Parvovirus B19 Infection in Fetal Deaths

Anita Riipinen,1 Elina Väisänen,2 Mika Nuutila,3 Markku Sallmen,4 Riitta Karikoski,5 Marja-Liisa Lindbohm,1 Klaus Hedman,2 Helena Taskinen,1,4 and Maria Söderlund-Venermo2

1Centre of Expertise for Health and Work Ability, Finnish Institute of Occupational Health, 2Department of Virology, Haartman Institute, and 3Central Pathology Laboratory, Helsinki University Central Hospital Laboratory Division, and 4School of Public Health, University of Helsinki, and 5Department of Obstetrics and Gynecology, Helsinki University Central Hospital, Helsinki, Finland

Background. Parvovirus B19 infection during pregnancy can lead to nonimmune fetal hydrops, miscarriage, and intrauterine fetal death (IUFD). Some studies have suggested that parvovirus B19 infection may surprisingly often result in nonhydropic fetal death during the third trimester, in the absence of maternal serological evidence of acute infection. This study was conducted to investigate the prevalence of parvovirus B19 DNA among fetuses from miscarriages and IUFDs.

Methods. We retrospectively studied 535 unborn fetuses, including 120 fetuses from miscarriages and 169 from IUFDs. The control fetuses were 246 fetuses from induced abortions. All fetuses were autopsied from July 1992 through December 1995 and from January 2003 through December 2005 in Helsinki, Finland. The period included a major epidemic of parvovirus B19 infection in 1993. Formalin-fixed, paraffin-embedded fetal tissues were studied with use of a highly sensitive and specific PCR that was capable of detecting all 3 parvovirus B19 genotypes and by histologic examination. In addition, maternal parvovirus B19 serological status was determined.

Results. Parvovirus B19 DNA was detected in 5 fetuses with gestational ages of 14, 22, 23, 30, and 39 weeks; these included fetuses from 4 (2.4%) of the 169 IUFDs and 1 (0.8%) of the 120 miscarriages. During the epidemic year 1993, the prevalence of parvovirus B19 DNA–positive fetal deaths was 6 times the prevalence during non-epidemic years. All 5 mothers of the parvovirus B19 DNA–positive fetuses had serological signs of acute parvovirus B19 infection close to the time of fetal death. The only nonhydropic fetus was full-term.

Conclusions. Our findings indicate that the prevalence of parvovirus B19 infection among fetuses from IUFDs is low. In particular, our findings did not verify the claimed high prevalence of third-trimester nonhydropic IUFDs associated with parvovirus B19.

Human parvovirus B19, a small, nonenveloped virus with a single-stranded DNA genome of 5.6 kb, is the type species of the genus Erythrovirus of the family Parvoviridae. The most typical manifestations of parvovirus B19 infection include arthropathies and erythema infectiosum, a common rash of childhood [1].

Parvovirus B19 replicates in RBC precursors, mainly in the bone marrow and fetal liver, resulting in hemolysis and RBC aplasia that can lead to a life-threatening aplastic crisis in patients with hemolytic disorders. Similarly, the fetus is particularly vulnerable, because of the diminished RBC survival combined with the increasing erythropoiesis and immature immune response. Aplastic crisis in the fetus can lead to cardiac failure, nonimmune hydrops, and death [2–6]. The main receptor of parvovirus B19, globoside or P antigen, occurs not only on RBCs but also on endothelial, placental trophoblast, and fetal hepatic and myocardial cells, in which parvovirus B19 capsids or DNA have also been detected [7–9].

Fifty percent to 70% of pregnant women are parvovirus B19 seropositive [4, 6, 10] and, thus, are immune to parvovirus B19 infection. Parvovirus B19 infection occurs in small outbreaks mostly during the spring; major epidemics occur every few years. The annual parvovirus B19 IgG seroconversion rate among women of childbearing age has been estimated to be 1.5% during nonepidemic periods and 13% during epidemic periods [10, 11]. Mothers of small children, school teachers, and day-care employees are at increased risk of parvovirus B19 infection [4, 11, 12].

