

# Detection of human parvovirus B19 in a patient with hepatitis

J.R.R. Pinho<sup>1,2</sup>, V.A.F. Alves<sup>1,3</sup>,  
A.F. Vieira<sup>4</sup>, M.O.S. Moralez<sup>4</sup>,  
L.E.P. Fonseca<sup>3</sup>, B. Guz<sup>5</sup>,  
A. Wakamatsu<sup>1</sup>,  
E.L.R. Cançado<sup>3</sup>,  
F.J. Carrilho<sup>3</sup>, L.C. da Silva<sup>3</sup>,  
A.P. Bernardini<sup>2</sup>  
and E.L. Durigon<sup>4</sup>

<sup>1</sup>Serviço de Virologia e Divisão de Patologia, Instituto Adolfo Lutz, São Paulo, SP, Brasil  
<sup>2</sup>Laboratório Bioquímico Jardim Paulista, São Paulo, SP, Brasil  
<sup>3</sup>Departamentos de Patologia e Gastroenterologia, Faculdade de Medicina and  
<sup>4</sup>Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brasil  
<sup>5</sup>Hospital do Servidor Público Estadual, São Paulo, SP, Brasil

## Abstract

Parvovirus B19 has been associated by some investigators with cases of severe hepatitis. The aim of the present study was to determine the presence of active parvovirus B19 infection among 129 Brazilian patients with non-A-E hepatitis. The patients were assayed for antibodies against parvovirus B19, IgM class, by ELISA. In IgM-positive cases, parvovirus B19 DNA was assayed by PCR in serum and liver tissue and parvovirus VP1 antigen in liver tissue was assayed by immunohistochemistry. Antibodies against parvovirus B19, IgM class, were detected in 3 (2.3%) of 129 patients with non-A-E hepatitis. Previous surgery and blood transfusions were reported by these 3 patients. One patient was a 56-year-old female with severe hepatitis, with antimitochondrial antibody seropositivity and submassive necrosis at liver biopsy, who responded to corticosteroid therapy. Strong evidence for active parvovirus B19 infection was found in this patient, with parvovirus B19 DNA being detected by PCR in liver tissue. Furthermore, parvovirus VP1 antigen was also detected in liver tissue by immunohistochemistry. The other two IgM-positive patients were chronic hepatitis cases, but active infection was not proven, since neither viral DNA nor antigen were detected in their liver tissues. This and other reports suggest a possible relation between parvovirus B19 infection and some cases of hepatitis.

## Key words

- Parvovirus B19
- Hepatitis non-A-E
- Autoimmune hepatitis
- Polymerase chain reaction
- Immunohistochemistry

## Correspondence

J.R.R. Pinho  
Serviço de Virologia  
Instituto Adolfo Lutz  
Av. Doutor Arnaldo, 355  
01246-902 São Paulo, SP  
Brasil  
Fax: + 55-11-3885-7955  
E-mail: jrpinho@usp.br

Research partially supported by  
FAPESP (No. 97/4955-0).

Received February 13, 2001  
Accepted May 29, 2001

## Introduction

Human parvovirus B19 is a member of the Parvoviridae family, genus *Erythrovirus*. This is a non-enveloped virus containing a single copy, single-stranded linear DNA molecule with approximately 5,000 nucleotides that codes for two major structural or capsid proteins, VP1 and VP2, and one nonstructural protein, NS1. The genome is

organized as a linear coding region bound on either end by terminal palindromic sequences, which fold into hairpin duplexes approximately 330 nucleotides in length (1).

Human parvovirus B19 infection can cause a wide spectrum of clinical manifestations. The most commonly recognized illness associated with viral infection is a mild childhood rash (erythema infectiosum), but this virus can also cause several other dis-

eases (transient aplastic crisis in patients with low red cell production, spontaneous abortions, hydrops fetalis, and fetal death). Parvovirus B19 has also been related to cases of hemophagocytic syndrome, myocarditis, neurological disease, systemic vasculitis, and hepatitis, though additional studies are needed to confirm the role of B19 in these diseases (2).

