JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1996, p. 603–608 Vol. 34, No. 3
0095-1137/96/$04.00+0

Comparative Evaluation of Virological and Serological Methods in Prenatal Diagnosis of Parvovirus B19 Fetal Hydrops

M. ZERBINI,[1]* M. MUSIANI,[1] G. GENTILOMI,[1] S. VENTUROLI,[1] G. GALLINELLA,[1] AND R. MORANDI[2]

Institute of Microbiology[1] and Pathophysiology Prenatal Section, II Department of Obstetrics and Gynecology,[2] University of Bologna, Bologna, Italy

Received 28 August 1995/Returned for modification 12 October 1995/Accepted 7 December 1995

Human parvovirus B19 infection in pregnancy represents a potential hazard to the fetus since fetal loss or fetal hydrops can occur. The risk of fetal loss due to transplacental B19 transmission has been evaluated in several studies using different diagnostic methods on maternal and fetal specimens. We analyzed the diagnostic value of virological and serological techniques on maternal serum, fetal cord blood, and amniotic fluid specimens obtained at the time of clinical diagnosis of fetal hydrops in 18 cases of B19 fetal hydrops. B19 DNA was detected by nested PCR, dot blot hybridization, and in situ hybridization assay. Anti-B19 immunoglobulin M and G antibodies were detected by immunoassays using recombinant B19 antigens. Our data suggest that for maternal sera, virological and serological methods have a complementary role in diagnosis, while for fetal specimens the in situ detection of B19 DNA in fetal cord blood is the most sensitive diagnostic system.

Since its discovery in healthy blood donors, B19 parvovirus has been associated with a variety of clinical syndromes (2, 3, 9, 12, 35–37, 41). In healthy adults, B19 infection generally occurs asymptomatically or can cause a mild disease (32), but maternal B19 infection in pregnancy is a potential hazard to the fetus (1, 16), since fetal loss or fetal hydrops can occur because of the ability of the virus to replicate in erythroid precursor cells and in fetal tissues (24, 25). Prospective studies, however, show that B19 infection in pregnant women, whether symptomatic or asymptomatic, most often leads to a normal pregnancy outcome with delivery of apparently healthy infants (18, 31).

The transplacental transmission rate of infection has been estimated at between 25 and 33%, and the incidence of fetal loss has been evaluated at 1.66 and 9% (15, 27) on the basis of laboratory investigations performed with infected mothers and fetuses. In mothers, the diagnosis of active or recent infection has been done most often by detecting anti-B19 immunoglobulin M (IgM) or rising concentrations of IgG antibodies in serum samples. In fact, virological findings for maternal serum have generally been considered transient markers of B19 infection, since specific symptoms of infection can appear in the mother after the acute phase of viremia and in the fetus 3 to 12 weeks after acute maternal infection. In view of recent data (8, 23), however, it should be considered that B19 DNA can be detected in serum samples by nested PCR between 2 and 6 months after acute-phase viremia and that virological findings are the only diagnostic support available for patients serologically unresponsive to B19 infection.

On the other hand, for the prenatal diagnosis of B19 infection in fetuses, a variety of virological methods have been applied to detect B19 parvovirus and B19 nucleic acid in different fetal specimens (19, 20, 28–30, 34), since specific IgG can represent passively acquired maternal immunity and IgM can be absent even in the presence of viral markers of B19 infection. Therefore, accurate laboratory diagnosis of B19 infection is essential in cases of nonimmune fetal hydrops both for the diagnostic value and for consideration of fetal treatment with intrauterine blood transfusions (26). In this study, we have evaluated the diagnostic value of virological and serological investigations of maternal serum, fetal cord blood, and amniotic fluid samples, using several diagnostic methods in 18 cases of fetal hydrops with laboratory diagnosis of B19 infection.

* Corresponding author. Mailing address: Institute of Microbiology, Via Massarenti 9, 40138 Bologna, Italy. Fax: 39-51-341632.

MATERIALS AND METHODS

Patients. From January 1992 to April 1995, 18 cases of nonimmune fetal hydrops with laboratory diagnosis of B19 infection were documented in the Virological Section of the University of Bologna. Fetal hydrops was evaluated by ultrasonographic examination by the Pathophysiology Prenatal Section of our regional hospital, which is a reference center in the country. Of the 18 patients observed, 7 were from the region and 11 were from elsewhere. Immunological hydrops had previously been excluded, since results of antibody screening were normal, fetal karyotypes also proved normal, and active or recent infections by toxoplasma, rubella virus, herpesvirus, or cytomegalovirus were excluded by serological data.

At the time of clinical diagnosis, blood samples were obtained from all 18 mothers, and within 1 week fetal cord blood samples were collected from all the hydropic fetuses. Amniotic fluids were obtained in 12 of the 18 cases, and pleuric fluids were also obtained from three hydropic fetuses.

Maternal serum samples were tested for B19 DNA by dot blot hybridization assay and nested PCR and for the presence of anti-B19 IgM and IgG antibodies. Serum samples from fetal cord blood were tested for B19 DNA by dot blot hybridization assay and nested PCR and tested for specific IgM and IgG; heparinized fetal cord blood was processed for in situ hybridization (ISH) assay. Amniotic fluids were tested for B19 DNA by dot blot hybridization assay and nested PCR, and cells from amniotic fluids were studied by ISH assay. Pleuric fluids were analyzed for B19 DNA by nested PCR. Tissue sections from the lungs, spleen, liver, and myocardium were obtained from one fetus with B19 fetal hydrops which died, and all the sections available were tested for B19 DNA by ISH.

