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Standardization of a PCR–ELISA in Serum Samples: Diagnosis of Active Parvovirus B19 Infection

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To standardize a PCR assay for the detection of parvovirus B19 DNA in serum samples three different sample treatments were evaluated on the basis of the efficiency of recovery, reproducibility, convenience of sample handling, and presence of PCR inhibitors. Moreover, the presence of an internal standard competitor as the working reagent at one defined concentration in a competitive PCR-ELISA has been suggested as a valid tool to standardize and validate the assay. The results indicated that serum sample treatment by rapid heating fulfilled the criteria for a routine practice in the diagnostic laboratory. Titration experiments carried out to define the optimal amount of the internal standard competitor to use in PCR-ELISA showed that at 2×10^2 competitor copies, any amplification interferences between target and competitor sequences were avoided. The internal standard competitor in a competitive PCR-ELISA allows the detection of false-negative results due to PCR inhibitors in the samples or large amounts of target DNA. Heating treatment and competitive PCR-ELISA for the detection of parvovirus B19 DNA were applied to the testing of 347 serum samples, which were submitted to the laboratory for B19 investigation. Of the 34 serum samples that were positive for B19 DNA, 15 were from adult patients and 19 from pediatric subjects. B19 infection was associated with haematological disorders, nonimmunological foetal hydrops, atypical rash, arthropathies, hepatic dysfunction, nonspecific symptoms, and congenital infections. ***J. Med. Virol. 59:239–244, 1999.***

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INTRODUCTION

The polymerase chain reaction (PCR) assay is a powerful diagnostic tool to detect minimal amounts of specific nucleic acid sequences in biological samples.

The first diagnostic applications in infectious diseases concern mainly the identification of causative agents [Podzorski and Persing, 1995]. In recent years, however, PCR assays have also been developed to quantify genome sequences of etiological agents in the course of persistent infections and to monitor the efficacy of therapeutic treatments [Clementi et al., 1995].

Since its discovery in healthy blood donors, B19 parvovirus has been associated with an increasing range of clinical manifestations [Brown, 1997].

In immunologically normal hosts, both children and adults, B19 can cause acute, generally self-limiting, diseases. B19 infection in pregnancy can be associated with nonimmunological fetal hydrops or fetal death. The infection leads to a viraemia that can be present at high titre for about 1 week, then the onset of a specific immune response controls the infection [Anderson et al., 1985]. Sometimes, however, B19 viraemia can still be detected at very low titres for some months after acute infection [Musiani et al., 1995b].

In immunocompromised hosts, B19 can persist over several months, and sometimes years, due to the impairment of the neutralising antibody response. Persistent or recurrent B19 infections are characterised by viraemia, ranging from very low to medium-high titres, and can be associated with chronic clinical manifestations or with transient clinical syndromes, generally related to the recrudescence of viral replication [Musiani et al., 1995a]. Since B19 parvovirus does not grow efficiently in continuous cell cultures, several techniques have been developed to detect B19 DNA sequences in clinical samples and several homemade PCR assays are used because of their high sensitivity in diagnostic laboratories [Salimans et al., 1989; Koch and Adler, 1990; Durigon et al., 1993; Musiani et al.,

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1993; Patou et al., 1993; Zerbini et al., 1995; Brown, 1997].

The need to monitor B19 viraemia until clearance and to evaluate the efficacy of immunoglobulin treatment in immunocompromised hosts prompted us to develop a quantitative PCR assay, based on the coamplification of target and internal standard competitor sequences. The internal standard competitor was constructed by recombinant PCR and cloned in a plasmid vector. The internal standard differed from the original genome sequences in a 21 bp mutagenised fragment internal to the region amplified by the same set of primers [Gallinella et al., 1997].

Since different treatments of serum samples for PCR assays have been described and several PCR protocols are used to detect B19 DNA in the diagnostic laboratory, we applied our quantitative PCR assay to evaluate various sample processings and their effects on the sensitivity of PCR in serum samples. Several reasons may lead to a failure of a PCR assay, such as loss or degradation of target DNA and/or copurification of PCR inhibitors present in clinical samples. The second purpose of the study was to evaluate the use of an internal standard competitor, at one defined concentration, as the working reagent to validate and standardize the PCR assay for B19 DNA detection in the routine diagnostic laboratory. This standardization procedure allowed us to verify the presence of inhibitors, which can only be detected by an internal amplification control, and may suit a routine diagnostic laboratory since quantitation can be achieved with a single PCR per sample by relating target signal to the signal of known amount of the internal standard competitor. The standardized PCR assay for B19 DNA detection was used in the routine laboratory to examine 347 serum samples submitted to our laboratory for B19 investigation.