Liver dysfunctions associated with parvovirus B19 have been described by several authors, especially involving acute hepatitis cases (3-12), and the transmission of parvovirus B19 to patients with liver diseases has possibly led to exacerbations of disease in patients with cirrhosis (13) and liver transplant (14).

In this study, we investigated the presence of human parvovirus B19 infection in 129 patients with non-A-E hepatitis followed up in São Paulo, Brazil, by the detection of anti-B19 antibodies (IgM) by ELISA. To confirm the presence of parvovirus infection in the liver, the patients were further assayed for B19 DNA by PCR and for VP1 antigen by immunohistochemistry.

## Patients and Methods

### Patients

We studied 129 patients with acute or chronic hepatic disorders for whom viral hepatitis was considered in the differential diagnosis. These patients were seronegative for HBsAg, anti-HBc and anti-HCV antibodies as determined with commercially available kits (Abbott Laboratories, Chicago, IL, USA). Acute cases also tested negative for anti-HAV IgM, anti-HBc IgM, anti-HEV antibodies (Abbott) and HCV-RNA by RT-PCR using previously described primers (15,16) after guanidine isothiocyanate extraction (17).

This research was approved by the Ethics Committee of the Hospital das Clínicas, Faculdade de Medicina, USP.

### Biopsies

Liver biopsies were performed using a Tru-Cut™ needle and liver histology was evaluated blindly by two specialized pathologists.

### Enzyme immunoassay for anti-parvovirus B19 antibodies (ELISA)

Serum samples from all patients were analyzed for IgM-specific antibodies specific for B19 as previously described by Erdman et al. (18), using as antigen the B19 capsid protein expressed in baculovirus (19).

### PCR for parvovirus B19 DNA

Paraffin-fixed liver biopsy specimens were cut into 0.7- $\mu$ m thick fragments with new blades. The fragments were transferred to 1.5-ml tubes and treated with xylene and absolute ethanol. After drying, the pellets were incubated at 37°C for 48 h in 180  $\mu$ l of sample buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, and 500  $\mu$ g/ml proteinase K). After digestion, the samples were extracted sequentially with equal volumes of phenol, phenol-chloroform-isoamyl alcohol, and chloroform-isoamyl alcohol and finally resuspended in 30  $\mu$ l of MilliQ water. To confirm the absence of *Taq* DNA polymerase inhibitors, extracted DNA was first amplified with  $\beta$  actin-specific primers. PCR/nested PCR assay was carried out as described by Durigon et al. (20). For PCR, the oligonucleotide primers used were P1 (5' AAT ACA CTG TGG TTT TAT GGG CCG 3') and P6 (5' CCA TTG CTG GTT ATA ACC ACA GGT 3'), nucleotide sequences 1399-1422 and 1682-1659, respectively. For nested PCR, the oligonucleotide primers used were P2 (5' CCA TTG CTG GTT ATA ACC ACA GGT 3') and P5 (5' CTA AAA ATG GCT TTT GCA GCT TCT AC 3'), nucleotide sequences 1498-1525 and 1600-1576, re-

spectively. The extracted DNA (10 µl) was added to the PCR mixture containing 10 µl of 10X reaction buffer (Applied Biosystems, Foster City, CA, USA); 200 µM each dATP, dCTP, dGTP, dTTP; 0.5 µM each oligonucleotide primer, and 2.5 U of *Taq* polymerase (Applied Biosystems). The amplification step was carried out on a thermocycler 2400 (Applied Biosystems) as follows: 35 cycles, 94°C for 45 s, 55°C for 60 s, 72°C for 90 s. After the first amplification, the second (nested) PCR was carried out, and 10 µl of the first PCR product was added to the nested PCR mix using the specific primers. The amplified products were submitted to 1.5% agarose gel electrophoresis and ethidium bromide staining.

#### Autoantibody profile

Anti-smooth muscle, anti-liver kidney microsome type 1, anti-liver cytosol type 1, and antimitochondrial antibodies were investigated by indirect immunofluorescence using unfixed tissue sections of rodent liver, kidney and stomach (21). Antinuclear and anti-actin antibodies were processed by the same technique in acetone-fixed human fibroblasts as the substrate (22). Western blotting to identify the antigens reactive to antimitochondrial antibodies was carried out using commercial enzyme (Sigma, St. Louis, MO, USA) and bovine heart antigens (23).

