Detection of Intrauterine Viral Infection Using the Polymerase Chain Reaction

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Intrauterine viral infection commonly presents as nonimmune hydrops fetalis or intrauterine growth restriction. Cytomegalovirus (CMV) and parvovirus are commonly recognized causes of fetal infection using serology and cultures. We used the polymerase chain reaction (PCR) to evaluate the frequency of fetal viral infection and the associated clinical course and outcome. Specimens (amniotic fluid, fetal blood, pleural fluid, tissue) from 303 abnormal pregnancies at risk for viral infection and 154 controls were analyzed using primers for CMV, herpes simplex virus, parvovirus B19, adenovirus, enterovirus, Epstein-Barr virus, and respiratory syncytial virus. Viral genome was detected in 144/371 samples (39%) or 124/303 patients (41%), with adenovirus (n = 74 patients; 24%), CMV (n = 30 patients; 10%), and enterovirus (n = 22 patients; 7%) most common. Only 4/154 (2.6%), unaffected control patients’ samples were PCR positive. We conclude that diagnosis of fetal viral infection by PCR is common in abnormal pregnancies. Adenovirus and enterovirus may cause fetal infection that have been previously unrecognized.

Key Words: polymerase chain reaction (PCR); fetal infection; nonimmune hydrops; viral infection.

Pregnancy is reportedly associated with decreased maternal resistance to viral infections due to depressed cell-mediated immunity (1). Therefore, the fetus is at risk to become infected by transplacental transmission during maternal viremia. Depending on the gestational age of the fetus, the involved virus, and the viral load, the spectrum of fetal involvement may range from asymptomatic to severe, causing either fetal or neonatal death or long-term sequelae in survivors. Rubella, cytomegalovirus (CMV), herpes simplex virus (HSV), and the varicella-zoster virus (VZV) are well-known teratogens (2–7). Congenital malformations after infection with coxsackie virus and echo virus (cardiac, urological, and gastrointestinal malformations), mumps (endocardial fibroelastosis), and influenza (central nervous system and heart defects) are also documented (8). Ultrasound findings during the second and third trimester of pregnancy consistent with intrauterine growth restriction (IUGR), nonimmune hydrops, isolated ascites, microcephaly, hydrocephaly, intracranial, or intrahepatic calcification raise the suspicion of a fetal viral infection affecting multiple organs (9).

Perhaps the most readily detectable ultrasound finding is nonimmune hydrops, defined as the accumulation of excessive fluid in at least two body cavities or edema and fluid collection in at least one body cavity. This nonspecific prenatal finding is associated with a perinatal mortality in excess of 50% (10,11). Fetal hydrops, isolated ascites or isolated pleural, and pericardial effusion, are believed to have many underlying causes and pathogenic
mechanisms including fetal anemia (11–15) and fetal heart failure. An infectious etiology is documented by viral culture or serologic methods in 5–15% of cases of fetal hydrops; in up to 22% of cases, the underlying cause is unknown (12,13). Fetal parvovirus infection causes nonimmune hydrops secondary to severe fetal anemia due to suppression of fetal erythroid progenitors (16); myocarditis has also been reported (17). Infections with other viruses such as CMV (18,19) or HSV (20), and more rarely with enteroviruses (14) or adenoviruses (15), are also reported to cause hydrops fetalis. With the recent advances in viral therapy and fetal medicine, some of these infections are now amenable to supportive or specific prenatal treatment (15,17,21,22). Therefore, rapid and accurate identification of the causative virus is increasingly important as a guide to prenatal management.

The detection of a fetal viral infection usually relies on documentation of maternal and fetal seroconversion for the specific virus and isolation of the virus from fetal body fluids, amniotic fluid, or autopsy specimens. However, serological diagnosis may lack sensitivity and can be difficult to interpret. In addition, a second convalescent sample 2–3 weeks after the initial study is required for diagnosis. Culture and subsequent identification of the virus is the “gold standard” of diagnosis, but this method is also generally slow, sometimes taking between 1 and 3 weeks to obtain a positive result. In addition, certain viruses (e.g., parvovirus B19), do not readily grow in standard culture systems (23). The limited availability of specimens for study of a suspected fetal infection commonly restricts the search to only a few probable causative viruses. Finally, the serologic diagnosis assumes a sustained fetal immunologic response, yet several case reports demonstrate this is untrue (24,25).

