RAPID DETECTION OF PARVOVIRUS B19 DNA IN CHILDREN WITH SICKLE CELL ANEMIA BY NESTED POLYMERASE CHAIN REACTION ("NESTED" PCR)

ABSTRACT

A highly sensitive "nested" PCR assay was applied to the rapid detection of parvovirus B19 DNA in 21 patients suffering from chronic hemolytic disease. Parvovirus B19 DNA was detected by this technique in a rare case (8-month-old boy) presenting transient aplastic crisis (TAC). This result was confirmed by the finding of B19 IgM in the acute phase serum sample and a significant rise in titer of specific IgG. False-positive PCR results were not observed in the present study. This is the 1st Brazilian study on the use of "nested" PCR for the rapid and efficient diagnosis of the parvovirus B19 infection.

INTRODUCTION

Human parvovirus B19 has been associated with a wide range of clinical illnesses, such as erythema infectiosum, transient aplastic crisis (TAC), fetal hydrops, chronic neuropathy and vasculitis, some of which result from its propensity to infect and lyse human erythroid precursor cells, leading to cessation of red cell production and a drop in hemoglobin levels. In persons with underlying chronic hemolytic disorders, like sickle cell anemia, B19 infection can lead to an acute, life-threatening anemia, which may require transfusion therapy. Parvovirus B19 is recognized as the primary etiologic agent of transient aplastic crisis (TAC) in patients with chronic hemolytic anemias.

The recent application of the highly sensitive polymerase chain reaction (PCR) for diagnosis and genetic characterization of B19 has expanded our knowledge of its clinical spectrum of illnesses, particularly in persistent infections. Because the uncharacteristic clinical manifestations of parvovirus B19 infection, laboratory diagnosis is necessary in most situations. Current methods to diagnose B19 infections include capture immunoassays (EIA) for antigen or specific IgM and IgG antibodies, "dot-blot" hybridization technique and PCR for the detection of parvovirus B19 DNA present in serum of patients. It was shown that PCR is the most sensitive technique for detecting viral DNA.

Keywords: Human parvovirus B19; Rapid DNA detection; Sickle cell anemia; Transient aplastic crisis; Polymerase chain reaction (nested PCR)
Recently, a second PCR amplification using internal primers ("nested" PCR) has improved assay sensitivity to 1 to 100 genome copies\(^1\). Here we describe the use of this technique, showing that "nested" PCR is highly sensitive and specific for the rapid detection of B19 DNA in children with sickle cell anemia.

**MATERIAL AND METHODS**

**Study group:** Twenty-one patients with sickle cell anemia received medical attention in the outpatient pediatric department at Hospital Santa Casa de Misericórdia of São Paulo City, Brazil, from August 1995 to January 1996. Diagnosis of sickle cell disease was based on a history of chronic hemolytic anemia and confirmed by hemoglobin electrophoresis.

**Samples collection:** Peripheral blood samples of 5 ml were collected in sterile tubes (without EDTA) from each child and referred for laboratory examinations and parvovirus B19 detection. Specific IgM and IgG antibodies to parvovirus B19 and viral DNA were detected in the Laboratories of Clinical and Molecular Virology of the Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo.

**Enzyme immunoassay (ELISA):** Serum samples from all patients were analyzed for specific IgM and IgG antibodies to B19 by an in-house EIA test described by ERDMAN et al (1991)\(^2\) with baculovirus B19 capsid protein as the antigen\(^3\).

**Primers:** location of primers was chosen by looking for regions of homology between the available sequences. The region of highest homology among these sequences was in the NS1 portion of the genome. Oligonucleotides were prepared by the beta-cyanoethyl phosphoramidite method on ABI DNA synthesizers, model 380A or 394, and synthesized with the 5'-dimethoxytrityl group attached to facilitate purification by reverse-phase, high-performance liquid chromatography (HPLC). The following primer sequences (5'-3') were used:

- P6 (CCATTGCTGGTTATAACCACAGGT);
- P2 (AATGAAACCTTCCATTTAAGTGATGAG);
- P5 (CTAAAATGGCTTTGCAGGCTTAC);
- P7 (TGCAGAAGCAGCATCGGTGCA);
- P11 (ATGGTCTCTAACATGCATAGGC);

"Nested" PCR: The serum (20 ul) was incubated at 37\(^\circ\) in 180 ul of sample buffer (10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate and 500ug/mL proteinase K). After the incubation, the sample was extracted sequentially with equal volumes of phenol, phenol-chloroform-isoamyl alcohol, and chloroform-isoamyl alcohol. The extracted DNA (10 ul) was added to the PCR mixture containing 10 mM Tris (pH 8.3); 2.0 mM MgCl\(_2\); 0.01% gelatin; 50 mM KCl; 200 uM each dATP, dCTP, dGTP, dTTP; 0.5 uM (each) oligonucleotide primer, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) in a total volume of 100 ul. Samples were overlaid with 75ul of mineral oil, heated to 95\(^\circ\)C for 3 min, and amplified through 35 cycles on a thermocycler (Perkin-Elmer Cetus) as follows: 94\(^\circ\) for 45 s to denature the DNA template, 55\(^\circ\)C for 60 s to allow annealing of primers, and then 72\(^\circ\)C for 90 s for extension. The 'Hot Start' method was done with Taq polymerase withheld until the reaction temperature had reached 94\(^\circ\)C at the initiation of cycling. The amplified products were examined by 1% agarose gel electrophoresis and ethidium bromide staining. Purified B19 plasmid pY103, kindly provided by Dr. Peter Tattevos, at concentration of 100 pg/ml and destilled water were amplified in parallel and used as positive and negative controls, respectively.