#### Detection of parvovirus B19 VP1 antigen in liver tissue by immunohistochemistry

VP1 antigen was detected on formalin-fixed paraffin-embedded 3-µm liver sections through a specific biotinylated monoclonal antibody against VP1 (MAB8292B, Chemicon International Inc., Temecula, CA, USA), diluted 1:800. Heat-induced epitope retrieval was achieved by incubation with a sodium citrate solution, pH 6.0, for 4 min in a pressure cooker. Endogenous biotin and peroxidase blocking and nonspecific protein bind-

ing were performed by incubation with 1:20 normal goat serum. The immunoreaction was amplified using the high-sensitivity streptavidin-biotin-peroxidase system (LSAB, Dako Corp., Carpinteria, CA, USA), with proper controls of specificity (24).

#### Results

Anti-B19 IgM antibodies were detected in three (2.3%) of 129 cases, as shown in Table 1. Previous surgery and blood transfusions were reported by the three positive patients.

Among these three cases, only the first biopsy of case 3 showed parvovirus B19 DNA (Figure 1) and VP1 antigen by immunohistochemistry (Figure 2) in the liver tissue collected at the time of the initial diagnosis of hepatitis in this patient, suggesting that parvovirus B19 was actively replicating in the liver tissue at that time. These two different viral markers were detected only in the first biopsy, but were negative in another biopsy collected several months later.

Anti-liver kidney microsome type 1, anti-liver cytosol type 1, antimitochondrial, anti-smooth muscle, antinuclear and anti-actin antibodies were assayed in the three cases positive for anti-B19 IgM or DNA. Only case 3 was positive for antimitochondrial antibody.

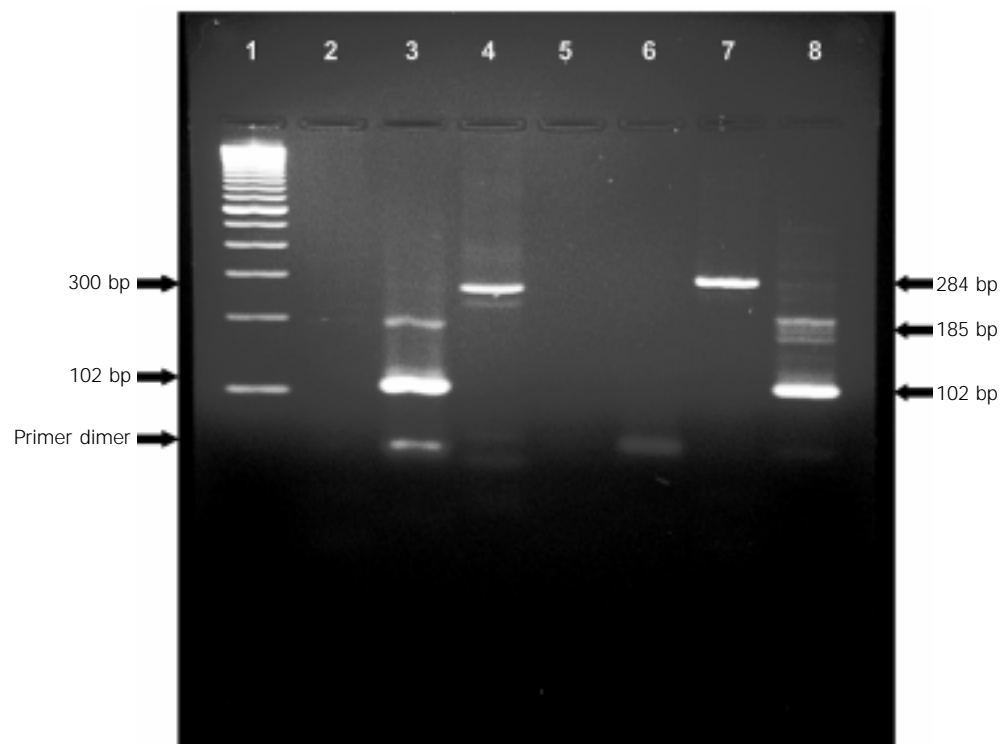
Cases 1 and 2 showed chronic liver dysfunction, with no other hepatitis-associated viruses being detected. These two patients were 70 and 78 years old, respectively, and the presence of liver disease was detected during surgery for another disorder (cholelithiasis and anovaginal fistula, for cases 1 and 2, respectively). Case 1 had normal levels of hepatic enzymes and a histological pattern of "nonspecific reactive hepatitis", with minor portal fibrosis and inflammation and with small amounts of lymphocytes, plasma cells and eosinophils. Portal triad and limiting plate were preserved. Focal hepatocytes were found to be swollen, rarely