Recent advances in molecular genetic diagnostic techniques, namely in situ hybridization (26) and the polymerase chain reaction (PCR) (27,28) permit rapid detection of viral nucleic acid in a variety of infected tissues and body fluids (15,29–35) from adults, children, and fetuses. Detection of fetal infection with CMV, toxoplasmosis, adenovirus, and human parvovirus B19 (36–38) has already been reported. In addition, PCR has been used to identify infection with a number of different viruses in adults or children with myocarditis and dilated cardiomyopathy (39–43).

In this study, the usefulness of PCR in the prenatal diagnosis of fetal viral infection was evaluated using oligonucleotide primers designed to amplify nucleic acid sequences from seven different viruses including CMV, parvovirus, adenovirus, enterovirus, HSV, Epstein-Barr virus (EBV), and respiratory syncytial virus (RSV). These viruses are common, are known to cause either fetal infection or myocarditis in infants and children, and infect individuals with marginal immune system function.

**MATERIALS AND METHODS**

**Patient Population**

Samples were obtained after written informed consent from mothers of 303 fetuses considered to be at risk for intrauterine viral infection. A variety of clinical presentations were used for inclusion criteria, including nonimmune fetal hydrops diagnosed by prenatal ultrasound, fetal pleural effusion, “stuck-twin” or presumed twin-to-twin transfusion syndrome, fetal ventriculomegaly, fetal intracranial calcification/microcephaly, fetal hepatic calcification or echogenic bowel, fetal myocarditis, maternal exposure to virus, polyhydramnios, oligohydramnios, IUGR, or combinations of these presentations. The mean gestational age at presentation and sample referral was 18 weeks with a range of 15 to 35 weeks. Control samples (n = 154) were obtained after informed consent at gestational ages ranging between 14 and 22 weeks, generally at the time of genetic amniocentesis in patients presumably at low risk for the presence of a viral infection.

**Sample Material**

Sample material (n = 371) was obtained for PCR analysis from “at risk” patients (n = 303) at 34 medical centers and included amniotic fluid (n = 253), fetal blood obtained by cordocentesis (n = 42), cord blood obtained at the time of delivery (n = 18), fetal ascites (n = 8), pleural effusion (n = 10), and tissue samples (n = 40), including 19 placental biopsies at the time of delivery. Other tissue samples received included autopsy specimens such as heart (n = 12), lung (n = 5), and fetal liver (n = 4).

Prenatally acquired samples were obtained during invasive diagnostic procedures (amniocentesis, cordocentesis, aspiration of other fetal body fluid accumulations) performed as part of an evaluation of either abnormal ultrasound findings or maternal exposure to viral infection. Fetal blood (2–4 ml) was
collected in sodium heparin collection tubes; body fluid (2–4 ml) and amniotic fluid (2–4 ml) samples were collected in sterile vials and transported either on ice or at room temperature before being stored at 4°C until analysis. Tissue specimens obtained at autopsy were formalin-fixed in the usual manner. Placental samples or other tissue specimens were frozen in liquid nitrogen (44) or analyzed after formalin-fixation. Viral culture and serology was obtained by the referring physician.

Control Samples

Amniotic fluid samples were collected for genetic amniocentesis from 154 patients with structurally normal fetuses. These patients had no history suggestive of a viral syndrome during pregnancy and were thus classified as low risk for fetal viral infection. These samples served as “negative patient controls” and were analyzed in blinded fashion concurrent with the other “diagnostic” samples. Nucleic Acid Extraction

Tissue samples were homogenized in RNAzol (45) using a Brinkmann Polytron homogenizer prior to nucleic acid extraction as previously described (42). Fluid specimens did not require homogenization and were extracted directly. Total RNA and DNA was isolated simultaneously from patient specimens using the method initially described by Chomczynski and Sacchi (45) and subsequently modified (42,43).