The transplacental transmission rate of parvovirus
B19 appears to be ∼30% [13]. Intrauterine parvovirus B19 infection can lead to both miscarriage (before week 22) and intrauterine fetal death (IUFD; at or after week 22) [5, 13–17]. The excess risk of fetal death is 5%–10% when maternal infection occurs during the first 20 weeks of gestation but is less thereafter [5, 13]. Currently, the most reliable diagnostic methods for primary parvovirus B19 infection during pregnancy and at the time of nonimmune fetal hydrops are serological testing (IgM and IgG, together with the VP2-IgG epitope-type specificity [18, 19] and VP1-IgG avidity assays [20]) and quantitative PCR of maternal serum samples [21, 22]. However, high prevalences of parvovirus B19 DNA positivity among nonhy-dropic fetuses have recently been reported in the context of third-trimester IUFDs, in the absence of serological evidence of acute parvovirus B19 infection in the mother [15–17], which challenges the current diagnostic guidelines [23, 24]. These conflicting findings prompted us to determine the prevalence of parvovirus B19 DNA positivity among a large number of fetuses from miscarriages and IUFDs, while also accounting for the maternal serodiagnostic status.

**METHODS**

**Clinical samples.** Formalin-fixed paraffin-embedded (FFPE) tissue samples from 550 fetuses that were collected from July 1992 through December 1995 and from January 2003 through December 2005 at the Helsinki University Central Hospital were retrieved. The former period was chosen because of a major epidemic of parvovirus B19 infection that occurred during 1993, and the latter period was nonepidemic. Samples from 5 fetuses were β-globin PCR negative because of DNA degradation or PCR inhibition and were thus excluded. In twin- or multiple-fetus pregnancies (n = 32), tissue samples were available mostly from only 1 fetus. Both fetuses were available from only 10 twin pregnancies, and all were found to be parvovirus B19 DNA negative. For conformity and statistical analysis, we considered each set of twins as 1 (10 exclusions). Thus, a total of 535 fetuses were included in the final study population. Parvovirus B19 DNA was amplified in samples from 3 organs (when available; placenta, heart, and liver) that were initially pooled from each fetus. The time of fetal death varied from 11 to 42 gestational weeks. Fetal loss was defined as an IUFD when it occurred during or after gestational week 22 and as miscarriage when it occurred earlier (according to the *International Classification of Diseases, Tenth Revision*). Our study comprised 120 miscarriages and 169 IUFDs (table 1). According to official statistical records, a total of 67,858 live births and 290 IUFDs occurred at the Helsinki University Central Hospital during the study period. Thereby, 169 (58.3%) of all 290 fetuses from IUFDs in the Helsinki region were available for our study. The proportion of miscarried fetuses included in our study, however, is unknown, because not all miscarriages are regis-tered. Two hundred forty-six fetuses from induced abortions (performed exclusively for medical reasons) were used as control fetuses.

**Extraction of viral DNA.** The tissue samples (volume, 2–4 mm³), available as FFPE blocks, were obtained by punch biopsy. This method provides more accurate sampling than does ordinary microsection slices. The biopsy instrument was sterilized between obtaining of each sample by successive treatments with 2% Deconex (Peror chemiek, water, 0.5 mol/L hydrochloric acid, water, ethanol, and flaming.

An FFPE fetal tissue sample and a parvovirus B19 DNA-positive FFPE placental tissue sample were used to select and optimize the deparaffinization and DNA extraction methods and the subsequent PCR. After examining several deparaffinization and DNA extraction methods, including the classic xylene-ethanol and phenol-chloroform extraction methods [25], the following procedure was chosen. Viral DNA was purified by protease K digestion for 48 h at 48°C (with protease K replenished at 24 h), followed by a salting-out extraction method, as described elsewhere but with modifications [26, 27]. In brief, tissue lysates were boiled for 10 min at 95°C and were centrifuged for 5 min at 13,200 rpm at 4°C. The solution under the paraffin layer was transferred into a new tube. Sodium chloride was added to a final concentration of 1.2 mol/L, and the sample was mixed for 20 s and then recentrifuged. The DNA in the supernatant was precipitated with absolute ethanol and was redissolved in 60 μL of water. The DNA solution was stored at −20°C until use. Water, as a negative control, was inserted between every 20 samples and was prepared with the tissue pools.