Table 1. Clinical features of three patients positive for parvovirus B19 IgM and DNA.

Clinical features	Case 1 (LMF)	Case 2 (EV)	Case 3 (NM)
Sex	Male	Female	Female
Age	70	78	56
IgM anti-B19	Positive	Positive	Positive
B19 DNA (liver)	Negative	Negative	Positive
VP1 antigen (liver)	Negative	Negative	Positive
Liver biopsy	Nonspecific reactive hepatitis	Cirrhosis	Submassive necrosis (first), and liver cirrhosis with interface hepatitis, rosettes and plasmocytes (second)
Autoantibody profile	Negative	Negative	Antimitochondrial positive
Surgery	Yes	Yes	Yes
Blood transfusion	Yes (14 years before)	No	Yes (12 years before)
AST (x UNV)	0.62	2.05	46.4
ALT (x UNV)	0.62	1.13	57.4
Alkaline phosphatase (x UNV)	0.25	2.70	2.20
$\gamma$ GT	0.58	5.88	2.80
$\gamma$ Globulins (g/100 ml)	1.70	1.28	2.66
HLA	ND	ND	DR7, DR8
Immunosuppressor therapy	ND	ND	Complete response
Score of international criteria for AIH diagnosis (Ref. 37)	ND	ND	15 (before treatment), 17 (after treatment, still under immunosuppressor therapy)

AST = aspartate aminotransferase; ALT = alanine aminotransferase; x UNV = times the upper normal value;  $\gamma$ GT =  $\gamma$ -glutamyl transpeptidase; HLA = human leukocyte antigen; AIH = autoimmune hepatitis; ND = not determined.

Figure 1. Agarose gel electrophoresis and ethidium bromide fluorescence of B19 DNA PCR and nested PCR amplification products. Lane 1, 100-bp DNA ladder. Lane 2, PCR product of a liver biopsy specimen (case 3). Lane 3, Nested PCR product of a liver biopsy specimen (case 3). Lane 4, PCR product of the control gene  $\beta$  actin. Lane 5, PCR negative control. Lane 6, Nested PCR negative control. Lane 7, PCR product of a B19 DNA-positive control (284 bp). Lane 8, Nested PCR product of a B19 DNA-positive control (102 bp).



presenting lipid droplets. Scarce mononuclear infiltration was seen along the sinusoids. Case 2 had elevated liver enzymes, especially alkaline phosphatase and  $\gamma$ -glutamyl transpeptidase. A liver biopsy showed cirrhosis with marked septal inflammation with lymphocytes and some histiocytes. Moderate piecemeal necrosis was found. In the parenchyma, liver cells were frequently swollen, with occasional "spotty necrosis" and extensive steatosis. Another serum sample obtained from this patient one year later exhibited the same enzymatic pattern. No evidence for parvovirus infection in liver tissue was found in these two cases.

Case 3 was a 56-year-old woman with clinically severe hepatitis associated with isolated 1:80 antimitochondrial antibody, whose specificity for the pyruvate dehydrogenase complex (anti-M2) was confirmed by Western blotting. A liver biopsy revealed submassive hepatic necrosis. Several groups of apoptotic and necrotic hepatocytes were seen among small lymphocyte aggregates, with some figures of emperipolesis (Figure 3). Occasional histiocytes and plasma cells were found interspersed. In other regions of the sample, only minor liver cell damage was seen.

Parvovirus VP1 antigen was detected by immunohistochemistry in the cytoplasm of several hepatocytes, especially in those presenting remarkable swelling or undergoing acidophilic retraction (apoptosis), as shown in Figure 3.

