

## Chemiluminescence Dot Blot Hybridization Assay for Detection of B19 Parvovirus DNA in Human Sera

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**A chemiluminescence dot blot hybridization assay was used for the detection of B19 parvovirus DNA in human sera by using digoxigenin-labeled probes. The probes were revealed immunoenzymatically by use of anti-digoxigenin Fab fragments conjugated with alkaline phosphatase. The chemiluminescence signal was obtained by reacting the labeled probe-target complex with an enzyme-triggerable dioxetane substrate. The emitted photons were detected with instant photographic films. In the search for B19 parvovirus DNA, 2,808 serum samples were analyzed.**

It has been suggested that human parvovirus B19 is the causative agent of a wide spectrum of diseases, including erythema infectiosum (27), aplastic crisis in hemolytic anemias (29, 32), hydrops fetalis (1, 3, 10), acute arthritis (13), and persistent anemias in immunocompromised hosts (22, 34).

Because B19 parvovirus cannot be routinely grown in stable cell lines, the methods of detection rely on counter-immunoelectrophoresis and electron microscopy (11, 15), radioimmunoassay (14), and more commonly, nucleic acid hybridization assay (2, 12, 16, 24).

In previous reports, we have described the detection of B19 infection and a rapid screening of B19 DNA in clinical specimens by use of digoxigenin-labeled probes. The hybridized DNA probes were immunoenzymatically visualized by a colorimetric reaction with antidigoxigenin Fab fragments labeled with alkaline phosphatase. The sensitivity of detection was comparable to that obtained with the same probes labeled with <sup>32</sup>P (4, 36).

Recently, some investigators have demonstrated that chemiluminescent enzyme substrates are the most sensitive tools available for detecting enzyme conjugates in a wide range of enzyme-based applications (6, 8, 19, 28, 30, 33). Therefore, in the present study, we aimed to develop a sensitive chemiluminescence hybridization immunoenzymatic assay for the detection of human B19 DNA in human sera by using digoxigenin-labeled probes. We used the recently synthesized compound adamantyl-1,2-dioxetane phenyl phosphate (PPD) as the chemiluminescent enzyme substrate (7, 31). In the presence of alkaline phosphatase, PPD is dephosphorylated and produces an unstable intermediate which decomposes further, emitting a glow of light in direct proportion to the amount of alkaline phosphatase present. The emitted photons were detected by use of Polaroid instant films, and the results that were obtained were compared with those of a hybridization assay with colorimetric detection.

A total of 2,808 serum samples received at our laboratory for serological viral studies were examined. Moreover, six B19 parvovirus-positive reference sera, which were previously tested by dot blot hybridization by the colorimetric assay (with a range of  $3 \times 10^6$  to  $1.5 \times 10^{10}$  viral genome

copies per  $\mu$ l of serum) and confirmed by electron microscopy, were tested by the chemiluminescence hybridization assay.

Five microliters of each serum sample was spotted with a Bio-Dot Apparatus (Bio-Rad Laboratories) onto a nylon membrane and treated as described previously (36). In each run, two reference serum samples positive for B19 parvovirus (a high-positive and a low-positive serum sample) were spotted as positive controls.

The B19 DNA probe was prepared from the molecular clone of a 700-bp *Bam*HI-*Hind*III insert in vector pGEM1, which was kindly donated by M. J. Anderson. Routine methods for large-scale preparation of plasmid were used (23). Digoxigenin labeling of the probe was performed as described previously (36) by using the randomly primed DNA-labeling method (17). The digoxigenin-labeled parvovirus DNA probe can detect 0.1 pg of homologous DNA with colorimetric detection. The parvovirus DNA probe can be stored at  $-20^\circ\text{C}$  for at least 4 months with no decrease in its activity.

Prehybridization and hybridization reactions were performed as described previously (36). After hybridization, membranes were washed under stringent conditions (4). After a brief wash in a 100 mM Tris hydrochloride buffer (pH 7.5) with 150 mM NaCl, the blocking reagent (Boehringer) was applied to the nylon membranes for 30 min at room temperature. Membranes were incubated at room temperature for 30 min with antidigoxigenin Fab fragments, which were conjugated to alkaline phosphatase (Boehringer). Membranes were then equilibrated for 2 min with equilibration buffer (100 mM Tris hydrochloride, 100 mM NaCl, 50 mM  $\text{MgCl}_2$  [pH 9.5]).

