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Detection of Human Parvovirus B19 DNA by Using the Polymerase Chain Reaction

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The polymerase chain reaction (PCR) was investigated for detecting human parvovirus B19 (B19) DNA in sera. Three pairs of oligonucleotides were evaluated as primers. The best oligonucleotide pair spanned 699 nucleotides, including the region common to VP1 and VP2. After PCR amplification of B19 DNA in serum, a 699-nucleotide DNA fragment was detected on agarose gels. This DNA fragment was B19 DNA, because after Southern transfer it hybridized to a 19-nucleotide internal probe and contained a single *PstI* cleavage site. Dot blot hybridization with a radiolabeled cloned portion of the B19 genome as a probe was compared with PCR. PCR was 10^4 times more sensitive than dot blot hybridization and, with an internal radiolabeled probe, 10^7 times more sensitive than dot blot hybridization. Of 29 serum specimens from 18 patients with proven B19 infections, 24 were PCR positive. None of 20 serum samples from uninfected controls were positive. Of 22 serum samples positive for immunoglobulin M to B19, PCR detected B19 DNA in 17. Seven serum samples lacking immunoglobulin M were PCR positive. PCR detected B19 DNA in urine, amniotic fluid, pleural fluid, ascites, and leukocyte extracts. PCR is a rapid and simple method for diagnosing infections with human parvovirus B19 but must be combined with serologic tests for immunoglobulin M to B19, especially when testing only a single serum sample.

Human parvovirus B19 (B19) causes several syndromes, including erythema infectiosum, chronic arthritis in adults, aplastic crisis in patients with hemolytic anemias, fetal death, and chronic anemia and neutropenia in immunocompromised patients (2, 3, 7, 10, 15, 17, 20, 22, 25). The virus replicates only in erythroid precursor cells derived from bone marrow (17, 19). In vitro culture systems producing human parvovirus B19 have not been developed. Consequently, diagnostic tests for this infection are not widely available. The few laboratories that do these tests must rely on antigen obtained from the serum of infected individuals (4). In addition, we and others have observed chronic B19 infection of immunocompromised patients whose serum lacked immunoglobulin G (IgG) and IgM to the virus (15, 16; W. C. Koch and S. P. Adler, J. Pediatr., in press). Diagnosis was made by detecting B19 DNA in their sera by dot blot analysis. To circumvent these problems in diagnosis and because the polymerase chain reaction (PCR) is sensitive and specific for detecting several other viruses, we evaluated the PCR for the detection of human parvovirus B19 in serum and other body fluids (1, 8, 13, 18, 23).

MATERIALS AND METHODS

Selection of sequences for primers and probes. B19 has a single-stranded DNA approximately 5,400 nucleotides long (12). The B19 genome encodes two capsid proteins, VP1 (84 kilodaltons) and VP2 (58 kilodaltons) and a nonstructural protein (77 kilodaltons) (11). VP1 and VP2 share common carboxy-terminal amino acids. VP2 composes 96% of the viral capsid. We evaluated three sets of primers and probes: one set located within VP1, one set within the nonstructural gene, and one set within VP2 (Table 1). Primers and probes were synthesized on a DNA synthesizer (model 380A; Applied Biosystems, Foster City, Calif.) and purified by high-pressure liquid chromatography before use.

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Amplification. Amplification of target sequences occurred in a 500-μl polypropylene microfuge tube with a total reaction volume of 100 μl. The reaction mixture contained 200 μM each dATP, dCTP, dGTP, and dTTP; oligonucleotide primers (each at 1 μM); 50 mM KCl; 10 mM Tris (pH 8.3); 2.0 mM $MgCl_2$; 0.01% gelatin; and 1 to 3 μl of sample. After the mixture was heated to 94°C for 3 min, 2.0 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was added. The mixtures were overlaid with 100 μl of mineral oil, and then thermal cycling was carried out in a programmable heat block (Perkin-Elmer). Each cycle consisted of 2 min at 94°C, 2 min at 37°C, and 3 min at 72°C. An additional 7 min was added at the end of the cycling to complete extension of the primers. Thirty-five cycles were performed in 6 h.