Primer Design and Synthesis

Oligonucleotide primer pairs were prepared on an oligonucleotide synthesizer (Applied Biosystems, Incorporated model 380) using published viral sequences (Table 1). One primer pair was designed to amplify a 151-bp region of the 5′-untranslated region conserved among all enteroviruses (including Coxsackie viruses and Echovirus), with the exception of echovirus 22 and 23 (46). A second primer pair, designed to amplify a 607-bp product was created from the immediate early gene of the AD169 strain of CMV, recognizing all CMV serotypes (33). The third primer pair was designed to amplify a 308-bp sequence of the hexon region of all adenovirus serotypes (except adenovirus 40,41) (29). The fourth primer pair amplifies a 241-bp product for HSV-2 or 229-bp product for HSV-1 (47), and the fifth primer pair amplifies a 699-bp sequence of the parvovirus genome, encoding the VP1 capsid protein (48). The sixth primer pair was designed to amplify a 375-bp amplimer from the EcoRI B fragment of EBV (49).

Finally the seventh primer set was designed to amplify a 391-bp RT-PCR amplimer from the F-glyco-protein of RSV, with a 207-bp product amplified by internal nested primers (50). In addition, a primer pair was designed to detect sequences from K-ras, a constitutive component of all tissues. A sequence spanning the 12th codon of K-ras which amplifies a 135-bp product was selected (49) to verify adequate nucleic acid extraction. PCR and RT-PCR were performed as described previously (15,29,30,33,42,43,47,49,50).

In order to demonstrate successful PCR amplification, all samples were analyzed with positive control viral nucleic acid; contamination was excluded by the lack of amplified product in a negative control sample (Fig. 1). All negative control amplifications were performed in the same reaction mixture used for positive controls and patient samples, but were devoid of nucleic acid template.

Template Preparation and PCR

Total RNA and DNA were isolated simultaneously from patient specimens using a modification of the RNAzol method first described by Chomczynski and Sacchi (69,70) as previously described (68,71,72). Tris-saturated phenol (pH 6.6) RNAzol solution was used in simultaneous isolation of both RNA and DNA, which was performed as follows.

Reverse transcriptase-PCR (RT-PCR) was employed to evaluate the RNA viruses, including the enteroviruses, and RSV. First strand cDNA, for use in detection of enterovirus, was generated from 3 μg of extracted total RNA in the presence of 20 units of an RNase inhibitor, RNasin (Amersham International). This mixture was heated to 95°C for 5 min then cooled in ice. To each reaction was added 5 pmol of primer no. 195, 4 μl of 5 mM each dNTPs, 4 μl of 5x reverse transcriptase buffer (5× buffer = 15 mM MgCl2, 50 mM dithiothreitol, 250 mM Tris, pH 8.3, and 375 mM KCl), 200 units of Maloney murine leukemia virus (MoMLV) reverse transcriptase (BRL-Gibco), and DEPC-treated dH2O to 20 μl; this reaction was incubated at 37°C for 1 h. Two microliters of this first-strand cDNA or 500 ng of control genomic DNA was combined with 25 pmol of each enterovirus primer, 5 μl 10× PCR buffer (100 mM Tris, pH 8.3, 500 mM KCl, and 15 mM MgCl2); and 5 μl 2 mM dNTPs each in a 50-μl reaction volume. Taq polymerase (Perkin-Elmer-Cetus) was added (2.5
TABLE 1
Viral PCR Primers