**PCR procedures.** Because the DNA in archival tissue samples can be deteriorated, PCR amplicons were designed to be short yet capable of detection of all known parvovirus B19 genotypes. The forward primer 9f (5'-TGACTTTAGGTATAG-
To minimize the effects of PCR-inhibiting factors, 3 disposable racks were observed to avoid false-positive results. Including the use of separate rooms, aerosol-resistant tips, and the reverse primer 3r (5′-CTTCTGCAAGAATTACTGAAGTTC-3′) were chosen to amplify a 248-base pair fragment of the VP1 gene. The analytical sensitivity and specificity were tested with 10-fold dilution series (both in water and in FFPE DNA extracts) of parvovirus B19 genotype 1, 2, or 3–containing plasmids (GenBank AY504945, AY044266, and AY083234, respectively) and of the genomic DNA extracted from the parvovirus B19-positive FFPE placenta samples.

Because of the high sensitivity of PCR, strict precautions, including the use of separate rooms, aerosol-resistant tips, and disposable racks, were observed to avoid false-negative results. To minimize the effects of PCR-inhibiting factors, 3 μL of a 1:10–dilution of the extract was used per PCR reaction. The reaction mixture consisted of 10× GeneAmp PCR buffer I (Applied Biosystems), 200 μmol/L of each deoxyribonucleotide triphosphate, 0.5 μmol/L of each primer (9f and 3r), and 1.25 U of AmpliTaq Gold (Applied Biosystems) for a total of 25 μL. The PCR reaction consisted of an initial step at 95°C for 10 min, followed by 40 cycles each at 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s, and a final step at 72°C for 5 min. The results were confirmed by Southern hybridization with use of a digoxigenin-labeled probe (nucleotides 3081–3328 of parvovirus B19 NAN; GenBank AY504945) designed to recognize all 3 parvovirus B19 genotypes. The constituent tissue samples from the parvovirus B19–positive tissue pools were reexamined individually.

Purified DNA from the same DNA extracts from the epidemic year 1993 was reamplified by the nested PCR that was used in a Swedish study [16, 17, 28]. However, a missing nucleotide A in the outer forward primer was corrected.

The human β-globin gene was amplified from each pool as a control to monitor PCR inhibition and DNA stability. Parvovirus B19 genotype 1 plasmid DNA was included as a positive control in each PCR set, and water was included as a negative control between every 20 samples. Total DNA concentrations of all IUFD extracts were measured with use of a spectrophotometer (Nanodrop Technologies).

Clinical data. Medical records corresponding to the 5 parvovirus B19 DNA–positive fetuses were reviewed (table 2) for gestational age, sex, cause of death, morphological changes, hydrops, and intrauterine blood transfusions. Information on the mothers included occupation, age, previous deliveries, and infectious diseases, including parvovirus B19 infection, which is routinely tested for when IUFD occurs. Serum samples obtained at gestational weeks 6–14 from 4 of the 5 mothers and at week 22 from the remaining mother were tested for parvovirus B19 antibodies. All parvovirus B19–positive tissue samples were histologically reexamined for signs of viral infection by a pathologist experienced in perinatal pathology. In addition, the medical information for all mothers who had received a diagnosis of parvovirus B19 infection during pregnancy in the Helsinki region during 1998–2007 was obtained from the medical records of the Helsinki University Central Hospital.

This study was approved by the Coordinating Ethical Committee of the Hospital District of Helsinki and Uusimaa. Permission for use of the medical records was obtained from the Ministry of Social Affairs and Health.