Detection of amplified B19 sequences in reaction products. After cycling, 10 μl of each amplified mixture was electrophoresed on a 4% agarose minigel (3% NuSieve, 1% SeaKem; FMC Corp., Rockland, Maine). After gels were stained with ethidium bromide, they were viewed under UV light and photographed. Southern transfer and hybridization with a B19-specific probe were performed by electrophoretically transferring DNA fragments from the agarose gels to nylon membranes (Nytran; Schleicher & Schuell Co., Keene, N.H.) in TAE buffer (10 mM Tris hydrochloride, 5 mM sodium acetate, 0.5 mM EDTA [pH 7.8]). Before transfer, the gels were soaked in 0.2 N NaOH–0.5 M NaCl for 30 min, followed by two washes of 10 min each in 500 ml of 4× TAE buffer and a final 10-min wash in 1× TAE buffer. After the membrane was presoaked in 1× TAE buffer for 5 min, the membrane and gel were assembled as a sandwich between filter paper and placed in the middle slot of a Trans-Blot tank (Bio-Rad Laboratories, Richmond, Calif.), and 2.5 to 3.0 liters of 1× TAE buffer was added. After electrophoretic transfer overnight at 250 mA, the membranes were baked at 80°C. The membranes were prehybridized at 42°C for at least 3 h in hybridization solution, which contained the following: 6× SSPE (20× SSPE is 3.6 M NaCl,

TABLE 1. Location in the B19 genome and sequences of oligonucleotide primer pairs and probes

Primer or probe	Sequence (5'–3')	Product length (base pairs)	Location	
			Nucleotide nos.	Gene
K-1 primer	ATAAATCCATATACTCATT	699	2936–2954	VP1
K-2 primer	CTAAAGTATCCTGACCTTG		3617–3635	
K-5 probe	CTAACTCTGTAACTTGTAC		3222–3240	
K-6 primer	AAACTATGGTAAACTGGTT	442	1085–1103	NS1
K-7 primer	TGCTACATCATTAAATGGA		1509–1527	
K-11 probe	TTACTTTGTCAAAACTATG		1321–1339	
K-8 primer	AGCTACAGATGCAAAACAA	308	4702–4720	VP2
K-9 primer	TAACCACAACAAATGTTTA		4992–5010	
K-12 probe	CTATAAGACAGCCTAACAC		4873–4891	

200 mM NaH_2PO_4 [pH 7.4], and 20 mM EDTA [pH 7.4]), 1% sodium dodecyl sulfate (SDS), 10× Denhardt solution (0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, and 0.2% Ficoll), 20 μg of tRNA per ml, and 50 μg of sheared salmon sperm DNA per ml. Then 0.2 μg of each probe was end labeled to a specific activity of at least 2×10^8 with gamma-labeled [^{32}P]ATP by using T4 kinase (Bethesda Research Laboratories, Gaithersburg, Md.). The labeled probe was purified on a Nensorb column (Nensorb 20; Du Pont Co., Wilmington, Del.). Labeled probe (5×10^7 cpm) was added to the hybridization solution (6× SSPE, 1% SDS) and incubated overnight at 5°C less than the melting temperature of each probe. After hybridization the membranes were washed in 6× SSPE–1% SDS three times at room temperature and once at hybridization temperature. After washing, the membranes were exposed to Cronex X-ray film (Du Pont) for 3 to 18 h at −70°C with an intensifying screen.

