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Characterization of a Nested Polymerase Chain Reaction Assay for Detection of Parvovirus B19

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The characterization and application of a nested polymerase chain reaction (PCR) assay for the detection of human parvovirus B19 DNA is described. The assay was evaluated with 149 diagnostic serum samples (collected up to 150 days after the onset of symptoms) previously tested by dot blot hybridization for B19 DNA and by class-specific capture radioimmunoassays for the detection of B19 immunoglobulin M (IgM) and IgG. B19 DNA was detectable by the PCR in 70% of the sera. There was a statistically significant association between the detection of B19 DNA by PCR and high B19 IgM values ($P < 0.005$), low B19 IgG values ($P < 0.05$), and a short interval between onset of symptoms and serum collection ($P < 0.005$). Serial serum samples, throat swabs, and peripheral blood mononuclear cells collected from 10 individuals during an outbreak of parvovirus B19 were also tested by the nested PCR. B19 DNA was detectable in the throat swabs at the time of the clinical illness and in the peripheral blood mononuclear cell fraction up to the end point of the study 6 months after infection. The location of the B19 DNA could not be determined in cytocentrifuge preparations of peripheral blood mononuclear cells with nonisotopic in situ hybridization and immunolabelling.

Human parvovirus B19, first recognized in 1975 (12), causes a variety of disease syndromes determined by the age and hematological status of the host. In healthy individuals it causes erythema infectiosum (5, 31, 35). In women, in particular, the rash illness is commonly accompanied by arthritis (1, 5). In subjects with hemolytic anemia, a more profound anemic episode occurs, with transient loss of erythrocyte precursors from the bone marrow, aplastic crisis (33, 40). Chronic anemia in immunocompromized individuals has been described (13, 22, 24). When infection occurs during pregnancy it may cause hydrops fetalis (6, 7) and/or loss of the fetus (17, 26, 30).

A striking feature of B19 infection is the profound viremia that occurs from days 6 or 7 to 12 postinfection (3, 37). Up to 10^{12} viral particles per ml can be detected at the height of the viremia by dot blot hybridization techniques for viral DNA (4, 28). Although B19-specific immunoglobulin M (IgM) appears just after the disappearance of B19 viremia (3, 37), the mechanism of B19 clearance has not been elucidated. Moreover, the sensitivity of the dot blot hybridization technique (lower limit of 10^5 viral particles per ml) has limited studies of the decline of B19 viremia.

We describe the development and application of a nested polymerase chain reaction (PCR) amplification assay for the detection of B19 DNA. The assay was used to examine the decline of B19 DNA in serum after infection and to determine whether there was any association between the presence of B19 DNA and the B19 IgM and B19 IgG content of the sample. The PCR was also used to determine whether B19 DNA is present in peripheral blood mononuclear cells (PBMNCs) and whether it persists in this cell population. The assay was also applied to throat swabs collected at the time of clinical illness (when B19 DNA is below the level of detection by dot blot hybridization) to determine whether the PCR could form the basis of a noninvasive diagnostic test.

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MATERIALS AND METHODS

Storage of samples and reagents. All sera, plasma, tissues, and throat swabs were stored at −20°C. Peripheral blood mononuclear cell fractions were stored at −70°C. Samples were thawed immediately before use. All commercial reagents were stored as recommended by the manufacturers. DNA probes were stored at −20°C.

Patients and samples. Three groups of sera were tested. (i) Twelve B19 DNA dot blot-positive sera from diverse geographical locations were collected between 1980 and 1987. (ii) Sera were collected from 127 patients up to 150 days (median 10 days) after the onset of symptoms. These had been submitted to the diagnostic parvovirus laboratory (University College & Middlesex School of Medicine) for B19 serological testing (Table 1). All negative sera collected from patients with a rash illness were tested for rubella IgM by an IgM antibody capture assay (41).

(iii) A set of sera, throat swabs, and PBMNCs collected longitudinally from 10 staff members involved in a nosocomial outbreak of B19 infection in a North London hospital in 1990 (34). Baseline sera, throat swabs, PBMNCs, and other serum samples were collected up to 6 months after the onset of symptoms and submitted to dot blot hybridization and B19 IgM and IgG testing. Negative control PBMNCs were collected from five B19 IgG-seropositive and two B19 IgG-seronegative individuals. A negative control throat swab was collected from an uninfected subject.

