

## Human Parvovirus B19 Infection and Hydrops Fetalis in Rio de Janeiro, Brazil

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*Formalin-fixed paraffin embedded lung and liver tissue from 23 cases of non immune hydrops fetalis and five control cases, in which hydrops were due to syphilis (3) and genetic causes (2), were examined for the presence of human parvovirus B19 by DNA hybridisation. Using in situ hybridisation with a biotinylated probe one positive case was detected. Using 32P-labelled probes in a dot blot assay format, five further positives were obtained. These were all confirmed as positive by a nested polymerase chain reaction assay. Electron microscopy revealed virus in all these five positive cases. The six B19 DNA positive cases of hydrops fetalis were from 1974, 1980, 1982, 1987 and 1988, four of which occurred during the second half of the year, confirming the seasonality of the disease.*

Key words: human parvovirus B19 - hydrops fetalis - virus detection

Human parvovirus B19 was first discovered in blood donors by Cossart et al. (1975) and has been identified as the causative agent of erythema infectiosum, an acute exanthem of childhood (Anderson et al. 1983). In adults, particularly women, the rash illness is less frequent, but there may be involvement of joints leading to acute arthritis (Reid et al. 1985). In addition, B19 is also a major cause of aplastic crisis in patients suffering from hemolytic anemias (Pattison et al. 1981).

Infection with the virus was first shown to be associated with fetal death in 1984 (Brown et al. 1984). Since then, several other reports have described B19 infection in pregnancy (Knott et al. 1984, Bond et al. 1986, Anderson et al. 1988). Fetal infection may cause hydrops fetalis and stillbirth, but normal delivery usually occurs (Kinney et al. 1988). Estimates of the risk of fetal death from intrauterine infection range from 1.66% to 9% (CDC 1989, PHLS 1990, Guidozi et al. 1994, Gratacós et al. 1995). More recent reports suggest that the risk of fetal loss in pregnancy may be higher with asymptomatic B19 infections (Smoleniec et al. 1994, Gratacós et al. 1995). The incidence of

congenital malformations after maternal infection is, however, no higher than the expected rate in the general population (Kinney et al. 1988). Thus, parvovirus B19 is considered to be embryocidal rather than teratogenic. The infection is diagnosed by the detection of IgM antibodies in both maternal and fetal blood (Knott et al. 1984) but, in some cases, maternal anti-B19 IgM can no longer be detected when hydrops fetalis develops (Bond et al. 1986, Anderson et al. 1988). In most of the reported cases, DNA detection in fetal tissues is the method of choice for diagnosis (Clewley et al. 1987, Salimans et al. 1989). Electron microscopy has also been used (Caul et al. 1988, Knisely et al. 1988, Naides & Weiner 1989, Field et al. 1991).

A serological survey of the Rio de Janeiro population showed that 30 to 60% of women of child-bearing age have antibodies (Nascimento et al. 1990) and thus at least 40% of women are still susceptible to infection during pregnancy. A previous study of hydrops fetalis done by the Pathology Department of the Instituto Fernandes Figueira (IF/FIOCRUZ) during 1954 to 1992 showed that infectious diseases predominate over immunologic causes in the aetiology of hydrops fetalis (Garcia et al. 1995). We report here the results of B19 detection in fetal tissues from non-immune hydrops fetalis (NIHF). In all but one, where syphilis has also been diagnosed, no other infectious agent was found.

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## MATERIALS AND METHODS

*Specimens* - Formalin-fixed paraffin embedded tissues. From a collection of 86 cases of NIHF analyzed during 38 years (1954 to 1992) at the Pathology Department in IFF/FIOCRUZ, 28 fetuses were selected because histology suggested intrauterine viral infection. For 23 of these 28 fetuses the aetiology of the NIHF was not known (Garcia et al. 1995). In five additional fetuses included as controls the hydrops was diagnosed as a result of syphilis (three) or genetic causes (two). Lung and liver tissues from all 28 cases were used for screening for B19 DNA. Other tissues, when available, were also examined from the six cases found positive for B19 DNA in lung or liver.

*In situ hybridisation (ISH)* - ISH was performed as described previously (Nascimento et al. 1991). The only modification was that a pBR322 plasmid containing citomegalovirus DNA was used as a negative control probe for each tissue.

