

A Simple and Sensitive DNA Hybridization Assay Used for the Routine Diagnosis of Human Parvovirus B19 Infection

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A dot blot hybridization assay for parvovirus B19 diagnosis was developed by using a PCR-generated probe, digoxigenin labelling, and chemiluminescence detection. Different labelling techniques and hybridization solutions were evaluated. From this analysis a protocol was devised for routine diagnostic use. The protocol enabled 1 pg of B19 DNA to be detected. The results of applying this method to 8,369 diagnostic samples collected during 1994 and 1995 are given.

Parvovirus B19 is associated with erythema infectiosum (2), transient aplastic crisis in patients with hemolytic anemias (14, 19), and arthritis (18). Infection during pregnancy may lead to hydrops fetalis and fetal death (4). Immunocompromised patients may develop chronic infection leading to persistent anemia (10, 11).

In immunocompetent individuals viremia is rapidly cleared following the production of anti-B19 antibodies, and diagnosis is most often made by specific immunoglobulin M (IgM) serology (1, 9). However, in some cases it is important to be able to detect B19 virus. For example, in immunocompromised patients rapid diagnosis is needed to allow immunoglobulin therapy to be started promptly (10). In high-risk groups, symptoms (if any) may appear before the antibody response, for example, transient aplastic crisis in patients with sickle cell anemia. Infection during pregnancy may be asymptomatic, but there are management implications if B19 virus is detected early after contact with B19 disease (17). Moreover, the early recognition of B19 virus may help prevent cross-infection (15). Because B19 virus is not readily grown in cell culture it must be detected by other means. B19 DNA can be detected by dot blot hybridization, which is quick, simple, and specific. Large numbers of specimens can be screened by this method, and it has a level of sensitivity appropriate for diagnosis (7). By running the dot blot assay in parallel with an IgM test (9), patients presenting earlier in the course of infection can be diagnosed.

Since 1986 a dot blot hybridization assay for B19 DNA has been routinely carried out in our laboratory. Initially, the probe used consisted of the B19 insert excised from a plasmid (pGEM) and labelled with ³²P (6) or biotin (13). Subsequently, digoxigenin labelling was used. However, preparation of plasmid-derived probe was laborious and there was also the disadvantage of possible cross-hybridization between residual bacterial plasmid and/or host chromosomal DNA and bacterial DNA present in a contaminated specimen (vector homology) (13). Here we describe a straightforward method for the detection of B19 DNA that uses a probe generated by PCR, with viral DNA used as a template. This probe is now used in the routine diagnosis of B19 in our laboratory.

Probe production. To generate template DNA for PCRs, B19 virion DNA was isolated from a virus-positive plasma sample as described elsewhere (12). By using this DNA as

template, a 4.5-kb DNA fragment representing about 80% of the B19 genome was produced by PCR. The following primers were used: forward, 5'-CCC GCC TTA TGC AAA TGG GCA G-3' (residues 217 to 238 of the B19-Au DNA sequence [16]), and reverse, 5'-TTG TGT TAG GCT GTC TTA TAG G-3' (residues 4893 to 4872). Reaction mixtures were made up with 15 ng of template DNA, 25 pmol of each primer, 0.25 mM (each) deoxynucleoside triphosphate, 1× buffer (10× buffer is 100 mM Tris-HCl [pH 8.8 at 25°C]), 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100, and 0.01% gelatin in a final volume of 50 μl. This was heated to 94°C, and then 2.5 U of *Taq* polymerase added. The mixture was maintained at 94°C for 2 min, and then 10 cycles were conducted as follows: denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and elongation at 72°C for 3 min. A further 20 cycles were carried out as described above, but 30 s was added to the elongation time at each cycle. To check that amplification had occurred, 5 μl of the reaction mixture was analyzed on an agarose gel. PCR products were purified by using silica matrix (Geneclean kit; Bio 101, Inc.). One microgram of the purified PCR product was labelled for 20 h with digoxigenin by random primed labelling (Digoxigenin DNA Labelling Kit; Boehringer Mannheim), and unincorporated nucleotides were removed by gel filtration (Nick columns; Pharmacia). Before use the labelled probe was denatured by heating to 95°C for 10 min; it was cooled on ice and then added to 10 ml of hybridization solution.

