

Dot Immunoperoxidase Assay for Detection of Parvovirus B19 Antigen in Serum Samples

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Received 2 December 1996/Returned for modification 8 January 1997/Accepted 21 February 1997

We describe a simple and rapid dot immunoperoxidase assay for the direct detection of parvovirus B19 capsid antigens in human sera. The assay was performed with serum specimens dotted onto nylon membranes. VP1 and VP2 B19 antigens, which represent 4 and 96% of the capsid, respectively, were detected with a pool of monoclonal antibodies directed against the two proteins, and the complex was visualized by immunoperoxidase staining. The assay could be performed in about 4 h, and positive results were revealed at the end of the reaction as dark blue spots on the nylon membrane at the site of positive specimens. A total of 541 serum samples from different subjects and with different laboratory evaluations with regard to B19 infection were analyzed. The results obtained by the dot immunoperoxidase assay were compared with the results obtained for the presence of B19 DNA by dot blot hybridization and nested PCR. With optimized working conditions, the dot immunoperoxidase assay was able to detect the presence of B19 with a sensitivity comparable or slightly higher than that achieved by dot blot hybridization but less than that achieved by nested PCR. Since the level of sensitivity of the dot immunoperoxidase assay proved to be appropriate for the detection of acute B19 infection, and since the cost, time to a result, and versatility of the assay are important issues, from our evaluation, the dot immunoperoxidase assay described may be particularly suitable for large-scale screening of samples and a good alternative to DNA detection methods in the routine laboratory evaluation of B19 infection.

Human parvovirus B19 is associated with a wide range of clinical manifestations such as erythema infectiosum in children, acute arthritis in adults, and aplastic crisis in patients with chronic hemolytic anemias; moreover, in immunocompromised patients it may cause chronic infections, leading to persistent anemias (1, 4, 18). Although many clinical symptoms of B19 infection have been clearly identified, other features have been proposed (10), and it is suspected that there are some others that remain unknown or unconfirmed. B19 has also been implicated as a cause of fetal hydrops and fetal loss during pregnancy, but the patient numbers in the work reported so far have been too small to determine the extent of fetal and congenital damage and the relative risk to the fetus in each trimester of pregnancy (3). Moreover, although B19 is normally spread via the respiratory route, parenteral transmission of B19 has been demonstrated via the transfusion of blood products from blood donated during the viremic stage of B19 infection. It must be indicated that the number of people needing parvovirus B19-free blood is very large, and screened blood should be considered, particularly for patients with sickle cell disease and other congenital anemias, immunocompromised hosts, and women during pregnancy (12). Large-scale screenings thus seem necessary to detect the presence of B19 in clinical samples to obtain more information about the clinical manifestations associated with B19 and to clarify the public health importance of B19 infection, in blood donors to reduce the risk of parvovirus B19 infection by transfusion of blood components and clotting factor concentrates, and in pregnant women to define the risks to the fetus associated with

maternal B19 infection and to study protocols for pregnancies complicated by B19 infection.

Currently, the methods used to detect B19-positive sera mainly consist of hybridization assays with labeled probes (2, 11, 21) or PCRs (5, 6, 15). The nucleic acid hybridization assay can detect the medium to high levels of viremia (above 10^4 genome copies of B19 DNA) which occur in the acute phase of B19 infection, and the PCR assay can detect even very low viral titers (between 1 and 100 B19 genome copies). The search for B19 by DNA hybridization techniques, however, is limited to certain laboratories, and the use of PCR with a large number of samples is expensive and cumbersome and can produce unreliable results. All of these data prompted us to develop a simple and rapid dot immunoperoxidase assay to detect the presence of B19 antigens in blood samples using commercially available monoclonal antibody against the two capsid proteins, VP1 and VP2, which represent 4 and 96% of the B19 capsid, respectively. Sera were treated in order to expose the antigenic sites present in the virions and to avoid the masking of the antigens by early specific host antibody.

MATERIALS AND METHODS

Samples. The sera to be tested by our B19 dot immunoassay for the detection of B19 antigens were chosen from different subjects with different laboratory evaluations with regard to B19 infection. In our study all serum samples had previously been examined for the presence of B19 DNA by dot blot hybridization and nested PCR. Dot blot hybridization was performed as described previously by using digoxigenin-labeled probes constructed and labeled in our laboratory (8), and the sensitivity of the assay with colorimetric detection was 3×10^4 genome copies. Nested PCR was performed as described previously with peripheral blood samples treated with a lysis solution, and the sensitivity of the assay was between 1 and 20 genomes (14). It must be emphasized that all the PCR studies were carried out by applying the precautions previously reported by our laboratory (14-16). Accordingly, false-positive results caused by contamination or carryover could practically be excluded.