Dot blot hybridization. (i) Specimen preparation on nylon membrane. Serum samples and amniotic fluids were prepared as previously described (39). In brief, 5 μl of each sample, mixed with 195 μl of distilled water, was filtered with a Bio-Dot apparatus (Bio-Rad Laboratories, Milan, Italy) onto a nylon membrane (Amersham Italia, Milan, Italy). After the samples were filtered, the nylon membrane was air dried and then treated with UV for 5 min. The specimens on the filters were then alkali denatured, neutralized, treated with pronase, and washed in 2× SSC buffer (0.3 M NaCl plus 0.03 M sodium citrate; pH 7.0) treated with diethylpyrocarbonate (DEPC). To test the specificity of the assay, B19 probes were hybridized with cellular DNA from human fibroblast cell cultures, with pGEM I plasmid DNA, and with reference sera negative for B19 DNA. To analyze the sensitivity of the assay, in all the experiments two reference serum samples positive for B19 parvovirus were tested undiluted and diluted up to 10^{-5}.

(ii) B19 RNA probe preparation. Plasmid pGEM I, which contains the T7 promoter, with a 700-bp *Bam*HI-*Hin*dIII B19 DNA insert was linearized by *Hin*dIII digestion in order to use T7 RNA polymerase to transcribe minus-sense

RNA probes (Promega, Madison, Wis.). Linearized DNAs were extracted with phenol-chloroform-isoamyl alcohol and precipitated with ethanol. Digoxigenin (Dig) labeling was performed by incorporating Dig-UTP (Boehringer GmbH, Mannheim, Germany) into transcripts from the T7 promoter (39). In brief, 1 μg of denatured DNA was added to a transcription reaction mixture consisting of 2 μl of 10× transcription buffer (Boehringer GmbH), 2 μl of ribonucleoside triphosphate (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM Dig-UTP), 1 μl of RNasine, and DEPC-treated distilled water in a final volume of 18 μl. The reaction was initiated by adding 2 μl of T7 RNA polymerase (40 U/μg of DNA) and carried out at 37°C for 2 h. After the transcription reaction was performed, the synthesized RNAs were incubated with 2 μl of RNase-free DNase (10 U/μl) for 15 min at 37°C. The digestion was stopped by adding 2 μl of EDTA (0.2 M; pH 8). The Dig-labeled RNA probes were precipitated with ethanol, washed with 70% ethanol, dried, and resuspended in DEPC-treated water. The RNA probes could be stored at −20°C for at least 4 months with no decrease in activity.

(iii) Hybridization reaction with RNA probes. Filters were sealed in a polypropylene bag with 200 μl of a DEPC-treated prehybridization mixture (5× SSC, 0.1% *N*-laurylsarcosine, 0.02% sodium dodecyl sulfate, 5% blocking reagent [Boehringer GmbH], 250 μg of denatured calf thymus DNA per ml, and 50% formamide) per cm^2. The filters were incubated at 50°C for 1 h in a shaking water bath. The prehybridization mixture was removed and replaced with 100 μl of DEPC-treated hybridization mixture (containing the same reagents as the prehybridization mixture plus 200 ng of strand-specific Dig-labeled RNA probe per ml) per cm^2. The reaction was allowed to proceed at 50°C for 6 h. The filters were washed after hybridization under stringent conditions.

(iv) Detection of hybridized RNA probes. After a short wash in a 100 mM Tris-HCl buffer (pH 7.5) with 150 mM NaCl, blocking reagent (Boehringer GmbH) was applied to the nylon membranes for 30 min at room temperature. The membranes were incubated at room temperature for 30 min with anti-Dig sheep Fab fragments conjugated to alkaline phosphatase (Boehringer GmbH). The membranes were then equilibrated for 2 min with equilibration buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ [pH 9.5]). The dry membranes were then treated with colorimetric alkaline phosphatase substrate as previously described (39). The development of a dark blue positive reaction was allowed to proceed for 2 h at room temperature.

ISH assay. (i) Sample preparation. Cell smears were prepared from fetal cord blood and amniotic fluid. Heparinized fetal cord blood was centrifuged on a Ficoll-Hypaque gradient (Organon Teknika), and the buffy coat was washed three times in phosphate-buffered saline (PBS) (0.15 M; pH 7.4). The cells were resuspended in PBS and smeared on glass slides pretreated as previously described (14).

Cells from amniotic fluid were obtained by centrifugation after three washes in PBS and spotted onto pretreated glass slides. The cells were air dried and fixed with 4% paraformaldehyde in PBS (0.15 M; pH 7.4) for 10 min. After fixation, the smears were washed three times in PBS for 10 min each and then dehydrated with ethanol washes (30, 60, and 95%) for 5 min each. The smears were then air dried and stored at 4°C. Cell smears could be stored for at least 1 month with no decrease in their interactivity with the hybridization probe. Sections (3 μm) were obtained from paraffin-embedded tissues from a fetus that had died, cut onto coated slides, dewaxed, and treated as previously described (22). Cell smears from fetal cord blood, amniotic fluid, and tissue sections were processed for in situ detection of B19 DNA, and at least three slides from each specimen were tested in each hybridization assay.

Moreover, the following controls were introduced: cells were (a) hybridized with labeled pGEM I control DNA probe and treated with anti-Dig Fab fragments conjugated with alkaline phosphatase, (b) hybridized with Dig-labeled B19 DNA probe, but primary incubation with anti-Dig Fab fragment was either omitted or replaced by incubation with nonimmune sheep serum, or (c) hybridized with unlabeled B19 DNA and treated with anti-Dig Fab fragments conjugated with alkaline phosphatase. Fetal specimens from pathologies unrelated to B19 infection were also tested with B19-specific probe and proved negative.