The sera were subjected to nested PCR, dot blot hybridization, and B19 IgM and IgG tests.

Serology. The B19 IgM capture radioimmunoassay (MACRIA) and the B19 IgG capture radioimmunoassay were performed as previously described (32). Briefly, polystyrene

TABLE 1. Characterization of sources of 127 serum samples tested by the nested PCR for B19 DNA

B19 diagnosis	No. of samples from patients with:						Total no. of samples
	Rash	Rash and arthralgia	Arthralgia	Pyrexia	No symptoms	Other	
Confirmed[a]	18	24	12	1	3	1	59
Negative	39	10	10	3		6	68

[a] DNA dot blot positive or B19 IgM positive.

beads (Northumbria Biologicals, Cramlington, United Kingdom) coated with anti-human IgM (Tago) or anti-human IgG (Dako, Denmark) were reacted with test serum. B19-viremic plasma and a mouse monoclonal anti-B19 antibody were added. Binding was assessed by using a radioiodinated sheep anti-mouse antibody (Amersham, United Kingdom). Radioactivity bound to the beads was measured in a gamma counter. B19 IgM results were expressed as MACRIA arbitrary units of B19 antibody compared with dilutions of a control positive serum. Five or more MACRIA units was considered to indicate recent parvovirus infection. For the B19 IgG test, sera binding more than twice as much label as the negative control were regarded as B19 IgG positive.

B19 DNA extraction. (i) Heat denaturation of serum for nested PCR. A modification of a method of Koch and Adler (20) was used to extract DNA for PCR amplification. One microliter of serum was added to the PCR reaction mix and heated at 95°C for 6 minutes before thermal cycling.

(ii) Detergent extraction of DNA from serum for nested PCR. The method used for detergent extraction of DNA from serum was adapted from that of Higuchi (18). A 50-μl sample of serum was added to a 100-μl lysis solution containing 50 mM potassium chloride, 10 mM Tris-HCl (pH 8.3), 2.5 mM magnesium chloride, 0.1 mg of gelatin per ml, 0.45% Nonidet P-40 (Sigma), 0.45% Tween 20 (Sigma), and 0.006 mg of proteinase K (no. 4914; Sigma). The preparation 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 centrifuged at 12,000 × *g* for 15 min, and the supernatant was used in the PCR assay.

(iii) Throat swabs. The throat swabs, collected in viral transport medium (VTM), were briefly vortexed to remove the DNA from the swab into the VTM. DNA was extracted as previously described (19). The concentration of DNA was determined by spectrophotometry. Three different quantities of DNA (0.05, 0.5, and 1 μg) were amplified by the PCR because of the potential presence of PCR inhibitors in saliva (15a). PCR products were alkali transferred and hybridized with pVTM-1 (4). Dot blot hybridization was also performed with 1 μg of DNA.

(iv) In vitro isolation of PBMNCs and extraction of PBMNC DNA. PBMNCs were isolated from 15 ml of heparinized (preservative-free) whole blood by the Ficoll-Paque (Pharmacia, Sweden) technique. The cells were resuspended in 1 ml of RPMI 1640 solution (Gibco) containing glutamine, antibiotics with 10% dimethyl sulfoxide, and 30% fetal calf serum and stored at −70°C.

Cryopreserved PBMNCs (ca. 0.33 ml) were thawed, washed with phosphate-buffered saline, and pelleted by centrifugation at 12,000 × *g* for 1 min. Then 100 μl of lysis solution (described above for serum extraction) was added to the cell deposit; the preparation was mixed and incubated at 65°C for 1 h. The buffer was inactivated by heating to 95°C for 10 min, and 5 μl of this reaction mix was then added to the PCR assay. PCR products were alkali transferred and hybridized with pVTM-1.

The PBMNC fractions were also subjected to dot blot hybridization with pVTM-1.

(v) Titration of throat swab and PBMNC DNA. The quantities of B19 DNA in throat swabs and PBMNCs were determined by PCR titration. DNA was extracted from these samples and diluted with water in $\log_{10}$ dilutions, and the nested PCR was performed.