*Dot blot hybridisation (DBH)* - A 10% homogenate of fetal tissue was prepared and DNA extracted as described by Clewley et al. (1987) except that the extracted DNA was applied to nitrocellulose filters and the blots were hybridised with a 32P-labelled B19 probe. The probe was made from the 5.2 Kb Eco RI fragment excised from the B19/pGem-1 plasmid (Mori et al. 1989). Diluted plasma from a viremic blood donor (Cruz et al. 1989) and normal human serum were used as positive and negative controls.

*Nested polymerase chain reaction (PCR)* - B19 DNA was recovered from 10% fetal tissue suspensions according to the method described by Boom et al. (1990). PCR amplifications were carried out as described by Clewley (1993). Two successive sets of amplification were performed using primers derived from the B19 parvovirus non-structural coding sequence. Primers pair H (1417-1424) and C (2160-2141) were used for the first round of reaction, primers pair F (1498-1520) and I (2029-2065) for the second round. The numbers indicate nucleotide positions relative to the clone sequenced by Shade et al. (1986), Genbank accession number, M13178. Ten µl of PCR products were analyzed by electrophoresis on a 4% Nusieve-agarose (3:1) gel. Deionized H<sub>2</sub>O extracted alongside the specimens was used in other to check for possible contamination. Positive and negative B19 DNA human sera were also used as controls. Amplifications were carried out in a suite of physically separated PCR rooms.

*Electron microscopy* - Direct electron microscopy (DEM) of the 10% fetal tissue suspensions were done as described before (Field et al. 1991). The tissue suspensions were negative stained with 2% PTA pH 7.2 and observed in a Philips 301 EM.

## RESULTS

Fifty-six lung and liver tissues from 28 fetuses were tested by ISH. Only one fetus was found to be positive for B19 DNA in both lung and liver tissues. From this case other tissues (placenta, heart, kidney, thymus and adrenal) were available. These were all found to be negative for B19 DNA, when tested by ISH. When DNA was extracted from these tissues and tested by DBH, using nitrocellulose membranes and a 32P-labelled probe, another two (kidney and adrenal) were found to be positive for B19 DNA. Electron microscopy of ultrathin sections was not able to demonstrate virus particles in any of these tissues (Table I). There was no material remaining for direct electron microscopy (DEM).

DNA was extracted from the lung and liver tissue from the other 27 cases and hybridized against a 32P-labelled probe. Five additional fetuses were found to be positive for B19 DNA for one of each of their tissues tested. A nested PCR was performed to confirm the five positive results found by DBH. Analysis of nested PCR products by agarose gel electrophoresis showed a DNA band of the expected size (591 bp), in at least one of the tissues for each of the samples tested. DEM was also performed on these tissues and parvovirus-like particles were observed in all of them. In one of these five positive cases (1757) the NIHF had been first diagnosed as syphilis.

The B19 positive fetuses were found during the years 1974, 1980, 1982, 1987 (one case each year) and 1988 (two cases) as shown in Table II. Of these, only two were not found during the second half of the year.

TABLE II

Cases of NIHF from 1968 to 1989 tested for the presence of human parvovirus B19

Year of death	No. positive/No. tested
1968/69	0/02
1974	1/02
1976/79	0/02
1980	1/01
1981	0/01
1982	1/03
1983	0/02
1987	1/03
1988	2/02
1989	0/05
Total	6/23

Five fetuses showing NIHF used as negative controls were selected in the years of 1980, 1986 and 1987.