MATERIALS AND METHODS

Samples and Experimental Design

To evaluate different serum sample treatments to provide target DNA efficient for the amplification reaction and the DNA yield, both cloned full-length B19 DNA and purified B19 virus were diluted in 6 different B19 negative reference serum samples at the final defined concentration of 1×10^4 genome copies per microliter [Gallinella et al., 1993], and each sample was processed with three different protocols, as described below. The reference negative serum samples were obtained from persons without serological and clinical evidence of B19 infection and proved negative for B19 DNA by standard nested PCR [Musiani et al., 1993]. The B19 DNA recovered from serum samples after the treatments was quantified by a competitive PCR-ELISA assay [Gallinella et al., 1997].

To evaluate one defined concentration of the internal standard competitor to use as the working reagent in the PCR-ELISA, the degree of amplification interference between target and competitor sequences was analysed by titration experiments. Cloned full-length B19 DNA genome [Gallinella et al., 1993] was used as the original target sequence. As internal standard competitor, a B19 DNA fragment was used that had been mutagenised by recombinant PCR in a 21-bp sequence (nts 1733–1753) and cloned in plasmid pUC19 as described previously [Gallinella et al., 1997]. Serial tenfold dilutions of the target sequence, carried out in reference negative serum samples, were amplified in the absence or presence of a constant amount of the internal standard competitor. The 347 serum samples submitted to the laboratory for B19 investigation were treated with the most efficient extraction method evaluated and then analysed by PCR-ELISA, using the concentration of the internal standard competitor defined as the optimal working reagent. In PCR-ELISA blank samples consisted of 50 μg ml^{-1} of herring sperm DNA in TE buffer (10 mM TrisCl pH 7.5, 1 mM EDTA). As positive controls, reference serum samples positive for B19 DNA at the same concentration of the internal standard competitor were used.

Serum Sample Preparation

Serum sample treatment was carried out following three different protocols.

Heat treatment. A volume of 50 μl of serum sample was heated at 70°C for 2 minutes and 5 μl was then directly used in the amplification reaction.

Lysis treatment. A volume of 50 μl of serum sample was mixed with 5 μl of 10X K buffer 0.5% Tween 20 in 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl pH 9.0), then 5 μl of proteinase K was added at a final concentration of 100 μg/ml. The sample was incubated at 50°C for 40 minutes, then at 95°C for 10 minutes to inactivate proteinase K. The sample was then centrifuged, and 6 μl of cleared supernatant was used in the amplification reaction.

Commercial kit (Qiagen). Briefly, using the QIAamp Tissue kit, a volume of 50 μl of serum sample was treated with proteinase K and then applied to the QIAamp spin column following instructions from the manufacturer (Qiagen, GmbH, Germany). The DNA was eluted from the column in a volume of 50 μl, and 5 μl was used in the amplification reaction.

B19 DNA Titration by Competitive PCR-ELISA Assay

A B19 DNA sequence of 184 bp (nts 1652–1835), located in the genomic region coding for NS protein, was chosen for competitive PCR amplification [Gallinella et al., 1997]. To quantify B19 DNA by competitive PCR, titration curves were carried out by coamplification of a constant amount of target DNA with increasing amounts of competitor sequences (from 1×10^0 to 1×10^6 competitor copies). To evaluate the degree of amplification interference between target and competitor sequences titration experiments were carried out by coamplification of increasing amounts of the target sequence (from 1×10^0 to 1×10^3 genome copies) with or without a constant amount of the competitor sequence (2×10^2 copies).

Both target and competitor were labelled during amplification reaction by incorporation of digoxigenin (dig)-labelled dUTP. The oligonucleotides used in the amplification reaction were 5′-CTGGAGTACCTGTGGTTA-3′ (nts 1652–1669) and antisense 5′-CACCATGTAAGCCACTGT-3′ (nts 1835–1818).