DNA transfer and hybridization. Alkali transfer of DNA from 2% agarose gels to Zeta probe membranes (Bio-Rad) was performed as previously described (38). DNA blotting of sera or extracted DNA and hybridization were performed as previously described (4) with probe pVTM-1. The 700-bp parvovirus DNA insert (corresponding to nucleotides 3141 through 3856) was excised with restriction enzyme *Pst*I and subcloned into *Pst*I-digested, phosphatase-treated vector pAT153. The probe was labelled with ^{32}P by random priming (14, 15).

Nested PCR method. Standard precautions (25) were taken to ensure that the PCR assay remained free from DNA contamination. In addition, all samples referred to this department for B19 testing were aliquoted in a class 3 cabinet for potential PCR testing before any diagnostic testing. Contamination was not seen in any of the PCR assays described here.

Oligonucleotides were prepared on an Applied Biosystems 381A DNA synthesizer according to the manufacturer's instructions.

A 5-μl sample of extracted serum was inoculated into a 50-μl PCR reaction volume 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 Cetus), 200 μmol of each deoxynucleoside triphosphate, and 300 ng of each of the oligonucleotide primers Parpat-1 and Parpat-3AS (see below). Tubes were overlaid with 100 μl of mineral oil (Sigma). After first-round PCR amplification, 1 μl of first-round PCR product was transferred into a second 50-μl PCR reaction mix. The second-round reaction mix contained the same constituents as the initial mix, but the first-round primers were substituted by 300 ng of B19-1 plus B19-2 (39).

The oligonucleotide primer sequences are as follows: Parpat-1, CTT TAG GTA TAG CCA ACT GG (nucleotides 2912 through 2931); Parpat-3AS, ACA CTG AGT TTA CTA GTG GG (nucleotides 4016 through 3997); B19-1, CAA AAG CAT GTG GAG TGA GG (nucleotides 3187 through 3206); B19-2, CCT TAT AAT GGT GCT CTG GG (nucleotides 3290 through 3271). The first round of amplification with primers Parpat-1 and -3AS yielded a 1,112-bp product. Second-round PCR was performed with previously published sequences (39), designated B19-1 and B19-2, giving a 104-bp product. Thirty-five cycles of both first- and second-round amplification were performed at 95°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min in a PHC-1 automated thermal cycler (Techne, Cambridge, United Kingdom). Control sera containing 10 and 1 copies of B19 DNA were included in each PCR assay.

Samples (15 μl) of the first- and second-round PCR products were then analyzed by electrophoresis on a 2% agarose gel. Bands were visualized by ethidium bromide staining.

Modified PCR with a 45°C annealing temperature. The stringency of the PCR conditions was reduced to determine whether B19 DNA with base pair mismatches to the primer sequences could be detected. The annealing temperature was reduced from 55°C to 45°C, so that the samples were cycled at 95°C for 1 min, 45°C for 1.5 min, and 72°C for 1 min for both rounds of amplification.

Combined biotin in situ hybridization and immunohistochemistry. To determine whether the B19 DNA present in the PBMNCs could be localized within a subpopulation of cells, thawed aliquots of PBMNCs, collected at 2 months from four subjects (one male and three females) and containing between 3.5×10^5 and 6.2×10^6 cells per ml, were analyzed. The PBMNCs were washed in phosphate-buffered saline and cytocentrifuged onto glass slides coated with 3'-aminotriethoxysilane. After fixation in 1:1 acetone-methanol for 3 min, samples were immunostained for either glycophorin A (JC159, Dako), an erythroid marker, or leukocyte common antigen (CD45; Dako) by the alkaline phosphatase-monoclonal anti-alkaline phosphatase technique with a Fast Red substrate (11).

Sequential in situ hybridization for B19 was then performed as previously described (36) with probe pYT104 (gift from P. Tattersall, Yale University, New Haven, Conn.) nick translated with biotin-II-dUTP. Controls included the use of biotinylated probe pHY2.1 (which detects 2,000 copies of a 2.1-kb repeat sequence present on the Y chromosome [10] as well as 200 autosomal homologous sequences) and biotinylated plasmid pBR322 (negative control). Cytocentrifuge preparations of fetal erythroid precursors (obtained from aborted fetal liver) infected with B19 in vitro were tested as positive controls in parallel.

RESULTS

Determination of the sensitivity of nested PCR. The sensitivity of the nested PCR was determined in two ways. First, the titer of a viremic plasma sample known to contain 7×10^{10} B19 particles per ml, as determined by electron microscopy (kindly performed by C. Potter, Oxford), was determined in B19-negative serum and assayed. The nested PCR detected a single copy of B19 DNA per microliter in this plasma sample.