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primer sequence</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterovirus (5' Untranslated region)</td>
<td>5'-TCCTCCGGCCCTCTGAAT-3'</td>
<td>151</td>
</tr>
<tr>
<td>Cytomegalovirus (Immediate early gene region)</td>
<td>5'-GGCCATGGGCCGATTGCGAACTTG-3'</td>
<td>607</td>
</tr>
<tr>
<td>Adenovirus (Hexon region)</td>
<td>5'-AGCACGCCGGGATGTCAAAG-3'</td>
<td>308</td>
</tr>
<tr>
<td>Herpes simplex virus (DNA pol gene region)</td>
<td>5'-TGGGTGACAGCTGCCAAGAT-3'</td>
<td>229 (HSV1)</td>
</tr>
<tr>
<td>Parvovirus B19</td>
<td>5'-ATAAATCCATATCTAATT-3'</td>
<td>699</td>
</tr>
<tr>
<td>Epstein-Barr virus (EcoRI B fragment)</td>
<td>5'-ACCTGGAGGCCATCGCAAGCTCC-3'</td>
<td>375</td>
</tr>
<tr>
<td>Respiratory syncytial virus (F-glycoprotein)</td>
<td>5'-GGTGTTGGATCTGCAATCGC-3'</td>
<td>392</td>
</tr>
<tr>
<td>Nested PCR</td>
<td>5'-AAGTGCTCTACTATCCACA-3'</td>
<td>207</td>
</tr>
<tr>
<td>K-ras</td>
<td>5'-TTACCTCTATTGATCATATCGTCA-3'</td>
<td>135</td>
</tr>
</tbody>
</table>

units) after an initial 5-min incubation at 95°C. Thirty-five (35) rounds of amplification were performed under the following conditions: 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min using a Perkin-Elmer-Cetus Thermocycler or Biometra Thermocycler. The same procedure was used for RSV genome amplification.

For adenovirus, CMV, HSV, and EBV (i.e., DNA viruses), 3 μl or 1000 ng of extracted viral DNA was combined with 25 pmol of appropriate primer, 5 μl PCR buffer, and 5 μl 2 mM dNTPs, each in a 50-μl reaction volume. Taq polymerase (2.5 units) was added after an initial 5-min incubation at 95°C. Forty (40) rounds of amplification were performed at the following conditions: 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min. For HSV, the conditions were 95°C for 1 min, 61°C for 1 min, and 72°C for 1 min, using 32 rounds of amplification. Ten microliters of each reaction was analyzed on a 1% ME agarose gel (FMC Biochemicals) or 3% Nu Sieve agarose (FMC Biochemicals) and 0.5% ME agarose gel containing 0.5 μg/ml of ethidium bromide (Sigma). The gels were then placed under UV light for visualization of the amplified products.

Parvoviral target DNA sequences were amplified in a total reaction volume of 100 μl containing 100 μM each dNTPs, 1 μM each of primers, 10 μl 10× PCR buffer, and 3 μl sample. Taq polymerase, 2.5 units (Perkin-Elmer, Branchburg, NJ), was added after an initial incubation at 94°C for 3 min. Thirty-three rounds of amplification were carried out at the following conditions: 94°C × 2 min, 42°C × 2 min, and 72°C × 3 min (72°C × 7 min extension).

All samples were run with a simultaneous positive and negative control (i.e., reaction mixture without sample nucleic acid) for the virus analyzed. If a band was visualized in the negative control lane, the PCR sample was considered contaminated and the sample was reanalyzed. All samples were run without knowledge of the clinical, culture, or serologic data, and were performed in duplicate. For the PCR amplifier to be considered positive, reproducibility of the product was required. Control PCR amplification to verify the presence of amplifiable nucleic acid extracted from each sample was performed using primers designed to amplify cellular nucleic acid; K-ras primers were chosen. If the K-ras primers failed to amplify the appropriate 135-bp amplimer, the sample was reextracted or excluded. Southern blotting and hybridization, as well as direct sequencing of the PCR product, were used to confirm positive results.

