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Detection of Human Parvovirus B19 DNA PCR Products by RNA Probe Hybridization Enzyme Immunoassay

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We have developed an RNA probe hybridization enzyme immunoassay for detection of human parvovirus B19 PCR-amplified DNA. The assay is easy to perform and increases assay sensitivity without the added inconvenience and risk of false-positive results associated with nested PCR.

Determination of acute infection with human parvovirus B19 can often be accomplished by detection of specific immunoglobulin M (IgM) antibodies (1, 8). Because of the occasional occurrence of false-positive IgM results (4) and the potential for false-negative results for persons with immune deficiencies, accurate diagnosis often requires confirmation by detection of virus or viral DNA. PCR assay combined with gel electrophoresis and ethidium bromide fluorescence or Southern blot is currently the most sensitive method for detecting B19 DNA (7, 10, 17). We and others have shown that a second amplification with nested primers provides added sensitivity essential for detection of B19 DNA in some clinical samples (5, 10, 14, 15, 20). However, this second amplification is costly and time-consuming and increases the risk of false-positive results from contaminating DNA.

In this report, we describe a nonradioactive microtiter plate-based RNA probe hybridization enzyme immunoassay (RPEIA) for detection of B19 PCR-amplified DNA that takes advantage of the fact that oligomeric primers can be labeled with a variety of haptens without adversely affecting the efficiency of the amplification reaction (9, 11–13). The sensitivity of the RPEIA was compared with those of ethidium bromide fluorescence for detection of the primary PCR product and of nested PCR, and its diagnostic value was determined with clinical samples.

Pooled sera positive for B19 antigen by EIA and positive for DNA by PCR were used to standardize the assays and compare assay sensitivities. Nineteen blood and/or bone marrow specimens obtained from patients of diverse geographical origins and various ages with clinical and/or serologic evidence of B19 infection were used to compare the assay systems by use of clinical material; ten of these specimens had previously been shown to be PCR negative and nested-PCR positive. Ten additional serum specimens obtained from persons without clinical or serological evidence of recent B19 infection were used to evaluate test specificity. For this study, we prepared new DNA extracts from all specimens and repeated the PCR and nested-PCR procedures.

Oligonucleotide primers specific to a conserved region of the NS1 gene coding for the B19 nonstructural protein were prepared (19). Primers P1 and P6 were used for primary PCR amplification, yielding a 284-bp product; nested primers P2 and P5 were used to amplify an internal 102-bp product and to prepare the RNA probe. The primer sequences and the method of synthesis have been previously described (9). For biotin labeling, dimethoxytrityl-biotin C6 phosphoramidite (Cambridge Research Biochemicals, Norwich, United Kingdom) was used during primer synthesis to incorporate a single biotin molecule at the 5′ end of P1.

Our standard PCR and nested-PCR assays were performed as previously described (9). For detection by RPEIA, biotin-labeled P1 was substituted for unlabeled P1 at an identical concentration (0.5 M). No differences in amplification efficiency between the biotin-labeled and unlabeled primers were noted. Positive B19 DNA controls containing 10 pg/ml (total B19 DNA, 100 fg) and 1 pg/ml (total B19 DNA, 10 fg) and negative water controls were included in each amplification reaction.

For preparation of the RNA probe, the TA Cloning System (Invitrogen Corp., San Diego, Calif.) kit was used to directly clone the 102-bp B19 nested PCR amplification product into plasmid vector pCR 1000 provided in the kit. The TA Cloning System takes advantage of the ability of *Taq* polymerase to add single deoxyadenosines to the 3′ ends of all duplex molecules. Because the vector provided in the kit is supplied as a linear molecule with single 3′ T overhangs at the insertion site, PCR products can be directly cloned immediately after amplification and their sequences can be transcribed from an available T7 polymerase promoter; subsequent modification of the version 1.0 vector used in this study has purportedly improved both the versatility and percent recovery expected with this procedure (11a). All reactions were carried out according to the manufacturer's instructions. Of 20 recombinant colonies recovered and screened by PCR with primers P2 and P5, two were positive for the 102-bp insert. Minipreparations of the plasmid DNA from these two colonies were prepared by the alkali lysis method of Sambrook et al. (18) and digested with restriction enzyme *Not*I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to confirm the insert size. Digestion of the 2,915-bp pCR1000 cloning vector with the restriction enzyme *Not*I yields fragments of 150 bp, 350 bp (plus the PCR insert), and 2,400 bp. Fragments of ≈450 bp were obtained from *Not*I digests of both clones, consistent with the expected size of the fragment (350 bp) plus the insert (102 bp).

