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Dot Blot Hybridization Assay of B19 Virus DNA in Clinical Specimens

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A nonradioactive dot blot hybridization assay for human parvovirus B19 DNA was set up by using a biotin-labeled DNA probe and streptavidin-alkaline phosphatase conjugate. The assay was used to examine 4,895 specimens referred for B19 virus diagnosis during 1987. Of 48 specimens that gave positive reactions for B19 DNA, 41 were confirmed virus positive by electron microscopy ($n = 36$), radioimmunoassay ($n = 26$), or counterimmunoelectrophoresis ($n = 20$). In 7 samples which were not confirmed and in 11 samples giving weak reactions for B19 DNA, there was serological or epidemiological evidence of recent B19 infection. A further 70 specimens gave weak, apparently false-positive reactions. By electron microscopy, 13 of 16 were contaminated by bacteria, and plasmid DNA was demonstrated in one specimen. Of 55 specimens tested, 52 reacted with streptavidin-alkaline phosphatase conjugate alone. These were probable sources of nonspecificity in an otherwise practical and economic screening method for B19 virus.

Human parvovirus B19 (B19 virus) was first detected in 1975 in the serum of healthy blood donors (10). It was later found to be the cause of aplastic crises in patients with sickle cell anemia (16) and other chronic hemolytic anemias (23) and of erythema infectiosum (fifth disease) in those with normal erythrocytes (3). When adults are infected, particularly females, they often develop acute arthritis (22). Another complication in this group is the risk of an unfavorable outcome to pregnancy should infection of the fetus occur (6).

B19 infection is most often diagnosed by demonstrating a specific immunoglobulin M (IgM) response in sera taken shortly after onset of illness (2, 9). It is less commonly diagnosed by detecting B19 virus, because viremia is usually transient and often precedes symptoms. The virus has been grown only in vitro in cultures of bone marrow enriched with erythroid cells (15). This technique is not applicable to the routine detection of B19 virus in clinical samples, and biophysical methods are used instead. These include electron microscopy (EM) for parvovirus particles and radioimmunoassay (RIA) and counterimmunoelectrophoresis (CIE) for B19 antigen. The introduction of hybridization assays for B19 DNA, with probes incorporating ^{32}P as a radioactive label, has provided a further, more sensitive means of detecting B19 virus in clinical specimens (4, 7).

Recently, Cunningham et al. (11) have described a nonradioactive hybridization assay for B19 DNA using biotinylated RNA probes. We now describe our experience with a similar assay using DNA probes. The assay was done on all specimens submitted to our laboratory for B19 testing during 1987. It was compared with EM, RIA, and CIE for virus detection and with specific IgM serology for diagnosis of B19 infection. The advantages and shortcomings of this nonradioactive system are discussed.

MATERIALS AND METHODS

Specimens examined. A total of 4,895 specimens were received at the Virus Reference Laboratory for B19 virus studies in 1987, including 4,753 serum samples, 24 throat swabs, 24 samples of postmortem fetal tissue, 18 samples of placenta tissue, and 76 miscellaneous samples. All specimens were tested by the dot blot hybridization assay described below. Those giving positive reactions were examined for parvovirus particles by EM and for B19 antigen by RIA (9) and CIE (8). All sera were also tested for anti-B19 IgM by RIA (9), and selected sera were tested for anti-B19 IgG by RIA (9).

Dot blot hybridization assay. (i) Specimen preparation. Portions (10 μl) of samples were mixed with 90 μl of 2× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) and 100 μl of 1 M NaOH–2 M NaCl and incubated for 10 min at room temperature. The samples were then applied to nitrocellulose filters (102 by 133 mm) by using a Minifold apparatus (Schleicher & Schuell, Inc.). (For proteinase K treatment [20], filters were neutralized in 1 M Tris hydrochloride [pH 7.0]–2 M NaCl and then immersed in 0.01 M Tris hydrochloride [pH 7.4]–0.005 M EDTA–0.5% [wt/vol] sodium dodecyl sulfate [SDS] for 10 min at 25°C. They were then transferred to the same solution containing 0.5 mg of proteinase K per ml and incubated for 1 h at 50°C.) The filters were washed briefly in 2× SSC and then baked in a vacuum oven at 80°C for 1 to 2 h.