The reaction was carried out as follows: 5 μl of both target and competitor B19 DNA, at the different concentrations tested, were added to a reaction mixture of 50 μl final volume containing 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM TrisCl pH 9.0, 0.1 mM dATP, dGTP, dCTP, 0.095 mM dTTP, 0.005 mM Dig-UTP, 0.1 μM of each primer and 2 units of Taq DNA polymerase (Boehringer GmbH). After an initial denaturation step at 95°C for 5 minutes, 40 cycles were carried out under the following conditions: 95°C for 30 seconds, 48°C for 30 seconds and 72°C for 1 minute, followed by an extension step at 72°C for 5 minutes.

Two biotin-labelled probes, specific for the B19 original sequence and the mutagenised sequence of the competitor, respectively, were used in two different hybridization reactions to detect the amplified products. The hybrids were then captured on streptavidin-coated microtitre plate. For each sample the following reactions were carried out in duplicate, using, respectively, probe T (5′-AAGCCTTAAAAGAGCGAATGG-3′), specific for the original B19 sequence and probe C (5′-GGATTCCGAAGAGATAGGCAA-3′), specific for the mutagenised sequence of the competitor. Both probes T and C were biotin-labelled at the 5′ end.

A volume of 10 μl of amplification reaction was added to 10 μl of denaturating solution (100 mM NaOH, 0.1% Tween-20) and incubated at 25°C for 10 minutes. Then, 200 μl of hybridization solution (300 mM NaCl, 100 mM TrisCl pH 6.5, 10 mM EDTA, 0.1% Tween-20) containing either probe T or probe C (10 pmol/ml) were added and hybridization was carried out at 50°C for 10 minutes. A volume of 200 μl of hybridization reaction was transferred to a streptavidin-coated microtitre plate well and incubated at 25°C for 30 minutes, then plates were washed 5 times with TBST (150 mM NaCl, 100 mM TrisCl pH 7.5, 0.1% Tween-20). To detect captured Dig-labelled hybrids a volume of 200 μl of anti-digoxigenin peroxidase (POD) conjugated solution (150 mU/ml in TBST) (Boehringer GmbH) was added and incubated at 25°C for 30 minutes, then plates were washed 5 times with TBST. Finally, a volume of 200 μl of ABTS substrate (Boehringer GmbH) solution was added to each well, and the reaction was allowed to proceed for about 30 minutes before the optical density was measured at 405 nm by spectrophotometer.

PCR-ELISA

A total of 347 serum samples were analysed by PCR-ELISA, using an internal standard competitor as working reagent. PCR-ELISA was carried out using the same reagents and the same experimental conditions described for the competitive PCR-ELISA, except that pretreated serum samples were coamplified with the constant amount of 2×10^2 genome copies of the internal standard competitor, which proved to be the optimal concentration with no interference with target sequences in the amplification reaction. For each run, blank and positive control samples were included.

Data Analysis

To evaluate the efficiency of different serum sample processing methods, quantitative determinations of B19 DNA were achieved by competitive PCR-ELISA. For each amount of internal standard competitor, the difference in absorbance values between competitor and target amplicons was obtained. These values were plotted against competitor genome copy number. Linear interpolation was carried out to obtain a value for which the difference in absorbance was 0. The interpolated value at the point of equivalence is the best estimate of target genome copy number present in the test sample. All data were analysed using Quattropro software (Borland International, California) [Gallinella et al., 1997]. Values were obtained for the six different samples, containing either plasmid B19 DNA or purified B19 virus, for each extraction method tested. Mean and standard deviation (SD) values were calculated. The coefficients of variation (CV) were calculated by the ratio between SD and mean values (CV = SD/mean). Reproducibility determinations, within-assay and interassay, were evaluated by testing the same sample three times in three different experiments.

In titration experiments OD values obtained for B19 target DNA, either alone or in presence of a constant amount of the competitor sequence, were plotted against the target genome copy number. In serum sample analysis, the OD values of target (ODt) and competitor (ODc) sequences were determined. Cutoff (co) was defined as ODt + ODc of blank sample, with the two single values not differing more than 20% from each other. For each sample ODt/co and ODc/co ratios were determined.

