in meerdere studies een oorzakelijk verband ontkracht wordt. In **hoofdstuk 6** werd de betekenis van B19V DNAemie onderzocht in een cohort van patiënten met cardiomyopathie met behulp van de endonuclease test uit hoofdstuk 5. Er werden 20 patienten geselecteerd met hoge waarden B19V DNA in het hart. In 6 van de 20 patienten bleek er tevens sprake te zijn van een DNAemie. De endonuclease test toonde in alle 6 patiënten aan dat er sprake was van virale overblijfselen. Dit cohort werd vergeleken met een cohort van 18 patiënten met verschillende uitingsvormen van acute infectie met B19V. In al deze gevallen bestond de DNAemie uit intacte viruspartikels (en was er dus sprake van een viremie). Dit maakt het onwaarschijnlijk dat een actuele cardiomyopathie door actieve B19V-infectie veroorzaakt wordt.

Hoofdstuk 7 onderzocht de prevalentie en de klinische betekenis van B19V DNAemie na niertransplantatie in een cohort niertransplantatie patiënten in een Nederlands academisch ziekenhuis. Er werd een puntprevalentie van B19V DNAemie vastgesteld van 1.5% (2 van de 134 gescreende patiënten). De 2 positieve patiënten werden nader onderzocht. Er werd met behulp van aanvullende monsters nagegaan hoe lang DNA aantoonbaar was en met behulp van de endonuclease test werd nagegaan wat de aard van het aangetoonde DNA was. Ook werd gekeken of het DNA mogelijk afkomstig was van de donornier door een donornierbiopt pretransplantatie te testen op B19V DNA. Bij 1 ontvanger was transmissie vanuit de donor zeer waarschijnlijk; de patiënt was seronegatief voor én na de DNAemie en het donornierbiopt was positief. Bij de tweede patiënt was het donornierbiopt

ook positief, maar gezien de ontvanger ook seropositief was, was niet met zekerheid te zeggen waar het DNA van afkomstig was. Deze studie toont aan dat B19V DNAemie met voorzichtigheid geïnterpreteerd moet worden in deze populatie. Het toont zowel aan dat DNAemie niet klinisch relevant hoeft te zijn en dat B19V DNA afkomstig kan zijn van een donororgaan, wat betekent dat DNAemie in deze patiënten niet gelijk staat aan een actieve of doorgemaakte infectie met B19V.

Wat in de hoofdstukken 5, 6 en 7 opvalt is dat aanwezigheid van 'naakt' DNA d.w.z. niet-replicerend virus correleert met de aanwezigheid van lage virale loads. Het is daarom belangrijk om virale loads betrouwbaar te kunnen kwantificeren. Daarnaast wordt (metagenomic) next-generation sequencing (NGS) steeds meer toegepast in de klinische virologie. Omdat B19V persisteert in bloed en weefsels, heeft dit als gevolg dat B19V steeds vaker wordt gedetecteerd als nevenbevinding in klinische samples. Om deze nevenbevinding te kunnen duiden, is het wenselijk dat dit gepaard gaat met goede kwantificering. Eerder is kwantificering d.m.v. NGS een tekortkoming gebleken. In **hoofdstuk 8** werd onderzocht of metagenomic NGS betrouwbare kwantificeerbare resultaten oplevert. Dit hoofdstuk toonde aan dat meerdere DNA-virussen, waaronder B19V, goed gekwantificeerd konden worden met een nieuwe NGS methode. Hiermee wordt een drempel weggenomen om NGS in te zetten in de klinische praktijk.

In **hoofdstuk 9** werd dieper ingegaan op het beloop van B19V viremie in immuungestoorde patiënten. Doordat zij door hun verminderde immuniteit de B19V infectie niet snel klaren, bestaat er een langdurige viremie. In dit hoofdstuk werd in drie patiënten met langdurige viremie nagegaan of er gedurende de infectie mutaties optraden in het viraal genoom. Bij één van de drie patiënten werden twee mutaties aangetoond gedurende het beloop. Door middel van eiwit modellering werd inzichtelijk gemaakt dat één van deze mutaties mogelijk ook leidt tot een structurele verandering in één van de oppervlakte-eiwitten van het virus. Deze patiënt had voorafgaand aan de mutatie zelf antilichamen tegen B19V ontwikkeld, maar was deze ook weer verloren. Dit zou erop kunnen wijzen dat er sprake is van een verandering in het virale eiwit onder druk van antilichamen. Deze bevindingen benadrukken het belang van het monitoren van het viraal genoom bij langdurige of terugkerende viremie, om meer inzicht te krijgen hoe het virus zich ontwikkelt tijdens een dergelijke infectie.