(ii) Probe preparation. B19 DNA was prepared from a molecular clone of a 5.2-kilobase-pair insert from isolate JB (13) in vector pGEM-1. It was labeled with biotin-11-dUTP (Bethesda Research Laboratories, Inc.) by nick translation, (nick translation reagent system; Bethesda Research Laboratories) and separated from unincorporated nucleotide by chromatography with Sephadex G-50.

(iii) Prehybridization. Usually two filters, placed back to back, were sealed in polypropylene bags with 10 ml of a prehybridization mixture containing 5× SSC, 50% formamide, 25 mM $NaPO_4$ (pH 6.5), 5× Denhardt solution, and 250 μg of sheared denatured salmon sperm DNA per ml. The filters were incubated at 42°C for 2 to 4 h in a shaking water bath.

(iv) Hybridization. The prehybridization mixture was removed and replaced with 10 ml of hybridization mixture containing 5× SSC, 45% formamide, 25 mM $NaPO_4$ (pH 6.5), 1× Denhardt solution, 250 μg of sheared denatured salmon sperm DNA per ml, 10% dextran sulfate, and 1 μg of probe DNA denatured by heating at 95°C for 10 min. The

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polypropylene bags were resealed and incubated for 18 to 22 h at 42°C in a shaking water bath.

(v) Posthybridization washes. The hybridization mixture was removed and stored at −20°C for reuse. The filters were washed in (i) 250 ml of 2× SSC–1.0% (wt/vol) SDS for 3 to 5 min at room temperature, (ii) 250 ml of 0.2× SSC–1.0% (wt/vol) SDS for 3 to 5 min at room temperature, and (iii) 250 ml of 0.16× SSC–1.0% SDS for 15 min at 50°C twice (for high-stringency washes, filters were incubated for 15 min, 25 min, and 2 h at 65°C in fresh solutions of 0.1× SSC–0.1% [wt/vol] SDS) and then were rinsed briefly in 2× SSC–0.1% SDS at room temperature.

(vi) Colorimetric DNA detection. Filters were washed for 1 min in 0.1 M Tris hydrochloride (pH 7.5)–0.15 M NaCl, and nonspecific binding sites were blocked by immersion in a 3% solution of bovine serum albumin in the same buffer for 20 min at 42°C. Streptavidin-alkaline phosphatase (SA-AP) conjugate (7 ml; 1 μg/ml in 0.1 M Tris hydrochloride [pH 7.5]–0.15 M NaCl) was then added for 10 min with gentle agitation, with occasional pipetting of the solution over the filters. The solution was decanted, and filters were washed twice in 250 ml of 0.1 M Tris hydrochloride (pH 7.5)–0.15 M NaCl for 15 min at room temperature and once in 250 ml of 0.1 M Tris hydrochloride (pH 9.5)–0.1 M NaCl–50 mM $MgCl_2$ for 10 min at room temperature. Filters were then sealed in polypropylene bags with 7.5 ml of freshly prepared dye solution containing 2.5 mg of Nitro Blue Tetrazolium and 1.25 mg of 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M Tris hydrochloride (pH 9.5)–0.1 M NaCl–50 mM $MgCl_2$. Development of a blue-black with positive samples was allowed to proceed for 1 to 2 h and then terminated by washing the filters in 20 mM Tris hydrochloride (pH 7.5)–0.5 mM disodium EDTA. Filters were air dried and resealed in polypropylene bags for storage.

Southern blotting was done as previously described (13).

EM. (i) Immune EM. A 50-μl volume of the test sample (serum or a clarified 10% tissue homogenate) was mixed with 50 μl of a human serum containing B19 antibody (IgG) and incubated at room temperature for 1 h or at 4°C overnight. The mixture was diluted with 3.0 ml of phosphate-buffered saline and centrifuged at 38,000 × *g* for 1 h. The pellet was drained and then suspended in 20 μl of distilled water, and 5 μl of the suspension was mixed with 5 μl of 3% phosphotungstic acid, pH 6.5. The mixture was applied to a Formvar-carbon-coated grid, and the excess was removed by blotting.

Grids were examined with an electron microscope at a screen magnification of ×60,000 for at least 20 min. If no virus particles were seen, three additional areas of the grid were examined at ×6,000 on the screen and by using 10× binoculars to check for the presence of bacteria. This last procedure occasionally also revealed aggregates of virus particles which were present in such low concentration that they had been missed in the high-magnification examination of a necessarily small area of the grid.

