Prevalence of Parvovirus B19 in Liver Tissue: No Association with Fulminant Hepatitis or Hepatitis-Associated Aplastic Anemia

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Parvovirus B19 has been proposed as the etiological agent of fulminant hepatitis (FH) or hepatitis-associated aplastic anemia (HAA). We studied the prevalence of parvovirus B19 in liver-tissue samples from patients with FH and HAA and from control subjects. In the first study, parvovirus B19 DNA was detected by nested polymerase chain reaction (PCR) in 4 of 15 livers from patients with FH and in 3 of 22 livers from patients with nonviral hepatic disease. In a second confirmatory study, livers were tested for parvovirus B19 and its variant erythroviruses, V9 and A6. Tissues were also tested by reverse-transcriptase PCR for the presence of parvovirus B19 transcripts as a marker of viral replication. There was no significant difference in the prevalence of parvovirus B19 DNA in livers from patients with FH or HAA, compared with liver-tissue samples from patients with hepatitis B virus (HBV) or hepatitis C virus (HCV) infection; parvovirus B19 transcripts were not detected. There was a significant increase ($P < .1$) in the prevalence of variant erythrovirus sequences in livers of patients with HBV or HCV hepatitis, the reason for which is currently unknown.

Parvovirus B19 is currently the only accepted member of the Erythrovirus genus and the only parvovirus known to be pathogenic in humans. The virus is the etiological agent of erythema infectiosum and transient aplastic crisis (TAC) and causes chronic anemia in immunocompromised patients [1]. Diagnosis of parvovirus B19 is done by serologic testing or viral DNA detection. Although the viral genome was thought to be well conserved, with only $\sim 2\%$ sequence variability between isolates [2], viral sequences, tentatively termed V9 [2, 3] and A6 [4], recently have been reported from patients with TAC and chronic anemia, respectively. V9 and A6 showed $\sim 10\%$ variability from each other and from the previously published parvovirus B19 sequences. More recently, French researchers have reported the identification of 3 parvovirus B19 genotypes in serum or bone marrow samples from France [5]; according to their classification, A6 sequence would be parvovirus B19 genotype 2. However, the true prevalence of these viruses is unknown, with no variant parvovirus B19 sequences detected in plasma pools from $>120,000$ Danish blood donors [4, 6].

Parvovirus B19 was first identified in a serum sample being tested for hepatitis B virus (HBV) [7], and liver tenderness was a feature of the illness in the first symptomatic and confirmed parvovirus B19 infection report.
ed in the literature [8]. Recently, detection of parvovirus B19 DNA in the liver of patients with hepatitis [9–11] has implicated parvovirus B19 as an etiological agent for hepatic inflammation [12]. Similarly, parvovirus B19 has been suggested as a possible cause of fulminant hepatitis (FH) [13, 14] and hepatitis-associated aplastic anemia (HAA) [15, 16], on the basis of polymerase chain reaction (PCR) results. However, these studies were either based on small numbers of samples or cases, or they lacked confirmatory tests for the significance of the parvovirus B19 DNA detection. We have developed reverse-transcriptase (RT) PCR assays to detect the presence of actively transcribing virus as a marker of viral replication [17]. We therefore studied the prevalence of parvovirus B19 and B19 variants in liver-tissue samples from patients with FH and HAA and from control subjects and analyzed positive tissues for the presence of viral RNA transcripts.

MATERIALS AND METHODS

Plasmids, serum samples, and plasma pools. Dr. Peter Tattersall (Department of Laboratory Medicine, Yale University, New Haven, CT) kindly provided the plasmid pYT103 containing the entire coding region of the Au isolate of B19 (GenBank accession no. M13178). A plasmid containing the complete coding sequence of V9 (V9-C22) was obtained from Collection National de Culture de Microorganisms (CNCM1-2066; Institute Pasteur, Paris; GenBank accession no. AJ249437). Plasmid A6-c8, which contains the complete coding sequence of A6, has been described elsewhere [4].