(ii) B19 parvovirus DNA probe. Plasmid pGEM I with a 700-bp DNA insert was prepared by routine methods, and B19 DNA probe preparation was performed as previously described (40). In brief, pGEM I vector DNA was separated from the *Bam*HI-*Hin*dIII B19 DNA fragment by electrophoresis in a 0.6% low-melting-point agarose gel. The B19 DNA fragment was then recovered from the gel and processed for labeling. Probe labeling was done by incorporating Dig-labeled dUTP by the random-primed DNA-labeling method. The reaction was stopped by the addition of 0.2 M EDTA (pH 8), and the labeled B19 DNA fragment was precipitated with ethanol. To check the sensitivity of the probe, it was hybridized with serially diluted, unlabeled parvovirus B19 DNA bound to filter membranes, and 0.1 pg of B19 DNA could be visualized on nylon filters. The B19 DNA probe could be stored at −20°C for at least 4 months with no decrease in its activity.

(iii) Hybridization reaction. Cell smears were hydrated in PBS and then placed in 0.02 N HCl for 10 min. After three washes with PBS, the cells were treated with 0.01% Triton X-100 in PBS for 2 min. After three further washes with PBS, the cell smears were treated with pronase (Boehringer GmbH) (0.5 mg/ml in 0.05 M Tris-HCl, pH 7.6) containing 5 mM EDTA for 5 min. The smear preparations were then washed twice with PBS containing 2 mg of glycine per ml and treated with pancreatic RNase (Boehringer GmbH) (100 μg/ml) and T_1 RNase (Boehringer GmbH) (5 μg/ml) in PBS and then washed twice with PBS to remove RNAs. After these treatments, the cell smears were postfixed with 4% paraformaldehyde in PBS and washed twice with PBS containing 2 mg of glycine per ml. The smears were then dehydrated by ethanol washes (30, 60, 80, 95, and 100%).

Dehydrated monolayers were overlaid with 10 μl of hybridization mixture consisting of 50% deionized formamide, 10% dextran sulfate, 250 μg of carrier calf thymus DNA per ml, and 2 μg of Dig-labeled probe DNA per ml in 2× SSC buffer. A clean glass coverslip was then applied, and the edges were sealed with rubber cement to prevent loss of the mixture during denaturation and hybridization. The cell smears and the hybridization mixture were denatured together by being heated in an 85°C water bath for 6 min and were then incubated at 37°C for 12 h. After hybridization, the coverslips were carefully removed and the cell smears were washed three times at 34°C with 50% formamide and 2× SSC for 3 min each, then twice in 2× SSC at 34°C for 3 min each, and finally once in 2× SSC at room temperature for 3 min. For the detection of hybridized probes, the slides were briefly washed in a 100 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. Normal sheep serum was applied to the cell smears for 30 min at room temperature. After two washings for 2 min each in Tris-HCl buffer, the smears were incubated for 30 min with sheep polyclonal anti-Dig Fab fragments conjugated to alkaline phosphatase, diluted 1/5,000 in Tris-HCl buffer. After incubation, the cell smears were subjected to two 15-min washes with Tris-HCl buffer and equilibrated for 2 min with equilibration buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$; pH 9.5), and then alkaline phosphatase substrate was added according to the manufacturer's instructions (Boehringer GmbH). The reaction was allowed to proceed for 10 min, and the development of a dark blue precipitate at the enzyme site in positive cells was monitored by microscopic examination. The cell smears were washed for 5 min with TE buffer (100 mM Tris-HCl, 1 mM EDTA; pH 8) and mounted with 50% glycerol in PBS.

Nested PCR assay. (i) Samples for PCR. For the PCR study, 50 μl of each sample (serum and amniotic fluid) was added to a 100-μl solution containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg of gelatin per ml, 0.45% Nonidet P-40, 0.45% Tween 20, and 0.006 mg of proteinase K. This mixture was incubated at 65°C for 1 h and then at 95°C for 10 min to inactivate the proteinase K. The precipitate formed was then centrifuged at 12,000 × *g* for 15 min, and 5 μl of the supernatant was used in the PCR assay. Samples for PCR study had been previously stored frozen at −20°C in aliquots of 50 μl each, with any contact with B19 probes and B19 viremic control sera avoided.

(ii) Nested PCR. The nested PCR assay was performed as previously described (21). In brief, 5 μl of each treated sample was added to PCR mix for a total volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.01% (wt/vol) gelatin, 1 U of recombinant *Taq* DNA polymerase (Perkin Elmer, Milan, Italy), 200 μM each deoxynucleoside triphosphate, and 300 ng of each primer. After an initial denaturation step of 5 min at 95°C, the first-round PCR amplification was performed. Then 2 μl of first-round product was transferred to a second 50-μl PCR mix. The second-round reaction mix contained the same constituents as the first-round mix, but 300 ng of each second primer was substituted for each first primer.

The oligonucleotide primers used in the first round of amplification were 5′-CTTTAGGTATAGCCAACTGG-3′ (nucleotides [nt] 2905 to 2924) and antisense 5′-ACACTGAGTTTACTAGTGGC-3′ (nt 4016 to 3997), yielding a product of 1,112 bp. Second-round PCR was performed with primers 5′-CAAAAGCATGTGGAGTGAGG-3′ (nt 3187 to 3206) and 5′-CCTTATAAT GGTGCTCTGGG-3′ (nt 3290 to 3271) to give a product of 104 bp. Thirty-five cycles of both first- and second-round amplification were performed under the following conditions: 95°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min. Ten-microliter samples of second-round PCR products were then analyzed by electrophoresis on a 2% agarose gel. Bands were visualized by ethidium bromide staining. The specificity of the PCR-positive products was confirmed by using an internal oligonucleotide probe as previously described (21). A number of precautions were taken to avoid carryover of PCR products into samples. Sample preparation and amplifications were performed in specially designated laboratories. All hybridization reagents were prepared with pipettes and containers which had never come into contact with amplified products or with B19-positive samples. Our success in avoiding contamination was monitored with negative controls which consisted of PCR tubes containing all PCR reagents and sterile bidistilled water instead of the sample; one negative control was inserted every three samples. As positive controls of the reaction, 10^{-3}, 10^{-9}, and 10^{-10} dilutions of a B19 viremic control reference serum were used throughout the experiments.