(ii) Direct EM. The direct EM procedure was the same as the immune EM procedure described above but omitted the addition of a B19 antibody-containing serum. Examination of the grids was carried out at ×60,000 magnification and was directed primarily towards detection of individual rather than aggregated parvovirus particles.

RESULTS

Positive dot blot reactions. A total of 48 specimens gave reactions in the dot blot hybridization assay that were stronger than that of the 100-pg B19 DNA control (Fig. 1). A total of 41 were confirmed positive for B19 virus by another method (Table 1). Parvovirus particles were seen in 35 specimens by immune EM and in 1 other specimen (Formalin-fixed postmortem fetal tissue) by direct EM. B19 antigen was detected in 26 specimens by RIA and in 20 specimens by CIE. Some samples could not be tested by all of the methods because there was insufficient material.

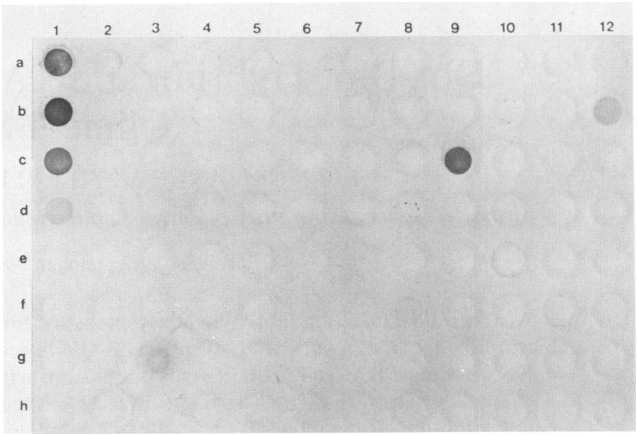

FIG. 1. Dot blot hybridization assay of B19 virus DNA in control samples and routine clinical specimens. Dots: a1, 100 ng of B19 DNA; b1, 10 ng of B19 DNA; c1, 1 ng of B19 DNA; d1, 100 pg of B19 DNA; e1, 10 pg of B19 DNA; f1, no B19 DNA; c9, positive reaction, confirmed by EM; b12, false-positive reaction; g3, coloration due to hemolyzed serum.

The other seven DNA-positive specimens were not reactive in another test for B19 virus. They were considered true positive reactions because there was serological or epidemiological evidence of recent infection (Table 2). In three cases with serological follow-up, anti-B19 IgM rose to high levels. A fourth case followed up was that of a patient who had persistent B19 infection with fluctuating viremia (12). There was no follow-up in three cases: that of a patient with sickle cell anemia involved in a family outbreak of aplastic crises; that of a child with leukemia and erythroid aplasia; and a case without clinical or epidemiological information or sufficient material for Southern blotting to be done. In three specimens the specificity of the DNA reaction was confirmed by Southern blotting (Table 2).

Weakly positive dot blot reactions. A total of 81 specimens gave reactions in the dot blot hybridization assay that were considered weakly positive, i.e., equal to or less than the reaction given by the 100-pg B19 DNA control but greater than that of the negative control. In 11 cases, there was serological or epidemiological evidence that these reactions

TABLE 1. B19 DNA dot blot positives which were EM, RIA, or CIE positive[a]

Test	No. of dot blots		
	Positive	Negative	Not tested
EM	36	1	4[b]
RIA	26	7	8[c]
CIE	20	11	10[c]

[a] $n = 41$.
[b] Includes 2 positive for B19 antigen by RIA but in insufficient amounts for EM.
[c] Includes 4 samples of postmortem fetal tissue in Formalin.

TABLE 2. B19 DNA dot blot positives which were not confirmed by EM, CIE, or RIA[a]

Data for patients			Result with:		
Age (yr)	Sex[b]	Clinical presentation	Southern blot	Anti-B19 IgM[c]	
				A	B
NA[d]	F	Rash in pregnancy	Positive	<1	>100 (10)
34	F	Rash, arthropathy	Negative	3.5	23 (60)
3	M	Leukemia, erythrocyte aplasia	Not done	<1	>100 (90)
2	M	Severe anemia, immunodeficiency	Positive	3.8	6.6 (3)
23	F	Sickle cell anemia, family outbreak of aplastic crisis	Not done	>100	NA
3	F	Leukemia, erythroid aplasia	Positive	<1	NA
75	M	NA	Not done	4.3	NA

[a] $n = 7$.
[b] F, Female; M, male.
[c] Data for DNA-positive samples (column A) and follow-up samples (column B; follow-up days in parentheses) are in arbitrary RIA units; <1 indicates a negative result.
[d] NA, Not available.

were significant (Table 3). In 3 of the 11 samples, the presence of B19 DNA was confirmed by another method (Table 3).

