Rapid Screening for B19 Parvovirus DNA in Clinical Specimens with a Digoxigenin-Labeled DNA Hybridization Probe

MARIALUISA ZERBINI,* MONICA MUSIANI, SIMONA VENTUROLI, GIORGIO GALLINELLA, DAVIDE GIBELLINI, GIOVANNA GENTILOMI, AND MICHELE LA PLACA

Institute of Microbiology, University of Bologna, 40138 Bologna, Italy

Received 19 March 1990/Accepted 21 August 1990

A rapid dot blot hybridization assay for the detection of B19 parvovirus DNA in human sera was developed. Small portions of four serum samples were mixed, filtered onto a nylon membrane, and hybridized with a digoxigenin-labeled DNA probe; for each membrane, 380 serum samples could be tested. When a dot was positive by the hybridization assay, the four serum samples dotted together were separately tested to identify the sample positive for B19 DNA. A total of 10,150 serum samples submitted for viral serological and laboratory investigation with no specific requests for B19 testing were analyzed. Nine serum samples were positive for B19 DNA by dot blot hybridization assay, and the results were confirmed by electron microscopy. This method has proven to be reliable, economical in terms of time and costs, and useful for large-scale screening of clinical specimens, both for diagnostic work and for a source of antigen.

Human parvovirus B19 was originally detected in the sera of healthy blood donors by Cossart et al. in 1975 (10). B19 parvovirus infection was later associated with widely differing syndromes, including erythema infectiosum, postinfection arthropathy, and aplastic crises in patients with a variety of hemolytic anemias (5, 6). Infection during pregnancy may result in fetal hydrops (1, 8), and recently, several authors have highlighted the risk of chronic B19 parvovirus infection in patients with an immunodeficiency (14–16). However, the infection also occurs asymptotically or associated with mild febrile illness (21).

The diagnosis of B19 virus infection is based either on serological assays to demonstrate a specific antibody response in the serum of the patient (2) or on viral detection by immunological methods and hybridization assays (3, 9, 11, 19). Serological assays are used for diagnosis more frequently than methods for detecting virus.

The viremic phase is usually transient, and clinical symptoms often develop as viremia wanes. In addition, B19 virus can be grown in vitro only in bone marrow cells (20), and viral isolation and propagation in cell cultures are not applicable to the routine screening of clinical specimens nor to antigen production. The source of viral antigen for serological assays is generally viremic human sera obtained by blood donations.

Several authors have described hybridization assays using DNA and RNA probes for B19 DNA detection (3, 11, 19). We have recently developed a nonradioactive dot blot hybridization assay to detect B19 parvovirus infection with a digoxigenin (Dig)-labeled DNA probe (7).

In this paper, we describe a rapid method for the large-scale screening of serum samples to detect B19-positive specimens both for diagnostic work and as a source of antigen. Specimen preparation was performed by mixing small portions of several serum samples and testing them for the presence of B19 DNA by dot blot hybridization with a Dig-labeled probe. The hybridization assay was applied to 10,150 serum samples, from outpatients and hospitalized patients, submitted for viral and laboratory investigations with no specific requests for B19 testing. This sampling allowed us to investigate the broad range of clinical manifestations associated with B19 parvovirus infection.

MATERIALS AND METHODS

Clinical specimens. A total of 10,150 serum samples were tested from June to December 1989 for B19 virus DNA with a dot blot hybridization assay using a Dig-labeled DNA probe. The sampling included 2,450 serum specimens submitted to our laboratory for viral serological studies, with no specific requests for B19 testing, and 7,700 serum specimens submitted by the Department of Clinical Pathology for laboratory testing.

Specimen preparation on nylon membrane. Four serum samples (5 µl each) were pooled, and 180 µl of distilled water was added. The 200-µl volume was filtered with a Bio-Dot apparatus (Bio-Rad) onto a nylon membrane (Amersham) equilibrated in distilled water. When hybridization assays gave positive reactions, the four serum samples present in the pool were tested separately. In that case, 5 µl of one serum sample was mixed with 195 µl of distilled water and filtered as described above. After filtering the samples, the nylon membrane was allowed to dry and then UV treated for 5 min. Specimens on the filters were alkali denatured and neutralized as described by Mason et al. (18), then soaked in 50 mM Tris hydrochloride (pH 7.6)–5 mM EDTA containing 1 mg of pronase (Merck) per ml, and incubated with gentle shaking for 60 min at 37°C. Pronase was removed by three 5-min rinses in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7).

In all experiments, a reference serum positive for B19 parvovirus was used at a dilution of 10⁻⁴. To test the sensitivity of the assay, 5 µl of this dilution of positive reference serum was mixed with 5 µl of each of three serum samples negative for B19 parvovirus and processed as described above. To test the specificity of the assays, the B19-labeled DNA probe was dot blot hybridized with cellular DNA from human fibroblast cell cultures and B19-positive reference sera were hybridized with a Dig-labeled cytomegalovirus DNA probe consisting of a Towne cytomegalovirus XbaI D fragment (20 kb) cloned in plasmid pACYC 184 (13).