Dot blot hybridization. Dot blot hybridization was performed as described by Clewley; plasmid pSP321, which contains the middle one-half (2.7 kilobases) of the B19 genome cloned into the *Pst*I site in the ampicillin resistance gene of pBR322 (9), was used as a probe. Briefly, 10 μl of sample was added to 200 μl of 2× SSC (0.3 M NaCl, 0.03 M sodium citrate [pH 7.0]), followed by the addition of 200 μl of 1 M NaCl–0.1 M NaOH. After 10 min at room temperature, 1.8 ml of 2× SSC was added and then filtered over a nitrocellulose membrane (Schleicher & Schuell) that had been prewet with 20× SSC for 30 min. The membrane was washed briefly in 2× SSC and baked at 80°C for 2 h in a vacuum oven. The membrane was prehybridized for 4 h at 65°C in 10 ml of hybridization solution (6× SSC, 0.5% SDS, 1× Denhardt solution, 100 μg of sheared salmon sperm DNA per ml). The membrane was transferred to fresh hybridization solution with 10^6 cpm of [^{32}P]dCT-labeled probe (2×10^6 cpm/μg). The filter was hybridized overnight at 65°C with mixing. After hybridization, the filter was washed at 65°C for 2 to 4 h with serial changes of 1× SSC–0.1% SDS, allowed to dry at room temperature for 10 to 20 min, and exposed to X-ray film. Autoradiograms were developed after 3, 18, and 72 h and 7 days at −70°C with an intensifying screen.

pBS321 was labeled with [^{32}P]dCT by nick translation (21). Unincorporated [^{32}P]dCT was removed, and the plasmid was concentrated with an Centricon 30 filter (W. R. Grace and Co., Danvers, Mass). Before use, the labeled plasmid was added to 0.5 ml of sheared salmon sperm DNA (10 mg/ml), placed in a boiling water bath for 3 min, and then placed on ice for 5 min.

Serologic assays. IgG and IgM to B19 in human sera were detected as previously described (4, 14).

Specimens. Serum samples for PCR were obtained from 18 patients at Medical College of Virginia Hospital with hematologic problems compatible with B19 infection. Six of the 18 patients had at least one specimen positive for B19 DNA by dot blot hybridization, including three patients with sickle cell anemia and aplastic crisis, two patients with acute lymphoblastic anemia, and one patient with systemic lupus erythematosus and autoimmune hemolytic anemia. The other 12 patients had at least one serum sample containing IgM to B19; 8 of these 12 had sickle cell disease, 3 had hereditary spherocytosis, and 1 adult had erthyma infectiosum with arthritis.

Additional B19-positive sera were provided by B. J. Cohen (Wi; London), A. M. Courouce (REM, DES, and LEC; Paris), and N. Young (Minor; Bethesda, Md.).

Other specimens included ascites and pericardial fluid from a hydropic fetus and urine and peripheral blood mononuclear cells from a patient with aplastic crisis. Peripheral blood mononuclear cells were obtained from heparinized blood samples by using Sepracell-MN (Sepratech Corp., Oklahoma City, Okla.); 5 μl of the cell suspension was used in the PCR assay without prior DNA extraction.

For amplification of sera and other body fluids, 1 to 3 μl was added directly to the PCR reaction mixture.

Control serum samples were obtained at random from 20 obstetric patients when blood was drawn for other purposes.

All serum was stored at −70°C.

RESULTS

Each of the three primers worked effectively for the detection of B19 DNA (Tables 1 and 4). Primers K1 and K2

TABLE 2. PCR results of B19 antigen-positive sera tested with three different sets of primer pairs

Serum	Yr	Location	Results with primer pair:		
			K1, K2	K6, K7	K8, K9
Wi	1973	United Kingdom	+	+	+
REM	1978	France	+	−	+
DES	1978	France	+	+	+
LEC	1979	France	+	+	+
Minor	1984	Ohio	+	+	+
KM	1987	Richmond	+	+	+
CS	1988	Richmond	+	+	+
KT	1988	Richmond	+	+	+
KA	1989	Richmond	+	+	−