Concluderend is één van de belangrijkste bevindingen in dit proefschrift het belang om onderscheid te maken tussen de aanwezigheid van replicerend virus

en virale overblijfselen. Dit onderscheid kan gemaakt worden met de endonuclease test. Het belang van dit onderscheid speelt in meerdere patiëntgroepen. Daarnaast is gebleken dat de hoogte van virale load (of DNA load) correleert met de aanwezigheid van replicerend virus. Bij een positieve B19V PCR bevinding in een patiënt moet men zich dan ook altijd afvragen wat deze voor de individuele patiënt betekent, en dit zou aanleiding moeten zijn tot het laagdrempelig inzetten van nadere B19V diagnostiek, zoals een virale load bepaling of een endonuclease test, om hier duidelijkheid over te krijgen.

Dit proefschrift laat ook zien dat B19V in epidemieën voorkomt die gepaard kunnen gaan met ernstige consequenties voor ongeborenen. Monitoring van en aandacht voor de epidemische verheffingen waarmee B19V voorkomt is aangewezen om infecties tijdig te kunnen opsporen en de consequenties hiervan te beperken.

Een van de belangrijkste vragen waarop nog geen definitief antwoord verkregen is, betreft het mechanisme dat verantwoordelijk is voor het langdurig aantoonbaar blijven van B19V in bloed en weefsels. Toekomstig onderzoek zou zich hierop moeten richten, niet alleen om definitief te kunnen begrijpen hoe B19V zich gedraagt in de gastheer maar ook omdat dit zou bijdragen aan inzicht in gastheer-pathogeen interacties voor virussen in het algemeen.

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CURRICULUM VITAE

Anne Russcher is geboren op 12 juli 1983 in Breda. Zij behaalde in 2001 haar gymnasiumdiploma *cum laude* aan het Johan van Oldenbarnevelt gymnasium te Amersfoort. Na een tussenjaar, dat werd doorgebracht in Andalusië en Mexico alwaar zij Spaans leerde en Spaanstalige reportages maakte voor een lokaal Mexicaans radiostation, begon zij in 2002 met de studie Engelse Taal en Cultuur aan de Universiteit van Leiden. Vanaf 2003 combineerde zij dit met de studie Geneeskunde in Leiden. In 2009 behaalde zij haar bachelordiploma Engelse Taal en Cultuur, afgesloten met de later gepubliceerde bewerking van haar bachelorscriptie onder supervisie van prof. dr. R. H. Bremmer. Tijdens haar studie Geneeskunde werd de interesse in infectieziekten al heel duidelijk, wat resulteerde in een wetenschapsstage naar virale respiratoire infecties in kinderen in het Reinier de Graaf ziekenhuis in Delft. In 2010 behaalde zij haar arts-examen *cum laude*. Vervolgens was zij gedurende een jaar werkzaam als ANIOS kindergeneeskunde in het Bronovo ziekenhuis in Den Haag. In dat jaar werd weloverwogen gekozen voor de opleiding tot arts-microbioloog waarmee in 2011 werd begonnen in het Leids Universitair Medisch Centrum (opleider: prof. dr. A. C. M. Kroes). Gedurende de opleiding werd een begin gemaakt met het onderzoek beschreven in dit proefschrift. Na het afronden van de opleiding tot arts-microbioloog in 2017, is zij sindsdien werkzaam als arts-microbioloog in de vakgroep Medische Microbiologie van het Meander Medisch Centrum/St Jansdal Ziekenhuis en heeft zij het werk aan het proefschrift gecombineerd met haar werkzaamheden als arts-microbioloog. Anne woont in Amersfoort, samen met Tom en hun kinderen Roos (2014) en Susanne (2016).