Although antimitochondrial antibody is a classical immunological marker for primary biliary cirrhosis, biochemical tests (elevated aminotransferases, hypergammaglobulinemia, and slight alterations of alkaline phosphatase and  $\gamma$ -glutamyl transpeptidase) and histological findings pointed to a diagnosis of hepatitis and not to cholestasis, in spite of the presence of mild pruritus. For these reasons, case 3 was treated for autoimmune hepatitis (or a hybrid form of autoimmune hepatitis with the serological marker

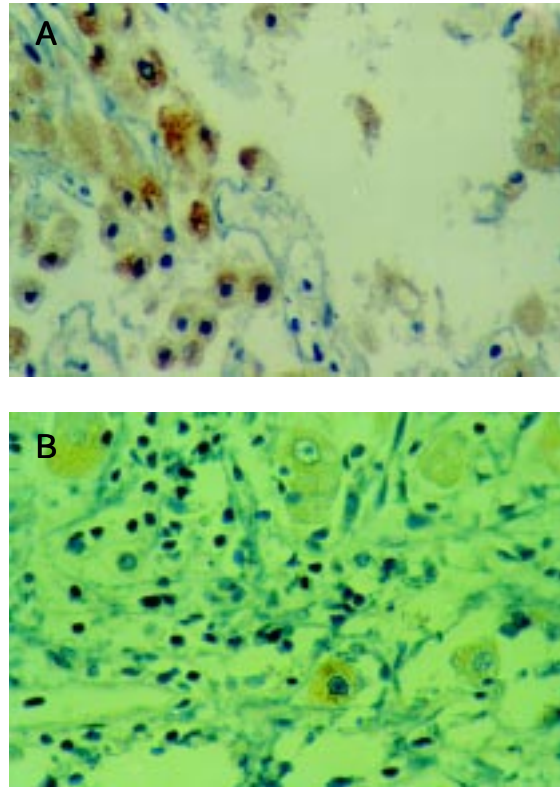


Figure 2. Immunohistochemical detection of parvovirus B19 VP1 antigen in a formalin-fixed paraffin-embedded liver biopsy (case 3). A, Several swollen or retracted centrilobular hepatocytes display VP1 antigen in granular, cytoplasmic distribution (LSAB-Peroxidase, 150X). B, High magnification view of a positive reaction for VP1 antigen in the cytoplasm of a hepatocyte immersed in mononuclear infiltrate (LSAB-Peroxidase, 600X).

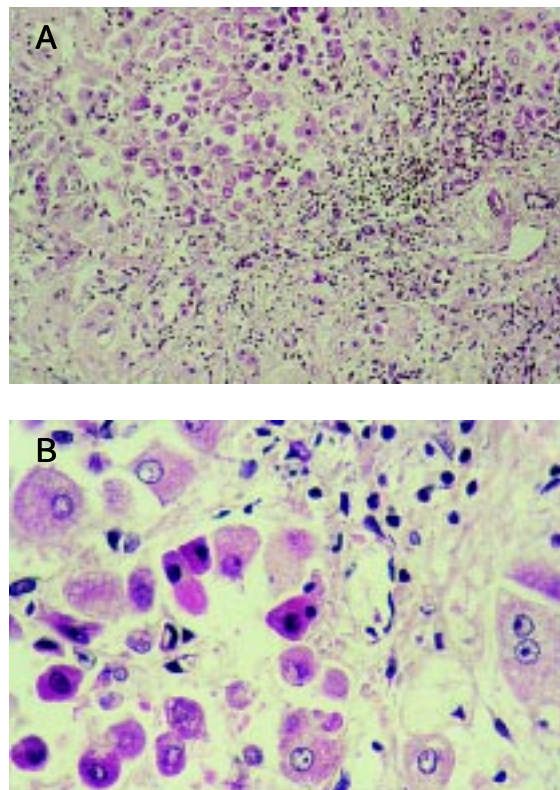


Figure 3. Histopathological aspects of extensive hepatic necrosis. A, Shrunken hepatocytes are detached from other swollen hepatocytes. Mononuclear inflammatory infiltrate is found, especially in the periportal area (case 3, H & E, 60X). B, Several acidophilic "Councilman-like" bodies are immersed in the loose, edematous liver tissue. At the center, one lymphocyte is found inside an acidophilic hepatocyte (emperipolesis) (case 3, H & E, 150X).

for primary biliary cirrhosis) with corticosteroid and azathioprine.