RESULTS

We found 5 parvovirus B19 DNA–positive fetuses; 4 (2.4%) of these fetuses were from the 169 IUFDs, 1 (0.8%) was from the 120 miscarriages, and none were from induced abortions (table 2). The fetal deaths had occurred during gestational weeks 14, 22, 23, 30, and 39. Tissue samples from all 3 sites (placenta, heart, and liver) were available for study for 4 of the 5 fetuses, and only a tissue sample from the placenta was available for 1 fetus. Parvovirus B19 DNA was detected in 5 placenta tissue samples, 3 myocardial tissue samples, and 1 hepatic tissue sample (table 2). Sequencing revealed that all were parvovirus B19 genotype 1. Moreover, we reamplified all tissue samples from the epidemic year 1993 with use of the nested PCR previously used by Tolfvenstam et al. [16], Norbeck et al. [17], and Broliden et al. [28] and obtained positive results for 2 of 3 parvovirus B19 DNA–positive fetuses. Our PCR, followed by hybridization, was able to detect 1–5 copies per reaction of serially diluted parvovirus B19 plasmid DNA, as was the nested PCR, when it was performed in parallel. In >99% of the IUFD extracts, the total DNA concentrations were >100 ng/μL (mean concentration, 882 ng/μL; median concentration, 802 ng/μL). The corresponding concentrations of total DNA in the parvovirus B19 DNA–positive extracts were 180–1541 ng/μL.

During the epidemic year, 3 (5.1%) of the 59 fetuses studied were parvovirus B19 DNA positive, including 2 (4.9%) of 41 from IUFDs and 1 (5.6%) of 18 from miscarriages. During the nonepidemic years, 2 (0.9%) of the 230 fetuses studied were parvovirus B19 DNA positive, including 2 (1.6%) of 128 from IUFDs and 0 (0%) of 102 from miscarriages. Four of the parvovirus B19 DNA–positive fetuses (from all trimesters) were hydropic (table 2). For 2 of these fetuses, the hydrops was known <4 weeks before death. The only nonhydropic parvovirus B19–positive fetus died on week 39. No malformations were observed in the parvovirus B19 DNA–positive fetuses. According to the medical records, 2 of the mothers had symptoms typical of acute parvovirus B19 infection (rash or arthralgia) 3 and 9 weeks before fetal death. All 5 mothers were serologically confirmed to have had an acute parvovirus B19 infection before fetal death; 4 of the 5 mothers had been seronegative during gestational weeks 6–14, whereas the remaining mother (fetus 2) had her first serum sample, which had an acute serological pattern, obtained at week 22. Four of the mothers were multiparous, and 1 worked with children.

For 3 of the fetuses, parvovirus B19 infection was the initially
Table 2. Characteristics of fetal deaths in the 5 parvovirus B19 DNA–positive fetuses and their mothers.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parvovirus B19 DNA-positive fetus</th>
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<td>1</td>
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<tr>
<td>Gestational age, weeks</td>
<td>14</td>
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<tr>
<td>Sex</td>
<td>Female</td>
</tr>
<tr>
<td>Type of fetal death</td>
<td>Miscarriage</td>
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<tr>
<td>Intrauterine blood transfusion</td>
<td>No</td>
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<tr>
<td>Assumed cause of fetal death</td>
<td>Parvovirus B19 infection</td>
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<td>PCR resulta</td>
<td>Parvovirus B19 DNA in paraffin-embedded tissue sample</td>
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<td></td>
<td>Placenta</td>
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<td>Heart</td>
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<td>Histopathological finding</td>
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<td>Maceration</td>
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<td>In the placenta sample</td>
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<td>In the heart sample</td>
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<td>Age, years</td>
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<td>Parvovirus B19 serological status</td>
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<td>Occupation</td>
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NOTE. IUFD, intrauterine fetal death; NA, not available.

a Based on medical records.
suspected cause of death. For fetuses 3 and 5, placental abruption was assumed to be the immediate cause of death (table 2). Whether a parvovirus B19 infection in utero or its treatment could predispose to placental abruption or whether parvovirus B19 was not the cause of death is not known. However, the prevalence of placental abruption among the parvovirus B19 DNA–positive fetuses from IUFDs (2 [50.0%] of 4 fetuses) was higher than that among the other fetuses from IUFDs (19 [11.5%] of 165), although the difference was not statistically significant ($P = .076$, by Fisher’s exact test). Fetuses 2 and 5 had been anemic and were treated with intrauterine transfusions. Large early erythroblasts were found in the placenta, myocardium, or lung (fetus 2) samples from fetuses 1, 2, and 5; this was suggestive of fetal anemia and, thus, parvovirus B19 infection.