In the 70 other weakly reactive samples, there was no further evidence to support a diagnosis of B19 infection. These were considered nonspecific reactions, and the false-positive rate (70 of 4,895) was therefore 1.4%.

To investigate the source of nonspecific reactivity, these 70 samples were reexamined in a number of ways. (i) Filters were treated with proteinase K before hybridization. (ii) Filters were washed with high-stringency washes after hybridization. Neither of these procedures had any appreciable effect. (iii) Sixteen samples were examined by EM. None contained parvovirus particles, but in 13 samples, bacteria or bacterial flagella were seen. (Bacterial contamination was also seen in 11 of 36 samples with parvovirus particles.) A DNA preparation from one contaminated serum sample revealed bands corresponding to bacterial chromosome and plasmid DNA (Fig. 2). An atypical strain of *Escherichia coli* that grew better at 30°C than 37°C was cultured from this specimen (G. Willshaw and H. Smith, personal communication). (iv) On separate probing with B19 and vector DNA labeled with ^{32}P, 1 of the 70 samples reacted with ^{32}P-labeled B19 DNA and 7 reacted with ^{32}P-labeled vector DNA. (v) On probing with biotinylated vector DNA, 62 of the 70 samples were positive, 3 were negative, and 5 were not tested because there was an insufficient amount of specimen. (vi) When applied to a filter without a hybridization step, 52 of the 70 samples reacted weakly with the SA-AP conjugate and the chromogenic substrate, 3 were negative, and 15 were not tested because there was an insufficient amount of specimen. (vii) When an alternative nonradioactive detection system (Boehringer Mannheim Biochemicals) was used, 32 of the 70 samples reacted, 23 were negative, and 15 were not tested because there was an insufficient amount of specimen.

Specific IgM serology. In addition to the 48 virus-positive specimens found by demonstrating viral DNA, there were 505 serum samples with anti-B19 IgM at diagnostically significant levels (>3 arbitrary RIA units). B19-specific antibodies were also found in one-third to one-half of dot blot-positive specimens, though frequently only in trace amounts (Table 4). B19 antigen was detected, in spite of the presence of antibody, in 15 samples by RIA and in 14 samples by CIE (Table 5).

TABLE 3. Other evidence of recent infection in B19 DNA dot blot weakly positive samples[a]

Data for patients			Result with:		
Age (yr)	Sex[b]	Clinical presentation	Southern blot	Anti-B19 IgM[c]	
				A	B
23	F	Rash, arthralgia	Negative	54	24 (14)
NA[d]	F	NA	Negative	18	NA
11	M	Hereditary spherocytosis, aplastic crisis	Not done[e]	48	NA
41	F	Rash, arthralgia	Negative	3.2	NA
32	F	Rash	Negative	19	40 (−14)
NA	F	Hydrops fetalis	Positive[e,f]	1.6	NA
NA	M	G6PD deficiency, aplastic crisis	Not done	75	NA
NA	M	Purpura	Not done[e]	9.2	NA
4	NA	Aregenerative anemia, erythroblastopenia	Not done	10	<1 (−93)
12	F	Hereditary spherocytosis, aplastic crisis	Not done	29.5	NA
32	F	Rheumatoid arthritis	Not done	3.5	54 (NK)

[a] $n = 11$.
[b] F, Female; M, male.
[c] Data for DNA-positive samples (column A) and follow-up samples (column B; follow-up days in parentheses) are in arbitrary RIA units; <1 indicates a negative result. NK, Not known.
[d] NA, Not available.
[e] Dot blot positive with ^{32}P probe.
[f] Single-stranded B19 DNA detected.