RESULTS

A critical step in PCR assay on serum samples is the DNA extraction methods [Saldanha et al., 1997]. The competitive PCR assay was used to quantify amplified products after three different treatments on serum samples.

To evaluate the efficiency of the treatments, six different reference serum samples negative for B19 DNA were spiked with both cloned, full-length B19 DNA and purified B19 virus particles at a final concentration of 1×10^4 genome copies per microliter. All the samples were extracted with: i) a heat treatment, ii) a lysis treatment with Tween 20/proteinase K, and iii) QIAamp tissue kit for extraction of sample. The competitive PCR assay showed that inhibitors of PCR assay were not present in any serum sample. The mean values of the different samples obtained for each treatment and the respective SD and CV values are reported in Table I.

Higher mean titration values, as well as higher CV values, were obtained in serum samples containing

TABLE I. Efficiency of Three Different Treatments (Heat, Lysis, and Commercial Kit) on Serum Samples Containing Either B19 DNA or B19 Virus Particles as Determined by Quantitative PCR

Treatments	Serum + B19 DNA			Serum + B19 virus particles		
	Mean[a] gen.cop./μl	STD[a]	CV[a]	Mean[a] gen.cop./μl	STD[a]	CV[a]
Heat	6.17×10^3	1.19×10^3	0.19	3.50×10^3	4.11×10^2	0.11
Lysis	4.30×10^3	1.26×10^3	0.29	3.63×10^3	4.50×10^2	0.12
Com. kit	4.66×10^3	4.75×10^3	1.01	1.05×10^3	6.38×10^2	0.60

[a]The mean values and the respective standard deviations (SD) and coefficients of variation (CV) of six different samples were determined for each treatment.

B19 DNA than in samples containing purified B19 virus. Both of the two different types of samples showed a better performance with heat, than with lysis and commercial extraction kit treatments respectively, with respect to mean titration values and CV. Reproducibility determinations by CV within-assay and interassays confirmed the data (data not shown). Therefore sample treatment by heating was used for the subsequent analysis of the 347 serum samples.

To define the concentration of the internal standard competitor to use as the working reagent in PCR-ELISA, titration experiments were performed. Since it was demonstrated previously [Gallinella et al., 1997] that in the competitive PCR assay there is a linear relationship between input genome copies and amplified products at a threshold value of 1×10^1–1×10^2 input genome copies and over a 3 log interval (10^2–10^5), the internal standard competitor was tested at the concentration of 2×10^2 genome copies. Titration experiments were carried out amplifying increasing amounts of target B19 DNA, from 1×10^0 to 1×10^3 genome copies, either in presence of 2×10^2 genome copies of the internal standard competitor, or alone (Fig.1). No amplification inteference between target and competitor sequences was observed, since no differences in titration curves of target sequences were shown either in absence or in presence of 2×10^2 genome copies of the internal standard competitor. Therefore 2×10^2 genome copies of the internal standard competitor were routinely used as the working reagent in PCR-ELISA for the analysis of the 347 serum samples.

A total of 347 serum samples submitted to the laboratory for specific B19 investigations were heat-treated and tested by PCR-ELISA method as described above. Each sample was characterised by two values: OD of target DNA/cut off and OD of competitor DNA/cutoff. Data were collected and grouped as follows: ODt/co < 1 and ODc/co < 1, no amplification occurred, sample was considered undetermined; ODt/co < 0.9 and ODc/co > 1, amplification occurred, sample was considered negative; ODt/co > 1.1 and ODc/co > 1, amplification occurred, sample was considered positive. Serum sample whose ODc/co was > 1 and ODt/co between 0.9 and 1.1 were considered borderline and the analysis was repeated, until unequivocal or concordant results were obtained. Of the 347 serum samples tested 275 were negative, 28 positive, 31 indeterminant (not amplified),