In comparison, according to the records from the Helsinki University Central Hospital during the period 1998–2007, 17 pregnant women received a diagnosis of parvovirus B19 infection; 5 of these women received the diagnosis during 2003–2005. These mothers had been examined weekly by ultrasound for ∼12 weeks after infection. Intrauterine transfusions had been given to 2 fetuses, 1 of which survived, whereas the other was detected in our PCR study (table 2). A third fetus had cardiac insufficiency and ascites without anemia and survived. Symptoms typical of parvovirus B19 infection did not develop in the other 14 fetuses during the follow-up period. Of the 17 parvovirus B19–infected mothers, 6 worked with children.

**DISCUSSION**

We found a low prevalence of parvovirus B19 DNA among fetuses from IUFDs (2.4%) and miscarriages (0.8%) during 1992–1995 and 2003–2005 in Helsinki; there were no parvovirus B19 DNA–positive fetuses from induced abortions during these periods. Even during the major epidemic of parvovirus B19 infection in 1993, the prevalence of parvovirus B19 DNA among IUFDs was low (4.9%), compared with the prevalence found in some previous studies, which revealed parvovirus B19 DNA–positive fetal or placental tissue samples in 7.5%–15% of fetuses from IUFDs [15–17]. However, our prevalence of hydropic parvovirus B19 DNA–positive fetuses from IUFDs (1.8%) was in accordance with the prevalence in Sweden (0%–2.2%) [15–17]. Therefore, the difference was solely attributed to nonhydropic fetuses, the prevalence of which, in our data, was 0.6% and, in the Swedish study, was 7%–13% [15–17].

The typical pattern of fetal death due to parvovirus B19 infection includes viral infection during the second trimester, hydrops, early erythroblasts in fetal circulation (either in internal organs or the placenta), PCR positive for and immunohistological signs of parvovirus B19 in multiple fetal tissue samples, and serological evidence of acute parvovirus B19 infection in the mothers. However, only a few of the fetuses from IUFDs that were described by Skjöldebrand-Sparre et al. [15], Tolfvenstam et al. [16], and Norbeck et al. [17] showed this pattern. Instead, the majority of their IUFDs occurred during the third trimester, with no hydrops, viral inclusions, or positive immunohistological examination results. In at least 7 of their 20 cases, only fetal tissue samples, not placental tissue samples, were PCR positive, which suggests lack of fetal or maternal viremia at the time of IUFD [16, 17]. Furthermore, in those studies, most of the mothers of the fetuses from late IUFDs had nondiagnostic parvovirus B19 serological evidence at the time of fetal death [15, 16].

Our IUFD samples included 58% of all IUFDs reported in the Helsinki region during the period. Three of our 4 parvovirus B19 DNA–positive fetuses from IUFDs were hydropic; the only nonhydropic fetus was full-term and had no apparent cause of IUFD other than the parvovirus B19 infection. Close to the time of fetal death, all 5 mothers showed a serological pattern of acute parvovirus B19 infection. Thus, our data confirmed neither the large numbers of parvovirus B19 DNA–positive nonhydropic fetal deaths during late pregnancy, nor the lack of maternal serodiagnoses in the parvovirus B19–induced fetal deaths, that were reported by the Swedish group [15–17]. Of interest, we observed a suggestive association between parvovirus B19 infection and the occurrence of placental abruption. However, this finding was based on a small number of cases and awaits confirmation in future studies.