IgM and IgG assays. Specific B19 antibodies in serum were detected by using an enzyme-linked immunosorbent assay based on the use of recombinant antigens (IDIA; Dako, Glostrup, Denmark), immunofluorescence tests based on recombinant viral VP1 antigen expressed in an insect cell line (Biotrin International), and Western blot (immunoblot) tests utilizing recombinant antigens (Mardx Diagnostics, Inc., Carlsbad, Calif.).

RESULTS

In 18 cases of B19 fetal hydrops, virological and serological signs of active or recent infection in maternal blood samples

TABLE 1. Virological and serological laboratory investigations of maternal serum samples

Patient no.	Assay result			
	Dot blot hybridization[a]	PCR[b]	IgM	IgG
1	−	+	+	+
2	−	+	+	+
3	−	+	+	+
4	−	+	+	−
5	−	+	−	+
6	−	+	−	+
7	−	+	−	+
8	−	+	−	+
9	−	+	−	−
10	−	−	+	+
11	−	−	+	+
12	−	−	+	+
13	−	−	+	+
14	−	−	+	+
15	−	−	−	+
16	−	−	−	−
17	−	−	−	−
18	−	−	ND[c]	ND

[a] Sensitivity of dot blot hybridization assay, 0.05 to 0.1 pg of B19 DNA, equivalent to 6×10^3 to 3×10^4 genome copies.
[b] Sensitivity of nested PCR assay, 1 to 10 genome copies.
[c] ND, not done (sample not available).

and paired fetal specimens were analyzed. The diagnostic value of different techniques for different specimens at the time of clinical diagnosis of fetal hydrops was evaluated. Of the 18 maternal serum samples tested for B19 DNA (Table 1), 9 proved positive by PCR but none were positive by dot blot hybridization, thus showing that viremia, at a low viral titer, could be detected in 50% of maternal serum specimens at the time of clinical diagnosis of fetal hydrops.

Of 17 maternal serum specimens tested, 9 proved positive for IgM anti-B19 antibodies (53%), and 4 of them were also positive for B19 DNA. Therefore, signs of active or recent B19 infection could be detected only by PCR for five samples, by both PCR and IgM for four samples, and by serological methods alone for another 5 serum samples. Of the nine PCR-positive maternal serum specimens, three proved positive concomitantly for B19 DNA and IgM and IgG antibodies, a pattern resembling a late viremic phase, and one sample was B19 DNA and IgM positive while IgG negative, a pattern compatible with an earlier viremic phase. Moreover, four maternal serum samples were B19 DNA and IgG positive but IgM negative, suggesting that maternal immunity is not always effective in controlling the infection (8, 34).

Of the nine PCR-negative maternal serum specimens, five proved positive for both IgM and IgG, one proved positive only for IgG, two were negative in all the virological and serological tests, and one was available only for virological assays and proved negative.

From the 18 hydropic fetuses (Table 2), 16 serum samples were obtained from cord blood for dot blot hybridization and PCR. Ten serum specimens proved positive for B19 DNA by PCR (62%); 4 of the 10 PCR-positive samples were also positive by dot blot hybridization, while 6 samples proved negative by both dot blot hybridization and PCR. Cell smears from 17 specimens of fetal cord blood were also tested by ISH, and all of them were positive for B19 DNA (Fig. 1a). Three of the 18 fetuses studied were found positive by all the virological tests performed, while 6 were proved positive by ISH but negative by PCR and dot blot hybridization.

Of 13 serum specimens from hydropic fetuses available for B19 serological tests, 2 (15%) showed IgM and IgG anti-B19 antibodies and 7 proved positive only for IgG; the remaining 4 serum samples were negative in all the serological tests, despite the fact that virological assays proved positive.

Amniotic fluids were obtained for 12 of the 18 cases of fetal hydrops: 10 specimens proved positive for B19 DNA by PCR (83%), and 2 of the 10 were also positive by dot blot hybridization assay. Moreover, cell smears obtained from amniotic fluids proved positive for B19 DNA by ISH (Fig. 1b) for 7 of the 10 PCR-positive samples and 1 PCR-negative sample (72%), while 1 specimen of amniotic fluid proved negative in

TABLE 2. Virological and serological laboratory investigations of fetal specimens

Patient no.[a]	Fetal cord blood					Amniotic fluid		
	Dot blot hybridization	PCR	ISH	IgM	IgG	Dot blot hybridization	PCR	ISH
1	+	+	+	+	+	+	+	+
2	−	+	+	−	+	−	+	+
3	−	−	+	−	+	−	+	ND[b]
4	−	−	+	ND	ND	−	+	−
5	−	+	+	−	+	−	+	−
6	+	+	+	−	−	−	+	+
7	−	+	+	−	+	−	+	+
8	−	+	+	−	+	ND	ND	ND
9	+	+	ND	−	−	+	+	+
10	−	−	+	+	+	ND	ND	ND
11	ND	ND	+	ND	ND	ND	ND	ND
12	+	+	+	−	−	−	−	+
13	ND	ND	+	ND	ND	−	−	−
14	−	+	+	−	+	ND	ND	ND
15	−	+	+	−	+	−	+	+
16	−	−	+	ND	ND	ND	ND	ND
17	−	−	+	−	−	ND	ND	ND
18	−	−	+	ND	ND	−	+	+

[a] Pleuric fluid samples were obtained from patients 6, 7, and 11 and proved positive for B19 DNA by PCR.
[b] ND, not done (sample not available).

