Parvovirus B19: diagnosis, distribution and disease associations

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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 autoimmune 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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