Liver-tissue samples. For the initial testing of parvovirus B19 in liver-tissue samples, anonymous coded samples of liver DNA, extracted as described elsewhere [18], were provided by Dr. Richard Sallie (Liver Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD). Only limited DNA was available, so liver-tissue samples were obtained from the Liver Tissue Procurement and Distribution System at the University of Minnesota or from our own program as part of our ongoing studies of the etiology of HAA, to conduct more-definitive DNA studies and to obtain RNA from tissues. HAA was defined as the development of severe aplastic anemia within 6 months after an episode of hepatitis [19]. Liver-tissue samples were immediately frozen at the time of hepatic transplant and stored frozen in liquid nitrogen. Frozen liver-tissue samples (50–100 mg) were ground in liquid nitrogen and used for either DNA or RNA extraction.

Parvovirus B19 PCR. DNA was extracted from liver tissues (25 mg) using the DNAEasy method (Qiagen), eluted in 100 μL of H2O, and tested for parvovirus B19 by nested PCR, as described elsewhere [20]. Specifically, the primary round (30 cycles) of PCR was performed using 30 pmoles of primers L (nt 1419–1435; [21]) and M (nt 2139–2123) in a 50-μL volume with ExTaq DNA polymerase (Panvera), and 5 μL of the product was reamplified using primer pair F (nt 1490–1515) and I (nt 2088–2065). Confirmation of the specificity of the PCR product was performed by Southern hybridization using a probe of the product of PCR amplification of pYT103 with primer pairs J (nt 1629–1650) and K (nt 1979–1958) labeled by either 32P-nick translation or alkaline phosphatase (Alkphos Direct Labeling with CDPStar kit; Amersham).

Variant parvovirus B19 PCR. Variant parvovirus B19 PCR was performed using primer set B [22], which is common to both parvovirus B19 and the V9 and A6 variants, as described elsewhere [4]. Specifically, PCR was performed using 12.5 pmoles of primers E1905F (nt 1905–1923) and E1987R (nt 2007–1987) in a 50-μL volume using AmpliTaq Gold (Perkin-Elmer) with 50 cycles of amplification (6 min at 94°C, 5 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C; 45 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by a 7-min extension at 75°C). Confirmation of specificity of the PCR product was performed by Southern hybridization using an alkaline phosphatase–labeled oligonucleotide, E1954P (nt 1954–1989).

To distinguish between parvovirus B19 and variant parvovirus B19 sequences, the PCR product was incubated directly with MfeI restriction enzyme for 2 h, and then the products were run on a 2.5% 3:1 Nusieve agarose gel (BioWhittaker). In addition, the sequence of the PCR product was determined by TA cloning of the PCR product into PCR2.1 TOPO (Invitrogen), transformation of Top10 cells (Invitrogen), and sequencing the resultant plasmids using BigDye terminator cycle sequencing (ABI–Perkin Elmer).

Confirmation of the variant sequences. Confirmation of the variant parvovirus B19 sequences was performed using nested primers common to the known parvovirus B19, V9, and A6 sequences. First-round PCR was performed using 20 pmoles of primers V1847 (nt 1847–1868) and V2925R (nt 2925–2904) in a 50-μL volume, using ExTaq DNA polymerase (Panvera) with 30 cycles of amplification (denatured at 94°C for 1 min, 30 cycles of 40 s at 92°C, 40 s at 60°C, 1.5 min at 75°C, and then extension at 75°C for 7 min). Second-round PCR was performed using the same conditions but with primers V1906 (nt 1906–1925) and V2799R (nt 2799–2780). PCR products were purified by Qiaquick (Qiagen) and directly sequenced using BigDye terminator cycle sequencing (ABI–Perkin Elmer).