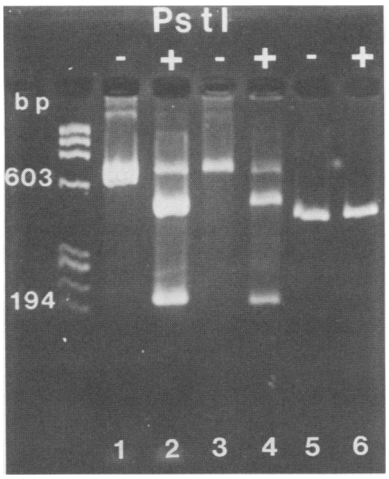

FIG. 1. Ethidium bromide-stained agarose gel of PCR amplified reaction products of two different sera (lanes 1 and 2 and lanes 3 and 4). The left lane (unlabeled) contains base pair (bp) marker fragments, with the sizes of two fragments indicated. Undigested (−) reaction products for both serum samples contained a 699-base-pair fragment when K1 and K2 were used as primers (lanes 1 and 3) and, for one of the two serum samples, a 442-base-pair fragment when K6 and K7 were used as primers (lane 5). For both serum samples after *Pst*I digestion (+), two fragments of the predicted length (194 and 505 base pairs) appeared when K1 and K2 were the primers (lanes 2 and 4), but the K6-K7-primed fragment lacked a *Pst*I restriction site (lane 6).

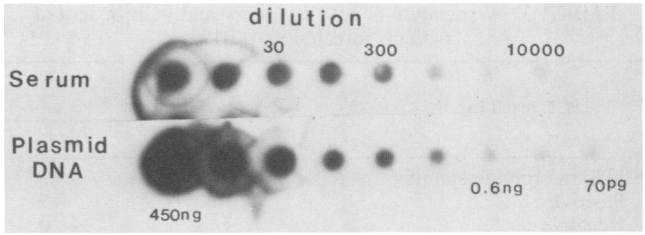

FIG. 2. Autoradiogram after dot blot hybridization of plasmid pSB321 and serum containing B19 virus. Serum and plasmid were diluted in 2× SSC, and 10 μl of each dilution was denatured and filtered over nitrocellulose. For the serum the fold dilution is indicated above three of the dots, and for the plasmid the quantity of DNA on each dot is indicated below three of the dots. ^{32}P-labeled pSP321 was the probe, and the nitrocellulose sheet was exposed to X-ray film for 1 week at −70°C.

were selected for complete characterization of the PCR reaction. To find optimal reaction conditions, three annealing temperatures (37, 40, and 42°C) and three different numbers of total cycles (25, 30, and 35) were investigated. The combination of 35 cycles and 37°C produced the best level of sensitivity and specificity. The expected amplified product of 699 base pairs was observed after amplification of a dot-blot-positive serum sample (Fig. 1). *Pst*I digestion of the 699-base-pair fragment generated by primers K1 and K2 in two serum samples yielded a 194-base-pair fragment and a 505-base-pair fragment, as predicted from the nucleotide sequence (Fig. 1). PCR performed with primers K6 and K7 yielded a DNA fragment of the predicted size, 442 base pairs, that lacked a PST1 site (Fig. 1). None of the primer sets produced B19-specific DNA fragments when either cytomegalovirus DNA, simian virus 40 DNA, or human cellular DNA (from MRC-5 fibroblasts and from lymphocytes) was used.

To determine the sensitivity of PCR, PCR was compared with dot blot hybridization. The serum from a patient with aplastic crisis due to human parvovirus B19 was serially diluted. The dot blot assay detected approximately 0.2 ng of B19 DNA in 10 μl of the diluted serum with an exposure of the nitrocellulose filter for 7 days at −70°C (Fig. 2). In contrast, when 1-μl samples of the same dilutions of this serum were amplified by using PCR and the agarose gels were stained with ethidium bromide, B19 DNA was detected in the $1{:}10^7$ dilution (Fig. 3A). When the agarose gel was probed with the internal probe K5, both single- and double-stranded fragments were detected in a $1{:}10^{10}$ dilution of this serum with an exposure at −70°C for 3 h (Fig. 3B). Therefore, with ethidium bromide staining the PCR detected approximately 0.02 pg of B19 DNA, and with a radiolabeled internal probe the PCR detected 0.02 fg of B19 DNA.