Sample preparation. Samples of 10 μl were added to 90 μl of 2× SSC (3 M NaCl plus 0.3 M sodium citrate [pH 7.0]) and 100 μl of 2 M NaCl-1 M NaOH in a microtiter plate, and the plate was incubated for 15 min at room temperature to allow the DNA to denature. As controls a B19 DNA-negative human serum sample and serial dilutions of cloned B19 DNA (pGEM1-B19 [8]) were used. The samples were applied to a membrane (positively charged nylon; Boehringer Mannheim) by using a 96-well filtration apparatus (minifold; Schleicher & Schuell). The membrane was rinsed briefly in 2× SSC and was baked at 120°C for 20 min. If a dot blot apparatus is not available, the 10-μl sample can be spotted directly onto the membrane and the DNA can be denatured by floating the membrane on 2 M NaCl-1 M NaOH solution for 15 min and then rinsing briefly in 2× SSC.

Test of probe sensitivity. To test the sensitivity of the probe and to compare its performance in standard hybridization solution (5× SSC, 1% blocking reagent [Boehringer Mannheim], 0.1% *N*-lauroylsarcosine sodium salt, 0.02% sodium dodecyl sulfate [SDS]) with that in Easy Hyb solution (Boehringer

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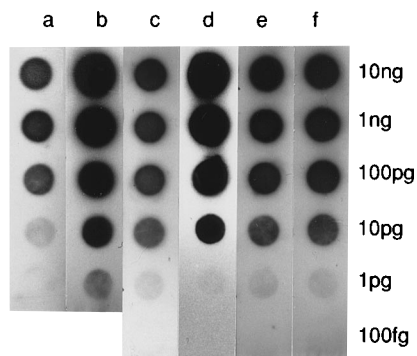


FIG. 1. Hybridization with the 4.5-kb B19 digoxigenin-labelled probe. Serial dilutions of pGEM1-B19 (a and b, 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg; c, d, e, and f, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, and 100 fg) were dot blot hybridized with the 4.5-kb B19 digoxigenin-labelled probe at 100 ng/ml. Lane a, random prime-labelled probe in standard hybridization solution; lanes b to f, random prime-labelled probe in Easy Hyb solution; b, random prime-labelled probe; c, High Prime-labelled probe, unpurified; d, High Prime-labelled probe, purified; e, PCR-labelled probe, unpurified; f, PCR-labelled probe, purified. The exposure times were 15 min for each lane except lane c, which was 5 min.

Mannheim), test membranes with serial dilutions of pGEM1-B19 from 1 ng/ μ l to 100 fg/ μ l were probed. Hybridization was carried out overnight at 68°C with standard hybridization solution or at 42°C with Easy Hyb. This solution allows hybridization to be carried out at a low temperature without the use of formamide. Posthybridization washes were two times for 5 min each in 2 \times SSC–0.1% SDS at room temperature and two times for 15 min each time in 0.1 \times SSC–0.1% SDS at 68°C. The membranes were then incubated with an anti-digoxigenin alkaline phosphatase antibody conjugate (digoxigenin detection kit; Boehringer Mannheim). Visualization was by the addition of a chemiluminescent substrate (CSPD; Boehringer Mannheim) and exposure to X-ray film (Fig. 1, lanes a and b). With both hybridization solutions, 1 pg of B19 DNA was detected after a 15-min exposure. With the Easy Hyb solution, the signal was clearer and the background was considerably lower than those with standard hybridization solution. Moreover, by using Easy Hyb solution 1 pg of B19 DNA was visible after only 5 min of exposure (data not shown). Earlier publications have described the use of carrier DNA and Denhardt's solution (bovine serum albumin, polyvinylpyrrolidone, and Ficoll) in the hybridization solution (3, 13), but we have found these to be unnecessary.