Another histopathological examination of a liver biopsy obtained after a few months of treatment detected liver cirrhosis with interface hepatitis, compatible with the autoimmune pattern. No viral DNA or antigen was detected in the liver tissue at that time. After one year of treatment, case 3 achieved a complete response to immunosuppression, reinforcing the initial hypothesis of autoimmune hepatitis. This time course is also consistent with infectious hepatitis in which the B19 infection was resolved.

## Discussion

In the present study, evidence for parvovirus B19 infection (monitored by anti-B19 IgM antibodies) was found in three (2.3%) of 129 cases of non-A-E hepatitis.

Although B19 IgM antibodies are rarely found in patients not associated with an outbreak and without an illness suggesting parvovirus infection (25), there always is the possibility of false-positive results among these cases, even when using highly accurate tests (26). Thus, we have confirmed the presence of parvovirus infection in the liver using two other methods, PCR and immunohistochemistry.

One patient (case 2) showed chronic liver dysfunction with the histological pattern of cirrhosis, whereas case 1 had only minor histological lesions. No parvovirus DNA or VP1 antigen was found in the liver tissue of these patients. The B19 diagnosis in these two cases could simply represent intercurrent infections. Both patients were older than 70 years, asymptomatic concerning liver disease, had undergone previous surgeries and their hepatic disease had been diagnosed by a liver biopsy performed during surgery due to other reasons. Parvovirus B19 infection in chronic hepatitis cases has been reported by several authors. Indeed, this virus was found originally in a patient with chronic hepatitis

B (27), but the finding of persistent asymptomatic infections initially ruled out a possible etiological role (28).

Case 3 was a 56-year-old woman with a severe hepatic clinical picture and severe liver damage (submassive necrosis). Parvovirus B19 DNA and VP1 antigen were detected in the liver biopsy confirming the presence of active viral infection. Remarkably, several liver cells positive for VP1 antigen were swollen or undergoing acidophilic retraction, raising the hypothesis of a possible direct role of the virus in these hepatocellular lesions. This patient also showed isolated antimitochondrial antibody and was treated for autoimmune hepatitis with corticosteroid and azathioprine, showing a good response to this therapy, at least regarding the levels of serum aminotransferases and the disappearance of submassive hepatic necrosis. However, the autoimmune cirrhosis has already been settled.

The results obtained for case 3 corroborate other data providing evidence that parvovirus B19 may be a cause of hepatic dysfunction by mechanisms that are not yet elucidated (3-12). Some authors suggest a direct effect of parvovirus B19 infection after interaction with its cellular receptors, the erythrocyte P antigens, which are present on erythroid and liver cells (29). Furthermore, B19 DNA has been detected in liver tissue from fulminant hepatic failure cases (5). The time course of hepatitis in case 3 is also consistent with infectious hepatitis in which the B19 infection was resolved.

Another possible mechanism may involve the immune system, as reported in the virus-associated hemophagocytic syndrome after infection with B19 (30). Viral infection may induce increased levels of cytokines such as interferon- $\gamma$  and tumor necrosis factor- $\alpha$ , that deregulate the phagocytic system, leading to pancytopenia and/or hepatic dysfunction (31). Parvovirus B19 has also been associated with other immunological diseases (32-34), and the induction of different autoanti-

bodies, including antimitochondrial ones, in patients infected with this virus has been reported (35,36).

This second hypothesis seems more likely since parvovirus B19 DNA and VP1 antigen were detected only in the first biopsy, but not in another one obtained several months later. Another clue for a possible immuno-medi-

ated pattern of liver lesion is the strict relation of lymphocytes to damaged hepatocytes, as demonstrated by the emperipolesis depicted in Figure 2B. This mechanism can also explain the association found in this case with antimitochondrial antibodies, even after viral clearance (i.e., in the absence of detectable virus in the liver).

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