The second method used was to determine the amount of B19 DNA in another viremic plasma sample, designated SM, by dot blot hybridization titration and then to compare this result with a titration endpoint for this sample in the nested PCR (21). Plasma SM contained 10^8 copies of B19 DNA per μl. The first-round PCR detected 10^6 copies of B19 DNA, and the second-round PCR detected 1 copy per μl (as detected by ethidium bromide staining of reaction products in an agarose gel; Fig. 1 and 2). The specificity of this result was confirmed by alkali transfer and hybridization. PCR amplification with only the internal primers B19-1 and B19-2 detected 10^3 B19 DNA copies (data not shown). Therefore, the nested PCR enhanced the sensitivity of B19 DNA detection by a thousandfold compared with that of single-round PCR amplification.

Although the nested PCR assay detected single copies of B19 DNA, the yield of specific PCR product, as determined by the intensity of the 104-bp product after gel electrophoresis and ethidium bromide staining, declined with decreasing copy number of target DNA in the plasma. This suggests that the kinetics of the nested PCR had not reached saturation. When the nested PCR was performed on dilutions of detergent-treated plasma SM, similar quantities of B19-specific PCR products were detected at each dilution, even in the dilution of plasma 1 log unit above the dilution producing no B19-specific product (Fig. 3). This result shows that detergent treatment of samples enables the nested PCR to reach saturation kinetics. Detergent extraction of serum samples was used in all subsequent experiments described herein.

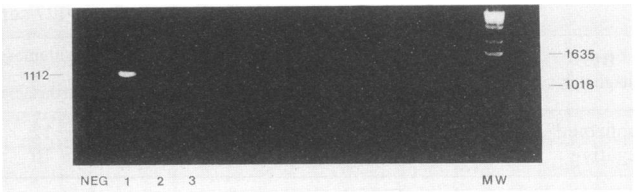

FIG. 1. Titration of B19 viremic plasma in a single-round PCR with primers Parpat-1 and Parpat-3AS. B19 viremic plasma SM was titrated in B19-negative serum and subjected to a single-round PCR with primers Parpat-1 and Parpat-3AS to produce a 1,112-bp product. The plasma was added directly to the PCR reaction mix and heated to 95°C for 6 min, *Taq* polymerase was added, and the reaction was subjected to thermal cycling. PCR products electrophoresed in an ethidium bromide-stained 2% agarose gel are shown. Lanes: NEG, no DNA added; 1, product from undiluted viremic plasma; 2, plasma at a dilution of 10^{-1}; 3, plasma at a dilution of 10^{-2}; MW, molecular size markers (1-kb DNA ladder: BRL).

Sera from diverse geographical locations and archival material. All 12 B19 DNA dot blot-positive serum samples collected from five different countries over 7 years were positive by both first- and second-round PCRs. These results indicate that the primers used in the nested PCR are from conserved regions of the B19 genome.

Sera submitted to the diagnostic laboratory. Sera were considered to have a laboratory-confirmed B19 infection if B19 DNA (detected by DNA dot blot hybridization) or B19 IgM was detectable. Eighty serum samples collected from 58 patients had a laboratory-confirmed B19 diagnosis; 79 samples contained B19 IgM, and the other sample was DNA dot blot positive. An additional B19 IgM-negative convalescent-phase serum sample, collected from a patient previously

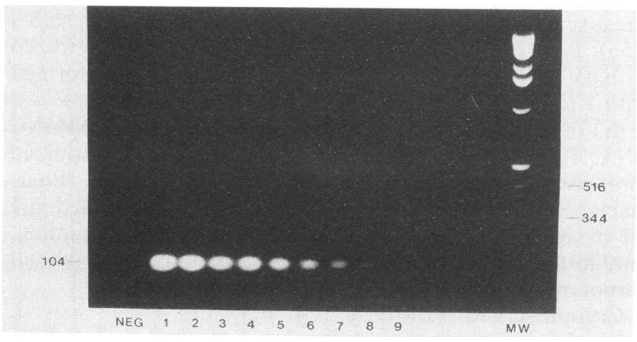

FIG. 2. Titration of B19 viremic plasma in the nested PCR. After first-round amplification, 1 μl of PCR products was subjected to a nested PCR with primers B19-1 and B19-2 to produce a 104-bp product. The PCR products after second-round amplification in an ethidium bromide-stained 2% agarose gel are shown. Lanes: NEG, no DNA added; 1 through 9, products of the titration of viremic plasma SM from 10^{-1} through 10^{-9}, respectively; MW, molecular size markers (1-kb DNA ladder).