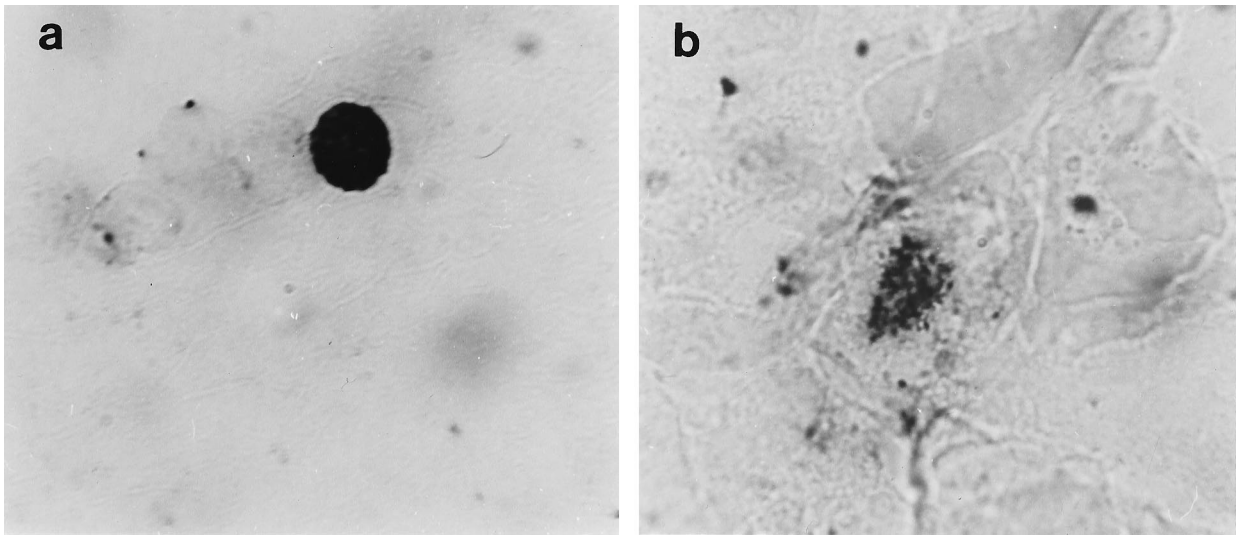

FIG. 1. Smears of cells from cord blood (a) and amniotic fluid (b). B19 DNA can be visualized in the nuclei of infected cells by ISH.

all the virological tests. Pleuric fluid samples from three subjects were analyzed and proved positive for B19 DNA by PCR.

Of the 18 mothers (mean age, 28.9 years), 5 complained of a flu-like illness between 2 weeks and 2 months before the clinical diagnosis of fetal hydrops (patients 2, 5, 10, 14, and 17), 1 had arthralgia (patient 13), 2 reported contacts with exanthematous children (patients 1 and 3), and 10 denied any symptoms. The gestation at diagnosis of fetal hydrops ranged from 20 to 35 weeks, with 9 cases in the second trimester and 9 cases in the third trimester of gestation. Four mothers opted for pregnancy termination, and two intrauterine fetal deaths occurred (patients 1 and 11), at 25 weeks gestation (3 weeks after the diagnosis) and at 22 weeks gestation (1 week after the diagnosis), respectively. From the first infant (patient 1), autoptic sections were obtained from the heart, spleen, liver, and lungs and B19 DNA-positive cells were detected by ISH (Fig. 2). Of the remaining 12 babies who were born, fetal manifestations spontaneously resolved in 9 without apparent sequelae, 1 presented meconium ileus, and 2 presented diaphragm hernias, which were surgically overcome.

DISCUSSION

Since 1984, B19 infection in pregnancy has been known to be a predisposing factor for adverse pregnancy outcome (5). Several studies have evaluated the incidence of poor fetal outcome or possible anatomical and functional abnormalities due to B19 infection (17, 33, 38). The data available concerning the risk of fetal loss due to transplacental B19 transmission vary widely, and discrepancies may be due to uncontrolled prospective studies and/or the different diagnostic methods applied to different maternal and fetal specimens.

We analyzed the diagnostic value of different virological and serological techniques for maternal serum, fetal cord blood, and amniotic fluid samples obtained at the time of clinical diagnosis of fetal hydrops in 18 cases of B19 fetal hydrops. B19 DNA detection was performed by nested PCR and dot blot hybridization for all the specimens. Nested PCR was used since nested viral gene amplification allows the detection of minute amounts of DNA (10 genome copies) and is even more sensitive than single PCR with hybridization detection of amplified products (7, 8). Samples for PCR have been treated to remove PCR inhibitors, an essential step at least for serum samples (7, 10, 13), but can represent a source of contamination in routine diagnostic laboratories.

A dot blot hybridization assay using Dig-labeled RNA probes was performed for a comparative evaluation of the viral loads in the samples. In fact, between 10^3 and 10^4 genome copies can be detected with this assay (39). Since the virus can remain cell associated without being detectable in body fluids (4, 8, 11), cell smears from fetal cord blood and amniotic fluid were also tested for B19 DNA by ISH using a Dig-labeled probe, which has proved highly sensitive and specific (20).

IgM and IgG antibodies to B19 infection were analyzed by commercially available immunoassays using recombinant B19 proteins. Positive results obtained by IDEIA were confirmed by both immunofluorescence and Western blot tests, which in our experience proved specific and sensitive assays, as recently shown by Bruu and Nordbø (6), and which can be a good alternative if the reference radioimmunoassay method is not routinely available in the diagnostic laboratory.