Dot blot hybridization and PCR were compared by using 15 serum samples from six patients with B19 infections. All six patients had at least one serum sample positive for B19 DNA by dot blot hybridization. Of 11 dot-blot-positive serum samples, all were also positive by PCR. Of four serum samples negative by dot blot hybridization, three were positive by PCR. One serum sample was negative by both tests. In addition we assayed 20 serum samples from 20 pregnant women selected randomly. All 20 serum samples lacked IgM to B19, and 8 contained IgG to B19. All 20 serum samples were negative by dot blot hybridization and also negative by PCR.

Twenty-nine serum samples from 18 patients with proven B19 infections were assayed for IgM and IgG to B19 and by PCR (Table 3). Of 22 serum samples that were IgM positive, 17 were PCR positive. All five serum samples (from five

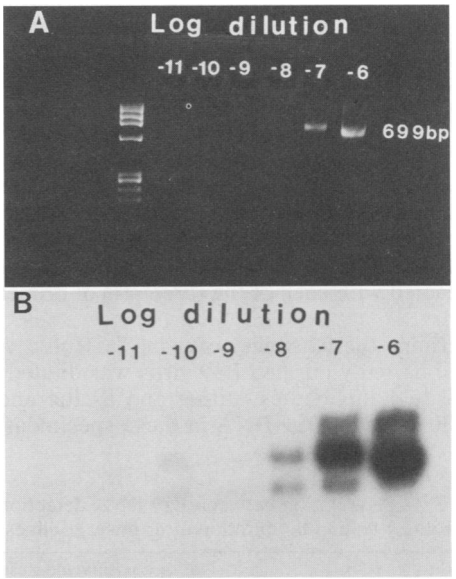

FIG. 3. PCR amplification of serially diluted patient serum. Samples (1 μl) of the same dilutions of the serum used for dot blot hybridization (Fig. 2) were amplified with primers K1 and K2. After amplification and agarose gel electrophoresis, the gel was stained with ethidium bromide (A). The DNA fragments of this gel were transferred to a nylon membrane and probed with ^{32}P-labeled internal probe K5 (B). Ethidium bromide stained only the double-stranded fragment (B) in serum diluted 10^{-7}. The radiolabeled probe detected single- and double-stranded fragments in serum diluted 10^{-10} after 3 h of exposure to X-ray film at −70°C.

TABLE 3. Association of IgM positivity and PCR in sera of patients infected with B19

PCR result by:	No. of serum samples by IgM status	
	Positive	Negative
Ethidium bromide staining		
Positive	13	7
Negative	9	0
Radiolabeled probe		
Positive	17	7
Negative	5	0

patients) that contained IgM but were PCR negative were obtained during the convalescent phase of illness (≥3 days after the onset of symptoms) and contained IgG to B19. Seven serum samples from five patients lacked IgM to B19, and all were PCR positive. One of these five patients was immunocompromised and had an impaired ability to make IgG and IgM to B19 (Koch and Adler, in press). The other four serum samples that lacked IgM but were PCR positive were from four patients with sickle cell disease and aplastic crisis. Each of these four serum samples was obtained during acute infection (<4 days after the onset of symptoms) and lacked IgG to B19.

Of 24 serum samples positive by PCR, 20 were positive when only ethidium bromide was used to detect B19 DNA after amplification (Table 3). Four serum samples were positive only after a radiolabeled probe was used for detection. These four serum samples (from four patients) were obtained during the convalescent phase, and each contained IgG and IgM to B19.