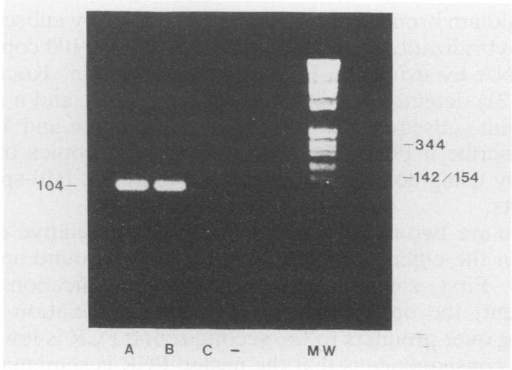

FIG. 3. Nested PCR of a titration of B19 DNA extracted from viremic plasma by a detergent method. DNA was extracted from B19-viremic plasma SM, and the titer was determined in B19-negative serum. Nested PCR products electrophoresed in an ethidium bromide-stained 2% agarose gel are shown. Lanes: A, B19-viremic plasma containing 10 copies of B19 DNA; B, B19-viremic plasma containing 1 copy of B19 DNA; C, dilution of B19-viremic plasma containing 0 copies of B19 DNA; −, B19-negative serum; MW, molecular size markers (1-kb DNA ladder).

shown to be IgM positive, was also included in this diagnostic group. A further 68 serum samples collected from 68 subjects did not contain markers of recent B19 infection.

Seventy percent of the sera with a laboratory-confirmed diagnosis of B19 infection contained B19 DNA; 16% of these exhibited a B19-specific product after both first- and second-round PCRs, whereas the other 84% contained detectable B19 DNA only after the nested PCR. B19 DNA was detected after both first- and second-round PCR amplification in the DNA dot blot positive sample. B19 DNA was not detected in any of the sera that did not fulfill the criteria of a laboratory-confirmed B19 infection (negative controls). Forty-nine of these serum samples were collected from patients with a rash illness, suggesting that it is unlikely that the B19 IgM test fails to detect cases of B19-associated rash illness. None of these samples contained rubella IgM.

Modified PCR with a 45°C annealing temperature. Sera collected from four patients with a recent (≤21 days) onset of symptoms were reassayed by the reduced-stringency nested PCR. Nonspecific DNA amplification occurred, manifesting as multiple ethidium bromide-stained bands in the agarose gel after electrophoresis. Alkali transfer and hybridization with pVTM-1 confirmed that only one of the sample products contained B19-specific DNA. This sample is included in the subsequent analysis of B19 PCR-positive and -negative sera (see below). Multiple banding with a 45°C annealing temperature limits the usefulness of this reduced-stringency PCR. No further testing was performed with this modified assay.

Analysis of the PCR patterns obtained with sera from patients with a laboratory-confirmed B19 diagnosis. Three patterns of PCR results (first- and second-round positive; first-round negative and second-round positive; and first- and second-round negative) were obtained from the 81 serum samples from patients with a laboratory-confirmed diagnosis of B19 infection. The PCR patterns were analyzed in terms of the B19 IgM and IgG contents of the sera (Fig. 4 and 5). A statistically significant variation by Fisher's F test (2) for B19 IgM ($P < 0.005$) and IgG ($P < 0.05$) values among the three PCR groups was detectable. B19 IgM values were lower in the PCR-negative group than in the PCR-positive

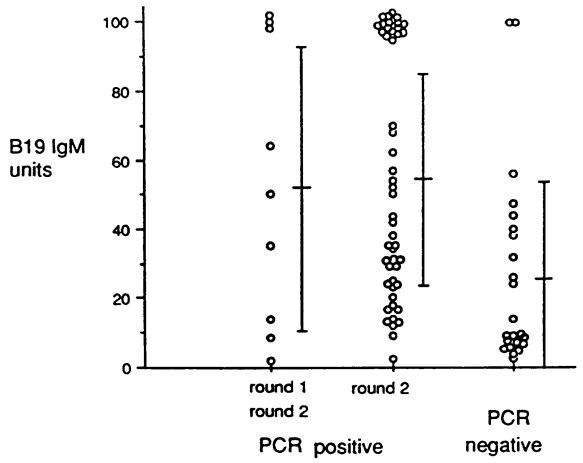

FIG. 4. B19 IgM values of 81 B19 PCR-positive and -negative sera from patients with a laboratory-confirmed diagnosis of B19 infection (F test, $P < 0.005$).

groups. B19 IgG values were lowest in the first- and second-round positive group, higher in the first-round negative, second-round positive group, and highest in the PCR-negative group.

