Quantitative Direct Probe Method for the Detection of Parvovirus B19

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Parvovirus B19 infection is associated with anemia and spontaneous abortions. While many qualitative assays are available, a few molecular-based quantitative methods have been described. This study reports the development and optimization of a quantitative direct-probe method for the detection of Parvovirus B19 DNA. Different concentrations of RNA probes were used to identify

the optimal conditions for hybridizing to the target DNA. Detection of DNA was linear between concentrations of 2 ng/ml to 200 pg/ml. Because this method requires no enzymatic amplification, it is not susceptible to amplifier contamination or enzymatic inhibitors, and it can be applied to serum samples or paraffin-embedded tissue. *J. Clin. Lab. Anal.* 14:38–41, 2000. © 2000 Wiley-Liss, Inc.

Key words: hybrid capture; Parvovirus B19; DNA; RNA

INTRODUCTION

Parvovirus B19 viral infection is characterized by malar rash, chills, myalgia, and arthralgia in healthy individuals (1,2). This virus invades and destroys erythroid progenitor and precursor cells (3). Aplastic crisis presents in patients with sickle cell anemia and other types of hemolytic anemias (4,5) or even in the absence of underlying hemolytic anemias (6). In previously infected pregnant women, this virus has been associated with spontaneous abortions and with hydrops fetalis (7,8). In immunocompromised patients, this is a treatable cause of anemia (9), and occasionally, parvovirus B19 DNA can persist in serum for a long period of time (10,11). The significance of this persisting virus is hampered by the lack of quantitative assays because molecular-based detection of parvovirus has used qualitative methods. We describe the optimization and development of a quantitative direct-probe detection system using the hybrid capture method (DIGENE Hybrid-Capture® System, Murex Diagnostic LTD, Dartford, UK) that is capable of detecting 200 pg/ml of complementary parvovirus B19 DNA.

MATERIAL AND METHODS

Cloned Viral DNA

Viral DNA was obtained from Peter Tattersall, Ph.D., and was amplified by the polymerase chain reaction (PCR) using previously described primers (Midland Certified Reagent Company, Midland, Texas) to generate a 900-base-pair (bp) sequence of Parvovirus B19 DNA from the “plus” sense strand (12). Then, the PCR product was cloned using Original TA Cloning® kit into a pCR® 2.1 vector (Invitrogen BV, NV

Leek, The Netherlands). The plasmid was transformed into E.coli Sure® Strain bacteria (STRATAGENE, La Jolla, CA) and incubated at 37°C for 48 hr. Extraction of the plasmid was performed following the manufacturer's indications, with a QIAprep Spin Plasmid kit purchased from QIAGEN (GmbH, Germany). The orientation of the clone was determined by sequencing. The transcription vector pCR® 2.1, with the B19 DNA insert, containing T7 promoter was linearized by Bam HI digestion as confirmed by gel electrophoresis. The linearized DNA was purified using QIA amp Viral RNA kits for plasma, serum, and body fluids (QIAGEN). Then RNase-free DNA was used as the template for RNA transcription and as positive controls.

In order to prepare the RNA probe, transcription of “minus” sense RNA was performed using T7 RNA polymerase, utilizing RNA Transcription kit from Stratagene Cloning System, (STRATAGENE). The DNA template was incubated with 10 U of RNase-free DNase/μg of DNA. High quality B19 RNA probe was obtained and resuspended in a buffer prepared with DEPC water (10 mM Tris, 15 mM NaCl, and 0.2% SDS) to a concentration of 30 nM/l. The probe mix was obtained by adding B19 RNA probe (30 nM/l) to probe diluent provided with the HPV-Human Papilloma Virus-hybrid capture kit (DIGENE Hybrid-Capture® System, Murex Diagnostic LTD, Dartford, UK).

Serial tenfold dilutions of plasmid-derived B19 DNA in

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50- μ l volume of RNase-free water were tested by direct hybridization based on "sandwich" capture of DNA/RNA hybrids (DIGENE Hybrid-Capture® System, Murex Diagnostic LTD, Dartford, UK).

Extracted serum from bone marrow aspirates, peripheral blood or cord blood—sent to our laboratory for PCR confirmation of parvovirus B19 infection—and DNA extracted from paraffin-embedded tissue were analyzed, to assess the applicability of the hybrid-capture test to both clinical samples and archival material. Some tissue samples were run in duplicate to compare the proteinase K DNA extraction to the heating method of DNA extraction.