Digoxigenin High Prime labelling. The digoxigenin High Prime labelling kit is a single-reagent random prime labelling kit optimized to provide a high yield of digoxigenin-labelled probes. By using this kit 1 μ g of the purified 4.5-kb PCR product was labelled for 20 h and a test membrane was probed as described before, except that the dilutions were from 1 ng/ μ l to 10 fg/ μ l (Fig. 1, lane c). After a 5-min exposure 1 pg of B19 DNA was visible. The manufacturer states that the removal of unincorporated nucleotides is not necessary, but we observed a high background even after a relatively short exposure. Therefore, a probe was prepared again by using the High Prime kit, purified by gel filtration, and used in a test hybridization (Fig. 1, lane d). In this case no background was seen even after a 15-min exposure, and 1 pg of DNA was detected. This sensitivity is similar to that obtained by the standard random primed method, but as a single tube the High Prime kit leaves less room for error.

PCR digoxigenin labelling. The PCR digoxigenin labelling kit (Boehringer Mannheim) directly incorporates the digoxi-

genin-dUTP into the PCR mixture. This was carried out with 15 ng of B19 DNA as a template, and an aliquot of the product was analyzed on an agarose gel. The product was divided in two, and one-half was purified by gel filtration. The probes were diluted in 10 ml of Easy Hyb solution, and the mixture was used in a test hybridization. Figure 1 (lanes e and f) shows that in both cases 1 pg of B19 DNA was detected after a 15-min exposure, as with the other probes. Purifying the probe only slightly diminished the background.

The quantity of labelled DNA present in a probe can be shown by spotting out dilutions of the probe onto a nylon membrane alongside labelled known standards and visualizing the digoxigenin as described earlier. In this way we have found that all the probes discussed above contained approximately 250 ng of labelled B19 DNA (data not shown).

Direct incorporation is the most rapid method for preparing a probe. However, for routine diagnosis, in which consistency is important and the requirement for probes is known in advance, we found High Prime labelling to be more convenient. Several PCRs can be carried out at the same time, and a stock of DNA ready for labelling can be prepared, standardized, and stored. PCR with digoxigenin-dUTP is less efficient than PCR with unlabelled TTP, and DNA sufficient for only one or two probes is produced by direct incorporation, whereas DNA sufficient for six or seven probes is produced in the standard way. Two filters, each containing 96 samples (including controls), can be hybridized with one probe. We have reused probes up to 10 times and obtained satisfactory results by extending the X-ray film exposure times. Thus, approximately 2,000 samples can be examined with one probe. The PCR product used here is relatively long (4.5 kb) compared with the more usual length of several hundred base pairs (20), so the PCR is less efficient. However, use of an almost full-length probe achieves a greater sensitivity in the hybridization assay (data not shown). We obtained 17 μ g of B19 DNA from 2 ml of infected serum. If 15 ng is used per PCR, this constitutes a large stock of template. A nested PCR could be performed on the 4.5-kb product, making the stock last even longer.

Vector homology. When preparing an insert probe, some plasmid DNA remains even after careful gel purification. In a test hybridization comparing the PCR-derived probe described above and an insert probe, the PCR probe hybridized only to B19 DNA and not to plasmid or bacterial DNA alone. The insert probe, on the other hand, hybridized to all samples (data not shown).

Optimized procedure. From the analysis described above, the following protocol was determined to be the most simple and efficient method for the B19 dot blot hybridization assay. It is now used in the routine diagnosis of B19 in this laboratory. (i) Perform the PCR with B19 viral DNA as the template. (ii) Purify the PCR product and measure the DNA concentration. (iii) Label 1 μ g with digoxigenin by using the High Prime labelling kit. (iv) Remove unincorporated nucleotides by gel filtration. (v) Prepare dot blot of 10- μ l samples. (Serum samples can be used directly, but tissue samples should be purified.) (vi) Hybridize overnight in Easy Hyb solution. (vii) Wash the filter and detect digoxigenin with an alkaline phosphatase-conjugated anti-digoxigenin antibody and a chemiluminescent reaction.