Colorimetric detection was performed as described previously (36).

For chemiluminescence detection, dry nylon membranes were sealed in a polypropylene bag with 100  $\mu$ l of a freshly prepared solution of the chemiluminescence substrate-enhancer solution (1:1) per  $\text{cm}^2$  (the substrate solution was Lumi-Phos 530 [Lumigen, Inc., Detroit, Mich.], which consisted of 0.33 mM PPD, 0.8 mM  $\text{MgCl}_2$ , and 1.13 mM cetyltrimethylammonium bromide, and 5.6  $\mu$ M fluorescence enhancer [5-*N*-tetradecanoyl-aminofluorescein in 0.75 M 2-amino-2-methyl-1-propanol buffer; pH 9.6] was used) and 50 mM sodium carbonate buffer (pH 9.5) containing 1 mM

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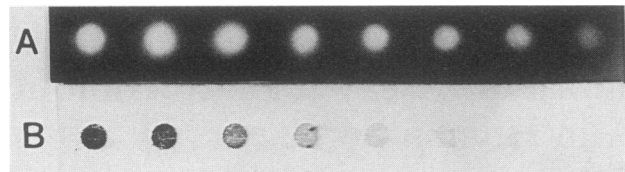


FIG. 1. Parvovirus B19 DNA dot blot hybridization assay with chemiluminescence detection (A) and colorimetric detection (B) of the digoxigenin-labeled B19 probe. In each row, serial dilutions of homologous B19 DNA were spotted (5, 2, 1, 0.5, 0.2, 0.1, 0.05, and 0.02 pg of DNA from left to right, respectively). A positive reaction could be visualized up to 0.02 pg of DNA by chemiluminescence detection and up to 0.1 pg of DNA by colorimetric detection.

MgCl<sub>2</sub>. The chemiluminescence signal was measured after a 2-h incubation with Lumi-Phos 530.

Polaroid type 667 instant black and white films in a Polaroid photocassette film holder (type CB 103) were used to record the chemiluminescence signal from the plastic-sealed assay membrane. The plastic-sealed membrane was placed in contact with the photographic film for about 15 s. Positive results were produced in the form of exposed spots.

To establish the sensitivity of our chemiluminescence assay, our digoxigenin-labeled B19 probe (used at a concentration of 50 ng/ml of hybridization mixture) was hybridized with dotted homologous DNA and was revealed by both chemiluminescence and colorimetric detection. These tests were performed in triplicate and gave the same results. After 2 h of incubation with their relative substrates, the detection limit of chemiluminescence was 0.02 pg of DNA, while 0.1 pg of DNA could be visualized by colorimetric detection (Fig. 1). In addition, different dilutions of a B19-positive reference serum sample were hybridized with the digoxigenin-labeled probe. By chemiluminescence detection, the signal was visualized up to a dilution of 1/2,560, while by colorimetric detection, it was visualized up to a dilution of 1/640. In the screening of the 2,808 serum samples tested by the chemiluminescence assay, 2,803 proved to be negative and 5 proved to be positive (Fig. 2). The same results were obtained by colorimetric detection of the hybridization assay, with a 100% accordance between the two tests.

The five positive results obtained by chemiluminescence and colorimetric detection after hybridization with digoxigenin-labeled probe were also confirmed by detection of viral particles by electron microscopy. Of the five serum samples

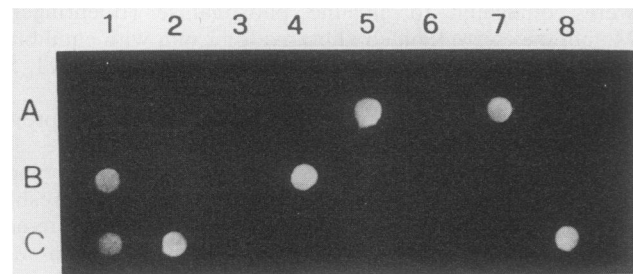


FIG. 2. Parvovirus B19 DNA chemiluminescence dot blot hybridization assay. A total of 24 serum samples were spotted on this nylon membrane. Of the 2,808 serum samples tested, 5 serum samples were found to be positive (coordinates A5, A7, B1, B4, and C2); also shown are 2 B19 parvovirus positive reference serum samples (coordinates C1 and C8) and 17 negative serum samples.

positive by the chemiluminescence assay and by colorimetric detection of the hybridization assay, two were from children with a diffuse rash, one was from a child with a congenital immunodeficiency, one was from an adult patient with a bone marrow transplant, and one was from an adult patient presenting with lymphadenopathy.