* Corresponding author.
Dig-labeled probe preparation. Plasmid pGEM 1 with a 700-bp B19 DNA insert (11) was kindly donated by M. J. Anderson and prepared by routine methods (11). pGEM 1 vector DNA was separated from the BamHI-HindIII B19 DNA fragment by electrophoresis in a 0.6% low-melting-point agarose gel. The B19 DNA fragment was then recovered from the gel by the method of Maniatis et al. (17) and processed for labeling as previously described (7).

Probe labeling was done by incorporating Dig-labeled dUTP by the random-primed DNA-labeling method developed by Feinberg and Vogelstein (12). The reaction was stopped by the addition of 0.2 M EDTA, pH 8, and the labeled B19 DNA fragment was precipitated by ethanol.

The B19 DNA probe could be stored at −20°C for at least 4 months with no decrease in its activity.

Hybridization reaction on nylon filter. Filters were sealed in a polypropylene bag with, per cm², 200 µl of a prehybridization mixture containing 4 X SET (20 X SET is 3 M NaCl, 0.4 M Tris hydrochloride [pH 7.8], and 20 mM EDTA), 5 X Denhardt solution (50 X Denhardt is 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin in distilled water), 100 µg of denatured calf thymus DNA per ml, and 0.5% sodium dodecyl sulfate. Filters were incubated at 65°C for 1 h in a shaking water bath.

The prehybridization mixture was removed and replaced with, per cm² of filter, 100 µl of hybridization mixture containing 4 X SET, 5 X Denhardt solution, 50% formamide, 0.5% sodium dodecyl sulfate, 100 µg of denatured calf thymus DNA per ml, and 50 ng of denatured B19 DNA probe per ml. The filters were then incubated in shaking water at 42°C for 18 to 20 h. The filters were carefully washed after hybridization as previously described (7).

Detection of hybridized probes. Filters were briefly washed in a 100 mM Tris hydrochloride buffer (pH 7.5) containing 150 mM NaCl (washing buffer). As a blocking reagent, normal sheep serum (10% in phosphate-buffered saline) was applied to the nylon filters for 30 min at room temperature.

After being washed for 2 min in washing buffer, filters were incubated for 30 min at room temperature with sheep anti-Dig Fab fragments, conjugated to alkaline phosphatase (Boehringer), and diluted 1:5,000 in washing buffer.

The filters were then equilibrated for 2 min with equilibration buffer (100 mM Tris hydrochloride, 100 mM NaCl, 50 mM MgCl₂, pH 9.5), and the alkaline phosphatase substrate was added. The alkaline phosphatase substrate consisted of 45 µl of solution A (75 mg of Nitro Blue Tetrazolium per ml of 70% dimethylformamide), 35 µl of solution B (75 mg of 5-bromo-4-chloro-3-indolylphosphate, toluidine salt [Sigma], per ml of dimethylformamide), and 10 µl of equilibration buffer per 100 cm². Development of the dark-blue color reaction was allowed to proceed for 2 h.

Filters were washed for 5 min with TE buffer (10 mM Tris hydrochloride, 1 mM EDTA), pH 8, air dried, and stored in a polypropylene bag.

Electron microscopy. Serum samples (0.5 ml) positive for B19 DNA by dot blot hybridization assay were purified on a 15 to 30% sucrose gradient in TEN (20 mM Tris hydrochloride, pH 7.5, 2 mM EDTA, 150 mM NaCl) by centrifugation at 50,000 × g for 4 h at 4°C. The pellet was suspended in 50 µl of TE (10 mM Tris hydrochloride, pH 7.6, 1 mM EDTA).

Viral particles were negatively stained with 2% phosphotungstic acid, pH 6.9, and examined by electron microscopy.

RESULTS

Rapid screening for B19 parvovirus was developed by using a Dig-labeled DNA probe in a dot blot hybridization assay. Four serum samples (5 µl each) were pooled and filtered on a nylon membrane so that 380 serum samples could be tested for each membrane (Fig. 1A). When a pool was positive in the hybridization assay, the four serum samples were separately tested to identify the specimens positive for B19 parvovirus DNA (Fig. 1B).

To determine the sensitivity of the Dig-labeled B19 probe, different concentrations of unlabeled B19 parvovirus DNA were dot blot hybridized with the Dig-labeled probe. A positive reaction was detected with 0.1 pg of B19 DNA and visualized as a dark blue dot with no background color.

The reference serum was positive for the parvovirus B19 up to a dilution of 10⁻⁴. Five microliters of reference serum at this dilution, mixed with 5 µl of each of three undiluted serum samples negative for B19 parvovirus, gave a positive result in the hybridization reaction with the Dig-labeled probe.