To prepare digoxigenin-labeled RNA probe, purified plasmid containing the 102-bp B19 insert was first linearized with *Eco*RI (Boehringer Mannheim) and then transcribed in the

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presence of digoxigenin-labeled uridine triphosphates by use of the Genius Nonradioactive RNA Labeling Kit (Boehringer Mannheim). The labeled RNA probe was precipitated for 2 h at −20°C by addition of 2.5 μl of 4 M LiCl and 75 μl of chilled ethanol. RNA was then pelleted by centrifugation at 12,000 × *g* for 30 min, washed once with 50 μl of 70% ethanol, and centrifuged again at 12,000 × *g* for 5 min. The pellet was then vacuum dried and resuspended in 50 μl of diethyl pyrocarbonate-treated water with 1 μl of RNase inhibitor and held at −20°C until use. When sized on a 1% agarose gel, the RNA probe was found to be approximately 200 bp in length, the expected size of the transcription product that includes the T7 promoter sequence (20 bp), polylinker (80 bp), and insert (102 bp).

To achieve consistency with other diagnostic assays performed in our laboratory, we chose assay reagents, diluents, and incubation conditions for our RPEIA that were similar to those of EIAs previously developed for detection of B19-specific antigen and antibodies (1). To capture biotinylated PCR products, Immulon II microtiter plates (Dynatech Laboratories, Chantilly, Va.) were coated overnight at 4°C with 200 ng of biotinylated bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) in 100 μl of 0.1 M phosphate-buffered saline (PBS), pH 7.2, per well, washed three times with PBS containing 0.15% Tween 20 (PBS-T), and then saturated with 1,000 ng of streptavidin (Sigma Chemical Co.) in 100 μl of PBS with 0.5% gelatin per well for 30 min at room temperature with shaking. The plates were washed three times with PBS-T and then used immediately or held at −20°C until use. We were able to achieve a significantly improved assay signal using a two-step method of coating microtiter plates with biotinylated bovine serum albumin followed by streptavidin and then coating streptavidin directly onto the microtiter plates (data not shown). Plates could be prepared in advance and held for several weeks at 4°C without appreciable loss of assay sensitivity.

To establish the optimal concentrations of amplification product and RNA probe, 20-, 10-, 5-, 2-, and 1-μl volumes of PCR product obtained from the standard positive control were each added to 200-μl volumes of hybridization buffer (4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] in diethyl pyrocarbonate-treated H_2O with 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 2 mM EDTA, and 0.15% Tween 20) containing serial twofold dilutions of the digoxigenin-labeled RNA probe (50 to 1,600 ng/ml). These mixtures were heated to 95°C for 5 min in a thermocycler and then cooled and held at 55°C for 30 min. The hybridization mixtures were then transferred to duplicate wells (100 μl per well) of a streptavidin-coated microtiter plate and incubated for 30 min at 37°C. A 1:5,000 dilution of peroxidase-labeled anti-digoxigenin Fab fragments (Boehringer Mannheim) in PBS-G-T (PBS with 0.5% gelatin and 0.15% Tween 20) was then added to all wells, and the mixtures were incubated for 1 h at 37°C. After a final wash, a 3,3′,5,5′-tetramethyl-benzidine chromogen solution was added and the color was developed. The reaction was stopped after 15 min with 2 M phosphoric acid, and the optical density (OD) was read at 450 nm. The optimal assay signal was obtained with 2 to 5 μl of product, whereas higher concentrations gave no improvement in assay signal or a reduced signal, presumably because of competition from excess biotinylated primers for the limited streptavidin-binding sites on the solid phase. Five-microliter volumes were chosen for the remainder of the study. A concentration of the RNA probe ranging between 100 and 200 ng/ml gave the best signal under the defined hybridization conditions. The cutoff value for a positive test was taken as 5 standard deviations above the mean value obtained with the 10 PCR-negative serum specimens (OD value, 0.035). Test results were reported as index values defined as the test value divided by the cutoff value.