TABLE I  
Positive B19 hydrops cases

Month Year	Gestacional age	Autopsy findings	Tissue	B19 Detection			
				ISH	DBH	EM	PCR
October 1974 (1447) <sup>a,b</sup>	33 wks	macerated, hydropic, fibro-elastosis, spleen and lung anomaly	lung	-	-	+	+
			liver	-	+	-	+
March 1980 (1757) <sup>a</sup>	28 wks	macerated, hydropic, syphilis lesional complex	lung	-	+	+	+
			liver	-	-	+	+
			cord	-	nd	+	nd
			placenta	-	nd	+	nd
November 1982 (2023) <sup>a</sup>	22 wks	macerated, hydropic, fibro-elastosis	lung	-	+	+	+
			liver	-	-	+	+
			cord	-	nd	+	nd
			placenta	-	nd	+	nd
			spleen	-	nd	+	nd
			adrenal	-	nd	+	nd
May 1987 (2387) <sup>a</sup>	27 wks	macerated, hydropic, hepatomegaly, esplenomegaly	lung	-	+	+	-
			liver	-	-	+	+
November 1988 (2595) <sup>a</sup>	31 wks	stillbirth, hydropic, hepatomegaly, esplenomegaly	lung	-	+	+	+
			liver	-	-	+	+
			cord	-	nd	+	nd
			placenta	-	nd	+	nd
			spleen	-	nd	+	nd
			kidney	-	nd	+	nd
			brain	-	nd	+	nd
adrenal	-	nd	+	nd			
December 1988 (2601) <sup>a,b</sup>	30 wks	macerated, hydropic, fibro-elastosis, hepatomegaly, esplenomegaly	lung	+	+	-	nd
			liver	+	+	-	nd
			placenta	-	-	nd	nd
			kidney	-	+	nd	nd
			adrenal	-	+	nd	nd
			heart	-	-	nd	nd
			thymus	-	-	nd	nd

a: registration number; b: mother with sickle cell disease; nd: not done

**DISCUSSION**

In the present study using DNA hybridisation we were able to detect six fetuses infected with human parvovirus B19.

In a previous study of a collection of fetuses in England, we found that ISH is as sensitive as DBH with 32P-labelled probe for formalin-fixed tissues (Nascimento et al. 1991). In this study of a collection of Brazilian tissues, DBH was much more efficient than ISH for detecting B19 DNA. One explanation for this could be that the fetuses were accumulated during a 21 year period compared to the five year period of the English collection. The quality of paraffin and the formalin may differ between the collections. For example, the forma-

lin used in the preservation of the tissues in Brazil is not buffered. This may contribute either to degradation of nucleic acids or prevention of probe access to the B19 DNA in the tissue. This would not interfere with DBH since DNA is concentrated by extraction from the tissue and treated with alkali before binding to the filter and subsequent hybridisation (Clewley 1985).

The difference between the detection of B19 DNA by DBH in different tissues from the same fetus could be explained by postmortem changes associated with the inevitable time lapse between fetal death and formalin fixation of the tissues (Lohr & Neremberg 1990). The site of B19 replication is erythroid progenitors which accumulate in fetal liver between 12 to 30 weeks of gestation (Clewley

et al. 1987, Knisely et al. 1988). Since liver is subject to greater autolysis than lung it is likely that differences in B19 DNA detection in these tissues is due to postmortem changes.

Recent reports have used sensitive PCR assay to detect B19 DNA in clinical specimens (Clewley 1989, Salimans et al. 1989), especially when studying B19 infection during pregnancy (Torok et al. 1992, Cassinotti et al. 1993). We used nested PCR to confirm the five positive cases found by DBH. Although a single-step PCR should be sufficient for detecting B19 DNA, a nested PCR may be necessary for maximum sensitivity, particularly when investigating fetal tissues in which partially degraded nucleic acids may be recovered (Clewley 1993).

Although a previous study showed that hybridisation is more sensitive than electron microscopy for testing formalin-fixed samples (Field et al. 1991), B19 parvovirus-like particles could be observed in this work, by DEM, in all five fetuses positive for B19 DNA by DBH.

A study carried out by the Pathology Department in IFF/FIOCRUZ showed that of 86 cases of NIHF, 31 were diagnosed as syphilis by identification of *Treponema pallidum*. One of these cases (1757) was used as a negative control in our study. B19 DNA was found in this fetus by DBH and PCR. Since congenital syphilis is also considered a cause of hydrops fetalis (Bulova et al. 1972) this may be a case of dual infection.

Four of the parvovirus B19 positive cases were found during the second half of different years. This would be consistent with the seasonality described in Rio de Janeiro for exanthematic disease (Schatzmayer 1985). Two of the positive cases occurred during November and December 1988, coinciding with the finding by chance of a B19 infected blood donor (Cruz et al. 1989). This supports the suggestion that 1988 was an epidemic year for B19 infection in Rio de Janeiro.

Human parvovirus B19 should be considered as a virus to be monitored during pregnancy, since it is clinically very similar to rubella (Shirley et al. 1987) and it has become evident as a cause of fetal infection in countries where rubella is controlled by vaccination (Cohen 1993). It would be particularly important to survey for B19 infection in some Brazilian states, for instance, São Paulo and Paraná which have started rubella vaccination programmes.

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