Viral PCR Electrophoresis

Ten microliters of each reaction was analyzed on a 1% ME agarose gel (FMC Biochemicals) containing
TABLE 2
Fetal Diagnosis Versus PCR Result

<table>
<thead>
<tr>
<th>Fetal abnormality</th>
<th>Patient number (n)</th>
<th>PCR + (%)</th>
<th>PCR – (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonimmune hydrops</td>
<td>91</td>
<td>50 (55)</td>
<td>41 (45)</td>
</tr>
<tr>
<td>Oligohydranios</td>
<td>11</td>
<td>4 (36.4)</td>
<td>7 (63.6)</td>
</tr>
<tr>
<td>Polyhydramnios</td>
<td>15</td>
<td>7 (46.7)</td>
<td>8 (53.3)</td>
</tr>
<tr>
<td>Hydrothorax/pleural effusion</td>
<td>18</td>
<td>6 (33)</td>
<td>12 (66)</td>
</tr>
<tr>
<td>“Stuck twin” syndrome</td>
<td>25</td>
<td>10 (40)</td>
<td>15 (60)</td>
</tr>
<tr>
<td>Ventriculomegaly</td>
<td>26</td>
<td>7 (26.9)</td>
<td>19 (73.1)</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>5</td>
<td>1 (20)</td>
<td>4 (80)</td>
</tr>
<tr>
<td>Cystic hygroma</td>
<td>7</td>
<td>0 (0)</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Maternal infection</td>
<td>22</td>
<td>12 (54.5)</td>
<td>10 (45.5)</td>
</tr>
<tr>
<td>Chorioid plexus cyst</td>
<td>5</td>
<td>0 (0)</td>
<td>5 (100)</td>
</tr>
<tr>
<td>Echogenic bowel</td>
<td>22</td>
<td>6 (27.3)</td>
<td>16 (72.7)</td>
</tr>
<tr>
<td>Liver calcification</td>
<td>3</td>
<td>1 (33)</td>
<td>2 (66)</td>
</tr>
<tr>
<td>IUGR</td>
<td>22</td>
<td>10 (45.5)</td>
<td>12 (54.5)</td>
</tr>
<tr>
<td>Thick placenta</td>
<td>7</td>
<td>0 (0)</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Hydronephrosis</td>
<td>3</td>
<td>0 (0)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>Multiple congenital anomalies</td>
<td>7</td>
<td>0 (0)</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Bradycardia</td>
<td>1</td>
<td>0 (0)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Tachycardia</td>
<td>1</td>
<td>0 (0)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Myocarditis (autopsy-proven)</td>
<td>12</td>
<td>10 (83.3)</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>Total</td>
<td>303</td>
<td>124 (41)</td>
<td>179 (59)</td>
</tr>
<tr>
<td>“Controls”</td>
<td>154</td>
<td>4 (2.6)</td>
<td>150 (97.4)</td>
</tr>
</tbody>
</table>

0.5 ml of ethidium bromide/ml of gel. The gels were placed under UV light for visualization of the amplified products. All samples were electrophoresed alongside a reference sizing ladder and a positive and negative control for the virus being analyzed. If a band was visualized in the negative control lane, the PCR sample was considered contaminated and the process repeated. All samples were analyzed without prior knowledge of clinical history or laboratory data results. PCR was considered positive only if the results could be duplicated. Southern blotting and hybridization (51,52), as well as direct sequencing of the PCR product (53), were used to confirm positive results. K-ras PCR was performed with each extraction.

RESULTS

Clinical Diagnosis

A total of 371 fetal samples obtained from 303 pregnant patients with an abnormal ultrasound or at risk for intrauterine fetal viral infection were analyzed by PCR. The clinical characteristics and reasons for referral for viral PCR analysis are listed in Table 2. The largest single referral diagnosis was nonimmune fetal hydrops (n = 91 patients, 30%). Ultrasound findings of a “stuck twin” was the reason for analysis in 25 patients (8%), and a pleural effusion was the reason in 18 patients (6%). A history of maternal exposure to potentially harmful viral disease was the only reason for analysis in 22 patients (7%). Other clinical indications are shown in Table 2.

PCR Analysis

One or more viral genome was detected in at least one sample from 124 of the 303 patients (41%). One hundred forty-four of the total of 371 samples analyzed (39%) were positive by PCR (Table 3). The most commonly amplified viral genome was adenovirus (74 patients; 51% of positives, 24% of patients), followed by CMV (30 patients), enterovirus (22 patients), HSV (9 patients), parvovirus (8 patients), EBV (4 patients), and RSV (2 patients). There were multiple (i.e., ≥2) viral genomes amplified concomitantly in 15 PCR-positive specimens from 13 patients; 9 of these fetuses died in utero.