A total of 541 serum samples were tested for B19 antigens. Of these 541 serum samples, 61 serum samples had proved to be positive for B19 DNA both by dot

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blot hybridization and by nested PCR, 25 serum samples had proved to be positive for B19 DNA by nested PCR but negative by dot blot hybridization, and 455 serum samples had proved to be negative for B19 DNA.

In particular the samples included (i) 61 serum samples that were submitted to our laboratory with a specific request for B19 testing and that had proved to be positive for B19 DNA both by dot blot hybridization and by nested PCR (28 samples were from bone marrow recipients with aplastic crises, 18 were from patients with hypoplastic anemias, 6 were from patients with acute leukemias, 4 were from liver transplant recipients, 3 were from patients with rash, 1 was from a patient with arthropathy, and 1 was from a patient with hyperpyrexia); (ii) 25 serum samples that were submitted to our laboratory with a specific request for B19 testing and that were negative for B19 DNA by dot blot hybridization but positive by nested PCR (8 samples were from patients with arthropathy with a persistence of symptoms between 1 and 3 months, 4 were from patients with fetal hydrops or fetal ascites, and 13 were from convalescent-phase patients after acute B19 infection); (iii) 15 serum samples that were submitted to our laboratory with a specific request for B19 testing and that were negative for B19 DNA both by dot blot hybridization and by nested PCR (6 were from patients with arthropathy, 5 were from patients with hyperpyrexia, and 4 were from patients with rash); (iv) 40 serum samples that were submitted to our laboratory for virological studies with no specific request for B19 testing and that were negative for B19 DNA both by dot blot hybridization and by nested PCR; and (v) 400 serum samples from healthy blood donors negative for B19 DNA by dot blot hybridization and by nested PCR.

Dot immunoperoxidase assay for the detection of B19 antigens. Aliquots of 10 μ l of each serum sample were treated with 20 μ l of sodium dodecyl sulfate (SDS) denaturing buffer (2.3% SDS, 5% β -mercaptoethanol, 0.015 M Tris hydrochloride [pH 6.8]) for 10 min at room temperature, and then they were added to 70 μ l of distilled water and were filtered onto a nylon membrane by using a Bio Dot Apparatus (Bio-Rad Laboratories, Milan, Italy). The membrane with dotted specimens was air dried and was then treated with 2% blocking buffer (Boehringer, Mannheim, Germany) in 0.1 M Tris HCl buffer (pH 7.5) containing 0.15 M NaCl for 1 h at room temperature. The membranes were then primarily incubated with monoclonal antibodies against VP1 and VP2 (anti-VP1 clone 829 and anti-VP2 clone LJF/6/88 from Biosoft, Paris, France) pooled 1:1 and used at the optimal dilution of 1/80 in blocking buffer. Incubation was for 1 h at room temperature with gentle agitation. After two washings of 10 min each in 0.3% Tween 20 in phosphate-buffered saline, the membranes were incubated with peroxidase-conjugated goat anti-mouse immunoglobulins (Dako, Glostrup, Denmark) used at the optimal dilution of 1/500 in blocking buffer. Incubation was for 30 min at room temperature with gentle shaking. Subsequently, two washings were performed as described above, and a rapid third washing was performed in distilled water. The membranes were then incubated in the dark for 10 to 30 min with the enzyme substrate for peroxidase, which consisted of a solution of 2 ml of 4-chloro-1-naphthol (Bio-Rad Laboratories) in methanol (3 mg/ml) that was added immediately before use to a solution of 10 ml of phosphate-buffered saline containing 6 μ l of 30% hydrogen peroxide. After incubation, dark blue spots were noted for positive samples. The membranes were then washed in distilled water, air dried, and stored for use as a permanent record.

The optimal working dilution of each immune reagent used in the reaction was determined by preliminary block titration.

Two positive and two negative controls, which consisted of positive and negative B19 reference sera, respectively, were included in every assay. Positive reference sera had previously proved to be positive by dot blot hybridization, nested PCR, and antigen detection, while negative reference sera had proved to be negative by the three tests.

RESULTS

In the dot immunoperoxidase assay for the detection of B19 antigens, which was performed in about 4 h, positive results were revealed at the end of the reaction as dark blue spots on the nylon membrane at the site of positive specimens (Fig. 1).

In our work we analyzed a total of 541 serum samples with different laboratory evaluations with regard to B19 infection. Of the 61 serum samples that were submitted to our laboratory with a specific request for B19 testing and that had proved to be positive for B19 DNA both by dot blot hybridization and by nested PCR, 60 also proved to be positive for B19 antigens by the immunoperoxidase assay and one proved to be negative. The discordant serum sample was from a patient with cutaneous rash.