B19 DNA decay in serum was also associated with the interval between the onset of symptoms and collection of serum. This was examined in 33 patients from whom only a single serum sample was collected. The interval was longest in those who were PCR negative (mean 39 days) and much shorter in those who were PCR second-round positive (mean 10 days) or PCR first- and second-round positive (mean 3 days). This trend was statistically significant ($P < 0.005$).

Longitudinal collection of sera, throat swabs, and PBMNCs. Only 4 of 10 subjects attended through the end of the study. None of the samples contained detectable B19 DNA by dot blot hybridization. B19 IgM was detectable in serum samples from all subjects at 1 to 2 weeks but undetectable by 4 months (Table 2). B19 DNA was detectable by the PCR in all

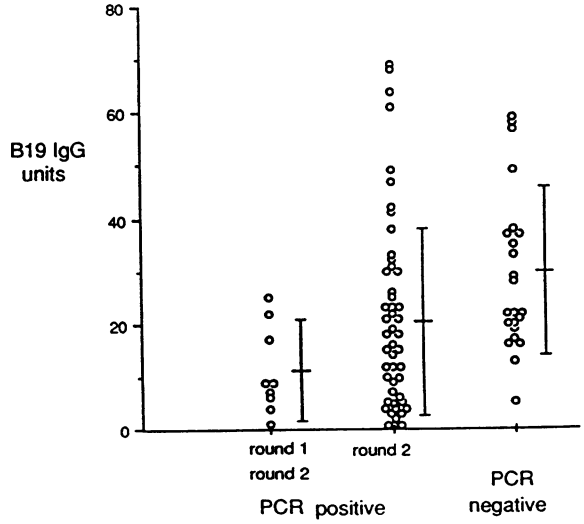

FIG. 5. B19 IgG values of 81 B19 PCR-positive and -negative sera from patients with a laboratory-confirmed diagnosis of B19 infection (F test, $P < 0.05$).

TABLE 2. Detection of B19 DNA in serum, PBMNCs, throat swabs by the PCR and serum B19 IgM by MACRIA collected from subjects during a nosocomial B19 outbreak

Interval between onset of symptoms and sample collection	No. positive (no. tested) in PCR			No. positive (no. tested) in MACRIA
	Serum	PBMNCs	Throat swabs	
1–2 wk	10 (10)	NT[a]	4 (4)	10 (10)
2 mo	1 (8)	4 (8)	3 (7)	2 (7)
4 mo	1 (5)	3 (4)	1[b] (4)	0 (5)
6 mo	0 (1)	3 (4)	0 (1[b])	NT

[a] NT, not tested.
[b] Samples from the same patient.

of the sera tested 1 to 2 weeks after the onset of symptoms. It was detectable in two serum samples after the first and second rounds of amplification, whereas in the other eight serum samples B19 DNA was detected only after both rounds of amplification. At subsequent time points, when B19 DNA was detectable in serum, it was only detected after both rounds of amplification. In general, B19 DNA was only present in sera containing B19-specific IgM; however, at 4 months, B19 DNA was detected in one serum sample that contained only 1 MACRIA unit of IgM. Too few serum samples were collected to draw firm conclusions about the temporal relationship between B19 DNA and IgM detection.

B19 DNA was detectable from all throat swabs collected at 1 week after two rounds of PCR amplification and was undetectable in all subjects by 6 months (Table 2). Two of 16 throat swabs were inhibitory to the PCR, so that B19 DNA was only detectable when diluted to 0.05 μg. One throat swab sample contained B19 DNA that was detectable only after alkali transfer and hybridization. B19 DNA was also detectable in the PBMNCs after two rounds of amplification and remained present in three of four subjects to the end of the study. Alkali transfer and hybridization with pVTM-1 confirmed the specificity of the PCR reaction products observed after agarose gel electrophoresis. B19 DNA was not detected in any of the PBMNC or throat swab controls.