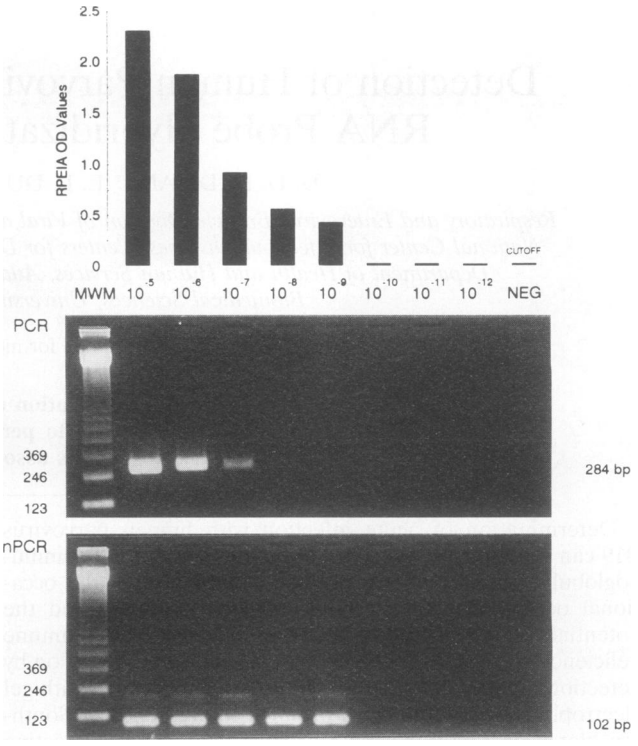

FIG. 1. Agarose gel electrophoresis and ethidium bromide fluorescence of PCR-amplified DNA (top gel) from serial 10-fold dilutions (10^{-5} to 10^{-12} are illustrated) of a pooled B19 DNA-positive serum specimen prepared in negative sera. Aliquots of PCR product were tested directly by the RPEIA (graph) and reamplified by nested PCR (bottom gel). A faint 284-bp band was detectable by PCR at a 10^{-8} dilution of the target. A 102-bp band was detectable by nested PCR at a 10^{-9} dilution; the additional band on the nested PCR gel represents primer carryover from the primary reaction. Similarly, the RPEIA was positive at a 10^{-9} dilution. MWM, molecular weight markers (sizes in base pairs are shown on the left).

The relative sensitivities of the different assay formats were determined by testing serial 10-fold dilutions of a B19 DNA-positive specimen prepared in negative sera. DNA extracts of this dilution series were amplified by PCR and nested PCR, and the amplified product was measured by RPEIA and ethidium bromide fluorescence. As shown in Fig. 1, the 284-bp primary PCR product was detectable at a dilution as great as 10^{-8}, whereas the 102-bp nested-PCR product was detectable at a 10^{-9} dilution (equivalent to a starting template number of approximately 3 to 30 B19 genomes). The RPEIA gave a level of detection equivalent to that obtained with nested PCR, with OD values ranging from >2.00 for the dilutions yielding the highest levels of PCR product to <0.02 for the negative controls.

Nineteen clinical specimens from patients with evidence of B19 infection were tested by PCR and nested PCR with ethidium bromide staining of the amplification product and by RPEIA. Twelve specimens were positive by all three assays, whereas two specimens were detectable only by RPEIA or after nested amplification (Table 1). Four specimens were

TABLE 1. Comparison of PCR product detection methods for 18 patients with clinical and/or serologic evidence of B19 infection[a]