The assay was performed according to the manufacturer's instructions for the HPV (Human Papilloma Virus) DNA Assay kit (DIGENE Hybrid-Capture® System). Briefly, 100 μ l of positive samples or negative controls were prepared in separate RNase-free centrifuge tubes in which 50 μ l of sample diluent and 50 μ l of denaturation agent were added. Tubes were incubated for 45 min at 65°C for denaturation of double-stranded DNA. Hybridization was carried out in the presence of strand-specific RNA probes in separate tubes incubated at 65°C for 60 min. DNA/RNA hybrids were then transferred to separate Capture Tubes coated with anti-DNA/RNA hybrid antibodies. Hybrid capture was undertaken after shaking for 60 min at room temperature on a rotary shaker. The tubes were decanted and Reagent 1 (alkaline phosphatase labeled anti-DNA/RNA hybrid antibodies) was added. The capture tubes were incubated at room temperature for 30 min then washed several times with buffer. The "sandwich" was then labeled with a substrate (Lumiphos® 530) protected from light for 30 min at room temperature. The substrate was transformed into a luminescent compound by alkaline phosphatase. The light emission that is proportional to the amount of parvovirus DNA was measured by a chemiluminometer (DCR-1™ Luminometer, DIGENE, Inc.) and the results were given in relative light units (RLU). The samples were then read using the raw data program of the chemiluminometer (DIGENE). According to the manufacturer's assay validation, acceptable-range positive results are considered reliable when the average of the positive control divided by the average of the negative control is equal to or greater than 1.5. For each run the positive cutoff value was estimated based on this formula. The manufacture's stated cutoff for HPV detection in clinical specimens was 10 pg/ml. Therefore, any given reading 1.5 times greater than the negative control was accepted as positive for that specific run.

For DNA extraction from paraffin embedded tissue, 3 to 4 10-micron sections were collected in sterile propylene tubes, from selected blocks. To avoid cross-contamination the negative tissue was cut first, and new disposable blades were utilized between blocks. Then, 1 ml of xylene was added to the tubes for 15 min. Xylene was then removed and 1 ml of ethanol was added for 15 min. Then, ethanol was removed and the tubes were sealed with parafilm. The film was perforated and the tis-

sue dried at 50°C. Next, 200 μ l of TE buffer and 50 μ l of Proteinase K were added. The tubes were placed at 37°C in a hybridization oven overnight. Then, the tubes were placed in boiling water for 10 min to inactivate the protease K. After cooling, they were briefly spun and the supernatant was removed. Finally, DNA was quantified using a spectrophotometer. For the heating extraction method, after removing the ethanol and sealing the tubes with parafilm, the tubes were placed at 95°C until the tissue dried out. Next, the tissue was resuspended in DEPC water and DNA was quantified using a spectrophotometer.

RESULTS

Detection Reagent 2 (Lumiphos® 530) blank readings and negative control readings (DEPC water or DNA carrier) fell within expected range in every run. In order to investigate the optimal concentration of RNA probe for hybridization, different concentrations of RNA were tested in the presence of a constant amount of parvovirus B19 DNA (Table 1). The best results were obtained with a 1:10 probe/diluent volume ratio (B19 RNA concentration of 3.8nM/l). Serial tenfold dilutions of DNA were carried out until negative readings were obtained (Fig. 1). Additional runs at 1–100 pg total DNA range (DNA concentration 2 ng/ml to 20 pg/ml) were performed to establish the cutoff for detecting target parvovirus B19 DNA. Reliable positive values were detected between 10 and 15 pg of total DNA (DNA concentration 200 pg/ml). However, occasional positive results at lower concentrations were obtained. Target parvovirus DNA detection was linear from 2 μ g/ml to 200 pg/ml (Fig. 2).

A cross-reactivity panel was tested using plasmid cloned DNA from bacteria, and from viruses that can be either part of the skin flora, urogenital tract, or blood-born infection agents in immunocompromised patients. With the exception of parvovirus B19, positive controls the results were negative in this group (Table 2).

Clinical data and results are shown in Table 3a,b. Hybrid-capture results were compared to PCR results in blood samples and bone marrow aspirates. For paraffin-embedded tissue, the hybrid-capture results were compared to previous PCR or immunohistochemistry results. In sample NB (Table 3a), there was a discrepancy between the results. The parvovirus B19 DNA concentration was greater than the negative control but less than the positive cutoff for this assay and thus yielded an indetermi-

TABLE 1. Different RNA probe concentrations^a

	Probe 1X (RNA 760 pM/l) (RLU) ^b	Probe 5X (RNA 3.8 nM/l) (RLU)	Probe 10X (RNA 7.6 nM/l) (RLU)
B19			
DNA			
1 μ g	27840	140238	39080
0.1 μ g	236497	769107	205126
Negative	3059	2149	1291

^aOptimal hybridization results were achieved with RNA at 3.8nM/l.

^bRLU, relative light units.