To assess whether our study’s lower prevalence of parvovirus B19 DNA could be attributable to a less sensitive PCR, we reexamined all samples from the epidemic year 1993 with the nested PCR of Tolfvenstam et al. [16], Norbeck et al. [17], and Broliden et al. [28]. We obtained positive results for only 2 of the 3 fetuses that were positive by our assay. It is possible that the shorter amplicon of our PCR could explain this difference. Of note, in a dilution series of a parvovirus B19–containing plasmid, the 2 assays were equally sensitive. Formalin fixation is known to decrease PCR sensitivity by deteriorating DNA. The tissue samples of Tolfvenstam et al. [16] were from freshly frozen biopsy samples. However, Norbeck et al. [17] used formalin-fixed tissue samples from the period 1993–1997 that were similar to our material. However, in that study, the tissue sections had been deparaffinized by xylene and ethanol [15], a classic approach shown to decrease the detection sensitivity of PCR [25]. Skjöldebrand-Sparre et al. [15] reexamined all 7 freshly frozen parvovirus B19–positive placenta samples as formalin-fixed and paraffin-embedded, and there was no noteworthy difference. We explored a variety of methods for deparaffination and DNA preparation, including the classic xylene-based method, and found that the paraffin-melting and salting-out procedures resulted in the best detection sensitivity. An important quality measure of our study, thereby, was the successful extraction of viral DNA from paraffin-embedded tis-
sue samples that had undergone long-term storage. Moreover, the human \(\beta\)-globin gene could be amplified from \(>99\%\) of the tissue pools, implicating good DNA preservation and a lack of PCR inhibition.

Another approach to assessing the impact of parvovirus B19 infection on the fetus has been to prospectively observe the pregnancies of parvovirus B19–infected women identified by serological testing [5, 13, 14]. These studies have shown an increased risk of fetal loss due to infection during the first half of gestation but have shown no or only a minimal risk thereafter. In our study, 1 mother most likely had been infected after week 20 of gestation. She was seronegative at week 14, and at the time of fetal death (week 39), she received a diagnosis of acute parvovirus B19 infection, with a low epitope-type specificity ratio of 1 [19]. Overall, our findings are still in accordance with the public health guidelines for management of parvovirus B19 infection during pregnancy [23].

Major epidemics of parvovirus B19 infection occur every few years. According to previous studies [6, 11], the risk of parvovirus B19 infection during epidemics is 10-fold higher than the risk during nonepidemic periods. Similarly, in Germany, the frequency of parvovirus B19–associated fetal hydrops was highest during the periods of high parvovirus B19 activity (1993 and 1997–1998; M. Enders, personal communication). The Swedish studies [15–17] revealed no obvious difference in activity between epidemic and nonepidemic periods, although some cyclic clustering could be seen. We found a 6-fold excess of parvovirus B19 DNA–positive fetuses during the 1993 epidemic, compared with the nonepidemic periods.

The risk of parvovirus B19 infection during pregnancy is greatest in women who are in contact with preschool children [11–13]. In accordance with this finding, 1 (20.0%) of our mothers with a parvovirus B19 DNA–positive fetus worked with children, and 6 (35.3%) of the 17 mothers with antenatal parvovirus B19 infection had occupational contact with children. However, our sample size was too small to address the occupational risk.

Our findings indicate that parvovirus B19–associated non-hydropic IUFDs are less prevalent than was suggested in some recent studies and that the prevalence of parvovirus B19 DNA among hydptic deaths is similar. Furthermore, we substantiated that fetal deaths caused by parvovirus B19 infection occur primarily during major epidemics of parvovirus B19 infection. All parvovirus B19–positive fetuses also had other markers of acute parvovirus B19 infection, including maternal serological patterns of acute parvovirus B19 infection. This indicates that noninvasive serodiagnostic tests (as complemented by the VP2-IgG epitope-type specificity and VP1-IgG avidity assays) and quantitative PCR assay remain the procedures of choice for the diagnosis of parvovirus B19 infection during pregnancy.

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