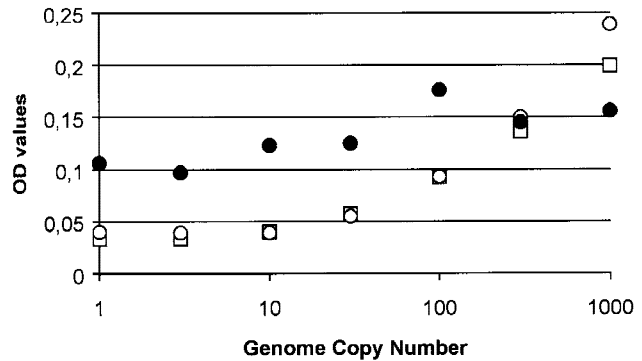

Fig. 1. Titration curves of B19 target DNA in presence or in absence of the internal standard competitor. (□) Titration curve of B19 target DNA (from 1×10^0 to 1×10^3) in absence of the internal standard competitor. (○) Titration curve of B19 target DNA (from 1×10^0 to 1×10^3) in presence of the internal standard competitor (2×10^2 genome copies). (●) Values of the internal standard competitor (2×10^2 genome copies) measured for each B19 target DNA dilutions (from 1×10^0 to 1×10^3).

and 13 borderline. When the 13 borderline samples were retested, 9 were found negative and 4 positive.

Since polymerase inhibitors could be present in the indeterminant serum samples, 14 were retested by extraction with the lysis treatment, which had resulted in a lower CV than the commercial extraction kit, and the PCR-ELISA was repeated. Lysis treatment proved efficient in 7 of the 14 serum samples treated, in which amplification occurred. The other 7 serum samples still remained undetermined. Of the 7 amplified serum samples, 2 were positive for B19 DNA and 5 negative. Of the 34 serum samples which were positive for B19 DNA, 15 were from 13 adult individuals and 19 from 17 paediatric subjects (Table II). The 13 adults had a clinical diagnosis of 2 cases of haematological disorders, 9 cases of nonimmunological fetal hydrops, 1 case of atypical rash, and the remaining patient had nonspecific symptoms which could have suggested a viral infection. Of the 17 paediatric subjects, 5 cases had a clinical diagnosis of haematological disorders, 3 of atypical rash, 4 of arthropathies/possible juvenile rheumatoid arthritis, 1 of hepatic dysfunction, and 2 of congenital infections and in the 2 remaining cases nonspecific symptoms were indicated.

Ig M and IgG antibodies against B19 VP2 structural protein were determined by ELISA (IDEIA, DAKO,

TABLE II. Serum Samples Proved Positive for B19 DNA by Standardized PCR-ELISA

Subjects		Clinical diagnosis	No. of serum samples positive for B19 DNA
Adult	13	2 haematologic disorders 9 fetal hydrops 1 atypical rash 1 aspecific symptom	15
Paediatric	17	5 haematologic disorders 3 atypical rashes 4 arthropathies 1 hepatic dysfunction 2 congenital infections 2 aspecific symptoms	19
Total	28		34

A/S, Glostrup, Denmark) in the 34 serum samples with active B19 infection. B19 infection was defined acute when PCR and/or IgM anti-VP2 proved positive in the absence of IgG anti-VP2. Recent B19 infection was identified by the presence of positive PCR and/or IgM anti-VP2 in association with IgG anti-VP2 and past infection was defined by the presence of IgG anti-VP2 without detectable B19 DNA and IgM anti-VP2 [Venturoli et al., 1998]. Of the 15 serum samples from adults, 4 were negative for IgM and IgG, showing a pattern of acute B19 infection; 11 showed a pattern of recent B19 infection: 4 were positive for IgM and IgG, and 7 were negative for IgM and positive for IgG [Venturoli et al., 1998]. Of the 19 serum samples from paediatric subjects, 7 were negative for IgM and IgG and 1 was positive for IgM and negative for IgG, showing a pattern of acute infection; 3 were positive for IgM and IgG and 8 were negative for IgM and positive for IgG, showing a pattern of recent infection [Venturoli et al., 1998].

DISCUSSION

Standardization and validation are essential features of PCR-based diagnostic methods in routine laboratory practice. Several reasons can lead to failure of amplification such as inadequate target DNA, PCR assay of a low sensitivity, or the presence of PCR inhibitors in clinical samples [Grundy et al., 1996; Haberhausen et al., 1998; Saldanha et al., 1997; Schweiger et al., 1997]. We evaluated several parameters to develop a model of standardization of PCR assay in serum samples for the detection of parvovirus B19 DNA.