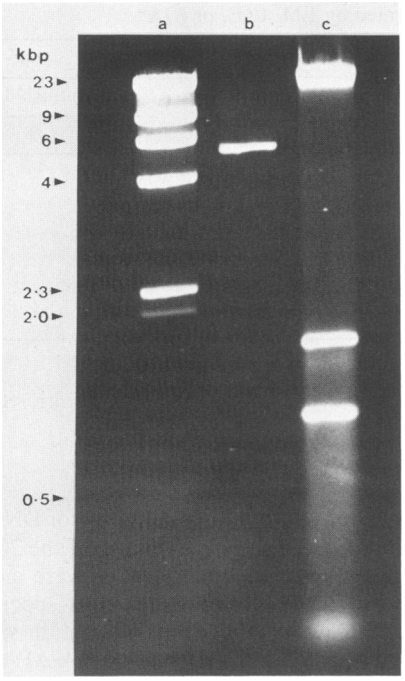

FIG. 2. DNA prepared from serum giving false-positive dot hybridization assay result (1% agarose gel, stained with ethidium bromide). Lanes: a, λ/*Hin*dIII fragments; b, serum with B19 virus; c, serum giving false-positive result.

DISCUSSION

Dot blot hybridization with biotin-labeled probes has several advantages for screening clinical samples for B19 virus. It is more sensitive than other currently available methods for detecting B19 virus (see below). The method is relatively fast, being completed in 2 days. It is also relatively inexpensive, mainly because of the long shelf life of the probe. In practice, we reused hybridization mixtures 10 or 12 times before they had to be replaced. This meant that, since two filters with approximately 90 samples each were hybridized together (back to back), up to 2,000 samples could be examined with each probe preparation. In our laboratory, therefore, new probe would have to be prepared only three times per year for sufficient reagent to screen all routine samples. The safety advantages of nonradioactive systems are well known, and it was convenient not to be obliged to do hybridization assays in areas of the laboratory set aside for radioactive work.

In addition to the problem of nonspecific reactions (discussed below), one possible shortcoming of this method was a prozonelike effect seen with strongly positive samples (Fig. 1). This may be due to steric hindrance, since it was not observed when probe DNA was labeled with ^{32}P, a moiety much smaller than biotin (results not shown). Whatever its origins, this effect reduced the confidence with which definite and weak positive reactions could be distinguished.

Apart from testing 70 serum samples which gave false-positive reactions, we made no attempt to compare biotin labeling with ^{32}P labeling. In most studies in which this has been done, ^{32}P has been shown to be more sensitive than biotin (17), often by testing dilutions of known positive material (21). While recognizing that the additional sensitivity of ^{32}P labeling is valuable for some applications (mainly research), we would argue that because the titer of B19 virus in clinical samples is usually high, biotinylated probes are sufficiently sensitive for diagnostic work.

We did not use nylon filters routinely, because in initial tests we found an unacceptable level of background coloration despite having followed the blocking procedure recommended by the manufacturers of the SA-AP conjugate (Bethesda Research Laboratories). High background levels sometimes develop with nitrocellulose filters when riboprobes for B19 virus (11) are used, but we did not find this a problem with DNA probes. It was also reported that the SA-AP conjugate supplied by Amersham International Plc was superior to that from other manufacturers (11). We did not investigate conjugates other than that made by Bethesda Research Laboratories.

Biotinylated probes have recently been used to detect B19 virus in fetal tissues by in situ hybridization (5, 18).

Although this study did not include a rigorous comparison of test methods, it is clear that the hybridization assay detected more samples with B19 virus than the other techniques used. The additional diagnostic yield was at least 7 (Table 2) and probably a further 11 (Table 3) samples. This was a third more than the yield by EM, the next most sensitive method.

In our hands, the least sensitive assays for B19 virus were RIA and CIE for viral antigen. It has previously been suggested that the presence of specific antibody may block the detection of B19 antigen in serum by these techniques (4, 9). Our findings did not support this suggestion, because the detection rate for B19 antigen was not significantly different when antibody was present (15 of 21 tested by RIA) or absent (11 of 17 tested by RIA) (Table 5).

In spite of the enhanced sensitivity provided by the dot blot hybridization assay, the diagnostic yield from anti-B19 IgM testing ($n = 505$) was approximately 10-fold greater than that from virus detection ($n = 48$). This is because viremia often precedes symptoms, whereas IgM is present at the onset of the rash, when a specimen is usually taken. The presence of anti-B19 IgM, sometimes in trace amounts, aided the interpretation of weak reactions in the hybridization assay.