Of the 25 serum samples positive for B19 DNA by nested PCR but negative by dot blot hybridization, 5 proved to be positive and 20 proved to be negative for B19 antigens. Of the five samples which proved to be positive by B19 antigen testing, two were from patients with fetal hydrops, two were from

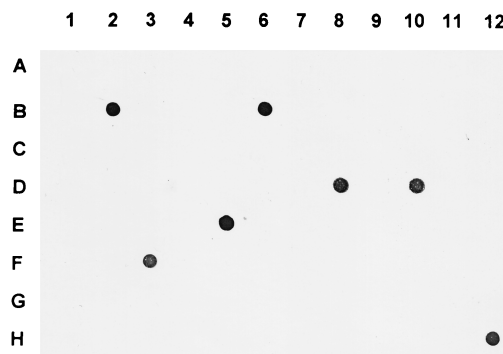


FIG. 1. Photographic record of dot immunoperoxidase assay for B19 parvovirus antigen detection. In this nylon membrane, 94 serum samples plus a negative reference serum (coordinate H11) and a positive reference serum (coordinate H12) were analyzed. Six serum samples (coordinates B2, B6, D8, D10, E5, and F3) and the positive control proved to be positive.

patients with arthropathy, and one was a convalescent-phase serum sample obtained from a patient 1 month after acute B19 infection.

Of the 15 serum samples that were submitted to our laboratory with a specific request for B19 testing and that had proved to be negative for B19 DNA both by dot blot hybridization and by nested PCR, 14 gave concordant negative results by searching for B19 antigens, while 1 proved to be positive. The serum sample with a discordant result was from a child with a rash with a slapped cheek feature. To establish whether this sample had falsely been considered negative for B19 DNA, we retested the sample with another B19 DNA probe of 700 bp that we had used in previous work for dot blot hybridization (13) and another set of primers for nested PCR (15). The negative results of dot blot hybridization and nested PCR were confirmed.

Among the 400 serum samples from blood donors and the 40 samples that had arrived at our laboratory without any specific request for B19 testing, all of which had proved to be negative for B19 DNA both by dot blot hybridization and by nested PCR, no discordant results were found; in fact all 440 serum samples proved to be negative by searching for B19 antigens.

The results obtained in the analysis of 541 serum samples are summarized in Table 1.

In order to determine the relative sensitivity of the immunoperoxidase assay, different serial dilutions of two reference B19-positive serum samples that were titrated by competitive PCR and that contained 7×10^4 and 9×10^4 genome copies/ μ l, respectively (9), were dotted onto a nylon membrane and

TABLE 1. Comparison of dot immunoperoxidase assay, dot blot hybridization, and nested PCR for detection of parvovirus B19 in serum specimens

Dot blot hybridization result/ nested PCR result	No. of samples	No. of samples with the following dot immunoperoxidase result:	
		Positive	Negative
Positive/positive	61	60	1
Negative/positive	25	5	20
Negative/negative	455	1	454
Total	541	66	475

analyzed. The immunoperoxidase assay could positively detect serum dilutions containing 2×10^3 genome copies, which was the endpoint.

Since in a previous work, in order to screen a large number of specimens, we demonstrated that four serum samples could be pooled to be tested by dot blot hybridization (20), we evaluated the possibility of whether four aliquots of 10 μ l each of four serum samples could be pooled to test for B19 antigens by the dot immunoperoxidase assay. Four pools of 4 positive serum samples, 4 pools of four negative serum samples, 16 pools of one positive sample plus three negative serum samples, and 8 pools of 2 positive and 2 negative serum samples were tested in duplicate by the dot immunoperoxidase assay, and we obtained the expected results without false positivity or false negativity.

A series of control experiments definitely proved that the dot immunoperoxidase assay was detecting B19 antigens specifically. In fact (i) a positive reaction was observed with B19-positive reference sera, (ii) a negative reaction was observed with B19-negative reference sera, (iii) no positive reaction was observed when, in an analysis of B19-positive reference sera, the primary incubation with the anti-B19 monoclonal antibody pool was either omitted or replaced with nonimmune mouse serum, and (iv) membranes were completely unstained after incubation of B19-positive reference sera with anti-B19 monoclonal antibody pool, by omitting the treatment with peroxidase-conjugated anti-mouse immunoglobulins.

To determine if the treatment of sera with SDS denaturing buffer could also inhibit any possible activity of cross-reactive endogenous peroxidase, we added to four B19-negative serum samples serial dilutions of horseradish peroxidase from 50 pg/ml to 500 μ g/ml. The samples to which peroxidase was added were treated with SDS denaturing buffer and were then processed by the dot immunoperoxidase assay, and all proved to be negative.

To test the specificity of our assay, five serum samples which had proved to be positive for B19 antigens were treated with monoclonal antibody to herpes simplex virus type 2 and with monoclonal antibody to respiratory syncytial virus, and they all proved to be negative.