B19 DNA copy numbers in throat swabs and PBMNCs were determined by PCR titration. The mean copy numbers of B19 DNA detected in the PBMNC containing B19 DNA were 220 per ml of whole blood at 2 months and 4 per ml at 4 and 6 months. A mean of 50 copies of B19 DNA per ml was detected in the throat swabs at 1 to 2 weeks. This declined to a mean of 1 copy per ml by 2 months in throat swabs still found to contain B19 DNA.

Characterization of the B19 DNA-containing cell type in PBMNCs by immunolabelling and in situ hybridization. Although the in situ hybridization technique was sufficiently sensitive to readily detect signal from the 200 autosomal copies of Y repeat sequence (data not shown), no specific B19 hybridization signal was detected in any of the PBMNC preparations examined. Immunolabelling demonstrated that although the vast majority of the cells in the PBMNC fraction were of leukocyte lineage, there were occasional scattered nucleated erythroid-lineage cells in which glycophorin A was labeled (data not shown).

DISCUSSION

The sensitivity of the nested PCR technique described here compares favorably with previously published PCR methods for B19 DNA detection. Salimans et al. (39) detected 10^3 to 10^4 copies of B19 DNA by gel electrophoresis and ethidium bromide staining and 100 copies by subsequent DNA hybridization. Clewley (8) detected 10 to 100 copies of B19 DNA by using the PCR and hybridization. Koch and Adler (21) detected 10^3 DNA copies with PCR and a single copy with subsequent hybridization. Frickhofen and Young (16) describe a PCR assay that detected 30 copies of B19 DNA by using dot blot hybridization to detect B19-specific products.

There are two direct consequences of the relative differences in the efficiencies of first- and second-round amplifications. First, since the first round of amplification is so inefficient, the opportunity for DNA contamination when carrying over products to the second round PCR is low. The second consequence is that the nested PCR is semiquantitative, distinguishing large "quantities" of B19 DNA from "small" quantities depending on whether the reaction product is detectable after first or second round PCR.

Initially, the nested PCR for the detection of B19 DNA was evaluated in plasma by adding serum directly by a modification of the method of Koch and Adler (21). However, the nested PCR assay failed to achieve saturation kinetics, so that a modification of a method of whole-blood DNA extraction (18) was developed. The method was quick (1.5 h of preparation time), simple to perform, and did not require the multiple decanting steps of phenol-chloroform extractions. This minimized the potential for cross-contamination of sample DNA during preparation. With this method, the nested PCR achieved saturation kinetics.

Nucleotide sequence differences in different viral strains have been implied from restriction enzyme digest studies (27, 29, 42). The nested PCR method was shown to be capable of detecting B19 DNA from widely varied geographic locations and archival sources. This suggests that the primers anneal to a relatively conserved region of the genome. However, one of four B19 IgM-containing serum samples that tested negative for B19 DNA with the nested PCR and a 55°C annealing temperature was shown to contain B19 DNA when the annealing temperature was reduced to 45°C. This result suggests sequence variation in this sample. Unfortunately, nonspecific products were also generated at this annealing temperature, and this makes it impractical to use these reaction conditions for routine diagnostic use.

For the testing of individual sera, the nested PCR assay fails to accurately predict the timing of infection, but it is predictive on a population basis. It detected B19 DNA in 70% of sera with a laboratory-confirmed diagnosis of B19 infection. PCR products were detectable after the first round of amplification in 16% of diagnostic sera (found to contain B19 DNA by PCR) collected up to 11 days after the onset of symptoms. However, there were a number of serum samples collected within 11 days of the onset of symptoms that either only demonstrated the specific PCR product after the nested PCR or were not found to contain B19 DNA. The other 84% of sera contained levels of B19 DNA that were only detectable after both rounds of amplification. The temporal relationship between the presence of the second-round PCR product and the timing of infection was even more variable. The second-round product was detected in sera collected a mean of 10 days from the day of onset of symptoms. However, both a serum sample collected on the day of onset of symptoms and another collected 150 days after the onset of symptoms contained B19 DNA.

