Parvovirus B19: diagnosis, distribution and disease associations

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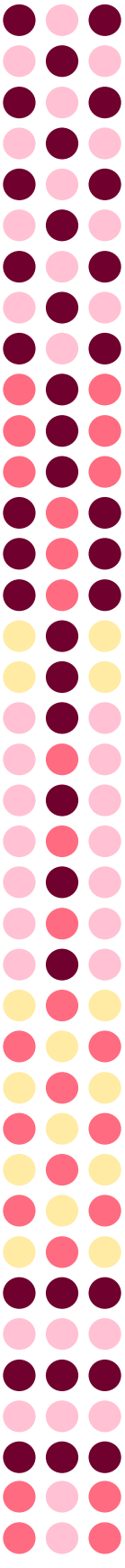

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Diagnosis of intrauterine parvovirus B19 infection at birth – value of DNA detection in neonatal blood and dried blood spots

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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).

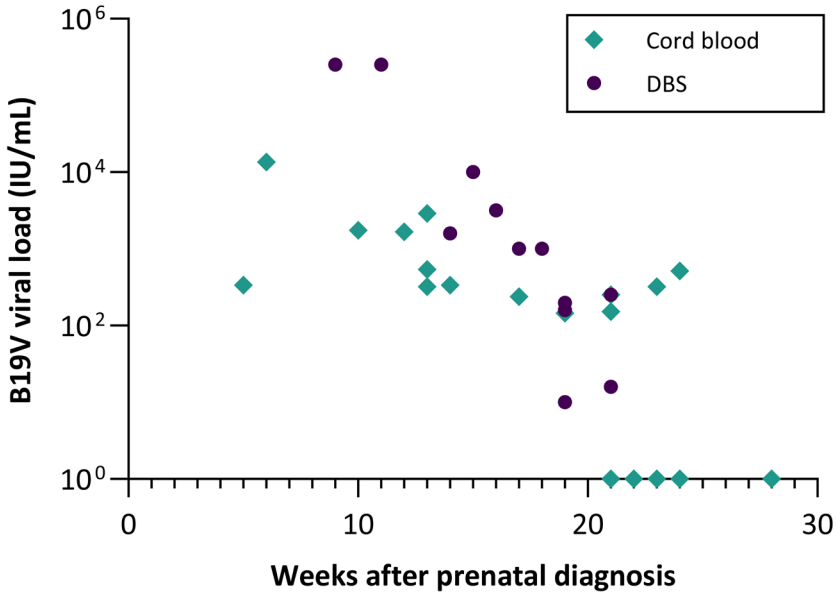

***Figure 1**. Viral load in CB and DBS according to weeks after prenatal diagnosis. Green diamonds represent cord blood, purple dots represent dried blood spots.*

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.

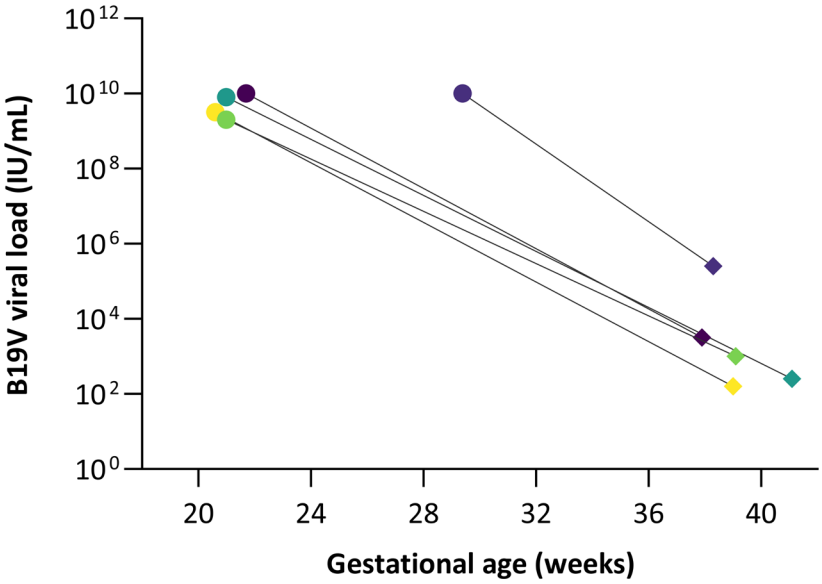

Figure 2. *Viral load in fetal plasma and DBS according to gestational age. Dots represent loads in fetal plasma; the newborn's corresponding load in DBS is indicated by diamonds in the corresponding colour.*