In our chemiluminescence assay, no false-positive results were obtained, although hemolyzed or bacterial-contaminated sera were present. Moreover, six reference serum samples that were previously tested by hybridization with colorimetric detection and by electron microscopy were tested by the chemiluminescence assay and gave positive results.

Our chemiluminescence assay proved to be specific on the basis of the following control reactions. (i) No chemiluminescence signal was observed when a B19-positive reference serum was hybridized with an unlabeled DNA probe and treated with antidigoxigenin Fab fragments conjugated with alkaline phosphatase followed by chemiluminescence detection. (ii) No chemiluminescence reaction was detectable when a B19-negative reference serum was hybridized with a B19 parvovirus-labeled probe treated as described above. (iii) No chemiluminescence signal was shown after hybridization of a B19-positive reference serum with a digoxigenin-labeled probe when the primary incubation with antidigoxigenin Fab fragments was either omitted or replaced by incubation with nonimmune sheep serum. (iv) Similarly, no light emission was detectable when pGEM1 vector DNA was hybridized with the B19-labeled probe.

In our assay, to avoid problems caused by bacterial contamination of sera, which could give false-positive reactions in the hybridization assay, we used as a viral probe only the 700-bp B19 insert, which was excised and separated from the pGEM1 vector. This avoided cross-reactions between the vector DNA and the plasmid DNAs present in contaminating bacteria.

Digoxigenin is the preferred nucleotide marker, because some reports have described problems that may arise with the use of biotin-labeled probes detected with avidin systems, not only in tissue sections (5, 20, 35) for use in *in situ* hybridization assays but also in sera for use in dot blot hybridization assays for the search for B19 DNA (24). Digoxigenin was also preferred for its high specificity (18, 25, 26) and sensitivity, similar to that obtained with radiolabeled probes (4, 21), without the potential health hazards, disposal problems, and instability associated with radioisotopes. PPD was used as the chemiluminescence substrate for alkaline phosphatase, because it has recently been described to be the most sensitive substrate for chemiluminescence alkaline phosphatase-based immunoassays (9, 30). Hence, in our assay the chemiluminescence detection of the probe, which was hybridized with dotted homologous DNA or serial dilutions of a B19-positive reference serum sample, proved to be about four- or fivefold more sensitive than colorimetric detection. These results are especially significant when we consider that colorimetric detection with digoxigenin-labeled probes reaches the same sensitivity as that obtained with radiolabeled probes (4, 21). In the chemiluminescence assay, the intensity of light emission for highly positive samples was so strong that in the darkroom one could see the sites of enzyme activity as bluish green luminescent spots, and short exposure times (about 15 s) were needed to obtain the signal intensities shown in the present study.

Black and white film exposure with camera systems is inexpensive and gives a permanent (hard-copy) photographic film record, while membranes stained with chro-

mogenic substrates can lose their color differentiation. Moreover, the membranes used with chemiluminescent substrates can be rehybridized several times, because chemiluminescence detection does not alter the color of the membranes.

In the screening of 2,808 serum samples in the search for B19 DNA, we did not find differences in the positivity or negativity of samples by the two tests, although chemiluminescence detection proved to be more sensitive than colorimetric detection in hybridization with both homologous DNA and serially diluted positive reference serum samples. Moreover, the six reference serum samples previously tested by colorimetric detection of the hybridization assay all proved to be positive in the chemiluminescence assay. This demonstrates an excellent accordance between the two tests.

In conclusion, our findings suggest that the chemiluminescence dot blot hybridization assay described here can be considered an additional, sensitive, and reliable method both for diagnostic work and routine screening of serum samples in the search for B19 parvovirus DNA.

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