The percentage of B19 IgM-containing sera found to contain B19 DNA and the long period during which B19 DNA could be collected were in accord with the findings of Clewley (8). Nevertheless, in contrast to that of Clewley,

this study did detect a statistically significant association between the amount of B19 IgM in serum and the PCR result and an inverse relationship with IgG serological status (Fig. 4 and 5). Clearly, the time since the onset of illness and B19 IgG and IgM values are not independent variables. This study did not determine which (if any) of these parameters was responsible for the B19 DNA decline in serum.

The association between B19 IgM seropositivity and the presence of B19 DNA was further analyzed in the investigation of a B19 outbreak at a local hospital. In only one serum sample could B19 DNA be detected in the absence of B19 IgM during the convalescent phase of infection. The number of sera tested were too small to enable us to draw more than tentative conclusions. Nonetheless, on the basis of observations on the level of B19 IgM and the presence or absence of B19 DNA and the detection of immune complexes in serum at the time of development of the IgM response to B19 infection (3), a unifying testable hypothesis can be proposed. We postulate the following. (i) B19 IgM is complexed with virus in the convalescent phase of the illness; although B19 IgM may be present in the absence of B19 DNA, the reverse is not usually the case. (ii) The presence of B19 DNA is a marker of the antigenic stimulation to sustain the B19 IgM response.

The relationship between the detection of B19 DNA by the nested PCR and the potential infectivity of the material under study remains to be determined. Preliminary unpublished observations on the ability of sequential sera collected from infected volunteers to infect fetal liver cell cultures correlates with B19 dot blot hybridization positivity (26a). Whether this reflects the situation in vivo is not known.

The nested PCR was applied to 49 serum samples collected from patients with an unexplained B19 IgM-negative, rubella IgM-negative rash illness. This was performed to determine whether the MACRIA assay missed cases of B19-related rash illness. None of these sera was found to contain B19 DNA by the PCR.

The hospital outbreak of B19 infection also facilitated a longitudinal study into the persistence of B19 DNA in throat secretions and PBMNCs. B19 DNA was detectable by the nested PCR in all the throat swabs collected within 1 week of the onset of symptoms and was only detectable in one throat swab at 4 months. This contrasts with the results obtained by DNA dot blot hybridization from experimentally infected volunteers (3), in whom B19 DNA was detected from days 7 through 11 postinfection. It was not possible to determine from these studies whether nested PCR or dot blot hybridization positivity is a marker of B19 infectivity.

Although three different starting quantities of DNA had to be assayed by the PCR to overcome the problem of PCR inhibitors in oropharyngeal secretions, this approach to diagnosis could form the basis of a non-serum-based test of acute B19 infection. This would be useful for investigating rash illnesses in children.

B19 DNA was also detectable in the PBMNCs of infected subjects and remained so in three of four individuals to the study endpoint 6 months after the onset of illness. Thus, it appears that, after infection, B19 DNA persists at this site after the disappearance of B19 DNA and IgM from serum. It was not possible to demonstrate by in situ hybridization which cell type contained the B19 DNA. This could be due to a lack of sensitivity of the in situ hybridization if very low copy numbers of B19 DNA were present in many cells or to a limitation in sampling if there were high copy numbers of B19 DNA in a very few cells. A further possibility is that the B19 DNA was present in a form attached to the cell surface, perhaps via a B19 antibody-Fc receptor or complement-Fc receptor interaction and that this complex dissociated during the labelling procedures.

Immunolabelling demonstrated the presence of nucleated erythroid precursors in the PBMNC fraction. The known tropism of B19 virus for erythrocyte precursor cells makes this the most likely cell candidate to contain the B19 DNA detected. Alternatively, the virus may have been phagocytosed by macrophages or neutrophils, with the B19 DNA remaining intact within the cells.

Kurtzman et al. (23) have shown that B19 DNA can be detected by dot blot hybridization in the high-density, granulocyte-enriched fraction of blood of acutely infected individuals. This was detected at a time when viremia was no longer detectable in serum by dot blot hybridization. In addition, this group was able to demonstrate that the B19 DNA was actively replicating, as indicated by the detection of intermediate replicative forms by Southern blot hybridization. Occasional granulocytes were seen in the PBMNC fractions collected in the hospital outbreak study, so these cells cannot be excluded as the potential host for the B19 DNA detected by the PCR. The question of whether the B19 DNA detected by the PCR represents persistent or latent B19 infection remains to be answered.

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