RT-PCR for parvovirus B19 transcripts. RT-PCR for viral transcripts was a modification of our method, described elsewhere [17], for detecting parvovirus B19 RNA transcripts in a parvovirus B19 neutralization assay. RNA was extracted from ground frozen liver-tissue samples by direct suspension in RNA STAT 60 (0.1g/mL; TelTest), and aliquots were kept at −80°C until use. RNA was precipitated by centrifugation, according to the manufacturer’s instructions, and redissolved in ~20 μL of H2O, and the concentration was calculated by optical density.
RNA in all samples were tested by detection of GAPDH tran-

ting of the products with an alkaline phosphatase–labeled probe

were analyzed by agarose gel electrophoresis and Southern blot-

a primer based in the capsid region of the genome. All products

Similarly, amplification of the spliced product of parvovirus

plification conditions as those used for parvovirus B19 PCR.

9 (nt 384–402) and B19-NS (nt 599–582), using the same am-

Technologies). Amplification of the nonspliced product of par-

random hexamers and Superscript II reverse transcriptase (Life

in a volume of 22

L of cDNA and primers B19-

measurement. As a positive control for parvovirus B19 infec-

tion, bone marrow cells from a healthy donor were infected

with parvovirus B19, the cells were harvested 3 days after in-

fection, and the RNA was extracted by use of RNA STAT 60.

Residual DNA from both the liver and the parvovirus B19–

infected bone marrow cells was removed by DNase I treatment

(final concentrations, 90 U/mL DNase I and 20 mg/mL RNA

in a volume of 22 μL) for 15 min before cDNA synthesis with

random hexamers and Superscript II reverse transcriptase (Life

Technologies). Amplification of the nonspliced product of par-

vovirus B19 was done using 5 μL of cDNA and primers B19-

9 (nt 384–402) and B19-NS (nt 599–582), using the same am-

plification conditions as those used for parvovirus B19 PCR.

Similarly, amplification of the spliced product of parvovirus

B19 was done using primer B19-9 and B19-1 (nt 2139–2120),

a primer based in the capsid region of the genome. All products

were analyzed by agarose gel electrophoresis and Southern blot-

ting of the products with an alkaline phosphatase–labeled probe

(parvovirus B19, nt 2103–2138). Extraction and integrity of

RNA in all samples were tested by detection of GAPDH tran-

scripts (GenBank NM_002046, nt 101–312).

**RT-PCR for variant parvovirus B19 sequences.** On the

basis of the genome similarity to parvovirus B19 and the known

sequences of V9 and A6, RT-PCR for the variant spliced viral

capsid transcripts also was developed [4]. In brief, RNA was

extracted from the livers, and RT-PCR was performed, as

described above, with amplification of the spliced product using

primers V9-9 (GTG-CTT-TGC-CTG-CTA-AGT-A) and V9-1

(CCA-CGA-GGC-GGC-TAC-CAC-CT) or A6-9 (TTT-GCC-

TGC-TAA-TTA-ACA) and A6-1 (ACT-GGT-CTG-CAA-GTG-

GAG-TG), and probing for the amplicon with an alkaline phos-

phatase–labeled probe (parvovirus B19, nt 2103–2138). As a positive control,

Cos-7 cells (ATCC) were transfected with plasmids V9-C22 or

A6-c8, and RNA was extracted from cells 3 days later.

**RESULTS**

**Results of testing samples for parvovirus B19.** For the initial

screening of liver-tissue samples for parvovirus B19, DNA was

obtained from 38 livers. Of these, 7 (18%) were positive for

parvovirus B19 DNA (table 1). Although there was an increased

prevalence of parvovirus B19 in the samples from patients with

FH, this difference was not significant, compared with that in

the nonviral hepatitis group. Only limited samples were avail-

able, and there was insufficient material for variant parvovirus

B19 PCR testing or RT-PCR analysis.