Fig. 1. Parvovirus DNA versus relative light units (RLU).

nate result that was reported as negative. Interestingly, in this patient, the diagnostic band of the PCR was borderline positive. Similar results were observed in paraffin embedded tissue. Sample #5 (Table 3b) was in the lowest positive range of detection by hybrid capture and slightly above the PCR positive threshold. Sample #11 was from a different section of the same paraffin block as sample #5 and also yielded an indeterminate value. In summary, 16 specimens were examined for parvovirus B19 DNA using a signal amplification method. Fourteen specimens positively correlated with results produced from other methodologies. The two discordant results yielded values that were in the indeterminate range of the assay and were therefore reported as negative.

In general, proteinase K-extracted DNA yielded higher results when compared to DNA extracted by heating method (data not shown). Sample #5 was repeatedly negative with the heating extraction method but positive with the proteinase K-extraction method (Table 3b).

DISCUSSION

Parvovirus B19 is a member of *Parvoviridae* known to cause human disease. Exposure to the virus can have innocuous to

TABLE 3a. Blood and bone marrow aspirates

Patient	Age/sex	Source	Hybrid capture	PCR ^a
PD	29 F	BM ^b	Neg.	Neg.
WD	1M	PB	Neg.	Neg.
DJ	1 F	PB	Neg.	Neg.
GC	Fetus	CB	Pos.	Pos.
NB	39 F	PB	Neg.	Pos.

^aPCR, polymerase chain reaction.

^bBM, bone marrow; PB, peripheral blood; CB, cord blood.

TABLE 3b. Paraffin-embedded tissue samples (proteinase K extracted DNA)^a

Patient	Sample no.	Age/sex	Source	HC	PCR	IMM
NN	1	24 F	PLA	Neg.	Neg.	ND
GJ	2	71 M	BM	Neg.	Neg.	ND
GJ	3	71 M	BM	Neg.	Neg.	ND
LJ	4	37 M	BM	Neg.	Neg.	ND
AH	5	23 F	PLA	Pos. ^b	Pos.	ND
AH	6	23 F	PLA	Pos.	Pos.	ND
AH	7	Fetus	Liver	Pos.	Pos.	Pos.
AH	8	Fetus	Lung	Pos.	Pos.	Pos.
GJ	9 ^c	71 M	BM	Neg.	Neg.	ND
LJ	10 ^c	37 M	BM	Neg.	Neg.	ND
AH	11 ^c	23 F	PLA	Neg. ^b	Pos.	ND

^aBM, bone marrow; PLA; placentas; HC, hybrid capture; PCR, polymerase chain reaction; ND, not determined.

^bSample #5 and #11 are from the same block.

^cHeating DNA extraction.

lethal consequences. The infection outcome is related to host factors; however, little is known about virulence factors.

In the last decade numerous methods have been used to detect parvovirus B19 in either serum or tissue samples: biotinylated RNA hybridization probes (13); PCR alone or combined with DNA dot hybridization (12,14); strand-specific in situ hybridization probes (15); digoxigenin-labeled DNA hybridization probe (16); in situ hybridization (17); in situ digoxigenin-labeled DNA hybridization probe (18); microplate-capture hybridization of amplified digoxigenin-labeled DNA (19); nested PCR (20); capture hybridization of digoxigenin-labeled B19 DNA amplicons (21), and synthetic oligonucleotides using dot blot (22). We describe a direct hybrid-capture method that is very simple, fast, and does not require expensive equipment. It is specific because the RNA probe can couple only with the target DNA forming a stable RNA/DNA hybrid. The antibodies directed against DNA/RNA hybrids do not bind to RNA/RNA targets that could result from secondary structures in the RNA probe. Target parvovirus DNA detection was linear from a concentration between 2 µg/ml and 200 pg/ml. The sensitivity limit is 10 pg/ml. Therefore, negative results generated from patient samples should be accompanied with a comment stating that the specimens containing less than 10 pg/ml of parvovirus B19 DNA will be reported as negative.

The sensitivity of the method can be improved by utilizing

TABLE 2. Cross-reactivity panel^a

Bacteria	Virus
Diphtheroids	Cytomegalovirus
Erlichia	Hepatitis B virus
<i>Escherichia coli</i>	Hepatitis C virus
<i>Listeria monocytogenes</i>	Human papillomavirus
<i>Pseudomonas aeruginosa</i>	Herpes simplex virus
<i>Staphylococcus aureus</i>	
<i>Staphylococcus epidermidis</i>	
<i>Streptococcus B-hemolyticus</i>	

^aAll the tested organisms did not cross-react with the B19 probe.

larger probes and second generation hybrid-capture reagents, designed to increase the amplification of the signal. Another potential application of this technique is in paraffin-embedded tissue, which proved to provide adequate material that can be used in morphologic correlation in retrospective studies using archival material. In conclusion, we describe a simple, direct, and quantitative assay that is not susceptible to amplifier contamination or enzymatic inhibitors.

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