Amniotic fluid was positive for one or more viral genomes in 95 of 233 samples (41%), while fetal blood was positive in 13 of 60 samples (22%). The PCR was also positive in 6 of 18 pleural fluid samples (31%) and 10 of the 20 (50%) ascites samples. From the samples obtained at delivery or at autopsy, a PCR-positive result was found in 5 of 19 placental biopsies (26%) and in 10 of 11 samples (91%) obtained from the right ventricle. A single sample obtained from the left ventricle did not amplify viral genome. Five of 9 samples (56%) obtained from other organs (such as liver and lung) were PCR-positive. Several patients had multiple sample sources (i.e., amniotic fluid plus tissue specimens, etc.) analyzed concomitantly. In some, viral amplification occurred in only one sample (i.e., amniotic fluid positive, blood negative, etc.). Most of these discordant findings occurred when amniotic fluid was positive while the fetal blood or ascites was negative.

Nonimmune hydrops were associated with a high incidence of viral infection (50/91 patients) and adenovirus (Fig. 1) was detected in 30 (60%) of these patients (Table 3). Adenovirus was also frequently amplified from ascites, pleural effusions, and miscellaneous tissues (i.e., myocardium).

CMV was associated with a broad range of sonographic abnormalities including nonimmune hydrops (Fig. 2), echogenic bowel, hepatic calcification,
TABLE 3
Viral Etiologies of Fetal Disease

<table>
<thead>
<tr>
<th>Fetal abnormality (patient no.)</th>
<th>PCR + patient no.</th>
<th>Adeno</th>
<th>Entero</th>
<th>Parvo</th>
<th>CMV</th>
<th>HSV</th>
<th>EBV</th>
<th>RSV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonimmune hydrops (91)</td>
<td>50</td>
<td>30*</td>
<td>7*</td>
<td>8*</td>
<td>8*</td>
<td>5*</td>
<td>2</td>
<td>1*</td>
<td>61*</td>
</tr>
<tr>
<td>Oligohydramnios (11)</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Polyhydramnios (15)</td>
<td>7</td>
<td>5*</td>
<td>3*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8*</td>
</tr>
<tr>
<td>Hydrothorax/pleural effusion (18)</td>
<td>6</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>“Stuck twin” syndrome (25)</td>
<td>10</td>
<td>4*</td>
<td>5*</td>
<td>0</td>
<td>2*</td>
<td>2*</td>
<td>2*</td>
<td>0</td>
<td>15*</td>
</tr>
<tr>
<td>Ventriculomegaly (26)</td>
<td>7</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
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<td>Microcephaly (5)</td>
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<td>0</td>
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<td>0</td>
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<td>1</td>
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<td>Cystic hygroma (7)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Maternal infection (22)</td>
<td>12</td>
<td>7*</td>
<td>0</td>
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<td>6*</td>
<td>1*</td>
<td>0</td>
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<td>15*</td>
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<td>Echogenic bowel (22)</td>
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<td>0</td>
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<td>Liver calcification (3)</td>
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<td>1</td>
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<td>IUOR (22)</td>
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<td>8*</td>
<td>1*</td>
<td>0</td>
<td>3*</td>
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<td>0</td>
<td>0</td>
<td>12*</td>
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<td>Thick placenta (7)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Myocarditis (autopsy-proven) (12)</td>
<td>10</td>
<td>6*</td>
<td>5*</td>
<td>0</td>
<td>1*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12*</td>
</tr>
<tr>
<td>Total (303)</td>
<td>124</td>
<td>74</td>
<td>22</td>
<td>8</td>
<td>30</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td>149</td>
</tr>
<tr>
<td>“Controls” (154)</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

Note. * Denotes multiple concomitant PCR-positive viral amplimers.

growth restriction, “stuck twin” syndrome, ascites, and maternal infection with CMV. In most cases of CMV infection, other viruses were also concomitantly identified, particularly adenovirus and enterovirus. The enteroviral genome was commonly found in “stuck twin” syndrome (where it was the single most common genome amplified), myocarditis, ascites with pericardial effusion, hydramnios, and nonimmune hydrops (Fig. 3).