Our investigations show that in B19 hydrops, 78% (14 of 18) of maternal serum samples showed evidence of acute or recent B19 infection at the time of clinical diagnosis. The diagnosis was made by PCR alone in 28% of cases, by only anti-B19 IgM detection in another 28% of cases, and by both PCR and specific IgM detection in 22% of cases. Of the remaining 22% (4 of 18 serum samples) in which active or recent infection was not documented, one serum sample had only B19 IgG, another serum sample was available only for virological assays and proved negative, and the last two samples were negative in all the virological and serological assays (34). This observation could have been explained by no prior infection, but B19 DNA was detected in fetal cord blood by ISH; a low sensitivity of the assays might be suspected, or a transient low-titer viremia might have occurred in the mother without inducing an immune response.

B19 infection in all 18 hydropic fetuses was documented in fetal cord blood and/or amniotic fluid. The greatest sensitivity was obtained by ISH: 100% of the fetal cord blood samples tested proved positive by ISH, while 62% proved positive by nested PCR. These data could mean that B19 DNA can remain associated with erythroid precursor cells and is undetectable by PCR in body fluids, as other observations suggest (4, 8, 11);

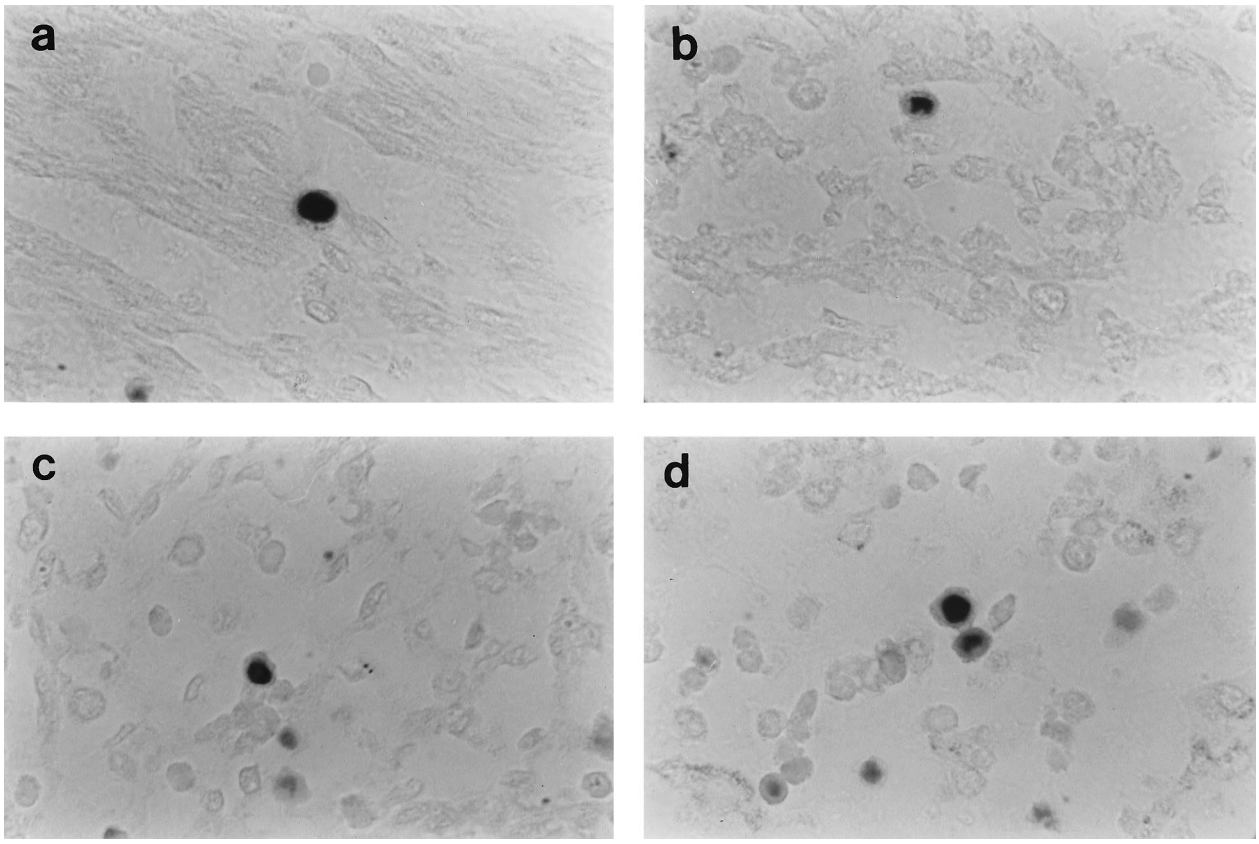

FIG. 2. Autoptic sections of fetal myocardium (a), lung (b), liver (c), and spleen (d) tissues. Elements of the erythroid series show a clear nuclear positivity for the presence of B19 DNA.

alternatively, some inhibitors could still be present in serum samples, but we discarded this hypothesis, since when serum samples from PCR-negative, ISH-positive patients were added to positive reference sera, the efficiency of amplification did not change (data not shown).

A total of 25% of fetal cord blood samples also proved positive by dot blot hybridization, showing that medium- to high-titer viremias are detectable in fetuses. A total of 15% of fetal serum samples tested for B19 antibodies were IgM positive, while 53% showed only IgG of maternal origin.

Virological assays performed on amniotic fluid proved positive at 83% by PCR and at 72% by ISH on the tested cell smears. The lower percentage of positive ISH results in comparison with those observed for fetal cord blood could be explained by a lower susceptibility to B19 infection of the cells present in the different specimens and/or by the limited number of cells available from amniotic fluid.