An example of a typical diagnostic dot blot result is shown in Fig. 2. Being nonradioactive, the digoxigenin detection system is safe and convenient. It is as sensitive as using radioisotopes (5) and is more specific than using biotin. Probes can be stored for many months at –20°C between used. Before reuse probes in Easy Hyb are denatured at 68°C for 10 min. Chemiluminescence was chosen for visualization of the digoxigenin in the dot

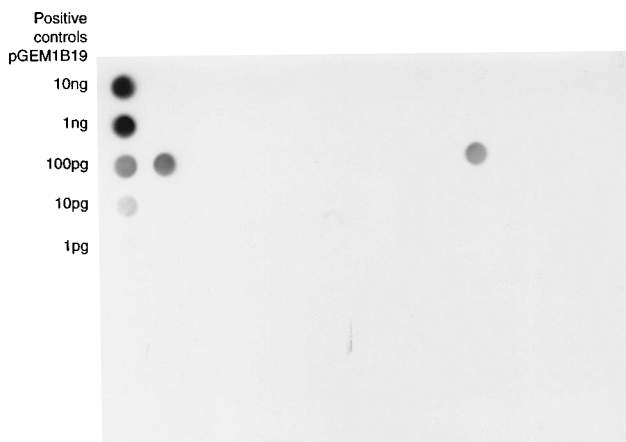


FIG. 2. Lumigraph of a diagnostic DNA dot blot assay. Positive controls are dilutions of pGEM1-B19, as indicated. A negative control (normal human serum) is in the top position on the second column. Of the samples tested, two were positive for B19 DNA.

blot assay of clinical samples because some samples (e.g., unpurified tissue extracts and hemolyzed blood) stain the membrane and interfere with the interpretation of results when color detection is used. The titer of B19 virus in clinical samples is frequently high (10^9 to 10^{11} particles per ml of serum [15]), and dot blotting provides an appropriate level of sensitivity for detecting this concentration of virus because its sensitivity, 1 pg of B19 DNA, is equivalent to approximately 10^5 B19 genomes (in 10 μ l of serum).

Diagnostic samples. The value of dot blot hybridization for B19 diagnosis has been documented previously (13, 21) and is further illustrated here by reference to the results of diagnostic testing during 1994 and 1995. In the 12 months following the introduction of the optimized procedure (1 April 1994 to 31 March 1995), which coincided with a trough in the B19 epidemic cycle, 65 (0.6%) of 10,341 samples referred for B19 testing were dot blot positive. During the same period, 767 (8.1%) of 9,442 serum samples were B19 IgM positive. Details for patients with dot blot-positive samples, taken from the laboratory request form, are summarized in Table 1. Of these 65 dot blot-positive samples, 20 were tissue samples and 45 were serum samples; 37 of the serum samples were also tested

TABLE 1. Clinical details of patients with B19 DNA dot blot-positive samples provided in 1994 and 1995

No. of samples	Clinical details
26.....	Embryopathy (hydrops fetalis, intrauterine death, etc.)
10.....	Human immunodeficiency virus positive, anemia
8.....	Rash and/or arthropathy
4.....	Leukemia (2 with rash)
4.....	Anemia
3.....	Transient aplastic crisis (1 sickle cell anaemia; 2 hereditary spherocytosis)
3.....	Bone marrow transplant (2 with erythrocyte aplasia)
1.....	Hemolytic anemia
1.....	Pancytopenia
1.....	Erythrocyte aplasia
1.....	Blood donor
3.....	Not known

for B19 IgM by radioimmunoassay; 7 were positive, 8 were equivocal, and 22 were negative. Of the dot blot-positive samples in which IgM serology was negative, three were confirmed by counterimmune electrophoresis for B19 antigen and two were confirmed by PCR; nine serum samples in this category were from immunocompromised patients with persistent B19, three were fetal samples from fetuses in which gestational B19 was confirmed by maternal IgM serology, and three more were from patients with acute infections confirmed by B19 IgM in follow-up samples, but for the remaining two patients no follow-up sera were available to assess the specificity of the dot blot reactions.

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