To assess the reproducibility of the dot immunoperoxidase assay, we retested five B19-positive serum samples and five B19-negative serum samples in five different runs and on 5 different days and reobtained the expected results. The optimal working dilution of monoclonal antibody used in the reaction mixture was determined by preliminary block titration, and no variation was seen between different lots.

DISCUSSION

We have described a simple and rapid dot immunoperoxidase assay for the direct detection of B19 antigens in human sera. The assay was performed with serum specimens dotted onto nylon membranes and was completed in about 4 h. VP1 and VP2 B19 capsid antigens, which represent 4 and 96% of the capsid, respectively, were detected by using a pool of monoclonal antibodies directed against the two proteins, and the complex was visualized by immunoperoxidase staining.

In our assay, serum specimens were treated with SDS denaturing buffer in order to expose the antigenic sites present in the virions and to avoid the masking of antigenic determinants due to the presence of host-specific antibody (19). This treatment was also able to inactivate up to 500 μ g of horseradish peroxidase per ml, thus avoiding any possibility of cross-reactivity due to endogenous peroxidase present in serum specimens. The dilutions of immunoreagents were optimized by

preliminary block titration, and the time of incubation was optimized by choosing the most rapid treatment which could give a positive result. In fact, at the working dilutions of the monoclonal antibody pool (1/80) and the labeled antisera (1/500), 1 h of incubation with the monoclonal antibody pool and 30 min of incubation with the labeled antisera were the minimum times required to produce optimal results. A further decrease in incubation times resulted in a decreased sensitivity of the assay. The use of a primary mouse monoclonal antibody and the use of labeled anti-mouse immunoglobulins avoided any possible cross-reaction with human immunoglobulins present in serum samples.

In our dot immunoperoxidase assay, 94 samples plus the controls could be tested on each membrane. Because it was demonstrated that four serum samples can be pooled without a decrease in sensitivity, 376 samples per membrane can be tested for large-scale screening, with an effective reduction in the cost of reagents.

With optimized working conditions, our assay was able to detect B19 antigens with a sensitivity comparable to or slightly higher than that achieved by dot blot hybridization. In fact of the 61 samples positive by dot blot hybridization, 60 also proved to be positive for B19 antigens, while of the 25 samples positive by nested PCR but negative by dot blot hybridization, antigenic detection proved to be positive for 5 samples; moreover, 1 serum sample, from a child with a clinically diagnosed slapped cheek rash, proved to be positive only for B19 antigens and negative by the search for DNA, probably because of the loss or damage of B19 DNA or the presence of inhibitors of *Taq* polymerase. A slightly higher sensitivity of the dot immunoperoxidase assay with respect to that of dot blot hybridization could be explained by the fact that the immunoperoxidase assay gave a positive endpoint reaction with serum dilutions known to contain 2×10^3 genome copies of B19 DNA, while the endpoint sensitivity of dot blot hybridization with colorimetric detection is 3×10^4 genome copies. Moreover, by the dot immunoperoxidase assay, a pool of monoclonal antibody directed against all of the capsid proteins (VP1 and VP2) was used in order to detect the whole capsid, and electron microscopy studies have shown that some B19 viral particles appear empty (7, 20), and in that case the capsid proteins but not the DNA can be revealed. The dot immunoperoxidase assay proved to be much less sensitive than the PCR-based assay when comparing just those samples that were PCR positive but dot blot hybridization negative, and this could be due to the different sensitivities of the two assays, but it must be stressed that it is very difficult to reach the sensitivity of a method able to detect as few as 1 to 100 B19 genome copies.

Since the sensitivity of the dot immunoperoxidase assay for B19 antigens is about 2×10^3 viral particles, the transmission of B19 with blood units cannot be excluded by testing samples only for B19 antigens, but surely it will be greatly reduced. Moreover, until every parameter of PCR for B19 is highly standardized to ensure a precise result and until PCR can be used to test hundreds of samples simultaneously, we think that our test can be appropriate for the routine screening of blood units for B19.

In conclusion, since during the acute phase of B19 infections it is known that B19 viremia can reach titers of 10^{12} particles/ml with a clearance of the virus in between 3 and 6 months (17), the level of sensitivity of the dot immunoperoxidase assay seems appropriate for the detection of acute B19 infection. Moreover, since the cost, time to a result, and versatility of the assay are important issues, from our evaluation the dot immunoperoxidase assay described here can be particularly suitable for the large-scale screening of samples and can be a good

alternative to DNA detection methods in the routine laboratory evaluation of B19 infection.

ACKNOWLEDGMENTS

The skillful technical help of Marinella Plazzi is gratefully acknowledged.

This work was partially supported by grant 60% Ministry of University and Scientific Research and by funds for selected research topics from the University of Bologna.

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