Among the 18 cases of fetal hydrops associated with B19 infection analyzed, two fetal deaths occurred, at 22 and 25 weeks gestation. As suggested by Schwarz et al. (29), the higher frequency of B19-associated fetal death observed between the 20th and 28th weeks is probably due to the hematopoietic status of the fetus at that time. Autoptic sections were obtained from several tissues of one fetus, and B19 DNA could be detected by ISH in all of the tissues examined. B19-positive cells were morphologically identified as erythroid precursors.

Our data suggest that virological methods are a useful tool for the prenatal diagnosis of B19 infection in both maternal and paired fetal specimens. For maternal sera, viral diagnosis does not exclude serological study, since our results show complementary roles for the different laboratory investigations. For fetal specimens, the detection of viral markers seems to be the most sensitive tool for the prenatal diagnosis of B19 infection. Both fetal cord blood and amniotic fluid specimens are suitable for diagnosis, but the detection of B19 DNA in fetal cord blood by ISH proved to be the most sensitive diagnostic system.

ACKNOWLEDGMENTS

The skillful technical help of Marinella Plazzi is gratefully acknowledged.

This work was partially supported by grant 60% from the Ministry of University and Scientific Research.

REFERENCES

1. **Anand, A., E. S. Gray, T. Brown, J. P. Clewley, and B. J. Cohen.** 1987. Human parvovirus infection in pregnancy and hydrops fetalis. N. Engl. J. Med. **316:**183–187.
2. **Anderson, M. J., S. E. Jones, S. P. Fisher-Hoch, E. Lewis, S. M. Hall, C. L. R. Barlett, B. J. Cohen, P. P. Mortimer, and M. S. Pereira.** 1983. The human parvovirus, the cause of erythema infectiosum (fifth disease)? Lancet **i:**1378.
3. **Boruchoff, S. E., B. A. Woda, G. A. Pihan, W. A. Durbin, D. Burstein, and N. R. Blacklow.** 1990. Parvovirus-B19-associated hemophagocytic syndrome. Arch. Intern. Med. **150:**897–899.
4. **Brown, K. E., S. W. Green, J. Antunez de Mayolo, J. A. Bellanti, S. D. Smith, T. J. Smith, and N. S. Young.** 1994. Congenital anaemia after transplacental B19 parvovirus infection. Lancet **343:**895–896.
5. **Brown, T., A. Anand, L. D. Ritchie, J. P. Clewley, and T. M. S. Reid.** 1984. Intrauterine parvovirus infection during pregnancy. Lancet **ii:**1033–1034.
6. **Bruu, A. L., and S. A. Nordbø.** 1995. Evaluation of five commercial tests for detection of immunoglobulin M antibodies to human parvovirus B19. J. Clin. Microbiol. **33:**1363–1365.
7. **Carrière, C., P. Boulanger, and C. Delsert.** 1993. Rapid and sensitive method