Testing of the second group of liver-tissue samples revealed a

similar prevalence of parvovirus B19, with viral DNA detected

in 20 (24%) of 83 samples (table 2). Although parvovirus B19

was present in 8 (35%) of 23 liver-tissue samples from patients

with FH, it was also found in 10 (33%) of 30 livers from patients

with known HBV and/or hepatitis C virus (HCV) infections.

Parvovirus B19 DNA was detected only in 1 (9%) of 11 samples

from patients with HAA and 1 (5%) of 19 samples from patients

with biliary atresia.

**Variant parvovirus B19 sequences and hepatitis.** The vari-

ant PCR amplifies a much smaller product (103 bp vs. 599 bp)

and is more sensitive at detecting parvovirus B19 sequences (au-

thors’ unpublished observations). By use of this assay, erythro-

virus sequences could be detected in 37 (45%) of 83 samples.

However, there was no significant difference in the prevalence

of erythroivirus sequence in the FH group (16/23 samples), com-

pared with the prevalence in patients with known hepatitis A

virus, HBV, or HCV infections (16/30 samples; table 2).

To identify variant sequences, all variant PCR products were

digested with MfI, and amplicons that were not cut with MfI

or that tested negative for parvovirus B19 PCR then were se-

quenced. Seven variant sequences were identified, and all were

confirmed by sequencing the products of the confirmatory

PCR. Six of the variant sequences, 5 A6-like sequences and 1

V9-like sequence (figure 1), were from livers of patients with

**Table 1. Results of testing liver DNA samples for parvovirus B19 by nested polymerase chain reaction.**

<table>
<thead>
<tr>
<th>Liver-tissue samples</th>
<th>Total</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH</td>
<td>15</td>
<td>4 (27)a</td>
<td>11</td>
</tr>
<tr>
<td>HBV infection</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Nonviral</td>
<td>22</td>
<td>3 (14)a</td>
<td>19</td>
</tr>
<tr>
<td>Primary biliary cirrhosis</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Primary sclerosing cholangitis</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Acetaminophen overdose</td>
<td>8</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Alcoholic liver disease</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Wilson disease</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of liver-tissue samples. FH, fulminant hepatitis; HBV, hepatitis B virus.

a P = .3.

**Table 2. Results of testing liver-tissue samples for parvovirus B19 DNA and variant erythrovirus DNA by polymerase chain reaction (PCR).**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>B19 PCR positive</th>
<th>Variant PCR positive</th>
<th>Variant (n)</th>
<th>RT-PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH³</td>
<td>8/23</td>
<td>16/23</td>
<td>V9 (1)³</td>
<td>0/7</td>
</tr>
<tr>
<td>HAA</td>
<td>1/1</td>
<td>3/11</td>
<td>NA</td>
<td>0/1</td>
</tr>
<tr>
<td>Biliary atresia</td>
<td>1/19</td>
<td>2/19</td>
<td>NA</td>
<td>0/1</td>
</tr>
<tr>
<td>HBV or HCV infection</td>
<td>10/30</td>
<td>16/30</td>
<td>V9 (1), A6 (5)</td>
<td>0/7</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. of samples positive/no. of samples tested, unless otherwise indicated. Samples that had either detectable parvovirus B19 DNA (by nested PCR) or variant erythrovirus DNA also were tested by reverse-transcriptase (RT) PCR for spliced RNA transcripts. FH, fulminant hepatitis; HAA, hepatitis-associated aplastic anemia; HBV, hepatitis B virus; HCV, hepatitis C virus; NA, not applicable.

³ One sample had not been tested to exclude HCV infection.
Figure 1. Sequence alignment (A) and phylogenetic tree (B) of the variant erythrovirus sequences obtained from liver-tissue samples. Nucleotides that are identical to those in the Au isolate of parvovirus B19 are indicated with a period. Nos. indicate nucleotide position of parvovirus B19.