Although less frequently identified, HSV (n = 9) and parvovirus (n = 8) were associated with predict-
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virus in this series, occurring twice as often as CMV (24 vs 10%). Also unexpectedly, the enteroviral genome was amplified frequently, being detected in 7% of patients. Enteroviruses, especially Coxsackievirus, are common causes of myocarditis in neonates and older children (39–41,54). Adenoviruses and, less frequently, CMV and HSV have also been reported to cause myocarditis using traditional methodologies (54–57). With the application of molecular-based techniques such as PCR (15,27,28,39–43) and in situ hybridization (58–60), it has become apparent that both the relative incidence of myocardial infection and the epidemiology of these viruses is different than previously believed. This is probably explained by the low sensitivity of standard viral culture methods and serology for many of these viruses. Enteroviral infections have occasionally been reported to cause hydrops, and there are reports of newborns with enteroviral myocarditis diagnosed so early in life that the infection could only have been contracted during the intrauterine period or during passage through the birth canal (61–63). To our knowledge, there have been only two cases reported of prenatally acquired adenoviral myocarditis (15,54), one of which was diagnosed by PCR; this fetus was successfully treated in utero with cardiac supportive therapy (digoxin) (15).

Regardless of the underlying cause, two etiopathogenic mechanisms are thought to underlay the develop-

able clinical findings. Nearly all cases of parvovirus (7/8) were found in fetuses with nonimmune hydrops (Fig. 4), while those fetuses with HSV infection had either nonimmune hydrops (4/9) or “stuck twin” syndrome (2/9).

PCR Analysis of Control Patients

Analysis of amniotic fluid from 163 patients with structurally normal fetuses thought to be at low risk for fetal viral infection revealed PCR-positive results in 13 pregnancies initially. In 9 PCR-positive pregnancies, the fetus subsequently became clinically affected (e.g., nonimmune hydrops) in 3 of these in utero death occurred. In the remaining unaffected fetuses, only 4/154 (2.6%) were PCR-positive (Table 3).

DISCUSSION

The rapid and accurate identification of an infectious agent in a pregnancy with a sonographic abnormality or the confirmation of transplacental transmission of a viral infection to the fetus after documented maternal viremia remains a difficult problem in perinatal medicine. The detection of viral genome in 41% of our study population is considerably higher than previously reported (1). These differences may be due to various factors. Our study is the first to systematically investigate viruses not usually considered important causes of intrauterine fetal infection in addition to investigating commonly reported causes of fetal viral infection such as CMV (3,5,9,18,19) and parvovirus B19 (16,17). Surprisingly, adenovirus was the most commonly detected

FIG. 3. Amniotic fluid from a fetus with nonimmune hydrops demonstrated an amplified fragment using enteroviral PCR primers. The positive control and amniotic fluid lanes had the 151-bp amplimer; the negative control was PCR negative.

FIG. 4. Parvovirus PCR analysis of nucleic acid extracted from the placenta of twins with nonimmune fetal hydrops. Left panel: Note the 699-bp amplimer in the parvovirus-positive control lane as well as in the lanes containing both twins (“Twin A”, “Twin B”) on this ethidium-bromide agarose gel. The parvovirus negative control and an endomyocardial biopsy from another patient (“Tissue Control”) were devoid of 699-bp bands. A nonspecific extraneous band is seen above the 699-bp parvovirus in the parvovirus positive control and “Tissue Control.” The source of the extraneous band is unknown. Right panel: Autoradiogram of the Southern blot obtained from the gel at left after hybridization with radiolabeled parvoviral probe. The parvovirus positive control and the lanes containing both twins hybridized to the probe (1 h exposure). Neither the parvovirus negative control or SNL RVEMB lane hybridize to this radiolabeled probe.
opment of nonimmune fetal hydrops as a final common pathway: (1) cardiac failure and (2) anemia (10–13). The significance of cardiac failure in nonimmune hydrops was previously suggested by Ruiz-Villaespesa (64) and has now been directly demonstrated by the measurement of umbilical venous pressures during diagnostic cordocentesis (11) and indirectly by the measurement of the cardiothoracic index during prenatal ultrasound examination. Based on these approaches, cardiac failure appears more common than previously suggested (11,64). In the majority of cases, cardiac failure is explained by structural anomalies, compression by cardiac or extracardiac intrathoracic tumors, or other reasons for impaired venous return (11,12,13,64). Occasionally, a fetal tachyarrhythmia of unknown underlying etiology is present (10,12,13). We speculate that in a substantial proportion of these fetuses, as well as in the 22% (12) of those fetuses classified as “idiopathic” nonimmune hydrops, an unrecognized fetal myocarditis affecting myocardial contractility or the conduction system is the origin of the cardiac failure. It is well documented that a rhythm disturbance may be the presenting finding of myocarditis postnatally.