Three different methods were used in sample treatments and examined by competitive PCR. Samples consisted of B19 negative reference serum samples with added B19 DNA or with purified B19 virus at a defined concentration to evaluate the ability of extraction methods to provide target DNA efficient for the amplification reaction and to determine the DNA yield. For this purpose, samples were extracted by rapid heating or lysis with Tween 20/proteinase K or QIAamp tissue kit. Serum samples containing B19 DNA showed that all three treatments allowed for target amplification, and therefore preserved DNA molecules from degradation. Heat treatment gave a higher DNA yield for the extraction sample than lysis and commercial kit. Moreover, the coefficient of variation proved that heat treatment gave the most reproducible results. These observations were confirmed with samples obtained by adding purified B19 virus particles. The latter samples gave a DNA yield slightly lower than samples obtained by adding B19 DNA; this could be explained by an incomplete extraction of the sample. Heat treatment proved to be rapid and technically not demanding and therefore suitable for routine practice, also preventing contaminations. Therefore the sample treatment by heating was chosen for the PCR analysis of 347 serum samples and the presence of PCR inhibitors in serum samples was evaluated.

For the PCR assay a sequence located in the nonstructural region of B19 genome was chosen, but other primers could have been used, since the sensitivity of the assay does not appear to depend on the different primers used [Saldanha et al., 1997]. PCR amplified products, identified by hybridisation, were detected by ELISA. The hybridisation reaction increases the sensitivity and the specificity of the assay [Merkelbach et al., 1997], while ELISA detection allows an exact quantification of the signal [Zerbini et al., 1995]. To standardize and validate the PCR-ELISA an internal standard competitor, as the working reagent, was used at one defined concentration in the assay. This concept of standardization offers several advantages over others: it omits any standard curves, one single sample for PCR is prepared and a quantification can be achieved by signal ratios of the internal standard competitor and target sequences by the internal standard concentration [Haberhausen et al., 1998]. The internal standard competitor we used, differs from the original B19 sequence by a 21-bp DNA fragment located inside the amplification region and in the competitive assay is identified by a specific probe. The presence of the internal standard competitor in the PCR-ELISA allows the identification of the presence of inhibitors in the sample or false negative results due to a great amount of target DNA in the sample [Grundy et al., 1996; Merkelbach et al., 1997]. Since the presence of the internal standard competitor could decrease the sensitivity of the assay for competition between target and competitor sequences, titration experiments were carried out and the optimal concentration of the internal standard was defined as 20 × the limit of sensitivity of the assay.

The standardized PCR-ELISA was carried out on 347 serum samples treated by heating. Of the 347 serum samples tested for the presence of B19 DNA, 31 (8.9%) were indeterminant, since the amplification of the internal standard competitor was inhibited. Of these 31 samples, 14 were still available, and PCR-ELISA was repeated after extraction with lysis treatment. In 7 samples the inhibition was removed, while the remaining 7 samples still remained indeterminant. This observation indicates that the lysis treatment was

more effective than heating in removing inhibitors present in serum samples. However, since only 8.9% of serum samples were indeterminant after heating extraction, heating treatment seems suitable for serum samples [Saldanha et al., 1997] in routine practice of diagnostic laboratory on account of its practical advantages, while lysis treatment can be carried out as a second step only on serum samples that are indeterminant. The commercial extraction kit, which gave a low DNA yield and a high coefficient of variation, therefore does not seem suitable for routine extraction from serum samples, but, since commercial matrices are expected to reduce most of the inhibitors [Merkelbach et al., 1997], they can be useful for samples, such as plasma or plasma pools, in which many inhibitors can be present [Saldanha et al., 1997]. Of the 347 serum samples tested by PCR-ELISA, 34 from 13 and 17 adult and pediatric subjects, respectively, were positive for B19 DNA, showing active B19 infection. Serological analysis of the 34 B19 DNA positive serum samples showed that active B19 infection was associated with a pattern of acute infection in 4 and 8 serum samples from adult and pediatric subjects, respectively, and with a pattern of recent B19 infection in 11 serum samples both in adult and pediatric patients.

These observations suggest that the standardization of a PCR assay is essential in the diagnostic laboratory since PCR can be used routinely to monitor active B19 infections in the course of acute and recent infection, and it is the only diagnostic tool valid to follow B19 persistent infection in immunocompromised hosts.

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