Sequences in alignment: B19 (GenBank accession no. M13178), V9-c22-1 (GenBank accession no. AJ249437), A6-c2 (GenBank accession no. AY064475), and A6-c8 (GenBank accession no. AY064476). Sequences LiverC25, LiverC26, LiverC32, LiverC41, LiverC45, and LiverC47 were obtained from livers of patients with hepatitis B virus and/or hepatitis C virus (HCV) infection; LiverH41 was from a patient with fulminant hepatitis, although the sample had not been tested for HCV.
confirmed HBV or HCV infections. The seventh isolate (V9-like) was from the liver of a patient with FH. Unfortunately the HCV status of this liver was unknown, and we were unable to obtain intact RNA from the liver for HCV RNA testing.

**RT-PCR results.** RNA was extracted from all samples that were parvovirus B19 positive, or variant parvovirus B19 sequences were isolated and, after confirming that RNA was successfully extracted by testing for GAPDH transcripts, were tested by RT-PCR for parvovirus B19, A6, or V9-like sequences. Although good-quality RNA was obtained from most of the samples, RNA could not be obtained from the liver sample from a patient with FH that contained V9-like sequence or from 3 livers from patients with hepatitis that contained parvovirus B19. However, parvovirus B19, A6, or V9 RNA transcripts could not be detected in any of the liver-tissue samples.

**DISCUSSION**

Despite the identification of HCV and hepatitis E virus as known causes of viral hepatitis, the etiology of hepatitis remains obscure in 3%–10% of cases in Europe [23] and the United States [24, 25] and, perhaps, up to 30% of cases in Asia [26, 27]. In addition, the cause of FH [18, 28–30] and HAA [19, 31] is unknown, although especially in the latter, the clinical picture and liver histologic testing suggest a viral etiology. The known tropism of parvovirus B19 for erythroid progenitor cells and the virus’ association with TAC makes parvovirus B19 an attractive culprit as the cause of more generalized marrow failure after hepatitis. However, in the present study, we detected parvovirus B19 sequences in only 3 of 11 liver-tissue samples, significantly less (P < .03) than in livers from patients with FH. Parvovirus B19 was present in some FH liver-tissue samples, but not at a significantly higher prevalence than in hepatic tissue from patients with confirmed HBV or HCV infections. In addition, we were unable to detect parvovirus B19 viral transcripts in any of the tissues, arguing against parvovirus B19 actively replicating in these tissues, although we cannot exclude the possibility that there were levels of parvovirus B19 transcripts below the sensitivity of our assay.

Although parvovirus B19 has been detected in individual cases and in series with small sample numbers, most investigators have not determined the prevalence of parvovirus B19 in healthy tissue. A German study determined the prevalence of parvovirus B19 by PCR in 44 liver transplant samples and 17 autopsy samples; parvovirus B19 DNA could be detected in 40% of the liver transplant samples and 24% of the autopsy samples, indicating, as in our study, that parvovirus B19 DNA can persist in liver tissue, perhaps for years following acute infection. Liver tissue is not unique in hosting persistent parvovirus B19 DNA. Parvovirus B19 DNA was detected in 13 (48%) of 27 synovial tissue samples from patients with traumatic knee injury [32], and parvovirus B19 could be detected in 4 (9%) of 45 bone marrow samples from healthy bone donors [33]. These data collectively emphasize the importance of appropriate controls and the difficulty in interpreting the presence of parvovirus B19 DNA in the etiology of hepatitis, because the presence of parvovirus B19 DNA in tissue cannot be used to infer causality.

The significance of the detection of the variant parvovirus B19 sequences in the livers from patients with known hepatitis viral infections is unclear. These variants clearly do not appear to be associated with either HAA or FH. Previously, we detected only 1 A6 sequence in 225 bone marrow and serum samples and no A6-like or V9-like sequences in 63 plasma pools, representing >120,000 donors [4]. The inability to detect viral transcripts in these tissues suggests that the variants may be “innocent bystanders,” although little is known of the pathogenicity or site of replication in the body of these variants.

**References**


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