for the detection of B19 DNA using the polymerase chain reaction with nested primers. J. Virol. Methods **44:**221–234.
8. **Cassinotti, P., M. Weitz, and G. Siegl.** 1993. Human parvovirus B19 infections: routine diagnosis by a new nested polymerase chain reaction assay. J. Med. Virol. **40:**228–234.
9. **Corman, L. C., and D. J. Dolson.** 1992. Polyarteritis nodosa and parvovirus B19 infection. Lancet **339:**491.
10. **Espy, M. J., R. Patel, C. V. Paya, and T. F. Smith.** 1995. Comparison of three methods for extraction of viral nucleic acids from blood cultures. J. Clin. Microbiol. **33:**41–44.
11. **Foto, F., K. G. Saag, L. L. Schatosch, E. J. Howard, and S. J. Naides.** 1993. Parvovirus B19 specific DNA in bone marrow from B19 arthropathy patients: evidence for B19 virus persistence. J. Infect. Dis. **167:**744–748.
12. **Frickhofen, N., and N. S. Young.** 1989. Persistent parvovirus B19 infections in humans. Microb. Pathog. **7:**319–327.
13. **Frickhofen, N., and N. S. Young.** 1991. A rapid method of sample preparation for detection of DNA viruses in human serum by polymerase chain reaction. J. Virol. Methods **35:**65–72.
14. **Gentilomi, G., M. Zerbini, M. Musiani, G. Gallinella, D. Gibellini, S. Venturoli, M. C. Re, S. Pileri, C. Finelli, and M. La Placa.** 1993. In situ detection of B19 DNA in bone marrow of immunodeficient patients using a digoxigenin-labelled probe. Mol. Cell. Probe **7:**19–24.
15. **Gratacós, E., P. J. Torres, J. Vidal, E. Antolin, J. Costa, M. T. Jimenez de Anta, V. Carach, P. Alonso, and A. Fortuny.** 1995. The incidence of human parvovirus B19 infection during pregnancy and its impact on perinatal outcome. J. Infect. Dis. **171:**1360–1363.
16. **Gray, E. S., A. Anand, and T. Brown.** 1986. Parvovirus infections in pregnancy. Lancet **i:**208.
17. **Hall, C. J.** 1994. Parvovirus B19 infection in pregnancy. Arch. Dis. Child. **71:**4–5.
18. **Koch, W. C., S. P. Adler, and J. Harger.** 1993. Intrauterine parvovirus B19 infection may cause an asymptomatic or recurrent postnatal infection. Ped. Infect. Dis. J. **12:**747–750.
19. **Kovacs, B. W., D. E. Carlson, B. Shahbahrami, and L. D. Platt.** 1992. Prenatal diagnosis of human parvovirus B19 in nonimmune hydrops fetalis by polymerase chain reaction. Am. J. Obstet. Gynecol. **167:**461–466.
20. **Morey, A. L., H. J. Porter, J. W. Keeling, and K. A. Fleming.** 1992. Nonisotopic in situ hybridization and immunophenotyping of infected cells in the investigation of human fetal parvovirus infection. J. Clin. Pathol. **45:**673–678.
21. **Musiani, M., A. Azzi, M. Zerbini, D. Gibellini, S. Venturoli, K. Zakrzewska, M. C. Re, G. Gentilomi, G. Gallinella, and M. La Placa.** 1993. Nested PCR assay for the detection of B19 parvovirus DNA in HIV seropositive patients. J. Med. Virol. **40:**157–160.
22. **Musiani, M., G. Gentilomi, M. Zerbini, D. Gibellini, G. Gallinella, S. Pileri, P. Baglioni, and M. La Placa.** 1990. In situ detection of cytomegalovirus DNA in biopsies of AIDS patients using a hybrido-immunocytochemical assay. Histochemistry **94:**21–25.
23. **Musiani, M., M. Zerbini, G. Gentilomi, G. Gallinella, and S. Venturoli.** 1995. Parvovirus B19 clearance from peripheral blood after acute infection. J. Infect. Dis. **172:**1360–1363.
24. **Nascimento, J. P., N. F. Hallam, A. M. Field, J. P. Clewley, K. E. Brown, and B. J. Cohen.** 1991. Detection of B19 parvovirus in human fetal tissue by in situ hybridization. J. Med. Virol. **33:**77–82.
25. **Ozawa, K., G. Kurtzman, and N. Young.** 1986. Replication of the B19 parvovirus in human bone marrow cell cultures. Science **233:**883–886.
26. **Peters, M., and K. Nicolaides.** 1990. Cordocentesis for the diagnosis and treatment of human fetal parvovirus infection. Obstet. Gynecol. **75:**501–504.
27. **Public Health Laboratory Service Working Party on Fifth Disease.** 1990. Prospective study of human parvovirus (B19) infection in pregnancy. Br. Med. J. **300:**1166–1170.
28. **Rogers, B. B., D. B. Singer, S. K. Mak, G. W. Gary, M. K. Fikrig, and P. N. McMillan.** 1993. Detection of human parvovirus B19 in early spontaneous abortus using serology, histology, electron microscopy, in situ hybridization, and the polymerase chain reaction. Obstet. Gynecol. **81:**402–407.
29. **Schwarz, T. F., A. Nerlich, B. Hottentragen, G. Jager, I. Wiest, S. Kantimm, H. Roggendorf, M. Schultz, K. P. Gloning, T. Schramm, W. Holzgreve, and M. Roggendorf.** 1991. Parvovirus B19 infection of the fetus: histology and in situ hybridization. Am. J. Clin. Pathol. **96:**121–126.
30. **Sevall, J. S., J. Ritenhous, and J. B. Peter.** 1992. Laboratory diagnosis of parvovirus B19 infection. J. Clin. Lab. Anal. **6:**171–175.
31. **Sheikh, A. U., J. M. Ernest, and M. O'Shea.** 1992. Long-term outcome in fetal hydrops from parvovirus B19 infection. Am. J. Obstet. Gynecol. **167:** 337–341.
32. **Shneerson, J. M., P. P. Mortimer, and E. M. Vandervelde.** 1980. Febrile illness due to parvovirus. Br. Med. J. **ii:**1580–1581.
33. **Tiessen, R. G., A. M. W. van Elsacker-Niele, C. H. R. Vermeij-Keers, D. Oepkes, J. Van Roosmalen, and M. C. B. Gorsira.** 1994. A fetus with a parvovirus B19 infection and congenital anomalies. Prenat. Diagn. **14:**173–176.
34. **Török, T. J., Q. Y. Wang, G. W. Gary, Jr., C. F. Yang, T. M. Finch, and L. J. Anderson.** 1992. Prenatal diagnosis of intrauterine infection with parvovirus B19 by polymerase chain reaction technique. Clin. Infect. Dis. **14:**149–155.
35. **White, D. G., A. D. Woolf, P. P. Mortimer, B. J. Cohen, D. R. Blake, and P. A. Bacon.** 1985. Human parvovirus arthropathy. Lancet **i:**419–421.
36. **Young, N. S.** 1988. Hematologic and hematopoietic consequences of B19 parvovirus infection. Semin. Hematol. **25:**159–172.
37. **Zachoval, R., M. Kroener, M. Brommer, and F. Deinhardt.** 1990. Numbness and tingling of fingers associated with parvovirus B19 infection. J. Infect. Dis. **161:**354–355.
38. **Zerbini, M., M. Musiani, G. Gentilomi, S. Venturoli, G. Gallinella, D. Gibellini, R. Morandi, B. Guerra, L. Bovicelli, and M. La Placa.** 1993. Symptomatic parvovirus B19 infection of one fetus in a twin pregnancy. J. Clin. Infect. Dis. **17:**262–263.
39. **Zerbini, M., M. Musiani, D. Gibellini, G. Gentilomi, S. Venturoli, G. Gallinella, and M. La Placa.** 1993. Evaluation of strand-specific RNA probes visualized by colorimetric and chemiluminescent reactions for the detection of B19 parvovirus DNA. J. Virol. Methods **45:**169–178.
40. **Zerbini, M., M. Musiani, S. Venturoli, G. Gallinella, D. Gibellini, G. Gentilomi, and M. La Placa.** 1990. Rapid screening for B19 parvovirus DNA in clinical specimens with a digoxigenin-labeled DNA hybridization probe. J. Clin. Microbiol. **28:**2496–2499.
41. **Zerbini, M., M. Musiani, S. Venturoli, G. Gallinella, D. Gibellini, G. Gentilomi, and M. La Placa.** 1992. Different syndromes associated with B19 parvovirus viraemia in paediatric patients: report of four cases. Eur. J. Pediatr. **151:**815–817.