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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REFERENCES

1. Mossong J, Hens N, Friederichs V, Davidkin I, Broman M, Litwinska B, et al. Parvovirus B19 infection in five European countries: seroepidemiology, force of infection and maternal risk of infection. Epidemiology and infection. 2008;136(8):1059-1068.
2. van Rijckevorsel GG, Sonder GJ, Schim van der Loeff MF, van den Hoek JA. Population-based study on the seroprevalence of parvovirus B19 in Amsterdam. J Med Virol. 2009;81(7):1305-1309.
3. Koch WC, Harger JH, Barnstein B, Adler SP. Serologic and virologic evidence for frequent intrauterine transmission of human parvovirus B19 with a primary maternal infection during pregnancy. Pediatr Infect Dis J. 1998;17(6):489-494.
4. Bonvicini F, Puccetti C, Salfi NC, Guerra B, Gallinella G, Rizzo M, et al. Gestational and fetal outcomes in B19 maternal infection: a problem of diagnosis. J Clin Microbiol. 2011;49(10):3514-3518.
5. Enders M, Weidner A, Zoellner I, Searle K, Enders G. Fetal morbidity and mortality after acute human parvovirus B19 infection in pregnancy: prospective evaluation of 1018 cases. Prenatal diagnosis. 2004;24(7):513-518.
6. Fairley CK, Smoleniec JS, Caul OE, Miller E. Observational study of effect of intrauterine transfusions on outcome of fetal hydrops after parvovirus B19 infection. Lancet. 1995;346(8986):1335-1337.
7. Nagel HT, de Haan TR, Vandenbussche FP, Oepkes D, Walther FJ. Long-term outcome after fetal transfusion for hydrops associated with parvovirus B19 infection. Obstetrics and gynecology. 2007;109(1):42-47.
8. Schild RL, Bald R, Plath H, Eis-Hubinger AM, Enders G, Hansmann M. Intrauterine management of fetal parvovirus B19 infection. Ultrasound in obstetrics & gynecology. 1999;13(3):161-166.
9. Jensen IP, Thorsen P, Jeune B, Moller BR, Vestergaard BF. An epidemic of parvovirus B19 in a population of 3,596 pregnant women: a study of sociodemographic and medical risk factors. BJOG. 2000;107(5):637-643.
10. Dembinski J, Haverkamp F, Maara H, Hansmann M, Eis-Hubinger AM, Bartmann P. Neurodevelopmental outcome after intrauterine red cell transfusion for parvovirus B19-induced fetal hydrops. BJOG. 2002;109(11):1232-1234.
11. Miller E, Fairley CK, Cohen BJ, Seng C. Immediate and long term outcome of human parvovirus B19 infection in pregnancy. British journal of obstetrics and gynaecology. 1998;105(2):174-178.
12. Rodis JF, Rodner C, Hansen AA, Borgida AF, Deoliveira I, Shulman Rosengren S. Long-term outcome of children following maternal human parvovirus B19 infection. Obstetrics and gynecology. 1998;91(1):125-128.
13. De Jong EP, Lindenburg IT, van Klink JM, Oepkes S, van Kamp IL, Walther FJ, et al. Intrauterine transfusion for parvovirus B19 infection: long-term neurodevelopmental outcome. American journal of obstetrics and gynecology. 2012;206(3):204 e201-205.