Table 4 compares DNA detection by dot blot hybridization and PCR with serologic tests for IgG and IgM to B19 by duration of illness. Of 10 serum samples obtained 3 days or less after the onset of illness, 9 were PCR positive but only 6 were IgM reactive. However, all 16 serum samples obtained after 3 days of illness from 16 patients contained IgM to B19.

Nine different serum samples with B19 antigen were subjected to PCR amplification with each of the three primer sets listed in Table 1. After amplification, one serum sample from France did not react with primers K6 and K7 and one Richmond isolate did not react with K8 and K9. All the other isolates reacted with each of the three sets of primers (Table 2).

To determine the feasibility of using PCR in a variety of specimens, partially purified B19 virus was diluted 1:10,000 and added to lymphocytes, urine, and ascitic and pleural fluids. PCR detected B19 DNA in these specimens. Pleural fluid, if used undiluted, inhibited PCR. PCR detected B19 DNA in lymphocyte lysates from a patient with aplastic crisis due to B19 and in the ascitic and pericardial fluids obtained from a hydropic fetus.

TABLE 4. Association between B19 DNA detection and serologic testing by duration after onset of illness

Days after onset of illness	No. of patients[a]	No. sera positive/no. tested			
		DNA detection		B19 antibody	
		Dot blot	PCR[b]	IgM	IgG
0–3	7	7/10	9/10	6/10	2/10
4–7	11	1/11	9/11	11/11	10/11
>7	5	0/5	3/5	5/5	5/5

[a] Includes 17 patients. One patient with leukemia who never made antibodies to B19 is excluded.

[b] All sera were tested with a radiolabeled probe.

DISCUSSION

The PCR with ethidium bromide for detection is at least 10^4 times more sensitive than dot blot hybridization for the detection of B19 DNA in serum and detects fewer than 10 genomes with a radiolabeled probe. This level of sensitivity agrees with a previous report with DNA and RNA probes (24). Besides sensitivity, PCR has other advantages for diagnostic laboratories over dot blot hybridization. First, a radiolabeled probe would be unnecessary for many serum samples. B19 infections typically produce high titers of virus in sera, especially during aplastic crisis (6). In this study, 20 serum samples were positive after ethidium bromide staining, and only 4 serum samples required a radiolabeled probe for detection of amplified B19 DNA. Second, the procedure is complete within 8 h. Third, an automated thermocycler for temperature shifts eliminates personnel time and allows multiple specimens to be tested simultaneously.

PCR has two disadvantages. First, specimen contamination occurs easily. Avoiding contamination requires meticulous care in the handling and transfer of specimens. Second, not all sets of probes will detect all isolates. Of nine isolates tested against all three sets of probes, two isolates each failed to react with one set of probes. However, we did find one set of probes that reacted with all of the isolates. This was expected, because epidemiologically different isolates of B19 have variations in restriction enzyme sites, indicating genetic heterogeneity (B. J. Cohen, personal communication).

PCR is specific and sensitive for detecting B19 viremia but should be used with IgM detection for the diagnosis of B19 infection, especially when only a single specimen is tested. Using dot blot hybridization to detect virus, Anderson et al. showed that B19 viremia precedes the appearance of B19-specific IgM and that IgM persists for weeks after viremia has cleared (5). Thus, patient sera obtained early in infection may lack IgM to B19 but be PCR positive. Later during infection, sera may be PCR negative but contain IgM. This agrees with our observations. Four serum samples obtained early in infection were PCR positive and lacked IgM and IgG. Five serum samples obtained late in infection were IgM and IgG positive and PCR negative.

Finally, we and others have recently observed immunocompromised patients with chronic B19 infections (15, 16; Koch and Adler, in press). These chronic infections occur because of the inability of these patients to produce adequate levels of IgG or IgM antibodies to B19; antigen or DNA detection is required for diagnosis. For these immunocompromised patients PCR, because of its excellent sensitivity and specificity, is likely to be the most useful of the currently available diagnostic tests.

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