Patient	Age (yr)	Sex	Clinical diagnosis	IgG EIA	IgM EIA	PCR-EBF	nPCR-EBF	RPEIA index, result
1	36	F	Aplastic crisis	+	+	+	+	5.1, +
2	9	F	Rash	+	+	+	+	6.3, +
3	8	F	Rash	+	+	+	+	5.1, +
4	12	M	Rash	+	+	+	+	10.9, +
5	37	F	Polyarthritis	+	+	+	+	5.6, +
6	26	F	Rash and arthritis	+	+	+	+	5.7, +
7	5	M	Anemia	−	+	+++	+	64.3, +
8	Fetus	ND	Fetal death	+	+	++	+	30.0, +
9	ND	F	Polyarthritis	−	+	++	+	18.9, +
10	16	F	Anemia	+	+	++	+	32.9, +
11	27	M	Anemia	−	+	+	+	8.0, +
12	Fetus	ND	Fetal death	+	+	+	+	6.9, +
13	ND	M	HIV, aplasia	Bone marrow		−	−	0.6, −
14	ND	M	HIV, aplasia	Bone marrow		−	+	2.6, +
15	6	M	Anemia	−	+	−	−	0.3, −
16	2	F	Polyarthritis	−	−	−	−	0.6, −
17	37	M	Aplasia	+	+	−	−	0.6, −
18	6	M	Polyarthritis	+	−	−	+	4.6, +

[a] PCR and nested-PCR (nPCR) products detected by gel electrophoresis and ethidium bromide fluorescence (EBF) and the RPEIA. PCR-EBF results were quantitated as follows: no band, −; a band present, but no more intense than the 10-fg control, +; approximately the same intensity as the 100-fg control, ++; and of a greater intensity than the 100-fg control, +++. The cutoff for a positive RPEIA was taken as the mean OD plus 5 standard deviations of the 10 B19 DNA-negative serum specimens (OD = 0.035). The RPEIA index is derived by dividing the observed OD by the cutoff value; index values of ≥1 are considered positive. Abbreviations: F, female; M, male; HIV, human immunodeficiency virus; ND, not determined.

negative by all three assays. The 10 B19 DNA-negative control serum samples were negative in all assays.

A variety of nucleic acid probes for use in Southern and dot blot hybridization assays for detection of parvovirus B19 DNA have been described (2, 3, 6, 9, 14, 16, 18–22). Because RNA probes offer greater sensitivity in hybridization assays owing to their single strandedness and the inherent greater stability of the RNA-DNA duplex, we attempted to identify a procedure for preparing a nonradioactive B19-specific RNA probe that would provide an unlimited supply of high quality reagent by a method more accessible to laboratories with limited molecular skills. The TA Cloning System (Invitrogen) used in combination with the Genius Nonradioactive RNA Labeling Kit satisfied these requirements. Although approximately 50% of our probe sequence consists of polylinker region from the pCR 1000 cloning vector, nonspecific hybridization with PCR-amplified target DNA would not be expected, given the specificity of the amplification reaction. Moreover, the added probe length likely increases the number of incorporated digoxigenin labels and therefore increases the potential specific activity of the probe.

Using this probe, we were able to devise an assay for detection of B19 PCR-amplified DNA that increased the sensitivity of our single-cycle PCR and eliminated the need for nested PCR, thereby reducing the risk of false-positive results associated with a second amplification. The RPEIA combines the advantages of incorporative labeling of the PCR product with biotin to facilitate capture of the product-probe hybrid with those of liquid-phase hybridization with the digoxigenin-labeled RNA probe for rapid and specific detection of the PCR product. We achieved at least a 10-fold increase in sensitivity over our standard PCR protocol for detection of B19 target DNA, giving us a level of detection of between 3 and 30 genome copies, equivalent to the sensitivity of our nested PCR assay (10). The diagnostic value of this increased sensitivity was best illustrated in this study by two cases: (i) patient 14, for whom a serum specimen was unavailable and for whom diagnosis was accomplished by PCR amplification of bone marrow samples, and (ii) patient 18, for whom B19 DNA was detected in serum negative for specific IgM antibodies. Although repeat extractions of the clinical specimens gave similar results, the possibility of B19 DNA contamination could not be ruled out in these cases. Moreover, by adapting the RPEIA to a microtiter plate format, coupled with colorimetric detection, we were able to simplify the procedure and make it more compatible with our current EIA protocols for detection of B19-specific antigen and antibodies (1).

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