TABLE 4. Specific antibodies in B19 DNA-positive specimens[a]

Result[b]	No. with result for anti-B19:	
	IgM	IgG
Positive (>3)	14	13
Equivocal (1–3)	3	10
Negative (<1)	23	13
Not tested	8	12

[a] $n = 48$.
[b] Numbers in parentheses are in arbitrary RIA units.

TABLE 5. Viral antigen in B19 DNA-positive specimens[a]

Result	No. of specimens with result for B19 antigen by:	
	RIA	CIE
Positive	26 (15)[b]	20 (14)
Negative	12 (6)	18 (7)
Not tested	10 (3)	10 (3)

[a] $n = 48$.
[b] Numbers in parentheses are number of specimens with anti-B19 IgM or anti-B19 IgG or both.

Even though we used a viral probe that had been purified from vector DNA, we obtained many false-positive reactions. These reactions were usually weak, less than that given by the 100-pg B19 control (Fig. 1). However, not all weak reactions were nonspecific, because some were obtained with samples thought to contain B19 virus (Table 3).

Attempts to reduce nonspecific reactions by treating filters with proteinase K and with high-stringency washes were not successful.

Bacterial contamination was observed in 13 of 16 samples giving false-positive reactions that were examined by EM. We demonstrated bacterial and plasmid DNA in one contaminated sample (Fig. 2) and in a few samples which also contained B19 DNA (results not shown). This raised the possibility that vector homology (1) was a cause of nonspecificity. However, it is unlikely to be a common cause; although 62 samples giving false-positive reactions reacted with biotinylated vector DNA, only 7 reacted when the same DNA was labeled with ^{32}P.

These findings, on the other hand, implicate the biotin-avidin detection system as a common source of nonspecific reactions. In experiments in which specimens giving false-positive reactions were simply bound to filters, reactions with the SA-AP conjugate were frequent. This suggests that some specimens contain biotin (or avidin-binding activity). The phenomenon has been found previously with other tissues by in situ hybridization (14) but not, as far as we know, with serum. It is not clear whether the serum avidin-binding activity is endogenous or (more likely) introduced by bacterial contamination. As a reference laboratory, we rarely receive fresh specimens: samples are first handled in other laboratories and then transmitted to us under conditions which may permit the growth of bacterial contaminants. If the avidin-binding activity is introduced this way, it can be anticipated that the rate of false-positives would be lower when fresh samples are tested, e.g., when donors are screened at a blood bank.

In addition to avidin-binding activity and vector homology, another problem was encountered with certain samples such as hemolyzed blood or tissue extracts that produced a strongly colored spot when applied to filters. This caused difficulties in interpreting the results of the color development stage of the assay.

These findings emphasize the difficulties of interpreting dot blot hybridization assays of clinical specimens. A limited number of tests with a second nonradioactive detection system (Boehringer Mannheim) produced fewer false-positive results. Further evaluation of this system would be worthwhile.

The nonradioactive dot blot hybridization assay described here provided a safe, economic, and relatively fast screening test for B19 virus and could be reliably used, in conjunction with serological testing for B19 antibodies and particularly IgM, in the clinical setting. Its sensitivity was greater than that of other assays routinely available in our laboratory for the detection of B19 virus and was adequate for diagnostic work, giving an increased yield of virus-positive samples. The accuracy of the assay for anti-B19 IgM has been discussed by us in previous publications (7, 9). IgM usually persists for 2 to 3 months. Since viremia usually precedes symptoms, timing of specimen acquisition is important in evaluating laboratory results. Recent studies have shown more persistent B19 infection in immunocompromised patients (12). In this circumstance the value of the assay for B19 DNA is enhanced, because the level of anti-B19 IgM may be too low to make a diagnosis. In the dot blot assay, some false-positive results were obtained, especially with specimens contaminated with bacteria, but this would not be such a problem when fresh samples were being tested. The rate of false-positives, about 1.4%, was not unacceptably high for a screening test, but it detracted from a system which otherwise had many advantages. In future work, this kind of assay may be replaced by one based on the polymerase chain reaction (19). This has already been developed for B19 virus (J. P. Clewley, unpublished observations; M. M. M. Salimans, S. Holsappel, F. M. Van de Rijke, N. M. Jiwa, A. K. Rapp, and H. T. Weiland, J. Virol. Methods, in press) but has not yet been fully evaluated in routine clinical specimens.

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