**RESULTS**

As shown in Table 1, 21 cases of sickle cell anemia were examined for the presence of parvovirus B19 DNA and B19 antibody. Only the case presenting transient aplastic crisis gave a positive result for DNA by "nested" PCR (Figure 1) and for B19 IgM antibody by ELISA in the acute phase serum sample. A significant rise in titer of specific IgG antibody was also detected by ELISA in a serum sample pair taken from the infant.
Figure 1: Agarose gel electrophoresis of PCR and nested-PCR amplification products of B19 DNA-positive serum, collected from 8-month-old boy with TAC, stained with ethidium bromide and photographed under UV light. **Lane 1:** molecular weight marker (100 bp DNA Ladder); **Lanes 2, 3:** PCR and nested-PCR of Clinical Samples; **Lanes 4 e 5:** PCR and nested-PCR positive control; **Line 7 and 8:** PCR and nested-PCR negative control.
TABLE 1


<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Cases</th>
<th>Age (years)</th>
<th>ELISA</th>
<th>&quot;nested&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sickle cell anemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ TAC</td>
<td>1</td>
<td>&lt;1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>20</td>
<td>1-12</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
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TAC: Transient aplastic crisis; VOC: recurrent vasocclusive crisis.
* IgG result of 2nd. serum sample taken 30 days later

This positive case, an 8-month-old boy, was admitted to the pediatric ward of Santa Casa de Misericórdia Hospital in August 1995, after presenting with a 4-month history of sickle cell anemia. On admission, his temperature was 39°C, heart rate 128 beats per min and respiratory rate 36 per min. The child was very pale and the spleen and liver were slightly enlarged. Peripheral blood was collected and examined for B19, HIV-I and Hepatitis B diagnosis. No HIV-I antibody or Hepatitis B surface antigen could be detected. Laboratory data of the patient is shown in Table 2. Low hematocryte (11.1%) and reticulocyte (2%) counts, as well as low hemoglobin concentration (3.8 g/dl) and sickle cells were observed, confirming aplastic crisis of sickle cell disease.
TABLE 2

Laboratory data of the 8-month-old boy presenting transient aplastic crisis (TAC).

<p>| | |</p>
<table>
<thead>
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<tbody>
<tr>
<td>Ht (%)</td>
<td>11.1</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>3.8</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>2.0</td>
</tr>
<tr>
<td>Leucocytes (x10^9 /l)</td>
<td>9.4</td>
</tr>
<tr>
<td>Sickle cells</td>
<td>+</td>
</tr>
<tr>
<td>Chest roentgenogram</td>
<td>Slight bilateral bronchial infiltration</td>
</tr>
<tr>
<td>B19 DNA and IgM antibody</td>
<td>positive</td>
</tr>
<tr>
<td>HBsAg</td>
<td>negative</td>
</tr>
<tr>
<td>HIV-1 antibody</td>
<td>negative</td>
</tr>
<tr>
<td>Hemoculture</td>
<td>negative for capsulate organisms</td>
</tr>
</tbody>
</table>

DISCUSSION

We believe this to be the first Brazilian study on the use of the "nested" PCR assay developed for the rapid detection (about 8 hours) of human parvovirus B19 DNA in peripheral blood specimens. To avoid false-positive results by "nested" PCR, we used a second set of primers (P7 and P11), that does not amplify the product from the first set, to ensure that the target is B19 genome. No false-positive PCR results were observed in sera of the 20 patients without TAC. PCR contamination could be circumvent by performing the first 3 steps of the test in different sterile environments (lamina flow devices): specimen preparation, PCR mixture preparation and performance of the test. Gel electrophoresis and ethidium bromide staining was done in a separate room. Amplification inhibitors leading to "false-negative" results were controlled collecting peripheral blood samples without EDTA or heparin, moreover both is the major inhibition source in blood specimens.

Human parvovirus B19 DNA was found only in the serum sample of the child presenting transient aplastic crisis. In earlier studies we have shown that "nested" PCR is highly sensitive, so that B19 DNA can be detected in serum 2 weeks or longer after onset of TAC illness. Here we give further evidence that "nested" PCR is reliable and has markedly improved laboratory diagnosis of human parvovirus B19 infection.

The first laboratory based notification of transient aplastic crisis in children with sickle cell anemia in Brazil was done by CUBEL et al. (1992) in the city of Rio de Janeiro. In 2 out of 3 cases they could not detect B19 DNA by "dot-blot" hybridization, using biotinylated B19/p GEM 1 recombinant plasmide as molecular probe. The investigators explained these negative results by the delay of about 1 week after onset of symptoms before a blood sample could be taken and examined. It was shown by FRIDELL et al. (1992) that the "dot-blot" hybridization technique is less sensitive than
"nested" PCR for the detection of B19 DNA in serum of patients with TAC12, which may explain the two negative results found by CUBEL, et al (1992). In general, the level of detectability of the "dot-blot" hybridization tests ranges from about 103 to 105 genome copies, whereas "nested" PCR ranges from 1 to 100 copies (5).

The 8-month-old boy suffering from TAC in sickle cell anemia is a very rare observation of a B19 infection among infants*(10). As with erythema infectiosum, TAC is most common in school age children and was so far observed only in some infants in one study carried out in Jamaica (1,8). Our observation indicates that parvovirus B19 infection may not be so rare in infancy and that the virus can cause life-threatening illness early in life, which requires urgent transfusion therapy. The present case was treated once with packed red blood cell transfusion and maintained at the ward during 5 days.

REFERENCES