The relative frequency of the individual viral genomes amplified from the clinical specimens in this study differs from previous fetal and neonatal studies which analyzed fetuses by culture and serologic methods. Studies using more sophisticated molecular analysis focused only on CMV and parvovirus. Recent studies of fetuses and children with myocarditis have demonstrated results similar to those reported here (15,42,65). In addition, viral genome was found in fetal conditions not formerly believed to be associated with viral infection. For example, adenoviral genome was found in some fetuses with intracranial findings such as ventriculomegaly, and adenovirus and enterovirus were found in a significant number of fetuses with a “stuck twin” syndrome. The exact significance of these results remains at this time speculative. However, the significant increase in prevalence of positive PCR results in abnormal pregnancies compared to amniotic fluid samples from uncomplicated pregnancies suggests a causative association. Additional support for a causative association is provided by the nine pregnancies initially considered to be at low risk for viral infection (i.e., control patients) in whom a PCR-positive result preceded fetal disease. Adenovirus and enterovirus are both common in the population and it may be expected that a significant percentage of pregnant women will contract a primary infection without any measurable direct pathological effect even with transmission of the virus to the fetus. Importantly, more than 50% of mothers with known infections were PCR-positive (usually CMV or adenovirus) in this study and a substantial percentage of their fetuses had postnatal abnormalities or fetal demise. This strengthens the likelihood of causation. The assessment of the role of these viruses in pregnancy requires further systematic investigations of large numbers of fetuses at risk, as well as pregnancies not thought to be at significant risk for either primary fetal disease or intrauterine infection.

Some of the fetuses studied had evidence of multiple viral genomes by PCR. In a high percentage of these cases the fetus was either severely affected or the pregnancy ended with in utero demise. The potential relevance of the molecular identification of viral genome(s) to disease is speculative, but the identification of multiple genomes appears to be a poor prognostic indicator. Larger patient numbers are required for study in order to clarify this relationship as speculation or fact.

Since PCR is a very sensitive technique the possibility of false positive results must be considered. Strict criteria and compulsive technique are required for the prevention of contamination of the samples under study. All studies in the present investigation included positive and negative control samples for each amplification, as well as amplification of a constitutive human gene for evaluation of the nucleic acid extraction method, and the confirmation of results with Southern blotting (51), liquid hybridization techniques (52), and/or direct sequencing (53). We believe these efforts minimize the likelihood of laboratory error as an explanation for our findings.

In conclusion, viral genomic material is commonly associated with abnormal pregnancies. Our data suggest that viral infection may have a much larger role in fetal pathology than previously noted. Future studies should include analyses for the viruses outlined in this report, as well as other candidates for fetal disease, such as the newly identified human herpes virus 6 which appears to cause disease in infants and young children (66). In addition, McLean et al. (67) evaluated 243 amniotic fluid specimens obtained from low risk pregnancies for adenovirus, CMV, parvovirus, and HSV using PCR and found no amplification of viral genome. This work supports
the notion that viral genome is not expected to occur in noninfected mothers and by extrapolation, amplified viral genome in affected fetuses is likely to be causative of the fetal disease.

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