14. McMichael G, MacLennan A, Gibson C, Alvino E, Goldwater P, Haan E, et al. Cytomegalovirus and Epstein-Barr virus may be associated with some cases of cerebral palsy. J Matern Fetal Neonatal Med. 2012;25(10):2078-2081.
15. Bogdanovic G, Pou C, Barrientos-Somarribas M, Bjerkner A, Honkaniemi E, Allander T, et al. Virome characterisation from Guthrie cards in children who later developed acute lymphoblastic leukaemia. Br J Cancer. 2016;115(8):1008-1014.
16. Lefrere JJ, Servant-Delmas A, Candotti D, Mariotti M, Thomas I, Brossard Y, et al. Persistent B19 infection in immunocompetent individuals: implications for transfusion safety. Blood. 2005;106(8):2890-2895.
17. Matsukura H, Shibata S, Tani Y, Shibata H, Furuta RA. Persistent infection by human parvovirus B19 in qualified blood donors. Transfusion. 2008;48(5):1036-1037.
18. Juhl D, Gorg S, Hennig H. Persistence of Parvovirus B19 (B19V) DNA and humoral immune response in B19V-infected blood donors. Vox Sang. 2014;107(3):226-232.
19. Molenaar-de Backer MW, Russcher A, Kroes AC, Koppelman MH, Lanfermeijer M, Zaaijer HL. Detection of parvovirus B19 DNA in blood: Viruses or DNA remnants? J Clin Virol. 2016;84:19-23.
20. Kerr JR, Curran MD, Moore JE, Coyle PV, Ferguson WP. Persistent parvovirus B19 infection. Lancet. 1995;345(8957):1118.
21. Musiani M, Zerbini M, Gentilomi G, Plazzi M, Gallinella G, Venturoli S. Parvovirus B19 clearance from peripheral blood after acute infection. The Journal of infectious diseases. 1995;172(5):1360-1363.
22. Barbi M, Binda S, Caroppo S. Diagnosis of congenital CMV infection via dried blood spots. Reviews in medical virology. 2006;16(6):385-392.
23. Knoester M, von dem Borne PA, Vossen AC, Kroes AC, Claas EC. Human parvovirus B19 genotype 3 associated with chronic anemia after stem cell transplantation, missed by routine PCR testing. J Clin Virol. 2012;54(4):368-370.
24. Croghan CW, Egeghy PP. Methods of dealing with values below the limit of detection using SAS. Presented at Southeastern SAS User Group, St. Petersburg, FL, September 22-24, 2003.
25. Beckhoff A, Steffen I, Sandoz P, Hirsch HH, Schaub S. Relapsing severe anaemia due to primary parvovirus B19 infection after renal transplantation. Nephrology, dialysis, transplantation. 2007;22(12):3660-3663.
26. Kim C, Katheria AC, Mercer JS, Stonestreet BS, Fluid Distribution in the Fetus and Neonate, in: Polin RA, Abman SH, Rowitch DH, Benitz WE, Fox WW (Eds.), Fetal and Neonatal Physiology. Vol 2. 5th ed. Philadelphia: Elsevier; 2017. pp 1081-1083
27. Pearson HA. Life-span of the fetal red blood cell. J Pediatr. 1967;70(2):166-171.
28. Bascietto F, Liberati M, Murgano D, Buca D, Iacovelli A, Flacco ME, et al. Outcome of fetuses with congenital parvovirus B19 infection: systematic review and meta-analysis. Ultrasound in obstetrics & gynecology. 2018;52(5):569-576.
29. Chen HL, Tseng HI, Lu CC, Yang SN, Fan HC, Yang RC. Effect of blood transfusions on the outcome of very low body weight preterm infants under two different transfusion

criteria. Pediatr Neonatol. 2009;50(3):110-116.
30. de Haan TR, Beersma MF, Oepkes D, de Jong EP, Kroes AC, Walther FJ. Parvovirus B19 infection in pregnancy: maternal and fetal viral load measurements related to clinical parameters. Prenatal diagnosis. 2007;27(1):46-50.