The specificity controls (DNA from human fibroblast cell cultures dot blot hybridized with the B19 Dig-labeled probe and B19 parvovirus-positive reference serum dot blot hybridized with a Dig-labeled cytomegalovirus DNA probe) gave negative results.

Of 10,150 serum samples tested by dot blot hybridization assay, 9 were positive for B19 DNA, and the results were confirmed by the detection of viral particles by electron microscopy (Fig. 2).

Of the 9 serum samples positive for B19 DNA, 5 were from the 2,450 samples submitted to viral laboratories (0.2%), while the remaining 4 were from the 7,700 serum samples submitted for clinical chemistry laboratory investigation (0.05%).

Two of the nine B19 parvovirus DNA-positive samples were from children aged 7 and 8 years, and seven were from adults aged between 30 and 43 years (Table 1). In the two positive children, a diffuse rash was present as a clinical manifestation. Among the adults, one pregnant woman re-
mained symptom free; two drug-immunodepressed patients had a short febrile illness (4 to 5 days) associated with leukopenia in the days immediately after the documented viremia; one patient had a nephropathy, and the remaining three patients positive for B19 DNA demonstrated involvement of the upper and lower respiratory tract.

**TABLE 1.** B19 DNA dot blot- and electron microscopy-positive serum samples

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Clinical presentation</th>
<th>Samplinga</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>F</td>
<td>Pregnancy</td>
<td>VL</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>M</td>
<td>Petechial rash</td>
<td>VL</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>F</td>
<td>Neurological disorders, rash</td>
<td>VL</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>M</td>
<td>Pneumonia</td>
<td>VL</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>M</td>
<td>Respiratory disease</td>
<td>VL</td>
</tr>
<tr>
<td>6</td>
<td>32</td>
<td>M</td>
<td>Liver transplant recipient (drug immunodepressed)</td>
<td>HP</td>
</tr>
<tr>
<td>7</td>
<td>37</td>
<td>M</td>
<td>Nephropathy</td>
<td>HP</td>
</tr>
<tr>
<td>8</td>
<td>43</td>
<td>M</td>
<td>Cardiomyopathy (drug immunodepressed)</td>
<td>HP</td>
</tr>
<tr>
<td>9</td>
<td>39</td>
<td>F</td>
<td>Respiratory disease</td>
<td>HP</td>
</tr>
</tbody>
</table>

a F, Female; M, male.

b VL, Serum samples submitted to viral laboratory; HP, serum samples of hospitalized patients, submitted for laboratory investigation.

**DISCUSSION**

Hybridization techniques have proven to be practical and reliable for the diagnosis of viral infections. They are particularly useful for B19 parvovirus detection in clinical specimens, since the virus cannot be isolated in cell cultures (3, 7, 11, 19).

The dot blot hybridization assay we have described is a specific and sensitive test. Moreover, the Dig labeling together with the immunoenzymatic detector system avoided the practical problems associated with both radiolabeling and nonspecific reactions observed with biotin labeling (19). The ability to perform the dot blot hybridization assay by mixing four samples of human serum without a loss of sensitivity increases the usefulness of the assay, which may be suitable for a rapid screening of large numbers of clinical specimens. Moreover, the assay provides an economical tool in terms of both time and costs for diagnostic work.

We have also evaluated the pooling of more than four samples of human serum (results not shown), but difficulties in filtering with the Bio-Dot apparatus made the sample spread out on the nylon filter rather than produce a well-defined dot.

Of the 10,150 serum samples tested, 2,450 were submitted to the viral laboratory with virological requests other than requests for B19 parvovirus testing. The remaining 7,700
serum samples came from the Department of Clinical Pathology and had been submitted for clinical chemistry laboratory investigation.

Among the 2,450 serum samples, 5 samples were positive for B19 DNA (0.2%), while only 4 of the 7,700 samples (0.05%) were positive. It therefore seems possible that in some instances, viral infections are suspected but there are no specific clinical indications of B19 parvovirus infection.

It should also be noted that all of the serum samples were collected from June to December, months in which epidemics due to B19 parvovirus are unlikely.

Our results indicate that the dot blot hybridization assay described is a practical, reliable test which can be performed for diagnostic work and the routine screening of serum samples with a great saving of time and costs. Moreover, serum samples submitted for virological investigations may also be useful in increasing the yield of B19-positive samples and in analyzing the clinical conditions associated with B19 parvovirus infections.

ACKNOWLEDGMENTS

The skilful technical help of Simona Lolli and Marinella Plazzi is gratefully acknowledged.

This work was partially supported by the Consiglio Nazionale delle Ricerche Progetto Finalizzato Biotecnologie e Biostrumentazioni and by Regione Emilia Romagna.

LITERATURE CITED